

Human assembloid model of the ascending neural sensory pathway

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Somatosensory pathways convey crucial information about pain, touch, itch and body part movement from peripheral organs to the central nervous system^{1,2}. Despite substantial needs to understand how these pathways assemble and to develop pain therapeutics, clinical translation remains challenging. This is probably related to species-specific features and the lack of in vitro models of the polysynaptic pathway. Here we established a human ascending somatosensory assembloid (hASA), a four-part assembloid generated from human pluripotent stem cells that integrates somatosensory, spinal, thalamic and cortical organoids to model the spinothalamic pathway. Transcriptomic profiling confirmed the presence of key cell types of this circuit. Rabies tracing and calcium imaging showed that sensory neurons connect to dorsal spinal cord neurons, which further connect to thalamic neurons. Following noxious chemical stimulation, calcium imaging of hASA demonstrated a coordinated response. In addition, extracellular recordings and imaging revealed synchronized activity across the assembloid. Notably, loss of the sodium channel $Na_v1.7$, which causes pain insensitivity, disrupted synchrony across hASA. By contrast, a gain-of-function *SCN9A* variant associated with extreme pain disorder induced hypersynchrony. These experiments demonstrated the ability to functionally assemble the essential components of the human sensory pathway, which could accelerate our understanding of sensory circuits and facilitate therapeutic development.

The ascending somatosensory pathways are responsible for conveying environmental and bodily sensory information from the peripheral organs to the central nervous system. Somatosensory neurons, whose cell bodies are located in the dorsal root ganglia (DRG) and trigeminal ganglia (TG), are pseudo-unipolar cells that innervate peripheral organs, including the skin, to detect somatosensory signals and transmit them to the spinal cord and hindbrain. Neurons in the dorsal spinal cord or hindbrain monosynaptically transmit this information to various brain structures, including the thalamus, that further relay these signals to the cerebral cortex^{1,2}. Genetic or environmental disruptions to these pathways can lead or contribute to various neurological disorders, including chronic pain and autism spectrum disorder, and cause both heightened and diminished sensory functions^{3–6}. Despite the clinical significance, how ascending sensory pathways form during human development and how functional defects arise in pathological conditions during development are still poorly understood. Challenges in bench to bedside translation of pain and sensory research are thought to be related, at least in part, to differences across species. Crucially, the discovery of new treatments and their translation is hindered by the lack of reliable human experimental models that reconstitute the ascending sensory

pathway⁷. In fact, concomitant probing of all components of these circuits through imaging or recording has not yet been achieved in animal models, which limits our understanding of the consequences of various sensory manipulations at the circuit level.

Recent advances in organoid and assembloid technologies hold promise for generating self-organizing stem-cell-based models to study and manipulate human neural circuits *ex vivo* in the context of development and disease states. We previously developed three-part assembloids to recapitulate the descending cortico-spino-muscle pathway connecting deep layer cortical glutamatergic neurons to cholinergic spinal cord motor neurons to muscle⁸. The generation of functional circuits in assembloids with more than three components, which would enable the assembly of the ascending sensory pathway from sensory neurons to the cerebral cortex, has not yet been achieved.

Here we focus on the spinothalamic and trigeminothalamic pathways, which are particularly prominent in humans⁹ and are crucial for conveying pain and other somatosensory information through the spinal cord and thalamus¹. In this circuit, sensory neurons in the DRG or TG connect with neurons in the dorsal spinal cord or hindbrain. These neurons relay information to the thalamus through monosynaptic

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connections and subsequently to the somatosensory cortex and other cortical areas (Fig. 1a). To reconstitute these pathways from their components, we separately generated somatosensory organoids, dorsal spinal cord organoids, diencephalic (thalamic) organoids and cerebral cortical organoids from pluripotent stem cells and then functionally assembled them to create four-part human ascending somatosensory assembloids (hASAs). We demonstrated that chemical stimulation or glutamate uncaging of sensory neurons elicited responses within hASA. Calcium imaging and electrophysiological recordings of the assembloid revealed synchronized activity across the ascending pathway. Finally, we applied this platform to study circuit-level consequences of loss- and gain-of-function pathogenic variants in the $\text{Na}_v1.7$ sodium channel encoded by the *SCN9A* gene, which have been associated with congenital insensitivity to pain and extreme pain disorder, respectively. We discovered that the loss of $\text{Na}_v1.7$ disrupted synchronized activity across the four-part hASA, whereas the gain-of-function mutation resulted in hypersynchrony throughout the sensory pathway in hASA.

Generation of key components of the somatosensory pathway

To derive the key parts of the ascending sensory pathway in vitro, we generated regionalized neural organoids from human induced pluripotent stem (hiPS) cells using guided differentiation with small molecules and growth factors (Fig. 1b and Extended Data Fig. 1). We first generated human cortical organoids (hCO) and human diencephalic organoids (hDiO) using previously described and validated methods^{10,11}. To generate human dorsal spinal cord (and hindbrain) organoids (hdSpO), where the soma of spinothalamic (or trigeminothalamic) projection neurons resides, we modified a protocol we have previously reported for deriving the ventral part of cervical spinal cord organoids⁸ by excluding ventralizing cues (Extended Data Fig. 1). Finally, to derive human somatosensory (also referred to as sensory) organoids (hSeO), which contain somatosensory neurons of the DRG or TG, we developed a protocol that leveraged cues identified in two-dimensional-based methods¹² (Extended Data Fig. 1). To verify that we have all key cellular components in the ascending sensory pathway, we combined the single-cell RNA sequencing (scRNA-seq) data of hCO and hDiO that we generated in previous studies^{11,13} with our newly obtained transcriptional datasets of hdSpO and hSeO (Fig. 1c–e). We found distinct clusters for key cell types in the ascending circuit: cortical glutamatergic neurons (*FOXP1*⁺ (ref. 14) and *SLC17A7*⁺), thalamic excitatory neurons (*TCF7L2*⁺ (ref. 15) and *SLC17A6*⁺), dorsal spinal cord and hindbrain projection neurons (*HOXB4*⁺ (ref. 16) and *PHOX2A*⁺ (ref. 17)) and primary afferent somatosensory neurons (*POU4F1*⁺ (ref. 18) and *PPP1R1C*⁺ (ref. 19)) (Fig. 1f). We used immunostaining to confirm the protein expression of key region-specific markers in the regionalized organoids (Fig. 1g). To further investigate cell identity in regionalized neural organoids, we performed label transfer analysis²⁰ from a human primary tissue atlas of the whole brain (PCW7–14)²¹. Given the paucity of publicly available scRNA-seq datasets of developing human DRG²², we procured human primary tissue (at PCW15) and freshly dissected DRG and performed scRNA-seq (Fig. 1h,i and Extended Data Fig. 2a–d). Overall, we found that cells derived from each regionalized organoid resembled the transcriptomic profiles of their corresponding in vivo counterparts (Fig. 1j and Extended Data Fig. 2e).

Characterization of hSeO and hdSpO

To further characterize the cellular identity within hSeO, we inspected the pattern of expression of several canonical markers using scRNA-seq data (Fig. 2a,b and Extended Data Fig. 3a,b). We found that approximately 30–40% of the neurons (*STMN2*⁺ (ref. 23)) expressed the peripheral

neuron-specific markers *POU4F1* and *SIX1* (ref. 24) (Fig. 2c), which we independently confirmed in other batches of organoids by reverse transcription–quantitative polymerase chain reaction (RT–qPCR) (Extended Data Fig. 3c). Co-localization of VGLUT2 and NeuN in immunostaining experiments demonstrated the excitatory neuronal identity of hSeO neurons (Extended Data Fig. 3d). We found a substantial proportion of cells expressing neural crest cell markers, such as *SOX10* and *FOXD3*, which is consistent with the neural crest origin of peripheral sensory neurons²⁵ (Fig. 2b,c and Extended Data Fig. 3b,e). We also examined the expression levels of several key sensory receptors^{26–28} (*P2RX3*, *TRPV1*, *TRPM8*, *TRPA1*, *PIEZO1* and *PIEZO2*) (Fig. 2d and Extended Data Fig. 3f). To further characterize the cellular composition of hSeO, we compared the single-cell transcriptome of hSeO with the scRNA-seq data of the human DRG. Integrated uniform manifold approximation and projection (UMAP) and cell composition proportions revealed similar cellular compositions, except for endothelial cells, macrophages and blood cells, which were absent in hSeO (Extended Data Fig. 3g–k).

A unique feature of the sensory neurons is their ability to respond to environmental stimuli. To verify whether neurons in hSeO respond to sensory stimuli, we established a live imaging system of intact three-dimensional (3D) organoids expressing a genetically encoded calcium indicator using a chamber equipped to inject chemicals. Consistent with the high abundance of mRNA encoding calcium-permeable P2X purinoceptor 3 (*P2RX3*) and capsaicin receptor *TRPV1* (Fig. 2d), bath application of their agonists, α,β -methyleneATP ($\alpha\beta$ -MeATP)²⁶ and capsaicin²⁹, respectively, induced calcium transients (Fig. 2e–h). Interestingly, the dynamics of the responses were similar between hSeO and intact ex vivo primary mouse DRG (Extended Data Fig. 4a–d); $\alpha\beta$ -MeATP induced a fast calcium spike, whereas capsaicin induced a slow and prolonged calcium transient, which is consistent with previous pharmacological studies using DRG neurons isolated from rat^{30,31} or mouse^{32,33}, as well as induced nociceptors in two-dimensional culture³⁴.

There are species-specific differences in the expression and function of some receptors on sensory DRG neurons, which may be responsible for sensory processing differences and challenges in translation from animal models to humans⁷. For instance, 2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate (TNP–ATP) is a potent antagonist of the rodent *P2X3* receptor³⁵; however, primate-specific amino acid substitutions in the *P2X3* receptor are thought to modify sensitivity to TNP–ATP in the macaque DRG³⁶. Consistent with previous reports³⁶, we found that TNP–ATP successfully blocked calcium responses in ex vivo mouse DRG in a dose-dependent manner. However, when applied to human sensory neurons in hSeO, TNP–ATP had no significant effect (Fig. 2i–m), which suggests differences in responsiveness across species. These results are consistent with a previous report showing the importance of primate-specific amino acid changes at two residues of the *P2X3* receptor (A197 and T202), which influence the TNP–ATP antagonism³⁶.

We further investigated the cellular identity of neurons in hdSpO using scRNA-seq data (Fig. 2n,o and Extended Data Fig. 5a). Similar to the ventral part of the spinal cord organoids⁸, hdSpO displayed high neuronal diversity (Fig. 2o,p and Extended Data Fig. 5a–c). Relevant to the ascending somatosensory pathway, we found clusters expressing the projection neuronal markers *PHOX2A*¹⁷, *LBX1* (ref. 17) and *TACR1* (encoding NKIR)³⁷ (Fig. 2p and Extended Data Fig. 5b). We also confirmed the expression of *TACR1* and *LBX1* by RT–qPCR in hdSpO (Extended Data Fig. 5d). We next annotated the dorsoventral neuronal clusters in hdSpO using combinatorial marker expression, as described previously^{38,39} (Extended Data Fig. 5e). We found overlap of *PHOX2A*⁺ neurons with d15 cluster, which is consistent with previous reports^{17,40} (Fig. 2q). We also performed label transfer analysis of scRNA-seq data from the developing human spinal cord d15 cells³⁸ onto the hdSpO scRNA-seq dataset. We found that the *SLC17A6*⁺PH

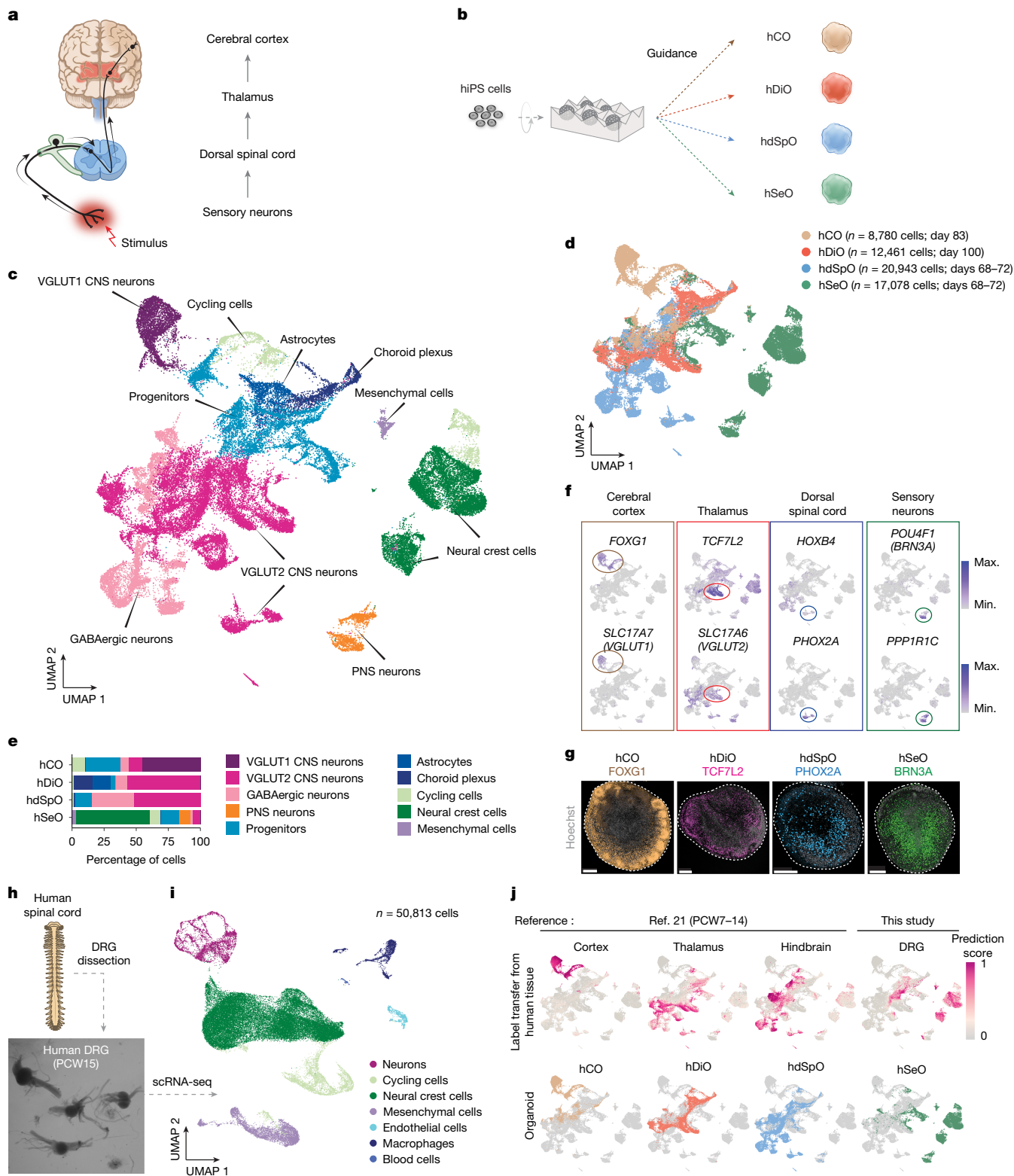


Fig. 1 | Generation from hiPS cells of components of human ascending sensory pathway. **a**, Schematic illustrating the key components of the ascending anterolateral sensory pathway. **b**, Schematic illustrating the generation of hCO, hDiO, hdSpO and hSeO from hiPS cells. **c**, UMAP visualization of single-cell gene expression of hCO (day 83; $n = 8,780$ cells from two hiPS cell lines¹³), hDiO (day 100; $n = 12,461$ cells from three hiPS cell lines¹¹), hdSpO (days 68–72; $n = 20,943$ cells from five hiPS cell lines) and hSeO (days 68–72; $n = 17,078$ cells from four hiPS cell lines). **d**, UMAP plot coloured by the organoid identity. **e**, Cell composition in each regionalized neural organoid based on scRNA-seq data. **f**, Gene expression level for regional identity markers. **g**, Immunostaining

for specific regional markers in hCO, hDiO, hdSpO and hSeO on day 63. **h**, Schematic illustrating the establishment of an scRNA-seq atlas of human DRG. **i**, UMAP visualization of scRNA-seq of human DRG ($n = 50,813$ cells from seven isolated DRG at PCW15). **j**, Label transfer of the human tissue datasets onto the organoid dataset. UMAP plots were coloured using the prediction score for each brain subregion (top) and organoid identity (bottom). Scale bar, 200 μm (**g**). CNS, central nervous system; max., maximum; min., minimum; PNS, peripheral nervous system. Credits: **b** adapted with permission from ref. 11, Elsevier; **h** adapted from ref. 54, Springer Nature America, Inc.

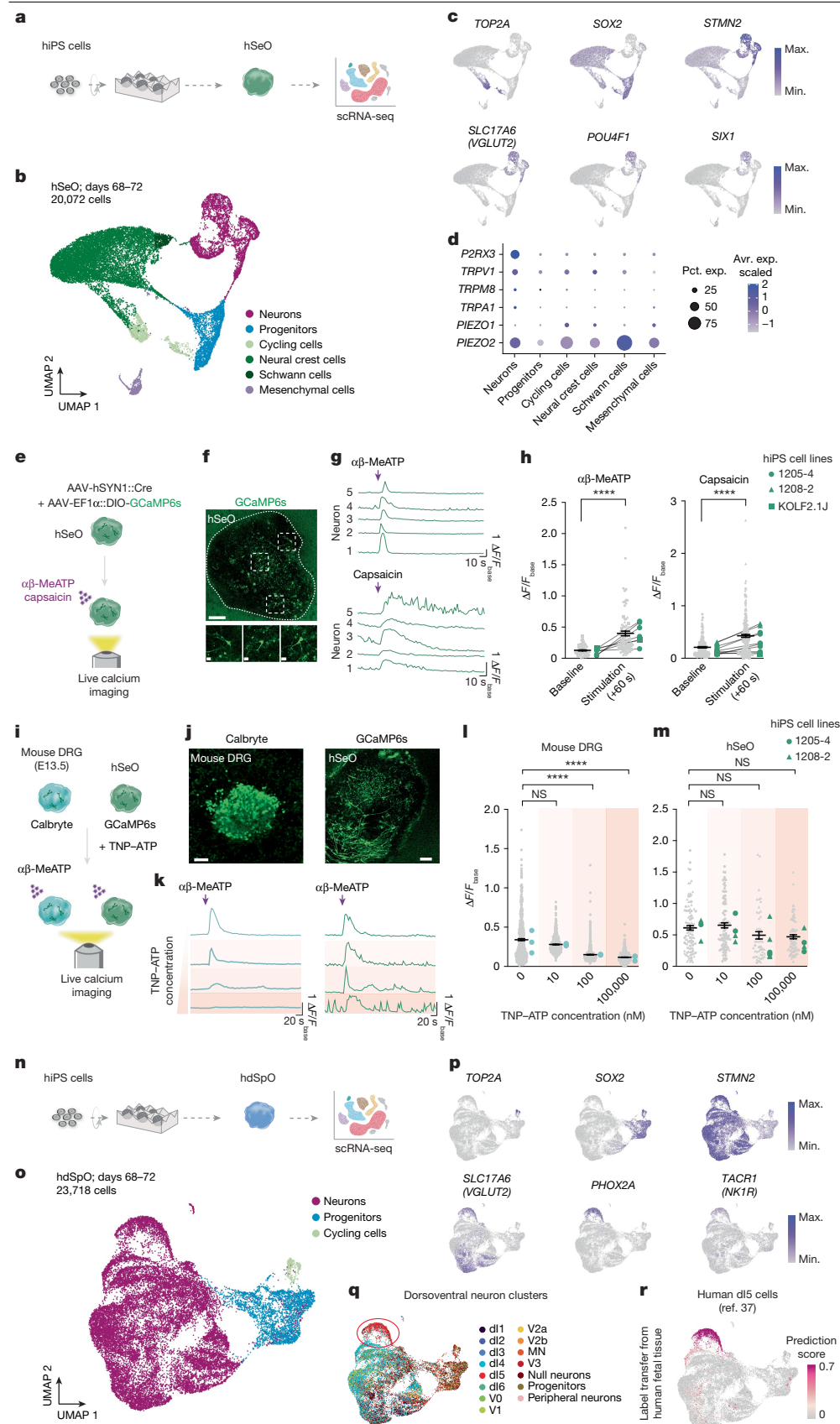


Fig. 2 | Characterization of hSeO and hdSpO. **a**, Generation of hSeO from hiPS cells and scRNA-seq analysis. **b**, UMAP of scRNA-seq of hSeO on days 68–72 ($n = 20,072$ cells; the same dataset as in Fig. 1 with optimized QC criteria). **c**, UMAP of cluster-specific markers. **d**, Expression of key sensory receptors. **e**, Calcium imaging for pharmacological studies. **f**, GCaMP6s expression in hSeO. **g**, $\Delta F/F_{\text{base}}$ traces showing $\alpha\beta$ -MeATP and capsaicin-evoked calcium responses. **h**, Mean $\Delta F/F_{\text{base}}$ values at baseline and after $\alpha\beta$ -MeATP ($n = 88$ neurons; seven hSeO; three differentiations) and capsaicin ($n = 205$ cells; ten hSeO; three differentiations) on days 54–82. Wilcoxon matched-pair signed-rank test; **** $P < 0.0001$. **i**, Calcium imaging following TNP-ATP and $\alpha\beta$ -MeATP exposure of ex vivo mouse DRG and hSeO. **j**, Calbryte-incubated mouse DRG and GCaMP6s-expressing hSeO. **k**, Response after $\alpha\beta$ -MeATP and TNP-ATP exposure of mouse DRG and hSeO. **l**, **m**, Responses after exposure to $\alpha\beta$ -MeATP with various TNP-ATP concentrations of mouse DRG (**l**) ($n = 518, 605, 652$ and $1,392$ cells from three, three, three and four mouse DRG, respectively) and hSeO (**m**) ($n = 110, 99, 50$ and 77 cells from three, four, six and four hSeO; two differentiations on days 75–82). Kruskal–Wallis test with Dunn’s test; **** $P < 0.0001$. **n**, Generation of hdSpO from hiPS cells and scRNA-seq analysis. **o**, UMAP of scRNA-seq of hdSpO on days 68–72 ($n = 23,718$ cells; the same dataset as in Fig. 1 with optimized QC criteria). **p**, UMAP of cluster-specific markers. **q**, UMAP showing dorsoventral clusters (circle; d15 cluster). **r**, Label transfer of human spinal cord datasets onto hdSpO (UMAP coloured using d15 prediction score). Data: mean $\pm$ s.e.m. Small dots, cells; large dots, organoids or mouse DRG. NS, not significant. Scale bars, $150 \mu\text{m}$ (**f** (top)), $20 \mu\text{m}$ (**f** (bottom)), $100 \mu\text{m}$ (**j**). Pct. exp., percentage expression; avr. exp., averaged expression. Credits: **a**, **n** adapted with permission from ref. 11, Elsevier; **e**, **i** adapted from ref. 48, Springer Nature America, Inc.

OX2A⁺*TACR1*⁺ neuron cluster demonstrated the highest prediction score values for the developing human d15 cells, indicating that hdSpO contains a neuronal cluster resembling the transcriptomic

identity of human d15 cells (Fig. 2r). These cells also express another d15 marker gene, *LMX1B*⁴⁰, but not the d14/6 marker gene *PAX2* (ref. 41) (Extended Data Fig. 5b,e). Immunostaining for *PAX2* and

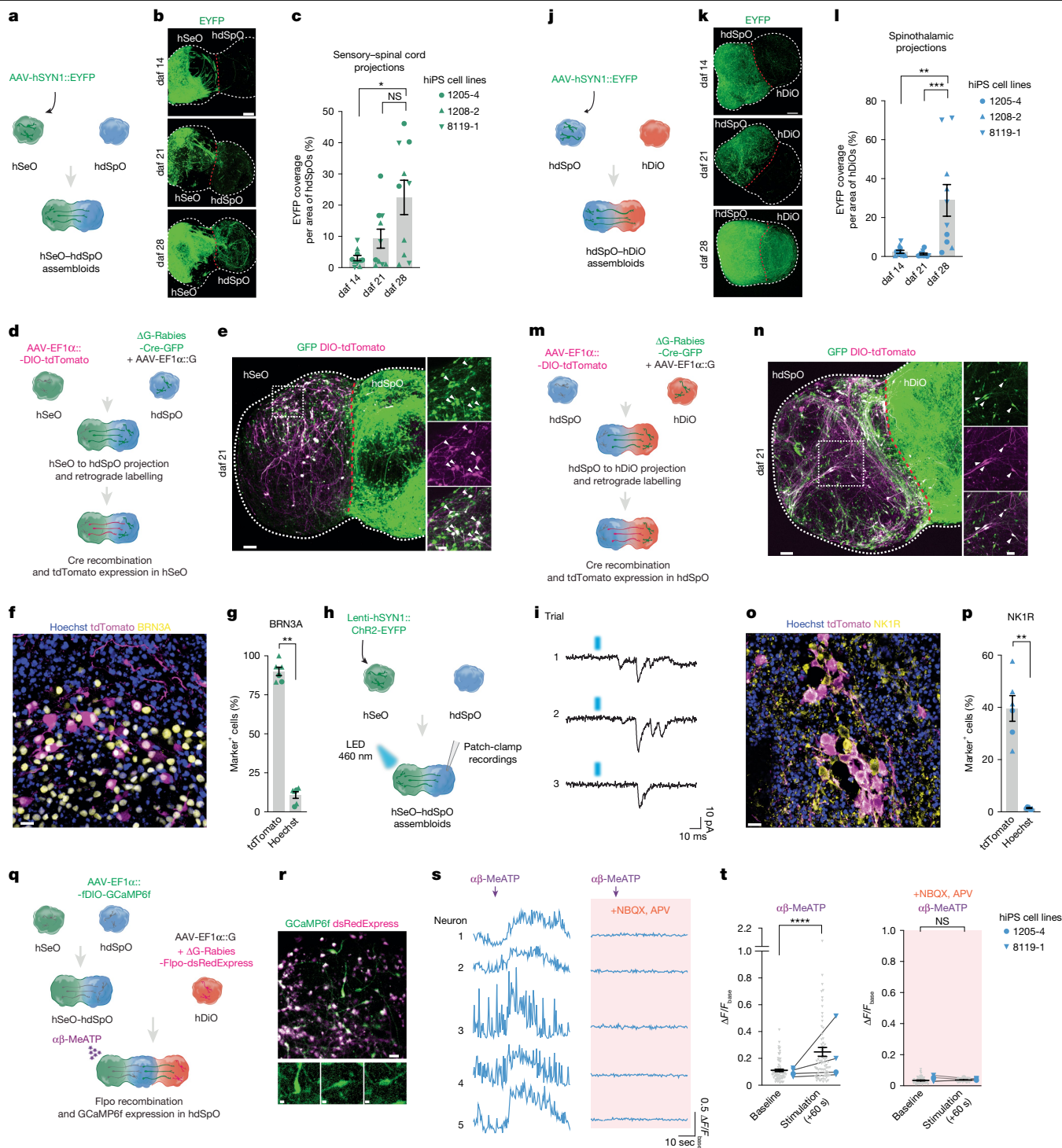


Fig. 3 | Connectivity in hSeO-hdSpO and hdSpO-hdDiO assembloids.

a, Generation of hSeO-hdSpO assembloids. **b**, Gradual increase in sensory-spinal cord projections in hSeO-hdSpO. **c**, Quantification of EYFP in the hdSpO side of assembloids ($n = 10$ assembloids; three differentiations; Kruskal-Wallis test with Dunn's test; $^{*}P = 0.0267$). **d**, Retrograde rabies viral tracing in hSeO-hdSpO. **e**, EGFP⁺tdTomato⁺ cells in hSeO side. **f**, BRN3A⁺tdTomato⁺ co-localization in hSeO side. **g**, Percentage of BRN3A⁺ cells of tdTomato⁺ or Hoechst⁺ cells in hSeO side of assembloids ($n = 6$ assembloids; three differentiations; Mann-Whitney test; $^{*}P = 0.0022$). **h**, Patch-clamp recordings with optogenetic stimulation of hSeO neurons. **i**, eIPSC traces from three trials in the same neuron. **j**, Generation of hdSpO-hdDiO assembloids. **k**, Gradual increase in spinothalamic projections in hdSpO-hdDiO. **l**, Quantification of EYFP in the hdDiO side of assembloids ($n = 10-11$ assembloids; three differentiations; Kruskal-Wallis test with Dunn's test; $^{*}P = 0.0050$; $^{***}P = 0.0003$). **m**, Retrograde rabies viral tracing

in hdSpO-hdDiO. **n**, EGFP⁺tdTomato⁺ cells in the hdSpO side of assembloids. **o**, NK1R⁺tdTomato⁺ co-localization in the hdSpO side of assembloids. **p**, Percentage of NK1R⁺ cells of tdTomato⁺ or Hoechst⁺ cells in the hdSpO side of assembloids ($n = 6$ assembloids; three differentiations; Mann-Whitney test; $^{*}P = 0.0022$). **q**, Calcium imaging of spinothalamic neurons with retrograde viral tracing in hSeO-hdSpO-hdDiO. **r**, GCaMP6f⁺dsRedExpress⁺ co-expression in the hdSpO side of assembloids. **s**, αβ-MeATP-evoked responses and blockade by NBQX and APV (50 μM each). **t**, Responses to αβ-MeATP with or without NBQX and APV ($n = 74$ cells; four assembloids; paired t -test; $^{****}P < 0.0001$) on daf 70-88. Data: mean ± s.e.m. Small dots, cells; large dots, assembloids. Scale bars, 100 μm (**b**, **e** (left), **k**, **n** (left)), 50 μm (**n** (right), **r** (top)), 20 μm (**f**, **o**), 10 μm (**r** (bottom)). Credits: **a**, **d**, **h**, **j**, **m**, **q** adapted from ref. 48, Springer Nature America, Inc.

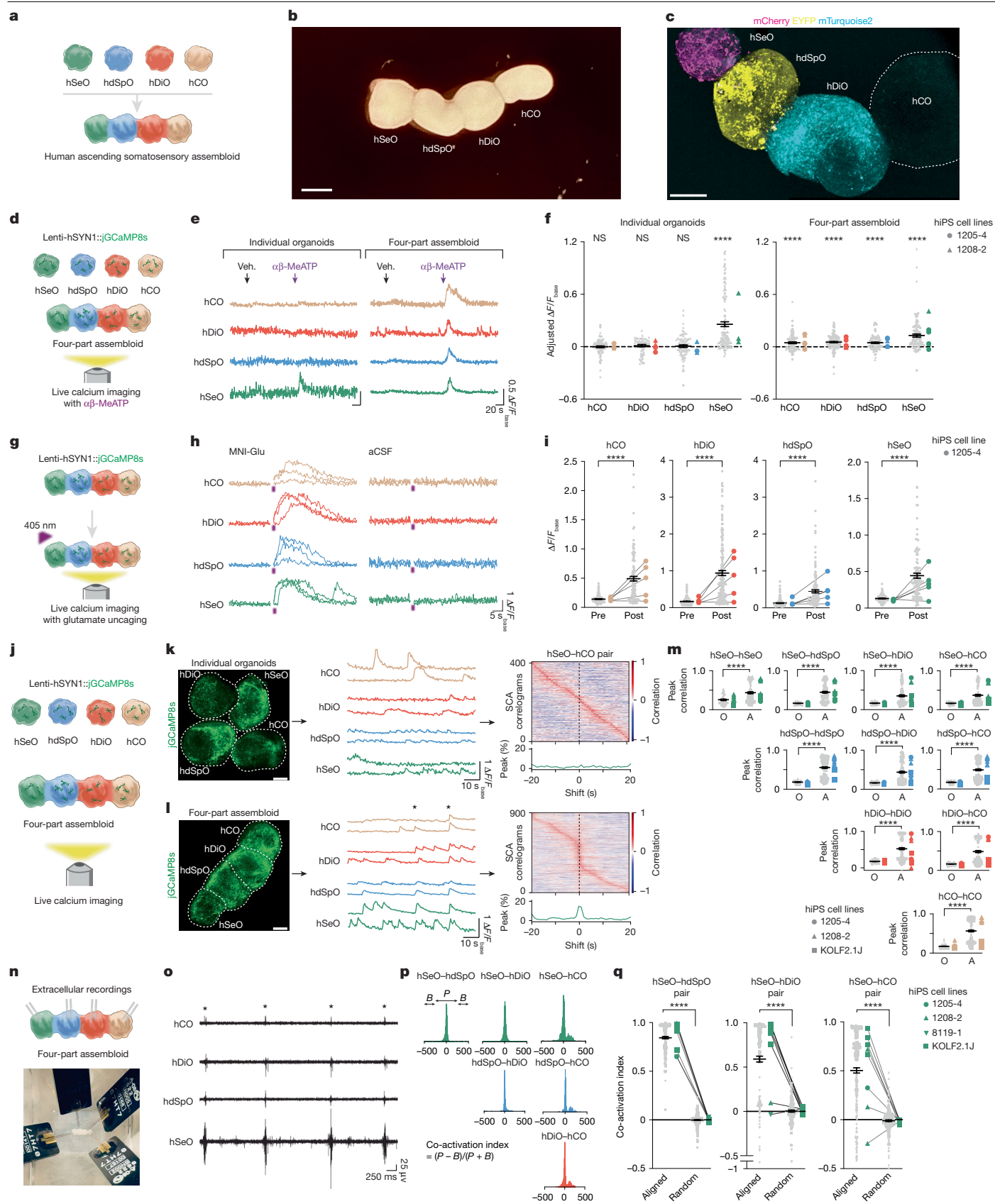


Fig. 4 | See next page for caption.

PHOX2A indicated that the distinct types of spinal neurons in hdSpO were intermingled without a clear spatial organization (Extended Data Fig. 5f). Taken together, these transcriptomic and functional

studies indicate that these regionalized organoids contain key cellular components of the developing human ascending sensory pathway.

Fig. 4 | Generation and functional characterization of hASA. **a**, Generation of hASA. **b,c**, Bright-field (**b**) and fluorescent image (**c**) of hASA. **d,e**, Calcium imaging (**d**) and traces (**e**) after $\alpha\beta$ -MeATP exposure. **f**, Responses following $\alpha\beta$ -MeATP exposure, compared to null value (0), in organoids (hCO, $n = 83$ cells; hDiO, $n = 52$ cells; hdSpO, $n = 84$ cells; hSeO, $n = 131$ cells from three to four organoids; two differentiations) and assembloids (hCO, $n = 91$ cells; hDiO, $n = 116$ cells; hdSpO, $n = 98$ cells; hSeO, $n = 95$ cells from six assembloids; one differentiation) on daf 74–78. Wilcoxon signed-rank test; **** $P < 0.0001$. **g,h**, Glutamate uncaging after ultraviolet application to hSeO (**g**) and $\Delta F/F_{\text{base}}$ traces (**h**). **i**, Response before and after ultraviolet exposure (hCO, $n = 113$ cells; hDiO, $n = 157$ cells; hdSpO, $n = 159$ cells; hSeO, $n = 109$ cells; five assembloids; one differentiation; Wilcoxon matched-pair signed-rank test; **** $P < 0.0001$) on daf 74–99. **j**, Calcium imaging. **k,l**, jGCaMP8s expression, spontaneous activity, SCA correlograms (hSeO–hCO pair) and peak percentage

correlations in organoids (**k**) and assembloids (**l**). Asterisks; synchronous events. **m**, Correlation values in organoids (O; hCO, $n = 96$ cells; hDiO, $n = 61$ cells; hdSpO, $n = 63$ cells; hSeO, $n = 47$ cells; seven organoids; three differentiations) and assembloids (A; hCO, $n = 208$ cells; hDiO, $n = 157$ cells; hdSpO, $n = 156$ cells; hSeO, $n = 110$ cells; ten assembloids; three differentiations) on daf 74–131. Mann–Whitney test; **** $P < 0.0001$. **n,o**, Four-part extracellular recordings (**n**) and traces (**o**). Asterisks; synchronous events. **p**, Representative cross-correlograms. **q**, Co-activation index for hSeO–hdSpO, hSeO–hDiO and hSeO–hCO pairs ($n = 223$, 180 and 222 channels, respectively; seven to eight assembloids; four differentiations; Wilcoxon matched-pair signed-rank test; **** $P < 0.0001$) on daf 80–99. Data: mean $\pm$ s.e.m. Small dots, cells or channels; large dots, assembloids. Scale bars, 1 mm (**b**), 500 μm (**c**), 400 μm (**k,l**). Veh., vehicle. Credits: **a,d,g,j** adapted from ref. 48, Springer Nature America, Inc.; **n** adapted with permission from ref. 11, Elsevier.

Projections in hSeO–hdSpO and hdSpO–hDiO

We next examined the specificity of projections between pairs of regionalized organoids. First, we investigated whether the sensory neurons in hSeO could send projections to spinal neurons in hSeO–hdSpO assembloids. We virally labelled hSeO with hSYN1::EYFP and integrated them with hdSpO to form hSeO–hdSpO assembloids (Fig. 3a). We found that the area of EYFP fluorescence on the hdSpO side, corresponding to projections from hSeO neurons, increased progressively over 28 days after fusion (daf) (Fig. 3b,c). These EYFP⁺ sensory projections co-localized with the axonal marker NF-H (Extended Data Fig. 6a). We next used rabies virus-mediated retrograde tracing combined with Cre recombination in hSeO–hdSpO assembloids (Fig. 3d). On daf 21, we observed tdTomato⁺GFP⁺ cells on the hSeO side, indicating successful retrograde tracing of rabies–Cre–GFP resulting in the recombination and expression of DIO–tdTomato (Fig. 3e). These tdTomato⁺GFP⁺ cells were not detected in unassembled organoids, which were kept in the same cell culture well and media, confirming projection-dependent retrograde transmission (Extended Data Fig. 6b–d). To investigate the specificity of these projections, we co-immunostained for the sensory neuronal marker BRN3A and found that nearly 80% of tdTomato⁺ cells were also BRN3A⁺ (Fig. 3f,g). To further verify functional connectivity between hSeO and hdSpO, we combined optogenetic stimulation with whole-cell electrophysiological recordings. Upon 460-nm light stimulation of channelrhodopsin 2-expressing hSeO, we observed optically evoked excitatory postsynaptic currents (oEPSCs), with a success rate of eight of 30 recorded cells (Fig. 3h,i and Extended Data Fig. 6e,f). Responsive neurons exhibited an average oEPSC amplitude of 52 pA and an onset delay of 32 ms (Extended Data Fig. 6f). These results demonstrate that hSeO sensory neurons can form functional connections with neurons in hdSpO. By contrast, when we generated assembloids by integrating hSeO with hCO, we observed only minimal projections from hSeO that did not increase over time (Extended Data Fig. 6g–i).

We took a similar approach to investigate spinothalamic projections in hdSpO–hDiO assembloids (Fig. 3j). We observed a significant increase in spinothalamic projections on daf 28 (Fig. 3k,l). Using rabies virus-mediated retrograde tracing (Fig. 3m,n), we found that approximately 40% of tdTomato⁺ cells co-expressed the spinal cord projection neuronal marker NK1R⁺ (Fig. 3p). We also observed co-localization of tdTomato and PHOX2A (Extended Data Fig. 6l). In addition, we examined whether hdSpO neurons preferentially project to hDiO compared to hSeO by using three-part hSeO–hdSpO–hDiO assembloids. Quantification of EYFP⁺ spinal projections within the same assembloid, in both the hDiO and hSeO regions, revealed more projections in hDiO, indicating a preferential for hdSpO targeting the hDiO (Extended Data Fig. 6k–m). These studies, in combination with our previous experiments on thalamocortical projections in hCO–hDiO assembloids¹¹, confirmed that the three pairs of assembloids (hCO–hDiO, hDiO–hdSpO and hdSpO–hSeO) built from the four regionalized neural

organoids intrinsically recapitulate some of the basic inter-regional projections.

We next investigated the functionality of these connections in three-part hSeO–hdSpO–hDiO assembloids by combining rabies virus-mediated retrograde tracing, recombinase Flpo-dependent expression of GCaMP6f and live calcium imaging following $\alpha\beta$ -MeATP treatment (Fig. 3q). We assembled AAV-EF1 α ::fDIO–GCaMP6f-infected hdSpO with hSeO to generate hSeO–hdSpO. On daf 43, we infected hDiO with rabies–Flpo–dsRedExpress and AAV-G and fused these onto the hdSpO side of the hSeO–hdSpO assembloid. On daf 70, we detected expression of fDIO–GCaMP6f, which is suggestive of transmission of rabies–Flpo–dsRedExpress from hDiO into hdSpO (Fig. 3r). These GCaMP6f⁺ neurons demonstrated increased calcium activity after $\alpha\beta$ -MeATP exposure, thereby indicating signal transmission from sensory neurons to spinal neurons (Fig. 3s,t). These calcium transients were blocked by the glutamate receptor antagonists 2,3-dioxo-6-nitro-7-sulfamoylbenzo[f]quinoxaline (NBQX) and 2-amino-5-phosphonovaleric acid (APV) (Fig. 3s,t). These results imply that chemical stimulation of sensory neurons in hSeO elicited calcium activity that was transmitted to and induced calcium increase in connected rabies–Flpo-labelled spinothalamic projection neurons in hdSpO. Moreover, hdSpO neurons projected axons into hDiO, as evidenced by rabies tracing.

Functional assembly of four-part hASA

Next, we generated hASA by linearly placing the four key neural regionalized organoids in a tilted cell culture plate (Fig. 4a,b). We measured success assembly rate and evaluated the shapes of assembloids. All samples were successfully assembled, with 87.7% forming linear or curved shapes, which are suitable for functional assays (Extended Data Fig. 7a). We performed viral labelling of each organoid with distinct hSYN1 promoter-driven fluorescent proteins before fusion and confirmed maintenance of distinct domains of these assembloids (Fig. 4c). To verify cellular composition after assembly, we performed scRNA-seq from hASAs and stage- and cell line-matched individual organoids on days 162–229 and found that the composition was comparable between individual organoids and four-part assembloids (Extended Data Fig. 7e,f).

We examined functional connectivity within hASA. In one continuous imaging session, we serially treated hASA with either vehicle or $\alpha\beta$ -MeATP while recording jGCaMP8s signal at single-cell resolution (Fig. 4d). We calculated the adjusted $\Delta F/F_{\text{base}}$ by subtracting the response to vehicle from the response to $\alpha\beta$ -MeATP. We found that $\alpha\beta$ -MeATP exposure significantly elevated the calcium levels in hASA neurons in all four regions on daf 74–78 (Fig. 4e,f and Supplementary Video 1). By contrast, in non-assembled organoids, only neurons in hSeO had a significant increase in calcium after the application of $\alpha\beta$ -MeATP, whereas neurons in other organoids did not (Fig. 4e,f). To

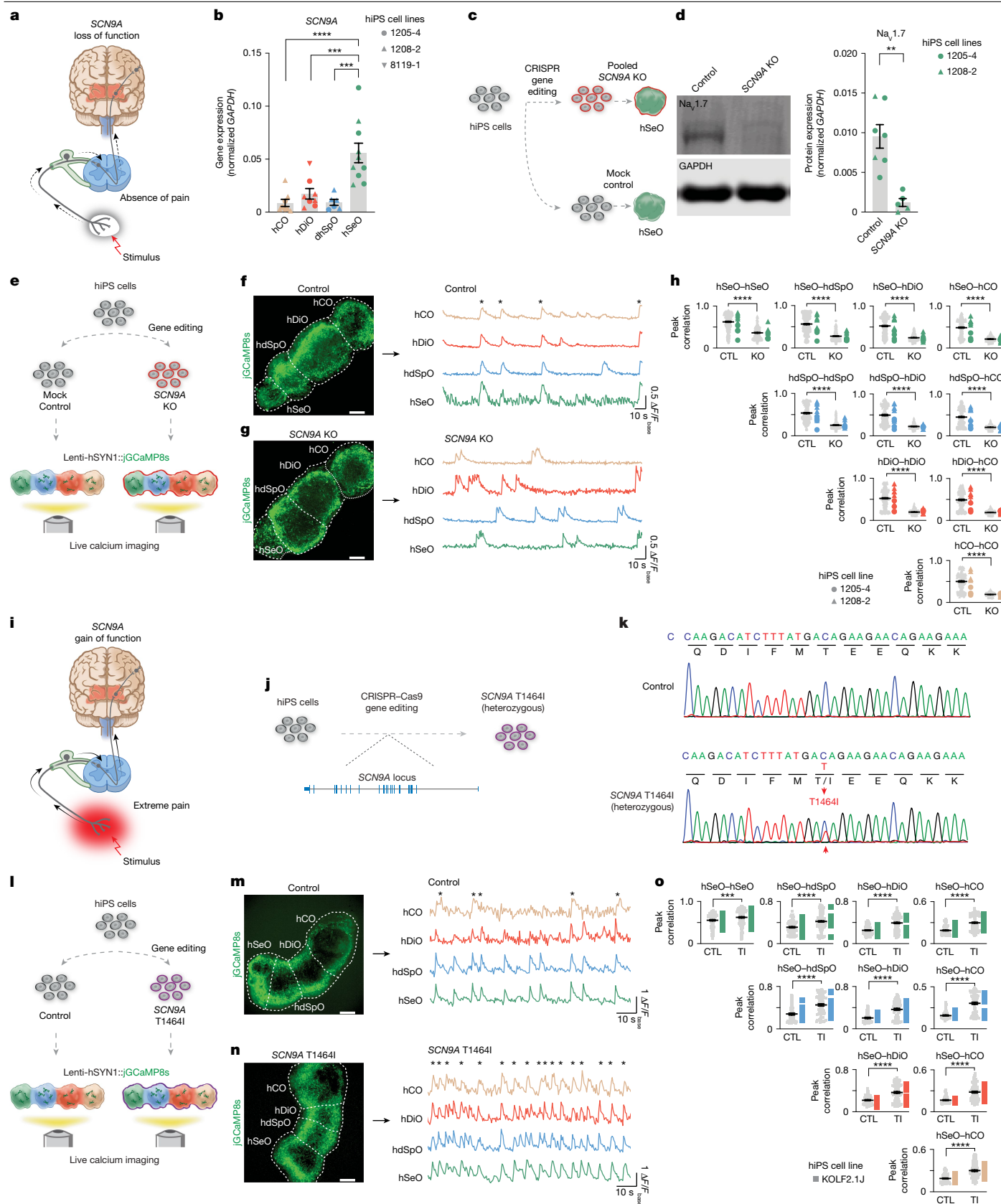


Fig. 5 | See next page for caption.

further verify whether specific stimulation of sensory neurons in hSeO elicited responses in other regions of the assembloid, we used 405-nm photostimulation in the presence of 4-methoxy-7-nitroindolyl

(MNI) caged glutamate to trigger rapid and localized release of glutamate in a specific area (Fig. 4g). We performed photostimulation of hSeO within hASA and detected a robust increase in calcium levels, not

Fig. 5 | Abnormal emergent calcium activity in hASAs with *SCN9A* pathogenic variants. **a**, *SCN9A* loss causes clinical absence of pain. **b**, RT–qPCR of *SCN9A* (hCO, $n = 8$ samples; hDiO, $n = 8$ samples; hdSpO, $n = 6$ samples; hSeO, $n = 10$ samples; four to six differentiations; one-way analysis of variance (ANOVA) ($F_{3,28} = 2.691$) with Tukey's test; *** $P = 0.0003$ for hdSpO versus hSeO; *** $P = 0.0010$ for hDiO versus hSeO; **** $P < 0.0001$) on days 45–100. **c**, Generation of *SCN9A* KO hSeO. **d**, Western blot of Na_v1.7 (control, $n = 7$ samples; KO, $n = 5$ samples; two differentiations; unpaired t -test; ** $P = 0.0011$) on days 48–69. **e**, Calcium imaging. **f**, **g**, jGCaMP8s expression and spontaneous calcium activity in control (**f**) and *SCN9A* KO (**g**) assembloids. **h**, Correlation values in control (hCO, $n = 182$ cells; hDiO, $n = 199$ cells; hdSpO, $n = 211$ cells; hSeO, $n = 218$ cells; ten assembloids; three differentiations) and KO (hCO, $n = 179$ cells; hDiO,

$n = 202$ cells; hdSpO, $n = 219$ cells; hSeO, $n = 208$ cells; 12 assembloids; three differentiations) on daf 74–111. Mann–Whitney test; *** $P = 0.0005$; **** $P < 0.0001$. **i**, *SCN9A* gain-of-function mutation causes excessive pain. **j**, Generation of *SCN9A* T1464I hiPS cells. **k**, Sanger sequencing of hiPS cells. **l**, Calcium imaging. **m**, **n**, jGCaMP8s expression and spontaneous activity in control (**m**) and *SCN9A* T1464I (**n**) assembloids. **o**, Correlation values in control (hCO, $n = 76$ cells; hDiO, $n = 61$ cells; hdSpO, $n = 60$ cells; hSeO, $n = 96$ cells; seven assembloids; two differentiations) and T1464I (hCO, $n = 97$ cells; hDiO, $n = 92$ cells; hdSpO, $n = 81$ cells; hSeO, $n = 145$ cells; eight assembloids; two differentiations) on daf 98. Mann–Whitney test; *** $P = 0.0005$; **** $P < 0.0001$. Data: mean $\pm$ s.e.m. Small dots, cells; large dots, qPCR, western blot samples or assembloids. Scale bars, 400 μ m. Credits: **c**, **e**, **j**, **l** adapted from ref. 48, Springer Nature America, Inc.

only in hSeO but also in all other regions, on daf 74–99 (Fig. 4h,i). These experiments suggest transmission of somatosensory signals within the assembloid from sensory neurons through spinal and thalamic neurons to cortical neurons.

We next explored whether the four-part assembly is associated with emergent network properties. We recorded baseline calcium activity at single-cell resolution and compared hASA with four non-assembled organoids placed in close proximity just before imaging (Fig. 4j–l). Notably, we found that neurons in assembloids exhibited synchronous activity across all four regions (Fig. 4l and Supplementary Video 2). We applied scaled correlation analysis (SCA) to minimize the effects of low-frequency noise signals⁴², which was established in our recent study to quantify synchronous activity across neural circuits⁴³. Representative SCA correlograms from all possible pairs between hSeO and hCO neurons in an assembloid showed that the correlation peaks were distributed around 0 s, whereas representative SCA correlograms from unfused organoids had equal distribution of correlation peaks (Fig. 4k,l). The peak correlation for all combinations of the four regions was significantly higher in assembloids than that in individual non-assembled neural organoids (Fig. 4m).

We further investigated activity patterns through concomitant extracellular recordings with 32 electrodes in each of the four hASA parts (Fig. 4n). We found spontaneous and synchronous activity across the entire assembloid (Fig. 4o). We calculated the co-activation indexes based on cross-correlograms⁴⁴ (Fig. 4p). When we compared the co-activation index aligned with the activity of hSeO, we found significantly higher values than alignment to random time points (Fig. 4q). Taken together, these results demonstrate that the assembly of intact 3D human regionalized neural organoids resembling the human sensory ganglia, dorsal spinal cord, thalamus and cerebral cortex enables the formation of connections capable of synchronized activity across the pathway and in response to somatosensory like (chemical sensory) stimuli.

SCN9A pathogenic variants alter synchrony in hASA

Finally, we studied whether hASA could be used to identify functional defects in models of neurologic and psychiatric disorders. Pathogenic variants in the gene *SCN9A*, which encodes the voltage-gated sodium channel Na_v1.7, cause pain insensitivity if the variant leads to loss of channel function, or hyperalgesia if the variant causes gain of function⁶ (Fig. 5a,i). Although defects in channel conductance and excitability of sensory neurons have been proposed as disease mechanisms in cellular and animal models^{6,45–47}, the circuit-level consequences have not yet been explored in a human model.

We first verified the expression of *SCN9A* and found high levels in hSeO (Fig. 5b). We then applied CRISPR-mediated gene editing to induce one or more frameshift variants in the *SCN9A* gene (Fig. 5c). We confirmed that hSeO derived from pooled *SCN9A* knockout (KO) iPS cell lines exhibited decreased *SCN9A* mRNA and Na_v1.7 protein levels (Fig. 5d and Extended Data Fig. 8a). The expression of the

sensory neuron marker *POU4F1* (BRN3A) was not affected by *SCN9A* KO (Extended Data Fig. 8b–d). Consistent with previous reports of hypo-excitability of sensory neurons in the absence of this key sodium channel⁴⁵, we found that *SCN9A* KO hSeO displayed reduced frequency of spontaneous calcium activity (Extended Data Fig. 8e,f). We then generated hASA from isogenic control and *SCN9A* KO hiPS cells to explore the circuit-level consequences. We recorded spontaneous activity patterns using jGCaMP8s in all regions from control and *SCN9A* KO hASA (Fig. 5e). We discovered that the synchronous pattern of activity across hASA was reduced in *SCN9A* KO, although non-synchronized events were found in all regions (Fig. 5f,g and Supplementary Videos 3 and 4). We then performed SCA analysis and found a significant reduction in correlation, indicating that *SCN9A* KO impaired the emergent synchronization across the assembloid (Fig. 5h and Extended Data Fig. 8g).

In addition, we investigated the circuit-level consequences of the T1464I *SCN9A* gain-of-function variant, which is associated with paroxysmal extreme pain disorder⁴⁷ (Fig. 5i). We applied CRISPR–Cas9 gene editing to introduce the heterozygous *SCN9A* T1464I into a hiPS cell line (Fig. 5j). We confirmed mixed gDNA of control and T1464I *SCN9A* by Sanger sequencing (Fig. 5k). We then generated hSeO from control and *SCN9A* T1464I hiPS cells and confirmed that *POU4F1* expression level was not altered by genetic engineering (Extended Data Fig. 8h). Calcium activity imaging of control and *SCN9A* T1464I hSeO demonstrated that the gain-of-function mutation produced hyperexcitability of sensory neurons, which is consistent with previous reports^{6,47} (Extended Data Fig. 8i,j). To investigate the effects of *SCN9A* T1464I variant on the emergent synchronized activity of the sensory ascending pathway, we derived hASA from control and *SCN9A* T1464I hiPS cell lines and imaged spontaneous calcium activity (Fig. 5l). Interestingly, SCA analysis revealed higher peak correlation values across the pairs in hASA, suggesting that *SCN9A* T1464I resulted in hypersynchrony (Fig. 5m–o and Extended Data Fig. 8k). These results highlight the capability of hASA to capture circuit-level dysfunction associated with the pathogenic variants of *SCN9A* and thus its utility to model aberrations in emergent properties within human sensory circuits.

Discussion

Reconstructing the ascending sensory pathway and circuits ex vivo holds significant promise for understanding the mechanisms involved in human sensory system development and thereby unravelling the pathophysiology of sensory-related disorders. We previously demonstrated the potential of assembloids composed of regionalized neural organoids derived from hiPS cells to model circuits in vitro^{8,48,49}. Despite previous efforts to derive neural organoids containing sensory neurons^{22,50,51}, a multiregional assembloid model recapitulating the human somatosensory ascending pathway has not yet been achieved. Here we leveraged this assembloid approach to generate a four-component ascending sensory circuit going from peripheral sensory neurons to cortical neurons. This hASA is an in vitro model encompassing the major components of the human sensory ascending pathway,

and includes peripheral sensory neurons, dorsal spinal cord (and hindbrain) projection neurons, thalamic neurons and glutamatergic cortical neurons. This allowed us to visualize transmission of chemically triggered calcium signals through the network following sensory stimulus application and to capture emergent properties of the sensory pathway that cannot be seen in individual organoids, such as synchronous activity across the four parts of the assembloid. We also discovered that *SCN9A* loss resulted in reduced synchronous activity, whereas gain-of-function *SCN9A* variant induced increased synchrony across the pathway. Therefore, hASA holds great promise for addressing disease pathophysiology and testing therapeutic approaches. A recent study identified that spontaneous synchronized activity has a crucial role for fine-scaling circuit assembly in neonatal mice, highlighting the importance of synchronized activity during circuit development⁵². Notably, hASA can also capture human-specific aspects of the sensory system, such as reduced responsiveness to TNP-ATP. Going forward, this multicellular platform can be applied to model a wide range of disorders, including sensory dysfunction in neurodevelopmental disorders or hyperalgesia, and could be helpful for drug screening.

Improvements at several levels would enhance the utility of hASA. First, hiPS cell-derived sensory neurons are still limited in receptor expression and thus in the repertoire of responses to sensory stimulation, which is partially attributable to their developmental stage⁵³. Future studies could improve the specification and maturation of sensory neurons, include other neurons in this pathway and functionally advance this model to study postnatal stages. Future efforts to develop comprehensive transcriptomic atlases of TG and DRG in the developing human will help with further elucidating the molecular identity of sensory neurons. Second, there are several neural pathways involved in sensory perception in addition to the anterolateral spinothalamic tract, which our current hASA models. We envision that these other pathways can be built in parallel with hASA or within the same system. Further studies should be conducted to investigate in detail the specificity of connectivity in these assembloids, including within individual organoids, and the role of activity-dependent processes in establishing and refining these neural networks. These efforts could ultimately be used to dissect the intrinsic rules for establishing connectivity in early human circuits, as well as suggest strategies for accelerating maturation in these in vitro models.

Overall, this new platform holds potential to yield insights into how the human sensory pathways assemble and therefore to develop and screen therapeutics for pain and other sensory system-related disorders.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41586-025-08808-3>.

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Characterization and maintenance of hiPS cells

The hiPS cell lines used in this study were validated using methods described in previous studies. Genome-wide single-nucleotide polymorphisms (SNP) genotyping was performed using the Illumina genome-wide GSAMD-24v2-0 SNP microarray at the Children's Hospital of Philadelphia. Cultures were tested for and maintained *Mycoplasma* free. A total of five control hiPS cell lines derived from fibroblasts collected from five healthy participants were used for experiments¹³ (Supplementary Table 1). Two of the control hiPS cell lines were used to generate *SCN9A* KO hiPS cell lines, and one control hiPS cell line was used to generate the *SCN9A* gain-of-function hiPS cell line. Approval for experiments was obtained from the Institutional Review Board panel of Stanford University. Informed consent was obtained from all participants.

Human primary tissue

Human brain tissue samples were obtained under a protocol approved by the Research Compliance Office of Stanford University. PCW15 tissue was delivered on ice, and DRG were dissected immediately after arrival.

Generation of organoids (hCO, hDiO, hdSpO and hSeO) from hiPS cells

For neural differentiation, hiPS cells were cultured on vitronectin-coated plates (A14700; Life Technologies) in Essential 8 Medium (A1517001; Life Technologies). Cells were passaged every 4–5 days with UltraPure 0.5 mM EDTA, pH 8.0 (15575; Thermo Fisher Scientific). For the generation of regionalized neural organoids, hiPS cells were incubated with Accutase (AT104; Innovative Cell Technologies) at 37 °C for 7 min and dissociated into single cells. Optionally, 1–2 days before organoid formation, hiPS cells were exposed to 1% dimethylsulfoxide (472301; Sigma-Aldrich) in Essential 8 Medium. For aggregation into organoids, about 3×10^6 single hiPS cells were seeded per AggreWell 800 plate well in Essential 8 Medium supplemented with the Rho-associated coiled-coil containing kinases (ROCK) inhibitor Y27632 (10 μ M; S1049; Selleckchem), centrifuged at 100g for 3 min and then incubated at 37 °C in 5% CO₂. On the next day (day 1), organoids consisting of approximately 10,000 cells were collected and transferred into ultra-low attachment plastic dishes (3262; Corning) in Essential 6 Medium (A1516401; Thermo Fisher Scientific) supplemented with patterning molecules, as shown in Extended Data Fig. 1.

The hCO were generated as described previously^{10,55}. For days 1–6, Essential 6 Medium was changed every day and supplemented with dorsomorphin (2.5 μ M; P5499; Sigma-Aldrich) and SB-431542 (10 μ M; 1614; R&D Systems), and was changed every day. On day 7, the organoids were transferred to neural medium containing Neurobasal-A Medium (10888022; Thermo Fisher Scientific), B-27 Supplement, minus vitamin A (12587010; Thermo Fisher Scientific), GlutaMAX Supplement (1:100; 35050079; Thermo Fisher Scientific), penicillin–streptomycin (1:100; 15070063; Thermo Fisher Scientific), supplemented with FGF2 (20 ng ml⁻¹; 233-FB; R&D Systems) and EGF (20 ng ml⁻¹; 236-EG; R&D Systems) until day 22. From days 23 to 46, the neural medium was supplemented with BDNF (20 ng ml⁻¹; 450-02; PeproTech), NT3 (20 ng ml⁻¹; 450-03; PeproTech), L-ascorbic acid 2-phosphate trisodium salt (200 μ M; 323-44822; Wako), N6, 2'-O-dibutyryl adenosine 3',5'-cyclic monophosphate sodium salt (cAMP; 50 μ M; D0627; MilliporeSigma) and *cis*-4, 7, 10, 13, 16, 19-docosahexaenoic acid (10 μ M; D2534; MilliporeSigma). From day 47, a neural medium containing Neurobasal-A Medium was used for changes in the medium (every 4 days).

The hDiO were generated as described previously¹¹. For days 1–6, Essential 6 Medium was changed every day and supplemented with dorsomorphin and SB-431542. On day 5, the medium was additionally supplemented with 1 μ M CHIR (S1263; Selleckchem). On day 7, organoids

were maintained in a neural medium supplemented with CHIR. On day 9 of differentiation, the neural medium was supplemented with 100 nM SAG (days 9–15; 566660-IMG; MilliporeSigma), in addition to the CHIR. Furthermore, on days 12–18 of differentiation, organoids were supplemented with 30 ng ml⁻¹ of BMP7 (120-03P; PeproTech), in addition to the compounds described above. From days 19 to 46, the neural medium was supplemented with BDNF, NT3, L-ascorbic acid 2-phosphate trisodium salt, cAMP and *cis*-4, 7, 10, 13, 16, 19-docosahexaenoic acid. For days 19–25, DAPT (2.5 μ M, 72082; STEMCELL Technologies) was added. From day 47, a neural medium containing Neurobasal-A Medium was used for changes in the medium (every 4 days).

To generate hdSpO, from days 1 to 6, organoids were maintained in Essential 6 Medium supplemented with SB-431542 and dorsomorphin. From days 5 to 6, CHIR (3 μ M) was added. On day 7, organoids were transferred to a neural medium supplemented with EGF, retinoic acid (0.1 μ M; R2625; Sigma-Aldrich) and CHIR (3 μ M; S1263; Selleckchem). On day 22, the medium was supplemented with BDNF, cAMP, L-ascorbic acid 2-phosphate trisodium salt, insulin-like growth factor 1 (IGF-1) (10 ng ml⁻¹; 100-11; PeproTech) until the end of experiments. From days 22 to 28, DAPT was added.

To generate hSeO, from days 1 to 6 in suspension, organoids were maintained in Essential 6 Medium supplemented with SB-431542. From days 1 to 4, BMP4 (5 ng ml; 120-05ET; PeproTech) was added. From days 3 to 5, CHIR (600 nM) was added. On day 6 in suspension, the organoids were transferred to a neural medium supplemented with SB-431542. From day 9, BDNF, GDNF (25 ng ml⁻¹; 450-10; PeproTech) and NGF (25 ng ml⁻¹; 450-01; PeproTech) were supplemented until the end of the experiment. From days 9 to 14, the medium was supplemented with SB-431542. From days 15 to 20, DAPT was included.

Cryosection and immunocytochemistry

Organoids and assembloids were fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) overnight at 4 °C. They were then washed in PBS and transferred to 30% sucrose/PBS for 2–3 days until the organoids/assembloids sank into the solution. Subsequently, they were rinsed in optimal cutting temperature (OCT) compound (Tissue-Tek OCT Compound 4583; Sakura Finetek) and 30% sucrose/PBS (1:1), embedded and snap-frozen using dry ice. For immunofluorescence staining, 30- μ m-thick sections were cut using a Leica CM1860 cryostat. Cryosections were washed with PBS to remove excess OCT compound from the sections and blocked in 10% normal donkey serum (NDS, S30-100ML; MilliporeSigma), 0.3% Triton X-100 (T9284-100ML; MilliporeSigma) and 1% BSA diluted in PBS for 1 h at room temperature. The sections were then incubated overnight at 4 °C with primary antibodies diluted in PBS containing 2% NDS and 0.1% Triton X-100. PBS was used to wash the primary antibodies, and the cryosections were incubated with secondary antibodies in PBS with the PBS containing 2% NDS and 0.1% Triton X-100 for 1 h. The following primary antibodies were used for staining: anti-FOXG1 (rabbit; M227; 1:400 dilution; Takara), anti-TCF7L2 (rabbit; 2569S; 1:200 dilution; Cell Signaling Technology), anti-PHOX2A (rabbit; ab155084; 1:200 dilution; Abcam), anti-BRN3A (mouse; MAB1585; 1:200 dilution; Sigma), anti-mCherry (goat; orb153320; 1:1,000 dilution; Biorbyt), anti-NK1R (rabbit; S8305; 1:200 dilution; Sigma), anti-VGLUT2 (mouse; MAB5504; 1:200 dilution; Millipore), anti-NF-H (mouse; NE1023; 1:200 dilution; Millipore) and anti-PAX2 (goat; AF3364; 1:200 dilution; R&D). The following secondary antibodies were used for staining: Alexa Fluor 647 AffiniPure donkey anti-rabbit IgG (H&L) (711-605-152; 1:1,000 dilution; Jackson ImmunoResearch), Alexa Fluor 568 donkey anti-goat IgG (H&L) (A11057; 1:1,000 dilution; Thermo Fisher Scientific), Alexa Fluor 568 donkey anti-rabbit IgG (H&L) (A10042; 1:200 dilution; Thermo Fisher Scientific), Alexa Fluor 647 donkey anti-mouse IgG (H&L) (A31571; 1:200 dilution; Thermo Fisher Scientific) and Alexa Fluor 568 donkey anti-mouse IgG (H&L) (A10037; 1:1,000 dilution; Thermo Fisher Scientific). Nuclei were visualized with the Hoechst 33258 dye (H3549;

1:10,000 dilution; Life Technologies). Cryosections were mounted for microscopy on glass slides using Aqua-Poly/Mount (18606; Polysciences) or VECTASHIELD (H-1000; Vector Laboratories) and imaged on a confocal microscope. Images were processed and analysed using Fiji (v.2.14.0) and Imaris (Oxford Instruments).

Real-time qPCR

Three to five organoids were collected in the same tube and considered as one sample. The RNA from samples was isolated using the RNeasy Plus Mini kit (74136; QIAGEN). Template cDNA was prepared by reverse transcription using the SuperScript III First-Strand Synthesis Super-Mix for qRT-PCR (11752250; Thermo Fisher Scientific). qPCR was performed using the SYBR Green PCR Master Mix (4312704; Thermo Fisher Scientific) on a QuantStudio 6 Flex Real-Time PCR System (4485689; Thermo Fisher Scientific). The primers used in this study are listed in Supplementary Table 2.

Preparation of scRNA-seq library and data analysis

Dissociation of organoids, assembloids and human DRG was performed, as described previously^{10,49,56,57}. For the organoid dissociation, four to five randomly selected organoids were pooled to obtain a single-cell suspension. For assembloids, one to two assembloids were pooled for dissociation. To match the relative cell numbers across different regions, one to two organoids from each of the four regions, totalling four to eight organoids, were pooled for dissociation only when comparing organoids to assembloids. For human primary tissue, one to two DRG were pooled for dissociation. Samples were incubated in 30 U ml⁻¹ of papain enzyme solution (LS003126, Worthington Biochemical) and 0.4% DNase (12,500 U ml⁻¹; LS2007, Worthington Biochemical) at 37 °C for 45 min. After enzymatic dissociation, organoids were washed with a solution containing protease inhibitor and gently triturated to obtain a single-cell suspension. Cells were resuspended in 0.04% BSA/PBS (B6917-25MG, MilliporeSigma) and filtered through a 70-µm Flowmi Cell Strainer (H13680-0070, Bel-Art), and the number of cells were counted. To target 7,000 cells after recovery, approximately 11,600 were loaded per lane on a Chromium Single Cell 3' chip (Chromium Next GEM Chip G Single Cell Kit; PN-1000127, 10x Genomics), and cDNA libraries were generated with the Chromium Next GEM Single Cell 3' GEM, Library & Gel Bead Kit v.3.1 (PN-1000128 and PN-1000269, 10x Genomics) according to the manufacturer's instructions. Each library was sequenced using the Illumina NovaSeq S4 2 × 150 bp (Admera Health) and NovaSeq X 28 + 90 bp (CZ Biohub). Unique molecular identifier (UMI) counting was performed using the 'count' function (-include-introns=TRUE) in Cell Ranger (v.7.0.1 and 7.1.0 for organoids; v.7.2.0 for the human DRG and organoid versus assembloid comparison) with GRCh38/Ensembl 98 reference (available at <https://cf.10xgenomics.com/supp/cell-exp/refdata-gex-GRCh38-2020-A.tar.gz>). Further downstream analyses were performed using the R package Seurat (v.4.3.0). Genes on the X or Y chromosome were removed from the count matrix to avoid biases in clustering due to the sex of the hiPS cell lines.

For comparison across four region organoids, hSeO samples ($n = 4$) and hdSpO samples ($n = 5$) from this study, along with hDiO samples¹¹ ($n = 3$; GSE224766) and hCO samples¹³ ($n = 2$; GSE145122), were aggregated using the 'aggr' function (-normalize=mapped) in Cell Ranger. Cells with more than 7,500 or less than 2,000 detected genes, with less than 2,000 UMI counts or with mitochondrial content higher than 15% were excluded. Genes that were not expressed in at least three cells were not included in the analysis. Gene expression was normalized using a global-scaling normalization method (normalization method; 'LogNormalize'; scale factor 10,000), and the 2,000 most variable genes were selected (selection method; 'vst') and scaled (mean = 0 and variance = 1 for each gene). The top 50 principal components (PCs) were used for clustering (resolution of 1.0) using the 'FindNeighbors' and 'FindClusters' functions and for visualization with UMAP. Clusters were annotated based on the expression of known marker genes.

For the analysis of hSeO and hdSpO, cells with more than 10,000 or less than 2,500 detected genes, with less than 2,000 UMI counts or cells with mitochondrial content higher than 15% were excluded. Genes that were not expressed in at least three cells in each library were not included. Normalization and variable gene detection were performed, as described above. The hSeO samples ($n = 4$) and hdSpO samples ($n = 5$) were each separately integrated using 'FindIntegrationAnchors' and 'IntegrateData' functions with the default parameter. The top 10 PCs were used for clustering (resolution of 1.0) using the 'FindNeighbors' and 'FindClusters' functions and for visualization with UMAP. The classification of dorsoventral neuronal cell types of hdSpO was performed based on the combinatorial expression of known markers, as described previously^{38,39}, by using 'doCellPartition' function (cell_level_min_step1 = 2, cell_level_min_step2 = 1; available at github.com/juliendelille/MouseSpinalCordAtlas).

For the organoid versus assembloid comparison, cells with more than 10,000 or less than 1,000 detected genes or cells with mitochondrial content higher than 15% were excluded. Genes that were not expressed in at least three cells in each library were not included. After normalization and variable gene detection, individual organoid samples ($n = 4$) and assembloid samples ($n = 4$) were integrated and clustered (30 PCs; resolution = 1).

For human DRG data, cells with more than 10,000 or less than 500 detected genes or cells with mitochondrial content higher than 10% were excluded. Genes that were not expressed in at least three cells in each library were not included. After normalization and variable gene detection, human DRG samples ($n = 7$) were integrated. UMAP visualization and clustering (30 PCs; resolution = 1) were performed. Human DRG samples ($n = 7$) and hSeO samples ($n = 4$) were also integrated. After removing putative stressed cells using Gruffy⁵⁸, an additional round of integration, UMAP visualization and clustering (30 PCs; resolution = 2) were performed.

As a reference for label transfer from the human tissue datasets to four region-specific organoid datasets, the Braun et al.²¹ reference (available at <https://github.com/linnarsson-lab/developing-human-brain>) was downsampled, as described in our previous study⁵⁹, and integrated with human DRG data using each donor as a batch. In the Braun et al. data, subregion labelling of hindbrain, pons and medulla was grouped into one as hindbrain. For label transfer of the dorsoventral subdomain information onto hdSpO, the Rayon et al. developing spinal cord data³⁸ (GSE171892) were quality control (QC) filtered with the same criteria, as described in the original paper, and integrated using each donor as a batch. The prediction scores for each subregion were computed with the 'FindTransferAnchors' and 'TransferData' functions with 50 PCs. For each cell, the subregion with the highest prediction score was assigned as its predicted subregion. If the highest score was less than 0.5, the predicted subregion was marked as uncertain. Prediction proportions were calculated by dividing the number of cells in each organoid condition with a given subregion prediction by the total number of cells with that subregion prediction.

Generation of assembloids

To generate assembloids, regionalized organoids were differentiated separately and later assembled by positioning them in close proximity. For two-part assembloids, the organoids of interest were fused by placing them in 1.5-ml Eppendorf tubes for 2–3 days inside an incubator. For three- or four-part assembloids, the organoids of interest were integrated by placing them in tilted six-well ultra-low attachment plates (no. 3471; Corning) for 1 week in an incubator. After assembly, the assembloids were maintained in ultra-low attachment plates (24-well or six-well plates) with medium change every 4–7 days. For three- or four-part assembloids, the six-well ultra-low attachment plates were kept tilted in the incubator. The neural medium used for four-part assembloids was supplemented with BDNF (20 ng ml⁻¹; 450-02; PeproTech), GDNF (25 ng ml⁻¹; 450-10; PeproTech), NGF (25 ng ml; 450-01;

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PeperoTech), L-ascorbic acid 2-phosphate trisodium salt (200 μ M; 323-44822; Wako), IGF-1 (10 ng ml⁻¹; 100-11; PeperoTech) and cAMP (50 μ M; D0627; MilliporeSigma). In the course of in vitro development, the timing of neuronal maturation differs along the rostro-caudal axis. This gradient has been taken into consideration especially when generating assembloids composed of organoids resembling spatially separated brain regions, such as hCO and hSpO⁸. Along the same line, we generally integrated more advanced hCO and hDiO (days 90–120) with earlier-stage hSpO and hSeO (days 40–70).

Viral labelling and rabies tracing

Viral infection of organoids was performed, as described previously⁶⁰. In brief, two or three organoids were placed in a 1.5-ml Eppendorf tube containing a 200- μ l medium with 0.5 μ l of the virus and incubated overnight at 37 °C and 5% CO₂. The next day, 800 μ l of fresh culture medium was added. The following day, neural organoids were transferred into the fresh culture medium in ultra-low attachment plates. For rabies virus retrograde tracing, organoids representing the 'presynaptic' part were labelled with AAV-DIO-tdTomato, and organoids representing the 'postsynaptic' part were separately labelled with Rabies- Δ G-Cre-GFP and AAV-EF1 α ::CVS-G. Two days after viral infection, the organoids were assembled. After 3 weeks of integration, assembloids were fixed with 4% paraformaldehyde and processed for immunocytochemistry. For rabies virus tracing and calcium imaging experiments, hSpO were labelled with AAV-EF1 α ::fDIO-GCaMP6f, and hDiO were separately labelled with both Rabies- Δ G-Flpo-dsRedExpress and AAV-EF1 α ::CVS-G. For GCaMP virus infection of four-part assembloids, one single assembloid was cultured overnight in 200- μ l medium containing 0.5 μ l of the virus of interest, at 37 °C and 5% CO₂. The next day, 4 ml of the fresh culture medium was added.

The viruses used in this study were AAV-DJ-hSYN1::EYFP (GVVC-AAV-16; Stanford University Neuroscience Gene Vector and Virus Core), AAV-DJ-EF1 α ::CVS-G (produced by Stanford University Neuroscience Gene Vector and Virus Core using Addgene plasmid no. 67528), AAV-DJ-EF1-DIO-tdTomato (GVVC-AAV-169; Stanford University Neuroscience Gene Vector and Virus Core), AAV-DJ-hSYN1::mTurquoise2 (produced by Stanford University Neuroscience Gene Vector and Virus Core using Addgene plasmid no. 99125), AAV-DJ-hSYN1::mCherry (GVVC-AAV-17; Stanford University Neuroscience Gene Vector and Virus Core), Rabies- Δ G-Cre-GFP (Salk), Rabies- Δ G-Flpo-dsRedExpress (Salk using Addgene plasmid no. 32650), AAV1-EF1 α ::fDIO-GCaMP6f (no. 128315-AAV1; Addgene), Lenti-hSYN1::jGCaMP8s⁶¹ (produced by VectorBuilder), AAV1-hSYN1::Cre (no. 105553; Addgene) and AAV-DJ-EF1 α ::DIO-GCaMP6s (GVVC-AAV-91; Stanford University Neuroscience Gene Vector and Virus Core).

Live calcium imaging from organoids or assembloids

To achieve single-cell-resolution live calcium imaging in intact 3D organoids or assembloids without significant sample movement during chemical injection, we acutely attached samples to ethyleneimine polymer (PEI)-coated coverslips before imaging. Autoclaved 12-mm coverslips were coated with 0.01875% PEI (no. 03880; Sigma) in water for at least 1 h at 37 °C and then washed three times with water before use. An organoid or assembloid was carefully positioned on top of a PEI-coated coverslip, and an L-shaped tubing-type Chamliide CMB chamber (CM-B12-1PB; Live Cell Instrument) was assembled. This chamber was connected to a peristaltic pump (no. 702027; Harvard Apparatus) to establish continuous suction through the outlet hole. To prevent sample drying, a vehicle solution was applied immediately after assembly. Organoids and assembloids with detectable expression of genetically encoded calcium indicators and displaying baseline activity were imaged.

The live imaging chamber was transferred to the confocal microscope stage (Leica STELLARIS 5 or SP8), and the samples were imaged using LAS X software (Leica) under controlled environmental conditions

(37 °C and 5% CO₂) using a $\times 5$ or $\times 10$ objective. We waited for 5–10 min and then started time-lapse live imaging at approximately 0.436 s per frame, with adjustments for each sample. For imaging spontaneous activity patterns, calcium activity was recorded for 3–5 min. To capture calcium responses after chemical injection, we recorded 60-s baseline activity. Following this, $\alpha\beta$ -MeATP (no. 3209; Tocris) or capsaicin (no. 0462; Tocris) dissolved in vehicle was injected over 30 s at a slow speed (approximately 2 ml min⁻¹) to minimize any potential movement of the samples.

For analysis, regions of interest (ROI) were manually drawn, and raw fluorescent intensities were exported using Fiji (v.2.14.0). Using MATLAB (v.R2023a), raw fluorescent intensities were transformed into relative changes in fluorescence: $\Delta F/F_{\text{base}} = (F(t) - F_{\text{base}})/F_{\text{base}}$, where F_{base} is the lower fifth percentile value of the session. The mean amplitudes of $\Delta F/F_{\text{base}}$ from each cell for 60 s were compared before and after chemical injection.

As shown in Fig. 2g,h, a neural medium supplemented with BDNF (20 ng ml⁻¹), GDNF (25 ng ml⁻¹) and NGF (25 ng ml⁻¹) was used as the vehicle solution. After 1 min of baseline imaging, 30 μ M of $\alpha\beta$ -MeATP or 3 μ M of capsaicin was injected into the chamber.

As shown in Fig. 2k,l, a neural medium supplemented with BDNF (20 ng ml⁻¹), GDNF (25 ng ml⁻¹) and NGF (25 ng ml⁻¹) was used as the vehicle solution. The hSeO were incubated with various concentrations of TNP-ATP (10, 100 or 100,000 nM) dissolved in the vehicle solution. After 1 min of baseline imaging, 30 μ M of $\alpha\beta$ -MeATP was injected into the chamber.

As shown in Fig. 3q,r, assembloids were acutely attached to a PEI-coated 12-well cell culture plate before the imaging. The plate was moved onto the microscope stage, and the imaging was done as the lid of the plate was open. A neural medium supplemented with BDNF, GDNF and NGF was used as the vehicle solution. After 1 min of baseline imaging in 1 ml of the vehicle solution, 500 μ l of $\alpha\beta$ -MeATP (90 μ M) was added during imaging to make 30 μ M of $\alpha\beta$ -MeATP. After 6 min of imaging, the samples were incubated with NBQX (50 μ M; no. 0373; Tocris) and APV (50 μ M; no. 0106; Tocris) for 30 min in the incubator. Calcium response by $\alpha\beta$ -MeATP in the presence of NBQX and APV was performed, as described above.

As shown in Fig. 4e,f, Dulbecco's phosphate-buffered saline (DPBS) with calcium, magnesium (SH30264.01; Cytiva) or artificial cerebrospinal fluid (aCSF; containing 124 mM NaCl, 3 mM KCl, 1.25 mM NaH₂PO₄, 1.2 mM MgSO₄, 1.5 mM CaCl₂, 26 mM NaHCO₃ and 10 mM D-(+)-glucose) with the addition of GlutaMAX (35050061; Gibco) was used as the vehicle solution. After 1 min of baseline imaging, 3 μ M of $\alpha\beta$ -MeATP was injected into the chamber.

As shown in Figs. 4k,l and 5f,g, aCSF was used as the vehicle solution.

The correlation between calcium traces was quantified using SCA⁴². Traces were first averaged over 1-s intervals and partitioned into 20-s-long segments. Pearson correlation coefficient (r) was computed between corresponding segments and averaged across all segments. This analysis was repeated for multiple time lags within the range of [-20, 20] s for each pair of calcium traces, with 1-s shift step. The peak correlation value across time lags was taken as a measure of synchrony between the calcium signals. This approach yields an accurate correlation estimate between signals on a short timescale, irrespective of common activations over longer periods of time.

Glutamate uncaging in assembloids

Assembloids were imaged under environmentally controlled conditions (37 °C) using a $\times 5$ objective in a confocal microscope (Leica SP8). For glutamate uncaging experiments, MNI-caged-L-glutamate (1490; Tocris) was used at a final concentration of 2.5 mM in aCSF. The FRAP software module of the Leica SP8 confocal microscope was used to uncage glutamate using ultraviolet light (405 nm). At a frame rate of 2.3 frames per second, one trial consisted of 50 frames for pre-stimulation, three frames of ultraviolet stimulation (in specified

ROI) and 100 frames for post-stimulation; three trials were applied for one sample. Calcium responses were calculated by comparing the mean of $\Delta F/F_{\text{base}}$ during 50 frames (21.8 s) before and after ultraviolet illumination, as described above, and averaged values of three trials were used for each sample.

Western blotting

Three to five organoids were collected in the same tube and considered as one sample. Whole-cell protein lysates for samples were prepared using the RIPA buffer system (sc-24948; Santa Cruz Biotechnology). Protein concentration was quantified using the bicinchoninic acid assay (Pierce; catalogue no. 23225; Thermo Fisher Scientific). For electrophoresis, 30 μg of protein per lane was loaded and run on a 4–12% Bis-Tris PAGE gel (Bolt 4–12% Bis-Tris Protein Gel; catalogue no. NW04122BOX; Invitrogen) and transferred onto a polyvinylidene difluoride membrane (Immobilon-FL; EMD Millipore). Membranes were blocked with 5% BSA in tris-buffered saline with Tween (TBST) for 1 h at room temperature and incubated with primary antibodies: anti-glyceraldehyde 3-phosphate dehydrogenase (mouse; 1:5,000; GTX627408; GeneTex) for 1 day and anti-Na_v1.7 (rabbit; 1:1,000, ASC-008; Alomone Labs) for 6 days. Membranes were washed three times with TBST and then incubated with near-infrared fluorophore-conjugated species-specific secondary antibodies (Goat Anti-Mouse IgG Polyclonal Antibody (IRDye 680RD; 1:10,000; 926-68070; LI-COR Biosciences) or Goat Anti-Rabbit IgG Polyclonal Antibody (IRDye 800CW; 1:10,000; 926-32211; LI-COR Biosciences)) for 1 h at room temperature. Following secondary antibody application, membranes were washed three times with TBST and once with TBS and then imaged using a LI-COR Odyssey CLx imaging system with Image Studio Software (v.5.2; LI-COR). Western blots were analysed using the Image Studio Software or Fiji (v.2.14.0) (see Source data for full blots).

Axon projection imaging and analysis

EYFP⁺ projections in target regions were imaged under environmentally controlled conditions in intact assembloids using a Leica TCS SP8 or Leica STELLARIS 5 confocal microscope with a motorized stage. Assembloids were transferred to a well in a 24-well glass bottom plate (P24-0-N; Cellvis) in a cell culture medium and incubated in an environmentally controlled chamber for 15–30 min before imaging. Images were taken using a $\times 10$ objective to capture the entire target area at a depth of 100 μm . Projections were quantified using Fiji (v.2.14.0). ROI were manually drawn to cover the target area to be measured in max projection confocal stacks. The percentage of fluorescence-positive pixels over the target region area was calculated in binary images.

Mouse primary tissue dissection and imaging

Mouse tissue samples were obtained using protocols approved by Stanford University's Administrative Panel on Laboratory Animal Care. Timed pregnant CD1 mice with 11 days of gestation were obtained from Charles River Laboratories and maintained on a 12-h light–dark cycle, an ambient temperature of 20–26 °C and a humidity of 30–70%. We anaesthetized pregnant mice with isoflurane on day 13 of gestation, and E13.5 embryos were used in this study without knowing their sex. DRG were dissected from E13.5 embryos, placed en bloc onto PEI-coated 12-well plates and cultured in DRG culture medium (neural medium supplemented with BDNF, GDNF, NGF and 10% FBS). Calcium imaging was performed the next day after dissection.

As shown in Extended Data Fig. 3a–d, mouse DRG were incubated with Calbryte 520 AM (10 μM ; no. 20653; AAT Bioquest). After 45 min, the medium was changed to 1 ml of the DRG culture medium. To image the calcium activity of mouse DRG, a whole-cell culture plate containing mouse DRG was moved to an environment-controlled confocal microscope stage. Imaging was performed when the lid of the plate was opened. After 1 min of baseline line imaging, 500 μl of $\alpha\beta$ -MeATP (90 μM) or capsaicin (10 μM) dissolved in the DRG culture medium

was added into the well by pipetting without touching the cell culture plate (to prevent XYZ movement). Imaging was performed for 6 min. The final concentrations of the agonists were 30 μM ($\alpha\beta$ -MeATP) and 3.3 μM (capsaicin).

As shown in Fig. 2l,m, mouse DRG were incubated with Calbryte 520 AM, as described above, and additionally incubated with various concentrations of TNP-ATP (10, 100 or 100,000 nM; no. 2464; Tocris) dissolved into DRG culture medium for 30 min in the incubator. After 1 min of baseline imaging with TNP-ATP, 500 μl of $\alpha\beta$ -MeATP (90 μM) in the DRG culture medium was added into the well. Imaging was performed for 6 min.

Extracellular recordings

Extracellular recordings were performed, as described previously¹¹. Assembloids were embedded into 3% low-melting gel agarose (IB70056; IBI Scientific). Embedded samples were placed on a Brain Slice Chamber-2 (S-BSC2; Scientific Systems Design) and perfused with aCSF (bubbled with 95% O₂ and 5% CO₂) at 37 °C. The temperature was controlled and retained at 37 °C by connecting to a Proportional Temperature Controller PTC03 (S-PTC03; AutoMate Scientific). Acute 32-channel P-1 probes with two shanks (ASSY-37 P-1; Cambridge NeuroTech) were connected to an Acute probe adaptor; 32-channel Samtec to Omnetics (ADPT A32-Om32; Cambridge NeuroTech). Baseline activities were acquired using the Intan 1024ch recording controller (Intan Technologies) at 30,000 Hz for 5 min using RHX software (v.3.1.0). Raw recording data were processed using Intan Technologies code and analysed using MATLAB (v.R2022a; MathWorks).

To analyse extracellular recording data, band-pass filtering (350–2,000 Hz) was performed followed by common median referencing. A threshold (six times multiplied by s.d.) for each channel was used to count neuronal activities. Activity from hSpO, hDiO or hCO was counted ± 500 ms of each activity of hSeO channels. Activity counts around the peak area (P) – activity counts in the base area (B)/($P + B$) were used as co-activation index⁴⁴. Parameters (bin size, 5 ms; plot size, ± 500 ms) were used to draw the representative cross-correlograms.

Genome editing of hiPS cells to generate SCN9A KO and gain-of-function hiPS cell lines

For generating SCN9A KO cells, three single guide RNAs (sgRNAs) were designed and synthesized by Synthego to induce one or more fragment deletions. The sgRNA sequences are as follows: 5'-AGCUC GUGUAGCCAUAAUCA-3', 5'-CGUGUGUAGUCAGUGUCCAG-3' and 5'-UUCUCUUGGUACUCACCUGU-3'. The hiPS cells were dissociated with Accutase, and 0.5 million cells were mixed with 300 pmol sgRNAs and 40 pmol Cas9 protein (SpCas9 2NLS Nuclease (1,000 pmol); Synthego). Nucleofection was performed using the P3 Primary Cell 4D-Nucleofector X Kit S (V4XP-3032; Lonza), a 4D-Nucleofector Core Unit and the X unit (program CA-137; Lonza). Cells were then seeded onto vitronectin-coated six-well plates in Essential 8 Medium supplemented with the ROCK inhibitor Y27632 (10 μM). Essential 8 Medium was used for daily medium change. The genotype of pooled cells was determined by PCR with the primer set Fw, 5'-CGAGAACTACCCA TATTATTAGTGATGG-3'; Rv, 5'-CCAAGAACTATCACAAACGTCTGT-3', and Sanger sequencing (GENEWIZ) was performed with the reverse primer.

SCN9A gain-of-function cells were generated by Bayspair using two-step scarless genome editing⁶². Genotyping was performed using PCR with the primer set Fw, 5'-GTGTTTGGAGACCCATGTTTC-3'; Rv, 5'-GAAGTAGTAGTGTCTGAGGGAGATC-3', and Sanger sequencing (GENEWIZ) was performed with the reverse primer.

Patch-clamp recordings

Patch-clamp recordings were conducted, as described previously¹¹. In brief, hSeO were first infected with Lenti-hSYN1::Chr2-EYFP before assembly and then integrated with hSpO to create hSeO–hSpO

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assembloids. These assembloids were subsequently infected with AAV-DJ-hSYN1::mCherry and placed onto cell culture inserts (0.4- μ m pore size; 353090; Corning) positioned in six-well plates to form flattened assembloids. Recordings were typically performed on days 148–164 (daf 99–110). mCherry-positive neurons within the hdSpO were identified using an upright microscope. For optogenetic stimulation, whole-field light-emitting diode illumination (460 nm; 5–20 ms duration; maximal power; CoolLED) was applied through a $\times 40$ objective, and oEPSCs were recorded at a holding potential of -70 mV.

Patch-clamp recordings in whole-cell configuration were performed at room temperature on spinal organoid neurons, which were visualized using an upright microscope (Scientifica) equipped with an INFINITY2 charge-coupled device camera and INFINITY Capture Software (Teledyne Lumenera). The external aCSF recording solution contained 124 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4 , 1 mM MgSO_4 , 2 mM CaCl_2 , 26 mM NaHCO_3 and 10 mM D-(+)-glucose. During recordings, the assembloids were perfused with the aCSF recording solution (bubbled with 95% O_2 and 5% CO_2). Thick-walled borosilicate pipettes (resistance of 6–9 M Ω) were filled with an internal solution consisting of 135 mM K-gluconate, 20 mM KCl, 0.1 mM EGTA, 2 mM MgCl_2 , 2 mM sodium-ATP, 10 mM HEPES and 0.3 mM sodium-GTP (pH adjusted to 7.28 with KOH; approximately 302 mOsm). Data were acquired using a MultiClamp 700B Amplifier (Clampex 10.7; Molecular Devices) and a Digidata 1550B Digitizer (Molecular Devices), low-pass filtered at 2 kHz, digitized at 20 kHz and analysed with Clampfit (v.10.7; Molecular Devices).

Statistics and reproducibility

Data are presented as mean $\pm$ s.e.m. unless described otherwise. Raw data were tested for normality of distribution, and statistical analyses were performed by two-tailed unpaired *t*-test, two-tailed Mann–Whitney test, two-tailed paired *t*-test, two-tailed Wilcoxon matched-pair signed-rank test, Wilcoxon signed-rank test, one-way ANOVA and Kruskal–Wallis test with multiple comparison tests depending on the dataset. Sample sizes were estimated empirically. GraphPad Prism v.10.0.0 or MATLAB (v.R2023a; MathWorks) was used for statistical analyses. Data shown in representative experiments were repeated with similar results in multiple independent experiments.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Gene expression data have been deposited in the Gene Expression Omnibus under accession number GSE251892. The data in this study

are available on request from the corresponding author. Source data are provided with this paper.

Code availability

The codes for SCA were described in a previous study⁴³ and are available at Zenodo (<https://doi.org/10.5281/zenodo.13921410>)⁶³.

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Author contributions J.-i.K., K.I. and S.P.P. conceived the project and designed the experiments. J.-i.K. carried out the differentiation, characterization of regionalized neural organoids, extracellular recordings and calcium imaging. K.I. performed neural differentiations, single-cell transcriptomics, calcium imaging and *SCN9A* gene editing. O.J. contributed to the analysis pipelines for synchronized activities. K.W.K. contributed to scRNA-seq analysis. D.W. performed patch-clamp recording experiments. M.V.T. performed neural differentiations, western blotting and characterization of the regionalized neural organoids. Z.H. analysed the calcium imaging results. N.D.A. and R.J.L. contributed to the primary tissue experiments. G.S. contributed to experimental design and data analysis. J.-i.K., K.I. and S.P.P. wrote the article with input from all the authors.

Competing interests Stanford University filed or is filing patent applications covering the generation of regionalized organoids and assembloids.

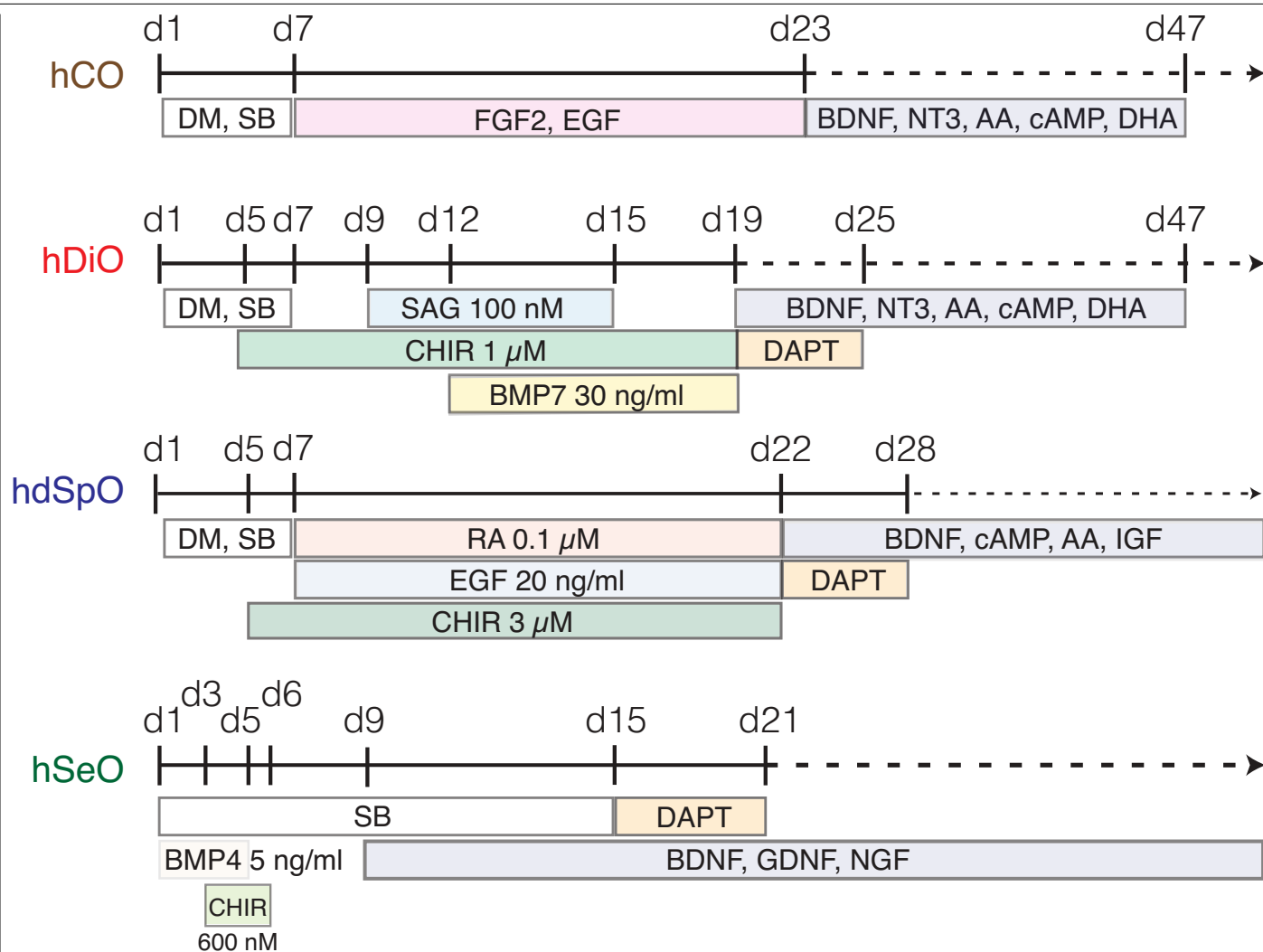
Additional information

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Peer review information *Nature* thanks the anonymous reviewers for their contribution to the peer review of this work.

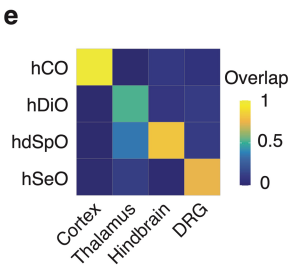
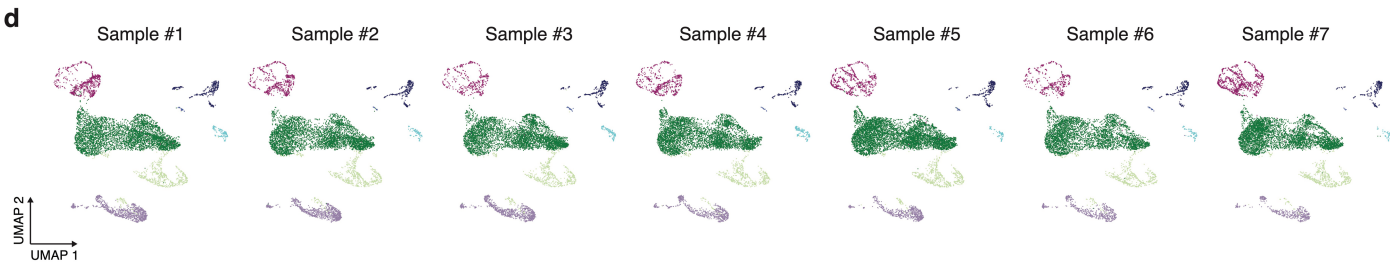
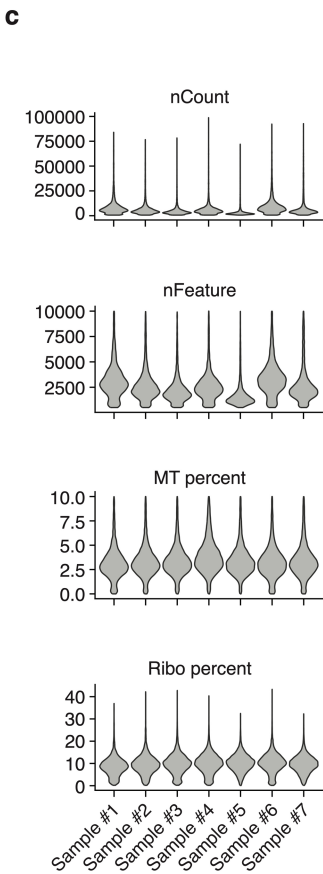
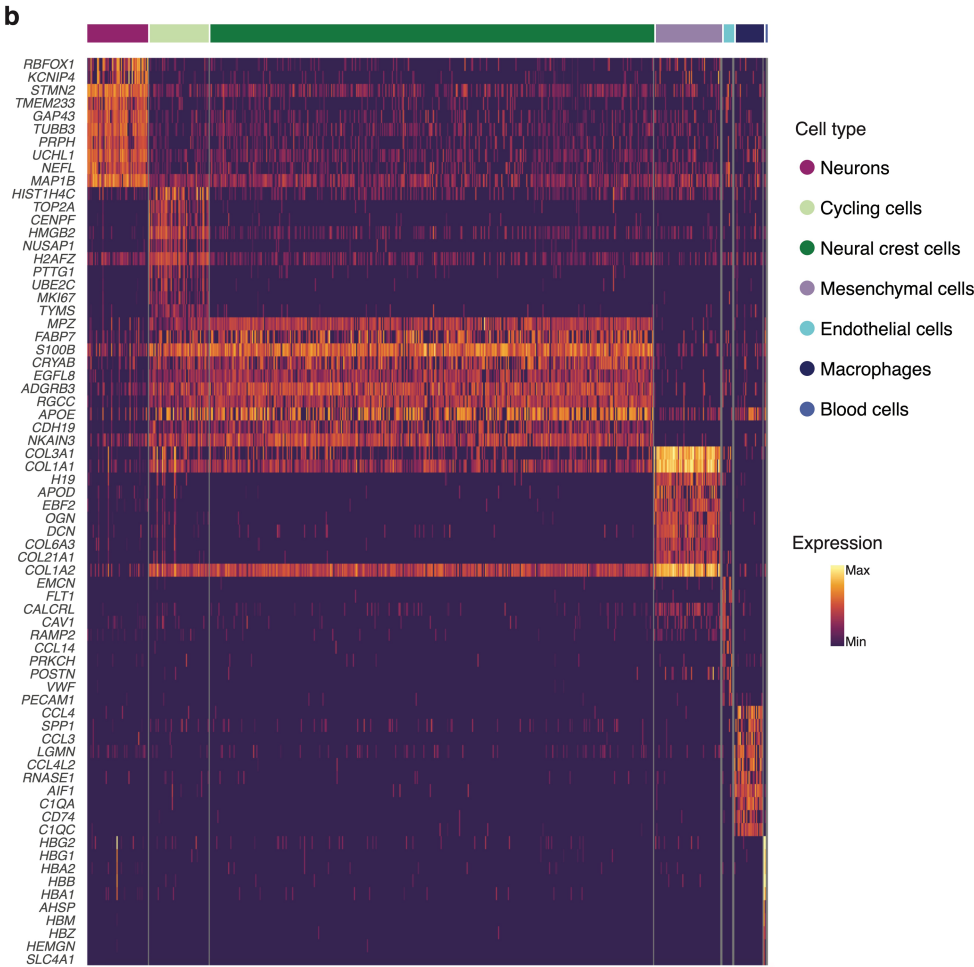
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Extended Data Fig.1 | Protocol for generating regionalized neural organoids. Protocols to generate hCO, hDiO, hdSpO, and hSeO from hiPS cells.

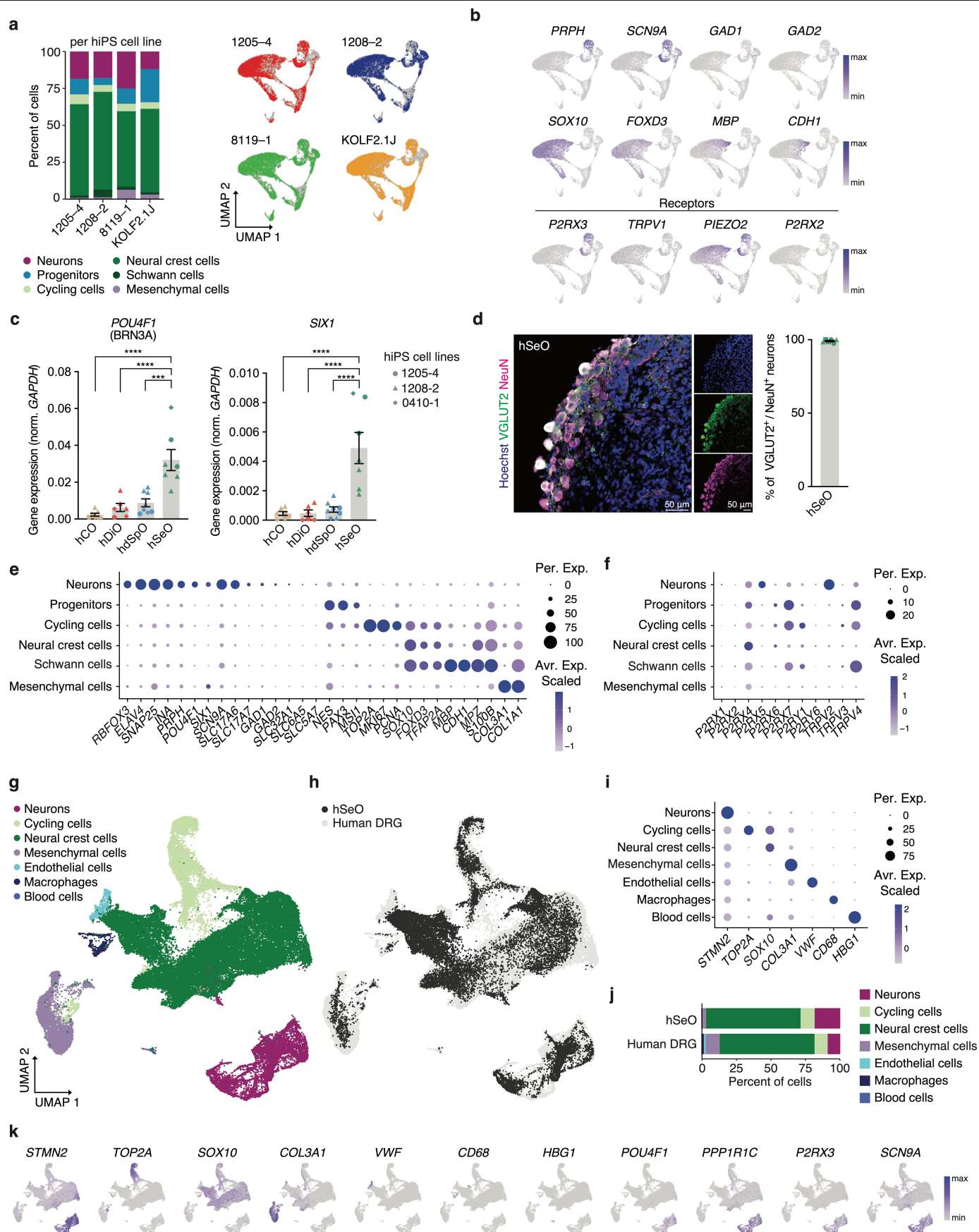
a scRNA-seq:
Human DRG (PCW15)

Sample #1	Sample #2	Sample #3	Sample #4	Sample #5	Sample #6	Sample #7	Total
7,630	7,104	7,110	7,097	8,531	6,006	7,335	50,813 cells



Extended Data Fig. 2 | Single cell transcriptome of human DRG. a. Table showing the number of single cells per sample. Numbers correspond to total numbers of cells recovered after QC and filtering. **b.** Heat map showing normalized expression of the top ten unique markers per cell type. **c.** Violin

plots showing total counts (nCount), total number of features (nFeature), percent of mitochondrial (MT) counts, and percent of ribosomal (ribo) counts separated by sample. **d.** UMAP plots split by sample. **e.** Proportion of cells with the indicated organoid identity for each brain subdomain label.

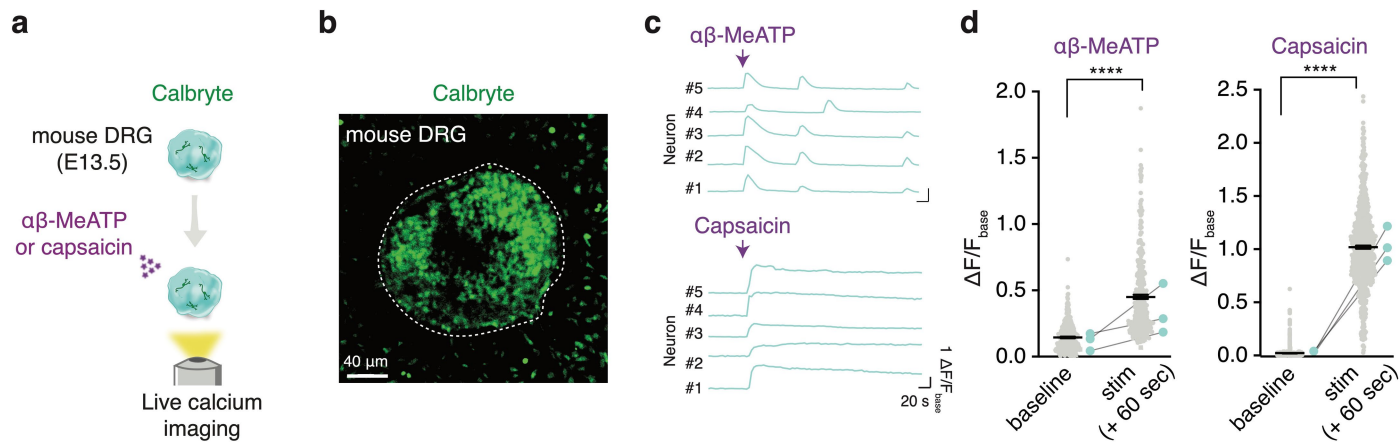


Extended Data Fig. 3 | See next page for caption.

Extended Data Fig. 3 | Transcriptomic and immunostaining analysis of hSeO.

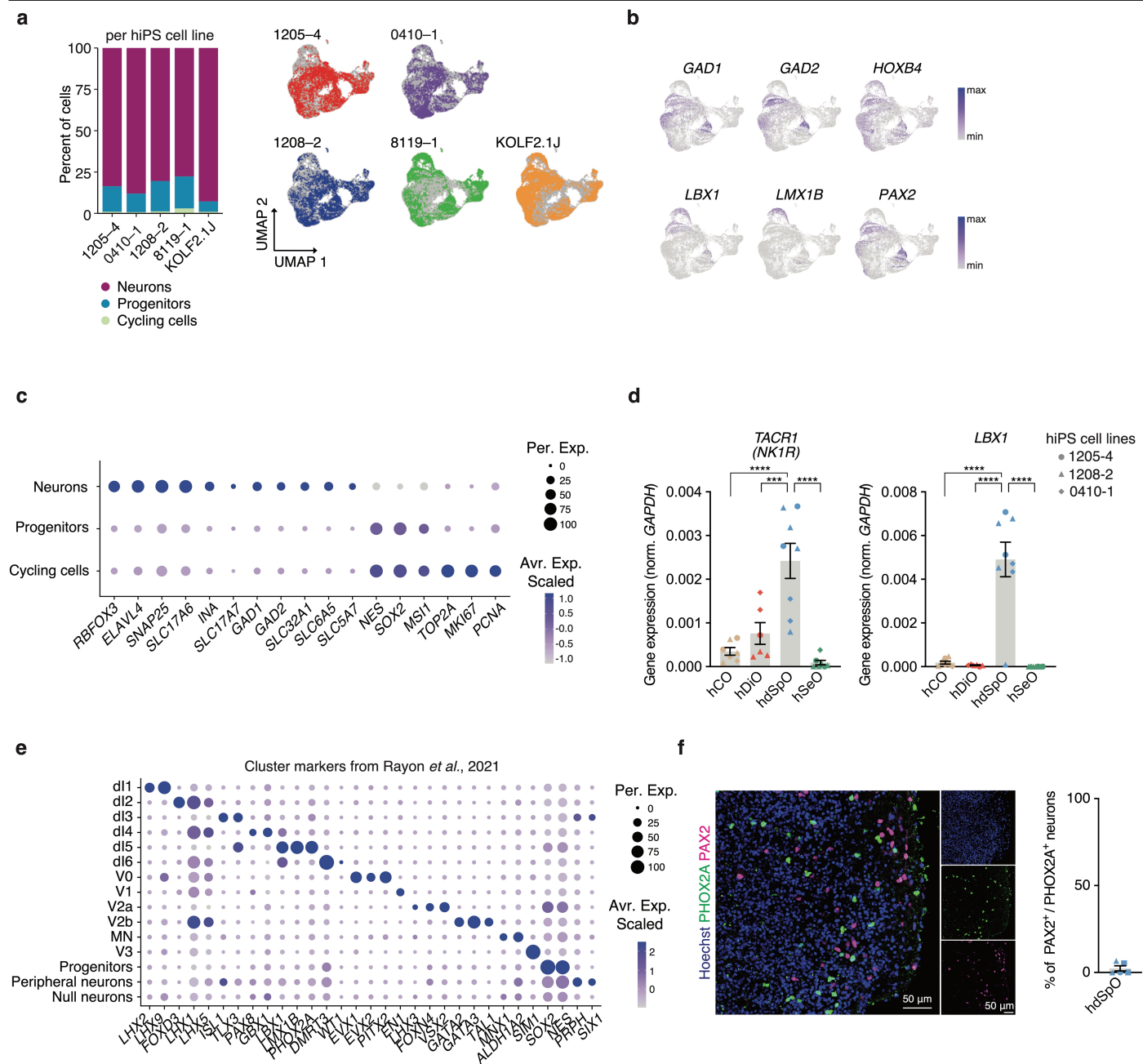
a. Cell type composition in hSeO colored by cluster (left), and UMAP plots of hSeO colored by the hiPS cell line (right). **b.** UMAP plots of hSeO samples showing selected cell type markers and receptors. **c.** RT-qPCR of *POU4F1* (hCO, n = 7; hDiO, n = 6; hdSpO, n = 8; hSeO, n = 7 samples, from 3 hiPS cell lines from 2–4 differentiation experiments, one-way ANOVA, $F_{3,24} = 16.59$ with Tukey's multiple comparisons test, *** $P = 0.0001$, **** $P < 0.0001$) and *SIX1* (hCO, n = 7; hDiO, n = 6; hdSpO, n = 8; hSeO, n = 7 samples, from 3 hiPS cell lines from 2–4 differentiation experiments, one-way ANOVA, $F_{3,24} = 15.49$ with Tukey's multiple comparisons test, **** $P < 0.0001$) expression across four regions at day 45–100. **d.** Immunostaining of VGLUT2 and NeuN in hSeO (left) and quantification of VGLUT2⁺/NeuN⁺ co-localization (right) at days 121–125

(n = 6 organoids from 3 hiPS cell lines from 4 differentiation experiments). **e.** Dot plot showing selected marker gene expression in each cluster. **f.** Dot plot showing gene expression of several receptors for sensory stimuli. **g.** Integrated UMAP visualization of scRNA-seq of human primary DRG (PCW15) and hSeO (human primary DRG, n = 49,705 cells from 7 DRG samples, one individual; hSeO, day 68–72, n = 18,382 cells from 4 hiPS cell lines, same dataset as in Fig. 1 with optimized QC criteria). **h.** UMAP plot colored by sample identity. **i.** Dot plot showing selected marker gene expression in each cluster. **j.** Cell composition of hSeO and human primary DRG based on scRNA-seq. **k.** UMAP plots showing selected cell type markers and receptors. Data shown as mean $\pm$ s.e.m. Dots indicate qPCR samples or organoids in c, d. Each shape represents a hiPS cell line: Circle, 1205-4; Triangle, 1208-2; Diamond, 0410-1; Square, KOLF2.1) in c, d.



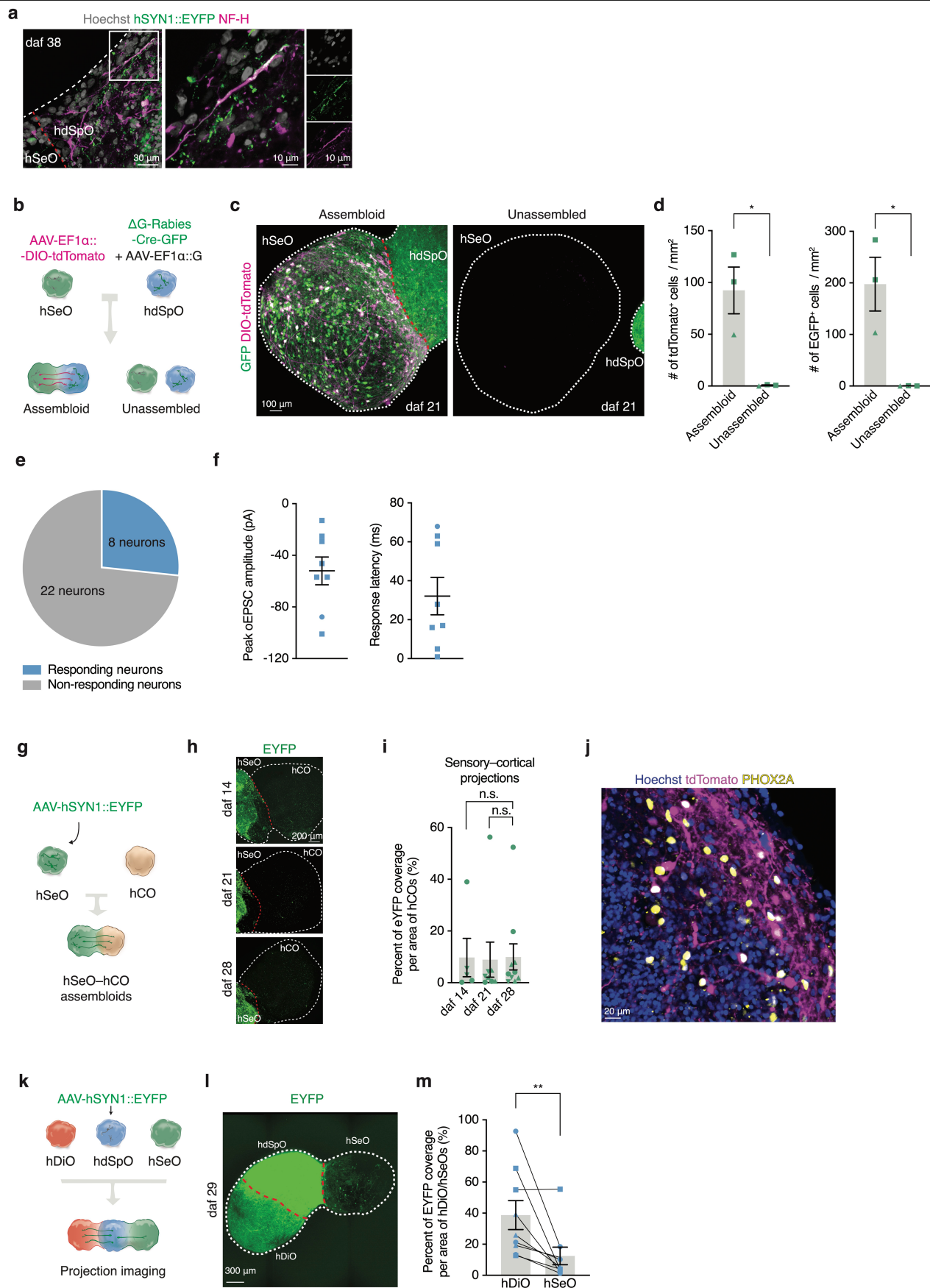
Extended Data Fig. 4 | Calcium imaging of isolated mouse DRG. **a.** Schematic of live calcium imaging of isolated mouse DRG following pharmacological treatment. **b.** Representative image showing GCaMP6s expression in mouse DRG. **c.** Example $\Delta F/F_{\text{base}}$ traces before and after agonist exposure showing calcium responses evoked by $\alpha\beta$ -MeATP (30 μM) and capsaicin (3.3 μM). **d.** Quantification of mean $\Delta F/F_{\text{base}}$ values at baseline and at 60 s after exposure to $\alpha\beta$ -MeATP (left)

($n = 384$ cells from 3 DRG, Two-tailed Wilcoxon matched-pairs signed rank test, **** $P < 0.0001$) and capsaicin (right) ($n = 762$ cells from 10 hSeO, 3 DRG, two-tailed Wilcoxon matched-pairs signed rank test, **** $P < 0.0001$). Data shown as mean $\pm$ s.e.m. Gray dots indicate cells, large colored dots indicate DRG in d. Credit: **a** adapted from ref. 48, Springer Nature America, Inc.



Extended Data Fig. 5 | Transcriptomic and immunostaining analysis of hdSpO. **a.** Cell type composition of hdSpO colored by cell clusters (left), and UMAP plots of hdSpO samples colored by the hiPS cell line (right). **b.** UMAP plots of hdSpO samples showing selected cell type markers. **c.** Dot plot showing selected marker gene expression in each cluster. **d.** RT-qPCR of *TACR1* (hCO, $n = 7$; hDiO, $n = 6$; hdSpO, $n = 8$; hSeO, $n = 7$ samples, from 3 hiPS cell lines from 2–4 differentiation experiments, one-way ANOVA, $F_{3,24} = 17.70$ with Tukey's multiple comparison test, $***P = 0.0009$, $****P < 0.0001$) and *LBX1* (hCO, $n = 7$; hDiO, $n = 6$; hdSpO, $n = 8$; hSeO, $n = 7$ samples, from 3 hiPS cell lines from 2–4 differentiation

experiments, one-way ANOVA, $F_{3,24} = 30.50$ with Tukey's multiple comparisons test, $****P < 0.0001$) expression across four regions at day 45–100. **e.** Dot plot showing dorsoventral cluster marker gene expression in each subcluster. **f.** Representative image showing distribution of PAX2⁺ and PHOX2A⁺ neurons in hdSpO (left) and quantification of co-localization of PAX and PHOX2A ($n = 5$ organoids from 3 hiPS cell lines, 4 differentiation experiments). Data shown as mean $\pm$ s.e.m. Dots indicate qPCR samples or organoids in d, f. Each shape represents a hiPS cell line: Circle, 1205-4; Triangle, 1208-2; Diamond, 0410-1; Square, KOLF2.1J in d, f.

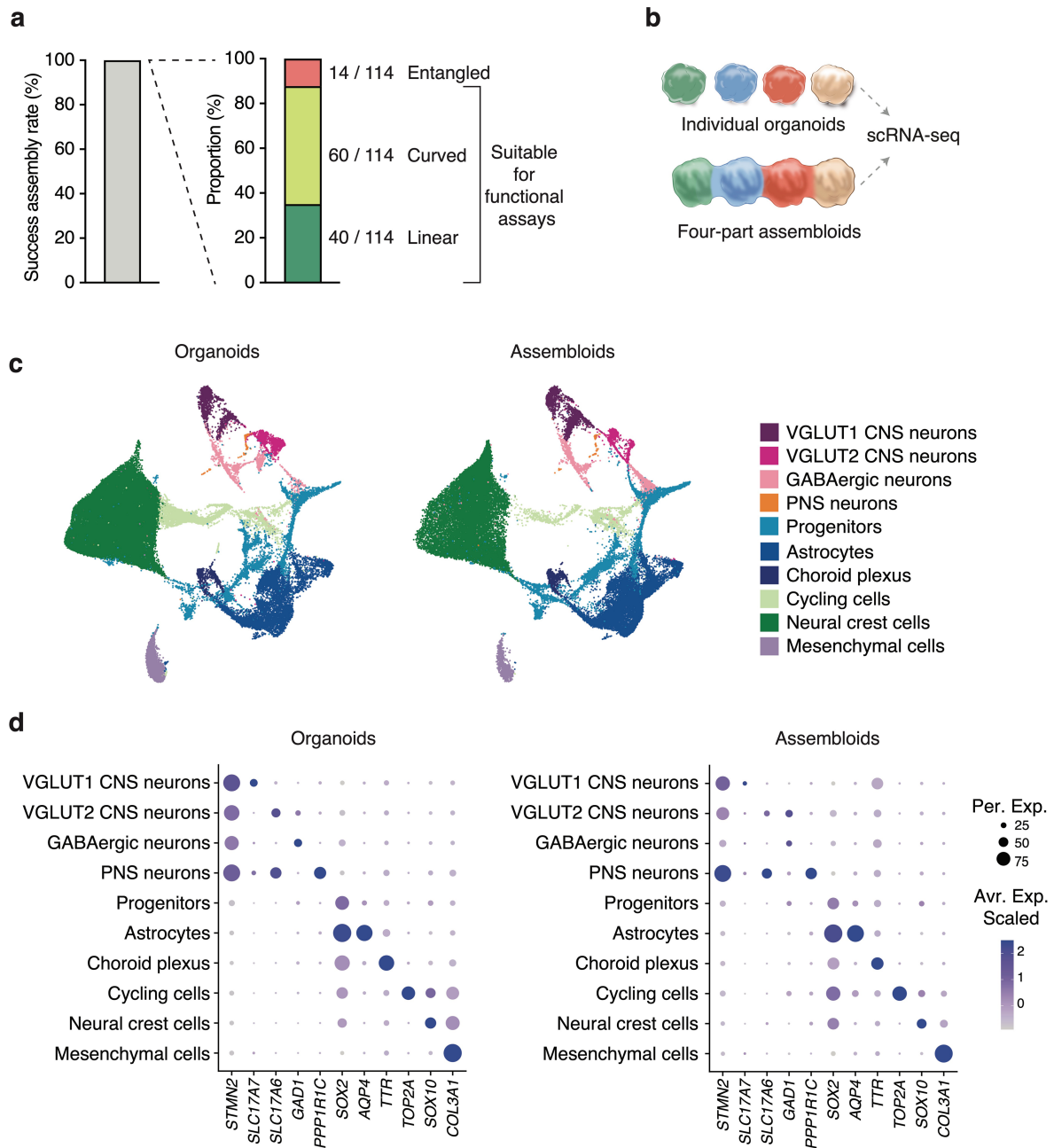


Extended Data Fig. 6 | See next page for caption.

Extended Data Fig. 6 | Characterization of projections in assembloids.

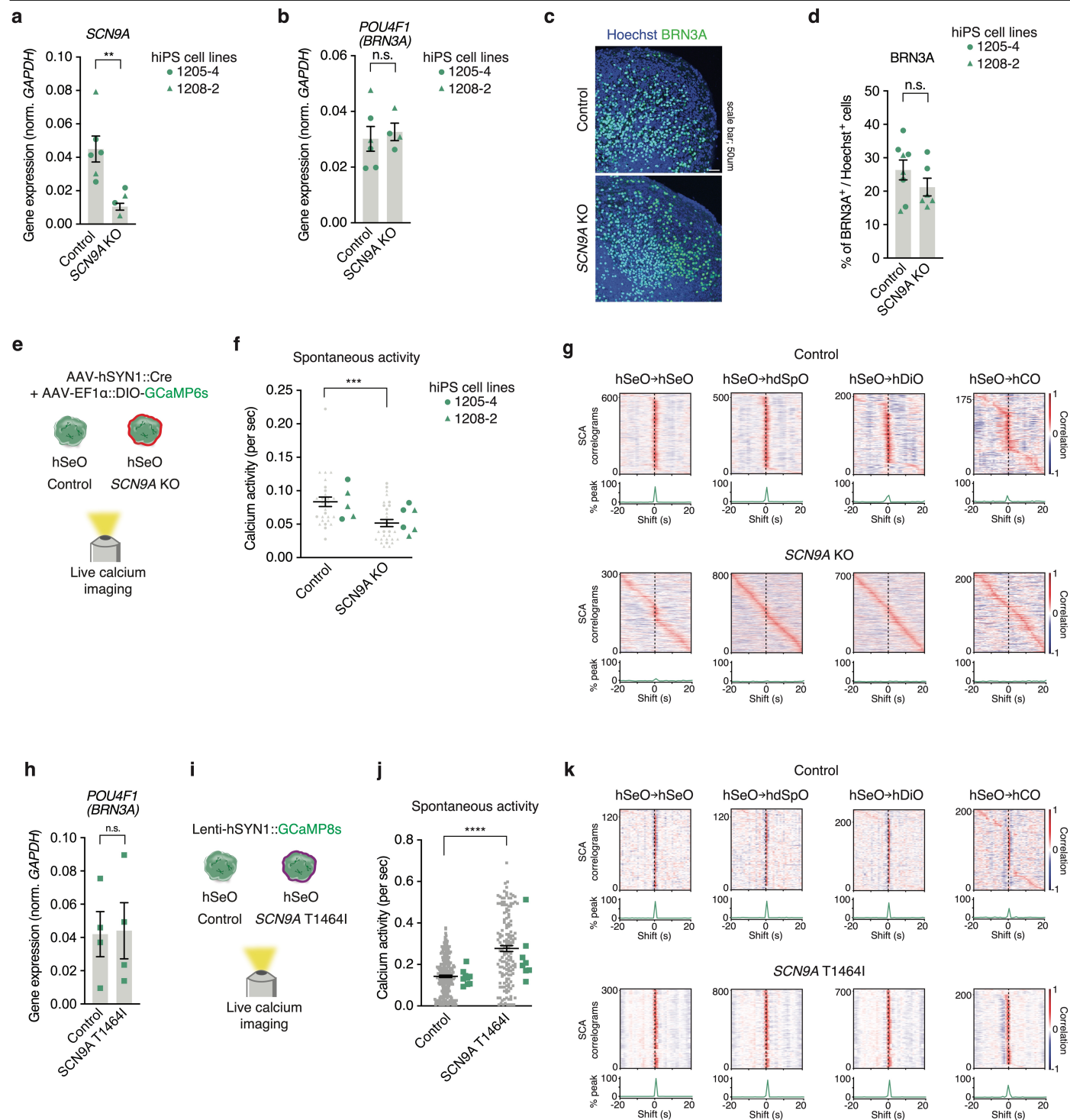
a. Immunostaining image showing co-localization of NF-H and EYFP⁺ projection in the hdSpO side of hSeO–hdSpO assembloids. **b.** Schematic illustrating rabies tracing experiments in assembloid and unassembled organoids. **c.** Representative images indicating presence of EGFP⁺/tdTomato⁺ cells on the hSeO side of the assembloid and absence in unassembled organoids at daf 21. **d.** Quantification of tdTomato⁺ and EGFP⁺ cells (n = 3 assembloids from 2 hiPS cell lines from 2 differentiation experiments, two-tailed unpaired t test, *P = 0.0155 for tdTomato⁺ cells and *P = 0.0193 for EGFP⁺ cells). **e.** Proportion of responding neurons (8/30 cells). **f.** Quantification of peak oEPSC amplitude (left) and response latency (right) (n = 8 neurons from 2 hiPS cell lines, 2 differentiation experiments). **g.** Schematic illustrating the fusion of hSeO and hCO to form hSeO–hCO assembloids. **h.** Representative images of a hSeO–hCO assembloid showing a limited number of hSeO-derived hSYN1::EYFP projections at 14, 21, and 28 daf. **i.** Quantification of hSeO-derived EYFP

fluorescence in hCO area at 14, 21, and 28 daf in hSeO–hCO assembloids (n = 5–10 assembloids from 3 hiPS cell lines from 3 differentiation experiments, Kruskal-Wallis test with Dunn's multiple comparisons test, n.s.; not significant). **j.** Immunostaining image showing co-localization of PHOX2A and tdTomato on the hdSpO side of hSeO–hdSpO assembloids. **k.** Schematic illustrating the integration of EYFP labeled hdSpO, hSeO and hDiO to form 3-part hSeO–hdSpO–hDiO assembloids. **l.** Representative image of a hSeO–hdSpO–hDiO assembloid showing preferential spinal projection into hDiO. **m.** Quantification of hdSpO-derived EYFP fluorescence in hDiO or hSeO at daf29 (n = 9 assembloids from 3 hiPS cell lines from 2 differentiation experiments, two-tailed Wilcoxon matched-pairs signed rank test, **P = 0.0078). Data shown as mean ± s.e.m. Dots indicate assembloids in d, i, m, or neurons in f. Each shape represents a hiPS cell line: Circle, 1205-4; Triangle, 1208-2; Inverted triangle, 8119-1, Square, KOLF2.1J in d, f, i, m. Credits: **b,g,k** adapted from ref. 48, Springer Nature America, Inc.



Extended Data Fig. 7 | Characterization of hASA. **a** Success of assembly (left) and distribution of shapes of assembloids (right). Approximately 87.7% (100/114) hASA were suitable for functional assays. **b** Schematic illustrating the scRNA-seq of individual organoids and four-part assembloids. **c** UMAP visualization of individual organoids (left) and four-part assembloids at day 162–229 (right) (individual organoids, $n = 59,267$ cells from 4 samples, 2 differentiation

experiments, one hiPS cell line; four-part assembloids, $n = 40,700$ cells from 4 samples, 2 differentiation experiments, one hiPS cell line). **d** Dot plot showing selected marker genes for each cluster of individual organoids (left) and four-part assembloids (right). Credit: **b** adapted from ref. 48, Springer Nature America, Inc.



Extended Data Fig. 8 | See next page for caption.

Extended Data Fig. 8 | Characterization of hSeO with *SCN9A* pathogenic variants. **a.** RT-qPCR analysis of *SCN9A* expression in control and *SCN9A* KO hSeO (Control, n = 6; *SCN9A* KO, n = 4 samples from 2 hiPS cell lines from 2 differentiation experiments, two-tailed unpaired t-test, **P = 0.0082) at day 48–69. **b.** RT-qPCR analysis of *POU4F1* expression in control and *SCN9A* KO hSeO (Control, n = 6; *SCN9A* KO, n = 4 samples from 2 hiPS cell lines in 2 differentiation experiments, two-tailed unpaired t-test, n.s.; not significant) at day 48–69. **c.** Immunocytochemistry images for BRN3A in control and *SCN9A* KO hSeO. **d.** Quantification of the percentage of BRN3A⁺ cells in control and *SCN9A* KO hSeO (Control, n = 8; *SCN9A* KO, n = 6 samples from 2 hiPS cell lines from 2 differentiation experiments, two-tailed unpaired t-test, n.s.; not significant). **e.** Calcium imaging of control and *SCN9A* KO hSeO. **f.** Quantification of the number of spontaneous calcium responses (Control, n = 28 cells from 5 organoids; *SCN9A* KO, n = 29 cells from 6 organoids, 2 hiPS cell lines from 2 differentiation experiments, two-tailed Mann-Whitney test, ***P = 0.0006) at day 64–82. **g.** Representative SCA correlograms between cells from hSeO–hSeO, hSeO–hdSpO, hSeO–hDiO, and hSeO–hCO for a control assembloid (top) and in a

SCN9A KO assembloid (bottom). The histograms of peak time-shifts (below each correlograms panel) show peaks around 0 in the control, but not in the *SCN9A* KO assembloid. **h.** RT-qPCR analysis of *POU4F1* expression in control and *SCN9A* T1464I hSeO (n = 4 samples from one hiPS cell line from 4 differentiation experiments, two-tailed unpaired t-test, n.s.; not significant) at day 25–31. **i.** Calcium imaging of control and *SCN9A* T1464I hSeO. **j.** Quantification of the number of spontaneous calcium activity (CTL, n = 244 cells from 9 organoids; T1464I, n = 149 cells from 8 organoids, one hiPS cell line from 3 differentiation experiments, two-tailed Mann-Whitney test, ****P < 0.0001) at day 103–111. **k.** Representative SCA correlograms between cells from hSeO–hSeO, hSeO–hdSpO, hSeO–hDiO, and hSeO–hCO for a control assembloid (top) and in a *SCN9A* T1464I assembloid (bottom). Data shown as mean ± s.e.m. Gray dots indicate cells, large colored dots indicate organoids or qPCR samples in a, b, d, f, h, j. Each shape represents a hiPS cell line: Circle, 1205-4; Triangle, 1208-2; Square, KOLF2.1J in a, b, d, f, h, j. Credits: **e, i** adapted from ref. 48, Springer Nature America, Inc.

Reporting Summary

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Statistics

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

- | | |
|-------------------------------------|--|
| n/a | Confirmed |
| ☐ | ☒ The exact sample size (<i>n</i>) 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. <i>F</i> , <i>t</i> , <i>r</i>) with confidence intervals, effect sizes, degrees of freedom and <i>P</i> 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 <i>d</i> , Pearson's <i>r</i>), indicating how they were calculated |

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

Software and code

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

Data collection	LAS-X (Leica) software was used for acquiring the microscopy data. Image Studio (V5.2) was used to acquire western blotting data. QuantStudio Real-Time PCR software (V1.7.1) was used to collect qPCR data. RHX software (V3.1.0) was used for acquiring the extracellular recording data. Infinity Capture software (Teledyne Lumenera) and Clampex (v10.7) was used for patch clamp recording.
Data analysis	The GraphPad Prism (V10.0.0) was used for statistical analyses. Fiji (ImageJ, V2.14.0) was used for image quantification and processing. MATLAB(R2022a and R2023a) was used to analyze calcium imaging and extracellular recording data. The codes for scaled correlation analysis (SCA) were described in Miura et al., bioRxiv (2024) and have been deposited at Zenodo (DOI: 10.5281/zenodo.13921410). ImarisViewer (V10.0.1) was used to process immunostaining images. CellRanger (7.0.1, 7.1.0, 7.2.0, 10x Genomics), R (4.2.2), and Seurat (V4.3.0, R package) were used for single-cell RNA-seq analysis. Clampfit (v10.7) was used to analyze patch-clamp recording data.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

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](#)

Source data were provided with this manuscript. Single cell RNA-seq data is available in GEO under accession number GSE224766. The following datasets were used in this study: GRCh38/Ensembl 98 reference for Cell Ranger (<https://cf.10xgenomics.com/supp/cell-exp/refdata-gex-GRCh38-2020-A.tar.gz>); single cell RNA-seq data of hCO by Khan et al. (GSE145122); single cell RNA-seq data of hDiO by Kim et al. (GSE224766); human fetal spinal cord single cell RNA-seq data by Rayon et al. (GSE171892); human fetal brain single cell RNA-seq data by Braun et al. (<https://github.com/linnarsson-lab/developing-human-brain>).

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	N/A
Reporting on race, ethnicity, or other socially relevant groupings	N/A
Population characteristics	N/A
Recruitment	N/A
Ethics oversight	N/A

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

Life sciences study design

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

Sample size	Sample sizes were estimated empirically, based on previous studies (Birey et al., Nature 2017; Marton et al., Nature Neuroscience; Pasca et al, Nature Medicine 2019; Yoon et al., Nature Methods 2019; Miura et al., Nature Biotechnology 2020; Birey et al., Cell Stem Cell 2022; Meng et al., 2023, Chen et al, Nature 2024).
Data exclusions	Organoids or assembloids displaying no activity were not included
Replication	Data shown in representative experiments were repeated with similar results in multiple independent experiments. Sample sizes for other experiments were indicated in Figure legends.
Randomization	Organoids and assembloids were randomly picked from cell culture plates for each assay.
Blinding	Blinding of experimental conditions was used for calcium imaging analysis, projection imaging analysis of assembloids. Blinding was not used or was not relevant for other experiments due to constraints in sample processing.

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

Methods

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

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

Antibodies

Antibodies used

Anti-GAPDH (mouse, GeneTex, GTX627408, 1:5000 dilution), lot#41323 Anti-FOXG1 (rabbit, Takara, M227, 1:400 dilution), lot#AM62662A
 Anti-TCF7L2 (rabbit, Cell Signaling Technology, 2569S, 1:200 dilution), lot#4Ref07/2019
 Anti-PHOX2A (rabbit, Abcam, ab155084, 1:200 dilution), lot#GR117345-3
 Anti-BRN3A (mouse, Millipore, MAB1585, 1:200 dilution), lot#3728208
 Anti-mCherry (goat, Biorbyt, orb153320, 1:1000 dilution), lot#VS2124
 Anti-NK1R (rabbit, Sigma, S8305, 1:200 dilution), lot#UD280591
 Anti-SCN9A (rabbit, 1:1,000, Alomone Labs, ASC-008), lot#3728208
 Anti-VGLUT2 (mouse, 1:200, Millipore, MAB5504)
 Anti-NF-H (mouse, 1:200, Millipore, NE1023)
 Anti-PAX2 (goat, 1:200, R&D Systems, AF3364), lot# XOT0823051

Goat Anti-Mouse IgG Polyclonal Antibody (IRDye 680RD, 1:10,000, LI-COR Biosciences, 926-68070)
 Goat Anti-Rabbit IgG Polyclonal Antibody (IRDye 800CW, 1:10,000, LI-COR Biosciences, 926-32211)
 Alexa Fluor 647 AffiniPure donkey anti-rabbit IgG (H&L) (1:1,000, Jackson ImmunoResearch, 711-605-152)
 Alexa Fluor 568 donkey anti-goat IgG (H&L) (1:1,000, Thermo Fisher Scientific, A11057)
 Alexa Fluor 568 donkey anti-rabbit IgG (H&L) (1:200, Thermo Fisher Scientific, A10042)
 Alexa Fluor 647 donkey anti-mouse IgG (H&L) (1:200, Thermo Fisher Scientific, A31571)
 Alexa Fluor 568 donkey anti-mouse IgG (H&L) (1:1,000, Thermo Fisher Scientific, A10037)

Validation

Anti-GAPDH: This antibody has been used in at least several hundreds of studies according to the manufacture's website.
 Anti-FOXG1: This antibody has been used by several studies, including Iwata et al., Science, 2020; Ideno et al., iScience, 2022.
 Anti-TCF7L2: This antibody has been used in more than 150 studies according to the manufacture's website.
 Anti-PHOX2A: This antibody has been used by Roome et al., Cell Reports, 2020.
 Anti-BRN3A: This antibody has been used in more than 50 studies according to the manufacture's website.
 Anti-mCherry: This antibody was used in our previous study (Andersen et al., Cell, 2020).
 Anti-NK1R: This antibody has been used in more than 60 studies according to the manufacture's website.
 Anti-SCN9A: This antibody has been used in more than 100 studies according to the manufacture's website.
 Anti-VGLUT2: This antibody was used in our previous study (Kim et al., Neuron 2024).
 Anti-NF-H: We have used this antibody in our previous study (Andersen et al., Cell, 2020). This antibody has been validated and used in 76 studies according to the manufacture's website.
 Anti-PAX2: This antibody has been used by Roome et al., Cell Reports, 2020.

Goat Anti-Mouse IgG Polyclonal Antibody: We have used this antibody in our previous study (Birey et al., Cell Stem Cell, 2022). Based on the manufacture's technical note, this antibody has been validated and used in multiple studies.
 Goat Anti-Rabbit IgG Polyclonal Antibody: We have used this antibody in our previous study (Birey et al., Cell Stem Cell, 2022). Based on the manufacture's technical note, this antibody has been validated and used in multiple studies.
 Alexa Fluor 647 AffiniPure donkey anti-rabbit IgG (H&L): This antibody has been used in 659 studies according to the manufacture's website.
 Alexa Fluor 568 donkey anti-goat IgG (H&L) : This antibody has been used in 855 studies according to the manufacture's website.
 Alexa Fluor 568 donkey anti-rabbit IgG (H&L): This antibody has been used in 1538 studies according to the manufacture's website.
 Alexa Fluor 647 donkey anti-mouse IgG (H&L): This antibody has been used in 1813 studies according to the manufacture's website.
 Alexa Fluor 568 donkey anti-mouse IgG (H&L): This antibody has been used in 898 studies according to the manufacture's website.

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s)

Four hiPS cell lines (1205-4, 1208-2, 8119-1, and 0410-1) were reprogrammed at Stanford University from fibroblasts harvested with IRB approval and following written consent. These hiPS cell lines were reported our previous publication (Khan et al., Nature Medicine, 2020)
 KOLF2.1J was derived at Jackson Laboratory and the line was obtained under an inter-institutional MTA.

Authentication

The hiPS cell lines (1205-4, 1208-2, 8119-1, 0410-1, and KOLF2.1J) were authenticated by SNP arrays.

Mycoplasma contamination

All cell lines were regularly tested and maintained Mycoplasma free.

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

No commonly misidentified lines used in this study.

Animals and other research organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals	E13.5 CD1 embryos were used in this study without knowing the sex of the embryos.
Wild animals	No wild animals were used in the study.
Reporting on sex	Mouse embryos were used in the experiment without knowing the sex of the embryos.
Field-collected samples	No field collected samples were used in the study.
Ethics oversight	Animal work was performed under protocols approved by the Stanford University's Administrative Panel on Laboratory Animal Care (APLAC).

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

Plants

Seed stocks	<i>Report on the source of all seed stocks or other plant material used. If applicable, state the seed stock centre and catalogue number. If plant specimens were collected from the field, describe the collection location, date and sampling procedures.</i>
Novel plant genotypes	<i>Describe the methods by which all novel plant genotypes were produced. This includes those generated by transgenic approaches, gene editing, chemical/radiation-based mutagenesis and hybridization. For transgenic lines, describe the transformation method, the number of independent lines analyzed and the generation upon which experiments were performed. For gene-edited lines, describe the editor used, the endogenous sequence targeted for editing, the targeting guide RNA sequence (if applicable) and how the editor was applied.</i>
Authentication	<i>Describe any authentication procedures for each seed stock used or novel genotype generated. Describe any experiments used to assess the effect of a mutation and, where applicable, how potential secondary effects (e.g. second site T-DNA insertions, mosaicism, off-target gene editing) were examined.</i>