Brain functions and cognition on transient insulin deprivation in type 1 diabetes

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BACKGROUND. Type 1 diabetes (T1D) is a risk factor for dementia and structural brain changes. It remains to be determined whether transient insulin deprivation that frequently occurs in insulin treated T1D individual alters brain function.

METHODS. We therefore, performed functional and structural magnetic resonance imaging, magnetic resonance spectroscopy, and neuropsychological testing at baseline and following 5.4 ± 0.6 hours of insulin deprivation in 14 T1D and compared to 14 age-, sex-, and body mass index–matched, nondiabetic (ND) participants with no interventions.

RESULTS. Insulin deprivation in T1D increased blood glucose, and β-hydroxybutyrate, while reducing bicarbonate levels. T1D participants showed lower baseline brain N-acetyl aspartate and myo-inositol levels but higher cortical fractional anisotropy, suggesting unhealthy neurons and brain microstructure. Although cognitive functions did not differ between T1D and ND at baseline, significant changes in fine motor speed as well as attention and short-term memory occurred following insulin deprivation in T1D participants. Insulin deprivation also reduced brain adenosine triphosphate levels and altered phosphocreatine/ adenosine triphosphate ratio. Baseline differences in functional connectivity in brain regions between T1D and ND were noted and on insulin deprivation further alterations in functional connectivity between regions especially cortical […]
Brain Functions and Cognition on Transient Insulin Deprivation in Type 1 Diabetes

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Brief Summary: Transient insulin deprivation in T1D individuals altered executive aspects of cognitive function and brain energy metabolites concurrent with functional connectivity between memory regions and the sensory cortex

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ABSTRACT

**Background:** Type 1 diabetes (T1D) is a risk factor for dementia and structural brain changes. It remains to be determined whether transient insulin deprivation that frequently occurs in insulin treated T1D individual alters brain function.

**Methods:** We therefore, performed functional and structural magnetic resonance imaging, magnetic resonance spectroscopy, and neuropsychological testing at baseline and following 5.4 ±0.6 hours of insulin deprivation in 14 T1D and compared to 14 age-, sex-, and body mass index–matched, nondiabetic (ND) participants with no interventions.

**Results:** Insulin deprivation in T1D increased blood glucose, and β-hydroxybutyrate, while reducing bicarbonate levels. T1D participants showed lower baseline brain N-acetyl aspartate and myo-inositol levels but higher cortical fractional anisotropy, suggesting unhealthy neurons and brain microstructure. Although cognitive functions did not differ between T1D and ND at baseline, significant changes in fine motor speed as well as attention and short-term memory occurred following insulin deprivation in T1D participants. Insulin deprivation also reduced brain adenosine triphosphate levels and altered phosphocreatine/ adenosine triphosphate ratio. Baseline differences in functional connectivity in brain regions between T1D and ND were noted and on insulin deprivation further alterations in functional connectivity between regions especially cortical and hippocampus-caudate regions were observed. These alterations in functional connectivity correlated to brain metabolites and to changes in cognition.

**Conclusions:** Transient insulin deprivation thus, caused alterations in executive aspects of cognitive function concurrent with functional connectivity between memory regions and the sensory cortex. These findings have important clinical implications as many patients with T1D inadvertently have periods of transient insulin deprivation.
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INTRODUCTION

There is a growing interest in the effects of type 1 diabetes (T1D) on brain development, cognition, and dementia, especially as the incidence and life expectancy of diabetic patients increases (1-4). Although the Diabetes Complications and Control Trial established a substantial reduction in microvascular complications, uncertainty remains on the impact of glycemic control and dementia (5). Recent work highlights the effects of chronic glucose excursions and variability on neuroanatomy and cognitive function (2;4;6;7). However, while past studies explored chronic dysglycemia (8-10) and the acute and selective effect of hyperglycemia in the presence of insulin (11), little is known about the effect of acute periods of insulin deficiency and resulting altered metabolic milieu on the brain (12). Although variability in insulin levels has no effect on brain glucose uptake in humans (13), preclinical studies in rodents indicate that insulin deprivation alters brain oxidative capacity and adenosine triphosphate (ATP) generation (14). Since neuronal conduction is ATP dependent (15) it remains unknown whether insulin deprivation has a detrimental effect on brain functional connectivity (FC) and cognitive performance in people with T1D.

Deliberate omission of insulin for weight loss has been reported in young T1D individuals (16;17). With the rapidly growing use of continuous subcutaneous inulin infusion pumps, it is common to have periods of insulin deprivation associated with missed insulin boluses, device malfunctions, or suspensions and likewise, people on multiple daily insulin injections also miss occasional injections, resulting in transient insulin deprivation (18-20). Although the adverse brain effects of acute hypoglycemia while on insulin treatment in T1D has been well described (21;22), the effects of acute insulin deprivation on the brain and cognition remain to be determined.
Here we sought to determine the impact of acute insulin deprivation on brain metabolites including ATP levels, functional connectivity and cognition. We hypothesized that the disruptions in oxidative phosphorylation/brain energy pathways resulting from insulin deprivation would alter brain functional connectivity and cognition. We assessed whether these biochemical and functional measurements are altered in T1D in comparison with nondiabetic (ND) participants using brain $^1$H and $^{31}$P magnetic resonance spectroscopy (MRS) and resting state-functional magnetic resonance imaging (rs-fMRI) as well as cognitive testing.
RESULTS

*Insulin deprivation, as anticipated, caused substantial biochemical changes in T1D participants (Table 1 and Figure 1)*

The study was designed to address whether transient insulin deprivation alters brain function. We compared the T1D (age range=14-28 years) to ND (16-29) participants who are matched for age, sex and body mass index (BMI). We did not include older T1D participants to delineate the effects of diabetes from age-related brain changes. Mean glycated hemoglobin (HbA1c) (6.7-8.8% in T1D vs 4.3-5.5% in ND groups, respectively) and Time 1 plasma glucose (7.68±0.67 mmol/L in T1D vs 5.15 ± 0.12) were different (P<0.001). All participants were studied following an overnight fast. In T1D participants, we administered insulin intravenously for 3 hours to maintain stable plasma glucose between 5-6.67 mmol/L by titrating the insulin infusion rates every 15-30 min, while ND participants received no insulin during the same period (Time 1). In response to discontinuing the insulin infusion for 5.4 ±0.6 hours, plasma glucose in T1D participants increased to an average 14.4 mmol/L which was higher than in ND of 4.72 mmol/L at the same time (P<0.001). As expected, beta hydroxy-butyrate (BOHB) concentrations were not different at Time 1 between T1D and ND, but significantly increased Time 2 (following insulin deprivation) in T1D, but not in ND, rendering BOHB to be higher in T1D than ND (P<0.001). Similarly, serum osmolalit was also higher at Time 2 compared to Time 1 in the T1D group and was higher than ND participants who had no change during Time 1 and Time 2. At Time 2, bicarbonate fell in the T1D group, while there was no significant change in ND. All of these changes are consistent with insulin deficiency in T1D.

*Impact of Insulin deprivation on Cognitive Function (Figure 2A)*
We sought to determine whether transient insulin deprivation has any adverse effect on cognitive function. There were no differences between the T1D and ND groups on any study measures including those of memory, verbal and nonverbal executive function, attention, or short-term memory at Time 1 (obtained while T1D were on insulin infusion). However, at Time 2 (following discontinuation of insulin infusion in T1D), the T1D group performed poorly compared with ND in 2 areas of cognitive function testing. The T1D group showed slower fine motor speed on (Delis-Kaplan Executive Function System) D-KEFS Condition 5 at Time 2 compared with ND. Also at Time 2, the T1D group showed less benefit from repeat exposure to tests of attention and working memory compared with ND participants. With the exception of the Ray Auditory Verbal Learning Test (RAVLT), there were no performance differences between adults and adolescents and so the results were pooled for analysis. On the RAVLT memory test, adolescents in both the T1D and ND groups did especially poorly at Time 2 compared to Time 1. T1D and ND adults showed a decline in performance at Time 2 \( (p<0.02) \) which was driven by the especially poor performance of ND adults. The overall decline in RAVLT may indicate the effect of fasting in adolescents and ND adults (Supplemental Table 1).

**Changes in brain metabolites by Proton MRS Data (Figure 2B)**

Brain lactate concentration was low at below the detectable levels in both T1D and ND. However, at Time 1 the T1D group had lower N-Acetyl Acetate (NAA)/ creatine (Cr), while having higher myo-inositol (mI)/Cr than in ND. Neither NAA/Cr nor mI/Cr changed from Time 1 to Time 2.

**Phosphorus MRS Data Showing reduced ATP levels and increase of Phosphocreatine (PCr)/ATP (Figure 2C)**
We hypothesized that ATP levels would decrease from Time 1 to Time 2 in T1D participants due to a shift to a low oxidative capacity in the setting of insulin deficiency, as noted in an animal model (14). We did indeed find that ATP levels decreased from Time 1 to Time 2 in T1D compared to ND, while PCr did not change in either group. An increase in PCr/ATP occurred in the T1D group from Time 1 to Time 2 compared to ND.

*Alterations in rs-fMRI Functional Connectivity following insulin deprivation* (Figure 3, Table 2)

rs-fMRI analysis was performed setting significance at $\alpha<0.01,|t|>2.576$, $P<0.01$ and a minimum cluster size more than 119 voxels (23;24). We found a total of 6 functionally-connected brain regions originating from 3 seed regions (Figure 3, numerical details in Table 2).

At Time 1, higher functional connectivity (FC) was demonstrated between the left hippocampus and the right early visual area (Figure 3A) in ND relative to the T1D group (Figure 3B), indicating that even when insulin treated abnormalities in FC existed in T1D. In T1D, Time 2 vs Time 1, the FC between the left hippocampus and bilateral sensorimotor cortex was also lower (Figure 3C) than ND showing that insulin deprivation was associated with lower FC. At Time 1, FC between the right hippocampus and right and left early visual areas was higher in ND but lower in the right putamen (Figure 3D and 3E). ND also had higher FC between the right hippocampus to left caudate and putamen (Figure 3F). The differences in FC between Time 1 and Time 2 of posterior cingulate cortex and right precentral gyrus were lower in ND than in the T1D group. (Figure 3G).

*Correlations between Cognitive Function and MRS/rs-fMRI Findings* (Table 3)
Correlations between the seed areas and cognitive testing results, as well as MRS metabolite findings were assessed (Table 3). Choline (Cho), an important neurotransmitter essential for brain functions including memory, correlated to FC between the hippocampus and sensorimotor cortex. mI and NAA, which are abundant in the brain, correlated to FC between the hippocampus and early visual area at Time 1 and also while PCr is correlated to FC between posterior cingulate cortex and pre-central gurus. In the diabetic group at Time 1, glutathione (GSH)/Cr was correlated to hippocampus and caudate and putamen regions, and a negative correlation with GSH/Cr was noted (Time 1 - Time 2) between the hippocampus and sensory motor cortex unlike in ND. In T1D at Time 2, GSH/Cr was higher, likely representing an oxidative stress response. There were significant correlations between the hippocampal region and sensorimotor cortex (Time 2-1), and mI/Cr levels.

FC between the hippocampus and visual areas, hippocampus and sensorimotor cortex and hippocampus and caudate and putamen are significantly either positively or negatively correlated in both diabetic and ND groups. In the diabetic group, FC (Time 2-Time 1) in the hippocampus and sensorimotor cortex, hippocampus and early visual area, as well as hippocampus and putamen are negatively correlated to important cognitive changes (Table 3).
DISCUSSION

The current study demonstrates significant differences in proton MRS-based biomarkers of neuronal health such as N-acetyl acetate, myo-inositol, and cortical fractional anisotropy in the T1D group while on insulin treatment in comparison with ND controls. Further, phosphorus MRS demonstrated alterations in bioenergetics, as shown by a decline in ATP levels and alteration the PCr/ATP ratio during insulin deprivation in the T1D group. Transient insulin deprivation (mean 5.4 hours duration) in the T1D group compared to ND group also resulted in diminished executive function including attention, short-term memory, and fine motor speed. Although the T1D group had baseline differences in FC, more important changes occurred following insulin deprivation, especially between the hippocampus-caudate-putamen regions and the sensory motor and early visual areas. We also noted significant correlations in regional brain FC to cognitive function, neurometabolites and energy parameters.

The rs-fMRI findings in the current study are intriguing from a number of perspectives. First, FC cluster analysis identified seed areas not reported in any major functional network (e.g., default mode network) (25). This identification is likely due to methodologic differences between seed-based analysis techniques versus non-constrained, predominantly independent components-based analyses (26). Of interest, other seed-based studies also reported FC correlations of the hippocampus to other regions such as the medial temporal lobe (27;28). Furthermore, Griffanti et. al. (29) performed seed-based FC correlations of the basal ganglia and found a significant negative correlation between age and the basal ganglia FC to other brain structures. In the current study, FC between different regions involved in memory differed between ND and diabetic groups. For example, lower FC between the left hippocampus and the right early visual area (Figure 3A) and between the right hippocampus and both early visual
areas (Figure 3D) are noted in the T1D group relative to ND (Table 2). In contrast, there was higher FC between the right hippocampus and right putamen (Figure 3E). After insulin deprivation, the most altered FCs were in the hippocampus-caudate to sensorimotor cortices (Figure 3 and Table 2) which are involved in memory. Overall, our data also suggests that in the T1D group at baseline and during insulin deprivation the accumulation of deleterious neurometabolites, including energy metabolites, altered FC, which then contributes to cognitive changes. Further studies with larger samples will be helpful to validate these important findings.

We noted that following insulin deprivation, the T1D group failed to demonstrate expected learning upon repeat cognitive testing in executive function domains. The T1D group slowed fine motor speed after insulin deprivation, whereas the ND group’s fine motor speed increased during the same period. The T1D group also benefited less from repeated exposure to tests of attention and working memory compared with the ND group. The alertness and attention required for success on this task are mediated by an interaction of brainstem-diencephalic structures which communicate through several pathways to structures involved in memory and sensori-motor regulations (30). Of interest, all of the above areas causing cognitive decline occurred in the T1D participants during insulin deprivation in brain regions with abundant insulin receptors (31).

While chronic poor glycemic control has been associated with changes in the hippocampus and memory, it is unknown whether transient insulin deficiency worsens brain functioning (32;33). The present study did not isolate insulin deficiency from hyperglycemia and other metabolic changes, including an increase in BOHB, non-esterified fatty acids (NEFA) and amino acids, as well as glucagon levels (34;35). However, it remains plausible that insulin deficiency independently produces neurometabolic changes. There is differential distribution
throughout the brain of insulin and IGF1 receptors and their post-translational signaling partners (31). Moreover, intranasal insulin administration in diabetic mice increased brain insulin concentrations without any systemic glucose concentration but activated downstream signaling such as PI3K/Akt and GDK3β and caused a notable effect on mitochondrial biogenesis and function in brain regions such as the hippocampus (14). The above results support a hypothesis that insulin directly acts on brain regions involved in memory by increasing energy metabolism. Some of the proposed downstream pathways of insulin receptors, such as the PI3K/Akt, mTORC1, GDK3β, and FoxO group of transcription factors play crucial roles in maintaining normal brain function (14;36-39). Likewise, insulin receptors directly interact with the MAP kinase cascades known to affect memory (40-42). Insulin is a key regulator of mitochondrial biogenesis and function in multiple tissues (14;43) including skeletal muscle, liver and brain. In diabetic mice (44) and humans with T1D (35), insulin deprivation directly and substantially inhibited mitochondrial ATP production and detrimentally increased oxidative stress in skeletal muscle. However, the effect of insulin deprivation on brain energetics in mice was more subtle and surprisingly enhanced the endogenous anti-oxidant defense system, thus protecting the brain against oxidative stress which appeared to be due to a beneficial effect of ketones and lactate (14). During transient insulin deprivation in the current study, a substantial BOHB elevation may have offered antioxidant defense and may explain why only subtle changes in brain energetics occurred. No comparable human studies, to our best knowledge, have explored brain changes related to transient insulin deprivation. Not only does our study illuminate potential mechanisms driving longitudinal changes seen in FC and cognitive functioning associated insulin deficiency and metabolic alterations, but also demonstrates the effects of acute insulin omission with important clinical implications on functional connectivity between brain regions.
and executive functions. The current study quantifies the neurologic changes associated with lapses in insulin administration commonly encountered in the growing number of people using insulin pumps (45). Results from the current study may inform further clinical practice and counseling for adults and children with T1D who are reliant upon continuous insulin infusion devices as well as self-administration of multiple daily insulin regimens.

In the current study in T1D participants the diabetes onset is variable and no conclusion on the impact of diabetes duration on our results. Previous studies have addressed structural and functional changes of brain changes that occur on chronic poor glycemic control (22;46). The current study demonstrated differences in MRS-based brain biomarkers of neuronal health such as N-Acetyl Acetate, myo-inositol, and cortical fractional anisotropy in the T1D group at the baseline state. Since the above parameters did not change following insulin deprivation in the current study, the differences in the above brain metabolites that we observed between T1D and ND participants represent potential suboptimal glycemic control of longer term. Although hyperinsulinemia with hypoglycemia also have adverse effect on brain (21;22), it has been shown that transient hypoglycemia does not alter energy rich phospho-metabolites in brain (21). Moreover, it appears that brain extract higher fraction of glucose so that its metabolic needs can be maintained during modest hypoglycemia (13). In contrast, the current we demonstrate that hypoinsulinemia with the concurrent metabolic changes altered brain energy metabolites. The current study measured static PCr and ATP levels representing the concentration of these metabolites within the cell but did not did not address the kinetics of these energy metabolites (47) which will require phosphorus MRS scans with different saturation transfer pulses requiring longer phosphorus MRS scans that was not practical with all measurements we performed. Our focus was not only energy metabolites but also acquiring other anatomical or functional imaging.
Our results must be interpreted within the context of several limitations. First, a larger sample size may have the ability to detect changes in brain volume associated with insulin deprivation. Second, as with the constraints of a human study, we were unable to further isolate the complex metabolic and hormonal changes occurring with insulin deprivation including hyperglycemia, increased NEFA, BOHB, amino acids and glucagon concentrations as well as volume changes, all of which could have potential independent effects on the brain. Further studies are required to differentiate the effect of each of these variables associated with insulin deprivation and isolate the effect of insulin deprivation alone on brain. However, pre-clinical and early clinical studies clearly established the selective effect of insulin on brain metabolism including cognition (14;48;49). We were limited in the insulin deprivation duration due to participant safety and risk for diabetic ketoacidosis, particularly in the pediatric group where even after 6 hours some adolescents had substantial metabolic derangements.

In conclusion, we found that transient insulin deprivation in T1D individuals altered brain connectivity between areas of memory and sensorimotor as well as early visual regions concurrent to alterations in cognition in the domains of executive functions and fine motor speed and subtle changes in brain bioenergetics and metabolites. Further understanding the neurocognitive effects of transient insulin deprivation, as highlighted in this study, will have important clinical implications given the growing number of people with diabetes and use of insulin pumps across the world.
METHODS

Study Participants

Adults and adolescents with T1D were recruited within Mayo Clinic, Rochester, Minnesota, and Olmsted County, Minnesota. Written informed consent or parental permission was obtained after Mayo Clinic Institutional Review Board approval. Adult ND controls were healthy age-, sex-, and BMI-matched while adolescent ND controls were the same sex sibling of similar age (±1 year) or a friend of similar BMI and age (±6 months). All participants were screened with a detailed history, physical table examination, and biochemical profile. Exclusion criteria included diagnosed cognitive delay, attention-deficit/hyperactivity disorder, learning disabilities, dementia, psychiatric disease, cardiovascular and cerebrovascular diseases, peripheral neuropathy, renal disease, substance use, or obesity.

T1D participants needed to have a hemoglobin A1c < 9%, BMI 20-30 kg/m² for adults and <95th percentile for adolescents, c-peptide <1 ng/mL, and no evidence of active diabetic complications, including renal disease, peripheral vascular disease, and neuropathy. The participants’ average diabetes duration was 10.7(±1.7) years (Range: 1-25 years).

Study Protocol (Figure 1)

Figure 1 outlines the study design. Prior to the study day, T1D participants using insulin injections held long-acting insulin 36 hours prior to study start to avoid any carryover effect, managing glucose with short-acting insulin corrections. T1D participants using insulin pumps continued on the pump until the study start day. All participants maintained typical sleep and exercise schedules the week prior to the study.
Participants were admitted after an overnight fast. Prior to Time 1 of the study, all participants had baseline blood biochemical measurements, including serum glucose. After these baseline measurements, participants entered Time 1. T1D participants were transitioned to intravenous regular insulin infusion titrated to target blood glucose of 5-6.67 mmol/L. Titration was performed using point of care glucose devices, as transportable devices were necessary in the MRI suite and allowed frequent glucose monitoring every 15-30 minutes to ensure each participant remained in range throughout the MRI study. Participants remained fasting with hydration from intravenous normal saline and water ad lib. Participants then underwent 30 minutes of cognitive testing followed by MRI scans as detailed below.

After Time 1, participants entered Time 2. During Time 2, T1D underwent insulin deprivation and ND continued hydration. Following >4 hours of Time 2, cognitive testing and MRI studies were repeated while insulin remained off for T1D, with a 6 hour maximal period of insulin deprivation as there was some variance within the study day on how long it took each subject to complete all study tests. Final blood sampling was performed before insulin was restarted. T1D participants were observed until clinically stable and the blood glucose was <11.1mmol/L, at which time they ate a meal and were discharged. ND participants underwent similar laboratory, cognitive, and MRI studies at Time 1 and Time 2 without any insulin treatment or deprivation.

**Hormones and Substrates**

Plasma glucomes were assayed in samples collected at baseline and following insulin deprivation enzymatically using Roche Modular platform. Plasma c-peptide, bicarbonate, BOHB, and electrolyte profiles were measured as previously described (35).
**Cognitive Testing**

All subjects underwent a cognitive battery before each MRI. This broad, multi-dimensional cognitive evaluation consisted of the RAVLT, the D-KEFS, the Trail Making Test and the Number Letter subtest of the Wide Range Assessment of Memory and Learning (WRAML).

The RAVLT is a memory test assessing acquisition, learning rate, susceptibility to interference, and forgetting (50). The RAVLT has marginal/adequate test-retest reliability with $r$ values about 0.60-0.70 (51-53). The D-KEFS measures various aspects of executive functions (54). The Trail Making Test assesses visual scanning, mental sequencing, thinking flexibility, and motor speed. Test-retest reliability ranges from $r=0.38$ to $r=0.77$. The Verbal Fluency test of the D-KEFS assesses an individual’s ready access to verbal stores of information. Test-retest reliability ranges from $r=0.36$ to $r=0.8$. The Number Letter subtest of the WRAML measures attention and short-term memory (55). Median coefficient alpha reliability is $r=0.83$. The combination of the above tests yielded a broad assessment of the subject’s cognitive function before and after insulin withdrawal.

**MRI Data Acquisition**

Brain imaging was performed on a single 3 Tesla Siemens Skyra equipped with a multi-nuclear option (MNO) running VE11C software. Each participant was scanned twice on the same day using the same scan protocol. A 32-channel proton-only head coil was used for all MR imaging and proton MRS. A dual-tuned proton/phosphorus flex coil (Clinical MR Solutions, Brookfield, Wisconsin: 16x18 cm diameter Helmholtz pair for proton, 12 cm diameter loop coil...
for phosphorus) was used for phosphorus MRS. A sagittal 3D Magnetization Prepared – Rapid Gradient Echo (MPRAGE) sequence with 0.7mm isotropic voxels (TR=2400ms, TE=2.57ms, TI=1100ms, FA=8deg) was acquired in order to obtain brain parcellations for subsequent rs-fMRI analyses.

To assess brain energy pathways, both proton and phosphorus MRS were performed in order to measure brain lactate (anaerobic metabolism) and ATP levels (aerobic metabolism). Proton MRS was acquired using the svs_se sequence with the voxel placed over the occipital lobes (TR=2000ms, TE=30ms, 128 averages). Phosphorus MRS was acquired using a multi-voxel chemical shift imaging sequence (WIP 1071, qa_csi_fid_31P) applied such that the phosphorus loop was positioned over the occipital cortices. An axial 1.5 cm CSI slab was acquired to encompass the occipital cortices (TR=1500ms, TE=30ms, 16x16 matrix over a 240 cm FOV, 1.5 cm nominal isotropic voxels, 2 averages).

To assess functional connectivity, rs-fMRI imaging was acquired using an axial 2D echo-planar imaging sequence with 3mm isotropic voxels (FA=90deg, TE=30ms, TR=3000ms, # slices=52, 116 volumes, 5m48s). Participants were instructed to lie still in the scanner, but given no other instructions besides that.

**MRI Data Processing**

Single voxel proton MRS data were processed using LCModel 6.3-1L (56). Spectra were visually inspected and any corrupted spectra were excluded from the analysis. Besides lactate, Cho, NAA, glutamate, glutamine, glutamate-glutamine, GSH, and mI ratios expressed using Cr were also tabulated. All metabolite values with Cramer Rao Lower Bound <= 20% were kept in
the analysis. No attempt was made to perform cerebrospinal fluid volume correction; we report only ratios.

Multivoxel phosphorus magnetic resonance spectroscopic imaging data were reconstructed and quantified using jMRUI 6.0 (http://www.jmrui.eu). Spectra were pre-processed by 1) truncating the data to 768 points, then zero-filling to 1024 points, 2) apodizing with a 5 Hz Lorenzian, and 3) aligning the data such that the PCr peak was set to 0 Hz. Next, two voxels from occipital cortex were selected, extracted and averaged together into a single free induction decay in order to reduce noise. Finally, as we were only interested in ATP and PCr levels, the average free induction decay was quantified using AMARES and a four metabolite basis set (PCr, γ-ATP, α-ATP, β-ATP) (52). The PCr/ATP ratio was calculated using the sum of the γ-ATP, α-ATP, β-ATP peaks for the denominator.

MPRAGE data were analyzed using Freesurfer 6.0 (http://surfer.nmr.mgh.harvard/edu). MPRAGE images were converted to a Neuroimaging Informatics Technology Initiative volume using mri_convert, then processed using recon_all with manual inspection of data between each recon step. The Freesurfer N27 template (57) was then used to obtain cortical and subcortical parcellations for functional connectivity matrix calculations. Resting-state functional MRI data were motion-corrected and denoised by a well-established preprocessing pipeline in the NIH rs-fMRI analysis tool (AFNI package) as previously described (8;9). Briefly, rs-fMRI data were despiked and corrected for physiologic noise, slice timing, head motion, and hardware artifacts. The corrected data were spatially smoothed with an isotropic Gaussian kernel (full-width-at-half-maximum at 6 mm), and then registered to the MPRAGE imaging (58) to get functional blood flow data for each cortical/subcortical brain region obtained from the N27 parcellation. Individual seed-based connectivity matrices for whole subjects were used for the group
comparison between diabetic and control groups, and also for correlation analysis between functional connectivity strength, clinical outcomes (behavioral scores) and metabolic data (proton and phosphorus metabolites) (10).

**Statistical Analysis**

We summarized measurements and paired differences using descriptive statistics. Data was inspected for normality, and transformations applied or nonparametric tests were used when necessary. A nonparametric robust analysis of variance-type statistic (F1-LD-F1 design in nparLD) was utilized to assess the effect of insulin deprivation in patients with T1D when compared to ND participants (59). Two-sample *t* tests were used to compare the T1D group to the ND group. All statistical tests were 2-sided with a significance threshold of $\leq 0.05$. No corrections were made for multiple comparisons. As our subjects had a narrow age distribution, no corrections were made for age as there was no need to eliminate age-related effects.
Author Contributions: AC and TC conducted the study, wrote the manuscript and researched the data, KSN designed the study, supervised the conduct and analysis of samples, researched and interpreted the data, revised and edited manuscript, JP supervised all MRI, MRS and rs-fMRI and interpreted the data and contributed to the manuscript, HJJ performed the analysis of rs-fMRI data, ARH performed and analyzed neuro-psychological tests, interpreted results and contributed to the manuscript, SD performed statistical analysis, JMT, and ANLA contributed to the participants screening and selection besides reviewing manuscript, KAK and GNR offered valuable technical help and contributed to the manuscript, YCK offered clinical support and contributed to manuscript, RCP contributed to the manuscript.

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Figure 1: Study Design: Both study groups (Non-diabetic - ND and T1D) started the study with a fasting blood sampling. T1D participants were then started on a continuous insulin infusion titrated to maintain blood glucose between 5-6.7 mmol/l (Time 1). Following a 3 hour insulin infusion in T1D or the corresponding time course for ND group, participants had cognitive testing. Following this the participants were taken to the Radiology and MR studies were performed. The insulin infusion was continued during both cognitive testing and MR studies. Then, blood sampling was performed and immediately after the insulin infusion was discontinued in the T1D participants, and 4 hours following after a period of insulin withdrawal (Time 2), or at the corresponding time course for the control participants, the cognitive testing (4-4.5 hours) and then MR studies were performed (5±0.6 hrs). Time 1 represents the insulin treated period in T1D and Time 2 represents the period following insulin deprivation in T1D. Both times represent no intervention in ND but specifically address the time related changes in the ND.
Figure 2: Cognitive test results showing changes in participants with type 1 diabetes (T1D) insulin treated (Time 1) to insulin-deprived state (Time 2) in comparison with the same period in participants without diabetes (ND). A, Compared with ND participants, T1D participants showed significantly poorer attention and working memory based on the Wide Range Assessment of Memory and Learning-2 (WRAML-2) Number Letter (*P<0.001) and fine motor speed based on Delis-Kaplan Executive Function System (D-KEFS) Trail Making (*P<0.02).

B, Baseline differences in myo-inositol (*P<0.05), N-acetyl aspartate (NAA) (*P<0.05) and cortical fractional anisotropy (FA) (*P<0.05) between the T1D group and ND controls and none of them significantly altered by insulin deprivation (data not shown).

C, Total adenosine triphosphate (ATP) levels that significantly decreased on insulin deprivation (Time 1 to Time 2) in T1D participants (P<0.04) and phosphocreatine (PCr showed no significant changes. The ratio of PCr to ATP increased in the T1D group (P<0.03).
Figure 3: Seed-based functional connectivity (FC) maps. Seed masks were extracted from the FreeSurfer parcellation of the N27 brain template. Three seed regions reached statistical significance (left and right hippocampi and bilateral posterior cingulate cortex). The FC of these seed regions was higher (yellow) or lower (blue) to 6 different brain regions (Table 2). At baseline (left column), there was appreciably higher FC in nondiabetic (ND) participants relative to type 1 diabetes (T1D) participants between the left hippocampus and the right early visual area (A), and between the right hippocampus and bilateral early visual areas (D). In contrast, there was significantly lower FC between the right hippocampus and right putamen (E) in ND participants. There were no significant FC differences with the bilateral posterior cingulate cortex in the baseline state. Following insulin deprivation (middle column), there was substantially higher FC in ND participants relative to T1D participants between the left hippocampus and bilateral sensorimotor cortices (B), but lower FC between both posterior cingulate cortices and the right precentral gyrus (G). There were no significant FC differences with the right hippocampus in the post-withdrawal state. Finally, assessing changes between the post-withdrawal and baseline states in T1D participants (right column), there was considerably decreasing FC between the left hippocampus and bilateral sensorimotor cortices (C) and the right hippocampus and the left caudate/putamen (F) following insulin withdrawal indicating that insulin deprivation adversely affected FC between these regions in T1D participants. No significant FC differences with the bilateral posterior cingulate cortex were found in diabetics between the baseline and post-withdrawal states. Note that the FC clusters for the bilateral sensorimotor cortex identified in B and C are similar, but not identical (see Table 2).
Table 1: Participant Characteristics and Biochemical Parameters

<table>
<thead>
<tr>
<th></th>
<th>T1D</th>
<th>ND</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y), mean ± SEM</td>
<td>21 ± 1.4</td>
<td>21 ± 1.4</td>
<td>1.0</td>
</tr>
<tr>
<td>M/F</td>
<td>6/8</td>
<td>6/8</td>
<td>1.0</td>
</tr>
<tr>
<td>HbA1c, mean ± SEM</td>
<td>7.9% ± 0.82</td>
<td>4.9% ± 0.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glucose Time 1 (mmol/L), a mean ± SEM</td>
<td>7.68 ± 0.67</td>
<td>5.15 ± 0.12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glucose Time 2 (mmol/L), b mean ± SEM</td>
<td>14.14 ± 1.42</td>
<td>4.72 ± 0.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Bicarbonate Time 1 (mmol/L), mean ± SEM</td>
<td>24.9 ± 0.4</td>
<td>24.4 ± 0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Bicarbonate Time 2 (mmol/L), mean ± SEM</td>
<td>21.2 ± 0.8</td>
<td>25.2 ± 0.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Osmolality Time 1 (mOsm/kg), mean ± SEM</td>
<td>289.3 ± 0.9</td>
<td>289.8 ± 0.9</td>
<td>0.79</td>
</tr>
<tr>
<td>Osmolality Time 2 (mOsm/kg), mean ± SEM</td>
<td>289.6 ± 0.9</td>
<td>286.6 ± 0.9</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>Beta hydroxybutyrate Time 1 (mmol/L), mean ± SEM</td>
<td>0.2 ± 0.03</td>
<td>0.2 ± 0.06</td>
<td>0.33</td>
</tr>
<tr>
<td>Beta hydroxybutyrate Time 2 (mmol/L), mean ± SEM</td>
<td>1.9 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Abbreviations: F, female; M, male; ND, nondiabetic; T1D, type 1 diabetes.

a Time 1 indicates baseline values; Time 2 indicates insulin deprived.

b Glucose value at the beginning of insulin treatment, all subjects then underwent frequent IV insulin titrations and glucose monitoring to achieve a glucose level of 5 – 6.7 mmol/dl throughout the first study phase.
Table 2: Talairach Coordinates of the 6 Functionally-Connected Brain Regions Detected by rsFMR

<table>
<thead>
<tr>
<th>Seed region</th>
<th>Timepointa</th>
<th>Cluster region</th>
<th>Talairach coordinate (mm)</th>
<th>Peak t value</th>
<th>Cluster size (mm³)</th>
<th>Cluster label (Figure 2)b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td>y</td>
<td>z</td>
<td></td>
</tr>
<tr>
<td>Left hippocampus</td>
<td>Time 1</td>
<td>Right early visual area</td>
<td>-23</td>
<td>-82</td>
<td>+11</td>
<td>+4.172</td>
</tr>
<tr>
<td></td>
<td>ND vs T1D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Time 1 ND vs T1D</td>
</tr>
<tr>
<td></td>
<td>Time 2</td>
<td>Left/right sensorimotor cortex</td>
<td>+8</td>
<td>-43</td>
<td>+62</td>
<td>+3.828</td>
</tr>
<tr>
<td></td>
<td>ND vs T1D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Time 2 ND vs T1D</td>
</tr>
<tr>
<td></td>
<td>Time 2 vs</td>
<td>Left/right sensorimotor cortex</td>
<td>+11</td>
<td>-34</td>
<td>+59</td>
<td>+4.078</td>
</tr>
<tr>
<td></td>
<td>Time 1 ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right hippocampus</td>
<td>Time 1</td>
<td>Left/right early visual area</td>
<td>-7</td>
<td>-70</td>
<td>-10</td>
<td>+3.669</td>
</tr>
<tr>
<td></td>
<td>ND vs T1D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Time 1 ND vs T1D</td>
</tr>
<tr>
<td></td>
<td>Time 2 –</td>
<td>Right putamen</td>
<td>+29</td>
<td>-4</td>
<td>+8</td>
<td>-3.739</td>
</tr>
<tr>
<td></td>
<td>Time 1</td>
<td>Time 1 ND vs T1D</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ND vs T1D</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Time 2 vs</td>
<td>Left putamen/caudate</td>
<td>-22</td>
<td>-1</td>
<td>+11</td>
<td>+4.759</td>
</tr>
<tr>
<td></td>
<td>Time 1 T1D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Posterior cingulate cortex</td>
<td>Time 1</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td>Posterior cingulate cortex</td>
</tr>
<tr>
<td></td>
<td>ND vs T1D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Time 1 ND vs T1D</td>
</tr>
<tr>
<td></td>
<td>Time 2 –</td>
<td>Right precentral gyrus</td>
<td>+23</td>
<td>-16</td>
<td>+44</td>
<td>-3.801</td>
</tr>
<tr>
<td></td>
<td>Time 1 T1D</td>
<td>Time 2 vs Time 1 T1D only</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>ND vs T1D</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ND, nondiabetic controls; rsFMR, resting-state functional magnetic resonance; T1D, type 1 diabetic participants.
aTime 1 signifies the insulin-treated state in T1D and baseline in ND, and Time 2 signifies the postinsulin deprivation period for T1D and time-related control for ND.
bLetters refer to parts of Figure 2.
Table 3: Correlation of Proton/Phosphorus Metabolite and Cognitive Data with Functional Connectivity (FC) Regions as in Figure 2.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>FC Regions (Figure 2)</th>
<th>Participant group</th>
<th>Time point</th>
<th>Correlation coefficient</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cho/Cr</td>
<td>Left hippocampus -bilateral sensorimotor cortex</td>
<td>ND</td>
<td>Time 1</td>
<td>0.561</td>
<td>0.046</td>
</tr>
<tr>
<td>NAA/Cr</td>
<td>Left hippocampus-right early visual area</td>
<td>ND</td>
<td>Time 1</td>
<td>0.566</td>
<td>0.044</td>
</tr>
<tr>
<td>ml/Cr</td>
<td>Left hippocampus-right early visual area</td>
<td>ND</td>
<td>Time 1</td>
<td>0.559</td>
<td>0.047</td>
</tr>
<tr>
<td>PCr/ATP</td>
<td>Bilateral posterior cingulate cortex – right precentral gyrus</td>
<td>ND</td>
<td>Time 2</td>
<td>0.725</td>
<td>0.012</td>
</tr>
<tr>
<td>GSH/Cr</td>
<td>Right hippocampus - right putamen</td>
<td>T1D</td>
<td>Time 1</td>
<td>−0.663</td>
<td>0.189</td>
</tr>
<tr>
<td>GSH/Cr</td>
<td>Right hippocampus - left caudate and putamen</td>
<td>T1D</td>
<td>Time 1</td>
<td>−0.645</td>
<td>0.024</td>
</tr>
<tr>
<td>GSH/Cr</td>
<td>Left hippocampus -bilateral sensorimotor cortex</td>
<td>T1D vs ND</td>
<td>Time 2</td>
<td>−0.760</td>
<td>0.004</td>
</tr>
<tr>
<td>ml/Cr</td>
<td>Left hippocampus -bilateral sensorimotor cortex</td>
<td>T1D</td>
<td>Time 2- Time 1</td>
<td>−0.733</td>
<td>0.007</td>
</tr>
</tbody>
</table>

**Cognitive Domain**

| Speeded word retrieval | Right hippocampus - right putamen | ND | Time 1 | 0.587 | 0.035 |
| Cognitive flexibility  | Left hippocampus-right early visual area | ND | Time 2 | −0.694 | 0.008 |
| Cognitive flexibility  | Left hippocampus -bilateral sensorimotor cortex | ND | Time 2 | −0.564 | 0.045 |
| Cognitive flexibility  | Left hippocampus -bilateral sensorimotor cortex | ND | Time 2 | −0.566 | 0.044 |
| Speeded word retrieval | Right hippocampus - right putamen | ND | Time 2 | −0.744 | 0.004 |
| Speeded word retrieval | Right hippocampus - left caudate and putamen | ND | Time 2 | −0.693 | 0.009 |
| Speeded visual processing | Left hippocampus-right early visual area | ND | Time 2-Time 1 | 0.641 | 0.018 |
| Cognitive flexibility  | Left hippocampus-right early visual area | ND | Time 2-Time 1 | −0.614 | 0.026 |
| Speeded verbal retrieval | Right hippocampus - left caudate and putamen | ND | Time 2-Time 1 | −0.565 | 0.044 |
| Speeded visual processing | Right hippocampus - right putamen | T1D | Time 1 | −0.628 | 0.021 |
| Fine motor speed       | Right hippocampus - right putamen | T1D | Time 1 | −0.672 | 0.012 |
| Fine motor speed       | Right hippocampus - left caudate and putamen | T1D | Time 1 | −0.560 | 0.046 |
| Cognitive flexibility  | Left hippocampus-right early visual area | T1D | Time 1 | −0.635 | 0.020 |
| Cognitive flexibility  | Left hippocampus -bilateral sensorimotor cortex | T1D | Time 2 | −0.686 | 0.010 |
| Speeded visual processing | Right hippocampus - right | T1D | Time 2 | −0.571 | 0.042 |
| D-KEFS, all error types, cumulative % rank | Right hippocampus - right putamen | T1D | Time 2 | −0.769 | 0.002 |
| Speeded word retrieval | Right hippocampus - right putamen | T1D | Time 2 | 0.576 | 0.039 |
| Attention and working memory | Right hippocampus - right putamen | T1D | Time 2 | −0.727 | 0.005 |
| Speeded mental sequencing | Right hippocampus - left caudate and putamen | T1D | Time 2 | 0.663 | 0.014 |
| Speeded mental flexibility | Right hippocampus - left caudate and putamen | T1D | Time 2 | −0.608 | 0.027 |
| Speeded verbal retrieval | Right hippocampus - left caudate and putamen | T1D | Time 2 | 0.573 | 0.041 |
| Speeded word retrieval | Right hippocampus - left caudate and putamen | T1D | Time 2 | 0.580 | 0.038 |
| Cognitive flexibility | Right hippocampus - left caudate and putamen | T1D | Time 2 | −0.623 | 0.023 |
| Cognitive flexibility | Left hippocampus - bilateral sensorimotor cortex | T1D | Time 2 - Time 1 | −0.582 | 0.037 |
| Cognitive flexibility | Left hippocampus-right early visual area | T1D | Time 2 - Time 1 | −0.694 | 0.008 |
| Speeded verbal retrieval | Right hippocampus - right putamen | T1D | Time 2 - Time 1 | −0.615 | 0.025 |
| Attention and working memory | Right hippocampus - right putamen | T1D | Time 2 - Time 1 | −0.563 | 0.045 |

Abbreviations: Cho/Cr: choline to creatine ratio; D-KEFS, Delis-Kaplan Executive Function System; GSH/Cr: glutathione to creatine ratio; mI/Cr: myo-inositol to creatine ratio; NAA/Cr: N-acetylaspartate to creatine ratio; ND, nondiabetic controls; PCr/ATP: phosphocreatine to adenosine triphosphate ratio; T1D, type 1 diabetes group.