iPSC-derived models of PACS1 syndrome reveal transcriptional and functional deficits in neuron activity

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ABSTRACT

PACS1 syndrome is a neurodevelopmental disorder characterized by intellectual disability and craniofacial abnormalities resulting from a *de novo* p.R203W variant in phosphofurin acidic cluster sorting protein 1 (PACS1). PACS1 plays roles in the endosomal pathway and nucleus, but little is known about how this variant affects developing neurons and patients have few therapeutic options. Here, we used stem cell-derived models to show that PACS1$^{(+/R203W)}$ neurons have impaired expression of genes enriched for synaptic signaling processes. We assessed the functional impact of this differential expression and find that PACS1$^{(+/R203W)}$ neurons have a striking prolongation of network burst duration resulting from an increased inter-spike interval. These results suggest that an aberrant regulation of ionic flux affecting spike frequency underlies the neurological phenotypes experienced by patients. This work is the first to investigate the impact of the PACS1 p.R203W variant on developing neural tissue, revealing electrophysiological mechanisms of disease and putative targets for pharmacological intervention.

INTRODUCTION

Neurodevelopmental disorders including autism spectrum disorder (ASD) and intellectual disability (ID) are highly prevalent in the population, both affecting 1-2% of individuals. Despite the pervasiveness of these disorders, few therapies exist due to a myriad of challenges including the genetic heterogeneity, historic inaccessibility of nervous tissue, and diagnosis typically occurring after the brain has developed. In certain instances, the genetic etiology of a neurodevelopmental disorder can be traced to a single source, providing a more constrained genetic background to study these disorders and work towards therapy development. PACS1 syndrome – a neurodevelopmental disorder caused by a single recurrent missense variant – represents one such condition.

PACS1 syndrome (MIM615009) was first reported in 2012 when two patients in an ID cohort presented with strikingly similar craniofacial abnormalities and comparable developmental delays.[1] Upon sequencing, both individuals were found to have the same *de novo* c.607C>T variant in the gene encoding phosphofurin acidic cluster sorting protein 1 (PACS1), which results in an arginine to tryptophan (R203W) substitution. Expression of the variant in a zebrafish model also produced craniofacial abnormalities, supporting the hypothesis that this was the variant responsible for patient symptoms.[1] Over 200 patients have since been identified with the same p.R203W pathogenic variant along with one report of a patient with an arginine to glutamine (R203Q) substitution.[2–6] All patients have ID and distinct craniofacial abnormalities, but patients experience frequent comorbidities including epilepsy, ASD, hypotonia, feeding dysfunction, and cardiac disorders.[3,7]

PACS1 is highly expressed in the brain during embryonic development and maintained at lower levels after birth, suggesting it plays an important role during neurodevelopment and continues to perform functions in adult tissues.[8] Genome-wide association studies (GWAS) have identified *PACS1* as a susceptibility gene in developmental delay,[9] bipolar disorder,[10] and severe early-onset obesity,[11] further suggesting a critical role for *PACS1* in neural development. However, most previous research on PACS1 function has focused on a non-neuronal context, revealing multifunctional roles in the endosomal pathway and nucleus. PACS1 was first discovered for its function as a cytosolic sorting protein that identifies targets with a specific phosphorylated acidic cluster motif and facilitates localization to the *trans*-Golgi Network (tGN) in concert with adaptor proteins such as AP-1 (adaptor protein 1).[12–15] The p.R203W variant characteristic of PACS1 syndrome lies within this acidic cluster identification region (called the furin binding domain, or FBR) and putatively interferes with its ability to identify targets. PACS1 was first reported and named in the context of shuttling furin to the tGN,[13] but since has been found to interact with dozens of targets that perform diverse roles in vesicle trafficking, ion transport, gene expression, and other processes.[14,16–26] More recently, PACS1 was also discovered to have nuclear functions including mediating DNA replication repair,[19,27] export of viral transcripts,[28] RNA binding,[20] and regulation of chromatin

stability through interaction with histone deacetylase (HDAC) proteins.[19] However, the role of PACS1 during human neurodevelopment and the impact of the p.R203W variant have never been reported, and very few therapeutic options exist for patients.[29]

In this study, we used induced pluripotent stem cell (iPSC)-derived neuronal models to begin to understand the impact of the PACS1 syndrome-causing variant in cortical development. We performed single-cell RNA sequencing on dorsal cortical organoids at multiple time points and found that mature excitatory neurons containing the p.R203W variant have impaired expression of genes regulating synaptic function. To characterize these effects, we did both calcium imaging in organoids and multielectrode array (MEA) recording of iPSC-derived neurons and found a consistent prolonging of network bursts in PACS1$^{(+/R203W)}$ cells. This is the first study to investigate the impact of the PACS1 variant in a knock-in model of the developing brain, revealing electrophysiological patterns that could be underlying patient symptoms and putative therapy targets for further characterization.

RESULTS

PACS1 p.R203W variant does not impact cytoarchitectural organization of cerebral organoids

To investigate how the p.R203W variant in PACS1 impacts developing neural cell types, we generated dorsal cortical organoids from three control and two patient lines. In addition, we introduced the heterozygous variant which leads to p.R203W into one control line and corrected it in two patient lines using CRISPR/Cas9 gene editing technology (**Fig. 1a; Fig. S1a-c;** see **Table S1** for breakdown of lines used in all experiments). PACS1$^{(+/+)}$ and PACS1$^{(+/R203W)}$ iPSCs were then directed towards excitatory dorsal cortical organoids through exposure to an established sequence of growth factors (**Fig. S2a**).[30,31] We chose to assess two early timepoints – day 40 and 80 – as PACS1 is highly expressed early in development and we wanted to capture this onset of upregulation.[8] After 40 days of differentiation, organoids of both genotypes strongly expressed dorsal forebrain markers such as FOXG1, EMX1, and PAX6, but lacked mature upper cortical layer marker SATB2 (**Fig. 1b-c*;* Fig. S2b-c**). PAX6^{+}FOXG1^{+} proliferative cells spontaneously developed ventricular zone (VZ)-like rosette structures surrounded by an intermediate zone containing CTIP2^{+} and TBR1^{+} excitatory neurons, supporting our organoids were differentiating as had been previously described (**Fig. 1b-c; Fig. S2b-c**).[30,31] By day 80, these rosette structures had largely dissipated in favor of more mature neuronal markers including SATB2 (**Fig. 1b-e; Fig. S2b-e**). There were no differences in ratios of these markers between genotypes (**Fig. 1d-e**, shown by line in **Fig. S2d-e**) suggesting that the p.R203W variant did not affect organoid gross cytoarchitectural organization. To assess if the variant impacted protein abundance, we quantified levels of PACS1 protein in day 40 organoids and did not find a difference between genotypes (**Fig. 1f-g**).

Since cortical organoids are composed of heterogeneous cell types and no previous studies had explored how the PACS1 syndrome-causing variant impacted developing human neural tissue, we performed single-cell RNA sequencing at day 40 and 88 to globally investigate cell type-specific transcriptomic changes in PACS1$^{(+/R203W)}$ developing neural cells (**Methods** and **Fig. S3**). Analysis of both time points revealed 16 total distinct cell groups (**Fig. 2a**) including radial glia at various states of proliferation and maturity; intermediate progenitors; newborn neurons; transcriptionally distinct immature neurons with higher expression than newborn neurons of markers such as *SLC17A6*; and day 88-enriched mature excitatory neural cell types that express markers such as *SATB2* (**Fig. 2a-d, Fig. S4**). Small clusters expressing features of inhibitory neurons, choroid plexus, and vascular leptomeningeal cells were also resolved in accordance to publicly available databases where these markers (such as *DLX6, GAD2,* and *COL1A2*) are sporadically present in organoids produced with the same (or very similar) protocols.[32,33] As expected, cell type composition drastically shifts to more mature cell types between day 40 and 88 (**Fig. 2b-c,** shown by line in **Fig. S5a-d**), though the transcriptomes of the same cell type across different time points hierarchically cluster together (**Fig. 2d**). *PACS1* is enriched in neurons but broadly expressed across all cell groups (**Fig. 2f,** shown by line in **Fig. S5e**) while a spatially restricted progression of proliferative to mature neuron markers is observed (**Fig. 2e-f; Fig. S4c**). Transcriptomic analysis showed no differences in the proportions of cell types between PACS1$^{(+/R203W)}$ and control organoids at either timepoint (**Fig. 2b-c,** shown by line in **Fig. S5b-c**), further supporting that they are developing similarly from a cell type composition perspective.

PACS1 p.R203W variant results in impaired expression of genes involved in synaptic signaling in mature glutamatergic neurons

To uncover molecular pathways dysregulated by the PACS1 p.R203W variant in distinct developing neural cell types, we performed differential expression (DE) analysis of cortical organoid transcriptomes for all cell groups. DE analysis was run separately for day 40 and 88 samples due to minor but consistent differences in the number of average counts and features per cell (**Fig. S5d**). Our conservative method of validating DEG hits identified by standard tools from the Monocle3 package with a DESeq2-based pseudobulk approach (see **Methods**) yielded a total of 277 combined DEGs across the 16 cell types and two time points (**Table S2; Fig. S6**) with the majority occurring between day 88 samples (**Fig. 3a**). Proliferative radial glia populations had the highest number of DEGs, followed by mature glutamatergic neuron groups. Top candidates include *CHODL,* which plays roles in mediating axon growth[34] and is upregulated in immature glutamatergic neurons at day 40, and *TRPC5,* a CNS-enriched Ca^{2+}-permeable cation channel[35] which is upregulated in all three mature glutamatergic neuron subpopulations at day 88 (**Fig. 3b**). Examples

of top candidates for each day and direction of change are shown in **Fig. 3b** (see **Table S2** for a complete DEG list).

Instead of focusing on a few candidates from our DEG list, we sought to determine patterns and enriched pathways across DEG functions using gene ontology (GO) analysis followed by reduction of redundant terms to assess the hierarchical GO result structure. All terms are collectively shown in the first panel of **Fig. 3c**. As indicated by the width of the pie section, which correlates to the negative log of the adjusted *p* value, the strongest patterns from these analyses included terms related to neuron projection development / organization and transmembrane ion / synaptic transport. These terms primarily resulted from DEGs upregulated in immature neurons for neuron projection development (from transcripts such as *SEMA3E, CHODL,* and *LHX2*) and DEGs upregulated in mature neuron groups for synaptic-related GO terms (from transcripts such as *SLC24A3, TRPC5,* and *CACNA2D3*) (**Fig. 3c**). This strong enrichment for synaptic transmission-related GO terms prompted us to further explore the relationship between mature neuron DEGs using Qiagen Ingenuity Pathway Analysis (IPA)[36,37] as this cell group has the most likely potential for therapeutic interventions. IPA specifically implicated GABA receptor signaling as an upregulated pathway in addition to functional predictions of depressed neurotransmission and increased dendritic growth/branching (**Fig. 3d**). These enrichments resulted from aberrantly expressed transcripts related to synaptic function and neurite outgrowth such as *CNTNAP4, GPRIN3, CACNA2D3, GABRG3,* and *TRPC5* (**Fig. 3d-e;** shown by line in **Fig. S7**).

PACS1$^{(+/R203W)}$ organoids have an increased spike rate not mediated by GABA-A receptors

A clear pattern observed in DEGs was aberrant expression of genes involved in synaptic signaling in the day 88-enriched mature neuron populations (**Fig. 3c-d**), suggesting the presence of p.R203W PACS1 may affect electrophysiological properties. We next sought to investigate the functional consequence of this differential expression (**Fig. 4a**). First, to see if synapses themselves were altered, we quantified glutamatergic synaptic abundance by staining for colocalization of a pre- and postsynaptic marker in day 80 organoids (**Fig. 4b**). We also probed for GABAergic synapse markers (**Fig. 4c**) as a top upregulated pathway predicted by Ingenuity Pathway analysis was GABAergic receptor signaling (**Fig. S8a**). PACS1$^{(+/R203W)}$ organoids have no change in glutamatergic synaptic puncta (**Fig. 4b,** shown by line in **Fig. S8b**), but an increase of GABAergic synapse density (**Fig. 4c,** shown by line in **Fig. S8c**), suggesting that any functional effects could be related to GABAergic transmission.

To investigate electrical activity in organoids we loaded sections with a calcium sensor Fluo4 and imaged on a confocal microscope (**Fig. 4a**). At both day 40 and 80, spontaneous TTX-susceptible calcium spikes were observed in PACS1$^{(+/+)}$ and PACS1$^{(+/R203W)}$ organoids (**Fig. 4d**). Strong differences were observed between the day 40 and 80 recording timepoints, including increased amplitude and number of

active cells (**Fig. 4e-f**), indicative of maturing neuronal tissue. Between genotypes, the most prominent effect was an increase in spike rate in PACS1$^{(+/R203W)}$ sections (**Fig. 4g**, shown by line in **Fig. S8d**). We hypothesized this could be GABA-A receptor mediated based on IPA predictions and our observation of increased GABAergic synapses in PACS1$^{(+/R203W)}$ organoids (**Fig. 4c**). Though GABA is typically thought of as inhibitory, organoids at day 88 still had higher expression of the gene encoding NKCC1 than KCC2 (**Fig. S8e**), which mediates a net efflux of Cl- ions in response to GABA early in development.[38] This switch from a depolarizing to hyperpolarizing has been shown to occur in the early postnatal period in animal models, whereas the epigenome of day 80 organoids generated with this protocol is more similar to post-conception week 10-17 human fetal tissue.[39] To test the hypothesis that GABA-A receptors were mediating the increased spike frequency observed in PACS1$^{(+/R203W)}$ organoids, we treated organoid sections with the competitive GABA-A receptor antagonist bicuculline. Contrary to our hypothesis, bicuculline exposure increased the spike frequency but was not differential between genotypes (**Fig. 4h**), suggesting a mechanism independent of GABA-A receptors was responsible for the change in spike frequency.

PACS1$^{(+/R203W)}$ neurons display a prolonged network burst duration

While the calcium imaging analysis effectively resolved gross measures of neuronal activity, metrics on a rapid timescale were completely obscured. To see if a higher resolution could yield further insights into the observed increase in spike rate in PACS1$^{(+/R203W)}$ organoids, we moved to MEAs. Since the phenotype was not differentially responsive to bicuculline across genotypes, we reasoned the effect was primarily excitatory-mediated and generated a more homogenous neuron population by overexpressing Neurogenin 2 (*NEUROG2*) in all three isogenic line pairs.[40] This model was chosen due to the strong overlap of *NEUROG2* on the top three most mature neuron clusters of interest (**Fig. 5a**), the homogeneity of the differentiation product, and the ability to culture in 2D for long periods of time due to the absence of proliferative cells that contaminate small molecule differentiation methods. Neurons generated with this approach (**Fig. 5b**) exhibited complex morphology and strong MAP2 expression (**Fig. 5c**). Sholl analysis was performed since neuron projection development was a strongly implicated pattern in DEGs (**Fig. 3c-d**) but did not reveal differences in dendritic arborization (**Fig. 5d-g**), suggesting any differences between genotypes would be due to synaptic or ionic alterations.

To record electrical activity, neurons were cultured along with rat astrocytes on a MEA system over a two-month time course. Synapsin-expressing cells were counted at the end of each of three experiments to ensure any differences were not due to number of surviving cells (**Fig. 5h**). Many parameters were the same between PACS1$^{(+/+)}$ and PACS1$^{(+/R203W)}$ neurons, including mean firing rate (**Fig. 5i**) and progression of active electrodes (**Fig. 5j**). Number of network bursts was different when analyzed as a whole, but not significant at any individual timepoint (**Fig. 5k**). However, PACS1$^{(+/R203W)}$ neurons displayed a striking

increase in the duration of the network burst that was consistent across all lines and differentiations (**Fig. 5l, Fig. S9a**). Single-electrode bursts were also prolonged (**Fig. S9b**), but to a lesser extent than network bursts. Despite the burst duration doubling in length, the number of spikes within a burst remained the same (**Fig. 5m**), with the difference arising from an increase in the inter-spike interval within bursts (**Fig. 5n**) and a delay in attainment of max spike rate (**Fig. S9c-d**). This strongly suggests that PACS1$^{(+/R203W)}$ neurons experience aberrant regulation of ion flux during synaptic transmission events that delay the fast afterhyperpolarization (fAHP) period, which occurs on a 2-5 ms timescale.[41] DEGs such as *KCNMB2, TRPC5,* or *CACNA2D3* could certainly underlie this phenotype. Closer investigation of spike trains (**Fig. 5p-q)** also reveals a consistent phasic, multimodel burst pattern in PACS1$^{(+/R203W)}$ neurons, potentially suggesting an additional component to fAHP at play such as change in intracellular calcium ion homeostasis, a top GO term for DEGs upregulated in PACS1$^{(+/R203W)}$ neurons (**Fig. 3c**).

In addition to drastic deficits in network bursting, PACS1$^{(+/R203W)}$ neurons also display aberrations in synchrony. As neurons matured on the MEA system, an increasing percentage of spikes occurred in network burst events. While this steadily increased in PACS1$^{(+/+)}$ neurons, PACS1$^{(+/R203W)}$ neurons lagged (**Fig. S9e**). This value converged by the end of our recording period but PACS1$^{(+/R203W)}$ neurons maintained a wider cross correlogram (**Fig. 5o**), indicating a weaker relationship between firing patterns than in controls. The proper coordination of synchronous neuron activity during development is critical for appropriate circuit formation and its disruption could certainly underlie PACS1 syndrome patient phenotypes, as has already been shown for other ASD and ID models.[42–49]

DISCUSSION

Here we present the first study investigating the impact of the PACS1 p.R203W variant on developing human neural tissue. Single-cell RNA sequencing of PACS1$^{(+/R203W)}$ cortical organoids revealed deficits in the expression of ion channels in mature glutamatergic neuron populations and downstream functional analysis showed a drastic increase in network burst duration. This could certainly underlie the epilepsy and ASD/ID experienced by patients, as impaired glutamatergic neuron network activity has been shown to be aberrant in many human iPSC-derived models of epilepsy and ASD including *KCNQ2, MECP2, FMR1, KCNT1, ATRX, AFF2, SCN2A, ASTN2, CNTN5, EHMT2,* and idiopathic cases.[50–61] Most of these studies did not report burst duration except the study by Quraishi and coworkers which documented shorter bursts in a KCNT1 p.P924L context,[57] and Sundberg and coworkers who observed no change in burst duration of 16p11.2 duplication or deletion models.[61] The observation of increased network burst duration and decreased synchrony in our system could be related to patient EEG reports (though rare and variable) for both PACS1 syndrome and a strikingly similar PACS2 syndrome of "excess discontinuity", "generalized slowing", and "slow wave bursts".[62–64] However, these are very different systems, and it is possible that

variable firing patterns in neurons initiate epileptic activity but are not reflected in EEG recordings. Further experiments with more diverse systems would be needed to fully make this connection.

Which channels are contributing the most to the increased inter-spike interval in mature glutamatergic neurons can be hypothesized by our DEG analysis but should be further investigated with pharmacological intervention studies. *KCNMB2,* a DEG upregulated in multiple cell types, is a promising candidate as it encodes an auxiliary subunit to BK channels, which are direct critical modulators of fAHP duration.[65] However, especially considering the phasic bursting patterns in addition to the increased interspike interval on a rapid timescale, it is also possible that DEGs which more generally contribute to calcium ion flux play a critical role as intracellular calcium levels have been shown to affect fast, medium, and slow afterhyperpolarizations.[66] Examples include *CACNA2D3* or *TRPC5*, the top-most upregulated gene in mature PACS1$^{(+/R203W)}$ neuron groups, a promising candidate as two reports have demonstrated a direct role of PACS1 in trafficking TRP channels[1,16] and TPRC5 activity has been suggested to prolong group I metabotropic glutamate receptor-mediated epileptiform discharges.[67,68] The p.R203W variant could impair binding to TRPC5 and result in a similar mechanism to what has been shown with TRPP2, which aggregates in the cell membrane resulting in increased ion channel activity upon disruption of the acidic cluster sorting motif identified by PACS1.[16] However, other top DEGs including *CNTNAP4, SLC1A2,* and *GABRG3* have also been shown to underly irregular firing patterns in the context of epilepsy or autism and could be contributing to the disease mechanism.[69–74] It should finally be considered that protein-level interactions could play a larger role as PACS1 has been shown to form a complex with Wdr37 and regulate ER Ca^{2+} homeostasis in lymphocytes.[24] Further exploration is needed as the elucidation of the mechanism would be a massive step towards identifying potential therapeutic compounds for PACS1 syndrome patients. Since a limitation of this model is the lack of functional circuits between diverse brain regions, collaborative efforts within the PACS1 Syndrome Research Foundation[29] are underway to generate a mouse model. This would greatly assist in characterizing the electrophysiological deficits across different brain regions, the resulting cognitive impact, and the safety of candidate therapies. It could also help elucidate the functional consequence of an increased density of inhibitory synapses, which this study did not fully characterize but has also been previously reported in other ASD models.[75–77]

In addition to pharmacological effectors of differentially expressed ion channels, another promising treatment is antisense oligonucleotides (ASOs), which traditionally are designed to bind in a complementary manner to the variant-containing mRNA and block translation. ASOs have the potential to systemically alleviate diverse toxic effects of the p.R203W variant in addition to correcting network deficits, but the variant's mechanism of action must be fully characterized to inform ASO design. Literature currently supports the p.R203W variant having a GOF effect, as zebrafish exhibited craniofacial deficits when overexpressing p.R203W – but not wild-type – PACS1 mRNA,[1] and a family has been reported with a multi-exon deletion in PACS1 but having little to no cognitive impairment, craniofacial abnormalities, or

structural brain abnormalities.[78] Future studies will involve crystallography to try and resolve the structure of the FBR in the mutant and control PACS1, immunoprecipitation assays in neurons to establish if binding partners are lost or acquired upon introduction of the variant, binding affinity assays to determine if p.R203W binds variably to targets, and immunohistochemistry analysis of PACS1 and its targets to assess how the variant impacts subcellular localization in order to better inform therapeutic development.

Therapies such as ASOs provide much promise for PACS1 patients. Unfortunately, most other genetic causes of ASD and ID are much more complex and developing therapies for each genetic origin is very challenging. A momentous leap forward for the field would be to develop a way to target common phenotypes across a multitude of etiologies, such as correction of convergent aberrant neuron firing patterns. This is a monumental task, but the potential provides hope for PACS1 patients and millions of others suffering from ID and ASD.

METHODS AND MATERIALS

Culture of iPSCs

Induced pluripotent stem cells were maintained in mTeSR+ medium (Stem Cell Technologies, 05825) on plates pre-coated with Matrigel (Corning, 354234) dissolved in DMEM/F12 at a concentration of 10 µg/mL. Stem cells were passaged as needed using ReLeSR medium (Stem Cell Technologies, 05872). Two control fibroblast lines GM03651 and GM03652 were purchased from Coriell and reprogrammed to pluripotency using Sendai viral vectors containing OCT4, SOX2, KLF4 and CMYC[79] by the Northwestern University Stem Cell Core. A third control line S033751*B/GM27160 and a patient line S033745*B/GM27159 were purchased as iPSCs from Coriell but are now available at Wicell (PACS1001i-GM27160 and PACS1002i-GM27159). An additional patient line, PACS1003i-GM27161, was purchased as iPSCs from WiCell. Pluripotency tests of GM27160 and GM27159 iPSC lines were performed by WiCell and demonstrated differentiation of all three germ layers. Karyotype of lines was done by WiCell and determined normal. An isogenic counterpart of each of these lines (except GM27160 and GM03652) was generated using CRISPR/Cas9. All cell lines were regularly tested for mycoplasma contamination and genotyped with Sanger sequencing.

CRISPR gene editing

Generation of isogenic cell lines was carried out utilizing the CRISPR/Cas9 system. Briefly, the Cas9 was delivered to cells as a px459-derived plasmid (Addgene, 62988) with a cloned guide that was designed using crispr.mit.edu software. The forward sequence was: caccgCAAGGTCTTATAGCCCAAGA and reverse: aaacTCTTGGGCTATAAGACCTTGc (lowercase letters are sticky ends to clone into the plasmid).

5 µg of plasmid and 5 µg of donor DNA (sequence TGCAGATCATGCTGCAAAGGAGAAAACGTTAC AAGAAT**T**GGACTATCTTAGGCTATAAGACCTTGGCCGTGGGACTCATCAAC) were delivered to 1 million cells using the NEON transfection system (Thermo Scientific, MPK5000). 24 hours after electroporation, cells were exposed to 0.5 µg/mL puromycin for 24 hours to select for cells without the plasmid. Cells were left to expand for roughly 10 days. When visible colonies had formed, they were manually isolated with a pipet and Sanger sequenced.

To check for off-target effects and confirm the genotype, DNA from clones of interest was sent to Novogene for whole genome or whole exome sequencing. The resulting bam files were imported to IGV (v2.8.10) and manually checked for off-target effects. Of the top 100 off-target effects predicted by the IDT CRISPR design tool, only two other sites were exonic (MVP chr16:-29844665; PACS2 chr14:-105355130) and did not have any mismatched base pairs in any of the three isogenic sets of lines. The top 5 off-target sites predicted by CHOPCHOP (v3) were all non-coding, but analysis of the CRISPR R203W and CRISPR A1 lines - which received whole genome sequencing - did not reveal any unintended edits.

Generation of cortical organoids

Cortical organoids were generated according to the previously described protocol by Sloan and coworkers.[30,31] In brief, iPSCs were grown on matrigel-coated plates in mTeSR medium until colonies were ~ 5 mm across. Colonies were detached from the plate by incubating at 37ºC with 0.35 mg/mL dispase for 20 minutes. Detached colonies were then transferred to an ultra-low attachment dish (Corning, 3262) with DMEM/F12 (1:1, Life Technologies, 1130-032) containing 20% vol/vol knockout serum replacement (Life Technologies, 10828-028), 1% vol/vol non-essential amino acids (Life Technologies, 1140-050), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, M3148), 5 µM of the SMAD inhibitor dorsomorphin (Sigma-Aldrich, P5499), 10 µM SMAD inhibitor SB-4321542 (R&D Systems/Tocris, 1614), and 10 µM Rock inhibitor Y-27632 (Selleckchem, S1049). Media was changed daily for the next five days with the same reagents as described above, minus the Y-27632. At day 6, media was changed to a Neurobasal-A base containing 1x B-27, 1x Glutamax, 20 ng/mL FGF2, and 20 ng/mL EGF (R&D Systems, 236-EG). This media was changed every other day until day 25. At day 25, growth factors were switched to 20 ng/mL BDNF (PeproTech, 450-02) and 20 ng/mL Neurotrophin 3 (PeproTech, 450-03). Organoids were transitioned to neurobasal-A medium supplemented with 1x B-27 beginning at day 43 and continuing until the desired endpoint. Later differentiations also utilized the kit developed based on this protocol (Stem Cell Technologies, 08620) according to manufacturer instructions.

Cryopreservation and sectioning

Organoids were fixed with 4% paraformaldehyde in PBS at 4ºC overnight. They were then washed with PBS and transferred to a 30% sucrose solution in PBS and incubated at 4ºC for at least 16 hours. To

cryopreserve, organoids were embedded in OCT medium using a mold and snap frozen in dry ice and ethanol. Organoids were stored at -80ºC indefinitely. Frozen organoids were sectioned 15-20 µm thick at -16ºC in a cryostat and applied to Thermo Scientific slides. Slides were left to dry overnight and then stored at -80ºC.

Immunohistochemistry

Organoid sections were dried for at least one hour either at room temperature or 37ºC. OCT was removed in a series of 3x10 min washes in PBS. An antigen retrieval step was performed by steaming slices at 99ºC for 10 minutes in a 10 mM citrate solution at pH 6.0. To remove the citrate buffer, slides were washed 1x5 min in dH_20 and 2x5 min in PBS. Tissue was blocked in a 10% NDS, 3% Triton X-100 solution in 1x PBS for 1-2 hours at room temperature. Following blocking, tissue was incubated in a 5% NDS, 1.5% Triton X-100 solution in 1x PBS containing primary antibodies at 4ºC overnight. Primary antibodies used in this study are: CTIP2 (Abcam, ab18465); PAX6 (BioLegend, 901301); TBR1 (Proteintech, 66564-1-Ig); SOX2 (EMD Millipore, AB5603); SATB2 (Synaptic Systems, 327004); FOXG1 (Abcam, ab196868); EMX1 (Atlas Antibodies, HPA006421); SYN1 (Cell Signaling Technology, 5297); PSD95 (NeuroMab, K28/43); vGAT (Thermo Scientific, PA5-27569); Gephyrin (Synaptic Systems, 147-021); TRA1-60 (Sigma, MAB4360); and OCT4A (Cell Signaling Technology, C30A3). The overnight incubation was followed by 3x10 min PBS wash, then Alexa Fluor secondary antibody incubation at 1:200 in a 5% NDS, 1.5% Triton X-100 solution in 1x PBS for 90 minutes at room temperature. Excess antibodies were removed with 3x10 minute PBS washes and a 20-minute incubation in 2 µg/mL DAPI. Slides mounted with Fluoromount-G (Thermo Scientific, 00-4958-02) and imaged on a Nikon W1 spinning disk confocal microscope or BZX710 Keyence epifluorescent microscope.

For quantifications in **Fig. 1d-e,** one field of view per section was imaged with up to five sections per organoid. Images were analyzed using ImageJ (Fiji v2.3.0) and CellProfiler (v4.2.4). To make visualizations in Fig. 1d-e comparable, the total number of positive cells was determined per image field and the fraction each marker contributing to the number of positive cells was calculated, then plotted as averages per genotype and day in **Fig. 1d** and per organoid in **Fig. S2d**. For FOXG1/TBR1/SATB2 statistics, distribution of each marker fraction (averaged per organoid) was first tested for normality using a Shapiro test. FOXG1 and TBR1 returned $p > 0.05$ in the normality test so a two-way ANOVA with Tukey HSD correction was run. This was not the case for SATB2 (Shapiro $p = 0.001$) and so a Kruskal Wallis test was done for genotype and day, then adjusted using a Benjamini Hochberg correction. In **Fig. 1e**, the ratio of SOX2+/CTIP2+ cells was calculated in R and averaged per organoid. A Shapiro test determined data did not follow a normal distribution ($p = 0.003$) and so a Kruskal Wallis test was done for genotype and day, then adjusted using a Benjamini Hochberg correction.

Protein extraction and Western blotting

Proteins were extracted from day 40 neural organoids using RIPA buffer (Cell Signaling Technology, 9806) supplemented with protease and phosphatase inhibitor cocktail (Thermo Scientific, 78440). Protein concentration was determined using the Pierce BCA protein assay kit (Thermo Scientific, 23227), and lysates were denatured by adding Laemmli buffer (Bio-Rad, 1610747) and boiling at 95°C. 30 µg of protein extract was resolved on 4-15% polyacrylamide gels (Bio-Rad, 4561085), transferred to PVDF membranes (Thermo Scientific, 88518), and then immunoblotted with specific antibodies anti-PACS1 (dilution 1:500, Sigma-Aldrich, HPA038914) and α-Tubulin (dilution 1:10,000, Abcam, ab7291). Membranes were then incubated with relevant near-infrared dyes-conjugated secondary antibodies (1:10,000, Li-Cor). Protein signal was detected using the Odyssey® Fc Imaging System (Li-Cor) and quantified with Image Studio (Li-Cor).

Dissociation of cortical organoids for single cell RNA sequencing

Dissociation was done using the Worthington Papain Dissociation System (LK003150). Steps were performed with sterile materials in a tissue culture hood. At day 40-42, two organoids between 3-4mm were selected per genotype. Due to limited availability of tissue at day 88 just one organoid was used per sample. Organoids were pooled and washed with PBS + 0.2% BSA, then chopped into ~0.5mm pieces using a #10 blade. Organoids were incubated in a sterile-filtered 5 mL solution containing 20 U/mL papain in 1x EBSS, 0.46% sucrose, 26mM NaHCO3 (included in kit), 0.5mM EDTA (included in kit), and DNase (included in kit) equilibrated with 95%O_2:5% CO_2. Organoid pieces were incubated for 75 minutes on a 95 RPM orbital shaker in a 37ºC incubator with triturated using wide bore tips (Rainin, 30389218) every 30 minutes to help break up pieces. Following incubation, organoids were triturated one more time and transferred to a 15 mL conical vial. Cells were spun at 300xg for 5 minutes. The pellet was resuspended in a 3 mL papain inhibitor solution containing 2 mg/mL ovomucoid inhibitor, 2 mg/mL albumin, 0.46% sucrose, and DNase in 1x EBSS (all from Worthington, kit LK003150). This cell suspension was gently applied on top of a 5 mL solution of 10 mg/mL ovomucoid inhibitor, 10 mg/mL albumin, and 0.46% sucrose to create a density gradient. The gradient was spun at 70xg for 6 minutes. The pellet was washed with 5 mL PBS + 0.2% BSA and spun again at 300xg for 5 minutes. The pellet was resuspended in 130 µL PBS + 0.2% BSA and filtered to remove cell clumps (Bel-Art, H13680-0040), resulting in the final single-cell suspension. In total, nine samples were processed in four distinct batches (three at day 40 and one at day 88).

Single cell RNA Sequencing and downstream analysis

Preparation of samples was carried out as described above. Samples were only processed if viability after the dissociation was >85%. The library prep was conducted by Northwestern's Center for Genetic

Medicine using the 10x Chromium library prep system. Sequencing was done using 100bp, paired-end reads on an Illumina HiSeq 4000 with a targeted 300-400 million reads per sample. Raw data was demultiplexed and reads were aligned to GRCh38 using the CellRanger pipeline.

After sequencing, we analyzed the data using Seurat (v4.0.0) and monocle3 (v1.0.0) packages in R (v4.0.3-v4.1.1).[80–82] In Seurat, data was filtered by number of features, counts, and percent mitochondrial reads (nFeature_RNA >500 & nFeature_RNA <6500 & percent.mt < 15 for day 40 and nFeature_RNA > 1000 & nFeature_RNA < 8000 & nCount_RNA > 3000 & nCount_RNA < 40000 & percent.mt < 7.5 for day 88 samples). For each sample individually, doublets were first identified by performing standard processing steps and then running two separate doublet finding packages: DoubletFinder (v2.0.3) and DoubletDecon (v1.1.6).[83,84] Barcodes were extracted for consensus cells that both packages identified as doublets (< 5% of each sample). Following doublet identification (a schematic is shown in **Fig. S3a**), analysis was re-started and filtered with the above parameters including removal of doublet barcodes. Samples were merged and processed with the following functions: NormalizeData (default parameters), FindVariableFeatures (nfeatures = 3000), FindIntegrationAnchors (dims = 1:20), IntegrateData (default parameters), ScaleData (regress out the effect of percent.mt), RunPCA (default parameters), FindNeighbors (reduction = pca), FindClusters (resolution = 0.8), and RunUMAP (reduction = pca, n.neighbors = 30, min.dist = 0.2, dims = 1:20). Cluster identification was bioinformatically aided with the clustifyr package (v1.2.0) [85] using a fetal human tissue dataset generated in the Kriegstein lab as a reference.[86] Cluster names were simplified to represent the broad cell type classification.

Following cell type identification, differential expression analysis was run between genotypes for each cell type and time point. A standard single-cell differential expression workflow was first performed using the fit_models function in the monocle3 package according to the standard workflow where each cell is treated as an n.[82] To increase confidence of results, we validated hits using a pseudobulk approach with DESeq2 (v1.30.1).[87] First the count matrix was aggregated for each day/cell time and features with <5 counts for any aggregated sample were discarded. DESeq2 was the run followed with an lfcShrink transformation (type = apeglm) of results. These results were then joined by cell type, day, and direction of change. Genes with < 0.5 absolute fold change or > 0.05 p_{adj} for either method were discarded. Genes with a base mean of < 60 were also discarded. Mitochondrial (MT-) and ribosomal (RPS/RPL) proteins were removed (only 5). 277 DEGs across all cell types and both time points remained in the final list.

After generation of the final DEG list, testing for enrichment of gene ontologies was performed with the topGO (v2.42.0) package.[88] Due to the stringency of our DEG list we primarily tested for GO enrichment by cell class, categorized by cycling radial glia, radial glia, intermediate progenitors, newborn neurons, immature neurons, mature neurons, or other. All features found in the combined Seurat object were used as the background gene list. To more wholistically visualize GO results, the ontology hierarchy was extracted and reduced using the rrvgo package (1.9.1)[89] with the score of the term being $-\log_{10}(p_{adj})$. They

were then visualized using the PieDonut function from the webr package (v0.1.5).[90] Additional downstream analysis was done using the Qiagen Ingenuity Pathway Analysis tool (v1.21.03). The filtered DEG list was uploaded using the mean logFC and p_{adj} between monocle3 and DESeq2 analyses. 55/65 DEGs mapped in the up + downregulated genes dataset and 36/43 mapped in the upregulated dataset. Neurotransmission (p_{adj} = 0.0035) and synaptic transmission (p_{adj} = 0.0039) were the top two enriched functions for the up+downregulated DEGs while dendritic growth/branching was 21st (p_{adj} = 0.0193), but with many similar terms such as "shape change of neurites" (p_{adj} = 0.0182) before this point. In the upregulated DEG dataset, G beta gamma signaling and GABA receptor signaling tied for the strongest enriched pathways at p_{adj} = 0.0155.

Synaptic quantifications

Day 79 organoid sections were stained for MAP2 and either SYN1/PSD95 or vGAT/GPHN primary antibodies as described in the above section. Confocal images were taken on a Nikon W1 Dual CAM Spinning Disk microscope at 60x resolution with a 1 µm step size with a Z stack of 15 images, then collapsed to one plane in ImageJ based on maximal intensity. Pre- and post- synaptic puncta channels were converted to binary and superimposed, then the number of colocalizations above 10 pixels in area was automatically counted using the Analyze Particles macro. MAP2 was used to confirm neuronal identity of the image field but was not factored into the colocalization scheme. Statistical analysis was then done in GraphPad Prism using a two-tailed Mann-Whitney test.

Calcium imaging

Calcium imaging was done on days 40-42 and 79-82 of organoid differentiation. To section, organoids were mounted in 3.5% low-melt agarose (Promega, V2111) dissolved in oxygenated aCSF (125 mM NaCl, 2.5 mM KCl, 26.2 mM $NaHCO_3$, 1 mM NaH_2PO_4, 11 mM glucose, 5 mM HEPES, 2 mM $CaCl_2$, 1.25 mM $MgCl_2$). Organoids were then cut in ice-cold oxygenated aCSF as thin as possible, between 150 µm-250 µm, using a vibratome (Leica, VT1200S) set to 0.2 mm/s speed and 1.00 mm amplitude. To fix slices in place for imaging, sections were plated on 2x Matrigel-coated 8-chamber dishes (Ibidi, 80806) in maintenance media and left to recover overnight at 37°C, 5% CO_2. 1 hour prior to imaging, sections were loaded with Fluo-4 Direct (Thermo Scientific, F10473) prepared according to kit instructions in oxygenated aCSF. Before imaging, media was changed to aCSF only. Sections were imaged in a live-cell chamber at 37°C 5% CO_2 with a Nikon W1 spinning disk confocal microscope for 5 minutes at 2 Hz. An equal volume of 100 µM bicuculline (Tocris) in aCSF (for 50 µM total) was then added to the section and incubated for 15 minutes at RT. Following incubation, sections were imaged 3 minutes at 2 Hz. For sections exposed to

TTX (Tocris Bioscience, CAS 18660-81-6), 1 μM total was applied to the section and imaged after 5 minutes incubation for 1 minute at 2 Hz.

Calcium signal was extracted in Fiji by calculating average intensity for each frame over regions of interest with the greatest Z score. Traces were processed in Matlab (vR2022a) using code developed by Marc Dos Santos.[91–93] In brief, data were normalized to a rolling median background of 25 seconds. Peaks were detected using the findpeaks function (MinPeakDistance = 3 seconds, MinPeakHeight = $\Delta 0.15\ F/F_0$, MinPeakProminence $\Delta 0.08\ F/F_0$, MaxPeakWidth = 10 seconds). Cells with no detected peaks were removed from the analysis. Output metrics were analyzed in R. A Shapiro test for normality was performed followed by either an ANOVA test with Tukey HSD correction if $p > 0.05$ or Kruskal Wallis of individual metrics with Bonferroni correction if $p < 0.05$. We note that for spike rate data in Fig. 4g, frequency values were log-transformed to meet the assumptions of ANOVA and statistics were run on log-transformed values.

Excitatory neuron differentiation

iPSCs were differentiated using an adapted protocol based on NEUROG2 overexpression.[40] Stem cells were dissociated using Accutase, then resuspended in mTeSR+ with 10 uM ROCK inhibitor. 600k cells in 1 mL media were transferred to an eppendorf tube and incubated for 15 minutes at room temperature with 2.5 μL rtTA virus (Iowa Viral Vector Core, FIV3.2CAG-rtTA VSVG), 1.25 μL NEUROG2 (produced at the Northwestern University Gene Editing, Transduction, & Nanotechnology Core), 8 μL and 8mM polybrene. This solution was then transferred to a matrigel-coated 10 cm plate with 7 more mL mTeSR+ with 10 uM ROCKi. Infected cells were maintained in mTeSR+ and expanded to confluency. When confluent, half the media was changed to Neurobasal-A with 0.5x B27, 1x N2, 1x NEAA, and 1x Glutamax (called NBM media). 3 μg/mL doxycycline was added to the NBM:mTeSR media to induce NEUROG2 expression. On days 2-5, media was changed daily with the following components: Neurobasal-A with 0.5x B27, 1x N2, 1x NEAA, 1x Glutamax, 2 μg/mL dox, 10 μM SB431542, 100 nM LDN-193189, 2 μM XAV939, 2 μg/mL puromycin, and 10 μM ROCKi (day 2 only). On day 6, cells were dissociated with accutase and frozen at 1 million per vial in freezing media (45% day 2-5 media without puro; 45% FBS; 10% DMSO). When thawed, neurons were maintained in Neurobasal-A with 1x B27, 1x N2, 1x NEAA, 1x Glutamax, 2 μg/mL doxycycline, 2% FBS, and 10 ng/mL BDNF with half media changes on a Monday, Wednesday, Friday schedule.

Sholl analysis

To perform Sholl analysis, NEUROG2-derived neurons generated using the protocol described above were plated at day 7 onto a glia-covered, matrigel coated 18mm coverslip at 300k/well. Neurons were maintained in Neurobasal-A with 1x B27, 1x N2, 1x NEAA, 1x Glutamax, 2 μg/mL doxycycline, 2% FBS, and 10 ng/mL BDNF. Half the media was changed on a Monday, Wednesday, Friday schedule. At day 43,

neurons were transfected with a pEGFP-N2 plasmid from Clontech. Prior to incubation, half the old media was removed and saved. In accordance with the Lipofectamine 3000 protocol, 125 µL per well of Opti-MEM was mixed with 2.5 µg/well of pEGFP-N2 plasmid and 4 µL/well of P3000 reagent. In a separate tube, 125 µL/well of Opti-MEM was mixed with 2 µL/well of Lipofectamine 3000. After thoroughly mixing both solutions and letting them sit at room temperature for 5 minutes, DNA and lipofectamine mixtures were added in a 1:1 ratio. The mixture was allowed to incubate at 37°C for 20 minutes to facilitate formation of DNA-lipid complexes. Following incubation, 250 µL of the mixture was added to each well and put back in the incubator for five hours. Neurons were then gently washed 2x with warm Neurobasal-A and ½ old media (saved in the initial steps) and ½ fresh media was re-applied to the cells.

After allowing three days for GFP expression to begin, neurons were fixed for 15 minutes at room temperature with 4% PFA. Neurons were washed three times with PBS for 10 minutes each, then blocked for 1 hour in 10% NDS + 0.25% TX100 + PBS. Neurons were stained with a GFP antibody, as well as GFAP and MAP2 in order to distinguish neurons and glia. Coverslips were imaged with a BZX710 Keyence microscope. Debris or other cells that would interfere with Sholl analysis were manually removed with Fiji. Images of isolated neurons were then converted to binary files and the Sholl plugin on Fiji was used. Concentric circles 10 µm apart were drawn, up to 800 microns as needed. The number of intersections for each concentric circle was exported to R where further analysis was performed. Length of longest dendrite was defined as furthest concentric circle with 2 intersections to avoid a skewed value due to a long axon. *p* values were determined with student's t-tests when Shapiro and Goldfeld-Quandt tests were not significant.

Multielectrode array plating and recording

48-well MEA plates from Axion biosystems (M768-tMEA-48W) were coated with 2x Matrigel for 16 hours at 37°C prior to plating. A 10 µL droplet of 20 µg/mL Matrigel (Corning, 354234) dissolved in DMEM/F12 in the center of each well was placed directly on top of the electrode grid and incubated at 37°C overnight. The following day, Day 6 neurons were thawed and re-counted. Enough for 50k/well neurons per well were transferred to a conical vial. P2 rat primary cortical astrocytes (Thermo Scientific, N7745100) at 25k/well were added to the same vial. After centrifugation, cells were resuspended at 50 µL per well in MEA media (Neurobasal-A with 1x B27, 1x N2, 1x NEAA, 1x Glutamax, 10 ng/mL BDNF, 2 µg/mL doxycyline, and 2% FBS). A 50 µL drop containing 50k neurons and 25k glia was placed in the center of each electrode grid in the 48 well plate and left in the incubator for 1 hour to facilitate attachment of neurons over the electrodes as opposed to the edges of the well. After 1 hour, 250 µL more media was gently added to each well. Half the media was changed on a Monday/Wednesday/Friday schedule. At day 10, 1 µM AraC was added for two days to inhibit proliferative cells.

Starting at day 10, spontaneous and stimulated activity was recorded prior to media changing. Spontaneous activity was recorded using Axion Biosystems Maestro 768 channel amplifier and Axion

Integrated Studios (AxIS) v2.5 software. The amplifier recorded from all channels simultaneously using a gain of 1200x and a sampling rate of 12.5 kHz/channel. After passing the signal through a Butterworth band-pass filter (300–5000 Hz), on-line spike detection (threshold = 6 x the root-mean-square of noise on each channel) was performed with the AxIS adaptive spike detector. All recordings were conducted at 37°C in 5% CO_2/95% O_2. Spontaneous network activity was recorded for 5 min each day starting on day 10 of differentiation. Starting day 10, neurons were also electrically stimulated with 20 pulses at 0.5 and 0.25 Hz after spontaneous recordings were made to facilitate maturation and migration of neurons to the electrode field.

Spike files were processed with the Axis Neural Metric Tool (v3.1.7). Electrode burst settings were algorithm = Poisson surprise, min surprise = 5. Network burst settings were algorithm = ISI threshold, min # spikes = 50, max ISI (ms) = 100, min % of electrodes = 18, synchrony window = 20. Average network burst settings were network burst window start = 0 ms, network burst window end = 100 ms, bin size = 1 ms. Metrics generated from these output parameters were further investigated in R. *p* values of metrics over time between genotypes were calculated by conducting an ANOVA between a linear regression model with and without genotype included as a covariate. This trend-based approach was taken as opposed to examining each of 12 recording timepoints independently so different timepoints were assessed together as related variables. Data did not always meet all the assumptions of linear regression, so when appropriate, individual significances at specific timepoints were reported with *p* values obtained using a Kruskal Wallis test and are reported in the legend.

Statistics

Statistical analyses for each experiment were performed in R (v4.0.3-v4.1.1) or GraphPad Prism (v8.4.2). For FOXG1/TBR1/SATB2 counts in **Fig. 1d,** the ratio of each marker to the total number of positive cells (averaged per organoid) was calculated in R using a two-way ANOVA with Tukey HSD correction if $p > 0.05$ in the Shapiro test for normality, which was true of FOXG1 and TBR1. This was not the case for SATB2 ($p = 0.001$) and so a Kruskal Wallis test was done for genotype and day, then adjusted using a Benjamini Hochberg correction. In **Fig. 1e,** the ratio of SOX2+/CTIP2+ cells was calculated in R and averaged per organoid. $p = 0.003$ in a Shapiro test for normality and so a Kruskal Wallis test was done for genotype and day, then adjusted using a Benjamini Hochberg correction. In **Fig. 1g,** *p* value for PACS1 protein levels was determined using a two-tailed Mann-Whitney test. To determine final *p* values for differentially expressed genes, we averaged *q* value results of the classic DEG approach with the Monocle3 fit_models function and a pseudobulk approach with DESeq in R. Synapse quantifications in **Fig. 4b-c** were done in Prism using a two-tailed Mann-Whitney test. For calcium imaging analysis, a Shapiro test for normality was performed followed by either an ANOVA test with Tukey HSD correction if $p > 0.05$ or

Kruskal Wallis of individual metrics with Bonferroni correction if $p < 0.05$. We note that for spike rate data in Fig. 4g, frequency values were log-transformed to meet the assumptions of ANOVA and statistics were run on these log-transformed values. For Sholl analysis, Shapiro and Goldfeld-Quandt tests were first performed to check for a non-normal distribution and heteroscedasticity. $p > 0.05$ in both tests so a student's t-test was run. For MEA analysis, p values were calculated by conducting an ANOVA between a linear regression model with and without genotype included as a covariate. This trend-based approach was taken as opposed to examining each of 12 recording timepoints independently so different timepoints were assessed together as related variables. Data did not always meet all the assumptions of linear regression so when appropriate, individual significances at specific timepoints were reported with p values obtained using a Kruskal Wallis test and are reported in the legend.

Data Availability

ScRNAseq data will be uploaded to the NCBI Gene Expression Omnibus repository.

Code Availability

All code is available upon request. Code used in this study is a compilation from the following sources. Reads were aligned using the 10X Genomics Cell Ranger pipeline found at https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/using/tutorial_ov. The output matrix files were first processed with Seurat following vignettes found at: https://satijalab.org/seurat/. DEG analysis was done with Monocle version 3 following the workflow described at: https://cole-trapnell-lab.github.io/monocle3/ and DESeq2 following tutorials specifically developed for single-cell analysis found at: https://hbctraining.github.io/scRNA-seq/lessons/pseudobulk_DESeq2_scrnaseq.html.

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AUTHOR CONTRIBUTIONS

This work was conceptualized by AGG and LER. Experiments, data analysis, and figures were done by LER unless otherwise specified. JR performed the western blot and quantification analysis in **Fig. 1f-g**, made **Fig. 1f**, and provided regular guidance. EGP performed many of the immunohistochemistry stains in **Fig. 1b-c** and **Fig. S2b-c** and did all the quantifications in **Fig. 1d-e**. Writing was done by LER and edited by JR and AGG.

DISCLOSURES

LER, JR, EGP, and AGG all declare that they have no competing financial interests or conflicts of interest in relation to the work described.

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FIGURES

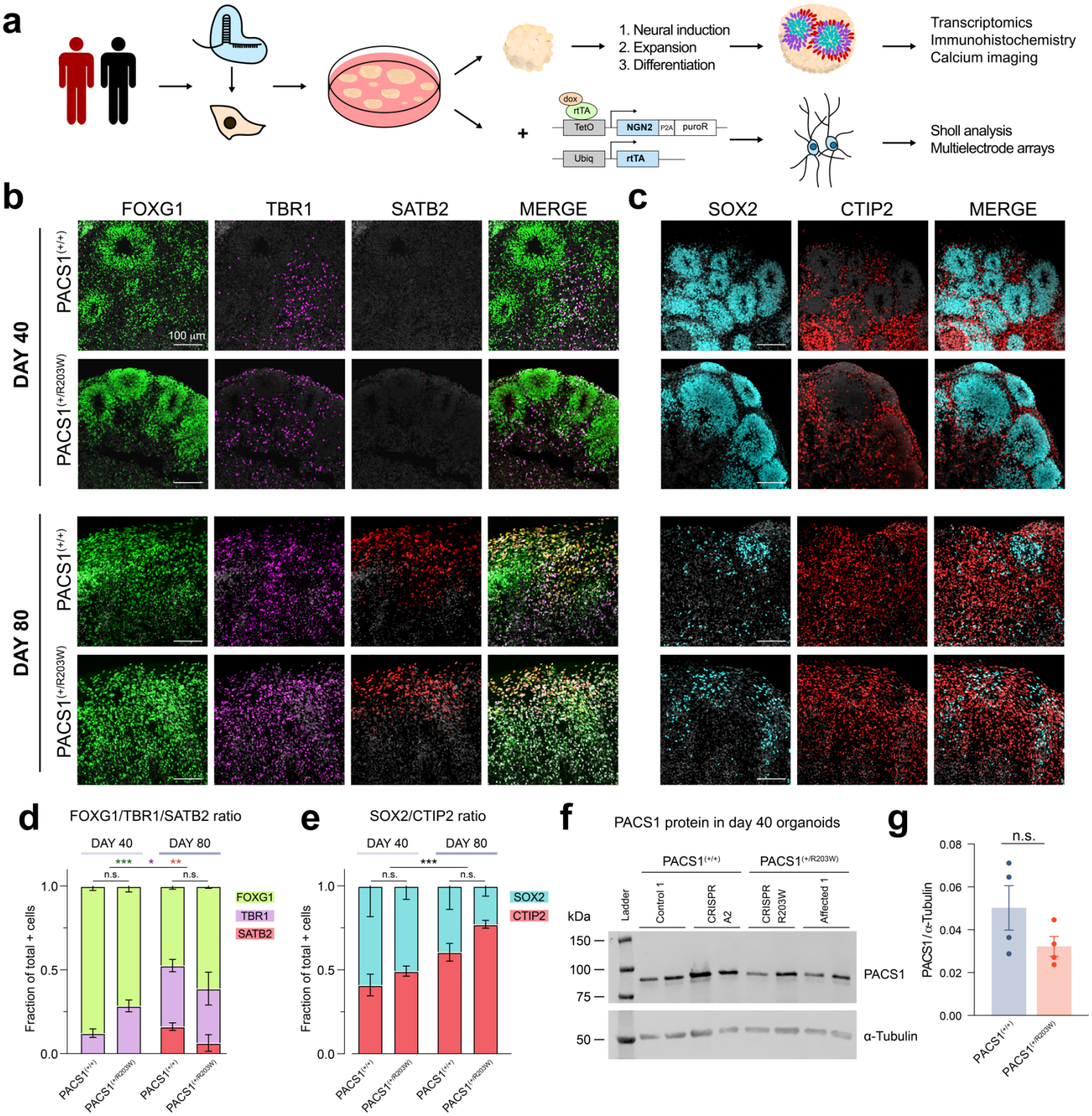

Figure 1. PACS1 p.R203W variant does not impact cytoarchitectural organization of cortical organoids. **a)** Experimental overview. Control, patient, and isogenic cell lines were generated using CRISPR/Cas9 and differentiated to dorsal cortical organoids through a series of growth factors. Differences between patient and control organoids were then assessed using immunohistochemistry and transcriptomic analysis. Downstream functional analysis was performed both with organoids and rapidly directed glutamatergic neurons. **b)** Expression of forebrain transcription factors FOXG1 and TBR1 and upper layer marker

SATB2 in day 40 and 80 organoids. Scale bar = 100 µm. DAPI is in greyscale. **c)** Expression of neural precursor protein SOX2 and layer V pyramidal neuron marker CTIP2 in day 40 and 80 organoids. Scale bar = 100 µm. DAPI is in greyscale. **d)** A quantification of **b**. Mean ± SEM fraction of FOXG1+, TBR1+, or SATB2+ cells contributing to the total number of positive cells per field of view. Stains are different by day (FOXG1 p_{adj} = 8.43E-04, TBR1 p_{adj} = 0.013, SATB2 p_{adj} = 0.004) but not genotype ($p_{adj} > 0.05$) using a two-way ANOVA unless $p < 0.05$ in a Shapiro test of normality (see **Methods**). $n = 3$ day 40 organoids for each condition. A complete list of cell lines and number of organoids used can be found in **Table S1** with results per organoid shown in **Fig. S2d**. **e)** A quantification of **c**. Mean ± SEM fraction of CTIP2+ or SOX2+ cells contributing to the total number of positive cells per field of view. The ratio of these two was quantified in the manner described in **d** (see **Methods**) and was significant between timepoints (p_{adj} = 6.90E-04) but not genotypes (p_{adj} = 0.067). A complete list of cell lines and number of organoids used can be found in **Table S1** with results per organoid shown in **Fig. S2e**. **f)** Western blot for the PACS1 protein abundance in day 40 organoid samples. **g)** Quantification of the ratio of PACS1 to α-tubulin signal in day 40 organoids shown in **f**. The bar represents mean ± SEM. $p = 0.200$ calculated using a two-tailed Mann-Whitney test.

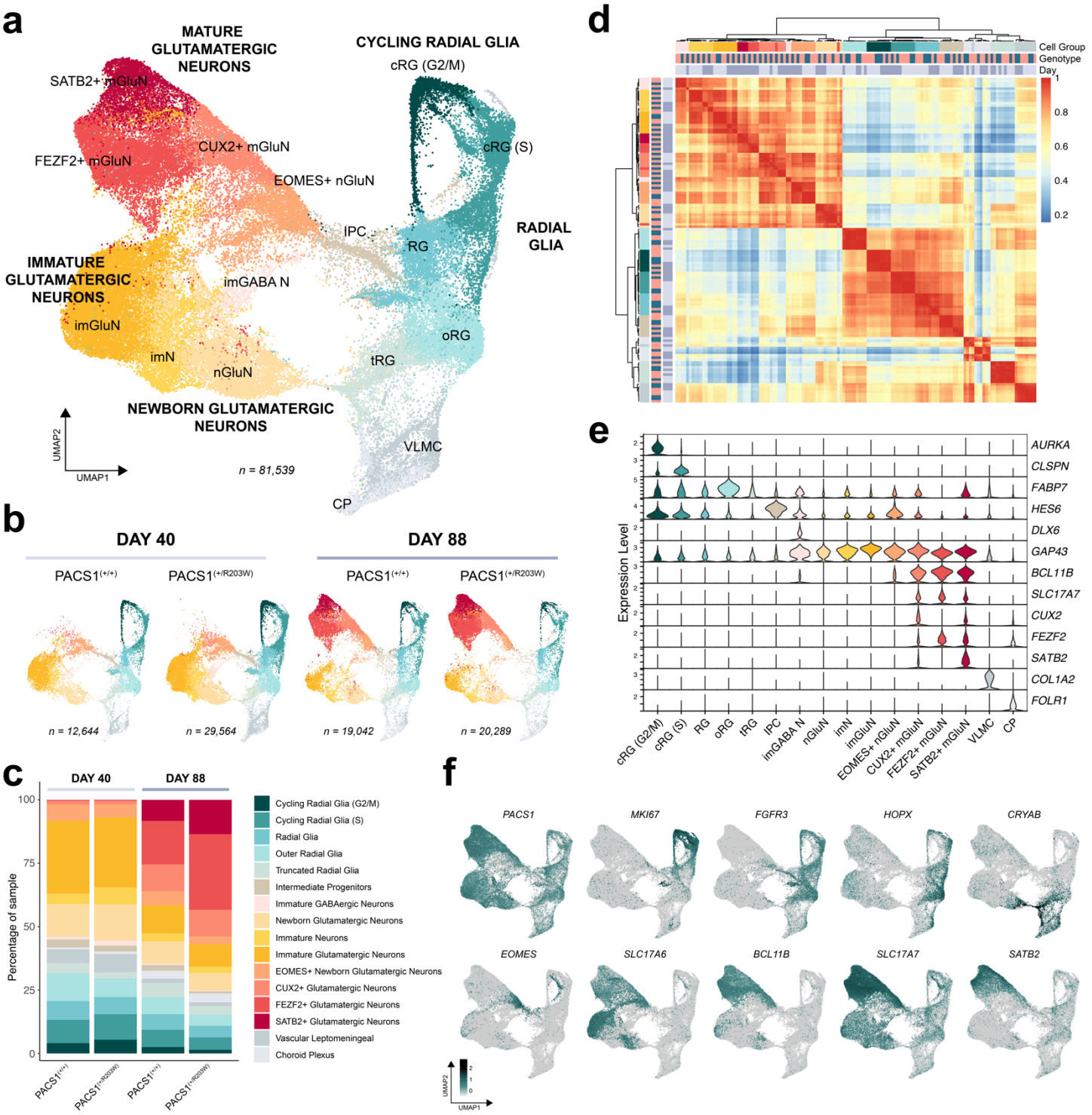

Figure 2. Single-cell RNA sequencing analysis reveals progression of neuronal maturity in cerebral organoids but no difference between genotypes. a) UMAP dimensionality reduction of all sequenced cells across nine samples from four independent differentiations (see **Table S1**) reveals distinct cell populations, including: cycling radial glia (cRG) in G2/M and S phases; non-cycling radial glia (RG); outer and truncated radial glia (oRG, tRG); vascular leptomeningeal cells (VLMC); choroid plexus (CP); intermediate progenitors (IPC); newborn glutamatergic neurons (nGluN, EOMES+ nGluN); immature neurons (imN, imGABA N, imGluN); and mature glutamatergic neurons (CUX2+ mGluN, FEZF2+

mGluN, SATB2+ mGluN). **b)** UMAP reductions divided by genotype and time point. Individual samples are shown in **Fig. S5a**. **c)** Bar chart showing the cell type composition of samples in each genotype and time point. Individual samples are shown in **Fig. S5b**. **d)** Hierarchical clustering of Pearson's correlation of variable feature expression across sample and cell type. **e)** Violin plots of feature expression. G2/M phase marker *AURKA* is restricted to cRG (G2/M) group and S phase marker *CLSPN* is strongly enriched in cRG (S). Radial glia marker *FABP7* is more generally expressed. *HES6* is enriched in IPCs. *GAP43* is expressed across young and mature neurons while *BCL11B* and *SLC17A7* are restricted to mature glutamatergic neurons. Enrichment of *CUX2, FEZF2,* and *SATB2* define mGluN subpopulations. VLMC-like cells express collagen markers and CP exclusively express *FOLR1.* **f)** Feature plots of all cells from both genotypes in UMAP space show expression of *PACS1*, proliferative marker *MKI67*, RG marker *FGFR3*, oRG marker *HOPX,* tRG marker *CRYAB,* IPC/newborn neuron marker *EOMES,* Immature neuron *SLC17A6* (vGLUT2)*,* more mature neuron markers *BCL11B, SLC17A7* (vGLUT1), and upper cortical layer marker *SATB2.* Scale is in units of $\log_{10}$(value + 0.1).

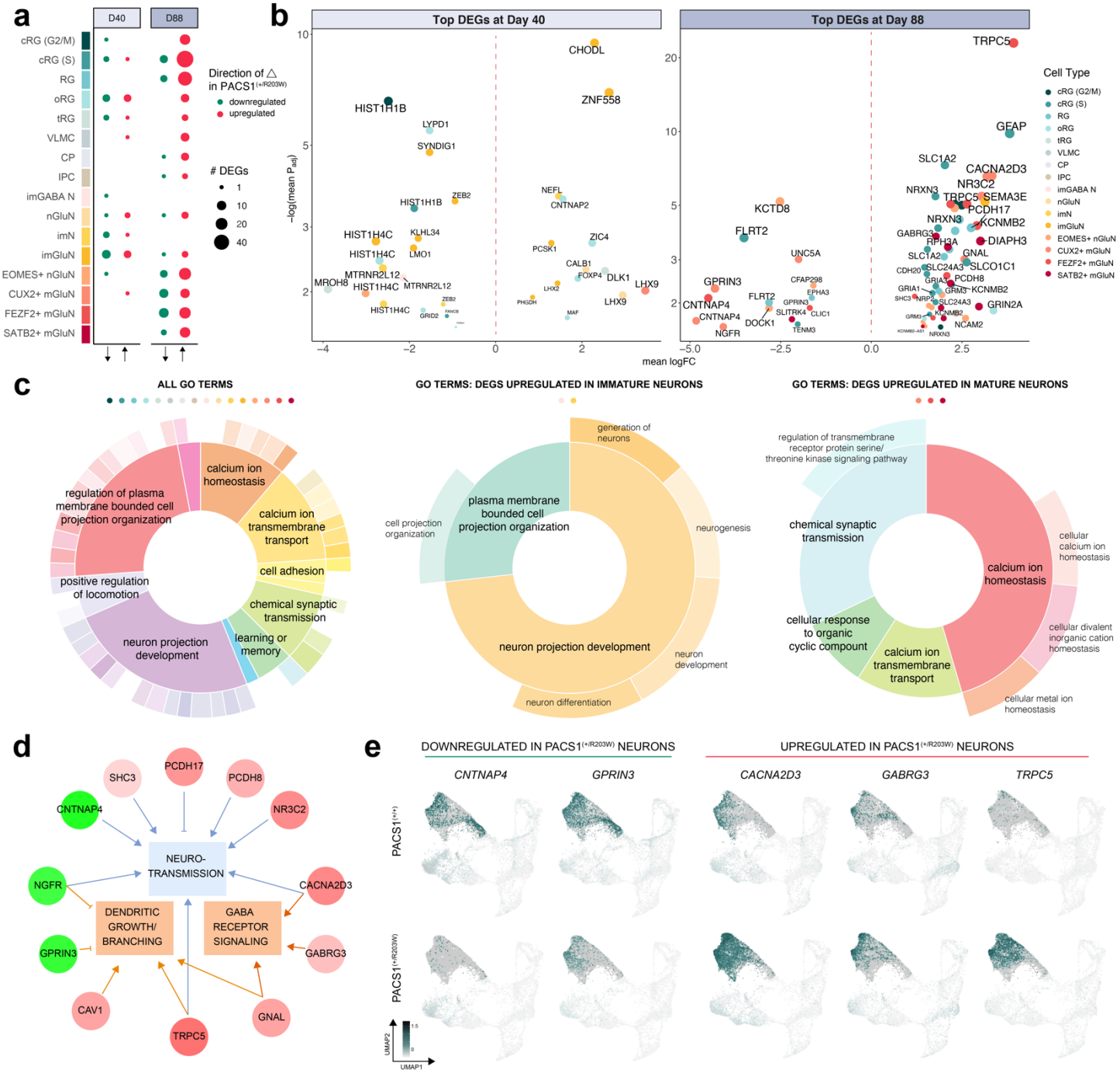

Figure 3. PACS1$^{(+/R203W)}$ mature neurons have dysregulated genes involved in ion transport and synaptic signaling. a) Dot plot showing the number of differentially expressed genes (DEGs) identified in each cell group at the two time points. Size indicates the number of DEGs while color indicates direction of change. **b)** A volcano plot showing select top DEGs. Color indicates cell type and size correlates to a combined metric of logFC and P_{adj}. All 277 DEGs are shown in **Fig. S4** and listed in **Table S2**. y axis is shown on a $\log_{10}$ scale. **c)** Nested pie chart shows root and leaf gene ontology terms with colored dot correlating to the cell types included in the analysis and width of the pie correlating to -log(p_{adj}) of the GO term. The first pie is collective GO terms for all cell classes; the second is GO terms for DEGs upregulated in immature neurons; and the third represents GO terms for DEGs upregulated in mature neuron groups. **d)** Functional

enrichment and pathway analysis results of DEGs in the mature glutamatergic neuron groups generated with IPA analysis. Gene color indicates fold change with green being downregulated and red upregulated. Orange indicates IPA predicts activation of the pathway, and blue repression. $p_{adj} = 0.0035$ for neurotransmission, $p_{adj} = 0.019$ for dendritic growth/branching, and $p_{adj} = 0.016$ for GABA receptor signaling. The GABA receptor signaling term refers to analysis of upregulated genes while the other terms include both up and downregulated genes. **e)** Feature plots of top DEGs implicated in IPA analysis. Cells have been down-sampled so the same number is shown for each genotype. Plots for each sample are in **Fig. S7**. Scale is in units of $\log_{10}(\text{value} + 0.1)$.

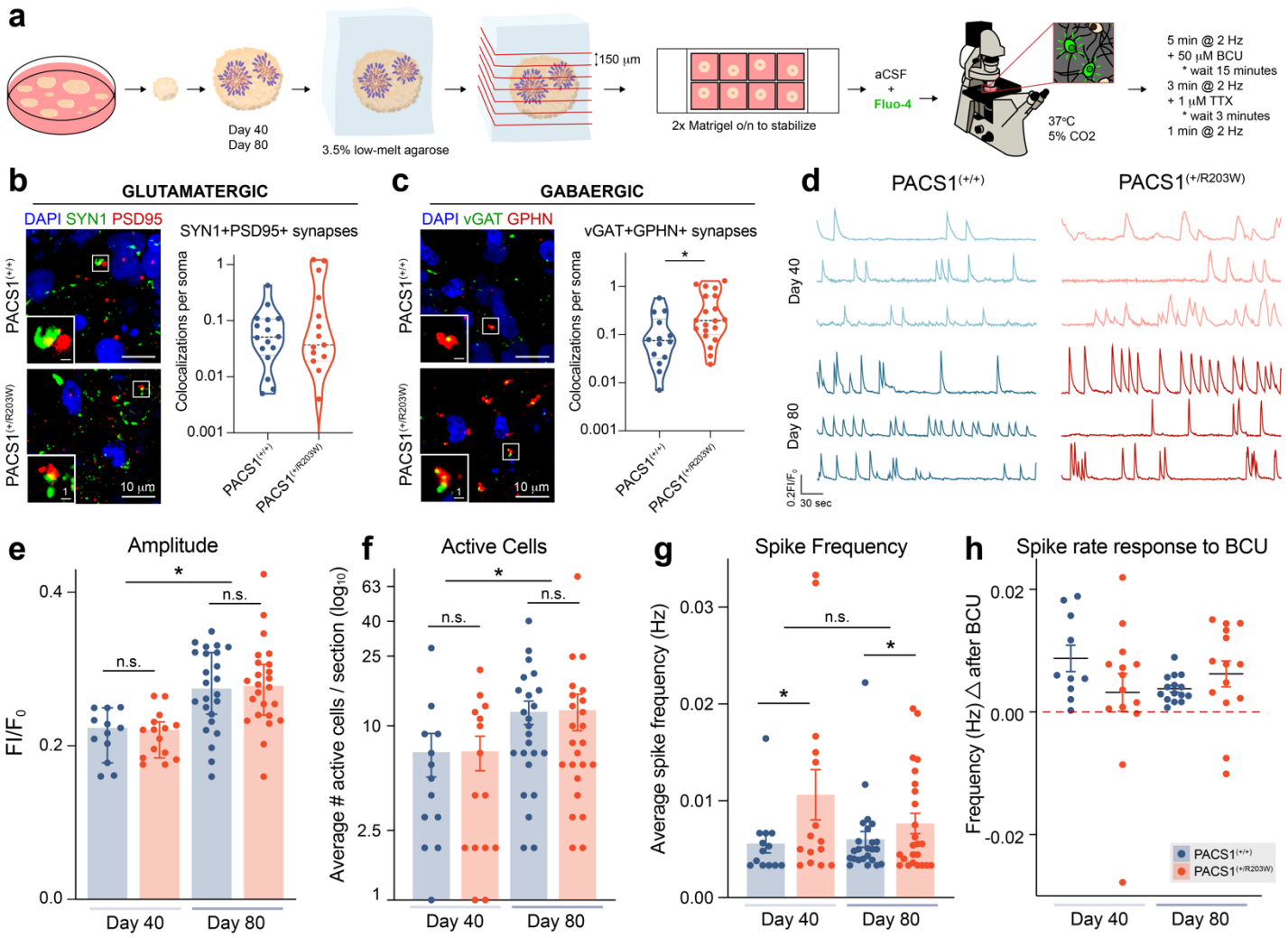

Figure 4. PACS1$^{(+/R203W)}$ organoids display a GABA-A receptor-independent increase in spike rate. a) Experimental workflow for calcium imaging in organoids from two independent differentiations. **b)** Expression of glutamatergic synapse markers in day 79 organoid sections. Co-localization of pre-synaptic marker synapsin 1 (SYN1) and post-synaptic density protein 95 (PSD95) was used to identify glutamatergic synapses. Scale bar = 10 µm in the main panels and 1 µm in the zoomed panel. Dashed lines indicate the median and the dotted lines are quartiles. Data is from n = 16 sections across 4 PACS1$^{(+/+)}$ organoids and n = 15 sections from 5 PACS1$^{(+/R203W)}$ organoids. $p = 0.953$ using a two-tailed Mann-Whitney test. y axis is on a $\log_{10}$ scale. Individual cell line results are shown in **Fig. S8b** and further details of which cell lines were used is in **Table S1**. **c)** Expression of GABAergic synapse markers in day 79 organoid sections. Co-localization of pre-synaptic marker vesicular GABA transporter (vGAT) and post-synaptic marker Gephyrin (GPHN) defines a synapse. Scale bar = 10 µm in the main panels and 1 µm in the zoomed panel. Dashed lines indicate the median and the dotted lines are quartiles. Data is from n = 14 sections from 4 PACS1$^{(+/+)}$ organoids and n = 20 sections from 5 PACS1$^{(+/R203W)}$ organoids. $p = 0.015$ calculated using a two-tailed Mann-Whitney test. y axis is on a $\log_{10}$ scale. Individual cell line results are shown in **Fig. S8c**. **d)** Representative calcium traces of PACS1$^{(+/+)}$ (blue) and PACS1$^{(+/R203W)}$ (red) cells

over 300 seconds of recording. **e)** Bar chart displaying the median ± 95% CI for calcium signal intensity of each spike event. p_{adj} = 0.494 between genotypes and p_{adj} = 0.016 between days using a two-way ANOVA test with a Tukey HSD correction. Dots are sections from n = 5 Day 40 PACS1$^{(+/+)}$ organoids, n = 6 Day 40 PACS1$^{(+/R203W)}$ organoids, n = 8 Day 80 PACS1$^{(+/+)}$ organoids, and n = 8 Day 80 PACS1$^{(+/R203W)}$ organoids. One data point (0.53; Day 40 PACS1$^{(+/+)}$) is not shown. see **Table S1** for further details on which cell lines were used. **f)** Bar chart showing mean ± SEM of number of active cells. Scale is $\log_{10}$ for clarity. Dots are sections from n = 5 Day 40 PACS1$^{(+/+)}$ organoids, n = 6 Day 40 PACS1$^{(+/R203W)}$ organoids, n = 8 Day 80 PACS1$^{(+/+)}$ organoids, and n = 8 Day 80 PACS1$^{(+/R203W)}$ organoids. p_{adj} = 0.528 between genotypes and p_{adj} = 0.027 between days using a Kruskal Wallis (data was not normally distributed) with a Benjamini Hochberg correction. **g)** Bar chart showing mean ± SEM of average frequency of calcium events in untreated conditions (results shown by cell line in **Fig. S8d**) and **h**) mean ± SEM response to bicuculline. p_{adj} = 0.033 between genotypes, p_{adj} = 0.144 between days, and p_{adj} = 7.8E-06 between treatments using a three-way ANOVA test with a Tukey HSD correction. Dots are sections from n = 5 Day 40 PACS1$^{(+/+)}$ organoids, n = 6 Day 40 PACS1$^{(+/R203W)}$ organoids, n = 8 Day 80 PACS1$^{(+/+)}$ organoids, and n = 8 Day 80 PACS1$^{(+/R203W)}$ organoids in spontaneous spikes and n = 5 Day 40 PACS1$^{(+/+)}$ organoids, n = 6 Day 40 PACS1$^{(+/R203W)}$ organoids, n = 7 Day 80 PACS1$^{(+/+)}$ organoids, and n = 7 Day 80 PACS1$^{(+/R203W)}$ organoids for paired recordings. Legend also applies to **e-g**.

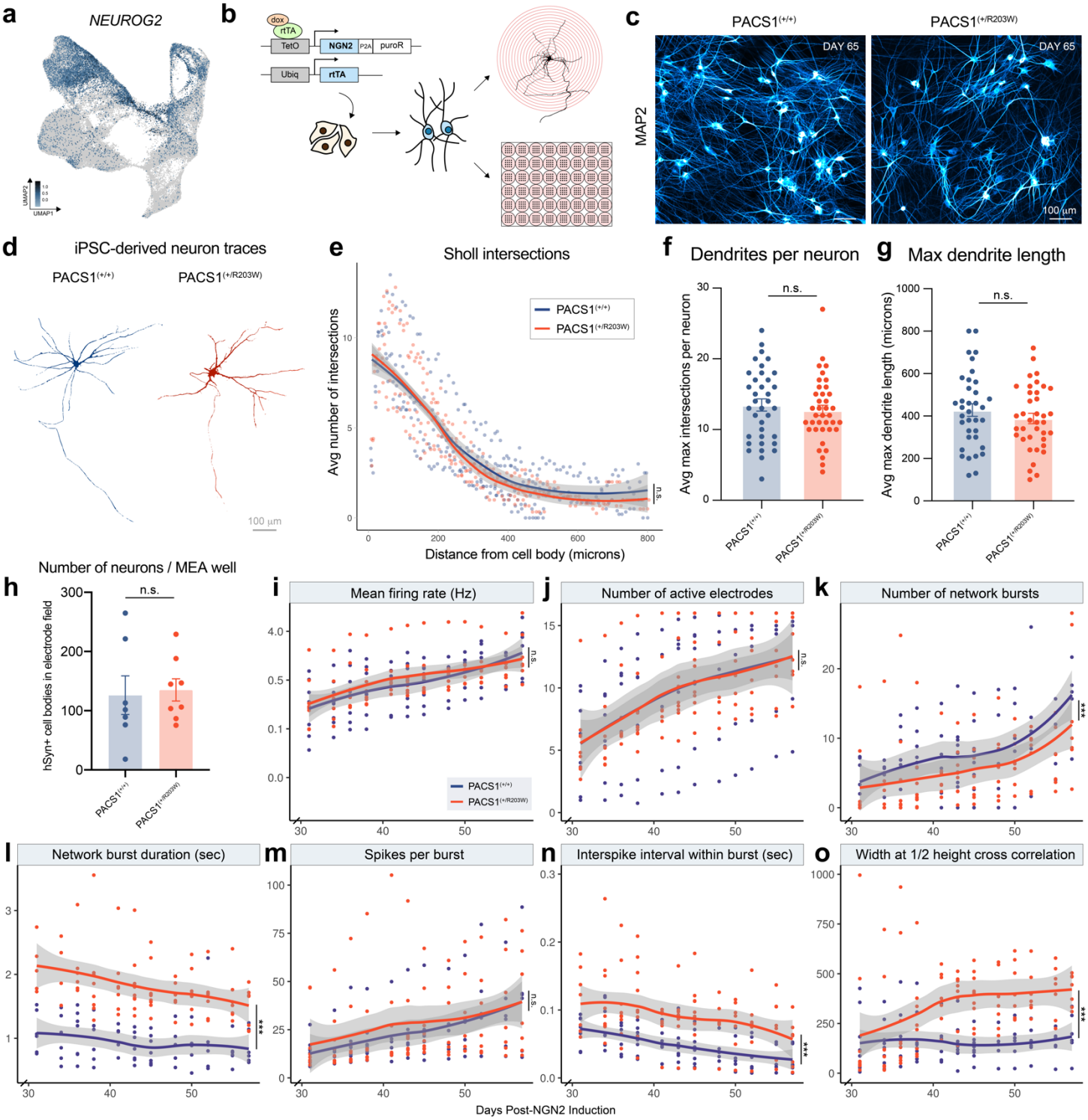
a
NEUROG2
UMAP2
UMAP1
b
dox
rtTA
TetO
NGN2
P2A
puroR
Ubiq
rtTA
c
PACS1(+/+)
PACS1(+/R203W)
DAY 65
DAY 65
MAP2
100 µm
d
iPSC-derived neuron traces
PACS1(+/+)
PACS1(+/R203W)
100 µm
e
Sholl intersections
Avg number of intersections
Distance from cell body (microns)
PACS1(+/+)
PACS1(+/R203W)
n.s.
f
Dendrites per neuron
Avg max intersections per neuron
n.s.
g
Max dendrite length
Avg max dendrite length (microns)
n.s.
h
Number of neurons / MEA well
hSyn+ cell bodies in electrode field
n.s.
i
Mean firing rate (Hz)
j
Number of active electrodes
k
Number of network bursts
l
Network burst duration (sec)
m
Spikes per burst
n
Interspike interval within burst (sec)
o
Width at 1/2 height cross correlation
Days Post-NGN2 Induction
p
PACS1(+/+) NEURON NETWORK BURSTS
10 msec
10 sec
150 msec
q
PACS1(+/R203W) NEURON NETWORK BURSTS
10 msec
10 sec
150 msec

Figure 5. PACS1$^{(+/R203W)}$ excitatory neurons display prolonged network bursts and decreased synchrony. **a)** *NEUROG2* expression in single-cell RNA sequencing data. Scale is in units of $\log_{10}$(value + 0.1). **b)** Experimental protocol for rapid induction of glutamatergic neurons dox-inducible overexpression of NEUROG2. **c)** MAP2 expression in neurons produced with the NEUROG2 overexpression protocol 65 days after viral induction. Scale bar = 100 µm. **d)** Representative day 46 neurons transfected and traced for Sholl analysis. Scale bar = 100 µm. **e)** Number of neurite intersections with concentric circles of increasing distance from the cell body from two independent differentiations. Dots are averages by cell line for each experiment. $p > 0.05$ for all micron bins. **f)** Average dendrites per neuron. Dots are replicates across n = 4 PACS1$^{(+/+)}$ coverslips and n = 4 PACS1$^{(+/R203W)}$ coverslips; bar represents the mean ± SEM. See **Table S1** for further details regarding which cell lines were used. $p = 0.853$ was calculated using a two-tailed t test. **g)** Furthest concentric circle from the cell body with 2 intersections, to avoid axon-related skewing. Dots are replicates across n = 4 PACS1$^{(+/+)}$ coverslips and n = 4 PACS1$^{(+/R203W)}$ coverslips; bar represents the mean ± SEM. $p = 0.865$ was calculated using a two-tailed t test. **h)** Dots are number of synapsin+ cell bodies visible within the multielectrode array (MEA) electrode field averaged by cell line for each of three independent experiments; bar represents the mean mean ± SEM. $p = 0.812$ using a two-tailed t test. **i-o)** MEA metrics for neurons days 30-57 of differentiation, averaged by cell line (see **Table S1**) for each of three independent experiments. *p* values were calculated by conducting an ANOVA between a linear regression model with and without genotype included as a covariate. In instances where *p* values are reported between specific timepoints, a Kruskal Wallis test was run. **i**) Mean firing rate in Hz. Genotype had no effect ($F_{0,2} = 0.933$, $p = 0.395$). y axis is on a log scale for clarity. **j**) Number of electrodes with 1+ spikes/minute. Genotype had no effect on progression of active electrodes ($F_{0,2} = 0.090$, $p = 0.914$). **k**) Number of network bursts over 5 minutes, as defined by 18%+ electrodes simultaneously active within a 100 msec window. Genotype had a significant effect on number of network bursts collectively ($F_{0,2} = 7.655$, $p = 5.63E-04$), but no individual timepoint achieved significance. **l**) Network burst duration. Genotype had a significant effect on network burst duration ($F_{0,2} = 123.17$, $p < 2.2E-16$). Results by isogenic pair and differentiation are shown in **Fig. S9a**. **m)** Spikes per burst. Genotype had no effect on spikes per burst ($F_{0,2} = 1.663$, $p = 0.192$). **n)** Inter-spike interval within a burst. Genotype had a significant effect on inter-spike interval ($F_{0,2} = 8.622$, $p = 2.35E-04$). **o)** Width of a correlogram at ½ height of cross correlation. Genotype had a significant effect ($F_{0,2} = 37.918$, $p = 3.11E-15$). Individual timepoints reached significance at day 41. **p-q)** Example of a neuron activity raster plot across 16 electrodes in PACS1$^{(+/+)}$ (**p**) or PACS1$^{(+/R203)}$ (**q**) wells. Above the raster plots is a spike histogram showing the collective density of activity over time. A network burst is highlighted in the light gray panels, which is zoomed in to the right. The pink dash below indicates the approximate location of the subsequent zoom panel.