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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

☐	☒	The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided <i>Only common tests should be described solely by name; describe more complex techniques in the Methods section.</i>
☐	☒	A description of all covariates tested
☐	☒	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted <i>Give P values as exact values whenever suitable.</i>
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒	Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Policy information about [availability of computer code](#)

Zeiss LSM 710
ZEN 2010
BD LSR II flow cytometer and FACSDiva
API 4000 tandem mass spectrometer
7900HT Real Time PCR System
Axion BioSystems Maestro Pro
Illumina NextSeq 550
NovaSeq 6000

FLOWJO software
ImageJ
Analyst software (v1.6.2)
Sequence Detection System software (v2.4)
Matlab R2012a
Axion BioSystems Neural Metrics and Plotting Tool software
GraphPad Prism 9
10x Genomics Cell Ranger
Seurat (version 4.0.2) toolkit in R (version 4.0.1)
DoubletFinder (version 2.0.3)

STAR aligner
FeatureCounts (Subread v1.6.3)
DESeq2 in R (v4.3.2)

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Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

Single nuclei RNA sequencing data in this study have been deposited in Gene Expression Omnibus under accession code GSE313106.

Bulk RNA sequencing data in this study have been deposited in EBI biostudies <https://www.ebi.ac.uk/biostudies/arrayexpress/studies/E-MTAB-16232>.

Research involving human participants, their data, or biological material

Policy information about studies with [human participants or human data](#). See also policy information about [sex, gender \(identity/presentation\), and sexual orientation](#) and [race, ethnicity and racism](#).

Reporting on sex and gender

Reporting on race, ethnicity, or other socially relevant groupings

Population characteristics

Recruitment

Ethics oversight

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

Data exclusions

Replication

Randomization

Blinding

Behavioural & social sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	Not applicable
Research sample	Not applicable
Sampling strategy	Not applicable
Data collection	Not applicable
Timing	Not applicable
Data exclusions	Not applicable
Non-participation	Not applicable
Randomization	Not applicable

Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	Not applicable
Research sample	Not applicable
Sampling strategy	Not applicable
Data collection	Not applicable
Timing and spatial scale	Not applicable
Data exclusions	Not applicable
Reproducibility	Not applicable
Randomization	Not applicable
Blinding	Not applicable

Did the study involve field work? ☐ Yes ☒ No

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	anti-ACTN2, Sigma, A7811 anti-BRN3A, Merck, MAB1585
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anti-CTNT, Abcam, ab45392
 anti-CD45, Abcam, Ab8216
 anti-CD68, Synaptic Systems, HS-460-008
 anti-DBH, Sigma, HPA070789
 anti-HCN4, Abcam, ab69054
 anti-Neurexin 1, Sigma, HPA071400
 anti-NF (H), Synaptic Systems, 171 106
 anti-NF (H), Biolegend, 822601
 anti-PAX6, Biolegend, 901301
 anti-PDGFRb, Cell Signalling, 28E1
 anti-PECAM1, DAKO, M0823
 anti-Peripherin, NOVUS, NBP1-05423
 anti-Phalloidin, Invitrogen, A12379
 anti-PHOX2B, Santa Cruz, sc-376997
 anti-Synapsin 1, Synaptic Systems, 106 011
 anti-TH, Synaptic Systems, 213 004
 anti-VACHT, Synaptic Systems, 139 103
 anti-VGLUT1, NanoTag, N1602-Abb635P
 anti-chicken Alexa Fluor 488, Invitrogen, A10039
 anti-rabbit Alexa Fluor 546, Invitrogen, A11035
 anti-mouse Alexa Fluor 546, Invitrogen, A11074
 anti-guinea pig Alexa Fluor 633, Invitrogen, A21052

Validation

All antibodies used were validated by the respective manufacturer. Antibody specificity were assessed with a positive and negative control.

Eukaryotic cell lines

Policy information about [cell lines and Sex and Gender in Research](#)

Cell line source(s)

Human induced pluripotent stem cell lines:
 RUCDRi002-A: Male, obtained from Institute of Pharmacology and Toxicology, University Medical Center Goettingen.
 RUCDRi002-A-71: Male, obtained from Institute of Pharmacology and Toxicology, University Medical Center Goettingen.
 UMGi093-A: Male, obtained from Lukas Cyganek (Stem Cell Unit, University Medical Center Goettingen).
 BIHi001-A-2: Male, obtained from Sebastian Diecke (Max Delbrück Center, Berlin).
 MDCi052_A26: Male, generated by Sebastian Diecke (Max Delbrück Center, Berlin).
 MDCi052_A27: Male, generated by Sebastian Diecke (Max Delbrück Center, Berlin).
 MDCi052_A30: Male, generated by Sebastian Diecke (Max Delbrück Center, Berlin).
 MDCi052_A31: Male, generated by Sebastian Diecke (Max Delbrück Center, Berlin).

Authentication

Cell lines that are well-established:
 RUCDRi002-A: <https://hpscreg.eu/cell-line/RUCDRi002-A>
 RUCDRi002-A-71: <https://www.sciencedirect.com/science/article/pii/S1873506124000151?via%3Dihub>
 UMGi093-A: <https://sfb1002.med.uni-goettingen.de/production/cellmodel/cell-line/view?tab=internal&line=125>
 BIHi001-A-2: https://www.cellosaurus.org/CVCL_CONJ

Cell lines established in the paper:
 MDCi052_A26: Sub-clone of RUCDRi002-A, mutation was validated by PCR and Sanger Sequencing. Pluripotency analysis and 3 germ layer differentiation were performed.
 MDCi052_A27: Sub-clone of RUCDRi002-A, mutation was validated by PCR and Sanger Sequencing. Pluripotency analysis and 3 germ layer differentiation were performed.
 MDCi052_A30: Sub-clone of RUCDRi002-A, mutation was validated by PCR and Sanger Sequencing. Pluripotency analysis and 3 germ layer differentiation were performed.
 MDCi052_A31: Sub-clone of RUCDRi002-A, mutation was validated by PCR and Sanger Sequencing. Pluripotency analysis and 3 germ layer differentiation were performed.

Mycoplasma contamination

All cell lines were routinely checked for mycoplasma contaminations and were tested negative.

Commonly misidentified lines
(See [ICLAC](#) register)

No misidentified lines have been used in this study.

Plants

Seed stocks	Not applicable
Novel plant genotypes	Not applicable
Authentication	Not applicable

Flow Cytometry

Plots

Confirm that:

- ☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- ☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- ☒ All plots are contour plots with outliers or pseudocolor plots.
- ☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	SNOs were dissociated into single cells using Collagenase Type I and Accutase dissociation reagent. Digested cells were fixed in 4% formaldehyde at room temperature for 15 min and stained for FACS. iEHMs were lysed with EZ prep nuclei lysis buffer and the pellet was resuspended in Nuclei Suspension buffer. Lysate was strained through 40 uM Flowmi Cell Strainers into FACS tube and stained. Cardiomyocytes were dissociated into single cells using Accutase dissociation reagent. Digested cells were fixed in cold 70% ethanol. Cells were subsequently stained for FACS.
Instrument	Cells were measured with BD LSR II Flow cytometer or BD FACSaria III.
Software	Data were analyzed with FACSDiva and FLOWJO.
Cell population abundance	Abundance of cell population is determined as a % of the parent population gating (gated after FSC/SSC plot and Hoechst-A/SSC-A). Isotype control were used to distinguish between the negative and positive signals.
Gating strategy	Cells were gated by the FSC/SSC plot and further gated by Hoechst-A/SSC-A or 7-AAD/SSC-A signal. Isotype control were used to distinguish between negative and positive signals.
☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.	

Innervated human cardiac muscle model reveals sympathetic drivers of KCNH2-associated arrhythmias

Corresponding Author: Dr Maria Patapia Zafeiriou

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I appreciate the authors clarifications to the points raised in my previous assessment.

The newly provided single-cell data indicate that the neuronal compartment of iEHM is heterogeneous, with noradrenergic neurons representing only a minority and a substantial fraction of cholinergic or mixed-identity neurons. This raises an important question about how the reported high noradrenaline and low acetylcholine levels should be interpreted, and whether the disease phenotype in KCNH2 mutant tissues can be attributed specifically to sympathetic neurons.

The pharmacological data are consistent with an autonomic contribution and may reflect partly sympathetic signaling. Atropine removes direct muscarinic parasympathetic effector effects, but it does not exclusively isolate noradrenergic signaling, because nicotine still activates nicotinic receptors in both sympathetic and parasympathetic ganglia. Therefore, these experiments do not conclusively establish that the phenotype is driven by a pure noradrenergic neuronal population, particularly in light of the single-cell data showing mixed neuronal identities. As a result, the mechanistic basis of the pro-arrhythmic phenotype remains less specific than implied.

Reviewer #2

(Remarks to the Author)

I believe the authors have thoroughly responded to all prior comments. The revisions meaningfully address my prior concerns, and I support publication in the journal pending minor clarifications.

The expanded snRNA-seq analysis in Supplementary Figure 10, with HOX gene-based lineage assignment and three biological replicates, now provides sufficient characterization of the neuronal component within the fused iEHM. The dual normalization strategy for bulk RNA-seq and the revised Discussion distinguishing nicotine-induced neuronal activation from direct β -adrenergic stimulation also resolve my previous concerns. The framing of the iEHM as a tractable multicellular model rather than a faithful replica of native myocardium is appropriately calibrated.

Remaining minor points:

The characterization of Cluster 4 as transitional based on TH/CHAT/GATA3/ISL1 co-expression is plausible; the authors should briefly note whether this cluster's relative abundance was consistent across biological replicates, as variability would be relevant for interpreting the functional data.

The cardiomyocyte cluster heterogeneity acknowledged in Supplementary Figure 11B warrants a brief prospective comment in the Discussion on whether extended culture or subtype-directed differentiation could address this in future iterations of the model.

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Point by Point response to the reviewers

We thank the reviewers for their time, insightful suggestions, and constructive criticism, which we believe have substantially strengthened this work.

Reviewer #1 (Remarks to the Author):

Schneider and colleagues present a 3D human innervated engineered heart muscle (iEHM) by fusing a sympathetic neuronal organoid (SNO) with engineered heart muscle (EHM) and apply this system to LQT2/KCNH2 disease modeling. The work is technically sound, and the data volume is substantial. The authors have made a serious effort to address the previous review, but the incremental novelty over existing approaches remains limited. Specifically,

1. As stated in my previous review, a number of groups have previously reported the generation of sympathetic neurons and their characteristics including most recently PMID 41386226. But what remains unclear in all these studies is a robust demonstration of physiological relevance including developmental origin. While the authors state that they made trunk neural crest-derived sympathetic neurons, they do not provide any proof. Moreover, given the heterogeneity in SNOs, which contain sensory, glutamatergic, and cholinergic neurons besides sympathetic neurons, it is important to demonstrate cell type composition of the progenitors as well as the end population. This has impact for disease modeling as the authors are evaluating a mixed population which may impact outcomes.

We thank the reviewer for this important comment. We agree that the developmental origin and cellular heterogeneity of the neuronal component are highly relevant for interpreting the physiological relevance of the model and its use in disease modeling.

Our SNO differentiation protocol was adapted from the previously established 2D protocol by Oh et al. (2016), which reported robust HOX gene expression consistent with trunk neural crest identity. In the original submission, we relied on this developmental framework and did not present this rationale with sufficient clarity. We have now clarified this point in the revised manuscript (Pg12, L29-37).

In addition, to address the reviewer's concern regarding the neuronal populations present in the disease model actually analyzed in this study, we have added new single-nucleus RNA-seq data from the iEHM in Supplementary Figure 10. These data provide a more detailed characterization of the neuronal populations present after fusion, including autonomic, cholinergic, noradrenergic, and sensory-like components (Pg6, L13-21, Pg12, L29-37).

We agree that direct profiling of the SNO progenitor population would provide complementary information. Because the present study is centered on the fused iEHM model and its functional consequences, we prioritized characterization of the final integrated system. We now explicitly acknowledge this in the revised Discussion (Pg11, L20-22).

To better position our work relative to recently published systems (PMID 41386226), we have also clarified the aspects in which our model extends beyond prior reports, including the vascularized and innervated 3D architecture, the ability to assess contractile performance in addition to beating rate, the application to KCNH2-associated arrhythmia modeling, and the observation that sympathetic innervation cannot be fully mimicked by acute β -adrenergic stimulation alone (Pg11, L10-16).

2. There is no data presented on the single nucleus sequencing of neurons except a UMAP in Figure 1D and some violin plots of ion channel genes in Supplementary Figure 10, which represents all neurons and not just sympathetic neurons. For transparency, and to address the issues mentioned above, the authors should provide single cell/nucleus gene expression profiles of SNOs and give an account of the cell composition.

We thank the reviewer for this helpful suggestion. We agree that a more transparent and unbiased account of neuronal composition is important.

To address this point, we have expanded the snRNA-seq analysis and now include data from three biological replicates, which allowed more robust resolution of neuronal subclusters in the iEHM. These new data are presented in Supplementary Figure 10 and identify multiple neuronal populations, including noradrenergic, cholinergic, sensory-like (glutamatergic), and additional autonomic neural crest-related clusters. We also include marker-based interpretation of these clusters together with HOX gene expression patterns to better define their likely identities.

We fully acknowledge that this analysis was performed on the fused iEHM rather than on isolated SNOs. We did not perform single-cell or single-nucleus profiling on SNOs alone in the present study, and we now state this explicitly in the revised manuscript (Pg11, L20-22). Because our main objective was to define the cellular composition of the final innervated cardiac model used for functional experiments, we focused our additional analysis on the iEHM.

Regarding the reviewer's question about the proportion of neurons that can be classified as sympathetic, our revised text now distinguishes more clearly between autonomic neuronal populations and the subset with noradrenergic features, while also acknowledging that some clusters remain incompletely resolved (Pg6, L13-21, Pg12, L29-37).

For example, based on Figure 1, there are an estimated 50 – 60% PHOX2B positive cells. This includes sympathetic neurons as well as any potential parasympathetic or cholinergic neurons. So, what is the % of neurons that can be classified as truly sympathetic? The authors should provide differential gene expression analysis of all clusters obtained from SNOs. They show selected marker expression by qPCR in supplemental figure 4A. Why can't this be done in a more unbiased way using their single cell data?

Specifically, the new data in Supplementary Figure 10 show that eight weeks after fusion, the iEHM contains peripheral autonomic identities in clusters 1, 4, 6, and 7, marked by *PRPH*, *CHRN3*, *PHOX2A/B*, and *ASCL1*, as well as non-autonomic identities in clusters 2, 3, and 5. Cluster 6 expresses *PHOX2B*, *TH*, and *SLC18A2* and is therefore most

consistent with a noradrenergic population, whereas cluster 1 expresses *CHAT* and represents a cholinergic component. Cluster 4 expresses both *TH* and *CHAT* together with *GATA3* and *ISL1*, suggesting a more immature population of mixed or transitional identity.

Moreover, *HOX* gene expression showed that *PHOX2A/B* marked the autonomic lineage (Clusters 1, 4, 6, 7), *HOXA3*, *HOXB3* defined vagal neural crest identity (Clusters 1, 4, 7) and *HOXB5*, *HOXD8* indicated a more posterior trunk neural crest identity (Cluster 6).

Such an analysis would also resolve whether cholinergic neurons are pre-ganglionic sympathetic or rather parasympathetic arising from cardiac neural crest cells.

The co-expression of *PRPH*, *CHRNA3*, *PHOX2A*, *KCNQ5*, and *CHAT* is most consistent with peripheral cholinergic autonomic neurons and therefore suggests a postganglionic identity. This data is now introduced in Supplementary figure 10.

3. For comparing SNOs generated in this study, the authors differentiated neurons as reported in Kirino et al. I do appreciate this but as the authors acknowledge themselves, this was done ad hoc through collaboration with an external party, which is not a neuronal differentiation group. The authors should consider comparing the RNA sequencing profiles of sympathetic neurons generated in different studies in order to better position their approach and also provide a transparent account of how the different approaches weigh against each other. I acknowledge that not all studies have performed this analysis but there are a few studies mentioned in Supplementary table 1 have done this.

We thank the reviewer for this thoughtful suggestion. We agree that cross-study comparison of transcriptomic profiles from stem cell-derived sympathetic neuron protocols would be valuable for positioning the present approach within the field.

We have not performed a systematic comparative reanalysis of published RNA-seq datasets across studies in the current revision. Our aim in including the 2D comparison was to provide additional context requested during review rather than to establish a comprehensive benchmark across all available differentiation strategies.

To address the spirit of the reviewer's comment, we have strengthened the discussion of how our model differs from previously published systems, including its integrated vascularized and innervated 3D architecture, its ability to assess contractile performance and arrhythmogenesis, and its application to *KCNH2*-associated disease modeling. We have also added text acknowledging that a systematic transcriptomic benchmarking analysis across studies would be an important future direction (Pg11, L18-19).

4. The cardiomyocyte identities in EHM remain confusing. There appears to be similar expression of ventricular markers *IRX4*, *MYL2*, and *MYH7* in atrial/pacemaker-like and the two ventricular clusters (Supplementary Figure 11B). It does appear that the atrial/pacemaker-like cluster expresses more *HCN4*, *SHOX2* but taken with the point above, it is still very mixed and indicates a suboptimal differentiation approach. The authors should check whether this is a line-specific issue or a protocol-dependent issue.

We thank the reviewer for raising this important point. We agree that the mixed identity of iPSC-derived cardiomyocyte populations requires careful interpretation. In our dataset,

the atrial/pacemaker-like cluster shows increased expression of nodal and atrial-associated regulators, including *HCN4* and *SHOX2*, together with relatively lower *MYL2* and *IRX4* expression compared with the ventricular clusters, supporting its classification despite residual overlap. At the same time, we agree that the overall population remains heterogeneous. We have not performed additional experiments in this revision to formally distinguish whether this heterogeneity is line-specific or primarily protocol-dependent. We now state this explicitly and discuss it as a limitation (Pg6, L25-28). We also note in the revised manuscript that mixed atrial-, ventricular-, and pacemaker-like populations are commonly observed with standard Wnt-modulation-based differentiation protocols, suggesting that the phenomenon is likely at least in part protocol-related (Galdos et al. 2023 eLife, Nakanishi-Koakutsu et al. 2024 Comm Biol).

The heterogeneous expression patterns detected among scRNA-seq cardiomyocyte clusters may, at least in part, arise from asynchronous maturation, in line with the EHM spontaneous beating observed at earlier culture stages that progressively declines and eventually stops. This represents a particularly interesting point that should be addressed systematically in future studies.

5. The title "Bioengineering of a human innervated cardiac muscle model" implies a novel engineering approach to create innervated cardiac tissue. Since the focus of the study is not on engineering itself but rather on functional integration and disease modeling, the title could be modified to reflect that.

We have now revised the title of our manuscript to: Innervated human cardiac muscle model reveals sympathetic drivers of *KCNH2*-associated arrhythmias.

Reviewer #1 (Remarks on code availability): I have not reviewed the code.

Reviewer #2 (Remarks to the Author):

The authors have comprehensively addressed most of my concerns with thoughtful revisions and additional analyses. I remain excited about the potential impact of their findings on cardiomyocyte maturation within the co-cultured 3D system. This aspect holds significant implications for advancing our knowledge in cardiac research. However, I would like to highlight a couple of points that still require clarification.

Firstly, I appreciate the authors' efforts to address my concerns, particularly their analysis of cardiomyocyte maturation and the detailed description of the EHM formation protocol. These enhancements significantly contribute to understanding their study and the complexities of the neuro-cardiac interface.

We thank the reviewer for these encouraging comments and for recognizing the additional analyses and clarifications included in the revision.

The authors' approach to bulk RNA-seq analysis and the normalization of certain gene expressions, such as for CasQ2, appears somewhat unconventional. Further explanation of their rationale behind this normalization method would be beneficial for understanding the implications of their findings.

We thank the reviewer for requesting clarification of this point. In the original analysis, we normalized selected expression data to a cardiomyocyte-enriched reference gene because our intention was to assess changes relative to the cardiomyocyte compartment rather than to total mixed-tissue input. We chose this reference based on our prior work, in which it showed stable expression in cardiac tissue.

We agree, however, that this normalization strategy may not have been sufficiently explained and could appear unconventional without additional context. To improve transparency, we now provide the corresponding data normalized to a conventional housekeeping gene as well, and we clarify the rationale for both normalization approaches in the revised Methods and Results (Pg33, L12-16).

Additionally, I noted that the authors have not provided a thorough explanation for the observed reduction in heart rate (HR) variability following nicotine addition. Given that isoproterenol is expected to stimulate heart rate increases and reduce variability, clarity on the mechanisms in their model that led to these observations is crucial for a comprehensive interpretation of their results.

We thank the reviewer for highlighting this point. We realize that our original presentation may not have made this aspect sufficiently clear.

In our dataset (Figure 5), nicotine increased heart rate variability rather than reducing it. We have now revised the text to state this more clearly and to better distinguish the effects of nicotine-induced neuronal activation from those of direct β -adrenergic stimulation with isoproterenol.

In the revised Discussion, we further clarify our interpretation that these differing responses may reflect the release of additional neuron-derived factors beyond noradrenaline, including candidates such as NPY, which may modulate the

arrhythmogenic phenotype in ways not reproduced by isoproterenol alone (Pg13, L20-33). If the reviewer was referring to another specific panel or analysis, we hope that the revised presentation resolves the ambiguity.

Moreover, a comment regarding the composition of their iEHM system is warranted. While the inclusion of neural and cardiac components is intriguing, the relevance and consistency of this composition with in vivo heart conditions remain uncertain. A discussion around the implications of these cell types and their interactions within the context of cardiac physiology should be included to provide readers with a clearer understanding of the potential limitations of the model.

We thank the reviewer for this important comment. We agree that the cellular composition of the iEHM and its relationship to the in vivo heart should be discussed more explicitly, particularly with respect to both the strengths and the limitations of the model.

We have therefore added text to the revised Discussion clarifying that the model is not intended to fully recapitulate the exact cellular composition of native human myocardium. Rather, it is designed as a tractable human multicellular system to study neuro-cardiac interactions in a controlled setting. We also now discuss more explicitly how the presence of neural, vascular, mural, and cardiac populations may influence interpretation of the observed phenotypes (Pg11, L22-30).

Finally, although the authors introduced additional marker analyses for neuronal subtypes, the completeness of their profiling and characterization of cholinergic neuron populations seems insufficient. While they acknowledge the limitations in distinguishing among sympathetic and parasympathetic neurons, a more detailed exploration of these neuronal subtypes would strengthen the foundation of their findings.

We thank the reviewer for this constructive suggestion. To strengthen the characterization of neuronal subtypes, we now include expanded snRNA-seq analysis of the neuronal component in the iEHM in Supplementary Figure 10.

These data identify multiple neuronal subclusters, including populations with cholinergic, noradrenergic, sensory-like, glutamatergic, and autonomic neural crest-related signatures. We have revised the manuscript to describe these clusters more clearly and to explain how marker combinations and HOX gene expression informed our interpretation (Pg6, L13-21, Pg12, L29-37).

Specifically, the new data in Supplementary Figure 10 show that eight weeks after fusion, the iEHM contains peripheral autonomic identities in clusters 1, 4, 6, and 7, marked by *PRPH*, *CHRN3*, *PHOX2A/B*, and *ASCL1*, as well as non-autonomic identities in clusters 2, 3, and 5. Cluster 6 expresses *PHOX2B*, *TH*, and *SLC18A2* and is therefore most consistent with a noradrenergic population, whereas cluster 1 expresses *CHAT* and represents a cholinergic component. Cluster 4 expresses both *TH* and *CHAT* together

with *GATA3* and *ISL1*, suggesting a more immature population of mixed or transitional identity.

Moreover, HOX gene analysis showed that *PHOX2A/B* expression marked the autonomic lineage (Clusters 1, 4, 6, 7), *HOXA3*, *HOXB3* defined vagal neural crest identity (Clusters 1, 4, 7) and *HOXB5*, *HOXD8* indicated a more posterior trunk neural crest identity (Clusters 4, 6).

Point by Point rebuttal letter

We thank the reviewers for their time, insightful suggestions, and constructive criticism, which we believe have substantially strengthened this work.

Reviewer #1 (Remarks to the Author):

I appreciate the authors clarifications to the points raised in my previous assessment.

The newly provided single-cell data indicate that the neuronal compartment of iEHM is heterogeneous, with noradrenergic neurons representing only a minority and a substantial fraction of cholinergic or mixed-identity neurons. This raises an important question about how the reported high noradrenaline and low acetylcholine levels should be interpreted, and whether the disease phenotype in KCNH2 mutant tissues can be attributed specifically to sympathetic neurons.

The pharmacological data are consistent with an autonomic contribution and may reflect partly sympathetic signaling. Atropine removes direct muscarinic parasympathetic effector effects, but it does not exclusively isolate noradrenergic signaling, because nicotine still activates nicotinic receptors in both sympathetic and parasympathetic ganglia. Therefore, these experiments do not conclusively establish that the phenotype is driven by a pure noradrenergic neuronal population, particularly in light of the single-cell data showing mixed neuronal identities. As a result, the mechanistic basis of the pro-arrhythmic phenotype remains less specific than implied.

We appreciate the reviewer's concern. Although the noradrenaline measurements were performed in SNO at day 40, not in iEHM, where the neurons are at approximately day 90 we would like to underline that innervation cannot be assessed solely by cell number, since a single sympathetic neuron may innervate multiple cardiomyocytes, as also indicated by the immunofluorescence shown in Figure 2. In line with this point, the functional data obtained in the presence of atropine showed only a minor contribution of endogenous acetylcholine to iEHM beating rate (Figure 3g). Moreover, because the effect on beating rate was abolished by beta blockers, it is unlikely that other neurotransmitters played a substantial role in beating rate rather than noradrenaline. Last but not least, optogenetic stimulation of all neurons was performed in the absence of atropine (Figure 3e). In these experiments, the beating rate increased again in most tissues, consistent with a stronger influence of sympathetic neurons rather than parasympathetic.

For KCNH2 modeling our data support that noradrenaline is not the main contributor for the recorded pro-arrhythmic phenotype. Although beta adrenoreceptor blockade reduced iEHM beating rate also in the mutant tissues, it clearly did not alleviate the beating rate variability. We added in the discussion that other neuropeptides as for example NPY may be responsible for this phenotype. Defining the driver of arrhythmia in KCNH2 is a very interesting question which we plan to address in a future study employing this model. We have now changed the wording in the discussion that

addresses that the neurotransmitter responsible is not known and it should be investigated highlighted in green (Page 13).

Reviewer #2 (Remarks to the Author):

I believe the authors have thoroughly responded to all prior comments. The revisions meaningfully address my prior concerns, and I support publication in the journal pending minor clarifications.

The expanded snRNA-seq analysis in Supplementary Figure 10, with HOX gene-based lineage assignment and three biological replicates, now provides sufficient characterization of the neuronal component within the fused iEHM. The dual normalization strategy for bulk RNA-seq and the revised Discussion distinguishing nicotine-induced neuronal activation from direct β -adrenergic stimulation also resolve my previous concerns. The framing of the iEHM as a tractable multicellular model rather than a faithful replica of native myocardium is appropriately calibrated.

Remaining minor points:

The characterization of Cluster 4 as transitional based on TH/CHAT/GATA3/ISL1 co-expression is plausible; the authors should briefly note whether this cluster's relative abundance was consistent across biological replicates, as variability would be relevant for interpreting the functional data.

We thank the reviewer for raising this point. We now added a brief note about the relative abundance of this population across the samples on pg 6 (Highlighted in green)

The cardiomyocyte cluster heterogeneity acknowledged in Supplementary Figure 11B warrants a brief prospective comment in the Discussion on whether extended culture or subtype-directed differentiation could address this in future iterations of the model.

We thank the reviewer for this comment. We now added a small paragraph on the discussion that addresses the CM heterogeneity in the discussion on pg 11 (Highlighted in green).

Title: Supplementary Movie 1.

Description: Spontaneous beating of iEHM 1 week after fusion

Title: Supplementary Movie 2.

Description: Representative immunofluorescence picture depicting neurofilament positive axons (green) interlacing alpha-actinin positive CMs (red) in iEHM

Title: Supplementary Movie 3.

Description: Representative immunofluorescence picture 1 depicting synapsin positive pre-synaptic terminals (red) of noradrenergic neurons (TH, blue) in between CMs (cTNT, green) in 8 week-old iEHM.

Title: Supplementary Movie4.

Description: Representative immunofluorescence picture 2 depicting synapsin positive pre-synaptic terminals (red) of noradrenergic neurons (TH, blue) in between CMs (cTNT, green)) in 8 week-old iEHM.

Title: Supplementary Movie5.

Description: Representative immunofluorescence picture 3 depicting synapsin positive pre-synaptic terminals (red) of noradrenergic neurons (TH, blue) in between CMs (cTNT, green)) in 8 week-old iEHM.

Title: Supplementary Movie6.

Description: Representative immunofluorescence picture 4 depicting synapsin positive pre-synaptic terminals (red) of noradrenergic neurons (TH, blue) in between CMs (cTNT, green)) in 8 week-old iEHM.

Title: Supplementary Movie7.

Description: Representative immunofluorescence picture 5 depicting synapsin positive pre-synaptic terminals (red) of noradrenergic neurons (TH, blue) in between CMs (cTNT, green) in 8 week-old iEHM.

Title: Supplementary Movie8.

Description: Representative immunofluorescence picture 6 depicting synapsin positive pre-synaptic terminals (red) of noradrenergic neurons (TH, blue) in between CMs (cTNT, green) in 8 week-old iEHM.

Title: Supplementary Movie9.

Description: Representative immunofluorescence picture 1 depicting PECAM1 positive vascular network (red) surrounded by PDGFRb positive pericytes (cyan) in close proximity to neurons (NF, green) in 8 week-old iEHM.

Title: Supplementary Movie10.

Description: Representative immunofluorescence picture 2 depicting PECAM1 positive vascular network (red) surrounded by PDGFRb positive pericytes (cyan) in close proximity to neurons (NF, green) in 8 week-old iEHM.

Title: Supplementary Movie11.

Description: Representative immunofluorescence picture 3 depicting PECAM1 positive vascular network (red) surrounded by PDGFRb positive pericytes (cyan) in close proximity to neurons (NF, green) in 8 week-old iEHM.

Title: Supplementary Movie12.

Description: Chronotropic response of EHM and optogenetic iEHM evoked by light stimulation.

Supplementary Information

Innervated human cardiac muscle model reveals sympathetic drivers of *KCNH2*-associated arrhythmias

#Lennart Valentin Schneider^{1,2,3}, #Michael Gani Setya^{1,2,3}, Guobin Bao¹, Daniel Härtter^{1,2}, Aditi Methi⁴, Dennis Krüger⁴, Marie Kristin Schreiber^{1,3}, Ole Jensen⁵, Elisavet Kanari¹, Kea Aline Schmoll^{1,3}, Narasimha Swamy Telugu⁶, Sadman Sakib⁴, Eun Seo Kim¹, Aminath Luveysa Fahud¹, Maham Novin^{1,2}, Aylin Seedorf^{1,2,3,7}, Sebastian Diecke⁶, André Fischer^{3,4}, Norman Y. Liaw^{1,2}, Wolfram-Hubertus Zimmermann^{1,2,3,4,8,9,10}, *Maria-Patapia Zafeiriou^{1,2,3,9,11,12,13}

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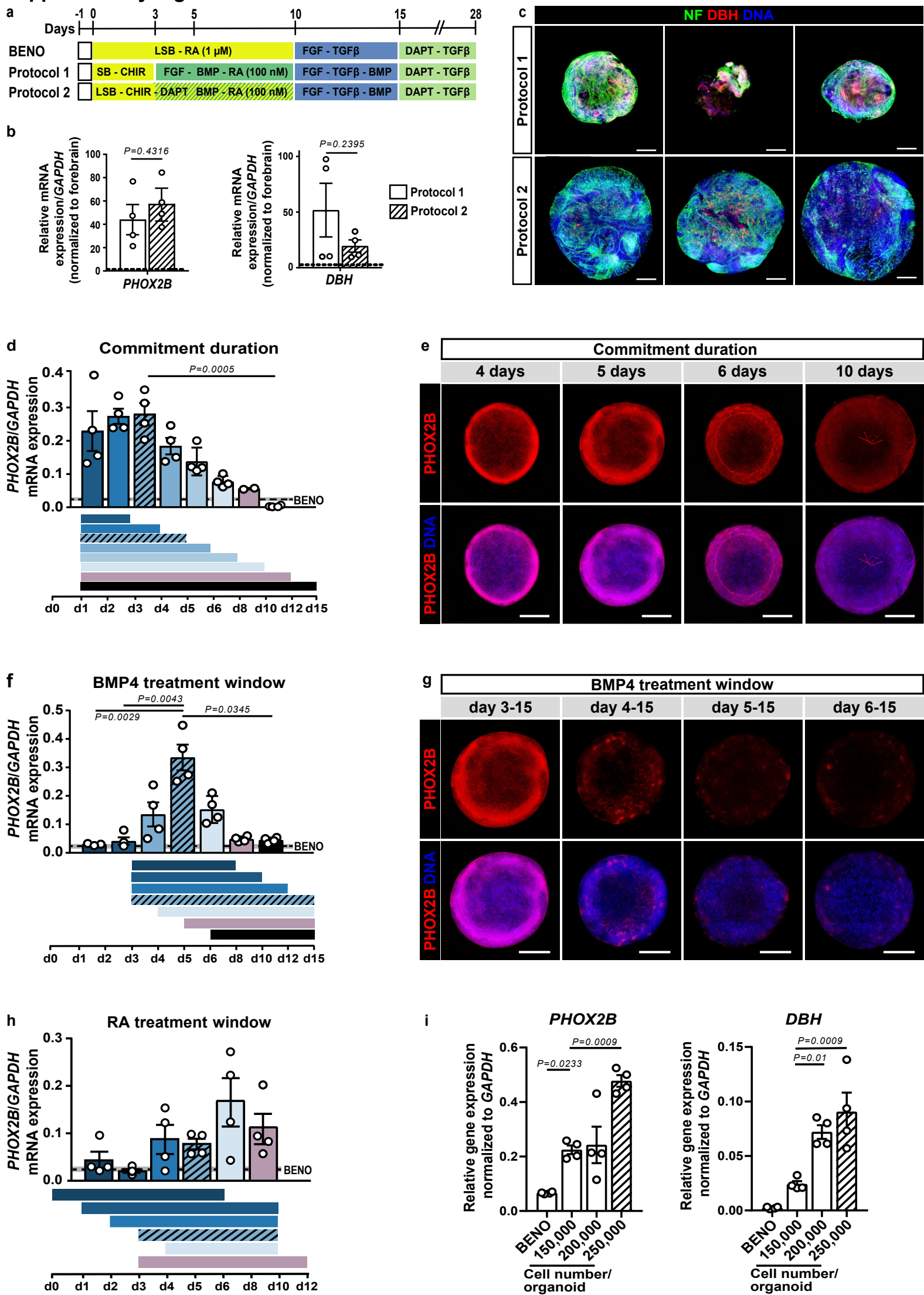
¹⁰ Campus-Institute Data Science (CIDAS), Göttingen, Germany

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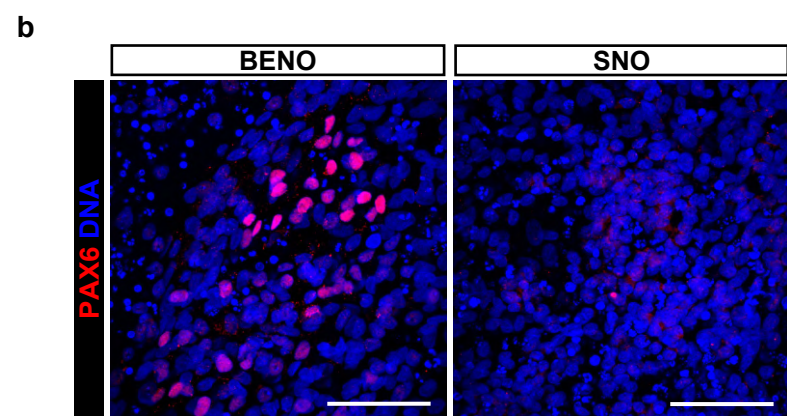
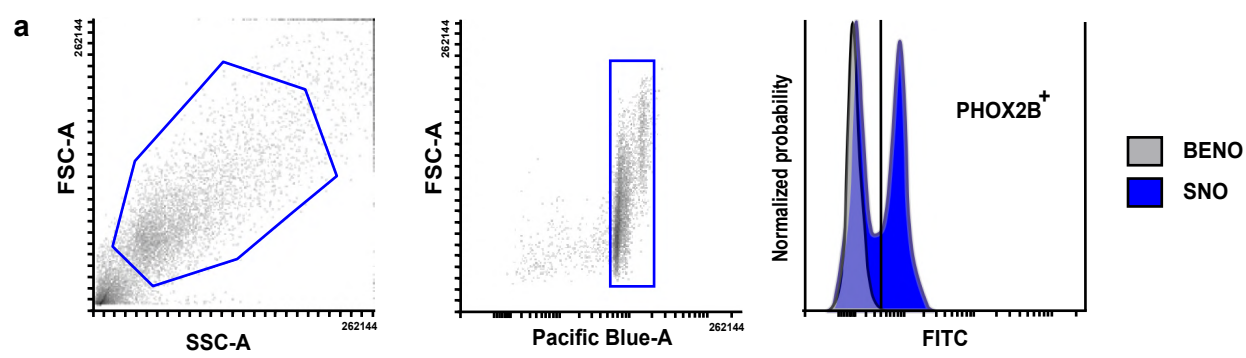
¹³ Collaborative Research Center 1690, University of Göttingen, Göttingen, Germany

Supplementary Figure 1



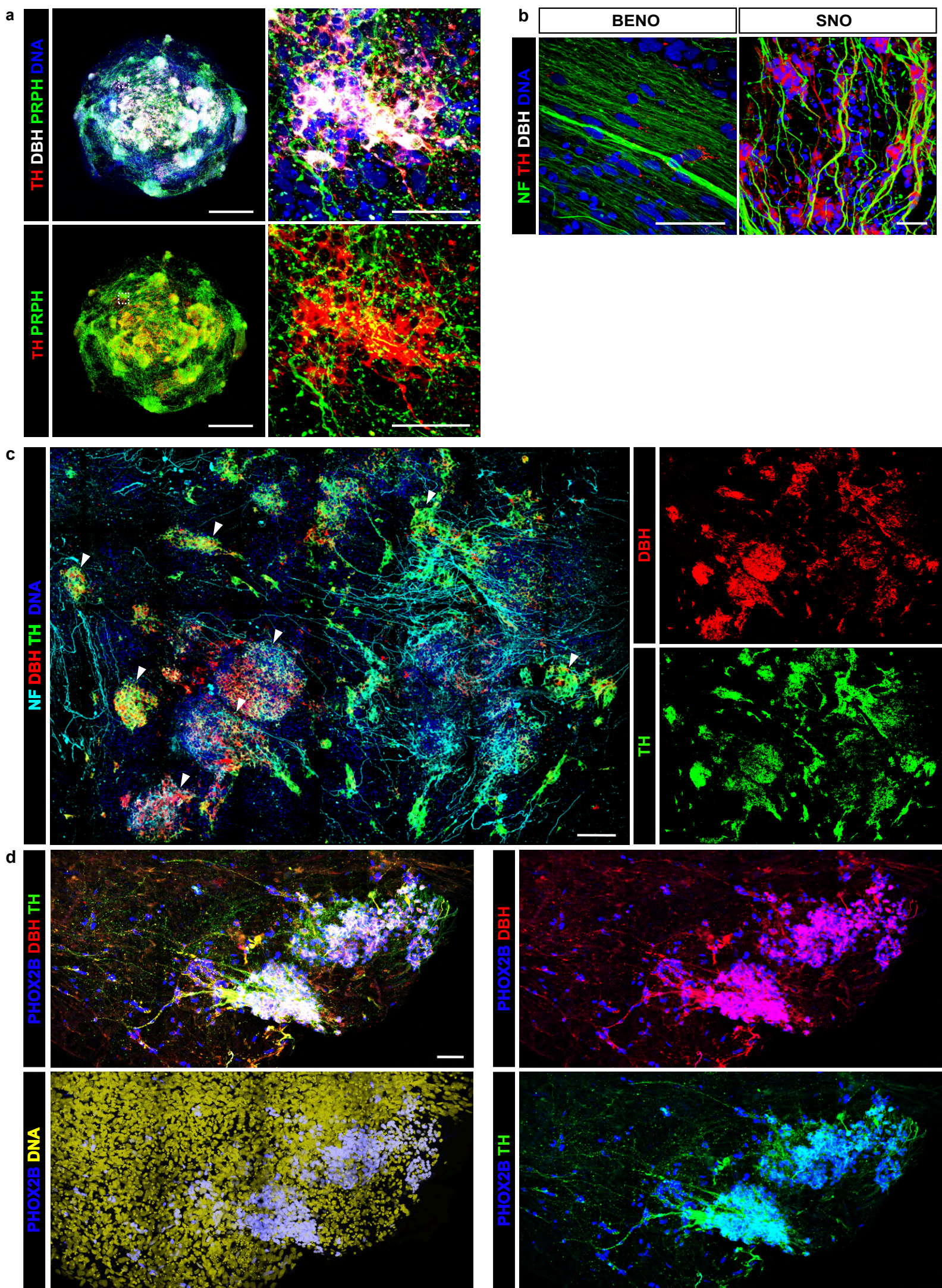
Supplementary Figure 1| SNO protocol optimization. **a** Schematic of two SN-differentiation protocols adapted from 2D-protocols (Kirino et al., 2018, Oh et al., 2016) to the BENO system. **b** *PHOX2B* (d15) and *DBH* (d30) expression in SNOs from both protocols, normalized to *GAPDH* and a forebrain organoid control (dashed line). 1 differentiation, n=4. **c** WmIF of d30 SNO stained for sympathetic marker DBH (red), neural marker NF (green) and DNA (blue). Scale bar, 500 μ m. **d** *PHOX2B* expression in d15 SNO with varying commitment durations based on protocol 2 (d1-10, see a), normalized to *GAPDH*. Treatment time windows are represented below with matching bar colors. 1 differentiation, n=4 (Except d1-d12 n=2). **e** WmIF of d15 SNOs stained for PHOX2B (red) and DNA comparing different commitment durations (10 days, see a). Scale bar, 500 μ m. **f** *PHOX2B* expression with varying BMP4 treatment windows, normalized to *GAPDH* (d3-10, see a). 1 differentiation, n=4 (Except d3-d8 n=3). **g** WmIF of d15 SNO stained for PHOX2B (red) and DNA comparing different BMP4 timing (starting d3, see a). Scale bar, 500 μ m. **h** *PHOX2B* expression SNO (d15) using varying RA treatment windows, normalized to *GAPDH* (d1-10, see a). 1 differentiation, n=4. **i** *PHOX2B* (d15) and *DBH* (d30) expression in SNO using different casting cell number, normalized to *GAPDH* (standard condition was 150,000 cells). 1 differentiation, n=4. Hashed bars indicate the new optimal condition. Data shown as mean $\pm$ s.e.m. with individual data points. One-way ANOVA or Kruskal-Wallis test with Dunn's multiple comparisons post hoc test was used for (**d**, **f**, **h**, **i**). Two-tailed unpaired Student's t-test or Mann-Whitney test was used for (**b**).

Supplementary Figure 2



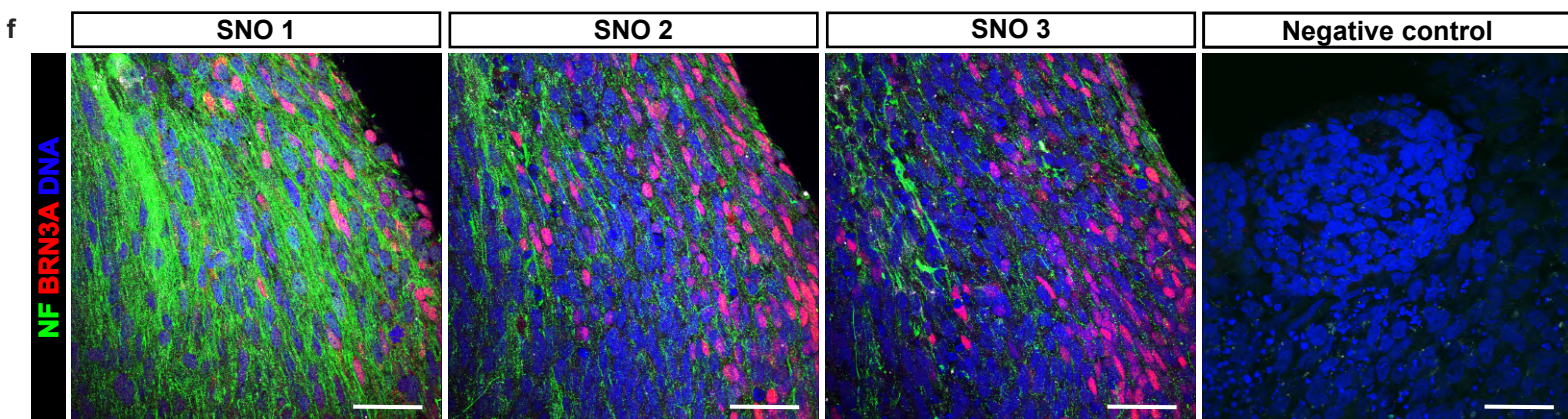
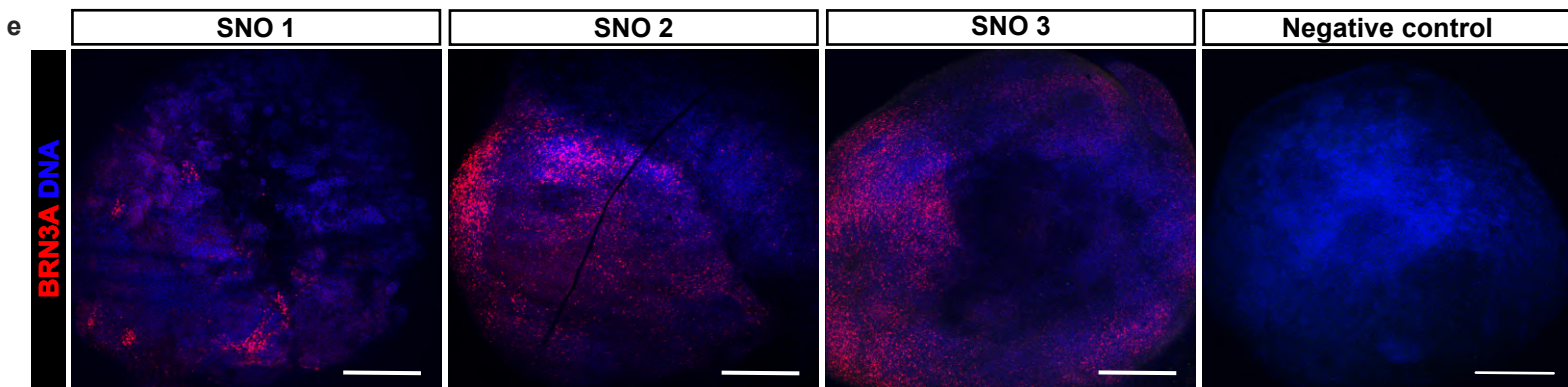
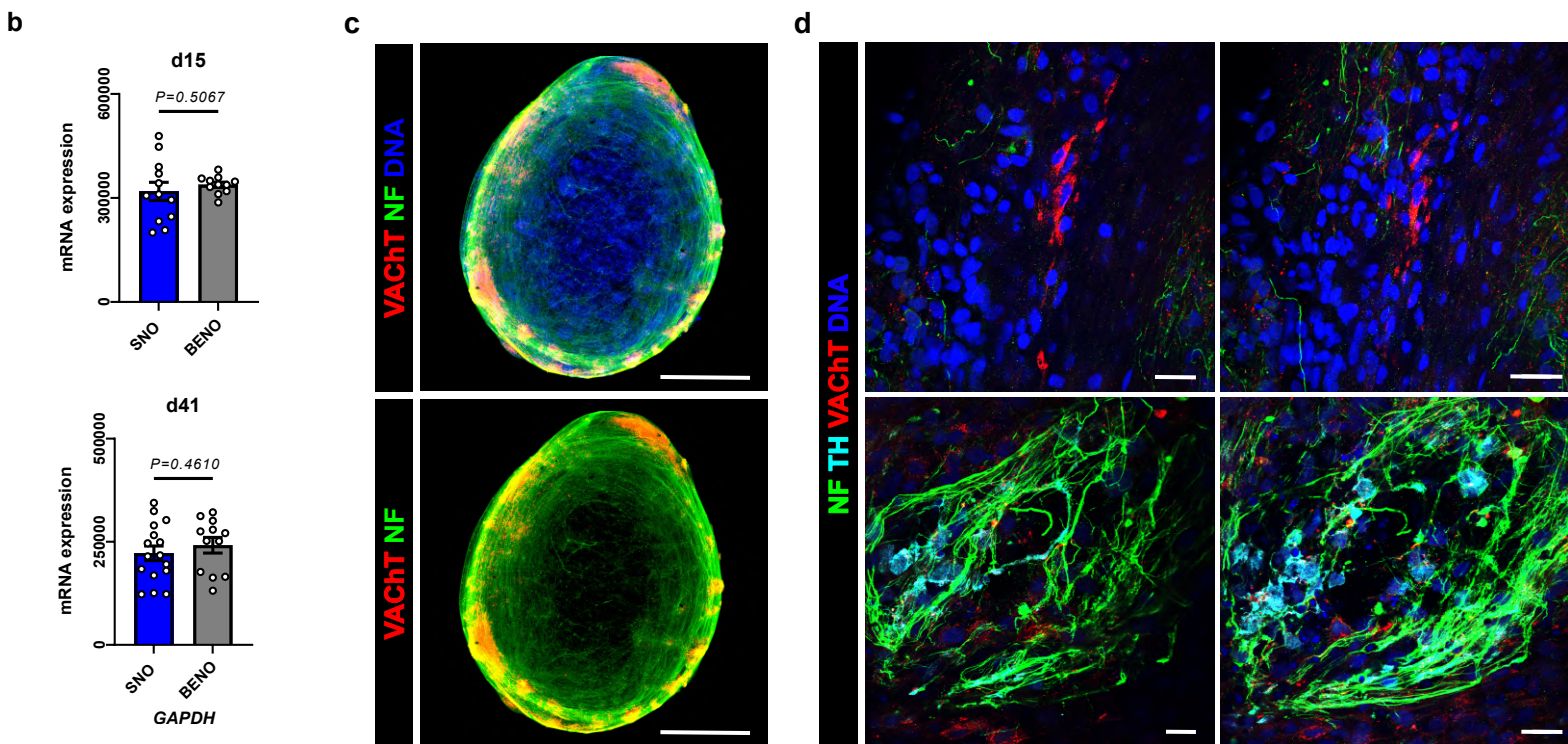
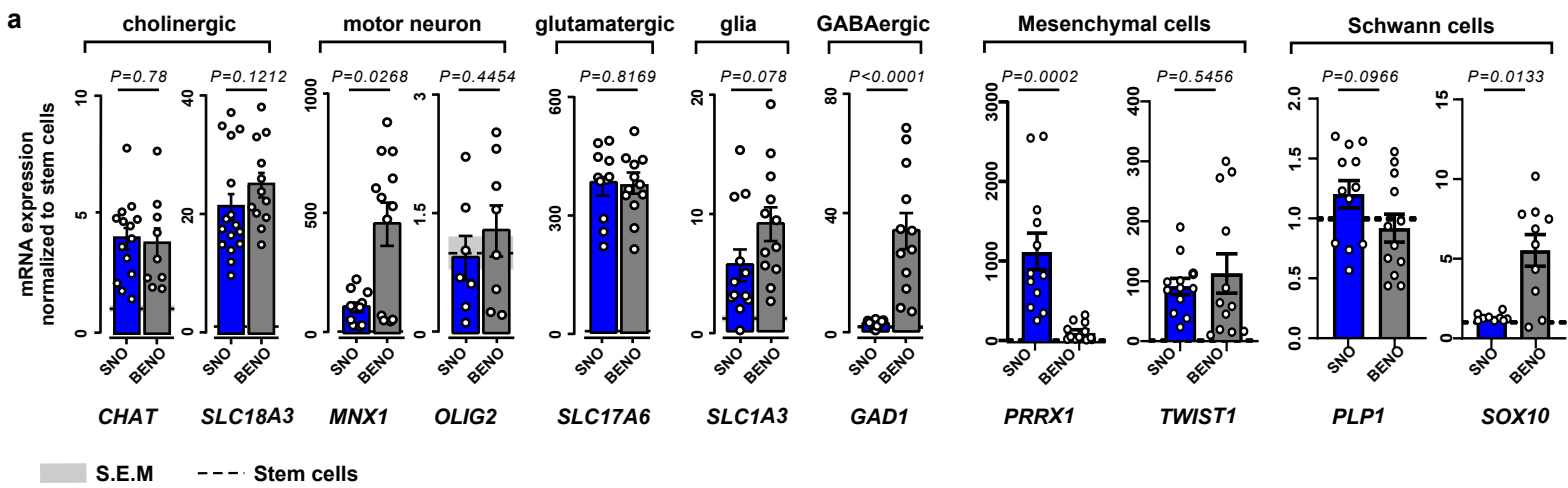
Supplementary Figure 2| Sympathetic neural progenitor analysis in d15 SNO. a Gating of flow cytometry analysis of d15 neural progenitor cells isolated from SNO (blue) and BENO (grey) stained for DNA and PHOX2B. SSC-A=side scatter-area, FSC-A=forward scatter-area. The right panel is the same as Figure 1b. **b** Immunofluorescence of d15 SNO and BENO stained for cortical marker PAX6 (red) and DNA (blue). Scale bar, 50 μ m.

Supplementary Figure 3



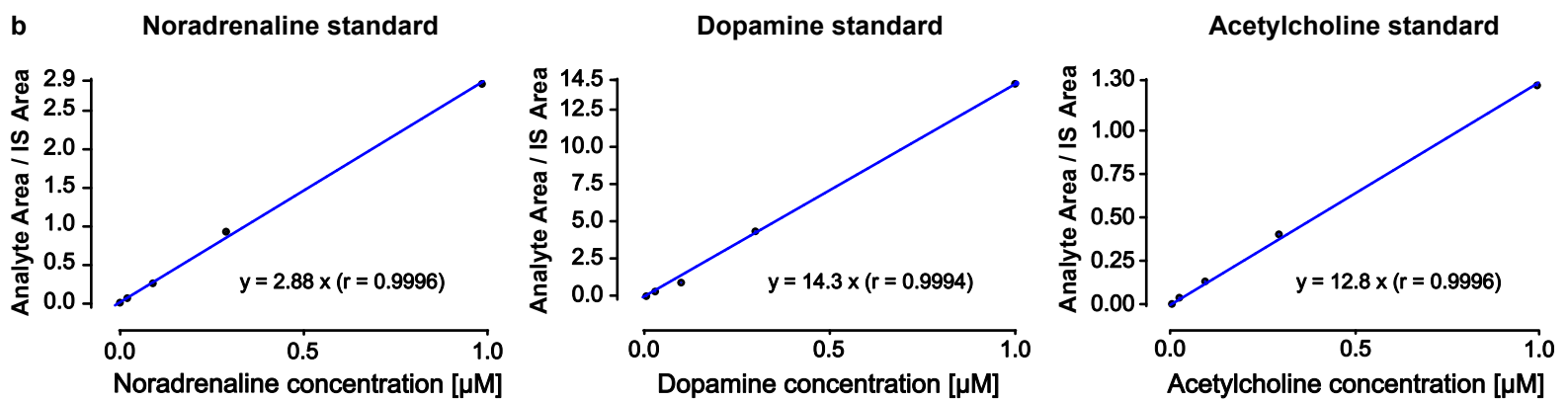
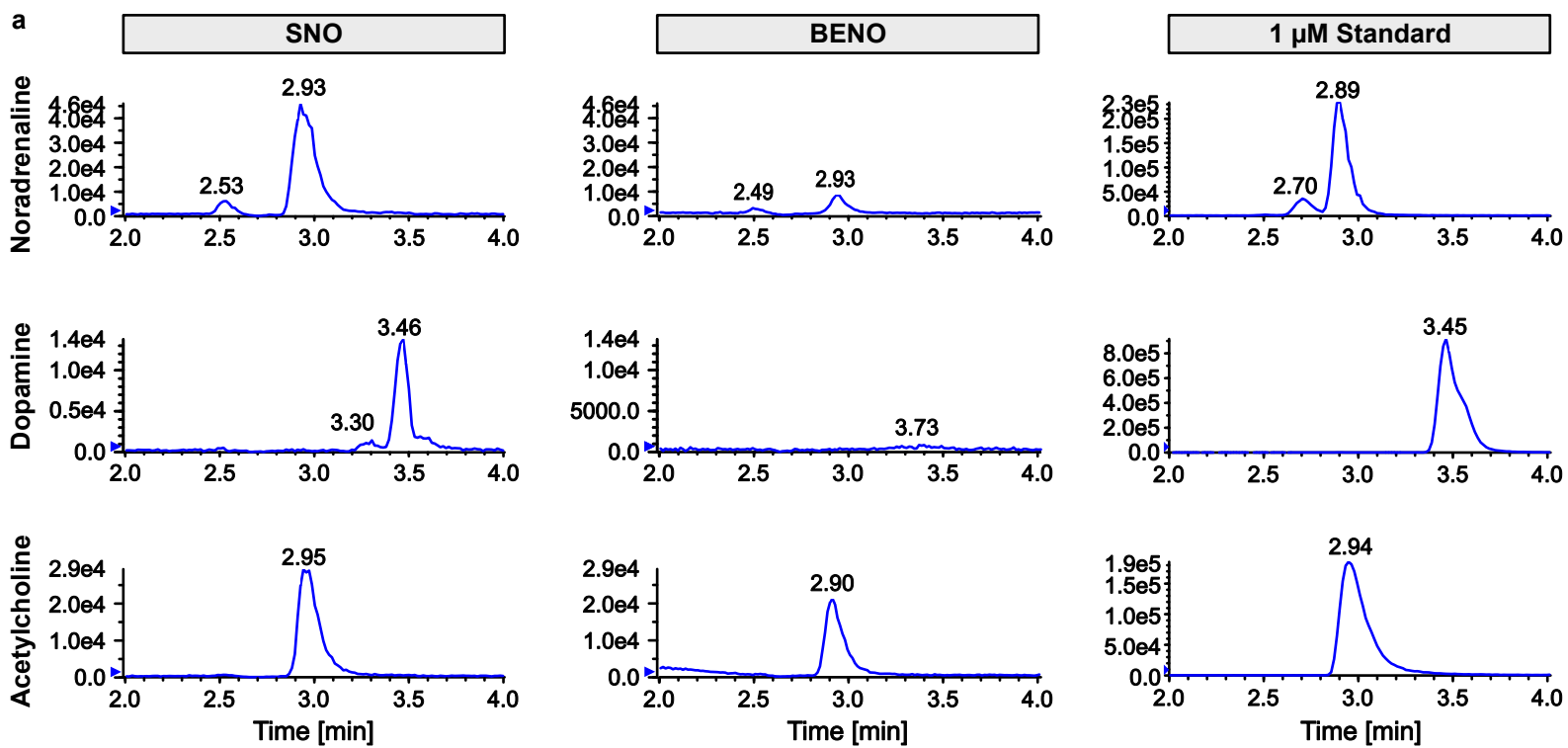
Supplementary Figure 3| Sympathetic neuron immunofluorescence analysis in SNO. a Representative image of d41 SNO stained for sympathetic marker TH (red), DBH (grey), peripheral neuron marker PRPH (green), and DNA (blue). Scale bar, 500 μ m. (Right) Close-up view. Scale bar, 50 μ m. **b** Close-up view shows SNs co-stained by TH (red), DBH (grey), NF (green) and DNA (blue) in SNO showing ganglia-like structures (Right). BENO did not contain SNs (Left). Scale bars, 50 μ m. **c** High resolution imaging of SNO stained for sympathetic marker TH (green) and DBH (red) as well as NF (cyan) and DNA (blue) showing widespread sympathetic ganglia-like structures (marked by white arrowheads). Scale bar, 100 μ m. **d** Close-up view of d93 SNO stained for sympathetic marker PHOX2B (blue), TH (green), DBH (red), and DNA (yellow). Scale bar, 50 μ m.

Supplementary Figure 4



Supplementary Figure 4| Identification of non-SN cells co-developing in SNO. **a** Expression analysis of cholinergic marker (*CHAT*, *SLC18A3* [aka *VACHT*]), motor (*MNX1*, *OLIG2*), glutamatergic (*SLC17A6* [aka *VGLUT2*]), GABAergic (*GAD1*) neuron marker, astrocyte (*SLC1A3* [aka *GLAST*]) marker, mesenchymal (*PRRX1*, *TWIST1*) markers, and Schwann cells (*PLP1*, *SOX10*) markers for SNO and BENO at d41 normalized to *GAPDH* expression. SNO and BENO were normalized to expression of undifferentiated stem cells. 3-4 independent differentiations, 3-4 tissues per differentiation (n=11-16). *CHAT*: n(SNO)=14 n(BENO)=9. *SLC18A3*: n(SNO)=16 n(BENO)=12. *MNX1*, *SLC17A6*: n(SNO)=11 n(BENO)=12. *OLIG2*: n(SNO)=7 n(BENO)=8. *SLC1A3*, *GAD1*, *TWIST1*, *PLP1*: n(SNO/BENO)=12. *PRRX1*: n(SNO/BENO)=10. *SOX10*: n(SNO)=9 n(BENO)=10. **b** *GAPDH* expression level of d15 (Top) and d41 (Bottom) SNO and BENO. 3 independent differentiations, 3-4 tissues per differentiation (n=11-16). d15: n(SNO)=12 n(BENO)=11, d41: n(SNO)=16 n(BENO)=12. **c** WmIF of d30 SNO stained for cholinergic marker VACHT (red), NF (green) and DNA (blue). Scale bar, 500 μ m. **d** Close-up view of cholinergic neurons VACHT (red) and SNs marked by TH (cyan) from two different SNO regions. Scale bar, 20 μ m. **e** IF-staining of three individual SNO at d41 for sensory neuron marker BRN3A (red) and nuclear counterstaining (blue). (Far right) d41 SNO as no primary antibody control. Scale bar, 200 μ m. **f** IF of three planes from a representative region of d41 SNO stained for sensory neurons (BRN3A (red) and NF (green)). No primary antibody control can be seen on the right. Scale bar, 20 μ m. Data shown as mean $\pm$ s.e.m. with individual data points. Two-tailed unpaired Student's t-test or Mann-Whitney test was used for (**a**, **b**).

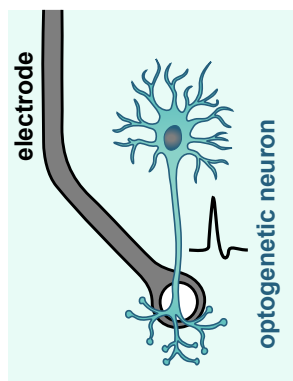
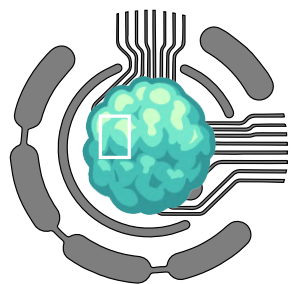
Supplementary Figure 5



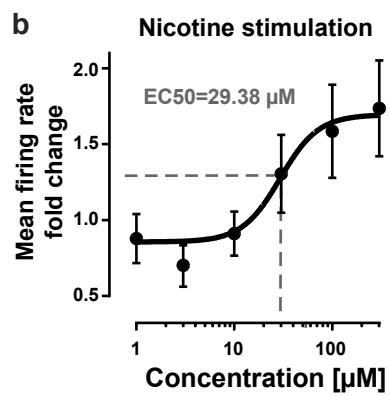
Supplementary Figure 5| Neurotransmitter quantification by LC-MS/MS analysis. a Representative LC-MS/MS spectra from lysed SNO and BENO for noradrenaline, dopamine, and acetylcholine compared to the respective standard at 1 μ M concentration. **b** Standard curves of all three neurotransmitters (concentrations from 0.001 μ M to 1 μ M). Linear regression (“through zero”) was calculated for the integrated peaks and used for quantification of the sample concentrations. Y-intercept and correlation coefficient r are indicated in the graphs.

Supplementary Figure 6

a Multi-electrode array

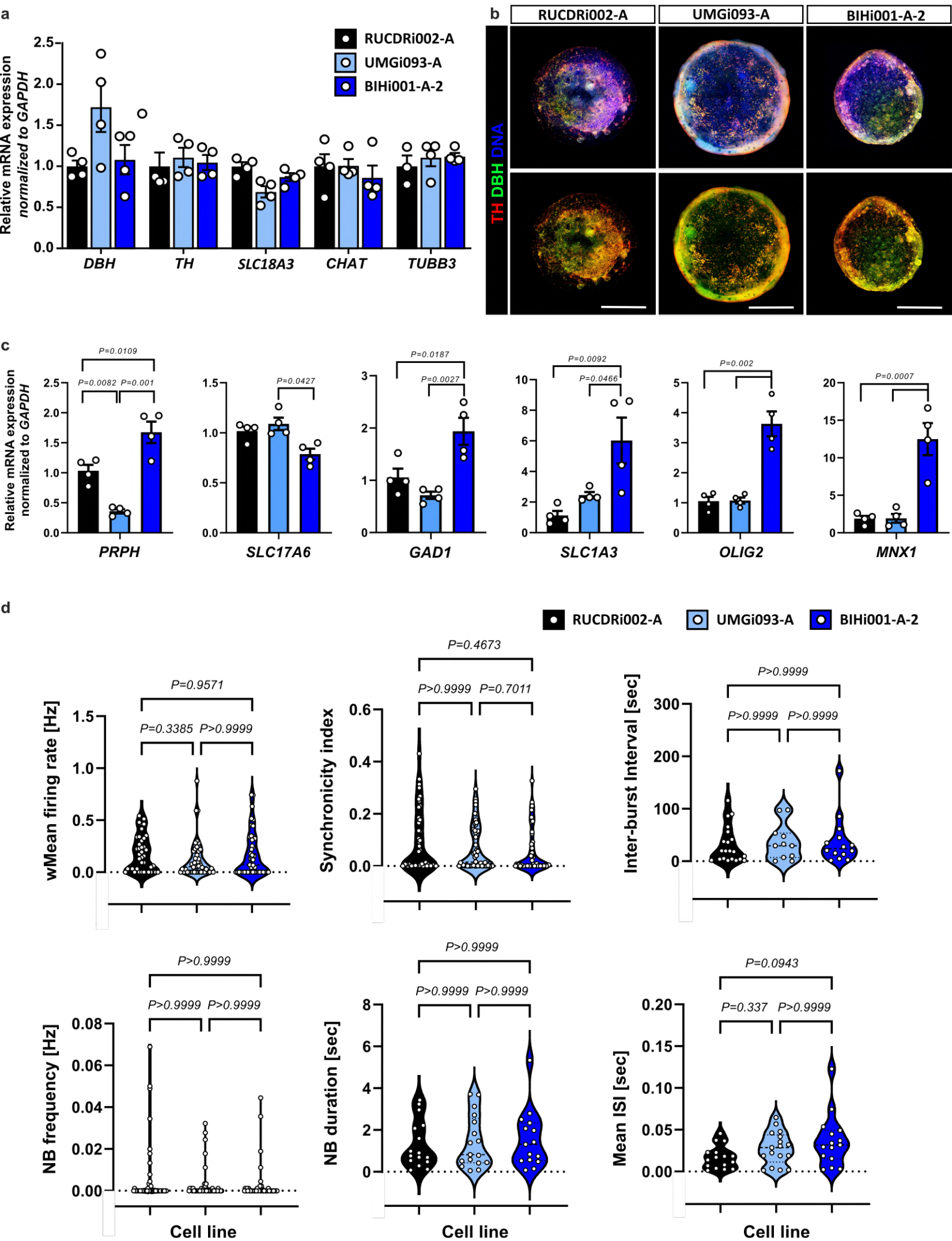


b



Supplementary Figure 6| Effect of nicotine on electrical activity of SNO d73-87. **a** Schematic of the placement of SNO on the multi-electrode array. **b** Dose response curve of mean firing rate of SNO upon stimulation with escalating concentrations of nicotine normalized to basal firing rate and untreated control. EC_{50} Nicotine=29.38 μ M, Nonlinear fit using variable slope (four parameters, ordinary fit). 3 independent differentiations, 6-7 tissues per differentiation (n=19), mean $\pm$ s.e.m.

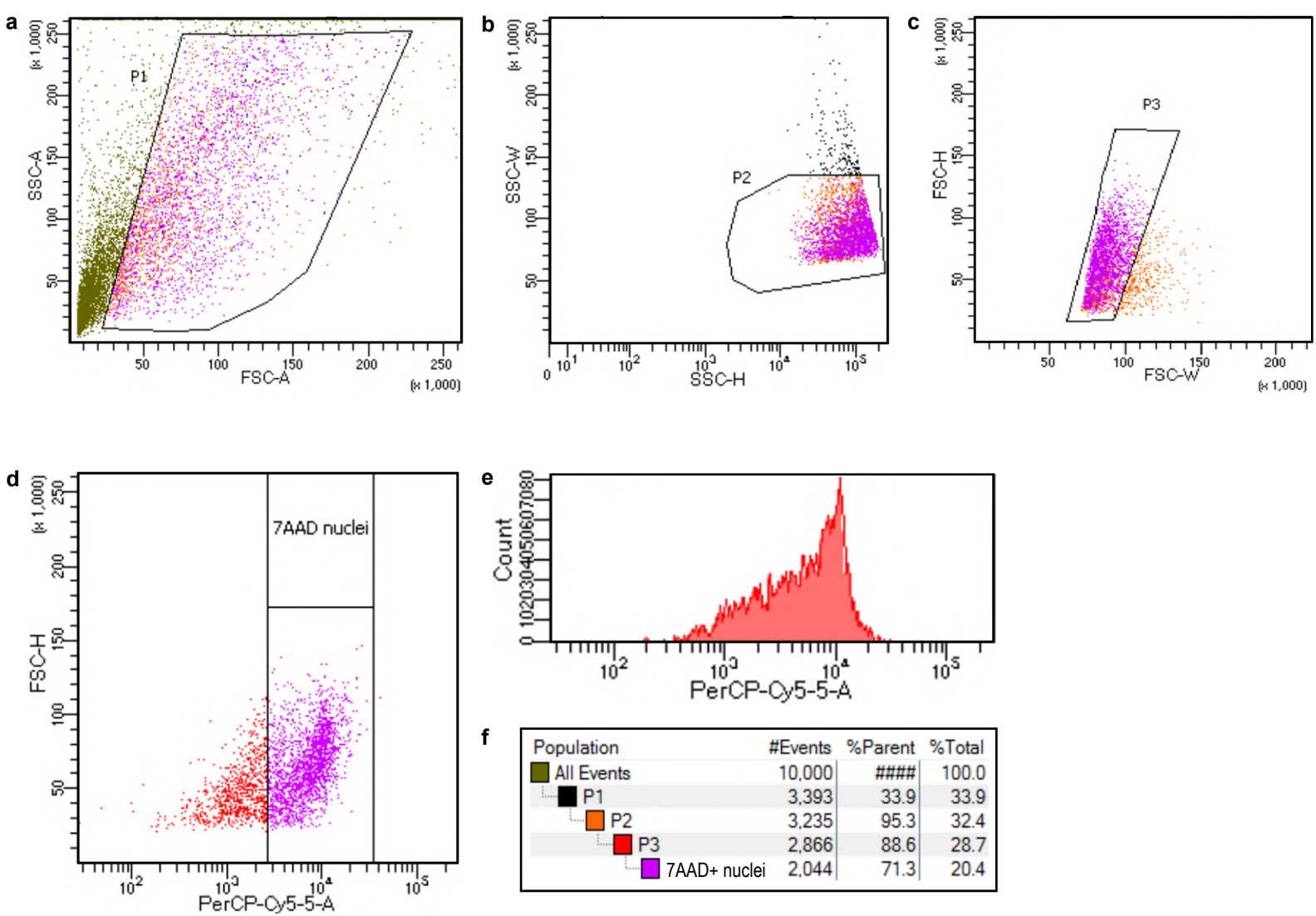
Supplementary Figure 7



Supplementary Figure 7| Reproducibility of SNO differentiation protocol in three iPSC-lines.

a qPCR analysis of SN-marker *DBH* and *TH*, cholinergic marker *SLC18A3* and *CHAT* as well as general neuron marker *TUBB3* between SNO from three different cell lines (RUCDRi002-A, UMGi093-A, BIHi001-A-2). Values were normalized to *GAPDH* and standard cell line RUCDRi002-A. 1 differentiation, n=4. **b** WmIF of SNO from three different iPSC lines stained for DNA (blue) and SN-marker DBH (green) and TH (red). Scale bar, 1 mm. **c** qPCR analysis of peripheral (*PRPH*), glutamatergic (*SLC17A6* [aka *VGLUT2*]), GABAergic (*GAD1*), motor (*MNX1*, *OLIG2*) neuron markers and astrocyte (*SLC1A3* [aka *GLAST*]) between SNO from three different cell lines (RUCDRi002-A, UMGi093-A, BIHi001-A-2). Values were normalized to *GAPDH* and standard cell line RUCDRi002-A. 1 differentiation, n=4. **d** Weighted (w) mean firing rate, synchronicity index, inter-burst interval, network burst (NB) frequency, NB duration and mean inter-spike-interval (ISI) of SNO from three different cell lines (RUCDRi002-A, UMGi093-A, BIHi001-A-2) weeks 4-8. 1 differentiation, n=16 / group. All data is presented as mean $\pm$ s.e.m. One-way ANOVA or Kruskal-Wallis test with Dunn's post hoc test was performed for (**a**, **c**, **d**).

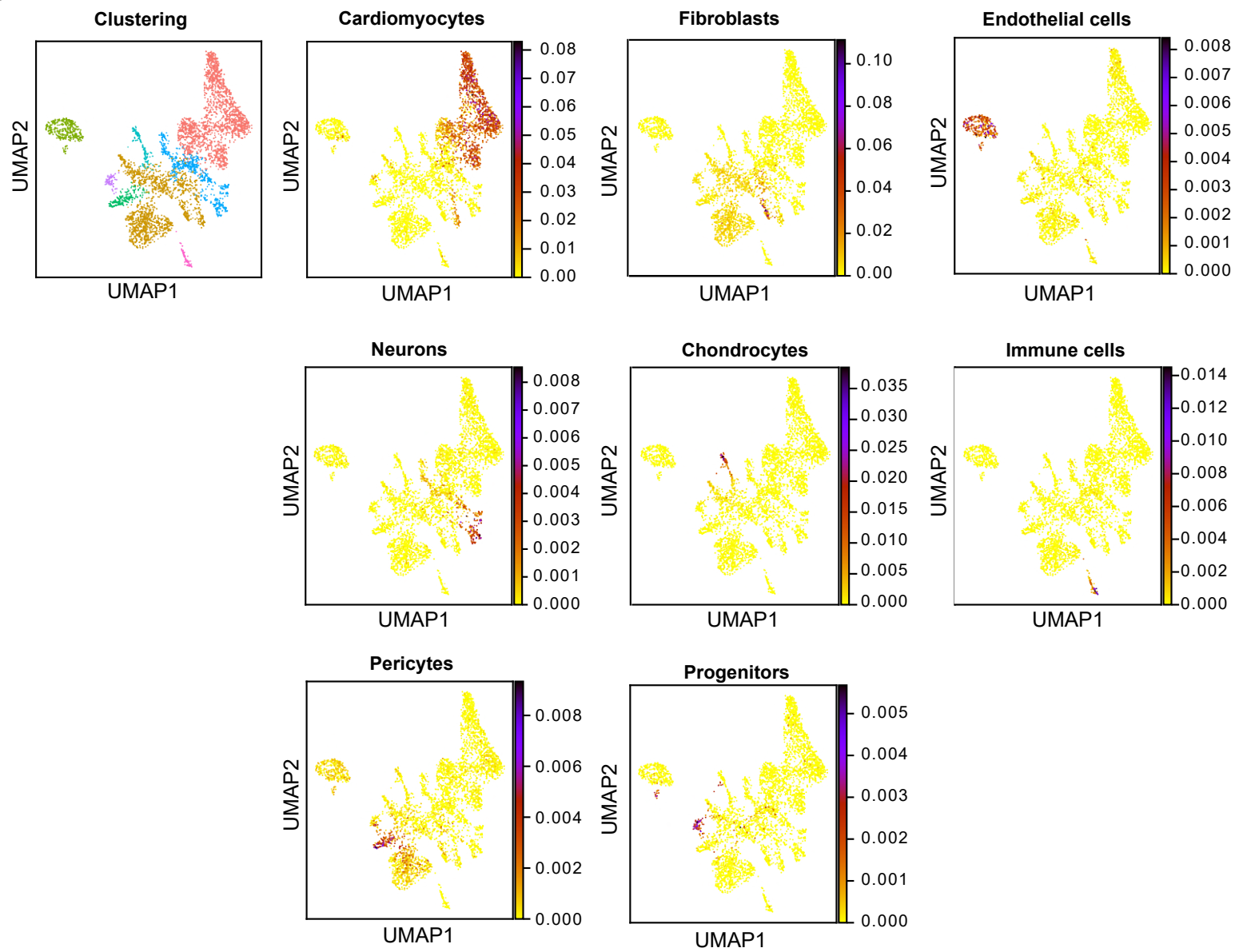
Supplementary Figure 8



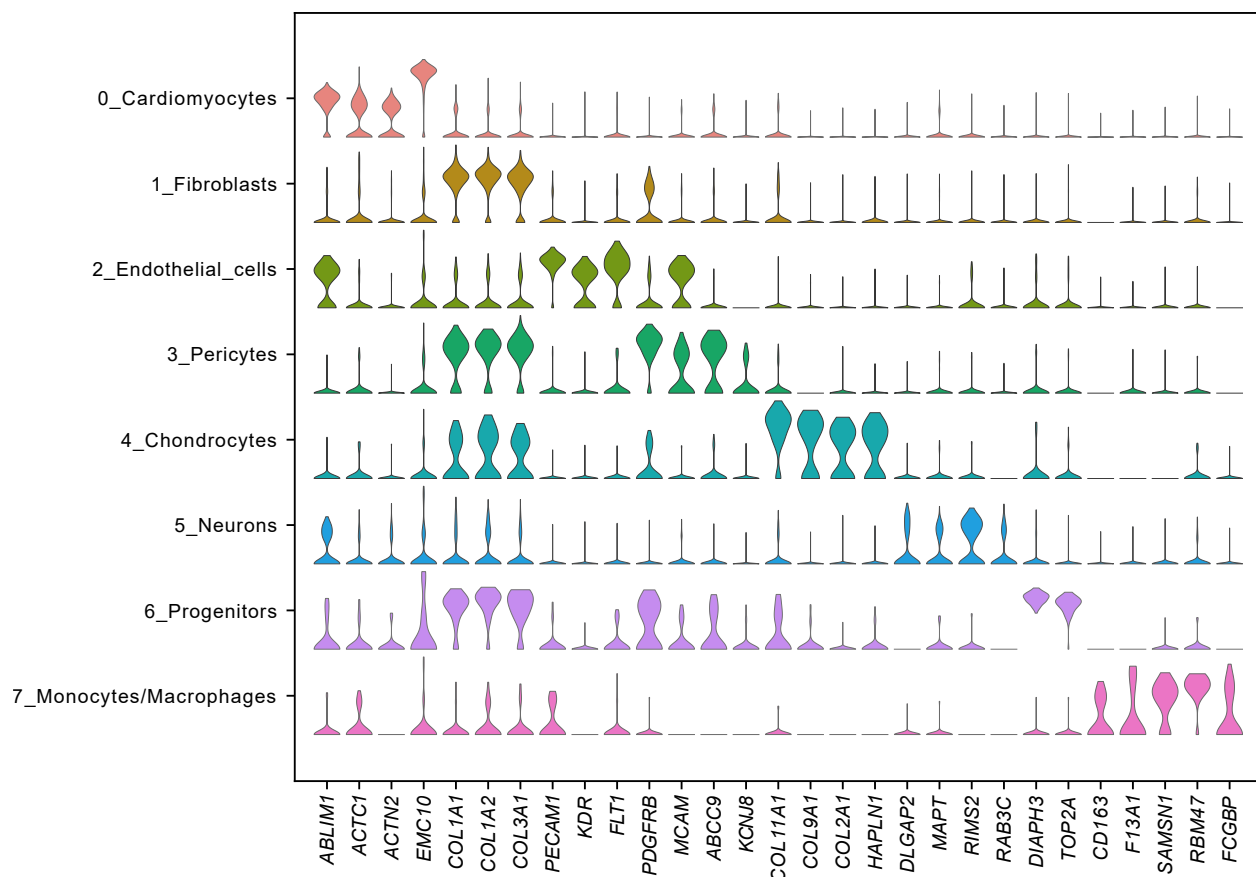
Supplementary Figure 8| Gating strategy for sorting nuclei from frozen iEHM lysates using 7AAD staining. a-c Gating strategy for exclusion of debris (P1, P2, P3) and for **d** 7AAD-stained nuclei from iEHM lysates. **e** Histogram of raw nuclei count (PerCP-Cy5.5 signal, 7AAD dye). **f** Hierarchy of the gated populations including proportions.

Supplementary Figure 9

a



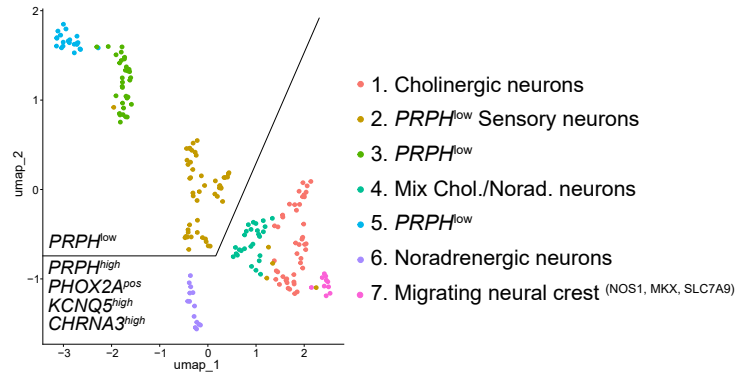
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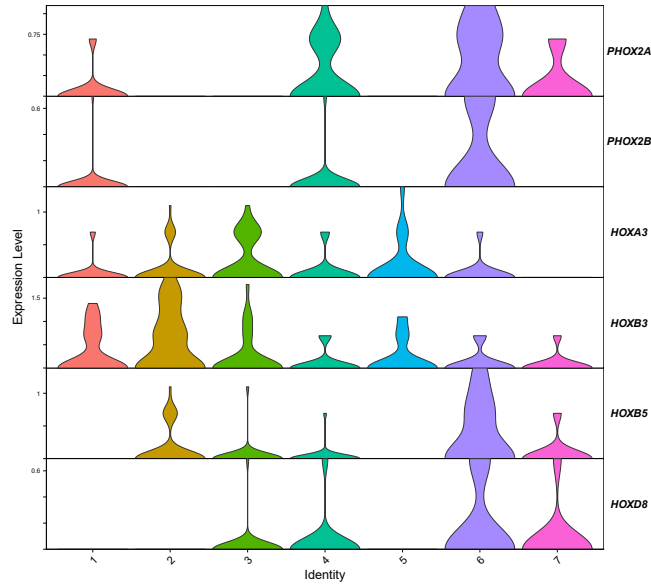
Supplementary Figure 9| Cell cluster annotation of snRNA-sequencing. **a** UMAP plots depicting the consensus expression (average module score) of cell-type specific marker genes used to annotate the clusters in the dataset. Gene names are highlighted in blue under the plot title of the corresponding cell-type. **b** Violin plots showing the normalized expression of individual marker genes from **a** across the annotated cell-types.

Supplementary Figure 10

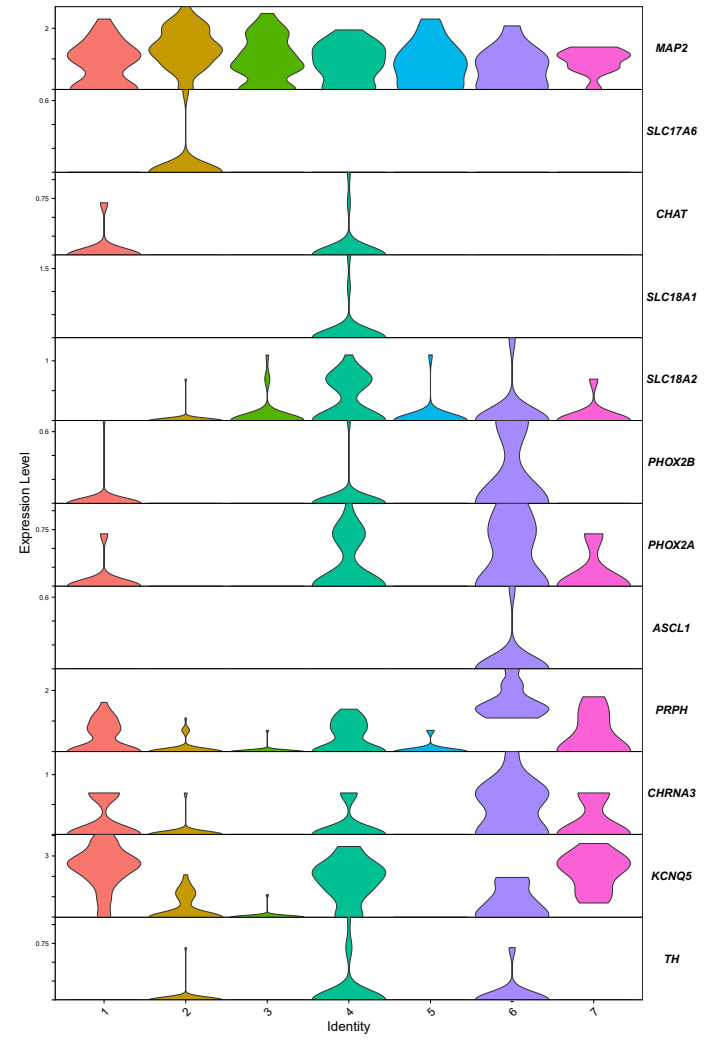
a



c



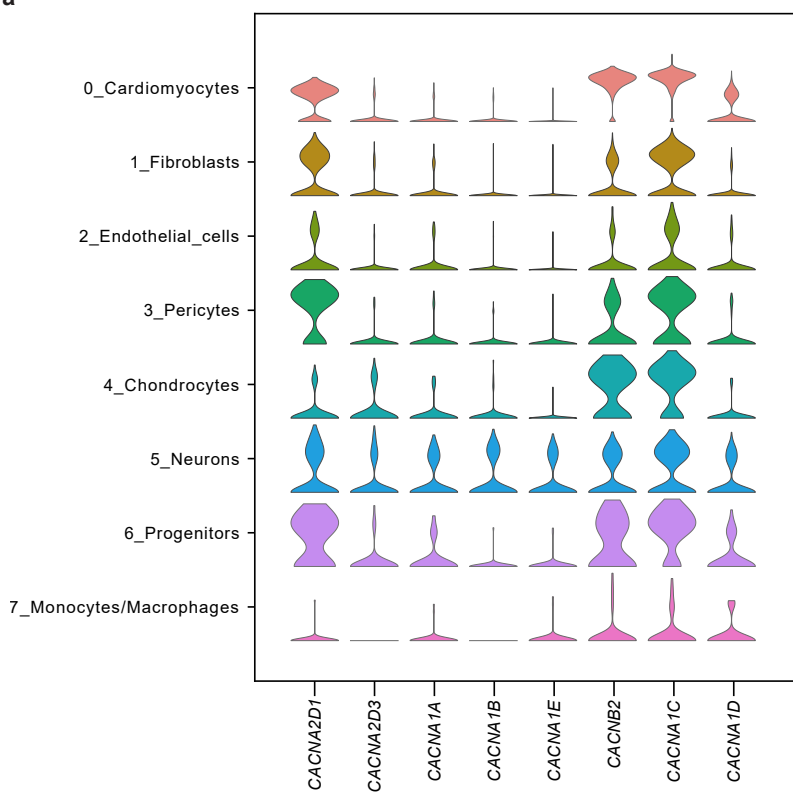
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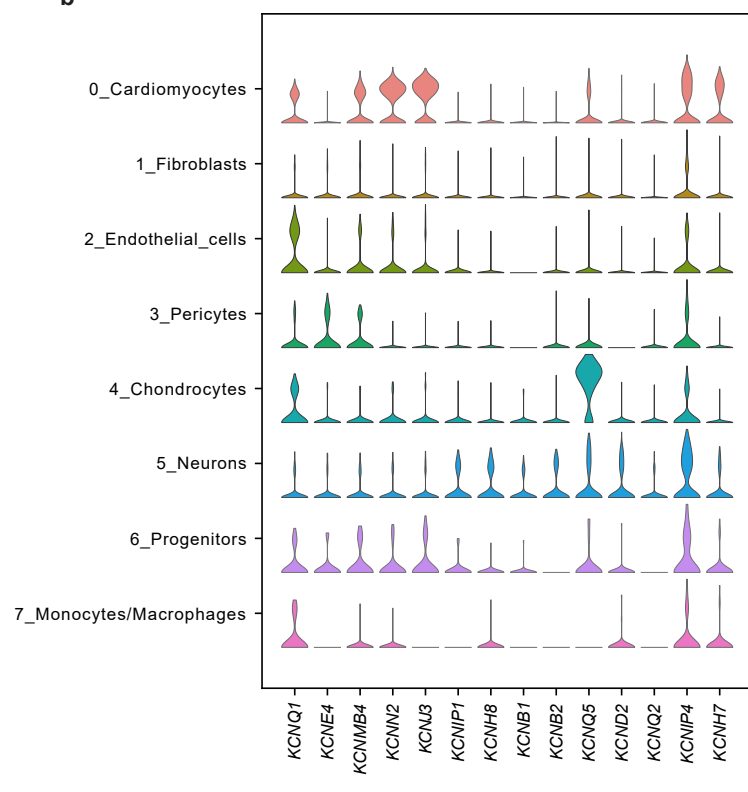
Supplementary Figure 10| Expression analysis of neuronal subclusters in iEHM by snRNA-sequencing. **a** UMAP visualization of neuronal subclusters identified in the dataset. Seven clusters were detected. Clusters 1, 4, 6, and 7 show high expression of peripheral autonomic markers *PRPH*, *KCNQ5*, *PHOX2A*, *CHRNA3*, whereas clusters 2, 3 and 5 display lower expression of these peripheral markers. **b** Violin plots showing the normalized expression of pan-neural marker *MAP2*; autonomic lineage *PRPH*, *ASCL1*, *PHOX2A/B*, *CHRNA3*; cholinergic marker *CHAT*; noradrenergic markers *TH* and *SLC18A1/2*; and glutamatergic marker *SLC17A6*. **c** Violin plots showing the normalized expression of HOX genes across neuronal clusters. *PHOX2A/B* mark the autonomic lineage, while *HOXA3*, *HOXB3* define vagal neural crest identity and *HOXB5*, *HOXD8* indicate a more posterior trunk neural crest identity.

Supplementary Figure 11

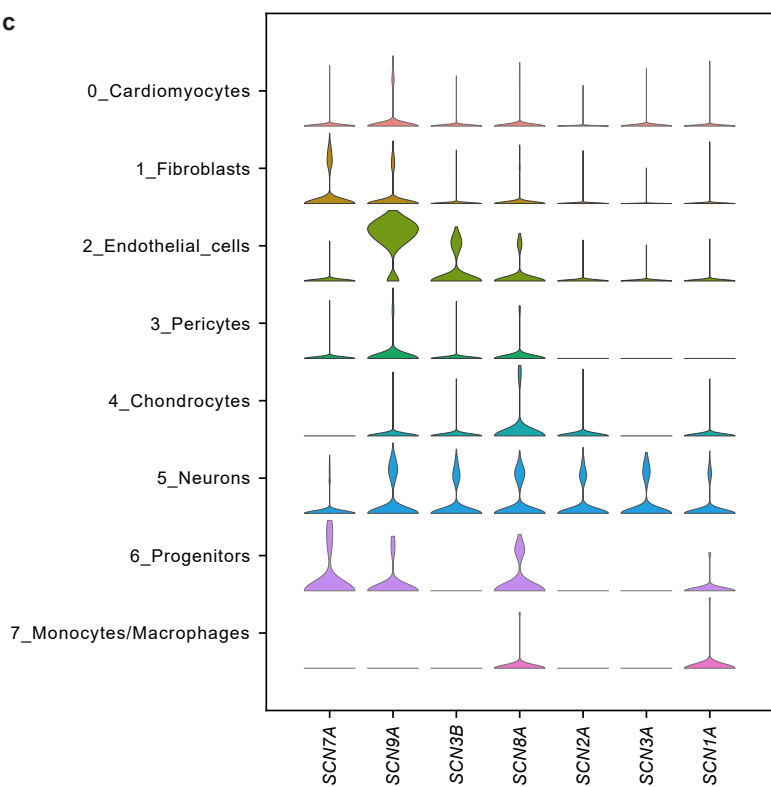
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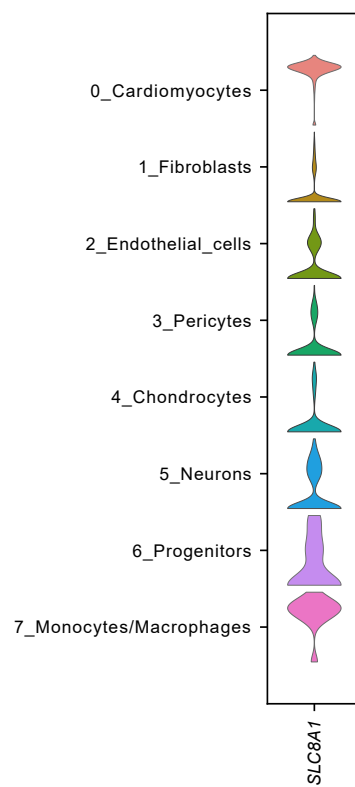
b



c

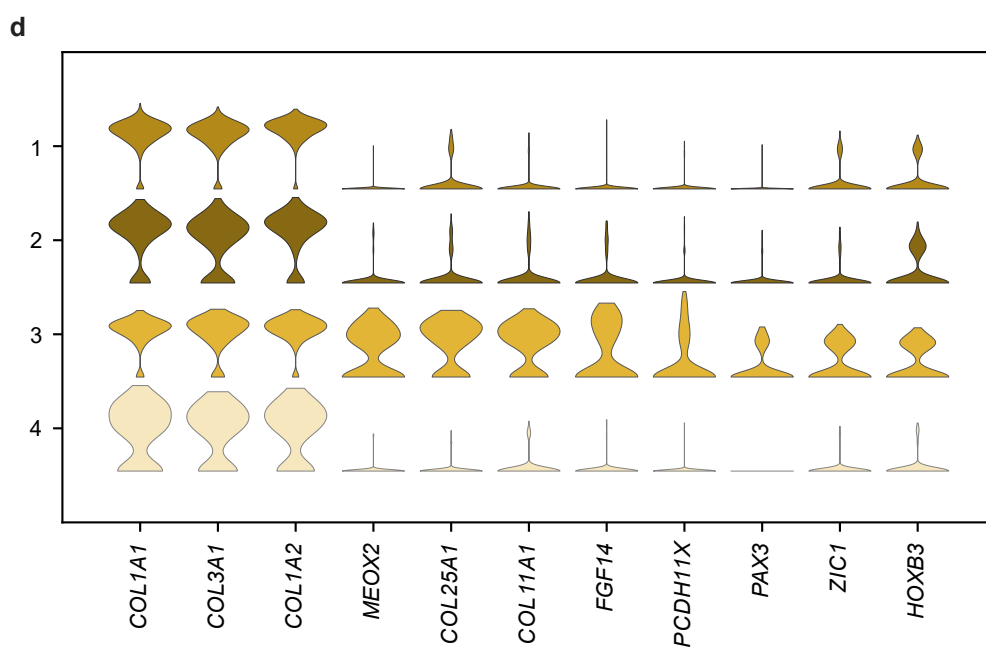
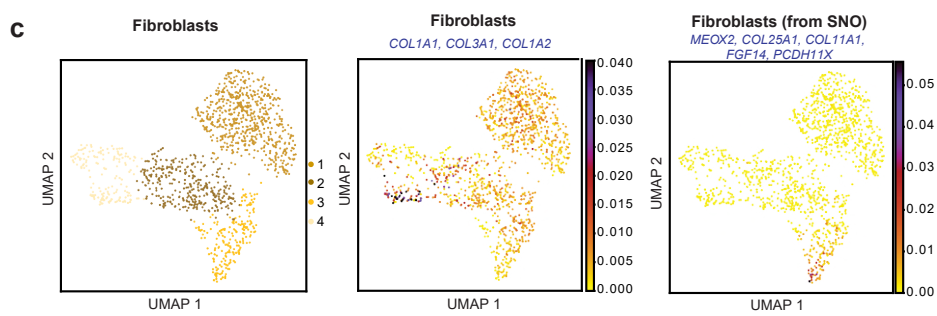
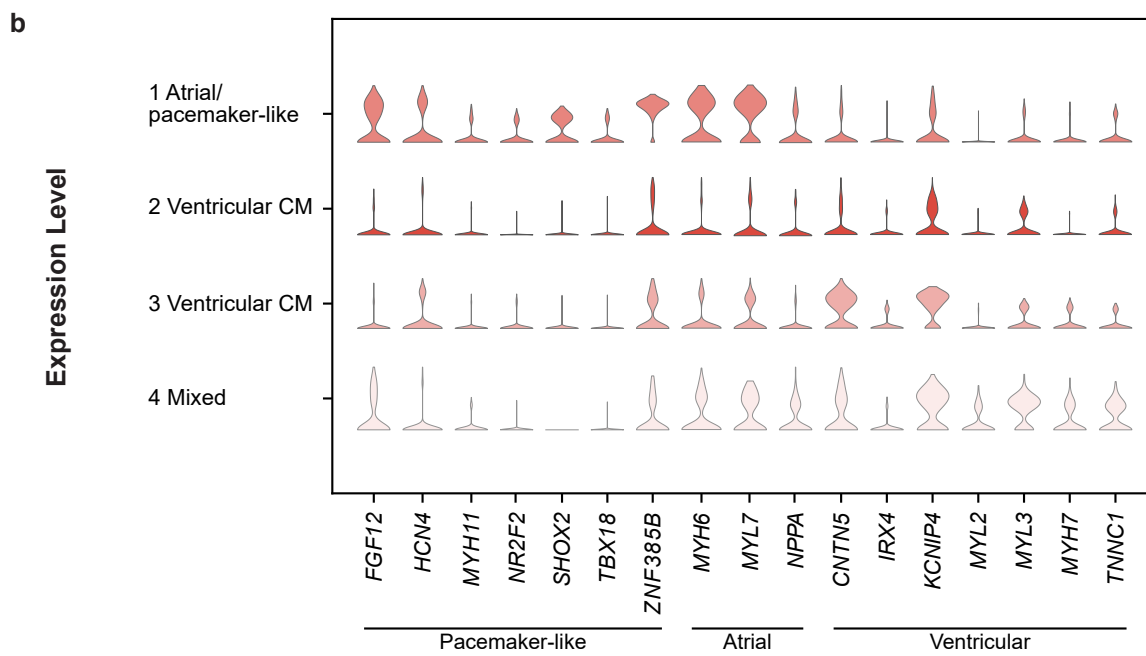
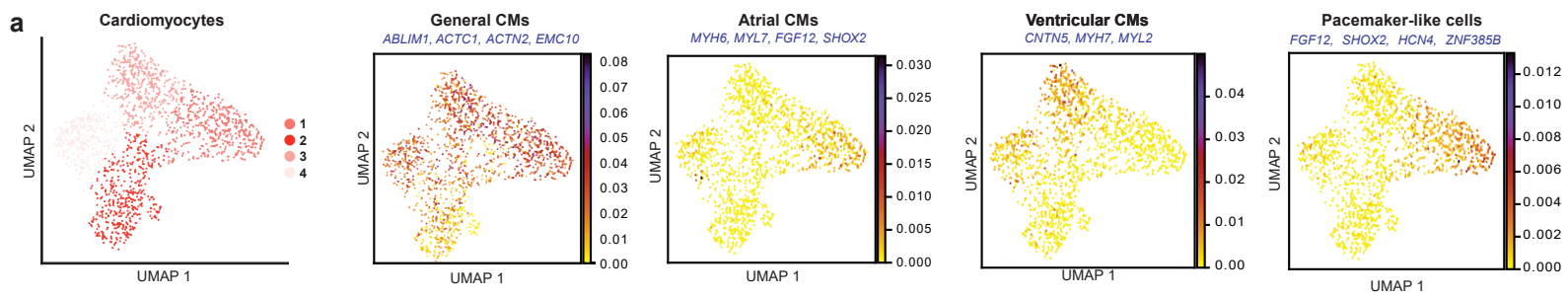


d



Supplementary Figure 11| Expression analysis of ion channels across cell types from snRNA-sequencing. Violin plots showing the normalized expression of **a** voltage-gated calcium (Ca^{2+}) channel transcripts, **b** voltage-gated potassium (K^+) channel transcripts, **c** voltage-gated sodium (Na^+) channel transcripts and **d** the $\text{Na}^+/\text{Ca}^{2+}$ exchanger *SLC8A1* transcript, across the annotated cell-types.

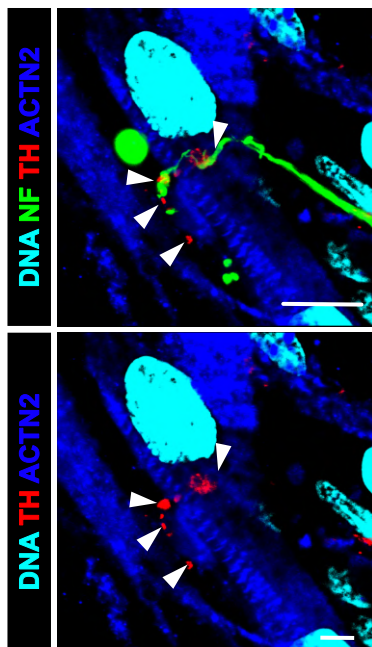
Supplementary Figure 12



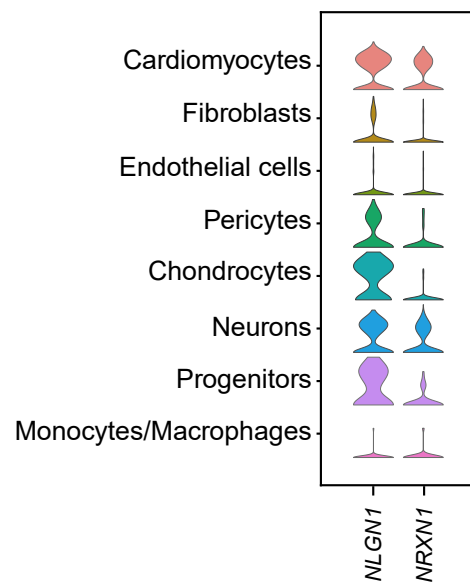
Supplementary Figure 12| Analysis of CM and fibroblast subcluster in the iEHM. **a** UMAP plots depicting the consensus expression (average module score) of atrial (*FGF12*, *MYH6*, *MYL7*), ventricular (*CNTN5*, *MYH7*, *MYL2*), pacemaker-like (*SHOX2*, *FGF12*, *HCN4*, *MYH11*, *ZNF385B*) and general CM marker genes across the CM sub-clusters (UMAP plot in the top-left panel). Gene names are highlighted in blue under the plot title of the corresponding sub-type. **b** Violin plots showing the normalized expression of individual atrial and ventricular CM marker genes from a across the three sub-clusters. **c** UMAP plots depicting the consensus expression (average module score) of fibroblast marker genes across the fibroblast sub-clusters (UMAP plot in the top-left panel). **d** Violin plots showing the normalized expression of individual fibroblast marker genes from c across the two sub-clusters.

Supplementary Figure 13

a

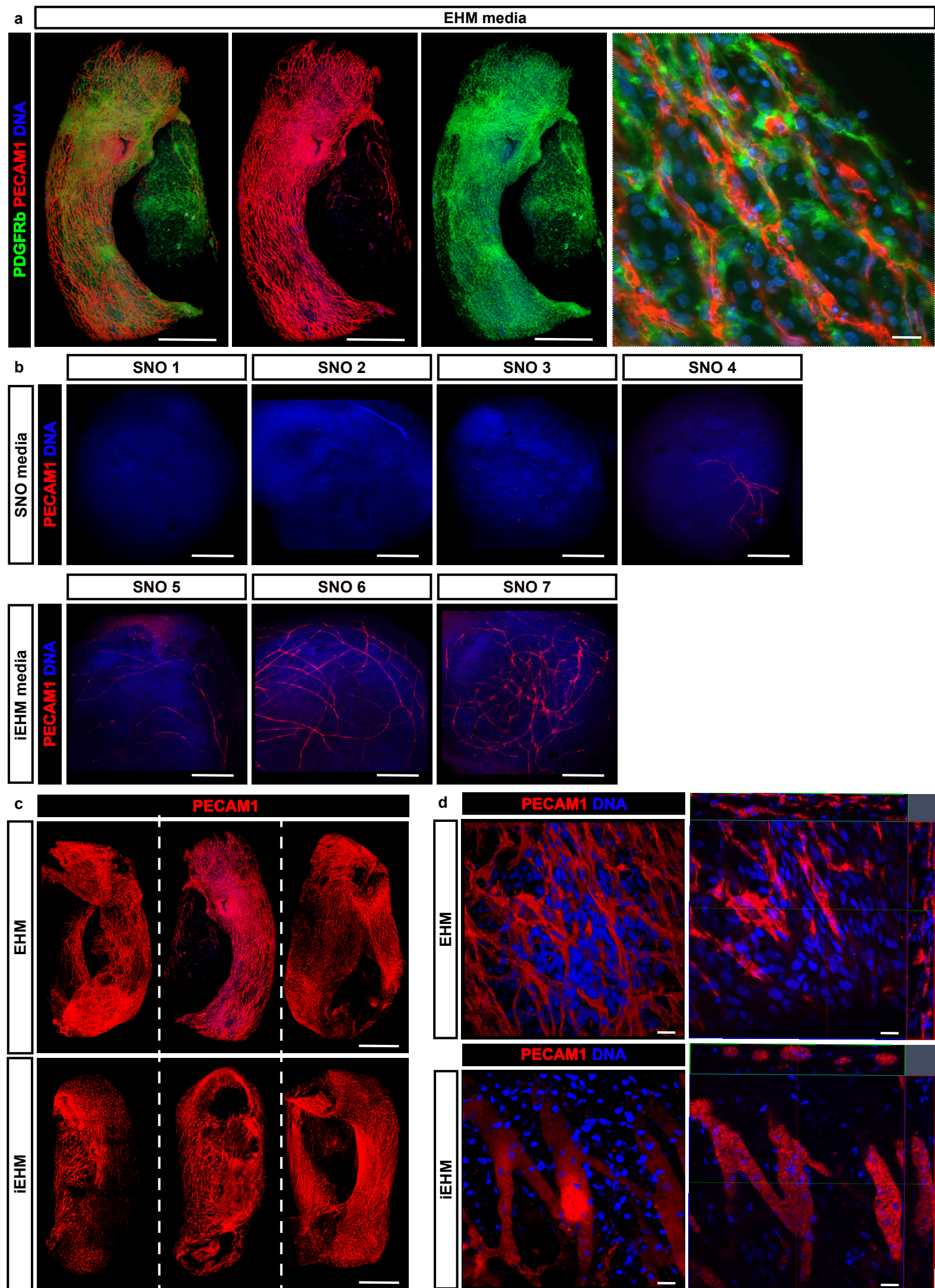


b



Supplementary Figure 13| Investigation of neuro-cardiac junctions in iEHM. a WmIF showing co-staining of synaptic bouton-like varicosity (NF, green) co-localizing with autonomic neuron marker TH (red) on the surface of CMs (ACTN2, blue) and DNA (cyan). Co-localization is indicated by white arrowheads. Scale bar, 10 μ m. **b** snRNA sequencing expression analysis of presynaptic marker neurexin 1 (*NRXN1*) and postsynaptic adhesion molecule neuroligin 1 (*NLGN1*) in the annotated iEHM cell clusters (see Supplementary Figure 9).

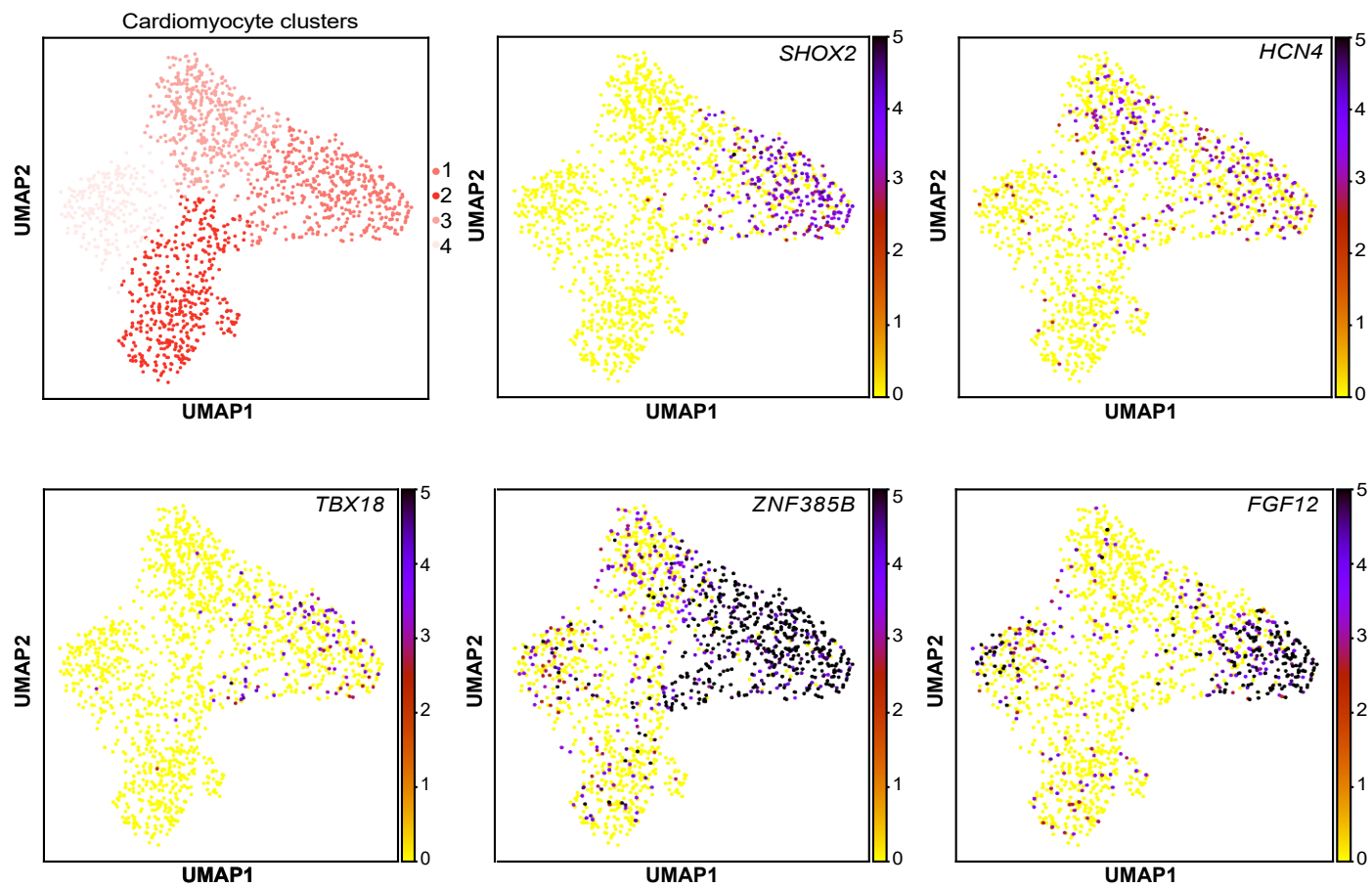
Supplementary Figure 14



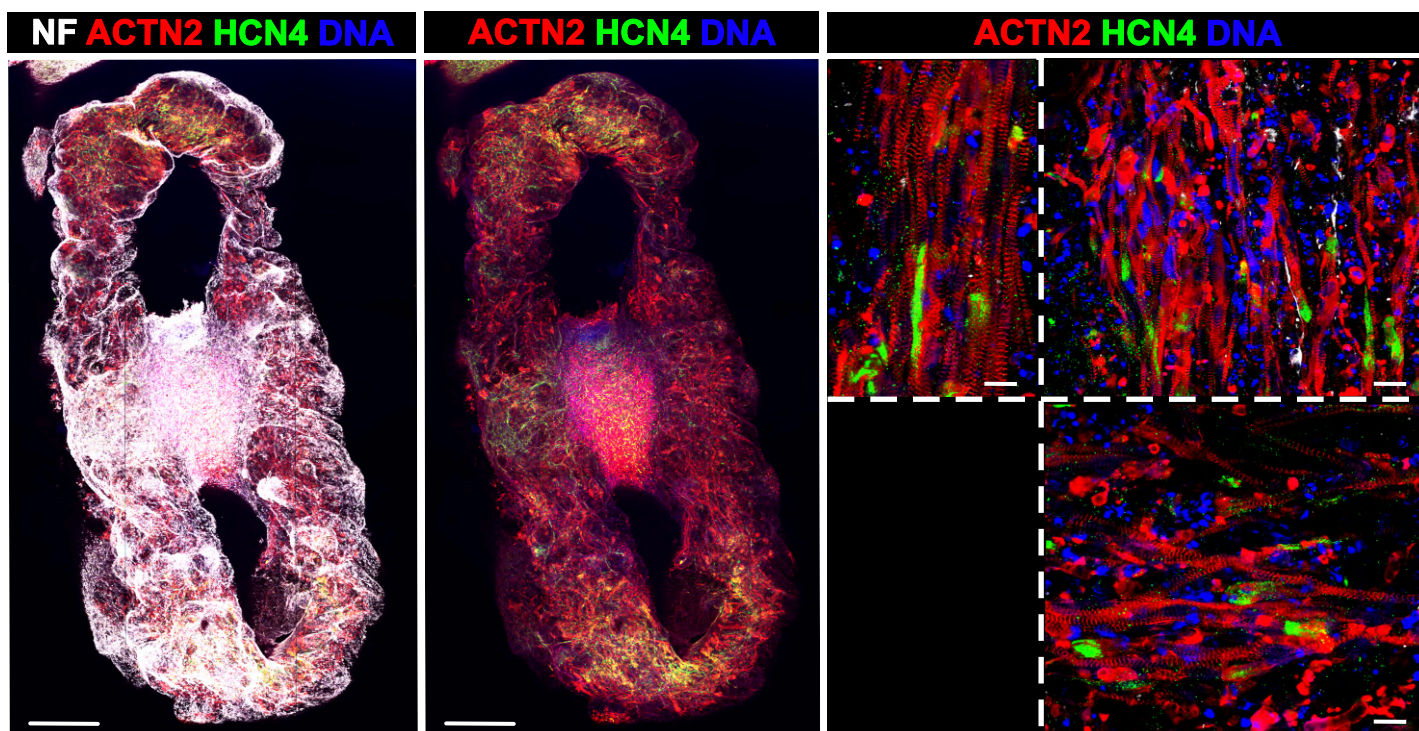
Supplementary Figure 14| Vascular network development in EHM, SNO and iEHM. **a** WmIF of 6 weeks EHM cultured in EHM medium reveals a dense endothelial network (PECAM1, red), presence of PDGFRb (green)-positive pericytes and DNA (blue). Scale bar, 1 mm. (Right) Close-up view showing close localization of pericytes and endothelial cells. Scale bar, 20 μ m. **b** (Top) WmIF of d93 SNO cultured exclusively in SNO media shows presence of endothelial cells (PECAM1, red) in only one out of four tissues. (Bottom) After eight weeks of culture in iEHM medium, d103 SNO show the presence of endothelial cells (PECAM1, red) in all of the screened tissues. Scale bar, 200 μ m. **c** PECAM1 (red) immunofluorescence analysis of three representative 6-week-old EHM and iEHM. Scale bar, 1 mm. **d** Close-up view of PECAM1 staining in EHM and iEHM (Left) and the 3D-reconstruction (Right) show a larger vessel diameter in some areas of the iEHM when compared to EHM. Scale bar, 20 μ m.

Supplementary Figure 15

a



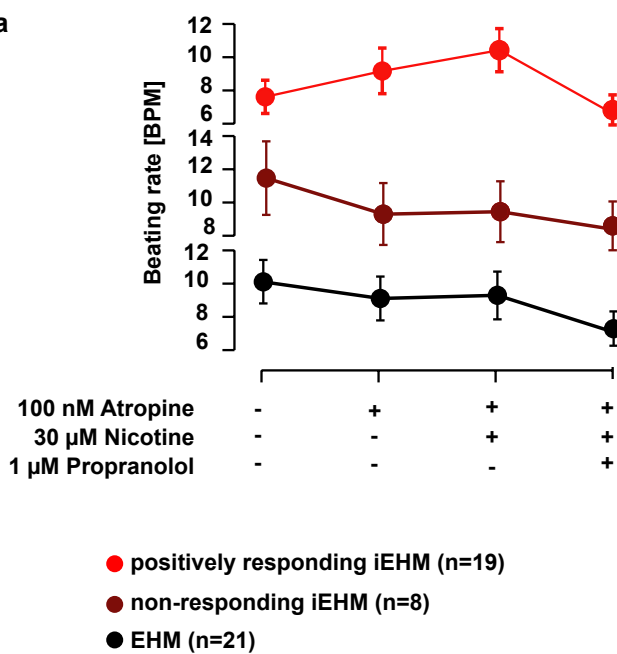
b



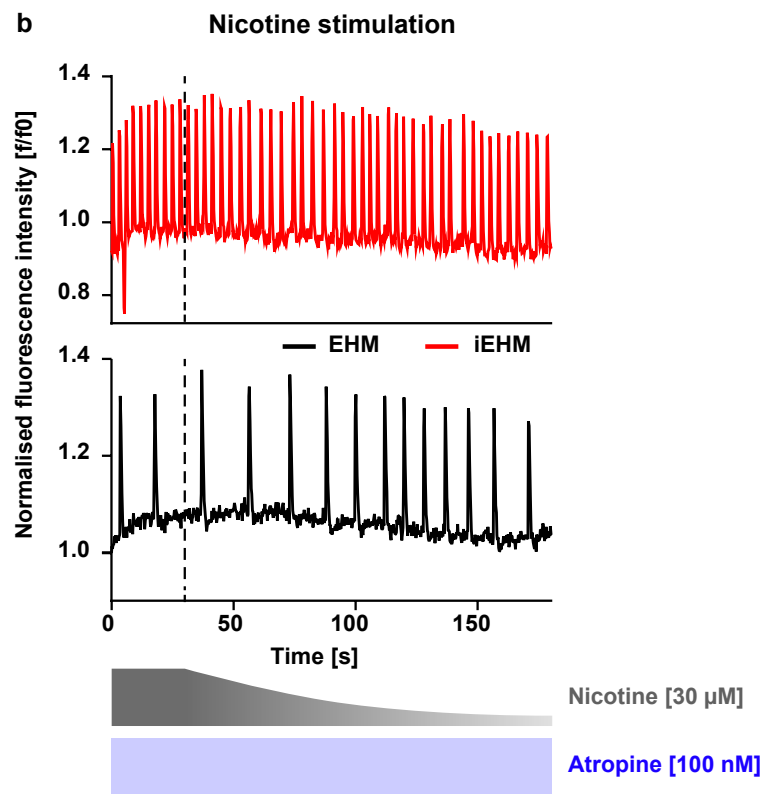
Supplementary Figure 15| Identification of pacemaker-like cells in iEHM. **a** UMAP plots showing the distribution of cells expressing the pacemaker-like-cell markers *HCN4*, *SHOX2*, *FGF12*, *TBX18*, and *ZNF385B* in the clusters in the dataset including cluster annotations (compare in the four CM cluster (Supplementary Figure 11)). **b** WmIF of iEHM stained for NF (grey), CM marker ACTN2 (red), pacemaker-like-cell marker HCN4 (green) and DNA (blue). (Left) Overview images show pacemaker-like-cells dispersed throughout the innervated areas of the EHM. Scale bar, 500 μ m. (Right) Close-up view show HCN4 positive cells in between ACTN2-positive CM co-localizing with NF-positive neurites (top left panel). Scale bar, 20 μ m.

Supplementary Figure 16

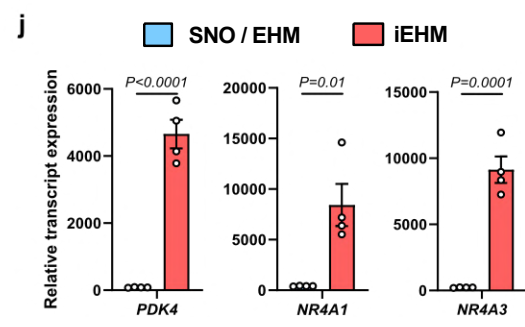
a



b



Supplementary Figure 16| Details of pharmacological stimulation of iEHM. **a** Chronotropic response to stimulation of EHM and iEHM with 100 nM atropine, 30 μ M nicotine and 1 μ M propranolol. iEHM could be separated into two distinct groups of responding (n=19) and non-responding tissues (n=8) that behaved similar to EHM controls (n=21). **b** Example traces showing the acute effect of nicotine stimulation on EHM and iEHM mean fluorescence intensity (f/f_0) of the ROI (see in Figure 3f) in the presence of 100 nM atropine.

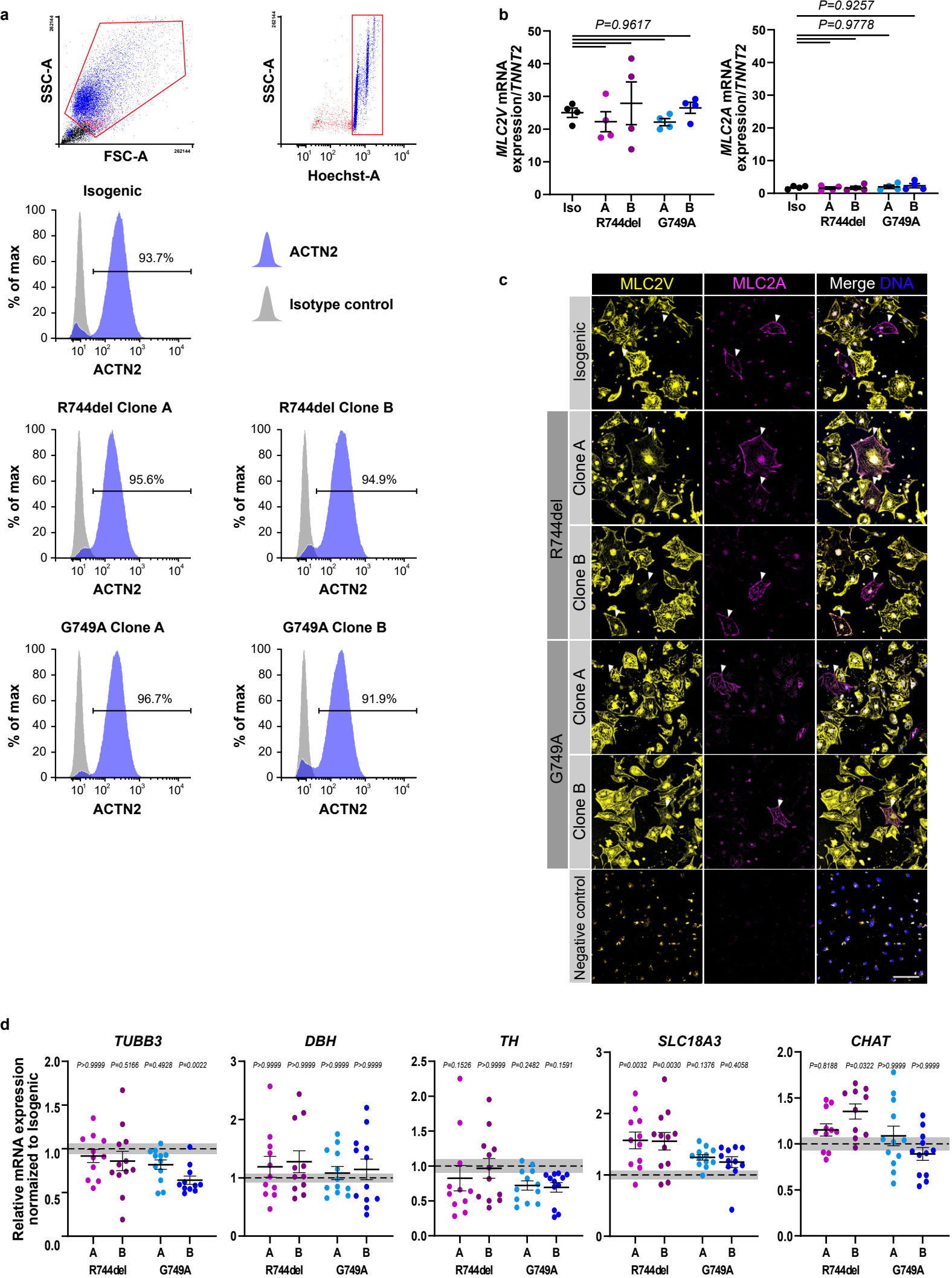


Supplementary Figure 17| Bulk RNA-sequencing of EHM+SNO vs iEHM. **a** Schematic diagram of bulk RNA-sequencing samples of pooled individual cultured neural (SNO) and cardiac (EHM) modules vs iEHM. **b** Volcano plot showing differentially expressed genes between iEHM and SNO+EHM organoids (adjusted $P < 0.05$, $|\log_2 FC| > 1$). **c** Heatmap of representative differentially regulated genes linked to cardiac function, metabolism, immune signaling, and hypertrophy. **d** WmIF of d71 EHM and iEHM stained for hematopoietic progenitors (CD45, cyan) and DNA (blue). Scale bar, 500 μm . (Right) Close-up views of CD45 progenitor cells in EHM and iEHM. Scale bar, 20 μm . **e** WmIF of d71 EHM and iEHM stained for immune cell marker CD68 (yellow), CM marker Phalloidin (magenta) and DNA (blue). Scale bar, 500 μm . (Right) Close-up views of CD68 immune cells in EHM and iEHM. Scale bar, 20 μm . **f** Cardiomyocyte (α -actinin) size of digested EHM and iEHM. Size is assessed by median fluorescence intensity of α -actinin signal. $n(\text{EHM})=3$ $n(\text{iEHM})=4$. **g** Cardiac specific transcript expression levels normalized to (Top) *CASQ2* and (Bottom) *DDB1*, $n=4$ tissues / condition. **h** WmIF of d71 EHM and iEHM stained for gap junction marker CX43 (green), cardiac marker CTNT (red) and DNA (blue). Scale bar, 20 μm . **i** Quantification of CX43 puncta from 6 representative close-up images of EHM and iEHM normalized to the number of nuclei. **j** Relative transcript expression of *PDK4*, *NR4A1*, and *NR4A3* in EHM/SNO vs iEHM. $n=4$ tissues / condition. All data is presented as mean $\pm$ s.e.m. Unpaired two-tailed Student's t-test was used for (**f**, **g**, **i**, **j**). Supplementary Figure 17a created in *BioRender*. Zafeiriou, M. P. (2026) <https://BioRender.com/w8pgnup>.

Supplementary Figure 18| Gene ontology from bulk RNA-sequencing of EHM+SNO vs iEHM.

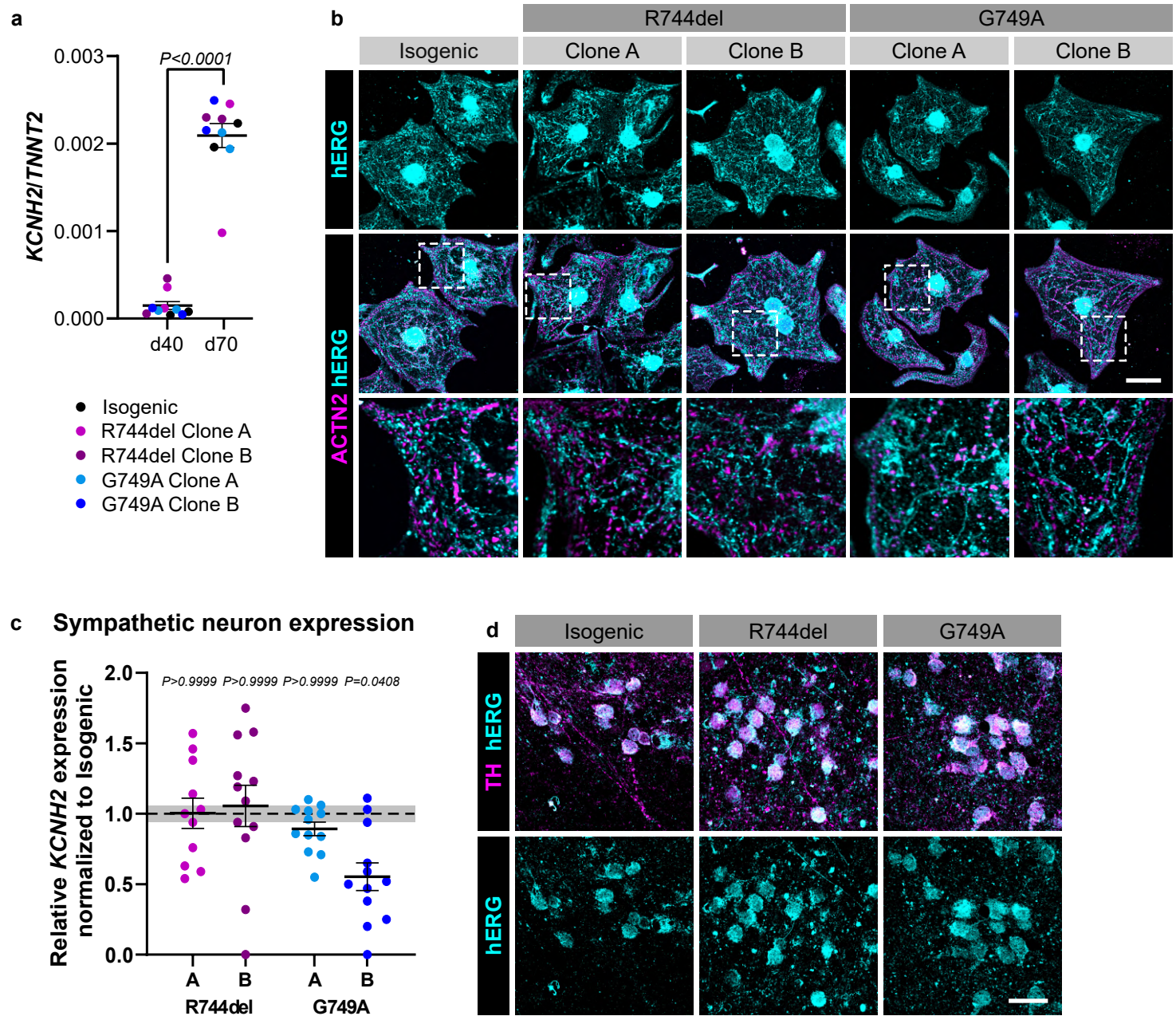
a Heatmap of variance-stabilized expression values for the top 500 variable genes across iEHM and SNO+EHM samples. Values are Z-score normalized per gene, and hierarchical k-means clustering ($k = 3$) is applied to visualize global expression differences between conditions. **b–c** Gene Ontology enrichment analysis for the transcriptional clusters. The most significantly enriched Biological Process (BP) terms (**b**) and Cellular Component (CC) terms (**c**) are shown, ranked by gene ratio. Bar length indicates the proportion of genes contributing to each term, and bar color reflects adjusted P values. **d–f**, Reactome pathway enrichment for each k-means gene cluster, highlighting distinct pathway signatures associated with the corresponding transcriptional module. **g**, STRING protein–protein interaction (PPI) networks generated from the genes in each expression cluster. Nodes represent proteins and edges denote predicted or experimentally supported functional associations. Network organization reflects cluster membership and node color indicates Reactome pathway enrichment significance.

Supplementary Figure 19



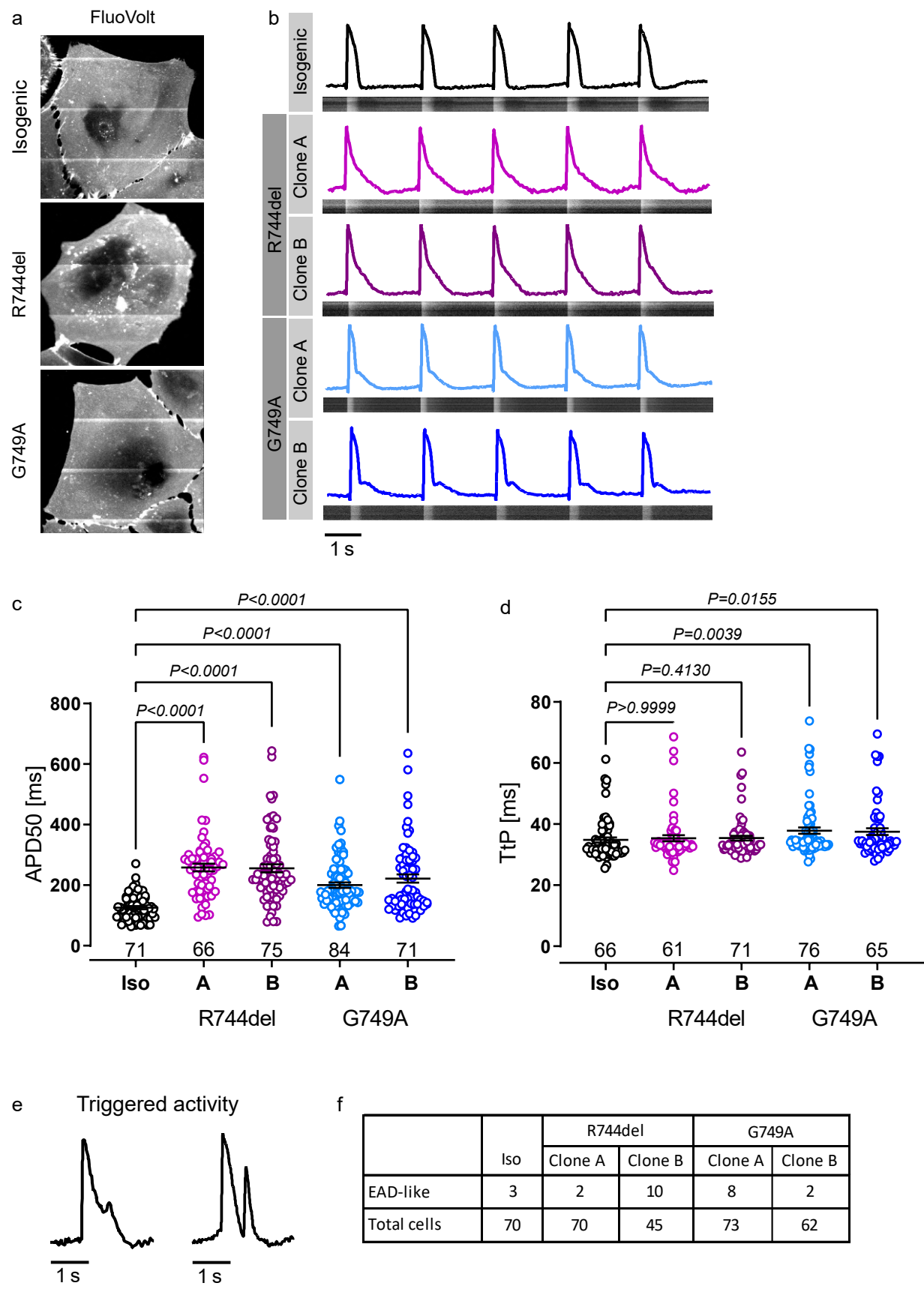
Supplementary Figure 19| Analysis of *KCNH2*-mutant differentiation efficiency to autonomic neurons and CMs. **a** Flow cytometry analysis of d30 CM stained for CM-marker ACTN2. (Uppermost panels). The gating strategy shows the exclusion of small debris based on size (forward scatter = FSC-A), granularity (side scatter = SSC-A), and DNA content (Hoechst-A). (Below) Histograms of ACTN2-staining fluorescence intensity distribution: ACTN2-stained CM are represented by blue curves and IgG1 isotype-stained negative controls are shown in grey. **b** qPCR expression analysis of myosin light chain's ventricular and atrial isoforms (*MLC2V* and *MLC2A*, respectively) in CM differentiation (d40-d60) from the *KCNH2* mutant lines and isogenic (Iso) control. 1 differentiation, n=4. **c** Immunofluorescent staining of MLC2A (magenta), MLC2V (yellow) and DNA (blue) in d60 CM shows predominant expression of the ventricular isoform. Atrial cells are marked by white arrowheads. Scale bar, 100 μ m. **d** mRNA expression of pan-neural marker *TUBB3*, SN markers *DBH* and *TH*, and cholinergic neuron marker *SLC18A3* and *CHAT* of d43 SNO from *KCNH2*-variant lines compared to isogenic control. All values are normalized to isogenic control. Isogenic control expression levels are represented by dashed lines, and grey shading indicates the s.e.m. 3 differentiations, n=3-4 tissues. *TUBB3*: n(Iso, R744del-A, G749A-B)=11, n(R744del-B, G749A-A)=11. *DBH*: n(Iso, G749A-A, G749A-B)=12, n(R744del-A, R744del-B)=11. *TH*: n(Iso, R744del-B, G749A-A, G749A-B)=12, n(R744del-A)=11. *SLC18A3*: n(Iso, R744del-B, G749A-A)=12, n(R744del-A, G749A-B)=11. *CHAT*: n(R744del-B)=10, n(Iso, R744del-A)=11, n(G749A-A, G749A-B)=12. All data is presented as mean $\pm$ s.e.m. One-way ANOVA with Holm-Šídák's multiple comparisons test was used for (**b**). One-way ANOVA or Kruskal-Wallis test with Dunn's multiple comparisons post hoc test was used for (**d**).

Supplementary Figure 20



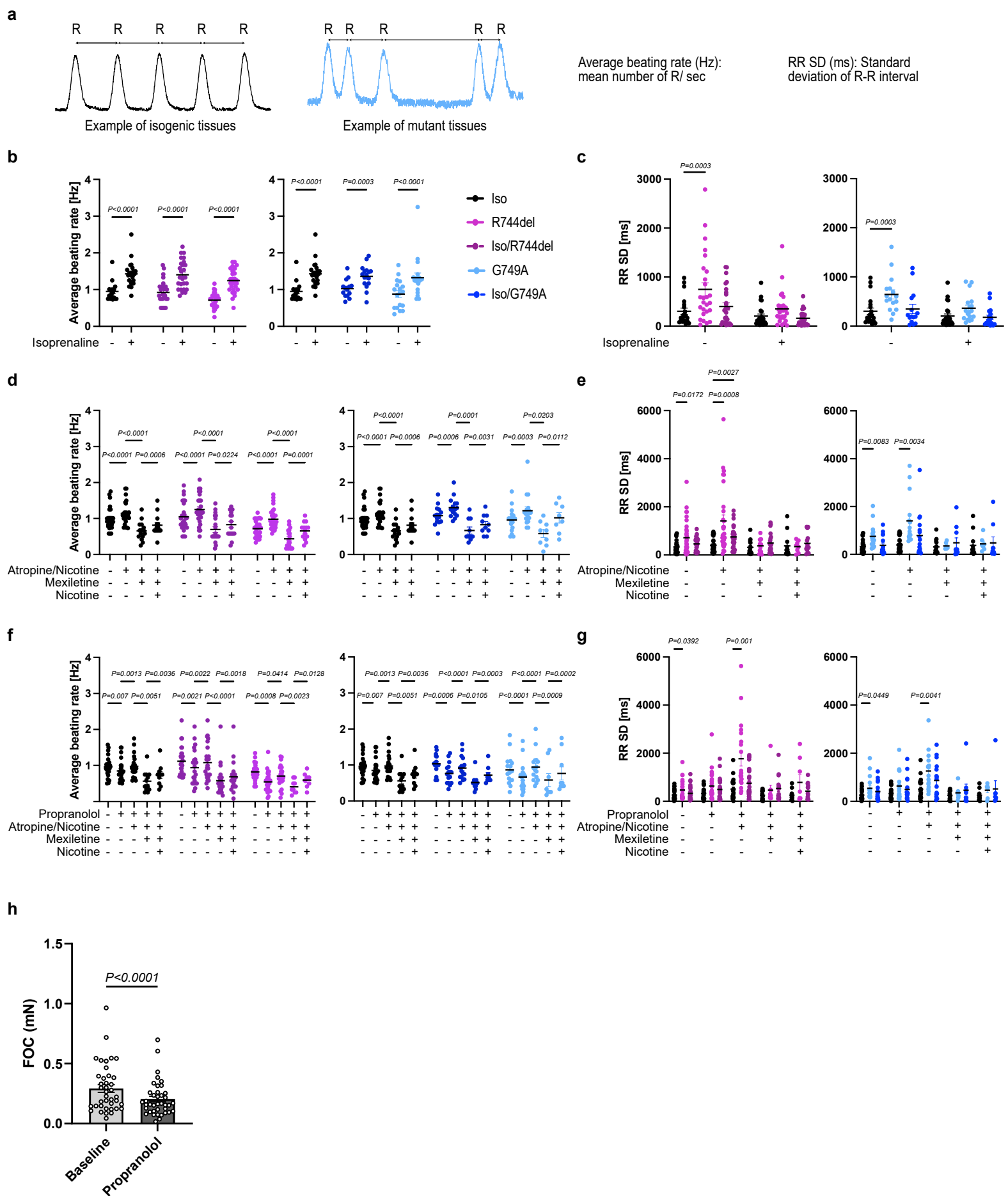
Supplementary Figure 20| *KCNH2* expression levels in CMs and sympathetic neurons. **a** qPCR analysis of *KCNH2* expression in *KCNH2*-mutant and isogenic control CM compared between d40 and d70 normalized to CM-marker gene *TNNT2*. n=10 / timepoint. **b** Immunofluorescence staining of hERG (cyan) and CM-marker ACTN2 (magenta) in hiPSC-CM at d70. (Lower panels) Detailed view illustrates endoplasmic reticulum and membrane localization of hERG channels. Detailed regions are indicated by white dashed rectangles. Scale bar, 30 μ m. **c** *KCNH2* transcript analysis in d43 SNO. Values were normalized to *KCNH2* expression in isogenic SNO. The dashed line and grey shaded area indicate isogenic mean expression and s.e.m., respectively. 3 differentiations, n(Iso, R744del-A)=11, n(R744del-B, G749A-A, G749A-B)=12. **d** hERG immunofluorescent staining (cyan) of SN marked by TH (magenta). Scale bar, 30 μ m. All data is presented as mean $\pm$ s.e.m. Unpaired two-tailed Mann-Whitney test was used for **(a)** and Kruskal-Wallis test with Dunn's multiple comparisons post hoc test was used for **(c)**.

Supplementary Figure 21



Supplementary Figure 21| Optical voltage imaging of *KCNH2* mutant vs isogenic CMs. a Example overview images of CM stained with voltage-sensitive FluoVolt™ dye. Scale bar, 50 μ m. **b** Representative traces of optical voltage imaging of ventricular CM from *KCNH2*-mutant lines and isogenic control at d60; 0.5 Hz electrical pacing rate. **c** Action potential durations at 50% repolarization (APD50) from voltage imaging analysis. 3 independent differentiations, n numbers are given in the graph. **d** Rise time (Time to peak = TtP) of *KCNH2*-mutant and isogenic CM calculated from FluoVolt™ voltage traces. 3 independent differentiations, n numbers are given in the graph. **e** Example traces from voltage imaging showing EAD-like events in p.R744del-mutant CM. **f** Quantification of EAD-like events for all lines. All data is presented as mean $\pm$ s.e.m. Kruskal-Wallis test with Dunn's multiple comparisons post hoc test was performed for (**c**, **d**).

Supplementary Figure 22



Supplementary Figure 22| Pharmacological modulation of rhythm variability and beating rate in iEHM carrying SUDEP-associated *KCNH2* mutations. **a** Representative contractility recordings from isogenic and hERG mutant (R744del, G749A) iEHM showing rhythmic force oscillations. Each peak corresponds to one contraction (R wave equivalent). The interval between consecutive contractions was used to calculate the R–R interval and its standard deviation (RR SD, in ms) as a measure of rhythm variability. The frequency of contractions was used to determine the average beating rate (Hz). **b, c** Quantifications of pharmacological responses showing the effects of β -adrenergic stimulation (isoprenaline) on beating rate and RR SD across isogenic and mutant iEHM. 3 independent differentiations, n(ISO)= 24, n(p.R744del)= 28, n(ISO/R744del)= 28, n(p.G749A)= 19, n(ISO/G749A)= 17. **d, e** Quantifications of pharmacological responses showing the effects of SN stimulation (atropine/nicotine), in the presence and absence of sodium channel inhibitor (mexiletine) on RR SD and beating rate across isogenic and mutant iEHM. 3 independent differentiations (Baseline and atropine/nicotine treatments: 3 differentiations, mexiletine and nicotine treatments: 2 differentiations). n(ISO)= 27, n(p.R744del)= 31, n(ISO/R744del)= 28, n(p.G749A)= 17, n(ISO/G749A)= 17. **f, g** Quantifications of pharmacological responses showing the effects of SN stimulation (atropine/nicotine), in the presence and absence of sodium channel inhibitor (mexiletine) and β -adrenoreceptor blocker (propranolol) on beating rate and RR SD across isogenic and mutant iEHM. 3 independent differentiations (baseline, propranolol and atropine/nicotine treatments: 3 differentiations, mexiletine and nicotine treatments: 2 differentiations). n(ISO)=26, n(p.R744del)= 26, n(ISO/R744del)= 27, n(p.G749A)= 18, n(ISO/G749A)= 17. **h** Force of contraction data showing decrease of contractile performance of iEHM treated with propranolol. 3 independent differentiations, n= 39. The data presented in c, e, g are single data points of each tissue corresponding to the graphs in Figure 5 d, f, h. Data is presented as mean $\pm$ s.e.m. Two-way ANOVA with Tukey's multiple comparisons post hoc test was performed for (**b-g**). Paired two-tailed Wilcoxon test was performed for (**h**).

Supplementary Table 1| Summarizing table comparing protocols and functional characterization of 2D and 3D SN culture systems.

Study	Starting cell population	Dimension	Differentiation factors		Cell contents	Functional analysis	Catecholamine content	Co-culture with hiPSC Cardiomyocytes	Functional connectivity
Oh et al 2016 ²	hESC (2 lines) hiPSC (1 line)	2D	AA BDNF BMP4 CHIR99021 DAPT db-cAMP FGF2	GDNF LDN193189 NGF PD173074 PMP SB431542 Shh C25il	SN* Enteric neurons NO Sensory neurons NO Motor neurons	Patch clamp (d18-d29) • Spontaneous firing • Response to Nicotine stimulation • Response to Optogenetic stimulation	ELISA: Secretion (d18-d29) • DA: 3.27 pmol/ml • NA: 0.27 pmol/ml	-	-
Frith et al 2018 ⁵⁰	hESC (3 lines) hiPSC (3 lines)	2D	AA BDNF BMP4 CHIR99021 DMH1 FGF2	GDNF NGF PMP SB431542 Shh C25il	SN**	Patch clamp (d19-d22) • Spontaneous firing	ELISA: Secretion (d19-d22) • DA: 2.94 pmol/ml • NA: 0.18 pmol/ml	-	-
Kirino et al 2018 ³	hESC (2 lines) hiPSC (2 lines)	2D	BDNF BMP4 CHIR99021 EGF FGF2	GDNF Heparin NGF RA SB431542	SN**	-	ELISA: Secretion (d40) • NA: 0.24-0.44 pmol/ml	-	-
Takayama et al 2020 ²⁴	hESC (1 line) hiPSC (2 lines)	2D	AA BDNF BMP4 CHIR99021 DM FGF2 Forskolin	GDNF IWR1 NGF NT3 SANT1 SB431542	SN PNS Enteric neurons Non-neuronal (Stromal cells, others) GABAergic neurons	MEA • Response to Optogenetic stimulation Calcium imaging (d45-d49) • Rare spontaneous firing • Response to Electrical stimulation • Response to Nicotine stimulation	-	2D	+
Winbo et al 2020 ⁵³	hiPSC (1 line)	2D	AA BDNF BMP4 CHIR99021 DAPT db-cAMP	GDNF LDN193189 NGF PD173074 PMP SB431542 Shh C25il	SN**	Patch clamp (d28-d76) • Spontaneous firing • Response to Tetrodotoxin	ELISA: Secretion (d75) • NA: 0-0.37 pmol/ml	2D	+
Wu & Zeltner 2020 ⁵¹	hiPSC (2 lines)	2D	AA BDNF BMP4 CHIR99021 db-cAMP	FGF2 GDNF NGF RA SB431542	SN PNS Sensory neurons Enteric neurons Motor neurons Myofibroblasts	MEA (d20-d30) • Spontaneous firing • Response to Nicotine stimulation • Response to Propranolol	-	-	-
Bernardin et al 2022 ⁵²	Rat PC12 hiPSC (1 line)	2D	Rat PC12: NGF hiPSC: Winbo et al 2020		SN** PNS	-	LCMS: Secretion (d14) • L-DOPA: 0.004 pmol/ml • DA: 0.215 pmol/ml • NA: 0.004 pmol/ml Fluorometric kit: Secretion (d14) • ACh: 24.39 pmol/ml	2D	-
Haekli et al 2022 ²²	hiPSC (1 line)	2D	AA BDNF db-cAMP FGF2	GDNF LDN193189 RA SB431542	SN** PNS	-	-	2D	+
Olmsted & Paluh 2022 ⁵⁷	hiPSC (1 line)	3D	AA CHIR99021 FGF2	HGF IGF1 VEGF	Peripheral progenitors Anterior foregut Motor neurons Sensory neurons Cardiac cells	-	-	3D	-
Wu et al 2024 ⁵⁴	hiPSC (1 line)	2D	AA BDNF BMP4 CHIR99021 db-cAMP	FGF2 GDNF NGF SB431542 Amphi	SN**	MEA (d20/d30+) • Spontaneous firing Calcium imaging (d30+)	-	-	-
Liu, Zhu, Lu et al 2026 ²⁶	hiPSC (1 line) hESC (2 lines)	3D	AA BMP4 CHIR99021 DAPT FGF2	GDNF NGF RA SAG SB431542	SN	MEA (d70-d80, d130-d150) • Spontaneous firing • Response to Acetylcholine & Nicotine	-	3D	+
Branco et al 2026 ²⁵	hiPSC (1 line)	3D	AA BDNF CHIR99021 DAPT GDNF	LDN193189 NGF SAG SB431542	SN**	MEA (d50-d62) • Spontaneous firing • Response to Nicotine	ELISA: Secretion (3 days culture) • DA: 1.76 pmol/ml • NA: 0.33 pmol/ml	3D	+
Schneider & Setya et al	hiPSC (3 lines)	3D	BMP4 CHIR99021 DAPT FGF2	LDN193189 RA SB431542 TGFβ1	SN PSN Sensory neurons Glutamatergic neurons NO Motor neurons	MEA (d28-d56) • Spontaneous firing • Response to Nicotine stimulation • Response to Optogenetic stimulation	LCMS: Cell lysate (d41) • DA: 6.18 pmol/mg • NA: 60.4 pmol/mg • ACh: 19 pmol/mg	3D	+

AA: Ascorbic acid, ACh: Acetylcholine, Amphi: Amphidicolin, BDNF: Brain-derived neurotrophic factor, BMP4: Bone morphogenetic protein 4, CHIR99021: GSK3 inhibitor/Wnt activator, DA: Dopamine, DAPT: γ-secretase inhibitor, db-cAMP: Dibutyl-γ-cAMP, DM: Dorsomorphin, DMH1: Dorsomorphin homolog 1, EGF: Epidermal growth factor, FGF2: Fibroblast growth factor 2, GDNF:

Glial cell-derived neurotrophic factor, hESC: Human embryonic stem cell, hiPSC: Human induced pluripotent stem cell, HGF: Hepatocyte growth factor, IGF1: Insulin growth factor 1, IWR1: Wnt pathway inhibitor, LDN193189: BMP pathway inhibitor, NA: Noradrenaline, NGF: Nerve growth factor, NT3: Neurotrophin-3, PD173074: FGFR1 inhibitor, PMP: Purmorphamine, RA: Retinoic acid, SAG: Smoothed agonist, SANT1: Sonic hedgehog pathway inhibitor, SB431542: TGF β pathway inhibitor, Shh C25II: Sonic hedgehog pathway agonist, TGF β 1: Transforming growth factor beta-1, VEGF: Vascular endothelial growth factor

*No PNS marker tested / **No other neuronal marker tested

Supplementary Table 2| List of primary and secondary antibodies

Antigen	Modification	Brand	Cat. No.	Host	Dilution	Type
ACTN2	None	Sigma	A7811	Mouse	1:1000	Primary
BRN3A	None	Merck Millipore	MAB1585	Mouse	1:500	Primary
CTNT	None	Abcam	ab45932	Rabbit	1:250	Primary
CD45	None	Abcam	Ab8216	Mouse	1:100	Primary
CD68	None	Synaptic Systems	HS-460 008	Rabbit	1:100	Primary
DBH	None	Sigma	HPA070789	Rabbit	1:100	Primary
HCN4	None	Abcam	ab69054	Rabbit	1:200	Primary
Neurexin 1	None	Sigma	HPA071400	Rabbit	1:200	Primary
NF (H)	None	Synaptic Systems	171 106	Chicken	1:100	Primary
NF (H)	None	Biologend	822601	Chicken	1:1000	Primary
PAX6	None	Biologend	901301	Mouse	1:500	Primary
PDGFRb	None	Cell Signalling	28E1	Rabbit	1:100	Primary
PECAM1	None	DAKO	M0823	Mouse	1:100	Primary
Peripherin	None	NOVUS	NBP1- 05423	Chicken	1:1000	Primary
Phalloidin	Alexa Fluor 488	Invitrogen	A12379	-	1:200	Coupled
PHOX2B	None	Santa Cruz	sc-376997	Mouse	1:250	Primary
Synapsin 1	None	Synaptic systems	106 011	Mouse	1:200	Primary
TH	None	Synaptic Systems	213 004	Guinea pig	1:500	Primary
VACHT	None	Synaptic Systems	139 103	Rabbit	1:500	Primary
VGLUT1	Abberior STAR 635P	NanoTag	N1602- Abb635P	Rabbit	1:250	Coupled
Mouse	Alexa Fluor 633	Invitrogen	A21052	Goat	1:400	Secondary
Chicken	Alexa Fluor 488	Invitrogen	A11039	Goat	1:400	Secondary
Rabbit	Alexa Fluor 546	Invitrogen	A11035	Goat	1:400	Secondary
Guinea Pig	Alexa Fluor 546	Invitrogen	A11074	Goat	1:400	Secondary

Supplementary Table 3| Detection parameters for a second compound-specific mass transition used as qualifier. Q1=first quadrupole, Q3=third quadrupole, DP=declustering potential, CE=collision energy, CXP=collision cell exit potential.

Compound	Mass Q1 [Da]	Mass Q3 [Da]	DP [V]	CE [V]	CXP [V]
NA	170.2	152.0 (107.0)	36	10 -28	10 -20
Dopamine	154.1	137.2 (91.0)	36	15 -33	10 -8
Acetylcholine	147.0	87.0 (88.0)	36	19 -20	16 -16
NA-d6	176.1	158.0 (111.0)	31	11 -29	10 -6
Buformin	157.91	60.9 (47.0)	36	35 -66	10 -8
Choline-d9	113.108	69.1 (66.1)	66	27 -44	12 -12

Supplementary Table 4| List of primer for quantitative real-time PCR

Gene	Forward primer	Reverse primer	Amplicon length (bp)
<i>ASCL1</i>	CGGTCTCATCCTACTCGTCG	GTTGTGCGATCACCTGCTT	150
<i>CHAT</i>	GGATCGCTGGACATGATTG	CCACACCGCCAGGTGCG	103
<i>DBH</i>	TGGGTGCCAAGGCATTTAC	ATGCCTGAGGAGTCGTTTCG	136
<i>GAD1</i>	AGGCAATCCTCCAAGAACC	TGAAAGTCCAGCACCTTGG	218
<i>GAPDH</i>	CCTCAAGATCATCAGCAATGCC	ATGTTCTGGAGAGCCCCGC	189
<i>GATA2</i>	GCAGAACCGACCACTCATCA	AGTGGCCTGTTAACATTGTGC	177
<i>GBX2</i>	GTTCCCGCCGTCGCTGATGAT	GCCGGTGTAGACGAAATGGCCG	118
<i>GLAST</i>	CCCCTTACAAAATCAGAAAAGTTGT	CCCATCTTGGGCTCTTCTCC	151
<i>MNX1</i>	TGCCTAAGATGCCGACTTC	AATCTTCACCTGGGTCTCGG	194
<i>OLIG2</i>	GCTGCGTCTCAAGATC	AGTCGCTTCATCTCCT	192
<i>PAX6</i>	CCCCACATATGCAGACACAC	TCACTTCCGGGAACCTTGAAC	112
<i>PHOX2B</i>	CGCCGCAGTTCCTTACAAAC	TGTCGGGGTAGTGAGTCTCC	140
<i>PLP1</i>	GGTTTCCCTGCTCACCTTCA	ATCAGAACTTGGTGCCTCGG	83
<i>PRPH</i>	TAAATATAAAGACGACTGTGCCTGA	GCAGAAAGACTTGTCCAGCTCA	151
<i>PRRX1</i>	CGCAGGAATGAGAGAGCCAT	GAGGGACGAGGATCTGTACG	165
<i>SLC17A6</i>	GTAGACTGGCAACCACCTCC	CCATTCCAAAGCTTCCGTAGAC	129
<i>SLC18A2</i>	TTCCGACTGTCCCAGTGAAG	TGCTGGAGAAGGCAAACATAAT	200
<i>SLC6A2</i>	CAGGTTCAAGCAACGACATCCAG	GTCGTAGGTGAGTGGCTTGAAG	142
<i>SOX10</i>	TCTGGAGGCTGCTGAACGAA	GTAGTGGGCCTGGATGGC	200
<i>TH</i>	CGGGGCTTCTCGGACCAGGTGTA	CTCCTCGGCGGTGTACTCCACA	111
<i>TUBB3</i>	CAGCGTCTACTACAACGAGG	AGGCCTGAAGAGATGTCCAA	121
<i>TWIST1</i>	GCCGGAGACCTAGATGTCATT	TTTTAAAAGTGCGCCCCACG	165
<i>SLC18A3</i>	CTGCTAGTGAACCCCTTGAGC	CAGGACTGTAGAGGCGAACAT	99