Influence of Age, Biological Sex and Body Weight on Brain Insulin Signalling

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Table of contents

Abbreviations

AD	Alzheimer's disease
a.m.	*ante meridiem* (lat.), (“before midday”)
ASL	Arterial spin labelling (measurement sequence in fMRI)
BBB	Blood-brain barrier
BMI	Body mass index
BOLD	Blood-oxygen-level-dependent (imaging)
CBF	Cerebral blood flow
CNS	Central nervous system
CSF	Cerebrospinal fluid
DLPFC	Dorsolateral prefrontal cortex
e.g.	*exempli gratia* (lat.), (“for example”)
FCR	Food cue reactivity (task)
[18F]-FDG-PET	18F-fluorodeoxyglucose positron emission tomography
(f)MRI	(functional) magnetic resonance imaging
HOMA-IR index	Homeostatic model assessment for insulin resistance index
i.e.	*id est* (lat.), (“that is”)
ISI	Insulin sensitivity index
$ISI_{Matsuda}$	Matsuda peripheral insulin sensitivity index
IU	International unit
ROI	Region of interest
SD	Standard deviation
T2D	Type 2 diabetes
TFEQ	German Three Factor Eating Questionnaire
vs.	versus („opposed“ or „in contrast to“)
VLPFC	Ventrolateral prefrontal cortex
WHO	World Health Organization

English Thesis Summary

Insulin is a pancreatic hormone, able to pass the blood-brain barrier (BBB) to act on the central nervous system. Insulin receptors are widely distributed across the brain and found in high densities in regions like the cerebellum, hypothalamus, olfactory bulb, hippocampus, frontal cortex and striatum. Brain insulin signalling plays a crucial role in regulating whole-body metabolism, cognitive processes and eating behaviour. Different factors and conditions, including obesity and ageing, were shown to influence either insulin availability in the brain through changes in the transport across the BBB or by directly influencing brain insulin action. Moreover, first evidence points to sex-specific differences in the insulin-induced modulation of eating behaviour or memory performances. The aim of this work was to clarify and disentangle the influence of obesity/peripheral insulin sensitivity, age and biological sex on central insulin signalling. To probe central insulin action, intranasal insulin administration, compared to placebo was used in combination with functional magnetic resonance imaging in healthy women and men of different weight status and age.

In the first study, we investigated whether brain insulin signalling influences appetite and reward regulation and if this process is modulated by sex, obesity and age. In response to high-caloric food cues, the amygdala, a region implicated in neural food reward pathways and eating behaviour showed higher insulin-mediated activity over all participants. The insulin-mediated insular cortex activity (besides other brain regions) was influenced by weight status and sex. Of interest, the insulin-mediated food cue reactivity in the insula was positively correlated to peripheral insulin sensitivity and this relationship was fully mediated by cognitive restraint, a measure for the cognitive self-control over food intake. In response to highly palatable food cues, brain insulin signalling in the prefrontal cortex, essential for decision-making and cognitive control of food intake, differed between women and men. In line with previous literature, central insulin action decreased perceived hunger over all participants and especially in men with normal weight, but also in women with overweight. Hence, insulin signalling in the brain modulates appetite and reward regulation, and this process is influenced by obesity and sex, but not age.

In study 2, we investigated the association between brain insulin action, peripheral insulin sensitivity and age in different regions known to be insulin sensitive and crucial for eating

behaviour, homeostasis and cognitive functions. We could show a region-specific relationship between brain insulin action, age and peripheral insulin sensitivity in different brain regions important for the regulation of eating behaviour, including food reward pathways (amygdala, insula, striatum) and memory functions (hippocampus) with more pronounced associations depending on sex. These findings underline brain insulin responsiveness as a possible link between systemic metabolism and neurocognitive functions.

In summary, our results confirm that central insulin signalling plays an important role in cognitive processes and in the regulation of homeostasis, appetite and reward regulation. Furthermore, our results demonstrate that insulin action is influenced and modulated by obesity, sex and age in a region-specific and task-specific manner. Brain insulin resistance was proposed as a joint pathological characteristic of neurodegenerative and metabolic disorders, including type 2 diabetes and Alzheimer's disease. Interestingly, epidemiological data suggests that the prevalence, especially for type 2 diabetes and Alzheimer's disease differs between women and men. Our results show that it is essential to consider and include different factors such as sex, age and weight status in further studies. Moreover, additional studies with larger numbers of participants and/or in suitable animal models are needed to understand the underlying (molecular) mechanisms of central insulin action in health and disease.

Deutsche Zusammenfassung

Insulin ist ein Hormon, das in der Bauchspeicheldrüse produziert wird und hauptsächlich für seine Rolle in der peripheren Energiestoffwechselregulation bekannt ist. Insulin gelangt über die Blut-Hirn-Schranke auch in das zentrale Nervensystem. Insbesondere Regionen wie das Kleinhirn, der Hypothalamus, der Riechkolben, der Frontalkortex und das Striatum besitzen eine hohe Dichte an Insulinrezeptoren. Dabei spielt Insulin im Gehirn eine entscheidende Rolle bei der Regulierung des Ganzkörperstoffwechsels, kognitiven Funktionen und des Essverhaltens. Es wurde bereits gezeigt, dass verschiedene Faktoren wie zunehmendes Alter oder Adipositas entweder die Insulinverfügbarkeit beeinflussen (durch einen verminderten Insulintransport über die Blut-Hirn-Schranke) oder mit einer reduzierten Insulinwirkung im Gehirn einhergehen. Darüber hinaus gibt es erste Hinweise auf geschlechtsspezifische Unterschiede bei der insulin-induzierten, zentralen Regulierung des Essverhaltens oder Gedächtnisleistungen. Ziel dieser Arbeit war es, den Einfluss von Übergewicht/peripherer Insulinsensitivität, Alter und biologischem Geschlecht auf die zentrale Insulinwirkung zu untersuchen. Die zentrale Insulinwirkung wurde mittels intranasaler Gabe von Insulin im Vergleich zu Placebo in Kombination mit funktioneller Magnetresonanztomographie bei gesunden Frauen und Männern mit unterschiedlichem Gewichtsstatus und Alter, gemessen.

In der ersten Studie wurde analysiert, ob die zentrale Insulinwirkung den Hunger oder das Verlangen nach Essen, sowie die Belohnungsaspekte von Essen beeinflusst und ob dieser Prozess durch Übergewicht, das biologische Geschlecht und Alter moduliert wird. Die Amygdala, eine zentrale Region in der Verarbeitung von Emotionen, aber auch Teil des Belohnungssystems und involviert in der Regulierung von Essverhalten, zeigte über alle Personen hinweg eine höhere insulin-vermittelte Aktivität als Reaktion auf hochkalorische Nahrungsmittel. Die insulin-vermittelte Aktivität im insularen Kortex (neben anderen Hirnregionen) wurde durch den Gewichtsstatus und das Geschlecht der Personen beeinflusst. Interessanterweise korrelierte die insulin-vermittelte Antwort auf Essensreize in der Insula positiv mit der peripheren Insulinsensitivität, sowie der kognitiven Kontrolle über das Essverhalten. Die insulin-vermittelte Antwort auf als sehr schmackhaft bewertete Essensreize unterschied sich zwischen Frauen und Männern im präfrontalen Kortex, einer Region die essenziell an der Handlungsplanung und Selbstkontrolle beteiligt ist. Darüber

hinaus gab es eine Abnahme des wahrgenommenen Hungergefühls über alle Personen hinweg und insbesondere bei Männern mit Normalgewicht, aber auch bei Frauen mit Übergewicht. Insulin im Gehirn moduliert also sehr wohl das Hungergefühl, sowie das Verlangen und die Belohnungsaspekte von Essen und dieser Prozess wird durch Übergewicht und das Geschlecht, nicht aber durch Alter beeinflusst.

In Studie 2 untersuchten wir den Zusammenhang zwischen Alter, der peripheren und der zentralen Insulinsensitivität in verschiedenen insulin-sensitiven Gehirnregionen, die an der Regulation von Essverhalten, Homöostase und kognitiven Funktionen beteiligt sind. Wir konnten einen regionsspezifischen Zusammenhang zwischen dem Alter, der peripheren Insulinsensitivität und der Insulinwirkung in verschiedenen Gehirnregionen nachweisen, wobei die Assoziationen je nach Geschlecht ausgeprägter waren. Bei diesen Gehirnregionen handelte es sich um Regionen die wichtig für die Regulierung des Essverhaltens oder Teil des Belohnungssystems sind (Amygdala, Insula, Striatum), sowie an Gedächtnisfunktionen (Hippocampus) beteiligt sind. Die Ergebnisse unterstreichen die Wichtigkeit der Insulinwirkung im Gehirn als mögliche Verbindung zwischen metabolischen und neurokognitiven Funktionen.

Zusammenfassend bestätigen unsere Ergebnisse, dass Insulin im Gehirn eine wichtige Rolle bei kognitiven Prozessen, sowie bei der Regulierung der Homöostase, Appetit und der Verarbeitung von Belohnungsaspekten von Essensreizen spielt. Des Weiteren zeigen unsere Ergebnisse, dass die Insulinwirkung regionsspezifisch und aufgabenspezifisch durch Übergewicht, Geschlecht und Alter beeinflusst und moduliert wird. Die zentrale Insulinresistenz wird als gemeinsames pathologisches Merkmal von neurodegenerativen und metabolischen Beeinträchtigungen, inklusive Typ 2 Diabetes und Alzheimer diskutiert. Interessanterweise zeigen epidemiologischen Daten, dass sich die Prävalenz, insbesondere für Typ 2 Diabetes und Alzheimer zwischen Frauen und Männern unterscheidet. Unsere Ergebnisse zeigen, dass es elementar ist unterschiedliche Faktoren wie Geschlecht, Alter und Gewichtsstatus in Studien zu berücksichtigen. Weitere Studien mit größeren Teilnehmerzahlen, sowie in passenden Tiermodellen sind erforderlich insbesondere auch um die zugrundeliegenden molekularen Mechanismen der zentralen Insulinwirkung in Gesundheit und Krankheit zu verstehen.

List of Publications & Contributions

Peer reviewed and accepted publications:

Study 1: Sex differences in central insulin action: Effect of intranasal insulin on neural food cue reactivity in adults with normal weight and overweight.

Wagner, L., Veit, R., Fritsche, L., Häring, H. U., Fritsche, A., Birkenfeld, A. L., Heni, M., Preissl, H., & Kullmann, S. (2022). **Sex differences in central insulin action: Effect of intranasal insulin on neural food cue reactivity in adults with normal weight and overweight**. *International journal of obesity* (2005), 46(9), 1662–1670. https://doi.org/10.1038/s41366-022-01167-3

Study 2: Brain insulin responsiveness is linked to age and peripheral insulin sensitivity.

Wagner, L., Veit, R., Kübler, C., Fritsche, A., Häring, H. U., Birkenfeld, A. L., Heni, M., Preissl, H., & Kullmann, S. (2023). **Brain insulin responsiveness is linked to age and peripheral insulin sensitivity.** *Diabetes, obesity & metabolism*, *25*(8), 2171–2180. https://doi.org/10.1111/dom.15094

Nr.	List of authors	Position of candidate in list of authors	Scientific ideas by the candidate (%)	Data generation by the candidate (%)	Analysis and Interpretation by the candidate (%)	Paper writing done by the candidate (%)
1	**Wagner, L.**, Veit, R., Fritsche, L., Häring, H. U., Fritsche, A., Birkenfeld, A. L., Heni, M., Preissl, H., & Kullmann, S.	1	50	60	80	90
2	**Wagner, L.**, Veit, R., Kübler, C., Fritsche, A., Häring, H. U., Birkenfeld, A. L., Heni, M., Preissl, H., & Kullmann, S.	1	50	50	75	90

Introduction

The brain as insulin sensitive organ

The hormone insulin was discovered and isolated a little over 100 years ago, in 1921, by Frederick Banting and Charles Best [1]. Insulin is produced in the pancreatic beta cells in response to rising blood sugar levels and has a seminal role in glucose metabolism by stimulating glucose uptake in muscles, the liver and adipocytes and by suppression of hepatic glucose production [2]. Approximately 50 years later, in 1978, insulin receptors were discovered in rodent brains [3]. However, the brain was for a long time considered as insulin-insensitive, as neuronal glucose uptake is thought to be mostly insulin-independent. Scientific interest only turned later to the role of brain insulin signalling. Especially in the last two decades, evidence is rapidly accumulating that brain insulin action is involved in metabolic and cognitive health, including memory, mood and olfaction as well as eating behaviour and peripheral metabolism [4-6]. Insulin receptors are found in high density in regions like the cerebellum, hypothalamus, hippocampus, frontal cortex, olfactory bulb and striatum [5, 6]. Besides targeting neuronal populations, insulin signalling also affects glia cells, including astrocytes. Astrocytes are functionally integrated and interconnected to neural networks and implicated in the regulation of synaptic transmission, neuronal proliferation and in mediating cerebral blood delivery linked to neural activity [7-9]. Recent findings suggest that brain-glucose uptake and energy balance regulation could nonetheless be regulated by insulin signalling in astrocytes [9]. Emerging evidence further indicates that insulin signalling in astrocytes, comparably to neurons, influences metabolic homeostasis and regulates cognition and mood [9].

As mentioned earlier, it is widely assumed that most of the insulin that reaches the brain, is produced in the pancreatic beta cells. The hormone is transported through the bloodstream and passes the blood-brain barrier (BBB) by a saturable receptor mediated transport mechanism [10-13]. In detail, peripheral insulin is transported from the blood stream through brain capillary endothelial cells in the brain interstitial fluid, where it can act on neuronal insulin receptors. From there insulin moves through the ependyma into the cerebrospinal fluid (CSF) [14, 15].

Previous studies showed that insulin concentrations in the CSF are lower than serum insulin concentrations in the periphery [16]. Under baseline conditions, insulin concentrations in the CSF were approximately 10 times lower than serum insulin concentrations [17-19]. Moreover, in healthy participants the serum and CSF insulin concentrations were highly correlated [20] and insulin concentrations in the CSF increased during systemic insulin infusion [21].

Insulin sensitivity: a link between cognitive and metabolic disorders?

The prevalence of metabolic disorders, including overweight and obesity is constantly rising. In brief, overweight and obesity arise from an energy imbalance between the consumed and expended calories [22]. In fact, the causes for overweight and obesity are multi-layered and not yet completely understood. Body weight is regulated by a complex interplay between the central nervous system (CNS) and the endocrine system, and inter alia, influenced by genetic and epigenetic factors [23, 24]. Moreover, an unhealthy lifestyle (e.g., reduced physical activity) and external factors including the continuous availability of processed, high-caloric food further promote excess weight gain [23, 24]. Obesity alone has almost tripled worldwide in the last 50 years [22]. Overweight and obesity are defined by an abnormal or excessive accumulation of body fat presenting a potential risk for health [25]. The most commonly used screening and classification tool for overweight and obesity is the Body Mass Index (BMI), a ratio between a person's weight and height. The cut-off value for overweight is 25 kg/m^2 and 30 kg/m^2 for obesity. Even though the BMI does not differentiate between fat mass and lean mass, the BMI is generally well correlated to percentage body fat [26].

A hallmark of obesity is a reduced peripheral insulin sensitivity or peripheral insulin resistance, defined as the diminished ability of insulin to exert its action on target tissues (peripheral will be used in contrast to brain/central), which can ultimately also lead to type 2 diabetes mellitus (T2D). Epidemiological evidence further suggests a strong link between reduced peripheral insulin sensitivity, including T2D, and age-related neurocognitive disorders [27-30]. The latter includes cognitive impairments as well as dementia (e.g., Alzheimer's disease (AD)). Besides pathological conditions, peripheral insulin sensitivity also slightly decreases with normal ageing [31, 32].

Not only peripheral insulin sensitivity is influenced by several conditions and factors, but also brain insulin action. Humans displaying an attenuated or absent response to insulin in the brain are often referred to as brain insulin resistant [33, 34]. The mechanisms behind altered brain insulin signalling may include a reduced number of insulin receptors, reduced insulin receptor sensitivity and/or an altered transport of insulin across the BBB from the periphery to the brain [35]. The causes for brain insulin resistance are diverse and potentially include genetic factors, but also obesity and ageing [34, 36]. Several factors including exercise, obesity, peripheral insulin resistance, ageing and AD have been demonstrated to influence the BBB transport leading to decreased CSF insulin levels [14, 37-40]. Moreover, first evidence points to a brain-region-specific association between peripheral and central insulin sensitivity, even though the underlying mechanisms are not completely understood [34, 38, 41-44].

Overall, there seems to be a close link between metabolic and cognitive impairments, whether reduced peripheral insulin sensitivity and/or brain insulin sensitivity is a common condition or a shared pathomechanism needs to be further elucidated.

Assessment of peripheral and central insulin sensitivity

The gold standard to assess peripheral insulin sensitivity in humans is the hyperinsulinemic-euglycemic clamp method. Hereby, insulin is infused at a constant rate, while glucose is infused at a variable rate to maintain euglycemia. Via the glucose infusion rate, glucose uptake of the different tissues in the body can be assessed, which serves as a measure of peripheral insulin sensitivity. A cheaper and less invasive method, is the estimation of different insulin sensitivity indices (ISI) from repeated insulin and glucose measurements during an oral glucose tolerance test (e.g., $ISI_{Matsuda}$ index). Further, in clinical practice often used indices, including the widely used HOMA-IR index, are based on a single fasted glucose and insulin measurement [45].

Compared to the periphery, the assessment of brain insulin sensitivity and also brain insulin action non-invasively in humans, is challenging. The intranasal application of insulin, -the delivery of insulin with a spray through the nose to the brain, combined with a functional imaging technique, like functional magnetic resonance imaging (fMRI) has been established over the last decades. As the intranasal application of insulin at the commonly used doses (40-160 IU), was shown to produce no or only little spill-over, no

peripheral hypoglycaemia or severe side effects [46], this method is widely used and allows to disentangle peripheral from brain insulin action. In contrast to the natural, peripheral insulin pathway via the bloodstream through the BBB to neuronal tissues, insulin is applied to the nasal cavity and enters the brain along olfactory, trigeminal pathways, bypassing the BBB [47-50]. The intranasally administered insulin is then distributed within several minutes along cerebral perivascular spaces in the brain [51, 52]. Following intranasal application, insulin levels in the CSF were shown to rise after 10 minutes, reaching a peak after 30 minutes and remain significantly elevated up to 80 minutes in humans [16].

Several functional neuroimaging techniques are available to non-invasively quantify and measure brain insulin sensitivity in humans, including fMRI [5]. fMRI measurements rely on the neurovascular coupling, a term used to describe changes in local perfusion (blood flow) that occur in response to neural activity. These local changes in blood flow are measured with fMRI as a proxy for neural activity. Brain activity can either be studied during resting-state or while performing a specific task. To investigate appetite and food-specific brain responses and their modulation by peripheral signals (including hormonal signals) or personality traits (e.g., eating habits, impulsivity), tasks with food cues are often employed [53]. Food cues can include visual or olfactory stimuli, or any cues associated with food-related memories [54]. Such cues can be considered as conditioned stimuli, able to elicit food-related psychological (e.g., craving) and physiological (e.g., salivation or hormone secretion) changes, accompanied by neurocognitive reactivity preparing for subsequent food intake [54, 55]. Food cue reactivity was shown to be a robust prospective and predictive measure for subsequent food-related outcomes, including eating behaviour and body weight changes over time (weight gain or weight loss) and is therefore commonly used to study appetite and obesity [56, 57].

Effects of brain insulin action on cognitive functions

Central insulin is essential in synaptic plasticity, neuronal survival, neurite growth and learning [50, 58-62]. In contrast, brain insulin resistance might constitute a pathological trait in cognitive impairments described in relation to obesity, T2D, aging and AD [5, 36, 63, 64]. Post-mortem studies in elderly patients demonstrated a decrease of insulin signalling in the brain of AD patients and this reduced central insulin sensitivity was directly associated with cognitive decline [64].

Beneficial effects of intranasal administration of insulin on cognition, memory functions and mood have been demonstrated in healthy and elderly, cognitively impaired humans [65]. The existing literature is quite inhomogeneous concerning the used insulin doses (10-160 IU), the study population (i.e., age, BMI, sex, health status) and the used study design and cognitive testing, which makes it difficult to directly compare the study outputs (reviewed here: [65, 66]). Most studies tested either executive functions (e.g., working memory, attention or inhibition) or memory functions (e.g., declarative, spatial or non-declarative memory). Acute doses of intranasal insulin were demonstrated to improve executive functions and memory in normal weight adults [67-70] and in cognitively impaired adults [71, 72]. First evidence points to sex-specific differences, as Benedict and colleagues [67] reported improved performances on declarative and working memory tasks only in women and not in men [67]. Longer-term (3-16 weeks) insulin administration was shown to improve memory functions in participants with normal weight and overweight [73, 74]. Moreover, declarative memory and executive functions were increased following intranasal insulin administration in participants with mild cognitive impairments or AD [75, 76].

Effects of brain insulin action on metabolism and eating behaviour

Brain insulin signalling plays a major role in the regulation of eating behaviour and peripheral metabolism. In physiological conditions, pancreatic insulin secretion and insulin concentration in the bloodstream are the highest after food intake. The hormone is transported into the brain [13] where it potentially acts as a satiety signal leading to reduced postprandial appetite. In the rodent model, a disruption or a reduction of CNS-specific insulin receptors led to hyperphagia and diet-induced obesity with higher body fat and peripheral insulin resistance [41, 77, 78]. In physiological conditions, brain insulin signalling was further shown to influence whole-body glucose metabolism by suppressing hepatic glucose production and to improve peripheral insulin sensitivity [5, 36, 79].

Moreover, the central administration of insulin led to reduced food intake in several animal models including rodents [80, 81], baboons [82], sheep [83] and young chicken [84]. In humans, previous behavioural studies showed decreases in appetite and food intake following acute intranasal insulin administration, but only in young men with normal weight [42, 67]. Longer-term intranasal insulin administration over 8-weeks led to

reductions in body weight and body fat content, again in men only. However, in women, intranasal insulin was shown to reduce the consumption of palatable food (i.e., cookies) in the postprandial state [85, 86]. Even though these studies point to sex-specific differences in central insulin action, such differences were not investigated or detected in fMRI studies so far.

In previous studies, central insulin was shown to modulate regional resting-state activity and functional connectivity especially in the amygdala, hippocampus, hypothalamus, insula, striatum and parts of the prefrontal cortex [42, 87-92] (*Figure 1*). These regions belong to an interconnected network modulating energy balance and food intake, are influenced by the sight, smell and taste of food and are responsive to postprandial hormones [93].

Based on actual knowledge and theories, eating behaviour and food intake are regulated by a crosstalk of two systems: the homeostatic system mainly influenced by the hypothalamus; and the reward or hedonic system including the mesolimbic-dopaminergic pathways and prefrontal areas [94]. The homeostatic system mainly gets input from peripheral hormones (e.g., insulin, leptin and ghrelin) and reacts to the current energy status and the metabolic needs of the body [95]. The reward system is regulated by physiological signals in response to food cues, including sight, taste, olfaction, palatability and probably further influenced by beliefs and expectations [4, 96]. A dysregulation of these systems can lead to the preference for high-caloric food, hyperphagia or eating in the absence of hunger, which in the long-term may promote obesity [4, 97].

In different neuroimaging studies, participants with overweight and obesity were shown to have higher food cue elicited brain responses, especially in regions linked to emotion and reward regulation, including the amygdala, insula, orbitofrontal cortex and striatum [54]. Such visual or olfactory food cues are able to elicit food related psychological (e.g., craving) and physiological (e.g., salivation or hormone secretion) changes, accompanied by neurocognitive reactivity preparing for subsequent food intake [54, 55]. Moreover, an incentive salience is often attributed to palatable food cues, leading to an activation of the reward neurocircuitry [98]. Of note, brain responses to food cues and craving can even be predictive for eating behaviour and weight gain [56] or, on the contrary, for the success of weight loss interventions [57].

In studies using a food cue reactivity (FCR) task and intranasal insulin, people with overweight and obesity showed an altered mostly increased response to visual food cues in brain regions linked to reward, associated with changes related to eating behaviour (e.g., food value and craving) [42, 90].

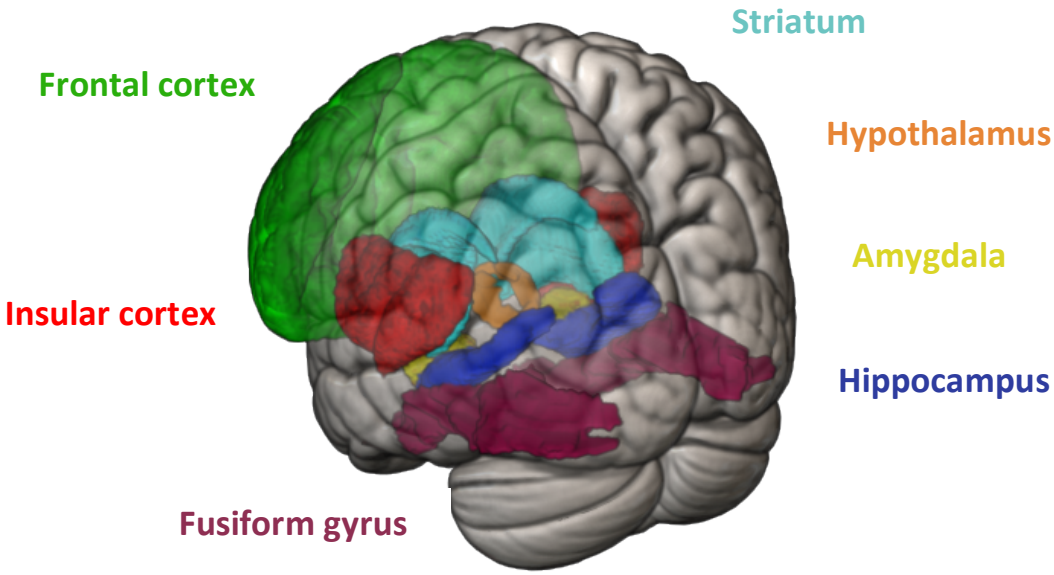

Figure 1: *Insulin sensitive brain regions displayed within a 3-dimensional standard anatomical template. In humans, central insulin action is mostly studied using intranasal administration of insulin, combined with a functional imaging technique (e.g., fMRI). The amygdala (yellow) plays an important role in the regulation of emotional behaviour and food intake. The frontal cortex (green) is responsible for decision-making and cognitive control of eating behaviour. The fusiform gyrus (violet) is involved in the recognition of visual stimuli including food cues and visual processing. The hippocampus (dark blue) has an essential role in learning behaviour and memory consolidation. The hypothalamus (orange) is vital for maintaining homeostasis (e.g., energy expenditure and food intake, thermoregulation, sleep…). The insular cortex (red) includes the primary taste cortex and plays an important role in sensory and homeostatic processes. The striatum (light blue), including the dorsal (i.e., caudate nucleus and putamen) and the ventral striatum (i.e., nucleus accumbens), is associated with reward-motivated behaviour. [36] (Figure 1 was created using MRIcroGL)*

Of interest, central insulin and dopamine action were shown to be closely linked and interconnected [4]. It was recently demonstrated in healthy humans that striatal dopamine levels [88] and functional connectivity of reward pathways [90, 91] were modulated by intranasal insulin administration. Moreover, in animal models, intranasal insulin directly modulated dopamine function in the midbrain and nucleus accumbens [99]. This shows that insulin signalling, besides being implicated in the modulation of energy homeostasis (satiety signal), is also involved in reward processing. Central insulin may act at the neural interface between metabolic and hedonic control and modulation of eating behaviour and food intake [90].

Thesis objectives

Insulin signalling in the brain has major implications in cognitive and metabolic processes. Several factors and conditions, including obesity and ageing have been shown to influence either insulin availability in the brain by altering the transport across the BBB or by directly affecting brain insulin action. First evidence further points to sex-specific differences in brain insulin action, particularly in the regulation of memory processes and eating behaviour. The aim of my thesis was to clarify and disentangle the influence of obesity/peripheral insulin sensitivity, age and sex on central insulin signalling.

Obesity, sex and age have previously been shown to influence appetite regulation and FCR. Compared to men, women demonstrated greater brain activity in prefrontal and reward areas, including the striatum and insula in response to high-caloric visual food cues [100]. Higher FCR in brain regions associated with emotion and reward regulation (i.e., amygdala, insula, orbitofrontal cortex) was also demonstrated in individuals with obesity [54] and increased activity in reward areas in response to food cues was a predictor of BMI in women [101]. Moreover, in middle-aged adults, brain activity elicited by food cues, in response to a meal decreased with age [102]. This suggests that increasing age may be linked to a reduced satiety effect, or a reduced rewarding effect of food.

Following intranasal insulin administration, individuals with obesity displayed an impaired response to food cues in reward-related brain areas [42, 90]. On behavioural level, central insulin failed to mediate eating behaviour (e.g., reduction of perceived hunger or food craving) in individuals with obesity [42, 90, 91]. In men, intranasal insulin led to a reduction of food intake and food craving [42, 67, 103]. Such an effect could not be observed in women. In women, however, intranasal insulin increased postprandial satiety, leading to a reduction of cookie consumption [85, 86]. Hence, first evidence suggests an influence of obesity and sex on central insulin signalling regarding appetite regulation and food intake.

Despite the extensive literature, to my knowledge, no study has investigated the influence of obesity, sex and age on central insulin-mediated neural responses to food cues. Therefore, the primary aim of study 1 was to clarify the influence of brain insulin signalling on appetite and reward regulation in healthy women and men of different age and weight status. We used a visual FCR task during fMRI measurements combined with intranasal

application of insulin or placebo. I expected a stronger insulin-mediated response to food cues in brain regions involved in eating behaviour in participants with normal weight, compared to participants with overweight. Moreover, I hypothesised to see sex-specific differences, with higher insulin-mediated brain activity in reward-related areas in women and particularly in women with overweight. In line with previous literature, I expected a reduction of perceived hunger and food craving in men with normal weight. Finally, with increasing age, I expected a reduced insulin-mediated response to food cues and a reduced effect on perceived hunger and food craving.

In study 2, I focused on the relationship between age, peripheral insulin sensitivity and central insulin action. Initial evidence suggests a regional association between peripheral and brain insulin sensitivity [38, 39, 41, 42]. However, no study to date has systematically analysed the association between peripheral and central insulin sensitivity in a larger sample of participants.

I expected to see region-specific correlations between peripheral insulin sensitivity and insulin-induced brain activity. I also expected brain insulin responsiveness to decrease with increasing age. Sex-specific differences in central insulin action have so far only been observed in behaviour-related measures (e.g., food intake or memory). I therefore explored whether sex has an effect on the association between age, peripheral and central insulin sensitivity.

Results and Discussion

In the following sections, the main results, mostly previously published [104, 105] will be outlined. Details concerning the study design (overview in *Figure 2*), statistics as well as the related figures can be found in the corresponding publications and in the *Appendix*.

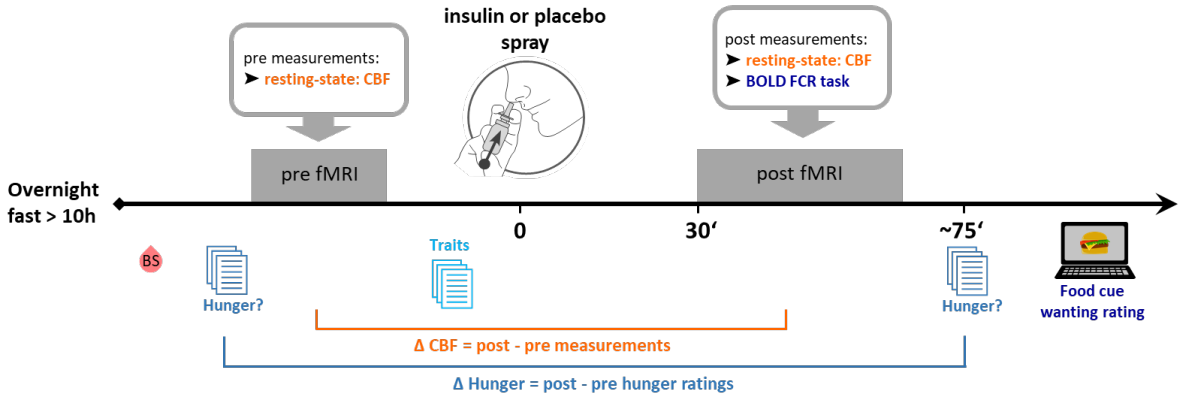

Figure 2: *Simplified and merged study overview. The studies consisted of a cross-over within-participant design with intranasal insulin or placebo in a pseudo-randomized order during two visits. Data analysed in study 1 are represented in blue, data analysed in study 2 in orange. The terms "pre" and "post" refer to the time points before and after application of the nasal spray.*
Upon arrival, the fasted state of the participants was controlled with a blood sample. Hunger was assessed with a visual analogue scale at arrival and approximately 75 minutes after nasal spray application. Trait eating behaviour characteristics were assessed with questionnaires, including the German Three Factor Eating Questionnaire (TFEQ) during the first visit.
Resting-state CBF data were recorded at each visit before and 30 min after nasal spray application (study 2). After nasal spray application and after the resting-state measurements, participants were shown 60 pictures of high- and low-caloric food cues during fMRI blood oxygen level-dependent (BOLD) measurements (study 1). The food cue viewing task in the fMRI was followed by a rating task: participants rated the food cues seen in the scanner on a laptop based on wanting (i.e., desire to eat).
When pre and post measures were available, we analysed the change (Δ), meaning we subtracted the pre measurement from the post measurement, leading to baseline-corrected data. To analyse specific intranasal insulin-induced responses, we used the difference between insulin and placebo measures in our statistical models.
BS: blood sample; BOLD: blood oxygenation-level dependent; CBF: cerebral blood flow; FCR: food cue reactivity task.

Study 1

– Sex differences in central insulin action: Effect of intranasal insulin on neural food cue reactivity in adults with normal weight and overweight

In the first study, we analysed whether central insulin action influences appetite and reward regulation in response to a food cue reactivity (FCR) task, where participants were exposed to visual high- and low-caloric food cues during BOLD-fMRI (*Figure 2*). Furthermore, we investigated whether brain insulin action differs between women and men (defined by biological sex) or weight status (defined by BMI). On a behavioural level, we were interested if central insulin reduces perceived hunger and the desire to eat specific food (wanting). 60 participants (30 women, BMI 18-32 kg/m^2, age 21-69 years) were measured with intranasal insulin and placebo spray in a pseudo-randomised, cross-over design (table with descriptive and metabolic data of the participants in *Table 1* in the Appendix [104]). Central insulin action was probed by intranasal insulin compared to placebo spray. Peripheral insulin sensitivity was assessed by an oral glucose tolerance test and the Matsuda peripheral insulin sensitivity ($ISI_{Matsuda}$) index was used as a measure for peripheral insulin sensitivity.

Results

Effect of central insulin on perceived hunger

Subjective feeling of hunger was assessed with a visual analogue scale before the fMRI measurements and approximately 75 minutes after spray administration. Hunger was significantly reduced after intranasal insulin administration compared to placebo over all participants and specifically in men with normal weight and women with overweight (*Figure 2* in the Appendix [104]).

Effect of central insulin on the desire to eat high and low-caloric food (wanting)

During the fMRI-FCR task, participants were shown 60 food cues, including high- and low-caloric sweet and savoury food (e.g., cakes, burger, pizza, vegetables, fruit and salads). Afterwards, outside of the scanner, participants were asked to rate the previously seen

food cues according to the question “how much they want to eat the food at that moment” on a 5-point Likert scale going from “not at all” to “very much”.

No main effect of insulin vs. placebo spray was found in the wanting ratings for high-caloric food cues. However, the differential wanting ratings (insulin-placebo) were significantly lower in men than in women. This indicates a reduction in wanting for high-caloric food in response to intranasal insulin in men, whereas women showed an insulin-induced increase. Moreover, the wanting ratings for high-caloric food cues correlated with the percentage of body fat. Participants with the highest percentage of body fat also displayed the highest wanting ratings for high-caloric food cues, in response to insulin compared to placebo spray. The wanting ratings for high-caloric food cues further correlated with cognitive restraint, which was assessed by the German Three Factor Eating Questionnaire (TFEQ).

In contrast, the wanting ratings for low-caloric food were significantly higher following intranasal insulin compared to placebo over all participants. No differences could be observed with respect to BMI or sex, and no correlations with physiological or behavioural measures were found.

Effect of central insulin on neural BOLD activity in response to high- vs. low-caloric food cues

The analysis of the FCR task was first performed according to the caloric content of the food cues (high-caloric vs. low-caloric food cues) and afterwards according to the individual participant’s wanting ratings for the 60 food cues.

Over all participants, higher BOLD-activity was found in the amygdala after insulin compared to placebo administration, specifically in response to high-caloric food cues, compared to low-caloric food (*Supplementary Figure 3* in the Appendix [104]). In the precuneus and insula (*Figure 3* in the Appendix [104]) men with normal weight and women with overweight displayed higher BOLD activity following intranasal insulin, compared to placebo in response to specifically high-caloric food, than women with normal weight and men with overweight. A comparable pattern was found in the cerebellum/lingual gyrus.

The differential (insulin minus placebo) BOLD response in the insula correlated positively with peripheral insulin sensitivity (*Supplementary Figure 4* in the Appendix [104]). Hence, with higher peripheral insulin sensitivity, participants showed increased BOLD activity in the insula following intranasal insulin administration. Furthermore, the insula

response was positively associated with the wanting ratings for high-caloric food cues and cognitive restraint (TFEQ).

On top, our mediation models showed a link between peripheral insulin sensitivity, cognitive restraint and central insulin-induced neural BOLD response in the insular cortex: cognitive restraint serves as a mediator between peripheral insulin sensitivity and central insulin action (*Figure 4* in the Appendix [104]). This indicates that cognitive restraint promotes the relationship between periphery and central insulin action in response to high-caloric food cues.

Effect of central insulin on neural food cue reactivity according to the individual wanting ratings

In addition to the analysis of the neural FCR in response to the calorie content of the food cues, we modelled brain activity according to the participants' individual wanting ratings for the different food cues (*Supplementary Figure 1* in the Appendix [104]). A significant interaction between condition and sex of the participants appeared in the dorsolateral prefrontal cortex. Women showed higher insulin-induced dorsolateral prefrontal cortex activity with increasing wanting ratings, while men showed lower activity with increased wanting ratings in response to intranasal insulin (*Figure 5* in the Appendix [104]).
Moreover, the differential dorsolateral prefrontal cortex response (insulin-placebo) correlated positively with percent body fat and cognitive restraint (TFEQ) over all participants.

Influence of age on central insulin action on reward and appetite regulation

Besides the results described above and published in [104], we analysed whether age influences central insulin action on appetite and reward regulation. In our study, participants between 20 and 70 years were recruited, ideally having led to a homogenous age distribution in our participant sample. Unfortunately, we had difficulties to recruit participants in the working-age group between 35 and 50 years. We think that the recruitment difficulties were due to the time load of our study including three visits in the morning from ~7-11 a.m. and in contrast to different medication studies, participants had no direct benefit from the study participation.

After all, to guarantee sufficient statistical power, we decided to use a median split, leading to two equi-sized age groups: below and over 40 years. The mean age of the younger group (15 women) was 29 ± 4.8 (SD) years and of the elderly group (15 women) 57 ± 7.8 (SD) years.

We did not observe significant main age group effects (or interaction effects with BMI or sex) with spray (insulin or placebo) on perceived hunger, wanting for high- or low-caloric food or on neural FCR (calorie-content or wanting-modulation analysis) ($p > 0.05$). In other words, no difference could be observed between the younger and elderly group after insulin compared to placebo spray on FCR or behavioural assessments.

Summary and Discussion

In study 1, we investigated the effect of central insulin action on perceived hunger, appetite and reward regulation in combination with an fMRI-FCR task in women and men of different weight status, specifically with normal weight or overweight and obesity. Over all participants, central insulin mediated an increase in BOLD activity in the amygdala in response to specifically high-caloric food cues. In other food-cue-responsive areas [106], like the insula, the central-insulin-mediated BOLD response differed between women and men, depending on the weight status. In addition, sex influenced the insulin-induced neural response to highly palatable food. Women showed an increase in insulin-induced BOLD activity in the dorsolateral prefrontal cortex with increasing palatability, whereas men showed a decrease. On the behavioural level, central insulin reduced perceived hunger. The desire to eat low-caloric food was increased in all participants, whereas the desire to eat high-caloric food was increased in women and decreased in men, in response to intranasal insulin. No effect of age was found on insulin-mediated neural food cue reactivity nor on behavioural level.

In previous animal and human studies, including different fMRI studies, the amygdala has been reported to be insulin responsive [107-109]. In line with the previously described results, Kullmann and colleagues [109] showed an activity increase in the amygdala following intranasal insulin compared to placebo administration in resting-state fMRI. The amygdala is implicated in neural food reward pathways [110, 111], depending on the nutritional status (hungry vs. satiated) [106, 112, 113], and including cognitive processes (memory/recognition of food cues) [114]. Several fMRI studies have shown

increased activity in the amygdala specifically in response to high-caloric food cues [113, 115, 116]. Likewise, in study 1, we observed greater BOLD activity in the amygdala following intranasal insulin administration in response to high- compared to low-caloric food cues. Our results further confirm that the amygdala responds to rewarding signals, including visual signals from the environment as well as internal hormonal signals.

Apart from the amygdala, several other previously reported brain regions known to be responsive to food cues [106, 117-119] displayed insulin-mediated activity, but depending on the sex and weight status of the participants. BOLD activity in the insula was increased in women with overweight and men with normal weight in response to intranasal insulin. The insula plays an important role in sensory and homeostatic processes and current evidence suggests that the insula is an important convergence region in the gut-brain axis, where internal states or signals (e.g., hunger, nutrient sensing pathways) are integrated with conscious sensory input or subliminal reward signals to influence food choice and intake [96, 120]. To our knowledge, the effects of BMI and sex on central insulin-mediated FCR have not been investigated so far. However, previous studies have reported that weight status and/or sex influence central insulin action in the insular cortex. Central insulin action led to an increase in regional cerebral blood flow (CBF) in the insula in young men with normal weight [89], whereas in men with overweight intranasal insulin led to a CBF decrease [121]. Moreover, central insulin action led to an increase in BOLD activity in the insula during a FCR task both in women with normal- and overweight [86]. Independent of central insulin action, several studies have investigated the effects of BMI and sex on neural responsiveness to food cues [113, 115, 122-124]. In one study [122], including men and women, participants with normal weight displayed greater activity in the insula in response to food cues than participants with overweight. In a second study [115] women with overweight revealed higher insula activity than women with normal weight. These results coincide with our results, where central insulin induced the highest activation in the insula in women with overweight and men with normal weight in response to high-caloric food cues.

Previous studies have indicated a partial, regional association between peripheral and brain insulin sensitivity [38, 39, 41, 42]. In study 1, we observed an association between peripheral insulin sensitivity and insulin-mediated FCR in the insula. Participants with high peripheral insulin sensitivity also showed the highest insulin-induced response in the

insula, and this relationship was fully mediated by cognitive restraint. Cognitive restraint is a measure of cognitive self-control over food intake and in general a good predictor for body weight maintenance or weight loss success [125]. Our results suggest that cognitive restraint may strengthen the association between peripheral insulin sensitivity and insulin-mediated FCR in the insula. Central insulin has previously been shown to act on the mesocorticolimbic dopamine system with a direct influence on dopamine signalling [4] leading to a reduction of the rewarding aspect of food [88, 90, 91]. Cognitive restraint may further influence the subjective value and hedonic aspects of food.

Based on these findings, we assume that in men with normal weight, the insulin-mediated BOLD response in the insular cortex is mainly driven by physiological signals (central insulin signalling/satiety signal). In women with overweight, we postulate that the insulin-mediated response in the insula was further influenced by environmental cues and cognitive processes. We will return to this assumption in the discussion below.

Notably, women with normal weight showed no response or if at all, a slight decrease in insulin-mediated BOLD response in the insula to high-caloric food cues. Central insulin effects in women with normal weight on appetite ratings and insulin-mediated FCR in the insula were previously described in the postprandial state [85, 86]. Thus, the nutritional state could further impact the insulin-mediated neural response to food cues in a sex- or weight-dependent manner [106, 113, 124, 126]. Moreover, in women, hormonal fluctuations associated with the menstrual cycle or menopause have been shown to affect peripheral insulin sensitivity as well as central insulin action [127, 128]. Further studies, including studies in suitable animal models, are needed to disentangle the complex interplay between nutritional status, sex hormones, cognitive and physiological cues on insulin-mediated neural activity and to evaluate the underlying molecular mechanisms.

On a behavioural level, intranasal insulin led to the most pronounced decrease in perceived hunger in men with normal weight and women with overweight. This is in line with previous studies, mainly conducted in men with normal weight, showing a central insulin-induced reduction in appetite or hunger ratings as well as reduced food intake [42, 67]. Moreover, Schneider and colleagues [86] showed a more pronounced reduction in appetite in women with obesity compared to lean women. Concerning the desire to eat high-caloric food, men displayed an insulin-induced reduction whereas women displayed an insulin-induced increase. Intranasal insulin has previously been shown to reduce

wanting for high-caloric food [42, 90, 129], and our results further demonstrate a sex-specific modulation.

Moreover, the insulin-mediated DLPFC activity was modulated by individual wanting ratings for the food cues and differed between women and men. With increasing desire to eat the different food items, women showed increased insulin-mediated DLPFC activity, whereas men showed decreased DLPFC activity. The prefrontal cortex is essential for decision-making and cognitive control of eating behaviour, such as food intake [130, 131], and is highly responsive to different hormones, including insulin [42, 132]. The sex differences in prefrontal cortex activity further support our findings discussed above, that insulin signalling differs between women and men, especially when cognitive processes related to eating behaviour are involved [5, 67]. In men, central insulin led to a reduction in prefrontal cortex activity for highly desired food cues and a decrease in perceived hunger, which could then lead to the previously described reduction in food intake in men [103] and male rats [80, 133]. In women, central insulin action seems to be strongly influenced by cognitive processes, linked to food choice. This hypothesis is further supported by the positive correlation between DLPFC activity and cognitive restraint. We postulate that in men physiological signals, e.g., central insulin, are the main regulators of homeostasis and appetite, whereas in women physiological and cognitive signals may be either dissociated or interrelated.

Limitations

It is known that peripheral insulin sensitivity [127], as well as eating behaviour and food preferences [100] vary throughout the female menstrual cycle. Due to the limited sample size, we could not consider the influence of menstrual cycle, contraceptive medication or menopause. Even though Krug and colleagues [68] did not find differences in food intake after insulin administration between postmenopausal and young women, the impact of sex hormones on central insulin-mediated FCR or eating behaviour should be addressed in further studies.

Conclusion

Overall, sex and obesity, but not age have a major influence on central insulin-mediated FCR. While previous studies have demonstrated an effect of obesity on central insulin

signalling, our results underline a complex interplay between obesity and sex on neural FCR. In addition, prefrontal cortex activity, modulated by the desire to eat specific food, displayed sex-dependent differences. Overall, this suggests that central insulin signalling differs between men and women, particularly in the regulation of cognitive and reward-related processes.

Study 2

– Brain insulin responsiveness is linked to age and peripheral insulin sensitivity

In the second study, we analysed whether central insulin action associates with peripheral insulin sensitivity or age. 110 participants including those of study 1; 54 women, BMI 18-49 kg/m^2, age 21-74 years) were measured with intranasal insulin and placebo spray in a pseudo-randomised, cross-over design (*Figure 2*). In contrast to study 1, the difference in cerebral blood flow (CBF) measurements after, compared to before nasal spray application, was used as a proxy for central insulin action. Peripheral insulin sensitivity was assessed by an oral glucose tolerance test, as in study 1, and the Matsuda peripheral insulin sensitivity ($ISI_{Matsuda}$) index was used as a measure of peripheral insulin sensitivity. Several regions of interest (ROI) known to be insulin sensitive and related to metabolism, eating behaviour or cognition were analysed: bilateral amygdala, hypothalamus, hippocampus, insula, dorsal and ventral striatum. (table with descriptive and metabolic data of the participants in *Table 1* in the Appendix [105]).

Results

Association of central insulin action and age

Age was negatively associated with insulin action in the hippocampus and caudate nucleus. With increasing age, the CBF response to central insulin decreased in these regions (*Figure 2* in the Appendix [105]). Moreover, the negative association of hippocampal insulin action and age was stronger in women. In men only, there were further associations between insulin action and age in the amygdala and insula. There were no associations between insulin action in the hypothalamus, putamen or ventral striatum and age.

Association of central insulin action and peripheral insulin sensitivity

Peripheral insulin sensitivity was negatively associated with insulin action in the amygdala (*Figure 3* in the Appendix [105]). Participants with low peripheral insulin sensitivity showed no response or a slight increase in response to intranasal insulin, whereas those with high peripheral insulin sensitivity showed the strongest decrease in the CBF response to

intranasal insulin. The negative association between peripheral insulin sensitivity and insulin action in the amygdala was more pronounced in men.

Additionally, we found an interaction between peripheral insulin sensitivity and age with insulin action in the insular cortex (*Figure 4* in the Appendix [105]). In younger participants (between 20 and 40 years), those with the highest peripheral insulin sensitivity showed the highest insulin action in the insula. In elderly participants, those with the lowest peripheral insulin sensitivity showed the highest response to intranasal insulin. A similar pattern was observed as a trend in the ventral striatum overall participants and this association was significant in men.

No association was found between peripheral insulin sensitivity and central insulin action in the hippocampus, hypothalamus, or dorsal striatum.

Summary and Discussion

In study 2, we revealed region-specific associations of central insulin responsiveness with age and peripheral insulin sensitivity. Insulin receptors are widely spread across different brain areas and brain insulin action has been shown to modulate metabolism and behaviour. Disturbances in central insulin responsivity affect different cell populations (including neurons and glia cells [9]) as well as different brain circuitries. This further induces changes in dopamine signalling, BBB function, synaptic plasticity in the hippocampus and amyloid β and microtubule-associated tau protein expression (reviewed in: [5]). Brain insulin resistance might be a joint pathological trait of psychiatric, neurodegenerative and metabolic diseases. In study 2, we showed that central insulin action is associated with age and peripheral insulin sensitivity in a region-specific way and dependent on sex.

Insulin action in different limbic brain regions was negatively associated with age. In the hippocampus and caudate nucleus, younger participants displayed the strongest CBF increase in response to intranasal insulin and this response declined with increasing age. Rebelos and colleagues [44] investigated insulin-stimulated brain metabolism using [^{18}F]-FDG-PET scans in a large study cohort. In line with our results, they could show that the insulin stimulated brain metabolism decreased with increasing age, especially in the limbic lobe. Nonetheless, further studies are needed to clarify the underlying mechanisms.

The hippocampus, also part of the limbic system, is known for its essential role in learning behaviour and memory consolidation and is very prone to age-related decline [134]. In rodents, intra-hippocampal administration of insulin led to increased performances in learning and memory tasks [135, 136]. In different previous human studies, intranasal insulin was shown to have a positive impact on memory performances in healthy young participants [67, 73, 137] as well as in patients with T2D or early AD (a recent review can be found here: [66]). Interestingly, in study 2 this age-related decrease in hippocampal insulin action seemed independent of peripheral insulin sensitivity. Such an independence between hippocampal and peripheral insulin sensitivity was already shown in rats and human post-mortem analyses [64, 138]. In rats with specifically downregulated insulin receptor expression in the hippocampus (i.e., "hippocampal insulin resistance") neural plasticity and spatial learning were impaired, while they had normal peripheral insulin sensitivity as well as normal body weight [138]. Furthermore, in human post-mortem studies, elderly patients with AD had decreases in insulin-induced signaling cascades in hippocampal tissue correlating with cognitive impairment scores, whereas they had no history of diabetes [64]. Even so, it needs to be considered that the prevalence of prediabetes (with reduced peripheral insulin sensitivity) rises with age and data of peripheral insulin sensitivity was not available in these post-mortem datasets [64]. Of interest, in study 2, especially women showed a reduced insulin response in the hippocampus with increasing age. Coincidentally, women have a higher prevalence of age-related AD [139] and first evidence points towards a relation between a reduction of brain insulin sensitivity and AD [140].

Moreover, insulin responsiveness was also reduced in the caudate nucleus, part of the striatum, with increasing age. Previous studies in healthy participants have shown that intranasal insulin increases striatal CBF, reduces striatal synaptic dopamine levels [88] and modulates mesostriatal connectivity [88, 90, 91, 99]. The striatum is not only involved in reward processing, but is also essential for motor functions. Besides a decline in cognitive functions, ageing is accompanied by a gradual decrease of motor performances [141]. To study and quantify motor functions, the gait, by means of walking speed, is often analysed and measured in studies. Gait is a complex task involving voluntary and automatic processes and a good predictor of disabilities and mortality [142, 143]. Moreover, Dumurgier and colleagues [144] demonstrated a direct linear relationship between

caudate nucleus volume and walking speed in elderly participants. First evidence suggests that gait functions can be improved using intranasal administered insulin [35, 49]. In elderly, healthy participants as well as in participants with T2D, gait speed was increased by a 24-week intranasal insulin intervention [49]. Furthermore, we recently showed that insulin responsiveness in the striatum can be improved by an 8-week exercise intervention and that this insulin-induced striatal activity positively correlates with improvements in metabolism and cognition [145]. Hence, enhancing central insulin sensitivity could constitute a potential new treatment option in ageing-associated or neurodegenerative disorders.

Besides the associations with age, we discovered a link between insulin action in the insular cortex and the amygdala, and peripheral insulin sensitivity. The amygdala and the insula are part of the mesolimbic reward system, and are involved in the regulation of non-homeostatic eating behaviour, emotion processing and the regulation of physical homeostasis [146, 147]. Following intranasal insulin administration, participants with the highest peripheral insulin sensitivity showed the strongest CBF decrease in the amygdala. Similarly, Wingrove and colleagues [148] described a decrease in the amygdala CBF in response to intranasal insulin in healthy young men. Hence, high insulin sensitivity might translate into an insulin-induced inhibition of blood flow in the amygdala, comparable to previous reports on insulin-induced inhibition of hypothalamic blood flow in insulin-sensitive individuals [42, 149]. Furthermore, a deletion of the insulin receptors in the amygdala led to an altered glucose metabolism in rodents [108] and the direct injection of insulin into the amygdala led to a reduction in food intake [150]. Consequently, amygdalar insulin signalling may be involved in the regulation of glucose metabolism and eating behaviour; and a resistance may lead to the development of T2D or mood disorders [108, 151]. Amygdalar insulin sensitivity may constitute a joint feature between whole-body metabolism and emotional behaviour in humans, although this needs to be further investigated.

Insulin action in the insula was related to peripheral insulin sensitivity and age. Younger participants with high peripheral insulin sensitivity showed the most pronounced insulin-induced CBF increase in the insula, whereas in elderly participants those with the lowest peripheral insulin sensitivity showed the highest insulin-induced CBF increase. Interestingly, such an association between insulin-induced insular activity and peripheral

insulin sensitivity was already found in study 1 in response to food cues [104]. Similar to the results in study 2, Schilling and colleagues [89] reported a CBF increase in the insula in response to intranasal insulin in healthy young men with normal weight, whereas Wingrove and colleagues [121] showed an insulin-induced decrease in men with overweight. In study 2, the relationship between insulin-induced insular activity and peripheral insulin sensitivity changed with increasing age, such that in young participants, those with the highest peripheral insulin sensitivity showed the greatest CBF increase, whereas in elderly participants, those with the lowest peripheral insulin sensitivity showed the highest CBF increase in response to intranasal insulin. This might constitute a compensatory effect linked to ageing. Lower CSF insulin concentrations have previously been reported in elderly participants with peripheral insulin resistance, caused by a reduction in insulin transport across the BBB [38, 39]. This reduction in insulin transport across the BBB could eventually lead to an insulin-deficiency in the brain. Through the intranasal administration of insulin, the BBB is bypassed and the pronounced insulin-induced response in elderly participants with low peripheral insulin sensitivity could represent a compensatory hyper-responsiveness in a relatively insulin deficient brain. In line with this hypothesis, early brain hypermetabolism has been proposed as a transient compensatory reaction in initial cognitive and neurodegenerative decline. This overstimulation and ultimately exhaustion of brain tissues and networks may then accelerate degenerative processes, ultimately and progressively leading to brain hypometabolism (reviewed in: [152]). This hypothesis and the underlying molecular mechanisms need to be confirmed and investigated in further studies (probably in suitable animal models).

Of note, several associations between brain insulin responsiveness, peripheral insulin sensitivity and age were more pronounced in either women or men. In rodents, oestrogen has been reported to modify brain insulin responsiveness [153, 154]. The steroid hormone is best known for its primary role in reproduction, but oestrogen also influences cognitive health, eating behaviour, energy expenditure and body weight regulation [155, 156]. In women, oestrogen levels drop dramatically during and post-menopause, which has been identified as an additional risk factor for neurodegenerative alterations [156]. In contrast to the cited animal studies, an influence of oestrogen on brain insulin responsiveness has not yet been confirmed in humans [68, 157]. Nonetheless, several previous studies (including study 1) have suggested sex-specific effects of brain insulin

action on eating behaviour, food intake, and cognitive functions, including memory, mood and olfaction [6]. Together with the results of study 2, this highlights the importance of studying central insulin action in both women and in men, in order to disentangle its role in metabolic, neurodegenerative and psychiatric disorders.

Limitations

Two different 3 Tesla fMRI scanners were used for data acquisition between 2013 and 2019. We minimised the variability by applying a global CBF correction and with our within-subject design, using the baseline-corrected difference between insulin and placebo CBF response.

We could not analyse the impact of sex hormones, menstrual cycle or menopause on central insulin responsiveness, as no exploitable data were available. Moreover, no standardised cognitive assessments were performed, hence we could not investigate the impact of hippocampal insulin responsiveness on memory or in general cognitive performance. Finally, we could not include prefrontal or parietal ROIs in our analysis due to the restricted field of view during the MRI acquisition.

Conclusion

The results of study 2 provide further evidence that cerebral functions, metabolism, age and sex are closely interconnected. It is essential to study and understand the underlying mechanisms to unravel the influence of the brain in healthy and unhealthy ageing including its consequences on neurocognitive functions. The findings of study 2 demonstrate that brain insulin signalling is region-specific, both in its responsiveness to insulin and in its associations with age or peripheral insulin sensitivity. Different interventions like exercise [145], weight loss [158] or pharmacological interventions [159] have been shown to restore brain insulin sensitivity. Whether these interventions may be tailored to counteract age-dependant alterations or improve peripheral metabolism needs to be investigated in further studies.

General discussion

Summary

In both previously discussed studies, we investigated central insulin action in participants of different weight status, age and biological sex. In study 1, a special focus was set on the central insulin-mediated influence of BMI, age and sex on perceived hunger, appetite and food-related reward regulation during a FCR task. Study 2 focused mainly on the influence of peripheral insulin sensitivity and age on central insulin action in the absence of a specific task.

We were able to show that central insulin signalling is involved in several neural and behavioural tasks and is mediated by sex, age, weight status and peripheral insulin sensitivity. On a behavioural level, central insulin action reduced perceived hunger and mediated wanting for high- and low-caloric food. On a neural level, central insulin influenced BOLD activity in the insula, amygdala, cerebellum/lingual gyrus, precuneus and dorsolateral prefrontal cortex in response to visual food cues, depending on sex and weight status. Insulin action during resting-state (measured by changes in CBF) in the hippocampus, caudate nucleus, ventral striatum as well as in the insula and amygdala was associated with age and/or peripheral insulin sensitivity and depending on sex.

Despite the methodological differences in the study designs and measurement techniques (resting-state CBF measurements vs. (FCR) task-related BOLD measurements), the insula and the amygdala, both part of the mesolimbic reward circuitry and involved in emotion processing and hedonic eating behaviour [146, 147], revealed central insulin specific activity in both studies. Central insulin signalling in the amygdala was increased in response to high-caloric food cues (study 1), whereas the insulin-induced CBF response in the amygdala, during resting-state, was associated with peripheral insulin sensitivity and age depending on sex (study 2).

Insulin-mediated activity in the insula was, in both studies (i.e., in response to food cues and during resting-state), associated with peripheral insulin sensitivity. In study 2, this association was influenced by age. Moreover, in response to food cues, obesity and sex influenced central insulin signalling in the insula (study 1). Hence, insulin signalling in the

insula and amygdala appears to be closely linked to peripheral insulin sensitivity and related to homeostatic/physiological functions (i.e., during resting state/in absence of a specific task) and involved in the processing of visual food cues (including reward processing). Besides the activity in the insula and the amygdala, obesity/peripheral insulin sensitivity, age and sex have a strong impact on central insulin signalling.

Influence of obesity on central insulin sensitivity

Overweight and obesity are defined by an abnormal or excessive accumulation of body fat presenting a potential risk for health [25]. The most commonly used screening and classification tool for overweight and obesity is the BMI, a ratio between a person's weight and height [22]. By its nature, the BMI does not take into account body composition (i.e., muscle or fat mass), nor metabolic parameters or anatomical/physiological sex differences. Nevertheless, the BMI is a non-invasive, inexpensive and universal index of overweight and obesity and is widely used in studies. Thus, the BMI was used in study 1, to separate our participants into groups in order to study differences between people with normal weight and people with overweight and obesity. In study 2, we used the Matsuda peripheral insulin sensitivity index, a whole-body insulin sensitivity index (composite estimate of hepatic and muscle insulin sensitivity) and estimated from a 5-point oral glucose tolerance test, as this is a stronger measure of metabolic health than BMI [160]. As peripheral insulin resistance is a hallmark of obesity, BMI and $ISI_{Matsuda}$ are in general highly correlated. Such a correlation can also be observed in our participants (study 2: $r= -0.268$, $p= 0.005$). The focus of study 2 was to investigate the link between peripheral insulin sensitivity and brain insulin action, as little is currently known about this relationship. It should be noted, that despite their correlation, BMI and $ISI_{Matsuda}$ were not interchangeable in study 2, and adjusting our statistical models for BMI had little or no effect on the results.

As mentioned above, the BMI is calculated based on a person's weight and height and does not take into account metabolic parameters. However, genetic factors (e.g., [161]), diet composition [162-164] and physical activity [165] have an impact on peripheral insulin sensitivity without necessarily influencing the BMI. Of note, up to 30% (depending on the classification criteria) of people with obesity (BMI > 30) are metabolically healthy with a higher prevalence in women [166]. These "metabolically healthy obese" were shown to have normal peripheral insulin sensitivity, greater cardiorespiratory fitness and a

characteristic body fat distribution, with a low amount of visceral and liver fat [167]. Consistently, recent findings showed that elevated visceral adipose tissue, rather than BMI is associated with changes in brain insulin responsiveness [149]. However, measures of visceral adipose tissue were not available in study 1 and 2. Still, this entails that a person with overweight (defined by BMI) adhering to an otherwise healthy lifestyle, including physical activity, may be metabolically healthy and even healthier than a sedentary person of normal weight. Depending on the study objectives, the criteria to measure and classify overweight and obesity should carefully be chosen and further parameters including metabolic measures should be considered in the analysis.

Brain insulin signalling is thought to act as a physiological signal to regulate homeostasis as well as a rewarding signal [168]. In study 1, our visual food cues also address homeostatic vs. hedonic aspects of food and eating. We therefore evaluated not only physiological, homeostatic functions of brain insulin signalling in response to food cues, but also psychological, hedonic aspects related to food and eating behaviour. In general, people with overweight and obesity demonstrate stronger responses to food cues [54] and less food-related inhibitory control [169]. In study 1, we could establish an association between cognitive restraint linked to eating behaviour (highest in women with overweight in study 1) with insulin-induced brain signalling in response to high-caloric and palatable food cues. Food cues can elicit strong conditioned responses (e.g., craving), but these responses can be suppressed by control regions, including the prefrontal cortex (DLPFC) [170]. In study 1, the insulin-mediated DLPFC activity in response to highly palatable food cues correlated positively with cognitive restraint. People with the highest cognitive restraint (i.e., women with overweight) displayed the strongest DLPFC activity in response to intranasal insulin. Whether this insulin-induced DLPFC activity would translate into changes in eating behaviour needs to be investigated into further studies.

Moreover, social stigmatisation of people with a high BMI, especially women with overweight and obesity might influence their eating behaviour, or the hedonic and psychological valuation of food cues. However, this needs to be evaluated in further studies. Nonetheless, our results confirm the role of central insulin signalling in the interaction between metabolic, reward and cognitive processes in appetite control [97, 170].

Influence of biological sex on central insulin sensitivity

Previous studies with intranasal insulin have yielded indicators of sex-specific differences [6]. These sex-specific effects of brain insulin action were found particularly in behaviour related measures, including food intake and appetite regulation, but also on olfaction, memory, mood and sleep [6]. First evidence suggests an influence of the nutritional state (fasted vs. satiated) on brain insulin signalling in men and women: reduced food intake was observed in fasted young men after intranasal insulin administration, whereas intranasal insulin reduced the postprandial consumption of cookies in women [67, 85]. To our knowledge, no neuroimaging studies have yielded indicators of brain insulin-induced sex-specific differences so far. However, most studies have been and are being conducted in either women or men, and/or with relatively small sample sizes.

In study 1 and 2, we revealed sex-specific differences in brain insulin signalling. These differences were mostly found in brain areas associated with reward or cognitive valuation of food. It could be speculated that differences in insulin signalling between women and men become apparent when cognitive valuation is involved or the rewarding aspects of food are addressed [100]. Moreover, most of the sex-specific effects were interrelated to obesity or peripheral insulin sensitivity and ageing. It remains to be investigated, whether there are sex-specific differences in healthy physiological brain insulin signalling, in the absence of a specific task, or whether such differences emerge when the optimal physiological state is disrupted, as in obesity or with increasing age (including menopausal changes in women). However, these speculations, need to be confirmed in further studies and the underlying mechanisms need to be elucidated.

Nevertheless, it is known that body composition, peripheral glucose homeostasis and metabolic functions differ between women and men, and that women generally appear to have better peripheral insulin sensitivity [171]. Consistently, epidemiological evidence shows a higher prevalence of T2D in men than in women [171]. Moreover, hormonal fluctuations during the menstrual cycle in women have an impact on both peripheral and also central insulin sensitivity [127, 128].

Previous studies [6] and study 1 and 2 clearly support a sex dependency in brain insulin signalling. Further systematic investigations in larger study populations are required to identify relevant sex differences in brain insulin signalling regarding physiological

differences (including influences of the nutritional state or menstrual cycle) and task-specific differences in the normal, physiological and pathological states.

Influence of age on central insulin sensitivity

Peripheral insulin sensitivity slightly decreases with increasing age [31, 32]. In a longitudinal study, Thambisetty and colleagues [172] were able to show that an impaired peripheral glucose tolerance in midlife was associated with changes in brain CBF in later life. Not only the periphery, but also brain insulin signalling is influenced by ageing, as ageing was associated with a decrease in brain insulin concentration and a decrease in brain insulin receptor density and binding [173]. Moreover, ageing seems to impact the transport of insulin across the BBB, as CSF insulin concentrations were reduced in comparison to serum insulin levels in elderly participants [39]. Rebelos and colleagues [44] further showed that the insulin-stimulated brain metabolism, measured by [^{18}F]-FDG-PET scans during a hyperinsulinemic-euglycemic clamp decreased with advancing age, particularly in limbic areas. Taken together, there seems to be a causal interaction between ageing, peripheral and central insulin sensitivity and signalling [31, 174-176]. Moreover, brain insulin resistance might constitute a pathological trait in cognitive impairments described in relation to obesity, T2D and AD [5, 36, 63, 64].

In a FCR task with visual food cues, Cheah and colleagues [102] demonstrated that ageing was associated with decreased brain activity, especially in the DLPFC and precuneus, and increased activation in the ventrolateral prefrontal cortex (VLPFC) and temporal lobe in the fasted state. In study 1, we did not find an influence of age on brain insulin action on perceived hunger or in response to visual food cues. Either ageing does not have a strong impact on insulin action in brain areas related to visual food cue processing. Or, our "elderly" group (57 ± 7.8 (SD) years) was too young or "too healthy" to see insulin-mediated age-specific effects.

In study 2, which included more participants, brain insulin action was associated with age, especially in several limbic brain regions including the hippocampus and the striatum. Increasing age was associated with a decrease in insulin-mediated CBF in these regions. Besides the direct associations with age, brain insulin action in the insula and the ventral striatum (in men) showed an association with age depending on peripheral insulin sensitivity. In young participants, those with the highest peripheral insulin sensitivity

showed the most pronounced insulin-mediated response whereas this relationship was reversed with increasing age. The results of study 2 suggest that age has a region-specific impact on brain insulin sensitivity. It also confirms an interaction between age, peripheral and central insulin sensitivity, as previously described in the literature.

Conclusion

Insulin acts in the CNS to modulate eating behaviour and energy homeostasis as well as cognition and memory functions. Study 1 and 2 highlight the broad influence and importance of brain insulin signalling in appetite and reward regulation. Moreover, our results corroborate that brain insulin acts as a physiological satiety signal and as a reward signal. The results of both studies further indicate that brain insulin action is task-specific and region-specific and that several factors such as body weight, peripheral insulin sensitivity and age significantly influence brain insulin signalling. Furthermore, our results add to the existing literature on sex-specificity in brain insulin signalling. In study 1 and 2, most of the sex-specific differences were observed in interaction with either BMI, peripheral insulin sensitivity or age. Depending on the investigated brain region, the health status or sex, insulin may have different effects on the brain [35]. Whether these effects can be explained by different insulin-sensitivity levels or insulin receptor densities in the concerned brain regions needs to be investigated and clarified in further studies.

Brain insulin resistance was proposed as a joint pathological characteristic of neurodegenerative and metabolic disorders. Further research with large participant samples and taking into account different participants' characteristics (e.g., weight status, age and sex) is needed to better understand the interactions and consequences of brain insulin signalling and resistance. As previously shown, brain insulin sensitivity can potentially be restored though exercise [145], weight loss [158] or pharmacological [159] interventions. Whether these interventions show BMI or sex-specific differences in their effectiveness needs to be further investigated. Overall, these interventions seem to improve age-dependent alterations or peripheral metabolism, potentially by targeting region-specific brain insulin action.

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Appendix- Study 1 (Wagner et al. 2022)

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ARTICLE OPEN

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Behavior, Psychology and Sociology

Sex differences in central insulin action: Effect of intranasal insulin on neural food cue reactivity in adults with normal weight and overweight

Lore Wagner[1,2✉], Ralf Veit[1,2], Louise Fritsche[1,2], Hans-Ulrich Häring[1,2,3], Andreas Fritsche[1,2,3,4], Andreas L. Birkenfeld[1,2,3], Martin Heni[1,2,3,5,6], Hubert Preissl[1,2,3,7] and Stephanie Kullmann[1,2,3]

BACKGROUND/OBJECTIVES: Central insulin action influences cognitive processes, peripheral metabolism, and eating behavior. However, the contribution of obesity and sex on central insulin-mediated neural food cue processing still remains unclear.
SUBJECTS/METHODS: In a randomized within-participant design, including two visits, 60 participants (30 women, BMI 18–32 kg/m^2, age 21–69 years) underwent a functional MRI task measuring blood oxygen level-dependent (BOLD) signal in response to visual food cues after intranasal insulin or placebo spray administration. Central insulin action was defined as the neural BOLD response to food cues after insulin compared to placebo administration. Afterwards, participants were asked to rate the food cues for desire to eat (i.e., wanting rating). For statistical analyses, participants were grouped according to BMI and sex.
RESULTS: Food cue reactivity in the amygdala showed higher BOLD activation in response to central insulin compared to placebo. Furthermore, women with overweight and obesity and men of normal weight showed higher BOLD neural food cue responsivity to central insulin compared to placebo. Higher central insulin action in the insular cortex was associated with better peripheral insulin sensitivity and higher cognitive control. Moreover, central insulin action in the dorsolateral prefrontal cortex (DLPFC) revealed significant sex differences. In response to central insulin compared to placebo, men showed lower DLPFC BOLD activity, whereas women showed higher DLPFC activity in response to highly desired food cues. On behavioral level, central insulin action significantly reduced hunger, whereas the desire to eat, especially for low caloric food cues was significantly higher with central insulin than with placebo.
CONCLUSIONS: Obesity and sex influenced the central insulin-mediated neural BOLD activity to visual food cues in brain regions implicated in reward and cognitive control. These findings show that central insulin action regulates food response differentially in men and women, which may have consequences for metabolism and eating behavior.

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INTRODUCTION

After the discovery and isolation of the hormone insulin, 100 years ago, the seminal role of insulin in the periphery was quickly recognized. Scientific interests only later turned to the role of the brain in insulin signaling [1–3]. Since then, evidence is rapidly accumulating that central insulin action plays a vital role in metabolic and cognitive health, including memory, mood and olfaction, eating behavior, and also peripheral metabolism (for review see: [4–6]).

Central insulin action can be assessed by intranasal application, -the delivery of the hormone with a spray through the nose to the brain. Combined with imaging techniques like functional magnetic resonance imaging (fMRI), this allows to study insulin action in the brain non-invasively in humans [6]. Several studies, using intranasal insulin, demonstrated changes in regional resting-state activity and functional connectivity in the hypothalamus, striatum, hippocampus, amygdala, insula, and parts of the prefrontal cortex [7–13]. These are all regions part of an interconnected network regulating eating behavior, which are responsive to a meal, postprandial hormones, and to the taste and sight of food [14]. People with obesity show higher food cue reactivity (FCR), particularly in regions important for emotion and reward regulation, including the insula, amygdala, and orbitofrontal cortex [15] and FCR is even predictive for the outcome of weight-loss interventions (e.g., [16]). In response to central insulin, persons with obesity showed altered activity in reward-related

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Table 1. Participants' characteristics.

	Normal weight (NW)		Overweight/obesity (OW)		*p*-value
	women	men	women	men	
N	20	17	10	13	–
Age [years]	42.55 (3.41)	40.29 (3.99)	42.9 (5.35)	47.08 (3.9)	0.514
BMI [kg/m^2]	22.9 (0.25)	22.66 (0.49)	27.5 (0.7)	27.01 (0.5)	<0.001
Fasting glucose [mmol/l]	4.96 (0.09)	5.12 (0.11)	4.95 (0.18)	5.09 (0.09)	0.415
Fasting insulin [pmol/l]	62.9 (4.66)	50.65 (5.71)	95.3 (37.26)	70 (8.9)	0.151
Body fat [%]	30.9 (0.96)	16.4 (0.93)	38.23 (1.09)	18.82 (1.04)	<0.001
Insulin sensitivity, OGTT-derived [AU] ($ISI_{Matsuda}$)	16.41 (1.6)	18.9 5 (3.13)	18.84 (4.78)	14.78 (2.37)	0.715
HbA1c [mmol/mol]	35.25 (0.62)	34.24 (0.69)	36.2 (0.83)	36.92 (1.14)	0.175
HbA1c [%]	5.39 (0.06)	5.29 (0.06)	5.48 (0.07)	5.52 (0.1)	0.162

Values in the table given as mean (SEM).
p-values: non-parametrical Kruskal-Wallis-H-Test between the 4 groups.
BMI Body mass index, $ISI_{Matsuda}$ Matsuda peripheral insulin sensitivity index.

brain regions with subsequent effects on eating behavior-related measures (e.g., failure to reduce food craving and hunger) [8, 11, 12]. A possible explanation could be the role of central insulin action on dopamine signaling. Recent findings demonstrated that intranasal insulin administration directly modulated striatal dopamine levels and functional connectivity of reward pathways in healthy humans [9, 11, 12] and directly modulates dopamine function in the midbrain and nucleus accumbens in animal models [17]. This leads to the assumption, that central insulin action is not only implicated in the regulation of energy homeostasis, but also in reward processing.

Sex also plays a prominent role in appetite regulation and FCR. Women, compared to men, displayed higher activity in frontal (PFC) and reward areas, including striatum and insula in response to high-caloric cues [18] and higher FCR in reward areas was a predictor for BMI in women [19]. Following intranasal insulin administration, a reduction of food intake [20, 21] and food craving [8] as well as slight reductions in body weight and adipose mass [22] were observed in men. In women with normal weight [23] and obesity [24], central insulin action decreased palatable food intake (i.e., cookies) in the postprandial state. Hence, first evidence points to sex-specific effects of central insulin action on eating behavior and appetite regulation.

However, no study thus far has evaluated whether obesity and sex determine central insulin effects on neural FCR. Therefore, our primary aim was to elucidate the effect of central insulin action on appetite and reward regulation by using an FCR task during fMRI in healthy volunteers. Central insulin action was probed by nasal insulin application compared to placebo. We hypothesized a stronger insulin effect on FCR in participants with normal weight compared to participants with overweight and obesity in brain regions involved in eating behavior. Furthermore, we expected sex-dependent effects on FCR in response to central insulin with increased activity in reward-related areas in women with overweight and obesity. On a behavioral level, we hypothesized that central insulin action results in a reduction in perceived hunger and wanting for high-caloric food, particularly in men with normal weight. Furthermore, we intended to explore whether central insulin response on FCR is associated with behavioral and peripheral measures.

METHODS

Subjects

Seventy participants were recruited for the study. Five participants had to be excluded from the analysis due to incomplete fMRI measurements (technical issues), three based on insufficient data quality (e.g., excessive movement (>2 mm or 2°) or participant fell asleep during the fMRI measurement), one participant was not in a fasted state and one participant had major anatomical abnormalities of the brain.

Datasets of sixty participants (30 women) were used for the final analysis: 37 participants with normal weight (NW group, 20 women, body mass index (BMI) range 18–25 kg/m^2, and age range 21–69 years), 23 participants with overweight and obesity (OW group, 10 women, BMI range 25–32 kg/m^2, and age range 24–65 years) (Table 1: shows complete descriptive and metabolic data).

Participants signed a written informed consent before participation and the study was approved by the local ethics committee of the medical faculty of the University of Tübingen. The study was registered as a clinical trial (NCT04372849).

Power calculation

In order to evaluate the effect of intranasal insulin versus placebo on neural FCR, we used medium effect size to calculate a total sample size of $n = 60$ using ANOVA repeated measures including within and between interactions (G*Power 3.1.9, $\alpha = 0.05$, power = 0.95). In previous studies between group differences based on BMI showed large effect sizes (Eta-squared of greater 0.2) for differences in insulin action in the prefrontal cortex [8, 25].

Experimental design and procedure

Prior to the experiment, all participants underwent a medical examination to assure that they did not suffer from psychiatric, neurological nor metabolic diseases or taking any kind of medication other than oral contraceptives. Insulin sensitivity was estimated from measurements during a 75-gram oral glucose tolerance test (oGTT) according to Matsuda and DeFronzo (ISIMats) [26]. This index mainly captures insulin effects in the liver and other peripheral organs (including skeletal muscle) [27]. We therefore used this index to capture peripheral insulin sensitivity (in contrast to brain/central insulin sensitivity). Body fat percentage was measured by Bioelectrical Impedance Analysis (BIA, single-frequency BIA device (50 kHz), manufacturer's protocol: BIA 101 BIVA, Akern, Germany).

After the screening and oGTT measurement day, all subjects participated in two fMRI visits (Fig. 1) with a time-lag of 3–28 days. After an overnight fast of at least 10 h, visits were scheduled between 7 a.m. and 11 a.m. with intranasal insulin or intranasal placebo in a pseudo-randomized order. Insulin or placebo nasal spray application will be referred to as condition. After blood sampling, fMRI measurements were recorded under baseline (pre) and 30 min after nasal spray application (post).

A questionnaire addressing subjective feeling of hunger was assessed before and approximately 75 min after spray application using a visual analogue scale from 0 to 10 (0 = not hungry at all; 10 = very hungry). For the analysis, hunger ratings were baseline corrected, meaning the rating of the pre measurement was subtracted from the post measurement (Fig. 1).

At the end of each fMRI visit, participants rated the food cues, seen before in the scanner, in a wanting and a recognition task (described below).

To address trait eating behavior characteristics, the German Three Factor Eating Questionnaire (TFEQ), with the three subscales 'Restraint eating/

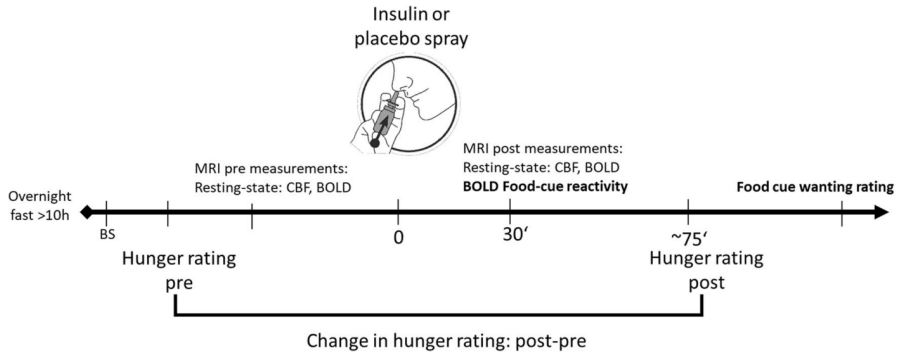


Fig. 1 Scheme of test procedure. Cross-over design with intranasal insulin or placebo in a counter-balanced order. Hunger ratings were assessed at arrival and approximately 75 min after nasal spray application. The food cue viewing task during fMRI was followed by a task on a laptop to rate the food cues seen in the scanner based on wanting (i.e., desire to eat) and recognizability. Resting-state functional data sets were recorded at each visit before and 30 min after nasal spray application (results not reported here). BS blood sample, CBF cerebral blood flow, BOLD blood oxygenation-level dependent.

cognitive restraint of eating', 'disinhibition' and 'hunger' [28], the eating disorder examination (EDE) [29] and the trait version of the Food Craving Questionnaire [30] was used (Supplementary Table 1).

Application intranasal insulin/ placebo. Participants received in total 160U of insulin (Insulin Actrapid; Novo Nordisk, Bagsvaerd, Denmark) or vehicle as placebo in a randomized fashion. The spray was administered over four minutes with two puffs per nostril every minute. Participants were blinded to the order of the conditions.

Imaging procedures. Scanning was conducted at a 3T whole-body Siemens scanner (Magnetom Prisma; Erlangen, Germany) with a 20-channel head coil. Neural food-cue reactivity using blood oxygen level dependent (BOLD)-fMRI was obtained after nasal spray application by using multi-band accelerated echo-planar imaging sequences, developed at the Center for Magnetic Resonance Research (CMRR) Minnesota, USA. The FCR consisted of two sessions, each lasting 5:30 min. Pictures were presented on a screen behind the scanner and were projected with a tilted mirror mounted on the head coil in the participant's field of view. For fMRI measurements the following sequence parameters were used: TR = 1.5 s, TE = 34 ms, FOV = 192 mm^2, matrix 96 × 96, partial Fourier = 6/8, bandwidth = 2264 Hz/pixel, echo spacing = 0.55 ms, flip angle 70°, voxel size 2 × 2 × 2 mm^3, slice thickness 2 mm, images were acquired in interleaved order with a multiband acceleration factor of 3. Each brain volume comprised 72 axial slices and each functional run contained 220 image volumes.

Food-cue task. An event-related design was used with high and low-caloric food cue pictures, presented in a pseudo-randomized order (software Presentation® (Version 10.2, www.neurobs.com)). Every picture was presented for 2 s with an interstimulus interval of 6–10 s. The pictures were separated by a grey screen with a black fixation circle or (every 6–7 pictures) a black fixation cross, in the middle of the screen. Participants were instructed to look at the pictures and immediately press a button when a cross appeared in between the pictures, to ensure attention and focus of the participants.

Stimulus material. A stimulus set of 60 food cues, 15 sweet high-caloric (e.g., donuts and cakes), 15 savory high-caloric (e.g., burger and pizza), 15 low-caloric sweet (e.g., fruit), and 15 savory low-caloric (e.g., vegetables and salads), was selected out of the freely available and standardized food cue database *food-pics* [31, 32] (*Supplementary Text and supplementary excel document*).

Recognition and wanting task. Approximately 15 min after the last fMRI measurement, participants performed outside of the scanner a computerized recognition (see *Supplementary Text*) and wanting task of the food cues seen during the fMRI measurement. The recognition task was used to control for attentiveness. For the wanting task (i.e., desire to eat), participants had to rate the food pictures by answering the question 'how much they want to eat the food at that moment', on a 5 point Likert scale going from '1-not at all' to '5-very much'. Wanting ratings were calculated as sum of the wanting ratings (scale 1–5) for the 30 pictures per category (high and low-caloric). Wanting rating values are reported in Supplementary Table 2 and Supplementary Fig. 2a.

Image Processing of food-cue reactivity task. Pre-processing and statistical analysis of the fMRI data were performed using SPM12 (Wellcome Trust Centre for Neuroimaging, London, UK). Standard pre-processing including slice-timing, realignment, coregistration to the anatomical T1 weighted image, normalization into MNI space, and Gaussian spatial smoothing (FWHM: 6 mm) was done. A threshold of 2 mm maximum head motion displacement or 2° of any angular motion was applied. Finally, fMRI data were highpass (cut-off period 128 s) filtered and global AR(1) auto correlation correction was performed.

The FCR task was first analyzed according to the caloric content (event-related) of the food cues and secondly according to the individual participants' wanting ratings.

Event-related analysis. For the calorie content, a design matrix was created individually for each participant for placebo and insulin day separately. For each condition, a separate regressor for low-caloric sweet, low-caloric savory, high-caloric sweet, and high-caloric savory was added in the model and convolved with a canonical hemodynamic response function and its time derivative. The movement regressors, separately for each session, were included as covariates in the model to account for possible movement-induced variance.

Individual contrast images were computed to estimate the activity changes for high-caloric pictures (sweet and savory together, 30 pictures in total) vs. low-caloric food cues (sweet and savory together, 30 pictures in total) (difference: high minus low-caloric food cue activity) on placebo and on insulin day. The individual contrasts for high minus low-caloric pictures were entered into a full factorial design for second level analysis.

Parametric modulation of the wanting ratings on food cue processing. For the analysis of the parametric modulation of the wanting ratings, a design matrix was created for each participant for placebo and insulin day separately. We used the individual wanting ratings for each food picture, independent of calorie content for parametric modulation of brain activity. Individual contrast images were computed according to the wanting ratings of the individual food cues. These contrast images were then entered into the full factorial models. We used the positive contrast, showing brain areas where the activity increased with increasing wanting ratings. (Three-dimensional representation in Supplementary Fig. 1).

Statistical analyses

Food-cue task. We used two separate full factorial design models to investigate the effect of central insulin action on neural BOLD food-cue

reactivity. The first model was based on the high minus low-caloric food contrasts; the second full factorial model was based on the wanting modulation contrasts. Both models included condition (insulin vs. placebo nasal spray) as a within-subject factor, BMI group (NW vs. OW) and sex group (female vs. male) as an in-between-subject factor, and age as a covariate.

A primary statistical threshold of $p < 0.001$ uncorrected and a $p < 0.05$ family wise error (FWE, based on Random Field Theory) corrected for multiple comparisons at a cluster level was applied. Additionally, small volume correction (SVC) was performed for regions recently identified as insulin sensitive [6], specifically the bilateral hypothalamus, the striatum, amygdala, hippocampus, insula and dorsolateral PFC. The masks were based on the *wfu* pick atlas (https://www.nitrc.org/projects/wfu_pickatlas/). For regions with SVC, reported *p*-values were adjusted by Bonferroni-correction for multiple comparisons (for the number of ROI's).

For post hoc analyses and correlation analyses, differential responses were calculated by subtracting the individual regional brain activity of the placebo day from the insulin measurement. A $p < 0.05$ was considered significant after Bonferroni-Holm correction (Holm) for multiple testing.

Behavioral data. Data are given as mean ± SEM. For the analyses of the behavioral data and questionnaires, SPSS (IBM SPSS Statistics Version 26.0. Armonk, NY: IBM Corp) and R (Version 4.1.1R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria; URL https://www.R-project.org/.) were used.

Linear mixed-effect models including sex, BMI group, and condition as well as age (covariate), with subject as random intercept, were used to analyze hunger and wanting ratings. *F* and *p*-values were obtained by the lmerTest package [33] (Satterthwaite approximation for degrees of freedom), pairwise comparisons were performed by the emmeans package [34] with Bonferroni-Holm correction (Holm) for multiple testing. For post hoc analyses of group differences, differential (Insulin-Placebo) wanting and hunger ratings were used and *p*-values adjusted by Bonferroni–Holm correction.

If data were normally distributed, paired and two-sided *t*-tests were used. Otherwise, we used non-parametrical Kruskal Wallis H-Tests and Mann–Whitney U-Tests.

Furthermore, Spearman and Pearson correlations (depending if data were normally distributed or not) were performed to identify associations between central insulin action and peripheral insulin sensitivity and eating behavior characteristics adjusted for sex, BMI, and age (referred to as r_{adj} and p_{adj}).

Mediation analysis of the relationship between peripheral insulin sensitivity, TFEQ-cognitive restraint and insular cortex activity was performed using PROCESS version 3.5 procedure in SPSS (www.afhayes.com). The significance of the mediation analysis (i.e., indirect effect *ab*) was estimated based on a bias-corrected bootstrap confidence interval (CI 95%, 5000 bootstrap samples).

For all analyses a $p < 0.05$ was considered significant.

RESULTS

Central insulin effects on subjective feeling of hunger (VAS)

We observed a significant main effect of condition ($F(1,56) = 10.712$, $p = 0.002$) as well as a significant interaction between condition x sex x BMI group for the hunger ratings ($F(1,56) = 11.494$, $p = 0.001$). Post hoc paired *t*-tests (Supplementary Table 3a*)* revealed a significant reduction of the hunger ratings by intranasal insulin compared to placebo over all participants ($T(56) = -3.273$, $p = 0.002$; Fig. 2A), specifically in NW men ($T(56) = -3.678$, $p = 0.002$) and OW women ($T(56) = -2.811$, $p = 0.020$). Between group post hoc analyses showed that NW women and NW men differed significantly ($T(55) = 3.034$, $p = 0.022$; Fig. 2B, Supplementary Table 3b).

Central insulin effects on wanting for high and low-caloric food cues

No main effect of condition was observed for high-caloric food wanting ratings (Supplementary Fig. 2b). However we observed a significant interaction between condition x sex ($F(1,56) = 7.148$, $p = 0.01$) and condition x sex x BMI ($F(1,56) = 7.639$, $p = 0.008$). Post hoc analyses showed that men displayed lower ratings than women ($T(55) = -2.643$, $p = 0.01$) and OW men lower wanting ratings for high-caloric food than OW women ($T(55) = 3.357$, $p = 0.009$; Supplementary Fig. 2c).

For low-caloric food, we observed higher wanting ratings in response to intranasal insulin compared to placebo (i.e., significant main effect of condition $F(1,56) = 8.025$, $p = 0.006$). No significant interaction effects were observed with condition (Supplementary Fig. 2d, e).

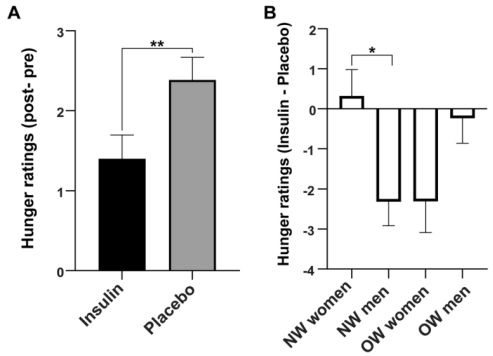


Fig. 2 Significant reduction of hunger in response to intranasal insulin compared to placebo. A Bar plot shows change in hunger rating from before to after insulin or placebo spray application (post minus pre nasal spray) based on a visual analogue scale (in cm). **B** Bar plot shows change in hunger rating for insulin compared to placebo spray application (insulin day $_{\text{post minus pre}}$ minus placebo day $_{\text{post minus pre}}$). When comparing the four groups, NW men and OW women respond differently compared to NW women and OW men, even though, only the difference between NW women and NW men remained significant after correction for multiple comparisons. NW with normal weight, OW, with overweight and obesity; $^{*}p < 0.05$ (Holm), $^{**}p < 0.01$.

Correlations with central insulin induced effects on wanting ratings

The differential (Insulin minus Placebo) wanting for high-caloric cues positively correlated with the percentage of body fat ($r = 0.278$, $p = 0.033$, $r_{adj.} = 0.295$, $p_{adj.} = 0.027$) and with TFEQ-cognitive restraint ($r = 0.344$, $p = 0.008$, $r_{adj.} = 0.283$, $p_{adj.} = 0.036$).

There were no significant correlations between wanting for low-caloric cues and percentage of body fat and cognitive restraint ($p > 0.05$).

Central insulin action on neural BOLD activity based on high minus low-caloric food

There was a significant main effect of condition in the left amygdala (peak-voxel (MNI) x: −24, y: −8, z: −14); $T(111) = 4.39$, $p_{FWE\text{-}corr.} < 0.05$, Supplementary Table 4 and Supplementary Fig. 3), with significantly higher BOLD activity after intranasal insulin compared to placebo.

Significant interactions between BMI, sex and condition were found in the cerebellum/lingual gyrus (peak-voxel (MNI) [x: −14, y: −60, z: −12]; *T*-value = 4.66, $p_{FWE\text{-}corr.} < 0.05$), precuneus (peak-voxel (MNI) [x: 8, y: −60, z: 60]; *T*-value = 4.51, $p_{FWE\text{-}corr.} < 0.05$) and the insula (peak-voxel (MNI) [x: 50, y: 6, z: −10]; *T*-value = 4.71, $p_{FWE\text{-}corr.} < 0.05$) (Supplementary Tables 4 & 5). Between group post hoc analyses showed that NW men and OW women displayed higher BOLD activations with intranasal insulin compared to placebo, whereas NW women and OW men showed lower activity in the insula and the precuneus ($p_{FWE\text{-}corr.} < 0.05$; Supplementary Table 5b*)*. Food-cue reactivity in the insula is displayed in Fig. 3.

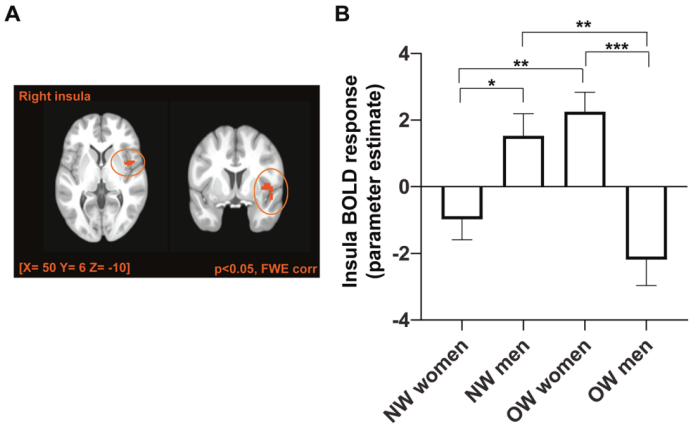


Fig. 3 Central insulin action on BOLD response in the insular cortex. A Overlay shows a significant 3-way interaction between BMI x sex x condition (insulin versus placebo) in the right insular cortex in response to high minus low-caloric food cues (pFWE-corr. < 0.05). **B** Bar plot shows insular cortex BOLD activity (insulin minus placebo) for NW women, NW men, OW women and OW men separately. NW, with normal weight; OW, with overweight and obesity; *$p < 0.5$; **$p < 0.01$; ***$p < 0.001$ (Holm).

Central insulin response in the insula correlates with behavioral and metabolic measures

The insula BOLD response correlated with peripheral insulin sensitivity ($r = 0.293$, $p = 0.024$ $r_{adj.} = 0.300$, $p_{adj.} = 0.030$) (Supplementary Fig. 4A). Hence, participants with higher peripheral insulin sensitivity showed an increased food-cue activation in the insular cortex in response to intranasal insulin.

Moreover, the insula response correlated positively with the TFEQ-cognitive restraint ($r = 0.419$, $p = 0.001$; $r_{adj.} = 0.466$, $p_{adj.} < 0.001$) (Supplementary Figs. 4B & 5) and the wanting ratings for the high-caloric cues ($r = 0.257$, $p = 0.048$; $r_{adj.} = 0.254$, $p_{adj.} = 0.068$). The correlation with the wanting ratings for high-caloric cues was driven by participants with overweight and obesity (NW: $r = -0.025$, $p = 0.884$, OW: $r = 0.551$, $p = 0.006$). No correlations were observed between behavioral measures and the central insulin response in the amygdala, cerebellum/lingual gyrus or precuneus.

Based on the correlations between the central insulin-induced BOLD response in the insula with peripheral insulin sensitivity and cognitive restraint, we tested, by mediation analyses, the process that underlies the observed relationships. We found a significant positive indirect effect (completely standardized indirect effect $ab = 0.11$, 95% CI [0.02 0.23]) of the TFEQ-cognitive restraint as a mediator between peripheral insulin sensitivity and differential insula BOLD activity. This indicates that cognitive restraint promotes the relationship between peripheral insulin sensitivity and central insulin action in the insular cortex (Fig. 4). Mediation models using the BOLD response in the insula or peripheral insulin sensitivity as a mediator did not indicate significant indirect effects. No direct effects were observed between peripheral insulin sensitivity and central insulin-induced BOLD response (see Fig. 4).

Central insulin action on neural BOLD food-cue reactivity based on parametric modelling by individual wanting ratings

No significant main effect of condition or interactions with BMI group were observed ($p > 0.05$) when we modelled brain responses according to the individual wanting ratings. We found a significant interaction between sex and condition in the dorsolateral frontal cortex (DLPFC) (right middle frontal gyrus, peak-voxel (MNI) [$x = 38$; $y = 24$; $z = 44$], T-value (peak) = 4.24, $p_{FWE\text{-}corr.} = 0.012$, Fig. 5).

Within group comparisons revealed significant insulin vs. placebo effects in the DLPFC, for both women ($T(29) = 2.542$, $p = 0.017$) and men ($T(29) = -3.968$, $p < 0.001$). Between group post hoc comparisons revealed significant differences between women and men in the DLPFC response ($T(58) = 4.634$, $p < 0.001$). Men showed lower DLPFC activity in response to central insulin with increasing wanting ratings, while women showed higher DLPFC activity with increased wanting ratings.

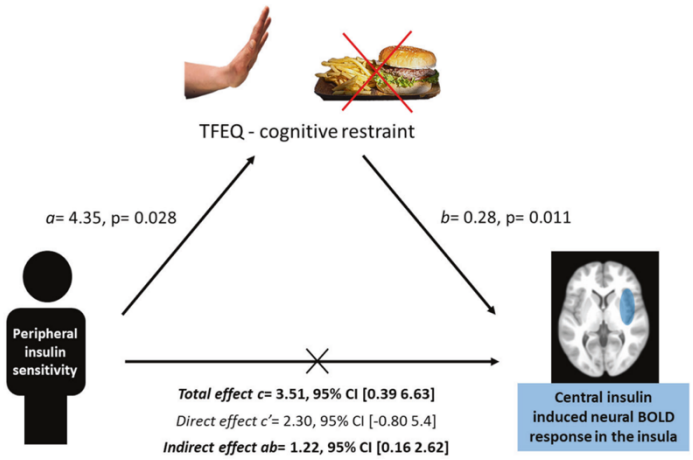
The DLPFC response positively correlated with percent body fat ($r = 0.444$, $p < 0.001$; $r_{adj.} = -0.103$, $p_{adj.} = 0.451$) and with TFEQ-cognitive restraint ($r = 0.373$, $p = 0.004$; $r_{adj.} = 0.220$, $p_{adj.} = 0.106$).

DISCUSSION

In this study, we investigated the effect of central insulin action on neural BOLD food-cue reactivity in men and women with normal weight, overweight and obesity. Overall, central insulin action increased the BOLD response in the amygdala, while several other food-cue responsive regions [35], as the insular cortex, showed interactions between sex and obesity on how insulin affected FCR. The response in the DLPFC was modulated by individual wanting ratings of food cues. Here women showed higher BOLD activity than men in response to central insulin. On the behavioral level, we found that central insulin decreased the feeling of hunger and increased the desire to eat low-caloric food.

The amygdala has been reported as insulin-responsive in previous studies [36, 37] and we recently reported an increase in the amygdala in response to intranasal insulin in resting-state fMRI data [38]. Furthermore, the amygdala is implicated in taste and vision-related neural food reward pathways [39, 40], with higher activity in response to high-caloric food [41–43], particularly in the fasted state [35, 42]. Likewise, in the current study, central insulin led to a higher BOLD activity in the amygdala, in response to high versus low-caloric food cues. This implies that the amygdala responds to rewarding signals, which includes hormones as well as rewarding sensory signals from the environment.

Apart from the central insulin action in the amygdala, we found an interaction between sex and BMI on central insulin BOLD responsivity in several previous reported food-cue responsive cortical regions [35, 44–46]. Specifically, men with normal weight and women with overweight showed an increase in central



Mediation model adjusted for BMI, sex and age

Fig. 4 Cognitive restraint as mediator between peripheral and central insulin action. Graphic illustrates mediation model adjusted for sex, BMI and age. Cognitive restraint (based on three factor eating questionnaire) positively mediated the relationship between peripheral insulin sensitivity and the insular cortex BOLD activity (insulin minus placebo) in response to high-caloric food cues. Path coefficients and corresponding *p*-values are shown next to the arrows; path a indicates the relationship between peripheral insulin sensitivity and cognitive restraint, path b indicates the relationship between the cognitive restraint and the insula BOLD activity in response to high-caloric food cues; path ab indicates the indirect effect (not standardized) of peripheral insulin sensitivity on the insular cortex activity via the cognitive restraint score; path c' indicate the direct effect of peripheral insulin sensitivity on the insular cortex activity.

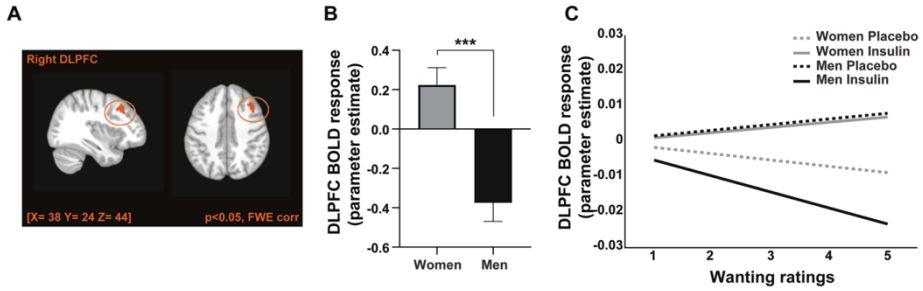


Fig. 5 Insulin action on BOLD response in the dorsolateral prefrontal cortex (DLPFC). BOLD activity in the DLPFC is modulated by the individual wanting ratings for food cues. **A** Overlay shows significant 2-way interaction between sex and condition in the DLPFC with increasing wanting ratings for food cues ($p_{FWE\text{-}corr.} < 0.05$). **B** Bar plot shows DLPFC BOLD response (insulin minus placebo) with increasing wanting ratings for women and men. Women showed significantly higher DLPFC BOLD activity than men. **C** Plot shows DLPFC BOLD activity for women and men with both insulin and placebo condition separately with increasing wanting ratings (1: low wanting; 5: high wanting, for visualization purposes only). ***$p < 0.001$.

insulin-induced BOLD response in the insula. Signals from the periphery and the environment converge in the insula to influence food intake [47]. Of note, several previous studies have identified either BMI or sex effects of central insulin action in the insular cortex. In young men with normal weight, central insulin induced an increase in regional blood flow [10], while men with overweight responded with a decrease [48]. Furthermore, in women with normal weight and obesity central insulin led to an increase in FCR in the insula [24]. Other studies, investigating food-cue reactivity, independent of insulin action, showed either BMI- or sex-related differences [41, 42, 49–51]. Two studies identified a similar food-cue response pattern in the insular cortex, with greater activation in the insula in participants with normal weight than in participants with overweight [49] and higher activity in the insula in women with overweight compared to normal weight [41]. This coincides with our study showing that women with overweight exemplify the greatest BOLD activity in the insular cortex in response to high-caloric cues, particularly in a fasted state.

Participants with high peripheral insulin sensitivity showed the highest central insulin-induced insular food-cue reactivity, which was fully mediated by cognitive restraint—a measure for the cognitive control of food intake. High scores in cognitive restraint correlate with a good maintenance of body weight or success in weight loss and lower BMI scores in people with overweight and obesity [52, 53]. Cognitive restraint may enhance the relationship between peripheral insulin sensitivity and central insulin BOLD response to food cues. Central insulin was shown to influence

dopamine signaling and reduce hedonic aspects of food [9, 11, 12]. Thus, cognitive restraint could further affect the subjective value and rewarding effect of food.

Based on our findings, we postulate that the insula BOLD response in men with normal weight was primarily driven by physiological signals (i.e., central insulin), while the response of women with overweight was additionally driven by environmental cues and cognitive processes [54]. Noteworthy, women with normal weight did not show an increase in insula BOLD activity with intranasal insulin. This could be due to the fact that we performed our study in the fasted state. Studies in the postprandial state identified a central insulin induced reduction in appetite ratings [23, 24] and increased insula activity [24] in women of normal and overweight. Hence, the nutritional state could additionally modulate the brain's response to food cues [35, 42, 51, 55–57], though, the detailed underlying mechanisms remain unclear. Furthermore, hormonal fluctuations during the menstrual cycle have shown to influence peripheral and central insulin sensitivity [58, 59]. Hence, further studies are needed to evaluate the complex interplay of sex hormones and nutritional state on the brain response to physiological and environmental cues.

On a merely behavioral level, central insulin action led to the strongest decrease in the hunger ratings in men with normal weight and women with overweight. Accordingly, previous studies, mostly in men with normal weight, described decreased ratings for appetite or hunger and food intake following intranasal insulin [8, 20]. Surprisingly, in the current study, we identified a general increase in low-caloric wanting ratings with intranasal insulin. This expands previous findings, showing that central insulin not only decreases hunger [8, 20] and food wanting for high-caloric food [8, 11, 60] but can also enhance wanting for low-caloric food. This could further corroborate that central insulin is a rewarding signal [54].

Furthermore, central insulin action led to a significant sex-dependent DLPFC BOLD response with increasing desire (i.e., wanting) for food cues. Women showed an increase in activity with increasing wanting following intranasal insulin, while men showed a decrease. The prefrontal cortex plays a crucial role in decision-making and cognitive control of food intake [61, 62] and is highly responsive to hormonal signals like insulin [8, 25]. The sex differences of central insulin action in the DLPFC further support the notion that women and men differ in central insulin signaling when eating behavior-related cognitive processes are involved [6, 20]. Meaning that in men, central insulin action reduces prefrontal activity for high wanted food cues and decreases hunger. This could lead to a decrease of food intake, as described in men [21] and male rats [63, 64]. Whereas in women, central insulin action seems to be influenced by cognitive processes related food choice, which is further supported by the positive association between DLPFC BOLD activity and cognitive restraint. Hence, physiological signals as central insulin regulate homeostasis and appetite in men, while in women there might be a dissociation between physiological and cognitive signals.

CONCLUSION

Obesity and sex seem to play a major role in central insulin-mediated neural BOLD food-cue reactivity. Our study shows a complex interaction between sex and obesity during neural FCR, which is associated with peripheral insulin sensitivity and cognitive restraint, which indicates that further factors likely contribute. Furthermore, neural activity modulated by the desire for food cues revealed pronounced sex differences in prefrontal activity. This further supports the hypothesis that insulin signaling in the brain differs between women and men, especially in the regulation of cognitive and hedonic processes.

Limitations

In our current study, we could not analyze the impact of menstrual cycle or contraceptive medication as the sample size was not large enough for further stratified analyses. Nonetheless, it is known that peripheral insulin sensitivity changes throughout the cycle [58] and also eating behavior and preferences may change [18] and should therefore be addressed in further experiments. Furthermore, peripheral insulin sensitivity was not assessed through hyperinsulinemic-euglycemic clamp but estimated with the widely-used Matsuda index from repeated insulin and glucose measurements during an oGTT [65].

DATA AVAILABILITY

The data are not publicly available due to them containing information that could compromise research participant privacy/consent. The authors will share them by request from any qualified investigator after the completion of a data-sharing agreement.

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AUTHOR CONTRIBUTIONS

LW analyzed the data, interpreted the data, and wrote the manuscript. LF provided metabolic data, RV and SK provided scientific guidance and contributed to the discussion. SK and MH designed the study. HP, HUH, AF and AB provided scientific guidance on the experimental design and contributed to the discussion. All authors read and approved the final manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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Supplemental material

Supplements: Sex differences in central insulin action: Effect of intranasal insulin on neural food cue reactivity in adults with normal weight and overweight

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Supplements Methods

Supplementary Text

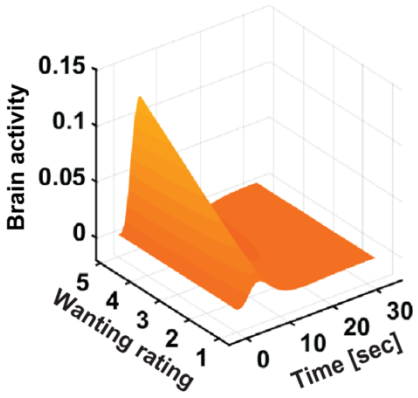
Stimulus Material

High and low-caloric pictures were matched for RGB-color distribution, intensity, contrast, complexity and object size. Concerning the nutritional values, the high-caloric cues had significantly higher amounts of protein, fat, carbs and kcal, as well per 100 g of the presented food as in total. The amount of presented food in the image (measured in grams/total) did not differ between high and low-caloric cues. (*please see excel sheet*)

Recognition task

Participants performed a recognition task of the food cues seen during the fMRI measurement on a laptop outside of the scanner. First, participants were shown 100 pictures (60 defined as old, as they were recently seen in scanner, and 40 new ones) for which they had to indicate on a 5 point Likert-scale ('1 – the picture is new for sure', '3- I do not know', '5- the picture is old for sure'), if they had seen them during the task in the scanner. The recognition task was used to control for the attentiveness of the participants and not further analyzed. The order of the pictures was randomized.

Parametric modulation of the wanting ratings on food cue processing



Supplementary Figure 1*: The individual wanting ratings for each food picture, independent of calorie content were used for parametric modulation of brain activity. We used the positive contrast, showing brain areas where the activity increased with increasing wanting ratings. Three-dimensional representation of parametric correlation of wanting (for the 60 food cues) with signal changes (example of one participant, for visualization purposes only) are shown in the figure.*

Supplements Results

Behavioral Results

Sex and BMI effects on Trait-Questionnaires of eating behavior characteristics

Supplementary Table 1: Trait- Questionnaires of eating behavior characteristics

	Normal weight (NW)		Overweight/obesity (OW)		
	women	men	women	men	p-value
Eating disorder examination (EDE) questionnaire					
- Restraint	3.6 *(0.73)*	2.63 *(1.12)*	9 *(2.36)*	2.54 *(1.16)*	**0.014**
- Eating concern	1.63 *(0.55)*	5.31 *(2.7)*	4.78 *(1.95)*	2.15 *(1.01)*	0.586
- Weight concern	1.6 *(0.55)*	0.25 *(0.11)*	5.9 *(2.17)*	1.38 *(0.87)*	**0.002**
- Shape concern	3.95 *(0.74)*	2.38 *(0.78)*	9.2 *(2.14)*	4.08 *(1.87)*	**0.028**
German three factor eating questionnaire (TFEQ)					
- Cognitive restraint	6.95 *(0.82)*	6.18 *(0.71)*	11.5 *(0.93)*	5.33 *(1.05)*	**0.002**
- Disinhibition	4.68 *(0.58)*	3.88 *(0.48)*	5.8 *(0.96)*	5.17 *(0.89)*	0.415
- Hunger	4.16 *(0.70)*	3.82 *(0.58)*	4.9 *(1.09)*	4.09 *(0.90)*	0.929
Food craving questionnaire trait (FCQ-T)					
-Total	80.89 *(5.30)*	76 *(4.22)*	85.1 *(10.14)*	64.92 *(10.31)*	0.132

Values in the Table given as mean (SEM).
p-values: non-parametrical Kruskal-Wallis-H-Test, uncorrected for multiple testing

For the EDE-weight concern, we found significant BMI x sex group differences ($\chi2(3)= 14.42$, $p= 0.002$). Post hoc tests showed that women with overweight and obesity had significantly higher scores than all three other groups ($p< 0.05$) and that women with normal weight had significantly higher scores than men with normal weight ($p= 0.031$). In the EDE, in the subscales 'Restraint' and 'Shape concern' as well as in the TFEQ- Cognitive restraint, women with overweight and obesity rated significantly higher than the three other groups (EDE-restraint: $\chi2(3)= 10.67$, $p= 0.014$, EDE-Shape concern: $\chi2(3)= 9.13$, $p= 0.028$, TFEQ-cognitive restraint: $\chi2(3)= 15.31$, $p= 0.002$). There was no significant difference between the three other groups. No significant differences were observed between groups in total food craving (FCQ-T).

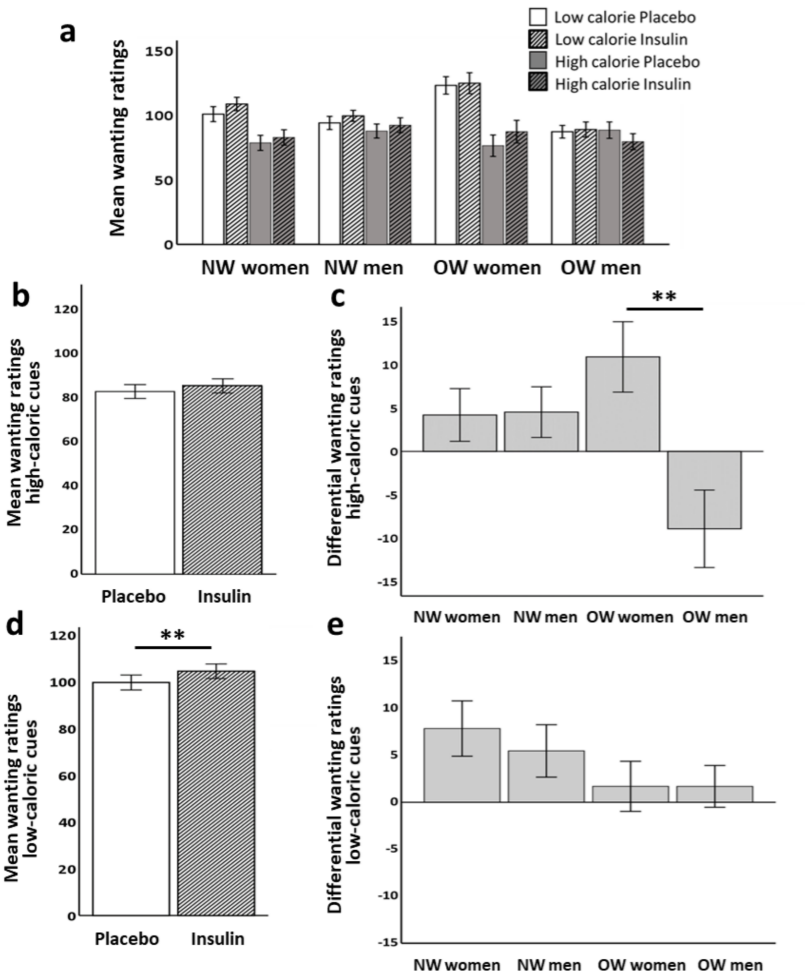
Central insulin effect on hunger ratings and wanting ratings of high and low-caloric food cues

Supplementary Table 2: Wanting and hunger ratings in the four BMI and sex groups (Mean (± SEM))

Note: For accuracy reasons, wanting ratings are shown as sum of the wanting ratings (scale 1-5) for the 30 pictures per category (high/low-caloric). If of interest, values can be divided by 30 (number of pictures per category) to obtain a mean wanting rating value.

			Normal weight (NW)		Overweight/obesity (OW)	
			women	men	women	men
Wanting ratings						
	Low-caloric	Placebo	100.85 *(5.82)*	94.06 *(5.13)*	123.1 *(6.76)*	87.23 *(4.91)*
		Insulin	108.7 *(5.19)*	99.53 *(4.3)*	124.8 *(8.12)*	88.92 *(5.86)*
		Delta	7.85 *(2.94)*	5.47 *(2.79)*	1.7 *(2.67)*	1.69 *(2.23)*
	High-caloric	Placebo	78.6 *(5.83)*	87.76 *(5.45)*	76.4 *(8.32)*	88.46 *(6.35)*
		Insulin	82.8 *(5.99)*	92.29 *(5.73)*	87.3 *(8.75)*	79.54 *(6.13)*
		Delta	4.2 *(3.04)*	4.53 *(2.92)*	10.9 *(4.06)*	-8.92 *(4.47)*
Hunger ratings [cm]	Placebo	Pre	2.67 *(0.54)*	2.9 *(0.6)*	3.07 *(0.72)*	3.66 *(0.72)*
		Post	4.83 *(0.58)*	5.41 *(0.65)*	6.62 *(0.87)*	5.32 *(0.73)*
		Change	2.17 *(0.53)*	2.51 *(0.37)*	3.55 *(0.62)*	1.65 *(0.73)*
	Insulin	Pre	3.36 *(0.52)*	4.66 *(0.67)*	4.08 *(0.88)*	2.94 *(0.58)*
		Post	5.85 *(0.53)*	4.86 *(0.66)*	5.32 *(0.9)*	4.35 *(0.71)*
		Change	2.49 *(0.6)*	0.19 *(0.45)*	1.24 *(0.49)*	1.42 *(0.58)*

Central insulin effects on wanting ratings of high and low-caloric food cues



***Supplementary Figure 2**: Wanting ratings for high and low-caloric food cues after intranasal insulin and placebo application. (a) For display only, bar plot shows mean sum of the wanting ratings (± SEM) in the four BMI and sex groups (b) Over all participants, bar plot shows mean wanting rating for high-caloric cues; no significant differences were observed after insulin compared to placebo application over all participants ($p>0.05$); however, significant interactions were observed between condition (insulin versus placebo), sex and BMI ($p<0.05$, see main document). For post hoc analyses, differential wanting ratings were used (insulin minus placebo). (c) Bar plot shows differential wanting ratings for high-caloric cues in the four*

*BMI and sex groups. Men with overweight and obesity revealed lower differential wanting ratings for high-caloric cues compared to all other groups, but after Holm-correction for multiple testing, only OW women and OW men differed significantly. (OW women > OW men: T(55)= 3.357, p= 0.009; NW men vs. OW men: T(55)= 2.482, p= 0.06; NW women vs. OW men: T(55)=2.573, p= 0.06, holm-correction for 6 tests). (d) Over all participants, bar plot displays mean wanting ratings for low-caloric food cues showing higher wanting ratings in response to intranasal insulin compared to placebo (T(56)= 2.833, p= 0.006). (e) Bar plot shows differential wanting ratings for low-caloric cues in the four BMI and sex groups. No significant interaction effects were found with condition. Abbreviations: NW, with normal weight; OW, with overweight and obesity. ** p < 0.01, error bars: ± SEM*

Central insulin effects on subjective feeling of hunger (VAS) based on significant 3-way interaction (BMI x sex x condition, adjusted for age)

Supplementary Table 3a: Within group post hoc comparisons: Main effect of condition (insulin versus placebo)

	NW women		NW men		OW women		OW men	
	T(56)	p-value	T(56)	p-value	T(56)	p-value	T(56)	p-value
Hunger ratings (VAS)	0.551	1	-3.678	**0.002**	-2.811	**0.020**	-0.331	1

Degrees-of-freedom method: Satterthwaite, p-value adjustment: Bonferroni-Holm method for 4 tests

Supplementary Table 3b: Between group post hoc comparisons of differential hunger ratings. i.e. insulin minus placebo (post-pre)

	NW women vs. NW men		NW women vs. OW women		NW women vs. OW men		NW men vs. OW women		NW men vs. OW men		OW women vs. OW men	
	T(55)	p-value	T(55)	p-value	T(55)	p-value	T(55)	p-value	T(55)	p-value	T(55)	p-value
Hunger ratings	3.034	**0.022**	2.592	0.06	0.616	1	-0.004	1	-2.096	0.163	-1.855	0.207

Degrees-of-freedom method: Satterthwaite, p-value adjustment: Boferroni-Holm method for 6 tests

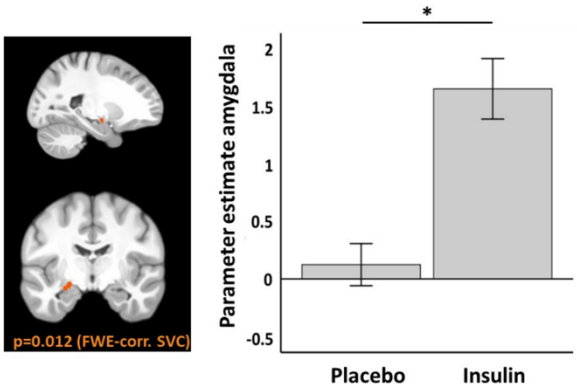
Imaging Results

Supplementary Table 4: Neural food cue BOLD reactivity in response to intranasal insulin compared to placebo for high- minus low-caloric food contrast

Regions	Hemisphere	T-value (peak)	P*	MNI-coordinates [mm] [a] x	y	z
Insulin > Placebo						
Amygdala	L	4.39	0.012 [b]	-24	-8	-14
Interaction BMI x sex x condition						
Insula	R	4.71	0.013	50	6	-10
Cerebellum/ Lingual	L	4.66	0.008	-14	-60	-12
Precuneus	R	4.51	<0.001	8	-60	60

** p < 0.05, FWE-corrected for multiple comparison (whole-brain); [a] Montreal Neurological Institute (peak-voxel); [b] Small volume corrected (mask) with Bonferroni correction for the number of ROI's: specifically the bilateral hypothalamus, the striatum, amygdala, hippocampus, insula and dorsolateral PFC. The masks were based on the wfu pick atlas (https://www.nitrc.org/projects/wfu_pickatlas/).*

Higher amygdalar food-cue reactivity in response to central insulin



Supplementary Figure 3: *Significantly higher BOLD activity in the amygdala in response to high versus low-caloric food cues after insulin compared to placebo application. * p<0.05, error bars: ± SEM*

Sex and BMI group effects on food cue-reactivity in response to central insulin (based on significant 3-way interaction: BMI x sex x condition, adj. for age)

Supplementary Table 5a: Within group post hoc comparisons: Main effect of condition (Insulin versus Placebo)

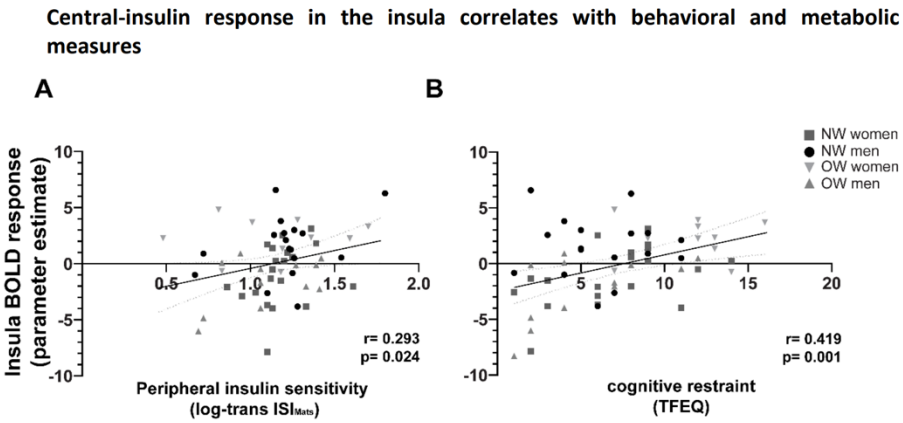
	NW women		NW men		OW women		OW men	
Brain region	**T(19)**	**p-value**	**T(16)**	**P-value**	**T(9)**	**p-value**	**T(12)**	**p-value**
Insula	-1.622	0.121	2.220	0.082	4.257	**0.008**	-2.560	0.075
Cerebellum/Lingual	-2.052	0.108	1.111	0.283	3.067	0.052	-2.848	0.052
Precuneus	-1.335	0.198	2.188	0.132	4.452	**0.008**	-1.956	0.148

p-value adjustment: Bonferroni-Holm method for 4 tests

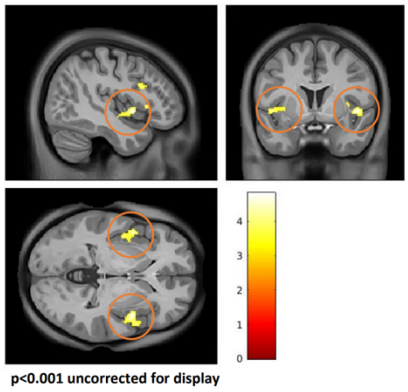
Supplementary Table 5b: Between group post hoc comparisons of differential brain response, i.e. insulin minus placebo

	NW women vs. NW men		NW women vs. OW women		NW women vs. OW men		NW men vs. OW women		NW men vs. OW men		OW women vs. OW men	
Brain region	**T(35)**	**p-value**	**T(28)**	**p-value**	**T(31)**	**p-value**	**T(25)**	**p-value**	**T(28)**	**p-value**	**T(21)**	**p-value**
Insula	-2.748	**0.027**	-3.554	**0.005**	1.108	0.554	-0.866	0.554	3.407	**0.008**	4.241	**< 0.001**
Cere-bellum/ Lingual	-2.234	0.1	-3.426	**0.01**	-0.208	0.836	-1.741	0.188	2.376	0.1	4.310	**< 0.001**
Precuneus	-2.315	**0.01**	-3.665	**0.005**	0.383	0.704	-2.272	0.081	2.934	**0.028**	4.507	**< 0.001**

p-value adjustment: Bonferroni-Holm method for 6 tests



Supplementary Figure 4: *(A) Central insulin action in the insular cortex (Insulin-Placebo) in response to high minus low-caloric food cues correlated positively with peripheral insulin sensitivity (B) and cognitive restraint over all participants. Hence, persons with higher peripheral insulin sensitivity and higher cognitive restraint show the highest central insulin action on BOLD response in the insular cortex. Abbreviations: ISIMats, Matsuda insulin sensitivity index; TFEQ, Three-factor eating questionnaire.*



Supplementary Figure 5: *Central insulin action (Insulin-Placebo) on BOLD response in the insular cortex in response to high minus low-caloric food cues shows a positive association with cognitive restraint (TFEQ) over all participants (right insula ~ cognitive restraint: [x:44, y:4, z:-2], T= 4.83, p_{FWE}=0.006, left insula ~ cognitive restraint: [x:-44, y:-4, z:0], T= 4.28, p_{FWE}=0.030, both small volume corrected). Here, a multiple regression model was performed with the difference of Insulin minus Placebo response for high minus low-caloric food pictures and cognitive restraint scores on a whole brain level (p<0.001 uncorrected for display). Abbreviations: TFEQ, Three-factor eating questionnaire.*

Study food cues (fMRI)

Food cue characteristics based on the freely available and standardized food cue database *food-pics* [1]

1: Blechert, J., et al., Food-pics: an image database for experimental research on eating and appetite. Front Psychol, 2014. 5: p. 617. & Blechert, J., et al., Food-Pics_Extended-An Image Database for Experimental Research on Eating and Appetite: Additional Images, Normative Ratings and an Updated Review. Front Psychol, 2019. 10: p. 307.

Picture number (fMRI)	Classification: low-caloric (savory:1, sweet: 2) high-caloric (savory:3, sweet:4)	Classification low-caloric:1 high-caloric:2	Image No	Item description english	Macronutrients									Image characteristics						
					protein per 100g	fat per 100g	carbs per 100g	kcal 100g per 100g	grams total	protein total	fat total	carbs total	kcal total	red	green	blue	object size	intensity (norm)	contrast (sd)	complexity (norm)
1	1	1	201	salad plate	2,6	0,3	3,9	31	50	1,3	0,15	1,95	15,5	0,393	0,393	0,214	0,546	114,62	67,91	0,404
2	1	1	212	veggie mix with dip	1,54	2,15	3,04	41,5	1000	15,4	21,5	30,4	415	0,447	0,348	0,205	0,373	104,87	61,42	0,450
3	1	1	229	salad plate	1,73	0,35	3,5	24,6	130	2,249	0,46	4,55	31,98	0,442	0,361	0,198	0,413	115,70	61,69	0,355
4	1	1	240	crisp bread with cottage cheese	8	1	25,5	150	30	2,4	0,3	7,65	45	0,412	0,367	0,220	0,271	99,70	54,28	0,485
5	1	1	242	veggie mix: cucumber, tomato, peppers...	0,57	0,2	3,02	17,8	670	3,819	1,34	20,23	119,26	0,450	0,377	0,173	0,333	112,57	60,36	0,453
6	1	1	275	tomatoes	0,9	0,2	2,47	16	455	4,095	0,91	11,24	72,8	0,543	0,264	0,193	0,381	105,15	50,69	0,320
7	1	1	332	mixed salad	1,16	0,14	1,74	13,74	230	2,668	0,32	4	31,60	0,460	0,360	0,180	0,478	95,99	60,44	0,387
8	1	1	361	carrots, cooked	1,3	0,2	5,2	26	150	1,95	0,3	7,8	39	0,580	0,307	0,113	0,417	116,77	33,44	0,266
9	1	1	411	mixed vegetables	3,53	0,38	5	39	380	13,414	1,44	19	148,2	0,459	0,408	0,133	0,419	128,58	57,17	0,389
10	1	1	447	mixed vegetables	3,3	0,42	9,28	56,62	130	4,29	0,55	12,06	73,61	0,403	0,444	0,153	0,474	102,34	56,03	0,350
11	1	1	482	mixed salad	1,02	0,18	2,78	17,52	270	2,754	0,49	7,51	47,30	0,439	0,349	0,212	0,436	85,03	60,58	0,399
12	1	1	502	mixed vegetables, cooked	7,3	0,2	8	44,3	35	2,555	0,07	2,8	15,5	0,429	0,372	0,198	0,453	85,06	53,14	0,240
13	1	1	526	plate of salad	1,58	2,29	4,93	47,3	230	3,634	5,27	11,34	108,79	0,418	0,381	0,200	0,485	103,64	65,06	0,417
14	1	1	558	asparagus with bechamel sauce	1,2	0,74	2	20,5	560	6,72	4,14	11,2	114,8	0,375	0,366	0,259	0,287	99,12	64,17	0,409
15	1	1	520	green chili	1	0,3	7,9	47	25	0,25	0,08	1,98	11,75	0,419	0,420	0,162	0,303	57,50	28,80	0,225
16	2	1	199	watermelon	0,26	0,09	3,64	17	300	0,78	0,27	10,92	51	0,572	0,279	0,149	0,216	115,28	50,39	0,309
17	2	1	214	wildberries mix	1	0,5	6	43	125	1,25	0,63	7,5	53,75	0,493	0,275	0,233	0,328	147,08	56,57	0,459
18	2	1	216	nectarines	0,83	0,09	11,41	52	300	2,49	0,27	34,23	156	0,525	0,305	0,171	0,399	115,20	55,32	0,356
19	2	1	217	fruit mix: kiwi, cherries, strawberries...	1	0,45	8,56	48	195	1,95	0,88	16,692	93,6	0,487	0,343	0,170	0,270	138,16	66,21	0,258
20	2	1	218	fruit mix: nectarines, raspberries, cherries ...	1	0,2	10,57	50	590	5,9	1,18	62,363	295	0,519	0,277	0,204	0,320	156,20	70,10	0,280
21	2	1	224	wildberries mix	1	0,5	6	43	100	1	0,5	6	43	0,496	0,263	0,241	0,388	162,82	67,38	0,353
22	2	1	234	strawberries	0,78	0,39	5,33	31	30	0,234	0,12	1,599	9,3	0,595	0,248	0,156	0,316	157,82	53,40	0,232
23	2	1	245	oranges	0,72	0,14	6,62	34	560	4,032	0,78	37,072	190,4	0,529	0,362	0,108	0,366	106,02	40,44	0,189
24	2	1	248	blueberries	0,6	0,6	7,3	41	60	0,36	0,36	4,38	24,6	0,309	0,319	0,372	0,232	130,49	75,46	0,387
25	2	1	280	cherries	1	0,3	13,3	63	60	0,6	0,18	7,98	37,8	0,559	0,220	0,221	0,219	169,11	65,83	0,378
26	2	1	284	grapes	1	0,3	15,6	71	30	0,3	0,09	4,68	21,3	0,521	0,266	0,213	0,278	174,46	46,81	0,254
27	2	1	325	fruit salad	0,6	0,3	11,6	53	150	0,9	0,45	17,4	79,5	0,460	0,356	0,184	0,447	104,80	59,96	0,351
28	2	1	404	apples	0,34	0	11,43	52	660	2,244	0	75,44	343,2	0,496	0,316	0,188	0,424	82,64	57,00	0,267
29	2	1	413	kiwis	0,87	0,55	9,37	53	195	1,70	1,07	18,27	103,35	0,439	0,363	0,198	0,405	113,13	40,01	0,155
30	2	1	528	peaches	0,7	0,1	9,4	41	312,5	2,19	0,31	29,38	128,13	0,514	0,289	0,197	0,473	106,49	56,34	0,183
31	3	2	32	pizza (with mushrooms)	6,55	5,64	21,27	165,45	550	36,03	31,02	116,99	909,98	0,541	0,304	0,155	0,531	137,45	61,05	0,444
32	3	2	37	pasta meal	5,39	3,97	18,71	133	250	13,48	9,93	46,78	332,5	0,549	0,297	0,155	0,365	126,06	66,70	0,461
33	3	2	60	french fries	3,75	17,04	34,10	295	220	8,25	37,49	75,01	649	0,491	0,345	0,163	0,325	106,01	46,17	0,421
34	3	2	65	cheese burger	12	12	14	217	350	42	42	49	759,5	0,477	0,368	0,155	0,381	114,06	63,19	0,312
35	3	2	68	doner kebab	12,11	4,91	24,31	192	350	42,39	17,19	85,09	672	0,518	0,312	0,170	0,367	130,15	62,16	0,424
36	3	2	69	fish sandwich	9,78	8,27	29,13	230	200	19,56	16,54	58,26	460	0,523	0,349	0,128	0,327	114,62	56,26	0,286
37	3	2	73	Spaghetti Bolognese	6	4,08	15,3	121	400	24	16,32	61,2	484	0,430	0,341	0,229	0,337	72,59	60,34	0,398
38	3	2	85	Pizza (with salami)	6,55	5,64	21,27	165,45	550	36,03	31,02	116,99	909,98	0,516	0,302	0,182	0,337	132,00	46,27	0,388
39	3	2	145	lasagna	5,7	5,1	13	123	350	19,95	17,85	45,5	430,5	0,534	0,318	0,148	0,345	117,06	56,76	0,361
40	3	2	310	pasta with bacon	14,07	3,92	52,46	306	65	9,15	2,55	34,10	198,9	0,494	0,336	0,170	0,460	76,92	46,46	0,417
41	3	2	311	Roast Beef	21	4	0	121	200	42	8	0	242	0,519	0,277	0,204	0,350	169,34	48,94	0,308
42	3	2	318	fried sausage with roll	12,6	18,2	9,2	248	197	24,82	35,85	18,12	488,56	0,487	0,310	0,203	0,245	112,02	58,63	0,402
43	3	2	350	quiche	9,3	26,1	12,8	321	800	74,4	208,8	102,4	2568	0,541	0,329	0,130	0,524	124,28	47,78	0,330
44	3	2	555	Viennese Schnitzel	26,1	9,3	6,5	230	500	130,5	46,5	32,5	1150	0,510	0,345	0,146	0,432	100,24	44,14	0,351
45	3	2	562	cutlet	22	5	0	133	300	66	15	0	399	0,525	0,318	0,157	0,316	124,31	52,16	0,382
46	4	2	6	cheese cake with strawberries and crumbles	9,03	12,67	28,03	264	150	13,55	19,01	42,05	396	0,408	0,322	0,270	0,277	68,64	52,70	0,311
47	4	2	41	Donut with chocolate sprinkles	7,82	23	42	454	50	3,91	11,5	21	227	0,438	0,327	0,235	0,443	125,94	63,37	0,412
48	4	2	88	stracciatella cake	3,1	21,2	28,8	320	90	2,79	19,08	25,92	288	0,435	0,310	0,256	0,392	105,17	67,46	0,292
49	4	2	103	raspberry cake	3,5	12	34,3	262	120	4,2	14,4	41,16	314,4	0,509	0,265	0,226	0,351	125,36	57,61	0,265
50	4	2	122	cake	5,4	18,5	33,5	320	100	5,4	18,5	33,5	320	0,448	0,365	0,186	0,382	82,66	43,22	0,265
51	4	2	127	Sacher cake	5,4	15,4	51,5	375	65	3,51	10,01	33,48	243,75	0,581	0,270	0,149	0,335	186,05	61,56	0,177
52	4	2	134	chocolate muffin	6,2	20,7	49	398	75	4,65	15,53	36,75	298,5	0,487	0,340	0,172	0,293	112,60	47,20	0,335
53	4	2	139	cake	6,2	20,7	33,3	346	100	6,2	20,7	33,3	346	0,512	0,302	0,186	0,255	153,55	67,35	0,274
54	4	2	163	chocolate cake	5,2	26	48	451	100	5,2	26	48	451	0,453	0,301	0,246	0,516	145,02	58,79	0,389
55	4	2	151	lemon cake	5,3	23	52	435	150	7,95	34,5	78	652,5	0,407	0,355	0,238	0,591	49,91	24,81	0,264
56	4	2	169	donut (chocolate topping)	6,9	27	36,4	420	55	3,80	14,85	20,02	231	0,428	0,306	0,266	0,481	152,27	45,64	0,179
57	4	2	178	German krapfen	6,4	13,9	41,5	333	126	8,06	17,51	52,29	419,58	0,412	0,334	0,254	0,255	86,54	41,72	0,385
58	4	2	351	croissant	7,08	33,57	44,86	508	65	4,60	21,82	29,16	330,2	0,577	0,320	0,103	0,341	130,45	34,72	0,204
59	4	2	400	éclair	5	15	40	294	160	8	24	64	470,4	0,537	0,313	0,149	0,321	123,68	53,39	0,295
60	4	2	421	raspberry cream cake	3,1	10,6	28,2	221	120	3,72	12,72	33,84	265,2	0,573	0,207	0,220	0,352	131,61	55,20	0,280

Appendix- Study 2 (Wagner et al. 2023)

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ORIGINAL ARTICLE

WILEY

Brain insulin responsiveness is linked to age and peripheral insulin sensitivity

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Abstract

Aims: Insulin action in the brain influences cognitive processes, peripheral metabolism and eating behaviour. However, the influence of age and peripheral insulin sensitivity on brain insulin action remains unclear.

Materials and Methods: We used intranasal administration of insulin and functional magnetic resonance imaging in a randomized, placebo-controlled within-subject design in 110 participants (54 women, body mass index 18-49 kg/m^2, age 21-74 years). Cerebral blood flow was measured before and after nasal spray application to assess brain insulin action. Peripheral insulin sensitivity was assessed by a five-point oral glucose tolerance test. Linear regressions were used to investigate associations between age and peripheral insulin sensitivity with brain insulin action in predefined region of interests (i.e. insulin-sensitive brain regions).

Results: We found significant negative associations between age and insulin action in the hippocampus ($\beta = -0.215$; $p = .017$) and caudate nucleus ($\beta = -0.184$; $p = .047$); and between peripheral insulin sensitivity and insulin action in the amygdala ($\beta = -0.190$, $p = .023$). Insulin action in the insular cortex showed an interaction effect between age and peripheral insulin sensitivity ($\beta = -0.219$ $p = .005$). Furthermore, women showed the strongest negative association between age and hippocampal insulin action, while men showed the strongest associations with peripheral insulin sensitivity and age in reward-related brain regions.

Conclusion: We could show a region-specific relationship between brain insulin responsiveness, age and peripheral insulin sensitivity. Our findings underline the need to study brain insulin action in both men and women and further substantiate that brain insulin sensitivity is a possible link between systemic metabolism and neurocognitive functions.

KEYWORDS
age, brain, cerebral blood flow, fMRI, insulin, sex

1 | INTRODUCTION

Insulin receptors are widely spread across different brain regions, even though neuronal glucose uptake is mostly insulin-independent.[1,2] Ever since the brain was identified as an insulin-sensitive organ, it was quickly appreciated that insulin action in the brain affects cognitive and metabolic processes. This includes memory, mood and olfaction, eating behaviour, body fat distribution and the brain-derived modulation of peripheral metabolism (for review see Kullmann et al., Kleinridders and Pothos, Hallschmid, and Rebelos et al.[3-7]) with first evidence pointing to sex-specific findings.[5,8-10]

Peripheral insulin resistance is a hallmark of obesity and one key mechanism in the pathophysiology of type 2 diabetes mellitus (T2D). Moreover, insulin sensitivity physiologically also slightly decreases with ageing.[11,12] Epidemiological evidence further suggests a strong link between reduced peripheral insulin sensitivity and age-related neurodegenerative processes such as cognitive impairments and dementia, including Alzheimer's disease (AD).[13-17]

Whether biologically active insulin can be produced locally in the brain is still controversial. Insulin mRNA and proteins were found in the nervous system of different chordates and local cerebral insulin expression was described also in rodents and in postmortem human choroid plexus epithelium cells.[18,19] Nonetheless, it is largely assumed that most insulin acting in the brain is produced and released from pancreatic beta cells and reaches the brain through the bloodstream. The hormone passes the blood-brain barrier (BBB) by a saturable receptor-mediated transport mechanism.[20,21] Previous studies in humans have indicated that there is a relationship between plasma and cerebrospinal fluid (CSF) insulin concentration.[22,23] In healthy participants, serum and CSF insulin levels were highly correlated whereas insulin concentrations in the CSF were relatively lower in insulin-resistant participants[22] and with increasing age.[23]

Several studies suggest that peripheral and brain insulin sensitivity are partially linked, although the underlying molecular mechanisms are still not completely understood.[2,22,24-26] To measure brain insulin action and discern peripheral from brain insulin effects is challenging in humans. The hyperinsulinaemic-euglycaemic clamp is considered the gold standard for the assessment of systemic insulin sensitivity. However, this technique results in insulin-stimulated effects in most tissues throughout the body and is not limited to brain-specific effects. Intranasal administration of insulin, on the other hand, has been established over the last decades to distinguish between peripheral and brain insulin effects. In combination with functional neuroimaging, it is possible to quantify brain insulin sensitivity non-invasively in humans.[6] As a proxy for neural activity, the functional magnetic resonance imaging (fMRI) signal measures the haemodynamic changes related to neuronal activity using blood-oxygen-level-dependent contrast imaging or cerebral blood flow (CBF). While the blood-oxygen-level-dependent contrast does not provide a measure of a single physiological parameter, the direct change in CBF provides absolute quantification of the neural signal, resulting in a well-characterized physiological parameter in physiological units (ml/100 g brain tissue/min). Hence, CBF measurements have been proposed to be ideally suited for pharmacological MRI studies.[27]

TABLE 1 Participants' characteristics

N (female/male)	54[a]/56
Age (years)	37 ± 1
BMI (kg/m^2)	25.68 ± 0.46
Fasting glucose (mmol/L)	5.0 ± 0.4
Fasting insulin (pmol/L)	62 ± 5
Insulin sensitivity ($ISI_{Matsuda}$), OGTT-derived (AU)	17.5 ± 1
HbA1c (mmol/mol)	34.9 ± 0.3
HbA1c (%)	5.35 ± 0.03

Note: values in the table are given as mean (±SEM).
Abbreviations: BMI, body mass index; HbA1c, glycated haemoglobin; $ISI_{Matsuda}$, Matsuda peripheral insulin sensitivity index (OGTT-derived).
[a]17 in the menopausal state.

Acute intranasal insulin results in regional specific CBF responses,[28-31] with no direct effect on the cerebral vasodilatory system.[29] This regional CBF responsiveness is affected by abdominal obesity, T2D and ageing, indicating alteration in brain insulin sensitivity.[6,30] Furthermore, recent studies show that brain insulin responsiveness can potentially be normalized in persons with obesity and a high risk to develop T2D through pharmacological,[32] exercise[33] and weight loss interventions.[34]

In the current study, we investigated the relationship between age, peripheral insulin sensitivity and brain insulin action in healthy women and men of different weight. Brain insulin action was defined as intranasal insulin induced change in regional CBF compared with placebo spray. We expected region-specific correlations between intranasal insulin-induced changes in CBF and peripheral insulin sensitivity and age. We hypothesized that insulin action in regions known to be insulin sensitive[6] (i.e. bilateral amygdala, hypothalamus, hippocampus, insula, striatum), is positively associated with peripheral insulin sensitivity and that brain insulin responsiveness is reduced with increasing age. Exploratory analyses for sex differences were performed, as recent findings revealed sex-specific effects of brain insulin action particularly on behaviour-related measures (for review see Hallschmid[5]) and neural food cue reactivity.[10] Furthermore, we explored whether brain insulin action was directly linked to its corresponding brain volume, as brain volume was shown to decrease with increasing age[35] and peripheral insulin resistance.[36]

2 | METHODS

2.1 | Subjects

Datasets of 110 participants (54 women, body mass index range 18-49 kg/m^2 and age range 21-74 years, measured between 2013 and 2019) were included in the analysis (Table 1 shows complete

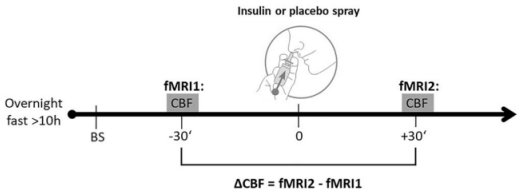

FIGURE 1 Scheme of study design. Cross-over design with intranasal insulin or placebo in a pseudo-randomized order. CBF was measured using arterial spin labelling at each visit before and 30 min after nasal spray application. BS, blood sample; CBF, cerebral blood flow; fMRI, functional magnetic resonance imaging.

descriptive and metabolic data, and there were no significant differences between women and men in those parameters; $p > .05$). Participants signed a written informed consent before participation, the studies were approved by the local ethics committee of the medical faculty of the University of Tübingen and the participants consented to the use of the data in combined studies (Clinical trial numbers: NCT04372849 and NCT01797601).

2.2 | Experimental design and procedure

All participants underwent a medical examination to rule out psychiatric, neurological, or metabolic diseases and document menopause status and medication use. Persons treated for chronic disease or taking any kind of medication other than oral contraceptives were excluded. Insulin sensitivity was estimated from measurements during a five-point 75-g oral glucose tolerance test according to Matsuda and DeFronzo ($ISI_{Matsuda}$).[37]

After the oral glucose tolerance test measurement day, the participants completed two fMRI measurements with a time-lag of 3-28 days. After an overnight fast of at least 10 h, visits were scheduled between 7 am and 11 am with intranasal insulin or intranasal placebo in a pseudo-randomized order. To ensure a counter-balanced study design, a randomization list was created beforehand by the study manager and participants were assigned in the order in which they were included in the study to either start with placebo or insulin spray. After blood sampling, fMRI measurements were recorded under baseline (fMRI1) condition and 30 min after nasal spray application (fMRI2) (scheme of study design in Figure 1).

2.2.1 | Application intranasal insulin/placebo

Participants received, in total, 160 U of insulin (Insulin Actrapid; Novo Nordisk) or vehicle as placebo in a pseudo-randomized fashion. The insulin dose was chosen based on previous work, as 160 U induced the most prominent acute effect on the different regions of interest (ROIs).[38] The spray was administered over 4 min with two puffs per nostril every minute. Participants were blinded to the order of the conditions.

2.2.2 | Imaging procedures

Scanning of the first 40 participants was conducted on a 3 T whole-body scanner (Magnetom Trio, A Tim System; Siemens Healthcare) equipped with a 12-channel transceiver head coil (as previously reported[24]). To acquire CBF maps, pulsed arterial spin labelling images were obtained with a PICORE-Q2TIPS (proximal inversion with control for off-resonance effects—quantitative imaging of perfusion by using a single subtraction) sequence by using a frequency offset corrected inversion pulse and echo planar imaging readout for acquisition.[39] In total, 16 axial slices with a slice thickness of 5 mm (1.00 mm gap) were acquired in ascending order. Each measurement consisted of 79 images with 78 alternating tag and control images with the following imaging parameters: inversion time (TI), TI1 = 700 ms, TI2 = 1800 ms, repetition time (TR) = 3000 ms, echo time (TE) = 19 ms, inplane resolution = 3×3 mm^2, field of view = 192 mm, matrix size 64×64 and flip angle = 90°. Scanning of the following 70 participants was conducted on a 3 T whole-body scanner (Magnetom Prisma) with a 20-channel head coil using the same sequence parameters, except slice thickness of 4.5 mm (0.90-mm gap) and TE = 13 ms. For all participants, the first image of the series (M0) was measured before the preparation scans and was used to estimate the equilibrium magnetization of the blood (M0B) for absolute CBF quantification. In addition, high-resolution T1 weighted anatomical images (MPRage: 176 slices, matrix: 256×224, $1 \times 1 \times 1$ mm^3) of the brain were obtained.

2.2.3 | Image processing of cerebral blood flow data

Image preprocessing was performed by using the ASLtbx[40] with SPM12 (Wellcome Trust Centre for Neuroimaging). Functional images were motion corrected, coregistered to the individual anatomical image and smoothed (full width at half maximum: 6 mm). Perfusion images were generated by calculating the control-tag differences by using surround subtraction. For accurate CBF quantification (ml/100 g/min), we used a unique M0 value extracted from an ROI in the cerebrospinal fluid. For absolute perfusion quantification the general kinetic model was applied. Possible outliers were cleaned using a slice-wise procedure based on priors.[41] The high resolution T1-weighted image was normalized in Montreal Neurological Institute space ($1 \times 1 \times 1$ mm^3) using SPM12's unified segmentation normalization, and the resulting parameter file was used with the individual co-registered CBF maps in normalized space ($3 \times 3 \times 3$ mm^3). A brain mask was used to exclude extracranial voxels in the normalized CBF images. CBF values of each measurement were extracted for insulin-sensitive ROIs: bilateral amygdala, hypothalamus, hippocampus, insula, ventral and dorsal striatum (putamen and caudate nucleus).[6] The masks were based on the *wfu* pick atlas (https://www.nitrc.org/projects/wfu_pickatlas). To reduce intersubject and interscanner variability, we normalized the regional CBF values by correcting for global CBF differences (CBF_n). This was done by dividing the CBF values of

the different ROIs with the individual global CBF values (separately for fMRI1 and fMRI2 measurements and both days). Brain insulin action was defined as the regional CBF change in response to intranasal insulin compared with placebo. For this purpose, normalized CBF maps of each participant were first corrected for baseline measurements ($\Delta CBF_n = CBF_n$ (fMRI2) $- CBF_n$ (fMRI1)) and then the difference between the insulin and the placebo measurement day for each individual ROI (ΔCBF_n (insulin day) $- \Delta CBF_n$ (placebo day)) was calculated (formula in the Data S1).

2.2.4 | Anatomical subfield brain volume segmentation

Segmentation of the insula, amygdala, caudate nucleus, putamen, nucleus accumbens (ventral striatum) and hippocampus was performed using the FreeSurfer image analysis software (https://surfer.nmr.mgh.harvard.edu/) version 7.2.0.[42] In brief, this process includes intensity normalization, removal of non-brain tissue, Talairach transformation, segmentation of the subcortical white matter, and tessellation and inflation of the surface.[43,44] The volume of the insular cortex was computed based on Killiany/Desikan parcellation.[45] The volume of the caudate nucleus, putamen and nucleus accumbens (ventral striatum) was computed using the aseg.stats function of FreeSurfer. Segmentation of the hypothalamus was done using the tool developed by Billot and colleagues.[46]

2.3 | Statistical analyses

We investigated the association of brain insulin action with age and peripheral insulin sensitivity. Age and peripheral insulin sensitivity ($ISI_{Matsuda}$) were mean-centred and an interaction term was calculated ($ISI_{Matsuda} \times$ age) to investigate interaction effects between peripheral insulin sensitivity and age. The change in normalized CBF (ΔCBF_n) response following intranasal insulin compared with placebo in the bilateral amygdala, hypothalamus, hippocampus, insula, ventral and dorsal striatum (caudate nucleus and putamen) was used as a proxy for brain insulin action.

We performed linear regressions with bootstrapping to identify associations between brain insulin action, peripheral insulin sensitivity and age. The *p*-values and confidence intervals were estimated using the BCa method (BCa: bias-corrected and accelerated; 95% confidence interval, 10 000 bootstrap samples). Exploratory analyses for specific associations depending on sex were performed by separate analyses for women and men. Furthermore, we investigated whether the CBF insulin response and its association to age or peripheral insulin sensitivity are directly linked to the corresponding cortical volume [hippocampus, amygdala, insula, hypothalamus, putamen, caudate nucleus and nucleus accumbens (ventral striatum)]. For that purpose, bilateral mean volume values were calculated for the different ROIs. We corrected for individual brain volume differences by dividing the ROI volume by the estimated total intracranial volume (eTIV). Pearson-correlations were calculated with ΔCBF_n (insulin-placebo) and the ROI-volume/eTIV.

All analyses are reported (partially in the Supporting Information), but only results with $p < .05$ (two-tailed) are considered significant. Statistics were performed using SPSS (Released 2021. IBM SPSS Statistics for Windows, Version 28.0; IBM Corp.).

The terms 'younger' and 'elderly' participants as well as 'lower' and 'higher' insulin sensitivity are used to describe the upper and lower limits of the linear relationship between age or peripheral insulin sensitivity and brain insulin action of our sample. The terms are used in a descriptive way.

3 | RESULTS

3.1 | Associations between age and brain insulin action

Age was negatively associated with insulin action in the hippocampus (Figure 2; $\beta = -0.215$, $p = .0165$) and in the caudate nucleus ($\beta = -0.184$, $p = .047$) (Table 2). The CBF response in these regions to intranasal insulin was lower with increasing age. The negative association between the hippocampus and age was driven by women (women: $\beta = -0.326$, $p = .019$; men: $p > .05$). In men only, age was negatively associated with insulin action in the amygdala ($\beta = -0.318$, $p = .025$) and positively associated with the insula ($\beta = 0.252$, $p = .039$). Insulin action in the hypothalamus, putamen and ventral striatum did not show significant associations with age (Table S1 in Data S1; $p > .05$).

3.2 | Associations between peripheral insulin sensitivity and brain insulin action

We observed a negative association between brain insulin action in the amygdala and peripheral insulin sensitivity ($ISI_{Matsuda}$) (Figure 3 and Table 2; $\beta = -0.190$, $p = .023$). Participants with high peripheral insulin sensitivity showed a decrease in CBF to intranasal insulin whereas participants with lower peripheral insulin sensitivity showed no response or a slight increase. The negative association between the amygdala and peripheral insulin sensitivity was driven by men (men: $\beta = -0.318$, $p = .025$; women: $p > .05$). Insulin action in the hypothalamus, hippocampus, insula, dorsal and ventral striatum did not show significant associations with $ISI_{Matsuda}$ (Table S1 in Data S1; $p > .05$).

3.3 | Interaction of age and peripheral insulin sensitivity with brain insulin action

We found an interaction between age and peripheral insulin sensitivity on insulin action in the insular cortex for the whole group (Figure 4 and Table 2; $\beta = -0.219$, $p = .005$), but not for men and women separately ($p > .05$). Younger participants with high peripheral insulin sensitivity showed an increase in CBF response in the insula, whereas in

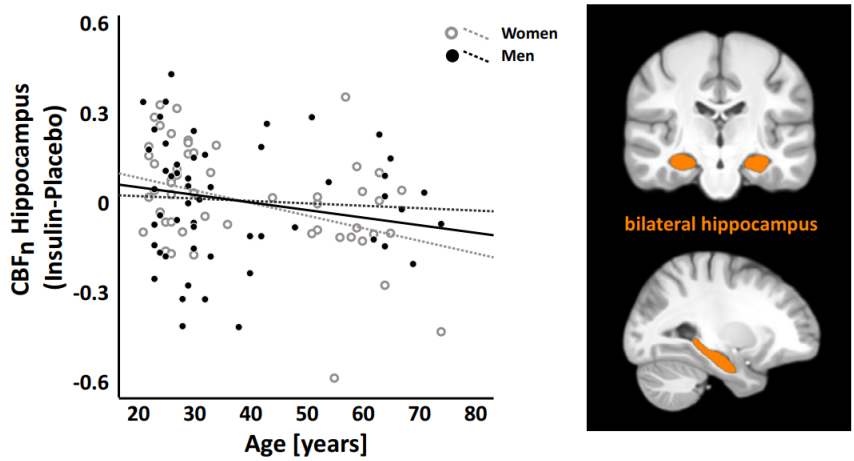

FIGURE 2 Hippocampal insulin action associates with age. Plot on the left shows correlation between age and insulin action in the bilateral hippocampus (black solid line, β = −0.215, $p = .017$). The negative association was more pronounced in women (dotted grey line, β = −0.326, $p = .019$) than in men (dotted black line, β = −0.120, $p = .301$). Overlay on the right shows hippocampal region of interest (in orange) on a standardized anatomical T1 image. CBF_n, normalized cerebral blood flow.

TABLE 2 Significant associations between brain insulin action, age, peripheral insulin sensitivity or their interaction

ROI		Standardized regression coefficient (β)	*p*-value[a]
Hippocampus	Age	All: −0.215	All: .017
		W: −0.326	W: .019
Caudate nucleus	Age	All: −0.184	All: .047
Amygdala	Age	M: −0.318	M: .025
Insula	Age	M: 0.252	M: .039
Amygdala	$ISI_{Matsuda}$	All: −0.190	All: .023
		M: −0.390	M: .001
Insula	Age × $ISI_{Matsuda}$	All: −0.219	All: .005
Ventral striatum	Age × $ISI_{Matsuda}$	M: −0.343	M: .038

Note: All other associations reported in Data S1, Table S1.
Abbreviations: $ISI_{Matsuda}$, Matsuda peripheral insulin sensitivity index; M, men; ROI, regions of interest; W, women.
[a]Based on 10 000 bootstrap samples.

elderly participants, lower peripheral insulin sensitivity was related to an increase in CBF response in the insula. A similar relationship was found in the ventral striatum (β = −0.223; $p = .055$), even though this association was only significant in men (β = −0.343; $p = .038$). Insulin action in the hippocampus, hypothalamus, amygdala and dorsal striatum did not show a significant association with the interaction of age × $ISI_{Matsuda}$ (Table S1 in Data S1; $p > .05$).

3.4 | Correlation between regions of interest volume and brain insulin action

We performed exploratory analyses to verify whether brain insulin action, based on the CBF response to intranasal insulin, was linked to the region's volume. None of the ROIs, in which the CBF response to insulin was associated with age or peripheral insulin sensitivity, showed a significant correlation with the corresponding volume ($p > .05$, Table S2 in Data S1).

4 | DISCUSSION

The brain is a major target for insulin action that results in multiple metabolic and behavioural effects. Alterations in brain insulin signalling affect various cell populations (as glia cells[47] and neurons) and brain circuitries, and include changes in dopamine signalling, blood-brain barrier function, hippocampal synaptic plasticity, expression of amyloid β and microtubule associated tau protein

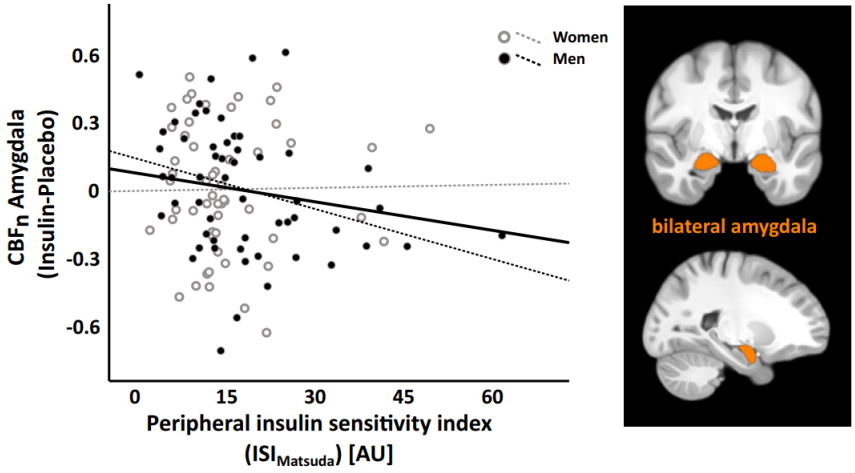

FIGURE 3 Amygdalar insulin action associates with peripheral insulin sensitivity. Plot on the left shows correlation between peripheral insulin sensitivity ($ISI_{Matsuda}$) and insulin action in the bilateral amygdala (black solid line, $\beta = -0.190$, $p = .023$). The association was more pronounced in men (dotted black line, $\beta = -0.318$, $p = .025$) than in women (dotted grey line, $\beta = 0.011$, $p = .928$). Overlay on the right shows amygdala region of interest (in orange) on a standardized anatomical T1 image. CBF_n, normalized cerebral blood flow.

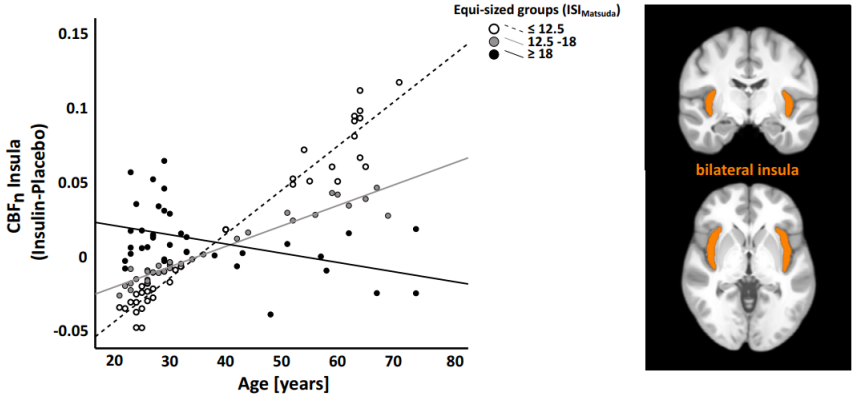

FIGURE 4 Insulin action in the insula cortex associates with age depending on peripheral insulin sensitivity. To visualize the interaction, plot on the left shows correlation between age and insulin action in the bilateral insula in three equi-sized groups based on peripheral insulin sensitivity. Younger participants with a high peripheral insulin sensitivity and elderly participants with lower peripheral insulin sensitivity showed the strongest CBF response in the insula cortex to intranasal insulin. Overlay on the right shows insula region of interest (in orange) on a standardized anatomical T1 image. CBF_n, normalized cerebral blood flow; $ISI_{Matsuda}$, Matsuda peripheral insulin sensitivity index [AU].

(for review see Kullmann et al.[6]). Hence, brain insulin resistance might constitute a joint pathological feature of metabolic, psychiatric and neurodegenerative diseases. In the current study, we investigated brain insulin action in healthy young and elderly participants by CBF responses to intranasal insulin. We identified that brain insulin responsiveness is linked to peripheral insulin sensitivity and age. Our findings indicate that the use of surrogate markers for brain insulin sensitivity are region-specific and may vary between sexes.

Age was negatively related to insulin action in limbic regions of the brain. In particular, in our study, young participants showed the most pronounced CBF increase to intranasal insulin in the hippocampus and caudate nucleus. This corresponds to a large cohort study investigating insulin-stimulated brain metabolism under clamp conditions using [^{18}F]-FDG-PET scans.[26] Rebelos and colleagues[26] showed that the insulin-stimulated brain metabolism during a hyperinsulinaemic-euglycaemic clamp decreased with advancing age. While the underlying mechanisms of this characteristic brain

metabolism is not clear, it is interesting to note that this insulin-stimulated decrease was particularly evident in the limbic lobe of the brain.

The hippocampus is part of the limbic system and plays an essential role in learning and memory; moreover, it is known to be very sensitive to age-related decline.[48] Multiple studies using intranasal insulin showed memory improving effects in young healthy participants,[8,49,50] but also an enhancement of hippocampal memory processes in patients with T2D and early AD (for review see Hallschmid[51]). Interestingly, in the current study, the decrease of insulin action in the hippocampus was predominantly found in women. This is of special interest, as the prevalence of age-related AD is higher in women[52] and a link between AD and reduced insulin action in the brain is assumed (for review see Kellar and Craft[53]).

Beside the hippocampus, in the current study, we observed decreased insulin responsiveness in the striatum (i.e. caudate nucleus) with higher age. In previous studies, we and others, could show that intranasal insulin can increase striatal blood flow,[30] reduce striatal dopamine levels[54] and modulate mesostriatal connectivity in healthy participants.[54-57] Besides reward processes, the striatum is implicated in motor behaviour (e.g. walking speed). The first evidence points to improvements on gait, a complex task and predictor of disabilities and mortality,[58,59] in response to intranasal insulin.[60,61] A 24-week intervention with intranasal insulin led to increased gait speed in elderly participants with and without T2D.[61] Furthermore, we were able to show improved central insulin responsiveness in the striatum after an 8-week exercise intervention, which was linked to improved cognitive and metabolic functions in middle-aged adults at high risk of developing T2D.[33] In elderly dieters, weight loss success was identified to be directly related to improved central insulin action in the striatum.[34] As brain insulin acts on striatal dopamine function, enhancing central insulin action in the striatum could lead to new treatment options in neurodegenerative and ageing-associated diseases.

Peripheral insulin resistance was significantly associated with insulin action in the insular cortex and amygdala. These regions are also part of the mesolimbic reward circuitry regulating non-homeostatic eating and are both involved in diverse functions linked to emotion and body homeostasis.[62] Of note, no associations were identified with hypothalamus insulin responsivity. This could be based on the fact that we investigated the link between brain and peripheral insulin sensitivity in healthy non-diabetic adults. Furthermore, we did not consider visceral fat accumulation, which has been shown to exacerbate hypothalamic insulin resistance.[63]

In our sample, participants with the highest peripheral insulin sensitivity showed the most pronounced decrease in amygdalar CBF following intranasal insulin. Likewise, Wingrove and colleagues reported an insulin-induced reduction in amygdalar CBF in healthy young men.[28,30] Hence, high brain insulin sensitivity would probably be accompanied by insulin-induced inhibition of amygdalar blood flow, similar to that reported in the hypothalamus.[24,63] This is also further substantiated by animal models showing that insulin receptor deletion in the amygdala also led to impaired glucose metabolism.[64] Hence, it is postulated that impaired insulin sensitivity in the amygdala is critical for the development of T2D and mood disorders.[64,65] Whether insulin sensitivity in the amygdala is a joint feature between peripheral metabolism and emotional behaviours in humans needs to be further investigated.

The insulin responsiveness of the insula cortex was related to both peripheral insulin sensitivity as well as age. In our study, younger participants with higher peripheral insulin sensitivity showed the greatest insulin response in the insula based on the CBF response. Likewise, we recently found intranasal insulin to change insular cortex activity in response to food cues depending on peripheral insulin sensitivity.[10] Moreover, young men with normal weight were reported to show an increase in intranasal insulin-induced CBF in the insula,[66] whereas men with overweight showed a decrease.[67] However, in our current study, elderly participants with lower peripheral insulin sensitivity (i.e. who were insulin resistant in the periphery) also showed increased brain insulin response. We hypothesize that this might represent a compensatory response specific to age-related decline. Insulin transport across the BBB is reduced in persons with advanced ageing and peripheral insulin resistance, resulting in lower insulin concentrations in the CSF.[22,23] This could ultimately lead to a relative insulin deficiency of the brain. As intranasal insulin application bypasses the BBB,[68] the 'insulin-deficient brain' could potentially show a compensatory hyper-responsiveness to central insulin. Similarly, early brain hyperactivity is seen as a compensatory phase in cognitive dysfunctions eventually accelerating neurodegenerative processes that result in later brain hypometabolism.[69] Clearly, more mechanistic work is necessary to test this hypothesis.

Interestingly, we found several associations between brain insulin action and age or peripheral insulin sensitivity to be more pronounced in either men or women. In animal studies, oestrogen seems to modify brain insulin action.[70] In women, oestrogen is not only crucial for reproductive functions, but it also influences cognitive function, food intake, energy expenditure and, in general, weight control.[71,72] In particular, during and post-menopause, oestrogen levels decline, which was identified as a further risk factor for neurodegenerative disorders.[72] Thus far, however, an influence of oestrogen on brain insulin action could not be confirmed in humans.[73,74] Several experimental findings suggest sex differences in response to intranasal insulin on eating behaviour and neurocognitive measures.[5] Our results exemplify that there is an ample need to study brain insulin signalling in both men and women to clarify the role of brain insulin sensitivity in metabolic, psychiatric and neurodegenerative diseases.

We need to acknowledge some limitations of the study. Because of the restricted field of view during the MRI acquisition, we could not include prefrontal or parietal brain regions in our analysis. Participants were measured at two different three Tesla MRI scanners, however the variability was minimized by global CBF correction and the within-subject design subtracting the placebo from the insulin CBF response. No data on sex hormones were available to elucidate the role of sex hormones on brain insulin responsivity in women, with respect to the menstrual cycle or menopause. Furthermore, no cognitive assessments were available to link hippocampal insulin sensitivity to cognitive and memory performance outcomes. Recent studies

showed that unfavourable fat distribution with elevated visceral adipose tissue is related to brain insulin resistance.[7] Unfortunately, no data on body fat distribution were available to evaluate whether brain insulin responsivity is associated with peripheral insulin resistance dependent on body fat distribution.

5 | CONCLUSION

Our results further corroborate a complex interplay between cerebral functions, metabolism, sex and age. Unravelling the underlying processes will be fundamental for a better understanding of brain effects of healthy versus unhealthy ageing and the clinical impact on neurocognitive functions. Our current findings show that brain insulin action is region-specific both in the responsiveness as well as in associations with peripheral insulin sensitivity or ageing. Furthermore, brain insulin sensitivity can potentially be restored through pharmacological,[32] exercise[33] and weight loss interventions.[34] These interventions may potentially be tailored to improve peripheral metabolism or age-dependent alterations by targeting regional-specific brain insulin action.

AUTHOR CONTRIBUTIONS

LW performed experiments, analysed and interpreted the data, and wrote the manuscript with contributions from all other authors. CK performed experiments and analysed data; RV was involved in preprocessing the CBF data and, together with SK, provided scientific guidance; CK and SK both further contributed to the discussion. SK and MH designed the study. HP, AF, HUH and AB provided scientific guidance on the experimental design and contributed to the discussion. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

Outside of the current work, MH reports research grants from Boehringer Ingelheim and Sanofi (both to the University Hospital of Tübingen), advisory board for Boehringer Ingelheim, and lecture fees from Lilly, Boehringer Ingelheim, Novo Nordisk and Amryt.

AF reports lecture fees from Sanofi Aventis, Novo Nordisk, Lilly, Boehringer Ingelheim, AstraZeneca and Synlab. All other authors have no conflicts of interest to declare.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/dom.15094.

DATA AVAILABILITY STATEMENT

The data are not publicly available due to them containing information that could compromise research participant privacy/consent.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Supplemental material

Supplements: Brain insulin responsiveness is linked to age and peripheral insulin sensitivity

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Supplements Methods

Calculation normalized brain insulin action

To reduce intersubject and interscanner variability, we normalized the regional CBF values by correcting for global CBF differences (CBF_n). This was done by dividing the CBF values of the different ROIs with the individual global CBF values (separately for fMRI1 and fMRI2 measurements and both days). Brain insulin action was defined as the regional CBF change in response to intranasal insulin compared to placebo. For this purpose, normalized CBF maps of each participant were first corrected for baseline measurements (ΔCBF_n= CBF_n(fMRI2) – CBF_n(fMRI1)) and then the difference between insulin and placebo measurement day for each individual ROI (ΔCBF_n(Insulin day) minus ΔCBF_n(Placebo day)) was calculated according to the following formula:

$$normalized\ brain\ insulin\ action = \left(\frac{CBF[ROI]_{fMRI2}}{global\ CBF_{fMRI2}} - \frac{CBF[ROI]_{fMRI1}}{global\ CBF_{fMRI1}}\right)_{Insulin\ day} - \left(\frac{CBF[ROI]_{fMRI2}}{global\ CBF_{fMRI2}} - \frac{CBF[ROI]_{fMRI1}}{global\ CBF_{fMRI1}}\right)_{Placebo\ day}$$

Supplements Results

Suppl. Table 1: Associations between brain insulin action*, age, $ISI_{Matsuda}$ or their interaction.

		Standardized regression coeff. (β)	p-value§	BCa Confidence interval (95%)§
Amygdala				
ISI	all	**-0.190**	**0.023**	**[-0.009526; -0.000727]**
	women	0.011	0.928	[-0.008306; 0.007034]
	men	**-0.390**	**0.001**	**[-0.015314; -0.005336]**
Age	all	-0.170	0.090	[-0.006381; 0.000559]
	women	-0.061	0.657	[-0.005607; 0.003573]
	men	**-0.318**	**0.025**	**[-0.010402; -0.000111]**
Age x ISI	all	0.019	0.865	[-0.000451; 0.000565]
	women	-0.030	0.865	[-0.000901; 0.000777]
	men	0.030	0.831	[-0.000599; 0.001121]
Caudate nucleus				
ISI	all	-0.128	0.090	[-0.004538; 0.000225]
	women	-0.112	0.183	[-0.005065; 0.000603]
	men	-0.148	0.238	[-0.006458; 0.001293]
Age	all	**-0.184**	**0.047**	**[-0.003626; -0.000050]**
	women	-0.160	0.272	[-0.004253; 0.001157]
	men	-0.210	0.085	[-0.004844; 0.000620]
Age x ISI	all	0.048	0.584	[-0.000183; 0.000277]
	women	0.167	0.220	[-0.000144; 0.000695]
	men	-0.066	0.654	[-0.000502; 0.000249]

Putamen				
ISI	all	-0.008	0.921	[-0.002316; 0.002041]
	women	-0.096	0.367	[-0.004235; 0.002104]
	men	0.071	0.529	[-0.002326; 0.004062]
Age	all	-0.026	0.786	[-0.001798; 0.001439]
	women	-0.118	0.401	[-0.003098; 0.001245]
	men	0.074	0.582	[-0.001801; 0.003591]
Age x ISI	all	-0.031	0.746	[-0.000252; 0.000173]
	women	-0.040	0.778	[-0.000380; 0.000237]
	men	0.009	0.947	[-0.000356; 0.000341]
Hippocampus				
ISI	all	-0.065	0.498	[-0.005095; 0.002206]
	women	0.114	0.336	[-0.002890; 0.008131)
	men	-0.195	0.173	[-0.008957; 0.000598]
Age	all	**-0.215**	**0.017**	**[-0.004877; -0.000421]**
	women	**-0.326**	**0.019**	**[-0.007209; -0.000855]**
	men	-0.120	0.301	[-0.004073; 0.001219]
Age x ISI	all	-0.034	0.752	[-0.00037; 0.00033]
	women	-0.046	0.782	[-0.00060; 0.00067]
	men	-0.025	0.831	[-0.00042 0.00041]
Hypothalamus				
ISI	all	-0.059	0.570	[-0.00600; 0.00231]
	women	0.016	0.884	[-0.00573; 0.00502]
	men	-0.141	0.411	[-0.01054; 0.00259]
Age	all	0.124	0.175	[-0.00071; 0.004242]
	women	0.113	0.447	[-0.00225; 0.00576]
	men	0.130	0.263	[-0.00104; 0.00516]
Age x ISI	all	-0.028	0.811	[-0.00042; 0.00039]
	women	-0.087	0.604	[-0.00075; 0.00045]
	men	0.064	0.730	[-0.00050; 0.00096]
Insula				
ISI	all	0.058	0.405	[-0.00124; 0.00264]
	women	-0.033	0.814	[-0.00476; 0.00240]
	men	0.126	0.202	[-0.00111; 0.00496]
Age	all	0.174	0.061	[-0.00000; 0.00318]
	women	0.105	0.484	[-0.00147; 0.00317]
	men	**0.252**	**0.039**	**[0.00020; 0.00518]**
Age x ISI	all	**-0.219**	**0.005**	**[-0.00042; -0.00005]**
	women	-0.250	0.063	[-0.00057; 0.00009]
	men	-0.165	0.168	[-0.00049; 0.00014]
Ventral striatum/Nucleus accumbens				
ISI	all	-0.066	0.435	[-0.00433; 0.0023]
	women	-0.188	0.089	[-0.00750; 0.00030]
	men	0.012	0.921	[-0.00446; 0.00753]
Age	all	-0.009	0.927	[-0.00281; 0.00265]
	women	-0.042	0.749	[-0.00346; 0.00227]
	men	0.028	0.864	[-0.00412; 0.00580]
Age x ISI	all	-0.223	0.055	[-0.00076; -0.00003]
	women	-0.058	0.657	[-0.00053; 0.00031]
	men	**-0.343**	**0.038**	**[-0.00130; -0.00002]**

Abbreviations: ISI - Matsuda peripheral insulin sensitivity index; BCa - Bias corrected and accelerated method;
* brain insulin action defined as the change in normalized cerebral blood flow (ΔCBF_n) after intranasal insulin compared to placebo
[§] based on 10'000 bootstrap samples

Suppl. Table 2: Correlations between brain insulin action* and the corresponding ROIs' volume.

ROI	Correlation coeff. r	p-value
Amygdala	0.106	0.270
Caudate nucleus	0.032	0.739
Putamen	0.116	0.227
Ventral striatum[#]	0.061	0.527
Hippocampus	0.016	0.865
Insula	-0.165	0.084
Hypothalamus	**-0.223**	**0.019**

* brain insulin action defined as the change in normalized cerebral blood flow (ΔCBF_n) after intranasal insulin compared to placebo

[#] Volume of the Nucleus accumbens used as proxy for the ventral striatum