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Article

Uncovering mirror neurons' molecular identity by single cell transcriptomics and microarray analysis

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SUMMARY

Mirror neurons (MNs) fire action potentials both when performing and seeing particular actions, ranging from grasping an object to observing a simple smile. During neuroimaging, the active cortical regions in both execution and observation are referred to as the mirror neuron system. It is composed of the superior temporal sulcus, posterior parietal cortex, and inferior frontal gyrus in humans. We aimed to find MNs' neuronal identity, or the cell cluster to which MN belongs. Such identification lays the groundwork for future MN analysis and understanding of the mechanism of action. Due to the uniqueness of the human MN system, we performed bioinformatics analysis instead of experiments on animal models. High-expression genes throughout the MN system became candidate genes derived from differential gene analysis, or microarray. Ten cell clusters fit the MN's trait as a layer three pyramidal excitatory neuron in the Allen Brain Atlas, human cortical single cell sequencing database. The cluster that had the highest sum of relative expression of candidate genes was cluster 85 with markers THEMIS and UBE2F. With MNs' likely molecular identity in cluster 85 identified, we further explored MNs' functional aspects. We gathered and analyzed data from single-cell sequencing and STRING (a functional protein association network) to uncover possible connections between typical neural degenerative diseases and psychiatric disorders with MN impairment. We found moebius facial syndrome, autism spectrum disorder, and amyotrophic lateral sclerosis to be potentially related to MN impairment.

INTRODUCTION

Imitation and empathy underlie our daily social interactions and are common cognitive features of higher primates. Mirror neurons (MNs) are vital in the investigation of these features' neuronal mechanism (1). MNs, initially discovered in area F5 of the rhesus macaques' ventral premotor cortex, are a class of neurons that modulate their activity both when an individual executes a motor action and when an individual observes the same action, or a similar action performed by another individual (2, 3). In particular, object grasping, scratching, yawning, and smiling are examples of MN-related behaviors. Human MN areas, which are different from those in macaques, are found using functional magnetic resonance imaging (fMRI) in the superior temporal sulcus (STS) for observation, the posterior parietal cortex (PPC) for motor processing, and the inferior frontal gyrus (IFG) for the goal and intention of imitation (4–6). These cortical regions, also referred to as the MN system, are active in both observation and execution of a class of actions (**Figure 1**) (7). In fact, the human MN system has been hypothesized to be involved in action understanding, intention attribution, language acquisition, and other advanced cognitive functions (8–12).

Previous studies on the MN system have mainly focused on electrophysiology and brain imaging which identifies MNs by their physiological function (13). It is unclear whether there is a group of cortical neurons that are as MNs. We intended to address this important, but less studied question in humans specifically. First, we hypothesized that MNs could be found in specific cell clusters that fit MNs' known traits via differential single cell gene expression analysis in human cortical areas. Second, we hypothesized that MN dysfunction would be related to disease-causing genes in neurodegenerative and psychiatric disorders. Our bioinformatic analysis results supported these two hypotheses. First, we determined cell cluster 85 found in brain layer 3 to 5 with marker genes *THEMIS* and *UBE2F* to be the most likely residence of MN. Second, we discovered that MNs are potentially connected to moebius facial syndrome (MBS), autism spectrum disorder (ASD), and amyotrophic lateral sclerosis (ALS).

RESULTS

Identification of the human MN in the Allen Brain Atlas database

To perform bioinformatic analysis, we sought to first identify components of the MN system within the Allen Brain Atlas database, a comprehensive model of mapped gene

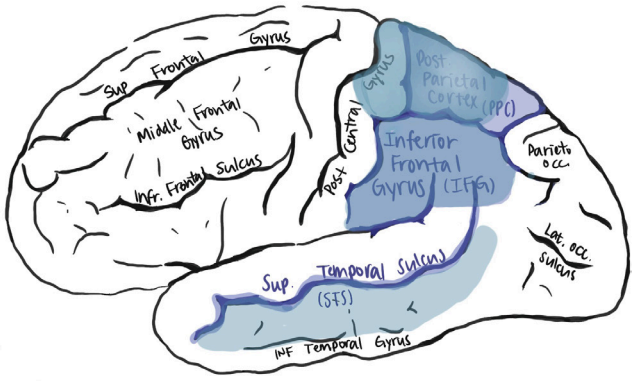

Figure 1: Brain cortical diagram showing the components of the Mirror Neuron System (MNS). The MNS consists of the three cortical brain regions where collections of mirror neurons are found: the superior temporal sulcus (STS), posterior parietal cortex (PPC) and inferior frontal gyrus (IFG) (16–18). MNS components derived from fMRI are in light purple. Their corresponding components in the Allen Brain Atlas database are in blue.

expression in human and mouse cortices (14, 15). The first component of the human MN system is STS, which is a sulcus, or groove, instead of a particular brain region. Because we were looking for specific brain regions, we had to choose between its two neighboring areas: the superior temporal gyrus (STG) and the middle temporal gyrus (MTG). Previous fMRI functional studies demonstrated a strong connection between the MTG and the MN system, so we chose MTG (16). The second component, the PPC, is absent within the Allen Brain Atlas database. This is likely because the MN system was studied primarily using fMRI and EEG (3), whereas the Allen Brain Atlas identifies regions based on micro-dissection and staining methods. The disparate methods mean that the same cortical areas were defined differently. Although we were unable to investigate with the exact regions referred to by the PPC, the paracentral lobule posterior part (PCLp) has the highest area of overlap anatomically among available options on the Allen Brain Atlas with PPC (**Figure 1**). PPC is known for higher-order association and integration across the cortex and subcortical area (17). PCLp corresponds functionally due to its dominant continuous morphology, enabling it to interact with regions across cell layers (18). PCLp was chosen as the substitute for PPC, because of its anatomical and functional similarity. The last component of the MNS, the IFG, is found in the Allen Brain Atlas.

Microarray analysis to uncover genes enriched in the MNS

With the MTG, PCLp, and IFG identified as components of the MNS, the next question we wanted to explore was which cell identity at the molecular level represents MNs. We started by identifying MN-associated genes to address this question. We first used the Allen Brain Atlas microarray data to find enriched genes differentially expressed in the MN system compared to the control (gray matter). The microarray analysis shows the relative expression levels of overlapping genes in the MN system in comparison with other cortical areas (**Figure 2**).

Genes with a fold change higher than 5 are highly expressed in all components of the MN system. The genes that were duplicated or had expression less than 0.03 throughout the brain, namely *NPAA* and *GALNTL5*, were ruled out. This resulted in a candidate gene pool of 18 genes. Their relative expression throughout the brain, derived from the human cortical single-cell RNA sequencing (SMART-seq) database of the Allen Brain Atlas, wholistically fit the MN's identity as an excitatory neuron (**Figure 3**). Expression levels of all 18 genes was higher in excitatory neurons than in inhibitory neurons, apart from cholecystokinin (*CCK*) which is expressed in the gastrointestinal tract and brain and functions in protein and fat digestion (19).

Single cell transcriptomics to find MN molecular identity

Next, we sought to uncover MNs' neuronal subtype, as defined by clusters in the Allen Brain Atlas's human cortical single cell sequencing database. MNs are cortical excitatory neurons that likely fall within the area of layer 3 to 4 pyramidal excitatory neurons (20–22). Therefore, our search targeted excitatory cell groups expressed in layers 3 and 4. Based on these criteria, 10 cell clusters in the Allen Brain Atlas single cell database were selected. The glutamatergic (excitatory) neurons were initially separated into four subtypes in accordance with their marker genes: *LINC00907*, *RORB*, *THEMIS*, and *FEZF2*. *LINC00507* may be involved in brain development of higher primates since it is age-dependent (23). *RORB* is vital in post-natal development of photoreceptors, as it modulates rod differentiation (24). *THEMIS* is associated with T-cell signaling, and its dysfunction is involved in the malfunction of both T cells and central nervous system neurons (25). *FEZF2* is linked with motor functions and is diversely expressed in layer 5 projection neurons with subtypes intratelencephalic (IT) and pyramidal tract (PT) (26, 27). All markers shared potential connections with MNs, so none could be directly eliminated. The corresponding expression levels of the 18 candidate genes in the 10 cell clusters were found using SMART-seq in the Allen Brain Atlas (**Figure 4**) (14). *TESPA1*, *GDA*, *CCK*, and *LY86-AS1* were highly expressed in various selected clusters, while the rest showed high expression in only one to three clusters.

To identify the most probable MN neuronal cluster from

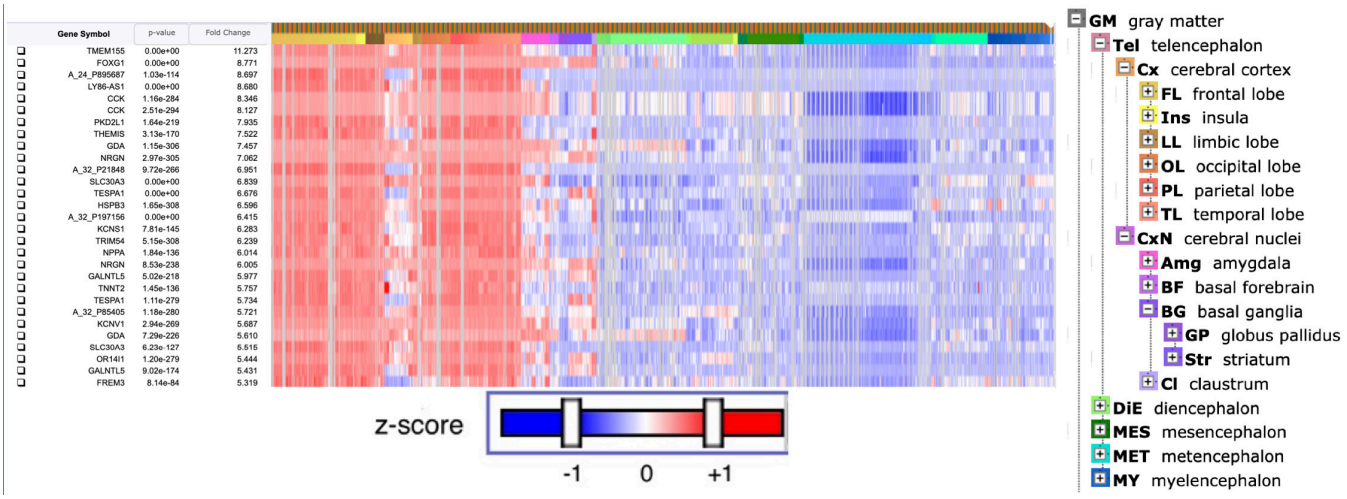

Figure 2: Differential analysis of genes comparing Mirror Neuron System to gray matter in Allen Brain Atlas. The results show the gene symbol (listed in decreasing value of fold change), p-value (probability of anomaly), fold change (relative concentration) throughout the MNS, and z-score (blue = negative, white = zero, red = positive). The top x-axis indicates the donor of each brain tissue. The bottom x-axis is the brain's gray matter, and the color code corresponds to the brain regions listed on the right.

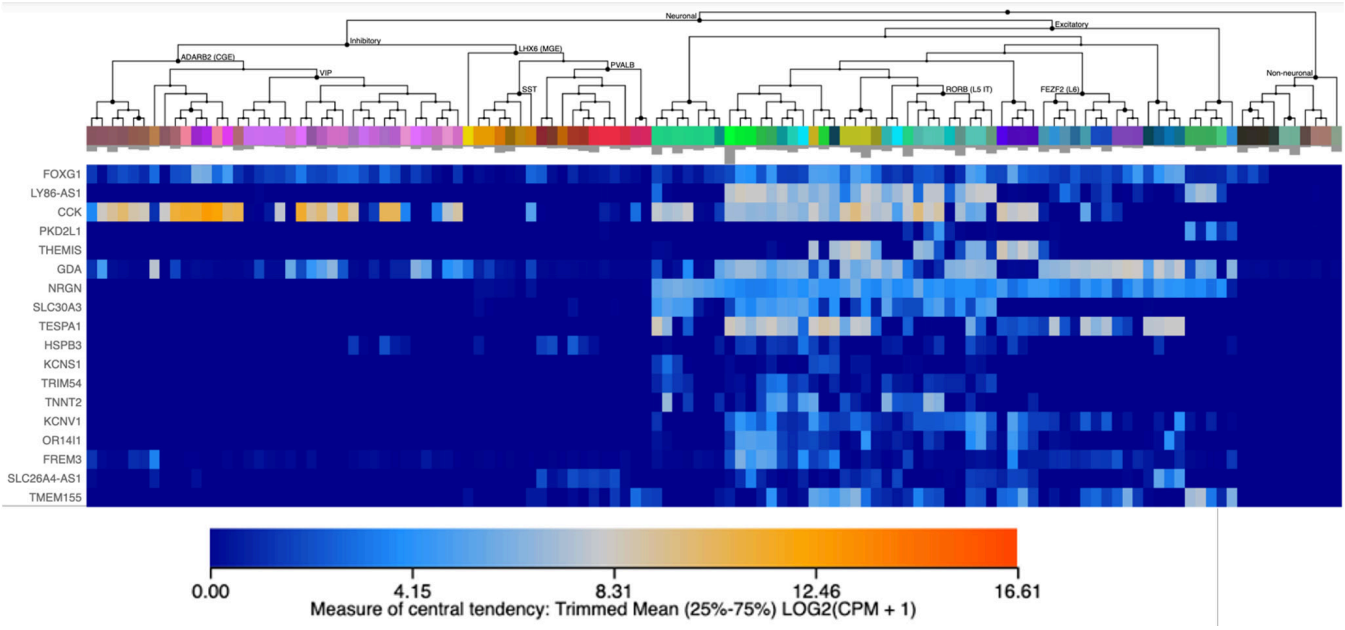

Figure 3: Microarray identified genes in human single cell RNA sequencing database. The heatmap shows the refined candidate genes' relative expression in human cortical layers (clusters). Genes that have extremely low expression in the excitatory section of the brain and genes that are repeated are not shown in the heatmap above. Blue indicates low expression and red low expression.

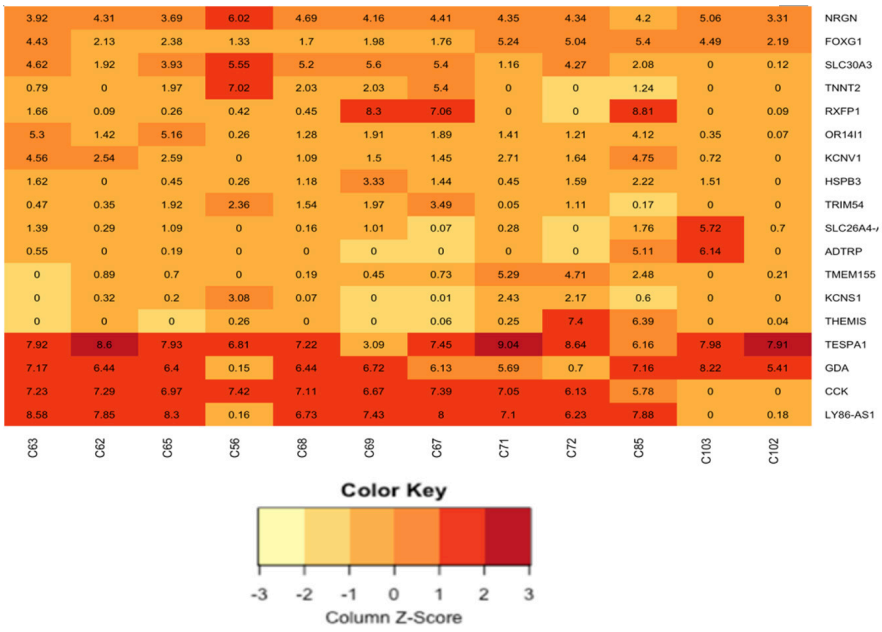

Figure 4: Relative expression of MN candidate genes in likely clusters. Heatmap shows the relative expression level of mirror neuron candidate genes (calculated using Log2 CPM +1) in clusters that fits known traits of MNs using single cell sequencing. The x-axis is the labelled cluster in Allen Brain Atlas data base that fits MN known traits. The y-axis is the name of the candidate genes.

cortical single cell transcriptomics in the Allen Brain Atlas, arithmetic sums of the relative expression levels of all the candidate genes within the same cluster were computed. In cluster 85, the sum was 76.31, which was much higher than that of all others. The second largest was cluster 67 with a sum of 62.14. The third was cluster 63 with a sum of 60.21. The difference between Cluster 85 and 67 was around 14, whereas the difference between Cluster 67 and 63 was around 2. This indicated that cluster 85 was the most likely cluster of MN identity.

To further characterize cluster 85, sampling analysis of single cell data was performed using Allen Brain Atlas (**Figure 5**) (14). Cluster 85 consists of excitatory neurons, is found in both genders with minor differences, and belongs to the IT cell subtype. IT cells and PT neurons are the two major types of striatum-targeting neurons, and the striatum is involved in motivational and motor functions (28, 29). In conclusion, based on unique expression, cell distribution, and related cell subtypes, cluster 85 is the most probable cell identity of MN.

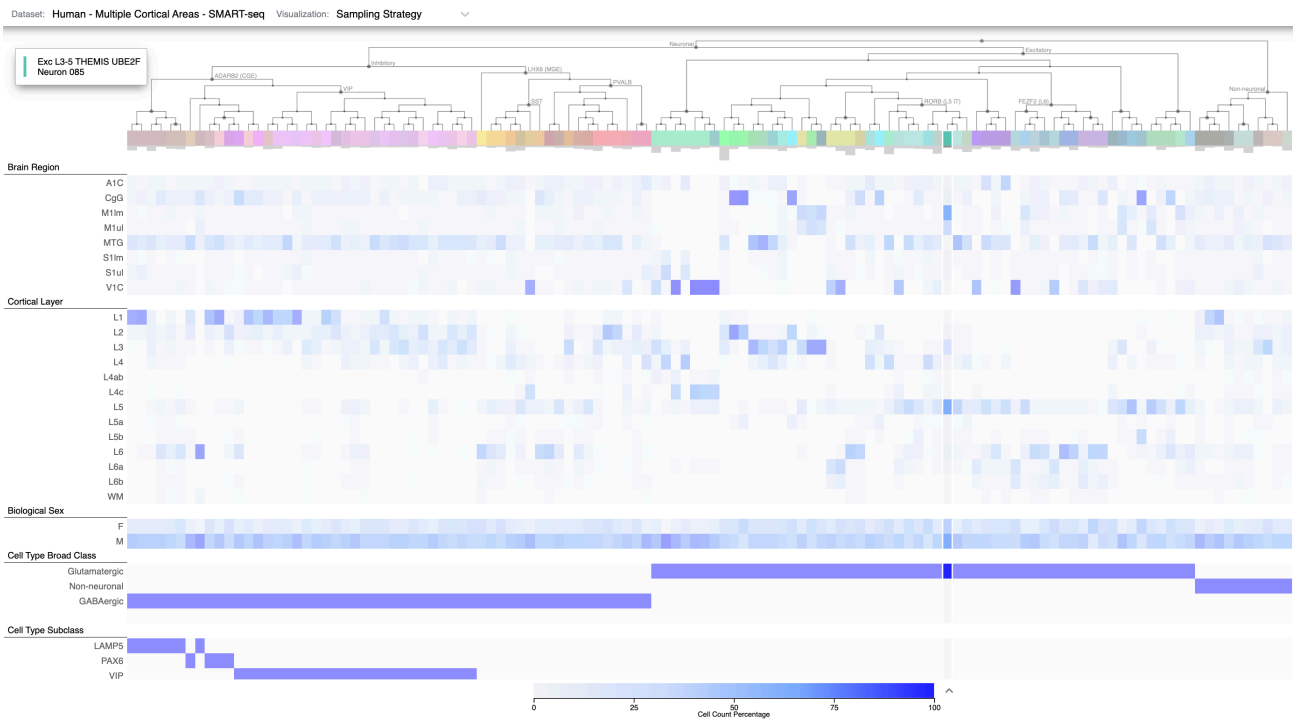

Figure 5: Traits of cluster 85 reflected in the sampling strategy of Allen Brain Atlas human single cell database. There are more cluster 85 cells in males then females; the cell count in females is 38.5%, while the cell count in males is 61.5%. All neurons in cluster 85 are glutaminergic, or excitatory. All cells in cluster 85 are intratelencephalic (IT) cells, which target the striatum. The striatum is responsible for motor and motivation, which corresponds with MN-involved actions like object grasping.

Uncovering diseases potentially related to MN dysfunction

MNs' identity includes not only corresponding cell clusters but also functional interactions within the MN system. To address the functional aspect of MN molecular identity, we sought to find diseases related to MN dysfunction. Since it is not clear which diseases might be associated with the MN system, we unbiasedly identified potential disorders connected to MNs. We first found 71 deterministic genes in major neurodegenerative diseases and psychiatry disorders which include autism spectrum disorder (ASD), Moebius syndrome (MBS), dopa-responsive dystonia, ataxia, bipolar disorder (BP), major depressive disorder, Alzheimer's disease, Huntington's disease, Lewy body disease, Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (30–44). We then screened all 71 genes via Allen Brian Atlas's single cell sequencing (**Figure 6**) (14). Among them, 18 had no expression in the neuronal excitatory section. The varying levels of expression across cell clusters suggest neurodegenerative diseases and psychiatry diseases are caused by genetic operations across distinct layers and cell types.

Our differential gene analysis of high expression genes in the MN system revealed that MNs are likely present in cluster 85, whose cells consistently express both *THEMIS* and *UBE2F* gene and are found in brain layer 3 to 5. Thus, we extracted all the candidate genes' expressions within cluster 85. Interestingly, the expression patterns of *LRRK1*, *SOD1*, *CASK*, *MAPT*, *ATRX*, *TMCC1*, *REV3L*, and *BASP1* trended to normal distributions with relatively high average expression (> 6.9 log2 CMP + 1) **(Figure 7).** The 8 genes' similar expression patterns indicated their potential co-expression within cluster 85. The high expression levels of these disease-related genes in an MN-like cell cluster implicated a potential connection with MNs**.** In contrast, *PODXL2* and *SHANK3* had low average expression and random distribution patterns, which can be viewed as negative controls. To check whether the eight identified disease-associated genes were connected, STRING analysis, which shows the interactions between proteins, was performed (**Figure 8)**.

STRING analysis suggested interactions between *REV3L, ATRX, SOD1*, and *MAPT* expressed proteins (45). *REV3L* is a catalytic subunit of the DNA polymerase ζ complex, that when mutated can cause MBS, which is a type of congenital facial palsy where the 6th and 7th cranial nerves are dysfunctional (46). *ATRX* is a transcriptional regulator that facilitates DNA replication and is influential in ASD (47). RNA expression pattern and protein co-regulation analyses showed that *REV3L* and *ATRX* are co-expressed in humans with a co-occurrence score of 0.083. *SOD1* mutations in exonic regions are commonly found in patients with ALS (48), where progressive degeneration of motor neurons responsible for controlling voluntary muscle movements occurs (49). *ATRX* and *SOD1* shared a co-expression score of 0.046. Overall, co-expression of the four disease-associated genes reflected correlations between MNS dysfunction and ASD, MBS, and ALS.

DISCUSSION

Using multiple bioinformatics analyses, we determined potential MN molecular and functional traits. By employing differential gene analysis within three cerebral regions associated with the MNS, we found cluster 85 (excitatory layer 3–5, con-

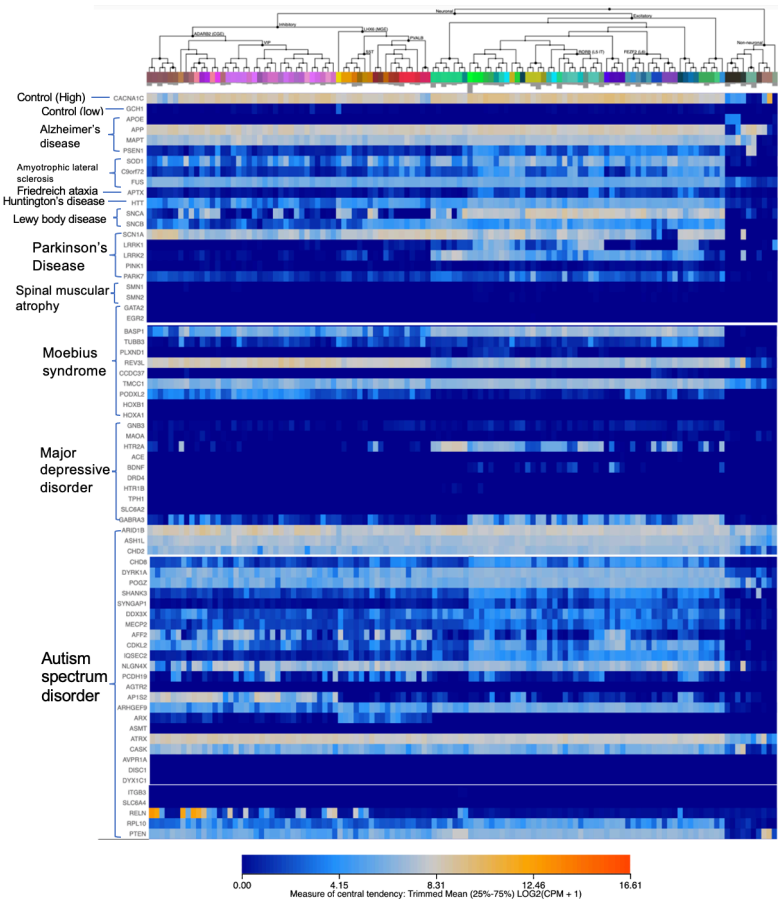

Figure 6: Neurodegenerative and psychiatry disease related gene expression in human single cell RNA sequencing database. The heatmap shows overview of diseases' relative gene expression in human cortices.

taining genes *THEMIS* and *UBE2F*) yielded the highest overall relative expression of MN candidate genes from a human single cell database. Therefore, we identified cluster 85 to be the most likely region where MNs are located. *THEMIS* and *UBE2F*, two crucial markers of cluster 85, may be utilized for future MN cell lineage tracing and/or functional characterization. We used Allen Brain Atlas's human cortical single-cell sequencing to find expressions of key disease-associated genes within cluster 85 (14). STRING analysis performed on these genes indicated that MBS, ASD, and ALS may be associated with MN system malfunction. The MN system's association with ASD and MBS points to emotional and cognitive circuits, while MNs' association with ALS points to voluntary motor circuits. The STRING analysis also revealed potential connections between the neurodegenerative diseases and psychiatric disorders (**Figure 8**). Co-expression of *REV3L* and *ATRX* indicates that MBS may relate to ASD, possibly because facial palsy would hinder emotion delivery, which is a vital part of social communication (50). Co-expression of *ATRX* and *SOD1* suggests ASD may be linked with ALS, possibly due to their shared deficit in the motor system. These potential connections may lead to better understanding of these diseases' pathologies, leading to improved treatment.

Analysis of gene expression in the MN system concluded that cluster 85 is the probable neuronal subtype for MNs. All cells in cluster 85 are excitatory and appear only in layers 3 to 5, corresponding with known MN traits. Characterization of cluster 85 also shows 60% of the cells were expressed in the region of the cortex controlling motor function of the lower limbs, while 30.8% were expressed in the region of the cortex controlling motor function of the upper limbs. This shows that cluster 85 cells are located within the motor cortex, consistent with MN attributes. This result is also contrary to common assumptions, as past MN research has focused on upper limb behavior like facial mimicry or hand grasping. In addition, higher cell counts of cluster 85 in males prompts consideration of a new confounding variable, sex, in future investigations. It is possible that MNs are not homogenous since they were initially discovered and grouped by function. A functional classification system means MN-specific genes could be expressed in multiple clusters. If so, cluster 85 could serve as a good entry point to the molecular identity of MNs.

Our study has certain limitations that invite future investigations. Firstly, current single cell RNA sequencing technology relies on the dissociation of cells from tissues, losing the spatial context of regulatory circuits. This feature limits understanding of neuronal interactions and tissue functional organization (51, 52). To address this, spatial transcriptomics that visualize mRNA distributions on brain slides like Slide-seq, *in situ* sequencing, and RNAscope can be performed to attain locational information of specific cell types and gain positional insight into cortical neurons (53–56). MNs' molecular identity in cluster 85 can be verified if regions derived from spatial transcriptomics correspond with regions active in observation

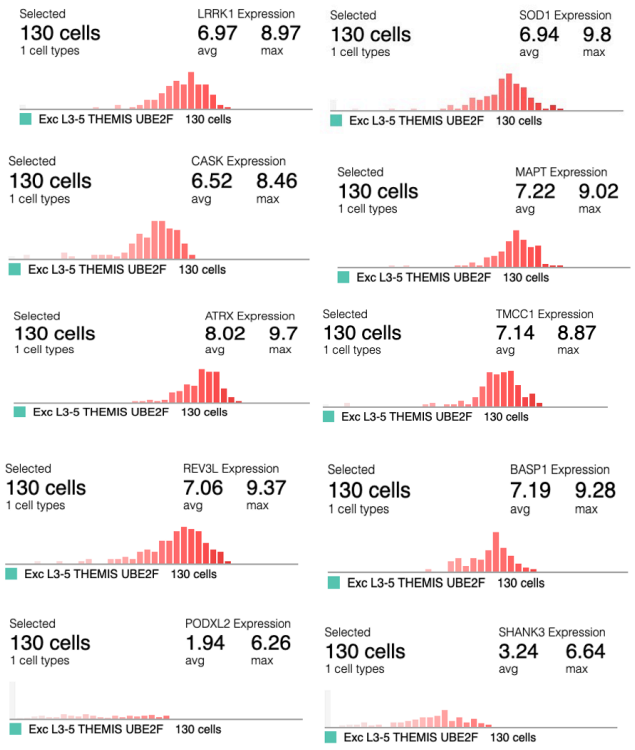

Figure 7: Possible MN-related disease gene expression in cell cluster 85 (Exc L3–5 THEMIS UBE2F). Varied gene expression of eight likely co-expressed genes and two controls within the most likely MN cluster.

and execution during behavioral studies. Secondly, disparate methods used in the Allen Brain Atlas data collection and the MN system component identification caused some cells that are present in the PPC but not in the PCLp to be left out. The excluded cells could be identified using single cell RNA or *in situ* sequencing and compared to known traits of MNs. Thirdly, the specific structural and/or functional deficits in the MN system that lead to MBS, ASD, and ALS call for further characterization. In fact, it would be interesting to evaluate the MN system's functionality in MBS, ASD, and ALS patients. Specifically, comparative studies of MN system activity between diagnosed patients and healthy controls via neuroimaging could be conducted. Lastly, our identified cell identity (cluster 85) cannot be attributed to the motor pathway (action imitation) or the limbic pathway (emotional mirroring) since connections between detailed molecular cell identities and macroscale brain regions are still unclear (57). Future connectomic studies may provide insight. Postmortem brain samples are stained, sliced, and viewed under high resolution imaging, like Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM), in refined pixels. Stained cells are initially manually traced, providing the ground truth to aid machine deep learning, often via segmentation, to sort through the whole data. This ideally results in a three-dimensional reconstruction of the stained cells (58).

Nonetheless, defining MNs on a genetic basis is a novel and significant attempt, as previous investigations of MNs were overwhelmingly focused on their physiology. In fact, identification of the MN's molecular identity enables research of MNs' anatomy and interaction on a neuronal level instead

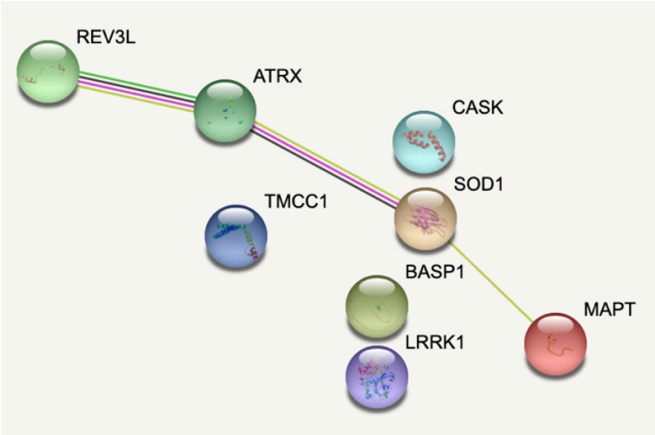

Figure 8: Interaction between proteins of diseases' genes that have similar expression pattern using the STRING network. Green lines indicate a shared a neighborhood, dark brown lines indicate co-expression, pink lines mean the relationship was experimentally determined, and the yellow-green lines mean the genes/proteins were mentioned together in text.

of a cortex level. In addition, it could potentially lead to discovering hidden symptoms of neurodegenerative diseases, besides the apparent features like cognitive impairment or muscle deterioration, derived from the shared pathological pathways within the MN system.

Initial MN transcriptomic analysis points to several interesting future directions. To confirm the identified MN markers, the initial step uses immunohistochemistry, staining the identified MN potential markers (*THEMIS*, *UBE2F*) within multiple postmortem patients' cortical areas. These brain slices can be reconstituted into a brain-wide 3D circuitry map using experimental methods such as brain-wide reduced-osmium staining with pyrogallol-mediated amplification (BROPA) or clarity (which preserves only proteins, tissue structure, and nucleic acids in specimens) (59, 60). Second, conditional knockout animal models, such as mice, can be developed using CRISPR/Cas9 genome editing technology, followed by tests for MN-like behavior. Gastrin-releasing peptide (GRP) and its receptor (GRPR) are found to be essential for contagious itching behavior in mice (61). However, both genes found in mice have no expression in human excitatory neuronal sections. The identified human MN markers from our study also have no concentrated expression throughout the mouse cortex. Hence, the MN system may not be conserved between mice and humans. An alternative model is macaques, where MNs were first discovered. In fact, investigations into neurodevelopmental disorders and neurodegenerative diseases have often used macaque models (62–65). Third, computational models using deep learning can be constructed based on traced genomic molecular identity data. Linked inference of genomic experimental relationships (LIGER) may be adopted to find shared and different cell identities across primates (humans and macaques) in the MN cluster or the MN system (66).

Based on our identified MN molecular identity, mapping of the MN system derived from immunostaining, knock-out transgenic animal models, and computational modeling could further advance our understanding of MN-like behaviors. It could potentially increase comprehension of neurodegenerative and psychiatric diseases, language acquirement, action

understanding, consciousness, and empathy (1, 3, 8, 9, 11, 67).

MATERIALS AND METHODS

Allen Human Brain Atlas

We employed the Allen Brain Atlas (14) for genetic analysis of the MN system. We used two parts of this database.

First, we used human multiple cortical area SMART-seq sub database to look at single cell sequencing data. Single cell sequencing is a scalable approach to provide gene expression of thousands of single-nucleus cells. This is achieved by micro-dissecting tissue from brain cortical layers or cell groups. Cell clusters were formed using the t-distributed Stochastic Neighbor Embedding (t-SNE) method to visualize high-dimensional data (68). Fifty-four of the clusters are GABAergic or inhibitory, 56 of them are excitatory, and 10 are non-neuronal. Data was visualized via heatmap showed trimmed mean (25 -75%) expression level (calculated using Log 2 CPM +1) of genes in various clusters. We used the data to create a more specific heatmap that shows a general overview and specific gene expression throughout the human brain at the same time. Data visualized via sampling strategy allowed us to better comprehend cluster 85.

The second part of the Allen Brain Atlas that we used was the microarray database. A differential search enabled us to find overlapping genes in the MN system compared with gray matter. It contained more than 62,000 gene probes per profile and around 500 samples per hemisphere across the cerebrum, cerebellum, and brainstem. The data was mapped with histology into a unified three-dimensional anatomic framework based on neuroimaging. We used a fold change value – the Log2 of the case-control ratio. A fold change larger than one indicated higher expression in the case than control. Genes with expression lower than 0.03 (Log 2 CPM +1) were statistically outliers in the data set (< Quartile one - 1.5 * Inter-Quartile Range), so we excluded them in further analyses.

STRING

We used STRING (45) to find connections between the protein products of likely candidate genes. It is an open database that finds and analyzes interactions between proteins. The interactions include both direct (physical) and indirect (functional) interactions. They are predicted based on computational modeling, knowledge transfer between organisms, and interactions collected from other databases (69). More specifically, functional links are inferred from genomic association supported by a unique scoring framework based on benchmarks of the different types of associations against a common reference set. STRING is updated continuously and highly accurate (45).

Data Analysis

We used R version 4.0.5 (2021-03-31) to visualize our collected single-cell RNA sequencing data from the Allen Brain Atlas in an interactive heatmap, which shows likely candidate genes' names and their relative expression in each potential cluster (70).

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