Singling out motor neurons in the age of single-cell transcriptomics

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Motor neurons are a remarkably powerful cell type in the central nervous system. They innervate and control the contraction of virtually every muscle in the body and their dysfunction underlies numerous neuromuscular diseases. Some motor neurons seem resistant to degeneration whereas others are vulnerable. The intrinsic heterogeneity of motor neurons in adult organisms has remained elusive. The development of high-throughput single-cell transcriptomics has changed the paradigm, empowering rapid isolation and profiling of motor neuron nuclei, revealing remarkable transcriptional diversity within the skeletal and autonomic nervous systems. Here, we discuss emerging technologies for defining motor neuron heterogeneity in the adult motor system as well as implications for disease and spinal cord injury. We establish a roadmap for future applications of emerging techniques – such as epigenetic profiling, spatial RNA sequencing, and single-cell somatic mutational profiling to adult motor neurons, which will revolutionize our understanding of the healthy and degenerating adult motor system.

Motor neurons – the conduit between brain and body

The fundamental purpose of the brain and spinal cord is to take in sensory stimuli, perform computations which incorporate that information into prior knowledge, and then plan and execute motor behaviors. Higher-order pathways that govern sensory processing and decision-making are almost always context and problem-dependent (for example, coordination between limbs when swinging a baseball bat). However, a shared feature is that all central nervous system (CNS) derived peripheral control must traverse the abyss between the brain and the body. Without the motor neuron, this task would be impossible.

Motor neurons are a class of neurons whose cell bodies reside in the CNS (spinal cord or brainstem), but whose axons project to the periphery. They receive excitatory, inhibitory, and neuromodulatory synaptic inputs from cells in the brain and spinal cord, and transmit the information they receive along extraordinarily long axons (for example, from the spinal cord all the way to the pinky toe) that form synapses with cells in the periphery [1,2]. Motor neurons can be roughly divided into two subtypes, depending on their innervation targets [3–5]. Skeletal motor neurons form synapses with skeletal muscle (neuromuscular junctions), whereas autonomic motor neurons control smooth muscle in the periphery by synapsing with postganglionic neurons in peripheral ganglia and effector cells in organs [6–8]. Motor neurons are thus the signaling conduits through which all information transmitted from the brain to the rest of the body must flow. This absolutely vital role has meant that motor neurons in vertebrates are an evolutionarily diversified and indispensable cell type [9]. Their physiological, morphological, and developmental identities are inextricably linked to the muscle and/or organ that they innervate. These properties make motor neurons exceptional candidates to unleash new technologies designed to illuminate cellular diversity [10–13].

Highlights

- Single-cell and single-nucleus transcriptomics have enabled rapid scaling in our understanding of the adult mammalian motor system.
- Characterizing motor diversity establishes links between cell types and causative dysfunction in disease using human genetics data.
- Transcriptional profiles of mature motor neuron subtypes are a new benchmark for what constitutes an induced motor neuron in vitro.
- Emerging technologies hold the potential to assess somatic mutations and epigenetic profiles along with the transcriptome in rare and powerful motor neurons.

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The genomics revolution has led to a proliferation of sequencing-based technologies that are uniquely suited to capturing diversity at the single-cell level. These techniques include single-nucleus RNA (snRNA) and single-cell RNA (scRNA) sequencing on droplet-based platforms [14,15] that can capture hundreds of thousands to millions of unique transcriptomes per experiment [16,17]. Coupled with these advances has been a revolution in spatial transcriptomics, which has made simultaneous detection of hundreds to thousands of transcripts in tissue sections feasible and cost-effective [18–20]. Beyond transcriptomics, single-nucleus epigenomics (in the form of chromatin-based transposition assays) has advanced in scale and quality, enabling high information content ‘multi-omic’ interrogations of cell types [21–23].

**Glossary**

**Autoimmune nervous system:** also called the visceral motor system, controls involuntary functions mediated by the activity of smooth muscle, cardiac muscle, and glands. Controls blood vessel dilation/contraction, heart rate, and function of sexual organs.

**Dysautonomia:** a group of medical conditions characterized by dysfunction of involuntary functions controlled by the autonomic nervous system.

**Extrafusal muscle fibers:** skeletal muscle fibers innervated by motor neurons; they contract to generate tension to control skeletal movement. Attached to bone by tendons.

**Genomic structural variation:** rearrangements comprising insertions, deletions, inversions, duplications, and interchromosomal translocations of genomic regions.

**Intramuscular muscle fibers:** act as specialized sensory organs (also called proprioceptors) by detecting changes in the length of a muscle; they make up the muscle spindle, and are innervated by sensory and motor neurons.

**Peripheral neuropathy:** a group of medical conditions characterized by damage to peripheral sensory or motor nerves, often manifesting in muscle weakness, autonomic dysfunction, and impaired sensation across modalities.

**TDP-43:** RNA-binding protein encoded by TARDBP whose cytoplasmic aggregation and nuclear depletion is the pathological hallmark of ALS.

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**Single-cell measurements of the nervous system in adult organisms**

Motor neurons are an ideal use case for these new techniques because they have been notoriously difficult to study in adult animals. They are fragile, enormous, and exceedingly rare (less than 1 out of every 500 cells in the human spinal cord is a motor neuron). The vast majority of their cell volumes reside outside of the CNS, but the mRNA that supports their functional heterogeneity derives from the nucleus. Historically, this has meant reliance on lower-throughput techniques to explore motor neuron diversity, such as laser-capture microdissection [24,147–150], bulk transcriptomics [25], and cell-type-specific pulldown of ribosome-associated transcripts using genetically engineered mice [26]. Yet each of these techniques has substantial limitations if the goal is to discover the full breadth of transcriptional diversity. Bulk transcriptomic studies have succeeded in defining the broad categories of changes in gene expression associated with diseases of the spinal cord [27,28], but this technique involves averaging over cellular heterogeneity [14]. In contrast, studying the gene expression profiles of individual cells enables refined characterization of neuronal diversity, as well as their individuated responses to disease-associated perturbations. Recent technological advances promise to give researchers unprecedented access to unlock motor neuron diversity.

One way to overcome the limitations of sequencing the transcriptomes of many cells all pooled together is to sequence them one by one. This is particularly important in the context of studying rare cell populations whose transcriptional state would otherwise be diluted by bulk analysis. scRNA and snRNA sequencing are techniques that enable reverse transcription reactions of mRNA transcripts that specifically label each molecule with an oligonucleotide tag that corresponds to its ‘cell of origin’. This idea was first implemented by placing each cell/nucleus to be profiled in an individual well and performing the reactions in small volumes [29]. However, the cost of reagents, as well as limitations of scale, meant that this approach could only be used to sequence thousands of individual cells at once, a paucity compared to the immense diversity of cells present in complex vertebrates. Recent efforts have been extraordinarily successful at tackling the scaling problem by using droplet-based strategies to effectively make pools of emulsified compartments that serve as microreactors for the barcoded reverse transcription step [14]. These techniques have empowered profiling hundreds of thousands to millions of cells by RNA sequencing in parallel per experiment [30]. This explosion in single-cell barcoding and sequencing technologies has been met with a coordinated effort to develop bioinformatic tools that are capable of dealing with the quality and character of information generated [31]. For fields that have historically relied on low-throughput morphological, electrophysiological, topological, and immunohistochemical techniques for the classification of distinct cell types, these advances represent a step change in our understanding of motor diversity (Figure 1).

A key challenge in the application of these techniques to the CNS is that, volumetrically, neurons consist mostly of distal processes, which are vital to their control of muscle contractions. But their elongated and ramified nature makes neurons notoriously difficult to dissociate from adult brain and spinal cord tissue, because that technique inevitably involves axotomizing and shearing off...
processes, which leads to unexpected transcriptional artifacts and cell death [32–35]. Motor neurons are a hyperbolic example of this conundrum: as little as 0.001% of their volume is contained within their cell bodies, with the rest residing in an immense axoplasm (Equation 1). Motor neurons also have strikingly large cell bodies, euchromatic nuclei, and an astonishing amount of RNA [36,37]. Most mammalian cells grown in culture typically contain ~10–30 pg of RNA. By comparison, a single motor neuron contains ~500–1300 pg of RNA [36,38]. Intuitively, it makes sense that motor neurons are transcriptionally supercharged: their axoplasms are packed with protein and are volumetrically equivalent to ~10–20 Caenorhabditis elegans flatworms, all of which requires an astounding level of gene transcription and protein synthesis [39]. These peculiarities specific to motor neurons make them exceptional candidates for study at the level of snRNA. In many other cell types the total RNA content per nucleus and low RNA capture efficiency can lead to an incomplete transcriptional picture, but this problem is heavily mitigated by the abundant transcriptional properties of motor neurons.

In contrast to the difficulties associated with adult neuron purification, isolation of nuclei is comparatively trivial. Recent studies have employed isolation of motor neuron nuclei to interrogate adult motor neuron diversity by genetically labeling and enriching for cholinergic nuclei prior to snRNA sequencing [10,11]. This technique overcomes the stoichiometric limitations of studying a rare cell population while leveraging the unique characteristics and RNA content of motor neuron nuclei to build comprehensive maps of motor neuron diversity in vivo. A key limitation thus far is that this enrichment strategy works only in animals with genetically encoded fluorescent proteins that can be localized to the nuclei of motor neurons (e.g., transgenic mice).
Equation 1 shows the relative proportion of cytoplasmic to whole motor neuron volume, assuming a spherical cytoplasm and a cylindrical axon. For a large motor neuron innervating a leg muscle, $r_{cyto} = 25 \, \mu m$, $r_{axon} = 5 \, \mu m$ and $l_{axon} = 2 \, m$, so the relative volume is 0.00130%.

$$Vol_{cyto/whole} = \frac{\left(\frac{4}{3}\pi r_{cyto}^3\right)}{\left(\frac{4}{3}\pi r_{cyto}^3 + \pi r_{axon}^2 l_{axon}\right)}$$

Elucidating the cellular etiology of neuromuscular disease

Because motor neurons serve as conduits between the CNS and the periphery, they are indispensable to the survival and proper function of vertebrate animals. When motor neurons get sick, cannot communicate with their targets properly, or die, the consequences are disastrous. Dysautonomia (see Glossary), peripheral neuropathy, multiple systems atrophy (MSA), and autonomic sequelae in Parkinson’s disease can result from autonomic motor neuron dysfunction [40]. Affected patients face a loss of sympathetic and parasympathetic innervation of diverse organ systems that impairs quality of life. On the other hand, loss of function in skeletal motor neurons leads to intractable devastating neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), progressive muscular atrophy (PMA), Kennedy’s disease, Charcot–Marie–Tooth disease (CMT), myasthenia gravis, among other rarer conditions [41]. Many of these diseases involve rapid neurodegeneration and paralysis, and there is no cure. The lifetime risk of developing ALS is around 1:400, and those who develop symptoms experience rapid, progressive paralysis over the course of 3–5 years that almost invariably leads to death [42]. Among most of these neuromuscular disorders, there is a remarkable specificity with which individual diseases affect just one subtype of skeletal or visceral motor neuron. Motor neuron diversity appears to make specific subtypes of motor neurons vulnerable to degeneration, while leaving others relatively unaffected. Defining the transcriptional and functional diversity of the adult motor system likely holds the key to unlocking the molecular underpinnings of motor neuron selective vulnerability in disease.

In ALS patients, not all spinal motor neurons are similarly affected. Rather, the disease is characterized by dysfunction and eventual death of skeletal, but not autonomic, motor neurons [43]. And even among skeletal motor neurons, those that innervate extrafusal muscle fibers (α) degenerate, while those that innervate intrafusal muscle fibers (γ) do not [25,44–46]. But not even all α motor neurons respond equally; those that innervate fast-twitch (type II) glycolytic muscle (fast-firing) are far more susceptible to dysfunction than their slow-firing, type I innervating counterparts [25,45,47]. Past efforts have attempted to address whether transcriptional differences in populations of autonomic and skeletal motor neurons underlie this differential susceptibility. These pioneering efforts utilized transcriptional profiling and laser-capture microdissection from predominantly vulnerable and resistant motor pools to find transcripts that are uniquely expressed in each population [25,147–150]. This approach yielded key insights into fast- and slow-firing transcriptional signatures, such as $Mmp9$, which is selectively expressed in vulnerable subtypes [25]. However, laser capture microdissection is a laborious and low-throughput process that is easily contaminated by transcripts in surrounding cell processes, limiting its usefulness to only dramatically different signatures [48]. Furthermore, the notion that individual motor pools are homogeneous is a necessary but flawed assumption that underlies motor pool-specific laser capture; it is well established that distinct subtypes exist within individual motor pools [49,50]. Thankfully, recent advances in single-cell (and single-nucleus) transcriptomics have empowered large-scale, less biased investigations of the underlying transcriptional heterogeneity of the skeletal motor system [13,51].
Interrogation of cell-type vulnerability in neurodegeneration

Single-nucleus transcriptomics enables interrogation of cell-type diversity at an unprecedented scale in the adult mammalian motor system. Recent work using these methods has demonstrated that α motor neurons can be independently distinguished based on the muscle type that they innervate (motor pool) as well as the fiber type within that muscle group (fast- versus slow-firing) [11]. The fact that these differences persist into adulthood and are not simply necessary while the nervous system is wiring during development suggests that gene expression differences encode persistent functional differences in differentially vulnerable cell types. Indeed, useful insights have already emerged from single-nucleus characterization of the adult motor system. Two recent studies identified a unique cluster of α motor neurons marked by the expression of Erbb4 [10,11], which encodes an ALS-associated receptor tyrosine kinase [52,53]. One of those groups demonstrated through retrograde tracing experiments that the cluster of Erbb4+ motor neurons is responsible for innervation of the diaphragm, that is, these motor neurons are responsible for voluntary breathing [10]. Subsequently, an analysis of genomic structural variation of ALS patients demonstrated that mutations in Erbb4 are responsible for almost all cases of ‘respiratory-onset’ ALS, a subtype of the disease where patients lose the ability to breathe very early in disease progression [52,53]. This example demonstrates the power of knowing which genes are expressed in vulnerable and resistant cell populations. It further points the way towards a tractable therapeutic avenue by upregulating expression of the genes present in ‘resistant’ cell types and downregulating the genes present in ‘vulnerable’ motor neurons. Combined with modern advances in gene-targeted therapies, this strategy could be utilized to convert vulnerable cells into a more resistant state in a living organism.

Beyond characterization of baseline differences in disease-affected cell types, single-nucleus transcriptomics also offers a window into the physiological process of neurodegeneration at a cell-autonomous level (Figure 2). It is worth noting, however, that this approach has been attempted in other neuronal populations that are differentially susceptible to degeneration, such as retinal ganglion cells [54]. However, establishing cell-type identity in the context of disease is difficult, especially when the transcriptional response to the disease itself is dramatic. A classical hallmark of neurodegeneration is that neuronal dysfunction and degeneration occur over a relatively large timescale, and often do not manifest symptomatically until late adulthood [42]. This is true despite compelling evidence that neurons begin to accumulate toxic protein aggregates long before they die, suggesting that the battle for neuronal survival in the face of stressful insults is a protracted one [55,56]. The pathological hallmark of ALS is aggregation of toxic proteins that can be readily identified in postmortem tissue derived from affected regions. But different forms of ALS have different pathologies. For ALS cases caused by mutations in the SOD1 gene, the protein that aggregates is mutant SOD1 [57]. ALS cases caused by FUS mutations have loss of FUS from the nucleus and its aggregation in the cytoplasm [58,59]. For virtually all the other cases (~98% of them), including sporadic ones for which there is no known genetic driver, the main pathology is nuclear depletion of the RNA-binding protein TDP-43 and its cytoplasmic aggregation [60]. These pathological signatures of disease have been fundamental to our understanding of cell-type vulnerability and disease progression [61–65]. Further, recent work by several groups has identified a loss-of-function mechanism in ALS, through which nuclear depletion of TDP-43 causes aberrant RNA splicing, premature polyadenylation, and inclusion of cryptic exons [66–70]. However, to date little is known about which of these RNA processing mechanisms are most relevant to motor neurons, or even whether TDP-43 activity is the same across all subtypes of neurons. Using snRNA is a feasible method for identifying RNA splicing defects in motor neurons with and without TDP-43 nuclear depletion, which should provide greater understanding of TDP-43’s role in the etiology of ALS.

Thus, the era of single-cell transcriptomics presents a novel and intriguing prospect: that we can define new pathological signatures based not on a low-dimensional phenotype (aggregated
proteins) but on gene expression portfolios of stressed and vulnerable cells. The phenotype of ALS is highly convergent (loss of motor neurons) despite vastly different genetics across familial and sporadic cases. Is it possible that motor neurons undergo a common stress/injury response, coupled with the same dysregulated transcriptional or epigenetic pathway, regardless of the underlying mutation that causes disease? Or that the principal insult has a large effect on...

Figure 2. Single-cell transcriptomic resolution enables interrogation of cell-specific disease states. Within an individual brain or spinal cord there is a diverse collection of neurons in various states of degeneration. A cross-sectional view of the degeneration process enables ordering of motor neurons by their stage of degeneration, and consequently a regression-based analysis of the physiological, transcriptional, and epigenetic drivers of disease. Abbreviation: TF, transcription factor.
motor neuron responses, meaning that each type of disease might need to be treated independently? These are the types of questions that single-cell measurements can readily address.

Because single-nucleus transcriptomics can only be performed using postmortem CNS samples and therefore cannot track the expression profiles of individual cells longitudinally, it provides a discrete cross-sectional view of the biological process of degeneration. Future studies will need to model this degenerative problem as a continuous ‘trajectory’ that individual cells experience to varying degrees, analogous to the approach that has been used to model developmental trajectories using single-cell techniques (Figure 2) [71]. When this technology reaches maturity, it should enable researchers to interrogate the epigenetic and transcriptional drivers that push vulnerable neurons along a trajectory of degeneration. Furthermore, it may illuminate analogous drivers that neurons utilize to stave off degeneration. Modifying each of these processes (getting motor neurons off the wrong track towards degeneration and back onto the right track of homeostasis) holds enormous potential in the treatment of neurodegenerative diseases [72], including those affecting skeletal motor neurons.

Towards understanding the motor neuron response to spinal cord injury

Neurodegenerative diseases such as ALS are hardly the only damaging insults that can result in motor neuron dysfunction. Indeed, spinal cord injury (SCI) affects close to 200,000 individuals in the United States alone [73] and typically results in a loss of skeletal and autonomic motor control, as well as sensory perception below the vertebral injury site [74]. Due to advances in transplantation and facilitated regrowth of descending axons past the SCI injury site [75], there is immense interest in understanding the factors that maintain and/or prolong motor neuron survival in areas that have lost functional innervation [76]. A recent study examined the transcriptional response of neurons and glia below an induced site of SCI in mouse, revealing endogenous mechanisms of spinal cord repair in adult animals [77]. Another possible application of snRNA is to selectively profile motor neurons from different spinal cord levels below an injury site. This approach would elucidate the pro- or anti-survival transcriptional response enacted by motor neurons in response to proximal or distal injury, and potentially nominate master-regulator genes involved in each of these processes. With a deeper understanding of these responses, future work could entail upregulating pro-survival genes or downregulating genes that negatively affect the subsequent degenerative process.

This approach is equally applicable to both skeletal and visceral motor neurons. Loss of voluntary motor control is a devastating consequence of SCI, but it is far from the only one. Autonomic dysreflexia is an extremely common and life-threatening symptom of SCI. Briefly, autonomic dysreflexia results from a loss in descending afferents that control the autonomic nervous system, leading to an exaggerated sympathetic response in various contexts. This occurs in 90% of patients with upper thoracic and cervical SCI, and can occur up to 40 times per day [78]. Symptoms include dramatic increases in blood pressure, severe headache, tachycardia, and alternative pallor, cold skin, and sweating in other parts of the body. By understanding and modulating the transcriptional response that alters visceral motor neuron function after SCI, future work could dramatically improve the lives and outcomes of affected patients.

Pay no attention to the neuron behind the curtain: the subtle and pervasive impact of autonomic dysfunction in health and disease

Thus far we have described the consequences of skeletal motor neuron dysfunction: disruptions lead to dramatic clinical consequences involving paralysis of individuals afflicted with these disorders. However, we have only briefly addressed the phenotypic consequences of autonomic motor neuron degeneration. Autonomic motor neurons have been the subject of substantially less basic research,
and readouts of small fluctuations in the autonomic nervous system are much less readily detectable than muscle restlessness or paralysis that results from dysfunction of the skeletal motor system. One fundamental limitation to the study of autonomic motor neurons is that until recently their transcriptional diversity was entirely uncharacterized. The paucity of information about which genes are specifically expressed in subtypes of autonomic motor neurons with known functions has made it difficult to link genetic dysautonomia to specific cell deficits. It also meant that there were no genetic tools available to precisely target and manipulate specific cell populations and interrogate their function in vivo. Several recent studies have begun to address these questions, dramatically restructuring the classical field of autonomic motor system research.

In one such example, researchers performed single-nucleus transcriptomics on vagal autonomic motor neurons to transcriptionally define seven subpopulations of neurons, and then used genetically defined Cre driver lines to establish the functional output and innervation targets of those cells [79]. These results strongly support the hypothesis that transcriptionally defined populations of adult motor neurons are functionally and physiologically distinct from one another and provide a first step towards building an integrated transcriptional, spatial, and functional map of autonomic motor neurons.

From recent work performed in various laboratories, it was observed that the sympathetic visceral motor system consists of 16 transcriptionally divergent cell clusters, several of which are uniquely located at specific levels (cervical, thoracic, lumbar) along the spinal cord [10,11]. These findings suggest that adult visceral motor neurons possess transcriptional properties that are tuned to their in vivo functional heterogeneity, including their specific innervation targets. Given the far-reaching impact of autonomic innervation on behaviors such as blood vessel dilation/contraction, heart rate, and sexual function [8,9,40], there is enormous utility in defining how the neural and transcriptional properties of each subpopulation affect their targets in vivo.

Characterization of visceral subtype heterogeneity will enable the generation of transgenic mouse lines that can be used for specific activation and repression of each cluster of visceral motor neurons. This approach presents the opportunity to address a core conceptual question of autonomic regulation: what role does each visceral subpopulation play in controlling autonomic function? One hypothesis is that each cluster controls a distinct autonomic behavior, as observed in parasympathetic control of digestion [79]. A competing theory is that each cluster may be responsible for controlling a single (or small group of) peripheral organ(s). This hypothesis is enticing because of its parallels to skeletal motor pool specificity for an individual motor unit, but it is unlikely to be true. Here is why. In the recent snRNA studies, both groups found several clusters that specifically express individual genes encoding bioactive neuropeptides such as neurotensin (NTS) [10,11]. However, past work has shown that numerous peripheral ganglia contain preganglionic NTS-positive fibers [80], contradicting the hypothesis that individual clusters (e.g., NTS-positive) exert autonomic control over just one organ system. With this new, easy-to-use, publicly available spinal cord atlas as a roadmap [10,11], future studies will be empowered to genetically label visceral motor populations, manipulate them in vivo (increasing/decreasing activity, ablating clusters), and measure the functional consequences of their activity in animal models.

Developing an integrated view of motor neuron differentiation

We know a great deal about the functional diversity of motor neurons and the muscles they control, thanks to decades of foundational research into the development of the spinal cord during embryogenesis [81–88]. This body of work has provided key insights into how temporally sensitive gene expression programs in progenitor cells and differentiated motor neurons enable appropriate patterning and development of motor networks [89,90]. This accumulated knowledge
sets the stage perfectly for single-cell transcriptomics and epigenomics by establishing an interpretive framework for these rich datasets. Several recent studies have sought to apply single-cell transcriptomics to classical paradigms used to study developmental neurobiology of the spinal cord [13,17,91]. Collectively, this work is the first step towards defining a shared language that effectively integrates years of developmental research along with recent advances in high-throughput collection of data from cells in the spinal cord [12,13,91].

An exciting future direction in the application of scRNAseq to developmental biology is in understanding differentiation of motor neurons during human development. While experiments involving nonhuman primates are costly, there is ample opportunity to perform descriptive postmortem single-cell transcriptomics and epigenomics during human development [92,93]. Using these techniques, we may garner key insights into the defining features that separate human motor neurons from those of other vertebrates. Furthermore, understanding the core logic of human motor neuron differentiation will enable us to differentiate motor neuron subtypes in vitro and may hold the key to unlocking the molecular etiology of neurodevelopmental diseases.

**Single-cell transcriptomics can be applied to diverse diseases to characterize causative cellular dysfunction**

An oft-overlooked element of designing and executing a single-cell transcriptomic study is the importance of using an appropriate technique to study the “right” cell types that are affected in disease. While there is some utility in performing wholesale single-nucleus transcriptomics on large brain/spinal cord regions in the context of the disease to find the cell type that is responsible for a phenotype, there is a growing appreciation for the necessity to enrich for cell types of interest prior to barcoding and sequencing the RNA from individual nuclei. This approach enables a far more detailed characterization of cell populations of interest, which might otherwise be diluted out by unfavorable stoichiometries [10,11,94].

An interesting illustration of this phenomenon is the study of Parkinson’s disease using single-cell transcriptomics. Recent advances have enabled the cell-type-specific enrichment of nuclei from the dopaminergic midbrain, a development that has provided an unprecedented look at the diversity that may underlie their differential susceptibility in Parkinson’s disease [34]. However, aside from motor dysfunction, the most debilitating symptoms associated with Parkinson’s disease involve the autonomic nervous system. Approximately 40–50% of Parkinson’s disease patients experience dysautonomia and progressive deterioration after diagnosis [35,96], but the mechanism underlying this phenomenon in patients is unresolved. In recent work autonomic motor neurons were identified as the highest expressors of Snca, the gene that encodes α-synuclein (the pathological hallmark of Parkinson’s disease). This finding raises the possibility that autonomic motor neurons rely heavily at baseline on α-synuclein function and therefore express it highly. The abundance of this aggregation-prone protein may prime autonomic motor neurons for dysfunction in Parkinson’s disease through a toxic gain-of-function or perhaps a loss-of-function mechanism. Future studies will aim to examine the role of autonomic motor neurons in Parkinson’s related dysautonomia.

Another devastating and intriguing recent development in autonomic nervous system research is the finding that a commonly reported ‘long-haul’ COVID-19 symptom is dysautonomia [97]. It is unclear whether severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is capable of directly infecting cells of the autonomic nervous system, but patients consistently report sympathetic and parasympathetic symptoms months after infection [97,98]. Similar findings were also reported with SARS, suggesting that coronaviruses might have convergent effects on the autonomic nervous system. It is critical to understand the diversity of autonomic motor neurons,
their innervation targets, and the consequences of their dysfunction to get to the bottom of autonomic symptoms reported in diverse patient populations.

**Single-cell measurements of motor neurons will inform and motivate human genetics**

Concomitant with advances in single-cell measurements at scale has been a revolution in the quantity and quality of genomic information collected from healthy subjects and individuals with common diseases. In particular, the UK BioBank is a vast resource that has amassed a collection of high-dimensional clinical and genomic data from subjects who are part of the UK government-sponsored universal healthcare system [99]. Because the majority of participants in the UK BioBank study are healthy individuals, this dataset offers a unique opportunity to discover genetic contributors to traits that fall within a nonpathological range with unprecedented statistical power [100]. The autonomic nervous system has far-reaching effects on normal human homeostasis [101]; thus, this genomic lens should empower the discovery of genetic regulators of autonomic function in healthy individuals [102].

Single-cell transcriptomics and genome-wide association studies are a powerful combination. Their ensemble analysis, known as ‘transcriptome-wide association study’, enables the simultaneous detection of both the genomic regions associated with disease and insights into what the principally affected cell type might be [103,104]. Recent advances in characterization of skeletal and autonomic motor neuron diversity [10,11,79], together with a growing push to capture genomic data from healthy research subjects, will converge to provide novel insights into human health and disease. While there are likely substantial differences between rodent and human motor neuron transcriptomes [147–150], the example of ERBB4 mutations causing respiratory ALS is an argument for intersectional analysis of datasets collected from different species. Still, future efforts should be aimed at characterizing the transcriptomic properties of other species, including Homo sapiens. This transcriptome-wide association study has been applied to many other diseases to great effect [103,105,106], and should prove useful in understanding ALS genetics more deeply.

**Induced motor neurons with single-cell phenotyping**

The primary utility of single-cell transcriptomics lies in investigating the functional diversity of cell types, as well as the epigenetic processes that underly their development and degeneration. However, another intriguing application of these technologies within the context of motor neurons is their use in characterizing and studying neurons *in vitro*. Reprogrammed induced pluripotent stem cells (iPSCs) can be derived from common cell types in healthy and diseased patients, and then differentiated in two-dimensional cultures to form motor-neuron-like cells. There are numerous protocols that enable induced motor neuron (iMN) differentiation, ranging from application of morphogen gradients and small molecules [107–109] to transcription factor perturbation [110,111]. These all result in the establishment of *in vitro* cell types that recapitulate elements of motor neuron identity.

The fidelity of differentiation methods has historically been established by measuring expression levels of motor-neuron-specific proteins such as CHAT, ISL1, and HB9, along with functional assays such as the innervation of muscle *in vitro* [110]. Motor neurons generated in this fashion can provide insight into the types of pathways and responses to cell stressors that are dysregulated in disease. However, single-nucleus transcriptomics now allows direct comparisons to be made between iPSCs and their *in vivo* counterparts, actual mature motor neurons. Using single-cell transcriptomics is a high-throughput and rigorous standard that may be used to establish *in vitro* models that phenocopy their *in vivo* counterparts (Figure 3). Furthermore, with increasing appreciation of motor neuron diversity in adult animals, it is essential that iPSC-
derived models evolve in parallel. There are numerous, functionally characterized differences between diverse classes of skeletal and autonomic motor neurons [7,10,11,84,112,113], yet in vitro models are typically defined generically as iMNs [110,111]. iMN differentiation protocols have been extraordinarily useful as a system to validate novel biology in patient-derived cells, but they fail to account for the substantial heterogeneity in transcriptional subpopulations of motor neurons found in mammals. Future work is needed to refine the differentiation of iPSCs to motor neurons such that it becomes possible to model specific cell types in culture. With single-nucleus and single-cell transcriptomes now in our arsenal, it should be possible to rapidly test the effectiveness of new combinatorial approaches to motor neuron generation in vitro.

Modeling motor neurons in vitro is no longer limited exclusively to two-dimensional culture systems. Rather, recent studies have outlined novel protocols to generate spinal-cord-like 3D organoids in vitro [114,115], an advance that leverages intrinsic developmental logic encoded in mammalian cells to recapitulate complex motor circuits and structures [116–118]. Importantly, these studies have used snRNA sequencing as a benchmark to determine the sensitivity and specificity of their approaches [114,118]. As we continue to collect data that outlines the transcriptional perturbations in disease-affected motor neurons, these models will provide a launching pad for testing whether in vitro systems can recapitulate physiologically relevant neurodegeneration.

**Single-cell transcriptomics: a transitional technology bridging the gap between target-driven research and high-throughput investigation of complex systems**

Emerging technologies are poised to reshape how neurodegenerative disease is classified and investigated (Figure 4) [23,119,120]. However, arguably the most impactful development in
Figure 4. Diverse, subtype-specific transcriptional responses from amyotrophic lateral sclerosis (ALS)-affected spinal cord (A) can be characterized by spatial transcriptomics (B) and single-nucleus profiling (C). Spatial transcriptomics (different RNA transcripts represented by yellow, blue, purple dots) enables preservation of pathological information (e.g., cytoplasmic TDP-43 aggregation, a hallmark of ALS, represented by blue protein aggregates) while nuclear isolation (C) enables somatic mutation profiling, epigenomic profiling, transcriptomics, and splicing analysis. Abbreviations: MN, motor neuron; snRNAseq, single-nucleus RNA sequencing; snATACseq, single-nucleus ATAC sequencing; snGENOMEseq, single-nucleus genome sequencing; TF, transcription factor.
studying neurodegenerative changes at single-cell resolution is the rapidly maturing field of spatial transcriptomics [119,121–123]. Leveraging the twin powers of oligonucleotide-based barcoding and in situ hybridization, numerous recent studies have demonstrated simultaneous recognition of hundreds to thousands of RNA species in morphologically intact brain tissue [18,19,124]. This technique is broadly applicable to the study of biological specimens, but motor neurons are particularly well suited owing to their stratification into spatially defined motor pools. Preserving the morphology of tissue maintains this informative topography, vastly increasing the information content of single-cell measurements. Furthermore, ALS and other musculoskeletal diseases are deeply characterized pathologically as well as clinically. Spatial transcriptomics will allow single-cell transcriptomics to be seamlessly integrated with morphological cell characteristics, as well as histopathology, providing codetection of protein aggregates and transcriptional responses in the same tissue. Furthermore, spatial transcriptomics enables detection of not only generic transcriptional signatures but also specific complements of mRNA splice variants [125,126]. This is especially important given the involvement of RNA splicing proteins such as TDP-43 in the genetic and pathological signatures of ALS, and the widespread splicing alterations observed in sick neurons and glia [66–70,127]. A huge promise of spatial transcriptomics is its capacity to distinguish cytoplasmic from nuclear localization of transcripts, including those that have been aberrantly spliced. This is a limitation of snRNA, which is easily handled by spatial transcriptomics. It should soon be possible to use spatial transcriptomics to look at alternative splicing, cell-type-specific markers, transcriptional responses to degeneration, and pathological hallmarks of disease all in the same morphologically intact tissue section (Figure 4B).

Another intriguing new approach is single-cell genotyping, which enables the discovery of somatic mutations in a diverse mix of cell types. Given the well-characterized relationship between DNA damage and ALS [128–130], as well as the age-related accumulation of DNA damage and somatic mosaicism in the nervous system [131–134], single-cell genome sequencing has the potential to unlock previously unappreciated mechanisms that underly sporadic disease (Figure 4C). Another common feature of neuromuscular diseases is the prevalence of genetic mutations that result from expansion of repetitive DNA sequences [135–138]. It is well documented that these germline repeats can expand somatically in adult brain cells [138–142], leading many to hypothesize that somatic repeat expansions may underlie neurodegenerative disease prevalence [138,142–145]. While it is currently challenging to measure repetitive sequences of DNA/RNA in individual cells at scale, such a technology would help answer fundamental questions about repeat-associated toxicity and selective vulnerability of neurons in the CNS to disease [146].

Concluding remarks

Being able to define the molecular differences between two adjacent motor neurons in the spinal cord – one with ALS protein aggregates and one without, one that is actively degenerating and the other that is spared – has the potential to unlock the secrets of cell-type vulnerability and disease progression (see Outstanding questions). Collectively, these techniques promise to help answer age-old questions in neurodegenerative research regarding the causal relationship between cell-type vulnerability, proteotoxic stress, protein aggregation, splicing, and neuronal degeneration.

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Declaration of interests

No interests are declared.
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Recent advances in understanding the pathogenesis of amyotrophic lateral sclerosis (ALS) and frontotemporal degeneration (FTD) provide insights into the complex interplay between genetic and environmental factors. TDP-43, a transcription factor, plays a central role in the disease process. The loss of stathmin-2 is a hallmark of TDP-43-dependent neurodegeneration. Premature polyadenylation-mediated exon inclusion in the FUS/TLS gene is associated with familial amyotrophic lateral sclerosis. The Stretch Rescue Promoter Network Organization and Synaptic Specificity of Cholinergic Partition Cells. Neuron 64, 456–472.

Key insights from the latest research include:

1. **Stathmin-2 Depletion**: The loss of stathmin-2 is a hallmark of TDP-43-dependent neurodegeneration. This suggests that targeting stathmin-2 could provide a therapeutic strategy for these diseases.

2. **Premature Polyadenylation**: Mutations in the FUS/TLS gene are associated with familial amyotrophic lateral sclerosis. This highlights the importance of understanding the mechanisms of polyadenylation-mediated exon inclusion.

3. **Cholinergic Network Organization**: The Stretch Rescue Promoter Network Organization and Synaptic Specificity of Cholinergic Partition Cells. This finding implies that cholinergic neurons play a crucial role in the pathogenesis of ALS.

These findings underscore the complexity of ALS and FTD and suggest potential avenues for future research and therapeutic interventions. Further studies are needed to validate these findings and to develop effective treatments for these devastating diseases.
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