Metabolic and cognitive outcomes of subchronic once-daily intranasal insulin administration in healthy men

Running title: *Subchronic once-daily intranasal insulin administration*

Yvonne Ritze[1], Werner Kern[2], Eva-Maria Ebner[1,3], Serena Jahn[1,3], Christian Benedict[4], Manfred Hallschmid[1,5,6]

[1]Department of Medical Psychology and Behavioral Neurobiology, University of Tübingen, Germany; [2]Endokrinologikum Ulm, Germany; [3]Department of Internal Medicine I, University of Lübeck, Germany; [4]Department of Neuroscience, Uppsala University, Sweden; [5]German Center for Diabetes Research (DZD), Tübingen, Germany; [6]Institute for Diabetes Research and Metabolic Diseases of the Helmholtz Center Munich at the University Tübingen (IDM), 72076 Tübingen, Germany.

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Corresponding author: Manfred Hallschmid, University of Tuebingen, Institute of Medical Psychology and Behavioral Neurobiology, Otfried-Müller-Straße 25, 72076 Tuebingen; Tel. +49 7071 2988925, Fax +49 7071 2925016; E-mail: manfred.hallschmid@uni-tuebingen.de

Abstract

Insulin acts in the brain to limit food intake and improve memory function. We have previously shown that eight weeks of intranasal insulin delivered in four daily doses of 40 IU decrease body weight and enhance word list recall. In the present study, we investigated the effect on body composition, endocrine parameters and memory performance of eight weeks of once-daily administration of 160 IU in healthy men. We assumed that intranasal insulin administered before nocturnal sleep, a period of relative metabolic inactivity that moreover benefits memory formation, would be superior to insulin delivery in the morning and placebo administration. After a two-week baseline period, healthy male normal-weight subjects (mean age, 27.1 ± 0.9 years) received either placebo, 160 IU intranasal insulin in the morning, or 160 IU in the evening (n=12 per group) for eight consecutive weeks. Throughout the experiment, we measured body weight and body composition as well as circulating concentrations of glucose, insulin, adrenocorticotropin, cortisol, growth hormone, insulin-like growth-factor 1, adiponectin, and leptin. Declarative and procedural memory function was repeatedly assessed by means of, respectively, word list recall and word-stem priming. We found that neither morning nor evening insulin compared to placebo administration induced discernible changes in body weight and body composition. Delayed recall of words showed slight improvements by insulin administration in the evening, and serum cortisol concentrations were reduced after two weeks of insulin administration in the morning compared to the other groups. The metabolic inefficiency of once-daily insulin indicates that catabolic long-term effects of central nervous insulin delivery necessitate repetitive, presumably pre-meal delivery schedules. In contrast, the observed memory improvements are in line with previous findings and suggest that sleep after intranasal insulin administration may support its beneficial cognitive impact.

Introduction

Insulin plays an important role in the central nervous control of metabolism and, moreover, improves cognitive function (for reviews, see Lee et al., 2016; Benedict & Grillo, 2018). While local insulin production in the cerebral cortex has been suggested based on animal experiments (Molnar et al., 2014), the hormone is not released in large amounts within the CNS and, after pancreatic release, rather reaches the brain via saturable transport mechanisms (Baura et al., 1993; Gray et al., 2014). Insulin receptors are expressed in high densities in the olfactory bulb, the hypothalamus and the hippocampal formation (Devaskar et al., 1994), i.e., structures that are relevant for sensory perception and metabolic regulation as well as the formation of declarative memory contents.

Experiments in animals (Woods et al., 1979; McGowan et al., 1992; Air et al., 2002) and humans (Benedict et al., 2008; Hallschmid et al., 2012) indicate that insulin administered directly to the brain reduces food intake. In the human setting, many insulin effects on brain function and behavior have been investigated by means of the intranasal route of administration, a non-invasive method of delivery that largely bypasses the blood-brain barrier (Born et al., 2002; Dhuria et al., 2010). In acute experiments, intranasal administration of 160 IU insulin reduced calorie intake in healthy male participants (Benedict et al., 2008). Young women who received 160 IU intranasal insulin after lunch showed enhanced postprandial satiety and consumed smaller amounts of palatable snacks (Hallschmid et al., 2012). Daily intranasal insulin administration of 4 × 40 IU (around 30 min before meals and again before going to bed) for eight weeks decreased body weight and body fat in men but not in women (Hallschmid et al., 2004). Further evidence for a distinct effect of insulin on food intake-regulatory networks has emerged in neuroimaging studies (see Heni et al., 2015; Kullmann et al., 2016 for reviews). Intranasal insulin has moreover been shown to improve memory function in healthy subjects when delivered acutely (Benedict et al., 2008; Brunner et al., 2015) or according to the eight-weeks, four-times-per-day schedule described above

(Benedict et al., 2004; 2007). Patients with mild cognitive impairments and early Alzheimer's disease (AD) likewise benefit from insulin administration (Reger et al., 2008; Craft et al., 2012; for review see de Felice, 2013).

Sleep has emerged as an important factor in energy homeostasis and food intake regulation (St-Onge, 2013). Habitually short sleep is associated with increased body weight (Magee & Hale, 2012; Vgontzas et al., 2014) and a greater risk of impaired glucose homeostasis (Gangwisch et al., 2007; Cappuccio et al., 2010). Acute sleep deprivation stimulates calorie intake on the subsequent day (Brondel et al., 2010) and leads to a deterioration in glucoregulation (Schmid et al., 2011). In the cognitive domain, the consolidation of memory contents markedly benefits from the brain's offline processing during sleep (Feld & Born, 2017). Neuronal ensembles that encode information during wakefulness are reactivated during subsequent sleep, thereby strengthening respective memory representations (Diekelmann & Born, 2010). We have previously demonstrated that intranasal insulin administration before nocturnal sleep may stabilize memory traces learned in the evening by limiting the interfering influence of encoding new information on the subsequent day (Feld et al., 2016). Moreover, the acute intranasal administration of 160 IU insulin before nocturnal sleep reduced breakfast intake on the following morning (Santiago & Hallschmid, 2017). Against this background, we hypothesized that sleep, a period of reduced metabolic activity and largely absent external input, facilitates the emergence of favorable metabolic and cognitive effects of intranasal insulin. We therefore expected the enhancement of brain insulin signaling during sleep to exceed the effects of repetitive administration of smaller insulin doses throughout the day (Hallschmid et al., 2004) and, in particular, of insulin administration in the morning. This assumption was tested in young, healthy male subjects who received 160 IU intranasal insulin in the evening or morning, or were treated with placebo for eight consecutive weeks.

Methods

Subjects and design

We included 36 healthy male subjects between 18 and 40 years of age (mean age ± SEM, 27.1 ± 0.9 years) with normal body weight (mean BMI, 23.5 ± 0.3 kg/m^2). They were all nonsmokers and any relevant psychiatric, neurological, cardiovascular, pulmonary, or gastrointestinal disease was excluded before participation by clinical examination and routine laboratory tests. Participants refrained from alcohol, caffeine or food intake 12 h before each experimental session. They provided written informed consent before the study, which conformed to the Declaration of Helsinki and was approved by the local ethics committee. Experiments were performed in a double-blind manner. Subjects were informed that the study was about the impact of insulin on cortical functions in dependence of body weight and body composition, but were left unaware of the expected memory-improving and catabolic treatment effects. Interviews at the end of the experiment ensured that they did not gain insight into the study purposes.

Subjects were randomly assigned to three groups (each n=12 men) that were comparable regarding age and BMI (p>0.46 for all comparisons). Body fat content averaged across baseline sessions did not significantly differ between the three groups (p>0.10). After two weeks of a baseline period of placebo administration in all groups, participants for eight weeks self-administered, respectively, intranasal insulin after awakening and placebo spray before going to bed ('morning insulin' group; mean age, 27.4 ± 1.1 years, mean BMI, 23.8 ± 0.5 kg/m^2), placebo spray in the morning and insulin spray before going to bed ('evening insulin' group; mean age, 27.1 ± 1.9 years, mean BMI, 23.3 ± 0.4 kg/m^2), or placebo spray in the morning and evening (control group; mean age, 26.8 ± 1.8 years, mean BMI, 23.4 ± 0.5 kg/m^2). Each daily dose was 160 IU insulin (Insulin Actrapid; Novo Nordisk, Mainz, Germany) dissolved in 0.4 ml carrier solution or vehicle administered within four 0.1-ml puffs (two per nostril). Sprays were stored in a refrigerator at 5°C and were replaced every week.

Note that before each individual examination, subjects were told to postpone their morning intake routine until after the examination, ensuring that long-term rather than acute effects were assessed. To ensure compliance, subjects kept a diary about their intake routine.

Four major test sessions (scheduled between 0700 and 0900 h) were conducted, i.e., at the start of the baseline period, after two weeks of baseline placebo administration, and after four and eight weeks of insulin or placebo treatment (see Figure 1A for an overview over the experimental design). Subjects were weighed (as well as on a weekly basis, see below) and their body composition was measured by standard bioelectrical impedance analysis (frequencies of 1, 5, 50, and 100 Hz; BIA 2000-M; Data Input, Frankfurt, Germany) indicating body fat, total body water, intracellular water, extracellular water, lean body mass, and body cell mass (Eurobody software; Data Input). Waist circumference was also measured, and subjects completed a questionnaire on their eating behavior (FEV; Pudel & Westenhöfer, 1989). Participants rated their hunger, thirst and tiredness on 10-point scales in the beginning and at the end of the session, yielding difference values indicating the current gradient of these parameters. In order to control for possible side effects, we also monitored blood pressure and heart rate, as well as routine laboratory measurements (serum electrolytes; creatinine; HDL, LDL, and total cholesterol; triglycerides; data not reported).

Psychological assessments

Word list. In this test of declarative memory, a list of 30 words was presented and recalled immediately as well as after a one-week delay. (Note that the final assessment of immediate recall took place after seven weeks of the insulin intervention in order to accommodate the final assessment of delayed recall after eight weeks of treatment.) The words belonged to three semantic categories, neutral (e.g., ‘wind’, ‘moss’), food-related (e.g., ‘pineapple’, ‘cheese’), and emotional (e.g., ‘joy’, ‘cock’), and were presented orally at a rate of one word/sec. Subsequently, subjects were told to remain silent for a break of 3 min and to keep the presented words in mind. For immediate recall, subjects wrote down all words they

remembered within 90 sec. For delayed recall, approximately one week later, subjects again had to write down all words they still remembered from this list (Greenwood et al., 2003; Benedict et al., 2004; 2007). Because the respective morning insulin or placebo administrations took place after the test session, the study design did not allow for testing acute insulin effects on immediate or delayed recall. In short post-treatment interviews none of the subjects stated to have learned or thought about the word list within the week before delayed recall, excluding major interfering influences of rehearsal effects.

Word-stem priming. Non-declarative memory was tested with a word-stem priming task based on a learning word list and a test list of two-letter word-stems. First, subjects rated the nouns of the learning word list according to their sound on a 5-point scale (from 1 = unpleasant to 5 = pleasant). This task was considered to induce implicit learning. Thereafter the subjects received the test list containing 52 two-letter word-stems (e.g. “ho” derived from “hotel”). Twenty-six word-stems of this list were derived from the (rated) learning list, whereas the other 26 word-stems were taken from a pool of new words not presented to the subject (new list). Subjects were instructed to complete the word-stems to the first noun that came to their mind. The difference between the number of word-stems completed to nouns from the learning list and the number of words accidentally completed to nouns of the new list was considered a measure of implicit memory (Plihal & Born, 1999; Benedict et al., 2004).

Mood. During each major test session, subjects filled in an adjective check list designed to assess current mood and feelings of activation on 15 dimensions (Eigenschaftswörterliste EWL-N; Janke & Debus, 1978). The adjective checklist consists of a total of 161 adjectives grouped into 15 dimensions, i.e., activation, concentration, deactivation, tiredness, numbness, extraversion, introversion, self-assuredness, mood, excitation, sensitivity, anger, anxiousness, depression, and dreaminess. For each adjective, the subject had to indicate whether or not it reflected aspects of his current state of mood. For each dimension, the number of adjectives

marked by the subject was counted and transformed to percentages of the respective achievable maximum value.

Blood parameters

Weekly, around 0800 h, subjects were weighed and blood samples were collected. Immediately after blood drawing, blood samples were centrifuged and plasma and serum were stored at -20°C. Concentrations of leptin, insulin, adrenocorticotropin, cortisol and adiponectin were assessed using standard radioimmunoassays (Human Leptin RIA KIT, Linco Research, St. Charles, MO; Pharmacia Insulin RIA100, Pharmacia Pharmacia & Upjohn, Uppsala, Sweden; Lumitest ACTH, Brahms Diagnostica, Hennigsdorf, Germany; Cortisol-RIA, DPC Biermann GmbH, Bad Nauheim, Germany; HADP-61HK adiponectin kit, Linco Research, St. Charles, MO). Serum concentrations of growth hormone (Immulite, DPC, Los Angeles, CA, USA) and insulin-like growth factor (IGF-I; Active IGF-I, Diagnostics Systems Laboratories, Inc., Sinsheim, Germany) were measured by ELISA. Plasma glucose was measured spectrophotometrically with the Hexokinase/G-6-PDH assay (Aeroset, Abbott, Wiesbaden, Germany). Intervals between weekly sessions were seven days but adjusted to minor extents in order to accommodate individual schedules of the participants.

Statistical analyses

Statistical analyses were based on analyses of variance (ANOVA) with the between-subject factor ‘group’ and the within-subject factor ‘time’. Analyses of psychological tasks included baseline values as covariates to take into account interindividual variations. Also, individual delays between sessions (expressed as number of days) were introduced into the analyses of delayed word list recall and word-stem priming to adjust for variations in retrieval intervals. In order to obtain a measure of declarative memory decay, immediate recall performance on the word list task was subtracted from delayed recall values. Student’s t tests for independent samples were used for pairwise post-hoc comparisons between groups. Values are expressed as means ± SEM and a p value < 0.05 was considered significant.

Results

Body weight, body composition and eating-related assessments

Body weight of the three groups, morning insulin, evening insulin and control, generally increased during the treatment period, i.e., between the last baseline examination and the session after eight weeks of insulin or placebo administration ($F(4,144)=2.41$, $p=0.046$ for *Time*; Figure 1B). There were no differences between groups ($F8,144)=1.26$, $p=0.26$ for *Group* × *Time*; $F(2,33)=0.16$, $p=0.86$ for *Group*), and neither any differences between morning and evening insulin administration ($p>0.39$). BMI values mirrored this pattern ($F(4,143)=2.32$, $p=0.055$ for *Time* and $p>0.31$ for treatment-related comparisons). Body fat content likewise displayed a general trend towards increased values between baseline and final examination ($F2,44)=2.5$, $p=0.09$) which, however, did not depend on insulin treatment ($p>0.13$). In the same time period, fat-free mass remained unchanged ($p>0.77$) and was likewise not altered by insulin treatment (all $p>0.10$) as were body cell mass, body water and intracellular water (all $p>0.10$). Even before the insulin intervention, extracellular water appeared generally decreased in the group receiving insulin in the morning compared to the evening insulin and the control group ($F(2,33)=4.65$, $p=0.017$ for *Group*), with no time-dependent changes to this pattern (all $p>0.31$). See Table 1 for a summary of body composition measures. Waist circumference did not change over time nor in dependence of insulin treatment ($p>0.30$).

Hunger ratings remained constant across the experimental period and were not modulated by treatment (all $p>0.32$). Values of the subscales ‘hunger’ and ‘suggestibility’ of the eating behavior questionnaire were likewise independent of time and treatment (all $p>0.09$). ‘Cognitive control’ according to this questionnaire was generally more strongly expressed in the morning insulin group (6.1 ± 0.8, averaged across the results obtained at the end of the baseline and after four and eight weeks of treatment; $F(2,33)=3.90$, $p=0.03$ for *Group*) than in the evening insulin (3.9 ± 1.0; $p=0.09$) and the control group (2.8 ± 0.7;

p=0.008), but did not change during the intervention (p>0.39 for *Time* × *Group* and *Time*). Thirst ratings were stable and unrelated to the intervention (p>0.20 for all comparisons). The analysis of tiredness ratings indicated a significant interaction between the factors *Group* and *Time* (F(4,61)=3.77, p=0.009; p>0.41 for the factors per se) that was due to an effect of insulin administration in the morning (F(2,33)=3.58, p<0.05; p>0.19 for comparisons between evening insulin and placebo). Thus, after four weeks of morning insulin administration, rated tiredness showed a steeper decline during the experimental session (-1.1 ± 0.3) than both after evening insulin (-0.2 ± 0.2; p=0.01) and placebo (-0.08 ± 0.4; p=0.04).

Control assessments of hemodynamic parameters did not indicate robust treatment effects or changes across time. Averaged across the experimental period, diastolic blood pressure reached values of 71.59 ± 1.63 mmHg (morning insulin), 72.11 ± 2.01 mmHg (evening insulin), and 72.30 ± 2.16 mmHg (placebo; p > 0.07 for all comparisons). Systolic blood pressure was 130.23 ± 2.02 mmHg (morning insulin), 129.10 ± 2.83 (evening insulin), and 127.88 ± 2.36 mmHg (placebo; p > 0.06). Heart rate averaged 63.77 ± 1.76 bpm (morning insulin), 67.60 ± 2.93 bpm (evening insulin), and 69.11 ± 1.99 bpm (placebo; p > 0.24).

Memory tasks and mood

Word list. Immediate recall of words was generally well comparable at baseline (p>0.16 for all overall comparisons), although for emotional words, the performance level in the morning insulin group tended to be below that of the placebo group (Table 2; note that post-baseline outcomes are baseline-adjusted). Insulin treatment did not have a systematic effect on the immediate recall of words. Delayed recall of words (assessed one week after encoding) appeared to benefit from insulin administration in the evening. For the sum of all words recalled after five weeks of treatment, the ANCOVA factor *Group* displayed a trend (F(2,30)=2.73, p=0.08), and participants of the evening insulin compared to the morning insulin group performed better on the recall of neutral as well as of all words, with the placebo group in-between. There were also signs of improvements in the delayed recall of

emotional words after five weeks of insulin administration in the evening vs. morning and placebo (Table 2).

We also analyzed the differences between immediate and delayed word list recall to obtain a measure of forgetting and found that memory decay was less pronounced in the evening insulin than the placebo group, with morning insulin in-between, in roughly half of sessions and categories combined (Figure 2). This pattern was corroborated on a tendency level for the sum of words recalled after one week of administration ($F(2,30)=2.61$, $p=0.09$), when it seemed particularly salient for emotional words, but was likewise visible after five weeks and, on a descriptive level, also eight weeks of treatment. Morning insulin administration appeared to curb the decay of memory for neutral words assessed after one week of treatment.

Word-stem priming. Performance on the word-stem priming task remained completely unaffected by insulin treatment both during immediate ($p>0.52$ for all overall comparisons) and delayed testing ($p>0.46$; see Table 3 for detailed results).

Mood. Results of the adjective scale provided to our participants to self-rate current mood on 15 dimensions indicated that self-rated concentration was enhanced in the participants of the insulin groups compared to those of the placebo group after four and eight weeks of treatment ($F(2,31)=5.54$, $p=0.009$ for *Group*; $p>0.12$ for *Time* and interaction), reaching mean values of 72.88 ± 5.80% (evening insulin), 73.39 ± 5.80% (morning insulin) and 48.76 ± 6.06% in the placebo group. All other scores remained unchanged, i.e., activation ($p>0.15$ for the factors *Group*, *Time* and respective interaction), deactivation ($p>0.12$), tiredness ($p>0.13$), numbness ($p>0.63$), extraversion ($p>0.20$), introversion ($p>0.64$), self-assuredness ($p>0.17$), mood ($p>0.57$), excitation ($p>0.21$), sensitivity ($p>0.21$), anger ($F(1,31)=3.73$, $p>0.06$ for *Time*; $p>0.31$ for *Group* and interaction), anxiousness ($p>0.28$), depression ($p>0.91$), and dreaminess ($p>0.32$).

Blood parameters

Circulating concentrations of glucose and endocrine parameters, except for serum cortisol, remained unaffected by insulin treatment (Figure 3). No group differences emerged for serum insulin and plasma glucose (p>0.16 for *Group* and *Group* × *Time*), which also remained stable during the experimental period (p>0.10). While plasma adrenocorticotropin was not altered by any of the insulin interventions (p>0.14) and temporal fluctuations failed to reach significance (F(6,188)=2.08, p=0.06), serum cortisol concentrations were suppressed in the morning insulin compared to both other groups after two weeks of administration (Figure 3D; F(15,242)=1.95, p=0.02; p>0.42 for *Group* and *Time*). Serum leptin concentrations remained unchanged (all p>0.10); plasma adiponectin concentrations did not respond to treatment (all p>0.49) but appeared to increase once a month independent of treatment, with a respective trough at the end of experiments (Figure 3F; F(5,177)=2.36, p=0.04). There were no robust treatment effects on serum concentrations of growth hormone (all p>0.09) and IGF-1 (p>0.41, p>0.06 for *Time*).

Discussion

Building on our previous studies in which we administered four daily doses of 40 IU intranasal insulin (Benedict et al., 2004; Hallschmid et al., 2004), here we investigated the metabolic and cognitive outcomes of eight-week once-daily administration of 160 IU insulin in healthy men. We expected to find superior effects of insulin administration in the evening compared to delivery in the morning and placebo, assuming that enhanced brain insulin signaling and sleep would interact to improve metabolic control and cognitive function. Neither the evening nor the morning schedule of insulin delivery exerted traceable effects on body weight and hunger regulation; in contrast, we found moderate signs of improved declarative memory consolidation during evening compared to morning insulin and placebo

administration. Insulin treatment also reduced circulating cortisol concentrations and exerted stimulating psychobehavioral effects, demonstrating the principal efficacy of our intervention.

Against the background of a slight general increase in body weight and body fat content across the experimental period, we did not detect robust effects on body weight and body composition of intranasal insulin delivered in the morning or evening, which stands in contrast to our previous observation of an insulin-induced loss of around 1.4 kg body fat in healthy men who received the peptide four times a day, i.e., before main meal intake and before going to bed (Hallschmid et al., 2004). Those subjects also displayed signs of insulin-induced reductions in hunger that were absent in the participants of the current study. Independent of insulin treatment, the subjects of the morning insulin group had higher levels of extracellular water than those of the other groups, so that subtle interactions between central insulin signaling and water homeostasis cannot be ruled out (Hallschmid et al., 2004; ter Maaten et al., 1999). However, such effects can be ruled out for the participants who received insulin in the evening and, nevertheless, did not show insulin-induced changes in body composition. This result is particularly puzzling because in previous experiments, the intranasal administration of 160 IU insulin to healthy men before bedtime led to an acute reduction of breakfast intake by 175 kcal (Santiago & Hallschmid, 2017). Since body weight in the evening insulin group was not affected even in the first weeks of treatment, this suggests that central nervous insulin administration before sleep might lose its catabolic impact rather quickly. Alternatively, counteracting mechanisms like centrally mediated increases in lipogenesis (Koch et al., 2008) might set in which, however, were not detectable in the present experiments. It should also be noted that animal experiments have not unanimously shown hypophagic effects of central insulin delivery (Jessen et al., 2010; Manin et al., 1988). Taken together and with a view to potential clinical applications, the current data indicate that the effects of intranasal insulin on body weight and body fat are clearly stronger

when smaller individual doses (e.g., 40 IU) are delivered before meal or snack intake (Hallschmid et al., 2004; Benedict et al., 2008; Hallschmid et al., 2012).

After two weeks of treatment onset, insulin administration in the morning compared to the evening, and to placebo, reduced cortisol concentrations. This finding replicates our previous observation in obese men receiving 4×40 IU/day (Hallschmid et al., 2008) and is in line with results found after eight weeks of 4×40 IU/day treatment in normal-weight men (Benedict et al., 2004), as well as with the acute insulin-induced suppression of nocturnal cortisol concentrations in elderly subjects (Thienel et al., 2017). Dampening effects of central insulin on hypothalamo-pituitary-adrenal (HPA) axis activity may be mediated by enhanced corticosteroid feedback processing in the hippocampus (de Kloet et al., 2018), which is assumed to exert inhibiting control over the HPA system via projections to the hypothalamus (Jacobson & Sapolsky, 1991). Considering that the participants of the morning insulin group were instructed not to administer insulin before blood sampling – excluding acute insulin effects –, these findings point to respective long-term changes. The absence of robust hemodynamic effects corroborates our previous finding that central insulin administration raises blood pressure acutely, but not after long-term delivery (Benedict et al., 2005).

In the cognitive domain, we detected relatively subtle insulin effects that nevertheless suggest greater efficacy of insulin delivery in the evening compared to the morning. Delayed recall of words presented one week earlier appeared to be generally enhanced after five weeks of evening insulin administration, which connects to our previous investigation into the acute impact of intranasal insulin given before bedtime on sleep-associated memory formation (Feld et al., 2016). In that study, insulin did not directly improve the consolidation of declarative memory contents, but impaired the acquisition of new, interfering contents learned on the subsequent day, suggesting that the peptide inhibits processes of active forgetting during sleep (Feld & Diekelmann, 2015). Across several weeks, such changes may yield the improvements seen in word list recall in the present study. Beneficial contributions of brain insulin to

memory function are likely mediated via insulin receptors located in the hippocampus and connected limbic brain structures (Unger et al, 1991) because down-regulating hippocampal insulin receptor function impairs long term potentiation and spatial memory (Grillo et al, 2015). Considering that long-term depression and potentiation support the establishment of hippocampal memory traces (Born & Feld, 2012; Goh & Manahan-Vaughan, 2015), insulin may exert some of its memory-improving effects by modulating these plastic processes. In the present experiments, memory decay between immediate and delayed word recall appeared mitigated already within one week of evening insulin administration, suggesting a rapid onset of respective effects. Notably, in our previous experiments based on the 4×40 IU/d paradigm, the beneficial effect of insulin emerged only after eight weeks of treatment (Benedict et al., 2004; 2007 – memory recall after five weeks was not tested in those studies), i.e., at a time when insulin's impact already appeared to wane in the present study. This pattern may imply different long-term dynamics of the memory effect depending on the exact insulin administration paradigm which, however, need to be specified in further investigations. Improved self-rated concentration and reduced tiredness due to insulin, which were not observed in the previous studies (Benedict et al., 2004; 2007), might have further enhanced cognitive function.

In line with our initial experiments (Benedict et al., 2004; 2007), the immediate recall of words and non-declarative memory function, as assessed by the word-stem priming task, were not affected by insulin. However, the morning insulin compared to the evening insulin and the control groups displayed signs of generally weaker immediate recall performance, which limits respective conclusions. While group sizes were generally comparable to those of previous studies (Benedict et al., 2004; 2007; Reger et al., 2005; 2006), larger samples might be needed to corroborate and expand the present findings. They should also include female subjects, although previous experiments suggest that the cognitive impact of intranasal insulin

differs between men and women after acute (Benedict et al., 2008) but not long-term administration (Benedict et al., 2004).

In sum, our finding that once-daily intranasal administration of 160 IU insulin does not affect body weight regulation but improves declarative memory function when scheduled in the evening may be of particular relevance for potential clinical applications in the metabolic as well as cognitive domain (Ott et al., 2012; Kullmann et al., 2016). The results imply that subchronic once-daily administration of high insulin doses is not superior to treatment regimens spread across the day. Considering that obese men treated with 4×40 IU/d of intranasal insulin show memory improvements but no change in body weight (Hallschmid et al., 2008), they also support the tentative assumption that the cognitive impact of intranasal insulin is generally more robust than its metabolic outcomes. Central nervous insulin delivery has long been proposed as a promising intervention to alleviate cognitive impairments for example in patients with AD (Craft et al., 2012; 2017). In this context, the timing of insulin administration certainly deserves a closer look, not least when taking into account interactions with sleep-associated processes.

Conflict of interest statement

The authors declare no conflict of interest

Author contribution statement

W.K., C.B. and M.H. designed the study. E.-M.E. and S.J. enrolled subjects, performed experiments and contributed to data analyses. Y.R. and M.H. analyzed and interpreted the data and wrote the manuscript.

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References

Air EL, Benoit SC, Blake Smith KA, Clegg DJ, and Woods SC (2002). Acute third ventricular administration of insulin decreases food intake in two paradigms. Pharmacol Biochem Behav 72:423-9.

Baura GD, Foster DM, Porte D Jr., Kahn SE, Bergman RN, Cobelli C, Schwartz MW (1993). Saturable transport of insulin from plasma into the central nervous system of dogs in vivo. A mechanism for regulated insulin delivery to the brain. J Clin Invest 92:1824-30.

Benedict C, Dodt C, Hallschmid M, Lepiorz M, Fehm HL, Born J, Kern W (2005). Immediate but not long-term intranasal administration of insulin raises blood pressure in human beings. Metabolism 54:1356-61.

Benedict C, Grillo CA (2018). Insulin resistance as a therapeutic target in the treatment of Alzheimer's disease: A state-of-the-art review. Front Neurosci 12:215.

Benedic C, Hallschmid M, Hatke A, Schultes B, Fehm HL, Born J, Kern W (2004). Intranasal insulin improves memory in humans. Psychoneuroendocrinology 29:1326-34.

Benedict C, Hallschmid M, Schmitz K, Schultes B, Ratter F, Fehm HL, Born J, Kern W (2007). Intranasal insulin improves memory in humans: superiority of insulin aspart. Neuropsychopharmacology 32:239-43.

Benedict C, Kern W, Schultes B, Born J, Hallschmid M (2008). Differential sensitivity of men and women to anorexigenic and memory-improving effects of intranasal insulin. J Clin Endocrinol Metab 93: 1339-44.

Born J, Feld GB (2012). Sleep to upscale, sleep to downscale: balancing homeostasis and plasticity. Neuron 75:933-5.

Born J, Lange T, Kern W, Mcgregor GP, Bickel U, Fehm HL (2002). Sniffing neuropeptides: a transnasal approach to the human brain. Nat Neurosci 5:514-6.

Brondel L, Romer MA, Nougues PM, Touyarou P, Davenne D (2010). Acute partial sleep deprivation increases food intake in healthy men. Am J Clin Nutr 91:1550-9.

Brunner YF, Kofoet A, Benedict C, Freiherr J (2015). Central insulin administration improves odor-cued reactivation of spatial memory in young men. J Clin Endocrinol Metab 100:212-9.

Cappuccio FP, D'Elia L, Strazzullo P, Miller MA (2010). Quantity and quality of sleep and incidence of type 2 diabetes: a systematic review and meta-analysis. Diabetes Care 33:414-20.

Craft S, Baker LD, Montine TJ, Minoshima S, Watson GS, Claxton A, Arbuckle M, Callaghan M, Tsai E, Plymate SR, Green PS, Leverenz J, Cross D, Gerton B (2012). Intranasal insulin therapy for Alzheimer disease and amnestic mild cognitive impairment: a pilot clinical trial. Arch Neurol 69:29-38.

Craft S, Claxton A, Baker LD, Hanson AJ, Cholerton B, Trittschuh EH, Dahl D, Caulder E, Neth B, Montine TJ, Jung Y, Maldjian J, Whitlow C, Friedman S (2017). Effects of regular and long-acting insulin on cognition and Alzheimer's disease biomarkers: a pilot clinical trial. J Alz Dis 57:1325-34.

de Felice FG (2013). Alzheimer's disease and insulin resistance: translating basic science into clinical applications. J Clin Invest 123:531-39.

de Kloet ER, Meijer OC, de Nicola AF, de Rijk RH, Joëls M (2018). Importance of the brain corticosteroid receptor balance in metaplasticity, cognitive performance and neuro-inflammation. Front Neuroendocrinol 49:124-45.

Dhuria SV, Hanson LR, Frey WH, 2nd (2010). Intranasal delivery to the central nervous system: mechanisms and experimental considerations. J Pharm Sci 99:1654-73.

Diekelmann S, Born J (2010). The memory function of sleep. Nat Rev Neurosci 11:114-26.

Feld GB, Born J (2017). Sculpting memory during sleep: concurrent consolidation and forgetting. Curr Opin Neurobiol 44:20-7.

Feld GB, Diekelmann S (2015). Sleep smart-optimizing sleep for declarative learning and memory. Front Psychol 6:622.

Feld GB, Wilhem I, Benedict C, Rudel B, Klameth C, Born J, Hallschmid M (2016). Central nervous insulin signaling in sleep-associated memory formation and neuroendocrine regulation. Neuropsychopharmacology 41:1540-50.

Gangwisch JE, Heymsfield SB, Boden-Albala B, Buijs RM, Kreier F, Pickering TG, Rundle AG, Zammit GK, Malaspina D (2007). Sleep duration as a risk factor for diabetes incidence in a large U.S. sample. Sleep 30:1667-73.
Goh JJ, Manahan-Vaughan D (2015). Role of inhibitory autophosphorylation of calcium/calmodulin-dependent kinase II (alphaCAMKII) in persistent (>24 h) hippocampal LTP and in LTD facilitated by novel object-place learning and recognition in mice. Behav Brain Res 285:79-88.
Gray SM, Meijer RI, Barrett EJ (2014). Insulin regulates brain function, but how does it get there? Diabetes 63:3992-7.
Greenwood CE, Kaplan RJ, Hebblethwaite S, Jenkins DJ (2003). Carbohydrate-induced memory impairment in adults with type 2 diabetes. Diabetes Care 26:1961-6.
Grillo CA, Piroli GG, Lawrence RC, Wrighten SA, Green AJ, Wilson SP, Sakai RR, Kelly SJ, Wilson MA, Mott DD, Reagan LP (2015). Hippocampal insulin resistance impairs spatial learning and synaptic plasticity. Diabetes 64:3927-36.
Hallschmid M, Benedict C, Schultes B, Fehm HL, Born J, Kern W (2004). Intranasal insulin reduces body fat in men but not in women. Diabetes 53:3024-9.
Hallschmid M, Benedict C, Schultes B, Born J, Kern W (2008). Obese men respond to cognitive but not to catabolic brain insulin signaling. Int J Obes (Lond) 32:275-82.
Hallschmid M, Higgs S, Thienel M, Ott V, Lehnert H (2012). Postprandial administration of intranasal insulin intensifies satiety and reduces intake of palatable snacks in women. Diabetes 61:782-9.
Heni M, Kullmann S, Preissl H, Fritsche A, Haring HU (2015). Impaired insulin action in the human brain: causes and metabolic consequences. Nat Rev Endocrinol 11:701-11.
Jacobson L, Sapolsky R (1991). The role of the hippocampus in feedback regulation of the hypothalamic–pituitary–adrenocortical axis. Endocr Rev 12:118-34.
Janke W, Debus G (1978). Die Eigenschaftswörterliste (EWL). Göttingen, Germany: Hogrefe.
Jessen L, Clegg DJ, Bouman SD (2010). Evaluation of the lack of anorectic effect of intracerebroventricular insulin in rats. Am J Physiol Regul Integr Comp Physiol 298:R43-50.
Koch L, Wunderlich FT, Seibler J, Konner AC, Hampel B, Irlenbusch S, Brabant G, Kahn CR, Schwenk F, Bruning JC (2008). Central insulin action regulates peripheral glucose and fat metabolism in mice. J Clin Invest 118:2132-47.
Kullmann S, Heni M, Hallschmid M, Fritsche A, Preissl H, Haring HU (2016). Brain insulin resistance at the crossroads of metabolic and cognitive disorders in humans. Physiol Rev 96:1169-1209.
Lee SH, Zabolotny JM, Huang H, Lee H, Kim YB (2016). Insulin in the nervous system and the mind: Functions in metabolism, memory, and mood. Mol Metab 5:589-601.
Magee L, Hale L (2012). Longitudinal associations between sleep duration and subsequent weight gain: a systematic review. Sleep Med Rev 16:231-41.
Manin M, Balage M, Larue-Achagiotis C, Grizard J (1988). Chronic intracerebroventricular infusion of insulin failed to alter brain insulin-binding sites, food intake, and body weight. J Neurochem 51:1689-95.
Mcgowan MK, Andrews KM, Grossman SP (1992). Chronic intrahypothalamic infusions of insulin or insulin antibodies alter body weight and food intake in the rat. Physiol Behav 51:753-66.
Molnar G, Farago N, Kocsis AK, Rozsa M, Lovas S, Boldog E, Baldi R, Csajbok E, Gardi J, Puskas LG, Tamas G (2014). GABAergic neurogliaform cells represent local sources of insulin in the cerebral cortex. J Neurosci 34:1133-7.
Ott V, Benedict C, Schultes B, Born J, Hallschmid M (2012). Intranasal administration of insulin to the brain impacts cognitive function and peripheral metabolism. Diabetes Obes Metab 14:214-21.
Plihal W, Born J (1999). Effects of early and late nocturnal sleep on priming and spatial memory. Psychophysiology 36:571-82.
Pudel V, Westenhöfer J (1989). Fragebogen zum Essverhalten: Handanweisung. Göttingen, Germany: Hogrefe.
Reger MA, Watson GS, Frey WH, 2nd, Baker LD, Cholerton B, Keeling ML, Belongia DA, Fishel MA, Plymate SR, Schellenberg GD, Cherrier MM, Craft S (2006). Effects of intranasal insulin on cognition in memory-impaired older adults: modulation by APOE genotype. Neurobiol Aging 27:451-8.

Reger MA, Watson GS, Green PS, Wilkinson CW, Baker LD, Cholerton B, Fishel MA, Plymate SR, Breitner JCS, Degroodt W, Mehta P, Craft S (2008). Intranasal insulin improves cognition and modulates beta-amyloid in early AD. Neurology 70:440-8.

Santiago JC, Hallschmid M (2017). Central nervous insulin administration before nocturnal sleep decreases breakfast intake in healthy young and elderly subjects. Front Neurosci 11:54.

Schmid SM, Hallschmid M, Jauch-Chara K, Wilms B, Lehnert H, Born J, Schultes B (2011). Disturbed glucoregulatory response to food intake after moderate sleep restriction. Sleep 34:371-7.

St-Onge MP (2013). The role of sleep duration in the regulation of energy balance: effects on energy intakes and expenditure. J Clin Sleep Med 9:73-80.

ter Maaten JC, Bakker SJ, Serne EH, ter Wee PM, Donker AJ, Gans RO (1999). Insulin's acute effects on glomerular filtration rate correlate with insulin sensitivity whereas insulin's acute effects on proximal tubular sodium reabsorption correlation with salt sensitivity in normal subjects. Nephrol Dial Transplant 14:2357-63.

Thienel M, Wilhelm I, Benedict C, Born J, Hallschmid M (2017). Intranasal insulin decreases circulating cortisol concentrations during early sleep in elderly humans. Neurobiol Aging 54:170-74.

Unger JW, Livingston JN, Moss AM (1991). Insulin receptors in the central nervous system: localization, signalling mechanisms and functional aspects. Prog Neurobiol 36:343-62.

Vgontzas AN, Fernandez-Mendoza J, Miksiewicz T, Kritikou I, Shaffer ML, Liao D, Basta M, Bixler EO (2014). Unveiling the longitudinal association between short sleep duration and the incidence of obesity: the Penn State Cohort. Int J Obes (Lond) 38:825-32.

Woods SC, Lotter EC, Mckay LD, Porte D Jr (1979). Chronic intracerebroventricular infusion of insulin reduces food intake and body weight of baboons. Nature 282:503-5.

Table 1. Body composition.

	Placebo	Morning insulin	Evening insulin
Baseline			
Body fat (kg)	11.38 ± 1.34	14.48 ± 1.05	12.05 ± 1.43
Fat free mass (kg)	67.08 ± 1.82	61.76 ± 1.58	65.28 ± 1.49
Total body water (kg)	49.12 ± 1.34	45.22 ± 1.15	47.78 ± 1.09
Intracellular water (kg)	28.98 ± 0.86	27.08 ± 0.74	28.31 ± 0.70
Extracellular water (kg)	20.14 ± 0.51	18.14 ± 0.44*	19.48 ± 0.43
Body cell mass (kg)	37.95 ± 1.14	35.14 ± 0.96	37.85 ± 0.87
4 weeks of treatment			
Body fat (kg)	11.66 ± 1.25	14.65 ±1.12	11.53 ± 1.38
Fat free mass (kg)	66.52 ± 2.17	61.77 ± 1.66	66.62 ± 1.73
Total body water (kg)	48.71 ± 1.59	45.23 ± 1.21	48.76 ± 1.27
Intracellular water (kg)	28.78 ± 0.97	27.16 ±0.79	28.78 ± 0.82
Extracellular water (kg)	19.93 ± 0.64	18.07 ± 0.45*	19.98 ± 0.49
Body cell mass (kg)	37.61 ± 1.31	35.28 ± 1.05	38.15 ± 0.92
8 weeks of treatment			
Body fat (kg)	11.65 ± 1.17	15.21 ± 1.13	12.48 ± 1.49
Fat free mass (kg)	66.94 ± 2.18	61.69 ± 1.61	65.71 ± 1.66
Total body water (kg)	49.02 ± 1.59	45.17 ± 1.18	48.10 ± 1.21
Intracellular water (kg)	28.94 ± 0.96	27.17± 0.76	28.63 ± 0.81
Extracellular water (kg)	20.08 ± 0.66	18.00 ± 0.45*	19.48 ± 0.44
Body cell mass (kg)	37.87 ± 1.21	35.35 ± 1.07	37.94 ± 1.02

Results are mean ± SEM. N=12 per group; * $p < 0.05$ for comparisons between the morning insulin and placebo/evening insulin groups.

Table 2. Immediate and delayed word list recall.

Immediate recall	**Placebo**	**Morning insulin**	**Evening insulin**
Baseline			
Food-related	3.79 ± 0.37	3.25 ± 0.37	3.29 ± 0.32
Emotional	4.42 ± 0.34	3.54 ± 0.26[a]	4.17 ± 0.37
Neutral	3.50 ± 0.40	3.08 ± 0.34	3.46 ± 0.34
All words	11.71 ± 0.89	9.88 ± 0.83	10.92 ± 0.71
4 weeks of treatment			
Food-related	3.73 ± 0.35	3.23 ± 0.34	3.37 ± 0.34
Emotional	4.35 ± 0.46	4.64 ± 0.47	4.34 ± 0.45
Neutral	4.75 ± 0.49	3.48 ± 0.49[a]	4.27 ± 0.49
All words	12.51 ± 0.89	11.80 ± 0.90	11.85 ± 0.88
7 weeks of treatment			
Food-related	3.83 ± 0.45	3.76 ± 0.45	4.07 ± 0.44
Emotional	4.63 ± 0.47	4.36 ± 0.47	3.93 ± 0.46
Neutral	4.15 ± 0.55	3.50 ± 0.55	4.26 ± 0.55
All words	12.40 ± 1.10	11.83 ± 1.10	12.27 ± 1.08
Delayed recall			
1 week of treatment			
Food-related	1.84 ± 0.41	0.93 ± 0.41	1.06 ± 0.41
Emotional	1.44 ± 0.46	1.39 ± 0.48	2.25 ± 0.46
Neutral	1.47 ± 0.30	1.47 ± 0.30	1.15 ± 0.31
All words	4.60 ± 0.82	3.84 ± 0.81	4.56 ± 0.82
5 weeks of treatment			
Food-related	1.26 ± 0.35	0.48 ± 0.35	1.10 ± 0.36
Emotional	1.19 ± 0.37	1.16 ± 0.37	2.15 ± 0.37[b]
Neutral	1.69 ± 0.48	0.81 ± 0.48	2.25 ± 0.48*
All words[c]	3.96 ± 0.91	2.56 ± 0.90	5.57 ± 0.91*
8 weeks of treatment			
Food-related	1.19 ± 0.33	0.72 ± 0.36	1.08 ± 0.33
Emotional	0.98 ± 0.31	1.31 ± 0.31	1.63 ± 0.32
Neutral	0.96 ± 0.41	1.05 ± 0.41	1.02 ± 0.42
All words	3.03 ± 0.78	3.13 ± 0.78	3.85 ± 0.80

Results are mean ± SEM. N=12 per group; [a] $p<0.10$ for comparison between the placebo and the morning insulin group, [b] $p<0.10$ for comparison between the evening insulin and the placebo/morning insulin groups, [c] $p<0.10$ for ANCOVA factor *Group*, * $p<0.05$ for comparisons between the evening and morning insulin groups.

555 **Table 3. Results of the word-stem priming task.**

	Placebo	Morning insulin	Evening insulin
Immediate recall			
Baseline	4.15 ± 0.67	3.47 ± 0.67	3.22 ± 0.67
4 weeks of treatment	3.75 ± 0.65	3.41 ± 0.65	3.34 ± 0.65
7 weeks of treatment	4.46 ± 0.82	4.94 ± 0.82	4.10 ± 0.82
Delayed recall			
1 week of treatment	0.82 ± 0.42	0.81 ± 0.42	0.62 ± 0.42
5 weeks of treatment	0.37 ± 0.46	0.59 ± 0.45	0.96 ± 0.46
8 weeks of treatment	1.05 ± 0.46	1.80 ± 0.46	1.15 ± 0.47

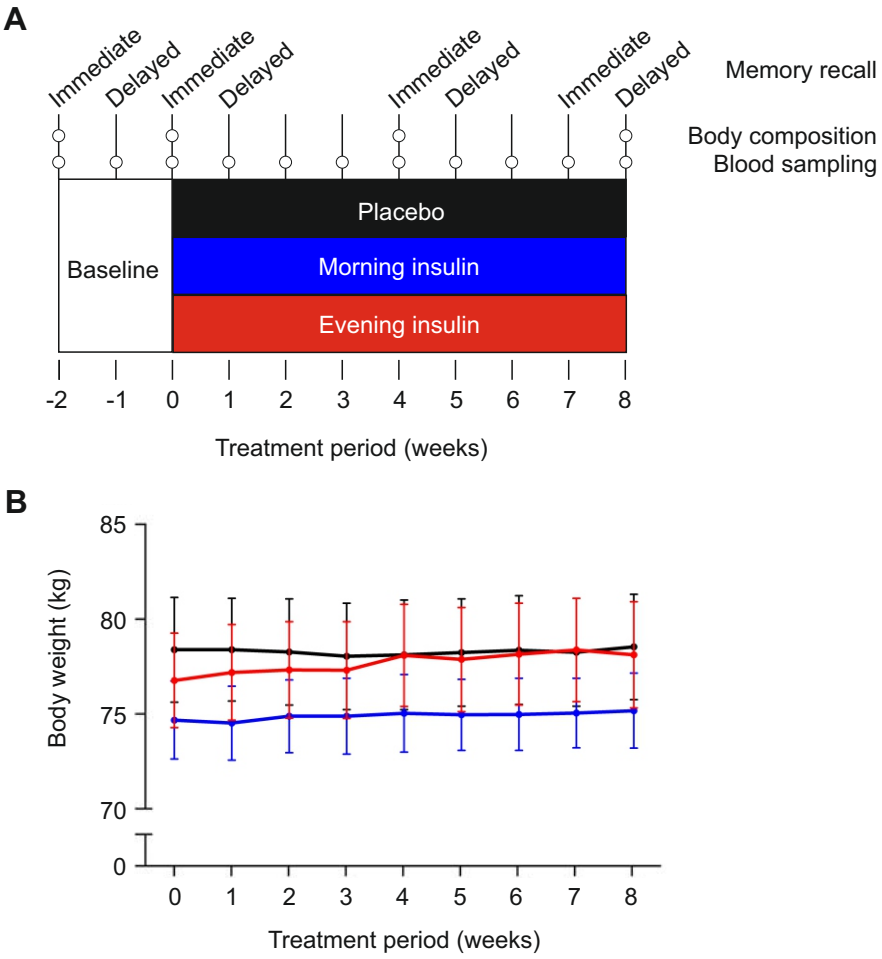
556 Results are mean ± SEM. N=12 per group.

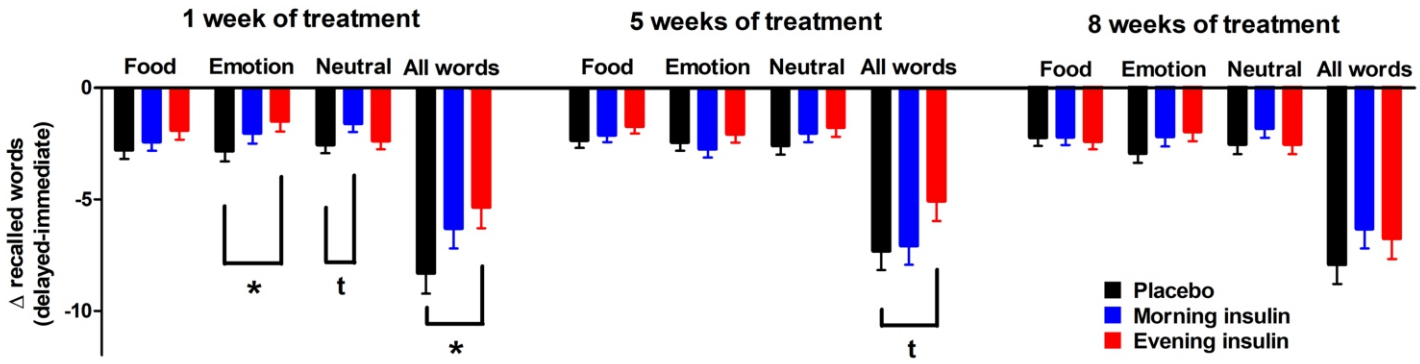
Figure legends

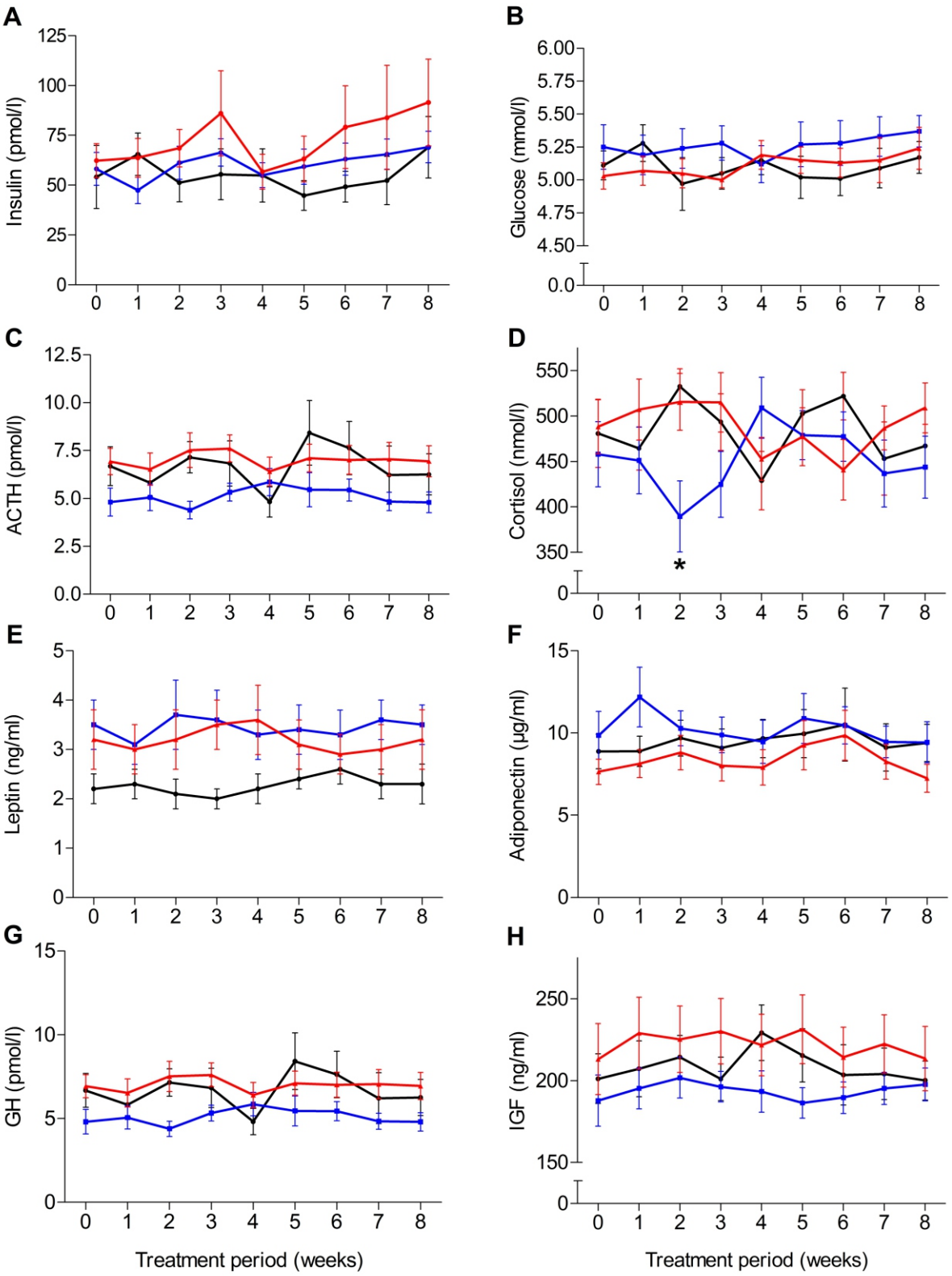
Figure 1. (A) Experimental procedure. After a placebo baseline period of two weeks, three groups of male subjects (each N=12) were submitted to eight weeks of intranasal insulin (160 IU) or placebo administration. The 'morning insulin' group self-administered insulin after awakening (or after the weekly examination) and placebo spray before going to bed; the 'evening insulin' group self-administered placebo spray in the morning and insulin spray before going to bed; the control group received placebo in the morning and evening. Metabolic and cognitive assessments took place as depicted; for methodological details, see text. **(B)** Average body weight (± SEM) in the three groups during insulin intervention or placebo treatment.

Figure 2. Memory decay between immediate and delayed word recall. Differences (± SEM) between the numbers of words (food-related, emotional, neutral and all words) from the word list recalled in the delayed and the immediate sessions, which took place roughly one week apart. Values were adjusted by ANCOVA for baseline differences and the individual delays between immediate and delayed recall. N=12 per group; * $p < 0.05$, $^{t}\,p < 0.10$.

Figure 3. Average (± SEM) serum or plasma concentrations of (A) insulin, (B) glucose, (C) adrenocorticotropin, (D) cortisol, (E) leptin, (F) adiponectin, (G) growth hormone and (H) insulin-like growth factor. N=12; * $p<0.05$ for comparisons between the morning and evening insulin/placebo groups.


A
Immediate
Delayed
Immediate
Delayed
Immediate
Delayed
Immediate
Delayed
Memory recall
Body composition
Blood sampling
Placebo
Baseline
Morning insulin
Evening insulin
-2
-1
0
1
2
3
4
5
6
7
8
Treatment period (weeks)
B
85
80
75
70
0
Body weight (kg)
0
1
2
3
4
5
6
7
8
Treatment period (weeks)


1 week of treatment
5 weeks of treatment
8 weeks of treatment
Food
Emotion
Neutral
All words
0
-5
-10
Δ recalled words
(delayed-immediate)
*
t
*
t
Placebo
Morning insulin
Evening insulin


A
Insulin (pmol/l)
125
100
75
50
25
0
0 1 2 3 4 5 6 7 8
B
Glucose (mmol/l)
6.00
5.75
5.50
5.25
5.00
4.75
4.50
0.0
0 1 2 3 4 5 6 7 8
C
ACTH (pmol/l)
12.5
10.0
7.5
5.0
2.5
0.0
0 1 2 3 4 5 6 7 8
D
Cortisol (nmol/l)
550
500
450
400
350
0
*
0 1 2 3 4 5 6 7 8
E
Leptin (ng/ml)
5
4
3
2
1
0
0 1 2 3 4 5 6 7 8
F
Adiponectin (µg/ml)
15
10
5
0
0 1 2 3 4 5 6 7 8
G
GH (pmol/l)
15
10
5
0
0 1 2 3 4 5 6 7 8
Treatment period (weeks)
H
IGF (ng/ml)
250
200
150
0
0 1 2 3 4 5 6 7 8
Treatment period (weeks)