The gene expression features reveal the connection between chloride-relevant gene and disease

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Research Article

Keywords: Neurodevelopment, Gene Expression Pattern, Chloride-Relevant Gene, Single-Cell Analysis, Central Nervous System Disorders.

Posted Date: May 26th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1673294/v1

Abstract

Chloride-mediated signals are involved in forming neural function during development and maintaining its stability in adulthood. The chloride homeostasis is synergistically controlled by various chloride-relevant genes, but their intrinsic relationships remain not fully understood. In this study, an expression profiling of the human brain with different developmental periods was used for the gene expression pattern analysis. Functional analysis for the different expression patterns was performed to explain the functional distinctions of chloride-relevant genes. The major distribution of chloride-relevant genes in single-cell and brain region were investigated to present their spatial expression features. Further exploration was conducted for the relationship between the above expression characteristics of those genes and central nervous system disorders. We found that the chloride-relevant genes regular the specific biological function rather than extensive functions. Additionally, the distribution of chloride-relevant genes is correlated with the pathological regions. Finally, we tested that one of the most well-known chloride-relevant gene *SLC12A2* may take an even more important effect on glial cells rather than neurons. Our findings provide a more comprehensive view to explain the temporal and spatial expression characteristics of chloride-relevant genes, which can help to understand the complex roles of chloride-relevant genes in both the development of the normal human brain and the etiology of brain disorders.

Introduction

Chloride, as the most abundant physiological anion, plays a crucial role in maintaining anion environment for the electrophysiological properties of neural activities. The intracellular concentration of chloride is in charge of communicating the signals of development in central nervous system (CNS), mediating the initiation of several neurophysiological functions (Fukuda 2020). Meanwhile, several studies have highlighted that abnormal chloride homeostasis is associated with brain disorders(Watanabe and Fukuda 2015). Thus, chloride homeostasis is regarded as an attractive therapeutic target, which has yielded promising outcomes in CNS disorders, including autism spectrum disorder (ASD), neonatal epilepsies and schizophrenia (De Koninck 2007). The chloride homeostasis is synergistically controlled by various chloride-relevant genes, while current studies focus more on the role of a single gene, therefore, it is necessary to further study the chloride-related mechanism. In this study, we tend to offer a more comprehensive view to explain the temporal and spatial expression features of chloride-relevant genes, and explore their intrinsic connections with CNS diseases.

Methods

Gene Expression Acquirement

To explore the specific expression of human brain in developmental stages, RNA-seq data of different developmental stages were downloaded from the ATLAS OF THE DEVELOPING HUMAN BRAIN(Miller et al. 2014) (http://www.brainspan.org/static/download). Brain single-cell transcriptome data were obtained from MULTIPLE CORTICAL AREAS-SMART-SEQ(Hodge et al. 2019) (https://portal.brain-map.org/atlases-and-data/rnaseq). The gene landscape of cell types heterogeneity and temporal dynamics across the human brain was obtained by STAB (a SpatioTemporal cell Atlas of the Human Brain)(Song et al. 2021). Heat maps of the gene expression profiles and enrichment analysis were generated using TBtools (Chen et al. 2020).

Gene Expression Pattern Analysis

The gene expression pattern during development was analyzed by Short Time-series Expression Miner (STEM) software(Ernst and Bar-Joseph 2006). The options were set to default since STEM had given optimal results with both biological and simulated data. The expression level used in this analysis was the FPKM value. The p value < 0.05 for the clustered profile was considered significant. R package and related packages were used to realize data visualization.

Function, Pathway, Disease Associated with Dynamic Expression Patterns

Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of different clusters were performed using the R package “clusterprofiler”, and *p* values for the representative GO and KEGG terms shown in this study were adjusted for multiple testing with the Benjamini-Hochberg correction. The functional categories and pathways with adjusted *p* values < 0.05 were deemed to be significantly enriched. Gene-disease associations(GDAs) analysis of differentially expressed genes were classified according to the DisGeNET association type ontology(Piñero et al. 2020). *q* value < 0.05 was considered significant.

Spatial Gene Expression Analysis

The spatial expression information of genes was obtained from the brain using the Allen Institute Human Brain Atlas(Hawrylycz et al. 2012). Whole brain gene expression maps for chloride-relevant genes were downloaded in the Montreal Neurological Institute space (https://neurosynth.org/genes/). Functional connectivity is offered by neurosynth.org, and we adopt this index to exhibit the relative strength of associations. This is the Pearson correlation coefficient(*r*) between the whole-brain (uncorrected) reverse-inference map for each term, and the functional connectivity map seeded at the current location. The mergence of gene maps and whole-brain maps was performed by Python.

Gene Expression in Specific Disease

The specific disease gene expression data was acquired from the Gene Expression Omnibus(GEO) database (GSE108000, GSE122228). To identify the chloride-relevant gene enrichment differences between the control and disease groups, Gene Set Enrichment Analysis (GSEA) was conducted. Differentially Expressed Genes (DEGs) between the control and disease groups were identified using the deseq2 R package. *p*-value < 0.05 and |log2 Fold Change| > 2.0 were set as the screening criteria for significantly differential expression.

Results

Temporal Expression Characteristics of Chloride-relevant Genes

To investigate the temporal expression characteristics of chloride-relevant genes in human brain development, we firstly identified the target genes as the term ‘chloride transmembrane transport (GO:1902476)’ in Gene Ontology database. After being selected by species, 104 genes were included in the final analysis. The RPKM values of those genes were obtained from the ATLAS OF THE DEVELOPING HUMAN BRAIN database. We focused on the transcriptomic changes during hippocampal development, as it was a representative biological process in the development of brain regions(Skutella and Nitsch 2001). A total of 10 time points representing different developmental stages were finally chosen for further analysis (**Table S1**). Multivariate analysis using clustering heatmaps described the classification of different stages (**Fig. 1**). Some genes, such as *CFTR*, *CLCN1* and *SLC22A1*, were maintained at a stable level or stayed at a relative low-level during the lifetime. While some genes, such as *GABRA1*, *CLIC6* and *GLRA2*, which were specifically expressed in the CNS with high levels in all developmental stages, presented different expression trends during development. This result indicates that these genes whose expression levels changed over time might be involved in different developmental stages of brain function formation.

A Dynamic Gene Expression Landscape of Brain Development

Since similar functions are often generated in a specific time window, and the associative transcriptomic states present consistent dynamics(Kang et al. 2011, Zhong et al. 2018). We additionally detected the gene expression patterns of human brain. We used STEM software to cluster the genes with similar expression pattern. In total, 10 distinct clusters were identified, which represented different gene expression kinetics. They were ordered by their statistical significance in Fig. 2.

The number of each cluster was marked in pie chart. Then, we further investigated into which clusters the chloride-relevant genes were grouped. As listed in Table 1, 41 chloride-relevant genes were screened for final analysis. They were evenly distributed among different clusters, and no obvious clustering was observed in one group.

Table 1 Chloride-relevant genes classification

cluster1	cluster2	cluster3	cluster4	cluster5	cluster6	cluster7	cluster8	cluster9	cluster10
SLC26A10	TTYH1	GABRG3	ANO3	GABRG2	LRRC8A	SLC1A1	TTYH2	GABRB2	TTYH3
SLC12A9	CLIC1	GABRA3	GABRB1	GABRG1	SLC12A6	CLCN6	FXYD1	GLRA3	
SLC26A6		SLC1A4	GABRA2	ANO5	SLC12A7		APOL1	GABRA1	
ANO8		CLCN7	GLRB				CLCA4	GABRA5	
PCYOX1			SLC4A8				SLC12A2	GABRA4	
GLRA2								SLC12A5	
ANO10								CLIC6	
								GABRD	
								GABRQ	

Pathways Associated with Expression Patterns

Genes with different expression patterns may get involved in time-dependent development functions. Therefore, we conducted GO and KEGG enrichment analysis with genes from 10 clusters. Each cluster manifested unique biological characteristics (**Fig. 3**).

The enriched GO terms of biological process (BP) included some CNS specific terms in cluster 4, 8, 6, 9. Most terms in the cluster 4 and 9 were related to signal regulation of CNS. The cluster 6 contained the terms for regulation of cell morphogenesis. The cluster 8 embodied the terms related to ensheathment formation, which belonged to non-neuronal components. The enriched cellular components (CC) analysis corresponded with BP associated location. What's more, KEGG analysis revealed that cluster 4, 6, 9 tend to trigger the pathways which were well-known to be involved in the regulation of neural signals. According to the above results, the genes in cluster 4, 6, 8, 9 may be of great importance in the neuronal functions, and we observed some chloride-relevant genes were contained in those clusters, suggesting they may regulate corresponding neuronal functions.

Heterogeneity of Chloride-Relevant Gene in Cell Subtypes

Since some clusters are closely related to CNS-specific functions, we supposed that they may be expressed in specific neural cells. To testify this, we assessed single-cell transcriptomics of chloride-relevant genes in those clusters. As shown in **Fig. 4**, the genes in cluster 4 and 6 showed no preference in certain cell types, nevertheless the genes in cluster 9 were expressed mainly in neurons with bare expression in non-neuronal cells. The genes in cluster 8 were mainly expressed in glial cells. To investigate whether those genes in the specific cell type were determined by early development, we selected some neural cell-relevant genes to detect their expression dynamics in different cell types using STAB. The representative genes selected from different clusters were shown in **Fig. S2**. The results indicated that the remarkable changes of expression level during development were detected in specific cell types, which were consistent with the distribution of the gene in adult.

Spatial Specificity of Chloride-Relevant Genes

Considering the brain is functionally organized into distinct regions that are composed of diverse molecularly defined cell types, we supposed that the chloride-relevant genes detected in diverse cell types may play special roles in brain function. To do this, we downloaded spatial gene expression maps for interested genes. The distribution of all interested genes was shown in **Fig. S1** and **Table S2**, and the representative genes from different clusters showed in **Fig. 5**. The members in cluster 4 had relatively higher expression in cortex area. The chloride-relevant genes in cluster 6 were mainly expressed at the brainstem and ganglia basal. In addition, the chloride-relevant genes in cluster 8 were also highly expressed in the brainstem, ganglia basal and thalamus. Genes in cluster 9 were highly expressed in the visual cortex, temporal lobe, parietal lobe and hippocampus. This result showed that there was anatomical homogeneity of chloride-relevant genes in different clusters.

The Correlation Between Chloride-Relevant Genes and Brain Disorders

We observed the chloride-relevant genes had the functional and anatomical heterogeneity in brain. Many diseases were related to impaired cell functions and connections of brain region, so we next examined the relationship between chloride-relevant genes and diseases. We observed that different clusters were enriched with distinct diseases, and most of them were CNS disorders (**Fig. 6**). In addition, the most common diseases of the CNS were enriched in cluster 4, 6, 8, 9. The cluster 4 and 9 were positively and similarly enriched for kinds of 'Epilepsy'. The cluster 6 was enriched for 'Neurodevelopmental Disorders'. 'Demyelinating Diseases' was glaringly enriched in cluster 8.

The Effects of *SLC12A2* and *SLC12A5* on Different Brain Disorders

NKCC1 and KCC2, encoded by *SLC12A2* and *SLC12A5*, are required for neural chloride homeostasis(Zhang et al. 2021). To further confirm their association with enriched diseases, we examined their heterogeneity in corresponding diseases. Firstly, we defined the chloride-relevant genes which with related neural function as a gene set. Then the GSEA was conducted to evaluate their performance in gene expression profiles of multiple sclerosis and epilepsy. The results showed that the defined gene set was significantly enriched in epilepsy ($p < 0.05$) and multiple sclerosis ($p < 0.05$), indicating chloride-relevant genes were involved in the mechanisms of diseases. Next, *SLC12A2* and *SLC12A5* were compared respectively. *SLC12A2* showed no significant difference compared with control in epilepsy, but it was increased significantly in multiple sclerosis, while *SLC12A5* expression was significantly changed in epilepsy, but not in demyelinating diseases (**Fig. 7**). This result implied that *SLC12A5* and *SLC12A2* weighed differently in the pathological mechanisms of these two diseases.

Discussion

The abnormal chloride homeostasis occurs in various CNS disorders, but the mechanisms are not fully understood. In this work, we explored the relationship between the chloride-relevant genes and neurological diseases according to gene temporal and spatial expression features, tend to provide a new view to illustrate the intrinsic connections between them.

As temporal demands drive the evolution of neural diversity, time is regarded as the key metric to all brain operations. With neural signals sending out specific programs to start or stop the neurogenesis process, similar functions are generated in specific time windows during development(Reh et al. 2020; Silva et al. 2018). Thus, understanding the expression dynamics of crucial genes may help to interpret the function heterogeneity underlying the developmental processes. Since chloride-mediated signals are so important, we investigated the temporal expression features of chloride-relevant genes in human brain development. We found some genes not only presented higher expression levels, but also showed changing trends in developmental periods. As we know, the emergence of distinct functions often occurs in critical periods (CPs) over development, which is driven by multiple molecular events(Reh et al. 2020). Thus, we suppose that the gene dynamics with developmental progress may play an important role in functional formation in neurodevelopment.

To illustrate the relevance between expression trend and biological function, we clustered the genes with similar expression dynamics and enriched their biological functions. We found those clusters were enriched for distinct biological functions, which are essential components to neurodevelopment. Some clusters targeted enrichment terms about transcription regulation, division regulation and energy metabolism, which belong to general cell functions. Beyond that, we observed some enrichment terms were related to some specific functions of neural cells. For instance, genes in cluster 4 were enriched for synaptic components, signal transmissions, and targeted neuron related pathways, such as the 'Oxytocin signaling pathway', which regulates synaptic plasticity and adaptively modifying neural circuits, and causes neurodevelopmental and psychiatric disorders(Jurek and Neumann 2018; Grinevich and Neumann 2021). The 'Phosphatidylinositol signaling system' regulates cell functions including receptor signaling, secretion, endocytosis in CNS. Deregulation of these pathways leads to several neurological diseases(Di Paolo and De Camilli 2006; Pacheco and Jope 1996). Therefore, we suggest that classifying the genes by their temporal expression dynamics may help screen the biological information related to neurodevelopmental processes.

We further paid close attention to the performance of the chloride-relevant genes in these clusters. Most chloride-relevant genes have been reported involved in the corresponding functions as their clusters were enriched. *ANO3* in cluster 4 was enriched for the function of regulating synaptic signaling, and it has been reported to control the excitability of synapse in hippocampus to response hyperthermy(Wang et al. 2021; Feenstra et al. 2014). *SLC12A6* and *SLC12A7* help maintain cell volume and intracellular chloride levels(Mount et al. 1999), which is concordant with the functions in cluster 6. The gene *TTYH2* in cluster 8, constitutes Volume-regulated Anion Channel (VRACswell) channel and brings intracellular volume to normal levels(Nalamalapu et al. 2021), which executes glial-related functions. As the chloride-relevant genes were scattered in various functional modules, we believe the chloride-relevant genes regular the specific biological function rather than extensive functions.

The similarities between genes in their expression patterns may reflect cell type specific expression(Huisman et al. 2017). Thus we suppose this distinction of functions from the GO enrichment might result from their differences in cell types. As mentioned before, cluster 4, 6, 9 were enriched for the neural functions in functional analysis, which was consistent with our finding that, the chloride-relevant genes in those clusters were mostly detected in neurons (Fig. 3), for example, the GABA receptor gene family genes in cluster 9(Fritschy and Mohler 1995). Furthermore, we found the expression of *GABRA5* and *GABRD* were mostly detected in excitatory neurons, indicating a more important role they play in that type of cells. It has been proved that *GABRA5* contributes to excitatory synapse development(Nuwer et al. 2021; Fischell et al. 2015). *GABRD* codes the delta subunit of GABAA receptor which generates tonic inhibition(Ahring et al. 2021; Whissell et al. 2016), and its effect on excitatory neurons is worthy of further study. What's more, the data from STAB indicated that the dramatic changes of the gene level in specific cell during developmental period also proved the important roles of chloride-relevant genes for neurological function formation in development. However, it should be admitted that the data from STAB only supply gene expression value with limited time points after birth, so more detailed studies in postnatal stage are necessary.

Brain is functionally organized into distinct regions. We speculate that chloride-relevant genes with similar functions in the same cluster may have similar spatial distribution in the brain. The result showed that the chloride-relevant genes in the same clusters presented higher expression in specific brain area, and the distribution differences were shown between clusters. Considering that abnormal connections between certain brain regions appear in different diseases, we further analyze the gene-disease association for clusters. Combining the results of expression characteristics in brain area and disease, we noticed the brain region with high gene expression level was in accordance with pathological sites of these diseases. For example, the genes in cluster 4 and 9 were mainly expressed in cortex, hippocampus and temporal lobe, which constitute the seizure circuits and regulate the different stages of seizures(Bertram 2013; Wang and Chen 2019). The pathogenesis of epilepsy is the disruption of synaptic transmission and membrane potential(Keezer et al. 2016), which also correspond with the enrichment of function analysis, indicating that the chloride-relevant genes in cluster 4 and 9 may play more important roles in the mechanism of seizure than other chloride-relevant genes. We also noticed that part of genes in these two clusters encoded GABA receptors. Those genes are associated with a broader spectrum of epileptic syndromes.

The variants in GABA receptor genes can either be associated with monogenic epileptic syndromes or constitute risk factors for the genetically complex generalized epilepsies (May et al. 2018). Interestingly, some genes in cluster 9 were distributed in hippocampus, while the genes in cluster 4 had less expression in this region. This may provide a clue that genes in cluster 9 might have higher therapeutic significance for the epilepsy in which the pathological process involved the hippocampus.

In previous studies, chloride is identified as a signal that broadly regulates neural activities in the CNS. So far, we suggest that chloride-relevant genes play more specific roles in certain diseases, for they have specific molecular characteristics, cell type distribution and brain region distribution. Moreover, disease-related chloride dyshomeostasis may not be caused by a single chloride-relevant gene, but caused by the genes with same expression characteristics.

Concretely, we focused on the best-known chloride-relevant gene *SLC12A2* and *SLC12A5*, encoding protein NKCC1 and KCC2. KCC2 is the major extruder of intracellular chloride, while NKCC1 mediates the influx of chloride ions(Nalamalapu et al. 2021). They are indispensable for neural chloride homeostasis. Dysfunction of them can cause depolarizing actions of GABA leading to neuronal hyperexcitability, which would not only result in the pathogenesis of several brain disorders (Huang et al. 2019), but also lead to GABAergic signal disorder during brain development(Watanabe and Fukuda 2015). In our temporal single-cell results, we found that *SLC12A2* expression mainly changed in oligodendrocytes but was stable in neurons. If dramatic change of mRNA occurs mainly in non-neuronal cells, how it regulates neuronal excitability in development, is worth further studying. Moreover, according to our data above, they were classified into completely different expression patterns (Fig. 8). *SLC12A5* belonged to the similar pattern with GABA receptors, and it was located in neurons and mainly involved in the regulation of neural functions, which accorded with the previous reports(Sedmak et al. 2016; Vu et al. 2000; Coull et al. 2003). *SLC12A2* mediated the function of non-neuronal cells, and its corresponding disease is myelin dysfunction, which were barely reported. However, *SLC12A2* is regarded as a target gene in epilepsy treatment(Dzhala et al. 2010; Hampel et al. 2021). We found it not significantly changed in epileptic models, but acted more sensitively in demyelinating diseases compared to *SLC12A5*. Overall, we suggest *SLC12A2* may play an even more important role in oligodendrocytes rather than neurons.

SLC12A2 in cluster 8, is enriched for the biological functions of non-neural cells, and is mainly expressed in oligodendrocytes. The brain region distribution concentrates in the brainstem and basal ganglia. The associated disease is Demyelinating disease.

SLC12A5 in cluster 9, is enriched for the biological functions of neural cells. The high expression is detected in neurons. The brain region distribution concentrates in the cerebral cortex, especially in visual cortex and parietal lobe. The associated disease includes Neuralgia and Epilepsy.

In summary, although the chloride disorder occurs in various neurological diseases, the chloride-relevant genes involved in homeostasis regulation are specific. The findings of our current study are compelling but have some limitations. The lack of abundant post-natal data on CPs bounds the interpretation of results in those periods. And it should not be ignored that the delicate regulations of post-protein modification in development, which can magnify functions of protein even when the mRNA level is relatively low. Therefore, we believe that more comprehensive studies for chloride-relevant genes are required to help decode the intricate mechanisms that underlying brain diseases.

Abbreviations

CNS Central nervous system

ASD Autism spectrum disorder

DEG Differentially expressed gene

CPs Critical periods

Declarations

Author Contributions Conceptualization: YW Wang, ZJ Hao, and YR Huang. Methodology and analysis: QH Wang, YS Peng, WJ Du, Q Wang and JT Qi. The first draft of the manuscript was written by YR Huang and QH Wang. All authors commented on previous versions of the manuscript and all authors read and approved the final manuscript.

Funding This work was supported by the National Natural Science Foundation, People's Republic of China (Grant Nos. 81730031 to YW Wang, Grant Nos. 82001455 to WJ Du) and the Foundation of Shanghai Municipal Key Clinical Specialty (shslczdzk06901 to YW Wang).

Data Availability Data will be made available on reasonable request.

The authors declare that they have no conflict of interest.

Consent for Publication All authors have revised and have agreed with the content of the manuscript. Also, all authors have given explicit consent to submit

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Figures

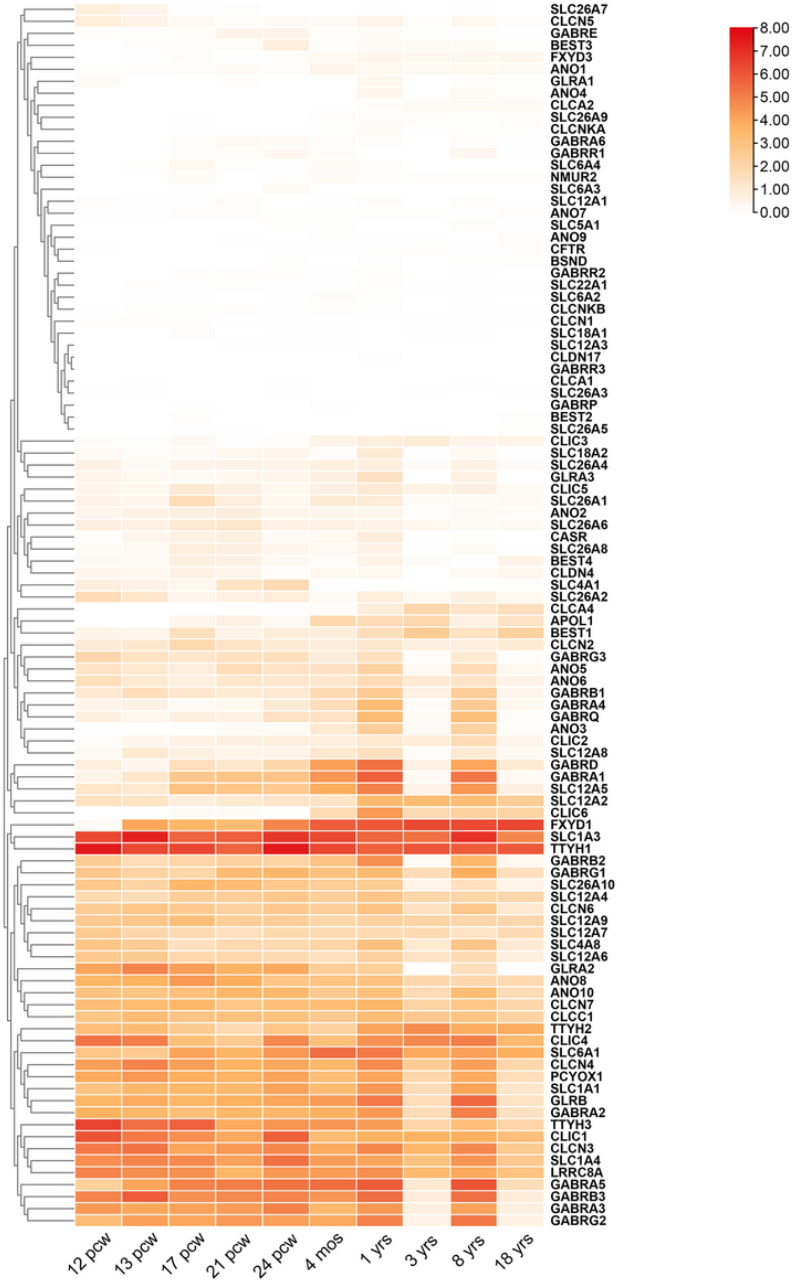

Figure 1

Chloride-relevant genes expression dynamics

Heat map of chloride-relevant genes with development.

pcw, postconception week, mos, months, yrs years.

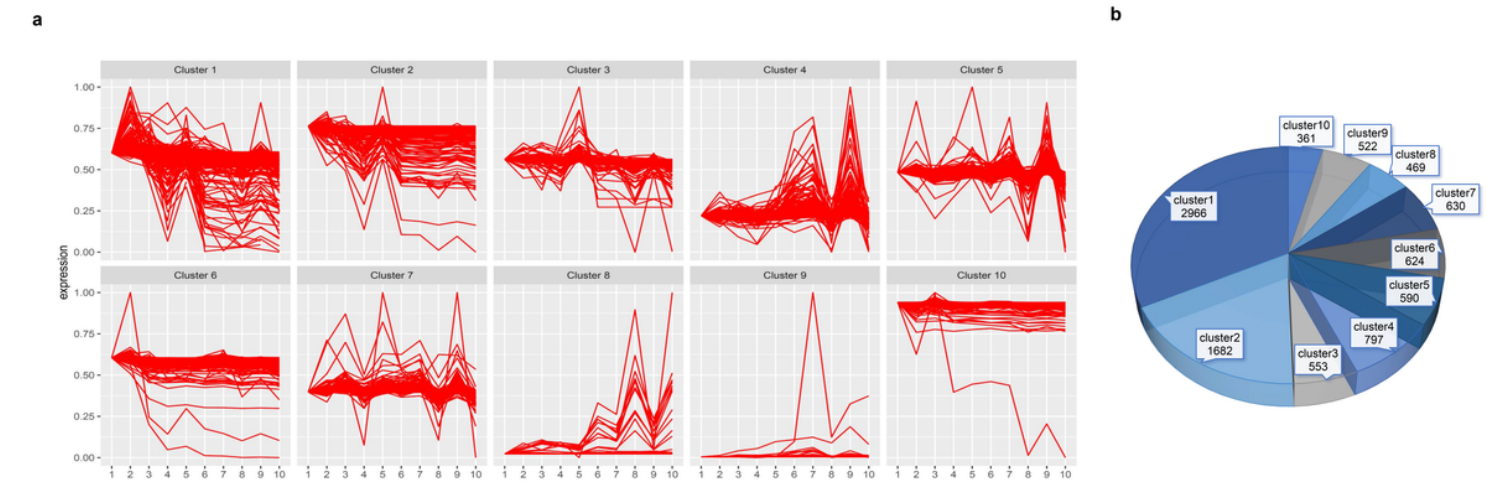

Figure 2

Temporal profiles of gene expression in developmental brain

a The series of diagrams illustrate the patterns of dynamic changes of genes during the developmental period determined by STEM. The x axis represents 10 developmental timing, 1 to 10 are 12 pcw, 13 pcw, 17 pcw, 21 pcw, 24 pcw, 4 mos, 1 yrs, 3 yrs, 8 yrs, 18 yrs, respectively. while the y axis represents normalized expression change in each stage. **b** The pie chart shows the number of genes in each cluster in A.

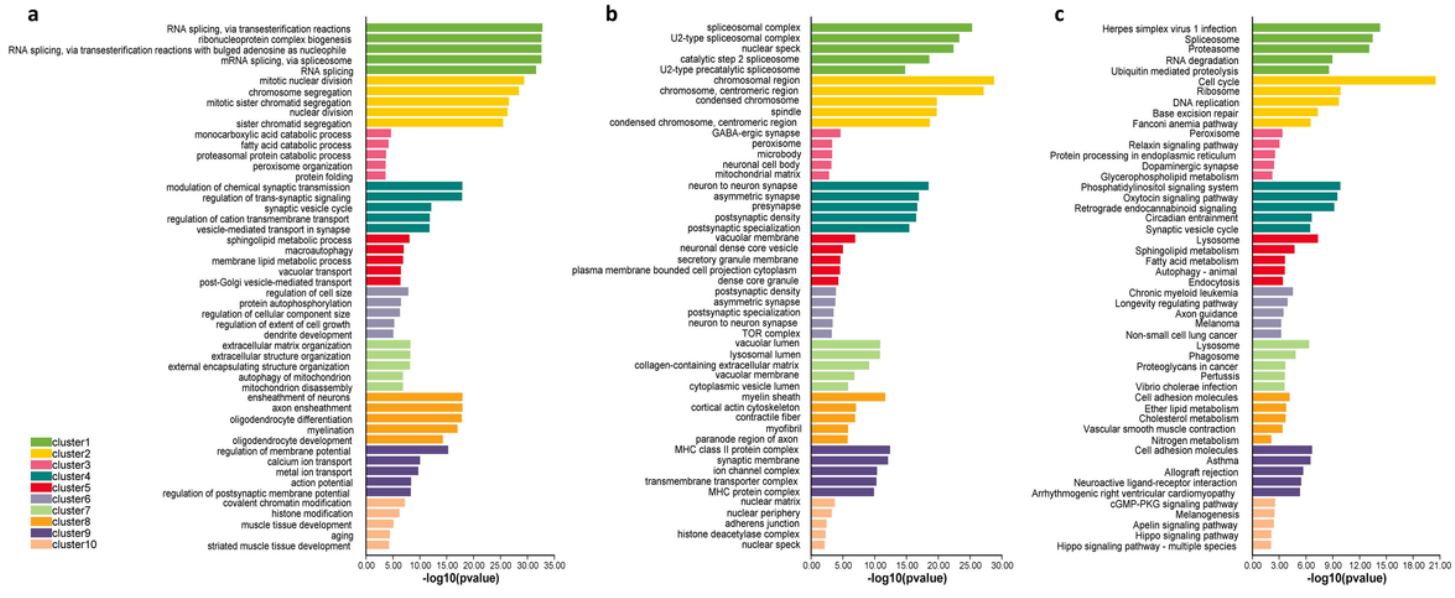

Figure 3

Molecular characteristics of dynamic expression patterns

a GO analysis (biologic processes, BP) of the genes in 1-10 clusters

b GO analysis (cellular components, CC) of the genes in 1-10 clusters

c KEGG analysis of the genes in 1-10 clusters

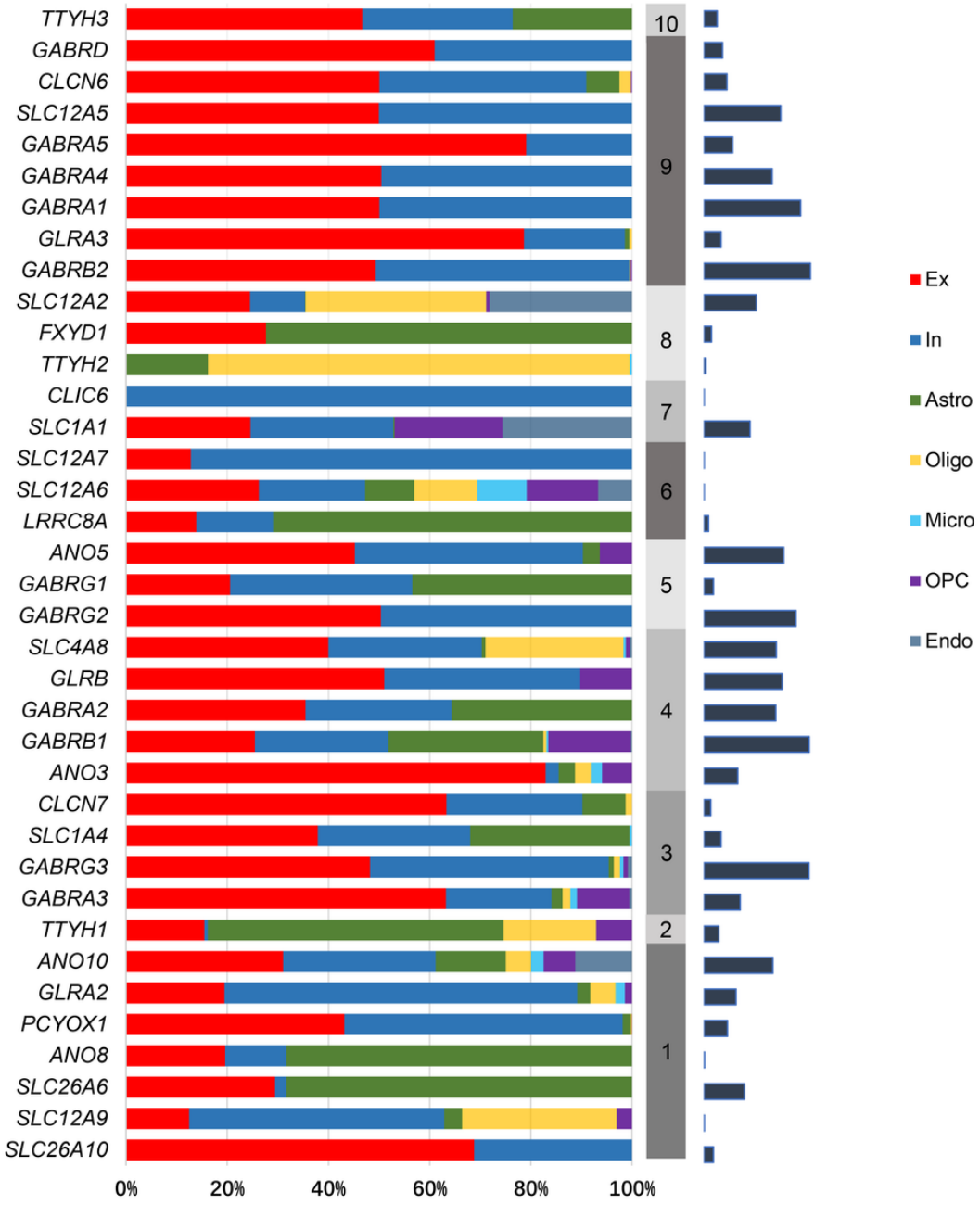

Figure 4

Expression levels of chloride-relevant genes in different cell types

The salmon-colored bars show the percentage of gene expression in each cell type. Bar with color gray indicates the 1-10 clusters. Bar with color dark blue on the right indicates the relative expression level of genes in brain. (Genes with extremely low expression are removed.)

Ex, Excitatory neuron, In, Inhibitory neuron, Astro, Astrocyte, Oligo, Oligodendrocyte, Micro, Microglial cell, OPC, Oligodendrocyte Precursor Cell, Endo, Endotheliocyte.

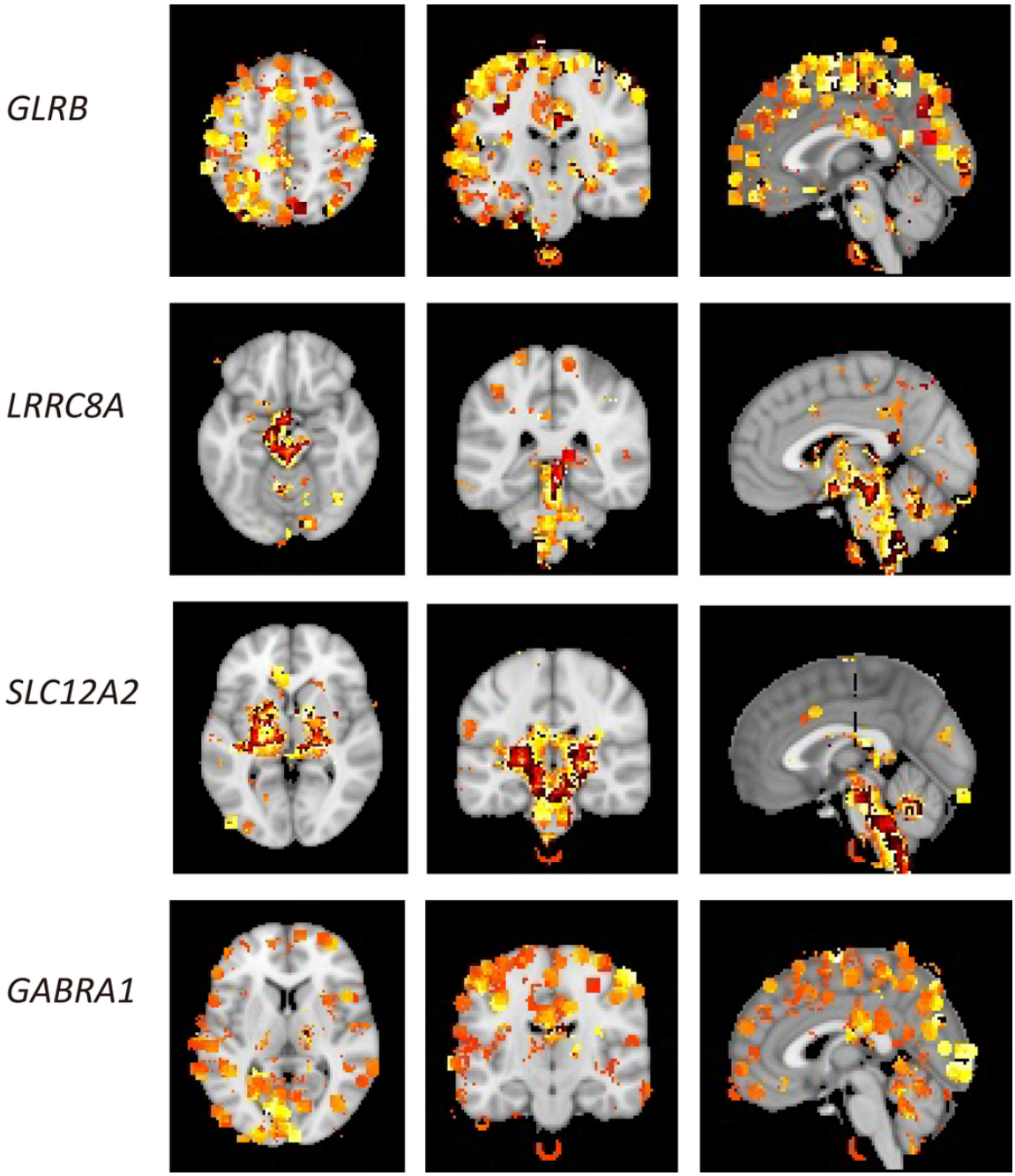

Figure 5

Regional gene expression in the brain for chloride-relevant genes

This figure shows brain regions with high levels of expression for chloride-relevant genes. Selected representative genes from different clusters for display. The heat map indicates the correlation. Yellow means relatively higher correlation. Red means relatively lower correlation. (Thresholds follow the setting in Neurosynth.org)

GLRB in cluster 4, is mainly expressed in cerebral cortex.

LRRC8A in cluster 6, is mainly expressed in brainstem, midbrain and thalamus.

SLC12A2 in cluster 8, is mainly expressed in thalamus, brainstem and basal ganglia.

GABRA1 in cluster 9, is predominantly expressed in cerebral cortex, particularly the visual cortex and parietal lobe.

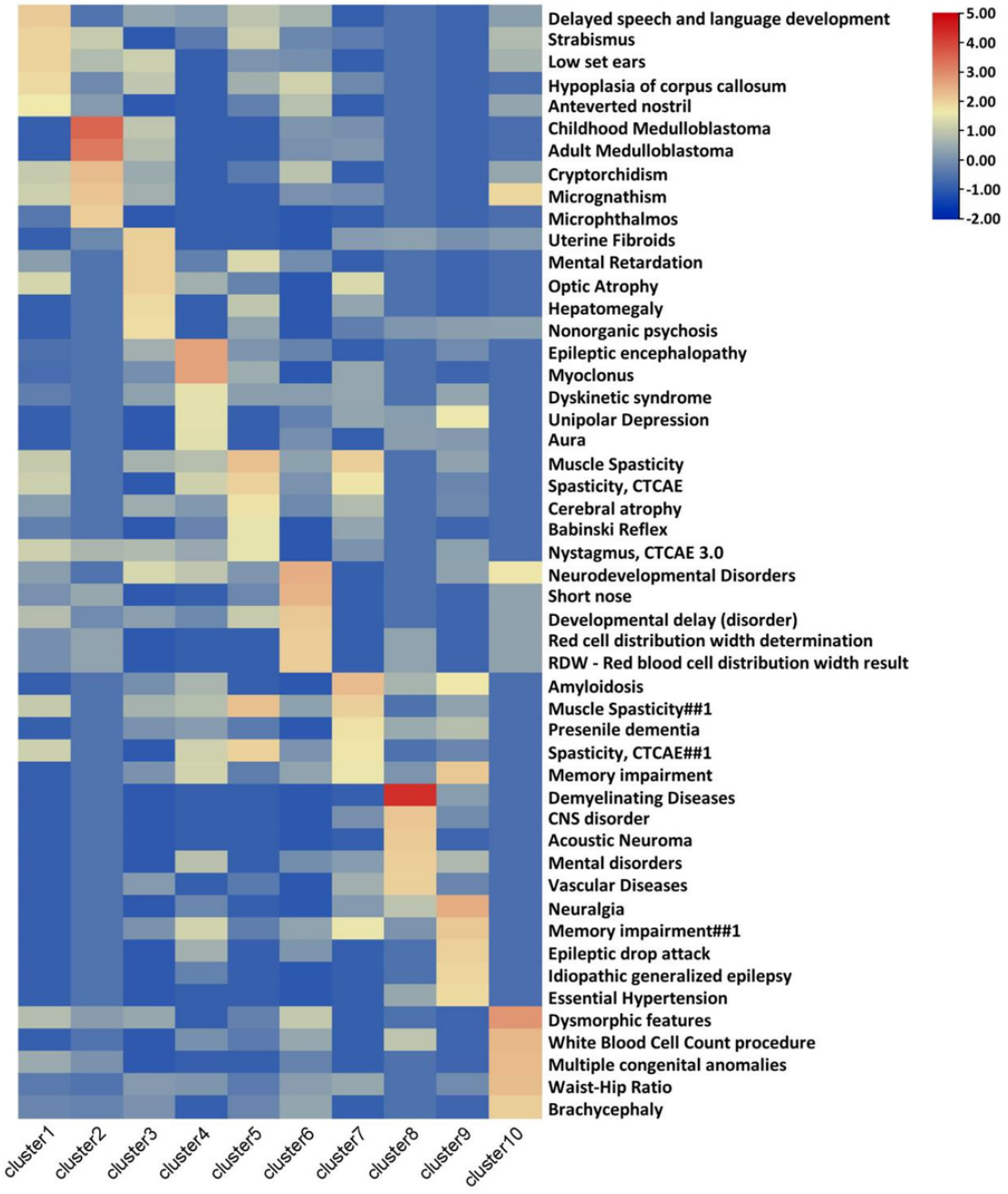

Figure 6

Gene-disease associated analysis of genes in different clusters

The heat map shows the -log10 transformed significance value of the disease terms in the gene-disease associated analysis describing each of the 10 clusters.

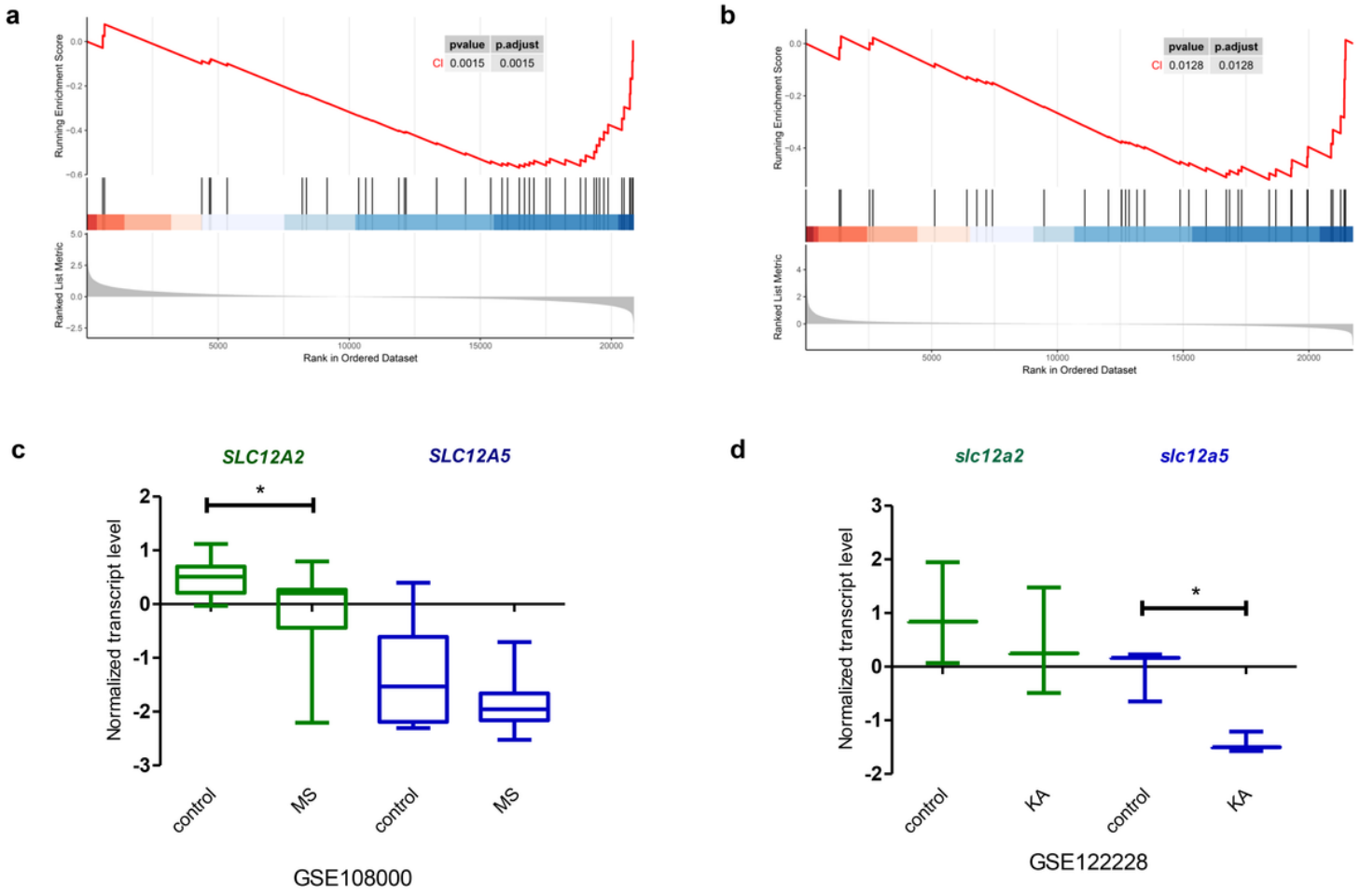

Figure 7

Schematic overview of the expression characteristics of *SLC12A2* and *SLC12A5*

SLC12A2 in cluster 8, is enriched for the biological functions of non-neural cells, and is mainly expressed in oligodendrocytes. The brain region distribution concentrates in the brainstem and basal ganglia. The associated disease is Demyelinating disease.

SLC12A5 in cluster 9, is enriched for the biological functions of neural cells. The high expression is detected in neurons. The brain region distribution concentrates in the cerebral cortex, especially in visual cortex and parietal lobe. The associated disease includes Neuralgia and Epilepsy.

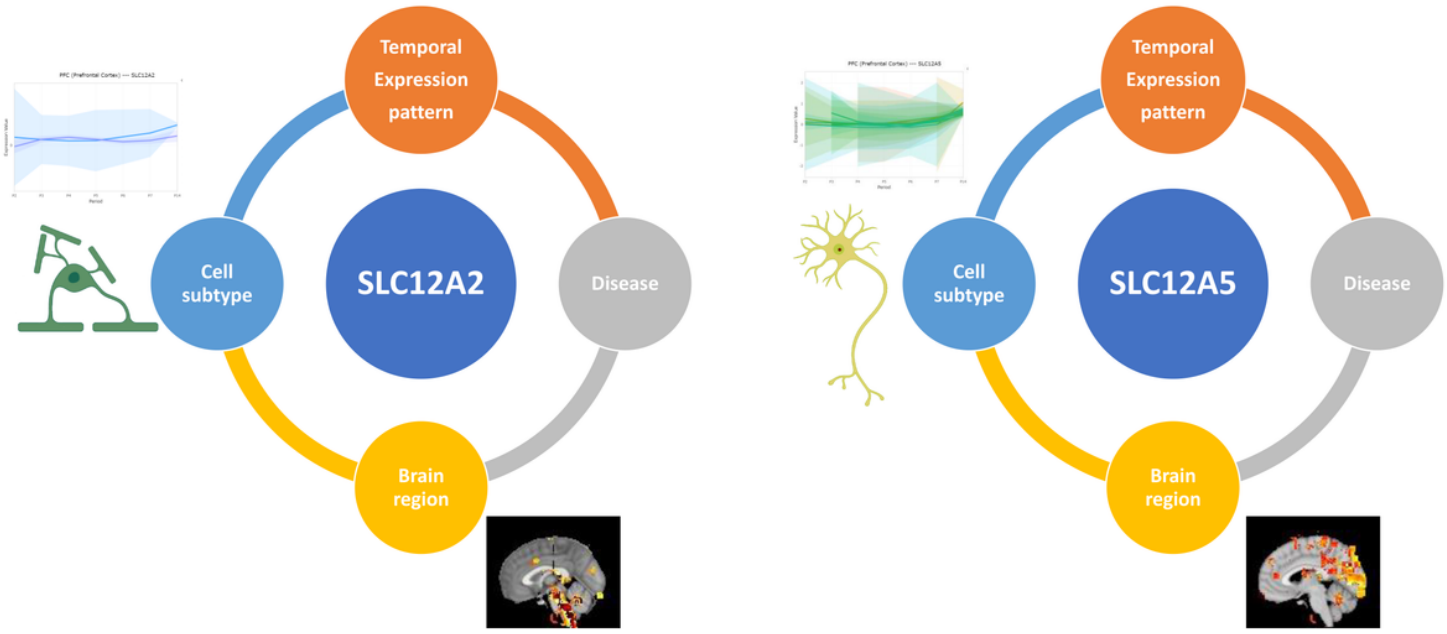

Figure 8

Schematic overview of the expression characteristics of *SLC12A2* and *SLC12A5*

Supplementary Files

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

- FigureS1.pdf
- FigureS2.pdf
- TableS1Periodsofhumandevelopmentdefinition.xlsx
- TableS2Theexpressiondynamicsofgeneswithindifferentcellsubtypesalongwithdevelopment.xlsx