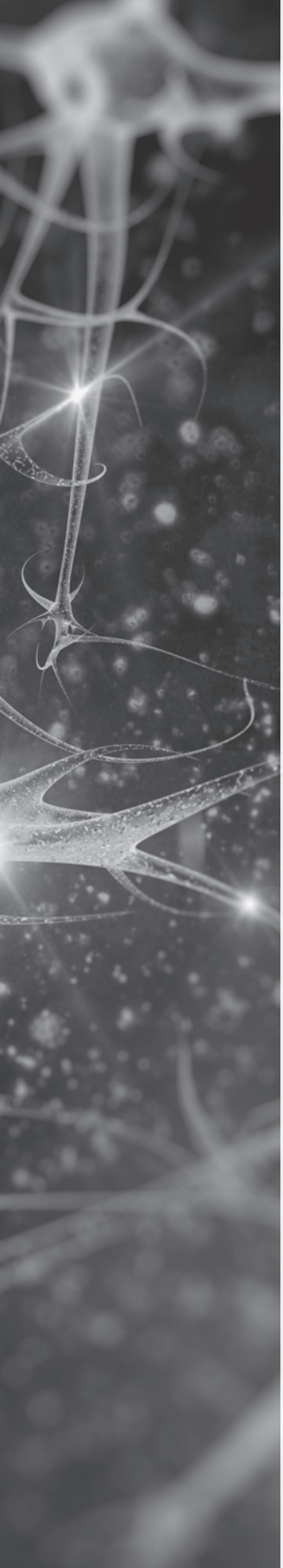
Neuronal identity and maturation



Summary

There exists a tremendous degree of diversity within the neuronal population, and even morphologically similar-looking neurons can be markedly different in function. The major aim of this doctoral dissertation was to stratify the functional heterogeneity of human stem cell-derived neurons *in vitro* by investigating the molecular features that define neuronal identity in terms of specific functional and morpho-structural properties.

CHAPTER 1 is a general introduction to this thesis, outlining the scientific background and rationale to the studies presented. Specifically, in this chapter, I introduce the concept of modeling neurological and psychiatric disease features *in vitro* using patient-derived neurons, and discuss the existence of cellular heterogeneity as a major challenge to accurately interpreting findings from research investigations which employ stem cell-derived neuronal models. I then describe how the technology of single-cell transcriptome sequencing can be used to dissect the biological heterogeneity of cell types, and obtain a detailed characterization of cellular state and function. Lastly, I detail the specific aims of the experimental work conducted, and provide an outline of the structure of the chapters of this thesis.

In **CHAPTER 2**, we provide an extensive literature review on the transcriptional and epigenetic mechanisms of somatic cell reprogramming to obtain a better understanding of the complex molecular regulation of cell identity and pluripotency induction in somatic cells. The technology of induced pluripotent stem cell (iPSC) generation is quite inefficient and research studies are only beginning to elucidate the molecular mechanisms involved. From the studies reviewed, it is clear that faithful reprogramming is greatly dependent on various technical factors. Notably, non-optimal culture conditions *in vitro* can bring about considerable variation in cell-molecular profiles that could impinge on cells' biological functionality.

In **CHAPTER 3A**, we present the first experimental evidence that *in vitro* neuronal culturing conditions may not be optimized to reflect fundamental principles of *in vivo* brain physiology. Specifically, in calcium imaging experiments, we found only very few neurons to be spontaneously active when imaged in Dulbecco's Modified Eagle's Medium (DMEM), a standard basal medium routinely used in neuronal cell culture. Yet, many cells became active when imaging in a neurophysiological solution of artificial cerebrospinal fluid (ACSF) that had the exact same inorganic salt concentration, pH and osmolarity as DMEM. Providing a first indication that various components in DMEM (acutely) interfere with neuronal function, we found that removing all of the amino acids, vitamins or extra components of DMEM avoided the basal medium-induced impairment of neuronal activity.

Further in-depth electrophysiological analyses, detailed in **CHAPTER 3B**, revealed a strong impairment in synaptic function and action potential (AP) firing of neurons in DMEM, as well as in Neurobasal (a modified version of DMEM basal), and serum. The detrimental alteration of essential electrophysiological neuronal properties in these

media stimulated us to design a new basal medium that better mimics the healthy brain's *in vivo* micro-environment and adequately supports essential physiological neural activity *in vitro*. Specifically in this new medium called BrainPhys™, we excluded or decreased the levels of neuroactive amino acids, and set the concentrations of inorganic salts and energetic substrates, as well as the osmolarity, to neurophysiological conditions. Notably, BrainPhys™ basal medium, with the appropriate supplements, supported long-term electrical activity and survival of human neurons in culture, and enhanced neuronal synaptic function. We suggest that this synaptic enhancement is due to strengthening of silent synaptic contacts, rather than to the formation of new synapses.

CHAPTER 4A describes the results of a multi-dimensional characterization of single human neurons by combined electrophysiological, morphological and transcriptome (i.e., "Patch-seq") analysis. We show that the inherent variation in basic electrophysiological profiles of human stem cell-derived neurons can be largely explained by two AP metrics (amplitude and firing rate), which together enable categorization of the neurons into five functional types that largely reflect stages of increasing neurophysiological maturity. For these functional neuronal types, we found strong correlations between their electrophysiological properties, morphological features and molecular profiles. Importantly, machine-learning strategies enabled the identification of new molecular biomarkers that efficiently predict which neurons are highly functional independently of time spent *in vitro*. As proof of concept, we selected one of the putative neurophysiological biomarkers—GDAP1L1—to effectively single out and enrich highly functional, live human neurons from heterogeneous cultures *in vitro*. This approach of predicting and studying neurophysiological cell types based on molecular signatures has higher throughput than patch clamping and may proof very useful for large-scale analyses in translational studies.

In **CHAPTER 4B**, we detail our Patch-seq pre-sequencing quality control (QC) pipeline, which we established to facilitate the identification and filtering out of poor-quality and contaminated single-neuron captures ahead of library preparation and deep sequencing. Captured neuron samples were subjected to a series of QC steps that included (i) expression profiling of common housekeeping genes (quantitative real-time PCR), (ii) fluorometric quantitation of cDNA yield (Qubit) and (iii) subjective qualitative analysis of cDNA fragment profiles (Agilent Bioanalyzer), and any sample not meeting predefined quality standards was filtered out. Our results indicated some significant correlations between these pre-sequencing QC measures and post-sequencing variables that are, at least partially, indicative of expression data quality. The presented framework thus forms a basis for better methodological designs, which may decrease variability and increase statistical power in single-cell gene expression experiments.

In **CHAPTER 5** of this dissertation, we conducted a systems-level analysis of the single-neuron transcriptome data by Weighted Gene Co-Expression Network Analysis

(WGCNA) to acquire further knowledge of the biology underlying the different functional neuron types. Our major finding is the identification of a cluster of 495 highly co-expressed genes that are strongly correlated with the functional properties and morphological features of highly functional human neurons. These genes were found to be involved in processes related to mitochondrial energy metabolism and cytoskeletal function, and Gene Set Enrichment Analysis (GSEA) indicated that they might play a general role in neuronal maturity. Another important result is the identification of a set of 21 putative transcriptional regulators of the highly functional, mature neuronal state. We suggest that these transcription factors represent promising targets for the development of novel chemical compounds that can accelerate the functional maturation of neurons.

The final chapter of this thesis, **CHAPTER 6**, critically discusses the experimental findings presented, and points out potential limitations and future avenues for investigation. Overall, the results of our studies provide a basis for biologically more relevant and accurate *in vitro* models of human neuronal cells derived from stem cells, which may facilitate the likelihood of translational success. The use of more physiological media to grow brain cells *in vitro*, and the stratification of functional neuronal heterogeneity to generate more homogeneous neuronal cultures, as detailed in this thesis, represent important means towards this end.