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Original Article

Differential excitatory vs inhibitory SCN expression at single cell level regulates brain sodium channel function in neurodevelopmental disorders

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ABSTRACT

The four voltage-gated sodium channels SCN1/2/3/8A have been associated with heterogeneous types of developmental disorders, each presenting with disease specific temporal and cell type specific gene expression. Using single-cell RNA sequencing transcriptomic data from humans and mice, we observe that *SCN1A* is predominantly expressed in inhibitory neurons. In contrast, *SCN2/3/8A* are profoundly expressed in excitatory neurons with *SCN2/3A* starting prenatally, followed by *SCN1/8A* neonatally. In contrast to previous observations from low resolution RNA screens, we observe that all four genes are expressed in both excitatory and inhibitory neurons, however, exhibit differential expression strength. These findings provide molecular evidence, at single-cell resolution, to support the hypothesis that the excitatory/inhibitory (E/I) neuronal expression ratios of sodium channels are important regulatory mechanisms underlying brain homeostasis and neurological diseases. Modulating the E/I expression balance within cell types of sodium channels could serve as a potential strategy to develop targeted treatment for Na_V-associated neuronal developmental disorders.

Abbreviations: DS, Dravet Syndrome; E/I, Excitatory/Inhibitory; GoF, Gain-of-function; LoF, Loss-of-function; NDDs, Neurodevelopmental Disorders; PTVs, Protein Truncating Variants; *SCN* (*Scn*), Sodium Channel Gene; SCN/Na_V, Sodium Channel Protein; scRNA-seq, Single-cell RNA Sequencing.

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1. Introduction

Voltage-gated Na^+ channels encoded by *SCNs* are intrinsic plasma membrane proteins that initiate the action potential in electrically excitable cells. The α-subunit genes encode the primary channel-forming pores and also play a critical role in the propagation of action potentials along neurons. Variants in the predominant Na_Vs expressed in brain, *SCN1A* ($Na_V1.1$), *SCN2A* ($Na_V1.2$), *SCN3A* ($Na_V1.3$), and *SCN8A* ($Na_V1.6$) [1], are one of the most frequent causes of Mendelian forms of epilepsy and neurodevelopmental disorders (NDDs). A hallmark of these disorders is the heterogeneity in clinical phenotypes, including age-of-onset, disease severity, and drug response.

In the past decades, integrative genetic and electrophysiology studies were conducted to assign *SCN* genetic variants to disease phenotypes. Differential mutational landscapes were observed among *SCNs*, in particular between *SCN1A* and *SCN2A/3A/8A*. On one hand, loss-of-function (LoF) variants in *SCN1A*, including protein truncating (PTVs) and missense variants, mainly cause Dravet Syndrome (DS), a severe form of developmental and epileptic encephalopathy that typically presents in the first year of life. DS patients are often refractory to treatment and conventional SCN blocking anticonvulsants can exacerbate the disorder [2]. LoF variants in *SCN2A* are mainly associated with autism spectrum disorder [3], characterized by global developmental delay (particularly of social and language skills), and LOF variants in *SCN8A* can result in isolated intellectual disability [4]. The phenotypic effects of LoF variants in *SCN3A* remain to be established, though this gene is strongly depleted for PTVs in the general population. On the other hand, *SCN1A* gain-of-function (GoF) variants are associated with familial hemiplegic migraine [5] and *SCN2A/3A/8A* GoF variants contribute substantially to early infantile epilepsy with/without intellectual disability [6,7].

Developmental and spatial Na_V expression patterns have emerged as molecular-level traits that influence the effects of genetic variants on electrical signaling. In the rat, the expression of $Na_V1.1$, $Na_V1.2$, and $Na_V1.6$ increases continually from birth, while $Na_V1.3$ peaks at birth but is maintained at low levels in adulthood [8]. In neo-cortex and hippocampus, inhibitory interneurons largely express $Na_V1.1$ whereas excitatory neurons predominately express $Na_V1.2$ and $Na_V1.6$,[9] suggesting expression variations of Na_Vs between neuronal types. However, it is not clear how these variations are driven by changes at the single-cell level.

Recently, technological advances in single-cell RNA sequencing (scRNA-seq) enable the transcriptomes of individual cells to be assayed in a high-throughput and high-resolution manner, which can clarify the distribution of *SCN* expression. In this study, we use scRNA-seq to evaluate Na_Vs expression profiles across excitatory and inhibitory neurons in human and mouse brain regions and reveal further insights into the molecular pathology of the *SCN*-associated NDDs.

2. Materials and methods

We first analyzed *SCNs* expression at single-cell level from postmortem adult human cerebral cortex using droplet-based scRNA-seq (Drop-seq) [10] (unpublished data, courtesy of Dr. Steven McCarroll). Reads alignment (human assembly hg19) and cell clustering were performed using Drop-seq software (v1.13, http://mccarrolllab.org/dropseq) and Seurat (v1.4.0.8, https://github.com/satijalab/seurat). Neuronal markers were used to identify clusters of pan-excitatory (*SLC17A7*$^+$) and pan-inhibitory (*GAD1*$^+$) neurons (*RBFOX3*$^+$). We also compared our study to previously published scRNA-seq studies on several human brain regions, including adult hippocampus [11], adult prefrontal cortex [11], mature fetal brain [12], and immature fetal brain [12].

We then performed plate-based single nucleus RNA-seq (sNuc-seq) [13] from several brain regions of mice, including the hippocampus of adult Pvalb-Cre mice (8–10 weeks, the Jackson Laboratory, #008069), whole cortex tissues of wild-type mice (C57BL/6) at postnatal day 1 (P1) and days 30 (P30) (unpublished data, courtesy of Dr. Joshua Z. Levin, Dr. Boaz Barak, and Dr. Guoping Feng). Single neuronal nuclei (Rbfox3$^+$) were isolated from freshly dissected tissues and enriched by fluorescence activated cell sorting (FACS). Libraries were constructed using a modified SMART-Seq2 method and sequenced on NextSeq500 (Illumina, read 1: 50bp; read 2: 25bp, 1 million reads/cell). We further integrated these three sNuc-seq datasets with previously published two scRNA-seq data sources, adult primary visual cortex [14] and somatosensory cortex combined with hippocampus (Hippocampus and SCx) [15]. The principal component analysis was performed to reduce the dimensionality of each dataset, followed by Louvain clustering to identify excitatory (*Slc17a6/7*$^+$ & *Rbfox3*$^+$) and inhibitory (*Gad1/2*$^+$ & *Rbfox3*$^+$) neurons. The second round of clustering was used to refine the neuronal clusters in the P30 cortex, after regressing out sort types (unsorted, *Rbfox3*$^+$, or *Rbfox3*$^-$ sorted cells). The scaled expression data from P1 cortex was projected onto a matrix of average gene expression from each cluster in P30 cortex and further clustered using the hclust function in R.

For both human Drop-seq and mouse sNuc-seq analysis, single-cells were visualized on a two-dimension space based on expression signatures of neuronal markers using t-distributed stochastic neighbor embedding (t-SNE) method (tsne v0.1.3, https://cran.r-project.org/web-/packages/tsne). The gene expression level was evaluated using transcripts per million (TPM). *SCN/Scns* were selected with MeanVarPlot function and the number of genes per cell was corrected for using the RegressOut function from Seurat. The differential gene expression analysis and FDR adjusted p-values were performed using MAST (v1.0.5, https://bioconductor.org/packages-/release/bioc/html/MAST.html).

3. Results

To investigate whether there is cell-type specific expression of the four *SCN* genes in the human brain, we analyzed Drop-seq profiling of cells derived from the adult cerebral cortex. We connected 80,000 cells to known cell types using a set of markers that are highly expressed in each cell type and visualized cells in cortex sections using t-SNE plots, in which individual points correspond to single cells identified by the expression of the marker gene (e.g. *RBFOX3*, Fig. 1A). In total, seven out of 15 clusters were identifiable as neuronal cells (*RBFOX3*$^+$), including excitatory (*SLC17A7*$^+$) and inhibitory (*GAD1*$^+$) neurons. We observed that all four *SCNs* displayed expression in both excitatory and inhibitory neurons. However, a lower expression ratio between excitatory/inhibitory (E/I) neurons was detected for *SCN1A* compared to *SCN2A/3A/8A* (Fig. 1B). Additionally, *SCN1A* expression level is lower in deep-layer excitatory cortical neurons (clusters 5–7) compared to up-layer excitatory neurons (clusters 1–2) suggesting a layer-specific distribution of *SCN1A* in the cortex, which might be associated to layer-specific neuronal excitability, suggesting the E/I expression ratio variations between *SCN1A* and *SCN2A/3A/8A* are maturity-related.

To validate our observation, we explored previously published scRNA-seq data on several human brain regions including adult hippocampus [11], adult prefrontal cortex [11], mature fetal brain [12], and immature fetal brain [12]. We compared expression of each *SCN* in excitatory vs inhibitory neurons and the ln(fold change) were plotted in Fig. 2A. We observed an enrichment of *SCN1A* in inhibitory neurons in the adult brain regions and a more

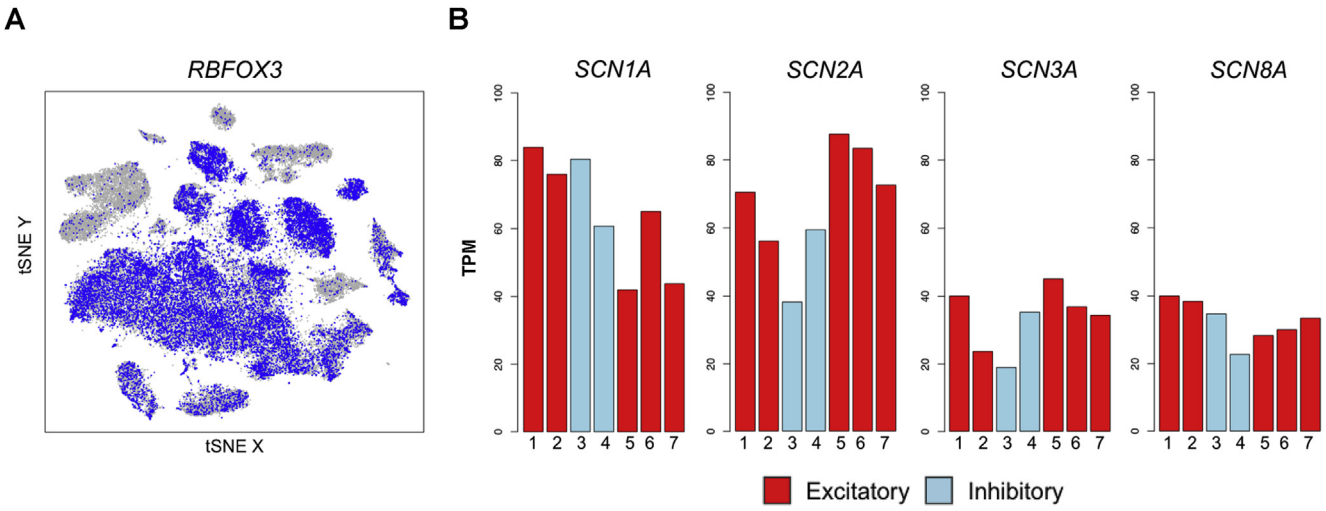

Fig. 1. Single-cell analysis of excitatory and inhibitory neuronal expression of *SCNs* using Drop-seq data from human cortex.
A) The two-dimensional t-SNE representation of the Drop-seq data with clusters colored in blue according to the expression of the neuronal marker (*RBFOX3*$^+$) in cortex sections. **B)** Differential *SCNs* expression in excitatory and inhibitory neurons in the cortex. Bar plots show the mean expression levels (TPM) of *SCNs* in 7 broad cell clusters identified in the t-SNE visualization, of which bars are colored in red and blue according to inhibitory (*GAD1*$^+$ & *RBFOX3*$^+$); cluster 3–4) and excitatory (*SLC17A7*$^+$ & *RBFOX3*$^+$) neurons. Clusters 1–2 are up-layer excitatory and clusters 5–7 are deep-layer excitatory neurons. A lower gene expression ratio between excitatory and inhibitory neurons is detected for *SCN1A* compared to *SCN2A/3A/8A*. *SCN1A* also displays a lower expression in the deep-layer excitatory neurons compared to up-layer excitatory neurons. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

profound expression of *SCN2A/3A/8A* in excitatory neurons in both adult and fetal brain regions.

We also performed integrative scRNA-seq analysis on normal mouse brain regions including adult hippocampus (342 neurons), adult somatosensory cortex combined with hippocampus (Hippocampus & SCx, 632 neurons) [15], adult primary visual cortex (1631 neurons) [14], P1 cortex (458 neurons), and P30 cortex (620 neurons). In alignment with the adult human data, an inhibitory neuronal enrichment of *Scn1a* and excitatory neuronal expression of *Scn2a/3a/8a* were detected (Fig. 2B). Moreover, we observed age-related changes in *Scns* expression and their E/I expression ratios in the cortex. At the very early P1 stage, no significant difference in the E/I expression ratio was observed for *Scn1a/8a*. From P1 to P30, a significantly lower E/I ratio for *Scn1a* and a higher E/I ratio for *Scn2a/3a/8a* were detected, suggesting the E/I expression ratio variations between *Scn1a* and *Scn2a/3a/8a* are maturity-related.

Taken together, *SCN1A* (*Scn1a*) is expressed predominantly in inhibitory neurons, whereas, *SCN2A/3A/8A* (*Scn2a/3a/8a*) are predominantly expressed in excitatory neurons. This expression similarity of *SCN2A/3A/8A* (*Scn2a/3a/8a*) and their divergence from *SCN1A* are in line with *SCN*-associated NDDs phenotypes.

4. Discussion

The two main types of neurons are excitatory and inhibitory neurons, which together maintain the homeostasis of neuronal excitability in the brain. By analyzing single-cell transcriptomes in human and mouse brain regions, we uncover differential expression profiles of Na_Vs in excitatory and inhibitory neurons. *SCN1A* (*Scn1a)* is highly expressed in inhibitory neurons across human/mouse brain, indicating its dominating function in inhibitory neuronal activity. Accordingly, LoF variants in *SCN1A* can lead to reduced activity of inhibitory neurons and result in hyperexcitable neuronal populations. This notion is supported by functional studies showing that deletion of $Na_V1.1$ in inhibitory interneurons causes seizures and premature death, whereas $Na_V1.1$ haploinsufficiency in excitatory neurons has an ameliorating effect in a mouse model of DS [9]. Second, *SCN2A/3A/8A* (*Scn2a/3a/8a*) show relatively enriched expression in excitatory neurons in multiple brain regions, suggesting their primary functions in excitatory conductance. GoF variants in *SCN2A/3A/8A* can influence excitatory neurons more than inhibitory neurons by elevating E/I expression ratio of these genes and inducing hyperexcitability. Third, an age-dependent change in E/I expression ratio is observed for four *Scns* in mouse cortex, suggesting that activities mediated by these four channels may be required in the cortex during restricted periods of development.

Several previous studies investigated functional links among Na_Vs. For example, reduced *in vivo* expression of *Scn8a* function was found to ameliorate seizure severity in *Scn1a*$^{+/-}$ DS mice and rescue their premature lethality [16]. However, this rescue effect cannot be explained by the existence of a compensatory up- or down-regulation of *Scn1a* and *Scn8a*, because *Scn8a* expression does not differ between *Scn1a* null mutants and wild type [9]. The Na_V blocker GS967, which functions by reducing persistent sodium current, revealed high efficacy in suppressing spontaneous seizures and prolonging survival time in *Scn1a*$^{+/-}$ DS mice [17], as well as in models of *Scn2a* [18] and *Scn8a* [19] epilepsy. The data suggests that impaired inhibition due to *Scn1a* LoF variants can be corrected via adjusting the activity of *Scn8a*, which restores E/I signalling balance and maintains neuronal activity. Previously, some NDDs, from autism spectrum disorder to schizophrenia, were found to exhibit alterations of E/I balance at the circuit level [20]. These findings could be relevant for Na_Vs-associated disorders since the disinhibition of inhibitory neurons and hyperexcitability of excitatory neurons are common mechanisms of these diseases. In the future, developing a more complete understanding of the single-cell transcriptome profiles of *SCNs* in normal and disease state will allow testing of the E/I balance theory.

In conclusion, we observed differential E/I neural expression patterns of Na_Vs at single-cell level, which could be a signature of brain neuronal activity. Modulating the E/I expression balance of NaVs may be a strategy for targeted therapy of NaV-associated NDDs.

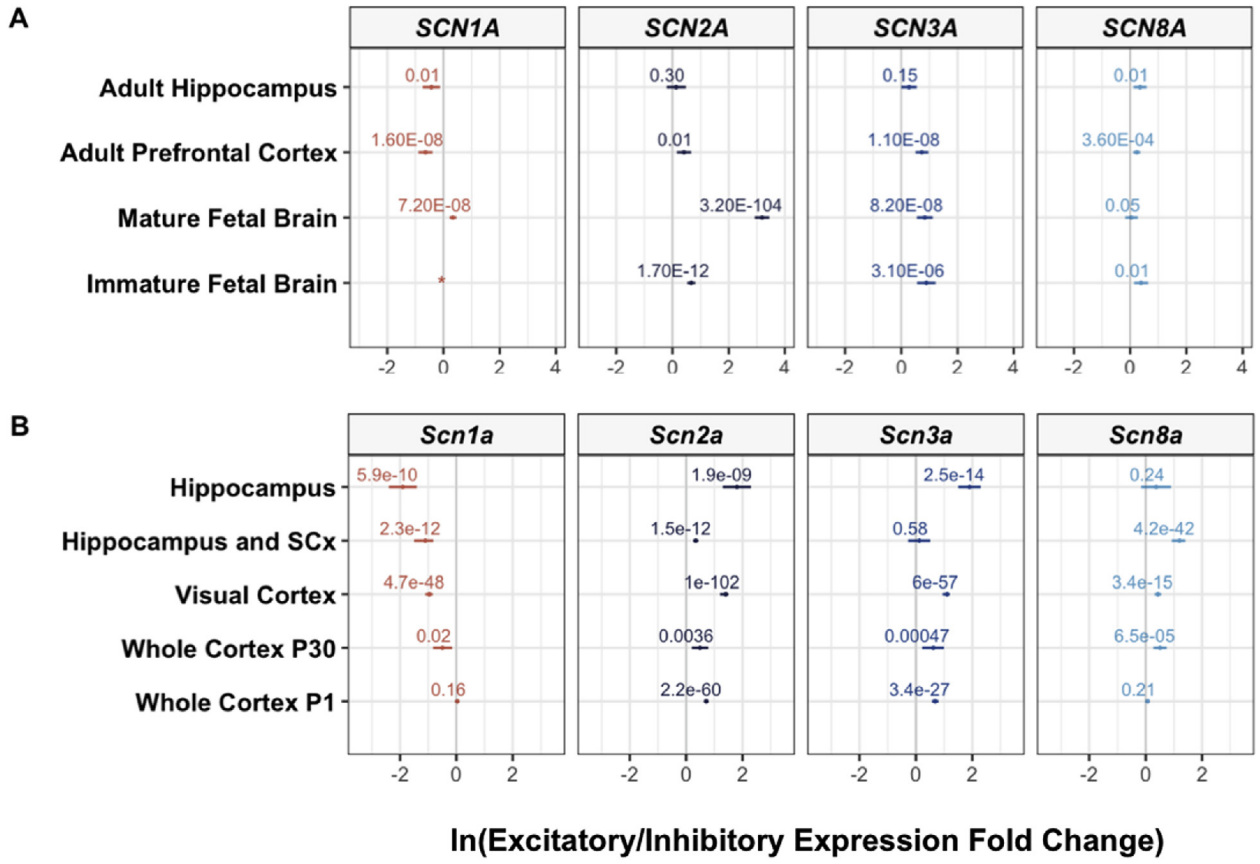

Fig. 2. Comparison of excitatory and inhibitory neuronal expression of *SCNs/Scns* using single-cell sequencing data from different human (A) and mouse (B) brain tissues.
A) Differential *SCN* expression in excitatory and inhibitory neurons in the human brain. Using previously published data, we examined single-cell expression of *SCNs* in several human brain regions, including the adult hippocampus, prefrontal cortex (PFC), mature fetal brain, and immature fetal brain. *GAD1* was used as the marker for inhibitory neurons and *SLC17A7* was used as the marker for excitatory neurons. Plotted is the natural logarithmic excitatory/inhibitory neuronal expression ratio of SCN1A/2A/3A/8A. P-values are shown above the fold change and confidence interval. Overall, *SCN1A* has a relatively higher expression in inhibitory neurons in adult tissues whereas the other three *SCNs* are enriched in excitatory neurons. For each gene, the E/I ratio of expression varies up to 4-fold in different brain regions. **SCN1A* was expressed a few newborn cells and was filtered out in differential expression analysis.
B) Differential *Scn* expression in excitatory and inhibitory neurons in the mouse brain. We examined single-cell expression of *Scns* in adult hippocampus, adult somatosensory cortex combined with hippocampus (Hippocampus & SCx), adult primary visual cortex, and whole cortex tissue (P1 and P30). We used *Gad1* as the marker for inhibitory neurons and *Slc17a7* as the marker for excitatory neurons (*Rbfox3*). Plotted is the natural logarithmic excitatory/inhibitory neuronal expression ratio of *Scn1a/2a/3a/8a*. P-values are shown above the fold change and confidence interval. Overall, *Scn1a* has a relatively higher expression in inhibitory neurons in adult tissues while the other three *Scns* are enriched in excitatory neurons.

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Author contributions

D.L. conceived the original idea and designed the study. S.M. provided the data from the human brain Drop-seq experiments. J.Z.L, B.B, and G.F. provided the data from the mouse brain sNuc-seq project. X.A., C.H. R.S., Z.F., and Y.L. carried out the mouse scRNA-seq experiments. S.S performed the analytic calculations. J.Du, A.B. and D.L. drafted the manuscript and designed the figures. M.M., H.L., R.S., J.Du, A.P., and A.J.C aided in interpreting the results and worked on the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

Declaration of competing interest

None to report.

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