Intrauterine hyperglycemia impairs memory across two generations

Kexin Zou1,2,3, Jun Ren4,6, Sisi Luo1,2,3, Junyu Zhang1,2,3, Chengliang Zhou1,2,3, Chengxi Tan1, Pingping Lv4, Xiao Sun1,2,3, Jianzhong Sheng4, Xinmei Liu2,3,5, Hefeng Huang1,2,3,4,5, and Guolian Ding1,2,3,5

© The Author(s) 2021

Studies on humans and animals suggest associations between gestational diabetes mellitus (GDM) with increased susceptibility to develop neurological disorders in offspring. However, the molecular mechanisms underpinning the intergenerational effects remain unclear. Using a mouse model of diabetes during pregnancy, we found that intrauterine hyperglycemia exposure resulted in memory impairment in both the first filial (F1) males and the second filial (F2) males from the F1 male offspring. Transcriptome profiling of F1 and F2 hippocampi revealed that differentially expressed genes (DEGs) were enriched in neurodevelopment and synaptic plasticity. The reduced representation bisulfite sequencing (RRBS) of sperm in F1 adult males showed that the intrauterine hyperglycemia exposure caused altered methylated modification of F1 sperm, which is a potential epigenetic mechanism for the intergenerational neurocognitive effects of GDM.

Translational Psychiatry (2021)11:434 ; https://doi.org/10.1038/s41398-021-01565-7

INTRODUCTION

Accumulating evidence suggests that an adverse in utero environment can increase the risk of chronic diseases in later life. Long-term postnatal health may be affected by metabolic experience in utero [1, 2]. Intrauterine hyperglycemia is a major characteristic of gestational diabetes mellitus (GDM) and is associated with a high risk of diabetes in offspring [3]. In humans, it is noteworthy that in addition to metabolic dysfunction, studies have documented that children of mothers with diabetes during pregnancy is associated with the impaired cognitive ability [4–7], although one study failed to detect such association in another population [8]. It is also controversial whether the association between maternal diabetes in pregnancy and offspring cognitive outcomes can be fully explained by shared familial environmental factors or by an intrauterine biological mechanism [9].

Experimental investigations in animals indicated that uncontrolled diabetes mellitus was associated with morphological and functional alterations in the brain [10–12]. Hippocampus, a structure critical to cognitive processes, has been shown to undergo apoptotic cell death when subjected to hyperglycemic insult [13–15]. Diabetes during pregnancy strongly influences the regulation of both insulin-like growth factor-1 receptor (IGF-1R) and insulin receptor (InsR) in the rat hippocampus [13]. Maternal diabetes mellitus can also reduce the expression of synaptophysin (SYP) in the developing hippocampus and cerebellar cortex of neonatal rats [16, 17]. However, the intergenerational effect on the F2 offspring and the underlying molecular mechanism are unclear.

Epigenetic alterations regulate tissue-specific gene expression during growth and development without altering the DNA sequence. DNA methylation primarily occurs on CpG dinucleotides and is generally associated with gene repression when positioned on the promoter region [2, 18]. Our previous research has demonstrated that intrauterine hyperglycemia changed DNA methylation levels on the imprinted gene Igf2/H19 in the F1 pancreatic islet, which was further transmitted to F2 through F1 germ cells [19]. Therefore, we hypothesize that the hyperglycemic intrauterine environment of GDM could result in a high risk of cognitive impairment in F2 offspring by affecting the development or function of the hippocampus through altering DNA methylation in F1 germ cells. We focused on the male offspring in this study because our previous GDM mouse model of intrauterine hyperglycemia indicates that male offspring was more susceptible to such intergenerational effects than females.

MATERIALS AND METHODS

Mice

All animal protocols were reviewed and approved by the Zhejiang University Animal Care and Use Committee. At the age of 8 weeks, virgin female ICR mice (n = 60) were mated with normal males. The onset of pregnancy was determined by the presence of a copulation plug after overnight mating (designated as day 0 [D0] of pregnancy). After a 12-h fast, the females were randomly divided into a control group and an intrauterine hyperglycemia group with GDM (GDM group). Mice in the GDM group were injected with a single intraperitoneal injection of streptozotocin (STZ; Sigma, St. Louis, MO) in 0.1 mmol/L citrate buffer.
Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). The probe set IDs were converted into the corresponding gene symbol using the annotation information derived from platform GPL6887. The limma package V3.34.9 in R was used to identify and visualize the Gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enriched by DEGs [21]. P value < 0.05 was considered as a significant enrichment. The KEGG enrichment analysis applied to all 1000 gene set permutations by clusterProfiler. The normalized enrichment score (NES) was regarded as the primary statistic for examining GSEA enrichment results.

Reduced representation bisulftite sequencing Reduced representation bisulftite sequencing (RRBS) was performed in sperm obtained from the caudal epididymis of 4-month-old F1 male mice (Genery Biotechnology Co., Ltd., Shanghai, China). Briefly, 5 μg genomic DNA was digested using the methylation-insensitive restriction enzymeMspI (New England Biolabs, Beverly, MA, USA). A Qiagen Mini Purification kit (Qiagen, Hilden, Germany) was used to purify the digested products. Then, the ends of each restriction fragment were filled in and adenosine was added at the 3'-end. Methylated paired-end Illumina adapters were ligation in the ends of the DNA fragments using T4 DNA ligase, and fragments sized 100–200 bp were purified by agarose gel extraction. The purified fragments were treated with sodium bisulfite and then amplified by PCR. The final PCR products were sequenced on HiSeq 2500 (Illumina Inc., San Diego, CA, USA). Differentially methylated loci (DML) and differentially methylated regions (DMRs) were analyzed based on a Bayesian approach [22], summarized as follows as our previous study: (23) two groups were modeled according to the Bayesian stratification method, and the Wald test was applied to test the posterior odds ratio value for each CpG site. For each CpG site, a difference in methylation value between two groups ≥5% and a posteriori probability of Wald test ≥0.95 was considered to be a DML. A methylation region was defined as a DMR when it met these three criteria: (1) the length of this region was at least 50 bp; (2) the region contained no less than three CpG sites, (3) the proportion of DMLs in this region was no less than 50%. When a DMR showed no less than 50% overlap with one element of the gene, it was defined as a differentially methylated gene (DMG). RRBS reads were mapped to the reference mouse genome (mm10) by Bismark (version 0.16.3). DSS V2.30.1 in R was used to detect DMRs. The RRBS data reported in this paper have been deposited in the GEO database with accession number GSE142502.

Statistical analysis Data were shown as the mean ± SEM. A priori sample size calculation was not performed, but our sample sizes are similar to those reported in previous publications. Shapiro–Wilk (n < 10) and D’Agostino and Pearson omnibus (n > 10) normality tests were performed to determine if values fit a Gaussian distribution. For all behavioral studies, a two-tailed unpaired Student’s t-test was used to analyze the significance between groups. The t-test and Benjamini–Hochberg method were used to calculate the P value and FDR of microarray, respectively. GO analysis was performed using a hypergeometric distribution test. All the statistical analyses were conducted with GraphPad Prism 7 (GraphPad Software, Inc) and R (version 3.6.2). Differences were considered statistically significant at p < 0.05.

RESULTS Intraterine hyperglycemia affects cognition of both F1 and F2 male offspring We induced moderate hyperglycemia during pregnancy through injection of STZ. Male F1 adults were then intercrossed to unexposed females to obtain F2 offspring (Fig. 1a). Both F1 and F2 offspring were analyzed in behavioral tests at 3–4 months old. The anxiety levels and locomotor activity was assessed in the OF test. Compared with the F1-Control group, F1-GDM mice showed normal total explorative distance, total explorative activity in the central area, and the index of thigmotaxis (Fig. 1b). Based on the normal explorative activity, we further investigate the spatial memory ability between F1-control and F1-GDM. F1-GDM mice showed less spontaneous alteration than F1-control in Y-maze (Fig. 1c), suggesting impaired spatial memory. A similar result was observed in the NOR test. In the NOR test, the amount of time
Fig. 1  Intrauterine hyperglycemia impairs spatial memory in F1 offspring. a Experimental design. b Open field test of F1 offspring of control (F1-C) and GDM (F1-GDM) mice (n_{F1-C} = 15, n_{F1-GDM} = 15 male 3-month-old mice). c Y maze test of F1-C and F1-GDM mice (n_{F1-C} = 12, n_{F1-GDM} = 11 male 3-month-old mice). d Novel object recognition test of F1-C and F1-GDM mice (n_{F1-C} = 13, n_{F1-GDM} = 13 male 3-month-old mice). e Open field test of F2 offspring of control (F2-C) and GDM (F2-GDM) mice (n_{F2-C} = 19, n_{F2-GDM} = 16 male 3-month-old mice). f Y maze test of F2-C and F2-GDM mice (n_{F2-C} = 17, n_{F2-GDM} = 17 male 3-month-old mice). g Novel object recognition test of F2-C and F2-GDM mice (n_{F2-C} = 15, n_{F2-GDM} = 15 male 3-month-old mice). h Object in place test of F2-C and F2-GDM mice (n_{F2-C} = 15, n_{F2-GDM} = 15 male 3-month-old mice). Data were analyzed by a two-tailed unpaired t-test from three independent experiments. * P < 0.05 vs. F1-C; ** P < 0.01 vs. F1-C; *** P < 0.001 vs. F2-C.
spent with the novel object compared with the total time spent exploring both objects represents an index of recognition memory. Compared to F1-control, F1-GDM mice spent less time investigating the novel object despite similar total exploration times, revealing a remarkable memory deficit (Fig. 1d).

Similar to F1 mice, F2-GDM mice showed normal locomotor activity and did not have an anxiety disorder (Fig. 1e), but displayed a significant deficit in spatial memory in the Y-maze test although there was no obvious difference in NOR test or OiPT associative recognition memory (Fig. 1f–h). These results suggest that intrauterine hyperglycemia impairs spatial memory in both F1 and F2 male offspring.

**Intrauterine hyperglycemia disrupts hippocampal transcriptome in F1 offspring**

To explore the long-term effects of intrauterine hyperglycemia on transcriptional reprogramming, we used microarray to analyze the transcriptome of hippocampi from 4-month-old F1 (n = 5 per group). Using a stringent threshold of adjusted P value < 0.05 and |log2FoldChange| > 1, we identified a total of 451 DEGs, including 218 upregulated and 234 downregulated genes, in F1-GDM compared to control (Table S2).

GSEA of the hippocampi transcriptome in F1-GDM vs. Control revealed the robust enrichment of curated gene sets for axon guidance (KEGG:ncmu04360) and dendrite extension (GO:0004819), indicating that the expression of member genes were decreased in the F1-GDM group (NES = -1.41, P value = 0.017) (Fig. 2a and Table S3). Axon guidance is a process by which axons stretch to their correct targets and plays a key role in building neuronal circuitry [24]. Dendrite growth and synapse formation occur concurrently during development, these processes may be coordinated and interdependent [25]. GO analysis revealed that down- and up-regulated DEGs were mainly enriched in “forebrain cell migration”, “synapse organization” and “cognition” (Fig. 2b, c). Heatmap shows the genes related to cognition (GO:0050890) were significantly up- or down-regulated in F1 offspring (Fig. 2d). Among them, mice overexpressing S100b show enhanced excitotoxicity, altered synaptic plasticity, and cognitive impairment [26]. Hrh3 encodes the histamine receptor H3, which is ubiquitously released from neurons and can regulate neurotransmitter release [27]. Researchers also reported that Drd2 has a role in neuronal maturation and dopaminergic synapse formation [28]. Gpr88 is implicated in a large repertoire of behavioral responses including spatial learning [29]. Meis2 is associated with impairments in working memory and cognition [30]. Neurotrophin-3 (Ntf3) belongs to the family of highly conserved dimeric growth factors that functions as a positive modulator of synaptogenesis involving TrkC and PTPσ [31]. In summary, intrauterine hyperglycemia altered gene expression patterns in the hippocampus of the adult F1 male offspring, and the DEGs were highly enriched in neural development and synapse function.

**Epigenetic changes in the F1 germ cells**

Since the F1-GDM male were directly exposed to hyperglycemia in utero, their cognitive impairment could be due to direct disruption of neurodevelopment by hyperglycemia rather than...
any epigenetic changes. Whether cognition impairment and gene expression changes in F2-GDM is more likely caused by epigenetic mechanisms, such as DNA methylation. We performed RRBS to search for DML in F1-GDM sperms vs. F1-control. Intrauterine hyperglycemia exposure resulted in 64,658 DML that were distributed in the upstream 2 k (6.36%), 5′-untranslated region (5′-UTR, 1.24%), coding sequence (CDS, 14.01%), introns (38.18%), 3′-UTR (3.17%), downstream 2 k (4.99%), and other elements (38.75%) of genes (Fig.3a). We also investigated the distribution across CpG islands (CGIs) and neighboring regions (Fig.3b). CpG island shore was defined as 2 kb regions flanking a CpG island, and CpG shelf as a 2 kb region outside a CpG shore (away from the CGI). GO analysis identified a cluster of DMGs that were strongly related to "neuron development", "neuron differentiation" and "organ growth" (Fig. 3c).

**Intrauterine hyperglycemia disrupts hippocampal transcriptome in F2 offspring**

To explore the intergenerational effects of intrauterine hyperglycemia on transcriptional reprogramming, we used microarray to analyze the transcriptome of hippocampi from 4-month-old F2 (n = 5 per group). Using a stringent threshold of adjusted P value <0.05 and | log2FoldChange | >1, we identified a total of 1050 DEGs, including 511 upregulated and 539 downregulated genes, in F2-GDM vs. control (Table S4).

GSEA of preranked genes in F2 offspring also indicated significant inhibition in dendrite extension (GO:0097484) (NES <−1, P value <0.05). GSEA also provided insights into changes of activated pathways in F2-GDM, including dopaminergic synapse (KEGG:mmu04728) pathway (NES >1, P value <0.05) (Fig. 4a and Table S5). GO analysis revealed that down- and up-regulated DEGs were also mainly enriched in "synapse organization", "postsynaptic membrane" and "neuron to neuron synapse", including several shared GO terms in both F1 and F2 hippocampus (Fig. 4b, c). Heatmap shows the genes related to cognition (GO:0050890) were significantly up- or down-regulated in F2 offspring (Fig. 4d).

**Overlapping DMGs in F1 sperm and differentially expressed genes in F2 hippocampus**

By overlapping DMGs of sperm in F1 offspring and expressed genes of the hippocampus in F2 offspring, we found 56 genes hypermethylated in F1-GDM sperm compared to Control and the tendency of 56 gene expression are downregulated (logFC <0) in F2-GDM hippocampus compared to Control (Supplementary Fig. 2). GO analysis showed that 56 genes were enriched in "neuron to neuron synapse", "postsynaptic density/specialization", "neuron projection development", "structural constituent of synapse/postsynapse", including Akap7, Cacn2, Dlgap1, Tanc1, Tubb2b, Wnt5a, and Zeb2, most of which were downregulated (logFC <0) in the hippocampus of both F1-GDM and F2-GDM. Additionally, within the nerve system development (GO:0007399) term in MGI database (http://www.informatics.jax.org/), a network of hypermethylated genes related to axon guidance (Htr7), positive
regulation of astrocyte differentiation (Fryl), negative regulation of oligodendrocyte differentiation (Nfx), regulation of postsynaptic density assembly (Hoxb3), had a tendency for low expression in the F2-GDM versus the Control samples (Table 1). Of these genes, hypermethylated CpGs was most frequent in CDS regions, followed closely by intron regions.

Common transcriptomic signatures across two generations
By overlapping DEGs of the hippocampus in F1 and F2 offspring, we found that there were 106 genes upregulated, and 117 genes downregulated, in both F1-GDM and F2-GDM mice compared to their respective control mice. All genes were changed in the same direction between these two generations (Fig. 5a). Among the overlapped dramatically altered genes of F1-GDM and F2-GDM offspring, the most biologically significant genes were further validated by RT-qPCR analysis, including the genes regulating hippocampal synaptic plasticity and learning (Camk2b and Drd1), regulating the density and activity of glutamate receptors (Dlgap1 and Tanc1), neurogenic Wnt signaling pathways (Wnt5a and Atp6ap2), and some other genes associated with the hippocampal function (Akap7, Tubb2b, Map1b, and Gpr88) (Fig. 5b). Enrichment analysis of 223 shared DEGs indicated that GO terms were mainly involved in the biological process such as “negative regulation of nervous system development”, “regulation of dopamine receptor signaling pathway”, “forebrain development”, “negative regulation of neuron differentiation”, “axon development”, and “regulation of neurotransmitter transport and synapse organization” (Fig. 5c). These results highlights that intrauterine hyperglycemia leads to persistent and consistent transcriptomic changes across two generations, which may affect hippocampal synaptic plasticity and contribute to memory impairment.

DISCUSSION
The association of GDM with offspring cognitive deficits has been investigated in a number of epidemiological studies [33, 34]. Further, a systematic review and meta-analysis found that according to 19 articles among 18,681 exposed and more than 2.8 million control participants, exposure to maternal preexisting diabetes in pregnancy was not only related to an impaired intelligence ability in the offspring, but also increase the risk of autism spectrum disorders [35]. The data suggest there is a signal that GDM may be associated with adverse neurocognitive and behavioral outcomes past the neonatal period. However, the underlying mechanisms leading to a higher susceptibility of the progeny to develop cognitive abnormalities later in life involve a complex pathophysiological change.

Maternal metabolic disorders could bring about sex-specific changes in the neurodevelopmental process of growing fetus. It is noteworthy that male offspring are at a higher risk of developing
neurodevelopmental disorders. Previous studies showed that fetal exposures to the adverse maternal environment are significant risk factors for neuropsychiatric disease predisposition, in particular in male offspring [36–38]. The most frequently used model of type 1 diabetes is the streptozotocin (STZ) model. STZ is a glucosamine-nitrosourea antibiotic that is similar structurally to glucose and is taken up preferentially by the GLUT2 glucose transporter in insulin-producing pancreatic β-cells [39]. Intraperitoneal treatment with STZ results in β-cell toxicity and necrosis, leading ultimately to insulin deficiency [40]. In our previous study, we also used the STZ-induced GDM model to find that the effect of intrauterine hyperglycemia on male offspring was more obvious than female, and that the effect of intrauterine hyperglycemia transfer to the brain has not been studied, thus further research is required. In both F1-GDM and F2-GDM male offspring of mice, there was no difference with control in the OF test, suggesting that the change of learning and memory was not confounded by the lack of locomotor activity. As the hippocampus-dependent memory test, the Y-maze showed the spontaneous spatial recognition was significantly decreased in F1-GDM offspring. In the NOR test, F1-GDM mice spent significantly less times investigating the novel object-in-place test between F2-GDM and control. All these phenotypes indicated that the intrauterine hyperglycemia exposure could result in impairment of cognition and memory not only in F1 but also in F2 male offspring.

The clinical and basical evidence have suggested that the disturbances in intellectual and behavioral functioning observed in the children of diabetic women are accompanied by modification of hippocampus structure and function. Investigation of the mechanisms responsible for maternal diabetes-related changes in the development of the hippocampus is helping to prevent impaired cognitive and memory functions in offspring [41]. In this study, the DEGs of F1-GDM and F2-GDM offspring were mainly enriched in “learning or memory”, “cognition”, and “neuron to neuron synapse”. By overlapping DEGs of the hippocampus in F1-GDM and F2-GDM offspring, totally we found the same 106 upregulated genes and 117 downregulated genes. It is interesting that there is no differentially expressed gene showing inconsistent tendency in F1-GDM and F2-GDM mice. The function of these DEGs included regulation of nervous system development, neuron differentiation, and axon development.

Beyond the shared genes associated with cognition (GO:00080890) mentioned above in the Result section, some important overlapped DEGs of F1-GDM and F2-GDM offspring were screened and verified in our study. Camk2, the calcium/calmodulin-dependent kinase type II, is the holoenzyme of the forebrain predominantly, which consists of heteromeric complexes of the Camk2a and Camk2b isoforms, regulating hippocampal synaptic plasticity and learning [42, 43]. Dopamine receptor Drd1 agonist could result in Camk2 activation, glutamate receptor exocytosis, synaptic reorganization, and expression of early markers of hippocampal synaptic plasticity [44]. Wnt5a regulates neuronal morphogenesis during embryonic development, and maintains dendritic architecture of pyramidal neurons in the adult hippocampus, through activating Wnt/JNK and Wnt/Camk2 signaling [45, 46]. As a core protein involved in neurogenic Wnt signaling pathways, Atp6ap2 is critical for proliferating adult neural stem cells and differentiating neuroblasts, essential in early brain development, adult hippocampal neurogenesis, and in cognitive functions. Lack of Atp6ap2 leads to cognitive impairment and neurodegeneration, and mutations of Atp6ap2 in humans are associated with intellectual disability [47, 48]. Postsynaptic density proteins (PSD) play a critical role in regulating the density and activity of glutamate receptors. As a scaffold protein localized at the PSD of glutamatergic neurons, Dlgap1 knockout leads to disruption of protein interactions in the PSD, and deficits in sociability [49]. And, TANC1 is a PSD-95-interacting synaptic protein that contains multiple domains for protein–protein interactions, important for dendritic spine maintenance and spatial memory [50]. The protein kinase A anchoring protein Akap7, a member of tubulin genes family Tub2b2, microtubule-associated protein Map1b, and some other genes associated with hippocampal function were also changed in F1-GDM and F2-GDM offspring.

There may be changes in the cellular composition of the hippocampus (for example, changes in the ratio of neurons:glia),

---

Table 1. The crucial genes associated with central nervous system development are hypermethylated in F1-GDM sperm and have a tendency to be downregulated (logFC <0) in the F2-GDM hippocampus.

| Symbol | RRBS of Sperm (F1-GDM vs. Ctrl) | Microarray of Hip (F2-GDM vs. Ctrl) |
|--------|---------------------------------|-----------------------------------|
|        | Start                           | End                               | mean Methy Ctrl | mean Methy F1-GDM | Element | logFC  | adj. P value |
| Tubb2b | 34127486                        | 3412764                           | 16.03%          | 81.88%            | CDS     | −3.0865 | 0.001     |
| Htr7   | 35969594                        | 35969756                          | 7.22%           | 73.70%            | CDS 3’-UTR | −1.7638 | 0.249     |
| Dlgap1 | 70516618                        | 70516771                          | 6.80%           | 86.87%            | CDS     | −1.2927 | 0.186     |
| Tanc1  | 59843298                        | 59843455                          | 65.81%          | 81.41%            | CDS     | −1.1135 | 0.026     |
| Zeb2   | 44988693                        | 44988893                          | 14.49%          | 85.39%            | CDS     | −0.7887 | 0.645     |
| Wnt5a  | 28518390                        | 28522909                          | 26.62%          | 76.36%            | CDS Intron | −0.5713 | 0.211     |
| Hoxb3  | 96345925                        | 96346054                          | 7.08%           | 28.65%            | CDS     | −0.4266 | 0.644     |
| Nfix   | 84784452                        | 84784657                          | 27.85%          | 45.75%            | Intron  | −0.2088 | 0.678     |
| Nfix   | 84704134                        | 84704384                          | 38.83%          | 64.20%            | Intron  | −0.2088 | 0.678     |
| Akap7  | 25251588                        | 25251727                          | 25.82%          | 77.69%            | Intron  | −0.2014 | 0.529     |
| Camk2b | 6010025                         | 6010230                           | 49.49%          | 89.20%            | Intron  | −0.1977 | 0.253     |

RRBS reduced representation bisulfite sequencing, Hip hippocampus, F1-GDM the first filial of gestational diabetes mellitus, F2-GDM the second filial of gestational diabetes mellitus, Ctrl/ control group.
and this may be also one of the potential mechanisms. Hippocampus is a complex brain structure embedded deep into the temporal lobe. It has a major role in learning, memory, and spatial navigation. Notably, the hippocampus has a mixed cellular environment with multiple cell types, including neurons (excitatory pyramidal cells, inhibitory interneurons) and glia (microglia, astrocytes, and oligodendrocytes) [51], and both neurons and glia display heterogeneous morphologies. Glia interacts with neuronal synapse can modulate synaptic transmission and plasticity, and both cell types impact learning and memory. As neurons and glia work together in complex, interdependent networks, it is difficult to isolate and disentangle the relative contribution and role of glia in neurological manifestations. Thus, the work described here, which briefly focuses on the transgenerational effect and hippocampal-related cognition, used the whole hippocampus for gene expression analyses as other studies do [52, 53].

The explicit mechanism of maternal effect on offspring is still unclear. A few studies indicated that maternal overnutrition could induce cognitive deficits across several generations [54]. For F1 offspring, as a mediator of stress effects on neurodevelopmental reprogramming, the placenta may play an important role in the transmission of the maternal adverse environment and effects on the developing brain [55]. Dysregulation of imprinted genes is a plausible mechanism linking maternal stressors with

---

**Fig. 5 Common transcriptomic signatures across two generations.**

a Venn diagram of differential genes overlapped between F1-GDM vs. control and F2-GDM vs. control. b The expression of meaningful shared DEGs in F1 and F2 offspring (nF1-Control = 4, nF1-GDM = 5, nF2-Control = 4, nF2-GDM = 5 male 4-month-old mice). c Enrichment analysis of shared DEGs in F1 and F2 offspring. Data were analyzed by a two-tailed unpaired t-test. *P < 0.05 vs. control; **P < 0.01 vs. control; ***P < 0.001 vs. control.
in F2 offspring [19, 23], we mainly investigated the methylation status of F1 sperm, finding 56 genes downregulated (logFC < 0) in F2-GDM hippocampus hypermethylated in F1-GDM sperm. Our result confirmed that the epigenetic memory carried by DNA methylation pattern could be reprogrammed in F1 germ cell during fetal development in the uterus.

In conclusion, in this study, with the mouse model, we firstly investigated the intergenerational effect of intrauterine hyperglycemia on cognition and memory in offspring and the potential molecular mechanism. The results showed that intrauterine hyperglycemia exposure could result in impairment of cognition and memory in both F1 and F2 male offspring. The DEGs in both F1 and F2 hippocampi were mainly enriched in learning or memory, cognition, and other neuron function. Further research found the altered methylated modification of sperm in F1 adult caused by intrauterine hyperglycemia exposure. Therefore, epigenetic alteration may play important role in the intergenerational transmission of GDM-induced abnormal neurodevelopment. It is essential that future studies focus on identifying the potential mechanism of the maternal effect on epigenetic regulation in the fetus and even their germ cells. To study the effect of intrauterine high glucose environment on the hippocampus of offspring mice more comprehensively, we plan to do the transcriptome sequencing and metabolomics in fetal mice hippocampi to better explain the direct effect of intrauterine hyperglycemia on the hippocampal development of offspring mice.

CODE AVAILABILITY
No custom code was used for analysis. RBBs and microarray analysis were performed using R function calls in the publicly available Bioconductor R packages. The datasets generated during this study are available at the Gene Expression Omnibus (GEO) Repository: GSE147039 and D GSE142502.

REFERENCES
1. Barker DJ, Eriksson JG, Forsen T, Osmond C. Fetal origins of adult disease: strength of effects and biological basis. Int J Epidemiol. 2002;31:1235–9.
2. Radford EJ, Ito M, Shi H, Corish JA, Yamasawa K, Igoianitis E, et al. In utero effects. In utero undernourishment perturbs the adult sperm methylene and intergenerational metabolism. Science. 2014;345:1255903.
3. Chandna AR, Kuhlmann N, Bryce CA, Greba Q, Capanu ME, Howland JG. Chonic maternal hyperglycemia induced during mid-pregnancy in rats increases RAGE expression, augments hippocampal excitability, and alters behavior of the offspring. Neuroscience. 2015;303:241–60.
4. Bytof F, Knorn S, Vlachova Z, Zheng RB, Mathiesen E, Beck-Nielsen H, et al. Long-term cognitive implications of intrauterine hyperglycemia in adolescent offspring of women with type 1 diabetes (the EPICOM Study). Diabetes Care. 2016;39:1365–66.
5. Nielsen GL, Dethlefsen C, Sorensen HT, Pedersen JF, Moltsted-Pedersen L. Cognitive function and army rejection rate in young adult male offspring of women with diabetes: a Danish population-based cohort study. Diabetes Care. 2007;30:2827–31.
6. Dahlquist G, Kallen B. School marks for Swedish children whose mothers had diabetes during pregnancy: a population-based study. Diabetologia. 2007;50:1826–31.
7. Rizzo T, Metzger BE, Burns WJ, Bums K. Correlations between antepartum maternal metabolism and intelligence of offspring. N Engl J Med. 1991;325:911–6.
8. Veena SR, Khilnani GV, Sinrivasan K, Kurpad AV, Muthayya S, Hill JC, et al. Childhood cognitive ability: relationship to gestational diabetes mellitus in India. Diabetesobiol. 2010;5:2314–8.
9. Fraser A, Almquist C, Larsson H, Langstrom N, Lawlor DA. Maternal diabetes in pregnancy and offspring cognitive ability: sibling study with 723,775 men from 579,857 families. Diabetologia. 2014;57:102–9.
10. Vuong B, Odero G, Rozbacher S, Stevenson M, Kerekuluk SM, Pereira TJ, et al. Exposure to gestational diabetes mellitus induces neuroinflammation, derangement of hippocampal neurons, and cognitive changes in rat offspring. J Neu- roinflammation. 2017;14:80.
11. He A, Zhang Y, Yang Y, Li L, Feng X, Wei B, et al. Prenatal high sucrose intake affected learning and memory of aged rat offspring with abnormal oxidative stress and NMDARs/Wnt signaling in the hippocampus. Brain Res. 2017;1669:114–21.
12. Kruse MS, Vega MC, Rey M, Corini H. Sex differences in LXR expression in normal offspring and in rats born to diabetic dams. J Endocrinol. 2014;222:53–60.
13. Hami J, Sadi-Nabavi A, Sankian M, Balali-Mood M, Haghir H. The effects of maternal diabetes on expression of insulin-like growth factor-I and insulin receptors in male developing rat hippocampus and cortical structure function. J Mol Neurosci. 2013;52:75–84.
14. Huang K, Sun M, Lv J, Li J, Wu C, Chen N, et al. Hippocampal apoptosis involved in learning deficits in the offspring exposed to maternal high sucrose diets. J Nutr Biochem. 2014;25:985–90.
15. Haghir H, Hami J, Loft L, Peyvandi M, Ghasemi S, Hosseini M. Expression of apoipoprotein-regulatory genes in the hippocampus of rat neonates born to mothers with diabetes. Metab Brain Dis. 2017;32:617–28.
16. Vafaee-Nezhad S, Hami J, Sadeghi A, Ghaimi K, Hosseini M, Abedini MR, et al. The impacts of diabetes in pregnancy on hippocampal synaptogenesis in rat neonates. Neuroscience. 2016;318:122–33.
17. Hami J, Vafaee-Nezhad S, Ivar G, Sadeghi K, Ghaemi M, Mostafavizadeh G, et al. Altered expression and localization of synaptophysin in developing cerebellar cortex of neonatal rats due to maternal diabetes mellitus. Metab Brain Dis. 2016;31:1369–80.
18. Golic M, Stojanovska V, Bendix I, Wehner A, Hesse H, Naes E, et al. Diabetes mellitus in pregnancy leads to growth restriction and epigenetic modification of the Srebf2 gene in rat fetuses. Hypertension. 2018;71:911–20.
19. Ding GL, Wang FF, Shu J, Tian S, Jiang Y, Zhang D, et al. Transgenerational glucose intolerance with Igf2/H19 epigenetic alterations in mouse islet induced by intrauterine hyperglycemia. Diabetes. 2012;61:1133–42.
20. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. Lmima powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015;43:e47.
21. Yu G, Wang LG, Han Y, He QY, clusterProfiler: an R package for comparing biological themes among gene clusters. Omics. 2012;16:284–7.
22. Feng H, Conneely KN, Wu H. A Bayesian hierarchical model to detect differentially methylated loci from single nucleotide resolution sequencing data. Nucleic Acids Res. 2014;42:e69.
23. Ren J, Cheng Y, Ming ZH, Dong XY, Zhou YZ, Ding GL, et al. Intrauterine hyperglycemia exposure results in intergenerational inheritance via DNA methylation reprogramming on F1 PGCS. Epigenetics Chromatin. 2018;11:20.
24. Shipecta T, Lu B, Holt CE. Cell biology in neuroscience: RNA-based mechanisms underlying axon guidance. J Cell Biol. 2013;202:991–99.
25. Vaughn JB, Barber RP, Sims TJ. Dendritic development and preferential growth in synaptogenic fields: a quantitative study of Golgi-impregnated spinal motor neurons. Synapse. 1982;6:29–78.
26. Gerfl R, Wojtowicz J, Marks A, Roder J. Overexpression of a calcium-binding protein, S100 beta, in astrocytes alters synaptic plasticity and impairs spatial learning in transgenic mice. Learn Mem. 1995;2:36–39.
27. Ellenbroek BA. Histamine H1 receptors, the complex interaction with dopamine and its implications for addiction. Br J Pharm. 2013;170:46–57.
28. Rani M, Kanungo MS. Expression of D2 dopamine receptor in the mouse brain. Biochem Pharmacol. 2006;344:981–6.
29. Meissner AC, Le Merrer J, Le Merrer LP, Daza J, Clesse D, Keiffer BL, et al. Mice lacking GPR88 show motor deficit, improved spatial learning, and low anxiety reversed by delta opioid antagonist. Biol Psychiatry. 2016;79:917–27.
30. Jakovcevski M, Ruan H, Shen EY, Dincar A, Jadavif BR, Ma Q, et al. Neuronal Kmt2a/Mill1 histone methyltransferase is essential for prefrontal plasticity and working memory. J Neurosci. 2015;35:1097–108.
31. Han KA, Woo D, Kim S, Choi G, Jeon S, Won SY, et al. Neurotrophin-3 regulates synapse development by modulating TrkC-Ptpo synaptic adhesion and intra-cellular signaling pathways. J Neurosci. 2016;36:4816–31.
32. Delthel T, Guedard BP, Cedern J, David DJ, Tanaka KF, Repérant C, et al. Behavioral and serotonergic consequences of decreasing or increasing hippocampus brain-derived neurotrophic factor protein levels in mice. Neuropharmacology. 2008;55:1006–14.
33. Adane AA, Mishra GD, Tooth LR. Diabetes in pregnancy and childhood cognitive development: a systematic review. Pediatrics. 2016;137:e20154234.
34. Fraser A, Lawlor DA. Long-term health outcomes in offspring born to women with diabetes in pregnancy. Curr Diab Rep. 2014;14:489.
35. Yamamoto J, Benham JL, Dewey D, Sanchez JJ, Murphy HR, Feig DS, et al. Neurotrophic and behavioural outcomes in offspring exposed to maternal pre-existing diabetes: a systematic review and meta-analysis. Diabetologia. 2016;59:1561–74.
36. Braithwaite EC, Pickles A, Shar H, Glover V, O’Donnell KJ, Tibu F, et al. Maternal prenatal cortisol predicts infant negative emotionality in a sex-dependent manner. Physiol Behav. 2017;175:31–36.
37. Lemaire V, Koehl M, Le Moal M, Abrus DN. Prenatal stress produces learning deficits associated with an inhibition of neurogenesis in the hippocampus. Proc Natl Acad Sci USA. 2000;97:11032–7.

K. Zou et al.
38. Misra P, Ganesh S. Sex-biased transgenerational effect of maternal stress on neurodevelopment and cognitive functions. J Genet. 2018;97:581–3.
39. Schnell WJ, Ferber S, Johnson NJ, Newgard CB. STZ transport and cytotoxicity. Specific enhancement in GLUT2-expressing cells. Diabetes. 1994;43:1326–33.
40. Bonnevie-Nielsen V, Steffes MW, Lemmark A. Major loss in islet mass and B-cell function precedes hyperglycemia in mice given multiple low doses of streptozotocin. Diabetes. 1981;30:424–9.
41. Hami J, Shojae F, Vafaee-Nezhad S, Lotfi N, Kheradmand H, Haghj H. Some of the experimental and clinical aspects of the effects of the maternal diabetes on developing hippocampus. World J Diabetes. 2015;6:412–22.
42. Borgesius NZ, van Woerden GM, Buitendijk GH, Keijzer N, Jaarsma D, Hoogenaard CC, et al. betaCaMKII plays a nonenzymatic role in hippocampal synaptic plasticity and learning by targeting alphaCaMKII to synapses. J Neurosci. 2011;31:10141–8.
43. Kool MJ, Proietti Onori M, Borgesius NZ, van de Bree JE, Elgersma-Hooisma M, Nio E, et al. CAMK2-dependent signaling in neurons is essential for survival. J Neurosci. 2019;39:5424–39.
44. Kern A, Mavrikaki M, Ullrich C, Albarran-Zeckler R, Brantley AF, Smith RG. Hippocampal dopamine/Drd1 signaling dependent on the ghrelin receptor. Cell. 2015;163:1176–90.
45. Arredondo SB, Guerrero FG, Herrera-Soto A, Jensen-Flores J, Bustamante DB, Ofate-Ponce A, et al. Wnt5a promotes differentiation and development of adult-born neurons in the hippocampus by noncanonical Wnt signaling. Stem Cells. 2020;38:422–36.
46. Chen CM, Orefice LL, Chiu SL, LeGates TA, Hattar S, Huganir RL, et al. Wnt5a is essential for hippocampal dendritic maintenance and spatial learning and memory in adult mice. Proc Natl Acad Sci USA. 2017;114:e628.
47. Schafer ST, Han J, Pena M, von Bohlen Und Halbach O, Peters J, Gage FH. The Wnt adaptor protein ATP6AP2 regulates multiple stages of adult hippocampal neurogenesis. J Neurosci. 2015;35:4983–98.
48. Bracke A, Schafer S, von Bohlen Und Halbach V, Klempin F, Bente K, Bracke K, et al. ATP6AP2 over-expression causes morphological alterations in the hippocampus and in hippocampus-related behaviour. Brain Struct Funct. 2018;223:2287–302.
49. Coba MP, Ramaker MU, Ho EV, Thompson SL, Komiyama NH, Grant S, et al. Dlgap1 knockout mice exhibit alterations of the postsynaptic density and selective reductions in sociability. Sci Rep. 2018;8:2281.
50. Han S, Nam J, Li Y, Kim S, Cho SH, Cho YS, et al. Regulation of dendritic spines, spatial memory, and embryonic development by the TANC family of PSD-95-interacting proteins. J Neurosci. 2010;30:15102–12.
51. Zeisel A, Muñoz-Manchado AB, Codeluppi S, Lönnerberg P, La Manno G, Juréus A, et al. Brain structure. Cell types in the mouse cortex and hippocampus revealed by single-cell RNA-seq. Science. 2015;347:1138–42.
52. Aguilar-Valles A, Haji N, De Gregorio D, Matta-Camacho E, Eslamizade MJ, Popic J, et al. Translational control of depression-like behavior via phosphorylation of eukaryotic translation initiation factor 4E. Nat Commun. 2018;9:2459.
53. Sankowski R, Strohl JJ, Huerta TS, Nasiri E, Mazzarello AN, D’Abramo C, et al. Endogenous retroviruses are associated with hippocampus-based memory impairment. Proc Natl Acad Sci USA. 2019;116:25982–90.
54. Sarker G, Peleg-Raibstein D. Maternal overnutrition induces long-term cognitive deficits across several generations. Nutrients. 2018;10:8.
55. Bronson SL, Bale TL. The placenta as a mediator of stress effects on neurodevelopmental reprogramming. Neuropsychopharmacology. 2016;41:207–18.
56. Argyraki M, Damidopoulou P, Chatzimeletiou K, Grimbizis GF, Tarlatzis BC, Syrrou M, et al. In-utero stress and mode of conception: impact on regulation of imprinted genes, fetal development and future health. Hum Reprod Update. 2019;25:777–801.

ACKNOWLEDGEMENTS
The work was supported by the National Key Research and Development Plan (2017YFC0101300 and 2018YFC0104500), National Natural Science Foundation of China (81971458, 82088102, and 31671222), the Municipal Human Resources Development Program for Outstanding Young Talents in Medical and Health Sciences in Shanghai (2017YQ047), the Innovative Research Team of High-level Local Universities in Shanghai, the Fundamental Research Funds for the Central Universities, and the Ferring Institute of Reproductive Medicine, a strategic collaborative research program of Ferring Pharmaceuticals and Chinese Academy of Sciences (FIRMA180309). The study is also supported by CAMS Innovation Fund for Medical Sciences (CIFMS) (No. 2019-2M-5-064) and the Collaborative Innovation Program of the Shanghai Municipal Health Commission (2020CXQ01).

AUTHOR CONTRIBUTIONS
G.-L.D. conceived the study. G.-L.D. and H.-F.H. designed research. K.-X.Z., J.R., C.-L.Z., C.-X.T., P.-P.L., X.S., J.-Z.S., H.-F.H., X.-M.L., and G.-L.D. interpreted the data. G.-L.D. and K.-X.Z. wrote the manuscript with input from other authors.

COMPETING INTERESTS
The authors declare no competing interests.

ADDITIONAL INFORMATION
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41398-021-01565-7.
Correspondence and requests for materials should be addressed to H.H. or G.D.
Reprints and permission information is available at http://www.nature.com/reprints
Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.