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14. ABSTRACT

Blast-related mild traumatic brain injury (mTBI) is widespread amongst service members and veterans who served in Iraq and Afghanistan. TBI has many long term adverse health consequences as summarized in a recent report by the Institute of Medicine[1]. However, it remains unclear if TBI increases the susceptibility to **diabetes** and other metabolic diseases. Given the tremendous impact that diabetes and metabolic diseases have on the quality of life and morbidity by fueling cardiovascular disease, cancer, impaired cognition and reducing life expectancy, it is important to establish if mTBI can indeed cause metabolic disease and if so, through which mechanisms. Here we tested if blast induced TBI impairs metabolism in a rats independent of food intake. In well controlled studies we established that indeed blast induced TBI causes profound and long lasting glucose intolerance. Importantly we were able to completely reverse this glucose intolerance through the treatment with an SGLT2 inhibitor, a commercially available diabetes drug which also prevented the brain inflammation. The biggest impact of these studies lie in the realization that TBI impairs metabolic control and increases the risk for type 2 diabetes. Secondly, they give hope to those that may suffer from metabolic disease and have TBI as we have begun to identify drugs that seem to be promising in treating TBI induced metabolic disease.

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1. Introduction

Blast-related mild traumatic brain injury (mTBI) is widespread amongst service members and veterans who served in Iraq and Afghanistan. TBI has many long term adverse health consequences as summarized in a recent report by the Institute of Medicine[1]. However, it remains unclear if TBI increases the susceptibility to diabetes (one of the FY17 PRMRP Topic Areas) and other metabolic diseases. Given the tremendous impact that diabetes and metabolic diseases have on the quality of life and morbidity by fueling cardiovascular disease, cancer, impaired cognition and reducing life expectancy, it is important to establish if mTBI can indeed cause metabolic disease and if so, through which mechanisms.

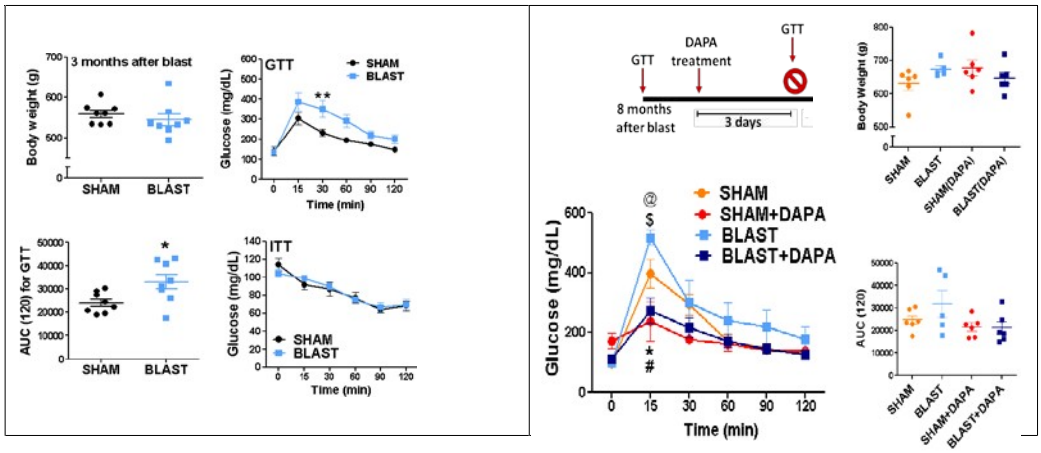
Here we find that TBI induces long lasting glucose intolerance, which can be completely reversed by an SGLT2 inhibitor.

2. Keywords

Diabetes, traumatic brain injury, insulin resistance, metabolic disease, glucocorticoid signaling, stress, metabolic phenotyping

3. Accomplishments

To dissect the mechanisms through which TBI disrupts brain control of metabolism we used a rat model of TBI created by the Naval RC. 8-week old rats were anesthetized and received a 74.5 kPa blast exposure daily for 3 consecutive days; with sham animals not receiving the blast. We followed these rats for a total of 10 months and as shown below did find consistent glucose intolerance (GTT) with significantly increased AUC, even though body weight and insulin tolerance was not altered.



Glucose tolerance at 3 months after the blast injury.	8 Months after the blast injury, the glucose intolerance persists. We decided to treat two subcohorts with an SGLT2 inhibitor and find that this treatment completely reversed the glucose intolerance.Glucose tolerance at 3 months after the blast injury.

When we originally submitted the application we had found that the GR blocker mifepristone ameliorated the TBI induced glucose tolerance and hence we proposed to study this drug as a therapeutic intervention to ameliorate TBI induced glucose intolerance. In independent studies we have found that glucose toxicity (effect of hyperglycemia, which can be caused by glucose intolerance) that worsens metabolic control and causes brain inflammation, can be ameliorated by a drug that allows one to excrete glucose through the urine, an Sglt2 inhibitor, prevented the brain inflammation. Hence we tested if an Sglt2 inhibitors ameliorated TBI induced glucose intolerance. We find that the glucose intolerance and the prediabetes is completely reversed through an SGLT2 inhibitor, and that the effect is much more profound than that of mifepristone.

Given that hyperglycemia results in several pathophysiological changes such as oxidative stress, DNA damage, and causes local and systemic inflammation as evidenced by expression of pro-inflammatory cytokines (*e.g.,* interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α [1] we examined the effects of hyperglycemia on neuroinflammation, a key pathway that contributes and maintains the brain damage after TBI. Indeed, hyperglycemia-induced brain inflammation results in tissue damage that can be particularly harmful to the central nervous system (CNS), since neurons are generally irreplaceable [5]. Microglia, antigen presenting brain immune cells (or macrophages), are the innate immune components in the CNS parenchyma. Under physiological conditions, they act as sentinels to maintain CNS parenchymal integrity. Upon activation, microglia change their morphology, express increased levels of MHC antigens,become phagocytic [6] and can release potent neurotoxins, which may cause neuronal damage [7-9]. They release inflammatory cytokines that amplify the inflammatory response by activating and recruiting other cells to the site of inflammation. For example, microglia can recruit bone-marrow derived macrophages, stimulate new blood vessel formation through direct and indirect effects on endothelial cells or create changes in extracellular matrix (ECM) composition to modify the local tumor microenvironment (TME) [10-12]. The TME is composed of microglia, macrophages, and endothelial cells (glioma vasculature). These stromal cells in turn can elaborate numerous other compounds that stimulate angiogenesis (*e.g;* vascular endothelial growth factors, cytokines), neoplastic cell growth (*e.g;* transforming growth factor-β, cytokines and interleukins) and modify the ECM (*e.g;* MMPs) to further promote glioma expansion and invasion [13, 14]. In TME, cancers stroma-tumor interactions involve immune system cells (*e.g;* leukocytes, mast cells, and macrophages) and fibroblasts, which secrete extracellular proteins, cytokines, and growth factors [15-17]. In the most common forms of brain tumors, gliomas, it appears that unlike other cancers, fibroblasts and mast cells are not important stromal cell types suggesting that other cell types may contribute to the tumor microenvironment and dictate tumor proliferation and growth. Neuroinflammation is induced by the production of interferon-γ (IFNγ) from Th1 cells, astrocytes and microglia which in turn drive microglia polarization to the M1 activated phenotype, thus initiating the formation of TME [18].
Microglia create a supportive stroma for neoplastic cell expansion and invasion by releasing growth factors and cytokines, thus facilitating tumor proliferation, survival and migration [8, 19-22]. Indeed, microglia can promote glioma growth by producing pro-inflammatory cytokines such as IL-1b and inflammatory mediators such as IL-10 and basic fibroblast growth factor and transforming growth factor-β [23-25].
Here we examine whether acute and chronic hyperglycemia can activate microglia, trigger neuroinflammation and induce gene expression changes that may contribute to neuroinflammation and the detrimental cognitive effects of TBI. We pursued this in detail, since our comprehensive analysis of insulin signaling studies in the liver failed to reveal any difference between sham and blasted rats, but demonstrated profound effects by dapagliflocin that insulin sensitized as judged by the molecular insulin signaling studies we performed (data not shown)

Results:

Study 1: Effects of acute systemic hyperglycemia on microglia activation.
1a) Acute hyperglycemia is a key driver of brain inflammation.
To test whether acute hyperglycemia can trigger brain inflammation we performed hyperglycemic clamps. Jugular and carotid catheters were implanted in rats (**Fig.1A**) and acute hyperglycemia (2h) was induced through a hyperglycemic clamp, where glucose was maintained in the diabetic range of 400-500mg/dl (**Fig. 1B**) through a variable glucose infusion rate (GIR). Glycemia was measured every 10 min to adjust the GIR to maintain hyperglycemia (**Fig. 1C**). As expected, the hyperglycemia induced hyperinsulinemia (**Fig. 1D**). In turn, the hyperinsulinemia suppressed lipolysis which resulted in decreased plasma non-esterified fatty acids (NEFA, **Fig. 1E**). Triglycerides level moderately increased due to the pro-lipogenic actions of hyperglycemia and hyperinsulinemia (**Fig. 1F**). At the end of the experiment, we sacrificed the animals after anesthesia with ketamine (70mg/Kg), collected the brains and dissected the hypothalami that were snap-frozen in

liquid nitrogen and stored at -80°C until further analysis. To assess brain inflammation we performed: 1) bulk RNA-sequencing and 2) qPCR analysis to survey inflammatory cytokines expression at the mRNA level, and 3) immunohistochemistry (IHC) to confirm inflammation at the protein level by using the gold-standard microglia marker Iba-1 (ionized calcium binding adaptor molecule-1), and the glia marker GFAP (Glial Fibrillary Acidic protein).

Remarkably, of the 39 differentially regulated genes (expression increased at least 2 fold) identified by bulk RNA-seq between control and diabetic groups (**Fig. 1G**), seven were INFγ target genes such as the chemokines CXCL9, CXCL10, CXCL11, Basic Leucine Zipper ATF-Like Transcription Factor 2 (Batf2), Guanylate Binding Protein-5 (Gbp5), Gpb3, Gpb1 and Immunity Related GTPase M (IRGM2) (see highlighted in **Fig.1G**). This suggests that INFγ signaling is prominent in hyperglycemia induced brain inflammation. Of note, acute hyperglycemia also suppressed growth hormone (Gh1) as anticipated, confirming that hyperglycemia was exerting the biological effects that one would expect, since hyperglycemia is clinically used to suppress growth hormone. As an independent validation of the bulk RNA seq data, we performed qPCR on 13 selected inflammatory genes. IFNγ, CXCL9, CXCL10, CXCL-11, monocyte-chemoattractant-protein-1 (MCP; also known as CCL2) and inducible Nitric Oxide Synthase (iNOS)[29, 30] were markedly increased in the hypothalamus (**Fig. 1H**) and prefrontal cortex (PFC; **Fig. 1I**) after 2h of hyperglycemia. The effect size of the fold increase in mRNA expression was comparable to the effect size seen in the bulk RNA-seq results (2 to 6 fold changes). Next we determined whether longer hyperglycemia, (i.e; 4h versus 2 h of hyperglycemia) was sufficient to increase pro-inflammatory cytokines expression in the brain. The expectation was that 4h of hyperglycemia induced and maintained by a hyperglycemic clamp would result in more pronounced expression of proinflammatory cytokine mRNAs (**Fig. 2A).** Surprisingly, the expression of cytokines was less after 4 hrs compared to 2 hrs of hyperglycemia compared to the respective euglycemic controls, suggesting that either the inflammation is decreasing or that the mRNA expression is not a reliable indicator of the inflammation.

Within the CNS, inflammation is prominently driven through microglia activation (*e.g; microgliosis*), because microglia is the major immune effector cell within the CNS. Microglia can be categorized either as *"resting microglia"*, characterized by a ramified morphology and a tiny body and *"activated microglia"*, characterized by swollen ramified cells with a larger cell body and shorter, thick processes, or alternatively microglia can adopt a *"reactive state",* typically small, spherical cells, but can also exhibit rod-shape or amoeboid-like morphologies. To characterize microglia activation we performed IHC with Iba-1, a microglia and macrophage-specific calcium binding protein that is involved with the membrane ruffling and phagocytosis that increases with activation. The number of Iba-1^{+}-cells was highly increased and the microglia morphology was consistent with activation in the paraventricular nucleus of the hypothalamus (PVN, **Fig.2 D,E**), medio-basal hypothalamus (MBH, **Fig. 2 G,H**) and in the PFC (**Fig. 2 J,K**) of rats after 4h of induced hyperglycemia compared to vehicle animals. This demonstrates that more prolonged hyperglycemia activates microglia and hence induces CNS inflammation, even though this is less obvious in the gene expression profiles of proinflammatory cytokines which limits the value of gene expression profiling as an approach to quantify hyperglycemia induced inflammation. Of note, our results show that 2h of hyperglycemia induced a more pronounced effect on proinflammatory cytokines and IFNγ gene transcription compared to the 4h hyperglycemia, even though the hyperglycemia was comparable and more sustained, suggesting that a more prolonged and chronic hyperglycemia may impair the transcription of pro-inflammatory cytokines. Of note, only CD279 (also known as PD-1 [Programmed Cell Death Protein-1]) was significantly upregulated in the hypothalamus (**Fig. 2B**) and CXCL9 expression was slightly increased in the PFC (**Fig. 2C**) after 4h of hyperglycemia. The upregulation of PD-1 is of importance to the potential of tumorigenesis of microgliomas, since PD-1 is a critical checkpoint mediator responsible for tumor-induced immune suppression and hence involved in promoting tolerance while also limiting tissue damage in the settings of chronic inflammation. Many human solid tumors express PD ligand 1 (PD-L1), and this is often associated with a worse prognosis [31]. Tumor-infiltrating lymphocytes from patients with cancer typically express PD-1 and have impaired antitumor functionality [31, 32].

Programmed cell death protein 1, also known as PD-1 and CD279 (cluster of differentiation 279), is a protein on the surface of cells that has a role in regulating the immune system's response to the cells of the human body by down-regulating the immune system and promoting self-tolerance by suppressing T cell inflammatory activity. This prevents autoimmune diseases, but it can also prevent the immune system from killing cancer cells [5]. PD-1 inhibitors, a new class of drugs that block PD-1, activate the immune system to attack tumors and are used to treat certain types of cancer [5][8]. Importantly, IFNγ induces PD-L1 expression that is secreted from infiltrating T-cells which leads to inactivation of T cells expressing PD-1, further enabling tumor cell evasion of immune destruction [33] .

Discussion/Results

In summary, our results demonstrate that hyperglycemia very rapidly, within 2h, induces a mostly IFNγ-driven inflammation. While the expression of pro-inflammatory cytokines was less after 4 hrs compared to 2 hrs of hyperglycemia, the IHC .showed that the number of microglia was markedly increased after 4h of hyperglycemia both in the hypothalamus and in the PFC. IFNγ levels positively correlate with and seem to directly drive the expression of the immune inhibitory receptor ligand PD-L1, which is highly expressed in murine gliomas [33]. IFNγ also induces PD-L1 expression on primary cultured microglia, bone marrow-derived macrophages, and GL261 glioma cells *in vitro* [33]. CCL2 can trigger the release of IL-6 from microglia, which in turn promotes the invasiveness of glioma cells [34]. This suggest that hyperglycemia activates microglia and induces predominantly an INFγ-driven inflammation that then favors malignant transformation through PD-1 PDL1 as well as by inducing cytokines such as IFNγ, CXCL9, CXCL10, CXCL-11, CCL2 and iNOS.

<u>Study 2:</u> Effects of chronic hyperglycemia on microglia activation and neuroinflammation.
In this study we characterized the effects of chronic hyperglycemia in the context of insulin deficient diabetes. We used a model of streptozotocin (STZ) induced beta-cells death, a well-established method to induce insulin-deficient diabetes and hyperglycemia.

2a) Chronic hyperglycemia drives inflammation in multiple brain areas.
Rats were body-weight matched and randomly assigned to two conditions: (1) non-diabetic controls rats; injected *ip* with sodium citrate (1mL/Kg); (2) STZ-treated rats; injected *ip* with STZ (60mg/kg). Here, we also included a non-responders (NR) group, which describes rats that received the STZ injection but did not develop hyperglycemia (which is commonly observed due to some variable toxicity of STZ). NR were included in this study to account for the potential toxic effect of STZ that may occur independent of the hyperglycemia. After the induction of hyperglycemia rats were monitored for 2 months during which hyperglycemia was persistent, body weight and plasma glucose were measured twice a week. The increase of body weight that healthy rats experience as they age was markedly reduced in the diabetic animals due to the hypermetabolic state induced by the hyperglycemia (**Fig. 3A**). Plasma glucose levels in the STZ ratswere in the diabetic range of 400-500mg/dl for all the 2 months of the study, while control and NR remained normoglycemic (120-150 mg/dl) (**Fig. 3B**). To assess brain inflammation after chronic hyperglycemia, we performed IHC analysis for Iba-1 (**Fig. 3C and F**), GFAP (astroglial markers, **Fig. 3E and H**) and MAP2 (neuronal marker, Microtuble Associated protein-2, **Fig. 3D and G**). Our analysis revealed that chronic hyperglycemia resulted in increased number of microglia cells (**Fig. 3I**) and augmented GFAP density (**Fig. 3K**), while no difference was detected in the number of neurons (**Fig. 3J**), suggesting that while hyperglycemia triggers micro- and astro-gliosis it is not toxic for neuronal cells. In addition to the hypothalamus, which is one of the major brain area involved in the regulation of energy balance, here we aimed to map the spatial distribution of microgliosis and gliosis in the entire brain. Therefore, we performed IHC analysis for Iba-1, GFAP and MAP2 across the whole rat brain and our results demonstrated that hyperglycemia-induced inflammation was detectable in several brain regions such as the hypothalamus, the hippocampus and the amygdala (**Fig. 4**). Indeed the number of microglia cells was markedly augmented in the hippocampus (**Fig.4G**), and trending to be increased in the amygdala (**Fig 4P**), while GFAP density was increased in both areas in STZ-treated rats (**Fig.4I and R**). STZ did not affect the total number of neurons neither in the hippocampus (**Fig.4H**) nor in the amygdala (**Fig. 4Q**). In summary, our findings demonstrated that chronic hyperglycemia induced microglia activation in multiple CNS areas such as hypothalamus, amygdala and hippocampus.

2b) Bulk RNA-seq failed to detect increases in cytokine expression, while IHC clearly showed microgliosis in the brain of diabetic rats. Even though the IHC clearly demonstrated microglia activation, the bulk RNA-seq failed to show differences in pro-inflammatory cytokines expression. This experiment was performed by our collaborators at Pfizer. Briefly, RNA was extracted, enriched and fragmented. cDNA was synthetized and a library (Biospyder TempOSeq Rat Whole Transcriptome Targeted Sequencing Library Prep) was prepared to obtain cDNA with adapters for sequencing. Quality Control was assessed via QuBit/Tapestation, sequenced on NextSeq500, then data were uploaded into BaseSpace with FastQ QC condensation (4 lanes into 1). Fast Q files were then imported and analyzed in Qiagen CLC Genomic Workbench with alignment to the Rat TempOSeq reference genome. Finally differential gene expression analysis was conducted in CLC with outlier removal. QC of sequencing of the rat hypothalamus and PFC indicated read counts and good mapping percentages indicative of a successful sequencing run (**Fig. 5A**). Our results did not detect any changes in pro-inflammatory cytokines in diabetes *versus* control neither at 4h nor during chronic hyperglycemia (*i.e;* diabetes) (**Fig.5B**). This is consistent with the notion that chronic hyperglycemia may impair the transcription of such pro-inflammatory cytokines [35, 36]. The corresponding RNA-seq gene expression to qPCR data for acute (**Fig. 5C**) and chronic (**Fig. 5D**) hyperglycemia revealed that at the 4h time point, or after prolonged diabetes, no clear induction of IFNγ genes were detected in both tissues from either experiment. Together, these results suggest that 4h hyperglycemia and diabetes (STZ) did not increase the transcription of proinflammatory genes in either the PFC nor the hypothalamus. However, our IHC analysis clearly showed that activated microglia were markedly increased after 4h hyperglycemia in STZ rats (See **Fig.2, 3** and **4**). One potential explanation could relay on the protocol used to perform bulk RNA-seq. Bulk uses ribosome-depleted total RNA, and it might not be a technique sensitive enough to detect transcript for lowly expressed genes. The “bulk” data represent an average of gene expression patterns across millions of cells and while for example proinflammatory genes are increased in one cell type, they may be decreased in others; this might obscure biologically relevant differences between cells.

2c) Chronic hyperglycemia dramatically altered the cell identity in the hypothalami and in isolated microglia of diabetic rats.
To overcome the limitation of bulk RNA-seq, we performed Single-cell RNA-seq (scRNA-seq). Rats were body-weight matched and randomly assigned to two conditions: (1) non-diabetic controls rats; injected *ip* with sodium citrate (1mL/Kg); (2) STZ-treated rats; injected *ip* with STZ (60mg/kg). Rats were maintained hyperglycemic for about two months, with body weight and plasma glucose level monitored twice a week. At the end of the experiment, animals were sacrificed with ketamine (70mg/Kg) and the hypothalami were immediately collected, snap-frozen in liquid nitrogen and delivered to the RNA-Core facility for RNA-seq. scRNA-seq works by isolating single nuclei, capturing their transcripts, and generating sequencing libraries in which the transcripts are mapped to individual cells. scRNA-seq allows assessment of fundamental biological properties of cell populations and biological systems at an unprecedented,(*i.e;* single cell resolution). Since microglia are the main driver of brain inflammation, here we performed an *in-depth* phenotypic characterization of the cell

identity of the whole hypothalami (to confirm our IHC studies showing increased Iba-1 immunofluorescence) and the isolated microglia from diabetic and control rats.
Through clustering analysis, we identified 4 non-neuronal clusters and 2 neuronal clusters (**Fig. 6A**). Data analysis revealed the transcriptional dynamics underlying cell proliferation, as well as transcriptional heterogeneity of microglia. Additionally, single-cell transcriptome analysis revealed highly divergent expression patterns of neurons (*e.g;* increased number of glutamatergic neurons in the diabetic group). Our results revealed that STZ-induced diabetes *per se* causes profound changes in the cellular identity within the hypothalamus, reflecting changes in the activation states of immune cells and neurons within the hypothalamus of diabetic rats. Interestingly, we observed a marked increased in microglia and proliferating cells in the diabetic group, consistent with our observations from the IHC. Next, in another cohort of animals, also induced with STZ and maintained hyperglycemic for about 2 months, we isolated microglia and performed scRNA-seq. Our results identified cell-type specific differences among the microglia populations, with an increased number of dendritic cells and macrophages in the microglia population isolated from diabetic rats compared to control (**Fig.6B**). Of note, we identified 13 IFNγ and T-cells related genes such ad IFNgR1, IFI30, CCR5, SLFN2,IL17RA,CD74,ARHGAP45,FYB1,DOCK8,PTPRC, LCP2,VISIRS and CORO1A in the micorlgia cluster 7 in the diabetic condition. Thus, our study provides a comprehensive gene expression map across divergent cell types in the hypothalamus and among isolated microglia.

Commented [BC1]: What is the percentage of these cells, as a way to normalize for differenig cell numbers in the cohorts

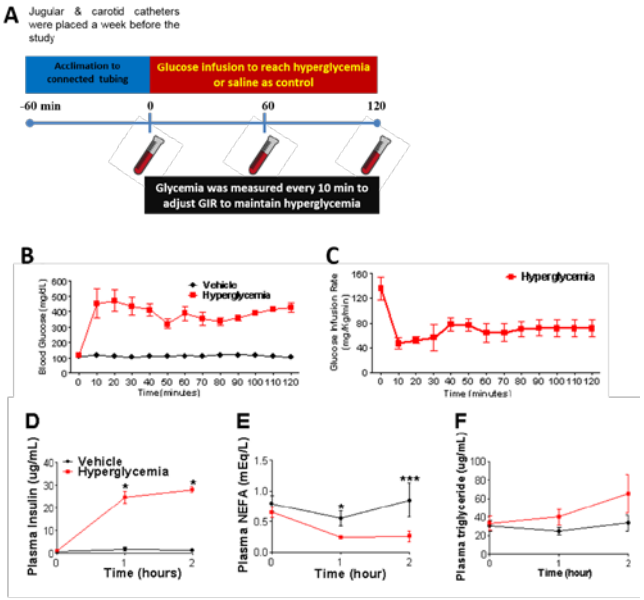
Commented [CL2]: What we have from X-10 Genomics is 3.15% control versus 3.88% diabetes for microglia cluster

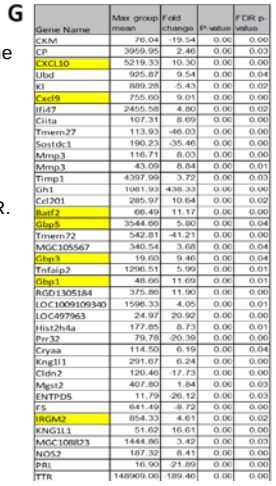
2d) Hyperglycemia is the key driver in a model of STZ-induced hyperglycemia for microglia activation.
Dapagliflozin (DAPA) is a sodium-glucose co-transporter-2 (SGLT2) inhibitor and is currently FDA-approved for the treatment of patients with type 2 diabetes. By inhibiting SGLT2 in the kidneys, DAPA reduces renal glucose reabsorption, leading to urinary glucose excretion and a reduction in blood glucose levels [37]. The efficacy of DAPA is independent of insulin secretion and action [37]. Here we used DAPA as a tool to examine the role of hyperglycemia in the STZ-induced insulin deficient diabetes associated brain inflammation.
We tested whether treatment with the SGLT2 can ameliorate hypothalamic inflammation even though the insulin deficiency remains unaltered. Rats were body-weight matched and randomly assigned to three conditions: (1) non-diabetic controls rats; injected *ip* with sodium citrate (1mL/Kg); (2) STZ-treated rats; injected *ip* with STZ (60mg/kg) and (3) STZ+ DAPA-treated rats; injected *ip* with STZ (60mg/kg), and once diabetes has developed were treated with DAPA (50mg/kg; mixed with standard chow) for the entire duration of the experiment. Body weight curves demonstrated that DAPA treatment is able to partially rescue the body-weight loss of the STZ (**Fig. 7A**) and the hyperglycemia (**Fig. 7B**). As expected, insulin levels were markedly low in the STZ-rats (**Fig. 7C**), since they are insulin-deficient, while fatty acids were increased (**Fig.7D**). Even though brain inflammation was present as per IBA1 IHC, the gene expression of proinflammatory cytokines again was not a reliable marker of brain inflammation (**Fig. 7E** and **F**). We performed IHC analysis for the microglia marker Iba-1(**Fig. 7G**), neuronal marker MAP2 (**Fig. 7H**) and glial marker GFAP (**Fig. 7I**). Our data demonstrated that the administration of DAPA is able to ameliorate the number of activated microglia (**Fig. J**) and GFAP density (**Fig. 7K**) in the hypothalamus, without affecting the total number of neurons (**Fig. 7L**). This suggest that hyperglycemia is the key driver of the microglia activation and DAPA treatment is a potential pharmacotherapy to prevent hyperglycemia-induced brain inflammation.

Dysfunction in glycolysis and mitochondrial impairments contribute to microglia activation [1]. An increase in glycolytic rate negatively affects mitochondrial functions by reducing oxidative phosphorylation and promoting the production of oxidative reactive species (ROS) thereby aggravating inflammation [38, 39]. Therefore we hypothesized that high concentrations of glucose can trigger microglia activation by increasing the glycolytic flux and disturbing mitochondrial function. We aimed to assess whether hyperglycemia in a cell autonomous fashion can directly activate microglia by reducing glycolysis. To examine the role of glucose in altering glycolytic flux underlying microglia activation, we profiled in primary microglia cultures basal oxygen consumption, glycolysis rates, ATP production and respiratory capacity, through a Seahorse Extracellular Flux Analyzer (**Fig. 7M and N**). Our results failed to detect any changes in mitochondrial activity between our groups.

In summary, chronic hyperglycemia increases the number and activation of microglia and number of proliferating cells consistent with hyperglycemia creating a prooncogenic tumor environment.

FIGURE 1





Gene Name	Max group mean	Fold change	P-value	FDR p-value
CKM	70.04	-19.54	0.00	0.00
CP	3959.95	2.46	0.00	0.03
CXCL10	5219.33	10.30	0.00	0.00
Ubd	925.87	9.54	0.00	0.04
Kl	889.28	-5.43	0.00	0.02
Cxcl9	755.60	9.01	0.00	0.00
Ifi47	2455.58	4.80	0.00	0.02
Ciita	107.31	8.69	0.00	0.00
Tmem27	113.93	-46.03	0.00	0.00
Sostdc1	190.23	-35.46	0.00	0.00
Mmp3	116.71	8.03	0.00	0.00
Mmp3	43.09	8.84	0.00	0.01
Timp1	4397.99	3.72	0.00	0.03
Gh1	1081.93	-438.33	0.00	0.00
Ccl201	285.97	10.64	0.00	0.02
Batf2	66.49	11.17	0.00	0.00
Gbp5	3544.66	5.80	0.00	0.04
Tmem72	542.81	-41.21	0.00	0.00
MGC105567	340.54	3.68	0.00	0.04
Gbp3	19.60	9.46	0.00	0.04
Tnfaip2	1296.51	5.99	0.00	0.01
Gbp1	48.66	11.69	0.00	0.01
RGD1305184	375.86	11.90	0.00	0.00
LOC100910340	1596.33	4.05	0.00	0.01
LOC497963	24.97	20.92	0.00	0.00
Hist2h4a	177.85	8.73	0.00	0.01
Prr32	79.78	-20.39	0.00	0.00
Cryaa	114.50	6.19	0.00	0.04
Kng1l1	291.67	6.24	0.00	0.00
Cldn2	120.46	-17.73	0.00	0.00
Mgst2	407.80	1.84	0.00	0.03
ENTPD5	11.79	-26.12	0.00	0.03
Fs	641.49	-8.72	0.00	0.00
IRGM2	854.33	4.61	0.00	0.02
KNG1L1	51.62	16.61	0.00	0.00
MGC108823	1444.86	3.42	0.00	0.03
NOS2	187.32	8.41	0.00	0.00
PRL	16.90	-21.89	0.00	0.00
TTR	148969.06	-189.46	0.00	0.00

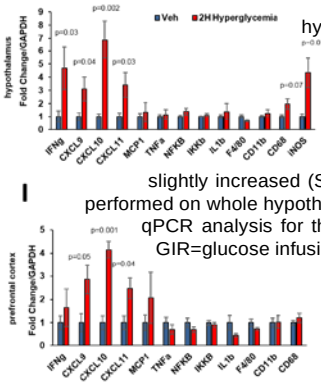


Figure1. Acute hyperglycemia triggered an Interferon-ϒ driven hypothalamic inflammation.
(A) Schematic representation of the experimental workflow. A jugular and a carotid catheters were placed in rats at least a week before the beginning of the study. Male rats (*n*=8 per group) were acclimated to connecting tubing for an hour before the hyperglycemic clamp. At time point 0, 60 and 120 minutes blood was collected, while glycemia was measured every 10 minutes to adjust GIR to maintain hyperglycemia. Acute hyperglycemia was induced through a hyperglycemic clamp, where **(B)** glucose was maintained at the diabetic range of 400-500mg/dl **(C)** through the infusion of a primed, followed by a variable GIR. Rats were **(D)** hyperinsulinemic, while **(E)** fatty acids were reduced and **(F)** triglycerides level slightly increased (Student t-test, P< 0.05). **(G)** Bulk RNA-seq was performed on whole hypothalami and genes of interests were confirmed by qPCR analysis for the (**D**) hypothalamus and (**E**) prefrontal cortex. GIR=glucose infusion rate. Data are expressed as mean ± S.E.M.

FIGURE 2

Figure2. 4h hyperglycemia markedly increased the number of microglia in the PVN, MBH and PFC. (A) Schematic representation of the experimental workflow. A jugular and a carotid catheters were placed in rats at least a week before the beginning of the study. Male rats (*n*=8 per group) were acclimated to connecting tubing for an hour before the hyperglycemic clamp. At time point 0, 60,120 and 240 minutes blood was collected, while glycemia was measured every 10 minutes to adjust GIR to maintain hyperglycemia. qPCR was performed on the **(B)** hypothalamus and **(C)** PFC. CD279 was markedly increased in the hypothalamus (Student t-test, P< 0.05). Immunohistochemistry for microglia marker Iba-1 was performed in the hypothalamus (PVH and MBH) and prefrontal cortex of **(D,G,J)** control and **(E,H,K)** diabetic rats. Microglia were markedly increased in the PVH, **(I)** MBH and **(L)** cortex of rats 4h after hyperglycemia. CD279= cluster of differentiation 279, MBH= medio-basal hypothalamus, PFC= prefrontal cortex, PVN= paraventricular nucleus of hypothalamus, 3V= third ventricle. Scale bar represents 50µm. Data are expressed as mean ± S.E.M.

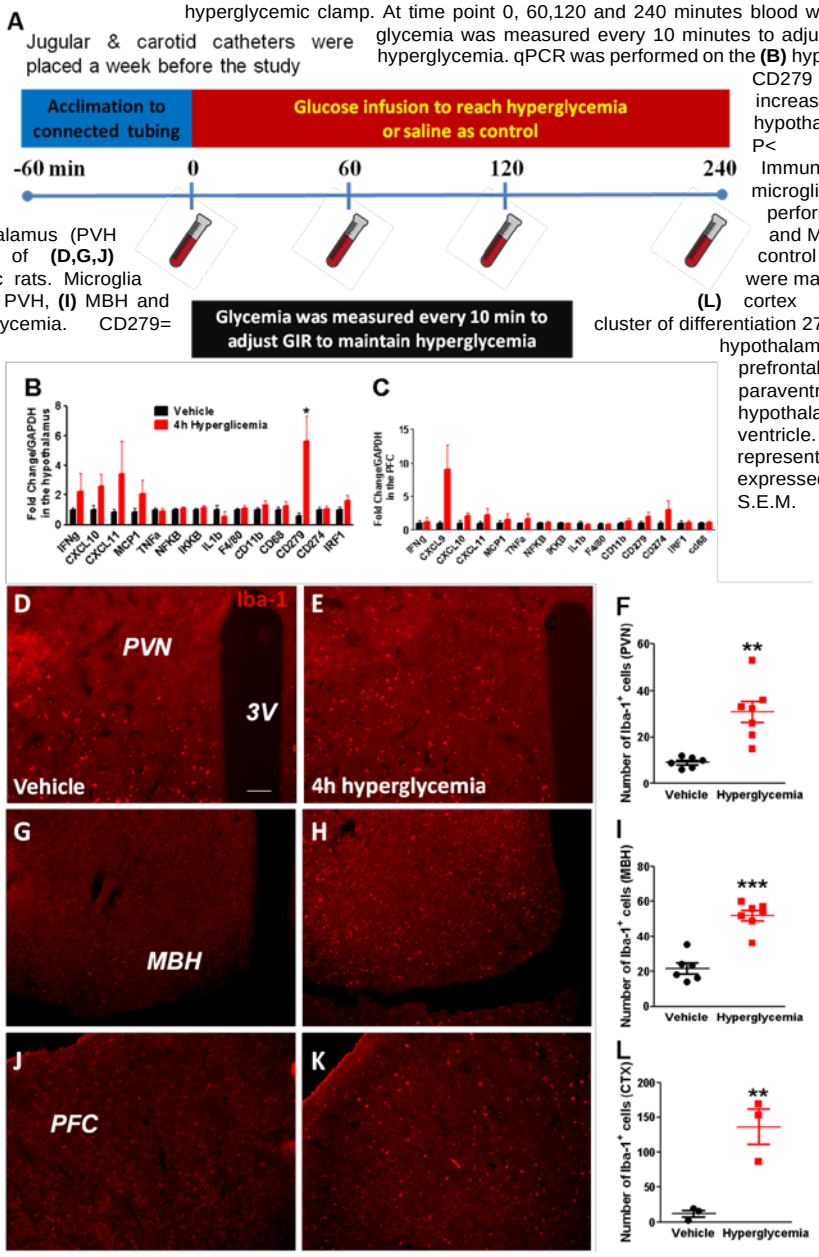


FIGURE 3

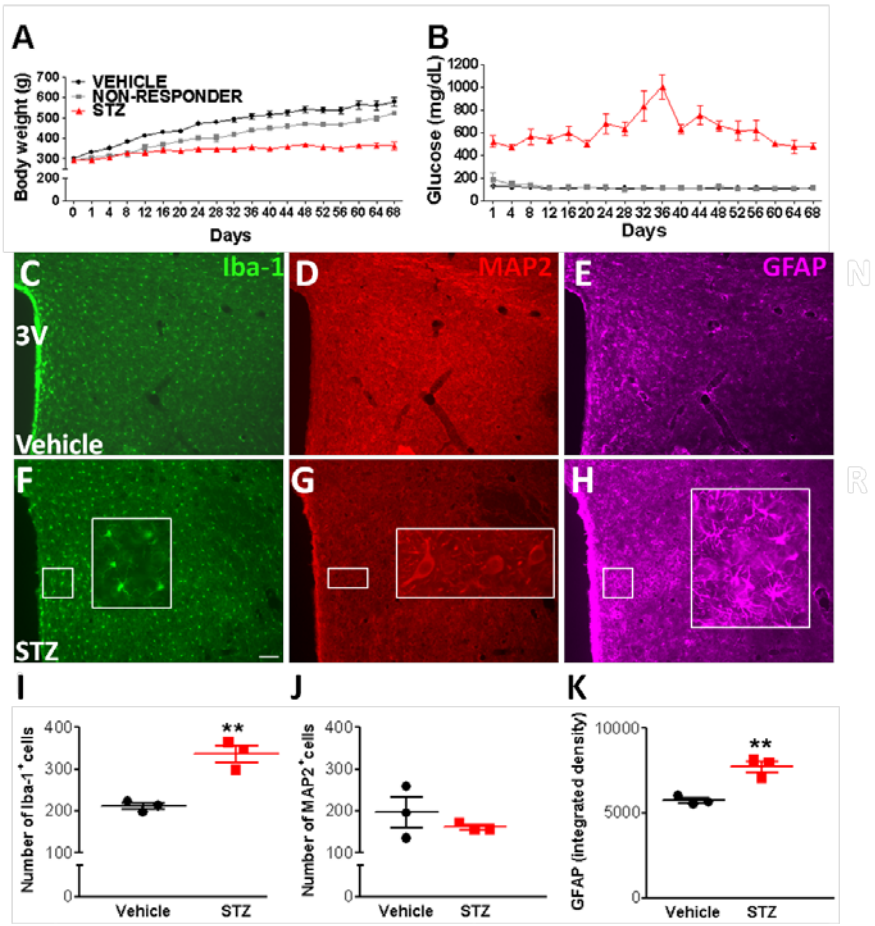


Figure3. Chronic hyperglycemia resulted in hypothalamic inflammation. Two days after STZ injection (60mg/Kg; ip), rats become hyperglycemic (*e.g;* plasma glucose level raised from ~120 mg/dl to ~ 600 mg/dl). Glucose levels and body weight were monitored every 2 days for about 2 months (**A**) Body weight was markedly reduced in STZ rats (*n*=6 per group), confirming their hypermetabolic state. STZ rats were (**B**) hyperglycemic compared to control and non-responders. Hypothalamic sections (*n*=3 per group) of vehicle (**C-E**) and STZ-treated (**F-H**); STZ (60mg/Kg) was given to induce hyperglycemia. Sections were stained for microglia (Iba-1; in **C** and **F**), neurons (MAP2; in **D** and **G**) and glia (GFAP; in **E** and **H**). Insets represent magnification of microglia, neurons and glial cells. Quantitative analysis of (**I**) microglia, (**J**) neurons and (**K**) glial cells. STZ-treatment significantly increased the number of microglia and the density of glial cells compared to vehicle (Student t-test, P< 0.05). The total number of neurons was not affected by the experimental conditions. Scale bar represents 50µm. Data are expressed as mean ± S.E.M.

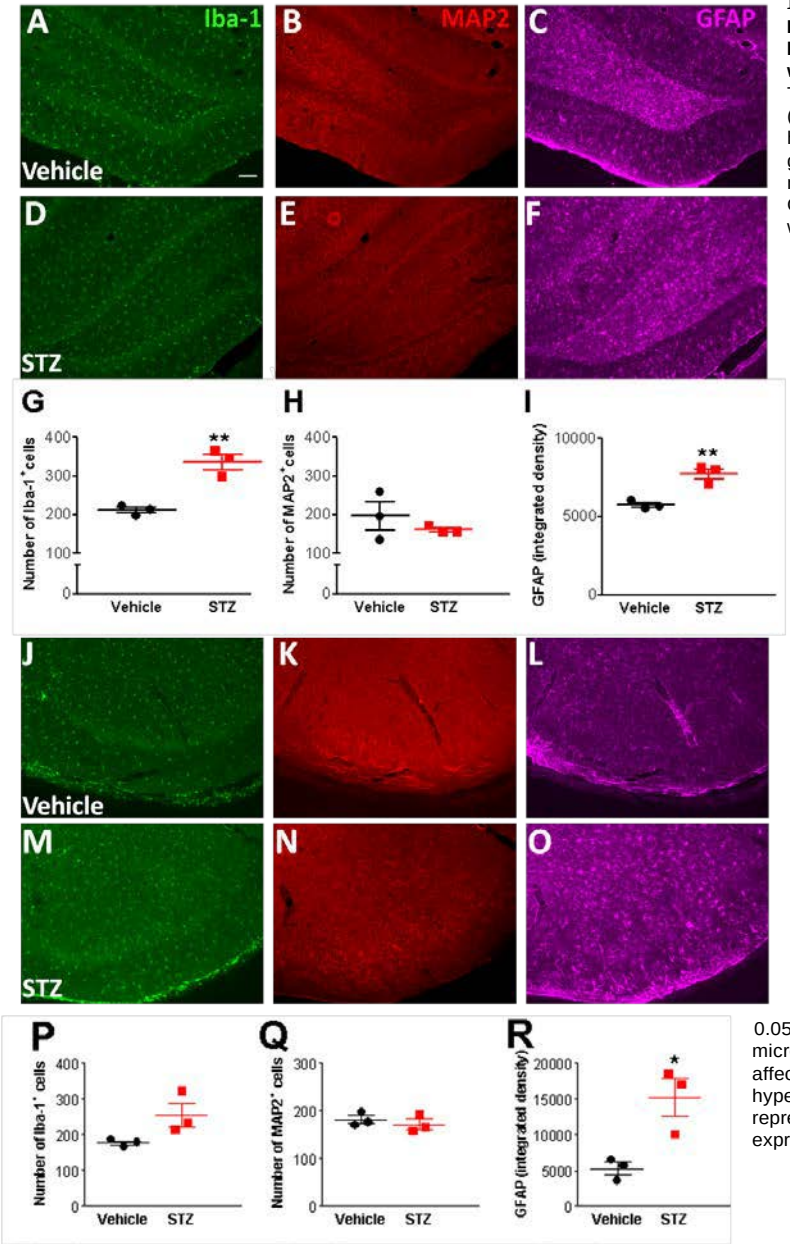


FIGURE 4

Figure4. Chronic hyperglycemia induced whole brain inflammation. Two days after STZ injection (60mg/Kg; ip), rats become hyperglycemic (*e.g;* plasma glucose level raised from ~120 mg/dl to ~ 600 mg/dl). Glucose levels and body weight were monitored every 2 days for about 2 months. Hippocampal sections (*n*=3 per group) of vehicle (**A-C**) and STZ-treated (**D-F**). Hippocampal sections (*n*=3 per group) of vehicle (**A-C**) and STZ-treated (**D-F**). Sections were stained for microglia (Iba-1; in **A** and **D**), neurons (MAP2; in **B** and **E**) and glia (GFAP; in **C** and **F**). Quantitative analysis of (**G**) microglia, (**H**) neurons and (**I**) glial cells. STZ-treatment significantly increased the number of microglia and the density of glial cells compared to vehicle (Student t-test, P< 0.05).The total number of neurons was not affected by chronic hyperglycemia. Amygdala sections (*n*=3 per group) of vehicle (**J-L**), STZ-treated (**M-O**). Sections are stained for microglia (Iba-1; in **J** and **M**), neurons (MAP2; in **K** and **N**) and glia (GFAP; in **L** and **O**). Quantitative analysis of (**P**) microglia, (**Q**) neurons and (**R**) glial cells. STZ-treatment significantly increased the density of glial cells compared to vehicle (Student t-test, P< 0.05). The total number of microglia and neurons was not affected by chronic hyperglycemia. Scale bar represents 50µm. Data are expressed as mean ± S.E.M

FIGURE 5

A

Read count statistics	Avg. Read count	Avg. Single, mapped %
Hypothalamus Rat Hyperglycemic	9,702,734	98.4
PFC Rat Hyperglycemic	7,995,547	99.1
Hypothalamus Rat STZ	12,522,839	97.5
PFC STZ Rat	10,852,347	98.8

B

	# of Significantly Changed Genes (FDR P-Value <= 0.05)	# of Significantly Changed Genes (P-Value <= 0.01)
2hr HG hypothalamus vs. Control	39	267*
4hr HG hypothalamus vs. Control	0	181*
4hr HG PFC vs. Control	1	48
STZ hypothalamus vs. Control	7	189
STZ PFC vs. Control	0	41

C

Gene Name	2hr glucose			4hr glucose		
	Fold change	P-value	FDR p-value	Fold change	P-value	FDR p-value
CXCL10	NA	NA	NA	1.57	0.34	0.90
Cxcl11	1.32	0.41	1.00	-2.60	0.04	0.90
CD68	1.22	0.85	1.00	1.13	0.67	0.97
CCL2	-1.08	0.86	1.00	-1.69	0.22	0.90
IL1B	NA	NA	NA	-1.16	0.72	0.98
SOCS3	-1.04	0.91	1.00	1.18	0.65	0.97
IL6	1.28	0.44	1.00	-1.10	0.73	0.98
IL4	NA	NA	NA	-1.18	0.74	0.98
IL10	-1.42	0.35	1.00	1.03	0.97	1.00
Pomc	-1.19	0.33	1.00	-1.10	0.82	1.00
Npy	-1.06	0.61	1.00	10.07	0.08	0.90
PPARG	-1.21	0.28	1.00	-1.74	0.14	0.90
IFNG	NA	NA	NA	-1.69	0.51	0.94

D

Gene Name	STZ (Prefrontal Cortex)			STZ (Hypothalamus)		
	Fold change	P-value	FDR p-value	Fold change	P-value	FDR p-value
CCL2	-1.01	0.98	1.00	-1.75	0.06	1.00
CD68	-1.24	0.28	1.00	-1.36	0.13	1.00
CXCL10	-1.42	0.34	1.00	-1.24	0.49	1.00
Cxcl11	-1.51	0.24	1.00	-3.14	0.0008	0.38
IFNG	-1.38	0.76	1.00	-1.36	0.64	1.00
IL10	-1.26	0.74	1.00	-1.49	0.49	1.00
IL1B	-1.23	0.45	1.00	-1.34	0.22	1.00
IL4	-1.23	0.72	1.00	1.55	0.24	1.00
IL6	1.22	0.48	1.00	1.03	0.87	1.00
Npy	NA	NA	NA	-5.88	0.16	1.00
Pomc	-1.20	0.74	1.00	-1.28	0.08	1.00
PPARG	1.16	0.65	1.00	-1.17	0.54	1.00
SOCS3	1.29	0.31	1.00	-1.74	0.002	0.60

Figure5. Bulk RNA-seq failed to detect changes in pro-inflammatory cytokines in hyperglycemic rats. QC via QuBit/Tapestation was performed to assess the quality of the run. (**A**) QC of sequencing indicated high read counts and good mapping percentage indicative of a successful sequencing run. (**B**) Number of significantly changed genes between groups using False Discovery Rate (FDR). Only 2 genes are in common between 2 and 4h hyperglycemia treatment. Corresponding RNAseq gene expression to qPCR data for (**C**) 2 and 4h hyperglycemia and (**D**) chronic hyperglycemia.

FIGURE 6

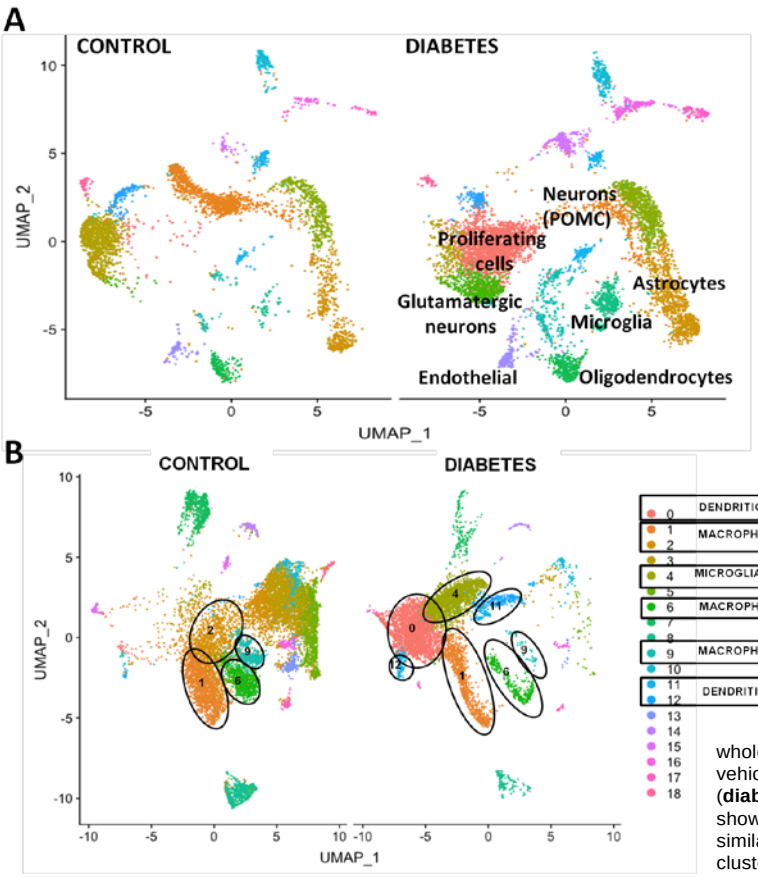


Figure6. ScRNA-sequencing of rat hypothalami and isolated microglia from whole rat brain revealed profound differences in cell identity. Hypothalami (*n*=3 per group) of vehicle (**control**) and STZ-treated (**diabetes**); STZ (60mg/Kg) was given to induce hyperglycemia. (**A**) UMAP plots showing genes clustered by similarity. We identified 16 different clusters that are color-coded. Microglia isolated from whole rat brain (*n*=6 per group) of vehicle (**control**) and STZ-treated (**diabetes**). (**B**) UMAP plots showing genes clustered by similarity. We identified 16 different clusters that are color-coded**.**

FIGURE 7

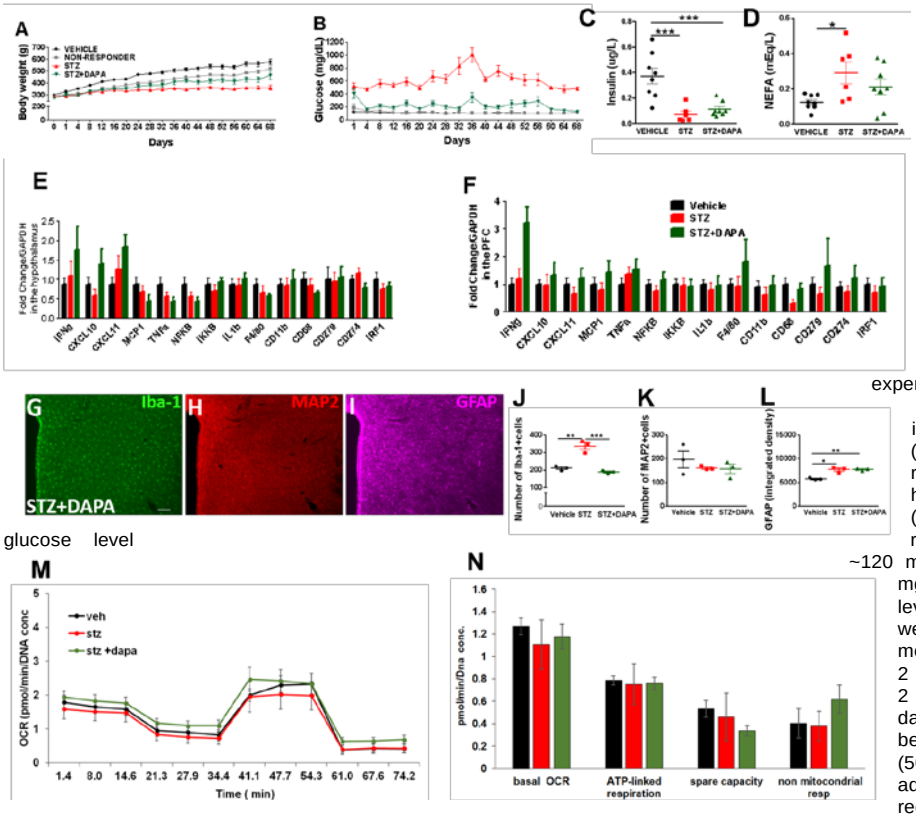


Figure7. Restoration of euglycemia through the SGLT2 inhibitor dapagliflozin (DAPA) reversed the microglial activation. Male rats (*n*=5-7 per group) were used in this experiment. Two days after STZ injection (60mg/Kg; *ip*), rats become hyperglycemic (*e.g;* plasma glucose level raised from ~120 mg/dl to ~ 600 mg/dl). Glucose levels and body weight were monitored every 2 days for about 2 months. Four days after the beginning DAPA (50mg/Kg) was administered with regular chow for the entire duration of the experiment. After four days of treatment, DAPA ameliorated (**A**) the body weight loss commonly observed in STZ and (**B**) markedly improved plasma glucose levels (*e.g;* restored basal glucose level of ~200 mg/dl). (**C**) Plasma insulin deficiency was pronounced in both STZ and DAPA-treated animals with no significant improvement by the DAPA. (**D**) DAPA prevented the marked increased in fatty acids induced by STZ. qPCR analysis in the (**E**) hypothalamus and (**F**) prefrontal cortex showed no difference in pro-inflammatory cytokines between the groups. Hypothalamic sections (*n*=3 per group) were stained for (**G**) microglia (Iba-1), (**H**) neurons (MAP2) and (**I**) glia (GFAP). Quantitative analysis of (**J**) microglia, (**K**) neurons and (**L**) glial cells. DAPA treatment was able to rescue the number of microglia (One-way ANOVA, P< 0.05) but did not decreased the density of glial cells. The total number of neurons was not affected by the experimental conditions. (**K**) The Seahorse XF Cell Mito Stress Test kit was used to assess mitochondrial function. (**L**) Multiple parameters were obtained in one assay, including: basal respiration (OCR), ATP production-coupled respiration, maximal and reserve capacities (spare capacity) and non-mitochondrial respiration. No functional differences were detected between the groups. Scale bar is 50µm. VEH= vehicle, NR= non-responders, STZ= diabetic and STZ+DAPA. Data are expressed as mean ± S.E.M.

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Opportunities for training and professional development was provided to the fellow supported by this awards Dr. Claudia Liberini as well as a summer student during the summer. This work gave Dr Liberini the opportunity to acquire the skills to perform euglycemic clamp studies in rats as well as perform insulin signaling studies, both methods and procedures that she had not performed before she joined this project.

The results so far have been distributed during talks presented at other institutions such as the Mayo Clinic, the NIH /Niddk intramural program, and Diabetes Grand rounds here at Sinai and at Einstein.

4. Impact

The biggest impact of these studies to be in the realization that TBI through brain damage impairs metabolic control and increases the risk for type 2 diabetes. This is an important Public Health insight that should guide providers of patients that suffered TBI and makes screening for diabetes imperative. This is a completely novel insight that before these Studies, had not been realized.

Secondly, they give hope to those that may suffer from metabolic disease and have TBI as we have begun to identify drugs that seem to be promising in treating TBI induced metabolic disease.

5. Changes/Problems

Studies that we replaced with the above described studies were: The euglycemic clamps from subtask 3 of aim 1 and from Subtask 2 from Aim 2. We further have forgone the treatment with mifepristone.

We replaced studies of mifepristone with Sglt2 inhibitors as a therapeutic option for TBI induced prediabetes. Because sglt2 Inhibitors seem to be much more potent for TBI induced prediabetes and are also very safe (more so than mifepristone). SGLT2 inhibitors are already commonly used for diabetes therapy, this makes clinical sense. It also could lead to a translational trial in humans with TBI as we have discussed. Preliminary data illustrating the benefit of dapagliflocin is shown above.
Instead we metabolically characterized these rats with glucose and insulin tolerance tests, and performed insulin signaling studies to define insulin action. In addition we will perform careful molecular analysis of tissues such as adipose and liver, and study brain inflammation through IHC and transcriptional studies and the effects of the Sglt2 inhibition on all of these read outs. While some studies were performed in non blasted rats, we believe these studies nervertheless provide

important insignt into how chronic glucose intolerance worsens brain inflammation as this was a key feature of the TBI model.

6. Products

A publication of these findings is under preparation. These studies also provide an important starting poiunt for a retrospective chart review in persons with TBI where we will explore if a history of TBI increases risk for diabetes. IRB approval has just been granted.

7. Participants & Other Collaborating Organizations

No change

8. Special Reporting Requirements

none

9. Appendices

none