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Cranial manipulation affects cholinergic pathway gene expression in aged rats

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Abstract

Context: Age-dependent dementia is a devastating disorder afflicting a growing older population. Although pharmacological agents improve symptoms of dementia, age-related comorbidities combined with adverse effects often outweigh their clinical benefits. Therefore, nonpharmacological therapies are being investigated as an alternative. In a previous pilot study, aged rats demonstrated improved spatial memory after osteopathic cranial manipulative medicine (OCMM) treatment.

Objectives: In this continuation of the pilot study, we examine the effect of OCMM on gene expression to elicit possible explanations for the improvement in spatial memory.

Methods: OCMM was performed on six of 12 elderly rats every day for 7 days. Rats were then euthanized to obtain the brain tissue, from which RNA samples were extracted. RNA from three treated and three controls were of sufficient quality for sequencing. These samples were sequenced

utilizing next-generation sequencing from Illumina Next-Seq. The Cufflinks software suite was utilized to assemble transcriptomes and quantify the RNA expression level for each sample.

Results: Transcriptome analysis revealed 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 were part of the cholinergic neurotransmission mechanism, which is known to affect cognitive function. In addition, 39.9% of 426 significant differentially expressed (SDE) genes (FDR<0.004) have been previously implicated in neurological disorders. Overall, changes in SDE genes combined with their role in central nervous system signaling pathways suggest a connection to previously reported OCMM-induced behavioral and biochemical changes in aged rats.

Conclusions: Results from this pilot study provide sufficient evidence to support a more extensive study with a larger sample size. 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.

Keywords: Alzheimer's disease; cholinergic pathway; dementia; neurotransmission; osteopathic cranial manipulation.

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Dementia is a progressive neurodegenerative disease primarily afflicting a rapidly growing older population [1], and it is 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. Although there are treatments to manage its symptoms, currently there is no cure for dementia, and there is no treatment to halt its progression or delay its onset [3].

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 (NMDA) receptor 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.

Osteopathic cranial manipulative medicine as a potential adjunct treatment for age-related dementia

CV4, an osteopathic cranial manipulative medicine (OCMM) technique, in which gentle pressure is applied to the occiput [10], 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 [11, 12] and lateral epicondylitis [13]. Observational studies have shown that craniosacral therapy leads to a reduction in symptoms associated with dementia [14] and multiple sclerosis [15]. Studies have also shown that OCMM affects cerebral blood flow [16, 17], tissue oxygenation in prefrontal lobes [18], brain cortex electrical activity [19], and reduces amyloid beta (A β) protein levels [20, 21]. 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 [22–24]. Simulated mechanical stimuli, similar to subtraumatic cranial pressure, was shown to induce cellular activity in neural cell cultures by modulating ion channels [22]. Mechanical compression of neural stem cells was shown to contribute to neurogenesis and neuronal migration [23]. Compression of the cerebral cortex simulated by epidural beads implanted in rats, showed a rapid increase in NMDA receptor concentration and postsynaptic activity [24].

In a previous pilot study, we showed that OCMM treatment improves spatial memory in aged rats, as measured by the Morris water maze assay ($p < 0.05$, $n = 6$) [20]. To identify a possible molecular mechanism for the effect of OCMM, in this continuation of the pilot study, we analyzed gene RNA expression in prefrontal cortex tissue samples from OCMM-treated aged rats and from untreated aged (control) rats. Transcriptional regulation is crucial for organogenesis, functional adaptation, and regeneration in adult tissues and organs [25]. Study of the

transcriptome can provide insights into changes that affect subsequent protein synthesis, trafficking, and cellular activity.

Methods

Aged rats

All animal experimental procedures and housing have been approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC) Protocol ID #15-099. Eighteen-month-old F344 male rats were obtained from Charles River Laboratories, Inc., and Envigo. The study included six treated rats and six controls [20]. Personnel involved in the study needed to be unaware of the individual rat's cognitive function to avoid bias on group assignment. Rats were selected for OCMM treatment before conducting any tests, as follows. Rats for this study were kept in cage numbers 1, 2, 7, 8, 9, and 10, with two animals in each cage. None of the animals had any visible distinguishing characteristics. Two animals from cage numbers 1 and 8, and one from cage numbers 7 and 9 were selected for the treatment group, without any additional consideration or forethought. The other six animals from above-mentioned cages were assigned to the untreated group. However, we were only able to extract high-quality RNA from brain tissue stored in formaldehyde for three treated and three control rats. With this sample size, we identified differential gene expression with $p < 6 \times 10^{-5}$ and false discovery rate (FDR) < 0.004 after correcting for multiple-testing, representing an average statistical power of 88.9% for $\alpha = 0.05$. The average statistical power was calculated from the average mean and standard deviation for gene expression levels for differentially expressed genes with FDR < 0.004 . All rats were provided with normal food and water *ad libitum* and housed with 12 h light-dark cycle. The involvement of the prefrontal cortex in cognitive function has been extensively studied in humans [26] and animal models [27]. In a previous study [21], we utilized cerebellar tissue samples for biochemical analysis, which largely reproduced results from prefrontal cortex samples.

OCMM protocol

All OCMM procedures were performed by an experienced Doctor of Osteopathy (HT). The protocol consisted of the following steps (see Tobey et al. [20] for details):

- All rats, including untreated rats, were anesthetized with 1.5–3.0% isoflurane.
- For OCMM treatment (CV4 technique), mechanical pressure (1.5–2.5 N) 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 cerebrospinal fluid (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 thus enabling the feeling of improved symmetry or fullness of the CRI (~5 min).

- Treatment was performed every day for 7 days.
- All rats were euthanized by cervical dislocation after 7 days of OCMM treatment.

Tissue sampling, RNA extraction, and sequencing

Utilizing a rat brain matrix, a 1 mm coronal section from the prefrontal cortex was obtained from the euthanized rats. The brain tissue samples were obtained from the animal studies conducted in August 2017. RNA was extracted utilizing the PureLink RNA Mini Kit. Extracted RNA was sequenced utilizing next-generation sequencing on Illumina NextSeq 500. Single-read sequencing was utilized to investigate highly expressed genes in the well-annotated rat genome.

Differential expression calculation

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

The Cufflinks software suite [28] was utilized to assemble transcriptomes and quantify the RNA expression level for each sample. The transcriptomes from all samples were merged into a master transcriptome utilizing the Cuffmerge program in Cufflinks. The Cuffdiff program in Cufflinks was then utilized to calculate the significance of differential expression (fold change, p value, and FDR) between samples from OCMM-treated and untreated animals. The two-sided t test statistic was utilized to calculate p value. The Benjamini–Hochberg correction for multiple-testing was then applied to calculate FDR.

Pathway analysis

Gene-pathway association data was downloaded from the Reactome database [29]. The 24 top-level Reactome pathways associated with each gene were identified. The percentage of all genes and significant differentially expressed (SDE) genes in each top-level pathway was utilized to calculate the significance of any differences. The chi-squared test statistic was utilized to calculate the 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 genes and neurological disorders.

Results

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 Section “Methods”.

The number of reads from RNA sequencing ranged from 58 to 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 Supplementary Table 1 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 of the 14,278 genes sequenced were differentially expressed with FDR <0.01, of which 426 had FDR <0.004 (Figure 1A). For the following analysis, we focus on the 426 genes with FDR <0.004, which we refer to as SDE genes. Of these 426 SDE genes, 314 were overexpressed and 112 underexpressed.

Pathways associated with SDE genes

The Reactome database contains a list of genes that have been associated with specific pathways [29]. 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 overrepresented in the SDE gene set with FDR=3E-14 (Figure 1B). Thirty-six (8.5%) of 426 SDE genes were associated with neuronal pathways (Figure 1C), compared to 343 (2.4%) of all 14,278 genes that were sequenced. The signal transduction pathway was also overrepresented with FDR=1E-5, while the gene expression pathway was underrepresented with FDR=5E-5. In addition, pathways for protein metabolism, cell cycle, and response to stress were underrepresented and the muscle contraction pathway overrepresented, with FDR <0.01.

SDE genes in the cholinergic neurotransmission pathway

Of the 36 SDE genes in the neuronal system pathway, 27 of them were overexpressed and nine of them underexpressed in OCMM-treated animals compared to untreated animals (Figure 1C). Twenty of the 36 neuronal SDE genes were associated with signal transmission across chemical synapses, 13 with potassium channels, and eight with protein-protein interaction at synapses. Five of these genes are associated with multiple pathways.

The five genes with the largest-fold change, *Slc5a7*, *Chat*, *Slc18a3*, *Adcy5*, and *Cacna2d2* (Figure 1C), are part of the acetylcholine (ACh) neurotransmission mechanism (Figure 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 [30, 31]. Choline

Figure 1: Significant differentially expressed (SDE) gene-pathways. (A) Volcano plot identifying the 426 SDE genes with false discovery rate (FDR) < 0.004 , p value $< 6 \times 10^{-5}$, t test, in three OCMM-treated and three untreated rats. (B) Distribution of pathways associated with all genes and SDE genes. ***FDR = 3×10^{-14} , $p = 1 \times 10^{-15}$. **FDR $< 5 \times 10^{-5}$, $p < 6 \times 10^{-6}$. *FDR < 0.01 , $p < 0.001$, chi-square test, $n = 14,278$ all genes and 426 SDE genes. (C) Thirty-six of the 426 SDE genes are in neuronal system pathways.

uptake is a rate-limiting step in ACh synthesis and thus ACh mediated neurotransmission [32]. The choline acetylase, *Chat*, catalyzes the biosynthesis of the ACh neurotransmitter from choline and acetyl-coenzyme-A [33]. The vesicular ACh transporter, *Slc18a3* (*VAcHT*), transports ACh into secretory vesicles for release into the synaptic cleft [34]. Neuronal action potentials activate the voltage-gated calcium channel [35], of which *Cacna2d2* forms the $\alpha 2/\delta 2$ subunit [36]. 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 α subunit from the G-protein complex. G_{α} binds to Adenylate Cyclase 5, *Adcy5*, activating downstream cyclic adenosine monophosphate (cAMP) signaling pathways,

whereas $G_{\alpha i}$ inhibits downstream signaling [37]. The above pathway is targeted by the Alzheimer's disease drug donepezil. Donepezil selectively inhibits AChE, which normally catalyzes ACh degradation.

The two most underexpressed genes based on fold change are *Chrna3* and *Chrb4* (Figure 1C). These genes code for the $\alpha 3$ and $\beta 4$ subunits of the pentameric nicotinic ACh gated ion channel receptor (nicotinic acetylcholine receptor [nAChR]) [38]. Some studies have suggested that nicotine desensitization (inactivation) of nAChR improves memory function in schizophrenia and Alzheimer's patients [38–40]. Reduced expression of nAChR, as suggested by the reduced expression of *Chrna3* and *Chrb4*, shown above, could contribute to improved cognitive function.

Figure 2: Pathway affected by the top five overexpressed genes. *CHT1*, *CHAT*, *VACHT*, *CaCN*, and *Adcy5* are part of the acetylcholine (ACh) neurotransmission mechanism in the neuronal pathway. (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 vesicular ACh transporter transports ACh into secretory vesicles. (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 postsynaptic terminal promotes the release of the G α subunit of the G-protein complex from the G-protein coupled receptor (GPCR). G α can promote or inhibit the cyclic adenosine monophosphate (cAMP) pathway (depending on the type of G α protein) by binding to Adenylate Cyclase 5 (*Adcy5*). Acetylcholinesterase (AChE) breaks down ACh so the Ch can be recycled.

Overall, 39.9% of the 426 SDE genes were associated with neurological disorders, with 17.1% being associated with dementia, 22.8% with movement disorders, and 28.4% with psychiatric disorders (Figure 3). See Supplementary Table 2 for a list of the 426 genes and references for associated disorders.

Discussion

The neurological importance of changes in the cholinergic pathway genes discussed above is evident from their role in neurological disorders, including Alzheimer's disease and dementia. *CHT1* was found to be overexpressed in Alzheimer's disease patients, presumably to compensate for the reduced cholinergic synaptic availability [41]. Polymorphisms in *Chat* have been associated with an increased risk of Alzheimer's disease [42, 43], and reduced *Chat* expression was found in Parkinson's dementia [44] and schizophrenia [45] patients. *VACHT* defects have been implicated in myasthenia syndrome [46], and reduced levels of *VACHT* have been associated with Alzheimer's disease [47]. Mutations in *Cacna2d2* have been linked to epileptic encephalopathy [48]. *Adcy5* mutations have been associated with dyskinesia, myokymia, chorea, and dystonia [49]. nAChR has been linked to schizophrenia, Alzheimer's disease, and other neurological disorders [38–40]. Together, these studies suggest that the cholinergic

pathway genes differentially expressed in OCMM-treated aged rats may play a role in the progression of Alzheimer's disease and dementia.

Mechanism by which OCMM may affect gene expression

The results obtained from this pilot study suggest that OCMM affects the expression of neuronal system genes in aged rats. These findings raise the question, how does OCMM affect gene expression? Two possible mechanisms could link OCMM to changes in gene expression: (1) improved lymphatic fluid circulation in the central nervous system; and/or (2) cellular stress induced by OCMM. Further studies will be required to determine if either of these mechanisms provide a valid explanation.

Studies have shown that enhanced CSF exchange can facilitate the clearance of accumulated toxic solutes, such as amyloid β [50, 51]. In addition, abdominal lymphatic pulse treatment utilized in osteopathic manipulation has been shown to improve lymphatic flux and immune response [52, 53]. It is possible that OCMM improves CSF exchange, which may alter gene expression.

In vivo and *in vitro* studies have shown that mechanical stress can affect cellular activity and growth, including in neuronal cells. Simulated mechanical stimuli, similar to

Figure 3: Significant differentially expressed (SDE) genes. Forty percent of the 426 SDE genes were associated with neurological disorders.

*Top five differentially expressed genes that are discussed in the text.

subtraumatic cranial pressure, was shown to modulate ion channel activity in neural cell cultures [22]. Mechanical compression of neural stem cells was shown to contribute to neurogenesis and neuronal migration [54]. Compression of the cerebral cortex, stimulated by an epidural bead implanted in rats, showed a rapid increase in NMDA receptor concentration and postsynaptic activity [24, 55], suggesting an influence upon gene expression. The OCMM technique utilized in this study was CV4, which consists of the treating physician applying pressure to the occipital

bone, medial to the occipital mastoid suture, and inferior to the lambdoid suture. The procedure applies pressure to the dural membrane around the fourth ventricle, which can cause a change in ventricular volume and affect intracranial pressure. This could translate into compression of brain tissue. Ventricular volume is highly variable and shown to change with cardiac rhythm [56], aging [57], dementia [58], and intoxication [59], which may affect intracranial pressure. Neck position and head elevation [60] and coughing [61] were also shown to alter intracranial

pressure. It is possible that cellular stress, due to the OCMM-induced change in intracranial pressure, causes the observed change in gene expression.

Study limitations

This study could benefit from a larger sample size and more control groups. Such a study is currently underway, supported by an NIH grant. Additional experimentation will be required to determine the validity of the previously mentioned explanations for how OCMM may affect gene expression. These experiments will involve measuring OCMM-induced changes in brain fluid dynamics and cellular activity, which is well beyond the scope of this work. From this pilot study, it is unclear whether our findings based on an animal model will translate to humans. Determining this will require clinical experiments, which is also well beyond the scope of this study.

Conclusions

This pilot study suggests that OCMM treatment affects the expression of genes associated with neurological pathways and disorders. This connection further suggests that OCMM could affect the progression of age-related dementia, providing support for a more extensive investigation with a larger sample size. With clinical validation utilizing robust placebo-controlled double-blind studies, OCMM may offer a much-needed low-risk adjunct treatment for age-related cognitive decline.

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of any part of the work are appropriately investigated and resolved.

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