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Progressions

Progressions on the Coexistence of Neuronal and Glial Precursor Cells in the Cerebral Ventricular Zone

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Heterogeneity is defined as the quality or state of being diverse in character or content. This article summarizes the natural progression from my studies, reported in the first issue of the *Journal of Neuroscience*, that identified molecular heterogeneity in precursor cells of the developing primate cerebral cortex to the current state in which differences defined at the molecular, cellular, circuit, and systems levels are building data encyclopedias. The emphasis on heterogeneity has impacted many contributors in the field of developmental neuroscience, who have led a quest to determine the extent to which there is diversity, when it appears developmentally, and what heritable and nonheritable factors mediate nervous system assembly and function. Since the appearance of the article on progenitor cell heterogeneity in the inaugural issue of the *Journal of Neuroscience*, there have been continuous advances in technologies and data analytics that are contributing to a much better understanding of the origins of neurobiological and behavioral heterogeneity.

My dissertation advisor at University of California, San Diego, Robert Moore, explained to me that biological scientists are either lumpers or splitters. In neuroscience, this characterization has been at the heart of the centuries-old pursuit to describe structure-function relations of the nervous system. At the time, I hadn't thought of myself as either one but, having spent much of my scientific career determining the unique functional, structural, and molecular properties (phenotypes) of cells that comprise the mammalian CNS, have come to realize that I am indeed a splitter. My pursuit has had a decidedly developmental focus, stemming from undergraduate courses undertaken at the University of Chicago, which revealed to me that organism diversity has its origins at the very beginning of embryogenesis, with unique genetic programs, cell-cell interactions, and environmental factors contributing to the emergence of the properties that define brain organization at the mesoscale and microscale. This focus was evident even in my very first publication (Levitt et al., 1976) reporting that single-cell dissociates of fetal mouse midbrain could be aggregated (to what we now refer as organoids). Remarkably, dopaminergic neurons, which comprise a minor subpopulation of this brain region, were sufficiently unique to recognize each other in the aggregate and, within a few days, reconstruct the substantia nigra *in vitro*.

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Fast forward a few years to 1979, when I joined the laboratory of Pasko Rakic at Yale University. I joined Pasko's laboratory because of his forward-thinking studies of cerebral cortical development, enamored with the concept that the progenitor cells of the ventricular zone mapped onto the developing cortical plate to form unique cortical areas (Rakic, 1978), a controversial but now widely accepted concept termed the protomap (Molnár et al., 2019). My main project as a postdoctoral fellow, however, was to determine the time of origin of brainstem monoamine neuron groups in rhesus monkey. Pasko had generated a remarkable library of paraffin sections prepared over the years from animals injected with ^{3}H-thymidine at different fetal and postnatal ages, with the goal of determining time of origin of different neuron populations. Counting thymidine-labeled neurons — not so exciting. How did I come to generate data demonstrating heterogeneity in progenitor cells in the developing cortex? I was having conversations with my friend, Mark Siegel, at the time a research technician in Lawrence Eng's laboratory at Palo Alto VA Hospital and Stanford. Larry had polyclonal antibodies to GFAP for use in human pathology (Eng and Rubinstein, 1978). Although others had generated antibodies that worked well in mature astrocytes, none were sufficiently sensitive to visualize the fine processes of developing radial glia and had not been developed for use in paraffin sections. Therefore, when Mark told me that their antibodies generated robust staining in pathology material prepared identically to the paraffin sections in Pasko's library, I was excited to approach Pasko with the idea of trying these new antibodies in the macaque cortex. Although he informed me that, indeed, Melitta Schachner had spent a year in his laboratory at Harvard trying, to no avail, available antibodies to GFAP, I did obtain a small aliquot of the antibodies from the Eng group. To be transparent, I cannot remember if Pasko agreed with this decision or not. Another obstacle: I had never used immunocytochemistry methods. Fortunately, Pietro DiCamilli, at the

time a postdoc in Paul Greengard's laboratory, was generating antibodies to synaptic vesicle proteins and developing sensitive methods of cellular immunostaining. I credit Pietro for giving me the tools and confidence to perform anti-GFAP labeling in the monkey material; and 1 year later, Pasko and I published a very detailed paper describing the organization of radial glial cell (RGC) processes at different fetal ages in the developing monkey neocortex using existing paraffin-embedded material from his library (Levitt and Rakic, 1980). The data replicated Golgi-based studies at the cellular level, demonstrating RGC soma location along the ventricle, but provided a level of detail that had not been visualized previously regarding the enormity of the radial fiber assembly that emerged and then expanded over the fetal time periods of major neuronal migration. At the light microscopic level, we could view counterstained fusiform-shaped cells (what appeared to be migrating neurons) hugging GFAP-stained radial fibers. This was just as Pasko had initially reported using electron microscopy (Rakic, 1972; Rakic et al., 1974).

An unexpected observation is what appeared to be heterogeneity of GFAP immunolabeling of cells in the ventricular zone, the transient embryonic structure that His and Fujita termed the germinal matrix (His, 1889; Fujita, 1963). Both classic studies had emphasized the homogeneity of cells giving rise to the neurons of the cerebral cortex, understandable given the near-identical morphology of cells traversing through different phases of the cell cycle. Our finding instead indicated a potential molecular heterogeneity of these progenitor cells. A nearly 100-year-old dogma was about to be revised, but we needed more than an interpretation of immunolabeling in single sections viewed by light microscopy. Given the limited methodology available at the time, the most convincing approach to demonstrate molecular heterogeneity was to perform immunostaining at the ultrastructural level, which would provide unequivocal identification of cell types. With my postdoc colleague, Michael Cooper, Pasko and I traveled to the New England Primate Center multiple times in 1980 to prepare fixed tissue collected from fetal rhesus monkeys. This was perhaps the most nerve-racking experiments in which I had participated, given the expense of time-pregnant monkeys and the need to generate suitable material for immunostaining that retained the highest quality ultrastructure in fetuses. As an aside, the sectioning and staining work was done in the basement of Sterling Hall on the Yale medical campus, with Dick Chen and his postdocs Aaron Fox and Martha Nowycky literally across the hall characterizing individual neuronal calcium channels by patch clamp, and Pietro Decamilli with Paul Greengard nailing the characterization of synapsin I on the third floor. The data that Michael Cooper and I produced formed the focus of the paper that appeared in the inaugural issue of the *Journal of Neuroscience* in 1981 (Levitt et al., 1981).

The paper made several important contributions. First, we discovered that there were at least two and probably more cell types in the precursor pool of the primate cortical ventricular zone. Second, we proposed immunocytochemistry as a way to address questions regarding molecular contributions to the generation of cellular diversity in the developing nervous system. Third, we proposed the hypothesis that neuronal and glial precursor cells formed separate lineages, beginning with the emergence of radial glial cells. The dogma of germinal matrix homogeneity first introduced by His had been challenged and views were changing. However, I believe that His and others would have discovered heterogeneity had they had access to the methods that were emerging in the 1970s and 1980s. Indeed, it is not possible for me to claim responsibility for promoting specific studies that followed our publication because the 1980s witnessed a flurry of activities related to understanding the molecular heterogeneity and lineage of cell types comprising invertebrate and vertebrate nervous systems. Yet it is the case that, within 6 years from the initial appearance in the *Journal of Neuroscience*, the paper already had been cited >100 times, the metric used to define a citation classic.

We ourselves provided a follow-up paper 2 years later (Levitt et al., 1983), in which we generated the quantitative data using immuno-electron microscopy, produced from the earliest time of visual cortical neuron production, embryonic day (E) 40, to the near exhaustion of neuronal precursors by midgestation in the rhesus monkey. The changing ratio of GFAP-immunolabeled:unlabeled precursor cells, which increased dramatically as neuron production peaked then terminated, supported the hypothesis that neurons and RGCs comprised separate lineages. The weakness, which we acknowledged, was the absence of a molecular marker for neuronal precursor cells. Indeed, it had been shown previously in the hypothalamus (Vitry et al., 1980), and became clear years later for the cerebral cortex, that our hypothesis regarding separate neuronal and radial glial lineages was not correct. Kriegstein and colleagues applied advanced methods in a series of papers (Noctor et al., 2001, 2004; Pollen et al., 2015; Molnár et al., 2019), using viral lineage tracing, live-cell imaging, and molecular profiling, to reveal that early radial glia (inner cerebral wall cells) do indeed, as we reported, divide asymmetrically. However, they additionally provided evidence that these early radial glia serve not only as precursors for the additional radial glia required as the developing cerebral wall expands tangentially, but also as the precursor cell for infragranular excitatory neurons. Later in development, as the subventricular zone appears, the outer radial precursor cells emerge and are responsible principally for generating supragranular excitatory neurons. Kriegstein and colleagues deserve the credit for establishing an even more complex mechanism responsible for generating neuronal diversity in the developing cerebral cortex.

What followed our work more immediately in the 1980s and 1990s was an avalanche of splitter-driven research, including that by Connie Cepko (Cepko, 1988) and others using retroviral tracing of cell lineages in the nervous system, as well as advanced molecular characterization of neural cells, driven substantially by the advent of broad access to the production of monoclonal antibodies (mAbs) and scalable molecular cloning and in situ hybridization (ISH) methods. Also at this time, Birgit Zipser, Ron McKay, and Susan Hockfield had already generated the first libraries of mAbs that revealed remarkable molecular diversity of cell types and axon "labeled lines" in leeches (Zipser and McKay, 1981; Hockfield and McKay, 1983). Corey Goodman and colleagues soon followed with developmentally focused mAb generation in grasshopper and *Drosophila* (Kotrla and Goodman, 1984). I recognized that, by isolating neurons from specific mammalian brain structures, mAbs offered a new way to immunize with a cell preparation to activate the mouse immune system, thus producing a library of antibodies against the expressed antigens. mAb technology thus served as a means of defining cellular and regional diversity in the nervous system. At the time of my work with Pasko, I initiated a brief visit to Max Cooper at University of Alabama, Birmingham Medical Center. Max, among the first Howard Hughes Investigators, along with John Kearney had developed one of the most reproducible and robust protocols for propagating antibody producing cells from an immunized mouse. This was done by fusing the immunogen-activated spleen cells with a cell line to create a novel hybridoma cell, each producing a single antibody type against a unique

antigen. From a developmental perspective, the efforts were forging the early assembly of data supporting Sperry's chemoaffinity hypothesis of synaptic specificity. The validity of the molecular hypothesis was soon to be advanced through forward and reverse genetic, cell biology, and protein biochemical studies by the laboratories of Goodman, Bonhoffer, Tessier-Lavigne, Jessell, and many others in the field of developmental neuroscience. Today, neuroscientists leverage molecular methods that emerged just a few years ago to again embrace splitting, rather than lumping, using single-cell RNA sequencing methods combined with advanced bioinformatics approaches to make sense of the complexity of the nervous system.

I left Yale and moved to a faculty position at the Medical College of Pennsylvania in late 1982 and pursued my efforts to advance the view that, like invertebrates, neurons comprising specialized circuits in the mammalian nervous system exhibited molecular specificity. I used rat hippocampal membranes as my immunogen of choice. From each immunized mouse, >1000 culture wells with a single hybridoma cell clone were generated. Expansion of the clones occurred rapidly, with the culture medium collected to serve as the primary antibody in an immunofluorescence analyses of staining patterns on tissue sections containing the hippocampus, and other regions of the rat forebrain. The hybridoma cells grew very rapidly, so the screening assay, using a fluorescence microscope located in my office, needed to be done rapidly, on tens of thousands of individual wells, to avoid the wells becoming overcrowded and the hybridoma cells dying. Each well had the potential to produce an mAb directed against a neuron subtype or circuit, with the screening revealing unique patterns of immunolabeling. Once unique neuroanatomical staining patterns were identified, the hybridoma cells were subcloned and then expanded to produce an immortal source of mAbs directed against a single antigen. I fulfilled my strong pull to relate anatomic specialization to unique molecular signatures by selecting those mAbs that labeled hippocampal neurons, followed with more extensive examination of staining patterns throughout the forebrain. The first experiment yielded mostly mAbs that stained all neurons or glial cells broadly. The second mouse I immunized led to the discovery of an mAb that labeled neurons in brain regions that I eventually recognized as interrelated structures of the limbic system, including enrichment in frontal, entorhinal, and perirhinal cortices, amygdala, septum and ventral striatum, medial dorsal thalamus, and some nuclei of the hypothalamus. Based on this work, the first independent publication from my new laboratory, a single-author paper in *Science* (Levitt, 1984) made the case that unique molecular properties were expressed by rodent neurons that were interrelated through connectivity. This was followed by mapping in the primate brain to demonstrate conservation of the expression of the antigen recognized by the mAb (Coté et al., 1995, 1996). A decade of molecular studies, led by Aurea Pimenta in my laboratory, isolated the mAb-directed protein and cloned the encoding gene, named the limbic system-associated membrane protein (LsAMP). It turned out to be a member of the Ig superfamily of cell adhesion molecules and was capable of regulating axon guidance of fetal and postnatal neurons (Keller et al., 1989; Zhukareva and Levitt, 1995; Zhukareva et al., 1997; Mann et al., 1998).

The protomap hypothesis that Rakic had popularized posited that the early developing neocortex contained region-specific information that served as the forerunner of canonical anatomically and functional unique cortical areas. The restricted expression of LsAMP in neurons of the medial and lateral mesocortical and allocortical regions prenatally aligned with the protomap hypothesis, and thus would provide a basis for determining the timing and plasticity of the molecular "code." In other words, were progenitor and postmitotic neurons, at different stages of maturation, rigid in their cortical area fate specification, or was there capacity to change molecular identities? Sue McConnell had taken on this issue with regard to laminar fate (McConnell, 1985). Sue used elegant transplant methods in the ferret to show that early cortical progenitors that normally produce infragranular neurons acquired cortical laminar fate based on the developmental state of the environment to which they were transplanted, retaining a deep laminar phenotype when transplanted at a time when deep layer neurons were being generated by the host but expressing a migratory and connectivity phenotype of superficial laminar neurons when transplanted into an older host. Later progenitors exhibited limited flexibility, expressing mostly superficial laminar phenotypes regardless of the age of the host. The transplantation studies of Brad Schlagger and Dennis O'Leary addressed regional fate and demonstrated that fetal posterior cortex had the ability to differentiate into somatosensory-like cortex, exhibiting morphologic features of barrels (Schlaggar and O'Leary, 1991). The studies that followed emphasized the concept that the cortical plate was an unspecified *tabula rasa*, rather than a protomap with early and distinct regional molecular properties. There was an added complexity to these experiments, however, namely, the heterogeneity of the transplanted tissue that comprised both progenitor cells and postmitotic neurons. This complexity influenced our thinking to use molecular features of mesocortex that normally expresses LsAMP to perform heterotopic and homotopic transplants derived from different embryonic ages into the newborn rat.

In another paper in the *Journal of Neuroscience* (Barbe and Levitt, 1991), Mary Barbe, a postdoc at the time, demonstrated that transplanted tissue derived early in development and containing only progenitor cells of mesocortex failed to express LsAMP in host heterotopic somatosensory cortex. Donor tissue taken a few days later contained both precursors and migrating postmitotic neurons, and we identified some ectopic expression of LsAMP in host somatosensory cortex. Finally, when tissue was taken later in prenatal development, when only postmitotic neurons were present, LsAMP was expressed in transplanted tissue regardless of the location. Connectivity studies (Barbe and Levitt, 1992, 1995) showed that the early input from the limbic thalamus to the host cortex and transplanted tissue mapped to the location of LsAMP expression, even when placed in host somatosensory cortex. In contrast, primary sensory dorsal thalamus innervated transplanted tissue when it was harvested to contain only progenitor cells, which produced neurons that did not express LsAMP. Connectivity to host cortical regions from the transplant (corticocortical connections) that contained LsAMP-expressing neurons projected specifically to host limbic regions expressing LsAMP. The combined results from the tissue transplant experiments made perfect sense to me; there was evidence accumulating that indeed the early developing cortex during fetal development expressed unique patterns of growth factors and axon guidance molecules that had been shown in *Drosophila* and in vertebrate explant studies to be responsible for controlling the growth of axons to their appropriate target areas. Thus, molecular cues were already present in the developing cerebral cortex before innervation by the dorsal thalamus, consistent with the growing number of developmental connectivity studies showing the specific targeting of afferent input, rather than nonspecific exuberant projections.

At the turn of the 21st century, developmental neuroscientists used advancing methods in synaptic electrophysiology combined with molecular biology and morphologic analyses to refine what we, as a field, mean by neuronal and glial heterogeneity and to subsequently begin to determine, in great detail, the specific molecular mechanisms that underlie the generation of cellular diversity. When I moved to the University of Pittsburgh, I had already been considering the new gene microarray technologies that were early in production by Affimetrix and Incyte for application to our developmental studies of molecular heterogeneity. Meetings with David Lewis, a clinician-scientist in psychiatry, changed my thinking to applying this technology instead to the study of brain diseases, particularly schizophrenia. David had demonstrated that there were cell type-specific changes in the dorsolateral PFC when postmortem brains from people with schizophrenia were compared with unaffected controls. Perhaps this reflected unique molecular properties of neuronal subtypes affected in schizophrenia, which could be identified by higher throughput methods, such as measuring the expression of thousands of transcripts at the same time. But could we demonstrate differential gene expression differences in cases and controls using David's collection of postmortem brains in the freezer? Karoly Mirnics, working as an instructor in the Department of Neurobiology, joined our nascent research team to take on the mRNA isolation and microarray project that seemed nearly impossible at the time. The goal was straightforward: modify protocols to enable isolation of intact mRNA from dead human tissue. Karoly, along with others in the laboratory, including Frank Middleton, who joined us a year later, did the heavy lifting to make two key advances. The first was to develop a method for consistent mRNA isolation with limited 5′ degradation. The second was advancing gene ontology methods applied to data comparing cases and controls. At the time, there were limited databases, such as KEGG, and no computational pipelines to discern significant gene groupings. Painstaking manual clustering was a cornerstone of the paper that appeared in *Neuron* (Mirnics et al., 2000), demonstrating that expression of transcripts encoding presynaptic, GABAergic, and glutamatergic family members was altered in every prefrontal cortical postmortem sample harvested from subjects with schizophrenia. What differed between subjects was the identity of the genes that comprised each functional cluster that exhibited reductions (including a new discovery of transcripts encoding proteins involved in energetics and metabolism) (Middleton et al., 2002). Microarray technologies became the rage: what followed were hundreds of molecular studies of various neurologic and psychiatric disease states, preclinical animal studies comparing gene KOs to WT, and the impact of environmental manipulations on gene expression profiles. Our studies had limited sample power, yet the core discoveries of the molecular heterogeneity that characterizes human brain diseases remain valid today, with far more advanced data analyses focusing on functional categories of genes. Advances in resolution in which the entire transcriptome of a dissected region from the development rodent or primate brain (Bakken et al., 2016), and more recently individual cells can be identified and measured (Lake et al., 2016; Rosenberg et al., 2018; Yuste et al., 2020), the reduced cost of sequencing, and the building of novel bioinformatics methods now makes it realistic, and even expected, to profile molecular phenotypes as part of an effort to understand neuronal and glial function and dysfunction in preclinical and clinical neuroscience studies. New discoveries of the prevalence of somatic mutations in the nervous system has led to what was a previously unthinkable contribution to cellular heterogeneity: nonheritable mechanisms to alter individual cell genomes over time (Westra et al., 2010; Lodato et al., 2015; D'Gama and Walsh, 2018; Kaeser and Chun, 2020). Whether stochastic or experience-driven, cellular mosaicism is becoming part of a greater understanding and more complex landscape of acquired acute and chronic epigenetic modifications of genomes across tissues and organ systems, including the brain (Aristizabal et al., 2020).

Our microarray studies in the early 2000s led me to where my research focus is today, considering heterogeneity as not only a normal state of development, but also typical of neurodevelopmental disorders that are categorically diagnosed, yet exhibit remarkable diversity of clinical symptoms. Kanner acknowledged this in his initial paper describing the features of 13 children that he defined as autistic (Kanner, 1943). Core behavioral and cognitive symptoms vary, and expression of associated medical conditions is quite heterogeneous in all neurodevelopmental disorders. We remain slow as a field in accepting that genes and environmental factors involved in increasing risk for brain disorders are likely to play a role in peripheral organ development and pathophysiological states (Boyce et al., 2021). With the heterogeneity bug persisting, our own studies of autism spectrum disorder at Vanderbilt University and the University of Southern California took this on with the characterization of children who have cooccurring medical conditions (Campbell et al., 2009; Gorrindo et al., 2013; Aldinger et al., 2015; Plummer et al., 2016). But the understanding of the origins of clinical heterogeneity remains elementary, in part because the field of experimental developmental neuroscience tends to be somewhat monolithic in using integrated strategies to address Gene × Environment × Time mechanisms. For example, in developmental neuroscience, there are limited experimental studies using genetic reference panels of mice, an approach that mimics an extended family pedigree for which members share various levels of their genomes. Using genetic reference panels, there is clear evidence that inherited genomic information contributes significantly to phenotypic heterogeneity (Williams et al., 2001; Williams and Mulligan, 2012). My colleague Allison Knoll has led our efforts to show the remarkable heterogeneity of complex social-emotional behaviors driven by heritable factors, including the mapping of genetic loci and candidate genes (Knoll et al., 2016, 2018).

My own focus on development, from early studies that attempted to define how things go right to the evolution of more recent studies of what and why things go wrong (particularly related to early postnatal environments), continues to include genes and cells but has advanced beyond those elements. Phenotypic heterogeneity changes over time, influenced by genetic programs, but also by context: environmental factors that are essential for experience-expectant development and the timing of when those experiences occur based on critical periods. Determining the molecular mechanisms that regulate critical period timing of specific functional phenotypes is high on the list of studies being pursued by my laboratory in collaboration with Shenfeng Qiu's group (Chen et al., 2020) and others (Reh et al., 2020). Our studies of early adversity in both animal models (Card et al., 2005; Heun-Johnson and Levitt, 2018; Eagleson et al., 2020) and in human infants (Pierce et al., 2019; Valdes et al., 2020) embrace these ideas.

The definition of development, change over time, has too often become a minor variable in experimental paradigms, with early manipulations that are not analyzed for phenotypic impact until adulthood. A deep mechanistic understanding requires insight into the adaptive processes that occur proximate to

developmental disruptions, as well as across prepubertal, peripubertal, and postpubertal epochs. These adaptations are different over time. But why is this so? Timing as a variable continues to be front and center in our studies of the mechanisms that may underlie the long-recognized fact that the timeline during which neuronal maturation occurs is not uniform. For example, my own quest for understanding the roots of phenotypic heterogeneity have included former and present members of the laboratory who have studied the c-Met receptor tyrosine kinase in cortical and brainstem circuit development and maturation. They have demonstrated neuronal subclass-specific and timing differences in expression and connectivity, temporally distinct patterns of neuronal and synaptic maturation, and even impact on timing of a critical period (Qiu et al., 2011; Kamitakahara et al., 2017; Kast et al., 2017, 2019; Chen et al., 2020). Heterogeneity is embedded in these discoveries; and yet, this is only one gene. Leveraging the remarkable progress of the field to build encyclopedias of cellular and circuit diversity now can be applied to the development of strategies to combinatorially manipulate genetic and environmental landscapes.

A final thought — it often seems a struggle to translate our collective basic research efforts into meaningful contributions to human well-being. But the contributions of the biology of heterogeneity should not be understated in its influence on evolving intervention research in health and education, with the promise of impact on policy (Shonkoff et al., 2021). Establishing what works, when it is optimal, and for whom are the next steps toward translation of what we know to what we do.

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