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ORIGINAL ARTICLE

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Transcriptomics analysis reveals potential regulatory role of nSMase2 (*Smpd3*) in nervous system development and function of middle-aged mouse brains

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Abstract

Neutral sphingomyelinase-2 (nSMase2), gene name sphingomyelin phosphodiesterase-3 (*Smpd3*), is a key regulatory enzyme responsible for generating the sphingolipid ceramide. The function of nSMase2 in the brain is still controversial. To better understand the functional roles of nSMase2 in the aging mouse brain, we applied RNA-seq analysis, which identified a total of 1462 differentially abundant mRNAs between *+/fro and fro/fro*, of which 891 were increased and 571 were decreased in nSMase2-deficient mouse brains. The most strongly enriched GO and KEGG annotation terms among transcripts increased in *fro/fro* mice included synaptogenesis, synapse development, synaptic signaling, axon development, and axonogenesis. Among decreased transcripts, enriched annotations included ribosome assembly and mitochondrial protein complex functions. KEGG analysis of decreased transcripts also revealed overrepresentation of annotations for Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington disease (HD). Ingenuity Pathway Analysis (IPA) tools predicted lower susceptibility to these neurodegenerative disorders, as well as predictions agreeing with stronger synaptic function, learning, and memory in *fro/fro* mice. The IPA tools identified signaling proteins, epigenetic regulators, and micro-RNAs as likely upstream regulators of the broader set of genes encoding the affected transcripts. It also revealed 16 gene networks, each linked to biological processes identified as overrepresented annotations among the affected transcripts by multiple analysis methods. Therefore, the analysis of these RNA-seq data indicates that nSMase2 impacts synaptic function and neural development, and may contribute to the onset and development of neurodegenerative diseases in middle-aged mice.

KEYWORDS
fro/fro, neurodegenerative diseases, neutral sphingomyelinase-2, RNA-seq analysis

Abbreviations: nSMase2, Neutral sphingomyelinase-2; Smpd3, Sphingomyelin phosphodiesterase-3; AD, Alzheimer's disease; PD, Parkinson's diseases; HD, Huntington disease; EV, Extracellular vesicle; *fro/fro*, Fragilitas ossium; IPA, Ingenuity Pathway Analysis; *Grin2b*/NR2B, ionotropic glutamate receptor subunit 2B; GEO, Gene Expression Omnibus database; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology.

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1 | INTRODUCTION

Sphingomyelinases (SMases) are classified into acid, neutral, and alkaline subtypes based on their pH optima of activity. Neutral sphingomyelinase 2 (nSMase2), one of the four neutral sphingomyelinases and the product of the sphingomyelin phosphodiesterase 3 (*Smpd3*) gene, is strongly expressed in the brain, where it generates ceramide, a bioactive lipid that is involved in diverse cellular processes,[1,2] including control of gene expression. nSMase2 is a therapeutic target for cancer and neurological disorders[3] and a key signaling molecule involved in cell growth arrest, differentiation, senescence, apoptosis and inflammation.[4–8] It has been reported that ceramide regulates gene expression critical for primitive streak formation.[9] Ceramide also regulates oxidative stress-related genes, such as mitogen-activated protein kinase, protein kinase ζ, stress-activated protein kinases, ceramide-activated protein kinases and protein phosphatase and phospholipases that also have impact on gene expression.[10–14] Ceramide levels in the brain are increased in neurodegenerative disorders,[15] including memory impairment[16] and cognitive impairment, in both Alzheimer's disease (AD)[17] and Parkinson's disease (PD).[18] In a cerebral ischemia model, inhibition of nSMase2-mediated ceramide production and accumulation primarily occurred in hippocampal astrocytes alleviated neuronal damage, indicating that the inhibition of ceramide production by targeting nSMase2 activity could be a promising approach to prevent pathological ceramide accumulation in neurological diseases.[19] Other studies have shown that nSMase2 is important for motor coordination in the striatum,[20] and synaptic function related to learning and memory.[20,21]

In addition, another remarkable function of nSMase2 is to generate ceramide that is a primary regulator in extracellular vesicle (EV) biogenesis and release. nSMase2 is critical for the secretion of exosomes that are enriched with small RNAs, which is important for antigen presentation.[22] nSMase2-produced ceramide is also critical for the release of EVs that transport and spread Aβ and α-synuclein, suggesting a pathological role of nSMase2 in aging-related neurodegenerative diseases, such as AD and PD.[23,24] Collectively, inhibition of nSMase2-mediated EVs release is a promising new therapeutic approach for cancer, heart disease, muscle disease and neurological disorders.[25]

An opportunity to better understand the function of nSMase2 is the mouse model of fragilitas ossium (the *fro/fro* mouse). In *fro/fro* mice, a chemically-induced deletion mutation of the *Smpd3* gene eliminates the C-terminal 33 amino acids of nSMase2, a region that includes a histidine residue crucial for enzymatic activity.[26] Our previous studies demonstrated that exosomes from Aβ-treated primary astrocytes are enriched with ceramide, which is critical for binding of these exosomes to Aβ. In contrast, the same phenomenon was not observed with astrocytes from the *fro/fro* mouse, which suggested that nSMase2 critically contributed to the secretion of Aβ-associated exosomes involved in AD pathogenesis.[23] Consistent with these findings, our studies also showed that treatment of 5 × FAD mice with GW4869, a specific inhibitor of nSMase2, decreased the number of exosomes in the brain as compared with untreated 5 × FAD mice. A similar result was obtained with *5 × FAD*; *fro/fro* mice, which were generated by crossing 5 × FAD mice with *fro/fro* mice. Our data strongly support the hypothesis that deactivation of nSMase2 is promising for therapy of AD, and may also be beneficial in aging related decline of cognitive function.

Our previous analysis of RNA-seq data from two pairs of *fro/fro* and heterozygous littermates +/*fro* revealed 1462 differentially abundant mRNAs in *fro/fro* as compared with +/*fro* brain cortex, comprising 891 increased mRNAs and 571 decreased mRNAs.[27] We demonstrated that protein levels of the ionotropic glutamate receptor subunit 2B (*Grin2b*/NR2B) increased by twofold in *fro/fro* versus *WT* cortex. We also showed that several species of ceramide were reduced in *fro/fro* at least 50% as compared with +/*fro* controls. In addition, we observed a threefold reduction in exosomes carrying miR-223-3p, a micro-RNA that downregulates *Grin2b*, which suggests a mechanism linking nSMase2 with *Grin2b* expression that is further supported by Ingenuity Pathway Analysis (IPA) of the transcriptome differences observed.[27] These preliminary analyses, along with the role of Grin2b in brain development, circuit formation, synaptic plasticity, learning, and memory,[28] indicate that more comprehensive analyses of the coordinated effects of nSMase2 deficiency will facilitate determining whether modulating nSMase2 activity will have beneficial effects on the progression of neurodegenerative conditions.

To provide a more comprehensive understanding of gene expression regulated by nSMase2, we revisited the groups of significantly affected transcripts using KEGG tools, GO analysis, and IPA bioinformatics tools. These analyses indicate that nSMase2 may play critical roles in neuronal synaptic function and processes of neuronal cell development, assembly of the mitochondrial protein complex and mitochondria function; and that nSMase2 may contribute to the onset and development of neurodegenerative diseases.

2 | METHODS

2.1 | Ethics statement

The use of experimental animals and protocol used in this study was approved by the Institutional Animal Care and Use Committee of University of Kentucky.

2.2 | Animal model

The *fro/fro* mouse strain was the gift from Dr. Christophe Poirier (Indiana University, Indianapolis). This mouse carries a deletion mutation in the sphingomyelin phosphodiesterase-3 (*Smpd3*) gene of a region that encodes the C-terminal 33 amino acids of neutral sphingomyelinase 2 (nSMase2) (NCBI gene ID: 58994.[17,26] In our previous study, we analyzed the ceramide and mRNA levels in the cortex of middle-aged (10-month-old) nSMase2-deficient (*fro/fro*) mice and control littermates (+/*fro*). We did not observe significant differences between +/*fro* and wild type cortex, especially in cytokine IL-6 and

IL-1β levels, prompting us to use +/*fro* littermates as controls throughout our analyses (see supplemental fig. 1A and B in Reference 27). Animals were group-housed and littermate mice were divided into 2 groups: 10 months-old +/*fro* (control) and 10 months-old *fro/fro* mice. For each group, 2 male animals were euthanized and brain cortex was dissected.

2.3 | RNA extraction, cDNA library preparation and sequencing

RNA was extracted from brain cortex using the miniRNeasy extraction kit (Qiagen, #74134). The total RNA was submitted to Novogene (https://en.novogene.com) for quality control and RNA-seq. The details of the library preparation (0.4 μg of RNA), sequencing, and identification of differentially abundant mRNAs (DeSeq2) were described in our previous publication.[27] The RNA sequencing and initial statistical analyses were provided by Novogene, using DeSeq2 for the identification of differentially expressed genes. The *P* values were adjusted using the Benjamin and Hochberg approach for controlling the false discovery rate. For detailed explanations on how DeSeq2 performs statistical analysis when replicates are ≤3 see Reference 29. The RNA-seq data are available in the Gene Expression Omnibus (GEO) database (accession number GSE179045).

2.4 | Functional bioinformatics analyses

The functional bioinformatics analyses were performed as previously described.[27] Briefly, lists of differentially abundant mRNAs (*fro/fro* vs. +/*fro*) were assessed using KEGG and GO analyses and IPA software (Ingenuity H Systems, www.ingenuity.com) to identify pathway annotations or functional annotations enriched among the proteins encoded by these mRNAs. IPA was also used to predict positively and negatively regulated nervous system functions and disease syndromes related to the affected mRNAs.

2.5 | Quantitative reverse transcription PCR (RT-qPCR)

RNA was extracted from the brain cortex using the mini RNeasy plus mini kit (Qiagen, #74134) as described before.[27] The primer sequences for qRT-PCR were selected from the PrimerBank website, http://pga.mgh.harvard.edu/primerbank/. The primers in Primerbank have broad linear dynamic ranges and greater sensitivity, and therefore are suitable for qPCR approaches to reanalyze changes in expression suggested by exploratory technologies such as microarrays and RNA-Seq.[30] qRT-PCR was performed to amplify cDNA fragments from the following genes: *Naa38*, *Halpn2*, *Mrpl27*, *Rnaset2a*, *Atp5k*, and *Gapdh*. The latter served as an internal control. Primers are listed in the following table. Fold changes of the targeted mRNA in *fro/fro* mice were calculated relative to +/*fro* mice using the $2^{-\Delta\Delta Cq}$ method.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
Hapln2	TCCACGAGGTCATTCACTCTC	CGTGCAGTCCGTTGGTAAT
Naa38	GGCTGTTATTACTTCTGATGGCA	ACACCACTTGTTCTACTCCCT
Rnaset2a	CCCCCAACAGTATGCAAGGA	AAGCTAGTTTTCCCAGACTGC
Atp5k	GTTCAGGTCTCTCCACTCATCA	CGGGGTTTTAGGTAACTGTAGC
Mrpl27	GAAGACAGGTGGCAGCTCTAA	GCCATCTAAACTGACGCTGAG
Gapdh	ACTCCACTCACGGCAAATTC	TCTCCATGGTGGTGAAGACA

2.6 | Immunoblotting

Tissue from mouse brain cortex was solubilized in 2× SDS sample buffer (Sigma-Aldrich, S3401-1VL) with sonication. The protein concentration was determined by the RC DC™ Protein Assay kit (Bio-Rad, Cat#5000121). The protein lysates were heated for 10 min at 95°C, and 30 μg of protein from each sample was loaded and resolved by SDS-PAGE gel electrophoresis on polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, Cat #1620112). Non-specific binding sites were blocked with 5% fat-free dry milk in PBS/0.1% Tween-20 (PBST) followed by overnight incubation with primary antibodies, such as Map1b (Santa Cruz Biotechnology, sc-8970), Gsk3α/β (Santa Cruz Biotechnology, sc7291), phospho-Gsk3α/β (Santa Cruz Biotechnology, sc135653) and GAPDH (Cell signaling tech, #5174) and secondary anti-rabbit (Jackson Immune Research, 715-035-152) and anti-mouse (Jackson Immune Research, 715-035-151) horseradish peroxidase (HRP) antibodies for 1 h at room temperature. After three times of washing with PBST, bands were developed using either pico or femto enhanced chemiluminescent (ECL) HRP substrate (Thermo Fisher, Cat #34096, and Cat #34580). Blot images were captured by Azure c600 system (Azure Biosystems, California, USA), and quantified by image J software (https://imagej.nih.gov/ij/download).

2.7 | Analysis of IPA upstream and downstream effects

IPA upstream analysis was used to predict regulatory molecules upstream of the genes encoding affected transcripts in our dataset. These predictions include regulators with network connections to affected genes as well as directions of effects unlikely to occur in a random model. In particular, an overlap *p*-value was applied to measure enrichment of network-regulated genes in the dataset, as well as an activation *Z*-score to identify regulating molecules and predict activation state (either activated or inhibited) based on a statistically significant pattern match of up- and down-regulation, to putative regulators.[31] Downstream effects analysis identified diseases and functions, biological processes associated with the differentially expressed mRNAs. This analysis determined whether cellular processes were driven up or down by correlating observed expression with reported experimental effects.

2.8 | IPA mechanistic networks

The mechanistic networks analysis identified possible causal mechanisms.[31] In brief, this analysis determined network edges between pre-determined upstream regulators for which there was statistical evidence that the corresponding relationship was likely relevant for causal mechanisms. The most significant causal edges between regulators were then used to construct networks downstream of a "master" regulator to indicate possible causal (e.g., signaling) mechanisms. Criteria for these expected biological functions or diseases were Ingenuity Knowledge Base Absolute *Z*-score ≥ 2.0 and *p*-value <0.05.

2.9 | IPA regulator effects analysis

IPA regulator effects analysis investigated how a phenotype, function or disease might be regulated in the data set by activated or inhibited upstream regulators. The analysis also investigated the impact of upstream molecules on downstream biology and potential mechanism for a phenotype of drug, defined drug targets and discovered novel factors that may regulate disease phenotype or function relationships.

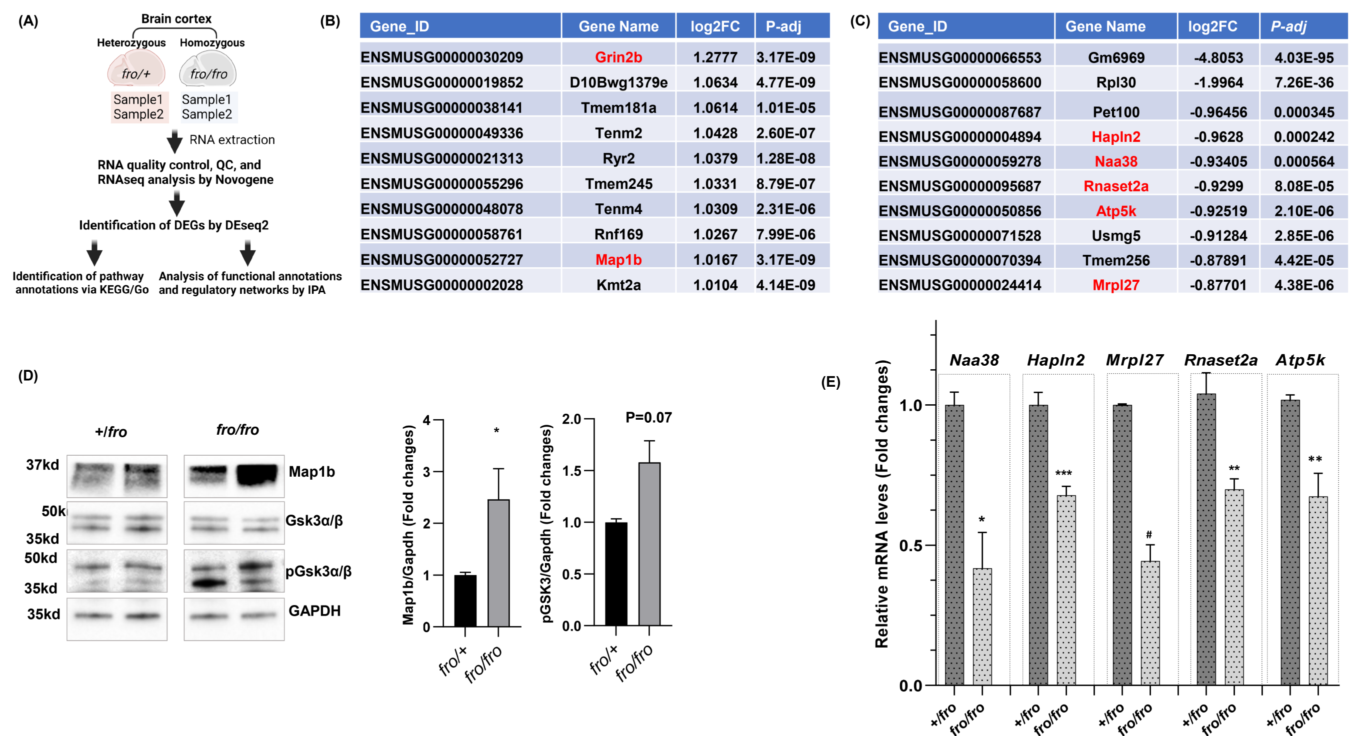
2.10 | Statistical analysis

Statistical analyses and graphing were performed using GraphPad Prism 9.0 software (GraphPad, San Diego, CA, USA). For IPA analyses, a Z score ($Z \le -2.0$ or $Z \ge 2.0$) was considered significant. The data are expressed as mean ± SEM and analyzed with GraphPad Prism 9 software. Differences between each group were analyzed by Student's *t*-test comparisons. *p*-Values below 0.05 were considered as significant. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; #$p < 0.0001$.

3 | RESULTS

3.1 | Identification of differentially abundant mRNAs and proteins in *fro/fro* versus *fro/+* brain cortex

In order to understand how nSMase2 affects the transcriptome in 10-month-old mouse cortex, RNA-sequence analysis was done on the cortices from 2 pairs of *fro/fro* and +/*fro* mice (Figure 1A). In total, 891 transcripts increased and 571 transcripts decreased in *fro/fro* versus +/*fro*.[27] The 10 most significantly increased and decreased transcripts are listed in Figure 1B,C, respectively. We previously assessed



(B)

Gene_ID	Gene Name	log2FC	P-adj
ENSMUSG00000030209	Grin2b	1.2777	3.17E-09
ENSMUSG00000019852	D10Bwg1379e	1.0634	4.77E-09
ENSMUSG00000038141	Tmem181a	1.0614	1.01E-05
ENSMUSG00000049336	Tenm2	1.0428	2.60E-07
ENSMUSG00000021313	Ryr2	1.0379	1.28E-08
ENSMUSG00000055296	Tmem245	1.0331	8.79E-07
ENSMUSG00000048078	Tenm4	1.0309	2.31E-06
ENSMUSG00000058761	Rnf169	1.0267	7.99E-06
ENSMUSG00000052727	Map1b	1.0167	3.17E-09
ENSMUSG00000002028	Kmt2a	1.0104	4.14E-09

(C)

Gene_ID	Gene Name	log2FC	*P-adj*
ENSMUSG00000066553	Gm6969	-4.8053	4.03E-95
ENSMUSG00000058600	Rpl30	-1.9964	7.26E-36
ENSMUSG00000087687	Pet100	-0.96456	0.000345
ENSMUSG00000004894	Hapln2	-0.9628	0.000242
ENSMUSG00000059278	Naa38	-0.93405	0.000564
ENSMUSG00000095687	Rnaset2a	-0.9299	8.08E-05
ENSMUSG00000050856	Atp5k	-0.92519	2.10E-06
ENSMUSG00000071528	Usmg5	-0.91284	2.85E-06
ENSMUSG00000070394	Tmem256	-0.87891	4.42E-05
ENSMUSG00000024414	Mrpl27	-0.87701	4.38E-06

FIGURE 1 Brain cortex deficient in nSMase2 has a different transcriptome and proteome. (A) Flow chart of the RNA-seq experiment and data analysis. (B) The top 10 most increased transcripts and (C) decreased transcripts. The transcripts in red color were selected to be analyzed either by immunoblotting or RT-PCR. (D) Immunoblots and quantification of protein levels of Grin2b, Map1b (antibody also detects Map1b light chain), phosphorylated Gsk3α/β (p-Gsk3α/β) and Gapdh in *fro/fro* versus *fro/+* cortex. (E) qRT-PCR to confirm differences in the amounts of *Naa38*, *Hapln2*, *Mrpl27*, *Rnaset2a*, *Atp5k* mRNAs in *fro/fro* versus *fro/+* cortex.

the protein levels of Grin2b in the brain cortex of both WT and *fro/fro* aging mice (10–15 months old). Grin2b protein in cortex was 2-fold higher in *fro/fro* versus WT controls.[27] In the present study, we re-analyzed the protein levels of Map1b, Gsk3α/β and phosphorylation of Gsk3α/β in both *fro/fro* and +/*fro* cortex. Unlike the other transcripts, Gsk3β was not a strongly affected transcript. However, because it shares with Grin2b a role in memory formation,[27,32,33] we decided to evaluate the levels of GSK3α/β protein and the phosphorylation of GSK3α/β. Immunoblot analysis to quantify protein levels confirmed a more than 2-fold increase in Map1b light chain protein[34] in *fro/fro* cortex, and a tendency for increased phosphorylation of Gsk3α/β (Figure 1D). In addition, qRT-PCR analysis confirmed that *Hapln2*, *Naa38*, *Rnaset2a*, *Atp5k*, and *Mrpl27* transcripts were decreased in *fro/fro* relative to +/*fro* cortex, as shown in Figure 1E ($n \geq 3$ pairs, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; # $p < 0.0001$).

3.2 | Evidence for improved synaptic function and resistance to neurodegenerative diseases

Our previous analysis using KEGG tools revealed that the increased transcripts are enriched for annotations associated with increased synaptic and signaling functions while decreased transcripts are enriched for transcripts annotated for neurodegenerative diseases and ribosome function (Figure 2A,B).[27] GO analysis gives highly similar results but its transcript cluster organization yields greater resolution of the types of mitochondrial compartments and ribosome functions in the overrepresented annotations among decreased transcripts (Figure 2C,D). The theme established by the increases in Grin2b mRNA and protein described above are represented in the enrichment for transcripts with synaptic annotations, especially glutamatergic synapses and numerous related annotations (Figure 2A,C). Corresponding with

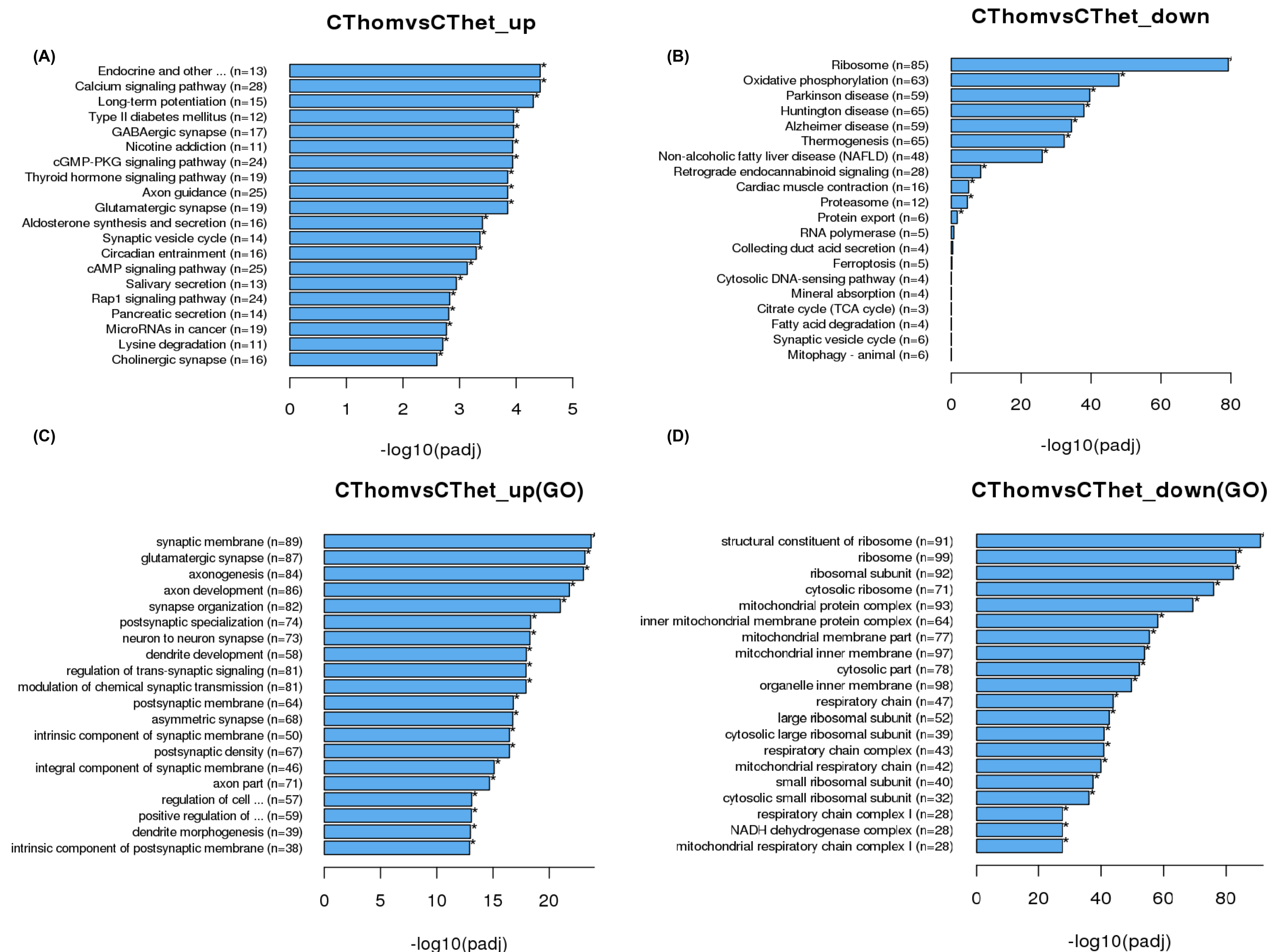


FIGURE 2 KEGG and GO function enrichment analysis. (A/C) Histogram of top 20 significant pathways enriched in the disease, biological process and signaling pathways that are predicted to be up-regulated (CThom vs. CTet-up) and (b/d) downregulated (CThom vs. CTet-down) in *fro/fro* versus *fro*/+ cortex. The top 10 significant pathways identified by KEGG, including the upregulated and downregulated biological function were also shown in our previous publication.[27] Moreover, the analysis of transcript abundance was done by either KEGG (A/B) or GO tools (C/D).

TABLE 1 Positively regulated nervous system development and function.

Diseases or functions annotation	*p*-Value	Activation state	Activation Z-score	#Molecules
Coordination	1.87E-18	Increased	5.538	62
Development of neurons	2.21E-37	Increased	5.22	203
Quantity of neurons	2.26E-15	Increased	4.382	93
Morphogenesis of neurons	9.41E-39	Increased	3.87	174
Neuritogenesis	2.33E-38	Increased	3.87	172
Long–term potentiation	2.96E-14	Increased	3.87	68
Potentiation of synapse	1.33E-14	Increased	3.661	69
Memory	1.26E-12	Increased	3.591	64
Long-term synaptic depression	9.16E-10	Increased	3.55	31
Migration of neurons	2.06E-10	Increased	3.426	53
Prepulse inhibition	1.70E-08	Increased	3.408	24
Neurotransmission	3.60E-24	Increased	3.39	108
Synaptic transmission	3.72E-22	Increased	3.05	91
Developmental process of synapse	6.69E-11	Increased	2.969	50
Extension of neurites	1.10E-10	Increased	2.767	42
Long-term synaptic depression of neurons	2.07E-08	Increased	2.709	24
Development of central nervous system	6.08E-22	Increased	2.601	146
Proliferation of neuronal cells	2.24E-18	Increased	2.566	118
Growth of neurites	4.50E-19	Increased	2.503	107
Formation of brain	9.74E-19	Increased	2.431	116
Dendritic growth/branching	6.39E-23	Increased	2.262	78
Long-term potentiation of brain	3.49E-10	Increased	2.204	34
Synaptic transmission of cells	7.46E-15	Increased	2.155	40
Long-term potentiation of cerebral cortex	1.20E-10	Increased	2.145	33
Synaptic transmission of nervous tissues	1.89E-09	Increased	2.12	28

TABLE 2 Negatively regulated nervous system development and function.

Diseases or functions annotation	*p*-value	Activation state	Activation z-score	#Molecules
Congenital neurological disorder	1.17E-13	Decreased	−4.688	143
Motor dysfunction of movement disorder	1.34E-31	Decreased	−4.595	238
Congenital encephalopathy	1.81E-11	Decreased	−4.009	103
Movement disorders	1.47E-30	Decreased	−3.999	233
Ataxia	8.16E-11	Decreased	−3.855	58
Congenital anomaly of central nervous system	8.01E-11	Decreased	−3.767	64
Congenital malformation of brain	8.14E-11	Decreased	−3.767	63
Seizures	2.09E-16	Decreased	−3.515	102
Seizure disorder	8.16E-17	Decreased	−3.497	112
Tremor	3.60E-11	Decreased	−2.411	38
Tonic–clonic seizure	2.49E-14	Decreased	−2.36	43
Epilepsy or neurodevelopmental disorder	2.42E-20	Decreased	−2.229	112
Epilepsy	1.36E-13	Decreased	−2.229	78
Glioma	2.59E-10	Decreased	−2.126	171

this evidence for strengthened synaptic connections among transcripts that increased is the evidence of enrichment for annotations related to PD, AD, and Huntington's disease (HD) among the transcripts that decreased in *fro/fro* relative to +/*fro* cortex. In other words, improved synaptic strength was accompanied by other changes that may reflect a decreased susceptibility to neurodegenerative diseases.

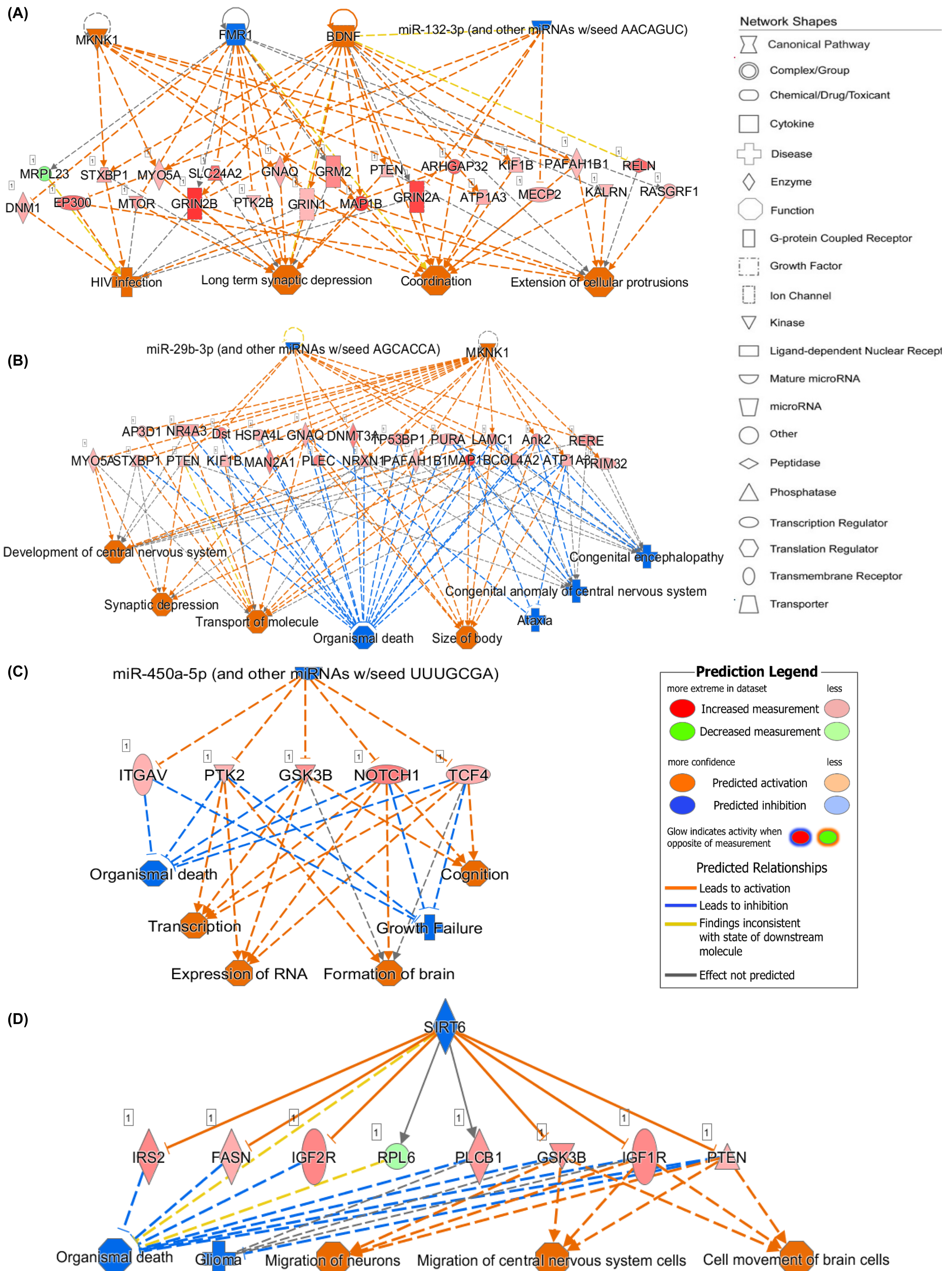


FIGURE 3 Legend on next page.

To further support these analyses, we determined whether IPA downstream effector analysis of the affected transcripts would predict increased synaptic strength and resistance to neurodegenerative diseases. Tables 1 and 2 present the lists of high-level annotation categories containing significantly overrepresented downstream functional categories among the affected cortical transcripts from *fro/fro* brain as compared with +/*fro* brain. Focusing on the most strongly affected categories to view more specific ontology categories we find that IPA predicts greater capacity for synaptic transmission, synaptic plasticity, memory, neural development, neuritogenesis, and other annotations related to a young, healthy nervous system, just as expected (Table 1). Also, as expected, it predicts decreased risk of several neural disorders due to decreased levels of transcripts encoding proteins that promote progression of these disorders (Table 2). Overall, the transcriptome of *fro/fro* mouse cortex appears to be functionally younger and have a correspondingly lower risk of neurological diseases than does +/*fro* mouse cortex.

3.3 | Causal network analysis of biological functions affected by nSMase2 (*Smpd3*) deficiency

To explore upstream events potentially explaining particular phenotypic or functional outcomes of nSMase2 deficiency we used IPA's mechanistic network analysis to build causal hypotheses. This identified five upstream regulator networks containing differentially abundant transcripts in our data. One of them, involving coordinate regulation of Grin2b and Bdnf, was described in our previous publication.[27] The remaining four are shown in Figure 3, which identifies eight major upstream regulators: Mknk1, Fmr1, Bdnf, Sirt6, and microRNAs miR-132-3p, miR-29b-3p, and miR-450-5p. These eight regulators represent several of the ways gene expression or translation is regulated, including signaling (Bdnf and Mknk1), mRNA splicing and translation (Fmr1 and the three microRNAs),[35–39] and epigenetic control of chromatin (Sirt6).[40] The predicted upstream regulators shown in Figure 3A explain effects such as the increases we observed in Grin2b, Grin2a, and Map1b transcripts in *fro/fro* cortex. As described above (Figure 2C), Grin2b and Map1b proteins are correspondingly increased in *fro/fro* cortex. Consistent with these data, Grin2a protein is increased in mouse brain treated with the nSMase2 inhibitor GW4869.[21] These coordinated changes in transcriptome and encoded proteins are predicted to affect the biological functions long term synaptic depression, coordination, HIV infection, and extension of cellular protrusions in *fro/fro* cortex (Figure 3A). Similarly, in Figure 3B–D, changes in Mnk1, Sirt6, miR-29b-3p, and miR-450-5p potentially explain other sets of transcriptome changes that the analysis predicts to be linked to downstream activation effects on processes such as neural development, brain formation, migration of neurons, transcription, cognition, synaptic depression, transport of molecules, and body size. This analysis also predicts simultaneous downstream inhibitory effects on growth failure, organismal death, congenital neural anomalies, ataxia, and glioma. Consistent with the simpler analyses of overrepresented annotations linked to genes whose transcripts are affected by nSMase2 deficiency, these analyses indicate that the transcriptome of *fro/fro* mouse cortex is more plastic and predicts a lower risk of neurological diseases than does *fro*/+ mouse cortex.

3.4 | The transcript clusters classified either as upregulated or downregulated in nervous system development and function

Genetic deficiency of nSMase2 (*Smpd3*) improved cognitive function in 10-month-old 5 × FAD mice, demonstrating that deficiency of nSMase2 in brain positively regulates a major nervous system function.[17] Therefore, we focused an additional analysis on the clusters of transcripts responsible for predicted changes in risk of neurological diseases or functions in *fro/fro* cortex. IPA returned seven clusters associated with the prediction of increased cognitive-related functions in *fro/fro* cortex: memory (Figure 4A), coordination (Figure 4B), conditioning (Figure 4C), learning (Figure 4D), cognition (Figure 4E), short-term memory (Figure 4F), and cued conditioning (Figure 4G) (Figure 4 and Table 1). The majority of transcripts in these clusters increased in abundance in *fro/fro* cortex and/or have positive effects on the associated functions, hence the prediction that these functions are enhanced.

IPA also returned transcript clusters associated with decreased risk of neurological diseases: movement disorders (Figure 5A), ataxia (Figure 5B), motor dysfunction (Figure 5C), seizure disorders (Figure 5D), epilepsy (Figure 5E), paralysis (Figure 5F), seizures (Figure 5G), tremor (Figure 5H), and tonic–clonic seizure (Figure 5I) (Figure 5 and Table 2). These transcript clusters are a mix of increased and decreased transcripts in in *fro/fro* cortex, but the vast majority act to decrease risk of these disorders.

4 | DISCUSSION

4.1 | nSMase2 appears to regulate cognition, learning, memory in the brain

Our previous study showed that global nSMase2 deficiency in 10-month-old 5 × FAD mice (*5 × FAD; fro/fro*) leads to improved cognition.[17] In another study, *Smpd3* knockout animals showed

FIGURE 3 Mechanistic network analysis of biological functions affected by nSMase2 deficiency. Major mechanistic networks identified among the affected transcripts. Upstream regulators predicted to be activated (orange-pink) or inhibited (blue) have known links to downstream transcripts that increased (red) or decreased (green). Predicted outcomes on biological processes or diseases are shown in the lower tier of each panel.

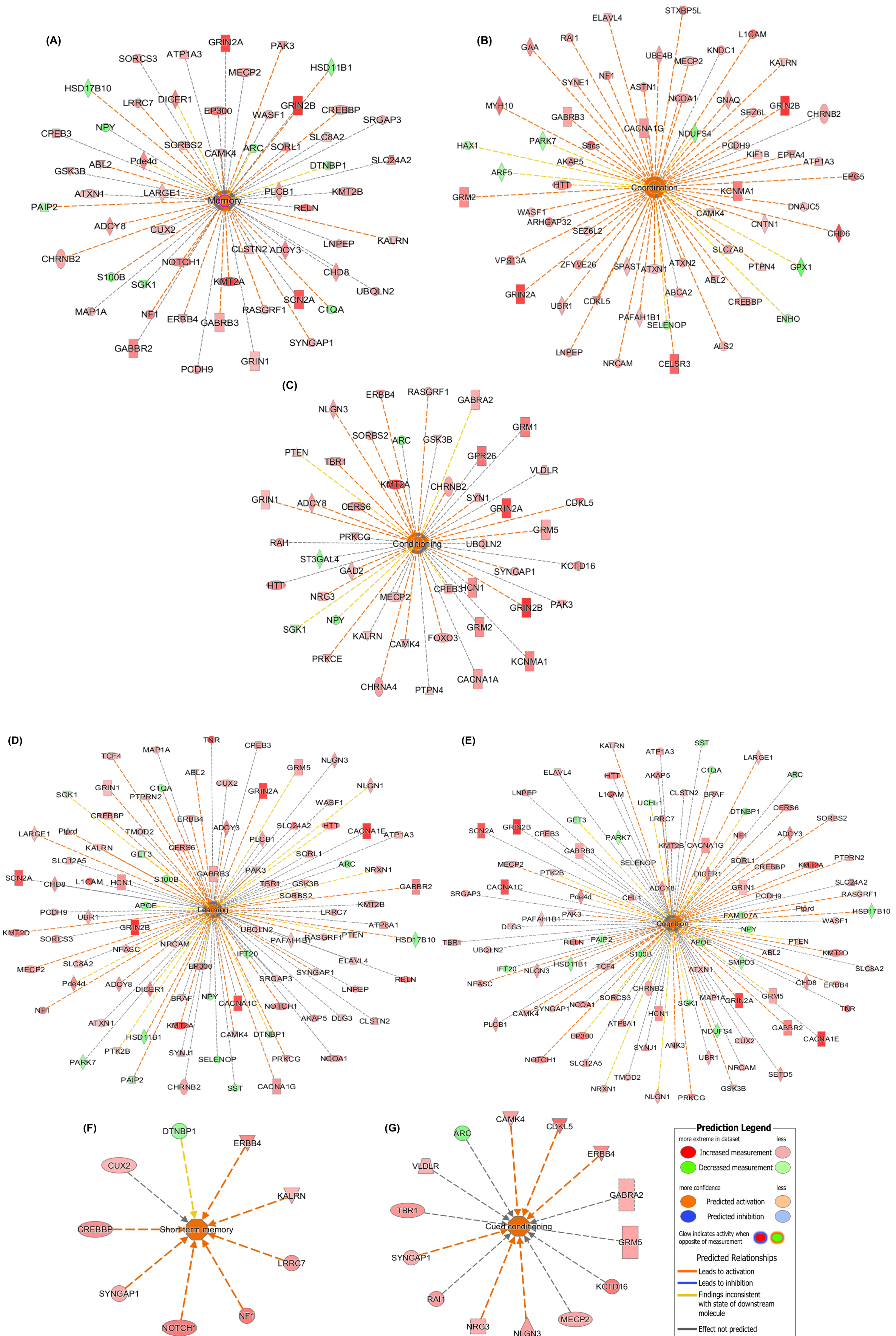


FIGURE 4 Legend on next page.

accumulation of APP, Aβ, and pTau in neurons, and impaired cognition in the cognitive related behavior tests.[41] These seemingly contradictory results may be resolved by understanding the different effects of lacking nSMase2 protein (*Smpd3* knockout mice) versus expressing nSMase2 that is enzymatically inactive (*fro/fro* mice). Both lead to an absence of nSMase2 enzymatic products but the latter may also allow unproductive interactions with upstream regulators, similar to how dominant negative proteins act. In this sense, and unlike nSMase2 deficiency, the *fro/fro* mouse phenotype is likely to be similar to that of pharmacological inhibition of nSMase2.

In our RNA-seq data from nSMase2 deficient, middle-aged *fro/fro* mice we find substantial numbers of mRNA abundance differences.[26] This further comprehensive analysis of the affected transcripts reveals how nSMase2 may contribute to gene expression and function in the aging brain. Because the data do not show evidence of changes in the number or proportions of the major brain cell types in these mice, we conclude that differences in mRNA abundance in these mice are mainly due to direct effects on gene expression. The increased abundance of mRNAs associated with neural development and neuritogenesis in *fro/fro* mice (Table 1) are primarily mRNAs linked to processes supporting increased synaptic contacts between neurons rather than increased numbers of neurons. However, that some differences could be due to shifts in the prevalence and changes of populations of certain cell subtypes remain possible.

Ten-month-old *fro/fro* mice show evidence of effects on gene expression consistent with improved cognition, learning, short term memory, cued conditioning, memory, and cued conditioning consistent with our previous study showing improved cognitive function in *5 × FAD: fro/fro* mice.[17] These results are different from the prior study showing the deficits of cognitive function in the *Smpd3* knockout animals.[41] A key change driving improved neural function in *fro/fro* mice appears to be increased expression of *Grin2b*, also known as N-methyl D-aspartate (NMDA) receptor subtype 2B (*Nr2b*) resulting in increased amounts of Grin2b/Nr2b protein in *fro/fro* cortex. Grin2b/Nr2b is a subunit of NMDA receptors that provide the associative property necessary for many instances of long-term potentiation and is important for maintaining cognitive function in aged mice.[42,43] Its expression is highest in young mice when learning is especially critical and diminishes along with Nr1 and Nr2a (Grin2a) NMDA receptor subunits during the transition from juvenile ages to adulthood.[44–46] Combining our new analyses with previous data comparing *fro/fro* versus +/*fro* cortex,[27] we identify changes in a set of transcripts and proteins capable of driving increased expression of Grin2b/Nr2b and other genes encoding proteins involved in neural plasticity and cognition. The predicted regulatory factors include Bdnf, Frmr1, Mknk1, and microRNAs miR-132-3p and miR-223-3P. The latter is particularly interesting because exosomes from brains of *fro/fro* mice carried less of this microRNA, a way in which cells that express nSMase2 could alter *Grin2b/Nr2b* expression in neighboring cells that do not express nSMase2.[27] Furthermore, *Grin2a* expression also appears increased in *fro/fro* cortex, consistent with upregulation of Grin2a protein in the mouse brain after administration of nSMase2 inhibitor.[21] Partial or total loss of Grin2a function is associated with the occurrence of epilepsy, seizures, learning disabilities, and speech abnormalities.[47–50] Coordinately increased expression of NMDA receptor subunits seems a likely contributor to improved cognition observed in nSMase2-deficient mice. A network of additional transcriptional changes probably also contributes to this phenotypic difference. For example, our analysis predicts that increased expression of *Adcy3*, *Adcy8*, *Atp8A1 Atp1A3*, *Atxn1 Camk4* and *Notch1*,[51–58] which are known to contribute to learning, memory, and cognition. In contrast, other genes believed to negatively affect cognition appear to have decreased expression in *fro/fro* mice, examples are: *Apoe*, *S100b*, *Arc*, *C1qa*, *Dtnbp1*, *Hsd11b1*, *Hsd17b10* and *Paip2*.[59–67]

4.2 | nSMase2 mediates exosome biogenesis linked to neurodegenerative diseases

Cd9 and Cd63 transcripts are less abundant in *fro/fro* mouse cortex compared with the +/*fro* control.[27] These transcripts encode plasma membrane tetraspanins abundant in exosomes. Ceramide produced by nSMase2 is important for budding of intraluminal vesicles to form multivesicular bodies including exosomes.[68] Exosomes are enriched in ceramide and their secretion is reduced through inhibition of nSMase2 with siRNA[69,70] or the small molecule inhibitor GW4869.[71–73] Conversely, generation of ceramide is critical for exosome biogenesis and secretion.[74]

This important role of nSMase2 in exosome biogenesis and secretion links nSMase2 with AD and PD. Our analyses predict that nSMase2 deficiency reduces susceptibility to neurodegenerative diseases such as these and evidence from other studies provide further support for this hypothesis. For example, exosomes propagate the spreading of extracellular tau in vivo[75] and mice injected with the nSMase2 inhibitor GW4869 exhibit a significant reduction of the amount of human-tau in the germinal cell layer weeks after its viral transduction in the entorhinal cortex.[76] According to our prior studies, prevention of nSMase2-directed exosome generation and release rescued Aβ-caused neuronal cell death in a mouse model of AD.[17] In a mouse model of PD reducing nSMase2 significantly decreases the transfer of oligomeric α-synuclein between neuron-like cells and reduces α-synuclein aggregation.[24] Exosomes are the critical vesicles

FIGURE 4 Clusters of transcripts that regulate nervous system behaviors predicted to be positively affected. IPA classified all of the activated (red) and inhibited molecules (green) in each nervous system function that is predicted to be enhanced in *fro/fro* cortex, such as (A) memory, (B) coordination, (C) conditioning, (D) learning, (E) cognition, (F) short-term memory and (G) cued conditioning.

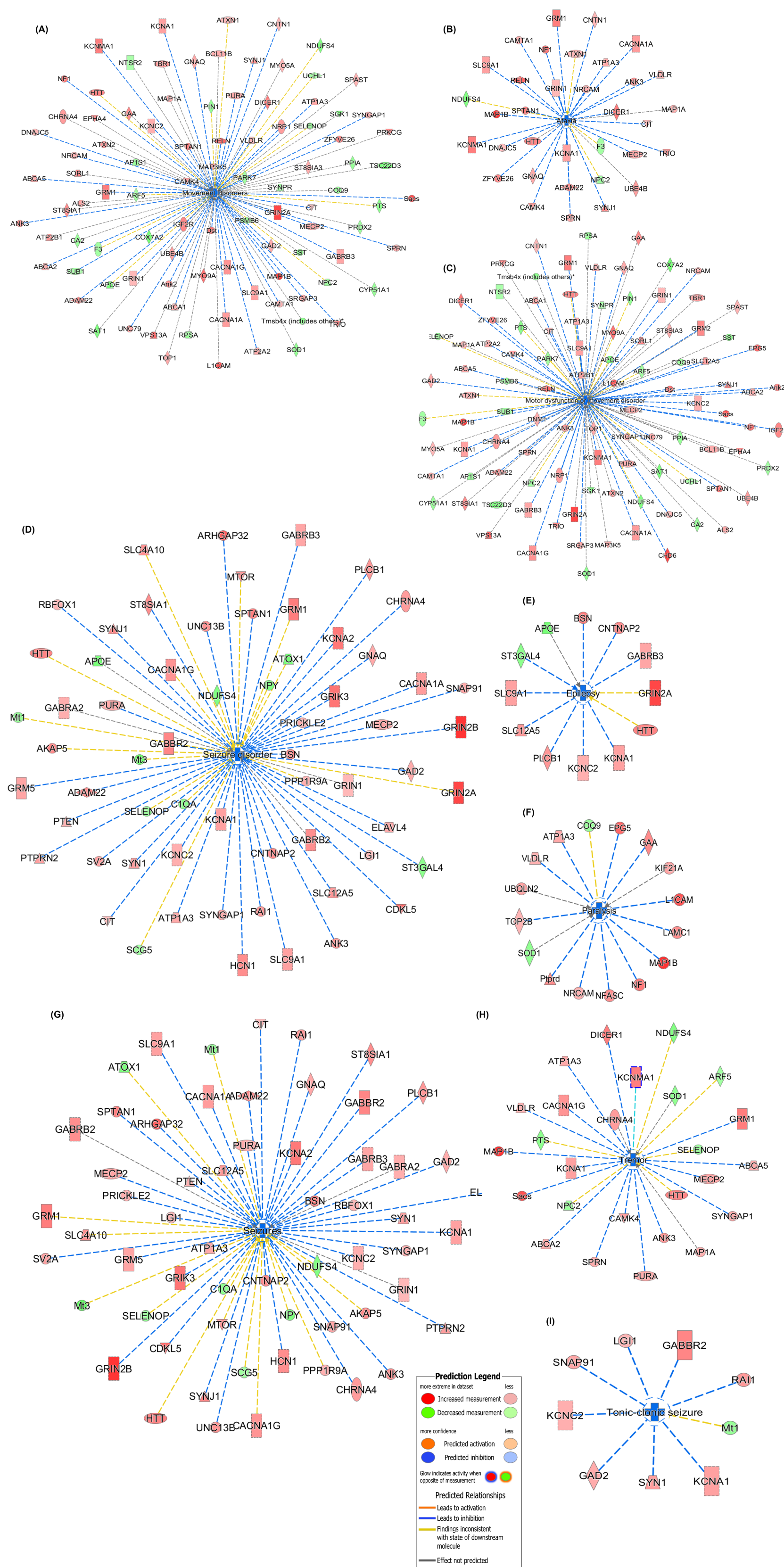


FIGURE 5 Clusters of transcripts that regulate nervous system behaviors predicted to be negatively affected. IPA classified all of the activated molecules (red) and inhibited molecules (green) which contribute to nervous system functions that are predicted be inhibited in *fro/fro* cortex, such as (A) movement disorders, (B) ataxia, (C) motor dysfunction of movement disorder, (D) seizure disorder, (E) epilepsy, (F) paralysis, (G) seizures, (H) tremor and (I) tonic–clonic seizure.

responsible for loading and transporting toxic Aβ and α-synuclein among brain cells. This argues that nSMase2 inhibition may be a strategy for reducing the pathogenesis of AD and PD. In future studies, we will investigate if altering gene expression by nSMase2 deficiency is due to decreasing ceramide levels in neurons, exosome-mediated effects, or both.

4.3 | Promising role of pharmaceutical inhibitors of nSMase2 to prevent and treat aging-related neurodegenerative diseases

Our analyses indicate that nSMase 2 deficiency in middle-age mice reduces risk of neurodegenerative diseases such as AD, PD, and Huntington's Disease (HD). These neurodegenerative diseases share common genetic, biological, and cellular mechanisms,[77] which provides a rationale for developing common approaches for disease prevention and therapy. For instance, small EVs such as exosomes are involved in neurodegenerative diseases, including AD, tauopathies, prion diseases, and aggregation of α-synuclein in PD pathology.[24] Inhibition of nSMase2 has already shown beneficial effects in animal models of primary tauopathy,[78,79] AD,[80] and PD.[72]

5 | CONCLUSIONS

The analysis of RNA-seq data from mouse brain cortex deficient in nSMase2 predicts that *fro/fro* mice have increased capacity for neural plasticity, as well as greater resistance to some neurodegenerative disorders. Modulating nSMase2 activity may therefore prove to be therapeutically advantageous. Genetically or pharmaceutically regulating nSMase2 is a promising approach to prevent and treat aging-related neurodegenerative diseases.

AUTHOR CONTRIBUTIONS

Drs. McClintock and Zhu performed the RNA-seq analysis, Dr. Zhu performed the RT-PCR and WB, and wrote the manuscript. Drs. Bieberich and McClintock revised the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article [and its supplementary information files. The RNA-seq data are available in the Gene Expression Omnibus (GEO) database (accession number GSE179045). The datasets used and/or analyzed during the current study are available from the corresponding author (zzh295@uky.edu) upon reasonable request.

ETHICS STATEMENT

Mice were handled according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All procedures involving mice were approved by the Institutional Animal Care and Use Committee of University of Kentucky. This study did not involve human subjects. The use of experimental animals and protocol used in this study was approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

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