

Profiling Basal Forebrain Cholinergic Neurons Reveals a Molecular Basis for Vulnerability Within the Ts65Dn Model of Down Syndrome and Alzheimer's Disease

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Abstract

Background: Basal forebrain cholinergic neuron (BFCN) degeneration is a hallmark of Down syndrome (DS) and Alzheimer's disease (AD). Current therapeutics have been unsuccessful in slowing disease progression, likely due to complex pathological interactions and dysregulated pathways that are poorly understood. The Ts65Dn trisomic mouse model recapitulates both cognitive and morphological deficits of DS and AD, including BFCN degeneration.

Methods: We utilized Ts65Dn mice to understand mechanisms underlying BFCN degeneration to identify novel targets for therapeutic intervention. We performed high-throughput, single population RNA sequencing (RNA-seq) to interrogate transcriptomic changes within medial septal nucleus (MSN) BFCNs, using laser capture microdissection to individually isolate ~500 choline acetyltransferase-immunopositive neurons in Ts65Dn and normal disomic (2N) mice at 6 months of age (MO).

Results: Ts65Dn mice had unique MSN BFCNs transcriptomic profiles at ~6 MO clearly differentiating them from 2N mice. Leveraging Ingenuity Pathway Analysis and KEGG analysis, we linked differentially expressed gene (DEG) changes within MSN BFCNs to several canonical pathways and aberrant physiological functions. The dysregulated transcriptomic profile of trisomic BFCNs provides key information underscoring selective vulnerability within the septohippocampal circuit.

Conclusions: We propose both expected and novel therapeutic targets for DS and AD, including specific DEGs within cholinergic, glutamate, GABAergic, and neurotrophin pathways, as well as select targets for repairing oxidative phosphorylation status in neurons. We demonstrate and validate an interrogative quantitative bioinformatic analysis of a key dysregulated neuronal population linking single population transcript changes to an established pathological hallmark associated with cognitive decline for therapeutic development in human DS and AD.

Background

Down syndrome (DS) is caused by triplication of human chromosome 21 (HSA21), observed in ~ 1 of 700 births (1). Estimates indicate > 250,000 persons with DS live in the USA (2). DS is the primary genetic cause of intellectual disability and results in a number of neurological conditions, including deficits in learning and memory (3–8). Individuals with DS also show age-dependent neurodegeneration early in mid-life associated with Alzheimer's disease (AD), including amyloid plaques, neurofibrillary tangles, and dementia in ~ 70% of individuals (9–15). In both AD and DS, age-related cognitive decline is associated with degeneration of the cholinergic basal forebrain system, including loss of cholinergic basal forebrain neurons in the nucleus basalis and cholinergic fiber projections to the hippocampus and neocortex (4, 16–18).

The Ts65Dn mouse model recapitulates many human DS neuropathological endophenotypes including AD-like hippocampal-dependent learning and memory deficits, basal forebrain cholinergic neuron (BFCN) degeneration and septohippocampal circuit dysfunction, notably CA1 pyramidal neuron and choline

acetyltransferase (ChAT) activity deficits (9, 19–23). While cognitive decline in DS is associated with degeneration of the BFCN projection system, the medial septal/ventral diagonal band, which projects to the hippocampus, is critical for memory (22–29). Degeneration in this projection system is a cardinal feature of the Ts65Dn mouse (21, 23, 26, 30–35). BFCN changes begin approximately at 6 months of age (MO) (21, 26, 27, 36). BFCN loss and changes in hippocampal innervation are consistently reported in Ts65Dn mice > 10 MO (26, 27, 29, 37, 38). Expression level changes in trisomic mice have been limited to regional analysis (37, 39) or specific neuronal subtype assessment by microarrays (35, 40–44).

Prior to RNA sequencing (RNA-seq) technologies, researchers queried RNA expression levels using qPCR, microarray analysis, and chip-based technologies (45–53). While these methods show high overlap with RNA-seq results (54), they have drawbacks, including the number of genes queried, cost effectiveness, input quantity, and sequence specificity (55, 56). RNA-seq has several key benefits, including identification of noncoding RNA (ncRNA) species, sequence variations, including single-nucleotide polymorphisms and highly homologous sequences, with virtually no background (57). Additionally, cost versus sequencing depth has been greatly reduced. RNA-seq advanced concomitantly with the ability to isolate individual cells through laser capture microdissection (LCM), microfluidics, and flow cytometry (58–61).

This study employs LCM and single population RNA-seq to profile vulnerable BFCNs, an approach that has decided advantages over regional-based tactics. We postulate by isolating vulnerable ChAT-immunopositive BFCNs from heterogenous medial septal nucleus (MSN) populations and identifying dysregulated genes therein, we will identify cell-type specific therapeutic targets that may help slow or stop the progression of BFCN degeneration associated with AD and DS. We hypothesize differentially expressed gene (DEG) changes are not solely due to the triplicated 'DS gene region' and involve novel pathways and targets that have not been previously identified or considered causative of BFCN dysfunction.

Methods

Mice: Animal protocols were approved by the Nathan Kline Institute/NYU School of Medicine IACUC in accordance with NIH guidelines. Breeder pairs (female Ts65Dn and male C57Bl/6J Eicher x C3H/HeSnJ F1 mice) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and mated at the Nathan Kline Institute. Mice were kept on a normal chow diet with *ad libitum* water access (42, 43). Mice were genotyped (62) at weaning and aged to ~ 6 MO.

Tissue Preparation: Brain tissues were accessed from Ts65Dn (Ts; n = 6) and normal disomic (2N; n = 6) male mice (age range: 5.8–6.4 MO, mean age 6.0 MO). Mice were perfused transcardially as previously described (40, 42–44). Brains were immediately flash frozen and 20 µm-thick tissue sections were cryostat cut in the coronal plane (CM1860UV, Leica, Buffalo Grove, IL, USA) and mounted on polyethylene naphthalate (PEN) membrane slides (Leica) (Fig. 1B). Slides were kept under desiccant at -80 °C until

used for immunohistochemistry. RNase-free precautions were employed, and solutions were made with 18.2 mega Ohm RNase-free water (Nanopure Diamond, Barnstead, Dubuque, IA, USA).

Immunohistochemistry: PEN membrane were equilibrated to room temperature (RT) under desiccant (-20 °C for 5 min, 4 °C for 10 min, RT for 5 min) prior to staining (Fig. 1B-C). A quick staining protocol (< 1 h) was utilized for RNA preservation in unfixed tissue. Slides were rinsed in phosphate-buffered saline (PBS, pH 7.4), blocked for 3 min in normal horse serum. Primary antibody against ChAT (AB144P, Millipore; 1:50 dilution) in normal horse serum with 20 U/ml of Suprase-In RNase inhibitor (Ambion, ThermoFisher Scientific, Waltham, MA, USA) was incubated for 25 min at RT, with 3x PBS washes after each subsequent step. Secondary antibody (ImmPRESS Polymer Reagent, Vector Labs, Burlingame, CA, USA) was incubated on slides for 20 min, and incubated for 3–5 min in peroxidase substrate solution (ImmPACT NovaRED, Vector Labs). Slides were air-dried prior to LCM or re-frozen at -80 °C under desiccant.

LCM: LMD7000 (Leica) was employed to identify individual ChAT-immunoreactive MSN BFCNs using a 40x objective (PL-Fluotar NA 0.60) and positive neurons were outlined using the draw/cut tool (Leica LMD version 8.0). Identified cells dropped by gravity into 50 µl Qiazol solution (Qiagen, Germantown, MD, USA) (Fig. 1C-D). Captured cells were counted and lysed cells were frozen until ~ 500 cells/brain/region were isolated. On average 17 sections were needed to collect ~ 500 ChAT-immunoreactive BFCNs.

RNA purification: RNA from ~ 500 BFCNs was purified using miRNeasy Micro kit (Qiagen) according to manufacturers' specifications. A DNase digestion was performed twice sequentially, before the final washes and RNA purification (Fig. 1E). RNA quality control (QC) was performed (RNA 6000 pico kit, Agilent, Santa Clara, CA, USA; Fig. 1F).

Library Preparation and RNA-seq: The SMARTer Stranded Total RNA-Seq Kit (Takara Bio, Mountain View, CA, USA; Fig. 1G) was employed with minor modifications to utilize full volume of RNA. Samples were quantified (Agilent 2100 HS DNA kit; Fig. 1H), and samples below 2 nM of library were excluded. Samples were pooled in equimolar concentrations and assayed on an Illumina HiSeq-4000 (San Diego, CA, USA) using a single read 50 cycle protocol at the Genome Technology Center, NYU School of Medicine.

Bioinformatics: FastQ files were generated and QC of the raw reads were performed by Fastqc version 0.11.8 (110). Read trimming was performed as necessary by Trimmomatic 0.39 (111). If QC passed and showed no adapter contamination, this step was skipped. Sequence reads were aligned to the reference genome (Gencode GRCm38-mm10) using STAR Aligner (2.7.0) (112). QC was performed on alignments using Rseqc (v3.0.0) and Picard (2.20.03) (113). Pseudo alignment and read quantification was performed by Kallisto version 0.44.0 (63) using mouse reference genome (Gencode GRCm38-mm10).

Statistical Analysis: Sample by gene count matrix obtained from Kallisto were further normalized using voom transformation (63, 64). Genes with at least 2 counts per million (CPM) in 50% of samples were employed for downstream analysis. The normalized gene expression matrix was used to select known covariates. RNA concentration was used as a covariate and quality weights were obtained. The weighted

multiple linear model was fit for each gene and contrasts were computed with Limma (65). Gene expression differences at ($p < 0.05$) were considered statistically significant. Protein coding genes were extracted using the R Bioconductor package AnnotationDbi (66). Multiple testing corrections were performed by false discovery rate (FDR) (67).

Pathway Analysis: Pathway analyses included Ingenuity Pathway Analysis (IPA; Qiagen) (68, 69), Kyoto Encyclopedia of Genes and Genomes (KEGG) (70), and STRING (71) in Cytoscape (cutoff 0.4) (72).

qPCR validation: MSN neurons were isolated from tissue sections after Nissl staining {0.1% thionin in sodium acetate (49.44 mM)/ acetic acid (3.6 mM) buffer}. LCM was performed on Nissl-positive MSN neurons based on morphology, enriching for BFCNs. Approximately 200 neurons/sample were collected and RNA was processed (TaqMan Gene Expression Cell to Ct kit, ThermoFisher). qPCR was performed utilizing 2 μ l cDNA, from 50 μ l reaction with 22.5 μ l input RNA. Taqman qPCR primers (Life Technologies, Grand Island, NY, USA) were employed (Supplemental Table 1) to assay samples in triplicate on a real-time qPCR cycycler (PikoReal, ThermoFisher). The ddCT method was used to determine relative gene level differences between groups (73–75). Glucuronidase Beta (*GusB*) qPCR products were utilized as a control, as *GusB* did not show significant changes in RNA-seq data obtained from BFCNs. Negative controls consisted of the reaction mixture without input RNA. The two study groups (Ts and 2N) were compared with respect to PCR product synthesis for each gene tested. qPCR log-fold change (LFC) were scored without significance measures.

Results

MSN BFCN single population RNA-seq

RNA-seq was performed on BFCNs from Ts65Dn (Ts) and 2N mice (Fig. 1 and Supplemental Table 2). RNA-seq reads from BFCNs were mapped and normalization and covariate analyses were performed (Fig. 2 and Supplemental Fig. 1). Principal component analysis (PCA) revealed minimal variability in 2N BFCNs while greater variability was observed in Ts BFCNs (Fig. 3A). This likely reflects the onset of BFCN degeneration and indicates BFCN degeneration may have an epigenetic component. Differential expression analysis revealed 1,443 of 13,523 genes were differentially expressed at $p < 0.05$, with 22 genes at FDR < 0.05 . Analysis revealed 84.54% of DEGs were protein coding. The remaining DEGs were ncRNAs, pseudogenes, and microRNAs (miRNAs) (Fig. 3B). Heatmaps and volcano plots show individual genes differentially regulated in Ts BFCNs by LFC in individual samples and mean LFC and p-value (Fig. 3C, D). DEGs exhibited both upregulated (777) and downregulated (666) gene expression level changes in Ts BFCNs with LFCs ranging from 0.32 to 10.25 (Fig. 3E and Supplemental Table 3). Select DEGs are represented by violin plots across genotypes (Fig. 3F).

DEGs linked to DS

We queried DS murine orthologs triplicated in the Ts mouse model (62, 76), and 64 protein coding trisomic genes were expressed in MSN BFCNs. Ten genes were differentially expressed, with 8

upregulated and 2 downregulated (Fig. 4). Due to the low number of expression differences, we hypothesize BFCN degeneration likely involves additional factors than simply triplicated gene expression. Accordingly, DEGs were analyzed utilizing pathway analysis.

IPA and KEGG analysis reveal novel pathways providing a molecular basis for vulnerability of trisomic BFCNs

Molecular pathways were examined that individual DEGs belong to by IPA and KEGG analysis (Figs. 5, 6). IPA analysis revealed 114 of the 161 significant pathways ($-\log(p\text{-value}) \geq 1.3$) were neuronal-relevant and dysregulated in Ts BFCNs (Table 1). We curated 20 pathways for in-depth analysis at the start of cholinergic degeneration (Fig. 5A). Several critical pathways were upregulated in trisomic BFCNs, including Glutamate Receptor Signaling (Fig. 5B), Synaptic Long-Term Potentiation (Fig. 5C), CREB Signaling in Neurons (Fig. 5D), and Calcium Signaling (not shown). Downregulated pathways were also identified, including Superpathway of Cholesterol Biosynthesis (not shown), Oxidative Phosphorylation (Fig. 5E), and Integrin Signaling (not shown). Not surprisingly, several pathways showed redundancy in specific gene expression, including the Glutamate Receptor Signaling, CREB Signaling in Neurons and Long-Term Potentiation pathway genes.

KEGG analysis enabled a second independent bioinformatics approach. KEGG analysis identified 41 neuronal pathways (Table 2). We curated critical key pathways (Fig. 6A) including dysregulation in the cholinergic synapse (Fig. 6B). Interestingly, phospholipase C -beta 2 (*Plcb2*) and protein kinase C gamma (*Prkcg*) were both upregulated and are positively linked to calcium signaling (identified as dysregulated by IPA; Table 1), while muscarinic receptor 2 (*Chrm2*) and G protein subunit beta5 (*Gnb5*) were downregulated and are involved in cell survival (Fig. 6B). The Alzheimer's Disease pathway was novel to KEGG analysis (Fig. 6C) and identified several key DEGs disrupted within trisomic BFCNs. The GABA receptor signaling pathway (Fig. 6D) was identified by both IPA and KEGG analysis. KEGG also identified the neurotrophin signaling pathway (Fig. 6E) as significantly dysregulated. IPA and KEGG revealed overlapping and differing differentially regulated genes within many of these pathways (Table 1 and Table 2).

Transcript Analysis

Comparing DEGs and transcript expression (TEG) ($p < 0.05$), over half of identified transcripts were also significantly affected at the gene level (Supplemental Fig. 2A). IPA analysis revealed 17 of the top 20 pathways identified by DEG IPA were also significant by TEG IPA (Supplemental Fig. 2B). DEG and TEG overlap was found for the majority of genes within discrete pathways (Supplemental Fig. 2C-F), with some pathways showing notable differences. In TEGs, pathway analysis revealed $-\log(p\text{-values})$ increased for many selected pathways, but the z-score indicating directionality of pathway changes tended to decrease (Supplemental Table 4). This effectively resulted in transcript analysis detracting from effect sizes, which is likely due to identified TEGs with low expressing isoforms in neurons. Therefore, we pursued validation using different methodological approaches.

Weighted gene co-expression network analysis (WGCNA)

We utilized a WGCNA approach to analyze the association of module expression (based on module eigengene) within BFCNs. The 13,523 genes expressed in the BFCN network were subdivided into 19 modules ranging from 78 to 2,988 genes. Analysis revealed 2 modules with differential gene expression by genotype (FDR < 0.05; Fig. 7A). The Blue module contains 2,124 genes, with ~ 68% of the genes upregulated in Ts BFCNs (Fig. 7B). The Black module contains 701 genes with ~ 66% of these upregulated in Ts BFCNs (Fig. 7B). STRING network analysis in Cytoscape was performed to determine physical interactions of encoded proteins within each module. Of the 2,124 genes in the Blue module, STRING analysis identified 1,721 proteins with 2,999 interactions (Supplemental Fig. 3A), with insets highlighting close interactions in detail (Supplemental Fig. 3B-E). To query the most relevant genes, the top 500 significant gene hits in the Blue module were analyzed by STRING, with 428 identified proteins and 614 connections (Fig. 7C). Top interactions were discs large MAGUK scaffold protein 4 (*Dlg4*; also known as PSD-95), glutamate receptor, ionotropic, AMPA1 (*Gria1*), syntaxin 1A (*Stx1a*), adenylylate cyclase 1 (*Adcy1*), and mitogen-activated protein kinase 3 (*Mapk3*). All 701 genes in the Black module were queried by STRING analysis, of which 586 proteins with 1,043 connections were observed (Fig. 7D). The top interactions in the Black module were block of proliferation 1 ribosomal biogenesis factor (*Bop1*), proliferation-associated 2G4 (*Pa2g4*), bystin like (*Bysl*), DEAD-box helicase 5 (*Ddx5*), and eukaryotic translation initiation factor 5B (*Eif5b*).

To compare the two methodologies (DEG and WGCNA) for functional pathway analysis, we performed IPA analysis on WGCNA Blue and Black modules. A total of 109 significantly affected neuronal pathways were found in the Blue module, of which 58 overlapped with DEG significant pathways (Fig. 8A). A total of 29 of 40 significantly affected pathways in the Black module replicated the DEG analysis (Fig. 8B). Therefore, the majority of significant pathways by DEG analysis are also significant by WGCNA analysis, confirming these methodologies. Importantly, 12 of the top 15 interesting pathways in the Blue module were also in the top 20 of the DEG IPA analysis (Fig. 8C, Supplemental Table 5), while in the Black module 4 of the top 10 were also in the top 20 of the DEG IPA analysis (Fig. 8D, Supplemental Table 6). Collectively, module data also revealed several significant pathways, including G beta gamma signaling and Neurotrophin/Trk Signaling for the Blue module (Fig. 8E, Supplemental Table 5) and focal adhesion kinase (FAK), Notch, and p53 signaling in the Black module (Fig. 8F, Supplemental Table 6).

qPCR Validation

qPCR results showed positive correlations with several dysregulated genes by RNA-seq ($R = 0.67$, $p = 0.024$; Fig. 9A). *Chrm2* and *Mapk8* were significantly downregulated by RNA-seq and correlated with qPCR results while *Prkcg*, *Grin2a*, and *Camk2a* were significantly upregulated by RNA-seq and correlated with qPCR results (Fig. 9A, B). In addition, phospholipase C beta 1 (*Plcb1*), nerve growth factor receptor (*Ngfr/p75^{NTR}*), and *Chrm1* were within dysregulated pathways identified by IPA or KEGG analysis, and while not significantly dysregulated by RNA-seq, they correlated with RNA-seq and qPCR (Fig. 9A). In contrast, kinase D interacting substrate 220 (*Kidins220/Arms*) and neprilysin (*Mme*) were not

differentially regulated by RNA-seq and did not correlate with qPCR findings (Fig. 9A). *Mapk3* was the only gene queried by qPCR which did not correlate to significant changes in RNA-seq findings (Fig. 9A, B), likely due to a combination gene change selectivity within BFCNs and low expression levels of this gene.

Discussion

We generated an expression profile of vulnerable MSN BFCNs in 6 MO trisomic mice without the confounding transcriptomic signal of glial or other neuronal populations. Previous studies identified BFCN degeneration in Ts65Dn mice at 6 MO or older (21, 24, 26, 38, 77). Therefore, our results suggest transcriptomic changes seen herein precede or pace subsequent neuronal degeneration, a key finding. Not surprisingly, the profile of trisomic BFCN degeneration is more complex than simply the triplication of the 'DS critical region', for which we provide pathway analysis from numerous genes expressed on non-triplicated chromosomes. Importantly, the present DEG and WGCNA bioinformatics platforms analyzed by IPA and KEGG highlight the critical need for multiple bioinformatics approaches to reveal the mechanistic potential of analyzing single population RNA-seq datasets within vulnerable cell types.

Dysregulated genes in the oxidative phosphorylation pathway are also implicated in AD pathology (78). These genes include mitochondrially encoded NADH dehydrogenases (*Mt-Nd1*, *Mt-Nd2*, *Mt-Nd 4*, and *Mt-Nd5*), along with NADH:ubiquinone oxidoreductase subunits (*Ndufa6*, *Ndufab1*, *Ndufb2*, *Ndufb4*, *Ndufs1*, *Ndufs2*, *Ndufs4*, *Ndusf7*, and *Ndufs8*) (Fig. 5E). Results indicate energy metabolism is strongly disrupted, identifying a direct mechanistic link previously postulated between oxidative stress (24) and neurodegeneration in DS/AD, and may serve as novel therapeutic target candidates to deter BFCN degeneration.

Trisomic MSN BFCNs display upregulation of select glutamate receptor transcripts (Fig. 5B), similar to findings within the hippocampus (19, 42, 43, 79–81), contributing to disruption of long-term potentiation (LTP) and long-term depression (LTD) in Ts65Dn mice (82–85). We also observed downregulation of genes involved in GABAergic neurotransmission. Paradoxically, the majority of hippocampal studies in Ts65Dn mice report upregulation of several GABA_A receptor subunits and increased inhibition of LTP (80, 82, 83, 86–88). Discrepancies in gene expression point to the key differences between our single population approach in BFCNs relative to mixed hippocampal population analyses.

CREB signaling, along with many downstream effectors, is also dysregulated in trisomic BFCNs (Fig. 5D). Increases in CREB signaling and *Mapk3* have been found after stimulation by brain derived neurotrophin factor (*Bdnf*) in the Ts65Dn model (89). We found increased CREB signaling and upregulation of several calcium channel and glutamate receptor subunits as well as downstream calcium calmodulin dependent kinases and phospholipase C isoforms. Further, while downstream Creb pathway changes follow *Bdnf* stimulation, changes in neurotrophin receptor signaling does not include neurotrophin receptor or *Bdnf* DEG changes. Rather, significant alterations in the neurotrophin pathway include several downstream key regulators of apoptosis, LTP, and cell survival. Interestingly, TrkA (*Ntrk1*) gene expression is not significantly different between genotypes but is a key gene in the Blue module with a median 1.7 fold

decrease in expression in trisomic MSN BFCNs. Downregulation of TrkA expression has been observed within human postmortem cholinergic basal forebrain neurons during AD progression and correlates with cognitive decline and Braak stage (17, 90–97). We also demonstrate downregulation of several downstream effector genes of the NGF-TrkA pathway crucial for BFCN survival including phosphatidylinositol 3-kinase catalytic subunit (*Pik3ca*), v-rel reticuloendotheliosis viral oncogene homolog A (*Rela*), and calmodulin 3 (*Calm3*) in trisomic BFCNs, indicating the relevance of this pathway in this established AD and DS model.

We identified vulnerabilities by KEGG within the cholinergic synapse, further indicating this target for therapeutic intervention. Notably, *Chrm2*, encoding the muscarinic M2 cholinergic receptor and primarily localized to ChAT-positive cholinergic neurons (98, 99), was downregulated along with downstream effectors *Gnb5*, which leads to downregulation of *Mapk3*, causing dampening of synaptic plasticity (100). In addition, presynaptic choline transferase is downregulated, along with *Chrm2* and *Gnb5* which block choline uptake and calcium to the presynaptic terminal (101). Conversely, *Plcb2* and *Prkcg*, downstream of the muscarinic M1 cholinergic receptor, are upregulated, likely driving the increases seen in the calcium signaling pathway (102, 103). These novel findings in vulnerable BFCNs have translational implications, as muscarinic cholinergic receptors have relatively limited expression throughout the forebrain, and represent realistic candidates to slow or stop the BFCN degeneration that is seen in both DS and AD.

KEGG analysis also revealed significant dysregulation of genes involved in AD pathogenesis including apolipoprotein E (*ApoE*), calpain 1 (*Capn1*), nitric oxide synthase 1 (*Nos1*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) (104, 105). Interestingly, there is a small but significant decrease in ApoE expression in Ts BFCNs. The ApoE e4 isoform is known to drive AD pathology (106). Low plasma levels of ApoE were observed in subjects with severe dementia, correlating with cognitive decline (107). Increases in Capn1 activation have previously been linked to AD pathology (108, 109). Increased active Capn1 results in increased cerebrospinal fluid levels of neurogranin in AD patients (110), while inhibition results in improved cholinergic function in rats (111). Capn1 activation is also linked to type II diabetes mellitus, and pathways linked to diabetes and metabolic syndrome are dysregulated in Ts BFCNs (108). *Nos1*, upregulated in Ts BFCNs, also shows increased expression in AD (112, 113) and GWAS studies implicate aberrant *Nos1* expression in AD (114). Reduction in *Gapdh* activity has also been shown in AD brain due to oxidative modification (115). A component of AD dysfunction involves deficits in mitochondrial activity and oxidative phosphorylation, paralleling the present findings. In Ts BFCNs, the ATP synthase H⁺ transporting mitochondrial F1 complex, alpha subunit 1 (*Atp5a1*), cytochrome c oxidase subunit 4I1 (*Cox4i1*) and *Ndufs4* were dysregulated (Fig. 6C), as well as numerous genes within the oxidative phosphorylation pathway (Fig. 5E). Moreover, several Alzheimer's Disease pathway dysregulated genes within Ts BFCNs replicate key findings in a human AD study examining miRNA and RNA expression profiling from the GEO database (116), indicating trisomic BFCN degeneration is likely caused by early AD-relevant gene expression changes.

Combined WGCNA and STRING analysis indicate *Dlg4* (PSD-95) has direct interactions with numerous significantly dysregulated genes and pathways, including glutamate receptor signaling, synaptogenesis signaling, and neurotrophin signaling. These results suggest that *Dlg4* may be a hub in the degenerative pathways of BFCNs. Changes in *Dlg4* have been linked to early synapse loss in AD mouse models and human postmortem AD studies (116, 117). WGCNA analysis also shows significant pathway dysregulation in amyloid processing, calcium transport, FAK, and Notch signaling in trisomic BFCNs. Importantly, these individual genes and key pathways would not have been identified without individual cell population RNA-seq, as the relatively low abundance and very specific expression patterns would have likely been masked in admixed cell type or regional RNA-seq analysis.

Bioinformatic pathway analysis of trisomic BFCNs shows significant changes in many relevant pathways including excitatory and inhibitory neurotransmission, resulting in LTP and LTD changes, synaptic plasticity, along with changes in oxidative states and mitochondrial dysfunction. These results point towards mechanisms underlying BFCN degeneration which have direct translational implications for both DS and AD pathobiology and therapeutic intervention. Limitations of the current work include variability in RNA quality. Genotype differences in RNA quality is unlikely, as previous qPCR studies from subregional dissections have not revealed genotype effects (40, 42–44), and RNA quantity was normalized as a covariate during analysis. This initial study was performed in male mice, and sex differences may exist in BFCN degenerative programs (22). A second cohort of trisomic female mice is currently in progress, although previous work from mixed sex studies have not revealed significant differences in select gene expression (40, 43, 44). Several mouse models recapitulate aspects of the human trisomic phenotype. These models have varying numbers of triplicated HSA21 orthologs, including Dp16/Dp17/Dp10 (168), Tc1 (125), Dp16 (102), Ts65Dn/Ts2 (90), and Ts1Cje (70) (76, 118–120). The Ts65Dn model is the most widely used, and is notable for septohippocampal degeneration and behavioral deficits that mimic DS and AD endophenotypes (119, 121–123). However, the Ts65Dn mouse model does not recapitulate the full pathobiology of DS or AD. Future assessments will consider evaluating BFCN degeneration from other models in relation to postmortem changes in cholinergic basal forebrain neurons from DS and AD brains.

Conclusions

We provide single population expression profiling of BFCNs at a key timepoint at the initiation of neurodegenerative programs to understand mechanisms driving AD pathogenesis utilizing a trisomic model. We uncovered select genes in key signaling pathways that likely underlie BFCN degeneration, which will help the field rationally design therapeutic strategies aimed at preserving the septohippocampal circuit without targeting ancillary neuronal and non-neuronal populations.

List Of Abbreviations

adenylate cyclase 1 (*Adcy1*)

Alzheimer's disease (AD)

apolipoprotein E (*ApoE*)

ATP synthase H + transporting mitochondrial F1 complex, alpha subunit 1 (*Atp5a1*)

ATP synthase H + transporting mitochondrial F1 complex, O subunit (*Atp5o*)

basal forebrain cholinergic neuron (BFCN)

block of proliferation 1 ribosomal biogenesis factor (*Bop1*)

brain derived neurotrophin factor (*Bdnf*)

bystin like (*Bysl*)

calcium/calmodulin dependent protein kinase II alpha (*Camk2A*)

calmodulin 3 (*Calm3*)

calpain 1 (*Capn1*)

choline acetyltransferase (ChAT)

counts per million (CPM)

cytochrome c oxidase subunit 4l1 (*Cox4i1*)

DEAD-box helicase 5 (*Ddx5*)

differentially expressed gene (DEG)

discs large MAGUK scaffold protein 4 (*Dlg4*; also known as *PSD-95*)

disomic (2N)

Down syndrome (DS)

dual specificity tyrosine phosphorylation regulated kinase 1A (*Dyrk1a*)

E26 avian leukemia oncogene 2,3' domain (*Ets2*)

eukaryotic translation initiation factor 5B (*Eif5b*)

false discovery rate (FDR)

focal adhesion kinase (FAK)

G protein subunit beta5 (*Gnb5*)

Glucuronidase Beta (*GusB*)

glutamate ionotropic receptor NDMA type subunit 2A (*Grin2A*)

glutamate receptor, ionotropic, AMPA1 (*Gria1*)

glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*)

human chromosome 21 (HSA21)

Ingenuity Pathway Analysis (IPA)

junction adhesion molecule 2 (*Jam2*)

kinase D interacting substrate 220 (*Kidins220/Arms*)

Kyoto Encyclopedia of Genes and Genomes (KEGG)

laser capture microdissection (LCM)

lebercilin congenital amaurosis 5-like (*Lca5l*)

log-fold change (LFC)

long-term depression (LTD)

long-term potentiation (LTP)

medial septal nucleus (MSN)

microRNAs (miRNAs)

mitogen activated protein kinase 8 (*Mapk8 aka Erk2*)

mitogen-activated protein kinase 3 (*Mapk3*)

months of age (MO)

muscarinic cholinergic receptor 1 (*Chrm1*)

muscarinic cholinergic receptor 2 (*Chrm2*)

N-6 adenine-specific DNA methyltransferase1 (*N6amt1*)

NADH dehydrogenases (*Mt-Nd1*, *Mt-Nd2*, *Mt-Nd 4*, and *Mt-Nd5*)

NADH:ubiquinone oxidoreductase subunits (*Ndufa6*, *Ndufab1*, *Ndufb2*, *Ndufb4*, *Ndufs1*, *Ndufs2*, *Ndufs4*, *Ndusf7*, and *Ndufs8*)

neprilysin (*Mme*)

nerve growth factor receptor (*Ngfr/p75NTR*)

nitric oxide synthase 1 (*Nos1*)

noncoding RNA (ncRNA)

phosphatidylinositol 3- kinase catalytic subunit (*Pik3ca*)

phospholipase C beta 1 (*Plcb1*)

phospholipase C -beta 2 (*Plcb2*)

polyethylene naphthalate (PEN)

Principal component analysis (PCA)

proliferation-associated 2G4 (*Pa2g4*)

protein kinase C gamma (*Prkcg*)

quality control (QC)

RNA sequencing (RNA-seq)

room temperature (RT)

SET domain containing 4 (*Setd4*)

Son DNA binding protein (*Son*)

syntaxin 1A (*Stx1a*)

T cell lymphoma invasion and metastasis 1 (*Tiam1*)

tetratricopeptide repeat domain 3 (*Ttc3*)

transcript expression (TEG)

TrkA (*Ntrk1*)

Ts65Dn (Ts)

v-rel reticuloendotheliosis viral oncogene homolog A (Rela)

Declarations

Ethics approval and consent to participate

Animal protocols were approved by the Nathan Kline Institute/NYU School of Medicine Animal Care and Use Committee (IACUC) in accordance with NIH guidelines. Human data and tissue are not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Data analyzed within this study are included in this body of the manuscript and within the supplementary information files. Data are also available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MJA, SCP, AH, PR, and SDG designed the experiments. MJA and SCP performed the experiments. MJA, SCP, PR, and SDG performed the statistical analysis. MJA and SDG wrote manuscript. All authors read and approved final manuscript.

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References

1. Parker SE, Mai CT, Canfield MA, Rickard R, Wang Y, Meyer RE, et al. Updated National Birth Prevalence estimates for selected birth defects in the United States, 2004–2006. *Birth Defects Res A Clin Mol Teratol.* 2010;88:1008–16.
2. Presson AP, Partyka G, Jensen KM, Devine OJ, Rasmussen SA, McCabe LL, et al. Current estimate of Down Syndrome population prevalence in the United States. *J Pediatr.* 2013;163(4):1163–8.

3. Mann DM, Yates PO, Marcyniuk B. Alzheimer's presenile dementia, senile dementia of Alzheimer type and Down's syndrome in middle age form an age related continuum of pathological changes. *Neuropathol Appl Neurobiol.* 1984;10:185–207.
4. Coyle JO-G, Reeves ML, Gearhart RH. JD. Down syndrome, Alzheimer's disease and the trisomy16 mouse. *Trends Neurosci.* 1988;11(9):390–4.
5. Hook EB. Issues pertaining to the impact and etiology of trisomy 21 and other aneuploidy in humans; a consideration of evolutionary implications, maternal age mechanisms, and other matters. *Prog Clin Biol Res.* 1989;311:1–27.
6. Hodgkins PS, Prasher V, Farrar G, Armstrong R, Sturman S, Corbett J, et al. Reduced transferrin binding in Down syndrome: a route to senile plaque formation and dementia. *Neuroreport.* 1993;5(1):21–4.
7. Roth GM, Sun B, Greensite FS, Lott IT, Dietrich RB. Premature aging in persons with Down syndrome: MR findings. *AJNR Am J Neuroradiol.* 1996;17(7):1283–9.
8. Chapman RS, Hesketh LJ. Behavioral phenotype of individuals with Down syndrome. *Ment Retard Dev Disabil Res Rev.* 2000;6:84–95.
9. Cataldo AM, Peterhoff CM, Troncoso JC, Gomez-Isla T, Hyman BT, Nixon RA. Endocytic pathway abnormalities precede amyloid beta deposition in sporadic Alzheimer's disease and Down syndrome: differential effects of APOE genotype and presenilin mutations. *Am J Pathol.* 2000;157:277–86.
10. Hartley D, Blumenthal T, Carrillo M, DiPaolo G, Esralew L, Gardiner K, et al. Down syndrome and Alzheimer's disease: Common pathways, common goals. *Alzheimers Dement.* 2015;11:700–9.
11. Hartley SL, Handen BL, Devenny DA, Hardison R, Mihaila I, Price JC, et al. Cognitive functioning in relation to brain amyloid-beta in healthy adults with Down syndrome. *Brain.* 2014;137:2556–63.
12. Leverenz JB, Raskind MA. Early amyloid deposition in the medial temporal lobe of young Down syndrome patients: a regional quantitative analysis. *Exp Neurol.* 1998;150:296–304.
13. Wisniewski KE, Dalton AJ, Crapper McLachlan DR, Wen GY, Wisniewski HM. Alzheimer's disease in Down's syndrome: clinicopathologic studies. *Neurology.* 1985;35:957–61.
14. Lai F, Williams RS. A prospective study of Alzheimer disease in Down syndrome. *Arch Neurol.* 1989;46(8):849–53.
15. Perez SE, Miguel JC, He B, Malek-Ahmadi M, Abrahamson EE, Ikonomic MD, et al. Frontal cortex and striatal cellular and molecular pathobiology in individuals with Down syndrome with and without dementia. *Acta Neuropathol.* 2019;137(3):413–36.
16. Sendera TJ, Ma SY, Jaffar S, Kozlowski PB, Kordower JH, Mawal Y, et al. Reduction in TrkA-immunoreactive neurons is not associated with an overexpression of galaninergic fibers within the nucleus basalis in Down's syndrome. *J Neurochem.* 2000;74(3):1185–96.
17. Mufson EJ, Bothwell M, Kordower JH. Loss of nerve growth factor receptor-containing neurons in Alzheimer's disease: a quantitative analysis across subregions of the basal forebrain. *Exp Neurol.* 1989;105:221–32.

18. Mann DM, Yates PO, Marcyniuk B, Ravindra CR. The topography of plaques and tangles in Down's syndrome patients of different ages. *Neuropathol App Neurobiol.* 1986;12:447–57.
19. Belichenko PV, Kleschevnikov AM, Masliah E, Wu C, Takimoto-Kimura R, Salehi A, et al. Excitatory-inhibitory relationship in the fascia dentata in the Ts65Dn mouse model of Down syndrome. *J Comp Neurol.* 2009;512:453–66.
20. Belichenko PV, Masliah E, Kleschevnikov AM, Villar AJ, Epstein CJ, Salehi A, et al. Synaptic structural abnormalities in the Ts65Dn mouse model of Down Syndrome. *J Comp Neurol.* 2004;480:281–98.
21. Granholm AC, Sanders LA, Crnic LS. Loss of cholinergic phenotype in basal forebrain coincides with cognitive decline in a mouse model of Down's syndrome. *Exp Neurol.* 2000;161:647–63.
22. Kelley CM, Powers BE, Velazquez R, Ash JA, Ginsberg SD, Strupp BJ, et al. Sex differences in the cholinergic basal forebrain in the Ts65Dn mouse model of Down syndrome and Alzheimer's disease. *Brain Pathol.* 2014;24:33–44.
23. Kelley CM, Powers BE, Velazquez R, Ash JA, Ginsberg SD, Strupp BJ, et al. Maternal choline supplementation differentially alters the basal forebrain cholinergic system of young-adult Ts65Dn and disomic mice. *J Comp Neurol.* 2014;522:1390–410.
24. Lockrow J, Prakasam A, Huang P, Bimonte-Nelson H, Sambamurti K, Granholm AC. Cholinergic degeneration and memory loss delayed by vitamin E in a Down syndrome mouse model. *Exp Neurol.* 2009;216(2):278–89.
25. Hunter CL, Bimonte-Nelson HA, Nelson M, Eckman CB, Granholm AC. Behavioral and neurobiological markers of Alzheimer's disease in Ts65Dn mice: effects of estrogen. *Neurobiol Aging.* 2004;25(7):873–84.
26. Holtzman DM, Santucci D, Kilbridge J, Chua-Couzens J, Fontana DJ, Daniels SE, et al. Developmental abnormalities and age-related neurodegeneration in a mouse model of Down syndrome. *Proc Natl Acad Sci USA.* 1996;93:13333–8.
27. Cooper JD, Salehi A, Delcroix JD, Howe CL, Belichenko PV, Chua-Couzens J, et al. Failed retrograde transport of NGF in a mouse model of Down's syndrome: reversal of cholinergic neurodegenerative phenotypes following NGF infusion. *Proc Natl Acad Sci U S A.* 2001;98(18):10439–44.
28. Powers BE, Kelley CM, Velazquez R, Ash JA, Strawderman MS, Alldred MJ, et al. Maternal choline supplementation in a mouse model of Down syndrome: Effects on attention and nucleus basalis/substantia innominata neuron morphology in adult offspring. *Neuroscience.* 2017;340:501–14.
29. Powers BE, Velazquez R, Kelley CM, Ash JA, Strawderman MS, Alldred MJ, et al. Attentional function and basal forebrain cholinergic neuron morphology during aging in the Ts65Dn mouse model of Down syndrome. *Brain Struct Funct.* 2016;221:4337–52.
30. Ash JA, Velazquez R, Kelley CM, Powers BE, Ginsberg SD, Mufson EJ, et al. Maternal choline supplementation improves spatial mapping and increases basal forebrain cholinergic neuron number and size in aged Ts65Dn mice. *Neurobiol Dis.* 2014;70:32–42.

31. Kelley CM, Ash JA, Powers BE, Velazquez R, Alldred MJ, Ikonomic MD, et al. Effects of maternal choline supplementation on the septohippocampal cholinergic system in the Ts65Dn mouse model of Down syndrome. *Curr Alzheimer Res.* 2016;13:84–96.
32. Strupp BJ, Powers BE, Velazquez R, Ash JA, Kelley CM, Alldred MJ, et al. Maternal choline supplementation: a potential prenatal treatment for Down syndrome and Alzheimer's disease. *Curr Alzheimer Res.* 2016;13:97–106.
33. Moon J, Chen M, Gandhi SU, Strawderman M, Levitsky DA, Maclean KN, et al. Perinatal choline supplementation improves cognitive functioning and emotion regulation in the Ts65Dn mouse model of Down syndrome. *Behav Neurosci.* 2010;124:346–61.
34. Hunter CL, Bimonte HA, Granholm A-CE. Behavioral comparison of 4 and 6 month-old Ts65Dn mice: Age-related impairments in working and reference memory. *Behav Brain Res.* 2003;138(2):121–31.
35. Kelley CM, Ginsberg SD, Alldred MJ, Strupp BJ, Mufson EJ. Maternal Choline Supplementation Alters Basal Forebrain Cholinergic Neuron Gene Expression in the Ts65Dn Mouse Model of Down Syndrome. *Dev Neurobiol.* 2019;79(7):664–83.
36. Cataldo AM, Petanceska S, Peterhoff CM, Terio NB, Epstein CJ, Villar A, et al. App gene dosage modulates endosomal abnormalities of Alzheimer's disease in a segmental trisomy 16 mouse model of Down syndrome. *J Neurosci.* 2003;23:6788–92.
37. Hunter CL, Isacson O, Nelson M, Bimonte-Nelson H, Seo H, Lin L, et al. Regional alterations in amyloid precursor protein and nerve growth factor across age in a mouse model of Down's syndrome. *Neurosci Res.* 2003;45(4):437–45.
38. Contestabile A, Fila T, Bartesaghi R, Ciani E. Choline acetyltransferase activity at different ages in brain of Ts65Dn mice, an animal model for Down's syndrome and related neurodegenerative diseases. *J Neurochem.* 2006;97(2):515–26.
39. Ahmed MM, Block A, Tong S, Davisson MT, Gardiner KJ. Age exacerbates abnormal protein expression in a mouse model of Down syndrome. *Neurobiol Aging.* 2017;57:120–32.
40. Alldred MJ, Chao HM, Lee SH, Beilin J, Powers BE, Petkova E, et al. CA1 pyramidal neuron gene expression mosaics in the Ts65Dn murine model of Down syndrome and Alzheimer's disease following maternal choline supplementation. *Hippocampus.* 2018;28(4):251–68.
41. Alldred MJ, Duff KE, Ginsberg SD. Microarray analysis of CA1 pyramidal neurons in a mouse model of tauopathy reveals progressive synaptic dysfunction. *Neurobiol Dis.* 2012;45:751–62.
42. Alldred MJ, Lee SH, Petkova E, Ginsberg SD. Expression profile analysis of vulnerable CA1 pyramidal neurons in young-middle-aged Ts65Dn mice. *J Comp Neurol.* 2015;523:61–74.
43. Alldred MJ, Lee SH, Petkova E, Ginsberg SD. Expression profile analysis of hippocampal CA1 pyramidal neurons in aged Ts65Dn mice, a model of Down syndrome (DS) and Alzheimer's disease (AD). *Brain Struct Funct.* 2015;220:2983–96.
44. Alldred MJ, Chao HM, Lee SH, Beilin J, Powers BE, Petkova E, et al. Long-term effects of maternal choline supplementation on CA1 pyramidal neuron gene expression in the Ts65Dn mouse model of Down syndrome and Alzheimer's disease. *Faseb j.* 2019;33(9):9871–84.

45. Eberwine J, Crino P, Dichter M. Single-cell mRNA amplification: implications for basic and clinical neuroscience. *The Neuroscientist*. 1995;1:200–11.
46. Eberwine J, Kacharina JE, Andrews C, Miyashiro K, McIntosh T, Becker K, et al. mRNA expression analysis of tissue sections and single cells. *J Neurosci*. 2001;21(21):8310–4.
47. Ginsberg SD, Crino PB, Hemby SE, Weingarten JA, Lee VM, Eberwine JH, et al. Predominance of neuronal mRNAs in individual Alzheimer's disease senile plaques. *Ann Neurol*. 1999;45:174–81.
48. Ginsberg SD, Elarova I, Ruben M, Tan F, Counts SE, Eberwine JH, et al. Single-cell gene expression analysis: implications for neurodegenerative and neuropsychiatric disorders. *Neurochem Res*. 2004;29:1053–64.
49. Baugh LR, Hill AA, Brown EL, Hunter CP. Quantitative analysis of mRNA amplification by in vitro transcription. *Nucleic Acids Res*. 2001;29(5):E29.
50. Colangelo V, Schurr J, Ball MJ, Pelaez RP, Bazan NG, Lukiw WJ. Gene expression profiling of 12633 genes in Alzheimer hippocampal CA1: transcription and neurotrophic factor down-regulation and up-regulation of apoptotic and pro-inflammatory signaling. *J Neurosci Res*. 2002;70(3):462–73.
51. Dafforn A, Chen P, Deng G, Herrler M, Iglehart D, Koritala S, et al. Linear mRNA amplification from as little as 5 ng total RNA for global gene expression analysis. *Biotechniques*. 2004;37(5):854–7.
52. Alldred MJ, Che S, Ginsberg SD. Terminal continuation (TC) RNA amplification without second strand synthesis. *J Neurosci Methods*. 2009;177:381–5.
53. Alldred MJ, Che S, Ginsberg SD. Terminal continuation (TC) RNA amplification enables expression profiling using minute RNA input obtained from mouse brain. *Int J Mol Sci*. 2008;9:2091–104.
54. Zhang W, Yu Y, Hertwig F, Thierry-Mieg J, Zhang W, Thierry-Mieg D, et al. Comparison of RNA-seq and microarray-based models for clinical endpoint prediction. *Genome Biol*. 2015;16:133.
55. Mantione KJ, Kream RM, Kuzelova H, Ptacek R, Raboch J, Samuel JM, et al. Comparing bioinformatic gene expression profiling methods: microarray and RNA-Seq. *Med Sci Monit Basic Res*. 2014;20:138–42.
56. Dong Z, Chen Y. Transcriptomics: advances and approaches. *Sci China Life Sci*. 2013;56(10):960–7.
57. Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. *Nature reviews Genetics*. 2009;10(1):57–63.
58. Tang F, Barbacioru C, Wang Y, Nordman E, Lee C, Xu N, et al. mRNA-Seq whole-transcriptome analysis of a single cell. *Nat Methods*. 2009;6(5):377–82.
59. Trombetta JJ, Gennert D, Lu D, Satija R, Shalek AK, Regev A. Preparation of Single-Cell RNA-Seq Libraries for Next Generation Sequencing. *Curr Protoc Mol Biol*. 2014;107(22 1–17):4.
60. Kim T, Lim CS, Kaang BK. Cell type-specific gene expression profiling in brain tissue: comparison between TRAP, LCM and RNA-seq. *BMB Rep*. 2015;48:388–94.
61. Farris S, Wang Y, Ward JM, Dudek SM. Optimized Method for Robust Transcriptome Profiling of Minute Tissues Using Laser Capture Microdissection and Low-Input RNA-Seq. *Front Mol Neurosci*. 2017;10:185.

62. Duchon A, Raveau M, Chevalier C, Nalesso V, Sharp AJ, Herault Y. Identification of the translocation breakpoints in the Ts65Dn and Ts1Cje mouse lines: relevance for modeling Down syndrome. *Mamm Genome*. 2011;22:674–84.
63. Bray NL, Pimentel H, Melsted P, Pachter L. Near-optimal probabilistic RNA-seq quantification. *Nat Biotechnol*. 2016;34(5):525–7.
64. Law CW, Chen Y, Shi W, Smyth GK. voom: precision weights unlock linear model analysis tools for RNA-seq read counts. *Genome Biol*. 2014;15(2):R29.
65. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic acids research*. 2015;43(7):e47.
66. Pages HC, Falcon M, Li S, AnnotationDbi N. Manipulation of SQLite-based annotations in bioconductor. 2019.
67. Broberg P. A comparative review of estimates of the proportion unchanged genes and the false discovery rate. *BMC Bioinformatics*. 2005;6:199.
68. Qiagen. <https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis>. 2020.
69. Krämer A, Green J, Pollard J, Jr, Tugendreich S. Causal analysis approaches in Ingenuity Pathway Analysis. *Bioinformatics*. 2013;30(4):523–30.
70. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic acids research*. 2000;28(1):27–30.
71. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, et al. STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic acids research*. 2018;47(D1):D607-D13.
72. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome research*. 2003;13(11):2498–504.
73. Ginsberg SD, Alldred MJ, Counts SE, Cataldo AM, Neve RL, Jiang Y, et al. Microarray analysis of hippocampal CA1 neurons implicates early endosomal dysfunction during Alzheimer's disease progression. *Biol Psychiatry*. 2010;68:885–93.
74. Jiang Y, Mullaney KA, Peterhoff CM, Che S, Schmidt SD, Boyer-Boiteau A, et al. Alzheimer's-related endosome dysfunction in Down syndrome is Abeta-independent but requires APP and is reversed by BACE-1 inhibition. *Proc Natl Acad Sci USA*. 2010;107:1630–5.
75. ABI. Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR. Applied Biosystems Product Guide. 2004:1–60.
76. Sturgeon X, Gardiner KJ. Transcript catalogs of human chromosome 21 and orthologous chimpanzee and mouse regions. *Mamm Genome*. 2011;22:261–71.
77. Chen Y, Dyakin VV, Branch CA, Ardekani B, Yang D, Guilfoyle DN, et al. In vivo MRI identifies cholinergic circuitry deficits in a Down syndrome model. *Neurobiol Aging*. 2009;30:1453–65.

78. Birnbaum JH, Wanner D, Gietl AF, Saake A, Kündig TM, Hock C, et al. Oxidative stress and altered mitochondrial protein expression in the absence of amyloid- β and tau pathology in iPSC-derived neurons from sporadic Alzheimer's disease patients. *Stem cell research*. 2018;27:121–30.
79. Rueda N, Llorens-Martin M, Florez J, Valdizan E, Banerjee P, Trejo JL, et al. Memantine normalizes several phenotypic features in the Ts65Dn mouse model of Down syndrome. *J Alzheimers Dis*. 2010;21:277–90.
80. Souchet B, Guedj F, Penke-Verdier Z, Daubigney F, Duchon A, Herault Y, et al. Pharmacological correction of excitation/inhibition imbalance in Down syndrome mouse models. *Front Behav Neurosci*. 2015;9:267.
81. Kaur G, Sharma A, Xu W, Gerum S, Alldred MJ, Subbanna S, et al. Glutamatergic transmission aberration: a major cause of behavioral deficits in a murine model of Down's syndrome. *J Neurosci*. 2014;34:5099–106.
82. Costa AC, Grybko MJ. Deficits in hippocampal CA1 LTP induced by TBS but not HFS in the Ts65Dn mouse: a model of Down syndrome. *Neurosci Lett*. 2005;382(3):317–22.
83. Kleschevnikov AM, Belichenko PV, Villar AJ, Epstein CJ, Malenka RC, Mobley WC. Hippocampal long-term potentiation suppressed by increased inhibition in the Ts65Dn mouse, a genetic model of Down syndrome. *J Neurosci*. 2004;24(37):8153–60.
84. Siarey RJ, Carlson EJ, Epstein CJ, Balbo A, Rapoport SI, Galdzicki Z. Increased synaptic depression in the Ts65Dn mouse, a model for mental retardation in Down syndrome. *Neuropharmacology*. 1999;38(12):1917–20.
85. Siarey RJ, Stoll J, Rapoport SI, Galdzicki Z. Altered long-term potentiation in the young and old Ts65Dn mouse, a model for Down Syndrome. *Neuropharmacology*. 1997;36(11–12):1549–54.
86. Block A, Ahmed MM, Rueda N, Hernandez MC, Martinez-Cue C, Gardiner KJ. The GABAA α 5-selective Modulator, RO4938581, Rescues Protein Anomalies in the Ts65Dn Mouse Model of Down Syndrome. *Neuroscience*. 2018;372:192–212.
87. Colas D, Chuluun B, Warriar D, Blank M, Wetmore DZ, Buckmaster P, et al. Short-term treatment with the GABAA receptor antagonist pentylentetrazole produces a sustained pro-cognitive benefit in a mouse model of Down's syndrome. *Br J Pharmacol*. 2013;169:963–73.
88. Kleschevnikov AM, Belichenko PV, Gall J, George L, Nosheny R, Maloney MT, et al. Increased efficiency of the GABAA and GABAB receptor-mediated neurotransmission in the Ts65Dn mouse model of Down syndrome. *Neurobiol Dis*. 2012;45(2):683–91.
89. Fukuda Y, Berry TL, Nelson M, Hunter CL, Fukuhara K, Imai H, et al. Stimulated neuronal expression of brain-derived neurotrophic factor by Neurotrophin. *Mol Cell Neurosci*. 2010;45(3):226–33.
90. Counts SE, Che S, Ginsberg SD, Mufson EJ. Gender differences in neurotrophin and glutamate receptor expression in cholinergic nucleus basalis neurons during the progression of Alzheimer's disease. *J Chem Neuroanat*. 2011;42:111–7.
91. Counts SE, Nadeem M, Wu J, Ginsberg SD, Saragovi HU, Mufson EJ. Reduction of cortical TrkA but not p75(NTR) protein in early-stage Alzheimer's disease. *Ann Neurol*. 2004;56:520–31.

92. Ginsberg SD, Che S, Wu J, Counts SE, Mufson EJ. Down regulation of trk but not p75NTR gene expression in single cholinergic basal forebrain neurons mark the progression of Alzheimer's disease. *J Neurochem*. 2006;97:475–87.
93. Latina V, Caioli S, Zona C, Ciotti MT, Amadoro G, Calissano P. Impaired NGF/TrkA signaling causes early AD-linked presynaptic dysfunction in cholinergic primary neurons. *Front Cell Neurosci*. 2017;11:68.
94. Mufson EJ, Li JM, Sobreviela T, Kordower JH. Decreased trkA gene expression within basal forebrain neurons in Alzheimer's disease. *Neuroreport*. 1996;8(1):25–9.
95. Ginsberg SD, Mufson EJ, Alldred MJ, Counts SE, Wu J, Nixon RA, et al. Upregulation of select rab GTPases in cholinergic basal forebrain neurons in mild cognitive impairment and Alzheimer's disease. *J Chem Neuroanat*. 2011;42:102–10.
96. Mufson EJ, Counts SE, Ginsberg SD. Gene expression profiles of cholinergic nucleus basalis neurons in Alzheimer's disease. *Neurochem Res*. 2002;27:1035–48.
97. Mufson EJ, Counts SE, Ginsberg SD, Mahady L, Perez SE, Massa SM, et al. Nerve Growth Factor Pathobiology During the Progression of Alzheimer's Disease. *Front NeuroSci*. 2019;13:533.
98. Mufson EJ, Ginsberg SD, Ikonomic MD, DeKosky ST. Human cholinergic basal forebrain: chemoanatomy and neurologic dysfunction. *J Chem Neuroanat*. 2003;26:233–42.
99. Levey AI, Edmunds SM, Hersch SM, Wiley RG, Heilman CJ. Light and electron microscopic study of m2 muscarinic acetylcholine receptor in the basal forebrain of the rat. *J Comp Neurol*. 1995;351(3):339–56.
100. Fajardo-Serrano A, Liu L, Mott DD, McDonald AJ. Evidence for M2 muscarinic receptor modulation of axon terminals and dendrites in the rodent basolateral amygdala: An ultrastructural and electrophysiological analysis. *Neuroscience*. 2017;357:349–62.
101. Khaziev E, Samigullin D, Zhilyakov N, Fatikhov N, Bukharaeva E, Verkhatsky A, et al. Acetylcholine-Induced Inhibition of Presynaptic Calcium Signals and Transmitter Release in the Frog Neuromuscular Junction. *Frontiers in physiology*. 2016;7:621.
102. Lee J, Hwang YJ, Shin JY, Lee WC, Wie J, Kim KY, et al. Epigenetic regulation of cholinergic receptor M1 (CHRM1) by histone H3K9me3 impairs Ca(2+) signaling in Huntington's disease. *Acta Neuropathol*. 2013;125(5):727–39.
103. Dippel E, Kalkbrenner F, Wittig B, Schultz G. A heterotrimeric G protein complex couples the muscarinic m1 receptor to phospholipase C-beta. *Proc Natl Acad Sci U S A*. 1996;93(4):1391–6.
104. Wang X, Wang W, Li L, Perry G, Lee HG, Zhu X. Oxidative stress and mitochondrial dysfunction in Alzheimer's disease. *Biochim Biophys Acta*. 2014;1842(8):1240–7.
105. Swerdlow RH. Mitochondria and Mitochondrial Cascades in Alzheimer's Disease. *J Alzheimers Dis*. 2018;62(3):1403–16.
106. Yamazaki Y, Zhao N, Caulfield TR, Liu CC, Bu G. Apolipoprotein E and Alzheimer disease: pathobiology and targeting strategies. *Nature reviews Neurology*. 2019;15(9):501–18.

107. Predecki M, Florczak-Wyspianska J, Kowalska M, Ilkowski J, Grzelak T, Bialas K, et al. APOE genetic variants and apoE, miR-107 and miR-650 levels in Alzheimer's disease. *Folia neuropathologica*. 2019;57(2):106–16.
108. Liu Y, Liu F, Grundke-Iqbal I, Iqbal K, Gong CX. Deficient brain insulin signalling pathway in Alzheimer's disease and diabetes. *J Pathol*. 2011;225(1):54–62.
109. Saito K, Elce JS, Hamos JE, Nixon RA. Widespread activation of calcium-activated neutral proteinase (calpain) in the brain in Alzheimer disease: a potential molecular basis for neuronal degeneration. *Proc Natl Acad Sci U S A*. 1993;90(7):2628–32.
110. Becker B, Nazir FH, Brinkmalm G, Camporesi E, Kvartsberg H, Portelius E, et al. Alzheimer-associated cerebrospinal fluid fragments of neurogranin are generated by Calpain-1 and prolyl endopeptidase. *Molecular neurodegeneration*. 2018;13(1):47.
111. Kling A, Jantos K, Mack H, Hornberger W, Drescher K, Nimmrich V, et al. Discovery of Novel and Highly Selective Inhibitors of Calpain for the Treatment of Alzheimer's Disease: 2-(3-Phenyl-1H-pyrazol-1-yl)-nicotinamides. *Journal of medicinal chemistry*. 2017;60(16):7123–38.
112. Lüth HJ, Münch G, Arendt T. Aberrant expression of NOS isoforms in Alzheimer's disease is structurally related to nitrotyrosine formation. *Brain Res*. 2002;953(1–2):135–43.
113. Lüth HJ, Ogunlade V, Kuhla B, Kientsch-Engel R, Stahl P, Webster J, et al. Age- and stage-dependent accumulation of advanced glycation end products in intracellular deposits in normal and Alzheimer's disease brains. *Cereb Cortex*. 2005;15(2):211–20.
114. Lukiw WJ, Rogaev EI. Genetics of Aggression in Alzheimer's Disease (AD). *Front Aging Neurosci*. 2017;9:87.
115. Butterfield DA, Hardas SS, Lange ML. Oxidatively modified glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Alzheimer's disease: many pathways to neurodegeneration. *J Alzheimers Dis*. 2010;20(2):369–93.
116. Chang WS, Wang YH, Zhu XT, Wu CJ. Genome-Wide Profiling of miRNA and mRNA Expression in Alzheimer's Disease. *Medical science monitor: international medical journal of experimental clinical research*. 2017;23:2721–31.
117. Hong S, Beja-Glasser VF, Nfonoyim BM, Frouin A, Li S, Ramakrishnan S, et al. Complement and microglia mediate early synapse loss in Alzheimer mouse models. *Science*. 2016.
118. Choong XY, Tosh JL, Pulford LJ, Fisher EM. Dissecting Alzheimer disease in Down syndrome using mouse models. *Front Behav Neurosci*. 2015;9:268.
119. Gupta M, Dhanasekaran AR, Gardiner KJ. Mouse models of Down syndrome: gene content and consequences. *Mamm Genome*. 2016;27(11–12):538–55.
120. Gardiner K, Fortna A, Bechtel L, Davisson MT. Mouse models of Down syndrome: how useful can they be? Comparison of the gene content of human chromosome 21 with orthologous mouse genomic regions. *Gene*. 2003;318:137–47.
121. Gardiner KJ. Pharmacological approaches to improving cognitive function in Down syndrome: current status and considerations. *Drug Des Devel Ther*. 2015;9:103–25.

122. Rachidi M, Lopes C. Mental retardation and associated neurological dysfunctions in Down syndrome: a consequence of dysregulation in critical chromosome 21 genes and associated molecular pathways. *Eur J Paediatr Neurol.* 2008;12:168–82.
123. Rueda N, Florez J, Martinez-Cue C. Mouse models of Down syndrome as a tool to unravel the causes of mental disabilities. *Neural Plast.* 2012;2012:584071.

Tables

Due to technical limitations, table 1-2 is only available as a download in the Supplemental Files section.

Figures

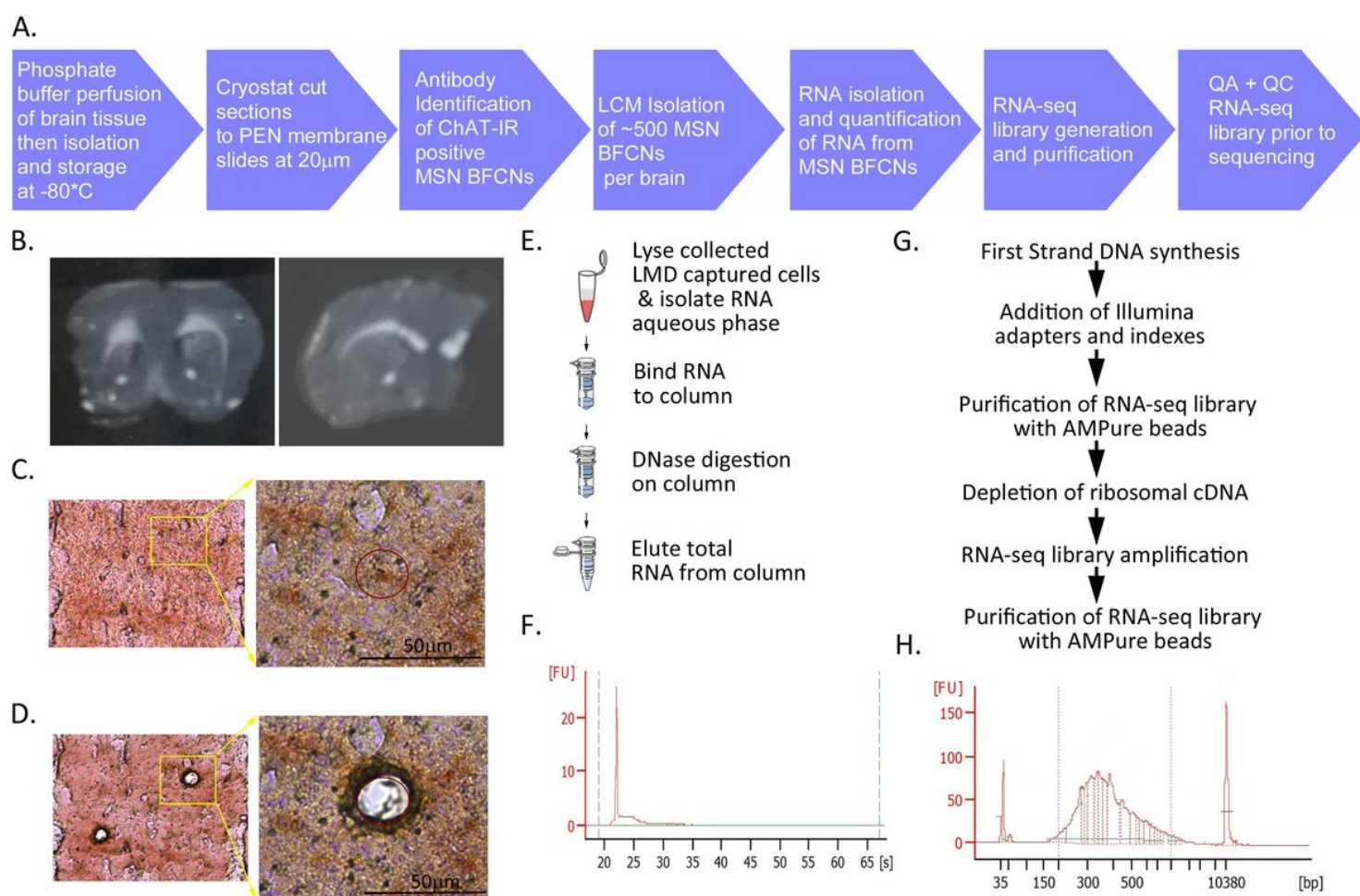


Figure 1

Overview of experimental workflow A. Flow chart illustrating isolation of MSN BFCNs, followed by RNA-seq library preparation B. Whole or biased-hemibrains including the midline were cryocut at 20 µm thickness and mounted on PEN membrane slides. C. ChAT immunopositive neurons were visualized at 10x and selected for isolation by LCM at 40x. D. LCM was used to isolate neurons and tissue was

checked for complete cutting of each cell and collection via gravity into tubes containing lysis reagent. E. RNA was isolated using the miRNeasy micro kit (Qiagen). F. RNA quantity was determined (Agilent RNA 6000 Pico). G. RNA-seq library prep was performed using isolated RNA from LCM-captured MSN BFCNs (Takara). H. RNA-seq library QC was performed for each sample (Agilent).

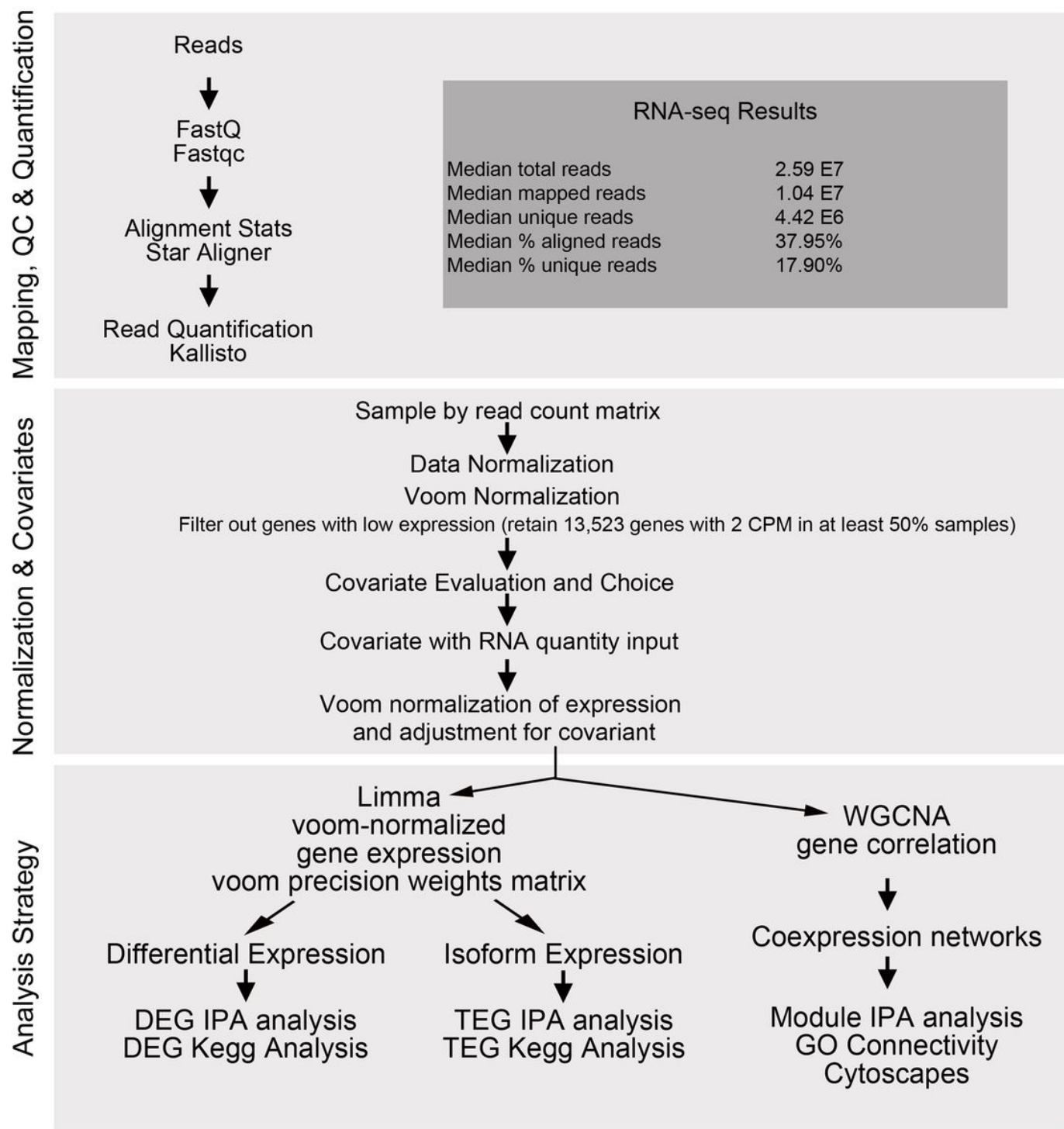


Figure 2

Pipeline for bioinformatic workflow using RNA-seq libraries derived from LCM-captured MSN BFCNs.

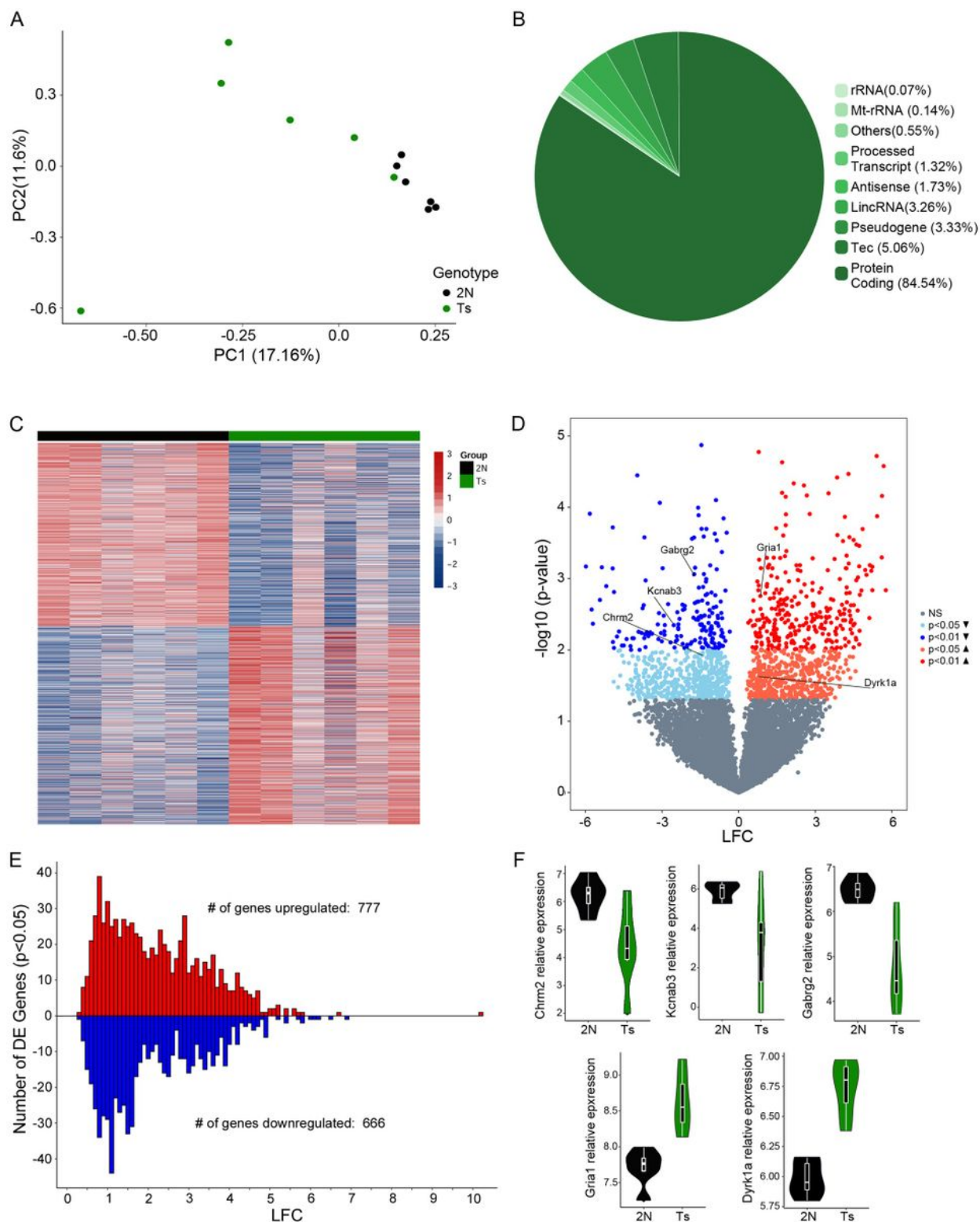


Figure 3

MSN BFCNs show significant differences in gene expression in 6 MO Ts and 2N mice. Study groups included trisomic (Ts, n=6) and normal disomic littermates (2N, n=6). A. PCA shows clear differences between the 2N and Ts MSN BFCNs, with Ts mice displaying a broader range of gene expression variability B. Pie chart indicates 84.54% of gene expression differences in ChAT-immunopositive BFCNs are protein coding. C. Heatmap illustrates DEGs in individual Ts and 2N mice. D. Volcano plots show

relative upregulation, downregulation, and LFC of Ts MSN BFCN gene expression differences on a $-\log(10)$ of p-value scale. Differential gene expression changes are expressed as log fold-change (LFC; red = Ts>2N, blue = Ts<2N). E. Distribution of LFC of differential expression for 1,443 DEGs. Ts MSN LFC for upregulated genes are plotted in red (n = 777, positive values), and Ts LFC for downregulated genes in green (n =666, negative values). F. Violin plots show relative gene expression for select DEGs.

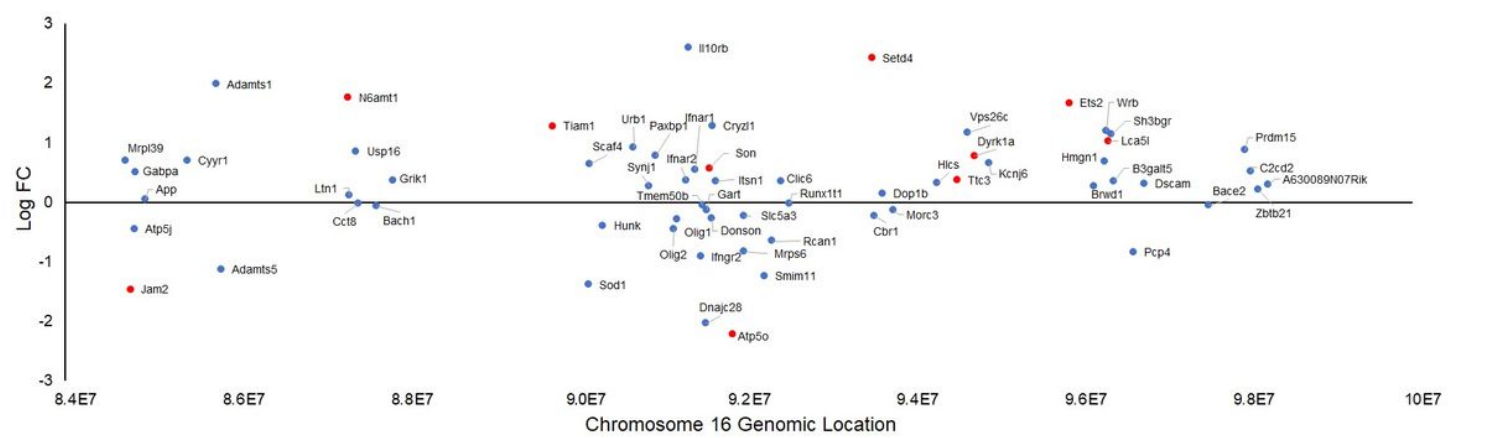


Figure 4

Trisomic protein coding genes do not necessarily match copy number within vulnerable cell types. In MSN BFCNs, only 64 genes (of 88) show quantifiable expression levels (2 CPM in 50% of samples) with 10 genes attaining statistical significance at 6 MO, including 8 upregulated {N-6 adenine-specific DNA methyltransferase1 (N6amt1), T cell lymphoma invasion and metastasis 1 (Tiam1), Son DNA binding protein (Son), SET domain containing 4 (Setd4), tetratricopeptide repeat domain 3 (Ttc3), dual specificity tyrosine phosphorylation regulated kinase 1A (Dyrk1a), E26 avian leukemia oncogene 2,3' domain (Ets2) and lebercilin congenital amaurosis 5-like (Lca5l)} and 2 downregulated genes {junction adhesion molecule 2 (Jam2) and ATP synthase H⁺ transporting mitochondrial F1 complex, O subunit (Atp5o)}.

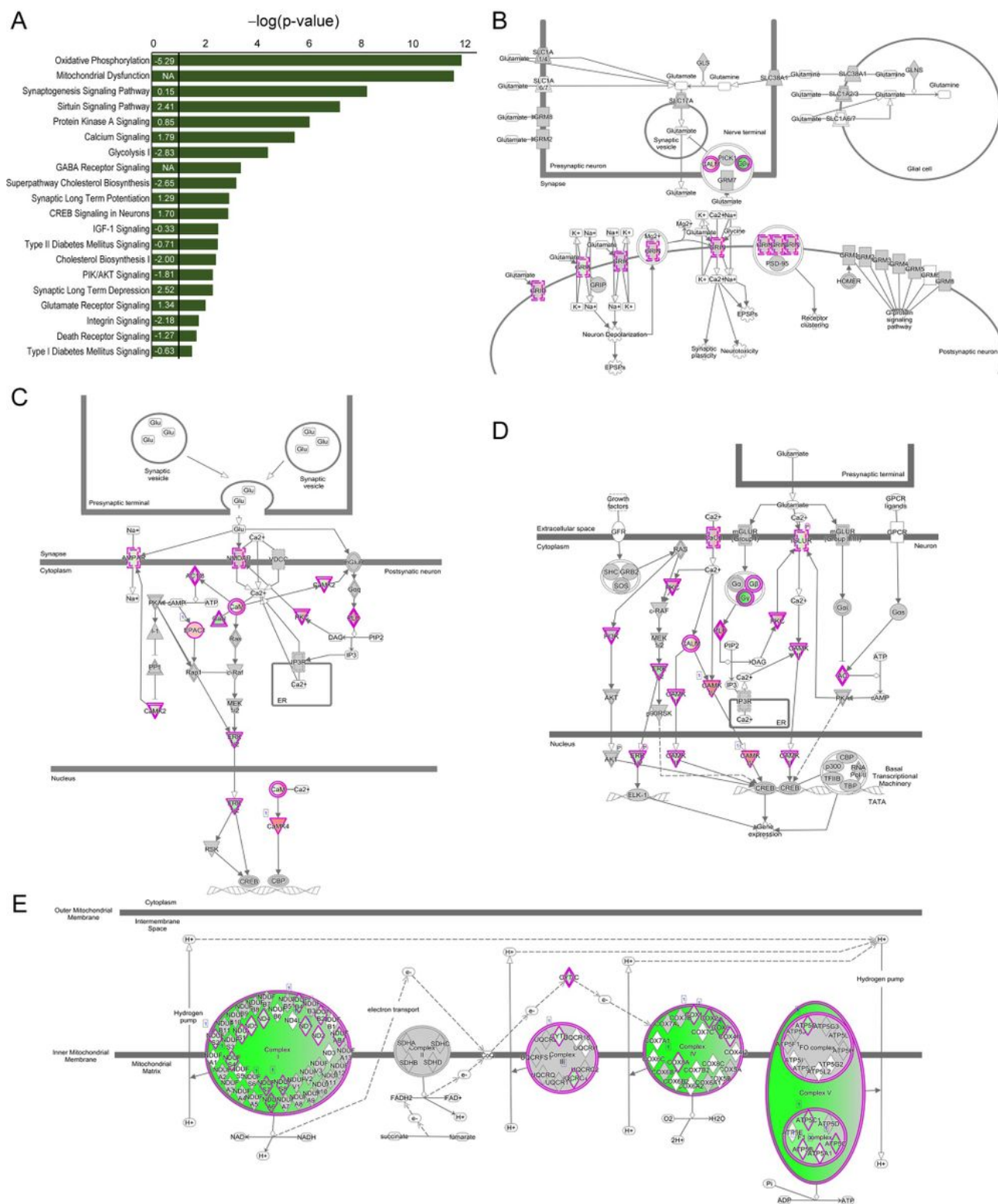


Figure 5

Bioinformatic assessment of vulnerable pathways in trisomic BFCNs by IPA. A. IPA identified significant effects on 161 pathways, with select pathways depicted ($-\log(p\text{-value})$): significance of pathway dysregulation; z-scores (white in bars): upregulation (+) or downregulation (-), not accessible (NA). B-E. Targeted pathways display significant dysregulation based on the highlighted gene expression changes (LFC, magenta outlines represent significant alterations, with pink fill indicating Ts>2N and green fill

indicating $T_s < 2N$). B. The glutamatergic pathway shows increased activation in trisomic BFCNs, which is also observed in (C) Synaptic Long-Term Potentiation and (D) the CREB signaling pathway. While the oxidative phosphorylation (E) pathway shows decreased activation in trisomic BFCNs.

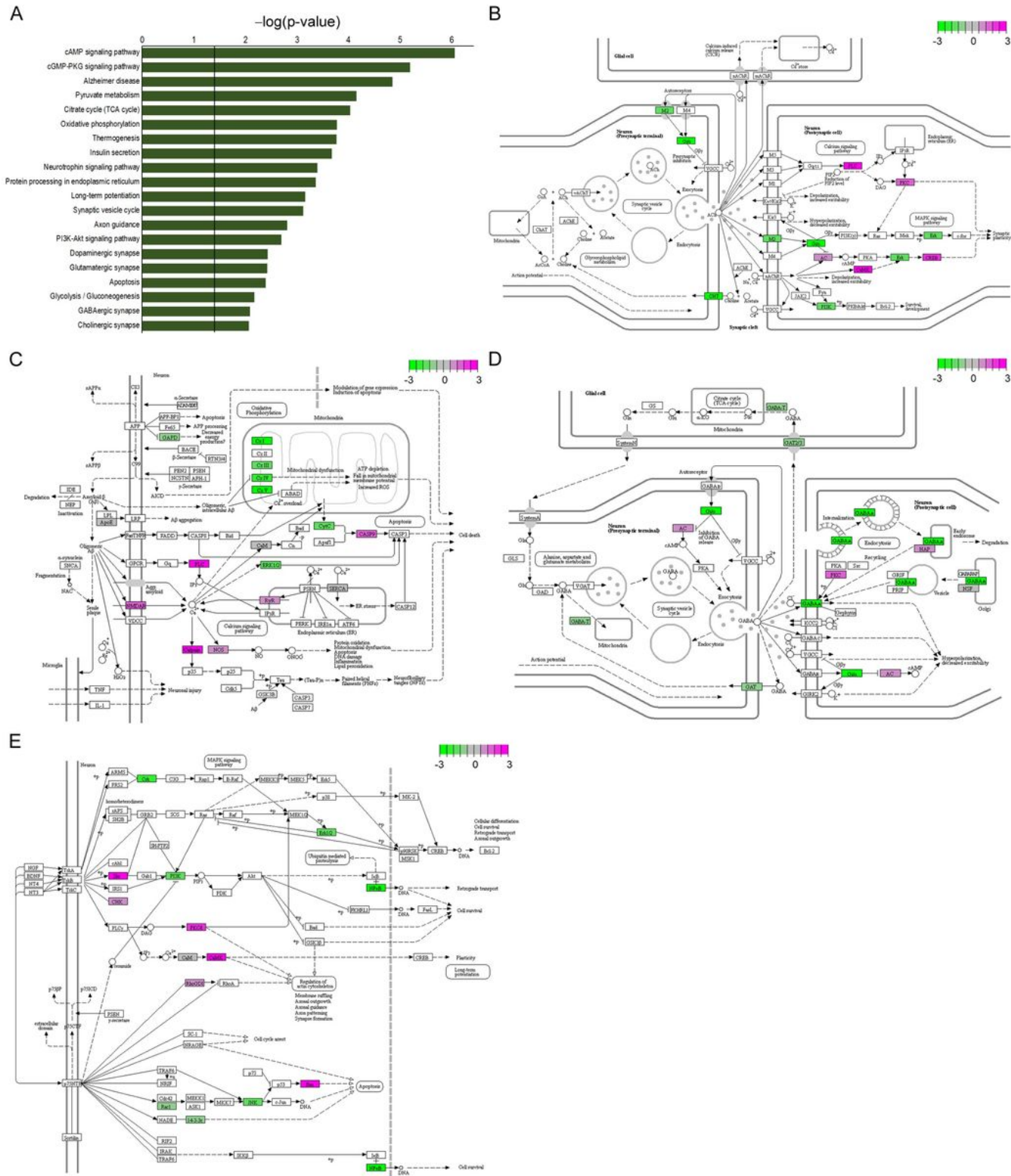


Figure 6

Bioinformatic assessment of vulnerable pathways in trisomic BFCNs by KEGG. A. KEGG analysis revealed novel dysregulated pathways as well as several that overlapped with IPA analysis. B. The

cholinergic synapse is dysregulated with the Chrm2 receptor downregulated, leading to changes in synaptic plasticity. C. The Alzheimer’s Disease pathway is dysregulated. D. GABAergic and glutamatergic pathways (see Fig. 5B, for IPA analysis) both showed differential expression in trisomic BFCNs (LFC; pink Ts>2N, green Ts<2N). E. Neurotrophin signaling pathway deficits implicate a decrease in cell survival signaling and an increase in apoptosis signaling in trisomic BFCNs.

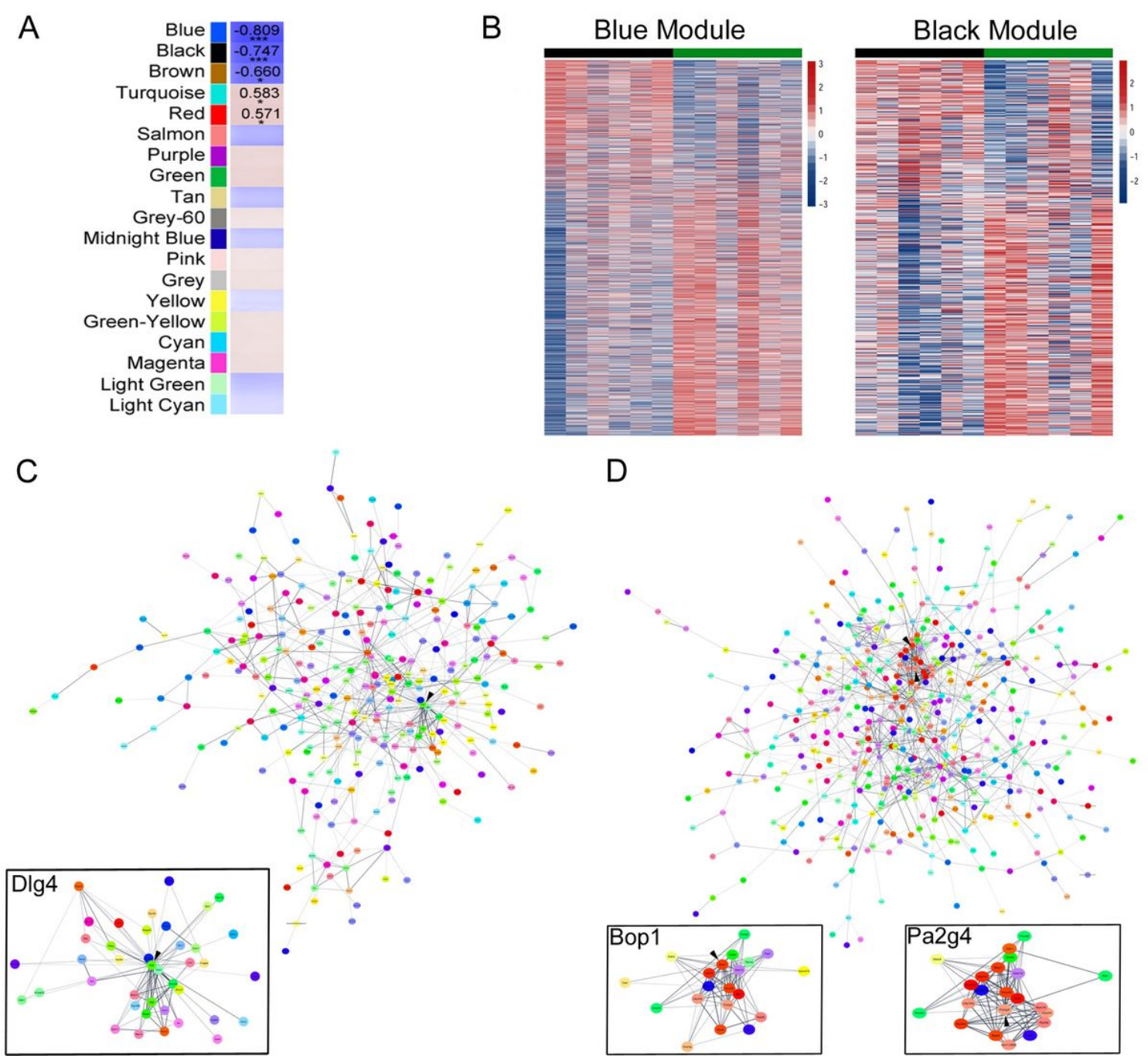


Figure 7

Whole Genome Coexpression Network Analysis in MSN BFCNs. A. Control-derived modules ranked by enrichment status. The Blue module (-0.809) and Black module (-0.747) revealed significant gene expression differences by genotype within MSN BFCNs at FDR (< 0.05). B. Heatmaps indicating the Blue

module contains 2,199 genes and the Black module contains 701 genes. C. The top 500 genes in the Blue module were queried by STRING in Cytoscape to examine protein-interactions. Dlg4 (PSD-95) showed 41 direct protein-protein interactions within the top 500 (inset). D. All 701 genes in the Black module were queried by STRING in Cytoscape. Bop1 and Pa2g4 had the highest number (17) of direct interactions (insets). * (p<0.05), ***FDR (<0.05).

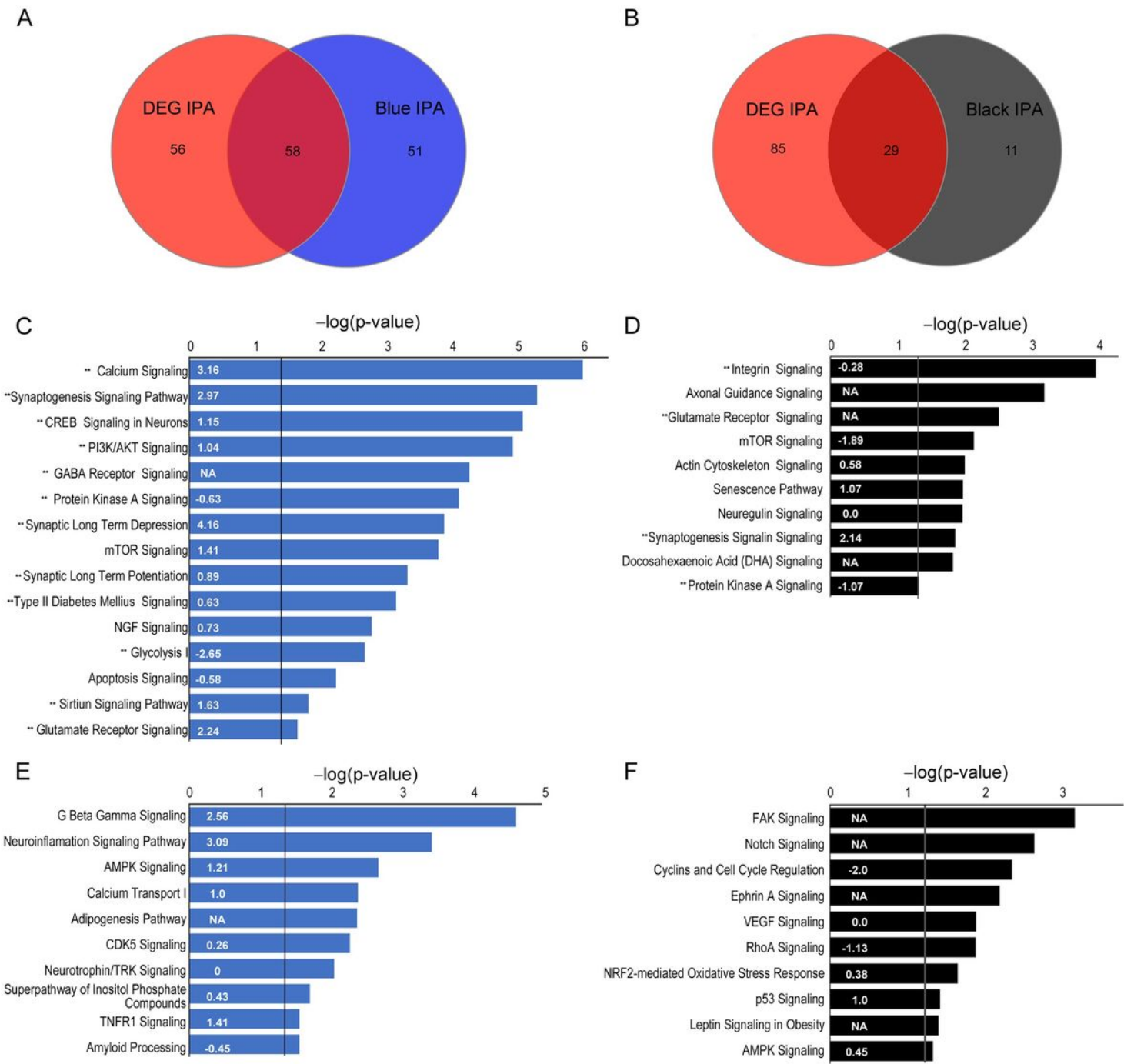


Figure 8

WGCNA Blue and Black modules were queried for pathway changes by IPA. A. Venn diagram shows overlap of genes and pathways identified by both DEG ($p < 0.05$) and the Blue module. B. Venn diagram shows majority of dysregulated pathways in Black module overlap with DEG pathways. C. Pathways significant to both the Blue module and DEG are shown with $-\log(p\text{-value})$ and z-scores in white text. D. A few pathways significant to both Black module and DEG were identified (** indicating pathways highlighted by DEG analysis). Pathways in the Blue module (E) and Black module (F) that were not significantly affected by DEG at $p < 0.05$ are depicted.

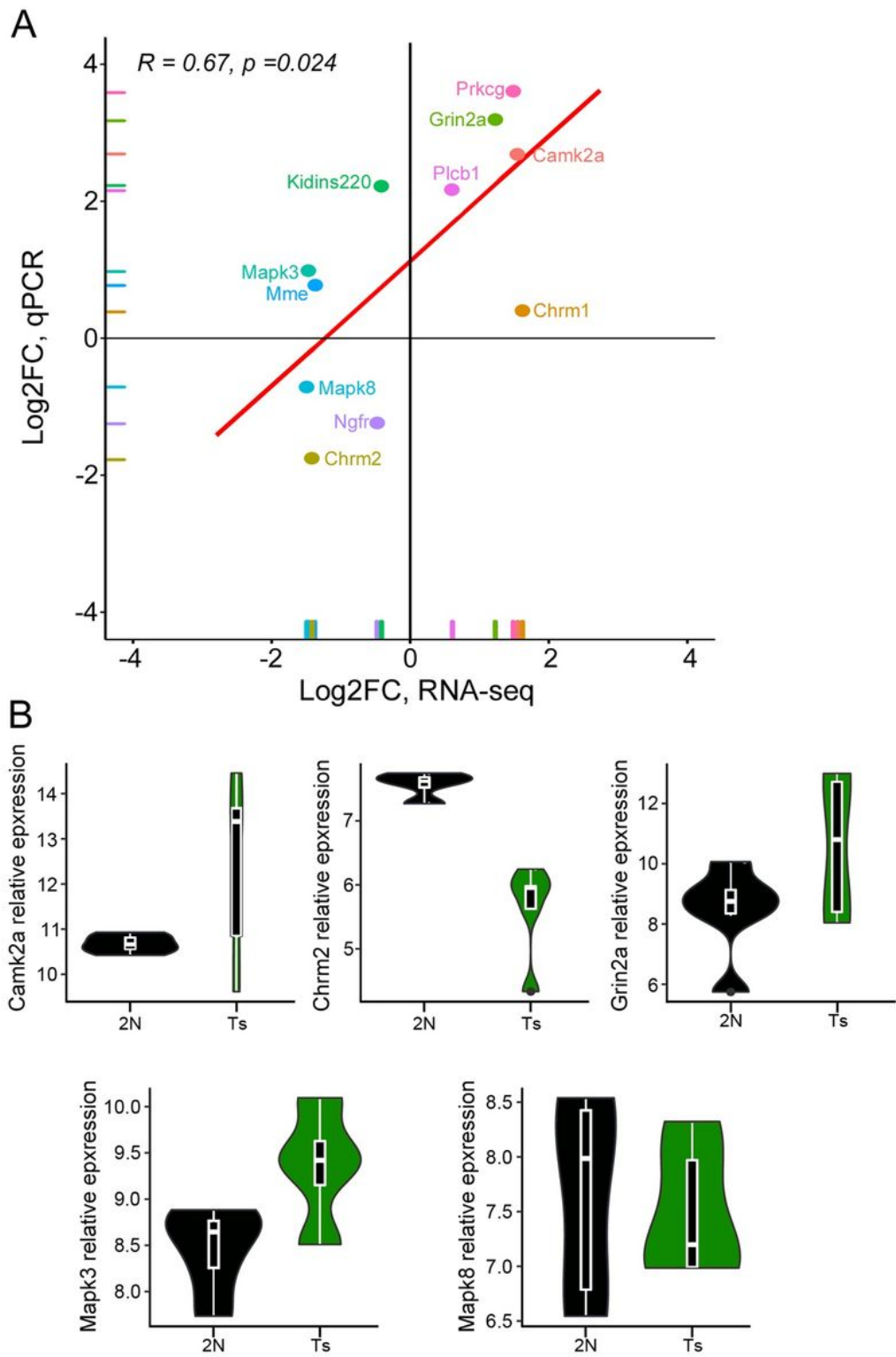


Figure 9

qPCR results for 11 genes {calcium/calmodulin dependent protein kinase II alpha (Camk2A), cholinergic receptor muscarinic 1 (Chrm1), Chrm2, glutamate ionotropic receptor NDMA type subunit 2A (Grin2A), Kidins220/Arms, Mapk3 (aka Erk1), mitogen activated protein kinase 8 (Mapk8 aka Erk2), Mme, Ngfr/p75NTR, Plcb1, and Prkcg} interrogated from Nissl-stained MSN neurons correlate strongly with RNA-seq data obtained from ChAT-positive MSN BFCNs ($R = 0.67$, $p=0.024$). B. Violin plots show relative gene expression values for a subset of the interrogated genes. Camk2a, Chrm2, Grin2a, Mapk3 and Mapk8 are all significantly dysregulated by RNA-seq in Ts MSN BFCNs. Of these, only Mapk3 qPCR does not replicate the directionality of the LFC seen in the RNA-seq data.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementalTable6MolNeurodeg.xlsx](#)
- [SupplementalTable5MolNeurodeg.xlsx](#)
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- [Table2MolNeurodeg.xlsx](#)
- [SupplementalTable3MolNeurodeg.xlsx](#)
- [SupplementalTable2MolNeurodeg.xlsx](#)
- [SupplementalTable1MolNeurodeg.xlsx](#)
- [SuppFigure3AlldredMolNeurodeg.tif](#)
- [SuppFigure2AlldredMolNeurodeg.tif](#)
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