

Intranasal Insulin Enhanced Resting-State Functional Connectivity of Hippocampal Regions in Type 2 Diabetes

Short title: Intranasal insulin effects on brain functional connectivity

Hui Zhang¹, Ying Hao¹, Bradley Manor^{1,2}, Peter Novak³, William Milberg⁴, Jue Zhang^{1,5}, Jing Fang^{1,5},
and Vera Novak⁶

¹ Academy for Advanced Interdisciplinary Studies, Peking University, Beijing, Beijing, China,

² Division of Gerontology, Beth Israel Deaconess Medical Center (BIDMC), Harvard Medical School,
Boston, Massachusetts, United States,

³ Department of Neurology, University of Massachusetts Medical School, Worcester, MA

⁴ New England Geriatric, Research, Education and Clinical Center–Boston Division, VA Boston
Healthcare, and Department of Psychiatry, Harvard Medical School, Boston, MA

⁵ College of Engineering, Peking University, Beijing, Beijing, China,

⁶ Department of Neurology, Beth Israel Deaconess Medical Center (BIDMC), Harvard Medical School,
Boston, Massachusetts, United States

Corresponding author:

Vera Novak, M.D. Ph.D.

Associate Professor of Neurology, Director Syncope and Falls in the Elderly Program

Department of Neurology, 185 Pilgrim Road, Palmer 127, Boston MA 02215, USA

Tel: 617-632-8680; Fax: 617-632-8805

Current word count: Abstract 200, MS 3639, 3 Figures, 3 Tables

Abstract

Type 2 diabetes mellitus alters brain function and manifests as brain atrophy. Intranasal insulin has emerged as a promising intervention for treatment of cognitive impairment. We evaluated the acute effects of intranasal insulin on resting-state brain functional connectivity in older adults with type 2 diabetes.

This proof-of-concept, randomized, double-blind, placebo-controlled study evaluated the effects of a single 40IU dose of insulin or saline in 14 diabetic and 14 control subjects. Resting-state functional connectivity between the hippocampal region and default mode network (DMN) was quantified using functional MRI (fMRI) at 3Tesla.

Following insulin administration, diabetic patients demonstrated increased resting-state connectivity between the hippocampal regions and the medio-frontal cortex (MFC) as compared to placebo (cluster size right, $p=0.03$), and other DMN regions. On placebo, the diabetes group had lower connectivity between the hippocampal region and the MFC as compared to controls (cluster size: right, $p=0.02$), but on insulin MFC connectivity was similar to controls. Resting state connectivity correlated with cognitive performance. A single dose of intranasal insulin increases resting-state functional connectivity between the hippocampal regions and multiple DMN regions in older adults with type 2 diabetes. Intranasal insulin administration may modify functional connectivity among brain regions regulating memory and complex cognitive behaviors.

Introduction:

Type 2 diabetes mellitus (DM) accelerates brain aging that manifests as a widespread generalized atrophy (1) and earlier onset of dementia and Alzheimer's disease (AD) (2). Aging, diabetes, and AD alter insulin transport and utilization in the brain (3). Central insulin is a neuromodulator involved in the key processes underlying cognition (4;5), energy homeostasis (6), synapse formation and neuronal survival (7).

Intranasal insulin administration delivers insulin directly to the brain (8), and therefore intranasal insulin administration is emerging as a promising tool to deliver therapeutics directly to the brain (9). Intranasal insulin increases regional perfusion (10;11) and improves cognition and memory (hippocampal function) in healthy young and older people (12;13), as well as in patients with cognitive impairment or mild AD (14).

Our proof-of-concept pilot study demonstrated that a single intranasal insulin dose of 40 IU acutely improved visuospatial memory in older people with type 2 DM and healthy controls (10). In diabetics, better cognitive performance following intranasal insulin administration correlated with regional vasodilatation in the middle cerebral artery territory and in the insular cortex. Still, the mechanisms for insulin-related improvement of memory (hippocampal function) remain unclear. Functional MRI (fMRI) studies have led to the characterization of a network, termed the default mode network (DMN), that is activated during wakeful rest and deactivated during the performance of cognitive tasks (15;16). Numerous brain regions within the DMN have been linked to higher cognitive processes (i.e., language and memory), including the medial temporal lobe, the medial pre-frontal cortex, anterior and posterior cingulate cortex and the medial, lateral and inferior parietal cortex (16;17). Older people with diabetes have worse functional connectivity among these regions, as compared to healthy controls, and the

abnormal neuronal connectivity may precede clinical manifestations of brain atrophy and cognitive impairment (18-20).

We hypothesized that intranasal insulin may acutely modify signaling between the hippocampus and the DMN regions that have been implicated in memory and cognitive processing. We acquired resting-state fMRI to identify functional connectivity between the hippocampus and DMN regions following the administration of intranasal insulin or placebo in older adults with and without type 2 DM.

Methods:

Design: We conducted a pilot, randomized, double-blind, placebo-controlled study with cross-over design of a single dose of INI or sterile saline in type 2 DM and healthy older adults (FDA-IND 107690; www.clinicaltrials.gov NCT01206322). Details of the study protocol have been reported and intranasal insulin administration was safe without affecting systemic glucose levels (10).

Subjects

The study was conducted at the Syncope and Falls in the Elderly (SAFE) Laboratory, the Center for Advanced MR imaging and the Clinical Research Center (CRC) at the Beth Israel Deaconess Medical Center (BIDMC). The protocol was approved by the BIDMC Committee on Clinical Investigation. Participants were recruited prospectively via advertisements in the local community. Diabetic participants were required to be diagnosed with type 2 DM for at least five years and treated with oral anti-diabetic agents. Controls were required to be normotensive, have fasting blood glucose <100 mg/dL and not be treated for any systemic disease. Of 262 participants who completed phone screen, 64 were eligible and provided written informed consent. Twenty-eight completed the protocol and were included in the

analyses: 14 diabetic (7 females, 61.7±8.1 years) and 14 healthy subjects (10 females, 60.1±9.9)

(Table 1).

Thirty-six participants were excluded for the following reasons: consent withdrawal (n=7), diagnosis of DM <5 years (n=3), insulin treatment (n=1), intranasal medication (n=1), abnormal laboratory results (n=3), controls with HbA1c >6% (n=4), uncontrolled hypertension (n=4), subthreshold MMSE scores (≤ 24 on age-adjusted norms) (n=2), psychiatric disorder (n=1), brain biopsy surgery (n=1), substance abuse (n=1), MRI-incompatible stents (n=1), hypoglycemic episodes during home monitoring (n=2), health care provider disapproval (n=1), lost to follow-up (n=3) and poor fMRI data quality due to motion artifacts (n=1).

On-site screening included: fasting laboratory chemistries, electrocardiogram, vital signs, detailed medical history and medication review, and anthropometric measurements. One control participant was excluded after randomization because of high blood pressure and one subject's data was excluded from analyses due to motion artifacts on the MRI scan. All other exclusions occurred before randomization during the screening phase. Glycemic control and other prescribed medications were taken during the study, but were held in the morning before the intervention, MRI and cognitive testing. Medications were administered at a usual dose after the completion of these procedures on Day 2 and Day 3. Participants had current prescriptions of one or more medications: glycemic control agents ((biguanides (metformin (n=11)), sulfonylureas (glyburide (n=4), glipizide (n=2) and thiazolidinediones (pioglitazone n=2)), antihypertensives (beta blockers n=5), angiotensin II receptors blockers (n=3), angiotensin-converting enzyme inhibitors (n=4), statins (n=10, controls n=0), hormone replacement (control=1). Women were required to be postmenopausal.

Protocol

Studies were conducted with at the BIDMC CRC. On CRC admission Day 1, participants completed a baseline cognitive assessment. On Day 2 and Day 3, protocols included safety monitoring for glucose and cardiovascular vital signs, insulin/placebo administration; anatomical and resting-state functional MR imaging and cognitive assessment. Resting-state fMRI was performed 26.5 ± 9.3 min after intranasal insulin administration. Vitals signs were also monitored during MRI using a Medrad® Veris® MR Vital Signs Monitor (Warrendale, PA).

Insulin/placebo administration

Each participant was treated with 40IU of insulin (Novolin^R, Novonordisk) or sterile saline in a random order on Day 2 and Day 3 using a ViaNase device (Kurve Technologies, Inc). Insulin administration contained 40 IU of insulin mixed with 0.4 ml of saline and an additional residual volume of 0.66ml (30IU of insulin mixed with 0.33 ml of saline). The placebo contained an equivalent volume of sterile saline.

Anatomical and functional MR imaging

Anatomical and functional studies were performed on a 3-Tesla GE HDx MRI scanner (GE Medical Systems, Milwaukee, WI) using the 3-D magnetization-prepared rapid gradient echo (MP-RAGE) (TR=6.6 ms, TE=2.8ms, FA=15°, Bandwidth=31.25 KHz, FOV=24, Slice Thickness =3mm, 52 slices, Matrix=192x256). Resting-state functional images were collected over a five minute period using a gradient-echo planar imaging pulse sequence sensitive to blood oxygenation level dependent (BOLD) contrast (TR=3000ms, TE=27ms, FA=60°, FOV=25, Slice Thickness=5mm, 30 slices, Matrix=64x64, NEX=1).

Neuropsychological Assessment

Baseline assessment (Day 1) included the Mini Mental State Exam (MMSE) and measures of verbal learning (Hopkins Verbal Learning Test-Revised (HVLTR)), executive function (Trail-Making Tests A and B, Digit Span). Cognitive assessment on insulin vs. placebo (Day 2 and Day 3) was done after MRI scan, and had to be completed within two hours after drug administration because of insulin pharmacokinetics (8;21;22). These assessments included a brief battery of parallel versions of the Brief Visuospatial Memory Test-Revised (BVMTR) and the verbal fluency measures (FAS, Category, and Switching conditions) of the Delis-Kaplan Executive Function System (D-KEFS) assessment (23;24).

Statistical Analyses

Statistical Parametric Mapping (SPM8, Wellcome Department of Cognitive Neurology, London; <http://www.fil.ion.ucl.ac.uk/spm>) was used to preprocess the raw functional MRI data, and resting-state fMRI Data Analysis (REST V1.8, <http://www.restfmri.net>) was used for the network correlation analysis.

The first two volumes of the scanning session were discarded to allow for T1 equilibration effects. The remaining images were corrected for timing differences between each slice using Fourier interpolation. The images were then corrected for motion effects, where the first volume of the scanning session was designated as the reference volume. One participant with head motion more than 2.0 mm maximum displacement in any direction of x, y, and z or 2.0 degree of any angular motion throughout the course of scan was excluded from the analyses. The mean EPI images were co-registered to the T1 images. Co-registered T1 images were normalized to the MNI (Montreal Neurological Institute Atlas) via SPM 8 tools. The resulting images were smoothed with a Gaussian kernel of 6mm×6mm×6mm (full-width half-maximum, FWHM). Linear trends were removed from the image time series and data

were band-pass filtered at 0.01–0.08 Hz.

A hypothesis-driven regions of interest (ROIs) approach was used to investigate the hippocampus and parahippocampus (hippocampal region) using the ROIs from the Wake Forest University PickAtlas (25). Bilateral hippocampus and parahippocampus were selected as seed regions and the correlations of time course between seed regions and the whole brain were calculated in a voxel-wise manner for each subject and condition (e.g. DM-insulin, DM-placebo, control- insulin, control-placebo). The Fisher transformation (r-to-z transformation) was used to normalize distribution of the Pearson correlation coefficient values (r) to standard z scores to represent the strength of connectivity (26). One-sample t tests (uncorrected, voxels with $p < 1 \times 10^{-9}$ and cluster size $\geq 270 \text{mm}^3$) were used to determine brain regions with significant connectivity to the seed regions in each state. Connectivity maps were compared between the insulin and placebo condition for each subject using a paired t-test. Two-sample t tests were used to compare the diabetes and control groups. The threshold was corrected with AlphaSim (AFNI, Bethesda, Maryland, USA; <http://afni.nimh.nih.gov/afni/>) in paired and two-sample t-tests ($p < 0.05$; minimum cluster size was set to 270mm^3).

Performances on the BVMT-R are reported as age-adjusted T scores for: the total learning score across the three immediate recall trials (Total Recall), delayed recall (Delayed Recall). Performances on the FAS, Category and Switching verbal fluency trials were also reported as age and education-adjusted T scores. Composite general cognitive function scores were calculated as average T Scores.

Least square (LS) models were used to evaluate the relationships between fMRI measures (regional Z scores) and cognitive measures (verbal fluency, BVMT-R, as dependent variables) with age and sex as model effects. A LS model for MMSE was adjusted for education years and race ((AA African/American). LS models were calculated separately within group and condition (e.g. diabetes

group on insulin) for each variable to minimize effects of multiple comparisons. Conservatively, we selected models with $R^2 > 0.25$, and $p < 0.05$, and we present R^2_{adj} adjusted for model covariates. Nominal observed p-values are reported without adjustment for multiple testing in this small proof of concept study.

Results

Demographic and baseline characteristics

Demographic group characteristics were similar (Table 1), but diabetic subjects had lower global gray matter volume ($p=0.03$), fewer years of education ($p=0.03$) and worse executive function ($p=0.004$) and verbal memory ($p=0.002$). Hippocampal volumes were similar between the groups.

Resting-state connectivity

Multiple regions within the DMN exhibited functionally connectivity to the right and left hippocampal regions. Figure 1 (A-F) depicts a summary of the DMN regions that were significantly correlated (voxels with $|t| \geq 15.4$, cluster size $\geq 270\text{mm}^3$) to bilateral hippocampal regions following intranasal insulin and placebo administration in the diabetes group (Figure 1A and 1B) and controls (Figure 1D and 1E).

In the diabetes group, insulin increased connectivity between the medial frontal cortex, right inferior parietal cortex, posterior cingulate gyrus and anterior cingulate cortex and hippocampal regions, as compared to placebo (Figure 1A-C, Table 2). The threshold was set at $p < 0.05$, voxel corrected, a minimum cluster size $= 270\text{mm}^3$.

Similarly, in the control group, insulin increased connectivity in the medial frontal cortex, posterior cingulate gyrus and anterior cingulate cortex (Figure 1 D-F, Table 2). Table 2 shows all regions connected

to the right or left hippocampal regions.

In addition, we calculated the strength of ipsilateral connections and an average regional cluster size for each subject, and compared the insulin and placebo conditions within each group (Table 3). In the diabetes group, insulin administration increased the average cluster size within the medio-frontal cortex that was functionally connected to the right hippocampal region, as compared to placebo ($p=0.03$). Following insulin administration, as compared to placebo, we also observed a trend towards an increase in cluster size within the left medio-frontal cortex (MFC) that was functionally connected to the left hippocampal region ($p=0.06$). The correlation between the right hippocampal region and the right inferior parietal cortex (R-IPC) also increased on insulin as compared to placebo (z value $p=0.03$). The group average peak z value range for all regions was 0.76-1.69 following insulin administration and 0.71-1.55 following administration of the placebo.

In the control group, insulin administration increased the average cluster size within the left posterior cingulate cortex (PCC) that was functionally connected to the left hippocampal region, as compared to placebo ($p=0.017$; z value $p=0.056$). Correlations between the left anterior cingulate cortex (ACC) and the left hippocampus also tended to be stronger (z value $p=0.056$).

The group average peak z value range for all regions was for insulin 0.71-1.69 and for placebo 0.73-1.64.

Figure 2 maps the differences between the diabetes and the control group after insulin (Figure 2A) and placebo (Figure 2B) administration. After insulin administration, the diabetes group still had worse functional connectivity in medio-frontal cortex as compared to healthy controls (Figure 2A), but these differences were less prominent than after the placebo administration (Figure 2B) (the threshold was set as Alphasim corrected $p<0.05$, a minimum cluster extent = 270mm^3).

Ipsilateral comparisons indicated that after placebo administration, the diabetes group had a smaller cluster of voxels within the medial frontal cortex that was functionally connected with the right hippocampus, as compared to controls (47% decrease; $p=0.019$; z value $p=0.31$). A similar trend was also observed for the connectivity between medial frontal cortex and the left hippocampus (58% reduction; $p=0.058$; z value $p=0.24$). However, diabetes group had a larger cluster of connectivity between right hippocampus and posterior cingulate gyrus as compared with controls (29% increase; $p=0.047$; z value $p=0.09$), and a similar trend for the increased connectivity between posterior cingulate cortex and the left hippocampus (23% increase, $p=0.1$; z value $p=0.17$).

After insulin administration, the cluster size differences between the diabetes and the control groups decreased by 44% in the medial frontal cortex and by 95% in the anterior cingulate cortex.

Resting State Connectivity and Cognition

Performances on verbal fluency and visuospatial memory (BVMT-R) tasks after insulin administration tended to be higher than on-placebo performances, and control subjects on insulin performed better than diabetic participants on insulin on FAS, switching and composite verbal fluency and BVMT-R T1-T3 trials and Total Recall (10).

In diabetic subjects on insulin, better performance on the Verbal Fluency Category (naming all words in same semantic category) was associated with stronger average connectivity (Z value) between the right hippocampal region and the anterior cingulate cortex ($R^2_{adj}=0.28$ $p=0.02$) (Figure 3A, B). Verbal Fluency Category Switching was associated with lower connectivity coefficient between the left hippocampal region and the medio-frontal cortex ($R^2_{adj}=0.43$ $p=0.04$) but not with cluster size. In controls on insulin, better scores on BVMT-R Delayed Recall tended to be associated with stronger average

connectivity between left hippocampal region and posterior cingulate cortex ($R^2_{\text{adj}}=0.41$, $p=0.07$).

In diabetic subjects on placebo, BVMT-R Total Recall scores were associated with lower average coefficients of connectivity between the left hippocampal region and the anterior cingulate cortex ($R^2_{\text{adj}}=0.45$, $p=0.04$), and the lower connectivity with the right inferior parietal cortex ($R^2_{\text{adj}}=0.44$, $p=0.03$) (LS models were adjusted for age and gender). BVMT-R Learning T scores were also associated with lower average coefficients of connectivity between the right hippocampal region and the inferior parietal cortex ($R^2_{\text{adj}}=0.60$, $p=0.01$) (Figure 3 C, D).

In controls on placebo, Composite General Cognitive Function scores were also associated lower average coefficients of connectivity between the right hippocampal region and the inferior parietal cortex ($R^2_{\text{adj}}=0.74$, $p=0.007$) (LS models adjusted for age, gender). HLVT-Recall T score was negatively associated with average connectivity ($R^2_{\text{adj}}=0.84$, $p=0.01$) and voxel size ($R^2_{\text{adj}}=0.84$, $p=0.01$) between right hippocampus and medio-frontal cortex and also between left hippocampus and medio-frontal cortex ($R^2_{\text{adj}}=0.81$, $p=0.02$), posterior cingulate cortex ($R^2_{\text{adj}}=0.73$, $p=0.04$) and right inferior parietal cortex ($R^2_{\text{adj}}=0.72$, $p=0.04$). These relationships were not observed after insulin administration in the diabetes and control groups.

Resting State Connectivity and Glycemic Control

There was no significant relationship between HbA1c and resting state connectivity after insulin administration. In the control group, after placebo administration, HbA1c was associated with stronger connectivity between right and left hippocampus and right inferior parietal cortex ($R^2_{\text{adj}}=0.76$, $p=0.03$).

Discussion

This study demonstrated that in diabetic and age-matched healthy subjects, intranasal administration of a

single dose of insulin acutely increased resting-state functional connectivity between the hippocampal region and multiple regions within the DMN (i.e., medial frontal cortex, interior parietal cortex and anterior and posterior cingulate cortex) that are linked to integrative higher cognitive function. After placebo administration, connectivity between hippocampal regions and these DMN regions was lower in diabetic subjects as compared to healthy controls in several brain regions. After insulin administration, the cluster size differences between the diabetes and the control groups decreased by 44% in the medial frontal cortex and by 95% in the anterior cingulate cortex. After administration of intranasal insulin, the differences in functional connectivity between the diabetic and control group were no longer significant. These findings suggest that acute administration of insulin via intranasal delivery route may acutely improve acutely functional connections between brain regions involved in memory and other cognitive domains processing.

The insulin resistance syndrome is associated with reduced brain insulin levels and sensitivity in age-related memory impairment and AD (5;27-29). Brain insulin plays an important role as a neuromodulator in cognition (4;5), energy homeostasis, food intake, sympathetic activity, neuron-astrocyte signaling, synapse formation, and neuronal survival (7;30). Insulin has been shown to reinforce signaling in the dopamine-mediated brain-reward system and modulate food intake and responses to reward stimuli (31-33). Intranasal insulin increases rapidly in cerebrospinal fluid and binds to insulin receptors (34;35) in the olfactory bulb, several regions in cerebral cortex including autonomic network (e.g., insular cortex, dorsal root ganglia, nigro-striatal neurons), cerebellum (36-38), hypothalamus, and hippocampus (34;35;39).

Type 2 diabetes is associated with impairment of hippocampus-dependent memory, and these effects are proportional to diabetes severity (2). Resting-state functional connectivity is also altered in type 2

diabetic subjects, and the severity of impairment correlates with the degree of insulin resistance (18;19). The effects of intranasal insulin on resting-state connectivity have not been studied. Diabetic subjects had worse baseline cognitive performance, especially in the memory and executive function domains. We have previously shown, in this cohort, that intranasal insulin may improve acutely visuospatial memory in older diabetic and healthy adults, and that this improvement of memory and verbal learning may be dependent upon vasodilatation response in the middle cerebral artery territory and in particular insular cortex (10). In diabetic subjects on insulin, better performance on the verbal fluency naming task was associated with stronger coefficient of connectivity between the right hippocampal region and anterior cingulate cortex and lesser connectivity between the left hippocampal regions and the medio-frontal cortex for a more difficult category switching task. In controls on insulin, better performance on the visuospatial memory task (BVMT-R) tended to correlate with stronger connectivity between the left hippocampal region and posterior cingulate cortex. Differences in relationships between cognition and connectivity between the right and left hippocampal regions are intriguing and reflect a complexity of the large scale verbal fluency network that comprises of verbal fluency and orthographic, discrimination sub-networks (40). Set switching is a complex operation involving a number of different brain structures that usually include various parts of the dorsolateral and dorso-medial prefrontal cortex, as well as temporal regions where hippocampus is located (41). Functional integration within verbal fluency network declines with age, and task difficulty, low productive-difficult tasks are associated with significant decreases in connectivity. Therefore, the decreased connectivity on the left may reflect the inhibition of the left hippocampus that might be part of the category switching process (42). After placebo administration, we have observed a “deactivation pattern” (15;16), which is characterized by task related decreases in activity and connectivity among several DMN regions. In other words, during a task,

a better task-related performance is associated with a decrease in functional connectivity within DMN.

In diabetic subjects, the worse performance on BVMT-R task was associated with stronger functional connectivity between hippocampal regions and anterior cingulate and inferior parietal cortex. Similarly in the control groups, negative associations were found between the general cognitive score, and verbal learning performance and connectivity between hippocampal regions and medio-frontal cortex, posterior cingulate cortex, and inferior parietal cortex. It has been demonstrated using magnetoencephalography and a two-step hyperinsulinemic clamp that resting state activity correlates with insulin disposal (43). Furthermore, intranasal insulin may improve peripheral insulin sensitivity, insulin sensitization was associated with increased hypothalamic blood flow and parasympathetic heart rate variability (44;45). Intranasal insulin also diminished saliva cortisol and stress-induced responsiveness along hypothalamus-pituitary-axis (46;47). These findings may suggest that intranasal insulin administration may enhance functional connectivity between DMN and other brain regions and may modulate central autonomic responses to stress.

This pilot study has several limitations. The small sample size in our pilot study may have limited the ability to observe the full extent of functional connectivity. Cognitive testing was done after completion of fMRI scan, and therefore could not assess acute responses in functional connectivity to different cognitive tasks that may involve different brain regions and range of difficulty. Eleven of 14 diabetic participants were treated with metformin, which may be associated with worse cognitive performance (48). Women were required to be postmenopausal, and only one participant received hormone replacement therapy, which minimized potential effects of estrogen levels on functional connectivity (49). Furthermore, the optimal dose on intranasal insulin to modulate brain function remains unknown, as no dose-response studies have been completed to date within this population. Larger and/or

more frequent doses may thus optimize the effects of intranasal insulin on brain function. Longer-term studies are also warranted to evaluate the potential for intranasal insulin for neuroprotection and improvement of cortical connectivity.

Conclusion

This study provided preliminary evidence that intranasal insulin may acutely increase functional connectivity between the hippocampal region and the DMN in older adults with type 2 diabetes and age-matched healthy controls. Furthermore, differences in post-insulin connectivity between diabetic and control subjects diminished. Cognitive performance on insulin was associated with regional changes in functional connectivity. Our findings provide insights how intranasal insulin acutely modulates resting state brain activity and its relationship to performance on higher cognitive tasks. Therefore, enhancement of functional connectivity may serve as a potential mechanism of acute intranasal insulin effect in the brain. However, larger prospective studies are needed to determine long-term safety and efficacy for prevention of cognitive decline in older people with type 2 diabetes.

Figure Legend:

Figure 1. Resting-state functional network regions (medio-frontal cortex, posterior cingulate cortex, inferior parietal cortex and anterior cingulate cortex) with significant connectivity (voxels with $|t| \geq 15.4$, cluster size $\geq 270\text{mm}^3$ and $p < 1 \times 10^{-9}$) to the right and left hippocampus in the diabetes and the control group after intranasal insulin and placebo administration.

A) Diabetes group: Intranasal insulin administration, B) Diabetes group: Placebo administration; C) Diabetes group: Differences in functional connectivity between insulin and placebo administration. Intranasal insulin administration was associated with increased connectivity between hippocampal regions and medial frontal cortex, right inferior parietal cortex and posterior cingulate gyrus, as compared with placebo (paired t-test, voxel corrected within subject comparisons, cluster size $\geq 270\text{mm}^3$, $p < 0.05$).

D) Age-matched healthy controls: Intranasal insulin administration; E) Control group: Placebo administration; F) Control group: Differences in functional connectivity between insulin and placebo administration.

Figure 2. Differences in connectivity between the diabetes and the control group after insulin (A) and placebo administration (B). After insulin administration, diabetic subjects had lower functional connectivity only in medio-frontal cortex as compared with controls. B) After placebo administration diabetic subjects had larger areas or lower functional connectivity in multiple regions the threshold was set as $p < 0.05$, a minimum cluster extent = 270mm^3 (corrected).

Figure 3: The relationship between functional connectivity measures and cognitive performance in the diabetes group after insulin and placebo administration. After insulin administration (A), the average

coefficient of connectivity between the right hippocampus and anterior cingulate cortex was associated with better Verbal Fluency Score, but not after placebo administration (B). Brief Visuospatial Memory Learning T Score showed a positive trend with coefficient connectivity between right hippocampus and right posterior inferior parietal cortex (RIPC) after insulin administration (A) and a strong negative association after placebo administration (B).

Acknowledgements

Authors contributions: V.N. has designed the study and overseen study conduct, data collection, analyses and MS preparation. H.Z. and Y.H. performed MRI analyses, MS preparation; P.N. overseen clinical aspects of the study, W.M. designed and overseen cognitive testing and contributed to MS preparation; B.M. contributed to study conduct and MS preparation; J.Z. and J.F. oversaw MRI analyses.

Conflict of interest: V.N. none; H.Z. none, Y.H. none, P.N. none, W.M. none; J.Z. none, J.F. none.

Funding Sources: V.N. has received grants from the NIH–National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) (5R21-DK-084463-02) and the National Institutes of Health (NIH)–National Institute on Aging (NIA) (1R01-AG-0287601-A2) related to this study, and V.N, B.M., P.N., W.M., received salaries from these grants. Y.H. received the grant support from China Scholarship Council (CSC) (No. 201206010220). B.M. received a KL2 Medical Research Investigator Training (MeRIT) award (1KL2RR025757-04) from The Harvard Clinical and Translational Science Center (National Center for Research Resources and the National Center for Advancing Translational Sciences, National Institutes of Health Award 8KL2TR000168-05). W.M. was also supported by the Translational Research Center for TBI and Stress Disorders (TRACTS), a VA Rehabilitation Research and Development Traumatic Brain Injury Center of Excellence (B6796-C) and VA Merit Review Award to Regina McGlinchey. This work was conducted with support from Harvard Catalyst | The Harvard Clinical and Translational Science Center (National Center for Research Resources and the National Center for Advancing Translational Sciences, National Institutes of Health Award 8UL1TR000170-05 and financial contributions from Harvard University and its affiliated academic health care centers). The

content is solely the responsibility of the authors and does not necessarily represent the official views of Harvard Catalyst, Harvard University and its affiliated academic health care centers, or the National Institutes of Health. The authors acknowledge contributions of Clinical Research Center nursing and MRI staff.

Registration:

This study was registered at ClinicalTrials.gov as trial number NCT01206322 and at federal Drug Administration IND number 107690.

Guarantor Statement:

V.N. is the guarantor of this work including the study design, access to data, and the decision to submit and publish the manuscript.

Reference List

1. Franke K, Gaser C, Manor B, Novak V: Advanced BrainAGE in older adults with type 2 diabetes mellitus. *Front Aging Neurosci.* 2013;**5**:90
2. Xu WL, Qiu CX, Wahlin A, Winblad B, Fratiglioni L: Diabetes mellitus and risk of dementia in the Kungsholmen project: a 6-year follow-up study. *Neurology* 2004;**63**:1181-1186
3. Banks WA: Brain meets body: the blood-brain barrier as an endocrine interface. *Endocrinology* 2012;**153**:4111-4119
4. Shemesh E, Rudich A, Harman-Boehm I, Cukierman-Yaffe T: Effect of intranasal insulin on cognitive function: a systematic review. *J Clin.Endocrinol.Metab* 2012;**97**:366-376
5. Freiherr J, Hallschmid M, Frey WH, Brunner YF, Chapman CD, Holscher C, Craft S, De Felice FG, Benedict C: Intranasal Insulin as a Treatment for Alzheimer's Disease: A Review of Basic Research and Clinical Evidence. *CNS.Drugs* 2013;**7**:505-514
6. Jauch-Chara K, Friedrich A, Rezmer M, Melchert UH, Scholand-Engler G, Hallschmid M, Oltmanns KM: Intranasal insulin suppresses food intake via enhancement of brain energy levels in humans. *Diabetes* 2012;**61**:2261-2268
7. Plum L, Belgardt BF, Bruning JC: Central insulin action in energy and glucose homeostasis. *J Clin.Invest* 2006;**116**:1761-1766
8. Born J, Lange T, Kern W, McGregor GP, Bickel U, Fehm HL: Sniffing neuropeptides: a transnasal approach to the human brain. *Nat.Neurosci.* 2002;**5**:514-516
9. Chapman CD, Frey WH, Craft S, Danielyan L, Hallschmid M, Schioth HB, Benedict C: Intranasal treatment of central nervous system dysfunction in humans. *Pharm.Res* 2013;**30**:2475-2484
10. Novak V, Milberg W, Hao Y, Munshi M, Novak P, Galica A, Manor B, Roberson P, Craft S, Abduljalil A: Enhancement of Vasoreactivity and Cognition by Intranasal Insulin in Type 2 Diabetes. *Diabetes Care* 2014;**3**:751-759
11. Schilling TM, Ferreira de Sa DS, Westerhausen R, Strelzyk F, Larra MF, Hallschmid M, Savaskan E, Oitzl MS, Busch HP, Naumann E, Schachinger H: Intranasal insulin increases regional cerebral blood flow in the insular cortex in men independently of cortisol manipulation. *Hum.Brain Mapp.* 2013;**35**:1944-1955

12. Benedict C, Hallschmid M, Hatke A, Schultes B, Fehm HL, Born J, Kern W: Intranasal insulin improves memory in humans. *Psychoneuroendocrinology* 2004;**29**:1326-1334
13. Benedict C, Hallschmid M, Schultes B, Born J, Kern W: Intranasal insulin to improve memory function in humans. *Neuroendocrinology* 2007;**86**:136-142
14. 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: Intranasal insulin therapy for Alzheimer disease and amnesic mild cognitive impairment: a pilot clinical trial. *Arch.Neurol.* 2012;**69**:29-38
15. Greicius MD, Krasnow B, Reiss AL, Menon V: Functional connectivity in the resting brain: a network analysis of the default mode hypothesis. *Proc.Natl.Acad.Sci U.S.A* 2003;**100**:253-258
16. Greicius MD, Supekar K, Menon V, Dougherty RF: Resting-state functional connectivity reflects structural connectivity in the default mode network. *Cereb.Cortex* 2009;**19**:72-78
17. Anticevic A, Cole MW, Murray JD, Corlett PR, Wang XJ, Krystal JH: The role of default network deactivation in cognition and disease. *Trends Cogn Sci* 2012;**16**:584-592
18. Musen G, Jacobson AM, Bolo NR, Simonson DC, Shenton ME, McCartney RL, Flores VL, Hoogenboom WS: Resting-state brain functional connectivity is altered in type 2 diabetes. *Diabetes* 2012;**61**:2375-2379
19. Chen YC, Jiao Y, Cui Y, Shang SA, Ding J, Feng Y, Song W, Ju SH, Teng GJ: Aberrant brain functional connectivity related to insulin resistance in type 2 diabetes: a resting-state FMRI study. *Diabetes Care* 2014;**37**:1689-1696
20. Hoogenboom WS, Marder TJ, Flores VL, Huisman S, Eaton HP, Schneiderman JS, Bolo NR, Simonson DC, Jacobson AM, Kubicki M, Shenton ME, Musen G: Cerebral white matter integrity and resting-state functional connectivity in middle-aged patients with type 2 diabetes. *Diabetes* 2014;**63**:728-738
21. Benedict C, Dodt C, Hallschmid M, Lepiorz M, Fehm HL, Born J, Kern W: Immediate but not long-term intranasal administration of insulin raises blood pressure in human beings. *Metabolism* 2005;**54**:1356-1361
22. Benedict C, Kern W, Schultes B, Born J, Hallschmid M: Differential sensitivity of men and women to anorexigenic and memory-improving effects of intranasal insulin. *J.Clin.Endocrinol.Metab*

2008;**93**:1339-1344

23. Benedict RHB, Schretlen D, Groninger L, Dobraski M, Sphritz B: Revision of the Brief Visuospatial Memory test: Studies of normal performance, reliability and validity. *Psychological Assessment* 1996;**8**:145-153
24. Yeudall LT, Fromm D, Reddon JR, Stefanyk WO: Normative data stratified by age and sex for 12 neuropsychological tests. *Journal of Clinical Psychology* 1986;**42**:918-946
25. Maldjian JA, Laurienti PJ, Kraft RA, Burdette JH: An automated method for neuroanatomic and cytoarchitectonic atlas-based interrogation of fMRI data sets. *Neuroimage*. 2003;**19**:1233-1239
26. van Duinkerken E, Schoonheim MM, Sanz-Arigita EJ, Ijzerman RG, Moll AC, Snoek FJ, Ryan CM, Klein M, Diamant M, Barkhof F: Resting-state brain networks in type 1 diabetic patients with and without microangiopathy and their relation to cognitive functions and disease variables. *Diabetes* 2012;**61**:1814-1821
27. Craft S: Insulin resistance and cognitive impairment: a view through the prism of epidemiology. *Arch.Neurol.* 2005;**62**:1043-1044
28. Craft S: Insulin resistance syndrome and Alzheimer's disease: age- and obesity-related effects on memory, amyloid, and inflammation. *Neurobiol.Aging* 2005;**26 Suppl 1**:65-69
29. Messier C, Teutenberg K: The role of insulin, insulin growth factor, and insulin-degrading enzyme in brain aging and Alzheimer's disease. *Neural Plast.* 2005;**12**:311-328
30. Plum L, Schubert M, Bruning JC: The role of insulin receptor signaling in the brain. *Trends Endocrinol.Metab* 2005;**16**:59-65
31. Figlewicz DP: Insulin, food intake, and reward. *Semin.Clin.Neuropsychiatry* 2003;**8**:82-93
32. Figlewicz DP, Benoit SC: Insulin, leptin, and food reward: update 2008. *Am.J Physiol Regul.Integr.Comp Physiol* 2009;**296**:R9-R19
33. Stice E, Figlewicz DP, Gosnell BA, Levine AS, Pratt WE: The contribution of brain reward circuits to the obesity epidemic. *Neurosci.Biobehav.Rev* 2012
34. Thorne RG, Pronk GJ, Padmanabhan V, Frey WH2: Delivery of insulin-like growth factor-I to the

- rat brain and spinal cord along olfactory and trigeminal pathways following intranasal administration. *Neuroscience* 2004;**127**:481-496
35. Hanson LR, Frey WH: Intranasal delivery bypasses the blood-brain barrier to target therapeutic agents to the central nervous system and treat neurodegenerative disease. *BMC.Neurosci.* 2008;**9** **Suppl 3**:S5
 36. Hopkins DF, Williams G: Insulin receptors are widely distributed in human brain and bind human and porcine insulin with equal affinity. *Diabet.Med.* 1997;**14**:1044-1050
 37. Albrecht J, Wroblewska B, Mossakowski MJ: The binding of insulin to cerebral capillaries and astrocytes of the rat. *Neurochemical Research* 1981;**7**:489-494
 38. Banks WA: The source of cerebral insulin. *Eur.J.Pharmacol.* 2004;**490**:5-12
 39. Abbott MA, Wells DG, Fallo JR: The Insulin Receptor Tyrosine Kinase Substrate p58/53 and the Insulin Receptor Are Components of CNS Synapses. *Journal of Neuroscience* 1999;**19**:7300-7308
 40. Marsolais Y, Perlberg V, Benali H, Joannette Y: Age-related changes in functional network connectivity associated with high levels of verbal fluency performance. *Cortex* 2014;**58C**:123-138
 41. Baldo JV, Schwartz S, Wilkins D, Dronkers NF: Role of frontal versus temporal cortex in verbal fluency as revealed by voxel-based lesion symptom mapping. *J.Int.Neuropsychol.Soc.* 2006;**12**:896-900
 42. Koch I, Gade M, Schuch S, Philipp AM: The role of inhibition in task switching: a review. *Psychon.Bull.Rev.* 2010;**17**:1-14
 43. Tschritter O, Preissl H, Hennige AM, Stumvoll M, Porubská K, Frost R, Marx H, Klosel B, Lutzenberger W, Birbaumer N, Haring HU, Fritsche A: The cerebrocortical response to hyperinsulinemia is reduced in overweight humans: a magnetoencephalographic study. *Proc.Natl.Acad.Sci U.S.A* 2006;**103**:12103-12108
 44. Heni M, Kullmann S, Ketterer C, Guthoff M, Linder K, Wagner R, Stingl KT, Veit R, Staiger H, Haring HU, Preissl H, Fritsche A: Nasal insulin changes peripheral insulin sensitivity simultaneously with altered activity in homeostatic and reward-related human brain regions. *Diabetologia* 2012;**55**:1773-1782

45. Heni M, Wagner R, Kullmann S, Veit R, Mat-Husin H, Linder K, Benkendorff C, Peter A, Stefan N, Haring HU, Preissl H, Fritsche A: Central insulin administration improves whole-body insulin sensitivity via hypothalamus and parasympathetic outputs in men. *Diabetes* 2014 pii: DB_140477
46. Veer IM, Oei NY, Spinhoven P, van Buchem MA, Elzinga BM, Rombouts SA: Endogenous cortisol is associated with functional connectivity between the amygdala and medial prefrontal cortex. *Psychoneuroendocrinology* 2012;**37**:1039-1047
47. Bohringer A, Schwabe L, Richter S, Schachinger H: Intranasal insulin attenuates the hypothalamic-pituitary-adrenal axis response to psychosocial stress. *Psychoneuroendocrinology* 2008;**33**:1394-1400
48. Moore EM, Mander AG, Ames D, Kotowicz MA, Carne RP, Brodaty H, Woodward M, Boundy K, Ellis KA, Bush AI, Faux NG, Martins R, Szoeki C, Rowe C, Watters DA: Increased risk of cognitive impairment in patients with diabetes is associated with metformin. *Diabetes Care* 2013;**36**:2981-2987
49. Ottowitz WE, Siedlecki KL, Lindquist MA, Dougherty DD, Fischman AJ, Hall JE: Evaluation of prefrontal-hippocampal effective connectivity following 24 hours of estrogen infusion: an FDG-PET study. *Psychoneuroendocrinology* 2008;**33**:1419-1425

TABLE 1

Demographic characteristics of the diabetes and control groups

	Diabetes(n=14)	Control(n=14)	p
Age(years)	61.7±8.1	60.1±9.9	0.7
Sex(men/women)	8,6	4,10	NS
Race(white/AA/Asian)	9,3,2	13,1,0	
Education(years)	14.1±3.9	17.1±3.2	0.03
Diabetes duration(years)	11.6±4.8		
HbA1c(%)	7.4±1.5	5.6±0.2	<0.0001
HbA1c(mmol/mol)	58.4±16.8	38±1.95	<0.0001
Fasting glucose	131.8±30.1	87.9±9.7	0.0004
Systolic blood pressure(mm Hg)	126.69±13.8	125.5±14.3	NS
Diastolic blood pressure(mm Hg)	73.2±8.92	72.1±10.9	NS
Hyperlipidemia(yes/no)	9,5	2,12	0.005
Total cholesterol (mg/dL)	160.7±37.0	213.1±45.6	0.003
Hypertension, N (%)	6,8	0	N/A
Mini-Mental State Examination	28.2±1.7	28.8±1.6	0.6 ^s
Hopkins Verbal Learning-Delayed Recall T Score	41.8±9.1	54.5±8.5	0.0018
Trail making Part B T Score	37.6±12.9	52.1±11.5	0.004
Global Gray Matter volume (cm ³)	635.5±29.0	691.3±27.5	0.03
Left Hippocampus volume (cm ³)	5.92±0.45	5.76±0.47	0.59

Right Hippocampus volume (cm ³)	5.69±0.43	5.62±0.42	0.55
Left MFC volume (cm ³)	21.2±0.7	22.9±1.0	0.08
Right MFC volume (cm ³)	21.8±0.9	23.3±1.3	0.18

Between groups comparisons, ANOVA, unadjusted. Mean± SD

* Pearson's chi-squared test, inclusion criteria: normotensive controls

\$ LS model adjusted for education years, race (AA African/American)

TABLE 2

Insulin vs. placebo connectivity within diabetes and control groups

Brain region	Brodmann area	cluster size(mm ³)		Average t value		Peak t value	
		Diabetes	Control	Diabetes	Control	Diabetes	Control
Medial frontal cortex	8//9	8073	3321	3.33	3.17	6.78	5.85
Right inferior parietal cortex(R-IPC)	40	2214	NS	3.33	NS	5.85	NS
Posterior Cingulate Gyrus(PCC)	23//31	1404	1188	3.49	2.84	5.53	4.17
Anterior Cingulate cortex(ACC)	24	4752	972	3.22	3.13	6.34	5.28

Comparisons of connectivity between hippocampal regions and DMN regions in both hemispheres between insulin and placebo conditions in the diabetes and the control group. Paired t-tests were used to compare insulin vs. placebo conditions within the diabetes and control groups, $|t| > 2.16$ (AlphaSim corrected $p < 0.05$).

TABLE 3

Insulin vs. placebo connectivity within diabetes and control groups

Brain region	Brodmann area	Diabetes Group				P value		Insulin	
		cluster size(mm ³)		Peak z value		Insulin vs. Placebo		DM vs. Control	
		Insulin	Placebo	Insulin	Placebo	cluster size	z value	cluster size	z value
Left hippocampal regions									
Medial frontal cortex	8//9	4900.5±3617.6	3276.6±2703.2	0.97±0.17	0.91±0.23	0.06	0.16	0.18	0.48
Left inferior parietal(L-IPC)	39	345.2±249.3	403.1±318.8	1.65±0.58	1.55±0.78	0.21	0.3	0.11	0.47
Right inferior parietal(R-IPC)	40	779.8±736.9	509.1.3±636.0	0.76±0.21	0.68±0.21	0.095	0.096	0.31	0.29
Posterior Cingulate Gyrus(PCC)	23//31	1824.4±744.3	1419.4±1060.3	1.07±0.27	0.94±0.33	0.32	0.38	0.49	0.27
Anterior Cingulate cortex(ACC)	24	2009.6±1664.0	1492.7±1289.3	0.91±0.22	0.87±0.24	0.24	0.40	0.35	0.26
Right hippocampal regions									
Medial frontal cortex	8//9	4142.6±3857.0	2684.6±2675.1	0.95±0.24	0.90±0.24	0.03	0.24	0.42	0.46
Left inferior parietal(L-IPC)	39	331.7±278.9	435.9±299.5	1.61±0.85	1.69±0.89	0.13	0.36	0.13	0.39
Right inferior parietal(R-IPC)	40	935.4±825.5	779.1±682.6	0.82±0.25	0.71±0.19	0.17	0.033	0.23	0.33
Posterior Cingulate Gyrus(PCC)	23//31	1982.6±809.8	1776.2±830.9	1.21±0.27	1.11±0.28	0.44	0.46	0.22	0.12

Control Group									
Brain region		Control Group					Placebo		
		Insulin	Placebo	Insulin	Placebo	Insulin vs. Placebo	DM vs.Control		
Anterior Cingulate cortex(ACC)	24	1776.2±14968	1018.3±12470	0.91±0.23	0.79±0.21	0.17	0.15	0.39	0.48
Left hippocampal regions									
Medial frontal cortex	8//9	6665.1±5254.0	5575.5±4496.5	0.97±0.24	0.97±0.22	0.10	0.16	0.058	0.24
Left inferior parietal(L-IPC)	39	439.7±131.6	468.6±99.3	1.67±0.65	1.64±0.47	0.21	0.44	0.24	0.35
Right inferior parietal (R-IPC)	40	636.4±764.0	501.4±734.9	0.71±0.29	0.64±0.28	0.31	0.29	0.33	0.28
Posterior Cingulate Gyrus(PCC)	23//31	1695.2±923.4	1147.5±1026.2	0.98±0.27	0.87±0.30	0.017	0.052	0.099	0.17
Anterior Cingulate cortex(ACC)	24	2131.1±1670.4	1886.1±1925.9	0.95±0.23	0.90±0.25	0.24	0.056	0.40	0.42
Right hippocampal regions									
Medial frontal cortex	8//9	5402.0±49617	5691.2±4349.1	0.93±0.25	0.94±0.23	0.23	0.44	0.019	0.31
Left inferior parietal(L-IPC)	39	428.1±131.4	459±100.0	1.69±0.69	1.65±0.46	0.2	0.42	0.39	0.44
Right inferior parietal (R-IPC)	40	709.7±784.4	692.4±778.9	0.78±0.24	0.73±0.26	0.24	0.24	0.19	0.38
Posterior Cingulate Gyrus(PCC)	23//31	1675.9±755.6	1373.1±1008.9	1.06±0.29	1.00±0.31	0.19	0.29	0.047	0.09
Anterior Cingulate cortex(ACC)	24	1807.1±1690.2	1940.1±1786.3	0.88±0.22	0.88±0.28	0.40	0.098	0.15	0.33

Comparisons of connectivity between right and left hippocampal regions and DMN regions in both hemispheres between insulin and placebo conditions within each group, and between the groups. Paired and two-tailed t-test.

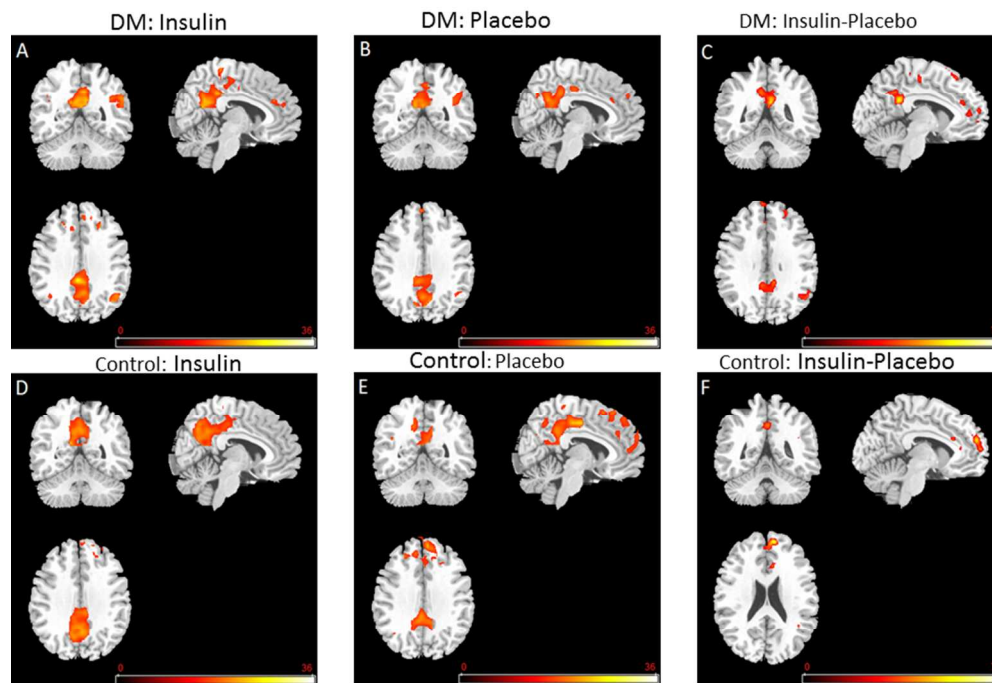


Figure 1

Figure 1. Resting-state functional network regions (medio-frontal cortex, posterior cingulate cortex, inferior parietal cortex and anterior cingulate cortex) with significant connectivity (voxels with $|t| \geq 15.4$, cluster size $\geq 270\text{mm}^3$ and) to the right and left hippocampus in the diabetes and the control group after intranasal insulin and placebo administration.

A) Diabetes group: Intranasal insulin administration, B) Diabetes group: Placebo administration; C) Diabetes group: Differences in functional connectivity between insulin and placebo administration. Intranasal insulin administration was associated with increased connectivity between hippocampal regions and medial frontal cortex, right inferior parietal cortex and posterior cingulate gyrus, as compared with placebo (paired t-test, voxel corrected within subject comparisons, cluster size $\geq 270\text{mm}^3$, $p < 0.05$).

D) Age-matched healthy controls: Intranasal insulin administration; E) Control group: Placebo administration; F) Control group: Differences in functional connectivity between insulin and placebo administration.

254x190mm (96 x 96 DPI)

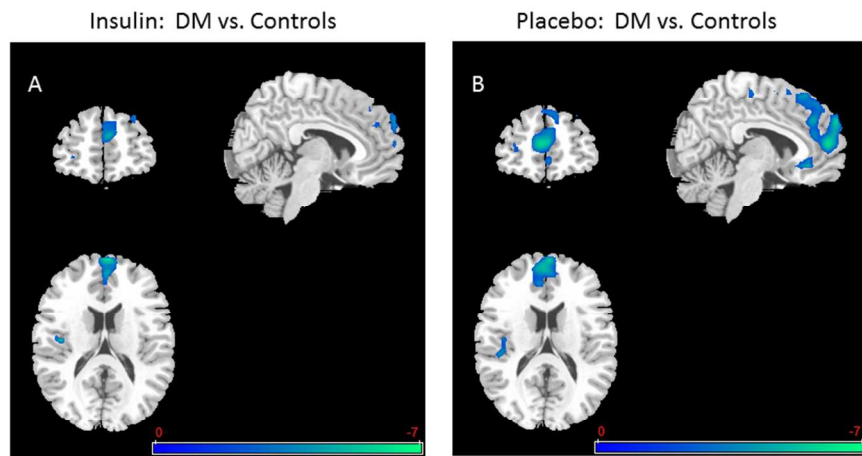


Figure 2

Figure 2. Differences in connectivity between the diabetes and the control group after insulin (A) and placebo administration (B). After insulin administration, diabetic subjects had lower functional connectivity only in medio-frontal cortex as compared with controls. B) After placebo administration diabetic subjects had larger areas of lower functional connectivity in multiple regions the threshold was set as $p < 0.05$, a minimum cluster extent = 270mm³ (corrected).
254x190mm (96 x 96 DPI)

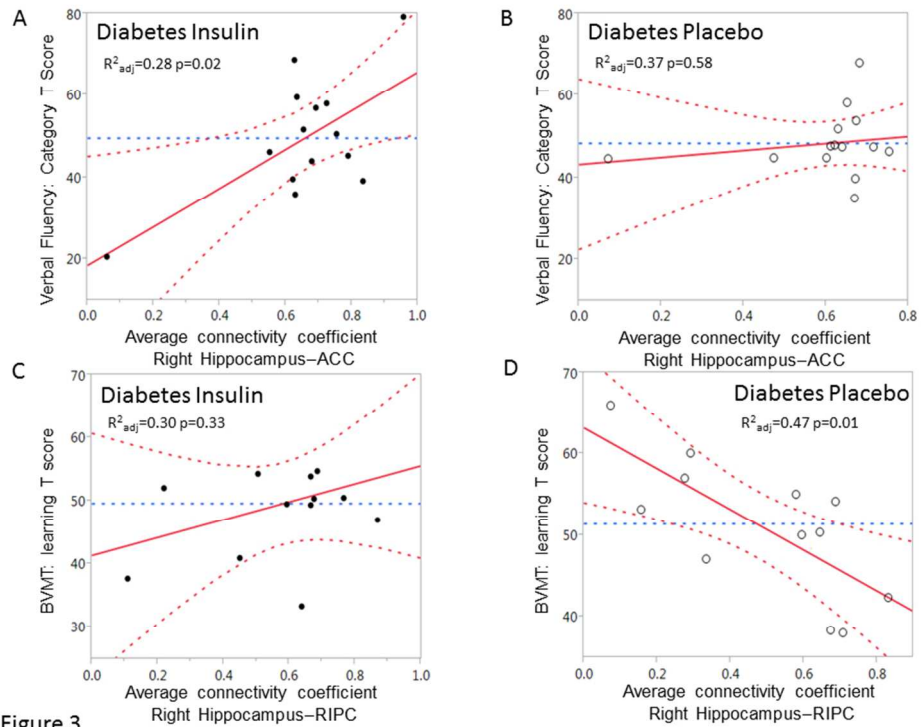


Figure 3

Figure 3: The relationship between functional connectivity measures and cognitive performance in the diabetes group after insulin and placebo administration. After insulin administration (A), the average coefficient of connectivity between the right hippocampus and anterior cingulate cortex was associated with better Verbal Fluency Score, but not after placebo administration (B). Brief Visuospatial Memory Learning T Score showed a positive trend with coefficient connectivity between right hippocampus and right posterior inferior parietal cortex (RIPC) after insulin administration (A) and a strong negative association after placebo administration (B).

254x190mm (96 x 96 DPI)