Dissecting cellular diversity of cortical GABAergic cells across multiple modalities: A turning point in neuronal taxonomy

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EVALUATION OF

Landmark

Integrated Morphoelectric and Transcriptomic Classification of Cortical GABAergic Cells.

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Decoding the complexity of the brain requires an understanding of the architecture, function, and development of its neuronal circuits. Neuronal classifications that group neurons based on specific features/behaviors have become essential to further analyze the different subtypes in a systematic and reproducible way. A comprehensive taxonomic framework, accounting for multiple defining and quantitative features, will provide the reference to infer generalized rules for cells ascribed to the same neuronal type, and eventually predict cellular behaviors, even in the absence of experimental measures.

Technologies that enable cell-type classification in the nervous system are rapidly evolving in scalability and resolution. While these approaches depict astonishing diversity in neuronal morphology, electrophysiology, and gene expression, a robust metric of the coherence between different profiling modalities leading to a unified classification is still largely missing. Focusing on GABAergic neurons of the cerebral cortex, Gouwens *et al.*[1] pioneered the first integrated cell-type classification based on the simultaneous analysis of the transcriptional networks, the recording of intrinsic electrophysiological properties, and the reconstruction of 3D morphologies of the same cell. Their comprehensive and high-quality data provide a new framework to shed light on what may be considered a "neuronal cell type."

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Background

The credit for what can be reasonably considered the earliest attempt to generate a cell-type classification should be assigned to the father of modern neuroscience, Santiago Ramon y Cajal, who — for the first time, and with remarkable precision — depicted in his vast collection of scientific illustrations the unparalleled cellular diversity that characterizes the cerebral cortex[2]. Cajal's classification was mainly based on morphological features, meticulously characterized thanks to his improved histological method, and precisely transferred to the page thanks to his artistic talent[2] (see Figure 1). However, the interest in systematic categorization became less pronounced over time as physiological and molecular methods advanced in the field of neuroscience, leaving the task of classifying neurons unattended for decades. Only recently has the lack of clear classification emerged as a major hurdle in understanding the functional role of neuronal types within the circuits, thus limiting full exploration of cerebral cortex complexity.

In a time when high-throughput technologies have been rapidly developed across multiple fields of application, overcoming the biases of the traditional methods and, to a certain extent, the associated costs, the quest for neuronal classification has inspired major efforts in the field of neuroscience. The renewed interest in this area has moved numerous studies to tackle the unresolved challenge and address the need for: i) studying different
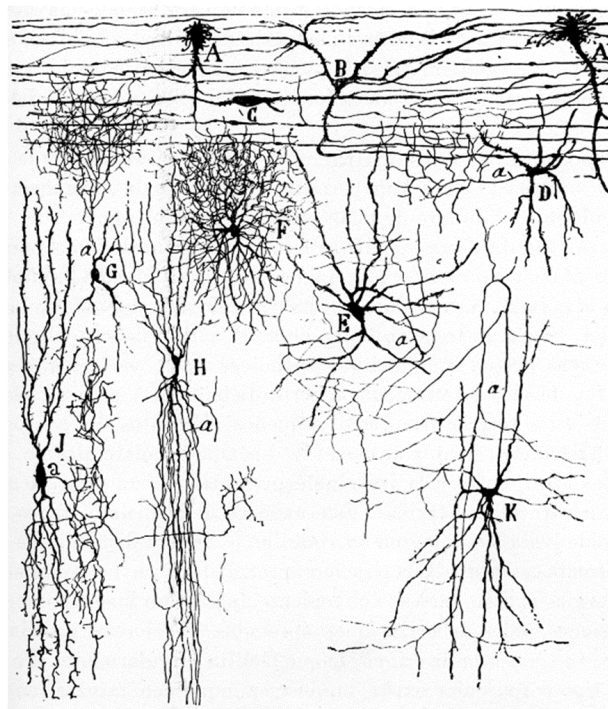

Figure 1. Illustration by Santiago Ramon y Cajal depicting short axon neurons

This image, originally published in 1899 (reproduced under Public Domain – for the English translation see [2]), nicely conveys the complexity/ diversity of cortical interneurons, mainly depicted by their distinct morphology and the wide range of radial and tangential expansion of their axon. It clearly shows a Martinotti cell (G) as it could be described today.
English translation of figure legend: "Cells with short axons of the plexiform and small- and medium-sized pyramidal cell layers from motor cortex of infant aged one month and few days. A, B, C, horizontal cells of the plexiform layer; D, cell with horizontal axon; E, large cell with very short diffusely subdivided axons; F , G, spider-shaped cells whose axons form dense plexus (G) up to plexiform layer; and H, J, bipanicled cells."[2].

neuronal populations in a reproducible and systematic manner; ii) genetically accessing specific cell types to selectively label or manipulate them; iii) revealing the existence of previously unrecognized cell types.

Neuronal cell types of the cerebral cortex have been often described based on morphology, electrophysiological features and, more recently, gene expression. Beyond this, cells have also been defined based on their spatial/anatomical location (i.e., cortical area and layers) and neurotransmitter identity, as well as projection/ connectivity patterns (i.e., long projecting neurons). Importantly, the recent explosion of single-cell transcriptomics datasets has allowed us to rapidly scale up the number of cells profiled, and to define cell types across many tissues and species. Applied to the mammalian cerebral cortex, these approaches have significantly extended our understanding of its complexity and heterogeneity. While individual modalities are critical to decode key aspects of neuronal identity and enable parsing of cortical neurons into distinct subtypes, they are unlikely to uniquely define a cell type. Indeed, an overall consensus about which are the best parameters (e.g., morphology, electrophysiology, transcriptome) to define neuronal identity is yet to be reached, and combination of the individual features can lead to a comprehensive definition. In addition, the differences in the number of distinguishable neuronal cell types inferred from each data modality and the challenge of decoding the relationships between those groupings identified based on distinct cellular features underscore the need for a more integrated experimental approach. For example, in the future it will become critical to include additional parameters that can account for the synaptic connectivity profiles (which neurons are wired together) and/or functional patterns measured in live performing animals in defined tasks: these additional features, some rather stable in time (connectivity), might establish robust links with circuit behavior and

cellular, molecular and electrophysiological traits of diversity.

Main contributions and importance

The Gouwens *et al.* paper represents a landmark study that opens a new pathway to identifying the essential features defining neuronal identity in the mammalian cerebral cortex at an unprecedented resolution. It will undoubtedly be a key reference point for the next generation of neuroscientists aimed at further defining neuronal identity at the intersection of multiple modalities and a prime example of a large-scale classification study[3–5].

Gouwens *et al.* are among the first to generate an integrated neuronal classification schema across morphology, electrophysiology, and the transcriptome, three leading modalities for defining neuronal identity. Their analysis focused on the heterogeneous populations of cortical GABAergic interneurons of the mouse, which have consistent transcriptomic profiles across cortical areas but significant discrepancies between the number of cell types defined by transcriptional profiling and the number defined by morphological or electrophysiological criteria.

By leveraging a comprehensive, high-quality dataset that integrates morphological, electrophysiological, and transcriptomic information of GABAergic neurons, the authors identified 28 MET-types that were highly consistent across modalities, embody a unified definition of cortical GABAergic interneuron subtypes, and provide for the first time a framework for capturing the defining features of neuronal subtype identity.

With different degrees of coherence between the three modalities in the cortical inhibitory population, this approach also allows predictive models of neuronal class identity for highly consistent neuronal groups.

While a robust and comprehensive strategy for integrating the data from different modalities fills a consistent gap of knowledge, this approach has also shown where the coherence falters. Since the early days of single-cell transcriptomics, there has been a general intuition that transcriptionally defined cell types match with morphology and electrophysiology. While Gouwens *et al*. find that transcriptomic subtypes broadly recapitulate morphological and electrical subtypes, there are exceptions. Of particular interest, some subtypes of somatostatin interneurons can have similar transcriptomic profiles but exhibit very different morphological or physiological properties. This finding might suggest that the precise spatial colocalization of transcribed protein (e.g., calcium buffers and ion channels) within cellular compartments can influence, for example, the emergent intrinsic properties. It is also worth mentioning that the number of transcriptional classes largely exceeds that of morphological and electrophysiological groups: technical and biological reasons might lie behind this difference. Simultaneous retrieval of several thousand (up to millions of) cells allows the classification of distinct cell types, defined as "stable" identity. However, beyond cell types, it is evident that single-cell transcriptomics also enables the detection of cell states, characterized by a more dynamic and plastic definition: clear discrimination between 'types' and 'states' is a topic of hot debate in the field. This consideration, in addition to other biologically relevant distinctions in developmental origin and connectivity profiles of the neurons, can impinge on the large number of transcriptionally defined clusters.

Interesting findings are also related to the localization of the somata of GABAergic subtypes: cell bodies of many MET and T (only identified by transcriptional profiling) types were found restricted to a single cortical layer or sublayer, underlying the link between topological distribution and transcriptional identity, so far largely not well recognized for inhibitory neurons.

In addition, the accurate and extensive 3D morphological reconstruction allowed the identification of a layer-specific axon innervation pattern as a defining feature that distinguishes different MET-types. These findings are likely to be extendable to other cortical regions, at least in the case of GABAergic interneurons.

Beyond the scientific impact of this work, the quality and the size of the collected data recorded for each modality is impressive. From an electrophysiological and morphological standpoint, the high caliber of the physiological assays and the precision of the detailed 3D reconstructions used to characterize the cell phenotypes meet the gold standard. The large number of cells analyzed through profiling, recording, and morphometric data are consistent with those that have been previously classified. Thus, this study has paved the road for novel integrated cell type annotations while setting a high methodological standard for neuronal taxonomic investigations and presenting an invaluable resource for the community. Moreover, performing multimodal measurements in the same set of single neurons can be harnessed in studies combined with gene expression perturbations or intersecting with disease risk genes to highlight select points of vulnerability.

Overall, the datasets generated here constitute an invaluable resource for the community. Beyond this, the integrated approach to identifying molecular correlates of physiological or morphological properties could serve as a novel paradigm of essential utility. The same datasets presented by Gouwens and colleagues have already been used to generate novel computational frameworks to align multimodal datasets and enable accurate cross-modal data prediction to consistently uncover neuronal identity of GABAergic interneurons[6].

Open questions

The integrated taxonomy provides a framework for addressing — at greater resolution and at a larger scale — the long-lasting challenge of understanding the diversity of GABAergic interneurons in the cerebral cortex, which is critically linked to function in these circuits in health and disease. While Gouwens *et al*. present a powerful schema to classify neuronal identity that influences our perspective on how a cell type census in the cerebral cortex might be reached, whether this logic can be applied to other brain regions where morphologies and intrinsic properties are less well-demarcated is still open for debate.

Inspired by the practical need of defining a "cell type," this work almost converges on a philosophical question: what is a cell type? And to what extent can it be reliably assumed that our strategies of parsing neuronal complexity (by the 'clustering' method) reflect neuronal identities?

Although unlikely because of the number of cells profiled and the robustness of the analysis, it cannot be excluded that some discrepancies between the annotations could be resolved by increasing the size of the morphological and physiological samplings to reach sufficient statistical power that can lead to predictions about additional consistent MET-types. Until then, how should we interpret as a community the transcriptional data alone for defining cell types? The cells that do not match predictions are potentially the most interesting. What evidence do we have that those are different cell types versus the possibility that, for example, the expression of subtype-specific genes in those cells is more susceptible to brain slice preparation or reflects a different state of function? These questions could serve as a springboard for future *in vivo* studies. On the other hand, the important finding that transcriptional clustering might not be the most accurate predictor for cell annotation in some cases naturally drives the question of which other modalities, not included in the study, should be considered to better capture neuronal identity. For example, the same framework presented in the study can be reasonably extended to other kinds of profiling: could variations in chromatin accessibility and proteomic landscape perhaps explain some incoherence between transcriptional and morpho-electric features? It has been shown that – at least during development – epigenetic signatures better predict cell type identity[7–9], as they retain a more stable profile compared to the transcriptional ones that are highly dynamic.

The transcriptional data also indicate a certain 'degree of freedom' that each GABAergic neuronal type exhibits, whose functional relevance is completely uncharted. Could this variation be shaped by their circuit integration and interaction with pre- and/or postsynaptic targets? Considering that transcriptional variations across T-subtypes are rather limited, could we exclude that the signatures identified to determine the classification could indicate subtle differences, perhaps influenced by specific circuit integration? Certainly, we know that network connectivity and activity patterns dictate intrinsic properties[10], but does that also influence the expression of T-types?

Programs of gene expression change with time, from development to adulthood, and in certain conditions, upon changes of internal states (i.e., circadian rhythm). This is a critical point to consider when classifying neuronal diversity in the adult, and developmental lineages could represent a further parameter to consider. Also, this highlights the need to be cautious when adult databases are used to understand or explore developmental processes.

It is evident that multiple profiling approaches undoubtedly represent a step closer to resolving the important question of cell-type classification in the

cerebral cortex, but which parameters would be best to prioritize with the goal of characterizing the functional meaning of the variability observed in the multiple domains? And which ones will be the best predictors?

At this stage, if one were to obtain a large unbiased dataset of morphology, and electrophysiology, and, potentially, the connectome, would it be reasonable to reverse the classification problem — i.e., predict transcriptomes from functional characteristics?

Future studies are indeed needed to determine to what extent the distinctions observed in some cells between transcriptomic profiles and morphological or physiological properties truly reflect cell type versus cell state distinctions, in particular those that may emerge in the context of a given animal behavior.

Lastly, is it reasonable to leverage the integrated taxonomy on consistent subtypes (i.e., MET subtypes) to identify the same cells in different species? Recent evidence seems to point to some divergences both at the transcriptional and functional levels in different species[11,12]. This point is of particular interest in light of evolutionary projects and/or those with translational impact.

Conclusion

Looking forward, the study authored by Gouwens *et al*. is reassuring for the validity of large-scale transcriptomic atlases generated by consortia such as the BRAIN Initiative Cell Census Consortium (BICCN)[3,13], which will contribute to accelerating discovery about cellular diversity in the brain.

With its large-scale and high-quality approaches, this work also extends neuronal characterization to multiple modalities and exemplifies how the outstanding cellular diversity of the cerebral cortex requires additional, or perhaps more refined, measures. Further investigation is needed to optimize the strategies for scalable collection of multi-modal data and integration, as well as to determine the best predictor of neuronal identity across different areas and species.

In conclusion, this study represents a turning point in understanding the complexity of brain cell types, whose classification is undoubtedly one of the most challenging tasks. It is indeed an enthralling idea that such complexity can be compared to that of a cosmic network of galaxies[14], and a comprehensive taxonomy of the neurons in the brain might be considered even more demanding than annotating the stars in the sky.

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