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Perspective

The Neuron Identity Problem: Form Meets Function

Gord Fishell[1,*] and Nathaniel Heintz[2,*]
[1]Department of Neuroscience and Physiology, Neuroscience Institute, New York University, New York, NY 10016, USA
[2]Laboratory of Molecular Biology, Howard Hughes Medical Institute, The Rockefeller University, 1230 York Avenue, New York, NY 10065, USA
*Correspondence: fisheg01@nyumc.org (G.F.), heintz@rockefeller.edu (N.H.)
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A complete understanding of nervous system function cannot be achieved without the identification of its component cell types. In this Perspective, we explore a series of related issues surrounding cell identity and how revolutionary methods for labeling and probing specific neuronal types have clarified this question. Specifically, we ask the following questions: what is the purpose of such diversity, how is it generated, how is it maintained, and, ultimately, how can one unambiguously identity one cell type from another? We suggest that each cell type can be defined by a unique and conserved molecular ground state that determines its capabilities. We believe that gaining an understanding of these molecular barcodes will advance our ability to explore brain function, enhance our understanding of the biochemical basis of CNS disorders, and aid in the development of novel therapeutic strategies.

Introduction

For over a century, neuroscientists have been fascinated by the diversity of cell types that comprise complex nervous systems (Ramon y Cajal, 1899). From these efforts, it is widely agreed that specific cell types serve as the building blocks of nervous systems and that exploring their diversity and determining how cells are assembled into circuits is essential for understanding brain function. Traditionally, efforts to explore this diversity have been achieved through the use of classical descriptors, wherein neural cells are categorized by shape, intrinsic physiological character, and immunomarkers with the hope of generating an all-inclusive accounting (Ramon y Cajal, 1899; Bota and Swanson, 2007; Masland, 2004; Sugino et al., 2006; Yuste, 2005; Bernard et al., 2009; DeFelipe et al., 2013; Ascoli et al., 2008). However, neurons exist neither in isolation nor as static entities, and, thus, more contextual classification schemes that recognize their dynamic nature are required. Over the last two decades, the development of a suite of new molecular, genetic, genomic, and informatics technologies have emerged to fill this gap. These methods have placed us at the threshold of an era of neuroscience in which a comprehensive analysis of complex nervous systems can be achieved. Genetic targeting of CNS cell types (Figure 1) with bacterial artificial chromosome (BAC) transgenic (Yang et al., 1997; Heintz, 2001; Gong et al., 2003), knockin (Jerecic et al., 1999; Taniguchi et al., 2011), and intersectional strategies (Branda and Dymecki, 2004; Luo et al., 2008; Awatramani et al., 2003) has resulted in the generation of engineered mouse lines that provide reliable and, more importantly, replicable resources for the comprehensive examination of the connectivity, activity, and function of specific cell types within circuits (www.gensat.org; www.brain-map.org; www.informatics.jax.org; http://gerfenc.biolucida.net/link/). Comparative cell-specific molecular profiling techniques (Rossner et al., 2006; Hempel et al., 2007; Cahoy et al., 2008; Heiman et al., 2008) have resulted in a deep appreciation for the fine-tuned molecular and biochemical properties of CNS cell types (Doyle et al., 2008; Hobert, 2011; Okaty et al., 2009; Chahrour et al., 2008; Schmidt et al., 2012). Moreover, the manipulation of neuronal activity with optogenetics (Fenno et al., 2011; Boyden, 2011; Yizhar et al., 2011) and other approaches (Auer et al., 2010; Lerchner et al., 2007; Rogan and Roth, 2011) has advanced our understanding of the contributions of specific cell types to behavior. Clearly, further expansion of large-scale efforts is needed in order to genetically target candidate "cell types," define them, and understand their unique properties. Nonetheless, the revolution has begun. At last, we are in a position to explore neuronal diversity comprehensively and directly in the context of the rapid modulations that are the essence of dynamic brain function.

Here, we argue that a cell type can be defined operationally as a shared, stable, molecular "ground state" that broadly dictates its functional capacities. Nonetheless, any individual cell of a given type can dynamically alter its precise molecular profile and corresponding physical and electrical properties in response to a variety of external cues (Curran and Morgan, 1985; Greenberg et al., 1985). Hence, although all cells of the same type stably express a common suite of genes, individual members of a cell type may vary in the precise profile of genes expressed depending upon context and activity. We argue also that this ground state is determined shortly after cells exit from their last mitotic cycle and that the execution and stabilization of neuronal gene expression programs require local events that occur in the final stages of maturation during what are commonly referred to as "critical periods" of development. Furthermore, although it is apparent that cell types can be defined molecularly, an understanding of the nervous system cannot be reached without comprehensive data regarding the circuits in which they are embedded, their connectivity, and their activity patterns in response to appropriate external stimuli. Only then can we begin to achieve the ultimate goal of providing an understanding of the contributions of discrete cell types to behavior.

What Is the Purpose of Cell-Type Diversity?

In a general sense, the number of cell types present within a given substructure of the nervous system reflects the computational

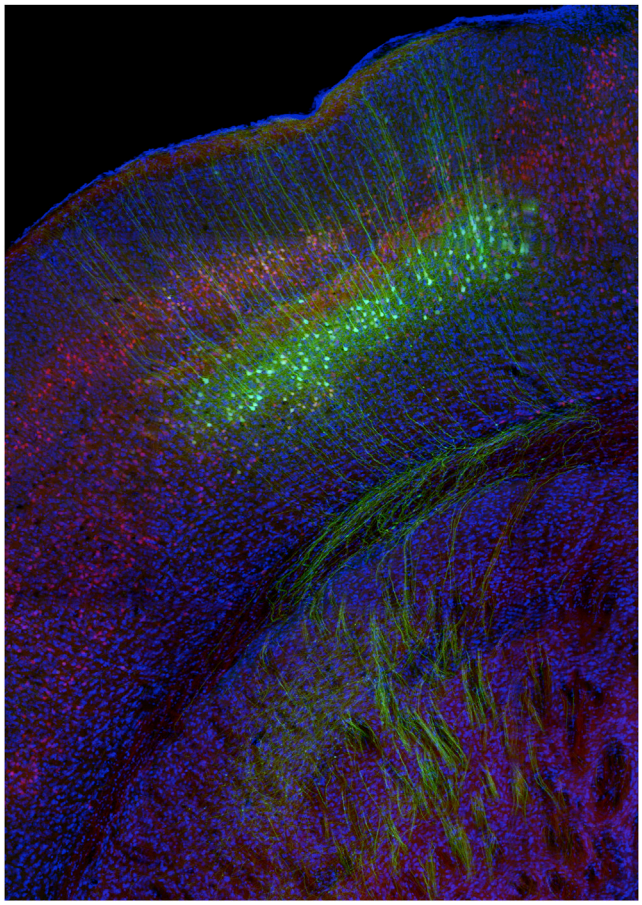

Figure 1. Genetic Targeting of CNS Cell Types: The Sim1 BAC-Cre Driver Line KJ18 Provides Genetic Access to Corticostriatal Projection Neurons

Developmental Cre expression is revealed by the recombination of the Rosa26_CAG-tdTomato Ai9 reporter line (shown in red). Adult pyramidal neurons expressing Cre activity is apparent after Cre-dependent AAV2/1.CAG.FLEX.EGFP.WPRE.bGH expression vector injections into primary motor cortex (shown in green) (Gerfen et al., 2013).

complexity of its functions. In simple organisms or in the context of the peripheral nervous system (Garcia-Campmany et al., 2010; Arber, 2012), the contributions of many specific cell types to behavior have been studied in great detail, and, in most cases, the reasons for their specialization are apparent. For example, specific sensory and motor neuronal classes with distinct anatomical and electrophysiological properties make up simple motor circuits that generate fixed action patterns (Schiff et al., 1999). Local neuron types modulate or generate rhythmic behaviors, allowing these cell types to execute discrete functions (Bargmann and Marder, 2013; Goulding and Pfaff, 2005). This general model may apply for even more complex circuits with a relatively large number of identifiable cell types. It is believed that a nearly complete accounting for all cell types present in the mammalian retina places the number at around 60 discrete types (Masland, 2012). Although the precise functions of each of these cell types are not known, the fact that they are tiled across the retina suggests that each of them contributes to specific aspects of visual perception. A particularly clear recent example of this idea comes from studies of the JamB retinal ganglion cell population in which the anatomy, physiology, receptive fields, and distribution of JamB cells are all tailored for their ability to perceive upward motion (Kim et al., 2008). The close correspondence between the functional requirements of these circuits and their cellular composition is inherently appealing. However, even in these relatively simple systems, specific elements of a functional repertoire may be distributed across multiple cell types—a circuit is certainly more than the sum of its parts.

As one moves from peripheral circuits that carry out relatively fixed routines to CNS circuits that mediate increasingly complex behaviors, the relationships between the number of cell types and function are less obvious. It is not immediately apparent how different structures utilize discrete cell types in order to mediate distinct but related forms of neural computation. For example, why do the entorhinal cortex and hippocampus organize at least several scores of distinct cell types into nested maps comprised of grid and place cells in order to mediate spatial learning (Parra et al., 1998; Thompson et al., 2008), whereas the cerebellar cortex can execute its complex procedural learning tasks with only a dozen or so discrete cell types (Llinás and Welsh, 1993; Gao et al., 2012)? We also lack an adequate explanation for the hundreds of distinct cell types thought to be present in the cerebral cortex, even considering its lamination, variations in local architectonic structure, and exceedingly complex functional properties.

One feature of nervous systems that may explain some of the cell-type diversity evident in complex systems is the ability of circuit activity to be modulated remotely by neuropeptides and other small mediators (Bargmann, 2012). Given the very specific expression patterns observed for a large number of neuropeptide and G protein-coupled receptors in the mammalian brain, segregation of these modulatory pathways into distinct circuit elements offers opportunities for simultaneous customized control of multiple circuits by the release of a wide variety of peptides, lipids, and other small molecules. Examples of this type of global modulation in response to internal states in mammals include the regulation of emotion by serotonin (Meneses and Liy-Salmeron, 2012) and neuropeptides (Love, 2013), the induction of "sickness behaviors" in response to prostaglandins (Pecchi et al., 2009), and the modulation of feeding behavior by peripherally produced peptides (Friedman, 2009). Given that the cell-surface receptors mediating these complex behavioral states converge onto a small number of intracellular effector pathways, their segregation into different cell types may be required in order to optimize their effects.

Consider the actions of serotonin in the cerebral cortex. Several serotonin receptors are expressed in the cortex, each with a different distribution across cortical cell types. Htr3a receptors, for example, are ionotropic and expressed in a range of interneuron classes that include neurogliaform cells that are thought to function for volume transmission of GABA (Oláh et al., 2009) and bipolar VIP-expressing populations that function selectively in disinhibition (Dávid et al., 2007). The concerted actions of serotonin through these cell types is thought to recruit these cells and their associated circuitry in order to modulate recurrent cortical signaling, resulting in increased discrimination

of sensory signals, heightened attention, and improved salience (Engel et al., 2013; Rudy et al., 2011). The expression, function, and regulation of cortical Htr4 receptors are clearly different. Htr4 receptors are G-protein coupled, and their expression is strongly and specifically increased in corticostriatal pyramidal cells as a result selective serotonin reuptake inhibitor (SSRI) treatment. This has led to the hypothesis that increased Htr4 expression heightens the sensitivity of corticostriatal pyramidal cells to SSRIs, thus improving communication between the cortex and the striatum and contributing to the therapeutic actions of these antidepressants (Schmidt et al., 2012). These two examples of cortical serotonin responses involve different receptors, signaling pathways, cell types, and behavioral outcomes, yet they are elicited by the same neuromodulator.

This suggests that any given neuromodulator has the possibility for a wide scope of action. For example, acetylcholine within the cortex has been shown to mediate attention (Froemke et al., 2007) and memory control (Hasselmo, 2006) as well as plasticity (Gil et al., 1997). However, the nucleus basalis is the primary source of acetylcholine to the cortex (Kilgard and Merzenich, 1998), raising the question of how signaling from a centralized source can mediate such disparate actions. Again, the answer lies in the fact that the receptor families for many modulatory substances are also scattered across distinct cell types and, conversely, that receptors with different signaling capacities can be coexpressed in the same cell type(s). For instance, both the neurogliaform and VIP-expressing interneurons express nicotinic acetylcholine (ACH) receptors (Lee et al., 2010) in addition to having Htr3a receptors. Other interneuron classes, such as the Martinotti (Kawaguchi and Shindou, 1998) and basket cells (Kruglikov and Rudy, 2008) as well as pyramidal cells, express muscarinic ACH receptors (McGehee, 2002). Hence, the release of acetylcholine can differentially engage and modulate distinct sets of cortical circuits. For instance, recent studies show that VIP-expressing bipolar cells function in the disinhibition of basket and Martinotti cells in fear association (Letzkus et al., 2011) or motor-sensory gating (Lee et al., 2013), respectively. The ability of these cells to increase their gain in response to ACH may begin to explain how they are effective in associating sensory and motor stimuli to behavioral associations. These are just a few of the myriad of possible recruitment strategies at the brain's disposal. Increased understanding of the specific neuron types recruited by acetylcholine and their functional connectivity is but one example of how our phenomenological understanding of neuromodulation and behavior is rapidly being taken to both a cellular and circuit level. Altogether, one begins to appreciate that, although the combinatorial possibilities for circuit modulation are vast, our ability to map circuits involved in neuromodulation in the context of behavior is rapidly leading to a functional understanding of the brain.

Although refinement of the electrical properties of individual neuronal cell types and their modulation by small molecules must contribute substantially to the number of distinct types of neurons in any animal, the amazing histological diversity of the mammalian brain discovered more than a century ago (Ramon y Cajal, 1899), remains unsettling. Expression profiling experiments of specific cell types has established that cell-surface proteins that generate or modulate neuronal activity and the transcription factors that regulate their expression are among the most important determinants of neuronal identity (Toledo-Rodriguez and Markram, 2007; Okaty et al., 2009; Doyle et al., 2008). However, the profile of cell-specific genes expressed by any given neuron type also includes a wide variety of proteins of unknown function and others that fine tune the biochemistry of that cell type (Heiman et al., 2008; Doyle et al., 2008). For example, among the most specifically expressed genes in cerebellar Purkinje cells are two carbonic anhydrases (Car7 and Car8), two centrosomal proteins (Cep76 and Cep72), a glucosyltransferase (b3gnt5), a ceramide kinase (Cerk), a subtilisin-like preprotein converstase (Pcsk6), and a whole host of encoding mRNAS of unknown function (1190004E09Rik, 2410124H12Rik, etc.). Although simple hypotheses can be formulated for many of the individually expressed proteins, we do not understand the properties conferred upon Purkinje cells (or any other neuron type) by the unique ensemble of genes whose expression is enriched in them. Nevertheless, we suspect that the evolution of such a rich variety of specialized neuronal cell types must be driven in part by the requirement for unique biochemical functions that we have yet to understand.

How Is Cellular Diversity Generated?

The past 20 years have seen broad inroads made in our understanding of the development of neurons in all regions of the nervous system of both invertebrates and vertebrates. The invariant lineages that give rise to the 302 neurons in nematodes (Hobert, 2010) and the stereotyped iterative production of *Drosophila* neurons derived from sensory organ precursors, the ventral nerve cord, and the ommatidia during embryonic development have been particularly informative (Jukam and Desplan 2010). Studies in these systems have provided a context for understanding how the broad classes of intrinsic and local determinants such as proneural genes and homeodomain proteins direct cell fate. Vertebrate studies have complemented this, most notably, those of the spinal cord, retina, and cerebral cortex of mammals. These have provided insight into the interplay between extrinsic morphogenic determinants and their impact on the less deterministic lineages seen in higher animals (reviewed in Briscoe and Novitch, 2008). A common theme of both is that, despite variations in how the ground state is established, cell identity becomes fixed when the cell exits the stem cell proliferative mode.

A wealth of experiments have demonstrated that, after the identity of a neuron has been established, it is maintained even after heterotopic transplantation or in vitro culturing (McConnell, 1992; Gaiano and Fishell, 1998). Similarly, perturbations in the transcription code occurring prior to or coincident with cell birth alter neuronal identity, whereas the same manipulations occurring postmitotically have a much less dramatic effect on neuronal phenotype (cf. Butt et al., 2008 and Nóbrega-Pereira et al., 2008). What then do we know about how ground states are determined during development? It appears that, in most cases, the strongest influence on cell identity occurs at or near the time at which cells become postmitotic (McConnell and Kaznowski, 1991). However, there are exceptions to this rule. For example, granule cells of the cerebellum and neural stem cells in the adult subependymal zone are both committed to their fate prior to their

last division. Although it is beyond the scope of this Perspective to comprehensively review mechanisms that establish neuronal identity, it is instructive to consider a few specific examples.

In *Drosophila*, neuronal ground state is established predominantly by intrinsic factors. Detailed studies over the last decade have established that neuroblasts express a succession of distinct to transcription factors in order to produce stereotypic cell types (Doe and Skeath, 1996). In the case of the *Drosophila* ventral nerve cord, an orchestrated program involving the sequential expression of *Hunchback*, *Kruppel*, *PDM*, and *Castor* produces particular cell types in a reliable series (Grosskortenhaus et al., 2005). In the *Drosophila* eye, an analogous progression of factors occurs within the visual laminae to produce discrete cell types with defined properties (Li et al., 2013). In other regions of the embryo, this general theme is upheld, in that daughter-cell-proliferative modes and changes in competence over time combine to generate specific neural cell types (Baumgardt et al., 2009). Therefore, it appears from these studies that the underlying logic of progressive changes in intrinsic neuroblast competence to generate diverse cell types is, at least in invertebrates, pervasive. In vertebrates, although lineage determination is less ordered, recent studies in the developing spinal cord (reviewed in Briscoe and Novitch, 2008), cerebral cortex (reviewed in Molyneaux et al., 2007), and retina (Livesey and Cepko, 2001) support a similar model whereby neural progenitors undergo temporally regulated changes in intrinsic competence through the expression of specific transcription factors, and these interact with extrinsic morphogen gradients to generate discrete lineages. As mentioned above, neuronal identity is attained as neurons become postmitotic. For example, in the spinal cord, a ventral-to-dorsal Sonic hedgehog (SHH) gradient is balanced by a competing inverse gradient of bone morphogenetic protein (BMP) (Tozer et al., 2013) and Wnts (Muroyama et al., 2002) that help establish a dorsoventral identity, whereas retinoic acid and fibroblast growth factor (FGF) act to establish the rostrocaudal axis (Diez del Corral and Storey, 2004). These gradients result in the expression of a Cartesian array of morphogen-responsive genes, such as the type 1 homeobox genes (e.g., *Nkx2.2* and *Nkx6.2*h) that are induced by SHH (e.g., *Nkx2.2* and *Nkx6.2*h), basic helix loop helix genes, such as *Ngn1* and *Athl*, that are induced by BMPs, and homeobox cluster genes that are expressed in the orthogonal axis and induced by FGF and retinoids (Philippidou and Dasen, 2013). Given the large number of transcription factors and extrinsic signals encoded in the mammalian genome, it appears that their coordinated and combinatorial expression could easily generate the large diversity of nervous system ground-state identities.

As neurons exit their last cell cycle, the expression of critical developmental factors is extinguished either immediately or gradually, and refinement programs that establish their mature differentiated state are executed (Figure 2). This is controlled by effector transcription factors that are generally induced within the cells during late mitosis but persist within cells in order to direct maturation. For instance, in the cerebral cortex, CTIP2 and Satb2 function in immature neurons to control the identity of particular pyramidal cell types (in this case, corticofugal versus commissural identity) (Molyneaux et al., 2007; Leone et al., 2008), whereas Lhx6, Sox6, and Satb2 function to promote the development of specific cortical interneuron subtypes (Bartolini et al., 2013). These factors, although critical for the development of specific cell types, are expressed much more broadly. Therefore, in addition to these differentiation determinants, there must be unique transcriptional codes that form the core of the ground-state identity of different neurons. Although high-throughput sequencing is rapidly providing transcriptome ground states for many different cell types, the outlines of these codes have perhaps only been deciphered in the retina (Siegert et al., 2012). Interestingly, at least in this case, although each cell type has at least one factor unique to specific retinal cell types, these genes are often found to be both expressed in and required for numerous other developmental and functional contexts. For instance, although *Ascl1* is unique to amacrine cells and *En2* is unique to horizontal cells within the mature retina, both these genes are iteratively used in numerous other contexts. What appears to provide specificity is the coordinate expression of other transcription factors that are more generally enriched in the retina, such as *Rax* in photoreceptors and *Vsx2* in bipolar cells.

The positioning and connectivity of neurons whose ground state has been determined appear to initially remain plastic. Recent findings suggest that their specialization most likely depends on processes that are largely stochastic in nature. Although they are not essential for determining cell type per se, local environment cues are essential for insuring that specified populations span the entire range of required cellular geometries and connectivity by selectively sampling the full range of available positional information. Explicit examples of this can be observed in the tiled distribution of amacrine cells in the retina or olfactory receptors in the nasal epithelium. For these classes to function properly, they must generate sufficient variations in connectivity in order to fully occupy the existing information space. Indeed, most diversity in the CNS reflects variance in synaptic connectivity and not intrinsic properties; hence, understanding how the selection of synaptic partners is determined is one of the next major challenges for neuroscientists. A growing number of adhesion molecules have been shown to be involved in the pre- and postsynaptic specificity of different cell types. In *Drosophila*, the DsCAM, leucine-rich repeat, and teneurin families of proteins (Kurusu et al., 2008; Matthews et al., 2007; Hong et al., 2012) have recently been implicated in controlling dendritic spacing, synaptic specificity, and target selection. In vertebrates, the contactin, protocadherin, and neurexin and neuroligin families have been shown to have considerable variation that can be linked to the specificity of synaptic connections in a variety of contexts, including the cortex, the cerebellum, and the retina (Brose, 2009; Yamagata and Sanes, 2008; Lefebvre et al., 2012). Consistent with the idea that neuronal ground states can have their synaptic connectivity controlled through local interactions, recent work has proposed a model whereby the activity-mediated regulation of the SAM68 splice factor results in the production of alternatively spliced forms of neurexin-1 numbering in the hundreds (Iijima et al., 2011). Similarly, the RBFox (A2BP) splice factor family has been implicated in the differential splicing of synaptic components, such as PSD95, as well as channel subunits (Gehman et al., 2011). Both of these examples provide intriguing mechanisms for the adaption of

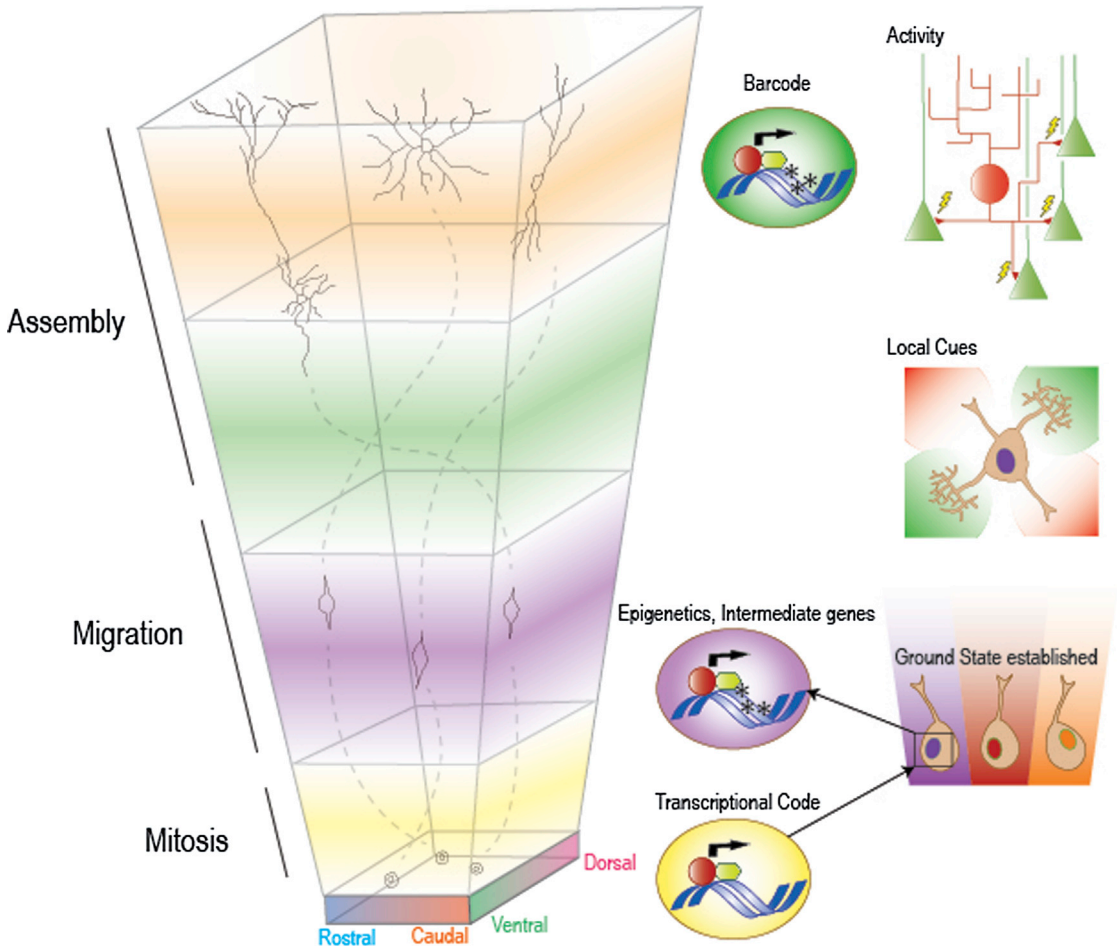

Figure 2. Schematic Showing the Progressive Steps Neural Progenitors Go Through in Order to Attain a Stable Cell Identity
As neurons move from the proliferative zone to become integrated into circuits, they go through three successive steps: (1) initial specification in the neuroepithelium, (2) establishment of a ground-state identity upon becoming postmitotic, and (3) refinement of cell characteristics by local cues as they establish their connectivity.

neurons to specific local environments on the basis of activity. At least in principle, this model provides sufficient variation to provide for a lock-and-key mechanism for explaining how a much smaller group of genetically specified neuronal subtypes could establish specific connectivity with the breadth and variation found in the nervous system.

How Is Cell-Type Identity Maintained?

Once neurons attain their identity and transit through the final stages of differentiation, they must remain responsive to a wide variety of internal and external cues while maintaining a stable ground state that defines their role in functional circuits. Foremost among the dynamic molecular responses of neurons are gene expression programs that are elicited by growth factors or altered electrical activity (Curran and Morgan, 1985; Greenberg et al., 1985; Cohen and Greenberg, 2008: West and Greenberg, 2011). These responses have been studied in great detail in a variety of different neuronal cell types, and the early regulatory steps have been defined. The general model that has emerged from this work is that these dynamic gene expression programs are regulated by a set of activity-dependent transcription factors that are posttranscriptionally regulated in response to changes in intracellular calcium levels and that these initiate a series of refined programs that alter dendritic and synaptic properties. The precise profile of downstream genes activated in response to specific cues can vary within or between cell types depending on the stimulus as well as its history of activation. Consequently, even neurons of the same cell type that we believe can be operationally defined by a common ground state can vary in their precise profile of gene expression depending on these dynamic, activity-dependent events. Although these programs are important for sculpting the synaptic and dendritic properties of developing neurons, in the context of this Perspective, it is important to emphasize that these programs must remain available to the cell so that it can be fine tuned to operate optimally as the animal continues to learn during its life. At the same time, we have argued that there is a characteristic set of genes that is stably expressed throughout the life of a cell that identifies it as a member of a specific cell type. Although recent experiments have demonstrated that neuronal identity can be induced by the activation

of transcriptional programs in induced pluripotent stem cells, transdifferentiation events have not been documented in adult neurons, which is consistent with the need for mechanisms to stably maintain identity for many years or decades in long-lived species. The concept of an "epigenetic landscape" (Waddington, 1940) that progressively restricts lineage and maintains the differentiated state clearly applies to neurons in this context. Classical epigenetic modifications to chromatin are present in neurons (Feng and Nestler, 2013; Telese et al., 2013), including those chromatin marks that identify poised genes that are not being expressed but are capable of activation in response to the appropriate stimulus.

The recent discovery that mammalian genomes contain 5-hydroxymethylcytosine (5hmC) (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009) and that this novel nucleotide is selectively enriched in neurons has added a dimension to epigenetic regulation in neurons that is not prevalent in many other cell types. 5hmC is produced from 5-methylcytosine by an oxidation reaction catalyzed by the TET protein family (Tahiliani et al., 2009). Although 5hmC can, in some cases, serve as an intermediate in active genomic demethylation (Tahiliani et al., 2009; Ito et al., 2011), the highly elevated levels of 5hmC in neuronal genomes suggested that 5hmC can serve as an epigenetic mark in neurons in order to regulate function (Kriaucionis and Heintz, 2009; Mellén et al., 2012). This hypothesis is supported by the discovery that MeCP2, a neuron-enriched protein that can bind to 5mC (Lewis et al., 1992) and whose loss of function causes Rett syndrome (Amir et al., 1999), also binds with high affinity to 5hmC. The fact that 5hmC is enriched in expressed neuronal transcription units (Song et al., 2011; Mellén et al., 2012) and that a loss of MeCP2 function in neurons results in a decrease in gene expression (Chahrour et al., 2008) has supported the idea that 5hmC is a neuron-enriched epigenetic mark that is bound by MeCP2 in active genes in order to relax chromatin structure and facilitate transcription. Although the magnitude of the transcriptional induction seen in Rett syndrome mouse models lacking MeCP2 is small (Chahrour et al., 2008), the fact that some Rett-syndrome-causing alleles of MeCP2 can preferentially impact 5hmC binding activity (Mellén et al., 2012) supports the notion that MeCP2 recognition of 5mC and 5hmC are both important for epigenetic control of neuronal function. One interesting possibility is that, during the evolution of long-lived organisms whose neurons must maintain a stable differentiated state for optimal function, the 5mC-5hmC-MeCP2 epigenetic mechanism was selected to provide protection against low-probability events that could either destabilize neuronal function or result in the induction of inappropriate programs used in other cell types to control population numbers (e.g., cell division, apoptosis, autophagy, etc.). In-depth characterization of gene expression and methylation status in specific cell types should accelerate our efforts to understand the stability of neuronal ground states and the contributions of these and other novel mechanisms to maintaining neuronal function and responsiveness in long-lived species.

How Can One Identify a Cell Type?

As we have argued above, a useful operational definition of a cell type is a cell or population of cells that share a molecular ground state that both identifies them as distinct from other cells and determines their functional capabilities. The reason we qualify this definition as "operational" comes from the realization that a consensus definition of cell type that applies universally has not been reached. This results from the fact that individual cells of a particular type need not be identical at an anatomical or molecular level in order to perform essentially the same function. B lymphocytes have evolved complex mechanisms that allow the diversification of antibody chains expressed by each clone of B cells (Neuberger, 2008). Olfactory sensory neurons have equally complex mechanisms for the stochastic expression of receptor genes that determine which odorants can be detected by each neuron and identify the specific glomerulus they will target (Buck and Axel, 1991). Purkinje cells express developmentally specific proteins that delineate conserved parasagittal domains with connectivity to specific nuclei deeper in the cerebellum or brainstem (Gravel and Hawkes, 1990). Indeed, the nervous system has evolved mechanisms for stochastic expression of a variety of cell surface proteins that can determine precise connectivity, fine tune neuronal function, and contribute to the "individuality" of neurons of many types (Yagi, 2013). It can be argued that these expressed molecules are critically important for cellular function and that, therefore, they identify a cell type. However, to our minds, it makes more sense to recognize these mechanisms as capable of providing fine-tuned functional diversification within individual cells of a type and to use the molecular ground state as the operational criterion for identifying them as a single cell type. In this way, one can both recognize the molecular individuality of single cells and maintain continuity with classical anatomical and electrophysiological studies.

The practical issue to be addressed is the determination of the molecular ground state of an individual cell or group of cells. We and others have argued that the most objective methodology for this purpose is to profile gene expression. Expression profiles can be obtained from genetically targeted cell populations or randomly chosen single cells with the use of a variety of technologies. Although a discussion of the strengths and weaknesses of these approaches is not possible here, there are certain features of these two broad categories of approach that must be considered if one hopes to obtain a complete account of cell types present in complex nervous systems. Strategies that employ genetic targeting allow repeated profiling of the same candidate cell type under a variety of different conditions (Heiman et al., 2008; Doyle et al., 2008), and they can provide genetic accessibility to that cell type so that additional anatomical, electrophysiological, and functional data can be incorporated into a understanding of the roles it plays in the nervous system. These features allow both technical and biological replicates to be collected in order to improve the quality of the profiles obtained and their comparative analysis. They enable the interrogation of that cell type during development, and they facilitate the incorporation of a wide variety of independent experimental data sets into cell-type-centered databases. This approach will necessarily improve our understanding of the functional diversity of CNS cell types, place them in the proper historical context, and enable molecular phenotyping experiments to identify their responses to a wide variety of genetic and environmental perturbations. The main advantages of single-cell profiling (Wichterle

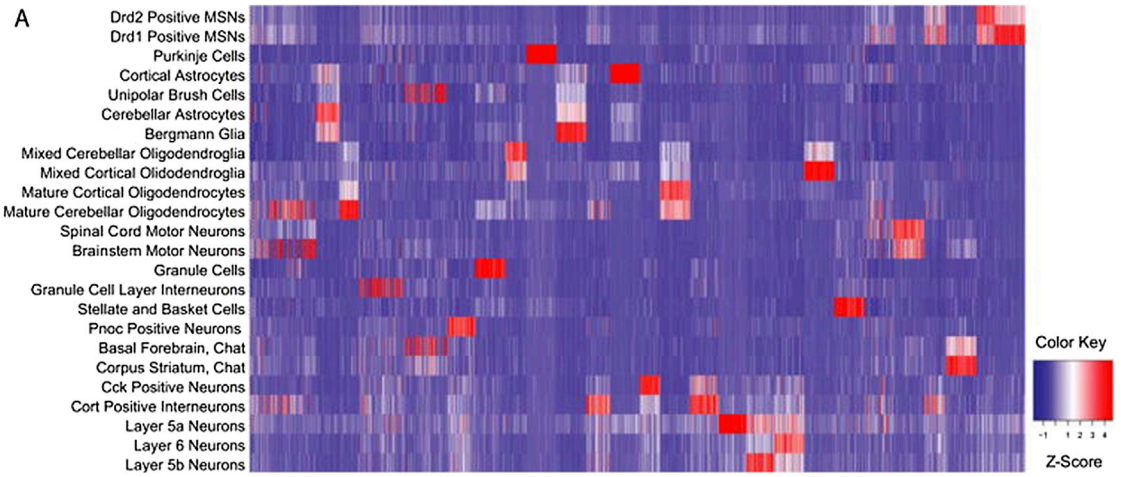

Figure 3. Comparative Analysis of TRAP Data Reveals Cell-Type-Specific Translational Profiles
A heat map showing the normalized expression of the top 100 ranked probesets from each sample across all samples. Note the blocks of genes detected as specific to each cell type (such as Pcp2) (Doyle et al., 2008).

et al., 2013) are that it is fast (i.e., it does not require specialized, stably targeted engineered lines), bar-coding can be used to obtain many profiles from individual cells in the same animal, and single-cell approaches can be pursued in organisms that are not genetically accessible. Although there is not yet enough data to place proper emphasis upon each of these strategies (or intermediate approaches that employ viral vectors to target cell types) within the broad goal of identifying and understanding cell type diversity in complex nervous systems, single-cell technologies will certainly play an important role in cell-type identification and analysis.

Given microarray or RNA sequencing (RNA-seq) data from candidate cell types, it is an operational matter to define a potential molecular ground state and determine whether it defines a cell type. As mentioned above, many microarray studies of defined cell types, as well as a few studies using more refined RNA-seq analysis, demonstrate that comparative computational analysis of profiling data from multiple cell types is capable of identifying genes with enriched expression in canonical cell types (Figure 3). Of course, this makes a great deal of sense, given that the specialized anatomical and functional features of cell types are encoded in these genes. As we have argued above, the defining molecular signature of specific cell types should include a suite of genes that are stably expressed within that cell type and exclude activity-dependent genes or those individual transcripts expressed stochastically in order to diversify fine-scale properties of individual cells. A simple experimental prediction should hold true if the candidate population is to be referred to as a cell type; i.e., the stably expressed, enriched mRNAs that characterize the ground state should be present in every cell in the population, and, in aggregate, they should be not be expressed of other cell types. In other words, it should not be possible to identify subprofiles that further subdivide the population into stable, defined subtypes of cells. For example, if one were to analyze the expression of a large number mRNAs that are thought to contribute to the molecular ground state of a cell type by in situ hybridization, single-cell quantitative PCR, or single-cell RNA-seq, then the cell-type-defining mRNAs should be shared by all cells of that type. Given these data, one could then go on to perform developmental studies in order to determine how early specific cell types defined in this manner evolve and whether a subset of transcription factors is sufficient to identify these cells as they exit their final cell cycle.

How Can the Identification and Molecular Characterization of Specific Cell Types Help Us to Understand and Treat CNS Disorders?

The tremendous diversity of cell types in the mammalian nervous system presents many challenges to our understanding of their function and dysfunction. It also provides unique opportunities for therapy. For example, we lack adequate explanations for the observations that, in many cases, very specific neuropathology can result from mutations in genes that are broadly expressed or for the finding that clinical disorders with specific defining characteristics can result from mutations in many, many different genes. How does a loss of function of a nearly ubiquitously expressed nuclear protein kinase result in death of a single CNS cell type (cerebellar Purkinje cells) in ataxia telangiectasia (AT) (Savitsky et al., 1995)? What is the explanation for the extreme genetic complexity of autism spectrum disorder (Geschwind, 2011) and other common afflictions of the nervous system? In cases like AT, the loss of one or a few key cell types due to the mutation of a common cellular protein must in some way reflect the rate-limiting nature of that protein in those few cell types as a consequence of their unique biochemistries. With regard to the astounding genetic complexity of many CNS disorders, one suspects that this must arise from both cell-specific consequences of alterations in the functions of the many causative genes and the ability of dysfunction in a variety of different cell types within specific brain circuits to result in a similar clinical outcome. Therefore, it seems evident that progress in understanding and treating these devastating disorders must include precise anatomic and functional characterization of CNS circuits. This will no doubt need to include the discovery

of the unique molecular properties of their component cell types and the investigation of the molecular phenotypes that arise in these cell types as a consequence of genetic and environmental influences.

Although the investigation of the detailed circuitry of nervous systems and their tremendous histological and functional diversity is a daunting challenge to many subfields of neuroscience, the nature of CNS circuits and cell types also offers unique opportunities for treatment. Every circuit is composed of many cell types, each distinguished by the presence of fine-tuned biochemical and signal-transduction pathways that govern activity. It follows that, if we can understand the development and molecular functions of the cell types that comprise the circuit, then we can generate and test hypotheses regarding mechanisms that modulate its output. Given the complexities of neural circuits, dysfunction in one element of the circuit can sometimes be compensated by the modulation of a second node in the circuitry. For example, Parkinson's disease (PD) is a late-onset neurodegenerative disease in which dopaminergic neurons in the substantia nigra degenerate, resulting in a loss of dopamine release into the striatum and the accompanying severe motor symptoms. The prevailing hypothesis (Feyder et al., 2011) for the actions of L-Dopa in PD patients is that its therapeutic benefits are primarily due its actions in replacing dopamine on dopamine receptor D2 (Drd2)-expressing medium spiny neurons (MSNs) that project indirectly to the substantia nigra. The L-Dopa-induced dyskinesia that results from long-term therapy is thought to be attributable to the stimulation of Drd1 in direct pathway MSNs. This is supported by observations of dramatic therapeutic effects from deep-brain stimulation (DBS) of the subthalamic nucleus (Kalia et al., 2013), an indirect pathway nucleus that receives input from Drd2 MSNs. If this hypothesis is correct, then the development of novel pharmacology for a specifically expressed alternative receptor that mimics the actions of dopamine on Drd2 neurons but is not expressed in Drd1 MSNs could prove to be an effective treatment strategy, given that it would achieve a similar therapeutic effect to L-Dopa without the negative side effects associated with the stimulation of Drd1-expressing MSNs. The fact that Drd2 and Drd1 MSNs differentially express ~350 genes (Heiman et al., 2008) offers a variety of potential targets for the execution of this strategy. Given recent evidence from optogenetic studies in rodents (Gradinaru et al., 2009) and DBS trials in humans (Kalia et al., 2013), the modulation of the activity of specific circuit elements for therapeutic benefit may be an effective approach for the treatment of a variety of neurological disorders. It follows that detailed studies of the cell types present in these circuits, and the expression of candidate therapeutic targets within them, holds great promise for symptomatic relief in these devastating disorders.

Of course, the most important information that can arise from comprehensive and detailed molecular studies of CNS circuits and cell types is the discovery of molecular mechanisms of disease. In some cases, this knowledge will lead to specific hypotheses for disease modification and new avenues for the development of appropriate therapy. As stated above, although many brain diseases result from genetic insults that affect broadly expressed genes, the resulting pathology often relates to the impact this has on a select number of cell types. The difficulty in recognizing which specific cells are affected often arises because the onset of the disease symptoms is temporally removed from the initial defect. For instance, many affective disorders can track their etiology to failures in development, and late-onset neurodegenerative conditions often arise from disturbances in molecular cascades whose consequences unfold over many years or decades. It seems apparent that detailed molecular profiling of the affected cell types during development and disease progression is a necessary step in understanding the molecular consequences of destructive genetic or environmental events. However, these studies cannot be pursued without comprehensive information regarding CNS cell types, their connectivity, and their contributions to behavior.

Concluding Remarks

In our view, the tremendous progress in targeting neural cell types in genetically accessible organisms has placed us at the verge of a new era in which neural circuitry can be investigated at levels spanning from systems neuroscience to molecular mechanism. Genetic targeting and molecular characterization of every cell type in the nervous systems of the worm, fly, and mouse is within reach. Although we have focused specifically on the diversity of neuron types present in complex nervous systems, equally compelling arguments can be made for an investigation of the variety of glial cell types, especially given the exciting new functions uncovered for glial cells in nervous system development and dysfunction (Clarke and Barres, 2013). Although we understand that it is difficult to identify and genetically target every cell type in complex nervous systems, we believe that deep knowledge of the developmental origins and molecular mechanisms that both create and govern the functions of specific cell types is essential.

In spite of the tremendous progress that has been made in the definition and functional analysis of specific cell types in the nervous system, progress in several areas would be advanced by new or improved experimental strategies. For example, the genetic targeting of specific cell types remains challenging even with all the currently available approaches and is restricted to a few accessible species. The development of genome-editing techniques that employ customized, chimeric nucleases in order to insert foreign DNA at a specific site in the genome has tremendous potential for improving the efficacy of genetic targeting in a variety of species (Gaj et al., 2013). Tests of the application of these methodologies for large-scale and comprehensive studies will be important. The generation of viral vectors, which are ideally suited for gene delivery, that are able to "read" the transcription code, thus providing a general solution to truly cell-type-specific targeting in adult animals, would strongly advance the field. Further development of clever strategies for the discovery and analysis of neurons responding to specific stimuli, such as phosphorylated ribosome capture (Knight et al., 2012), or for RNA-based biological regulation, similar to crosslinking and immunoprecipitation (Ule et al., 2003), will play increasingly important roles in advancing our understanding of neural circuitry and molecular mechanisms of CNS function. Continued improvements in DNA- and RNA-seq methodologies as well as price and quality control will be necessary in order to

bring these powerful methodologies into common usage in neuroscience laboratories. The refinement of existing informatics techniques and, in particular, the further development of user-friendly interfaces for the interrogation of these data will be required for leveraging the tremendous biological intuition of neuroscientists for the interpretation of these very powerful yet complex data sets. Continued efforts to properly annotate each cell type and agree on common conventions for naming those that are newly identified are essential for progress. The organizing principle for all of these efforts must hearken back to the founding of modern neuroscience by Ramon y Cajal (1899), who first saw and understood the fundamental importance of identification, characterization, and comparative analysis of the great diversity of cell types present in complex nervous systems.

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