

NGN2 induces diverse neuron types from human pluripotency

Hsiu-Chuan Lin,^{1,2,9} Zhisong He,^{3,9} Sebastian Ebert,^{3,4,9} Maria Schörnig,⁴ Malgorzata Santel,³ Marina T. Nikolova,³ Anne Weigert,⁴ Wulf Hevers,⁴ Nael Nadif Kasri,^{5,6} Elena Taverna,^{4,8} J. Gray Camp,^{1,2,4,*} and Barbara Treutlein^{3,4,7,*}

¹Institute of Molecular and Clinical Ophthalmology Basel, Basel, Switzerland

²Department of Ophthalmology, University of Basel, Basel, Switzerland

³Department of Biosystems Science and Engineering, ETH Zürich, Basel, Switzerland

⁴Max Planck Institute for Evolutionary Anthropology, Leipzig, Germany

⁵Department of Human Genetics, Donders Institute for Brain, Cognition, and Behaviour, Radboudumc, Nijmegen, the Netherlands

⁶Department of Cognitive Neuroscience, Donders Institute for Brain, Cognition, and Behaviour, Radboudumc, Nijmegen, the Netherlands

⁷Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany

⁸Human Technopole, Milan, Italy

⁹These authors contributed equally

*Correspondence: jarrettgrayson.camp@unibas.ch (J.G.C.), barbara.treutlein@bsse.ethz.ch (B.T.)

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SUMMARY

Human neurons engineered from induced pluripotent stem cells (iPSCs) through neurogenin 2 (*NGN2*) overexpression are widely used to study neuronal differentiation mechanisms and to model neurological diseases. However, the differentiation paths and heterogeneity of emerged neurons have not been fully explored. Here, we used single-cell transcriptomics to dissect the cell states that emerge during *NGN2* overexpression across a time course from pluripotency to neuron functional maturation. We find a substantial molecular heterogeneity in the neuron types generated, with at least two populations that express genes associated with neurons of the peripheral nervous system. Neuron heterogeneity is observed across multiple iPSC clones and lines from different individuals. We find that neuron fate acquisition is sensitive to *NGN2* expression level and the duration of *NGN2*-forced expression. Our data reveal that *NGN2* dosage can regulate neuron fate acquisition, and that *NGN2*-iN heterogeneity can confound results that are sensitive to neuron type.

INTRODUCTION

Human cell types engineered from induced pluripotent stem cells (iPSCs) through transcription factor overexpression are widely used to study the mechanisms controlling cell fate differentiation, to model human diseases, and to identify potential therapies (Guo and Morris, 2017). Human neurons can be generated through the forced expression of the transcription factor neurogenin 2 (*NGN2*) with high efficiency and reproducibility (Zhang et al., 2013). These *NGN2*-induced neurons (*NGN2*-iNs) functionally mature into morphologically complex and electrophysiological active neurons after approximately 3–4 weeks of co-culture with astrocytes. The *NGN2*-iN system has been used extensively to understand neuron development and model disease (Lin et al., 2018). However, the characterization of *NGN2*-iNs so far has generally been limited to functional assays, biomarker expression, and bulk transcriptomics. There is a lack of comprehensive transcriptomic comparison with primary neuron subtypes and it is unclear whether any off-target fate emerges during the differentiation process. Single-cell sequencing methods provide powerful resolution into the heterogeneity of directed differentiation culture systems (Bidy et al., 2018, 2018, 2018; Camp et al., 2018; Karow et al., 2018). Previously, we have used single-cell mRNA sequencing (scRNA-seq) to dissect the differentiation path from mouse embryonic

fibroblasts and human pericytes to neurons and identified previously undescribed heterogeneity generated by the overexpression of the pioneer factor *ASCL1* (Karow et al., 2018; Treutlein et al., 2016). Here, we set out to characterize *NGN2*-iNeuron heterogeneity, identify the cell states that are generated during differentiation, and analyze the dynamics of the differentiation process using scRNA-seq.

RESULTS

Heterogeneity of *NGN2*-induced neurons dissected by scRNA-seq

We generated a stable iPSC line expressing *NGN2* that can be induced by doxycycline (Dox) and drives the differentiation toward iNeurons (Zhang et al., 2013). We then performed scRNA-seq (10× Genomics) at multiple time points during directed differentiation (Figure 1A). After filtering the data of astrocytes, multiplets, and cells with insufficient unique molecular identifiers (UMIs), a total of 6,764 cells (day 0 [d0], 1,412 cells; d1, 2,688 cells; d5, 524 cells; d14, 1,515 cells; d28, 625 cells) were included in the analysis. We combined all time course data and reconstructed the differentiation path of *NGN2*-iNs (Figures 1B–1D). Surprisingly, we found at least four transcriptionally distinct cell populations at the d28 time point. One population is marked by *DCN/COL5A1* and we interpret this cluster as



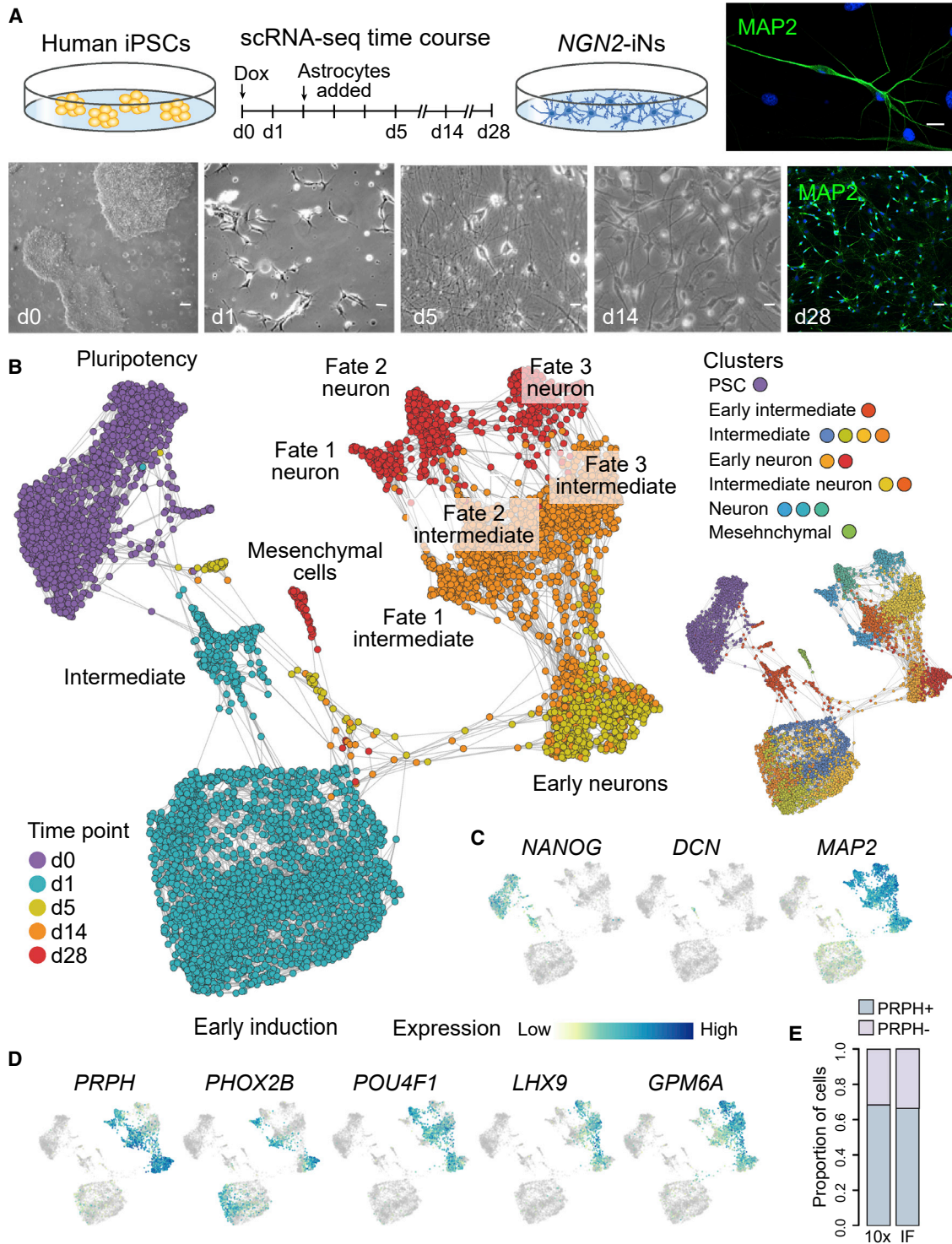


Figure 1. Diverse subpopulations emerge during NGN2-directed neuron differentiation into iNs from human iPSCs

(A) Schematic of scRNA-seq time course experiment and representative images from human iPSCs differentiating into NGN2-iNs. Cells were analyzed with scRNA-seq (10× Genomics) at multiple time points during differentiation. Immunohistochemical staining of NGN2-iNs at d28 with MAP2 (green) and DAPI (blue). Scale bars, 10 μm.

(B) SPRING embedding shows the developmental relationships of 409B2-derived NGN2-iNs with cells colored by time points (left) or cluster (right).

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an off-target mesenchymal population (Figure 1C). In addition, we observed three different neuronal clusters that express high levels of pan-neuronal genes (*MAP2*, *NCAM1*) yet are molecularly distinct (Figure 1C). Two clusters have high expression of *PRPH*, an intermediate neurofilament that is highly expressed in neurons of the peripheral nervous system and some central nervous system regions that have neural projections toward peripheral structures (Yuan et al., 2012). These two *PRPH*⁺ clusters segregate into a *PHOX2B*⁺ cluster and a *POU4F1*⁺ cluster (Figure 1D). The other neuronal cluster is marked by *GPM6A* expression, which is expressed throughout both the central nervous system (CNS) and the spinal cord during mouse development (Figure 1D) (Diez-Roux et al., 2011).

We characterized the neural identity and presence of molecular heterogeneity in our *NGN2*-iN culture using immunofluorescence of TUBB3 and *PRPH* (Figures S1A and S1B). The percentage of *PRPH*⁺ cells was quantified to compose 67% of neural cells, comparable with the percentage estimated by scRNA-seq (Figures 1E and S1B). We examined the presence of common makers that were used to characterize *NGN2*-iNs and how they overlap with *PRPH* expression (Figures S1C–S1E). Most *NGN2*-iNs and *PRPH*⁺ cells express *CUX1* and *VGlut1*, but not *GAD1/2*, supporting their cortical excitatory feature as reported previously (Zhang et al., 2013). However, unlike *PRPH* and other identified cluster markers, common neural markers are not able to resolve the heterogeneity in our dataset. We noticed that the percentage of *PRPH*⁺ cells in our dataset is higher than previous reports (Chen et al., 2020; Nickolls et al., 2020; Schörnig et al., 2021). This can be due to differences in protocol, the particular readout of neural identity used in the previous reports, or thresholds for assigning positive staining from immunohistochemistry (Figure S2). Together, these data suggest that *NGN2*-iNs generated from our protocol are comparable with other published reports of induced neurons resulting from *NGN2* overexpression, and differences in the method of readout (selected markers versus whole transcriptome) can influence the interpretations of heterogeneity.

Transcriptome trajectory analysis along the path of *NGN2*-iN development

We further analyzed *NGN2*-iN developmental trajectories after additional integration and clustering of all time course data (Figures S3A and S3B). *NGN2* induction resulted in major gene expression changes early on in programming (d0,

d1, and d5), likely driven by the immediate downstream targets of *NGN2*. Based on the observation of rapid transition from iPSCs to cells committed to a neuronal fate (Figures 1B and S3B), we hypothesized that directed differentiation bypasses early transitional states that are usually observed *in vivo* to reach neuronal states. To test the hypothesis, we ordered *NGN2*-iNs in pseudotime based on transcriptome similarities and compared the resulting trajectories with development of neurons in brain organoids (Figures S3C and S3D) (Kanton et al., 2019). We observed an escape from the early developmental stages from pluripotency directly into neural precursor stages, skipping multiple intermediate stages, including neuroectoderm and neuroepithelium induction, supporting a more direct differentiation model (Figure S3D).

To investigate the molecular events underlying the dramatic developmental changes, we identified 3,231 genes with significant expression changes along the course of *NGN2*-iN development and segregated them into six clusters with their expression peak at different stages (Figure S3E). We performed functional enrichment analysis on these differentially expressed (DE) genes and recovered gene ontology terms related to neural development (Figure S3F; Table S1). We cross-referenced DE genes with annotated transcription factors (TFs) (Hu et al., 2019) to identify potential drivers of gene expression changes during *NGN2*-iN development (Figure S1G). We focused on TFs that changed from 6 to 12 h to d1 after Dox induction and constructed a gene-regulatory network (Aibar et al., 2017), incorporating transcription factor binding site prediction in promoters with TF-target co-expression (Figure S3H). *NGN2* was predicted to connect with some TFs with the highest centrality in the constructed regulatory network, including *POU5F1*, *HES6*, and *SOX11*, supporting its role in driving direct reprogramming from iPSCs to induced neurons.

While neural induction begins before d1, the *NGN2*-iN heterogeneity emerges later as the expression of *NGN2*-iN subtype markers were detected after d1 (pseudotime Pt ~0.4) (Figures S3B and S3I). Interestingly, we found that *PHOX2B* and *POU4F1* had divergent expression from the beginning of their activation, while *POU4F1* and *GPM6A* bifurcated later at d5 (pseudotime Pt ~0.75) (Figures S3J and S3K). *PRPH*, on the other hand, was detected only after the *PHOX2B* and *POU4F1* bifurcation, suggesting that it was activated independently in *PHOX2B*- and *POU4F1*-expressing cells (Figure S3I).

(C) Expression feature plots stem cell, mesenchymal, and neural marker genes.

(D) Expression feature plots of *NGN2*-iN clusters.

(E) Proportion of *PRPH*⁺ and *PRPH*[–] cells quantified using scRNA-seq (10×) or immunofluorescence.

See also Figures S1A and S1B.

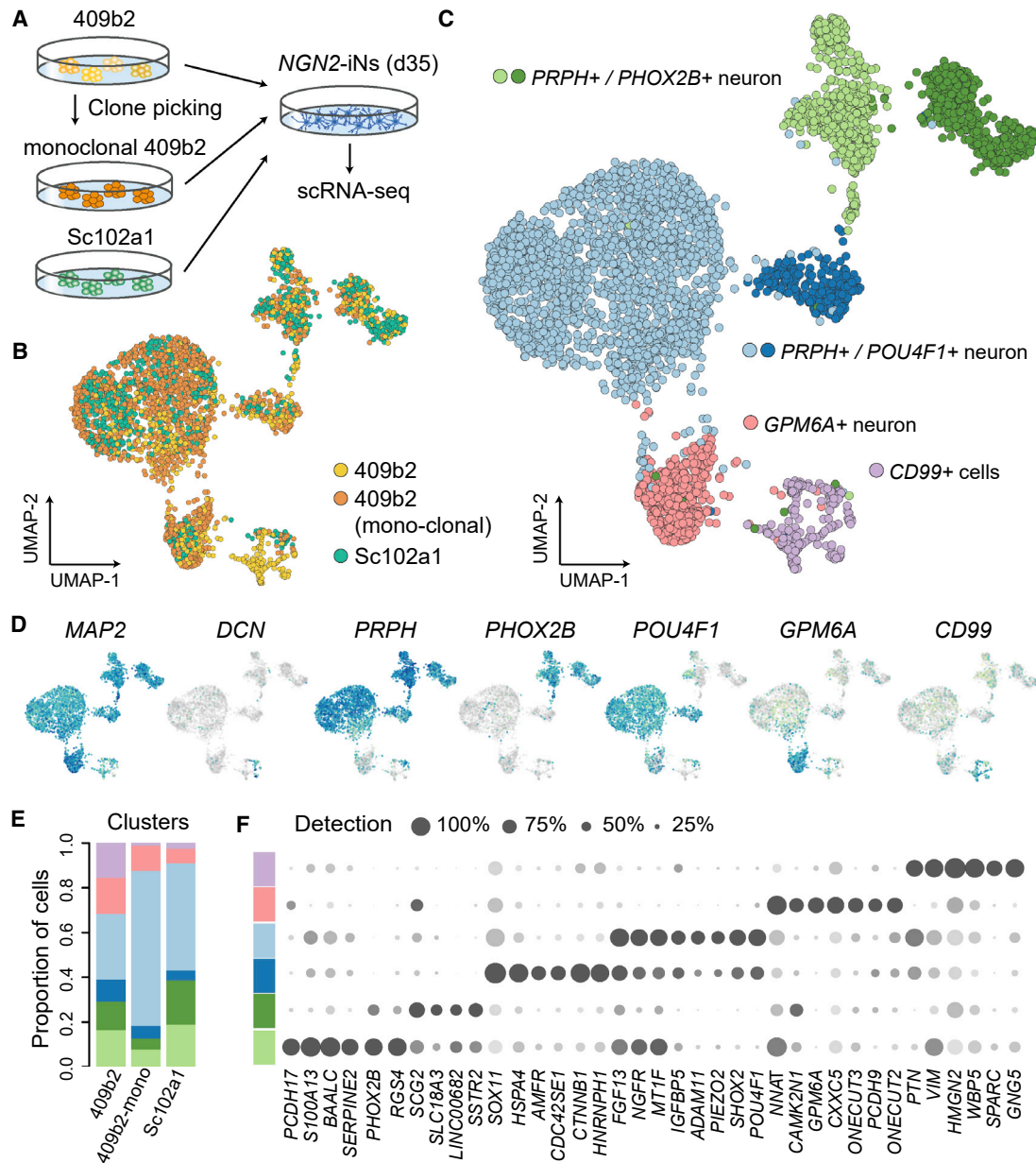


Figure 2. *NGN2*-iN neuron diversity is recapitulated in multiple iPSC lines

(A–C) (A) scRNA-seq was performed on d35 *NGN2*-iNs from polyclonal 409B2, monoclonal 409B2, and polyclonal Sc102a1 iPSCs. (B and C) UMAP embedding of Seurat 3.0 integrated scRNA-seq data, with cells colored by cell source (B) or cluster annotated by marker genes (C). (D) Feature plots showing the expression of marker genes. (E) Stacked bar plot showing proportions of clusters in each sample. (F) Dot plot of marker gene expression patterns and detection rates across clusters.

NGN2-iN heterogeneity is commonly detected

We next determined if the *NGN2*-iN heterogeneity results from heterogeneous iPSC populations used to induce iNs, or if the heterogeneity was specific to the particular iPSC line. We established a single iPSC clone (409B2 monoclonal) from the parent 409B2 line and additionally gener-

ated a polyclonal *NGN2*-inducible line from another individual (Sc102a1). We induced these lines and analyzed the resulting transcriptomes at d35 of differentiation (Figure 2A). We found that significant heterogeneity is still observed in an integrated analysis of the scRNA-seq data, with six molecularly distinct clusters containing cells

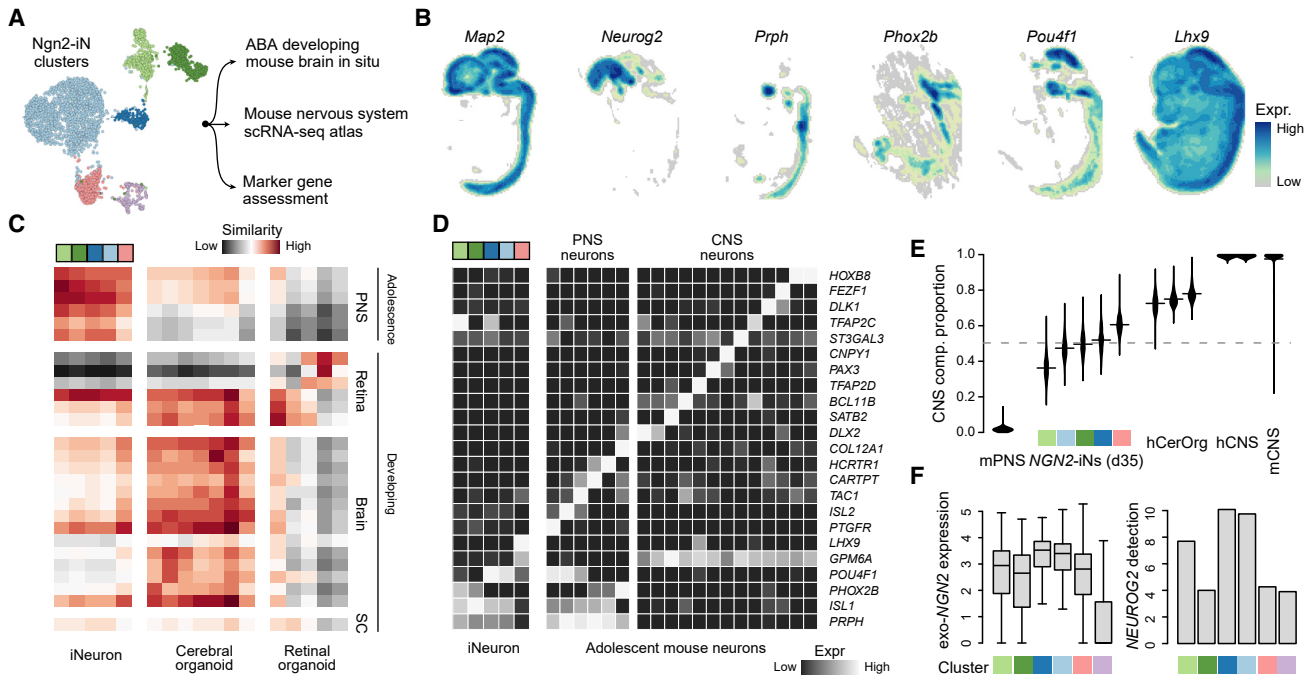


Figure 3. Molecular signatures of *NGN2*-iN compared with primary neuronal cell types mouse reference atlases

- (A) *NGN2*-iN subpopulation signatures were compared with diverse reference atlases.
- (B) Spatial expression patterns of selected markers as maximum intensity projections across sagittal sections in the embryonic day 13.5 mouse brain from the Allen Developing Mouse Brain Atlas.
- (C) Transcriptomic similarities between *NGN2*-iNs, other iPSC-derived neurons, and primary neurons represented as Pearson correlations between expression profiles. SC, spinal cord.
- (D) Average expression of various marker genes of primary neuron subtypes in *NGN2*-iN clusters and primary mouse PNS and CNS neuron subtypes.
- (E) Proportions of the estimated CNS component in *NGN2*-iNs, cerebral organoid neurons, and human/mouse primary mature PNS/CNS neurons.
- (F) Expression of exogenous (left) and endogenous (right) *NGN2* in different *NGN2*-iN clusters. The boxes show the lower and upper quartiles of the distributions. the bars extend to the min/max or 1.5x interquartile range.

from each of the starting iPSC lines. We note that the three main neural subtypes and off-target cells are all observed for each line (Figures 2B–2E). We searched for the top DE genes in the six clusters, and observed distinct gene expression patterns for each cluster (Figure 2F; Table S2). These data suggest that *NGN2*-based neural reprogramming is intrinsically heterogeneous, independent of the purity of the starting cell population, and that the heterogeneity is detected in neurons from multiple iPSC lines.

Molecular features of *NGN2*-iN subpopulations

We next analyzed the molecular signatures that distinguished each *NGN2*-iN cluster. We compared the signatures with *in situ* hybridization (Ravasi et al., 2010) data from the Allen Developing Mouse Brain Atlas, and with single-cell transcriptome atlases containing primary neural cells (Figure 3A). We also assessed the expression of neurotransmitters and other markers of neuron specialization. Based on

whole-embryo mouse ISH data (Thompson et al., 2014), we find that *Ngn2* is expressed in progenitor zones in the developing telencephalon and many other brain structures (Figures 3B and S4). *Prph* is expressed in the neural retina, trigeminal nerve, and nuclei within the gray horn of the spinal cord. *Phox2b* is expressed in rhombencephalon/brain stem neurons as well as neurons in the peripheral nervous system (PNS). *Pou4f1* is expressed in the retina, mesencephalon derivatives, trigeminal nerve, and gray horn nuclei. We next compared each *NGN2*-iN cluster with PNS and CNS neurons from primary reference cell atlases (Clark et al., 2019; La Manno et al., 2020; Zeisel et al., 2018) (Figure 3C). Unlike neurons in the iPSC-derived cerebral and retinal organoids, the *NGN2*-iN clusters did not show specific transcriptomic similarity to any CNS neuron subtypes (Figures 3C and S4D). Some *NGN2*-iN clusters were relatively similar to PNS neurons, especially the *PRPH*+ clusters, although they did not specify any of the

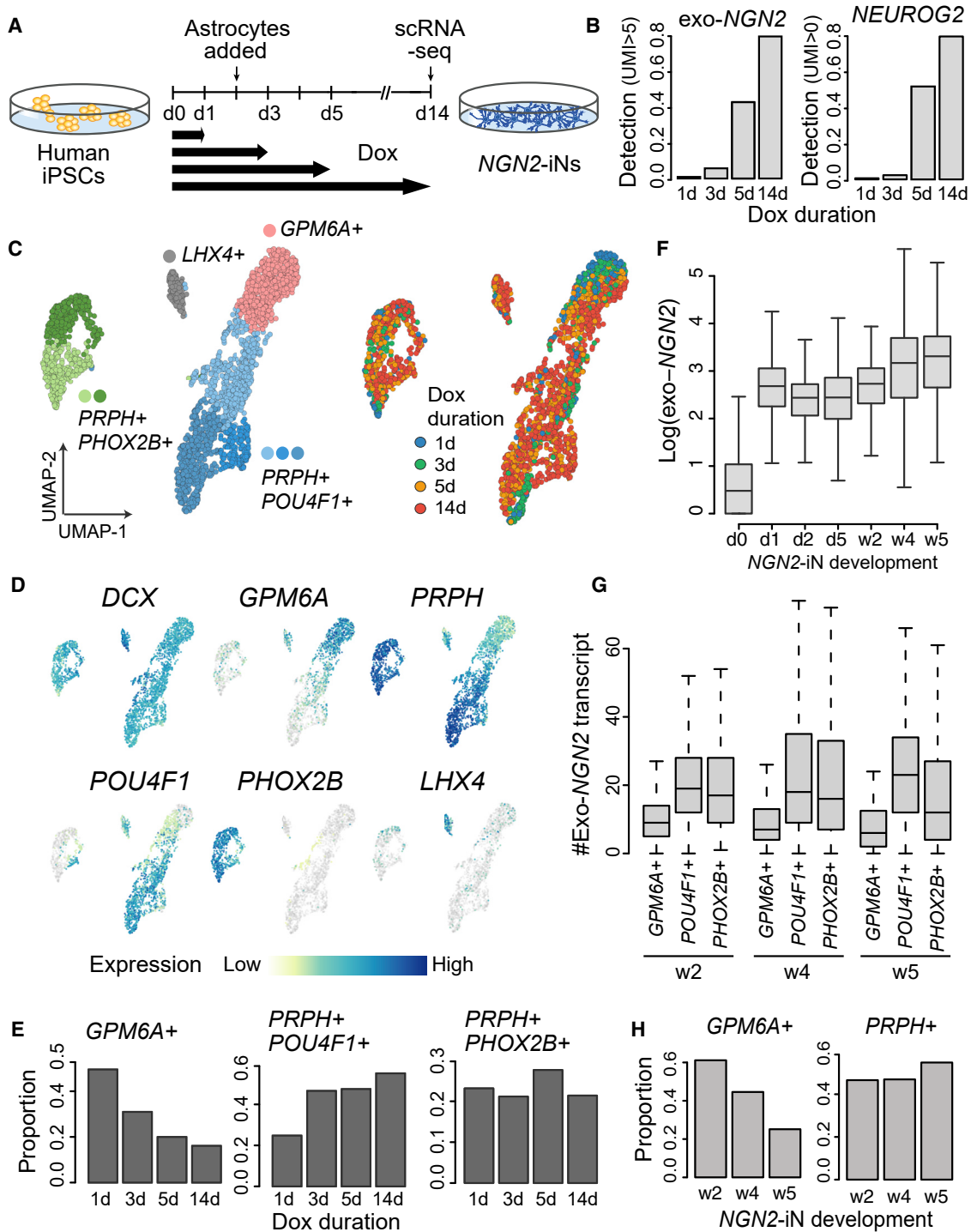


Figure 4. Neuronal fate specification is sensitive to *NGN2* dosage

(A) Schematic for the doxycycline (Dox) treatment duration experiment.

(B) Detection rates for cells expressing exogenous *NGN2* (>5 UMI) and endogenous *NGN2* (>0 UMI) from each sample.

(C) UMAP embedding of *NGN2*-iN cells from each sample. scRNA-seq data were integrated using cluster similarity spectrum-based integration (He et al., 2020).

(D) UMAP plots colored by marker gene expression. *DCX* marks the neural lineages. *LHX4* marks the identity of the off-target cluster.

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PNS neuron subtypes. We explored if *NGN2*-iN expressed markers of primary neuron subtypes (Figures 3D and S4E). There is no clear *in vivo* neuron population as the counterpart of any *NGN2*-iN cluster. We deconvoluted the ratio of PNS/CNS identity for each cluster and found that all *NGN2*-iNs have mixed signatures of CNS- and PNS-derived neurons without a clearly established identity (Figure 3E). We find that the *GPM6A*⁺ cluster has more CNS features while the *PRPH*⁺ clusters have a biased PNS signature, in line with *GPM6A* and *PRPH* showing high expression in CNS and PNS neurons, respectively. Altogether, our data suggested that *NGN2*-iNs have a mixture of neuronal signatures, and we were not able to establish a clear identity of *NGN2*-iN populations. We note that this lack of *in vivo* counterpart could be due to incomplete reference cell atlases, as well as discrepancies between human and mouse neurons.

The expression level of reprogramming factors could affect the outcome of reprogramming (Sommer et al., 2012) and lead in part to the heterogeneity of *NGN2*-iNs. We thus analyzed the relationship between *NGN2* expression and the molecular identity of corresponding *NGN2*-iNs. The *NGN2* expression level and proportion of *NGN2*-expressing cells was indeed lower in the off-target cluster (*CD99*⁺), in line with previous studies that failed reprogramming is linked to silenced reprogramming factors (Treutlein et al., 2016) (Figure 3F). Among the successfully reprogrammed neural clusters, we observed variable expression levels of *NGN2* and proportion of *NGN2*-expressing cells, prompting us to examine whether *NGN2* dosage affects the *NGN2*-iN reprogramming heterogeneity.

Duration of *NGN2* induction affects *NGN2*-iN subtype configuration

We manipulated *NGN2* expression by shortening the duration of Dox treatment and analyzed the resulting cells at d14 using scRNA-seq (Figure 4A). A total of 2,767 cells (d1, 311 cells; d3, 378 cells; d5, 727 cells; d14, 1,351 cells) were included in the analysis. As expected, the expression level of exogenous *NGN2* is correlated with the duration of Dox treatment (Figure 4B). Endogenous *NGN2* expression is also positively correlated with Dox treatment duration, likely as a result of positive autoregulation (Figure 4B) (Ejarque et al., 2013). Each of the previously identified major clusters were detected in all samples; however, the duration of Dox treatment affects the proportion of samples among

each cluster (Figures 4C–4E). Specifically, we found that the *GPM6A*⁺ population was enriched in samples with shorter Dox treatment, while the *PRPH*⁺/*POU4F1*⁺ population was more abundant in samples with increased Dox treatment (Figure 4E). Given the observation that *NGN2* expression could affect *NGN2*-iN fate specification, we revisited the time course data for *NGN2*-iN development. The expression of *NGN2* remained nearly consistent during *NGN2*-iN development, with a slight increase of *NGN2* expression at weeks 4 and 5 (Figures 4F and S3B). Interestingly, the expression level of *NGN2* is consistently lower in *GPM6A*⁺ cells, independent of the duration of development (Figure 4G). Supporting this data, the proportion of *GPM6A*⁺ cells also decreased with longer *NGN2*-iN culture (Figure 4H). This could imply that prolonged expression of *NGN2* potentially steers *NGN2*-iN fate away from CNS lineages. Altogether, these data show that the duration of *NGN2* induction impacts the proportion of neuron subtypes that emerge in this single factor reprogramming paradigm.

DISCUSSION

Cell fate engineering of neural subtypes from human iPSCs using defined TFs provides extraordinary new inroads into disease modeling and therapy screening using human cells. Methods to rapidly generate mature human neurons are exciting and transformative for these endeavors. It has been established that the *NGN2*-iN protocol is able to reprogram stem cells to general neural fates, with less heterogeneity and higher consistency across multiple stem cell lines compared with traditional reprogramming strategies mediated by small-molecule inhibitors/activators. However, our analysis suggests that the emergent neuron population is heterogeneous, with the heterogeneity being consistent across different cell lines. We are unable to assign the neuron populations to a particular identity with high confidence. We note that this may be due to the fact that current single-cell and spatial transcriptome reference atlases are incomplete. However, without a specific matrix and guiding molecules it may be expected that neurons are not able to establish the molecular profile observed *in vivo* with high precision. Our data show that multiple *NGN2*-iN subpopulations are more similar to neurons of the PNS than CNS, and it is unclear if this culture paradigm is indicative of CNS functionality. Modifications of the *NGN2*-iN

(E) Proportion of cells per Dox treatment time point in each of the three neural clusters.

(F) Expression level of exogenous *NGN2* from the time course experiment presented in Figure S1A. The boxes show the lower and upper quartiles of the distributions. the bars extend to the min/max or 1.5x interquartile range.

(G) Numbers of exogenous *NGN2* transcripts in the three neural clusters from different time points of *NGN2*-iN development. The boxes show the lower and upper quartiles of the distributions. the bars extend to the min/max or 1.5x interquartile range.

(H) Proportion of cells from different time points of *NGN2*-iN development.



protocol by adding developmental patterning factors to the culture medium can steer neuron differentiation to a desired path (Nehme et al., 2018). Our data support a continued effort into identifying combinatorial transcription factor overexpression systems (Ravasi et al., 2010) and medium conditions that can support precise neuron cell type engineering. Furthermore, comprehensive human nervous system reference cell atlases are required to understand the identity of cell states that emerge in *in-vitro*-engineered neuron systems. Single-cell genomics and comparisons with high-dimensional reference atlases should become a field gold standard to assess the heterogeneity and precision of *in vitro* engineered neurons.

EXPERIMENTAL AND COMPUTATIONAL PROCEDURES

Cell culture

All cells described in this work were incubated at 37°C, 5% CO₂, and 90% humidity unless otherwise stated. 409B2 (RIKEN BRC Cell Bank), Sc102a1 (System Biosciences) stem cells, and corresponding rtTA/NGN2-derivatives were cultured in standard feeder-free conditions in mTeSR1 (STEMCELL Technologies) on plates coated with Matrigel (Corning). Primary cortical rat astrocytes (Gibco) were cultured in high-glucose DMEM containing 10% fetal calf serum and 1% pen/strep on plates coated with poly-D-lysine (Sigma-Aldrich). Fresh medium was added to the astrocytes every 4–5 days and passaged once a week with trypsin-EDTA digestion at a standard ratio of 1:2. Astrocytes were used up to passage 10, with passage 0 being the culture of initial isolation. rtTA/NGN2 double-positive stem cell lines were generated and differentiated into NGN2-iNs as described previously (Frega et al., 2017).

scRNA-seq library preparation and sequencing

To prepare scRNA-seq libraries from NGN2-iN single-cell suspensions, Chromium Single-cell 3' Reagent Kits (10× Genomics, Pleasanton, CA, USA) were applied according to the manufacturer's instructions. The Chromium Single-cell 3' Reagent Kits v.2 was employed on NGN2-iN generated from 409B2 time course experiments, monoclonal 409B2 and Sc102a1 iPSCs with approximately 3,000 cells loaded per lane on a 10x microfluidic chip device. Chromium Single-cell 3' Reagent Kit v.3 was used on Dox treatment duration experiments with nearly 8,000 cells loaded per lane. Quantification and quality control of the 10× library was carried out on a Bioanalyzer (Agilent) using high-sensitivity DNA chips. Libraries prepared from the 10× v.2 kit and v.3 kit were respectively sequenced on the Illumina HiSeq 2500 and Illumina NovaSeq S1 platform.

Data analysis of the scRNA-seq experiments

Cell Ranger was used to demultiplex raw base call files to FASTQ files, align reads to the reference genome and transcriptome with the default alignment parameters, demultiplex human and mouse cells, and generate the count matrices for the human cells. Seurat (v.3.1) was then applied to the human scRNA-seq data for further preprocessing. The scRNA-seq data of all 409b2 cells from iPSCs to NGN2-iN was integrated with cluster similarity spectrum (CSS) (He et al., 2020). The scRNA-seq data of NGN2-iN cells at day 35 of samples from the 409b2 and Sc102a1 human iPSC lines was integrated with Seurat. Generation of UMAP embeddings, clustering, and pseudotime analysis was done on the integrated spaces. Marker genes of different NGN2-iN populations were identified as genes with BH corrected $p < 0.01$ and expression fold change >1.2 . The benchmark of NGN2-iN populations was done by comparisons with the adolescent mouse nervous system atlas (Zeisel et al., 2018), the developing mouse brain (La Manno et al., 2020), and the developing mouse retina (Clark et al., 2019). The NGN2-iN scRNA-seq data with varied Dox treatment durations was processed similarly and integrated with CSS. Details of the computational analysis are described in the supplemental experimental procedures.

Data and code availability

The accession number for the processed scRNA-seq data and computational codes reported in this paper is ArrayExpress E-MTAB-10632.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.stemcr.2021.07.006>.

AUTHOR CONTRIBUTIONS

M. Schörnig established the NGN2 iPSC lines. M. Schörnig and S.E. generated iN cultures with assistance from A.W. S.E. and W.H. established the selective single-cell dissociation. S.E. generated the time course and d35 scRNA-seq NGN2-iN scRNA-seq data with support from M. Schörnig, M. Santel, and W.H. M. Schörnig, H.-C.L., and M.T.N., with support from S.E. and A.W., generated IHC data. N.N.K. provided guidance to establish the NGN2 iPSC lines. E.T. provided guidance for IHC and iN culture. H.C.L. generated the NGN2 induction time course scRNA-seq data with support from S.E. and M. Santel. Z.H., S.E., and H.C.L. analyzed the scRNA-seq data. H.C.L., Z.H., S.E., B.T., and J.G.C. designed the study and wrote the manuscript.

CONFLICT OF INTERESTS

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