Nested oscillatory dynamics in cortical organoids model early human brain

network development

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SUMMARY

Structural and transcriptional changes during early brain maturation follow fixed developmental programs defined by genetics. However, whether this is true for functional network activity remains unknown, primarily due to experimental inaccessibility of the initial stages of the living human brain. Here, we analyzed cortical organoids that spontaneously developed periodic and regular oscillatory network events that are dependent on glutamatergic and GABAergic signaling. These nested oscillations exhibit cross-frequency coupling, proposed to coordinate neuronal computation and communication. As evidence of potential network maturation, oscillatory activity subsequently transitioned to more spatiotemporally irregular patterns, capturing features observed in preterm human electroencephalography (EEG). These results show that the development of structured network activity in the human neocortex may follow stable genetic programming, even in the absence of external or subcortical inputs. Our model provides novel opportunities for investigating and manipulating the role of network activity in the developing human cortex.

KEYWORDS: brain organoids, network oscillations, stem cells, phase-amplitude coupling, preterm electroencephalography, Methyl-CpG-binding protein 2 (MECP2).

HIGHLIGHTS

- Early development of human functional neural networks and oscillatory activity can be modeled *in vitro*.

- Cortical organoids exhibit phase-amplitude coupling between delta oscillation (2 Hz) and high-frequency activity (100-400 Hz) during network-synchronous events.

- Differential role of glutamate and GABA in initiating and maintaining oscillatory network activity.

- Developmental impairment of MECP2-KO cortical organoids impacts the emergence of oscillatory activity.

- Cortical organoid network electrophysiological signatures are similar to human preterm neonatal EEG features.

IN BRIEF

Brain oscillations are a candidate mechanism for how neural populations are temporally organized to instantiate cognition and behavior. Cortical organoids initially exhibit periodic and highly regular nested oscillatory network events that eventually transition to more spatiotemporally complex activity, mimicking features of late-stage preterm infant electroencephalography. Functional neural circuitry in cortical organoids exhibits emergence and development of oscillatory network dynamics similar to features found in the developing human brain.

1   **INTRODUCTION**

2   Diverse and hierarchical cellular networks develop into circuits with patterns of functional

3   spatiotemporal activity to form the human brain. Neural oscillations, a prominent, rhythmic brain

4   signal found across species, robustly track cognitive, behavioral, and disease states (Buzsáki and

5   Draguhn, 2004; Fries, 2005; de Hemptinne et al., 2015; Henriques and Davidson, 1991; Khan et

6   al., 2013; Uhlhaas and Singer, 2010) and have long been leveraged in cognitive and systems

7   neuroscience due to their ubiquity and accessibility. These complex network dynamics emerge

8   early in development, and is unclear if shaped exclusively by biological programming prenatally

9   (Blankenship and Feller, 2010; Johnson, 2001; Power et al., 2010). *In vitro* and *in vivo* rodent

10   studies have shown that a conserved repertoire of organized network activity, such as traveling

11   waves, giant depolarizing potentials, and early network oscillations, develop according to a

12   consistent timeline prior to and immediately after birth (Allene et al., 2008; Khazipov and

13   Luhmann, 2006; Uhlhaas et al., 2010). However, due to an inability to interrogate the

14   electrophysiology of intact embryonic brains, it remains unknown whether the same happens in

15   humans. As a result, our knowledge about human brain functional development rests upon

16   extrapolations from nonhuman model systems (Power et al., 2010).

17   Organoids generated from induced pluripotent stem cells (iPSC) have emerged as a

18   scaled-down and three-dimensional model of the human brain, mimicking various developmental

19   features at the cellular and molecular levels (Camp et al., 2015; Lancaster and Knoblich, 2014;

20   Lancaster et al., 2013; van de Leemput et al., 2014; Luo et al., 2016; Mariani et al., 2012; Paşca

21   et al., 2015; Qian et al., 2016; Renner et al., 2017). Despite recent advances in the understanding

22   of their vast cellular diversity, there is no evidence that these organoids show complex and

23   functional neural network activity that resembles early human brain formation (Birey et al., 2017;

24   Quadrato et al., 2017). Therefore, researchers have not yet clearly determined whether organoids

25   are a suitable model for neural network dynamics (Kelava and Lancaster, 2016; Paşca, 2018).

26   Here, we use human iPSCs to generate cortical organoids that exhibit evolving and nested

27 oscillatory network dynamics over the span of several months. We subsequently investigated the

28 molecular basis of human brain oscillatory activity formation, maintenance, and temporal control

29 by gene targeting. Finally, we applied unsupervised machine learning to evaluate the similarity

30 between electrophysiological activity patterns of the *in vitro* model and human preterm neonatal

31 electroencephalogram (EEG). Our findings suggest that organoid models are suitable for the

32 investigation of the physiological basis of network formation at early and late stages of the human

33 brain development. This prolonged evaluation of cortical organoid activity expands our

34 understanding of the emergence of network-level neurodynamics in humans.

35

36 **RESULTS**

37 **Development of functional cortical organoids**

38   Despite the structural and transcriptional similarities between brain organoids and the

39 developing nervous system, the emergence of higher-level complex network activity comparable

40 to the living human brain remains largely untested (Figure 1A). To investigate the formation of a

41 functional network, we promoted cortical specification by modifying previously described

42 protocols (Paşca et al., 2015; Thomas et al., 2016) (Figure 1B, see Methods for details). At the

43 beginning of differentiation, an abundance of proliferative neural progenitor cells (NPCs) (Ki67+,

44 SOX2+ and Nestin+) that self-organized into a polarized neuroepithelium-like structure was

45 observed. Similar to human cortical development *in vivo*, the proliferative zone around a lumen

46 delimited by β-catenin+ cells was surrounded by progenitor cells. Progressively, the organoids

47 increased in size and in the proportion of mature neurons (NeuN+ and MAP2+) to ultimately

48 develop into concentric multi-layer structures composed of NPCs, intermediate progenitors

49 (TBR2+, also known as EOMES), and lower (CTIP2+, also known as BCL11B) and upper

50 (SATB2+) cortical layer neurons (Figure 1B-E and S1A-C). Although the initial fraction of glial

51 cells was less than 5%, this population increased to about 30-40% after 6 months of differentiation

52 (Figure 1D, 1E and S1D-H). The maturation level of the cells is reflected by the presence of

53   pyramidally-shaped neurons and formation of dendritic spine-like protrusions and synaptic

54   structures (Figure 1F and 1G).

55   To further characterize the cellular diversity of a cortical organoid, we performed single-

56   cell gene expression profiling in 6-month-old organoids and used unbiased clustering to classify

57   the main existing cell types. From two independent differentiation replicates (Figure S2), seven

58   distinct clusters were characterized based on their differential gene expression patterns (Figure

59   S3, S4 and Table S1) including: progenitors, glia, and cortical neurons, which could be further

60   subdivided into lower and upper layer based on the expression of the layer-specific markers

61   CTIP2 and SATB2, respectively (Figure 1H-K and Figure S1).
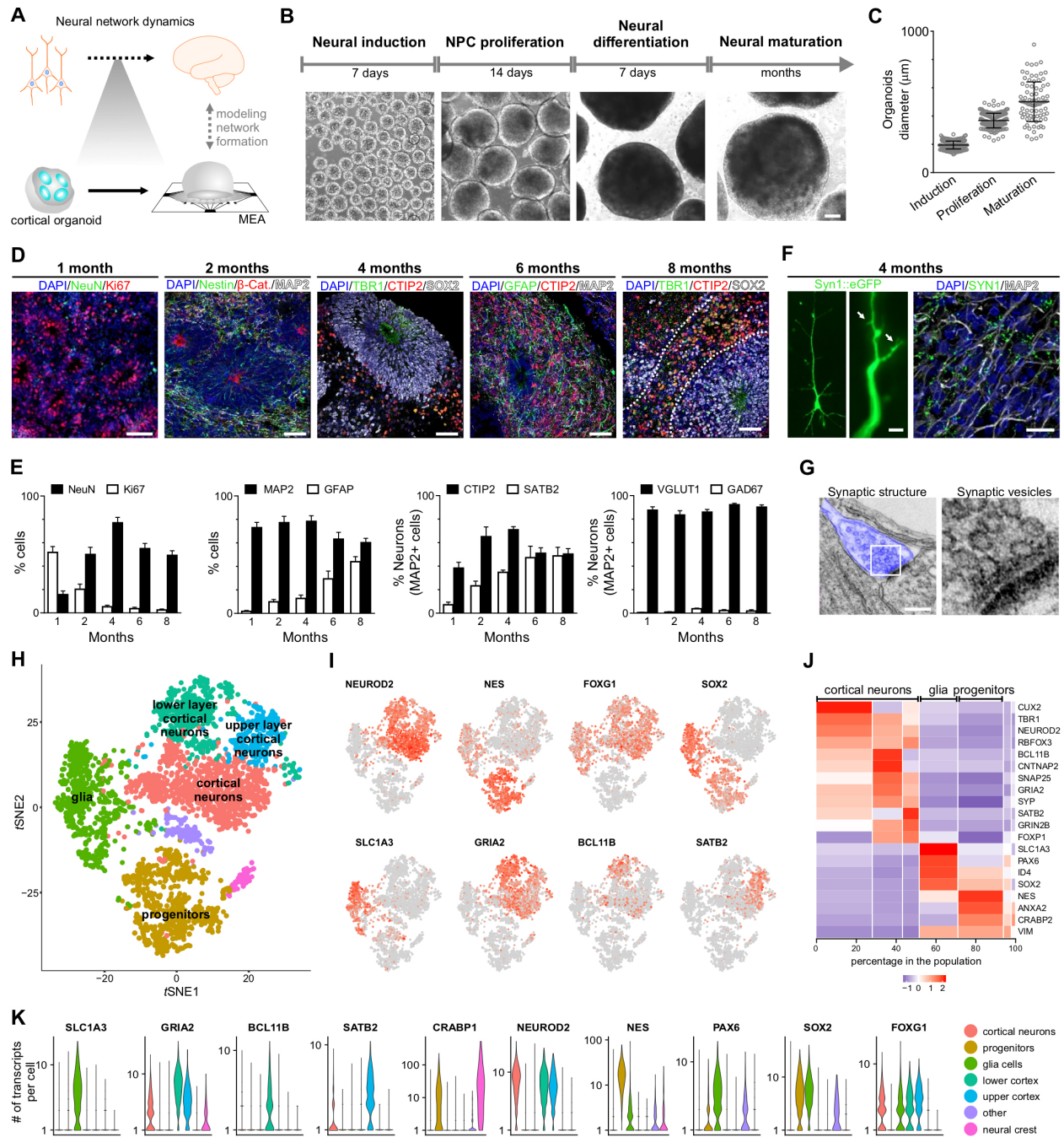
Figure 1. Cellular and molecular development of human cortical organoids. (A) Overview of

human neural network formation and dynamics evaluation using organoids. (B) Schematic of the

protocol used to generate cortical organoids. Scale bar, 200 µm. (C) Organoid growth during

different developmental stages. (D) Representative immunostainings showing proliferating NPCs

(Ki67+ and Nestin+), lower (TBR1+ and CTIP2+) and upper (SATB2+) cortical layer neurons and

68   glial cells (GFAP+) overtime. Scale bar, 50 µm. (E) Population analysis of specific markers

69   indicating stages of maturation and multiple neuronal subtypes. The data are shown as mean ±

70   s.e.m. (*n = 8*). (F) Representative image of a pyramidal neuron (left panel); dendritic spine-like

71   structures (arrow) are observed in cells transduced with the SYN:EGFP reporter (middle panel;

72   scale bar, 5 µm). Immunohistochemical detection of the synaptic protein Syn1 (right panel; scale

73   bar, 50 µm). (G) Electron microscopy of synaptic structures in 4-month-old cortical organoids

74   (blue). (H) *t*-distributed stochastic neighbor embedding (*t*SNE) plot of 3,491 cells from 6-month-

75   old organoids. Colors denote seven main cell clusters. (I) *t*SNE plots depicting cell-type specific

76   marker expression levels (red denotes higher expression). (J) Heatmap of average expression

77   for representative gene markers by cluster and cell-type (see also Figure S4). (K) Violin plots

78   showing transcript levels for representative markers of each cluster (see Figure S3 for additional

79   markers).

80

81   **Emergence of nested oscillatory network activity**
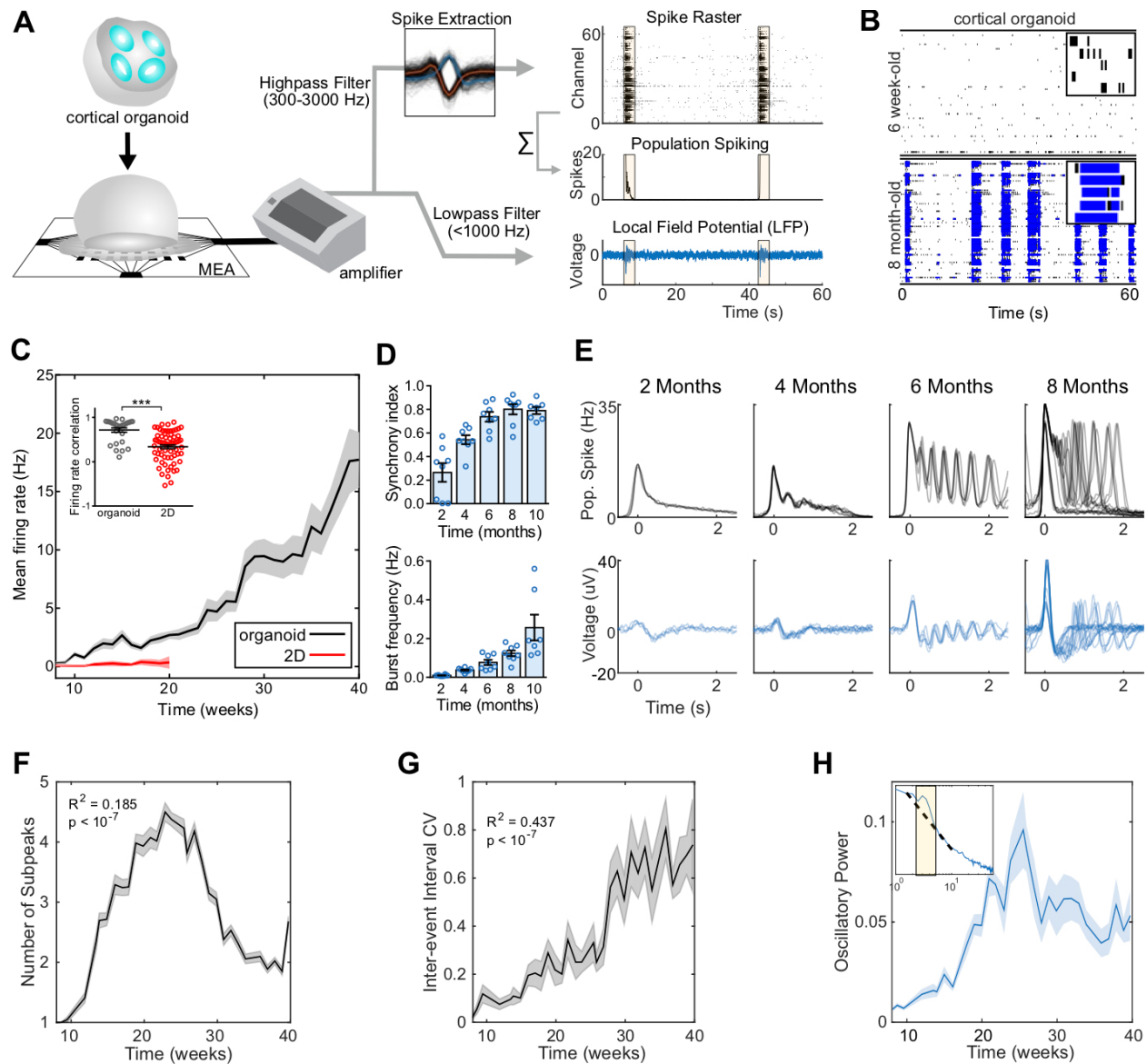
82   Considering the observed cellular diversity and expression of synaptic markers, we further

83   interrogated the presence of functional network activity. We performed weekly extracellular

84   recordings of spontaneous electrical activity using multi-electrode arrays (MEA). We separately

85   analyzed single-channel and population firing characteristics derived from channel-wise spike

86   times, and the local field potential (LFP); a measure of aggregate synaptic currents and other

87   slow ionic exchanges (Buzsáki et al., 2012) (Figure 2A). The spikes from each channel do not

88   represent putative single-unit action potentials. Since the spatial resolution of MEA electrodes

89   was sparse, the total population spiking of a well was submitted for further analysis, rather than

90   individual spike trains. Over the course of 10 months, organoids exhibited consistent increases in

91   electrical activity, as parametrized by channel-wise firing rate, burst frequency, and spike

92   synchrony (Chen et al., 2009; Lisman, 1997), which indicates a continually-maturing neural

93   network (Figure 2B-D and S5). Organoid firing rates were far higher than previously observed in

94   studies using iPSC-derived neurons or cerebral organoids (Figure S6). Additionally, the variability

95   between replicates over 40 weeks of differentiation was significantly lower compared to iPSC-

96   derived neurons in monolayer cultures (Figure 2C inset and S5E).

97   Population-level signals typically observed in *in vivo* electrophysiology were analyzed to

98   further probe the network properties of cortical organoids. During individual recordings, cultures

99   displayed a robust pattern of activity, switching between long periods of quiescence and short

100 bursts of spontaneous network-synchronized spiking (hereafter referred to as "network events").

101 These network events are periodic but infrequent early in development (~2 months), occurring

102 roughly every 20 seconds and decayed monotonically after the initial onset, similar to previously

103 reported network "oscillations" in primary cultures and organoids (Figure 2E). From 4-months

104 onwards, a secondary peak emerged 300-500 ms after the initial network activation, leading to

105 the presence of a nested fast oscillatory (2-3 Hz) pattern up to 6-months in culture (Figure 2F and

106 Figure S7). Notably, this robust fast timescale oscillation was not observed in 3D neurospheres,

107 suggesting that the spherical arrangement of neurons is insufficient for the emergence of nested

108 oscillations (Figure S8). The regular oscillatory activity during network events transitioned to

109 stronger, yet more variable, oscillations over time. To quantify this network complexity, we tracked

110 the regularity (coefficient of variation of inter-event intervals, CV) and the spatial and temporal

111 correlation between spontaneous network events. The inter-event interval CV consistently

112 increased over 10 months of differentiation (Figure 2G), from extremely regular latencies (CV $\cong$

113 0) at 2 months to irregular, Poisson-like (CV $\cong$ 1) at 10 months. This indicates increased variability

114 between consecutive network events initiation. Additionally, spatial and temporal irregularity on a

115 shorter time-scale (within-event) also increased with development, suggesting a breakdown of

116 deterministic population dynamics from the onset of network events (Figure S7G).

117   Periodic oscillatory activity is often defined as a "bump" over the characteristic *1/f*

118 background noise in the power spectral density (PSD) of extracellular signals above-and-beyond

119 the aperiodic *1/f* signal (Buzsáki et al., 2013; Gao et al., 2017). In organoid LFPs, we observed

120   both prominent oscillatory peaks in the low-frequency range (1-4 Hz) and in the aperiodic signal

121   characteristic of neural recordings (Ben-Ari, 2001; Voytek et al., 2015). The development of

122   oscillatory activity in cortical organoids over time was quantified by computing the PSD for each

123   LFP recording (Figure 2H, inset). Oscillatory power in the delta range (1-4 Hz) increased for up

124   to 24 weeks in culture, tapering off slightly in subsequent recordings and plateauing during the

125   last 10 weeks. This inverted-U trajectory reflects the network's initial acquisition of oscillatory

126   modes at steady frequencies and the dispersion of this regularity at later time points. The LFP

127   results reveal the development of the cortical organoid cultures across different network states:

128   from sparse activity with extreme rigidity and regularity, to one that acquires repetitive, perhaps

129   overly-regular oscillatory patterns (Voytek and Knight, 2015), until it finally reaches a stage of

130   higher spatiotemporal complexity and variability that is reminiscent of self-organized critical

131   networks (Tetzlaff et al., 2010) (Figure S7C-G).

Figure 2. Oscillatory network dynamics in long-term cortical organoids. (A) Schematic of the organoid signal processing pipeline. Raw MEA data is analyzed as population spiking and LFP separately. Synchronous network events are highlighted in yellow. (B) Raster plot of network spiking activity after 1.5 and 6 months of maturation. A 3-s interval of activity over 5 channels is shown in the upper right corners. (C) Cortical organoids show elevated and continuously increasing mean firing rate compared to 2D monolayer neurons (*n = 8* organoid cultures, and *n = 12* for 2D neurons). Inset, correlation of the firing rate vector over 12 weeks of differentiation (from 8 to 20) between pairs of cultures showing reduced variability among organoid replicates. (D)

141   Temporal evolution of cortical organoid network activity. Detailed definitions and further

142   parameters are presented in Figure 5B and 5C. (E) Time series of population spiking and LFP

143   during network events in cortical organoid development. Each trace represents a single event

144   during the same recording session. (F) Oscillatory dynamics within network events develop

145   nonlinearly, following an inverted-U trajectory. (G) Increase of network variability dynamics

146   throughout development. (H) Oscillatory power increases up to the 25th week in culture and

147   plateaus at 30 weeks. Inset, Oscillatory power is calculated by fitting a straight line (dashed) over

148   the aperiodic portion of the PSD and taken as the height of narrow peaks rising above the linear

149   fit. The data shown in C, D, F, G and H are presented as mean ± s.e.m. *P < 0.05, **P < 0.01,

150   ***P < 0.001, unpaired Student's *t*-test (C), quadratic (F) and linear (G) regression.

151

Oscillatory coordination of neural ensembles and its synaptic mechanisms

153   Oscillatory dynamics in the functioning brain have been postulated to coordinate spiking

154   across neural ensembles[1]. In the LFP and other mesoscopic brain signals, this manifests as a

155   phenomenon known as cross-frequency phase-amplitude coupling (PAC) (Voytek and Knight,

156   2015), wherein the high-frequency content of the LFP is entrained to the phase of slow oscillations

157   (Manning et al., 2009; Miller et al., 2007; Mukamel et al., 2005). PAC in the neocortex and

158   hippocampus has been shown to be functionally relevant in a range of behaviors and neurological

159   disorders (de Hemptinne et al., 2015; Voytek and Knight, 2015; Voytek et al., 2015). In the

160   organoids, we observed greater PAC between oscillatory delta (1-4 Hz) and broadband gamma

161   activity (100-400 Hz, see Methods) during network events compared to quiescent periods (Figure

162   3A-C). This result suggests that oscillations in the organoid may reproduce dynamics relevant for

163   the intact brain and could serve as a model to understand the fundamental mechanisms behind

164   the emergence of oscillatory networks in the developing human brain.

165   We further evaluated the role of glutamatergic and GABAergic synaptic transmission in

166   forming oscillations by pharmacological intervention. Organoid neural networks were susceptible

167   to both glutamate receptor antagonists (AP5 and CNQX; NMDA and AMPA/kainate, respectively)

168   and GABA receptor agonists (muscimol, $GABA_A$; baclofen, $GABA_B$) by significantly reducing the

169   number of spikes and bursts, with a subsequent extinction of synchronous activity. The electrical

170   activity was abolished in the presence of tetrodotoxin (TTX) (Figure 3D and 3E). Notably,

171   blockade of GABAergic transmission by bicuculline increased the number of network-

172   synchronized events and did not affect peak population firing rates, but abolished nested 2 Hz

173   oscillatory activity by erasing subsequent reverberant peaks (Figure 3F). The findings suggest

174   that GABA transmission is crucial for the maintenance, but not the initiation of faster oscillatory

175   activity. This is consistent with accounts of inhibition rhythmically coordinating pyramidal

176   populations activity during early development (Opitz et al., 2002).
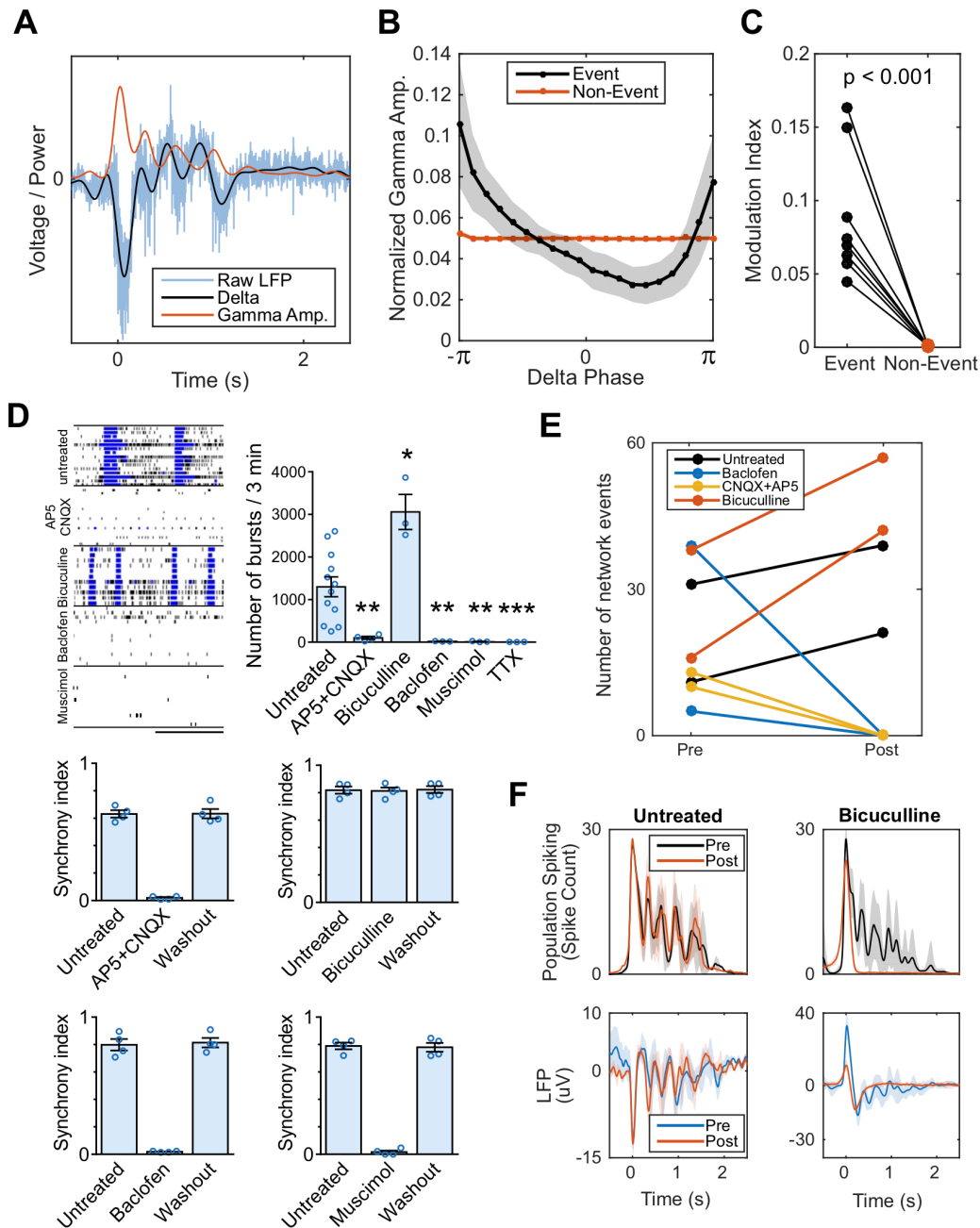
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Figure 3. Cortical organoid serves as a model of functional oscillations and their synaptic mechanisms. (A-C) Phase-amplitude coupling is observed in organoid LFP during network events, a phenomenon proposed to mediate neural communication *in vivo*. (A) Example of raw LFP during a network event decomposed into its low-frequency component (1-4 Hz delta) and the amplitude envelope of the high-frequency, broadband gamma component (200-400 Hz). Analysis was repeated for 100-200 Hz with near identical effect size and significance. (B) Normalized

184   gamma amplitude binned by delta phase during network events (black) shows greater modulation

185   depth by low frequency delta than during non-event periods (red). (C) Phase-amplitude coupling

186   during network events is significantly greater than non-event periods in all batches. (D) Effect of

187   selective drug treatments on neuronal electrical activity in 6-month-old organoids. Representative

188   raster plots and burst measurements of untreated and treated organoids. Scale bar, 20 s.

189   Exposure to AP5 + CNQX, baclofen and muscimol reversibly extinguish the network bursts

190   (synchrony), while no changes were promoted by bicuculline. (E-F) Pharmacological perturbation

191   of oscillatory activity during network events in 6-month-old organoids. Application of bicuculline

192   increases the number of network events, while CNQX + AP5 and baclofen completely abolish

193   synchronized network events. Bicuculline blocks oscillatory network activity but not the network

194   event itself. Data are shown as mean ± s.e.m.; unpaired Student's *t*-test.
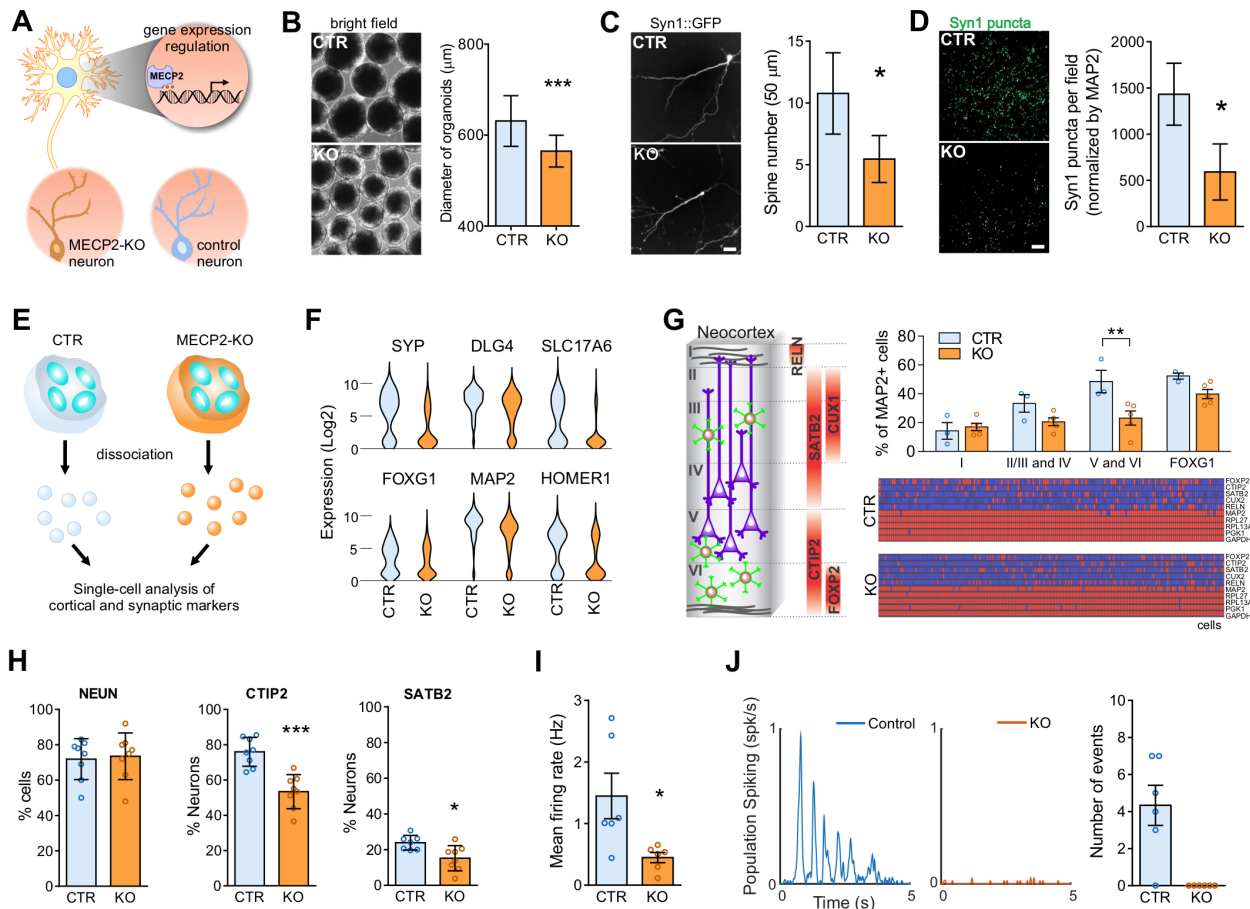
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196   **MECP2 is essential for the timely emergence of network oscillations**

197   In addition to modeling the typically-developing brain, cortical organoids can also shed

198   light on the mechanism behind functional deficits in neurodevelopmental disorders (Birey et al.,

199   2017; Lancaster et al., 2013; Thomas et al., 2016). Normal oscillatory network dynamics in the

200   brain are often shown to break down in psychiatric and neurological conditions (Uhlhaas and

201   Singer, 2010). However, the mechanisms by which that happens and its impact on the circuit are

202   difficult to elucidate. Thus, we next investigated whether cortical organoids could be used to model

203   oscillatory network defects. Previous work evidenced that patients with autism spectrum disorder

204   exhibit reduced alpha oscillation power (8-12 Hz) and evoked gamma (40-60 Hz) response, as

205   well as reduced PAC (Khan et al., 2013; Mohammad-Rezazadeh et al., 2016). Mutations in the

206   Methyl-CpG-binding protein 2 (*MECP2*) gene lead to a severe disruption in cortical development

207   that account for many symptoms of Rett syndrome, autism, schizophrenia and other neurological

208   disorders (Amir et al., 1999; Cohen et al., 2002; Du et al., 2016; Liu et al., 2016; Wen et al., 2017).

209   MECP2 is involved in the epigenetic regulation of target genes by binding to methylated CpG

210   dinucleotides promoter regions, acting as a transcriptional modulator (Figure 4A).

211   To model MECP2 deficiency during neurodevelopment, we a generated pluripotent stem

212   cell model with two different cell lines, each carrying a distinct *MECP2* mutation that lead to a

213   nonfunctional protein (Figure S9). Human *MECP2*-mutant neurons *in vitro* exhibit fewer synapses,

214   smaller soma size, altered calcium signaling and electrophysiological defects compared to

215   controls (Marchetto et al., 2010). Based on the observed reduction in the number of layer V

216   neurons in *Mecp2*-mutant mice (Stuss et al., 2012) and documented clinical data of microcephaly

217   in Rett syndrome patients (Amir et al., 1999), we sought to examine transcriptomics, cellular and

218   structural differences using MECP2-KO cortical organoids. The delay in the maturation process

219   was accompanied by a significant decrease in the diameter of MECP2-KO organoids, spine-like

220   density and synaptic puncta at later stages of differentiation (Figure 4B-D). Additionally, a

221   significant reduction in the proportion of CTIP2+ and SATB2+ neurons was observed by targeted

222   single-cell analysis (Figure 4E-G) and corroborated by immunostaining (Figure 4h). MECP2-KO

223   cortical organoids also showed reduced neural activity leading to an absence of network

224   oscillations, which supports a delay in the maturation process (Figure 4I and 4J). The inability to

225   entrain into a functionally connected network at early stages of development might underlie the

226   core deficits found in MECP2-deficient related disorders. More importantly, these results highlight

227   the contribution of specific genes in the formation of a network circuitry and the emergence of

228   oscillatory activity.

Figure 4. MECP2 contribution to the emergence of network oscillations. (A) MECP2-knockout neurons (MECP2-KO) show reduced spine-like density and soma size compared to controls. (B) Organoid diameter quantification (CTR, *n = 210* organoids; KO, *n = 333* organoids). (C) Spine-like density and (D) synaptic puncta are reduced in MECP2-KO neurons. Scale bar, 50 µm. (E-H) Targeted single-cell analysis of neural markers and cortical layer-related genes over defined control *Ct* value. In 3-month-old cortical organoids, a significant decrease in the number of CTIP2+ and SATB2+ neurons was observed. (I) MECP2-KO cortical organoids show decreased mean firing rate after 5 months of maturation (*n = 6* organoid cultures). (J) Lack of oscillatory network events in 5-month-old MECP2-KO organoids. Each trace represents a single event during the same recording session. For B, C, D, G, H, I and J, data are shown as mean ± s.e.m.; *P < 0.05, **P < 0.01, ***P < 0.001, unpaired Student's *t*-test.

Organoid network development recapitulates preterm EEG

Despite similarities between the complex oscillatory network activity in organoids and the *in vivo* brain, it is unclear whether the spontaneous developmental trajectory observed is representative of programmed early neurodevelopment. While network activity from organoids does not exhibit the full temporal complexity seen in adults, the pattern of alternating periods of quiescence and network-synchronized events is similar to electrophysiological signatures present in preterm human infant EEG. During *trace discontinu* (Tolonen et al., 2007), quiescent periods are punctuated by high-amplitude oscillations (spontaneous activity transients, SATs) lasting a few seconds. Intervals of complete quiescence disappear as infants become of term, and the EEG is dominated by continuous and low-amplitude desynchronized activity in adult brains (Figure 5A). The time-frequency representation of network events in organoids also resembled the oscillatory bursts in preterm EEG, with power localized in the low frequencies and often accentuated within a narrow oscillatory band (Figure S10).

To quantitatively compare network activity in cortical organoids to preterm human EEG, we trained (with cross-validation) a regularized regression model (L1 & L2 regularized, ElasticNet) on a subset of features relating to SATs from a dataset of 567 preterm neonatal EEGs (Stevenson et al., 2017) (24-38 post-conception weeks, PCW). We emphasize that the regression model was thus only optimized to predict preterm infant age based on their own brain features, and has not seen any organoid data whatsoever up to this point. After training, we submitted analogous features computed from organoid LFPs to the model and asked it to predict organoid "brain age" over time (Figure 5B). Notably, the mean model-generated organoid "brain age" was indistinguishable from its "true age" (*in vitro*) after 28 weeks (Figure 5C). In other words, organoids past 28 weeks in culture exhibit similar developmental trajectories of electrophysiological features as preterm neonates. Next, we examined the similarities between brain organoids and preterm humans by looking at each specific feature (Figure 5C and 5D). Of all features, "SATs per hour" ("events per hour" in organoids) showed strikingly similar values and growth, while "root-mean-

268   square SAT duration" showed a similar decline (but not in absolute value) over 25 to 38 weeks in

269   both datasets (Figure 5E, S10B and 10C). Therefore, while the developmental trajectory of

270   cortical organoids is not identical to that of the fetal brain, a machine learning model trained only

271   on preterm neonatal EEG features was able to predict organoid culture age, demonstrating that

272   the observed network electrophysiological features may share similarities representative of

273   genetically programmed developmental timelines.
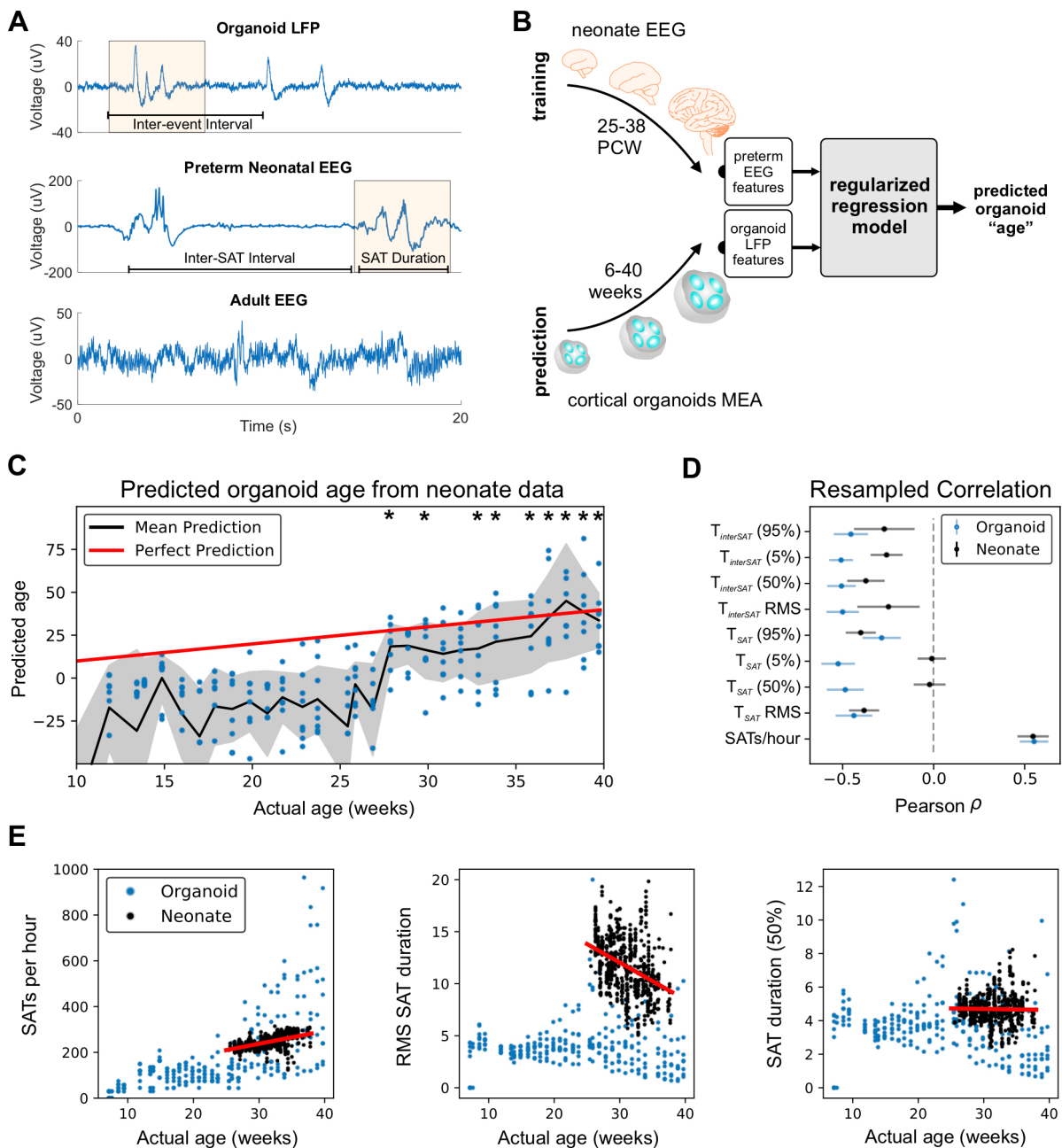
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275   **Figure 5. Organoid network dynamics mimic premature neonates after 28 weeks of**

276   **maturation.** (A) Representative LFP trace from cortical organoid, highlighting instances of

277   network events (yellow). Comparable events between periods of quiescence (discontinuous

278   network dynamics) are shown in human preterm neonate EEG at 35 weeks gestational age, while

279   a different pattern of continuous activity is observed in adult EEG. SAT: spontaneous activity

280   transient. (B) Schematic of machine learning pipeline for organoid "brain-age" prediction: 9 EEG

281   features from 39 premature babies (*n* = 567 recordings) between 25 and 38 PCW were used to

282   train and cross-validate a regularized regression model (ElasticNet) to optimally predict neonate

283   brain age, which was then applied directly to organoid LFP features to predict organoid "brain-

284   age". (C) Predicted organoid "brain age" plotted against actual organoid age. Black stars denote

285   time points where mean predicted age is not significantly different from actual age under 1-sample

286   *t*-test (P < 0.05, *n = 8*). (D) Resampled Pearson's correlation coefficient between age and

287   electrophysiological features for both organoid and premature neonates show different degrees

288   of developmental similarity for individual features. (E) EEG/LFP features over time for organoids

289   and premature neonates show various levels of similarity.

290

291   **DISCUSSION**

292   Development of functional human brain networks is an activity-dependent process guided

293   by genetic and molecular programs, shaped by emerging cellular diversity. Neonatal neural

294   networks share many features with adult brains, despite the fundamental structural differences

295   (Power et al., 2010). Even though the chronological stages of the human cortical network

296   formation are not well understood, it is suggested that emerging cognitive functions during infancy

297   are a result of different brain regions and environmental cues (Johnson, 2001). However, *in utero*

298   development is vital for the establishment of neuronal circuitry and healthy functioning of the brain.

299   The second and third trimester of gestation are when the corticothalamic network is formed *via*

300   transient connections of the subplate GABAergic neurons and the emergence of synchronized

301   network activity (Kostović and Judaš, 2010). Thus, early cortical functional maturation follows an

302   independent sensory-input pathway, guided by spontaneous activity and associated with synaptic

303   regulating mechanisms (Uhlhaas et al., 2010).

304   Here we report the formation of small-scale functional electrophysiological networks in

305   cortical organoids, similar to those observed in the developing brain. While we do not claim

306   functional equivalence between the organoids and a full neonatal cortex, the current results

307   represent the first step towards an *in vitro* model that captures the complex spatiotemporal

308   oscillatory dynamics of the human brain. Robust extracellular electrical activity was established

309   at earlier stages and progressively developed into an organized oscillatory network similar to that

310   observed in human EEG. As such, we show that features of early functional network dynamics

311   (e.g., spontaneous activity transients) can be recapitulated by an *in vitro* model of the developing

312   cortex, with no additional constraints other than structural and genetic similarities. This offers

313   strong evidence for a convergent experience-independent neurodevelopmental program of the

314   neocortex prior to birth. Given the potential roles of synchronized and oscillatory network

315   dynamics in coordinating information flow between developed cortical brain regions during human

316   cognition (Uhlhaas et al., 2010), these results highlight the potential for cortical organoids to

317   advance our understanding of functional electrophysiology, brain development, and neuro-

318   genetic disorders. Finally, our findings may ultimately reframe the ethical discussions on human

319   brain organoid research and offer an innovative link between microscale organoid physiology and

320   cognitive neuroscience.

321

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550 **SUPPLEMENTARY INFORMATION**

551 Supplemental information includes Supplemental Experimental Procedures, 10 Figures, 2 tables

552 can be found with this article online.

553

567

568 **AUTHOR CONTRIBUTIONS**

569 C.A.T., R.G. and P.D.N. should be considered co-first authors as they each designed the

570 experiments and conducted the analyses with input from A.R.M., B.V.; P.D.N. and C.A.T.

571 generated and characterized the cortical organoids and performed the MEA recordings. C.A.T.

572 performed C1 single-cell analyses and synaptic quantification. I.A.C. performed and analyzed

573 10X Genomics single-cell experiments. A.Do. processed 10X Genomics single-cell data. G.W.Y.

574 led and funded the single-cell RNA-seq analyses. M.V. and A.De. performed the functional

575 experiment. P.D.N. analyzed the MEA data using the Axion Biosystems Neural Metrics Tool. R.G.

576   performed the custom MEA and EEG analyses. A.R.M. and B.V. should be considered co-senior

577   authors as they contributed equally to directing the overall study design, with A.R.M. leading the

578   cortical organoid development and analyses, and B.V. leading the electrophysiological design

579   and analyses. C.A.T., R.G., P.D.N., B.V. and A.R.M wrote the manuscript. All authors reviewed

580   the manuscript for publication.

581

582   **CONFLICT OF INTERESTS**

583   Dr. Muotri is a co-founder and has equity interest in TISMOO, a company dedicated to genetic

584   analysis focusing on therapeutic applications customized for autism spectrum disorder and other

585   neurological disorders with genetic origins. The terms of this arrangement have been reviewed

586   and approved by the University of California San Diego in accordance with its conflict of interest

587   policies.

588

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599   **FIGURE LEGENDS**

600   **Figure 1. Cellular and molecular development of human cortical organoids.** (A) Overview of

601   human neural network formation and dynamics evaluation using organoids. (B) Schematic of the

602   protocol used to generate cortical organoids. Scale bar, 200 µm. (C) Organoid growth during

603   different developmental stages. (D) Representative immunostainings showing proliferating NPCs

604   (Ki67+ and Nestin+), lower (TBR1+ and CTIP2+) and upper (SATB2+) cortical layer neurons and

605   glial cells (GFAP+) overtime. Scale bar, 50 µm. (E) Population analysis of specific markers

606   indicating stages of maturation and multiple neuronal subtypes. The data are shown as mean ±

607   s.e.m. ($n = 8$). (F) Representative image of a pyramidal neuron (left panel); dendritic spine-like

608   structures (arrow) are observed in cells transduced with the SYN:EGFP reporter (middle panel;

609   scale bar, 5 µm). Immunohistochemical detection of the synaptic protein Syn1 (right panel; scale

610   bar, 50 µm). (G) Electron microscopy of synaptic structures in 4-month-old cortical organoids

611   (blue). (H) *t*-distributed stochastic neighbor embedding (*t*SNE) plot of 3,491 cells from 6-month-

612   old organoids. Colors denote seven main cell clusters. (I) *t*SNE plots depicting cell-type specific

613   marker expression levels (red denotes higher expression). (J) Heatmap of average expression

614   for representative gene markers by cluster and cell-type (see also Figure S4). (K) Violin plots

615   showing transcript levels for representative markers of each cluster (see Figure S3 for additional

616   markers).

617

618   **Figure 2. Oscillatory network dynamics in long-term cortical organoids.** (A) Schematic of

619   the organoid signal processing pipeline. Raw MEA data is analyzed as population spiking and

620   LFP separately. Synchronous network events are highlighted in yellow. (B) Raster plot of network

621   spiking activity after 1.5 and 6 months of maturation. A 3-s interval of activity over 5 channels is

622   shown in the upper right corners. (C) Cortical organoids show elevated and continuously

623   increasing mean firing rate compared to 2D monolayer neurons ($n = 8$ organoid cultures, and $n =$

624   *12* for 2D neurons). Inset, correlation of the firing rate vector over 12 weeks of differentiation (from

625   8 to 20) between pairs of cultures showing reduced variability among organoid replicates. (D)

626   Temporal evolution of cortical organoid network activity. Detailed definitions and further

627   parameters are presented in Figure 5B and 5C. (E) Time series of population spiking and LFP

628   during network events in cortical organoid development. Each trace represents a single event

629   during the same recording session. (F) Oscillatory dynamics within network events develop

630   nonlinearly, following an inverted-U trajectory. (G) Increase of network variability dynamics

631   throughout development. (H) Oscillatory power increases up to the 25th week in culture and

632   plateaus at 30 weeks. Inset, Oscillatory power is calculated by fitting a straight line (dashed) over

633   the aperiodic portion of the PSD and taken as the height of narrow peaks rising above the linear

634   fit. The data shown in C, D, F, G and H are presented as mean ± s.e.m. *P < 0.05, **P < 0.01,

635   ***P < 0.001, unpaired Student's *t*-test (C), quadratic (F) and linear (G) regression.

636

637   **Figure 3. Cortical organoid serves as a model of functional oscillations and their synaptic**

638   **mechanisms.** (A-C) Phase-amplitude coupling is observed in organoid LFP during network

639   events, a phenomenon proposed to mediate neural communication *in vivo*. (A) Example of raw

640   LFP during a network event decomposed into its low-frequency component (1-4 Hz delta) and the

641   amplitude envelope of the high-frequency, broadband gamma component (200-400 Hz). Analysis

642   was repeated for 100-200 Hz with near identical effect size and significance. (B) Normalized

643   gamma amplitude binned by delta phase during network events (black) shows greater modulation

644   depth by low frequency delta than during non-event periods (red). (C) Phase-amplitude coupling

645   during network events is significantly greater than non-event periods in all batches. (D) Effect of

646   selective drug treatments on neuronal electrical activity in 6-month-old organoids. Representative

647   raster plots and burst measurements of untreated and treated organoids. Scale bar, 20 s.

648   Exposure to AP5 + CNQX, baclofen and muscimol reversibly extinguish the network bursts

649   (synchrony), while no changes were promoted by bicuculline. (E-F) Pharmacological perturbation

650   of oscillatory activity during network events in 6-month-old organoids. Application of bicuculline

651   increases the number of network events, while CNQX + AP5 and baclofen completely abolish

652   synchronized network events. Bicuculline blocks oscillatory network activity but not the network

653   event itself. Data are shown as mean ± s.e.m.; unpaired Student's *t*-test.

654

655   **Figure 4. MECP2 contribution to the emergence of network oscillations.** (A) MECP2-

656   knockout neurons (MECP2-KO) show reduced spine-like density and soma size compared to

657   controls. (B) Organoid diameter quantification (CTR, *n = 210* organoids; KO, *n = 333* organoids).

658   (C) Spine-like density and (D) synaptic puncta are reduced in MECP2-KO neurons. Scale bar, 50

659   μm. (E-H) Targeted single-cell analysis of neural markers and cortical layer-related genes over

660   defined control *Ct* value. In 3-month-old cortical organoids, a significant decrease in the number

661   of CTIP2+ and SATB2+ neurons was observed. (I) MECP2-KO cortical organoids show

662   decreased mean firing rate after 5 months of maturation (*n = 6* organoid cultures). (J) Lack of

663   oscillatory network events in 5-month-old MECP2-KO organoids. Each trace represents a single

664   event during the same recording session. For B, C, D, G, H, I and J, data are shown as mean ±

665   s.e.m.; *P < 0.05, **P < 0.01, ***P < 0.001, unpaired Student's *t*-test.

666

667   **Figure 5. Organoid network dynamics mimic premature neonates after 28 weeks of**

668   **maturation.** (A) Representative LFP trace from cortical organoid, highlighting instances of

669   network events (yellow). Comparable events between periods of quiescence (discontinuous

670   network dynamics) are shown in human preterm neonate EEG at 35 weeks gestational age, while

671   a different pattern of continuous activity is observed in adult EEG. SAT: spontaneous activity

672   transient. (B) Schematic of machine learning pipeline for organoid "brain-age" prediction: 9 EEG

673   features from 39 premature babies (*n = 567* recordings) between 25 and 38 PCW were used to

674   train and cross-validate a regularized regression model (ElasticNet) to optimally predict neonate

675   brain age, which was then applied directly to organoid LFP features to predict organoid "brain-

676   age". (C) Predicted organoid "brain age" plotted against actual organoid age. Black stars denote

677   time points where mean predicted age is not significantly different from actual age under 1-sample

678   *t*-test (P < 0.05, *n = 8*). (D) Resampled Pearson's correlation coefficient between age and

679   electrophysiological features for both organoid and premature neonates show different degrees

680   of developmental similarity for individual features. (E) EEG/LFP features over time for organoids

681   and premature neonates show various levels of similarity.

682

683

684   **EXPERIMENTAL PROCEDURES**

685   **Cell source.** iPSC lines derived from control individuals have been previously characterized

686   elsewhere (Gore et al., 2011; Nageshappa et al., 2016). Human embryonic stem cell (ESC) and

687   iPSC colonies were expanded on Matrigel-coated dishes (BD Biosciences, San Jose, CA, USA)

688   with mTeSR1 medium (StemCell Technologies, Vancouver, Canada). The cells were routinely

689   checked by karyotype and CNV arrays to avoid genomic alterations in the culture. The study was

690   approved by the University of California San Diego IRB/ESCRO committee (protocol 141223ZF).

691

692   **Teratoma formation.** iPSC colonies were dissociated, re-suspended in PBS-Matrigel, and

693   injected subcutaneously in NOD SCID mice. The tumor was dissected, fixed in and paraffin

694   embedded after 8 weeks. Sections of 10 µm thickness were stained with hematoxylin and eosin,

695   and analyzed for the presence of the three germ layer tissues. Protocols were approved by the

696   UCSD Institutional Animal Care and Use Committee.

697

698   **MECP2-KO cell line generation.** MECP2-deficient cell lines were generated by inducing

699   pluripotency in fibroblasts derived from a male patient. Additionally, we used H9 human ESC with

700   the CRISPR/Cas9 genome-editing system to induce frameshift mutations in the *MECP2* locus.

701   This incorporation resulted in the creation of early stop codons rendering a non-functional MECP2

702   protein. Mutagenesis and off-targets were confirmed by exome sequencing techniques. The

703   CRISPR-Cas protocol can be found elsewhere (Thomas et al., 2017). Once we confirmed the

704   pluripotency state of the cellular models, we differentiated them into 2D neuronal monolayer

705   cultures (Thanathom et al., 2016) and cortical organoids.

706

707   **Generation of cortical organoids.** Feeder-free iPSCs were fed daily with mTeSR1 for 7 days.

708   Colonies were dissociated using Accutase (Life Technologies, Carlsbad, CA, USA) in PBS (1:1)

709   for 10 minutes at 37 °C and centrifuged for 3 minutes at 150 x *g*. The cell pellet was resuspended

710   in mTeSR1 supplemented with 10 µM SB431542 (SB; Stemgent, Cambridge, MA, USA) and 1 µM

711   Dorsomorphin (Dorso; R&D Systems, Minneapolis, MN, USA). Approximately 4 × 10⁶ cells were

712   transferred to one well of a 6-well plate and kept in suspension under rotation (95 rpm) in the

713   presence of 5 µM ROCK inhibitor (Y-27632; Calbiochem, Sigma-Aldrich, St. Louis, MO, USA) for

714   24 hours to form free-floating spheres. After 3 days, mTeSR1 was substituted by Media1

715   [Neurobasal (Life Technologies) supplemented with Glutamax, 2% Gem21 NeuroPlex (Gemini

716   Bio-Products, West Sacramento, CA, USA), 1% N2 NeuroPlex (Gemini Bio-Products), 1% MEM

717   nonessential amino acids (NEAA; Life Technologies), 1% penicillin/streptomycin (PS; Life

718   Technologies), 10 µM SB and 1 µM Dorso] for 7 days. Then, the cells were maintained in Media2

719   [Neurobasal with Glutamax, 2% Gem21 NeuroPlex, 1% NEAA and 1% PS] supplemented with

720   20 ng/mL FGF2 (Life Technologies) for 7 days, followed by 7 additional days in Media2

721   supplemented with 20 ng/mL of FGF2 and 20 ng/mL EGF (PeproTech, Rocky Hill, NJ, USA).

722   Next, cells were transferred to Media3 [Media2 supplemented with 10 µg/mL of BDNF, 10 µg/mL

723   of GDNF, 10 µg/mL of NT-3 (all from PeproTech), 200 µM L-ascorbic acid and 1 mM dibutyryl-

724   cAMP (Sigma-Aldrich)]. After 7 days, cortical organoids were maintained in Media2 for as long as

725   needed, with media changes every 3-4 days.

726

727   **Mycoplasma testing.** All cellular cultures were routinely tested for mycoplasma by PCR. Media

728   supernatants (with no antibiotics) were collected, centrifuged, and resuspended in saline buffer.

729   Ten microliters of each sample were used for a PCR with the following primers: Forward:

730   GGCGAATGGGTGAGTAAC; Reverse: CGGATAACGCTTGCGACCT. Only negative samples

731   were used in the study.

732

733   **Immunofluorescence staining.** Cortical organoids were fixed with 4% paraformaldehyde

734   overnight at 4°C and then transferred to 30% sucrose. After the 3D structures sink, they were

735   embedded in O.C.T. (Sakura, Tokyo, Japan) and sliced in a cryostat (20 µm slices). Following air

736   dry, the slides containing the sliced samples were permeabilized/blocked with 0.1% triton X-100

737   and 3% FBS in PBS for 2 hours at room temperature, and incubated with primary antibodies

738   overnight at 4°C. Primary antibodies used in this study were: mouse anti-Nestin, Abcam

739   (Cambridge, UK) ab22035, 1:250; rat anti-CTIP2, Abcam ab18465, 1:500; rabbit anti-SATB2,

740   Abcam ab34735, 1:200; chicken anti-MAP2, Abcam ab5392, 1:2000; rabbit anti-Synapsin1, EMD-

741   Millipore AB1543P, 1:500; mouse anti-NeuN, EMD-Millipore MAB377, 1:500; rabbit anti-Ki67,

742   Abcam ab15580, 1:1000; rabbit anti-SOX2, Cell Signaling Technology 2748, 1:500; rabbit anti-

743   GFAP, DAKO Z033429, 1:1000; rabbit anti-TBR1, Abcam ab31940, 1:500; rabbit anti-TBR2,

744   Abcam ab23345, 1:500; rabbit anti-beta-catenin, Abcam E247, 1:200; mouse anti-GABA, Abcam

745   ab86186, 1:200; rabbit anti-PROX1, Abcam ab101651, 1:250. Next, the slices were washed with

746   PBS and incubated with secondary antibodies (Alexa Fluor 488-, 555- and 647-conjugated

747   antibodies, Life Technologies, 1:1000) for 2 hours at room temperature. The nuclei were stained

748   using DAPI solution (1 µg/mL). The slides were mounted using ProLong Gold antifade reagent

749   and analyzed under a fluorescence microscope (Axio Observer Apotome, Zeiss).

750

751   **Synaptic puncta quantification.** Pre-synaptic Syn1+ puncta were quantified after 3D

752   reconstruction of z-stacks of random images from randomly selected regions of all lines and from

753   two independent experiments. Only puncta overlapping MAP2-positive processes were scored.

754

755   **Immuno-gold electron microscopy (EM).** Immuno-gold EM was performed at the CMM Electron

756   Microscopy Facility at University of California San Diego. Four-month-old organoids were fixed

757   using 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Fixed cells were pelleted and

758   washed with 0.15 M glycine/phosphate buffer, embedded in 10% gelatin/phosphate buffer and

759   infused with 2.3 M sucrose/phosphate buffer. Blocks of cells with 1 mm^3 were mounted onto

760   specimen holders and snap frozen in liquid nitrogen. Ultracryomicrotomy was carried out at –

32

761   100°C on a Leica Ultracut UCT with EM FCS cryoattachment (Leica, Bannockburn, IL) using a

762   Diatome diamond knife (Diatome US, Hatfield, PA). 80 to 90 nm frozen sections were picked up

763   with a 1:1 mixture of 2.3 M sucrose and 2% methyl cellulose (15cp) and transferred onto Formvar

764   and carbon-coated copper grids. Briefly, grids were placed on 2% gelatin at 37 °C for 20 min,

765   rinsed with 0.15 M glycine/PBS and the sections were blocked using 1% cold water fish-skin

766   gelatin. Grids were analyzed using a Tecnai G2 Spirit BioTWIN transmission electron microscope

767   equipped with an Eagle 4k HS digital camera (FEI, Hilsboro, OR).

768

769   **Targeted single-cell qRT-PCR and analysis.** Specific target amplification was performed in

770   individual dissociated cortical organoids using C1 Single-Cell and BioMark HD Systems (Fluidigm,

771   San Francisco, CA, USA), according to the manufacturer's protocol and as previously described

772   (Thanathom et al., 2016). Briefly, cortical organoids were mechanically dissociated after 30

773   minutes of incubation in Accumax (Innovative Cell Technologies, San Diego, CA, USA) at 37 °C

774   under rotation. After passing through 100-μm and 40-μm strainers, cells were centrifuged and

775   resuspended in Media2 (see *Generation of cortical organoids*). Single cortical cells were captured

776   on a C1 medium chip and cell viability was assessed using a LIVE/DEAD Cell Viability/Cytotoxicity

777   kit (Life Technologies). The targeted single-cell qPCR was performed using DELTAgene primer

778   pairs in the 96.96 Dynamic Array IFC chip. The results were analyzed using Fluidigm Real-time

779   PCR Analysis Software and Singular Analysis Toolset 3.0 (Fluidigm).

780

781   **10X genomics single-cell and analysis.** After organoid dissociation, single cells were processed

782   through the Chromium Single Cell Gene Expression Solution using the Chromium Single Cell 3'

783   Gel Bead, Chip and Library Kits v2 (10X Genomics, Pleasanton) as per the manufacturer's

784   protocol. In brief, single cells were resuspended in 0.1% BSA in PBS. Five thousand cells were

785   added to each channel with an average recovery rate of 1,746 cells. The cells were then

786   partitioned into Gel Beads in Emulsion in the Chromium instrument, where cell lysis and barcoded

787   reverse transcription of RNA occurred, followed by amplification, shearing and 5′ adaptor and

788   sample index attachment. Libraries were sequenced on an Illumina HiSeq 2500. De-multiplexing,

789   alignment to the hg19 transcriptome and unique molecular identifier (UMI)-collapsing were

790   performed using the Cellranger toolkit (version 2.0.1) provided by 10X Genomics. A total of 3,491

791   cells with approximately 53,000 reads per cell were processed. Analysis of output digital gene

792   expression matrices was performed using the Seurat R package. Matrices for replicates were

793   merged with the MergeSeurat function and all genes that were not detected in at least 5% of all

794   single cells were discarded, leaving 10,594 genes for further analyses. Cells with fewer than 600

795   or more than 8,000 expressed genes as well as cells with more than 50,000 UMIs or 0.1%

796   mitochondrial expressed genes were removed from the analysis. Data were log normalized and

797   scaled to 10,000 transcripts per cell. Variable genes were identified with the FindVariableGenes

798   function. Principal components were evaluated for statistically significant gene expression signals

799   using the JackStraw function. PCA was carried out, and the top 36 principal components were

800   retained. With these principal components, t-SNE was applied with the RunTSNE function to

801   visualize the cells in two dimensions and identified distinct cell clusters with the FindClusters

802   function with resolution = 0.30. Differential expression to identify cluster markers was performed

803   using the FindAllMarkers function.

804

805   **Data availability.** All data and/or analyses generated during the current study are available from

806   the corresponding author upon reasonable request. Single-cell RNA sequencing data that support

807   the findings of this study have been deposited at NCBI GEO: GSE113089.

808   **Multi-electrode array (MEA) recording.** Six-week-old cortical organoids were plated per well in

809   12-well MEA plates (Axion Biosystems, Atlanta, GA, USA). Each well contains 64 platinum

810   microelectrodes with 30 μm of diameter spaced by 200 μm, yielding a total of 512 channels. The

811   plate was previously coated with 100 μg/mL poly-L-ornithine and 10 μg/ml laminin, and we

812   performed four independent experiments in duplicates. Cells were fed twice a week and

813 measurements were collected 24 hours after the medium was changed, once a week, starting at

814 two weeks after plating (8 weeks of organoid differentiation). Recordings were performed using a

815 Maestro MEA system and AxIS Software Spontaneous Neural Configuration (Axion Biosystems)

816 with a customized script for band-pass filter (0.1-Hz and 5-kHz cutoff frequencies). Spikes were

817 detected with AxIS software using an adaptive threshold crossing set to 5.5 times the standard

818 deviation of the estimated noise for each electrode (channel). The plate was first allowed to rest

819 for three minutes in the Maestro device, and then four minutes of data were recorded. For the

820 MEA analysis, the electrodes that detected at least 5 spikes/min were classified as active

821 electrodes using Axion Biosystems' Neural Metrics Tool. Bursts were identified in the data

822 recorded from each individual electrode using an inter-spike interval (ISI) threshold requiring a

823 minimum number of 5 spikes with a maximum ISI of 100 ms. A minimum of 10 spikes under the

824 same ISI with a minimum of 25% active electrodes were required for network bursts in the well.

825 The synchrony index was calculated using a cross-correlogram synchrony window of 20 ms.

826 Bright-field images were captured from each well to assess for neural density and electrode

827 coverage over time.

828

829 **Custom MEA analysis.** Custom MEA analysis and neonatal EEG/organoid LFP regression

830 model can be found in: https://github.com/voytekresearch/OscillatoryOrganoids. Raw MEA

831 recordings were converted to *.mat* files using Axion-provided functions and analyzed offline using

832 custom MATLAB functions and scripts. Local field potential signals (LFP) from each of the 64

833 electrodes were generated by low-pass filtering (FIR filter) and downsampling raw signals from

834 12,500 Hz to 1,000 Hz (*resample.m*). Multi-unit spikes were detected as follows: each channel

835 was first referenced to the well median (64 channels). The median was used instead of the mean

836 to avoid biasing the reference during high firing rate periods. Next, the re-referenced signal was

837 bandpass filtered for 300-3,000 Hz with a 3rd-order Butterworth filter (*butter.m*). The spike

838 threshold was set to be 5.5 standard deviations, where the standard deviation was estimated as

839 previously described (Quiroga et al., 2004) to avoid biasing the threshold for channels with high

840 firing rates (thus an artificially high threshold). Spike timestamps were taken as the peak time

841 after the absolute value of the signal crossed the threshold, but at least 1 ms from another spike

842 (*findpeaks.m*). Spike timestamps were then converted into binary vectors (1 ms bin size), summed

843 across 64 channels, and smoothed (*conv.m*) with a normalized 100-point Gaussian window

844 (*gausswin.m*) to create a population spiking vector for each MEA well. Note that spikes from each

845 channel do not represent putative single-unit action potentials, as the spatial resolution of MEA

846 electrodes were too sparse. Multi-unit spiking were not sorted since total population spiking (of

847 well) was submitted for further analysis, rather than individual spike trains.

848

849 **Network event analysis.** A network event was detected when population spiking was i) greater

850 than 80% of the maximum spiking value over the length of the recording; ii) at least 1 spike/s; and

851 iii) 1 second away from any other network events. The first peak after all 3 criteria was satisfied

852 was marked as t = 0, and the window of data from 0.5 s before to 2.5 s after the peak was collected

853 as the network event. Nearly all spiking channels experienced a significant firing rate increase

854 during network events. LFP data from all 64 channels from the same timeframe were also

855 collected for analysis. All events from different MEA wells obtained on the same recording day

856 were aggregated for statistical analysis and plotting. Subpeaks within an event were identified

857 using *findpeaks.m*, where a subpeak must satisfy the following: i) peak height of at least 25% of

858 the first peak; ii) peak width of at least 50 ms; iii) at least 200 ms away from the previous peak;

859 and iv) peak prominence of 1 over Peak 1 height. Subpeak time and the height relative to the

860 initial peak were recorded. The inter-event interval coefficient of variation (IEI CV) was calculated

861 as the standard deviation of the inter-event interval divided by its mean, where IEI is the time

862 between consecutive network events within the same MEA well. Event temporal correlation was

863 calculated as the mean Pearson correlation coefficient of population spiking vector during each

864 network event with every other network event in the same MEA well across a single recording

865   session. Event spatial correlation was calculated as the mean Pearson correlation coefficient

866   between all pairs of 64 LFP channels during each 3-s network event.

867

868   **Oscillatory spectral power analysis.** Power spectral density (PSD) estimates were computed

869   using Welch's method (*pwelch.m*), with a window length of 2 s and overlap of 1 s. Oscillatory

870   power was defined as peaks in the PSD above the aperiodic *1/f* power law decay. Thus, for each

871   channel, a straight line was fit over the PSD in double-log space between 0.5-20 Hz using robust

872   fit (*robustfit.m*), and oscillatory power was computed as the difference between the mean log PSD

873   power and the mean log fitted power (baseline), over 2.5-4.5 Hz.

874

875   **Regression models.** For analysis in Figure 2F, G and S7C, F, G, we fit regression models

876   (*LinearModel.fit,MATLAB*) using organoid age (in days) as input and electrophysiological features

877   as output. Order-1 (linear) models were fit for Figure 2G and and S7C, G, and order-2 (quadratic)

878   models were fit for Figure 2F, 3C and Figure S7F. Reported R^2 and p values are model statistics

879   over the entire dataset. All events from different MEA wells on the same recording day were

880   aggregated as samples drawn from the same distribution. To predict culture age, we used 3

881   electrophysiological features as input: event latency, event peak spiking, and oscillatory power;

882   and their square roots to account for the nonlinear inverted-U features. These were used to build

883   a regression model. Within-well models were fit over all data points of the same well, and

884   goodness-of-fit was reported as the model R^2 and the RMSE. Across-well models were trained

885   and evaluated using leave-1-out cross-validation, and goodness-of-fit is reported as the R^2 and

886   the RMSE computed over the validation set, not the training set.

887

888   **Phase Amplitude Coupling (PAC).** LFP data from all 64 channels of each well was first

889   lowpass/bandpass filtered (*eegfilt.m*, EEGLAB) for delta (0-4 Hz) and high-frequency, broadband

890   gamma (100-400 Hz) activity. Delta phase was extracted by taking the phase angle of the

891   bandpassed delta signal Hilbert transform (*hilbert.m, angle.m*), while gamma power was extracted

892   by taking the squared magnitude of the filtered gamma. Gamma power was smoothed with the

893   same delta-band filter for display purposes, but not for subsequent analysis. Note that analysis

894   was done for 100-200 Hz and 200-400 Hz separately, as LFP spectrum follows an inverse power

895   law (1/f), and grouping a wide frequency band (100-400 Hz) together will bias power estimates

896   towards lower frequency limits (~100 Hz). To compute PAC, instantaneous delta phase was

897   binned into 20 equidistant bins between -π and π, and gamma power was sorted based on the

898   corresponding delta phase at the same sample time and averaged across the same phase bin.

899   This procedure was performed separately for event and non-event indices, where event indices

900   are the same 3-s windows as described above in *Network Event Analysis*. Modulation Index was

901   computed as the Kullback-Leibler divergence between the sum-normalized distribution of gamma

902   power across phase bins and a uniform distribution (Tort et al., 2010). Figure 3C presents well-

903   averaged MI across all 64 channels. For visualization in Figure 3b, the binned gamma vector for

904   each channel was circularly shifted such that the phase of maximum gamma power was -π.

905

906   **Pharmacology**. The pharmacological manipulation was performed using the following drugs: 10

907   μM bicuculline, 50 μM muscimol, 20 μM CNQX, 20 μM AP5, 25 μM baclofen and 1 μM TTX. In

908   this assessment, baseline recordings were obtained immediately before and 15 min after the

909   addition of the compound. Three washes with PBS for total removal of the drug were performed

910   in washout experiments; fresh media was added and another recording was conducted after 2

911   hours.

912

913   **Preterm neonatal EEG.** A preterm neonatal EEG dataset was obtained elsewhere (Stevenson

914   et al., 2017). Raw recordings were not available due to patient confidentiality concerns. The

915   dataset includes 567 recordings from 39 preterm neonates (24-38 weeks old conception age),

916   consisting of 23 EEG features computed from the entirety of each recording, as well as during

917   "low-activity periods" (46 features in total), and the post-conception age in weeks.

918

919   **Neonate-organoid age prediction model.** To compare the developmental trajectory of cortical

920   organoids and the preterm human brain, we trained an Elastic Net (L1- and L2- regularized)

921   regression model on only the preterm neonatal EEG features and used that model (with all

922   parameters held the same) to generate an equivalent organoid "brain-age" for each recording

923   time point over 40 weeks in culture. Specifically, the training dataset consists of a subset of the

924   preterm EEG data; of the 46 included features, we discarded all "low-activity-period" features

925   (Lisman, 1997) since there was no equivalent period for organoid recordings, as well as features

926   for which we could not sensibly compute from organoid LFPs, such as interhemispheric

927   synchrony. This selection was done *a priori*, and 13 features remained, including 4 features for

928   relative spectral power in distinct frequency bands, which were further discarded due to

929   frequency-dependent filtering properties of the skull and difference in spatial integration of

930   currents in macroscopic EEG electrodes compared to microscopic planar MEA electrodes. The

931   remaining 9 features correspond to aspects of spontaneous activity transient (SAT) timing, such

932   as SATs per hour and SAT duration, which were similarly computed on organoid LFPs after

933   network event detection described earlier (see Supplementary Table 2 for a full list of included

934   and rejected features). This latter organoid LFP test dataset was never seen by the regression

935   model until prediction time. Training was performed using scikit-learn linear model module

936   [(ElasticNetCV (Pedregosa et al., 2011)], with K-Group shuffle split cross-validation on

937   regularization hyperparameters, where K = 25% of groups, N = 200 shuffles. In other words, we

938   found the best regularized linear model possible for predicting the conception age of preterm

939   neonates using those 9 precomputed EEG features. This model was directly applied on organoid

940   LFP features to determine the corresponding "brain age" of the organoids during 40 weeks in

941   culture. 1-sample *t*-tests were performed from every time point to test whether the mean predicted

942   "brain age" was significantly different from the organoid culture age.

943

Resampled feature correlation. We computed Pearson's correlation coefficient between neonate age and each of the 9 EEG features, after a leave-K-groups-out resampling procedure N times, where K is the number of neonates from whom all recordings were left out in computing the correlation (50% of all neonates, resampling N = 100). An identical procedure was performed to compute the correlation between organoid culture age and LFP features (K = 4 out of 8, 50%, N = 100). Mean and standard deviation were then computed over all resampled draws in order to compare between organoid LFP and neonatal EEG.

951

Statistical analysis. Data are presented as mean ± s.e.m., unless otherwise indicated, and it was obtained from different samples. No statistical method was used to predetermine the sample size, and no adjustments were made for multiple comparisons. The statistical analyses were performed using Prism software (GraphPad, San Diego, CA, USA). Student's *t*-test, Mann–Whitney-test, or ANOVA with post hoc tests were used as indicated. Significance was defined as P < 0.05(*), P < 0.01(**), or P < 0.001(***). Blinding was used for comparing affected and control samples.

958

959   **SUPPLEMENTARY FIGURE LEGENDS**

960   **Supplementary Figure 1. Cellular and molecular characterization of human cortical**

961   **organoids.** (A) Schematic of the protocol used to generate cortical organoids. Scale bar, 200 µm.

962   (B) Reproducibility of organoid size at 2 months of maturation (*n = 20* independent experiment, 7

963   different cell lines). (C) Organoids are composed of a proliferative region surrounded by

964   intermediate progenitor cells, cortical and GABA+ neurons. Scale bar, 50 µm. (D) Principal

965   component analysis (PCA) of cells projected onto the first two components. Overlaid populations

966   of 2- and 10-month-old cortical organoids are compared to 2-month-old 2D monolayer neurons.

967   All timelines for this and the subsequent experiments consider the iPSC stage as day 0 (*n = 2*

968   independent cell lines for each cortical culture; *n = 3* for 2D monolayer neurons). (E-F) Violin plots

969   illustrate the differences in single-cell expression of target genes in cortical organoids and 2D

970   neurons. (G-H) Unsupervised hierarchical clustering single-cell analysis. Genes were clustered

971   using the Pearson correlation method and cells were clustered using the Euclidean method.

972

973   **Supplementary Figure 2. Reproducibility and single-cell characterization.** (A) Schematic

974   showing the single-cell approach performed to access reproducibility of organoid generation using

975   two control iPSC lines. (B) *t*SNE plot of single-cell mRNA sequencing data from 6-month-old

976   organoids color-coded by replicate. (C) Split Dot Plot depicting the correlation between expression

977   patterns of representative markers and cell populations identified within the dataset. The size of

978   the dots represents the percentage of cells expressing a given gene, while the intensity of the

979   color denotes the average expression level (grey, low expression; red/blue, high expression). (D)

980   Population ratio of each cluster by replicate.

981

982   **Supplementary Figure 3. Cell diversity in cortical organoids.** Violin and *t*SNE plots of selected

983   genes depicting the proportion of cells contributing to each cluster. For the violin pots, the dot

984   denotes a cell while colors correspond to their cluster identity. The *t*SNE plots show the

985   contribution of an individual cell-type marker within each cluster (red denotes higher expression).

986

987   **Supplementary Figure 4. Cell type identity of 6-month-old cortical organoids.** Heat map

988   reports scaled expression of discriminative gene sets for clusters defined in Figure 1h with AUC

989   cutoff ≥ 0.80. Color scheme is based on z-score distribution from –2.5 (blue) to 2.5 (red). For gene

990   lists and AUC values see Supplementary Table 1.

991

992   **Supplementary Figure 5. Long-term MEA network activity.** (A) Representative bright-field

993   images of cortical organoids over time on the MEA plate. (B) Schematic representation of the

994   electrical activity features analyzed from the MEA recordings. Each bar represents a spike; and

995   a spike cluster (in blue) represents a burst. Bursts occurring at the same time in different channels

996   characterize a network burst. The synchrony index is based on the cross-correlogram and

997   represents a measure of similarity between two spike trains. (C) Temporal evolution of network

998   activity characterized by different parameters. (D) Raster plots illustrating the development of

999   network activity. (E) Consistent and reproducible development of electrical activity in cortical

1000   organoids over time. The data are shown as mean ± s.e.m ($n = 8$, independent experiments

1001   performed in duplicates using two clones of a control iPSC line).

1002

1003   **Supplementary Figure 6. MEA electrical activity comparison of cortical organoids with**

1004   **available published data from iPSC-derived neurons, organoids, rodent primary cultures**

1005   **and primate models.** (A) Long-term network activity of our cortical organoids is shown for

1006   individual wells. Comparison of network activity between cortical organoids and iPSC-derived

1007   cortical neurons (B), rodent primary neuronal cultures and primate models (C). The data shown

1008   in B and C for cortical organoids are presented as mean ± s.e.m. ($n = 8$, independent experiments

1009   performed in duplicates) (Amin et al., 2016; Bardy et al., 2015; Barz et al., 2017; Brown et al.,

1010   2016; Cesca et al., 2015; Clements et al., 2016; Cotterill et al., 2016; Harrill et al., 2015; Marchetto

1011   et al., 2016; McSweeney et al., 2016; Odawara et al., 2014, 2016; Roy et al., 2016; Strickland et

1012   al., 2016; Tukker et al., 2016; Uesaka et al., 2007; Vessoni et al., 2016; Wallace et al., 2015; Xu

1013   et al., 2017; Yang et al., 2016).

1014

1015   **Supplementary Figure 7. Extended characterization of network electrophysiology.** (A)

1016   Spikes detected on 9 channels. Black traces represent single spikes, blue and red traces

1017   represent the average of positive and negative spikes, respectively. Spike trains are not sorted

1018   for their polarity in the subsequent analyses, as total population spiking is the main feature of

1019   interest. (B) Representative oscillatory network events. Each overlapping trace represents a

1020   single occurrence of an event recorded on the same channel. LFP polarity of events differs

1021   between channels due to the spatial configuration of cells around the electrode. (C) Event onset

1022   peak (Peak 1) increases in amplitude until 30 weeks, while (D) subpeak amplitude continues to

1023   increase (for the 2nd-4th peak) throughout development. (E) Subsequent peaks occur with a

1024   consistent latency of ~400 ms after the previous peak, particularly for Peak 3 and 4. (F) Temporal

1025   similarity of network events during the 3-s window is high at early time points, but decreases with

1026   development, acquiring more variable dynamics within an event. (G) Temporal similarity of

1027   network events during the 3-s window is high at early time points, but decreases with

1028   development, acquiring more variable dynamics within an event. The data showed in C, F and G

1029   are presented as mean ± s.e.m., linear (C, G) or quadratic (F) model regression.

1030

1031   **Supplementary Figure 8. MEA recording from 3-month-old neurospheres.** (A) Comparison

1032   of the protocol for neurosphere and cortical organoid generation. (B) Network-wide giant

1033   depolarizing potentials occur at a similar rate to those found in organoids recordings, and visible

1034   perturbations are observed in the LFP trace. However, the network recruitment in neurospheres

1035   is lower than in organoids (less than 8 spikes/s), and events have significantly shorter duration.

1036   No coherent low-frequency depolarizations are observed in filtered LFP events (C).

1037   **Supplementary Figure 9. MECP2-KO cell line characterization.** (A) Schematic overview of the

1038   *MECP2* locus in iPSCs derived from fibroblasts of a male patient (Q83X), and CRISPR/Cas9

1039   induced *MECP2* mutation in an embryonic stem cell line (K82fs, H9 ESC). Q83X cell line

1040   characterization is shown elsewhere (Zhang et al., 2016). DNA sequence chromatogram shows

1041   the nucleotide deletion in the *MECP2* gene leading to a frameshift mutation (K82fs) and a

1042   predicted premature stop-codon at the end of exon 3 (asterisk). The WT82 and WT83 were used

1043   as controls. The Q83X and K82fs do not express MECP2 protein. Blue line represents the guide

1044   RNA target locus. (B) Gel images showing Surveyor nuclease assay of genomic DNA extracted

1045   from FACs sorted H9 ESC. Expected PCR products were 278 bp and 220 bp. (C) Exome

1046   sequencing analysis to evaluate CRISPR off-target mutations. Numbers indicate the amount of

1047   reads across the lines. Off-target gene mutations induced by *MECP2* CRISPR/Cas9 are shown

1048   in the lower table. (D) Isogenic pairs of *MECP2*-mutant and control cell line colonies showing the

1049   expression of the pluripotency marker Nanog. Scale bar, 100 µm. (E) Eosin and Hematoxylin

1050   stains of teratomas showing the presence of all three germ layers. Scale bar, 200 µm. (F) Western

1051   blot of the isogenic pluripotent stem cells showing the absence of MECP2 in the mutant line. (G)

1052   Karyotypes of cell lines displaying no chromosomal abnormality. (H and I) Expression of

1053   pluripotency markers and *MECP2* by qPCR. GAPDH was used as housekeeping gene.

1054

1055   **Supplementary Figure 10. Network activity in cortical organoids mimics oscillatory**

1056   **features in the developing human brain.** (A) Spectral representation of time series data from a

1057   6-month-old cortical organoid, demonstrating oscillatory phenomenon. Spectrogram (left) of

1058   organoid LFP shows bursts of activity localized at low frequencies, while power spectral density

1059   (PSD, right) displays canonical *1/f* power law decay and a narrow oscillatory peak at 3 Hz. (B)

1060   Comparison of 9 preterm neonate EEG and cortical organoid features over time. For included

1061   EEG features, see Table S2. (C) Distributions of resampled Pearson correlation coefficients

1062   between feature and age for preterm neonate and organoid.

1063

1064 **SUPPLEMENTARY TABLES**
1065
1066 **Supplementary Table 1. Top expressed genes of each cell cluster.**

Cluster	Gene	myAUC	avg_diff	power	avg_logFC	pct.1	pct.2	p_val_adj
Cortical neurons	SOX11	0.889	1.29996	0.778	1.29996	0.996	0.864	NA
Cortical neurons	NEUROD2	0.886	1.366603	0.772	1.366603	0.944	0.382	NA
Cortical neurons	GPM6A	0.885	1.12235	0.77	1.12235	0.992	0.679	NA
Cortical neurons	SOX4	0.879	0.985057	0.758	0.985057	1	0.965	NA
Cortical neurons	MLLT11	0.856	0.916514	0.712	0.916514	0.998	0.869	NA
Cortical neurons	CCNI	0.843	0.707003	0.686	0.707003	1	0.994	NA
Cortical neurons	SLA	0.833	1.424488	0.666	1.424488	0.821	0.303	NA
Cortical neurons	MARCKSL1	0.832	0.578678	0.664	0.578678	0.999	0.981	NA
Cortical neurons	DCX	0.806	0.81224	0.612	0.81224	0.969	0.576	NA
Progenitors	NES	0.976	1.785855	0.952	1.785855	0.997	0.303	NA
Progenitors	ANXA2	0.972	2.407607	0.944	2.407607	0.976	0.149	NA
Progenitors	GYPC	0.963	1.587675	0.926	1.587675	0.954	0.065	NA
Progenitors	SPARC	0.958	1.745727	0.916	1.745727	0.978	0.24	NA
Progenitors	SDC2	0.944	1.388854	0.888	1.388854	0.924	0.08	NA
Progenitors	CRABP2	0.942	1.564316	0.884	1.564316	0.939	0.11	NA
Progenitors	NTRK2	0.941	1.61904	0.882	1.61904	0.912	0.057	NA
Progenitors	CCND1	0.941	1.505771	0.882	1.505771	0.909	0.046	NA
Progenitors	LGALS1	0.938	2.0633	0.876	2.0633	0.94	0.17	NA
Progenitors	SERF2	0.934	0.95158	0.868	0.95158	1	0.903	NA
Progenitors	MDK	0.933	1.279235	0.866	1.279235	0.996	0.832	NA
Progenitors	VGLL3	0.931	1.237999	0.862	1.237999	0.887	0.032	NA
Progenitors	S100A13	0.917	1.839522	0.834	1.839522	0.893	0.12	NA
Progenitors	PDLIM7	0.916	1.185298	0.832	1.185298	0.94	0.25	NA

Progenitors	ANXA5	0.902	1.221643	0.804	1.221643	0.926	0.184	NA
Progenitors	PRSS23	0.901	1.501512	0.802	1.501512	0.836	0.06	NA
Progenitors	RPL41	0.897	0.625131	0.794	0.625131	1	0.999	NA
Progenitors	NPC2	0.895	1.182853	0.79	1.182853	0.951	0.407	NA
Progenitors	SEC11A	0.894	0.828695	0.788	0.828695	0.98	0.64	NA
Progenitors	PRDX6	0.892	0.981071	0.784	0.981071	0.98	0.555	NA
Progenitors	TPM1	0.887	1.731952	0.774	1.731952	0.938	0.518	NA
Progenitors	RHOC	0.887	0.962424	0.774	0.962424	0.907	0.206	NA
Progenitors	NEAT1	0.883	1.352256	0.766	1.352256	0.948	0.285	NA
Progenitors	RPL12	0.882	0.706881	0.764	0.706881	0.999	0.992	NA
Progenitors	RPL7A	0.881	0.613375	0.762	0.613375	1	0.997	NA
Progenitors	EEF1A1	0.879	0.651914	0.758	0.651914	1	1	NA
Progenitors	RPL28	0.876	0.592999	0.752	0.592999	1	0.995	NA
Progenitors	RPS6	0.871	0.711767	0.742	0.711767	0.997	0.994	NA
Progenitors	RPL23A	0.867	0.568383	0.734	0.568383	0.999	0.994	NA
Progenitors	TIMP1	0.865	0.772749	0.73	0.772749	0.895	0.173	NA
Progenitors	RPL8	0.864	0.572428	0.728	0.572428	0.999	0.997	NA
Progenitors	METRN	0.863	0.835332	0.726	0.835332	0.907	0.229	NA
Progenitors	WLS	0.859	0.916782	0.718	0.916782	0.736	0.023	NA
Progenitors	RPL27A	0.858	0.534803	0.716	0.534803	1	0.998	NA
Progenitors	CTGF	0.857	1.335629	0.714	1.335629	0.727	0.017	NA
Progenitors	RCN1	0.857	0.806462	0.714	0.806462	0.967	0.377	NA
Progenitors	PFN1	0.857	0.738107	0.714	0.738107	0.99	0.844	NA
Progenitors	PMP22	0.855	1.601169	0.71	1.601169	0.778	0.101	NA
Progenitors	ITGB8	0.855	1.138802	0.71	1.138802	0.868	0.201	NA
Progenitors	SERPINH1	0.854	0.713982	0.708	0.713982	0.846	0.143	NA
Progenitors	VIM	0.853	1.146246	0.706	1.146246	1	0.77	NA
Progenitors	NME4	0.852	0.813408	0.704	0.813408	0.945	0.457	NA
Progenitors	RPS7	0.852	0.558045	0.704	0.558045	0.999	0.997	NA
Progenitors	MYL12A	0.85	0.739735	0.7	0.739735	0.84	0.157	NA
Progenitors	RPS20	0.849	0.558658	0.698	0.558658	1	0.991	NA

Progenitors	RPS2	0.848	0.524557	0.696	0.524557	0.999	1	NA
Progenitors	RPLP1	0.848	0.514123	0.696	0.514123	1	0.998	NA
Progenitors	RAB13	0.846	0.808108	0.692	0.808108	0.86	0.204	NA
Progenitors	TUBB6	0.845	0.757197	0.69	0.757197	0.806	0.121	NA
Progenitors	CRNDE	0.843	0.802352	0.686	0.802352	0.954	0.472	NA
Progenitors	TTYH1	0.84	0.971997	0.68	0.971997	0.963	0.416	NA
Progenitors	RPL23	0.84	0.546833	0.68	0.546833	1	0.997	NA
Progenitors	RPS19	0.84	0.516859	0.68	0.516859	1	1	NA
Progenitors	RPL29	0.84	0.464037	0.68	0.464037	1	0.997	NA
Progenitors	RPS14	0.839	0.462974	0.678	0.462974	1	0.999	NA
Progenitors	RPL3	0.838	0.497988	0.676	0.497988	1	0.998	NA
Progenitors	SLC25A6	0.835	0.71474	0.67	0.71474	0.995	0.891	NA
Progenitors	SPATS2L	0.831	0.9606	0.662	0.9606	0.811	0.193	NA
Progenitors	QPRT	0.83	0.651751	0.66	0.651751	0.855	0.198	NA
Progenitors	RPL35	0.83	0.470972	0.66	0.470972	0.999	0.993	NA
Progenitors	RPS18	0.828	0.49256	0.656	0.49256	1	1	NA
Progenitors	CLIC1	0.827	0.723496	0.654	0.723496	0.937	0.427	NA
Progenitors	RPS3	0.827	0.526597	0.654	0.526597	1	0.997	NA
Progenitors	RPL10A	0.827	0.523565	0.654	0.523565	1	0.994	NA
Progenitors	RPS28	0.825	0.506021	0.65	0.506021	1	0.993	NA
Progenitors	CD63	0.824	0.710828	0.648	0.710828	0.991	0.76	NA
Progenitors	PDPN	0.824	0.65191	0.648	0.65191	0.699	0.046	NA
Progenitors	ACTG1	0.824	0.488252	0.648	0.488252	1	1	NA
Progenitors	CCNG1	0.823	0.727443	0.646	0.727443	0.924	0.38	NA
Progenitors	CD99	0.82	0.68859	0.64	0.68859	0.953	0.405	NA
Progenitors	B2M	0.817	0.787786	0.634	0.787786	0.947	0.392	NA
Progenitors	CHCHD10	0.817	0.645222	0.634	0.645222	0.84	0.211	NA
Progenitors	RPLP0	0.817	0.469741	0.634	0.469741	1	0.997	NA
Progenitors	RPS27L	0.816	0.744701	0.632	0.744701	0.995	0.664	NA
Progenitors	COL1A2	0.815	0.896012	0.63	0.896012	0.647	0.019	NA
Progenitors	PFN2	0.815	0.622861	0.63	0.622861	0.991	0.763	NA

Progenitors	UBB	0.813	0.783749	0.626	0.783749	0.978	0.588	NA
Progenitors	RPL37	0.813	0.465752	0.626	0.465752	1	0.995	NA
Progenitors	CRABP1	0.811	1.087669	0.622	1.087669	0.737	0.133	NA
Progenitors	RPL7	0.811	0.467728	0.622	0.467728	1	0.998	NA
Progenitors	FSTL1	0.81	0.761788	0.62	0.761788	0.737	0.123	NA
Progenitors	RPL36	0.81	0.434708	0.62	0.434708	1	0.992	NA
Progenitors	RPL19	0.81	0.401045	0.62	0.401045	1	1	NA
Progenitors	FGFR1	0.809	0.608769	0.618	0.608769	0.839	0.204	NA
Progenitors	ENO1	0.808	0.582772	0.616	0.582772	0.996	0.869	NA
Progenitors	RPS15	0.806	0.381154	0.612	0.381154	1	0.999	NA
Progenitors	MYL6	0.805	0.530039	0.61	0.530039	1	0.986	NA
Progenitors	GSTP1	0.804	0.634369	0.608	0.634369	0.996	0.92	NA
Progenitors	PODXL	0.804	0.622402	0.608	0.622402	0.67	0.06	NA
Progenitors	CNN3	0.804	0.616921	0.608	0.616921	0.992	0.669	NA
Progenitors	GNG11	0.803	0.751478	0.606	0.751478	0.668	0.064	NA
Progenitors	RPS4Y1	0.803	0.680093	0.606	0.680093	0.963	0.62	NA
Progenitors	AHNAK	0.803	0.651782	0.606	0.651782	0.64	0.036	NA
Progenitors	CST3	0.802	0.645478	0.604	0.645478	0.963	0.567	NA
Progenitors	RPS23	0.801	0.419435	0.602	0.419435	1	0.998	NA
Progenitors	RPL13A	0.801	0.409875	0.602	0.409875	1	1	NA
Glia	SFRP1	0.94	2.001041	0.88	2.001041	0.952	0.385	NA
Glia	SOX2	0.909	1.356804	0.818	1.356804	0.946	0.321	NA
Glia	C1orf61	0.893	1.500525	0.786	1.500525	0.984	0.749	NA
Glia	FABP7	0.887	1.707591	0.774	1.707591	0.985	0.736	NA
Glia	SLC1A3	0.88	1.56662	0.76	1.56662	0.807	0.119	NA
Glia	SYNE2	0.876	1.218806	0.752	1.218806	0.919	0.445	NA
Glia	PAX6	0.871	1.251984	0.742	1.251984	0.83	0.186	NA
Glia	HMGN3	0.866	0.91378	0.732	0.91378	0.978	0.849	NA
Glia	ID4	0.851	1.407158	0.702	1.407158	0.875	0.381	NA
Glia	MYO10	0.85	1.025069	0.7	1.025069	0.857	0.338	NA
Glia	DBI	0.842	1.242287	0.684	1.242287	0.958	0.709	NA

Glia	PTN	0.836	1.579917	0.672	1.579917	0.948	0.752	NA
Glia	QKI	0.83	0.909655	0.66	0.909655	0.891	0.502	NA
Glia	LINC01158	0.818	0.903364	0.636	0.903364	0.901	0.546	NA
Glia	ZFHX4	0.817	1.004117	0.634	1.004117	0.707	0.132	NA
Glia	HES1	0.812	1.171994	0.624	1.171994	0.718	0.17	NA
Glia	HMGB2	0.809	1.270445	0.618	1.270445	0.921	0.594	NA
Glia	LHX2	0.806	0.931067	0.612	0.931067	0.846	0.398	NA
Lower cortex	SNAP25	0.942	1.540645	0.884	1.540645	0.987	0.415	NA
Lower cortex	GRIA2	0.892	1.185847	0.784	1.185847	0.96	0.352	NA
Lower cortex	CNTNAP2	0.88	1.447084	0.76	1.447084	0.876	0.272	NA
Lower cortex	CELF4	0.863	1.071334	0.726	1.071334	0.886	0.265	NA
Lower cortex	NSG2	0.851	1.031537	0.702	1.031537	0.96	0.403	NA
Lower cortex	SYT1	0.85	0.985569	0.7	0.985569	0.983	0.61	NA
Lower cortex	YWHAH	0.841	0.785308	0.682	0.785308	0.973	0.805	NA
Lower cortex	SNCA	0.839	0.953942	0.678	0.953942	0.914	0.451	NA
Lower cortex	BASP1	0.838	0.734567	0.676	0.734567	1	0.943	NA
Lower cortex	DOK6	0.831	1.000188	0.662	1.000188	0.814	0.264	NA
Lower cortex	RTN1	0.823	0.898627	0.646	0.898627	0.985	0.519	NA
Lower cortex	RUNX1T1	0.82	0.94366	0.64	0.94366	0.852	0.281	NA
Lower cortex	FAM49A	0.817	0.920672	0.634	0.920672	0.821	0.28	NA
Lower cortex	MAP1B	0.817	0.603691	0.634	0.603691	1	0.995	NA
Lower cortex	SYT4	0.816	0.939245	0.632	0.939245	0.821	0.262	NA
Lower cortex	B3GALT2	0.815	1.017411	0.63	1.017411	0.757	0.2	NA
Lower cortex	GABRB2	0.815	0.991632	0.63	0.991632	0.675	0.062	NA
Lower cortex	LMO3	0.814	1.36195	0.628	1.36195	0.688	0.101	NA
Lower cortex	SCG3	0.811	0.757346	0.622	0.757346	0.939	0.415	NA
Lower cortex	UCHL1	0.809	0.66339	0.618	0.66339	0.99	0.906	NA
Lower cortex	VAMP2	0.809	0.606955	0.618	0.606955	0.994	0.939	NA
Lower cortex	TMEM161B-AS1	0.808	0.816917	0.616	0.816917	0.941	0.63	NA
Lower cortex	LY6H	0.806	0.807691	0.612	0.807691	0.88	0.34	NA
Lower cortex	MAPT	0.805	0.73704	0.61	0.73704	0.962	0.486	NA

Lower cortex	CDKN2D	0.802	0.762383	0.604	0.762383	0.878	0.4	NA
Lower cortex	RAB3A	0.801	0.697869	0.602	0.697869	0.924	0.413	NA
Upper cortex	MEF2C	0.954	2.051853	0.908	2.051853	0.986	0.369	NA
Upper cortex	STMN2	0.885	1.043931	0.77	1.043931	1	0.644	NA
Upper cortex	NSG2	0.883	1.126043	0.766	1.126043	1	0.441	NA
Upper cortex	ARPP21	0.88	1.115469	0.76	1.115469	0.883	0.189	NA
Upper cortex	STMN4	0.874	0.982886	0.748	0.982886	1	0.696	NA
Upper cortex	MAPT	0.87	0.908315	0.74	0.908315	1	0.518	NA
Upper cortex	GRIN2B	0.869	1.002716	0.738	1.002716	0.9	0.246	NA
Upper cortex	CALM1	0.868	0.733117	0.736	0.733117	1	0.988	NA
Upper cortex	NELL2	0.861	0.95751	0.722	0.95751	0.973	0.409	NA
Upper cortex	SCD5	0.855	0.913699	0.71	0.913699	0.931	0.478	NA
Upper cortex	SATB2	0.853	0.902036	0.706	0.902036	0.811	0.125	NA
Upper cortex	PKIA	0.849	0.808509	0.698	0.808509	0.952	0.445	NA
Upper cortex	MAP1B	0.849	0.669352	0.698	0.669352	1	0.995	NA
Upper cortex	INA	0.847	0.831367	0.694	0.831367	0.966	0.437	NA
Upper cortex	STMN1	0.845	0.783568	0.69	0.783568	1	0.979	NA
Upper cortex	NEUROD6	0.843	1.007963	0.686	1.007963	0.986	0.502	NA
Upper cortex	VAMP2	0.843	0.689091	0.686	0.689091	0.993	0.943	NA
Upper cortex	DOK5	0.841	0.93379	0.682	0.93379	0.935	0.559	NA
Upper cortex	RASL11B	0.841	0.930199	0.682	0.930199	0.821	0.209	NA
Upper cortex	SNCA	0.841	0.896556	0.682	0.896556	0.952	0.482	NA
Upper cortex	R3HDM1	0.84	0.924861	0.68	0.924861	0.89	0.386	NA
Upper cortex	TTC9B	0.84	0.868857	0.68	0.868857	0.959	0.435	NA
Upper cortex	RAC3	0.83	0.70783	0.66	0.70783	0.945	0.624	NA
Upper cortex	CXADR	0.827	0.785512	0.654	0.785512	0.993	0.719	NA
Upper cortex	HN1	0.827	0.602815	0.654	0.602815	1	0.961	NA
Upper cortex	CAMK2B	0.822	0.749623	0.644	0.749623	0.897	0.279	NA
Upper cortex	RTN1	0.819	0.807888	0.638	0.807888	1	0.553	NA
Upper cortex	CHL1	0.819	0.775621	0.638	0.775621	0.918	0.374	NA
Upper cortex	NSG1	0.818	0.708593	0.636	0.708593	0.997	0.528	NA

Upper cortex	TUBB2A	0.817	0.659235	0.634	0.659235	1	0.946	NA
Upper cortex	GABBR2	0.815	0.777596	0.63	0.777596	0.79	0.182	NA
Upper cortex	RBFOX2	0.814	0.677032	0.628	0.677032	0.99	0.662	NA
Upper cortex	CRMP1	0.813	0.666121	0.626	0.666121	0.979	0.79	NA
Upper cortex	GAP43	0.811	0.737576	0.622	0.737576	0.997	0.816	NA
Upper cortex	UCHL1	0.809	0.645161	0.618	0.645161	1	0.911	NA
Upper cortex	CDKN2D	0.808	0.694482	0.616	0.694482	0.935	0.43	NA
Upper cortex	NCAM1	0.805	0.694452	0.61	0.694452	0.955	0.551	NA
Upper cortex	MSRA	0.804	0.734229	0.608	0.734229	0.814	0.288	NA
Upper cortex	GPR85	0.801	0.76111	0.602	0.76111	0.766	0.189	NA
Upper cortex	DAAM1	0.801	0.628961	0.602	0.628961	0.993	0.776	NA
Other	ALDOA	0.917	1.757415	0.834	1.757415	0.963	0.838	NA
Other	EIF1	0.888	0.999198	0.776	0.999198	1	0.999	NA
Other	FTL	0.883	1.541462	0.766	1.541462	1	0.997	NA
Other	BNIP3	0.87	1.504624	0.74	1.504624	0.844	0.345	NA
Other	FAM162A	0.857	1.366057	0.714	1.366057	0.881	0.459	NA
Other	ARF4	0.848	1.242187	0.696	1.242187	0.889	0.715	NA
Other	ENO1	0.845	1.199331	0.69	1.199331	0.978	0.894	NA
Other	P4HA1	0.832	1.239505	0.664	1.239505	0.741	0.175	NA
Other	TRMT112	0.825	0.918451	0.65	0.918451	0.926	0.735	NA
Other	RPS13	0.822	0.756328	0.644	0.756328	0.993	0.998	NA
Other	TPT1	0.817	0.840456	0.634	0.840456	0.993	0.998	NA
Other	SEC61G	0.812	0.841716	0.624	0.841716	0.963	0.881	NA
Other	PGK1	0.809	1.333477	0.618	1.333477	0.881	0.803	NA
Other	GADD45A	0.802	1.332596	0.604	1.332596	0.741	0.3	NA
Other	ST13	0.801	0.866714	0.602	0.866714	0.963	0.878	NA
Neural crest	TAGLN3	0.922	1.681741	0.844	1.681741	1	0.686	NA
Neural crest	PBX3	0.917	1.457984	0.834	1.457984	0.878	0.154	NA
Neural crest	CRABP1	0.886	2.63702	0.772	2.63702	0.892	0.257	NA
Neural crest	MEG3	0.872	2.436136	0.744	2.436136	0.824	0.289	NA
Neural crest	ACTG1	0.851	0.573491	0.702	0.573491	1	1	NA

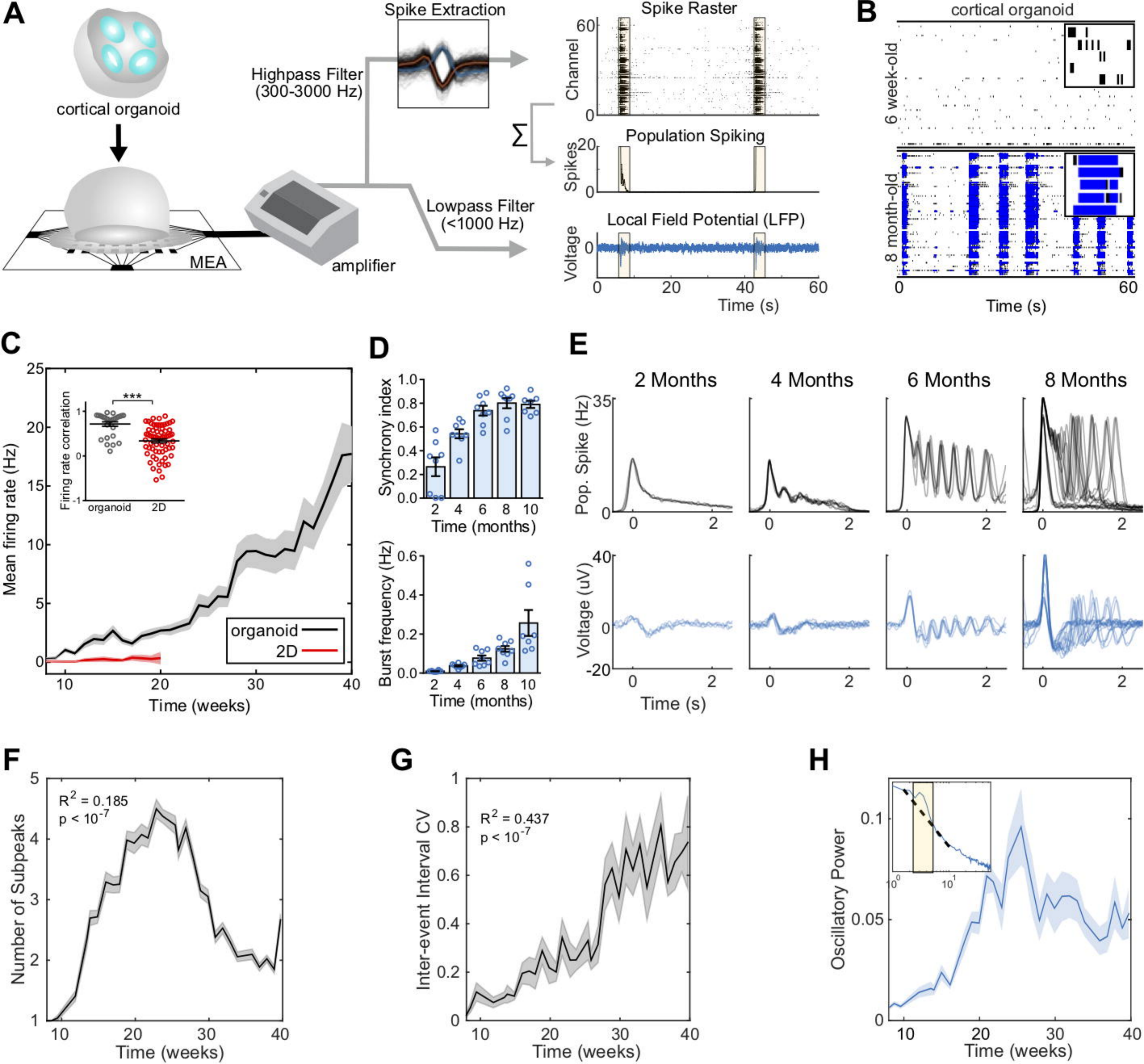|								
Neural crest	MIAT	0.82	1.008958	0.64	1.008958	0.932	0.671	NA
Neural crest	KCNQ1OT1	0.818	1.242528	0.636	1.242528	0.905	0.547	NA
Neural crest	NEAT1	0.806	0.991861	0.612	0.991861	0.865	0.427	NA
Neural crest	ELAVL2	0.806	0.728978	0.612	0.728978	0.932	0.464	NA
Neural crest	RGMB	0.804	1.190676	0.608	1.190676	0.703	0.168	NA

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1074 **Supplementary Table 2. Electrophysiological features in preterm neonatal EEG dataset and**

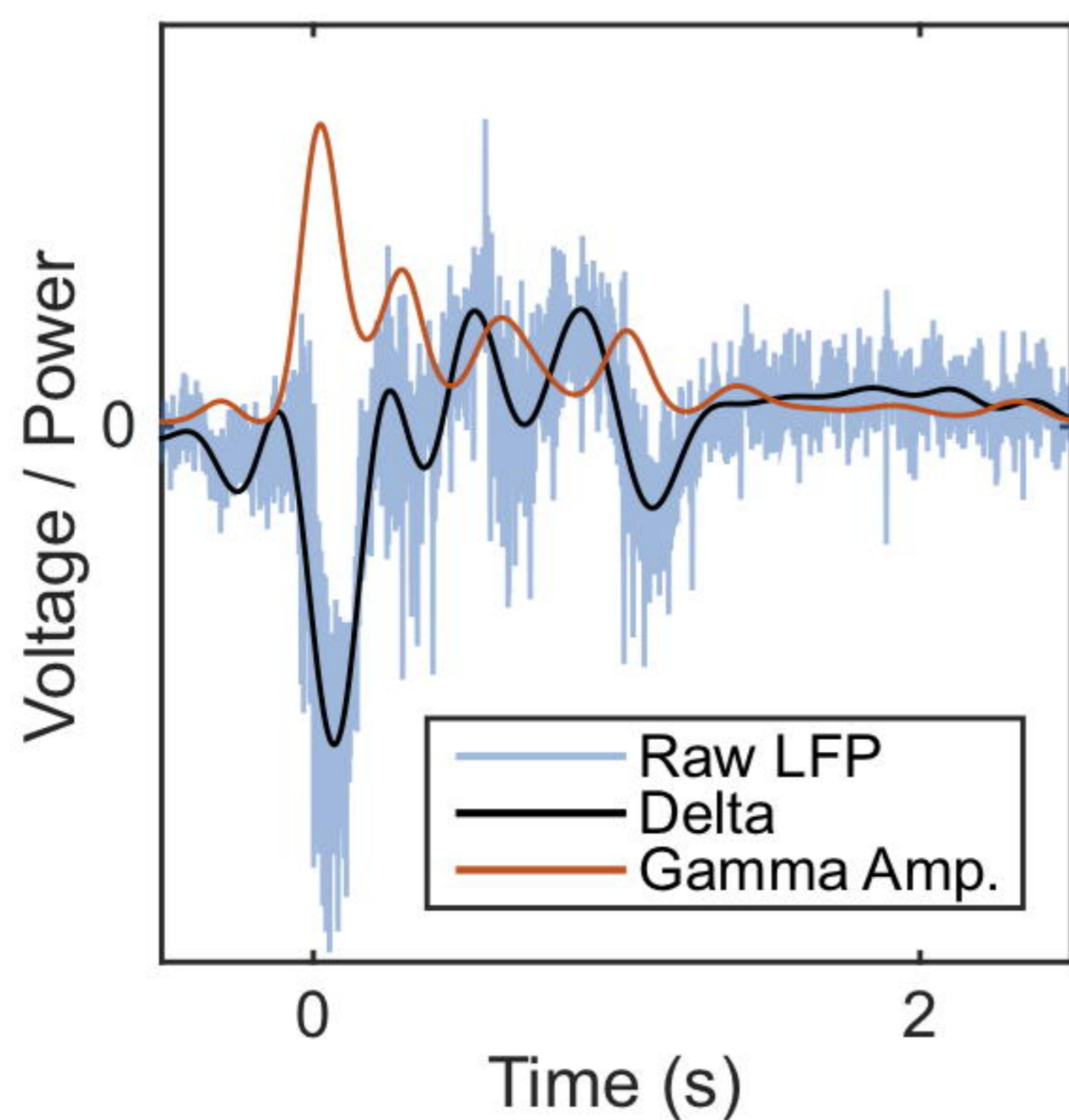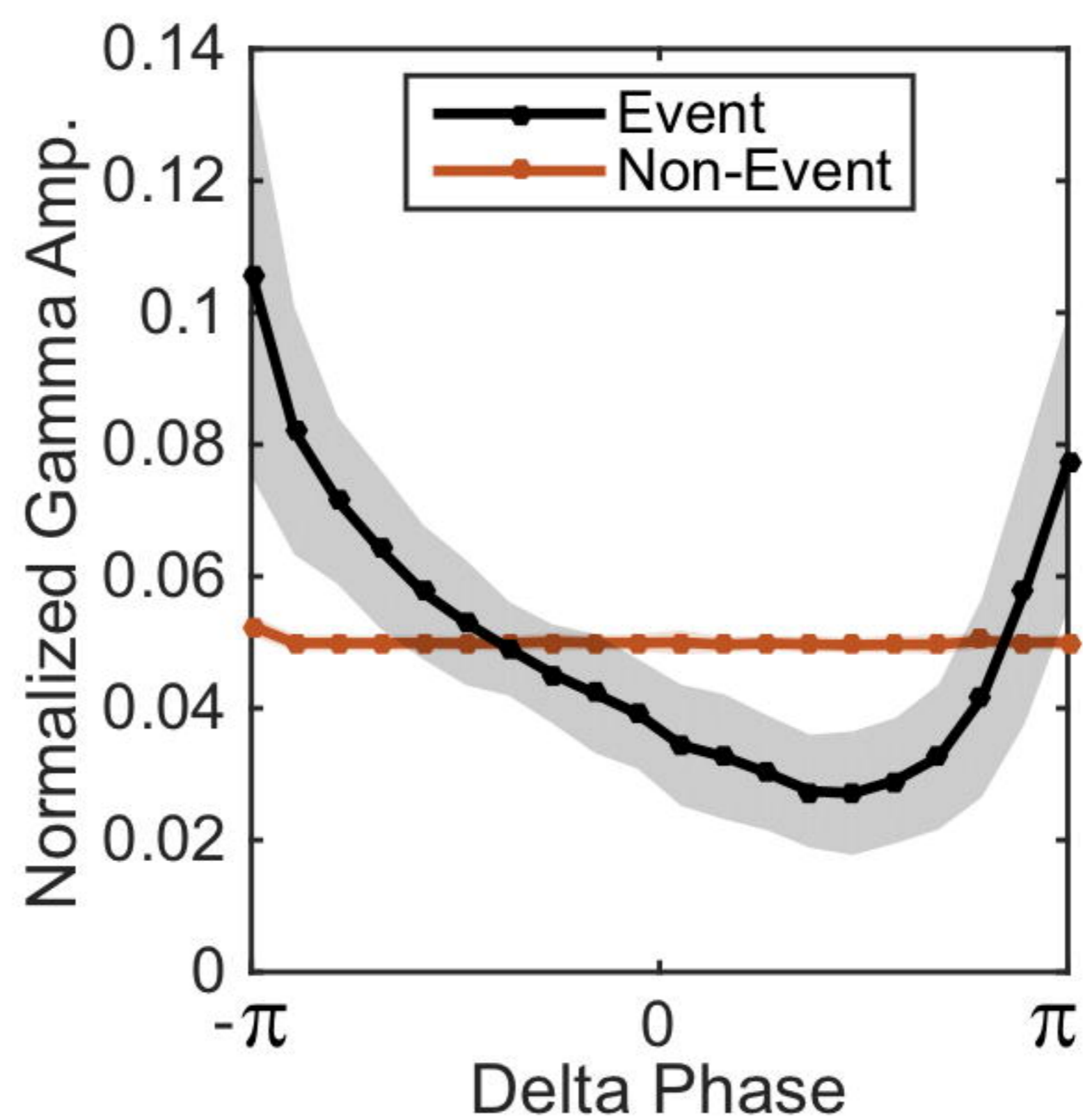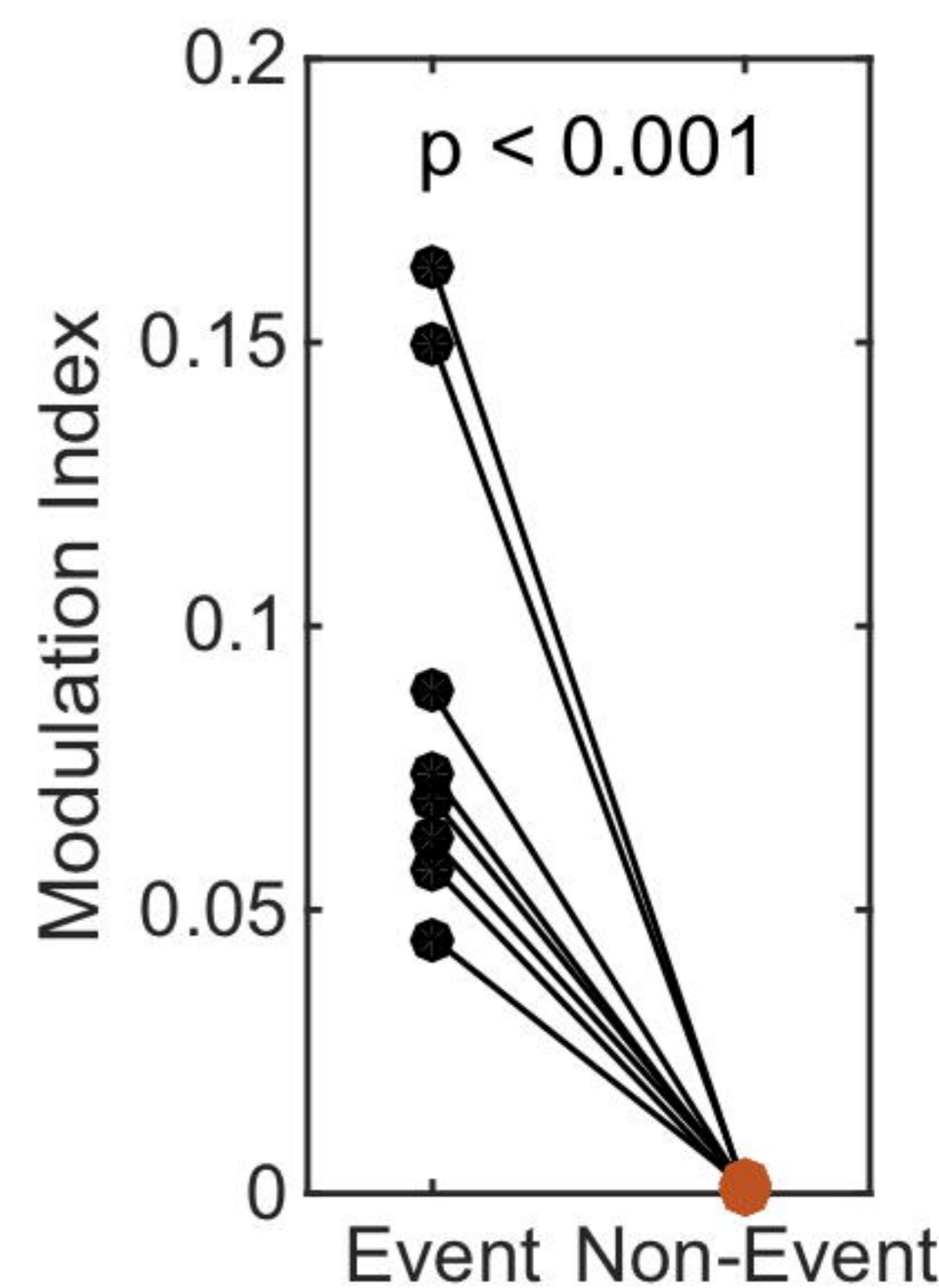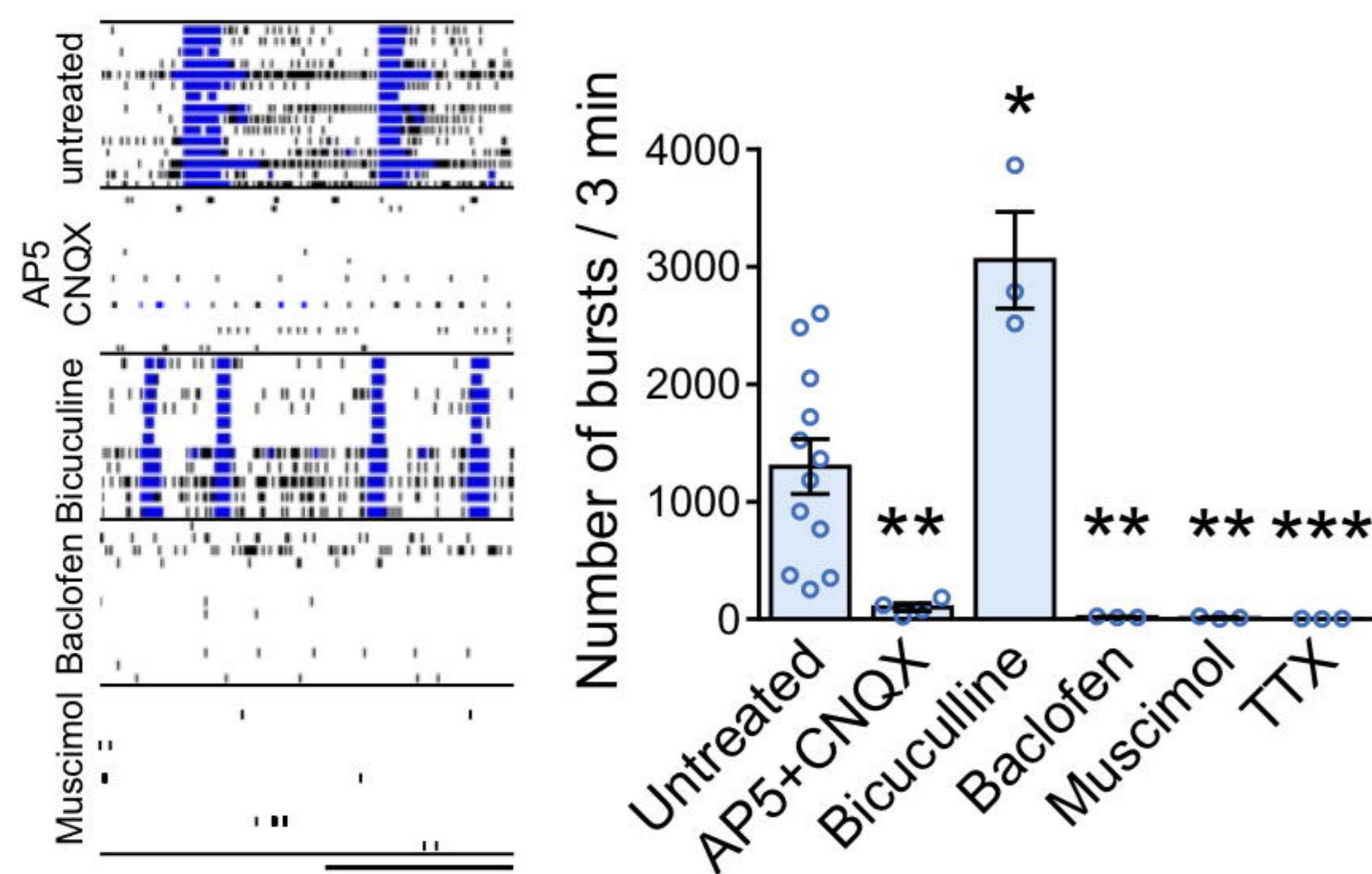1075 **analogous features computed in organoid LFP.**

Neonatal EEG features	Computed organoid LFP features
Envelope (50%)	None
Envelope (5%)	None
Envelope (95%)	None
rEEG (50%)	None
rEEG (5%)	None
rEEG (95%)	None
SATs per hour	Network Events per hour
RMS SAT duration	RMS network event duration
SAT duration (50%)	Network event duration (50%)
SAT duration (5%)	Network event duration (5%)
SAT duration (95%)	Network event duration (95%)
RMS Inter-SAT Duration	RMS Inter-event Duration
Inter-SAT duration (50%)	Inter-event duration (50%)
Inter-SAT duration (5%)	Inter-event duration (5%)
Inter-SAT duration (95%)	Inter-event duration (95%)
Temporal Theta Power	None
Activation Synchrony Index	None
Interhemispheric Correlation	None
Total Spectral Power	None
Relative Delta Power	Relative Delta Power
Relative Theta Power	Relative Theta Power
Relative Alpha Power	Relative Alpha Power
Relative Beta Power	Relative Beta Power

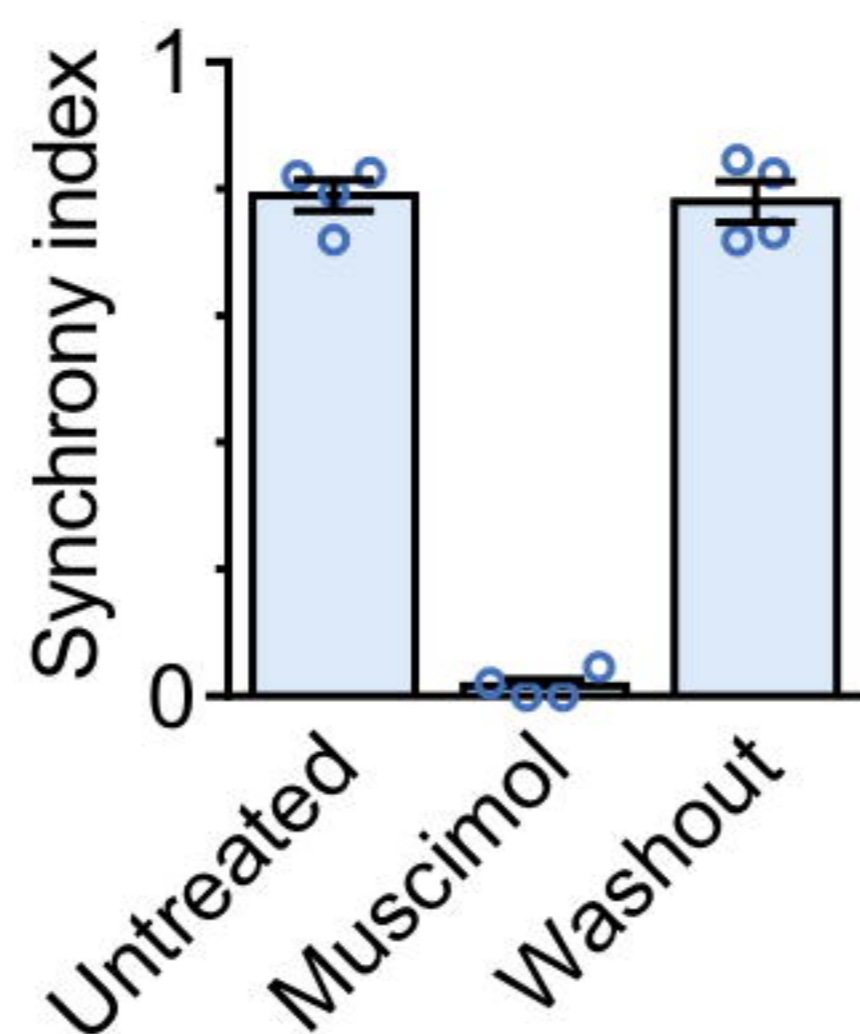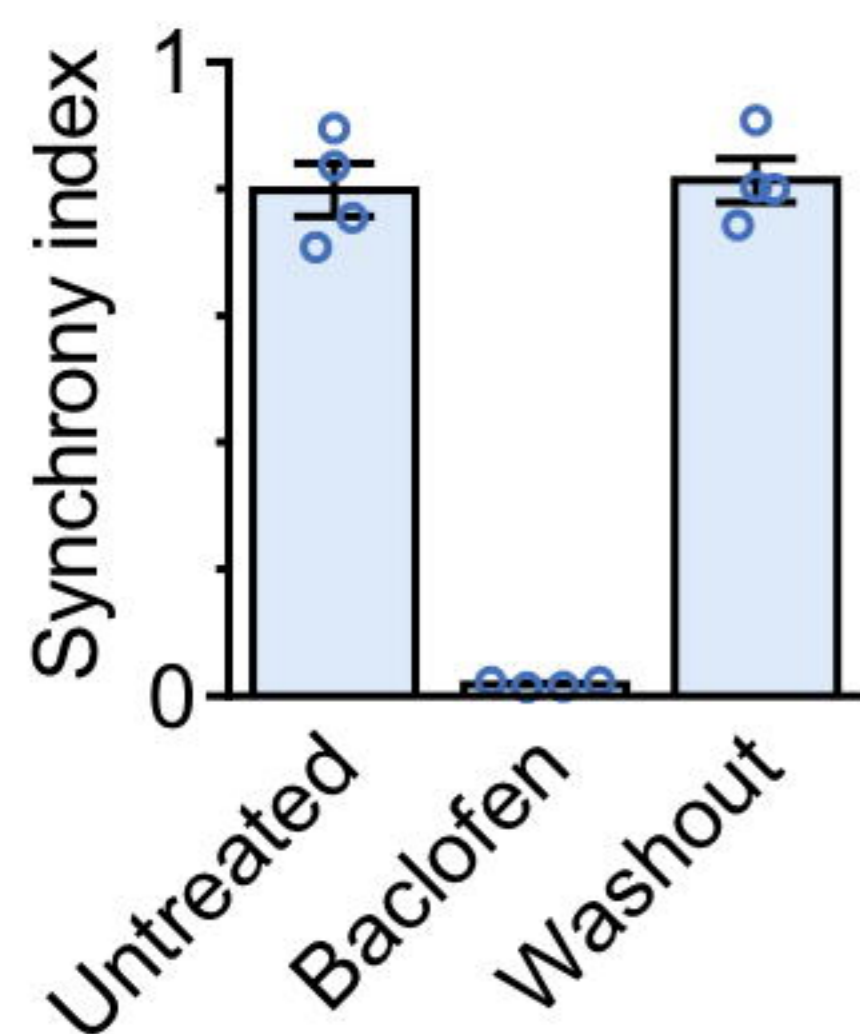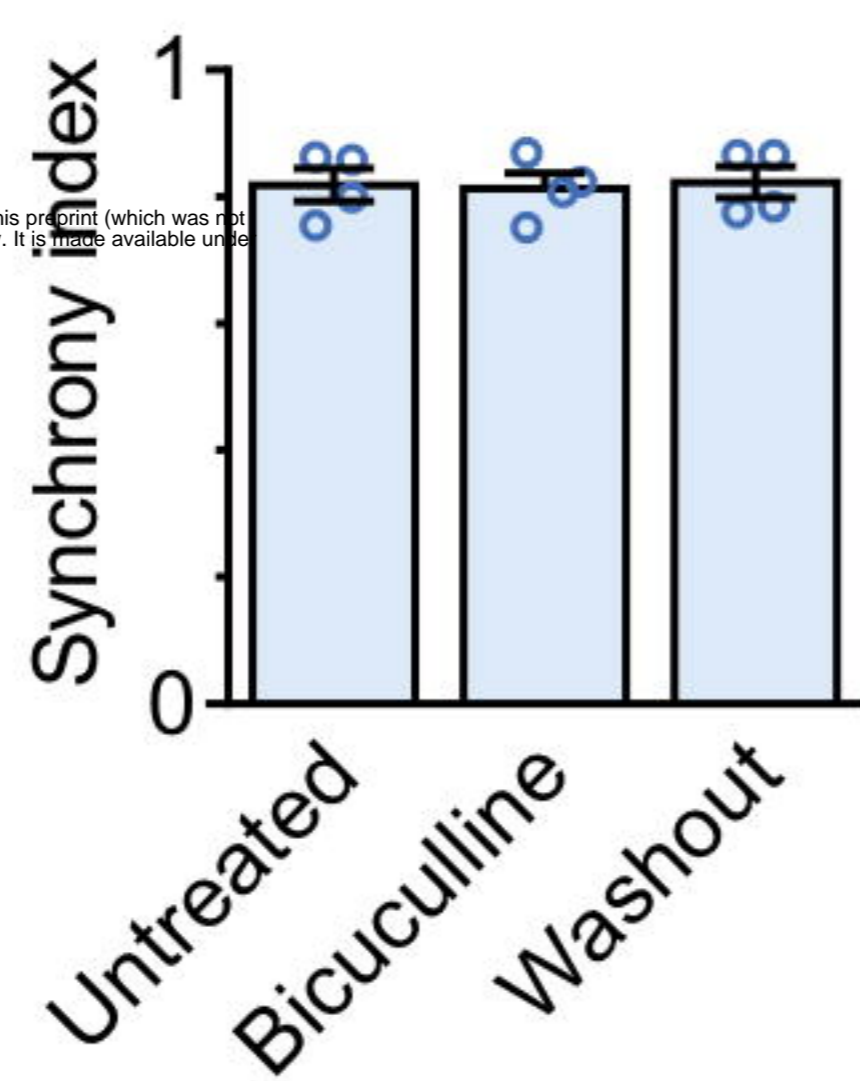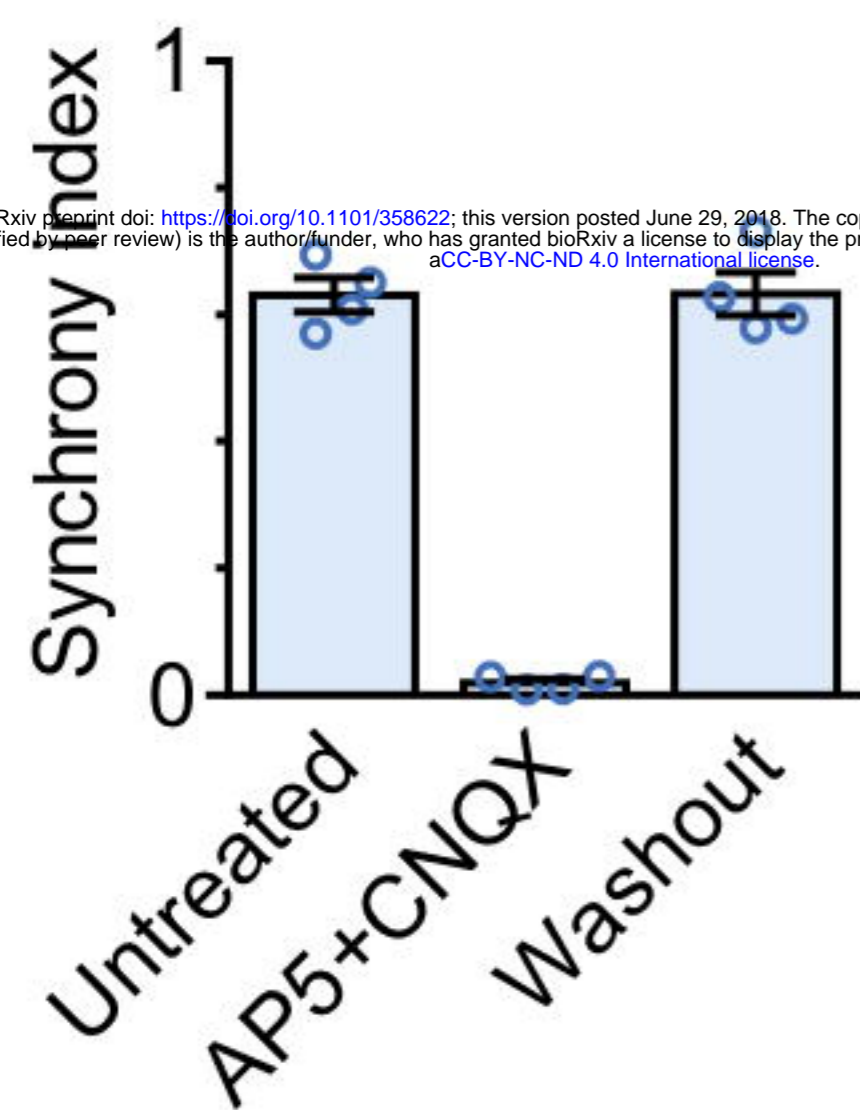1076 Shaded cells indicate features used in the age-prediction model.

1077

A Neural network dynamics

modeling
network
formation

cortical organoid

MEA

B

Neural induction	NPC proliferation	Neural differentiation	Neural maturation
7 days	14 days	7 days	months

C

Organoids diameter (µm)

Induction Proliferation Maturation

D

1 month	2 months	4 months	6 months	8 months
DAPI/NeuN/Ki67	DAPI/Nestin/β-Cat./MAP2	DAPI/TBR1/CTIP2/SOX2	DAPI/GFAP/CTIP2/MAP2	DAPI/TBR1/CTIP2/SOX2

F 4 months

Syn1::eGFP   DAPI/SYN1/MAP2

E

% cells
NeuN Ki67
Months 1 2 4 6 8

% cells
MAP2 GFAP
Months 1 2 4 6 8

% Neurons (MAP2+ cells)
CTIP2 SATB2
Months 1 2 4 6 8

% Neurons (MAP2+ cells)
VGLUT1 GAD67
Months 1 2 4 6 8

G

Synaptic structure   Synaptic vesicles

H

*t*SNE2

lower layer cortical neurons
upper layer cortical neurons
glia
cortical neurons
progenitors

*t*SNE1

I

NEUROD2   NES   FOXG1   SOX2

SLC1A3   GRIA2   BCL11B   SATB2

J

cortical neurons   glia   progenitors

CUX2
TBR1
NEUROD2
RBFOX3
BCL11B
CNTNAP2
SNAP25
GRIA2
SYP
SATB2
GRIN2B
FOXP1
SLC1A3
PAX6
ID4
SOX2
NES
ANXA2
CRABP2
VIM

percentage in the population

-1 0 1 2

K

# of transcripts per cell

SLC1A3   GRIA2   BCL11B   SATB2   CRABP1   NEUROD2   NES   PAX6   SOX2   FOXG1

cortical neurons
progenitors
glia cells
lower cortex
upper cortex
other
neural crest

A

cortical organoid

MEA

amplifier

Highpass Filter
(300-3000 Hz)

Spike Extraction

Lowpass Filter
(<1000 Hz)

Spike Raster

Channel

Population Spiking

Spikes

Local Field Potential (LFP)

Voltage

Time (s)

B

cortical organoid

6 week-old

8 month-old

Time (s)

C

Mean firing rate (Hz)

Firing rate correlation

organoid   2D

***

organoid
2D

Time (weeks)

D

Synchrony index

Time (months)

Burst frequency (Hz)

Time (months)

E

2 Months   4 Months   6 Months   8 Months

Pop. Spike (Hz)

Voltage (uV)

Time (s)

F

Number of Subpeaks

R² = 0.185
p < 10⁻⁷

Time (weeks)

G

Inter-event Interval CV

R² = 0.437
p < 10⁻⁷

Time (weeks)

H

Oscillatory Power

Time (weeks)

A

Organoid LFP

Inter-event Interval

Preterm Neonatal EEG

Inter-SAT Interval   SAT Duration

Adult EEG

Time (s)

B

training

neonate EEG

25-38 PCW

prediction

6-40 weeks

cortical organoids MEA

preterm EEG features

organoid LFP features

regularized regression model

predicted organoid "age"

C

Predicted organoid age from neonate data

* * * * * * * * *

— Mean Prediction
— Perfect Prediction

Predicted age

Actual age (weeks)

D

Resampled Correlation

$T_{interSAT}$ (95%)
$T_{interSAT}$ (5%)
$T_{interSAT}$ (50%)
$T_{interSAT}$ RMS
T_{SAT} (95%)
T_{SAT} (5%)
T_{SAT} (50%)
T_{SAT} RMS
SATs/hour

● Organoid
● Neonate

Pearson ρ

E

● Organoid
● Neonate

SATs per hour

Actual age (weeks)

RMS SAT duration

Actual age (weeks)

SAT duration (50%)

Actual age (weeks)