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Cranial Manipulation Affects Cholinergic Pathway Gene Expression in an Animal Model of Age-related Cognitive Decline

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Short report

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Abstract

Age dependent dementia is a devastating disorder afflicting the growing older population around the world. Although pharmacological agents improve symptoms of dementia, age related co-morbidities combined with adverse effects often outweigh their clinical benefits. Therefore, non-pharmacological therapies are being investigated as an alternative. Randomized controlled trials and observational studies have shown promising results for cranial manipulation as a treatment for dementia and other nervous system disorders. In this study we examine the effect of osteopathic cranial manipulative medicine (OCMM) on gene expression, in an animal model for age-related cognitive decline (aged rats). We found that OCMM significantly affected the expression of 36 genes in the neuronal pathway (False Discovery Rate (FDR) < 0.004). The top five neuronal genes with the largest fold-change (Slc5a7, Chat, Slc18a3, Adcy5 and Cacna2d2, >2-fold change, FDR<0.004) are part of the cholinergic neurotransmission mechanism, which is known to affect cognitive function. Slc5a7, the highest overexpressed neuronal gene (3-fold change) encodes a sodium and chloride ion-dependent high-affinity transporter that mediates choline uptake for acetylcholine synthesis in cholinergic neurons. This is the pathway enhanced by the clinically used Alzheimer's disease drug Donepezil, which selectively inhibits acetylcholinesterase, an enzyme that catalyzes endogenous acetylcholine degradation. In addition, 40% of significant differentially expressed (SDE) genes (FDR<0.004), have been previously implicated in neurological disorders. Overall, SDE genes and their role in central nervous system signaling pathways suggest a connection to previously reported OCMM induced behavioral and biochemical changes in rat models of age-dependent dementia. Further investigation in this direction will provide a better understanding of the molecular mechanisms of OCMM and its potential in clinical applications. With clinical validation, OCMM could represent a much needed low-risk adjunct treatment for age-related dementia including Alzheimer's disease.

Introduction

Dementia is a progressive neurodegenerative disease primarily afflicting a rapidly growing older population[1], and one of the primary causes of disability and dependency in this population[2]. Over 50 million people suffer from dementia worldwide, with approximately 10 million new cases per year[2]. Alzheimer's disease contributes to 60–80% of dementia cases[3]. Although there are treatments to manage its symptoms, currently there is no cure for dementia or a treatment for halting its progression or delaying its onset.

Two classes of drugs have been approved for treating dementia symptoms: acetylcholinesterase (AChE) inhibitors for mild to moderate symptoms and an N-methyl-D-aspartate receptor (NMDAR) antagonist for moderate to severe symptoms[4–6]. Although these drugs have been effective in managing the symptoms in the short-term (3–6 months), their longer term benefits are questionable[7–9]. Combined with age-related comorbidities, the undesirable side effects of these drugs often outweigh their benefits.

OCMM as a potential adjunct treatment for age-related dementia

Osteopathic cranial manipulative medicine (OCMM) represents a potential low-risk adjunct treatment to minimize the dosage or use of pharmaceutical treatments. Randomized controlled trials have shown that cranial manipulation can reduce pain in patients with fibromyalgia[10, 11] and lateral epicondylitis[12]. Observational studies have shown a reduction in symptoms associated with dementia[13] and multiple sclerosis[14]. Studies have also shown that OCMM affects cerebral blood flow[15, 16], tissue oxygenation in prefrontal lobes[17], brain cortex electrical activity[18] and reduction in Amyloid beta (Aβ) protein levels[19, 20]. How might OCMM produce these effects? *In vivo* and *in vitro* studies have shown that mechanical stress can affect cellular activity and growth in neuronal cells[21–23]. Simulated mechanical stimuli, similar to sub-traumatic cranial pressure, was shown to induced cellular activity in neural cell cultures by modulating ion channels[21]. Mechanical compression of neural stem cells was shown to contribute to neurogenesis and neuronal migration[22]. Compression of the cerebral cortex simulated by epidural bead implanted in rats, showed a rapid increase in NMDA receptor concentration and postsynaptic activity[23].

In a previous study we showed that OCMM treatment improves spatial memory in aged rats (an animal model for age-related cognitive decline), as measured by the Morris water maze assay[19]. To identify a possible molecular mechanism for the effect of OCMM, in this study, we analyzed gene RNA expression in prefrontal cortex tissue samples from OCMM treated aged rats and from untreated (control) rats. Transcriptional regulation is crucial for organogenesis, functional adaptation, and regeneration in adult tissues and organs[24]. Study of the transcriptome can provide insights into changes that affect subsequent proteins synthesis, trafficking and cellular activity.

Results And Discussion

OCMM significantly affects gene expression

RNA from tissue samples from the prefrontal cortex of three OCMM treated rats and three untreated control rats were extracted and sequenced as described in *Methods*. The number of reads from RNA sequencing ranged from 58–74 million with average read lengths of 73–75, across the six samples. Ninety six percent of the reads were successfully mapped to the rat genome. See Table S1 for a detailed breakdown of read mapping.

We compared gene expression levels calculated from the sequencing data for OCMM treated and untreated animals. The comparison showed that 688 genes were differentially expressed with FDR < 0.01, of which 426 had FDR < 0.004 (Fig. 1a). For the following analysis we focus on the 426 genes with FDR < 0.004, which we refer to as significant differentially expressed (SDE) genes. Of these 426 SDE genes, 314 were over expressed and 112 under expressed.

Neuronal system pathways were over-represented in SDE genes

The Reactome database contains a list of genes that have been associated with specific pathways[25]. We compared the distribution of Reactome top-level pathways associated with all genes that were sequenced, to the distribution of these pathways for SDE genes. Genes associated with neuronal system pathways were most significantly over-represented in the SDE gene set with FDR = 3E-14 (Fig. 1b). Thirty six (8%) of 426 SDE genes were associated with neuronal pathways (Fig. 1c), compared to 343 (2%) of all 14278 genes that were sequenced. The signal transduction pathway was also over-represented with FDR = 1E-5, while the gene expression pathway was under-represented with FDR = 5E-5. In addition, pathways for protein metabolism, cell cycle, and response to stress were under-represented and the muscle contraction pathway over-represented, with FDR < 0.01.

Cholinergic neurotransmission pathways were affected by SDE genes

Of the 36 SDE genes in the neuronal system pathway, 27 of them were over-expressed and 9 of them under-expressed in OCMM treated animals compared to untreated animals (Fig. 1c). Twenty of the 36 neuronal SDE genes were associated with signal transmission chemical synapses, 13 with potassium channels and 8 with protein-protein interaction at synapses.

The five genes with the largest fold change, Slc5a7, Chat, Slc18a3, Adcy5 and Cacna2d2 (Fig. 1c), are part of the acetylcholine (ACh) neurotransmission mechanism (Fig. 2), suggesting increased cholinergic neurotransmission activity in OCMM treated rats. The high affinity choline transporter, Slc5a7 (CHT1), mediates choline uptake at the presynaptic neuron terminal[26, 27]. Choline uptake is a rate limiting step in ACh synthesis and thus ACh mediated neurotransmission[28]. The choline acetylase, Chat, catalyzes the biosynthesis of the ACh neurotransmitter from choline and acetyl-Coenzyme-A[29]. The vesicular ACh transporter, Slc18a3 (VAChT), transports ACh into secretory vesicles for release into the synaptic cleft[30]. Neuronal action potential activates the voltage-gated calcium channel[31], of which Cacna2d2 forms the alpha2/delta2 subunit[32]. The influx of calcium ions, promotes ACh secretion. ACh binding to the acetylcholine receptors (AChR), a G-protein coupled receptor (GPCR), on the postsynaptic neuron, triggers the release of the Gα subunit from the G-protein complex. Gαs binds to Adenylate Cyclase 5, Adcy5, activating downstream cAMP signaling pathways, whereas Gαi inhibits downstream signaling[33]. The above pathway is targeted by the Alzheimer's disease drug Donepezil. Donepezil selectively inhibits acetylcholinesterase which catalyzes acetylcholine degradation.

The two most under-expressed genes based on fold change are Chrna3 and Chrnb4 (Fig. 1c). These genes code for the α3 and β4 subunits of the pentameric nicotinic ACh gated ion channel receptor (nAChR)[34]. Some studies have suggested nicotine desensitization (inactivation) of nAChR improves memory function in Schizophrenia and Alzheimer's patients[34–36]. Reduced expression of nAChR, as

suggested by the reduced expression of Chrna3 and Chrnb4 shown above, could contribute to improved cognitive function.

SDE genes have been implicated in neurological disorders

The neurological importance of the genes discussed above is evident from their role in neurological disorders. CHT1 was found to be overexpressed in Alzheimer's disease patients, presumably to compensate for the reduced cholinergic synaptic availability[37]. Polymorphisms in Chat have been associated with increased risk of Alzheimer's disease[38, 39], and reduced Chat expression was found in Parkinson's dementia[40] and Schizophrenia[41] patients. VAChT defects have been implicated in myasthenia syndrome[42] and reduced levels of VAChT have been associated with Alzheimer's disease[43]. Mutations in Cacna2d2 have been linked to epileptic encephalopathy[44]. Adcy5 mutations have been associated with dyskinesia, myokymia, chorea and dystonia[45]. nAChR has been linked to schizophrenia, Alzheimer's and other neurological disorders[34–36].

Overall, 40% of the 426 SDE genes were associated with neurological disorders, with 17% being associated with dementia, 22% with movement disorders, and 28% with psychiatric disorders (Fig. 3). See Table S2 for a list of the 426 genes and references for associated disorders.

Conclusions

This study has shown that OCMM treatment affects the expression of genes associated with neurological pathways and disorders, based on an animal model of age-related dementia. This connection suggests that OCMM could affect the progression of age-related dementia, providing further support for investigating OCMM as a potential adjunct treatment. With clinical validation using robust placebo-controlled double-blind studies, OCMM may offer a much needed low-risk adjunct treatment for age-related cognitive decline.

Materials And Methods

Aged rats

Six 18-month-old F344 male rats were obtained from Charles River Laboratories, Inc, and Envigo. Three of them were randomly selected for OCMM treatment. All rats were provided with normal food and water ad libitum and housed with 12-hour light-dark cycle. All methods were performed in accordance with the relevant guidelines and regulations. All animal experimental procedures and animal housing were approved by the Institutional Animal Care and Use Committee of Virginia Tech (protocol No. 15–099).

OCMM protocol

All OCMM procedures were performed by an experienced Doctor of Osteopathy (H.T.). The protocol consisted of the following steps:

- All rats, including untreated rats, were aestheticized with 1.5–3% isoflurane
- For OCMM treatment (CV4 technique), mechanical pressure (3–4 newtons) was applied over the rat's occiput, medial to the junction of the occiput and temporal bone and inferior to the lambdoid suture to place tension on the dural membrane around the fourth ventricle. This gentle pressure was applied to resist cranial flexion with the aim of improving symmetry in the cranial rhythmic impulse (CRI), initiating a rhythmic fluctuation of the CSF, and improving mobility of the cranial bones and dural membranes. This rhythmic fluctuation is thought to be primarily due to flexion and extension that takes place at the synchondrosis between the sphenoid and basiocciput. The treatment end point was achieved when the operator identified that the tissues relaxed and improved symmetry or fullness of the CRI was felt (~ 7 minutes).
- Treatment was performed every day for seven days.
- All rats were euthanized by cervical dislocation after seven days of OCMM treatment.

Tissue sampling, RNA extraction and sequencing

Using a rat brain matrix, a 1 mm coronal section from the dorsal end prefrontal cortex was sampled from the euthanized rats. RNA was extracted using the PureLink RNA Mini Kit. Extracted RNA were sequenced on the Illumina NextSeq 500 at Virginia Tech's sequencing center. Single-read sequencing was used to investigate highly expressed genes in the well annotated rat genome.

Differential expression calculation

The STAR v2.7.3a software package was used to map the fastq format sequencing data. STAR was run with runThreadN = 8, outSAMtype = BAM SortedByCoordinate, quantMode = GeneCounts, outSAMstrandField = intronMotif, and outFilterIntronMotifs = RemoveNoncanonicalreads.

The Cufflinks software suite[46] was used to assemble transcriptomes and quantify RNA expression level for each sample. The transcriptomes from all samples were merged into a master transcriptome using Cuffmerge program in Cufflinks. The Cuffdiff program in Cufflinks was then used to calculate significance of differential expression (fold-change, p-value and FDR) between samples from OCMM treated and untreated animals.

Pathway analysis

Gene-pathway association data was downloaded from the Reactome database[25]. The 24 top-level Reactome pathway associated with each gene was identified. The percentage of all genes and SDE genes in each top-level pathway was used to calculate the significance of any differences. The chi-squared test statistic was used to calculate p-value. The Benjamini-Hochberg correction for multiple-testing was then applied to calculate FDR.

Neurological disease association analysis

A literature search for each of the 426 SDE genes was performed to identify any associations between the gene and neurological disorders.

Abbreviations

OCMM
Osteopathic Cranial Manipulative Medicine
SDE
Significant Differentially Expressed
CSF
Cerebrospinal Fluid
FDR
False Discovery Rate
SDE
Significant differentially expressed
Ach
Acetylcholinesterase
Ch
Choline
AChR
ACh receptors
AChE
Acetylcholinesterase
VAChT
Vesicular acetylcholine transporter
CHT1
Choline transporter-1
CHT2
Choline transporter-2
Chat
Choline acetylate
nAChR
Nicotinic ACh gated ion channel receptor

Declarations

Ethics approval and consent to participate

All animal experimental procedures and animal housing were approved by the Institutional Animal Care and Use Committee of Virginia Tech (protocol No. 15-099.

Consent for publication

Not applicable

Availability of data and materials

The datasets supporting the conclusions of this article are available in the Gene Expression Omnibus (GEO) repository, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE157736.

Competing interests

The authors declare that they have no competing interests

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Author contributions

RA analyzed and interpreted RNA sequencing data and wrote the manuscript. TH performed the OCMM procedures on the rats. SN and OGS conducted the literature search to identify neurological disorders associated with SDE genes. BGK contributed to study design and manuscript preparation. BMC designed the study, participated in interpreting the results and in writing the manuscript. All authors reviewed and approved the manuscript.

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Figures

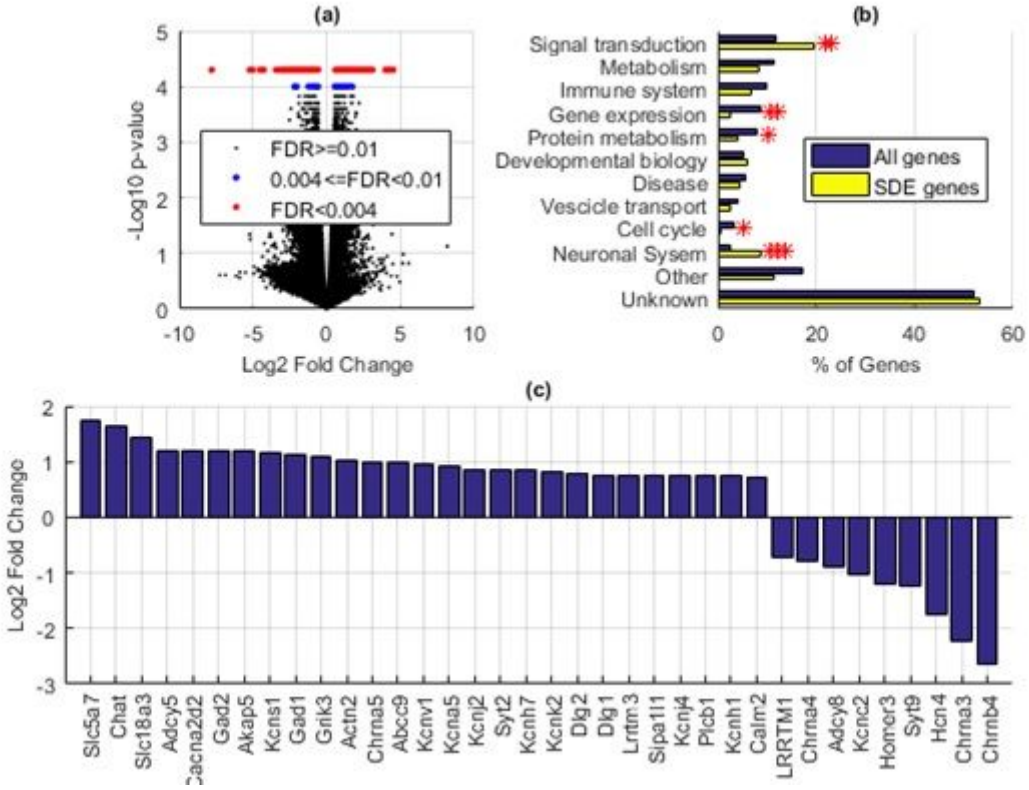


Figure 1

Significant differentially expressed (SDE) gene pathways. (a)Volcano plot identifying the 246 SDE genes with FDR < 0.004, p-value < 6E-5, t-test, N=3 OCMM treated and 3 untreated rats. (b) Distribution of pathways associated with all genes and SDE genes. Total can be > 100% because genes can be associated with multiple pathways. ***FDR=3E-14, p-value=1E-15 **FDR < 5E-5, p-value<6E-6 *FDR < 0.01, p-value<0.001, chi-squared test, N=14278 all genes and 426 SDE genes. (c) Thirty six of the 426 SDE genes are in neuronal system pathways.

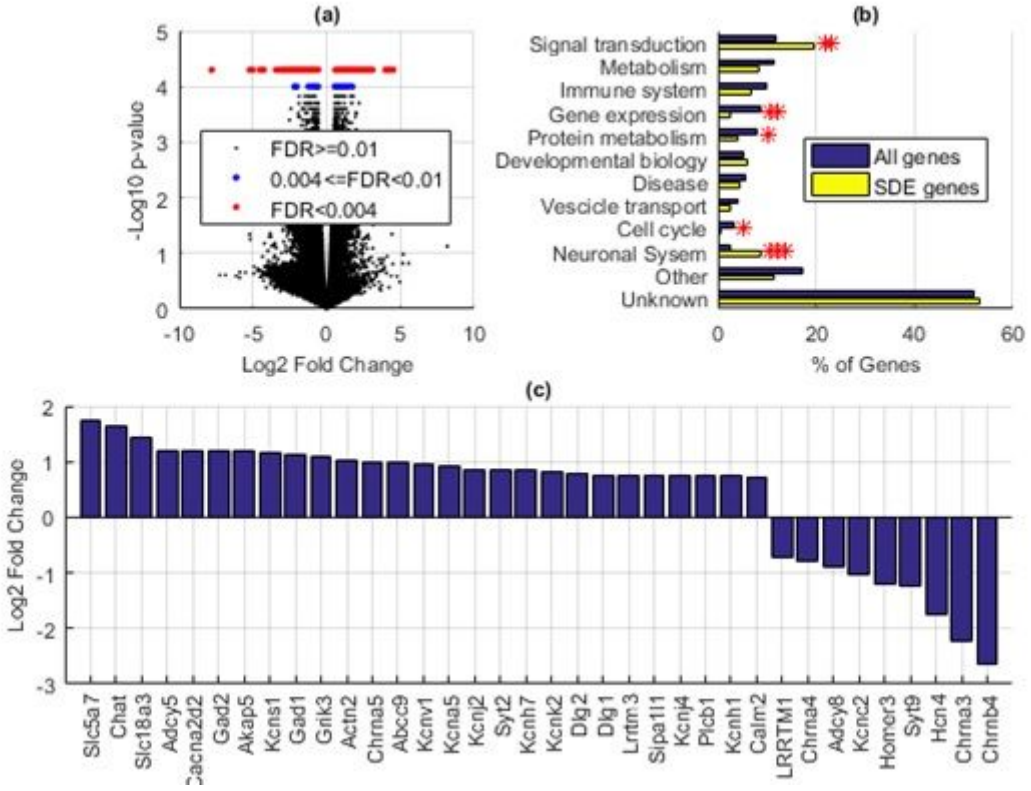


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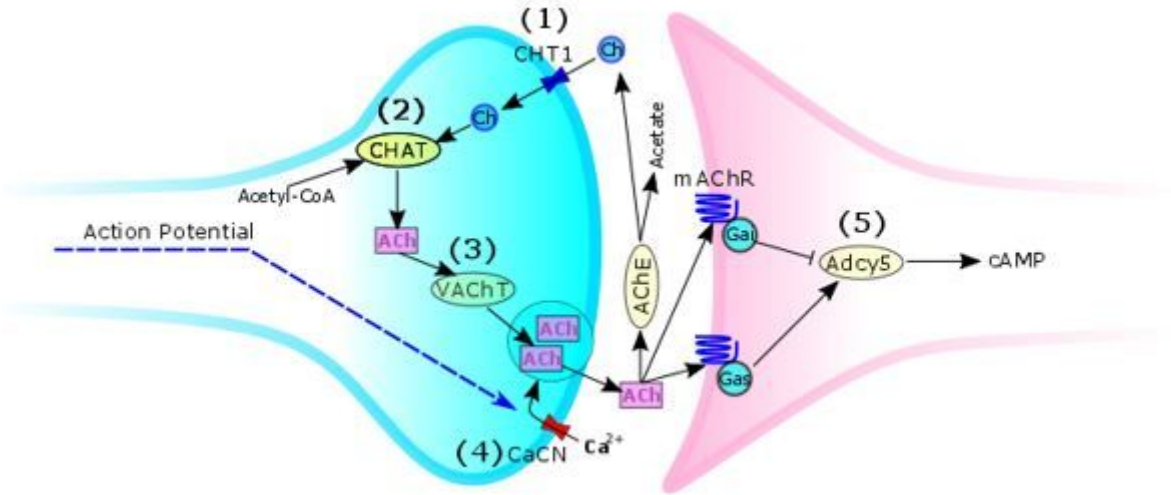


Figure 2

The top five over-expressed genes, CHT1, CHAT, VAChT, CaCN and Adcy5, in the neuronal pathway are part of the acetylcholine (ACh) neurotransmission mechanism. (1) The high affinity choline (Ch) transporter CHT2 transports Ch into the presynaptic terminal. (2) The choline acetylase Chat catalyzes the synthesis of ACh from Ch and acetyl-Coenzyme-A. (3) The vescicular ACh transporter transports ACh in to secretory vescicles. (4) Neuronal action potential opens the voltage gated calcium channel (CaCN). Ca ions promote the secretion of Ach into the synaptic cleft. (5) ACh binding to ACh receptors (AChR) on the post synaptic terminal, promotes the release of the Gα subunit of the G-protein complex from the GPCR. Gα can promote or inhibit the cAMP pathway (depending on the type of Gα protein) by binding to Adenylate Cyclase 5, Adcy5. Acetylcholinesterase (AChE) breaks down ACh so Ch can be recycled.

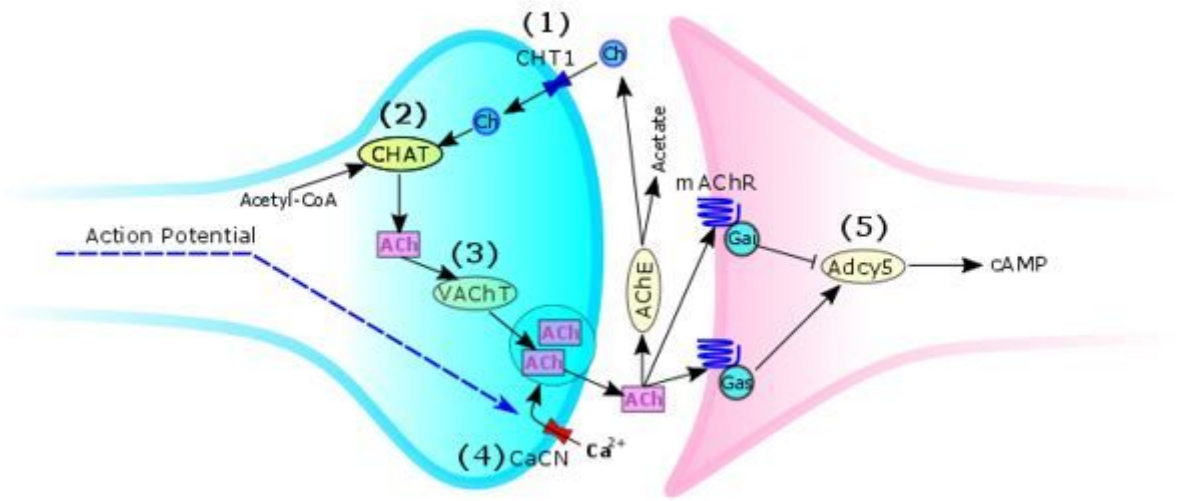


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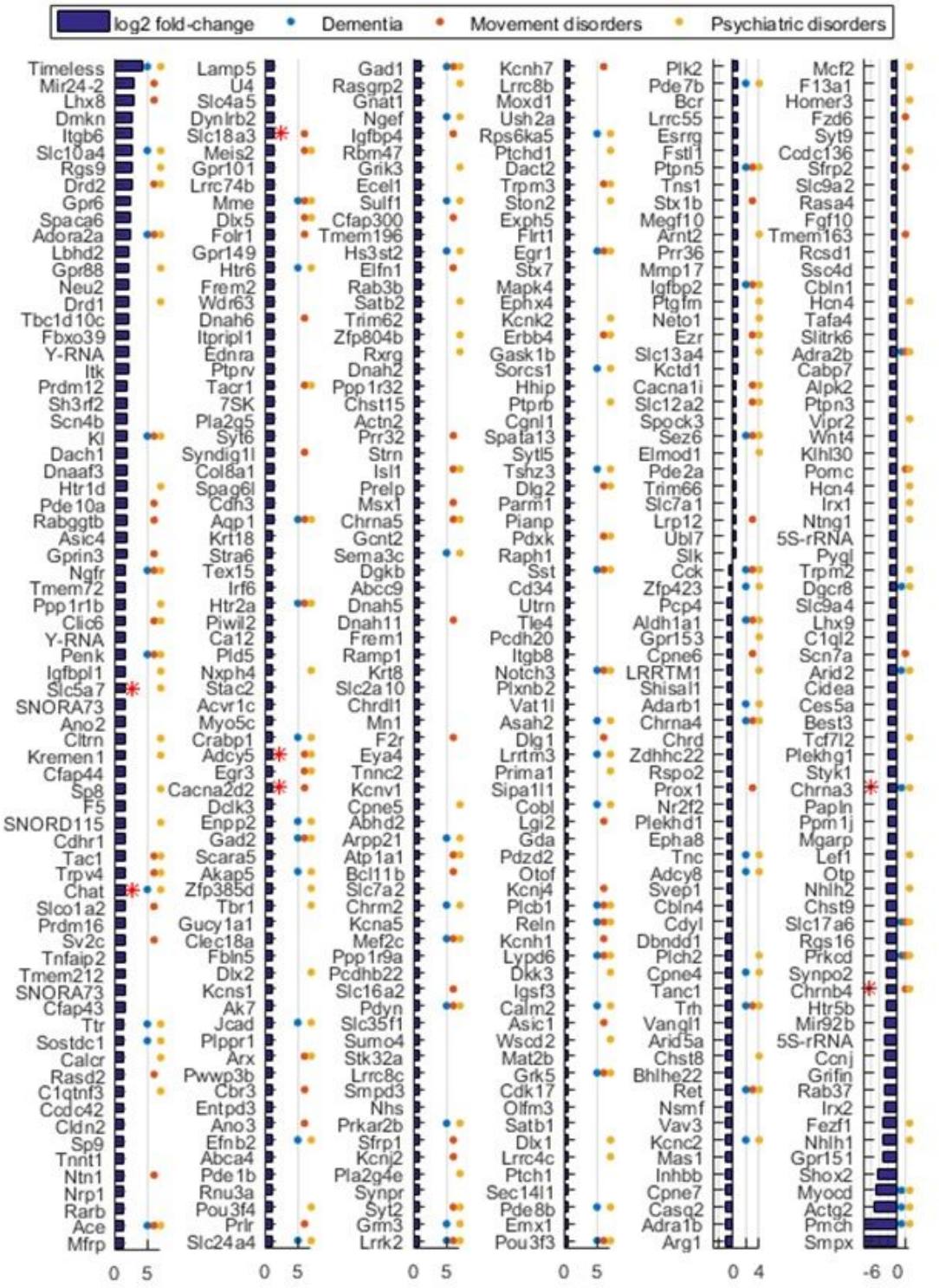


Figure 3

Forty percent of the 426 SDE genes were associated with neurological disorders. * Genes discussed in the text.

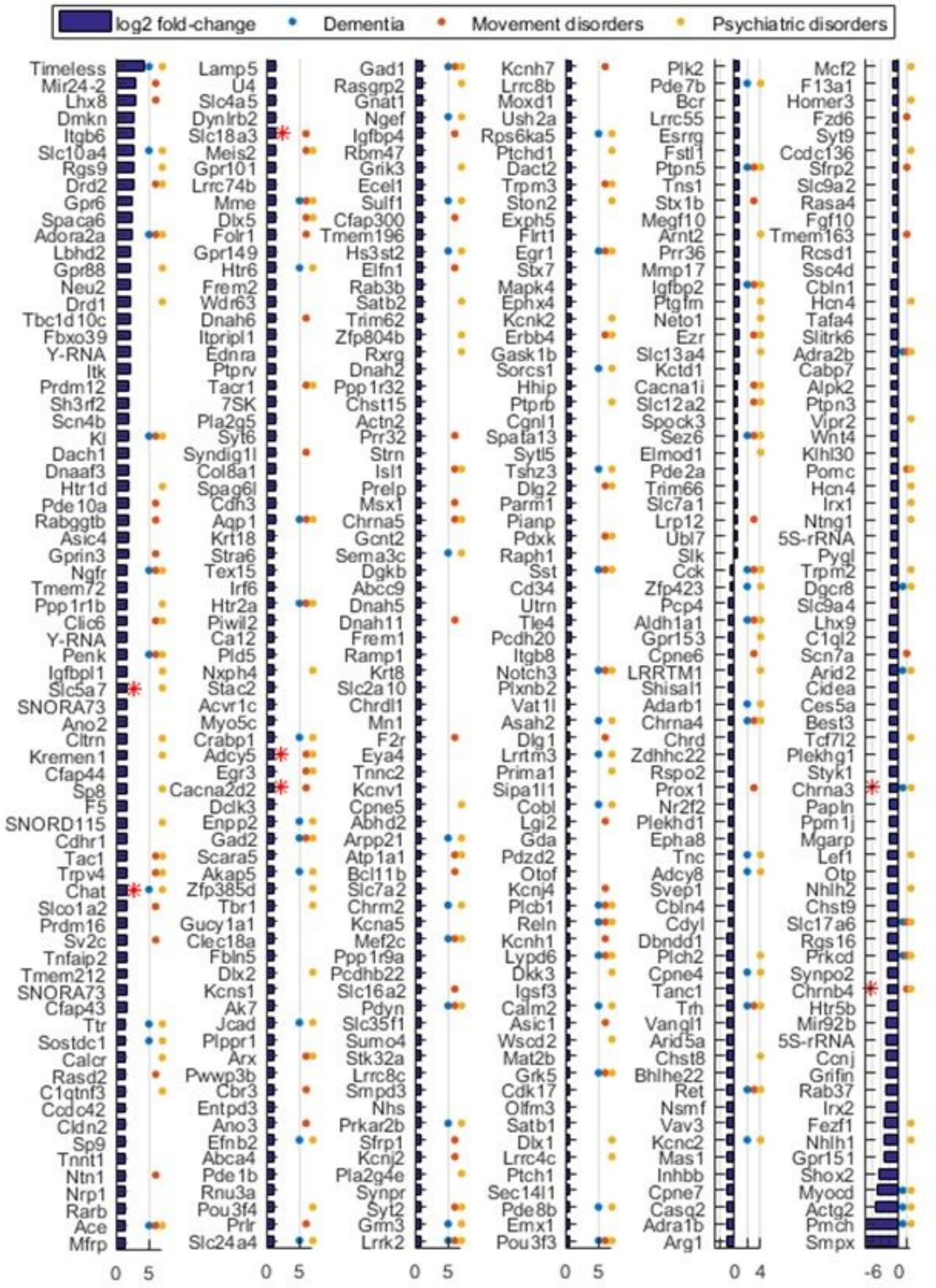


Figure 3

Forty percent of the 426 SDE genes were associated with neurological disorders. * Genes discussed in the text.

Supplementary Files

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

- tables2.xlsx
- tables2.xlsx
- tables1.xlsx
- tables1.xlsx