Sex-Specific Effects of Early-Life Iron Deficiency and Prenatal Choline Treatment on Adult Rat Hippocampal Transcriptome

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Short title: Iron and sex-specific effects on hippocampal transcriptome

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Author's contribution: PVT and MKG developed concept and obtained funding for the study; SXL and TKF performed experiments and collected data; SXL and PVT analyzed and interpreted results; SXL and PVT drafted the manuscript; All authors reviewed, edited, and approved the manuscript.

Keywords: Iron deficiency, choline, sex difference, hippocampus, transcriptome

ABSTRACT:

Background: Fetal-neonatal iron deficiency (ID) causes long-term neurocognitive and affective dysfunctions. Clinical and preclinical studies showed that early-life ID produced sex-specific effects. However, little is known about molecular mechanisms behind the sex-specific effects of early-life ID on neural gene regulation. Objective: To illustrate sex-specific transcriptome alteration in adult rat hippocampus induced by fetal-neonatal ID and prenatal choline treatment. Methods: Pregnant rats were fed iron-deficient (4 mg/kg Fe) or iron-sufficient (200 mg/kg Fe) diet from gestational day (G) 2 to postnatal day (P) 7 with or without choline supplementation (5 g/kg choline) from G11-18. Hippocampi were collected from P65 offspring of both sexes and analyzed for changes in gene expression. Results: Both early-life ID and choline treatment induced more transcriptional changes in female rat hippocampus than males with a less than 10 percent overlap between sexes. While both sexes showed alterations in gene networks related to increased risks of neurocognitive disorders, males also showed enhanced anti-inflammatory activities in response to ID, contrary to activated pro-inflammatory responses in females. Prenatal choline treatment to ID animals partially rescued ID-induced dysregulations in males but showed less effects in females. However, choline treatment to iron-sufficient rats showed more negative effects on the regulation of genes associated with cognition and affective behaviors, especially in females. Conclusions: This study provided unbiased global assessments of gene expression regulated by iron and choline in a sex-specific manner, with greater negative effects in female than male rats. Our new findings highlight potential sex-specific gene networks for further investigation.

INTRODUCTION:

Iron deficiency (ID) during fetal-neonatal periods can result in long-term neurocognitive and emotional abnormalities (1-4). In the clinical population, there is evidence supporting sex-specific outcomes for iron-deficient individuals or its treatment during early life, including differential effects on fecal microbiota (5), brain development (6), verbal fluency (7), and risks of cognitive disorders such as schizophrenia (2). Sex-specific physiologic and behavioral effects are also observed in preclinical models (8-11). Early postnatal ID anemia causes sex-specific neuroinflammatory responses (8), seizure vulnerability (9), social and affective behavioral abnormality (10), and vasculature dysfunction (11). However, analysis of sex-specific changes in gene transcriptional regulation that might underlie the neurodevelopmental changes remains limited to a report of alterations to the hippocampal transcriptome from our phlebotomy-induced ID anemia (PIA) mouse model (8) and to the placental transcriptome from a maternal ID mouse model (12). Thus, additional unbiased global transcriptomic studies are needed to establish the sex-specific molecular changes induced by early-life ID anemia that may explicate the differential long-term neurobehavioral effects and vulnerabilities between sexes (13).

Hippocampus is an important brain structure that mediates learning, memory, and cognitive function and can be influenced by sex (14, 15). The concurrent effects of early-life ID anemia on the development and function of the rat hippocampus have been well documented (16-18). However, long-term changes in transcriptional regulation induced by early-life ID anemia among specific cell types and between sexes within the hippocampus remain grossly understudied, in part due to the technical challenges in isolating various hippocampal cell types with minimal perturbations that could introduce artificial effects. Targeted studies within and

beyond the hippocampal structure have demonstrated the effects of early-life ID anemia on the development and function of oligodendrocytes (19, 20) and astroglia (21, 22). Additional analyses are needed to unravel the long-term consequences of early-life ID anemia on these specific cell types.

Transcriptomic analysis using the Next Generation Sequencing (NGS) technology offers a unique unbiased approach to identify global changes in transcriptional regulation. The NGS findings can be annotated into changes in relevant biological processes, canonical pathways, and disease states utilizing the knowledge-based database such as Ingenuity Pathway Analysis (IPA, Qiagen Inc.)(23). In addition, coupled with recent advances in single-nucleus sequencing(24), the NGS data can be further indexed onto gene expression profile of specific cell types. While this approach limits the findings to a correlative manner, it permits a more in-depth analysis beyond a normal routine of the NGS data analysis and avoids a prohibitive cost of single-cell sequencing. In this regard, the present study analyzed and compared long-term changes in hippocampal gene regulation between adult male and female rats that were iron-deficient during fetal and neonatal periods and between those with and without prenatal choline treatments. The study also attempted to ascribe specific effects of ID anemia on various cell types by utilizing a publicly available cell-specific gene expression profile derived from single-nucleus sequencing of the adult mouse hippocampus (24).

MATERIALS AND METHODS:

Animals

Gestational day (G) 2 pregnant Sprague–Dawley rats were purchased from Charles River Laboratories (Wilmington, MA, USA). Rats were maintained in a 12-hr:12-hr light/dark cycle with ad lib food and water. ID was induced by dietary manipulation as described previously (25). In brief, to generate iron-deficient pups, pregnant dams were given a purified iron-deficient diet (4 mg Fe/kg, TD.80396; Harlan Teklad) from G2 to postnatal day (P) 7 when lactating dams were switched to a purified iron-sufficient diet (200 mg Fe/kg, TD.09256; Harlan Teklad). Control iron-sufficient pups were generated from pregnant dams maintained on the iron-sufficient diet. Both diets were similar in all respects except for the iron (ferric citrate) content. Half of the dams on the iron-sufficient or iron-deficient diet received dietary choline supplementation (5.0 g/kg choline chloride supplemented; iron-sufficient with choline: TD.1448261, iron-deficient with choline: TD.110139; Harlan Teklad) from G11-G18, while the other half of the dams received their iron-sufficient or iron-deficient diet with standard choline content (1.1 g/kg) as previously described (25). Thus, dams and their litters were randomly assigned to one of four groups based on maternal diet: iron-deficient with choline supplementation (IDch), iron-deficient without supplemental choline (ID), always iron-sufficient with choline supplementation (ISch), always iron-sufficient without supplemental choline (IS, control group). Details of diet contents have been described in our previous study (26). All litters were culled to 8 pups with equal sexes at birth. Rats were weaned at P21. Littermates were separated by sex and housed in groups. To avoid litter-specific effects, two rats per litters and ≥3 litters/group were used in the experiments. The University of Minnesota Institutional Animal Care and Use Committee approved all experiments in this study (Protocol # 2001-37802A).

Hippocampal dissection

Rats were euthanized on P65 by an injection of pentobarbital (100 mg/kg, intraperitoneal). Brains were removed and bisected along the midline on an ice-cold metal block. Hippocampus was dissected and immediately flash-frozen in liquid nitrogen, banked and stored at –80°C for further use.

RNA isolation and sequencing

Total RNA was isolated from the hippocampal lobes of P65 females of all four groups (n=4/group) using RNAqueous kit (Invitrogen). RNA samples were submitted to the University of Minnesota Genomics Center for library preparation and sequencing. RNA quantity and quality were determined using the RiboGreen RNA Assay kit (Invitrogen) and capillary electrophoresis (Agilent BioAnalyzer 2100, Agilent), respectively. RNA samples with RIN score > 8.0 were used for library preparation. Barcoded libraries were constructed for each sample using the TruSeq RNA v2 kit (Illumina). Libraries were size-selected for ~200bp fragments. Sequencing was performed using Illumina NovaSeq to generate 150bp pair-end reads to generate NGS data. Sequencing depth was >25 million reads per sample for all samples.

NGS data and Differentially Expressed Genes (DEGs) selection

NGS reads from the female hippocampi were aligned to the rat reference genome (*rn6*) using HISAT2 (27). Gene quantification was done using Feature Counts for raw read counts. DEGs were identified using the EdgeR feature in CLCGWB (Qiagen, Red Wood City, CA) and raw read

counts were adjusted for multiple comparisons with absolute fold change ≥ 1.25x and $p < 0.05$. NGS reads from the P65 male hippocampi used in prior study (28), were reanalyzed with updated bioinformatic tools (i.e., *rn6* reference genome) to provide a comparable transcriptomic dataset.

Ingenuity Pathway Analysis (IPA)

DEGs were mapped onto known canonical pathways and biological processes using IPA, which has > 8.5 million findings (Qiagen, Redwood City, CA). Overlapping genes between groups were identified and annotated using the "Compare" feature of IPA. DEGs were used to identify altered signaling pathways, upstream regulators, and biological processes. Changes in activity of upstream regulators and top gene networks were further annotated to biological processes and dysfunctions using the "Overlay" feature of IPA. DEGs were further filtered for known biomarkers using brain, cerebrospinal fluid (CSF), blood, serum, and salivary as criteria. Significant findings were determined using an absolute z-score ≥ 2.0 and $p < 0.05$ computed by Fisher's exact test.

Results

Fetal-neonatal ID altered distinct patterns of hippocampal gene expression in a sex-specific manner

To assess the long-term sex-specific effects of fetal-neonatal ID anemia on transcriptional regulation in the adult hippocampus, comparisons were made between sexes for gene expression changes in ID vs IS (control) rats. ID females showed a greater number of

DEGs compared to ID males (635 vs 401) with 41 shared genes (Figure 1A). The 41 shared gene expression changes indicate activation of S100 Calcium Binding Protein A9 (S100A9) and inhibition of Amyloid Precursor Protein (APP) and Huntingtin (HTT); these changes indicated apoptosis and higher transport of molecules (Figure 1B). The 360 male-specific DEGs indicated activation of Estrogen Receptor 1 (ESR1) and inhibition of Glutamate Ionotropic Receptor NMDA Subtype Unit 3A (GRIN3A), Synuclein Alpha (SNCA), and Transforming Growth Factor Beta 1 (TGFB1) (Figure 1C); all are important for synaptic plasticity by increased microtubule dynamics, quantity of Ca2+, and branching of neurites (Figure 1B, male). The 594 female-specific DEGs were linked to activation of DNA Endonuclease (APEX1), Forkhead Box O3 (FOXO3), and Methyl-CpG Binding Protein 2 (MECP2), but inhibition of Immunity Related GTPase M (Irgm1), Phospholipase C Gamma 2 (PLCG2), and Ribonuclease H2 Subunit B (RNASEH2B) (Figure 1D). These female-specific changes indicate gained-in-activities of RNA transcription and translation (expression of RNA, promoter activation), neuronal cell death, inflammation, and metabolism of protein (Figure 1B, female). The ID-induced DEGs were further concatenated to gene networks and biological dysfunctions. The top gene network in ID males contains three major nodes (Protein Inhibitor of Activated STAT1 (PIAS1), GRIN3A, and SNCA), regulating cognition, pain, and movement disorders (Figure 2A). The top gene network in ID females contains five key regulators centering on Chromodomain Helicase DNA binding Protein 4 (CHD4), Iodothyronine Deiodinase 2 (DIO2), O-Linked N-Acetylglucosamine Transferase (OGT), SRY-Box Transcription Factor 2 (SOX2), and Teashirt Zinc finger Homeobox 3 (TSHZ3); these changes indicated higher vasculogenesis as well as reduced axonogenesis, movement disorders, and seizures (Figure 2B).

Sex-specific effects of prenatal choline supplementation on hippocampal gene regulation in adult male and female rats

Prenatal choline supplementation to the ID group resulted in 91 shared as well as 459 and 887 unique DEGs for males and females, respectively (Figure 3A). The expression changes of the shared genes indicated increased neuroinflammation and decreased beta-oxidation associated with activation of S100A in both sexes and inhibition of PPARa/RXRa in IDch females (Figure 3B). The sex-specific changes included activation of GRIN3A and inhibition of Lysine Methyltransferase 2D (KMT2D) activity in males; these changes suggested lower apoptotic cell death and higher transport of metal ion (Figure 3B, 3C). DEGs in IDch females showed activation of SNCA, TSHZ3, and STAT1 as well as inhibition of Irgm1 and RNASEH2B (Figure 3B, 3D). These changes are associated with decreased secretion of molecule and cell-cell contact as well as increased inflammatory response, neuronal cell death, neurotransmission, and protein kinase cascade (Figure 3B). The top gene network in IDch males centers on the Integrin Subunit Beta 1 (ITGB1), SOX2, and Transcription Factor 7 Like 2 (TCF7L2); changes in these activities are linked to activation of axonogenesis and recognition memory concomitant with inhibition of neuroglia proliferation (Figure 4A). The top network in IDch females has two major regulators centering on BDNF and Corticotrophin Releasing Hormone (CRH); changes in these regulators indicate increase in locomotion, fear and feeding behaviors and decrease in anxiety, epilepsy, learning and memory, and nociception (Figure 4B).

Prenatal choline supplementation to the IS group resulted in even more robust gene expression changes. 123 DEGs were common in both sexes but linked to sex-specific effects. In

ISch males, these gene expression changes indicate activation of APP and Apolipoprotein E (APOE) and inhibition of BDNF and Endoribonuclease DICER (DICER1) (Figure 5A-B). In contrast, the effects in ISch females were activation of BDNF and inhibition of APP, DICER1, and MTOR (Figure 5B). The male-specific DEGs (621 genes) revealed activation of Glycoprotein Hormone Alpha Polypeptide (CGA/TSHA), MECP2, and Prostaglandin E Receptor 4 (PTGER4) as well as inhibition of Bone Morphogenic Factor 7 (BMP7) and GRIN3A (an effect observed ID rats) (Figure 5B-C). These changes indicate increased activity of microtubule dynamics, neuritogenesis, release of lipid, and neurotransmission. The female-specific DEGs (916 genes) were linked to activation of CREB1, MAPK1, and Serum Response Factor (SRF1) accompanied by inhibition of Transcription Factor 20 (TCF20) (Figure 4B). These changes indicated increased activity of transactivation of RNA, long-term memory, branching of neurites and decreased feeding (Figure 5B, 5D). The top gene network in ISch males centers on S100A9 and SOX2 and indicates increased emotional behavior, movement disorders, and analgesia concurrent with decreased vasculogenesis, learning, cognition, and progressive encephalopathy (Figure 6A). The top gene network in ISch females has three key nodes (BDNF, cAMP Responsive Element Modulator (CREM), and Achaete-Scute Family BHLH Transcription Factor 1 (ASCL1)) and are associated with increased anxiety, learning, memory, and decreased in seizures, movement disorders, and progressive neuropathy (Figure 6B).

Sex-specific interaction between ID and choline differentially alter long-term hippocampal transcriptome in adult rats

To differentiate the effects of ID, choline supplementation, and their interaction on gene expression changes in male and female adult rat hippocampus, comparisons of IPA analyses were made among all treatment groups. Altered biological processes linked to DEGs were clustered into three distinct clades with specific group effects (Figure 7A), including changes in cell death/organ degeneration (ID and ISCh males), synaptic depression (ISch group), and neural differentiation (ISch females). Differentially affected canonical pathways among the comparison groups include CREB signaling in neurons and G-protein coupled receptor signaling (Figure 7B). In particular, activity of GP6 signaling, which regulates platelet activation (29), was different between sexes with activation in IDch males (Z-score = 1.89), inhibition in ID and IDch females (Z-scores = -2.24 and -1.89), and no significant effect in ID males (Figure 7B). Analysis of upstream regulators further identified unique patterns of activity changes among the treatment groups. These changes were hierarchically clustered into three major clades with ID and ISch females exhibiting the most significant changes compared to other groups (Figure 7C).

Fetal-neonatal ID and prenatal choline supplementation induced sex-specific changes in biomarkers

Leveraging our available P15 iron-deficient hippocampal transcriptomic dataset (30), we determined the long-term changes in expression of IPA-defined biomarkers persisted in male and female P65 hippocampal transcriptomes. While 8 biomarkers were identified in the P15 iron-deficient hippocampus (Figure 8A), Neuregulin 1 (*Nrg1*) in both sexes and Opioid Receptor Mu 1 (*Oprm1*) in males were reversibly changed in the P65 transcriptomes (Figure 8A). Prenatal choline treatment prevented the ID-induced changes in *Nrg1* and *Oprm1* expression; however,

it also led to new persistently altered biomarkers, including *Vwf1*, *Chl3l1*, *Itga4*, and *Tf* (Figure 8B-C, Table 1).

Discussions and Conclusions

By assessing global changes in hippocampal gene expression, this study presents novel findings pertaining to long-term sex-specific hippocampal effects of early-life ID and prenatal choline supplementation on gene regulation and changes in pre-defined neurologic biomarkers. Consistent with our previous findings in a neonatal model of PIA (8), substantially more gene expression changes in the hippocampal transcriptome were found in female than male rodents, indicating a more robust gene regulatory response to early-life ID in female rats. Prenatal choline supplementation, a promising adjunct therapy that prevents some long-term behavioral abnormalities following early-life ID, led to even more DEGs in both sexes of iron-sufficient compared to iron-deficient groups, further supporting previous observations of the interaction between choline and iron status in regulating hippocampal gene expression (25, 28). Collectively, both ID and choline supplementation caused sex-specific changes in hippocampal gene regulation, affecting distinct gene networks and biological processes.

Fetal-neonatal ID induced more gene expression changes unique to male and female adult rats than shared ones (6-10%). The expression changes of the shared genes indicated measurable effects associated with apoptotic cell death and small molecule transport. The inhibitory effect on cell death in the formerly ID hippocampus is intriguing given a known risk of progressive neuropathology such as Alzheimer's disease (AD) associated with early-life ID anemia (4, 31). Thus, additional analyses in older (e.g., 12-18 months old) formerly iron-

deficient rats is warranted to determine whether neural cell death contributes to AD pathogenesis.

Sex-specific gene expression changes indicate effects on distinct signaling pathways. For instance, activation of ESR1 was unique to ID male, whereas activation of FOXO3 and MECP2 was specific in ID female rats. In the formerly ID male rats, activation of ESR1 is a novel discovery and may have a role in reducing hippocampal-dependent cognitive impairment (32, 33). These male-specific effects on gene regulators could explain the sex-specific cognitive impairments associated with the interaction between early-life neuroinflammation and iron status in both preclinical models and human infants (7, 8, 34, 35). In the formerly ID female rats, activation of FOXO3 and MECP2 gene network could improve the overall metabolic activity and neuronal differentiation via recruitment of relevant target genes, including EGR4, FOS, ENO2, and NPAS4, thereby compensating for the reduced capacity of iron-mediated cellular metabolism (36-41). Moreover, the MECP2 gene network plays an important role in regulating synaptic plasticity genes, including *Bdnf* (42-45), a gene known to be downregulated in the long-term by early-life ID (46, 47). As such, notable sex-specific changes in the formerly ID hippocampus include increased vasculogenesis in female and pain (discomfort) in male rats. While ID-induced vasculogenesis has been reported previously (48), the effect on nociception, which is likely associated with a higher expression of *Oprm1* or an altered inhibitory-excitatory balance (Figure 2A) (49-51), has not been described. Collectively, these observations indicate that early-life ID could alter separate biological processes in male and female rats.

Leveraging publicly available adult mouse hippocampal single-nucleus RNAseq data, changes in gene expression of adult rat hippocampus could be indexed onto specific groups of

cells in the adult rodent hippocampus (Supplemental Figure 1). The findings revealed neuronal-specific changes suggesting increased serotonin receptor signaling in the formerly ID male as well as increased synaptogenesis and Insulin Receptor (InsR) signaling in the formerly ID female rats (Supplemental Figure 1A, 1B). Oligodendrocyte-specific gene expression changes potentially highlight novel gene candidates modified by early-life ID (Supplemental Figure 1C). Astrocyte-specific gene expression changes indicate increased neuroinflammation in female formerly ID rats (Supplemental Figure 1D). This preliminary exercise revealed potentially differential effects of early-life ID on various neural cell types, collectively contributing to the long-term sex-specific neurobehavioral risks associated with early-life ID anemia. Future work could determine the cell-specific long-term changes in gene expression and chromatin structure within the adult rat hippocampus using the single-nucleus RNAseq technology.

Little is known about long-term effects of prenatal choline supplementation on the female rat hippocampal transcriptome. Here we showed that prenatal choline supplementation exhibited long-term sex-specific gene expression changes in adult hippocampal transcriptomes with greater effects in female vs male and IS vs ID rats. In the formerly ID rats, the shared DEGs (91 genes) between both sexes showed differential effects on lipid metabolism (e.g., beta oxidation) but a similar effect on activation of neuroinflammation. Such an effect of choline supplementation could improve the lower fatty acid metabolism observed in early-life ID rats (52) and infant Rhesus monkeys (53). Choline activates the GRIN3A pathway in IDch male rats, whereas it was inhibited in ID male rat, suggesting choline could enhance activity of glutamatergic neurons to improve learning and memory function in the hippocampus. Choline supplementation may lower neurogenic potentials of neural stem cell pools via the

SOX2/TCF7L2 gene network (54-57), thus reducing the proliferation of neuroglia (Figure 4A). Unlike IDch males, choline supplementation did not rescue the active neuroinflammation in the ID female hippocampus, contributing to a lower expression of GRIN2A and 2B, and thus lower activity of glutamatergic system. Consistent with previous reports (58, 59), choline recruited BDNF gene network as an actionable mechanism to compensate for a lower glutamatergic system (Figure 4B), suggesting an alternative pathway to improve cognitive function. IDch females showed reduced expression of genes identified with anxiety disorder, but a higher expression of genes involved in locomotion behavior via an activation of the hormonal system (e.g., CRH, GHSR, NPY, STAR, CYP11A1). These findings highlight relevant molecular pathways and associated neurobehaviors modulated by prenatal choline supplementation. Thus, the interaction of choline and ID in each sex deserves further investigation.

Choline supplemented IS (ISch) rats showed even more robust long-term gene expression changes than IDch rats. Particularly in females, genes that were activated included ones negatively associated with cognition, emotion, and anxiety. The findings further confirm our previous speculation that choline and iron interact to regulate hippocampal gene expression (25, 28) and highlight differential effects of such an interaction between sexes. For instance, BDNF and APP gene networks showed opposite effects in male and female ISch rats, respectively (Figure 5B), illustrating the sex-specific effects of prenatal choline supplement in the iron-sufficient rats. In the ISch males, choline might have beneficial effects on recognition memory and social behavior by increased activity of MECP2/GRIN2A and CGA/HOMER2 (42-45, 60) as well as on vasculature phenotype by reducing ectopic vasculogenesis (Figure 6A). As such, choline could also impose undesired effects such as inhibition of the GRIN3A pathway

analogous to an effect seen in ID male rats. ISch female rats also showed similar dual effects of prenatal choline supplementation, including activation of BDNF, CREM, and SRF gene networks, which modulate hippocampal-dependent learning and memory (61, 62); however, they also exhibited activation of ADCYAP1 (PACAP) signaling, which could influence anxiety behavior (63-65). These findings indicate both benefits and potential risks of prenatal choline supplementation to the IS rats, thus the use of choline as a therapeutic agent deserves additional investigation, particularly the long-term gene expression changes modulating neurobehavioral functions (28).

Comparison analyses across treatment groups revealed that fetal-neonatal ID and choline supplementation exhibit distinct patterns of effects on gene regulation, signaling pathways, and biological processes. Gene expression changes indicating altered synaptic plasticity and neurotransmission are noteworthy as they have been established in previous reports (28), thus confirming the validity of the ID anemia model. The sex-specific effects on unsuspected mechanisms (e.g., GP6 signaling, differentiation of stem cells, MECP2 gene network) are noteworthy as they highlight novel molecular underpinnings of the long-term risks for progressive neuropathology and psychiatric disorders associated with early-life ID anemia. For instance, GP6 signaling is known to regulate platelet function (29, 66); however, its role in the hippocampus is completely unknown. In the context of early-life ID, formerly ID females showed gene expression changes indicating lower GP6 signaling accompanied by increased vasculogenesis. GP6 signaling could modulate such a change as a mechanism to maintain vasculature structure and function. Such a long-term change of GP6 signaling could also indicate an increased vulnerability of hippocampal damage following a second injurious event

(67). The role of GP6 signaling in ID and choline treated hippocampal function will require additional studies to further reveal its implication on non-neural cell functions and their contributions to the overall hippocampal health in adulthood.

Finally, a potentially important finding of the present study was the identification of biomarkers that are readily accessible in bodily fluids. Long-term specific changes due to ID (e.g., *Nrg1*), sex (e.g., *Oprm1*), or choline treatment (e.g., *Chl3l1*) in the rat hippocampus indicate that these genes can be utilized as potential markers to gauge the status of the hippocampal as well as the brain health. Future studies can determine the relationship of these markers in blood and brain compartments following the development of ID anemia. In this regard, our PIA model would be an ideal system to determine the utility of these biomarkers as we could generate various degrees of ID severity (i.e., 25 vs 18% hematocrit). Determining the predictive values of these potential biomarkers in an easily accessible source (e.g., blood) would be critically important in the prevention and treatment of brain ID in order to reduce the life-long risks of poor neurobehavioral outcomes.

In conclusion, the power of the present study is in the global unbiased approach to identify differential gene expression changes due to ID, sex, and choline treatment. The long-term changes in gene expression, and thus regulation, encompass various biological processes (neuritogenesis, myelination, inflammation, vasculogenesis) that are mediated by multiple cell types. The present study achieved our immediate goals in uncovering sex-specific effects of early-life ID and prenatal choline treatment on gene expression, laying down the groundwork by providing targets for future studies to address the causal relationship between these specific

gene expression changes and hippocampal-dependent behavioral outcomes as well as more broadly functional consequences of the cortical-hippocampal-striatal circuitry.

Acknowledgements: We thank Dr. Juan E. Abrahante Lloréns for help with RNAseq data analysis. We apologize to colleagues whose work on this subject were not cited due to space constraint.

Sources of funding: This work was funded by NIH R01NS099178 to P.V.T. and R01HD29421 to M.K.G.

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Figure Legends:

Figure 1: Comparative analysis of long-term gene expression changes in P65 male and female rat hippocampus that were iron-deficient during fetal-neonatal periods. (A) Venn diagram showing overlap (41) and sex-specific changes in gene expression in P65 rats. (B) Table showing IPA-annotated changes in activity of upstream regulators and altered biological processes based on the gene expression changes. (C, D) IPA-annotated changes in activity of upstream regulators in the hippocampus of male and female rats. Blue = Inhibition, Orange = Activation, Yellow = Finding inconsistent with state of downstream molecule, Grey = Finding not predicted finding, Solid and Dashed arrows = direct and indirect interactions, Green-to-Red = Downregulated-to-Upregulated genes.

Figure 2: IPA-annotated top gene networks associated with changes in gene expression in the formerly ID hippocampus. (A) Formerly ID males showed a gene network with three key nodes centering on GRIN3A, SNCA, and PIAS1. These changes indicate activation of pain (discomfort) and cognition as well as inhibition of movement disorders and seizures. (B) Formerly ID females showed a gene network with four key nodes including DIO2, OGT, SOX2, and TSHZ3. These changes suggest increased vasculogenesis and decreased movement disorders, seizures, and axonogenesis. Blue = Inhibition, Orange = Activation, Yellow = Finding inconsistent with state of downstream molecule, Grey = Finding not predicted finding, Solid and Dashed arrows = direct and indirect interactions, Green-to-Red = Downregulated-to-Upregulated genes.

Figure 3: Comparison of long-term changes in hippocampal gene expression between male and female rats that were iron-deficient during fetal-neonatal period with choline treatment during the prenatal period. (A) Venn diagram showing overlap and sex-specific changes in gene expression. (B) Table showing changes in activity of upstream regulators and associated biological processes. (C, D) IPA-annotated changes in activity of upstream regulators in adult male and female rats based on gene expression changes. Blue = Inhibition, Orange = Activation, Yellow = Finding inconsistent with state of downstream molecule, Grey = Finding not predicted finding, Solid and Dashed arrows = direct and indirect interactions, Green-to-Red = Downregulated-to-Upregulated genes.

Figure 4: Top gene networks altered by prenatal choline treatment of fetal-neonatal ID rats compared to IS rats. (A) IDch males showed a gene network with three key nodes centering on ITGB1, SOX2, and TCF7L2. These changes indicate activation of axonogenesis and recognition memory concomitant with inhibition of neuroglia proliferation. (B) IDch females showed a gene network centers on BDNF and CRH. These changes suggest activation of locomotion, fear, and feeding accompanied by inhibition of anxiety, learning and memory, epilepsy, and nociception. Blue = Inhibition, Orange = Activation, Yellow = Finding inconsistent with state of downstream molecule, Grey = Finding not predicted finding, Solid and Dashed arrows = direct and indirect interactions, Green-to-Red = Downregulated-to-Upregulated genes.

Figure 5: Long-term changes in hippocampal gene expression between iron-sufficient male and female rats that were treated with choline during the prenatal period (ISch) compared to IS

rats. (A) Venn diagram showing overlap and sex-specific changes in gene expression with a nearly 2-fold gene expression changes in female compared to male rats. (B) Table showing IPA-annotated changes in activity of upstream regulators and associated biological processes. Changes in expression of the overlap genes between both sexes indicate opposing effects for specific regulators between male and female rats. (C, D) IPA-annotated changes in activity of upstream regulators in adult male and female rats showing sex-specific effects on regulators and associated biological processes. Blue = Inhibition, Orange = Activation, Yellow = Finding inconsistent with state of downstream molecule, Grey = Finding not predicted finding, Solid and Dashed arrows = direct and indirect interactions, Green-to-Red = Downregulated-to-Upregulated genes.

Figure 6: Top gene networks altered by prenatal choline treatment of iron-sufficient (ISch) rats compared to untreated IS rats. (A) ISch males showed a gene network with two key nodes centering on SOX2 and S100A9. These changes indicate activation of emotional behavior, movement disorders, and analgesia as well as inhibition of cognition, learning, vasculogenesis, and progressive encephalopathy. (B) ISch females showed a gene network centers on BDNF, CREM, and ASCL1. These activity changes suggest activation of anxiety, learning, and memory accompanied by inhibition of movement disorders, seizures, and progressive encephalopathy. Blue = Inhibition, Orange = Activation, Yellow = Finding inconsistent with state of downstream molecule, Grey = Finding not predicted finding, Solid and Dashed arrows = direct and indirect interactions, Green-to-Red = Downregulated-to-Upregulated genes.

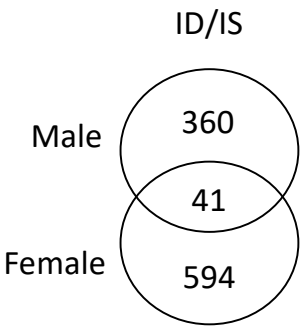
Figure 7: Comparison analyses of IPA-analyzed DEGs from male and female ID, IDch, and ISch rats. All data were normalized to the IS group of each sex. Findings were hierarchically clustered and selected for an absolute z-score ≥ 2.0. (A) Effects of treatment and sex on biological processes, which were clustered into three major clades with lower cell death (ID and ISch males), higher synaptic depression (ISch males), and higher neural differentiation (ID males and ISch females). (B) Effects of treatment and sex on canonical pathways showing distinct changes in signaling between sexes (wound healing and GP6 signaling). (C) Changes in activity of upstream regulators showing sex specific effects, including Irgm1 and RNASEH2B in females and SNCA, BMP7, and GRIN3A in males. Most robust changes were found in the choline treated female rats. Activation z-score ranges are shown. Dotted squares indicate findings with z-score<2.0.

Figure 8: Venn diagrams showing comparison of identifiable biomarkers. (A) Among P15 ID male, P65 formerly ID male and female rats, Neuregulin 1 (*Nrg1*, male and female) and Opioid Receptor Mu 1 (*Oprm1*, male) were lowered in P15 ID and higher in P65 formerly ID rats. (B, C) Prenatal choline treatment removed biomarker changes induced by ID and introduced new changes in IDch (*Vwf*) and ISch (*Itgα4*) males (B) as well as in IDch (*Tf*) females (C).

Table 1: Potential biomarkers identified by IPA from the rat hippocampal transcriptomes. *Asterisk denotes factors overlapped with and ∂ denotes opposite expression changes from those of P15 ID males.

Supplemental Figure 1: Preliminary analysis of long-term cell-specific effects in the formerly ID hippocampus of adult male and female rats utilized available cell-specific gene profiles derived from the adult mouse hippocampus (24). Formerly ID male rats showed changes in neurons indicating increased serotonin signaling, whereas female rats showed changes in neurons suggesting increased insulin receptor and synaptogenesis signaling. In addition, the formerly ID female rats showed changes in expression of astrocyte-specific genes indicating increased neuroinflammation signaling.

A



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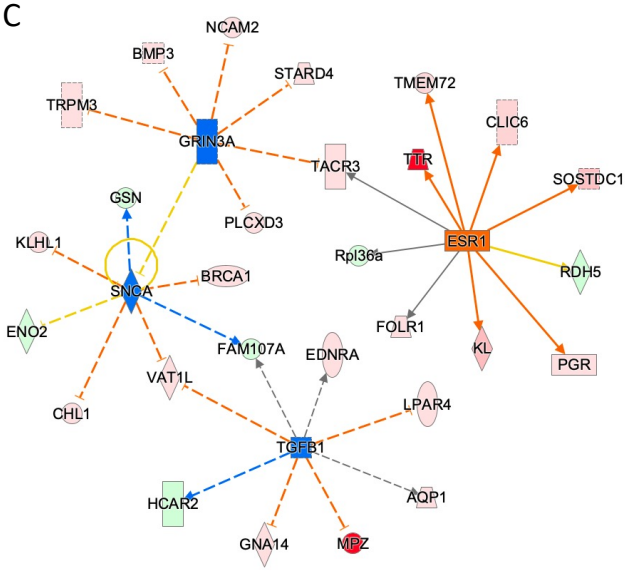

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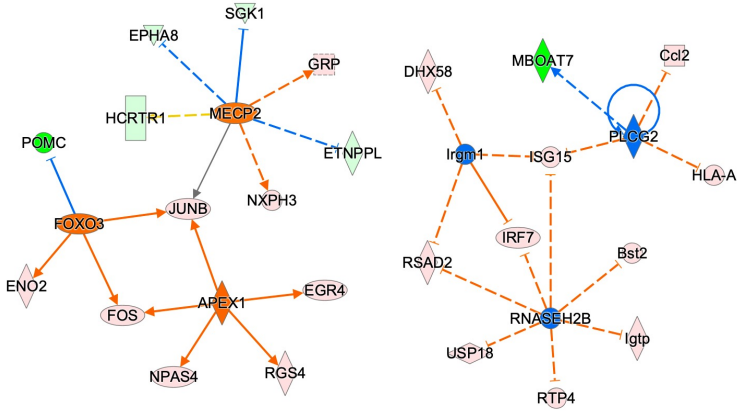
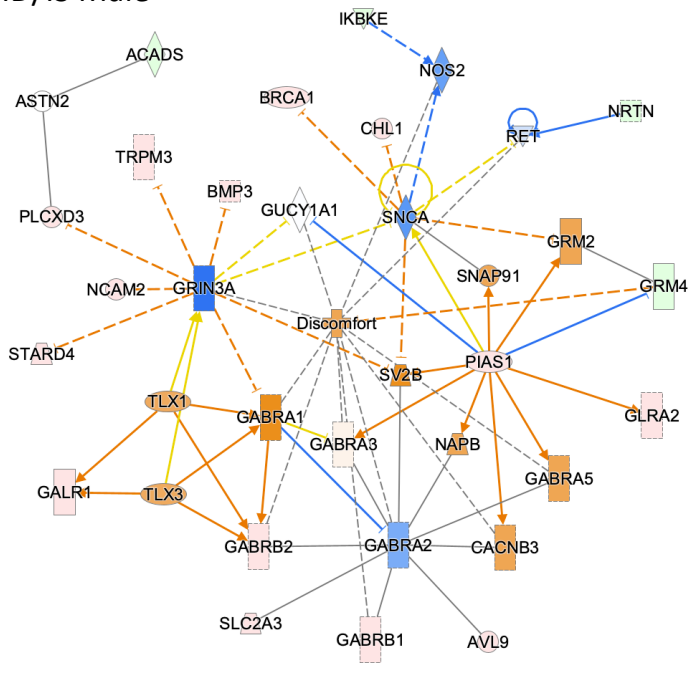


B

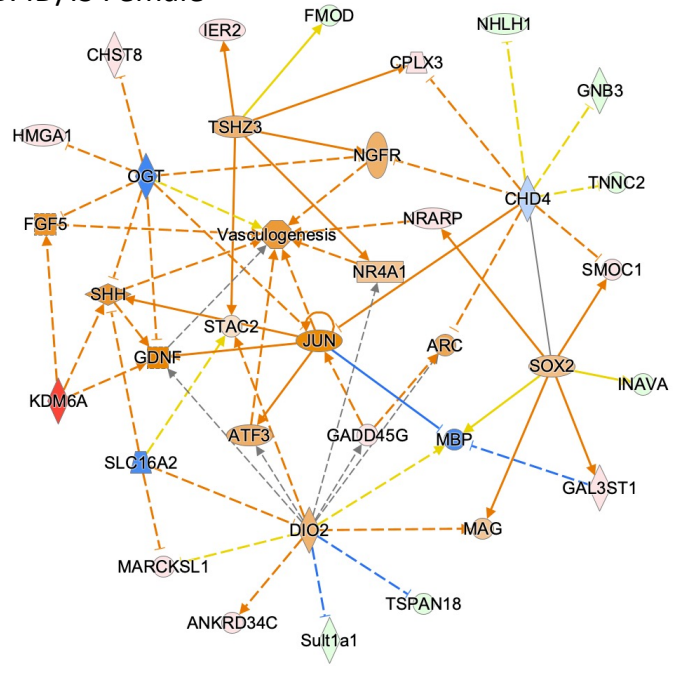
ID vs IS Sex	Regulators Activation	 Inhibition	Biological Processes
All (41)	S100A9	APP, HTT	Apoptosis, Transport of molecules
Male (360)	ESR1	GRIN3A, SNCA, TGFB1	Concentration of lipid, Microtubule dynamics, Quantity of Ca2+, Branching of neurites
Female (594)	APEX1, FOXO3, MECP2	Irgm1, PLCG2, RNASEH2B	Concentration of lipid, Expression of RNA, Neuronal cell death, Inflammation, Metabolism of protein, Promoter activation

A. ID/IS Male



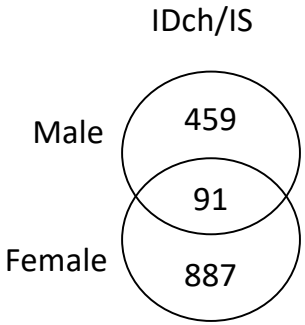
IPA-Predicted Disease State	
Activation	Inhibition
Cognition, pain/discomfort	Movement Disorders, Seizures

B. ID/IS Female



IPA-Predicted Disease State	
Activation	Inhibition
Vasculogenesis	Movement Disorders, Seizures, Axonogenesis

A



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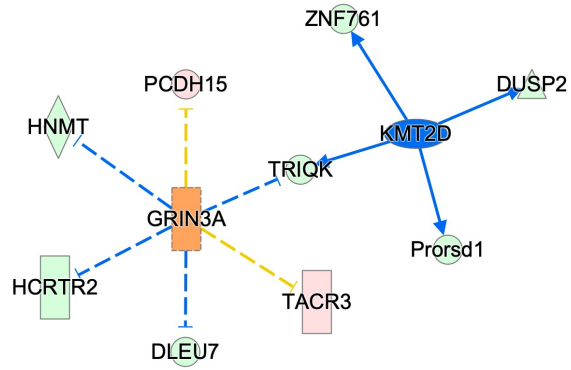

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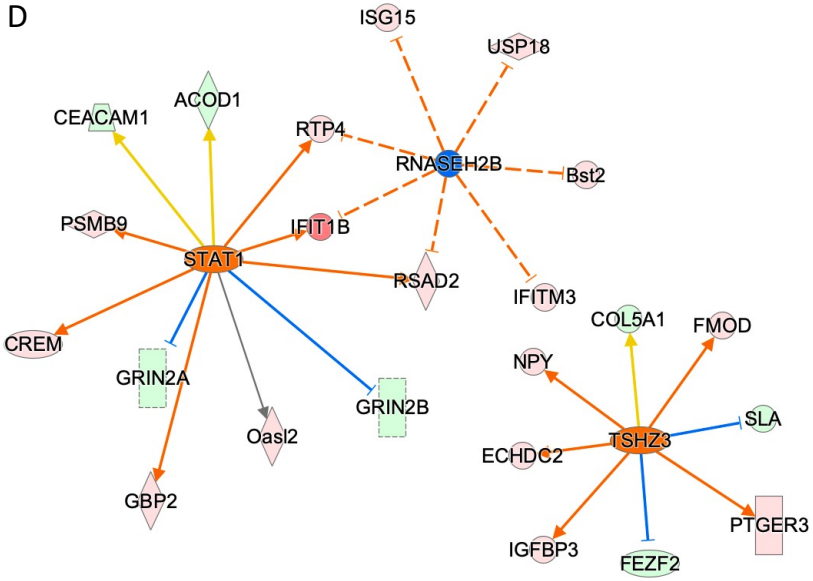
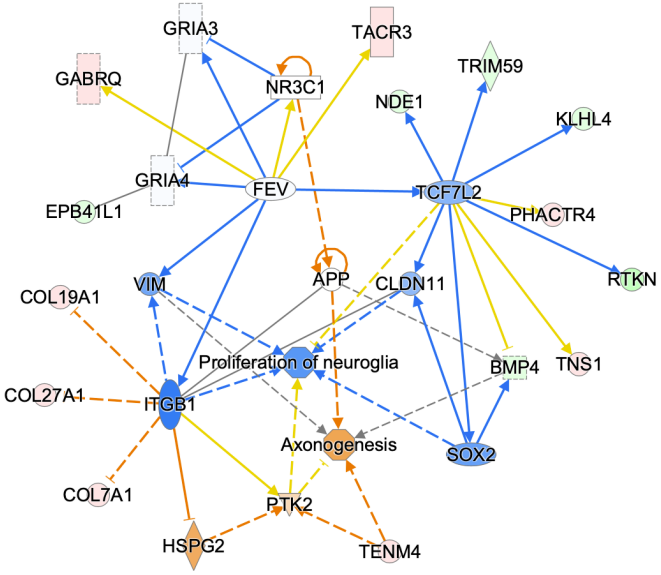


B

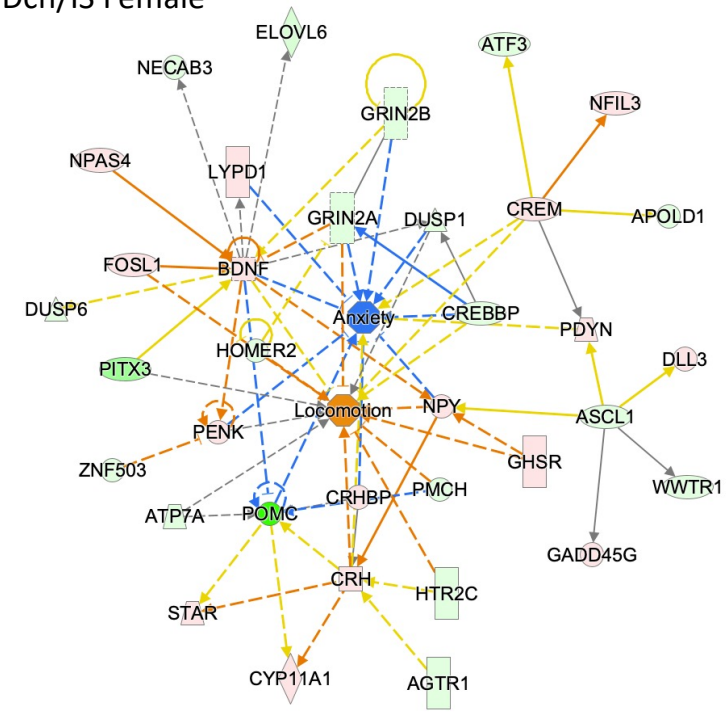
IDch vs IS Sex	Regulators		Biological Processes
	Activation	Inhibition	
All (91)	S100A8/A9	PPARa/RXRa (F)	Neuroinflammation, b-Oxidation
Male (459)	GRIN3A	KMT2D	Apoptosis, Transport of metal ion
Female (887)	SNCA, TSHZ3, STAT1	Irgm1, RNASEH2B	Secretion of molecule, Cell-cell contact, Inflammatory response, Neuronal cell death, Neurotransmission, Protein kinase cascade

A. IDch/IS Male



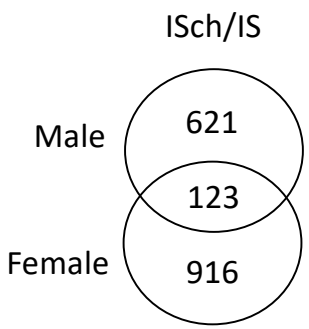
IPA-Predicted Disease State	
Activation	Inhibition
Axonogenesis, Recognition memory	Proliferation of neuroglia

B. IDch/IS Female

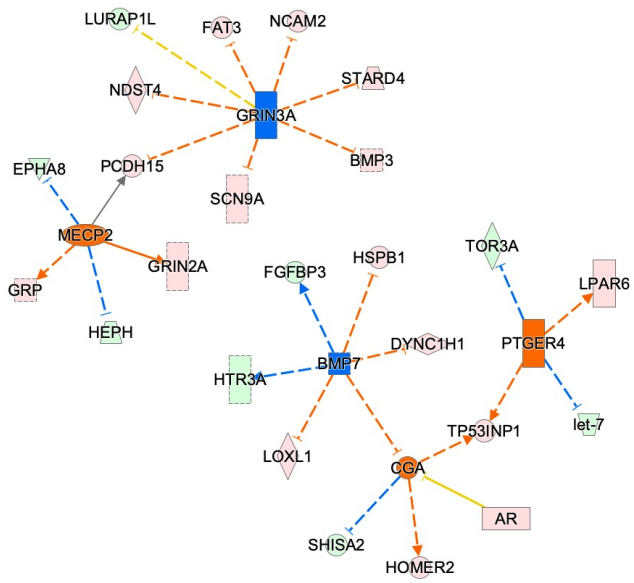


IPA-Predicted Disease State	
Activation	Inhibition
Locomotion, Fear, Feeding	Anxiety, Learning and memory, Epilepsy, Nociception

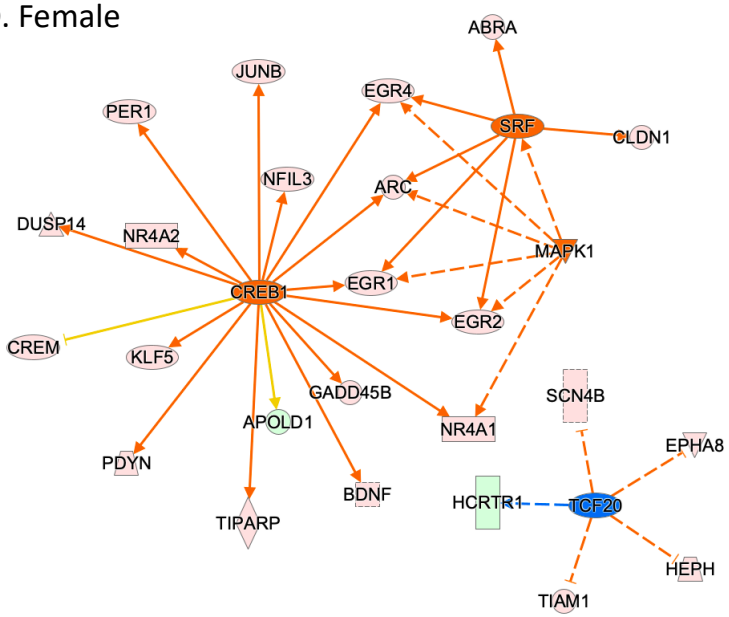
A



C. Male



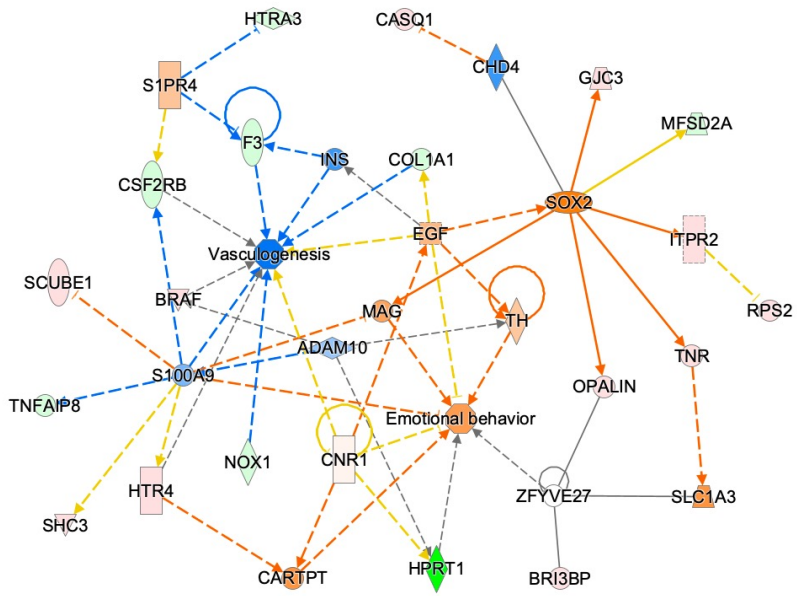
D. Female



B

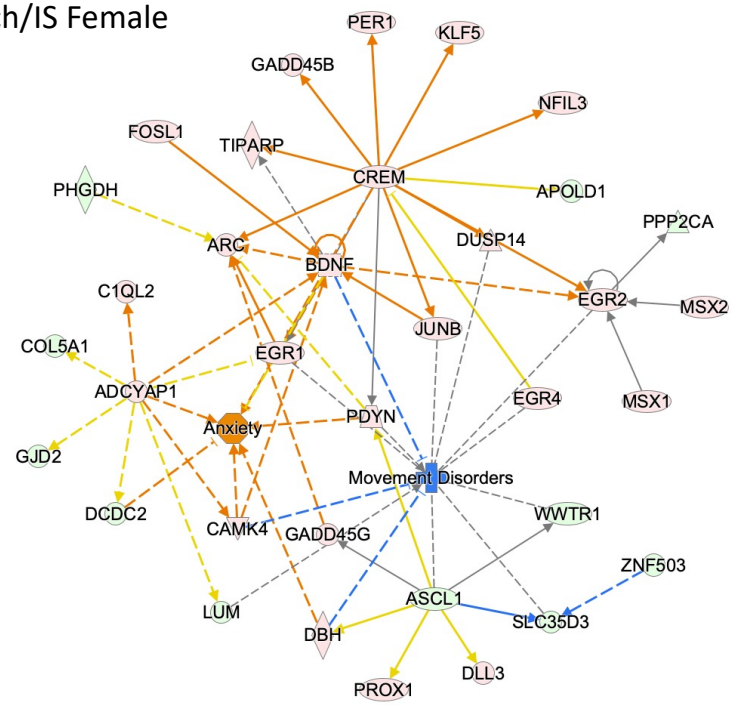
ISch vs IS Sex	Regulators		Biological Processes
	Activation	Inhibition	
All (123)	M: APOE, APP	M: BDNF, DICER1	M: Neuronal cell death, Inflammatory response, Vasculogenesis, Morphogenesis of neurons, Fatty acid metabolism
	F: BDNF	F: APP, DICER1, MTOR	F: Neuronal apoptosis, Morphogenesis of neurons, Myelination, Quantity of microglia, Long-term memory,
Male (621)	CGA, MECP2, PTGER4	BMP7, GRIN3A	Microtubule dynamics, Development of neurons, Neuritogenesis, Release of lipid, Neurotransmission
Female (916)	CREB1, MAPK1, SRF	TCF20	Transactivation of RNA, Conditioning, Long-term memory, Branching of neurites, Microtubule dynamics, Cell cycle progression, Feeding

A. ISch/IS Male

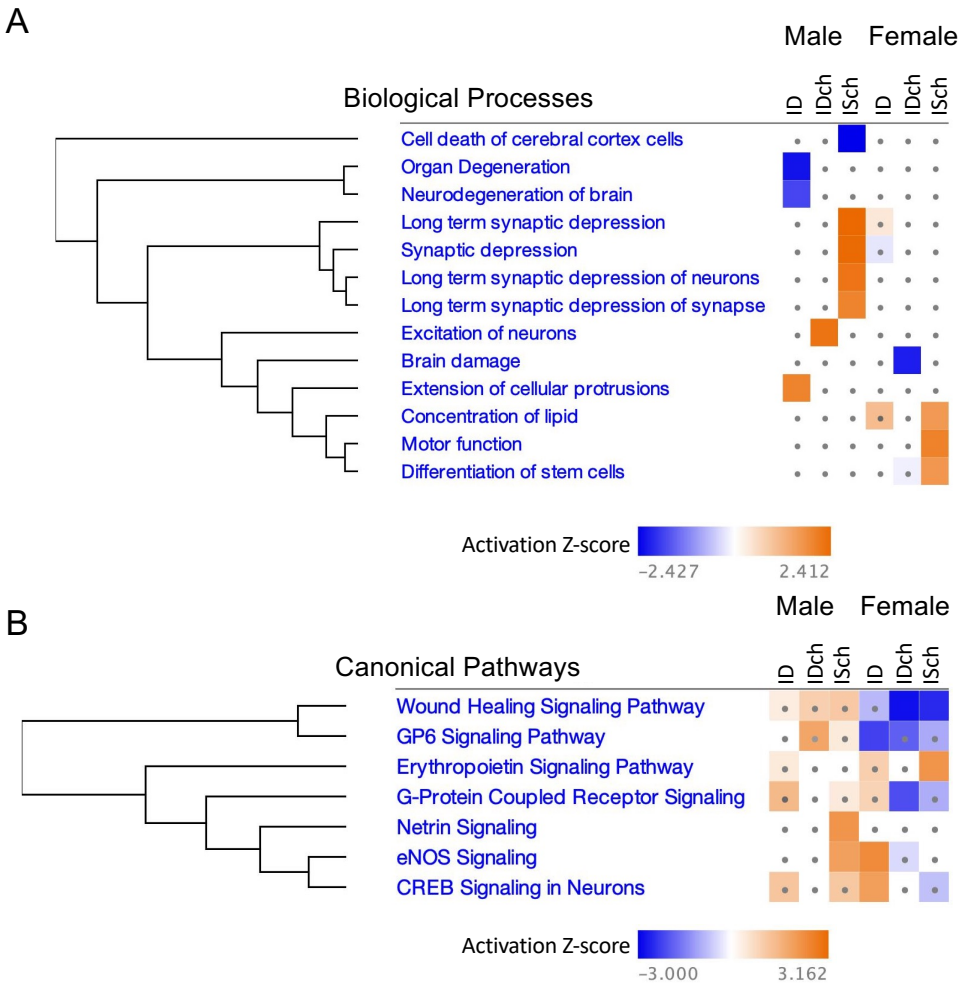


IPA-Predicted Disease State	
Activation	Inhibition
Emotional behavior, Movement Disorders, Analgesia	Progressive Encephalopathy, Cognition, Learning, Vasculogenesis

B. ISch/IS Female



IPA-Predicted Disease State	
Activation	Inhibition
Anxiety, Learning, Memory,	Seizures, Movement Disorders, Progressive neuropathy


A
Male
Female
Biological Processes
ID
IDch
ISch
ID
IDch
ISch
Cell death of cerebral cortex cells
Organ Degeneration
Neurodegeneration of brain
Long term synaptic depression
Synaptic depression
Long term synaptic depression of neurons
Long term synaptic depression of synapse
Excitation of neurons
Brain damage
Extension of cellular protrusions
Concentration of lipid
Motor function
Differentiation of stem cells
Activation Z-score
-2.427
2.412
B
Male
Female
Canonical Pathways
ID
IDch
ISch
ID
IDch
ISch
Wound Healing Signaling Pathway
GP6 Signaling Pathway
Erythropoietin Signaling Pathway
G-Protein Coupled Receptor Signaling
Netrin Signaling
eNOS Signaling
CREB Signaling in Neurons
Activation Z-score
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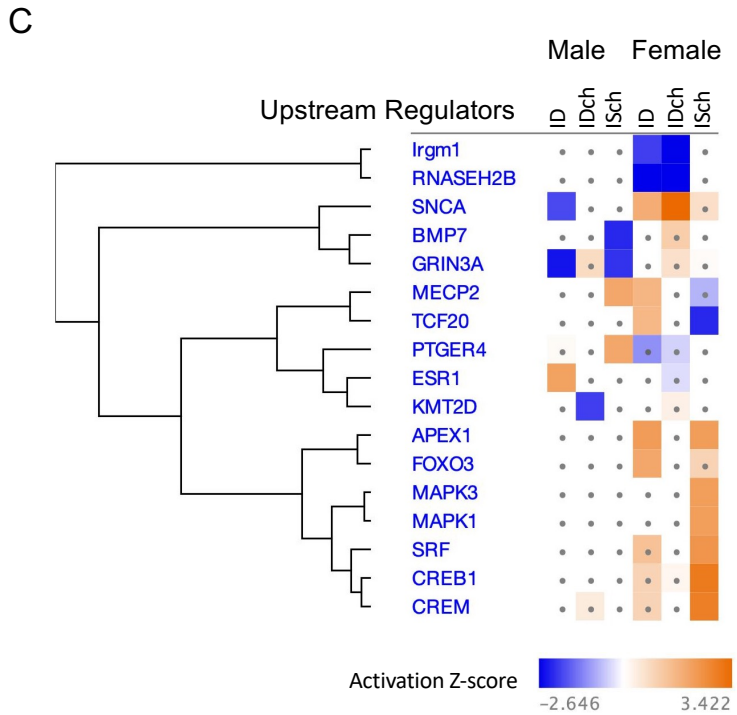
3.162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Male
Female
Upstream Regulators
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ID
IDch
ISch
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SNCA
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MECP2
TCF20
PTGER4
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CREM
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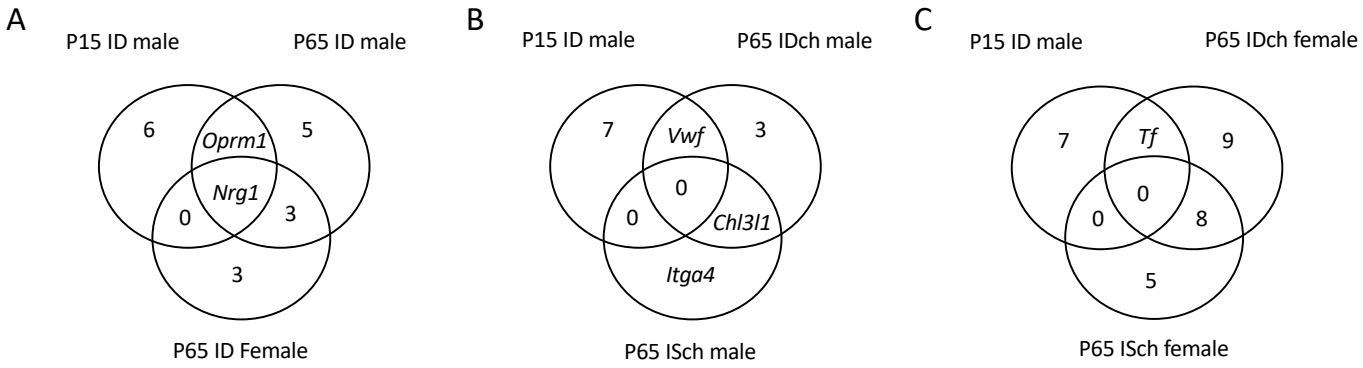
A
P15 ID male
P65 ID male
6
Oprm1
5
Nrg1
0
3
3
P65 ID Female
B
P15 ID male
P65 IDch male
7
Vwf
3
0
0
Chl3l1
Itga4
P65 ISch male
C
P15 ID male
P65 IDch female
7
Tf
9
0
0
8
5
P65 ISch female

Table 1: Potential biomarkers identified by IPA from rat hippocampal transcriptome. *Asterisk denotes factors overlapped with and ∂ denotes opposite expression changes from those of P15 ID male.

Group/Sex	***Entrez Gene Name***	***Symbol***	***log_2 (ratio)***	***p-value***	***Family***	***HC***	***Blood***	***CSF***	***Serum***	***Saliva***	***Urine***
ID male	Endothelin 1	*Edn1*	-1.78	0.002	Cytokine	x	x		x		
	Epidermal growth factor	*Egf*	-2.70	0.031	Growth factor	x	x		x		x
	Enolase 2	*Eno2*	-1.33	0.000	Enzyme	x	x		x		
	Integrin subunit alpha 4	*Itga4*	1.36	0.020	Transmembrane receptor	x	x		x		
	Leukotriene C4 synthase	*Ltc4s*	1.64	0.035	Enzyme	x	x				
	*∂Neuregulin 1	*Nrg1*	1.57	0.007	Growth factor	x	x				
	*∂Opioid receptor mu 1	*Oprm1*	1.79	0.025	G-protein coupled receptor	x	x		x		
	Protein tyrosine phosphatase receptor type C	*Ptprc*	1.44	0.018	Phosphatase	x	x				
	Transforming growth factor beta 1	*Tgfb1*	-1.33	0.025	Growth factor	x	x		x	x	
	Transthyretin	*Ttr*	55.69	0.000	Transporter	x	x	x	x		x
ID female	Actin alpha 2, smooth muscle	*Acta2*	-1.31	0.022	Other	x	x				
	CF transmembrane conductance regulator	*Cftr*	1.97	0.049	Ion channel	x	x		x		
	Endothelin 1	*Edn1*	1.59	0.025	Cytokine	x	x		x		
	Enolase 2	*Eno2*	3.70	0.001	Enzyme	x	x		x		
	*∂Neuregulin 1	*Nrg1*	1.42	0.041	Growth factor	x	x				
	Proopiomelanocortin	*Pomc*	-24.54	0.000	Other	x	x		x		x
	Transthyretin	*Ttr*	1.57	0.040	Transporter	x	x	x	x		x
IDch male	Chitinase 3 like 1	*Chi3l1*	-1.26	0.003	Enzyme	x	x		x		x
	Epidermal growth factor	*Egf*	-2.89	0.037	Growth factor	x	x		x		x
	Enolase 2	*Eno2*	-1.42	0.000	Enzyme	x	x		x		
	Toll like receptor 2	*Tlr2*	-1.38	0.044	Transmembrane receptor	x	x		x	x	
	*Von Willebrand factor	*Vwf*	1.29	0.001	Other	x	x		x		x

IDch female	ABL proto-oncogene 1, non-receptor tyrosine kinase	*Abl1*	4.31	0.007	Kinase	x	x				
	Angiotensin I converting enzyme	*Ace*	-1.28	0.015	Peptidase	x	x	x	x		x
	Apolipoprotein A1	*Apoa1*	2.85	0.012	Transporter	x	x	x	x		x
	Brain derived neurotrophic factor	*Bdnf*	1.40	0.000	Growth factor	x	x		x		
	Complement C3	*C3*	-1.39	0.013	Peptidase	x	x	x	x		x
	Corticotropin releasing hormone	*Crh*	1.76	0.004	Cytokine	x	x	x	x		
	Dual specificity phosphatase 1	*Dusp1*	-1.34	0.033	Phosphatase	x	x				
	Endothelin 1	*Edn1*	-1.89	0.009	Cytokine	x	x		x		
	Enolase 2	*Eno2*	-3.80	0.004	Enzyme	x	x		x		
	Heparin binding EGF like growth factor	*Hbegf*	1.47	0.010	Growth factor	x	x				x
	Insulin like growth factor binding protein 3	*Igfbp3*	1.29	0.028	Other	x	x		x		x
	Leukotriene C4 synthase	*Ltc4s*	1.67	0.009	Enzyme	x	x				
	Matrix metallopeptidase 2	*Mmp2*	-1.32	0.024	Peptidase	x	x		x		x
	Neuropeptide Y	*Npy*	2.22	0.044	Other	x	x	x	x		
	Neurotrophin 3	*Ntf3*	1.31	0.003	Growth factor	x		x			
	Proopiomelanocortin	*Pomc*	-32.06	0.000	Other	x	x		x		x
	*Transferrin	*Tf*	1.25	0.000	Transporter	x	x	x	x		x
	Transthyretin	*Ttr*	-2.06	0.000	Transporter	x	x	x	x		x
ISch male	Chitinase 3 like 1	*Chi3l1*	-1.32	0.000	Enzyme	x	x		x		x
	Integrin subunit alpha 4	*Itga4*	1.36	0.012	Transmembrane receptor	x	x		x		
ISch female	Brain derived neurotrophic factor	*Bdnf*	1.56	0.000	Growth factor	x	x		x		
	CD4 molecule	*Cd4*	-1.37	0.040	Transmembrane receptor	x	x		x		
	Cyclin dependent kinase inhibitor 1A	*Cdkn1a*	1.46	0.003	Kinase	x	x		x		
	Endothelin 1	*Edn1*	-2.20	0.004	Cytokine	x	x		x		

	Enolase 2	*Eno2*	-3.67	0.006	Enzyme	x	x		x		
	Heparin binding EGF like growth factor	*Hbegf*	1.54	0.000	Growth factor	x	x				x
	Hepatocyte growth factor	*Hgf*	-1.47	0.034	Growth factor	x	x		x		
	Interleukin 18	*Il18*	-1.49	0.026	Cytokine	x	x	x	x		
	Leukotriene C4 synthase	*Ltc4s*	2.24	0.000	Enzyme	x	x				
	Matrix metallopeptidase 2	*Mmp2*	-1.34	0.029	Peptidase	x	x		x		x
	Neurotrophin 3	*Ntf3*	1.25	0.013	Growth factor	x		x			
	Proopiomelanocortin	*Pomc*	-31.82	0.000	Other	x	x		x		x
	Protein tyrosine phosphatase receptor type C	*Ptprc*	-1.75	0.001	Phosphatase	x	x				