Leptomeningeal Neural Organoid (LMNO) Fusions as Models to Study Meninges-Brain Signaling

Vivian Gama (✉ vivian.gama@vanderbilt.edu)
  Vanderbilt University   https://orcid.org/0000-0002-1201-1394

Hannah Jones

Gabriella Robertson

Rebecca O'Rourke
  University of Colorado Anschutz Medical Campus   https://orcid.org/0000-0003-1198-6963

Alejandra Romero-Morales

Julie Siegenthaler

Article

Keywords:

Additional Declarations: There is **NO** Competing Interest.

Leptomeningeal Neural Organoid (LMNO) Fusions as Models to Study Meninges-Brain Signaling

Hannah E Jones[1,2*], Gabriella L Robertson[3*], Alejandra Romero-Morales[3], Rebecca O'Rourke[1], Julie A Siegenthaler[1,2#], Vivian Gama[3#]

[1]University of Colorado Anschutz Medical Campus, Department of Pediatrics, Section of Developmental Biology, Aurora, CO 80045 USA, [2]University of Colorado Anschutz Medical Campus, Cell Biology, Stem Cells and Development Graduate Program, Aurora, CO 80045 USA, [3]Vanderbilt University School of Medicine, Department of Cell and Developmental Biology, Nashville, TN 37232 USA

*These authors contributed equally to this work

#Corresponding Authors

Contact Information:

Vivian Gama, PhD
Associate Professor
Vanderbilt University
Department of Cell and Developmental Biology
Nashville, TN 37232 USA
e-mail: vivian.gama@vanderbilt.edu

Julie A Siegenthaler`
Associate Professor
Department of Pediatrics
Section of Developmental Biology
Aurora, CO 80045
e-mail: julie.siegenthaler@cuanschutz.edu

Abstract

Neural organoids derived from human induced pluripotent stem cells (iPSCs) provide a model to study the earliest stages of human brain development, including neurogenesis, neural differentiation, and synaptogenesis. However, neural organoids lack supportive tissues and some non-neural cell types that are key regulators of brain development. Neural organoids have instead been co-cultured with non-neural structures and cell types to promote their maturation and model interactions with neuronal cells. One structure that does not form *de novo* with neural organoids is the meninges, a tri-layered structure that surrounds the CNS and secretes key signaling molecules required for mammalian brain development. Most studies of meninges-brain signaling have been performed in mice or using two-dimensional (2D) cultures of human cells, the latter not recapitulating the architecture and cellular diversity of the tissue. To overcome this, we developed a co-culture system of neural organoids generated from human iPSCs fused with fetal leptomeninges from mice with fluorescently labeled meninges (*Col1a1-GFP*). These proof-of-concept studies test the stability of the different cell types in the leptomeninges (fibroblast and macrophage) and the fused brain organoid (progenitor and neuron), as well as the interface between the organoid and meningeal tissue. We test the longevity of the fusion pieces after 30 days and 60 days in culture, describe best practices for preparing the meninges sample prior to fusion, and examine the feasibility of single or multiple meninges pieces fused to a single organoid. We discuss potential uses of the current version of the LMNO fusion model and opportunities to improve the system.

Introduction

The development of the human central nervous system (CNS) is a complex process requiring the precise coordination of signaling molecules and various cell types. Our understanding of human CNS development is mostly derived from rodent and non-human primate models, along with investigation of post-mortem human brain tissue. While informative, studies using these approaches do not adequately mimic the *in vivo* processes of human brain development. Thus, neural organoids derived from human pluripotent stem cells, both embryonic stem cells (ESCs) and iPSCs, have become useful tools for understanding various stages of human brain development. Neural organoids can be used to model aspects of human CNS development, such as neurogenesis and synaptogenesis, and produce a heterogenous mixture of cell types[1–3]. Neural organoids can form distinct layers reminiscent of cortical layers found *in vivo* and bear an organizational and transcriptomic resemblance to the gestational human cortex[3–5]. Additionally, small molecules and other morphogens can be used to pattern the organoids into specific cell fates or encourage the patterning into different developmental regions, such as telencephalic or cerebellar-like neural organoids[2,6,7].Thus, neural organoids have become central tools for studying early human CNS developmental processes and for translational applications.

One major drawback of current human neural organoid platforms is their lack of support structures that are required for proper CNS development and function *in vivo*[2,8,9]. These are structures or cell types that are not derived from the neuroectoderm that give rise to the CNS and include vasculature, microglia, and meninges. Some strides have been made to incorporate microglia and vasculature into neural organoids[9,10] While these systems have improved our understanding of interactions between neural and non-neuronal cell types within neural organoid models of human CNS development, these platforms still lack many of the characteristics and spatial organization of human brain morphology. A structure that is key to proper formation and maintenance of the CNS *in vivo* is the meninges, a tri-layer structure composed of fibroblasts, blood vessels, and immune cells that encases the entire CNS. It has been well established that

88   the meninges play an essential role in brain development by secretion of important factors:

89   meningeal retinoic acid is required for cortical and cerebellar development in mice and humans[11],

90   meningeal CXCL12 directs Cajal-Retzius cell migration[12], and the pia layer of the meninges forms

91   the basement membrane interface which is required for radial glial cell attachment[13]. Despite this,

92   few attempts have been made to incorporate meningeal tissue into neural organoid models and

93   much of the work to understand interactions between the meninges and neuronal cell types during

94   development have been conducted in rodent models or meningeal cell lines.

95   Overall, there is a need for human neural organoid systems to better recapitulate the

96   structure and organization of the human brain during development, and we propose the meninges

97   as a key component to achieve this complexity. Here, we describe a three-dimensional (3D) co-

98   culture system created from iPSC-derived human neural organoids fused with embryonic

99   leptomeninges from *Col1a1-GFP* mice, which we named Leptomeningeal Neural Organoid

100   (LMNO) fusions. We show that the meningeal compartment of LMNOs retains key characteristics

101   of leptomeninges *in vivo*, such as layer-specific fibroblast markers and resident immune cells, up

102   to 60 days in culture. We also find that neurons in the LMNO fusions undergo less cell death

103   compared to neurons in non-fused organoids. Finally, we find evidence of iPSC-derived human

104   brain organoid Cajal-Retzius-like neuronal cells migrating into the meningeal compartment of

105   LMNOs, potentially responding to Cxcl12 ligand derived from meningeal cells. This proof-of-

106   concept study provides an important initial framework for incorporation of meninges into brain

107   organoid platforms.

108

109   **RESULTS**

110   **Cellular composition of neural organoids and leptomeningeal tissue.**

111   Prior to fusing, day 15 human neural organoids have begun to develop ventricular-like

112   zones, which recapitulate the developing cytoarchitecture of the human cortex (**Fig. 1A**). The cells

113   surrounding these ventricular-like zones express the expected markers of neural progenitors such

114   as PAX6 and SOX2 (**Fig. 1A**). By day 15, MAP2^{+} neurons have started to differentiate and migrate

115   away from the ventricular zone (**Fig. 1A**). This organization in neural organoids is intrinsic and

116   develops prior to our fusion; however, minimal neurogenesis has occurred at this timepoint.

117   The leptomeninges is comprised of the inner two layers of the meninges, the pia and

118   arachnoid. It contains the pial and arachnoid fibroblasts that produce ECM, retinoic acid, CXCL12,

119   and BMPs vital for brain development. The mouse leptomeninges can be isolated as a separate

120   structure from the brain and the outer layer of the meninges, the dura. We isolated meninges from

121   the *Collagen1a1-GFP (Col1a1-GFP)* reporter mouse line in which all fibroblasts are labeled with

122   GFP (**Fig. 1B, schematic**); using previously described whole-mounting techniques[14] combined

123   with immunofluorescent labeling we can visualize *Col1a1-GFP+* fibroblasts and the associated

124   meningeal vasculature (**Fig. 1B**). Single-cell profiling studies of the meninges reveal unique layer-

125   specific fibroblast subtypes and tissue-resident macrophages called border associated

126   macrophages (BAMs). These cell populations can be visualized in the dissected leptomeninges

127   using specific markers: pia-layer fibroblasts labeled by S100a6, arachnoid fibroblasts labeled with

128   RALDH2 and PDPN, pseudo-epithelial arachnoid barrier cells labeled by ECAD and CRABP2,

129   and BAMs labeled by CD206 and LYVE1 (**Fig. 1C**).

130   We previously showed that some meningeal fibroblast layer specific markers (e.g.,

131   S100a6, CRABP2) are shared between fetal mouse and human meninges[15]. To further test this,

132   we used published single nuclear RNA sequencing data sets from early embryonic (human

133   gestational week 6-10) and later fetal (gestational week 16-25) human brain development to

134   identify cell clusters that are representative of putative meningeal mesenchymal/fibroblast,

135   meningeal macrophage, and vascular cells (**Fig. 1D, E**). At early embryonic stages, three

136   mesenchymal cell clusters were identified with one of the clusters (mes 3) showing enriched gene

137   expression of previously identified mouse fetal pia (*NGFR, LAMA1, CYP1B1, LUM*) and arachnoid

138   cells (*CLDN11, ALCAM*)[15] (**Fig. 1D**). The analysis of clusters at later stages of fetal human brain

139   development identified a meningeal fibroblast enriched cluster, likely representing a mixture of

140 arachnoid and pial cells (**Fig. 1E**) as first identified in mouse fetal meninges. At both stages, we

141 identified endothelial cell clusters with CNS specific gene expression profiles, (low *PLVAP*, high

142 *CLDN5, SOX17*) and macrophage clusters with *MRC1* and *LYVE1* (**Fig. 1D, E**), consistent with

143 studies in mouse on vascular and macrophage development. This indicates that mouse

144 leptomeninges contain meningeal fibroblasts, macrophages, and vasculature populations that are

145 transcriptionally like human embryonic and fetal meninges. Further, these data provide supportive

146 evidence that mouse leptomeninges are a reasonable proxy for human-derived tissue.

147

148 **Generation of LMNO fusions.**

149 Neural organoid platforms mimicking human development have yet to incorporate the meninges,

150 a structure essential for proper brain development. To develop LMNO fusions, neural organoids

151 were first generated from iPSCs as previously described[3,16] and grown up to day 15 in culture,

152 resembling approximately 6-9 gestational weeks in human (**Fig. 2A**). Then, we isolated

153 leptomeninges from embryonic day (E)16 *Col1a1-GFP+* mice, in which meningeal fibroblasts

154 express GFP under the *Col1a1* promoter[17]. Prior to isolation of the meninges, embryos were

155 perfused with ion-free HBSS on ice to remove blood contents (**Fig. 2A**). Then, brains were

156 removed and the leptomeninges were dissected from each hemisphere of the forebrain (**Fig. 2A**),

157 as previously described[14]. Isolated meninges were collected in sterile, ion-free HBSS on ice until

158 ready to be transferred into culture with neural organoids (**Fig. 2A**). We transferred one neural

159 organoid along with 2-4 pieces of *Col1a1-GFP+* forebrain leptomeninges into one well of a static

160 culture system consisting of a round-bottom Eppendorf tube secured with hot glue to the bottom

161 of each well of a 6-well plate (**Fig. 2A**). Each well was filled with approximately 300-500uL of

162 organoid differentiation media and given complete media changes every other day (**Fig. 2A**). We

163 monitored neural organoid-meninges fusion progression by capturing bright-field images every

164 other day (**Fig. 2A-B**). At day 3 in culture, the organoid and meninges components remain mostly

165 separate, but by day 6 we observe fusion between the neural organoid and meninges, thus

6

166   generating LMNOs (**Fig. 2B**). LMNOs remained fused throughout the course of the experiment,

167   including after collection for staining at day 30 or 60 (**Fig. 2A-B**).

168

169   **Characterization of specialized meningeal cell types within LMNO fusions.**

170   The meninges are a complex tissue consisting of a diverse array of cell types including

171   specialized fibroblasts and tissue resident immune cells. Thus, we next sought to understand the

172   cellular composition of the meningeal tissue compartment of the LMNO fusions.

173   The meninges contain tissue-resident yolk-sac derived macrophages called border

174   associated macrophages (BAMs) that play an important role in immunological surveillance and

175   response to infection and injury[18,19]. BAMs appear in the meninges embryonically persisting

176   through adulthood and are identified by the markers CD206 (also known as mannose receptor C-

177   type 1 (MRC1)) and lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1). We conducted

178   fluorescent immunolabeling for CD206 and LYVE1 on sections from LMNO fusions at day 30 and

179   day 60 and found an abundance of CD206 and LYVE1 labeling of BAMs in the meningeal

180   compartment of the LMNOs at both timepoints (**Fig. 3A**). The CD206 and LYVE1 labeling

181   remained within the meningeal compartment and no labeling was observed in the organoid

182   compartment, suggesting that any BAMs that persist in the LMNO fusions stay confined in the

183   meninges. At day 60, we observed that much of the *Col1a1-GFP+* signal colocalized with CD206

184   and/or LYVE1 (**Fig. 3A**), suggesting that BAMs may be phagocytosing cells within the LMNOs.

185   The meninges also contain specialized fibroblasts called arachnoid barrier (AB) cells that

186   are critical for maintaining the barrier properties of the meninges[20]. AB cells are specified and

187   form elaborate cell-cell adhesions utilizing the molecules E-cadherin (ECAD) and claudin 11

188   (CLDN11) during embryonic development[20]. We conducted fluorescent immunolabeling for AB

189   cell junctional proteins ECAD and CLDN11 on sections from LMNO fusions at day 30 and day 60

190   and saw labeling for these markers within the meningeal compartment at both timepoints (**Fig.**

191   **3B-C**). At day 30, we observed 'ribbons' of co-localized ECAD/CLDN11 labeling reminiscent of

192 the AB layer *in vivo* (**Fig. 3B**, **Fig. 3C inset**). By day 60, these structures were mostly absent and

193 most of the ECAD/CLDN11 labeling was seen in ameboid-like structures (**Fig. 3B-C**); given the

194 abundance of BAMs we suspect that any AB structures that remain in the meningeal compartment

195 of the LMNO fusions are phagocytosed by this timepoint. Taken together, this data shows that

196 specialized meningeal cell types (BAMs and AB cells) are able to persist in LMNO fusion cultures

197 at day 30 and 60 and retain functional and structural characteristics.

198

199 **Neural organoids in the LMNO fusion model display reduced apoptosis.**

200 LMNO fusions retain characteristic meningeal cell types (*Col1a1-GFP+* fibroblasts, AB

201 cells, and BAMs) 30-60 days into culture, though we observe a significant amount of cell

202 fragmentation and what appears to be phagocytosed cellular components by resident BAMs by

203 day 60. Thus, we next quantified the relative frequency of cell death as a readout for overall health

204 of the culture system. To do so, we conducted immunofluorescent labeling for the apoptosis

205 pathway component activated Caspase-3, along with CRABP2, which labels both meningeal

206 fibroblasts and neurons, in LMNO fusions at D30 and D60 (**Fig. 4A, LMNO**). We also conducted

207 the same staining in neural organoids not fused to meninges but kept in the same culture

208 conditions as the LMNO fusions (**Fig. 4A, org. only**). We then quantified cell death by measuring

209 the percentage of the area labeled with Caspase-3 of each LMNO compartment (organoid,

210 meninges) (**Fig. 4B**). At D30, we find that the organoid compartment of LMNOs contain

211 significantly less Caspase-3 labeling compared to the meninges compartment, and the organoid

212 compartment had significantly less Caspase-3 labeling compared to organoids lacking meninges

213 (**Fig. 4B**). This effect was only observed at D30; at D60 we find that the amount of Caspase-3

214 labeling is not significantly different between the organoid compartment of LMNO fusions and

215 organoids alone (**Fig. 4B**). From these results, we speculate the meninges component of LMNOs

216 are protective against cell death within the organoid compartment, potentially via secretion of pro-

217 survival signals.

218 **Human REELIN+ neurons migrate into meningeal tissue in LMNO fusion model.**

219   Given the evidence for neuronal cell migration in other iPSC-derived organoid models[21],

220 we examined whether any human cells migrated into the meningeal compartment of the LMNO

221 fusion using a marker specific for human mitochondria. Human mitochondria were observed in

222 the mouse meningeal tissue in some instances (**Fig. 5A-B**). These migratory human cells express

223 REELIN (**Fig. 5C**), a marker of Cajal-Retzius-like cells known to be present in iPSC-derived neural

224 organoids and to migrate within the developing human brain. These data suggest that there is

225 integration of the two tissues and that human neural cells migrate into the meningeal tissue.

226 Conversely, PAX6+ neural progenitors remain in the neural organoid around ventricular-like

227 zones (**Fig. 5A**).

228   It is well established that meningeal-derived factors are essential for proper cortical

229 development. One essential factor, CXCL12, is secreted by the meningeal fibroblasts

230 embryonically and is required for Cajal-Retzius cell migration *in vivo*[12] . Given that we observe

231 organoid-derived REELIN+ Cajal-Retzius-like cells within the meningeal compartment of LMNOs,

232 we next sought to determine if the LMNO meningeal compartment acts as a source for CXCL12

233 ligand. To do so, we conducted RNAscope *in situ* hybridization for *Cxcl12* mRNA on LMNO

234 fusions at D30 and D60. At both timepoints, we observe abundant *Cxcl12* signal within the

235 meningeal compartment of LMNOs, and signal is notably lower in the organoid compartment (**Fig.**

236 **5C**). This may suggest a CXCL12-dependent mechanism is driving Reelin+ cell migration into the

237 meningeal compartment of LMNO fusions.

238

239 **Discussion**

240   We present a method for co-culturing human iPSC-derived neural organoids with embryonic

241 mouse meninges, referred to as LMNO fusions. In this system, embryonic mouse leptomeninges

242 placed in culture with neural organoids undergo fusion in six days and can remain in culture

243 through 60 days. We find that the leptomeningeal compartment of LMNO fusions retain key

244 characteristics of the meninges *in vivo*, with tissue-resident macrophages and specialized

245 arachnoid barrier cells persisting in culture through 30 days. Furthermore, the cell types known to

246 be present in neural organoids were present after fusion, suggesting that fusion did not disrupt

247 neural organoid development. We also find that the leptomeninges are potentially neuroprotective

248 in the LMNO organoid compartment within the first 30 days in culture. Finally, we show that the

249 LMNO organoid compartment recapitulates characteristics of cortical development *in vivo*,

250 including producing migratory REELIN+ Cajal-Retzius-like cells that potentially respond to LMNO

251 meninges compartment-derived CXCL12.

252   Our method provides a novel framework for the co-culturing of neural organoids with

253 leptomeningeal tissue. In the LMNO system, whole meninges are placed into culture with neural

254 organoids and undergo spontaneous fusion. Although the meninges in LMNOs do retain key cell

255 types (**Fig. 3**), they do not retain their overall organization or form key structures at the interface

256 between the meningeal and organoid components. *In vivo*, the meninges consist of three layers—

257 the pia, arachnoid, and dura—with the pia layer residing closest to the brain surface, forming the

258 glia limitans and acting as a crucial attachment point for radial glial cells[13]. Future meninges-

259 neural organoid platforms should aim to retain this organization, via scaffolding and/or methods

260 to self-organize the meninges into the proper layer order around organoids. A dissociated

261 meningeal cell line has been used to coat neural organoids with meningeal fibroblasts[22]; future

262 systems could employ similar methods with primary embryonic meningeal cells to maintain the

263 cellular diversity.

264   Human leptomeningeal tissue has yet to be derived from iPSCs *in vitro*. However, the LMNO

265 fusion model allows us to study the interaction of human neural organoids and mouse

266 leptomeningeal tissue *in vitro*, which may give insight into key signaling pathways that are

267 essential to deriving these tissues. Furthermore, this model provides evidence that meningeal

268 tissue can be cultured *in vitro* and retain the cellular diversity of the tissue. These future, improved

269   systems will not only be important for modeling brain development *in vitro* but will also be useful

270   models for studying meningeal development and brain-meninges cross-talk.

271   Within the meningeal component of LMNO fusions, we observed the survival of meningeal

272   tissue-resident macrophages, BAMs, marked by expression of CD206 and LYVE1. The BAMs

273   appeared to remain confined to the meninges and were not observed in the organoid

274   compartment of LMNO fusions, mirroring the homeostatic localization of BAMs *in vivo.* We also

275   observed GFP+ fluorescence within the CD206+/LYVE1+ cells, suggesting that the macrophages

276   present in the LMNO fusions may actively phagocytose fragments of dead fibroblasts. We also

277   detect cells expressing the junctional proteins E-cadherin and Claudin-11, which are expressed

278   by AB cells *in vivo.* These AB-like structures are seen at day 30 but appear to break down by day

279   60 in culture. It has been established that the AB layer begins establishing cell-cell junctions along

280   with its barrier function embryonically, however it is unknown what signals contribute to

281   maintenance of the AB layer *in vivo.* Potentially, other meningeal fibroblasts present in the LMNO

282   meninges compartment produce pro-survival signals or the BAMs act to reduce cytotoxicity by

283   phagocytosing dead cells.

284   During development, the meninges secrete factors (CXCL12, retinoic acid, BMPs) that are

285   essential for proper cortical development. In the LMNO fusion system, we observed that the

286   meninges component produces CXCL12 ligand, which *in vivo* is necessary for driving Cajal-

287   Retzius cell migration. We also found evidence that the meninges promote cell survival in the

288   organoid of the LMNO fusion through day 30 in culture. Potentially, other meningeal-derived cues

289   promote neuronal survival in LMNO fusions, though these mechanisms do not seem to persist

290   through day 60 in culture given the widespread cell death seen at this timepoint.

291   Developmental apoptosis is an evolutionary conserved pathway critical for

292   neurodevelopment[23], that is actively regulated during the generation of neural organoids[24].

293   However, increased cell death can also arise from long-term culturing conditions. At day 30,

294   LMNO fusion organoids show less caspase activation than neural organoids alone, suggesting

295 that the meningeal tissue secretes factors that could prevent cell death during the static conditions

296 of the LMNO fusion protocol and that the factors generated from mouse tissue affect human cells.

297   The LMNO fusion system may benefit from experimentation with other aspects of the fusion

298 model as well. For example, both the time post-differentiation of the organoid and the

299 developmental stage of the mice can be manipulated to interrogate different questions. Fusing

300 leptomeningeal tissue from an earlier time in development with a more developed neural organoid

301 may be used to examine how signals from the developing brain impact leptomeningeal

302 maturation. Further, fusion starting with an older neural organoid may allow for examination of

303 interactions between human iPSC-derived glia and leptomeningeal tissue. Overall, the LMNO

304 fusion model shows that human neural organoids can functionally integrate into mouse meningeal

305 tissue and that this platform can be used to reveal the intricate meninges-brain signaling networks

306 that underlie early brain development.

307
308 **Methods**

309 **Animals**. All mice were housed in specific-pathogen-free facilities approved by AALAC and were

310 handled in accordance with protocols approved by the IACUC committee on animal research at

311 the University of Colorado, Anschutz Medical Campus. The following mouse line was used in this

312 study: Col1a1-GFP[17] on a C57/BLK6J background. GFP+ embryos were identified by

313 fluorescence detection using a stereoscope equipped with a fluorescent light source.

314

315 **Bioinformatic Analysis.** Published human embryonic and fetal brain single nuclei data sets were

316 generously provided by Dr. Aparna Shah[25] and Dr. Alexandro Trevino[26] as R objects (Dr. Shah)

317 or were available as raw single cell data from the NeMO Repository at[20] and GEO: GSE162170.

318 From individual data sets containing nuclei isolated from Carnegie Stage (CS) 12-22 (correspond

319 to gestational weeks 6-10) cortex/telencephalon, hindbrain, midbrain or thalamus, clusters with

320 expression of putative meningeal mesenchymal cell enriched genes (*COL1A1, COL1A2, LUM,*

321 *S100A6, CRABP2, TBX18, FOXC1, DCN, NNAT, ALCAM, and BGN)*, endothelial genes (*CLDN5*,

322 *PECAM1, SOX17*), mural cell genes (*RGS5, ABCC9, KCNJ8*) or

323 macrophages/monocytes/microglia (*MRC1, CD74, AIF1, P2YR12*) were subset, integrated and

324 re-clustered. This dataset was further subset to remove a cluster of putative neural cells and

325 reclustered at 0.8 resolution leaving clusters of putative mesenchymal (combined meningeal

326 mesenchymal and pericyte markers), endothelial and macrophages/monocytes/microglia. From

327 a data set containing nuclei isolated from GW16-22 human cortex, previously annotated clusters

328 16 ('microglia'), 19 ('pericyte'), 20 ('endothelial cell)', and 22 ('vascular and leptomeningeal cell')

329 were subset and cluster analysis was performed. Analysis of putative meningeal mesenchyme,

330 pericyte/mural cell, monocyte/macrophage/microglia and endothelial markers in the data set

331 showed the presence of clusters with neural identity. This dataset was further subset to remove

332 a cluster of putative neural cells and reclustered at 0.8 resolution leaving clusters of meningeal,

333 pericyte/mural cell, monocyte/macrophage/microglia and endothelial cells.

334

335 **Human iPSCs**. The WTC11 iPSC line used in this study was maintained in E8 medium in plates

336 coated with Matrigel (Corning, cat # 354277) at 37°C with 5% CO2. Culture medium was changed

337 daily. Cells were checked daily for differentiation and were passaged every 3-4 days using Gentle

338 cell dissociation solution (STEMCELL Technologies, cat # 07174). All experiments were

339 performed under the supervision of the Vanderbilt Institutional Human Pluripotent Cell Research

340 Oversight (VIHPCRO) Committee. Cells were checked for contamination periodically.

341

342 **Neural organoids.** Cerebral organoids were generated as previously described[3,4,16] with some

343 modifications. Briefly, organoids were generated using the STEMdiff™ Cerebral Organoid Kit

344 (STEMCELL Technologies; Cat# 08571, 08570), supplemented with dual SMAD inhibitors. iPSCs

345 were dissociated into single cells using Gentle Cell Dissociation Reagent (STEMCELL

346 Technologies, cat # 07174) for 8 minutes at 37°C. Homogeneous and reproducible EBs were

347 generated by using a 24-well plate AggreWell™ 800 (STEMCELL Technologies, cat # 34815).

348 On Day 7, high-quality EBs were embedded in Matrigel (Corning, cat # 354277). On Day 10, the

349 Matrigel coat was broken by pipetting up and down and the healthy organoids were transferred

350 to a 60mm low attachment culture plate (Eppendorf, cat # 003070119). The plates were then

351 moved to a 37°C incubator and to a Celltron benchtop shaker for CO2 incubators (Infors USA,

352 cat # I69222) set at 85rpm. Full media changes were performed every 3–4 days for 15 days.

353

354 **Isolation of Leptomeninges.** Embryos were collected from *Col1a1-GFP* pregnant dams at E16.

355 Leptomeningeal tissue was dissected using previously established methods[14]; briefly, a single

356 lateral cut is made from the back of the head towards the eye, and the skin and calvarium is lifted

357 dorsally exposing the brain surface and GFP+ leptomeninges. Whole pieces of leptomeninges

358 were then peeled off the forebrain (telencephalon) using a dissecting scope and then stored in

359 ion-free HBSS on ice until fusion with organoids.

360

361 **Generation of LMNO fusions.** Leptomeninges from mice at E16 of embryonic development were

362 isolated (described above) and seeded together with neural organoids in a round-bottom tube

363 placed within a modified 6-well plate. The leptomeninges fused to forebrain neural organoids

364 within 6 days and were kept in culture for 30 to 60 days, with complete media changes and

365 imaging using a fluorescent stereoscope every other day.

366

367 **Immunohistochemistry and RNAscope.** At day 30 and 60, leptomeningeal-neural organoid

368 fusions were collected and sectioned prior to immunohistochemistry and detection of mRNA using

369 RNAScope. Briefly, LMNO fusions were fixed in 4% paraformaldehyde for 15 minutes at 4°C,

370 washed in PBS, then incubated in a 20% sucrose gradient at 4°C prior to embedding and flash-

371 freezing in a solution of 7.5% gelatin/10% sucrose. LMNO fusions were sectioned on a cryostat

372 (Leica CM1950) at a thickness of 15µm. For detection of leptomeningeal components and

14

373 assaying for cell death, the following antibodies were used at a dilution of 1:100: CD206 (goat,

374 R&D Systems, AF2535), Lyve1 (rat, R&D Systems, MAB2125), E-cadherin (mouse, BD

375 Biosciences, 610181), Claudin-11 (rabbit, Invitrogen, 36-4500), CRABP2 (mouse, Sigma-Aldrich,

376 MAB5488), Cleaved Caspase-3 (rabbit, Cell Signaling Technology, 9661) . For immunolabeling

377 of Cajal-Retzius like cells the following antibodies were used at a dilution of 1:200: Reelin (mouse,

378 Sigma-Aldrich, MAB5366), and Mitochondria (mouse, Abcam, ab92824). For detection of *Cxcl12*

379 transcript, the RNAScope Manual Detection Kit v.2.0 from ACDBio (Cat. No. 323100) and probe

380 against mouse *Cxcl12* (Cat. No. 422711-C3).

381

382 **Image acquisition**

383 Confocal images were acquired on a Nikon Ti2 inverted light microscope equipped with a

384 Yokogawa CSU-X1 spinning disk head, with 488 nm, 561 nm, and 647 nm excitation LASERs,

385 and Photometrics Prime 95B sCMOS camera.

386

387 **Acknowledgements**

394

395 The authors declare no competing financial interests.

396

397

References

1. Lancaster, M. A., Renner, M., Martin, C.-A., Wenzel, D., Bicknell, L. S., Hurles, M. E., Homfray, T., Penninger, J. M., Jackson, A. P. & Knoblich, J. A. Cerebral organoids model human brain development and microcephaly. *Nature* **501,** 373–379 (2013).

2. Arlotta, P. & Paşca, S. P. Cell diversity in the human cerebral cortex: from the embryo to brain organoids. *Curr. Opin. Neurobiol.* **56,** 194–198 (2019).

3. Velasco, S., Kedaigle, A. J., Simmons, S. K., Nash, A., Rocha, M., Quadrato, G., Paulsen, B., Nguyen, L., Adiconis, X., Regev, A., Levin, J. Z. & Arlotta, P. Individual brain organoids reproducibly form cell diversity of the human cerebral cortex. *Nature* **570,** 523–527 (2019).

4. Lancaster, M. A., Corsini, N. S., Wolfinger, S., Gustafson, E. H., Phillips, A. W., Burkard, T. R., Otani, T., Livesey, F. J. & Knoblich, J. A. Guided self-organization and cortical plate formation in human brain organoids. *Nat. Biotechnol.* **35,** 659–666 (2017).

5. Camp, J. G., Badsha, F., Florio, M., Kanton, S., Gerber, T., Wilsch-Bräuninger, M., Lewitus, E., Sykes, A., Hevers, W., Lancaster, M., Knoblich, J. A., Lachmann, R., Pääbo, S., Huttner, W. B. & Treutlein, B. Human cerebral organoids recapitulate gene expression programs of fetal neocortex development. *Proc. Natl. Acad. Sci. U. S. A.* **112,** 15672–15677 (2015).

6. Uzquiano, A., Kedaigle, A. J., Pigoni, M., Paulsen, B., Adiconis, X., Kim, K., Faits, T., Nagaraja, S., Antón-Bolaños, N., Gerhardinger, C., Tucewicz, A., Murray, E., Jin, X., Buenrostro, J., Chen, F., Velasco, S., Regev, A., Levin, J. Z. & Arlotta, P. Proper acquisition of cell class identity in organoids allows definition of fate specification programs of the human cerebral cortex. *Cell* **185,** 3770-3788.e27 (2022).

7. Meng, X., Yao, D., Imaizumi, K., Chen, X., Kelley, K. W., Reis, N., Thete, M. V., Arjun McKinney, A., Kulkarni, S., Panagiotakos, G., Bassik, M. C. & Paşca, S. P. Assembloid CRISPR screens reveal impact of disease genes in human neurodevelopment. *Nature* **622,** 359–366 (2023).

424   8. Cerneckis, J., Bu, G. & Shi, Y. Pushing the boundaries of brain organoids to study Alzheimer's

425   disease. *Trends Mol. Med.* **29,** 659–672 (2023).

426   9. Garcia-Epelboim, A. & Christian, K. M. Modeling neuro-immune interactions using human

427   pluripotent stem cells. *Curr. Opin. Neurobiol.* **79,** 102672 (2023).

428   10. Hong, Y., Dong, X., Chang, L., Xie, C., Chang, M., Aguilar, J. S., Lin, J., Lin, J. & Li, Q. Q.

429   Microglia-containing cerebral organoids derived from induced pluripotent stem cells for the

430   study of neurological diseases. *iScience* **26,** 106267 (2023).

431   11. Siegenthaler, J. A., Ashique, A. M., Zarbalis, K., Patterson, K. P., Hecht, J. H., Kane, M. A.,

432   Folias, A. E., Choe, Y., May, S. R., Kume, T., Napoli, J. L., Peterson, A. S. & Pleasure, S. J.

433   Retinoic acid from the meninges regulates cortical neuron generation. *Cell* **139,** 597–609

434   (2009).

435   12. Borrell, V. & Marín, O. Meninges control tangential migration of hem-derived Cajal-Retzius

436   cells via CXCL12/CXCR4 signaling. *Nat. Neurosci.* **9,** 1284–1293 (2006).

437   13. Radakovits, R., Barros, C. S., Belvindrah, R., Patton, B. & Müller, U. Regulation of radial glial

438   survival by signals from the meninges. *J. Neurosci. Off. J. Soc. Neurosci.* **29,** 7694–7705

439   (2009).

440   14. Jones, H. E., Abrams, K. A. & Siegenthaler, J. A. Techniques for visualizing fibroblast-vessel

441   interactions in the developing and adult CNS. *Neurophotonics* **9,** 021911 (2022).

442   15. DeSisto, J., O'Rourke, R., Jones, H. E., Pawlikowski, B., Malek, A. D., Bonney, S., Guimiot,

443   F., Jones, K. L. & Siegenthaler, J. A. Single-Cell Transcriptomic Analyses of the Developing

444   Meninges Reveal Meningeal Fibroblast Diversity and Function. *Dev. Cell* **54,** 43-59.e4 (2020).

445   16. Romero-Morales, A. I., Robertson, G. L., Rastogi, A., Rasmussen, M. L., Temuri, H., McElroy,

446   G. S., Chakrabarty, R. P., Hsu, L., Almonacid, P. M., Millis, B. A., Chandel, N. S., Cartailler,

447   J.-P. & Gama, V. Human iPSC-derived cerebral organoids model features of Leigh syndrome

448   and reveal abnormal corticogenesis. *Dev. Camb. Engl.* **149,** dev199914 (2022).

449   17. Yata, Y., Scanga, A., Gillan, A., Yang, L., Reif, S., Breindl, M., Brenner, D. A. & Rippe, R. A.

450   DNase I-hypersensitive sites enhance alpha1(I) collagen gene expression in hepatic stellate

451   cells. *Hepatol. Baltim. Md* **37,** 267–276 (2003).

452   18. Rebejac, J., Eme-Scolan, E., Arnaud Paroutaud, L., Kharbouche, S., Teleman, M., Spinelli,

453   L., Gallo, E., Roussel-Queval, A., Zarubica, A., Sansoni, A., Bardin, Q., Hoest, P., Michallet,

454   M.-C., Brousse, C., Crozat, K., Manglani, M., Liu, Z., Ginhoux, F., McGavern, D. B., Dalod,

455   M., Malissen, B., Lawrence, T. & Rua, R. Meningeal macrophages protect against viral

456   neuroinfection. *Immunity* **55,** 2103-2117.e10 (2022).

457   19. Riew, T.-R., Hwang, J.-W., Jin, X., Kim, H. L. & Lee, M.-Y. Infiltration of meningeal

458   macrophages into the Virchow-Robin space after ischemic stroke in rats: Correlation with

459   activated PDGFR-β-positive adventitial fibroblasts. *Front. Mol. Neurosci.* **15,** 1033271 (2022).

460   20. Derk, J., Como, C. N., Jones, H. E., Joyce, L. R., Kim, S., Spencer, B. L., Bonney, S.,

461   O'Rourke, R., Pawlikowski, B., Doran, K. S. & Siegenthaler, J. A. Formation and function of

462   the meningeal arachnoid barrier around the developing mouse brain. *Dev. Cell* **58,** 635-

463   644.e4 (2023).

464   21. Miura, Y., Li, M.-Y., Revah, O., Yoon, S.-J., Narazaki, G. & Pașca, S. P. Engineering brain

465   assembloids to interrogate human neural circuits. *Nat. Protoc.* **17,** 15–35 (2022).

466   22. Jalilian, E. & Shin, S. R. Novel model of cortical-meningeal organoid co-culture system

467   improves human cortical brain organoid cytoarchitecture. *Sci. Rep.* **13,** 7809 (2023).

468   23. Ke, F. F. S., Vanyai, H. K., Cowan, A. D., Delbridge, A. R. D., Whitehead, L., Grabow, S.,

469   Czabotar, P. E., Voss, A. K. & Strasser, A. Embryogenesis and Adult Life in the Absence of

470   Intrinsic Apoptosis Effectors BAX, BAK, and BOK. *Cell* **173,** 1217-1230.e17 (2018).

471   24. Joshi, P., Bodnya, C., Rasmussen, M. L., Romero-Morales, A. I., Bright, A. & Gama, V.

472   Modeling the function of BAX and BAK in early human brain development using iPSC-derived

473   systems. *Cell Death Dis.* **11,** (2020).

474   25. Eze, U. C., Bhaduri, A., Haeussler, M., Nowakowski, T. J. & Kriegstein, A. R. Single-cell atlas

475   of early human brain development highlights heterogeneity of human neuroepithelial cells and

476   early radial glia. *Nat. Neurosci.* **24,** 584–594 (2021).

477   26. Trevino, A. E., Müller, F., Andersen, J., Sundaram, L., Kathiria, A., Shcherbina, A., Farh, K.,

478   Chang, H. Y., Pașca, A. M., Kundaje, A., Pașca, S. P. & Greenleaf, W. J. Chromatin and

479   gene-regulatory dynamics of the developing human cerebral cortex at single-cell resolution.

480   *Cell* **184,** 5053-5069.e23 (2021).

481

482

483

484

485

486

487

488

489

490

491

492

493

494

495

496

497

498

499
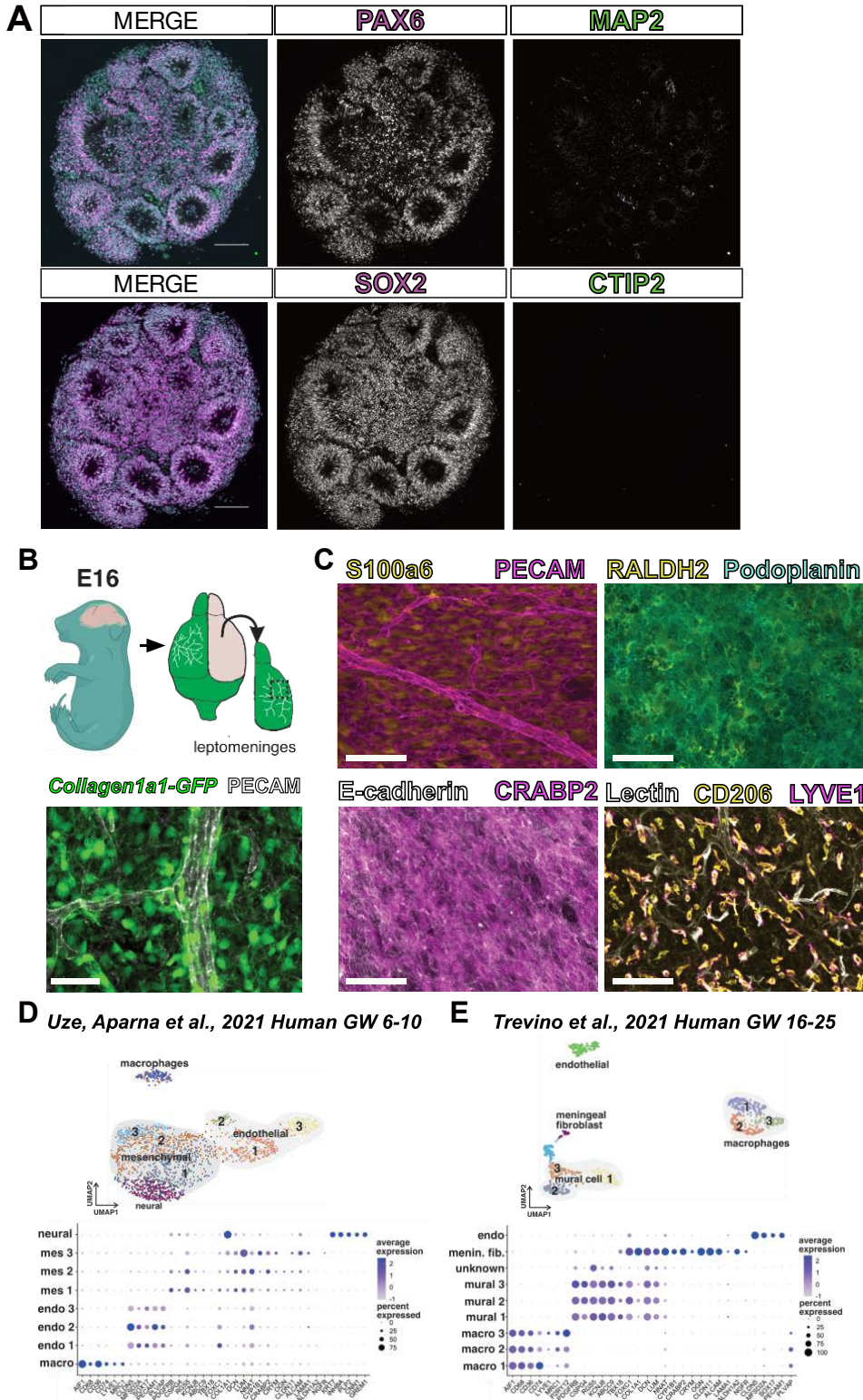
Figure 1: Characterization of neural organoids and mouse fetal meninges prior to fusion.
(**A**) Representative images of day 15 neural organoid labeled with PAX6/MAP2 or SOX2/CTIP2, scale bars 100 µm. (**B**) Schematic and maximum-projection image depicting isolation and whole-mounting of mouse embryonic *Col1a1-GFP+* leptomeninges, image shows GFP+ leptomeningeal fibroblasts (green) and vasculature labeled with PECAM (white). (**C**) Identification of cell types in mouse leptomeningeal whole-mount tissue, images show labeling of S100a6 (fibroblasts)/PECAM (vasculature), RALDH2 (fibroblasts)/Podoplanin (fibroblasts), E-cadherin (arachnoid barrier)/CRABP2 (fibroblasts), and CD206 (macrophages)/LYVE1 (macrophages)/Lectin (vasculature); all scale bars 100µm. (**D-E**) Dot-plots showing conservation of mouse meningeal cell types and markers in human brain data sets at (**D**) GW 6-10 (from Uze, Aparna et. al. 2021) and (**E**) GW 16-25 (from Trevino et. al. 2021).

A

B

	D3	D6	D12	D22	D30
Brightfield	org. men.	fusion			
Col1a1-GFP					

513
514
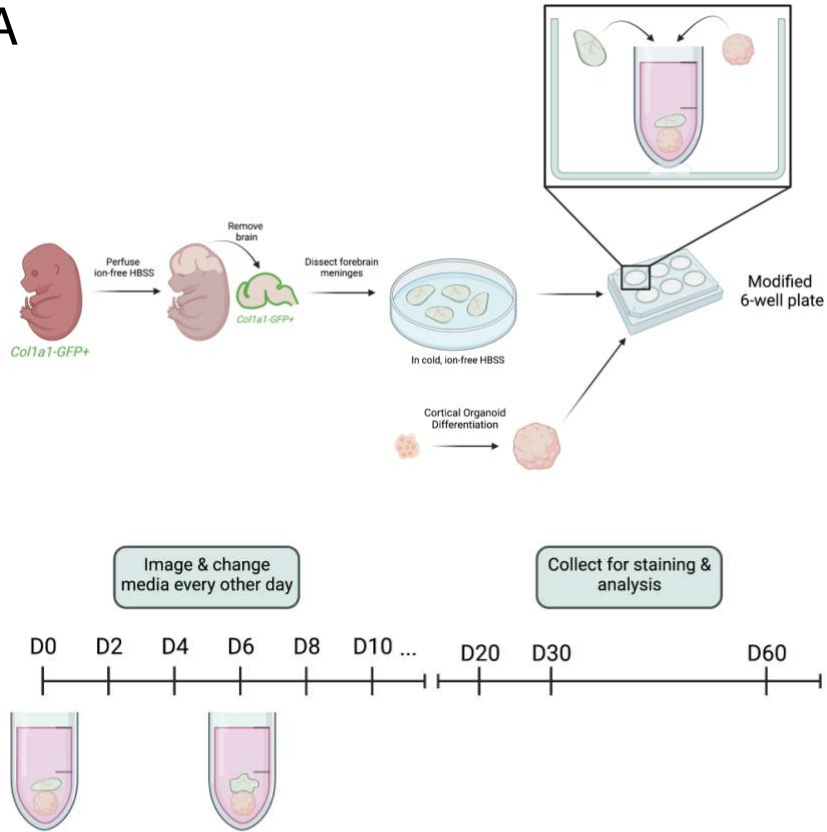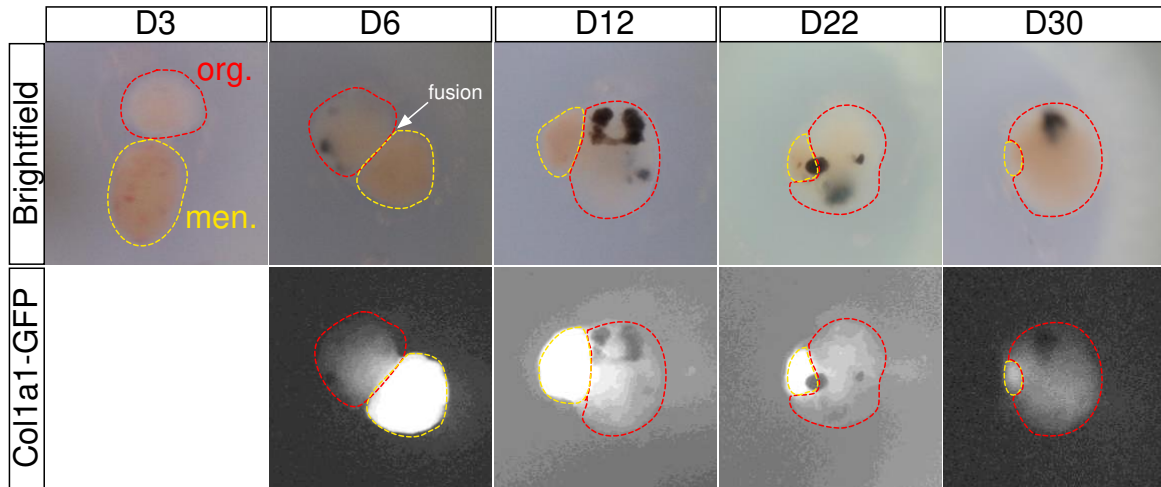
Figure 2: Leptomeningeal-neural organoid (LMNO) fusion culture establishment and growth.
(**A**) Graphical depiction of leptomeningeal dissection and establishment of LMNO fusions cultures. (**B**) Representative stereoscope images of LMNO fusions throughout time in culture. Red-dashed lines highlight organoid component, yellow-dashed lines and *Col1a1-GFP* fluorescence distinguishes meningeal component.
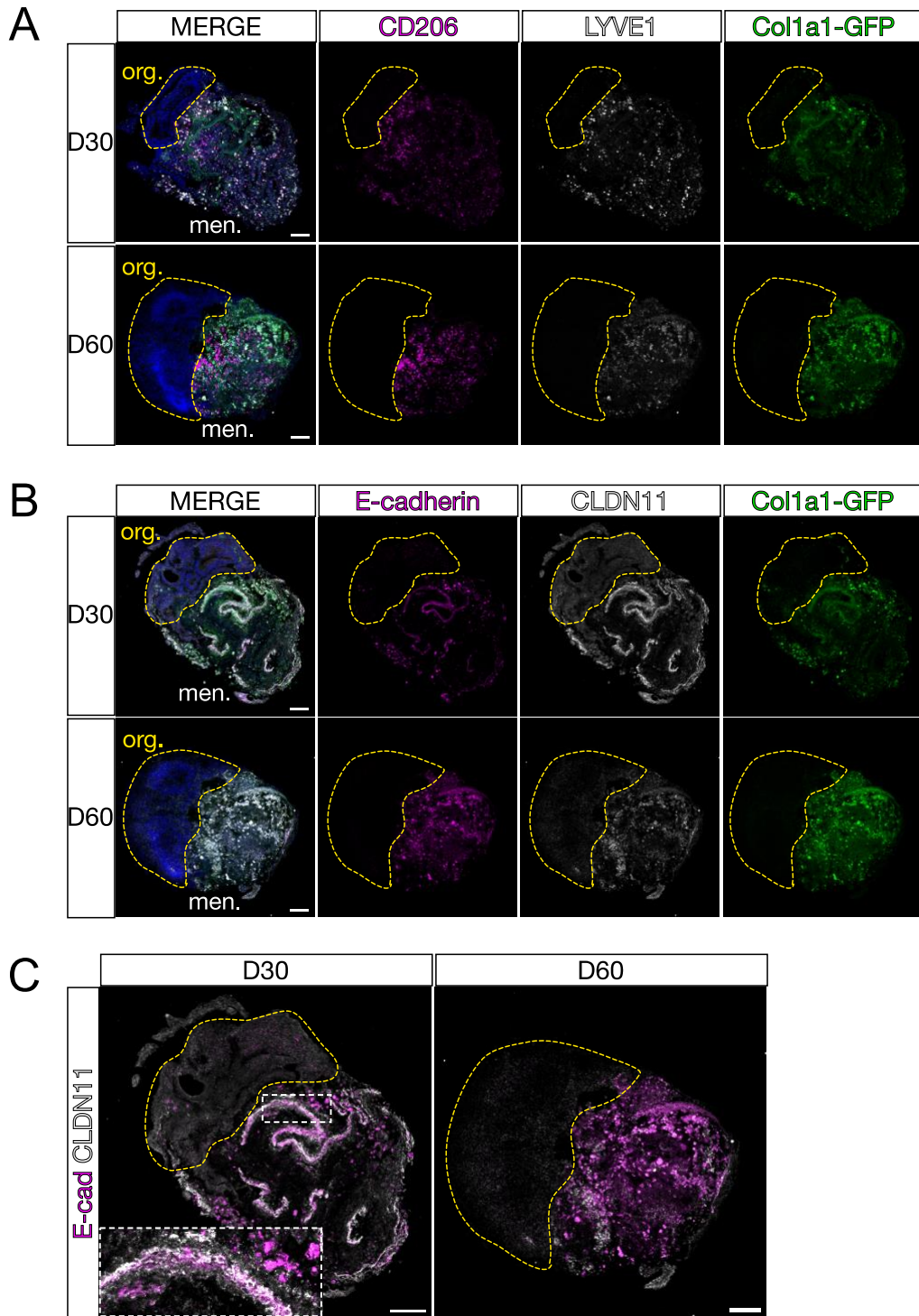
521
522
523 **Figure 3: Characterization of meningeal components in LMNOs after 30 and 60 days**
524 **in culture.**
525 Images showing LMNOs after 30 or 60 days in culture, labeled with markers for meningeal
526 cell types; (**A**) macrophages labeled with CD206 (magenta) and LYVE1 (white), (**B**)
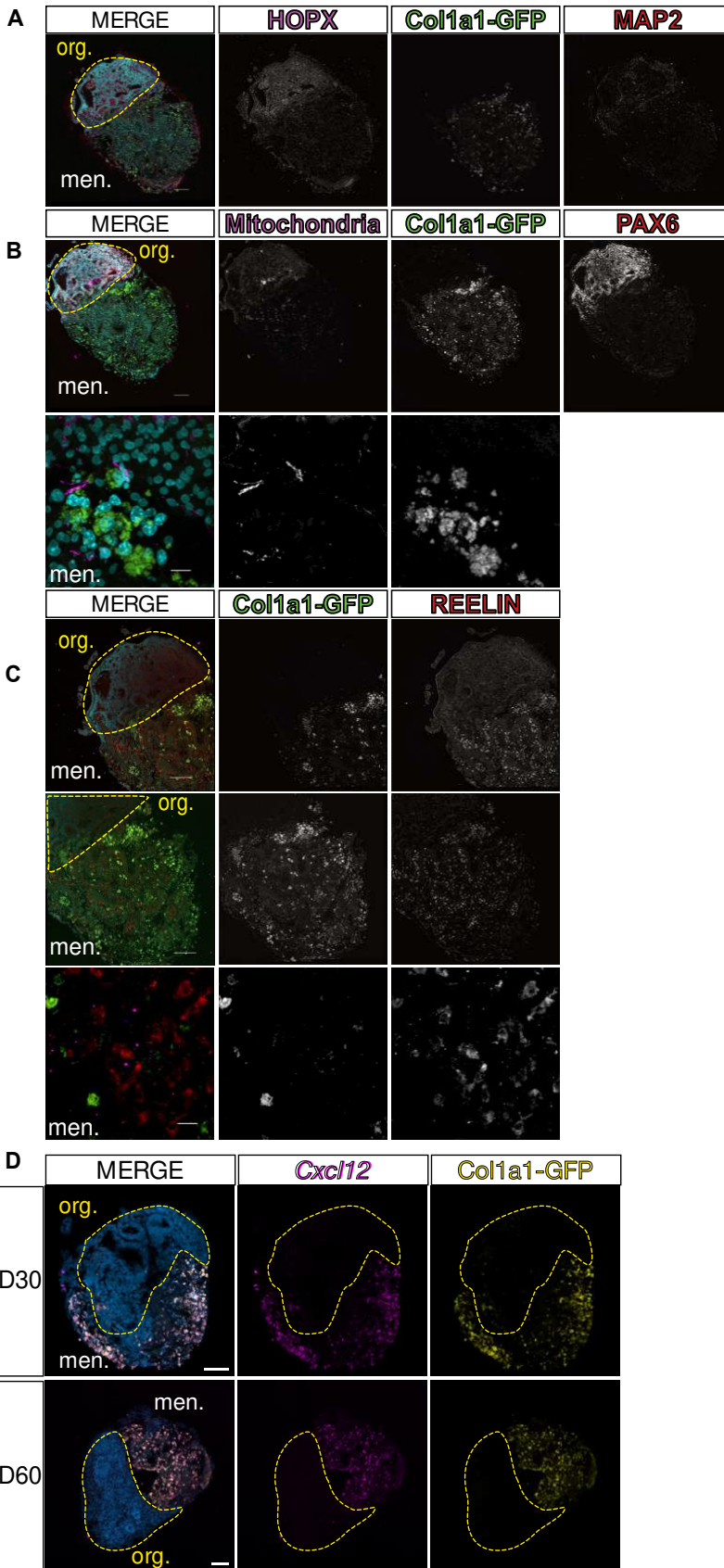
527   arachnoid barrier cells labeled with E-cadherin (magenta) and CLDN11 (white); all
528   fibroblasts labeled with *Col1a1-GFP* (green). (**C**) Images from **B** with E-cadherin/CLDN11
529   overlaid; inset shows overlap between markers. Yellow-dashed lines distinguish organoid
530   compartment (org.) from meninges (men.). All scale bars 100μm.
531
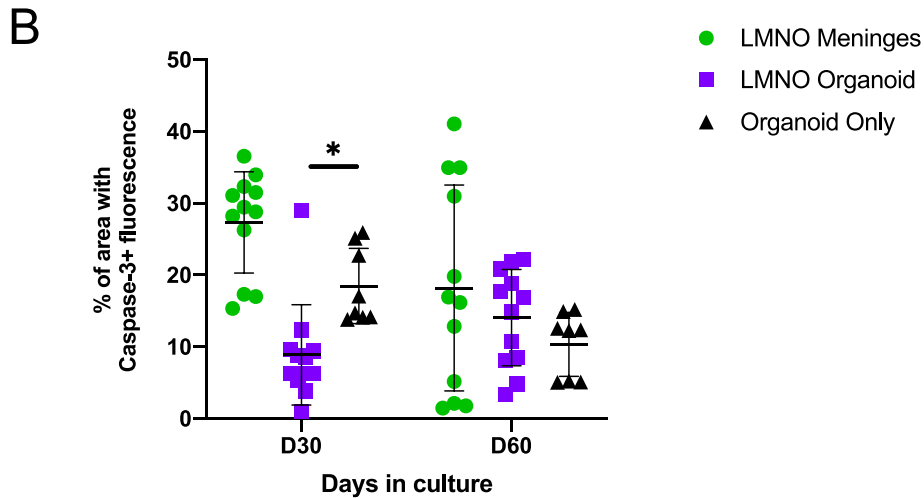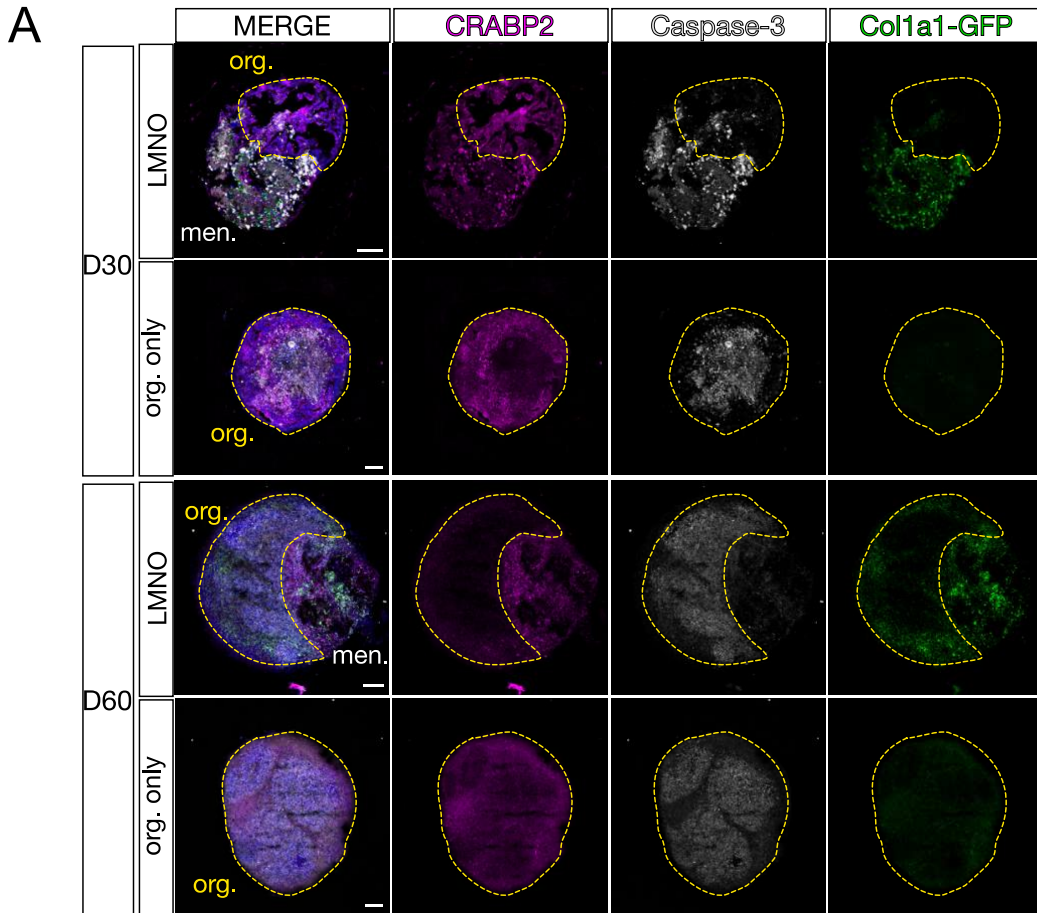
Figure 4: Neural organoids in LMNO fusions show reduced levels of apoptosis.
(**A**) Images showing Caspase-3 (white) and CRABP2 (magenta) staining in LMNO fusions and non-fused organoids (org. only) at 30 and 60 days in culture, *Col1a1-GFP* (green) labeling fibroblasts. Yellow-dashed lines outline organoid compartment. Yellow-dashed lines distinguish organoid compartment (org.) from meninges (men.). All scale bars 100μm. (**B**) Quantification of

539 percentage of area of organoid and meningeal compartments with Caspase-3 fluorescence at 30
540 and 60 days in culture; meninges in the LMNO fusion (green circles), LMNO fusions (purple
541 squares), non-fused organoid (black triangle). Two-way ANOVA with multiple comparisons
542 revealed significant differences between LMNO fusion versus organoid only at D30 ($p = 0.0423$)
543 ($p < 0.05$, *).

Figure 5: LMNO fusions recapitulate *in vivo* Cxcl12-dependent REELIN+ Cajal-Retzius cell migration.

(**A-B**) Images of LMNO fusions labeled with (**A**) neuronal markers HOPX (magenta)/MAP2 (red), (**B**) progenitor marker PAX6 (red) and human mitochondria (magenta). Top image scale bar 100μm and bottom image scale bar 10μm. (**C**) Cajal-Retzius cell marker REELIN (red); *Col1a1-GFP* marking meningeal fibroblasts (green). Top 2 images scale bar 100μm and bottom image scale bar 10μm. (**D**) Images of *in situ* detection of *Cxcl12* (magenta) in LMNO fusions at 30 and 60 days in culture, *Col1a1-GFP* marking meningeal fibroblasts (yellow). Yellow-dashed lines distinguish organoid compartment (org.) from meninges (men.). All scale bars 100μm.