

1 **Human-specific enrichment of schizophrenia risk-genes in callosal neurons of the**
2 **developing neocortex**

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39 **Summary**

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41 Human genetic studies have provided a wealth of information on genetic risk factors
42 associated with neuropsychiatric diseases. However, whether different brain cell types are
43 differentially affected in disease states and when in their development and maturation
44 alterations occur is still poorly understood. Here we generated a longitudinal
45 transcriptional map of excitatory projection neuron (PN) and inhibitory interneuron (IN)
46 subtypes of the cerebral cortex, across a timeline of mouse embryonic and postnatal
47 development, as well as fetal human cortex and human cortical organoids. We found that
48 three types of gene signatures uniquely defined each cortical neuronal subtype: dynamic
49 (developmental), adult (terminal), and constitutive (stable), with individual neuronal
50 subtypes varying in the degree of similarity of their signatures between species. In
51 particular, human callosal projection neurons (CPN) displayed the greatest species
52 divergence, with molecular signatures highly enriched for non-coding, human-specific
53 RNAs. Evaluating the association of neuronal class-specific signatures with
54 neuropsychiatric disease risk genes using linkage disequilibrium score regression showed
55 that schizophrenia risk genes were enriched in CPN identity signatures from human but
56 not mouse cortex. Human cortical organoids confirmed the association with excitatory
57 projection neurons. The data indicate that risk gene enrichment is both species- and cell
58 type-specific. Our study reveals molecular determinants of cortical neuron diversification
59 and identifies human callosal projection neurons as the most species-divergent population
60 and a potentially vulnerable neuronal class in schizophrenia.

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71 **Introduction**

72 Large-scale human genetic studies of neurodevelopmental and neuropsychiatric
73 disorders, such as Autism Spectrum Disorders [ASD], Bipolar Disorder [BD], and
74 Schizophrenia [SCZ], have implicated hundreds of loci, including a variety of genes
75 involved in neuronal development and function¹⁻¹⁰. To date, the specific neuronal
76 subtypes affected by these genetic variants are still poorly defined.

77 Comparing transcriptional profiles of neuronal subtypes with disease-enriched
78 gene sets can potentially provide information on cell-type susceptibility; this approach
79 has already begun to indicate links between individual disorders and cortical neurons,
80 including associations of SCZ and BD with excitatory pyramidal neurons^{11,12}. However,
81 these experiments have to date largely relied on broadly-defined cell classes, with little
82 subtype resolution. For example, most studies have not attempted to parse the diversity of
83 cortical pyramidal excitatory cells, instead reporting broad pan-neuronal or pan-
84 pyramidal associations. In addition, these experiments have largely examined adult cell
85 types, or a limited set of developmental timepoints, which presents a limitation as the set
86 of genes that are distinctive to a cell type and therefore important to its identity may
87 change over development^{13,14}. Finally, the gene sets specific for individual neuronal
88 subtypes may differ between species¹⁵, thus requiring direct study of human neurons.

89 Here, we generated a high-resolution transcriptional map of multiple subtypes of
90 both cortical excitatory projection neurons (PNs) and inhibitory interneurons (INs) over
91 development, including embryonic and postnatal mouse neocortex, human fetal cortex,
92 and human cortical organoids. We identify developmental, terminal, and stable molecular
93 signatures for each of the major PN and IN subtypes in the mouse, and developmental
94 signatures for the homologous human PNs classes. Notably, we find that expression of
95 PN-subclass gene signatures diverges between the two species, with callosal projection
96 neurons, the most evolutionarily recent PN class¹⁶, showing the greatest divergence.
97 Leveraging these molecular signatures to identify neuronal types susceptible to genetic
98 risk factors for various neuropsychiatric diseases, we show that polygenic risk for SCZ is
99 significantly enriched in signature genes of the CPN class in human fetal cortex. This

100 enrichment was specific to human CPNs, suggesting that SCZ risk genes may have
101 species-specific effects on neurodevelopment.

102

103 **Results**

104 **Molecular signatures with distinct temporal dynamics collectively define pyramidal** 105 **neuron and interneuron subtype identity in the neocortex**

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107 In order to trace the dynamic molecular signatures of individual neuron classes,
108 we profiled the major subtypes of cortical excitatory and inhibitory neurons at six
109 different time points along a timeline spanning phases of neuronal development from
110 early postmitotic fate decisions and neuronal migration to synaptic integration and circuit
111 maturation.

112 We first transcriptionally profiled the three major subtypes of excitatory PNs,
113 corticothalamic PNs (CThPNs) of layer 6, subcerebral PNs (ScPNs) of layer 5, and CPNs
114 of both deep and upper layers, in the mouse cortex (Figure 1A and Table S1). To
115 simultaneously and systematically purify multiple PN subtypes from the same sample, we
116 employed an optimized version of the MARIS technique^{17,18}, where the combinatorial
117 expression of three transcription factors, BCL11B, TLE4, and SATB2¹⁹⁻²², is used to
118 isolate the three major cortical PN subtypes. Although this approach requires pre-defining
119 the target cell populations, it allows deeper sequencing compared to single-cell RNA
120 sequencing. We FACS-purified ScPNs as BCL11B^{high}/TLE4^{low}/SATB2⁻, CThPNs as
121 BCL11B^{low}/TLE4^{high}/SATB2⁻, and CPNs as BCL11B⁻/TLE4⁻/SATB2⁺ from the mouse
122 somatosensory cortex at six different time points (E16.5, E18.5, P1, P3, P7, and P30, n≥3
123 per stage, 48 FACS-purified and transcriptionally profiled samples) (Figure 1A and
124 Figure S1A-C) (Table S1).

125 To begin to explore the transcriptional and temporal dynamics defining PN
126 diversity, we visualized the overall variation in the dataset using dimensionality reduction
127 by Multidimensional Scaling (MDS) on average gene expression between the PN
128 subtypes along their development. This showed that sample dissimilarity was primarily
129 driven by time (Dimension 1) and neuronal identity (Dimension 2) (Figure 1B).

130 Consistent with their known expression patterns *in vivo*, we observed increased
131 expression of *Bcl11b*, *Tle4*, and *Satb2* in ScPNs, CThPNs, and CPNs, respectively, while
132 markers of other cell types (e.g., oligodendrocytes, endothelial cells, interneurons,
133 astrocytes) showed negligible expression (31 out of 39 genes had FPKM < 2.5), thus
134 validating the specificity of our FACS-purification strategy (Figure S1G-L).

135 We considered both time and neuronal subtype identity to determine
136 differentially-expressed genes between all pairwise comparison (DEGs; Figure S1d-f,
137 see Methods for criteria). We then analyzed the resulting dataset to extract
138 *developmental*, *stable*, and *terminal* molecular signatures uniquely identifying ScPNs,
139 CThPNs, and CPNs (Figure 1C-D,F and Figure S4A). To discover developmental
140 signatures, we applied stringent criteria to filter out transcriptional changes reflecting the
141 asynchronous maturation of cortical PN (see Methods), retaining transcripts whose level
142 of expression was enriched in only one subtype for at least two consecutive time points
143 (1,008 genes, Table S2). Clustering the resulting set using partitioning around medoids
144 (PAM, k-medoids) identified seven clusters of genes with distinct temporal and subtype-
145 specific expression patterns, corresponding to early, mid, and late stages of development
146 of each neuronal class (Figure 1C-D and Figure S2A). These clusters contained both
147 established (e.g., *Ntng2* and *Inhba* for CPNs; *Wnt7b* and *Kcnab1* for CThPNs; *Lum*,
148 *Pex5l*, and *Grik2* for ScPNs)²³⁻²⁵, and novel (e.g., *Fstl4*, *Nr1d2*, *Cadps2* for CPNs; *Ssrt2*,
149 *Lig2* and *Fap* for CThPNs; *Tox2*, *Coll2a1*, for ScPNs) subtype-specific markers (Figure
150 1D and Figure S2A and Table S3). Gene ontology analysis of the developmental
151 signatures for each PN class revealed expected enrichment of developmental processes
152 such as axonogenesis in early- to mid-developmental signatures, and synaptic maturation
153 and function in late-developmental signatures (Figure 1E, Figure S2B, and Table S4).

154 We next examined this gene set to identify *stable* signatures for PN subtypes, i.e.,
155 genes consistently enriched in a single PN class at all time points, regardless of
156 developmental stage. For both CPNs and CThPNs, stable signature genes included novel
157 coding transcripts and unmapped loci (*Lcorl*, *Shank2* and *6530402F18Rik*), as well as
158 canonical markers already known to be involved in PN development (i.e., *Cux1*, *Hspb3*,
159 *Foxp2*, *Tbr1*)^{18,26} (Figure 1F, Figure S2C, and Table S5).

160 Finally, we identified *terminal* signatures, which we strictly defined as subtype-
161 specific transcripts whose enrichment emerged exclusively at P30, after definitive
162 transcriptional identity is established, and in only one neuronal subtype (Figure S4A and
163 Table S5). We identified 136 subtype-specific terminal signature genes, which included
164 genes associated with processes such as dendritic spine development and plasticity (e.g.,
165 *Baiap2l2* in ScPNs)²⁷, synaptic connection and trans-synaptic signaling (e.g., *Teneurin*
166 and *Tcap* in CPNs), and neuro-immune response (e.g., *Ill1ral* in CThPNs) (Figure S4A
167 and Table S4). The subtype-specific expression pattern of these genes was consistent
168 with single-cell resolution data from mature cortical subtypes in the Allen Mouse Cell
169 Types Database²⁸ (Figure S4C). These results show that the molecular class identity of
170 terminally-differentiated neurons is defined largely by the genes used to execute their
171 functional properties.

172 All PN types wire into a local cortical microcircuitry with distinct classes of
173 cortical inhibitory interneurons (INs)²⁹. Because INs display a high degree of cellular,
174 molecular and functional diversity^{30,31}, we sought to also define the molecular signatures
175 of cortical IN populations through time. Cortical interneurons derive from two main
176 germinal zones, the medial ganglionic eminence (MGE), and the caudal ganglionic
177 eminence (CGE)³⁰. We used genetically-labeled mouse lines to isolate MGE-derived
178 (Lhx6-GFP) and CGE-derived (5Ht3aR-GFP) cortical INs from the somatosensory cortex
179 at the same developmental stages used for PNs, as well as SST-dtTomato and VIP-
180 tdTomato reporter lines to isolate mature somatostatin (SST) and vasoactive intestinal
181 polypeptide (VIP) IN subtypes at P30 (n \geq 3 per stage, Table S1, 35 libraries, Figure 1G).
182 As for PNs, dimensionality reduction using MDS indicated that sample dissimilarity was
183 primarily driven by time (Dimension 1) and neuronal type (Dimension 2) (Figure 1H).

184 From this dataset, we performed pairwise differential gene expression analysis
185 across all developmental stages (filtering criteria in Methods, Figure S3a, see Methods)
186 and extracted *developmental*, *terminal*, and *stable* signatures using the same approach
187 described for PNs (Figure 1I-J,L, Figure S3C, and Table S2-3,5). Divergence between the
188 two cardinal IN subclasses (Lhx6- and 5Ht3aR-lineage) was evident from the earliest
189 stages of development (Figure 1G, Figure S3B-C, and see Methods). In contrast to PNs,

190 however, IN developmental signatures (1,789 genes, Table S2) included a large fraction
191 of genes stably enriched in one population alone (Figure 1L and Figure S3E), confirming
192 that the two cardinal subdivisions (5Ht3aR and LHX6) follow largely non-overlapping
193 identity programs from the earliest stages of development. GO analysis revealed
194 processes appropriate for each developmental stage for both IN subdivisions.
195 Interestingly, late signature genes of Lhx6-positive INs displayed specific enrichment for
196 genes involved in synaptic transmission, while signatures of 5HT3aR-positive INs were
197 enriched for axonogenesis genes (Table S4).

198 Terminal signatures of interneurons emerged at later stages, by P30, when
199 distinctive molecular features of each IN subtype have appeared (such as *Crhb4*, *Htr1a*,
200 and *Oprm1* for SST-INs and *Tac2*, *Frem1*, and *Npy1r* for VIP-INs). These remained
201 enriched in the adult cortex (Figure S4B, D, Allen Mouse Cell Types Database²⁸ and
202 Table S5). This observation supports a late specification of IN subtypes, likely following
203 recognition and pairing with their PN partners and integration into the cortical local
204 circuit.

205 All together, these data indicate that transcriptional signatures that define
206 individual neuron class identity in the neocortex are mostly composed of genes that
207 change expression over development, rather than invariant signatures. The fact that cell
208 identity signatures are mostly dynamic indicates that no single time point exemplifies a
209 cell type, highlighting the need to include more complete timelines of development and
210 maturation to comprehensively capture signatures of cell identity.

211

212 **Molecular diversity of ScPNs in distinct functional areas**

213

214 The cerebral cortex is divided into multiple functional areas^{32,33}. To begin to
215 examine whether the signatures we identified might describe individual PN types across
216 multiple regions of the cortex, we took ScPNs of layer Vb as a test case and compared
217 them across areas. For ScPNs, the only stable signature gene (i.e., differentially expressed
218 at all ages) was *Tcerg1l*, a previously identified ScPN marker^{18,25}. We therefore
219 employed a tamoxifen-inducible *Tcerg1l*-CRE driver mouse line³⁴ crossed to a

220 Sun1:GFP nuclear reporter³⁵ to label and isolate mature layer V ScPNs across different
221 functional areas. We FACS-isolated and profiled *Tcerg1l*-lineage nuclei from P56
222 *Tcerg1l*/Sun1:GFP mice (n ≥ 4 replicates per cortical region, each representing a pool of
223 5-6 mice) from motor (M), somatosensory (SS), auditory (AUD) and visual cortex (VIS),
224 each sampled at 1 or 2 defined anterior-posterior (AP) levels, and performed single-
225 nucleus RNA sequencing (snRNA-seq) (Figure 2 A-B, Figure S5).

226 We first confirmed that the sorted nuclei expressed the ScPN late-developmental
227 signature; as expected, the majority of the nuclei recovered expressed this signature
228 (Figure 2B and Figure S5D). This line also labels a small number of CPNs and
229 GABAergic interneurons, which we correctly detected in our dataset (*Rorb*+CPNs,
230 8.8%, and GABAergic interneurons, 11.7%); these cells were excluded from further
231 analysis (Figure S5D).

232 We then assessed the degree of consistency of the ScPN late-developmental
233 signature among all ScPN nuclei isolated from the primary somatosensory cortical area,
234 and found that it was comparable across all nuclei (Figure 2C, Figure S5E). Moreover,
235 this signature was equivalently expressed at different AP levels and in all functional
236 primary areas sampled (Figure S5F), indicating that it represents a core molecular feature
237 of ScPN identity across multiple areas of the mature brain.

238 In addition to this shared signature, we found that each functional area displayed a
239 unique molecular identity, with ScPN nuclei clustering according to their anatomical
240 location (Figure 2J-M and Figure S5H). We found 14 gene modules (see Methods) that
241 varied between ScPNs of different cortical functional areas (e.g., motor cortex ScPNs
242 differentially express *Lmo4*, *Socs2*, *Mgat4c*, and *Gpc5*), and 18 gene modules that varied
243 over both functional area and AP position (Figure 2L and Table S6). The data highlights
244 an additional level of molecular heterogeneity of the ScPN population in the adult brain,
245 both between different areas and between different AP positions within the same area.

246 The findings indicate that the core molecular programs that define the late-
247 developmental stages of neuronal subtype-specific identity continue to be broadly active
248 in the adult cortex, but that area-specific signatures are also present.

249

250 **Cross-species comparison reveals that human callosal projection neurons display**
251 **the greatest transcriptional divergence**

252

253 The cerebral cortex has diverged substantially during primate evolution, including
254 molecular differences between corresponding cell types in different species^{15,36,37}.
255 Although a number of reports have profiled adult human pyramidal neurons to
256 disentangle their transcriptional and epigenetic heterogeneity³⁸⁻⁴⁰, the molecular
257 signatures of developing human PN subtypes are poorly defined. These are needed to
258 investigate linkage to human disease, to allow cross-species comparative analysis, and for
259 validation of the class identity of PN subtypes generated *in vitro*⁴¹.

260 We therefore sought to define the molecular identity of PN types from the human
261 fetal cortex. We applied the same experimental approach used for murine tissues to
262 FACS-isolate and transcriptionally profile three molecularly distinct excitatory
263 populations from the human developing cortex at six gestational stages (gestational week
264 [GW]16, 17, 18, 19, 20 and 21, $n \geq 2$ per stage; Figure 3A-B, Figure S6A and Table S1),
265 based on the subtype-specific expression of the three TFs *BCL11B*, *TLE4*, and *SATB2*
266 (Figure 3C-D; Figure S6B-C, and Table S1). This dataset embodies ground-truth
267 information on pre-defined cortical PN diversity through human mid-gestation.

268 Dataset visualization using MDS showed that during the sampled developmental
269 window, the three neuronal populations clustered based on their identity, with CPNs
270 (*SATB2*^{high}/*BCL11B*^{low}/*TLE4*^{low}) distinctly segregated from the two corticofugal
271 (CFuPNs) populations (Figure 3E), and CThPNs (*TLE4*^{high}/*BCL11B*^{low}/*SATB2*⁻) separated
272 from ScPNs (*BCL11B*^{high}/*TLE4*^{low}/*SATB2*⁻), although they were less resolved from each
273 other than their murine counterparts. We confirmed the expression of genes known to be
274 PN subtype-specific, while expression of glial, oligodendrocyte and non-neuronal
275 markers were virtually absent, validating our isolation approach (Figure S6E-J). Of note,
276 contrary to the mouse neurons, human samples did not show a clear separation based on
277 gestational age, suggesting that, consistent with the prolonged length of human brain
278 development, the selected six-week time window may only cover limited developmental
279 progression within each neuronal subtype (Figure 3E).

280 We identified subtype-specific signatures using criteria similar to those used to
281 define developmental signatures in our mouse analysis, omitting the requirement for
282 specific enrichment in two consecutive time points, as time was not a major driver of
283 variation in this dataset. This identified 2,299 DEG; PAM clustering identified multiple
284 gene clusters with distinct subtype expression patterns, including clusters specific for
285 putative (p) pCThPNs, pScPNs, pCPNs, and corticofugal projection neurons (pCFuPNs)
286 (Figure 3F,H) (Table S7). These signatures contained both known markers of the
287 homologous murine PN subtypes (e.g., *CUX2* and *LHX2* for pCPNs, *NFIA* and *PAPPA2*
288 for pCThPNs, and *TCERGIL* and *SI00A10* for pScPNs), as well as genes with human-
289 specific expression (Figure 3H), which we validated using two single cell transcriptomic
290 datasets of human fetal cortex^{42,43} (Figure 3I and Figure S7A-B, see Methods). Using our
291 gene signatures, we were able to identify pScPN and pCThPN populations in these
292 datasets, as well as the previously annotated upper-layer PNs (Figure 3L and Figure
293 S7A). For all PN subtypes, a subset of the signature genes identified in our fetal dataset
294 also retained subtype-specific expression in the adult human cortex, as assessed by *in situ*
295 hybridization (Figure S8A)⁴⁴ and spatial transcriptomics of human dorsolateral pre-
296 frontal cortex (DPFC)⁴⁵ (Figure S8B) (*LPL* and *NTNG2* for CPNs, *HS3ST2* and
297 *COL24a1* for ScPNs, and *PP1R1B* and *ST18* for CThPNs, respectively).

298 These data indicate that during mid-gestation, human PN subtype signatures
299 already comprise genes that define their adult identity. Although human late-fetal and
300 postnatal molecular PN development is yet to be mapped, the persistent subtype-specific
301 expression of fetal signature genes in the adult suggests the existence of stable signatures
302 for human cortical PN classes.

303 To investigate the phylogenetic conservation of PN molecular identity between
304 mouse and human, we next compared their respective PN signature gene sets. We
305 identified 1803 genes with a corresponding ortholog in the other species, while 577 genes
306 in the human signatures had no mouse ortholog, mainly non-coding RNAs or annotated
307 pseudogenes (Table S8). Notably, 60.3% of those genes belonged to the human CPN
308 signatures, while 39.7% were found in the ScPN and CThPN signatures. The human CPN

309 signature was correspondingly the most enriched for human-specific genes (56% of CPN
310 signature genes, 43% of ScPN, 43% of CThPN, 31% of CFuPN).

311 Extending this analysis to other members of the primate clade (including Apes,
312 Old World Monkeys, New World Monkeys, and Promisians) showed that over 90% of
313 the genes not shared with mouse (i.e., *Nlgn4x*, *Diras3* and *Zim2*) were not conserved in
314 other primates and are exclusively present in the human genome (Figure 4A). CPNs
315 evolved relatively recently compared to the other cortical PN subtypes and have
316 undergone a disproportionately large population expansion from mouse to human¹⁶. Our
317 data suggest that many of the genes that define the molecular identity of this neuronal
318 class in humans are of an evolutionarily recent origin.

319 Next, we systematically performed cross-species comparison between
320 transcriptional signatures of mouse and human cortical PNs during development (Figure
321 4 B-C and Figure S9A). We first applied MDS on all PN samples from both species using
322 the genes from the orthologous signature gene lists, which revealed sample separation
323 based primarily on molecular identity (Figure S9A). To address the degree of homology
324 of the signature sets and identify conserved and human-specific developmental pathways,
325 we directly quantified the percentage of developmental signature genes shared between
326 the two species. For each human subtype, approximately 20% of signature genes were
327 shared with the mouse counterpart (20.5% for CPN, 16.4% for ScPN, 19% for CThPN),
328 while 7-18% of the mouse developmental signature genes were shared with the
329 corresponding human neuronal subtype (18% for CPN, 7% for ScPN, 8% for CThPN),
330 suggesting a limited conservation of subtype-specific gene programs between the two
331 species (Figure 4B).

332 To assess whether this low overlap might stem from differences in the
333 developmental stage of the human samples compared to the mouse, we computed
334 Pearson correlation coefficients on the orthologous genes. This analysis showed that
335 human PN subtypes isolated from midgestation cortex (GW16-21) are transcriptionally
336 more similar to PN subtypes from P1 mouse cortex (Figure 4C). The correlation to the
337 P1 mouse samples was consistent across all PN classes, indicating that, although human
338 fetal cortex development spans a longer period than in mice, it also occurs earlier relative

339 to birth. This may reflect molecular events which unfold postnatally in mice but occur
340 during fetal stages in human cortical development.

341 Given this specific correlation between human midgestation and the murine P1
342 stage, we focused on this stage for further comparative analysis. We sought to investigate
343 the degree of overlap of cellular features, molecular pathways, and biological processes
344 shared among the two species at this specific stage, by employing an established pipeline
345 for pathway enrichment (PE) analysis⁴⁶. We examined the genes with orthologs in both
346 species and determined P1-enriched DEGs for each PN type (Table S8, see Methods). PE
347 analysis on these genes identified a dense core of nodes (enriched gene ontology terms)
348 and edges (common genes among nodes) in support of shared pathways underlying
349 multiple developmental processes (Figure 4D). This indicates that human and mouse
350 PNs rely on different genes (as shown by their divergent signatures) to execute similar
351 biological functions at this stage. However, our approach also revealed species-specific
352 enrichment for some terms: cell communication for human CPN signatures and
353 extracellular matrix for human ScPN signatures (Table S9, Figure 4D). This suggests the
354 formation of a cellular environment unique to human corticogenesis.

355

356 **Human fetal CPNs display a specific enrichment for SCZ risk genes**

357 To determine whether the subtype-specific molecular maps we built for human
358 and mouse cortical development could be used to assess disease susceptibility in distinct
359 neuronal subtypes and along multiple developmental stages, we employed linkage
360 disequilibrium (LD) score regression⁴⁷ in combination with a large panel of GWAS
361 summary statistics for neuropsychiatric diseases and other complex diseases⁴⁸. Several
362 non-neuropsychiatric traits were included as controls (Table S10). For each of our mouse
363 developmental and human fetal signature gene sets, we identified a window of 100kb
364 upstream and downstream of the canonical transcriptional start site (TSS) for each gene,
365 and calculated risk association via prior heritability enrichment, as previously
366 described⁵². For mouse, we analyzed the corresponding human orthologs (Table S8).

367 For the mouse datasets, this analysis showed no enrichment in any signature gene
368 set of either PN or IN subclasses, for any of the traits examined. To verify whether
369 individual associations existed when subpopulations of mouse PNs and INs were
370 analyzed individually, we next clustered each of them separately. Again, we did not
371 observe any significant associations. (Figure 5A-B and Figure S10).

372 By contrast, when we analyzed signature gene sets from the human samples, a
373 significant and specific enrichment for SCZ was detected in one neuron class, CPNs
374 (Figure 5C and Figure S10). These data indicate that developmental signatures of human,
375 but not mouse, CPNs are enriched for genes associated with genetic risk for
376 schizophrenia, unearthing a previously unappreciated species-specific association of this
377 neuropsychiatric disorder with a defined cortical neuronal subtype.

378 We next tested whether this disease associations held true in a model of human
379 corticogenesis, human cortical organoids^{36,49-51}. Because manual annotation using small
380 lists of known marker genes had not previously been able to discriminate between
381 corticofugal subtypes in organoids^{49,52}, we first applied our gene signatures from human
382 projection neuron subtypes (CPNs, CFuPNs, CThPNs, and ScPNs) to infer neuronal
383 subtype identity in our previously published single-cell RNAseq dataset of human
384 cortical organoids⁵².

385 We used our human developmental signatures as input to Pathway-Level
386 Information Extractor (PLIER)⁵³, a matrix decomposition technique that optimizes latent
387 variables using prior biological knowledge to assign each cell a new identity (see
388 Methods). Among the excitatory neurons, we were able to clearly resolve CThPNs and
389 ScPNs in the organoid dataset, as well as the previously-annotated CPNs (Figure 5D-F,
390 Figure S10A-B). We then performed differential gene expression analysis to define the
391 molecular subtype-specific signatures of organoid-derived PN subtypes (Table S10). We
392 validated that these newly annotated ScPNs and CThPNs were enriched for known
393 subtype-specific signature genes (i.e. LMO3, TLE4, PPP1R1B for CThPNs, LDB2,
394 CRYM, ETV1 for ScPNs).

395 We next investigated whether CPNs produced within human cortical organoids
396 recapitulated the same disease association with SCZ found in endogenous human fetal

397 neurons. We included the DEG sets emerging from our PLIER analysis of organoid PN
398 subtypes (Table S10), or the top 5% of DE genes (by signed t-score ranking) for each
399 subtype, and partitioned heritability analyses to the associated genomic loci⁴⁸. We found
400 enrichment for SCZ risk genes in organoid CPNs, consistent with our findings on human
401 fetal CPNs. Of note, organoids also revealed association of SCZ with organoid ScPNs
402 and CThPNs. We also found significant enrichment for BD risk genes in organoid ScPNs
403 and CThPNs, and neuroticism risk genes in organoid CPNs and CThPNs (Figure 5G and
404 Figure S11), possibly reflecting a different maturation stage of the organoid neurons
405 compared to the endogenous ages we tested.

406 These data identify a species-specific susceptibility of a defined neuronal subtype,
407 callosal projection neurons, for schizophrenia. This association is not observed in mouse
408 neurons and is identifiable during midgestation of the human fetal cerebral cortex,
409 pointing to an early developmental effect that precedes the earliest clinical manifestations
410 of the disease, during late human adolescence⁵⁴.

411 412 **Discussion**

413
414 The emerging wealth of genetic information on neurodevelopmental and
415 neuropsychiatric disorders offers an opportunity to link disease-risk associated genes to
416 the specific cell types and developmental processes they may affect. Due to the diversity
417 of cell types present in the brain and the dynamic nature of their gene expression
418 programs over time, attempting such associations requires a detailed understanding of the
419 transcriptional profiles of different cell classes over multiple developmental stages.

420 Here we present a high-resolution, longitudinal transcriptional atlas encompassing
421 multiple neuronal subtypes of the neocortex in both mouse and human. The data unearths
422 notable features of the molecular programs associated with acquisition of cell type
423 identity and neuronal diversification in the neocortex. For example, it is evident that gene
424 signatures defining neuronal classes vary across time, indicating that the molecular
425 identity of a neuron cannot be defined by its molecular makeup at any one age; rather,
426 each type is represented by a collection of transitional states unfolding as development
427 progresses.

428 Cross-species comparison also highlighted salient features of human brain
429 development. Our finding that mid-gestation human fetal neurons are most similar to P1
430 mouse subtypes, points at accelerated cortical neuron development relative to organismal
431 development in humans. In addition, human PN classes shared only 10-20% of their
432 genes with their mouse counterparts, with long non-coding RNAs and unannotated loci
433 being highly enriched in the human-specific signatures. Beyond supporting a role for
434 non-coding loci in human brain evolution^{55, 56}, the data are intriguing in the context of
435 human disease. Genomic studies have identified the majority of risk variants associated
436 with SCZ within non-coding regions of the genome, suggesting that they alter risk by
437 changing levels of gene expression or splicing⁵⁷.

438 Prior studies on adult cerebral cortex have suggested a common, pan-neuronal and
439 glutamatergic enrichment for SCZ risk variants^{1-11,48,58,59}. Our association data, across
440 multiple types of neurons in mouse and human neocortex, now show that there is both
441 neuron-type and species-specific enrichment for SCZ-associated variants in signature
442 “core” genes of a single PN subtype of the human fetal cortex: callosal projection
443 neurons. Structural changes in the corpus callosum and reduced spine density in layer
444 II/III neurons have been reported in schizophrenia patients and experimental models⁶⁰⁻⁶²,
445 consistent with this new association. This is also interesting in light of our finding that
446 human CPNs retained the least conservation of molecular signatures compared to mouse,
447 showing the largest enrichment of human-specific molecular features among the neurons
448 sampled.

449 Collectively, the data point to developing human callosal projection neurons as a
450 potential target cell for functional investigation of genetic risk factors in SCZ pathology,
451 and contributes to decode the complex cellular and molecular underpinnings of
452 schizophrenia.

453
454

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473

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673 **Figure Legends**

674

675 **Figure 1: FACS-Purification and transcriptional profiling of mouse cortical PN and**
676 **IN subtypes along embryonic and postnatal development. A)** Schematic diagram of
677 samples collection. CPNs (green), ScPNs (red), and CThPNs (violet) were
678 simultaneously isolated from dissected somatosensory cortices across six developmental
679 stages from E16 to P30. Representative images of immunofluorescence for SATB2 (CPN
680 marker, green), BCL11B (ScPN marker, red), and TLE4 (CThPN marker, violet) on
681 mouse cerebral cortex sections at P7. Details on biological replicates, library sample size,
682 quality and RNA sequencing parameters are reported in Table S1. **B)** Dimensionality
683 reduction by multidimensional scaling (MDS) of the average gene expression shows that
684 PN sample dissimilarity is primarily driven by time (Dimension 1) and identity
685 (Dimension 2). **C)** Line plots of average gene expression illustrating cluster association
686 with distinct subtype identities and developmental stages. PN subtype-specific
687 developmental signatures - obtained as explained in detail in the Methods - were
688 classified in early, mid, and late, reflecting the time-specific expression dynamic along
689 development. Differentially expressed signature genes of PN subtypes are reported in
690 Table S2. Clusters 1, 5, and 7 identify early developmental signatures for CPNs, CThPNs
691 and ScPNs, respectively; cluster 2 represents CPN mid-developmental signature genes;
692 clusters 3, 6, and 4 represent CPN-, CThPN, and ScPN- late developmental signatures,
693 respectively. Gene clusters are reported in Table S3. **D)** Heatmap showing expression
694 pattern of PN subtype-specific developmental signature genes (5 top-ranked/cluster genes
695 are shown). **E)** Cnet plots displaying gene concept network analysis for GO terms
696 enriched in CPN- (green square), ScPN- (red square), and CThPN- (violet square)
697 subtype specific late-developmental signatures. Gene Ontology (GO) analysis reveals
698 enrichment of genes associated with circuit establishment and maintenance. Statistical
699 details and full description of GO terms is reported in Table S4. **F)** z-scores expression
700 heatmap of PN subtype-specific stable signatures. **G)** Schematic overview of purification
701 strategy of 5HT3aR- (pink) and Lhx6- (light green) expressing cortical INs.
702 Representative images of P7 cerebral cortex from genetically labeled 5HT3aR-GFP and
703 Lhx6-EGFP mouse IN reporter lines. **H)** Dimensionality reduction by multidimensional

704 scaling (MDS) of average gene expression shows that IN sample dissimilarity is
705 primarily driven by time (Dimension 1) and class identity (Dimension 2). **I**) Line plots of
706 average gene expression illustrating cluster association with distinct subtype identities
707 and developmental stages. Early, mid, and late developmental signatures reflected the
708 expression dynamics along development. IN subtype-specific developmental signatures -
709 obtained as explained in detail in the Methods - were classified in early, mid, and late,
710 reflecting the time-specific expression dynamic along development. Differentially
711 expressed signature genes of IN subtypes are reported in Table S2. Clusters 1 and 4
712 identify early-development signatures for 5HT3aR and Lhx6, respectively; cluster 2
713 represents 5HT3aR mid-developmental signature genes; clusters 3 and 5 represent late
714 developmental signatures of 5HT3aR and Lhx6, respectively. Gene clusters are reported
715 in Table S3. **J**) Heatmap showing expression pattern of IN subtype-specific
716 developmental signature genes (5 top-ranked/cluster genes are shown). **K**) Cnet plots
717 displaying gene concept network analysis for GO terms enriched in 5HT3aR- (pink
718 square) and Lhx6- (light green square) subtype specific late-developmental signatures.
719 Enrichment of genes associated with axonal development (5HT3aR) and synaptic
720 transmission (Lhx6) were found. Statistical details and full description of GO terms is
721 reported in Table S4. **L**) z-scores expression heatmap of IN subtype-specific stable
722 signatures.

723 Abbreviations: E, embryonic day; P, postnatal day; CPN, Callosal Projection Neurons;
724 ScPN, Subcerebral Projection Neurons; CThPN, CorticoThalamic Projection Neurons;
725 IN, Interneurons; SST, Somatostatin; VIP, Vasointestinal peptide. Scale bars: 250 μ m.

726
727 **Figure 2: ScPN molecular diversity across functional areas and along the anterior-
728 posterior axis.** **A**) Schematic diagram of experimental design. *Tcerg11-
729 CreERT2/Sun1:GFP* mice were injected with Tamoxifen at P7 and cortices analyzed at
730 P56. Representative images of double immunofluorescence of BCL11B (red) and SATB2
731 (violet) showing colocalization with GFP-expressing cells (proxy for *Tcerg11-
732 CreERT2/Sun1:GFP*). Scale bars: 250 μ m. Zoom-in inset: scale bar: 100 μ m. **B**) Uniform
733 Manifold Approximation and Projection (UMAP) plots of *Tcerg11-GFP* expressing nuclei
734 isolated by FACS confirming that the majority of the recovered nuclei in all the areas are

735 ScPNs, while a smaller fraction also contains CPNs and CthPNs, as well as INs. **C)**
736 UMAP of computationally extracted ScPNs from **B)** (only SS area), showing a
737 homogenous enrichment of late ScPN signature genes among nuclei isolated from
738 somatosensory cortex. **D)** UMAP of ScPNs in distinct functional areas of the cerebral
739 cortex. **E)** Heatmap of area-specific expression modules derived from differential gene
740 expression analysis of ScPNs between cortical functional areas. **F)** Representative violin
741 plots of ScPN signature genes of motor (red), somatosensory (blue), visual (green) and
742 auditory (yellow) areas. Differentially expressed signature genes of ScPN across areas are
743 reported in Table S6. **G)** UMAP of ScPNs in distinct anterior-posterior levels of
744 different cortical areas. **H)** Heatmap of AP level-specific expression modules derived
745 from differential gene expression analysis of ScPNs between distinct rostro-caudal
746 locations. **I)** Representative violin plots of ScPN signature genes of motor (red/pink),
747 somatosensory (blue/light blue), visual (green) and auditory (yellow) areas. Differentially
748 expressed signature genes of ScPN across AP levels are reported in Table S6.
749 Abbreviations: Mo, Motor; SS, Somatosensory; AUS, Auditory; VIS, Visual; CPN,
750 Callosal Projection Neurons; ScPN, Subcerebral Projection Neurons; CThPN,
751 CorticoThalamic Projection Neurons; AP, anterior-posterior.

752

753 **Figure 3: Identification, FACS-isolation, and transcriptional profiling of PN**
754 **subtypes from human fetal cortices at midgestation.** **A)** Schematic of sample
755 collection. **B)** Representative images of human fetal cortical sections isolated from GW16
756 and immunostained for SATB2 (labeling pCPNs in green), BCL11B (marker of ScPNs in
757 red), and TLE4 (labeling CThPNs in violet). Arrows indicate pScPNs expressing high
758 levels of BCL11B and low levels of SATB2, while arrowheads represent pCThPNs
759 expressing high levels of TLE4 and low levels of BCL11B. Scale bars: 250 μ m. Zoom-in
760 inset: scale bar: 100 μ m. **C)** FACS-isolation gating strategy of molecularly identified
761 human cortical populations. **D)** Representative FACS plots identifying pCPNs (BCL11B⁻
762 /TLE4⁻/SATB2⁺), pScPNs (BCL11B^{high}/TLE4^{low}/SATB2⁻), and pCThPNs
763 (BCL11B^{low}/TLE4^{high}/SATB2⁻). Details on biological replicates, library sample size,
764 quality and RNA sequencing parameters are reported in Table S1. **E)** Dimensionality
765 reduction by multidimensional scaling (MDS) of average gene expression shows that the

766 samples dissimilarity is driven by identity in the first two dimensions, clearly separating
767 pCPNs (represented in green) from pCFuPN in the first dimension and to a lesser degree
768 CThPN (represented in violet) and pScPN (represented in red) in the first dimension. The
769 second dimension shows a less evident trend in sample separation by time. **F)** Line plots
770 representing the expression profile of *Tle4*, *Satb2*, and *Bcl11b* transcripts. Gene
771 expression is in accordance with sample identity and consistent over time. **G)** Line plots
772 of average gene expression illustrating cluster association with distinct subtype identities
773 and developmental stages. PN subtype-specific signatures - obtained as explained in
774 detail in the Methods - are reported in Table S2. Clusters 1 and 2 represent pCFuPN
775 signatures, with genes highly expressed in both pScPN (red line) and pCThPN (violet
776 line), while depleted in pCPN (green line); Clusters 3, 4, and 5 were representing genes
777 enriched in pCPN; Clusters 6 and 7 pCThPN genes; and Clusters 8 and 9 genes enriched
778 in pScPN. Gene clusters are reported in Table S3. **H)** z-score heatmap representing the
779 expression pattern of human subtype-specific developmental signature genes at different
780 ages (5 top-ranked/cluster genes are shown). **I)** UMAP of a subset of human fetal cortex
781 cells from the previously published single cell study⁴³. **J)** Re-annotation of dataset in **(I)**
782 using our subtype-specific signatures (identified in **G)**. Subpopulation of pScPNs and
783 pCThPNs, previously annotated as “CFuPN”, are now clearly mapped and indicated with
784 arrows.

785 Abbreviations: GW, gestational week; pCPN, putative Callosal Projection Neurons;
786 pScPN, putative Subcerebral Projection Neurons; pCThPN, putative CorticoThalamic
787 Projection Neurons; pCFuPNs, putative Corticofugal Projection Neurons; Im CPN,
788 immature CPN.

789

790 **Figure 4: Interspecies comparison of human and mouse subtype-specific PN**
791 **signature genes.** **A)** Occurrence of the human-specific (no ortholog in mouse) signature
792 genes in the various primate clades. Assignment of the 577 human-specific signature
793 genes (Table S8) to a primate clade, based on the primate genome(s) in which an ortholog
794 was found in the present analysis (BioMart database). Clades are specified in top panel.
795 The color-coding indicates the degree of conservation among species in each clade: the
796 number reported in blue represents the frequency of genes that are present both in human

797 and the given primate species; the number in violet represents the frequency of genes
798 present in multiple primate genomes within the prosimian and simian groups. **B)** Upset
799 plot representing gene set intersection between human and mouse ortholog subtype-
800 specific signature genes. Only a subset of identified signature genes for projection neuron
801 populations intersect, while the majority of projection neuron signature genes do not
802 intersect. **C)** Pearson's correlation of gene expression of subtype-specific ortholog
803 signatures in human and mouse at the different sampled ages. Highest correlation
804 between samples of corresponding subtype across species was observed at P1. **D)** G-
805 profiler based enrichment analysis of human signature and mouse DEG at P1, and
806 network visualization of shared and species-specific enriched terms by Cytoscape
807 analysis (see Methods). Statistical details and enrichment term description is reported in
808 Table S9.

809 Abbreviations: GW, gestational week; E, embryonic day; pCPN, putative Callosal
810 Projection Neurons; pScPN, putative Subcerebral Projection Neurons; pCThPN, putative
811 CorticoThalamic Projection Neurons; pCFuPNs, putative Corticofugal Projection
812 Neurons; Im CPN, immature CPN; FDR, false discovery rate.

813
814

815 **Figure 5: Linkage Disequilibrium Regression score for GWAS hits of**
816 **neurodevelopmental and neuropsychiatric disorders in human and mouse cortical**
817 **neuron signatures.** Genetic correlations (estimated by LD Score Regression) between
818 mouse (**A, B**) and human (**C**) neuronal subtype-specific signature genes and Height,
819 Coronary Artery Disease (CAD), Autism Spectrum Disorder (ASD), Schizophrenia
820 (SCZ), Bipolar Disorder (BD). * represents significant genetic correlation. (**D-F**)
821 Computationally extracted PN subtypes from the 3 month organoids dataset previously
822 published⁵² were re-annotated using the PLIER algorithm which employed our signature
823 gene sets (**E-F**). (**G**) Genetic correlations (estimated by LD Score Regression) between
824 5% top expressing genes of cortical organoid subtypes at 3 mo in vitro and Height,
825 Coronary Artery Disease (CAD), Autism Spectrum Disorder (ASD), Schizophrenia
826 (SCZ), Bipolar Disorder (BD).

827 * represents significant genetic correlation. 5% top expressed genes and sum statistics of
828 disease traits are listed in Table S10.

829 Abbreviations: pCPN, putative Callosal Projection Neurons; pScPN, putative Subcerebral
830 Projection Neurons; pCThPN, putative CorticoThalamic Projection Neurons; pCFuPNs,
831 putative Corticofugal Projection Neurons; Im CPN, immature CPN.

832 **Methods**

833 **Animals.**

834 All animals were handled according to protocols approved by the Institutional Animal
835 Care and Use Committee (IACUC) of Harvard University. All mice were maintained in
836 standard housing conditions on a 12-h light/dark cycle with food and water ad libitum.
837 No more than four adult animals were housed per cage. Both females and males were
838 included in the study. Time pregnant CD1 females have been purchased at Charles River
839 Laboratories and embryonic and postnatal litters have been collected at the desired time
840 points (E16, E18, P1, P3, P7, P30). All the transgenic mouse lines used in this study
841 were imported and housed in our Animal Facility: Vip-IRES-cre (Jax Stock No 010908);
842 Sst-IRES-cre (Jax Stock No 018973); Lhx6-GFP (Lhx6-EGFP)BP221Gsat⁶³; 5HT3aR-
843 GFP⁶⁴ kindly provided by Hanna Monyer; and *Tcerg11-2A-CreER*³⁴, kindly provided by
844 Joshua Huang, was crossed with nuclear reporter line SUN1-sfGFP-myc-pA (Jax Stock.
845 021039).

846 Sequences of PCR primers employed to genotype reporter mouse lines are as follows: for
847 5HT3aR-GFP we used a common Forward (FW) primer
848 GCAAGATGTGACCAAGCCACCTATTT and as Reverse (RV)
849 TGAAGTTGTGGCCGTTTACGTCG for the mutant and
850 CAGCCCTCAGCCCTTTGAGACTTAAG to detect the wt; for Lhx6-GFP: FW
851 GCTGAAGCACTGCACGCCGTAGG and RV GTTTGTCGGGACCTTCTTCA; For
852 SST-Tom: TCTGAAAGACTTGCGTTTGG FW for the wt,
853 TGGTTTGTCCAAACTCATCAA FW to detect the transgenic, and
854 GGGCCAGGAGTTAAGGAAGA as a common RV primer; For VIP-IRES-Cre and
855 *Tcerg11-2A-CreER* GTCCAATTTACTGACCGTACACC and
856 GTTATTCGGATCATCAGCTACACC.

857

858 **Tissue samples.**

859 Mouse samples

860 For each biological replicate of the bulk sequencing experiment, the somatosensory
861 cortex was dissected from one litter of mouse embryos or pups (six to ten pups per litter)
862 or from 8-10 littermates P30 mice. For single-cell analysis, we pooled cortical regions

863 (motor, somatosensory, auditory, visual) at distinct anterior-posterior locations (A-P). 5-6
864 Tcerg11-2A-CreER P56 mice, previously injected intraperitoneally with a single dose of
865 Tamoxifen (100 μ l at 5 $\text{mg} \cdot \text{ml}^{-1}$) at P7. The Allen Mouse Brain framework version 3
866 ontology⁶⁵ was used to define cortical primary areas.

867

868 Human fetal samples

869 De-identified human fetal tissue samples (GW16-GW21) were obtained from ABR upon
870 patient consent in strict observance of the legal and institutional ethical regulations.
871 Protocols were approved by the Harvard Institutional Review Board. Fetal cortices were
872 microdissected on ice in HibernateA and dissociated into single cell suspension as
873 previously described⁶⁶.

874

875 **Tissue processing for cell and nuclei sorting.**

876 Enzymatic (Papain; Worthington Biochemical Corporation) and mechanical digestion
877 were used to dissociate cortex into single cell suspension, following manufacturer's
878 protocol. Briefly, tissue samples were cut into small pieces and placed in a vial
879 containing a pre-warmed solution of Papain and triturated following manufacturer
880 protocol. After removing the dissociation media, cells were resuspended in PBS and
881 processed for intracellular staining and FACS-sorting¹⁸. Somatosensory cortices obtained
882 from P30 mice were dissociated into single cell suspension by enzymatic and mechanical
883 digestion using the Papain dissociation kit, according to the manufacturer's protocol
884 (Worthington, cat. #LK003153). For nuclei preparation, somatosensory, motor, auditory
885 and visual cortex from wild-type animals at P56 was dissected and dissociated. Each
886 library was made from tissue pooled from at least 8 animals, and a balanced sex ratio was
887 used. Tissue dissociation was performed as previously described, and live cells were
888 isolated by FACS sorting as DAPI-negative, Vybrant DyeCycle Ruby (Thermo Fisher)-
889 positive events. Libraries were prepared using the 10x Genomics Chromium Single Cell
890 3' kit v2 (10x Genomics) according to the manufacturer's protocol.

891

892 **FACS-purification of mouse and human cortical projection neurons.**

893 The protocol for intracellular staining and RNA isolation was previously described¹⁸.
894 Single cell suspension was centrifuged for 5 minutes at 250g. Cell pellet was resuspended
895 in 4% paraformaldehyde (Electron Microscopy Science), 0.1% saponin, and 1:25 RNasin
896 in PBS (2×10^7 cells/ml) and incubated at 4°C for 30 minutes. Cells were pelleted at
897 3000g for 30 minutes, washed twice with wash buffer (0.1% saponin, 0.2% BSA, 1:100
898 RNasin in PBS), resuspended in primary antibody (0.1% saponin, 1% BSA, 1:20 RNasin
899 in PBS), and incubated for 30 minutes at 4°C. Cells were then washed twice, incubated in
900 secondary antibody for 30 minutes, washed twice more, and resuspended in PBS with 0.5%
901 BSA and 1:40 RNasin for FACS purification on a BD FACS Aria II into PBS with 0.5%
902 BSA and 1:40 RNasin. RNase free BSA was from Gemini Bio-Products. Primary
903 antibodies were mouse anti- β SATB2 1:250 (Abcam), rat anti-CTIP2 1:500 (Abcam), and
904 rabbit anti TLE4 1:2000 (gift from S. Stifani)³⁰. Secondary antibodies were goat anti-
905 mouse A488, goat anti-rat A546, and goat anti-rabbit A647 at 1:1000 (Molecular Probes).
906 Appropriate gates for FACS were set based on relative levels of SATB2, CTIP2, and
907 TLE4 expression to isolate CPN, ScPN, and CThPN, as previously described¹⁸.

908

909 **FACS-isolation of mouse interneuron classes.**

910 Single cell suspensions obtained from somatosensory cortices isolated from interneuron
911 reporter lines (Vip-IRES-cre; Sst-IRES-cre; Lhx6-GFP; 5HT3aR-GFP) were analyzed by
912 FACS and interneuron classes were FACS-purified based on the expression of the
913 reporter gene. Appropriate gates on FACS were set based on wild type cortex, which do
914 not express the reporter gene.

915

916 **FACS-isolation of mouse cortical nuclei.**

917 Single nuclei suspensions obtained from primary cortices isolated from *Tcerg1l-2A-*
918 *CreER* P56 mice were analyzed by FACS and Tomato positive neurons isolated.
919 Appropriate gates on FACS were set based on wild type cortex, which do not express the
920 reporter gene.

921

922 **Immunohistochemistry.**

923 Mice were anesthetized and transcardially perfused with ice-cold PBS followed by ice-
924 cold 4% paraformaldehyde in PBS. Dissected brains were post-fixed overnight in 4%
925 paraformaldehyde at 4 °C, and stored in PBS-Azide 0.0025%. Brains were processed as
926 previously described^{23,30}. Vibratome brain slices were incubated with blocking media (8%
927 goat serum, 3% BSA, 0.3% Triton in PBS) for 1hr, then with primary antibodies
928 overnight at 4°C. Secondary antibodies were applied with 1:800 dilution in the blocking
929 media and incubated for 2hr at room temperature, after washes. DAPI staining was
930 performed for 3 mins before mounting with Fluoromount G (Invitrogen, #00-4958-02).
931 The antibodies: Chicken anti-GFP antibody (ab16901, 1:500; Millipore), Mouse anti-
932 Satb2 (ab51502, 1:50; Abcam), Rat anti-Ctip2 (ab18465, 1:100, Abcam), Rabbit anti-
933 Tle4 (a kind gift from Stefano Stifani). Appropriate secondary antibodies were from the
934 Molecular Probes Alexa Series. All images were acquired using a Nikon Eclips 90i
935 fluorescence microscope and analysed with Volocity v6.0.1 software. Confocal images
936 were obtained using an LSM 700 inverted confocal microscope (Zeiss) and analysed with
937 a Zen Blue/Black 2012 Image-processing software.

938

939 **RNA extraction, library preparation, and sequencing.**

940 RNA was extracted from molecularly identified PN subtypes using the RecoverAll Total
941 Nuclear Isolation Kit (Thermo Fisher Scientific) following manufacturer's instruction
942 except for crosslinking reversion, which was performed by incubating the nuclear pellet
943 in Digestion Buffer and Protease mixture (100 µl buffer and 4 µl protease) for 3 h at 50°C.
944 Isolated interneuron subtypes were subjected to RNA extraction with Trizol, following
945 manufacturer's protocol. RNA was quantified using Nanodrop and Qubit and quality
946 assessed by Agilent 2100 Bioanalyzer. Purified RNA served as input for the cDNA
947 library preparation with SMART-Seq v.4 Ultra Low Input RNA kit (Takara Bio, Kusatsu,
948 Japan) and Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA)
949 according to the manufacturer's protocol. The cDNA library fragment size was
950 determined by the BioAnalyzer 2100 HS DNA Assay (Agilent, Santa Clara, CA, USA).
951 The libraries were sequenced as paired-end reads on HiSeq 2500 or NextSeq 500 to
952 retrieve 1.02×10^8 mapped reads. For single nuclei RNA sequencing, libraries were

953 prepared using the Chromium Single Cell 3' Reagent Kit v2 (10x Genomics). Briefly,
954 FACS-purified nuclei were loaded into a channel of the Chromium single cell 3' Chip,
955 following manufacturer's protocol, and partitioned into droplets in the Chromium
956 Controller, before library construction. Libraries were then deep-sequenced to
957 approximately 50,000 reads/nucleus.

958

959 **Computational analysis.**

960 Computational analysis was performed using R package version 3. More specifically, we
961 used v. 3.4.4 for the analysis of bulk sequencing, v. 3.6.1 for the analysis of single cell
962 sequencing, and v3.6.0 for the analysis of single nuclei sequencing.

963

964 **Quality control and data processing for bulk sequencing.**

965 RNA-Seq reads were mapped using STAR/2.5.0⁶⁷ with the following settings:

```
966 --outFilterType BySJout --outFilterMultimapNmax 20 --outFilterMismatchNmax 999  
967 --outFilterMismatchNoverReadLmax 0.04 --alignIntronMin 20 --alignIntronMax  
968 1000000 --alignMatesGapMax 1000000 --alignSJoverhangMin 8 --  
969 alignSJDBoverhangMin 1 --sjdbScore 1 --quantMode GeneCounts
```

970 Against the GRCh38 or mm10 genome respectively using Gencode based gene
971 annotation version 24 for human and 16M for mouse. Raw gene level quantification from
972 STAR was used for all further analyses and Input to DESeq. Technical replicates were
973 merged and afterwards conditional quantile normalization from cqn R package version
974 1.24.0 was applied to all three data sets, correcting for effects of sequencing depth, gene
975 length and GC content. GC content information was obtained from ENSEMBL, using
976 homo sapiens data set version 83 and mus musculus data set version 91. Gene length was
977 defined as the average length of all listed transcripts for each gene, based on the gencode
978 ENSEMBL transcript annotations version 24 for human and version M16 for mouse.
979 Subsequently, batch effects introduced by flow cell association were corrected for with
980 ComBat from sva package version 3.26.0 in both projection data sets. ComBat was not
981 applied to mouse interneurons because sequencing flow cell and conditions of interest

982 were confounded for this data set. In order to validate sample identity and quality,
983 dimensional reduction and further analyses were implemented. Principal component
984 analysis was conducted based on the `prcomp` function from `stats` package version 3.4.4
985 and used to check the first components explaining the majority of the observed variance
986 for outlier samples. To further support the sample selection process, classical
987 multidimensional scaling based on `stats cmdscale` function was used. Results for two
988 different distance metrics were evaluated, euclidean distance and centered Pearson.
989 Additional sample identity confirmation was attained through clustering of the
990 dissimilarity matrices based on `hclust` and `ward.D2` criterion for agglomeration. Lastly,
991 samples were validated by checking the expression of a previously established set of
992 known subtype marker genes. The final curated data sets encompassed 48 samples for
993 human projections, 43 for mouse projections and 31 for mouse interneurons.

994

995 **Gene signature set definition.**

996 Differential expression was tested for with the `DESeq2` package v. 1.18.1. Normalization
997 factors were derived from conditional quantile normalization and added to the `DESeq2`
998 object, correcting for transcript length, sequencing depth and gc content. For mouse and
999 human projection neuron data sets, flowcell identity was considered as a covariate in the
1000 design formula. The batch distribution of the mouse interneuron samples did not allow
1001 for inclusion of flow cell information in the design formula for this data set. The
1002 significance threshold for adjusted p values was set to 0.01. Subsequently, the resulting
1003 list of differentially expressed genes was filtered, using an empirical approach to identify
1004 meaningful parameters to pinpoint cell type specific marker genes. To that end, reference
1005 lists of known marker genes for projection and interneurons were used to define
1006 thresholds on normalized gene expression, filtering based on average \log_2 fold change
1007 and cell type specificity (defined as entropy). Thresholds were chosen so that the marker
1008 genes present in our preliminary lists were retained in the final sets as well. The results
1009 are listed in Table 2. Resulting signature genes were then clustered using `PAM` (`pam()`
1010 function from R package `cluster` v. 2.1.0). The input was defined as the average gene
1011 expression per subtype and age for each gene retained after filtering. Optimal k were
1012 determined using gap statistics as implemented in `clusgap` version 2.1.0. Genes resulting

1013 from human (k 23), mouse projection (k 7) and the two different lists of mouse
1014 interneuron genes (k 18 for 5HT3aR vs Lhx6 and k 24 for all four subtypes at P30) were
1015 all clustered separately. To each of the resulting clusters a subtype identity was assigned.
1016 Different clusters assigned to the same subtype showed different temporal expression
1017 patterns.

1018 Two strategies were implemented to merge the clusters to obtain the final gene sets. First,
1019 all clusters of the same subtype were merged together to obtain “subtype sets”. Second,
1020 all clusters within a subtype were clustered hierarchically (centered Pearson) using the
1021 overall average expression of all genes within a cluster for each subtype and age. All
1022 clusters derived from the same data set were merged according to the same distance
1023 cutoff. This second strategy was not applied to the mouse projection clusters, because
1024 they were already bigger in size than the clusters derived from the other two data sets
1025 where the chosen k were generally larger. Instead, all 7 clusters were treated as individual
1026 gene sets in the disease association analysis. All mouse derived sets were mapped to
1027 one2one human orthologues. The results are listed in Table 3.

1028

1029 **Single-nuclei RNA sequence analysis.**

1030 Single-nuclei RNA-seq reads were aligned to mm10 pre-mRNA reference, and gene
1031 expression matrix was obtained using CellRanger software v3.1.0 with the default
1032 parameters. R v3.6.0 and Seurat package v3.1.2 was used to perform downstream
1033 analyses. During the analysis, cells from the piriform region were removed, in order to
1034 focus our study on the ScPNs. Low-quality cells (with percent mitochondrial gene
1035 expression (percent.mt) >0.5%) were removed from the analyses. SCTransform was
1036 performed to normalize and scale the gene expression matrix. During the SCTransform,
1037 the number of UMIs per cell and percent.mt were treated as variables to regress out.
1038 Since we want to remove sex effects linked to the presence of chromosomes, X and Y
1039 chromosome genes were removed from the list of variable genes identified. The resulting
1040 list of variable genes was used to perform the principal component analysis. Using the
1041 top 30 principal components (ordered by the fraction of the total variance explained) and
1042 Louvain clustering algorithm (FindClusters function in Seurat with resolution set to 0.7),
1043 16 different cell clusters were identified. This dimension of 30 PCs is further reduced to

1044 two Uniform Manifold Approximation and Projection (UMAP) dimensions (RunUMAP
1045 function in Seurat with dims parameter set to top 30 PCs) for visualizations. Using
1046 known marker genes (listed in Table: Markers), clusters were classified into four broad
1047 cell types: ScPN, upper layer CPN, inhibitory neurons and low quality.

1048

1049 Markers

ScPN	UL CPN	Inhibitory Neurons
Fezf2	Rorb	Synpr
Parm1	Cux1	Sst
Tcerg1l	Cux2	Gad1
Bcl11b		Gad2

1050

1051 **Finding area-specific markers of ScPN.**

1052 ScPNs were selected from the broad cell type annotation, and were processed separately
1053 by applying the same workflow described above with percent.mt cutoff of 0.2%. Treating
1054 brain slice/functional area annotations as cell clustering, differentially enriched genes for
1055 each section were identified using MAST v1.10.0⁶⁸ (using FindAllMarkers function with
1056 test.use parameter set to MAST, latent variable set to the number of UMIs and percent.mt,
1057 min.pct set to 0.3). To group DEGs together by their patterns of expression, we first
1058 computed the average expression of these genes in each brain slice/functional area (using
1059 the AverageExpression function in Seurat). Z-scores were then computed to find enriched
1060 or depleted genes in each region. To group differentially expressed genes into groups
1061 based on their expression pattern, K-means clustering (Hartigan-Wong algorithm
1062 implemented in R base package named stats) was used. The number of clusters was
1063 determined by benchmarking several peaks from the gap statistics result (obtained from
1064 clusGap function in cluster package v2.0.8 with parameters K.max=20 and B=60).

1065

1066 **Organoid cell type re-annotation**

1067 We took the dataset produced in Velasco et al., 2019⁵² to test the performance of our gene
1068 signatures to accurately identify subtypes of PNs. To do so, we selected cells labeled as

1069 CPNs and CFuPNs according to the published annotation and processed only those cells.
1070 Normalized count matrix containing the selected cells was taken from the published
1071 dataset and processed using Seurat package in R using the default parameters unless
1072 otherwise specified. Variable genes were found using FindVariableFeatures function, and
1073 the ScaleData function was used to scale the dataset. Principal component analysis was
1074 performed using RunPCA function on the scaled data, and the top 18 PCs were chosen
1075 based on an elbow plot generated using ElbowPlot function. RunUMAP function was
1076 used on the top PCs to visualize the variation in cells. As the original authors did,
1077 Harmony (version 1.0) was used to remove the batch effect among different cell lines and
1078 replicates. The dataset contained HUES66 and PGP1 cell lines, and PGP1 line had two
1079 replicates. To identify specific PN subtypes (CPN, CThPN and SCPN) in this processed
1080 organoid dataset using our signatures gene lists, we applied PLIER (PLIER function from
1081 PLIER package version 0.99.0) specifying our signature lists as the prior knowledge
1082 (gene sets). Among the resulting latent variables that align to the prior knowledge, only
1083 the ones with AUC > 0.5 and p value < 0.005 were considered in the reannotation. The
1084 new annotation of each cell was chosen by the latent variable with the highest cell
1085 loading.

1086

1087 **Validation of human cell type signature genes in a published dataset.**

1088 We validated our curated human gene modules with a previously published dataset
1089 Nowakowsky et al., 2017⁴³. This scRNA-seq dataset was processed from the downloaded
1090 gene count matrix, using a similar downstream pipeline as specified in the single-nuclei
1091 RNA sequence analysis. Note that percent.mt was not used to filter out the cells and
1092 when scaling the dataset, because the original authors had already removed mitochondrial
1093 genes by only keeping genes expressed in at least 30 cells. Using our gene signature
1094 modules curated for each neuronal subtype of interest (CPN, CfuPN, CThPN and ScPN),
1095 we calculated gene signature scores to identify clusters expressing these modules at high
1096 levels. AddModuleScore() function from Seurat was used to compute gene signature
1097 scores, setting the control parameter to 30. Based on our cell cluster definition and the
1098 module scores, we applied cluster-based re-annotation strategy, where cell type
1099 annotations were reassigned if cells from a specific cluster showed enrichment with one

1100 of our modules. Enriched clusters were determined by looking at both the overall module
1101 scores in clusters and the expression of individual genes.

1102

1103 **Ortholog identification.**

1104 Human orthologs for mouse genes were obtained based on the mus musculus gene data
1105 set from ENSEMBL version 91 and biomaRt package version 2.34.2. Genes of mouse
1106 homology types (one to many; many to many) mapping were excluded and only “one-to-
1107 one” orthologs were considered for downstream analyses. Mitochondrial genes were
1108 excluded resulting in a total set of orthologs encompassing 15,974 genes. 6,336 of those
1109 genes were found to be differentially expressed both in human and in mouse projection
1110 neurons. 1,804 orthologs passed gene list filtering in at least one species and were thus
1111 identified as potential signatures and 302 of those passed in both species, with 234 of
1112 them being included in the final signature sets. 165 of those were assigned to the same
1113 neuronal subtype signature in both species and consequently identified as convergent
1114 signatures.

1115

1116 **Interspecies comparison analysis.**

1117 Raw counts for 48 human and 43 mouse samples were combined by retaining expression
1118 values for 15,974 ENSEMBL human mouse orthologs. Based on the combined count data,
1119 a DESeqDataSet object was created. Neuronal subtype and age were considered in the
1120 design formula as a joint variable in addition to the sequencing flow cells. Conditional
1121 quantile normalization was applied to remove the effect of GC content, and gene length
1122 was modelled as a smooth function to account for the lengths effect on gene count. The
1123 results were set as normalization factors before estimating size factors and dispersions
1124 and fitting a negative binomial GLM with DESeq2. A blinded variance stabilizing
1125 transformation (VST) was computed for the 15,966 genes that did converge in the
1126 previous step. ComBat from sva package version 3.26.0 was run to correct for batch
1127 covariate flow cell on the basis of a parametric empirical Bayes framework. Biological
1128 replicates were summarized by their mean transformed and corrected expression values.
1129 For classical metric multidimensional scaling (MDS), analyses were implemented with R
1130 package stats version 3.4.4. Distance matrices were computed with amap version 0.8-16

1131 choosing centered Pearson (1-corr(x,y)) as the distance metric. Supplementary Figure 9
1132 shows MDS for all 15,966 orthologs and for the subset of 1,803 orthologs that were
1133 identified as potential signatures in at least one species. Correlation maps were created on
1134 Pearson correlated data, Figure 4 c) shows the results of correlation analysis for 302
1135 genes that were identified as potential neuronal projection signatures both in human and
1136 in mouse.

1137

1138 **Functional annotation and Enrichment Map pathway analysis visualization.**

1139 Pathway enrichment analysis was carried out by searching for enriched gene-sets (e.g.
1140 pathways, molecular functional categories, biological processes) in the human signature
1141 gene set versus the murine DEGs at P1 for each neuronal subtype by mapping functional
1142 information from pathways annotation collection (updated version April 2019) using
1143 gProfiler. The collection comprises Gene Ontology annotations (*Biological Process*,
1144 *Cellular Component*, *Molecular Function*) as well as Reactome, Panther Pathway,
1145 Msigdb C2 and Wikipathways terms. The resulting enrichment results were visualized
1146 with the Enrichment Map plugin for the Cytoscape network visualization and analysis
1147 software. We loaded gProfiler results using a FDR cut-off of 0.05. In these maps, each
1148 gene set is symbolized by a node in the network. Node size corresponds to the number of
1149 genes comprising the gene-set. The enrichment scores for the gene-set are represented by
1150 the node's color intensity (green for CPN, red for ScPN, violet for CThPN and yellow for
1151 CFuPN). The color of the node upper hemisphere indicates the enrichment score for
1152 mouse gene sets, and the lower hemisphere indicates the score for human gene sets. To
1153 intuitively identify redundancies between gene sets, the nodes are connected with edges if
1154 their contents overlap by more than 50%. The thickness of the edge corresponds to the
1155 size of the overlap. The edge belonging to human dataset were represented in dark gray,
1156 those corresponding to mouse dataset were represented in light gray. We used version
1157 1.1.0 of the Enrichment Map software in Cytoscape 3.8.0⁶⁹.

1158

1159 **Gene ontology and functional enrichment analysis.**

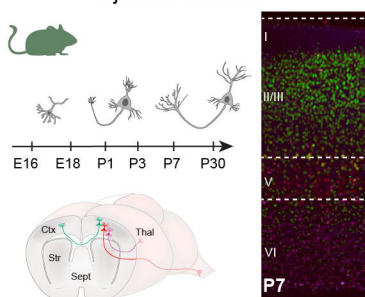
1160 From curated lists of genes, enriched biological pathways and molecular functions were
1161 identified using enrichGO function from an R package clusterProfiler (v3.14.3). We
1162 queried different databases for different organisms by setting OrgDb parameter
1163 (org.Hs.eg.db for human dataset and org.Mm.eg.db for mouse dataset, respectively) and
1164 reported only the significant terms and pathways by setting pvalueCutoff=0.05.
1165 Background list of genes were prepared for each dataset to include all the genes that are
1166 expressed in the dataset.

1167

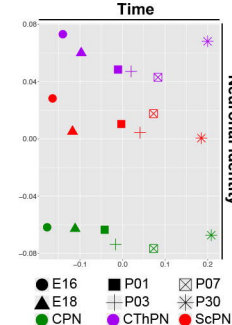
1168 **LDscore regression and partition heritability analysis.**

1169 Human gene coordinates corresponding to different cell types and cell type clusters
1170 (categories) were overlapped using bedtools (Version: v2.27.1-1-gb87c465) with the
1171 SNPs in the .bim file used for the computation of LD scores (1000 Genomes Phase 3
1172 downloaded from <https://data.broadinstitute.org/alkesgroup/LDSCORE/>), the resulting
1173 annotation files were used to calculate the annotation specific LD scores separately for
1174 each category using LD Score Regression (LDSC) Version 1.0.0. To assess each
1175 category's contribution to h^2 , a cell type specific analysis was performed for multiple
1176 disease traits, including Schizophrenia, Autism, Bipolar Disorder, Coronary Artery
1177 Disease (Table S 10) using their corresponding GWAS summary statistics (obtained here:
1178 <https://data.broadinstitute.org/alkesgroup/LDSCORE/>) and a modified baseline model
1179 v1.1 which contains 52 categories. To that end, we followed the CTS workflow according
1180 the software recommendations. This modification was done to add an extra category as a
1181 control (similar to the Finucane et al. 2018⁴⁸) that included all analyzed genes in our
1182 RNA-seq. After adjusting each category's p-value together with the baseline model 52+1
1183 categories to account for multiple testing, significant heritability enrichment was
1184 determined using $FDR < 0.05$.

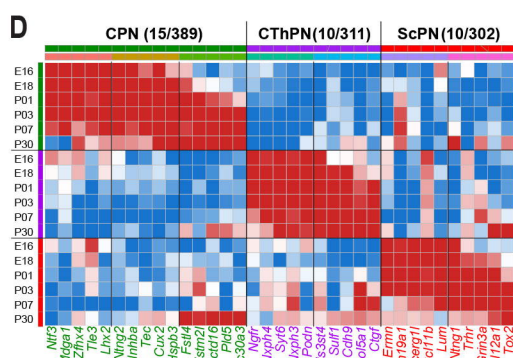
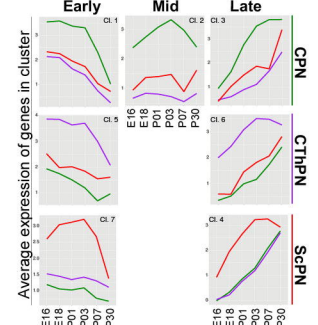
A Mouse Cortical Excitatory Projection Neurons



B Time

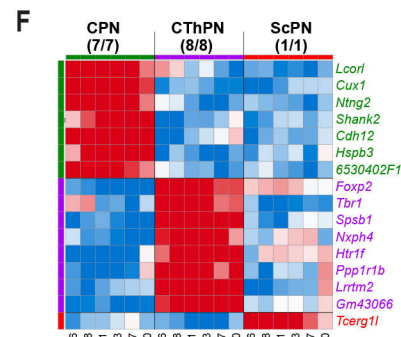
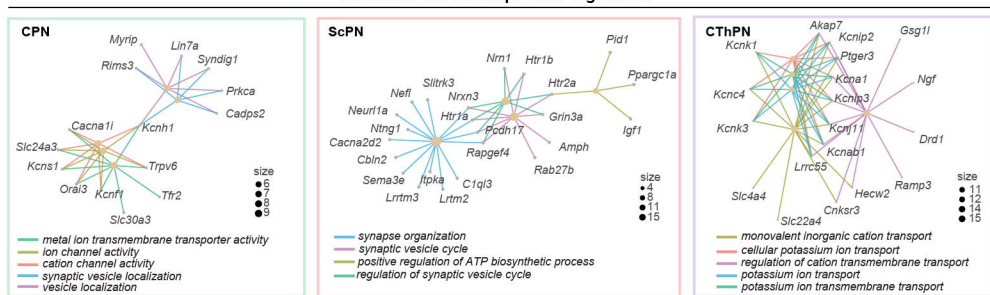


C Early Mid Late

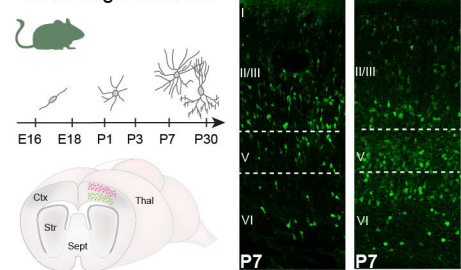


E

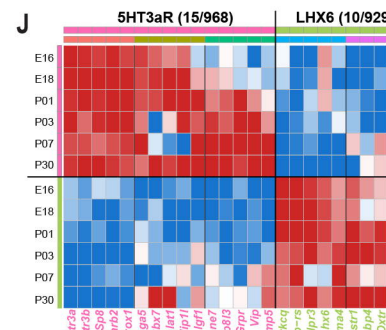
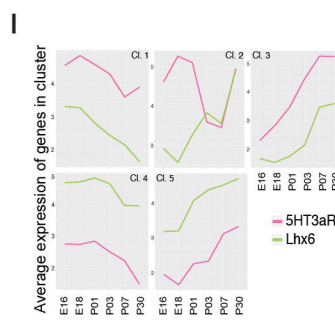
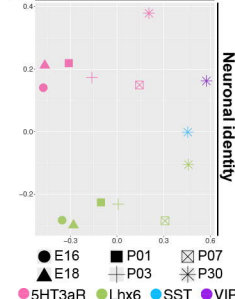
GO of PN Late Developmental Signatures



G Mouse Cortical GABAergic Neurons

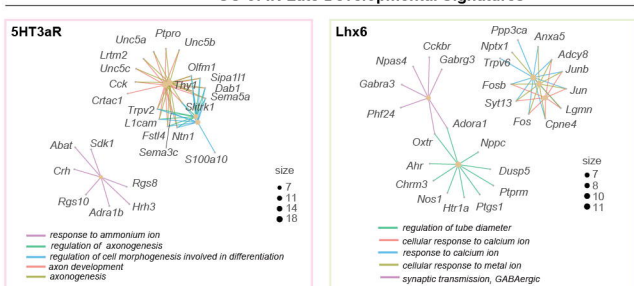


H Time

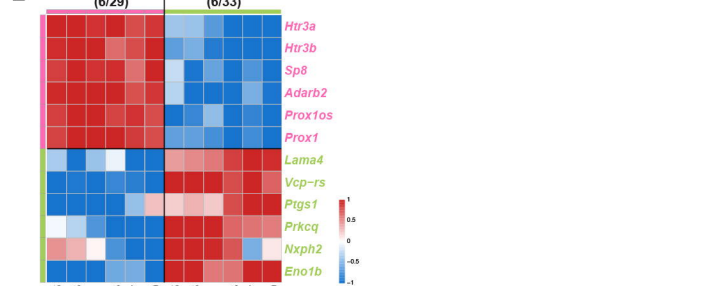


K

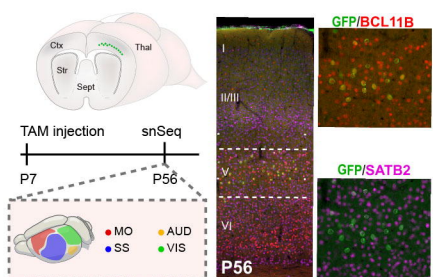
GO of IN Late Developmental Signatures



L 5HT3aR (6/29) LHX6 (6/33)

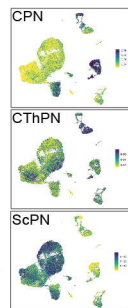
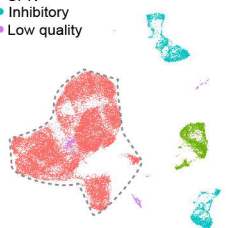


A



B Cell type annotation

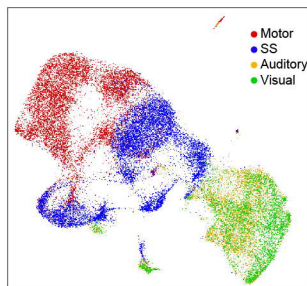
- ScPN
- CPN
- Inhibitory
- Low quality



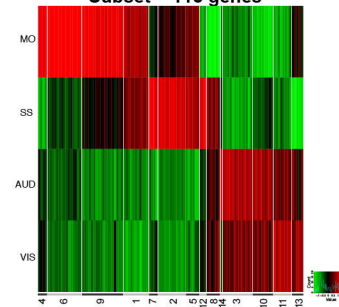
C ScPN late dev. signatures in ScPNs from SS area



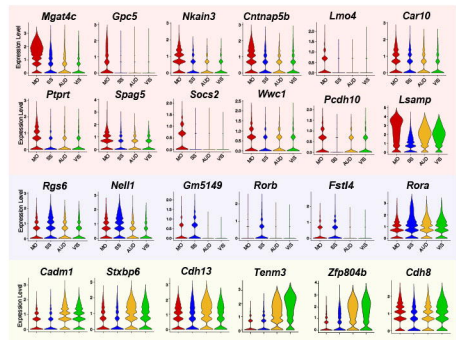
D ScPN by Functional area



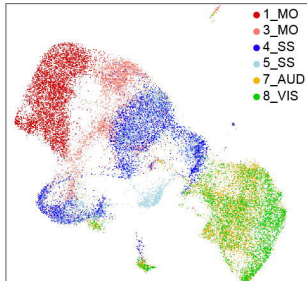
E Area-Specific Gene Modules Subset - 115 genes



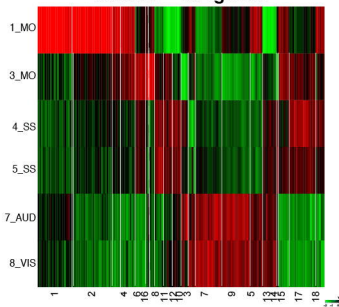
F



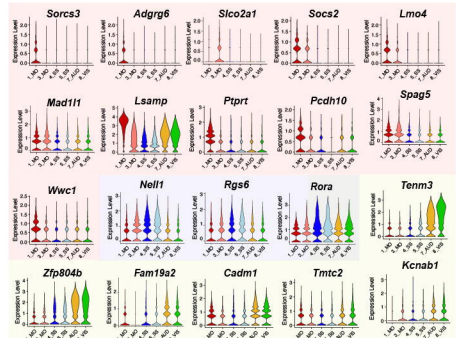
G ScPN by area and AP level



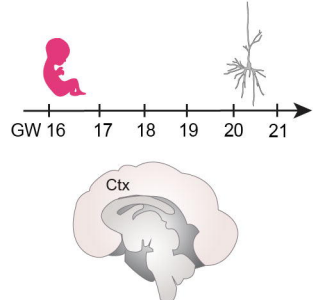
H AP level-Specific Gene Modules Subset - 162 genes



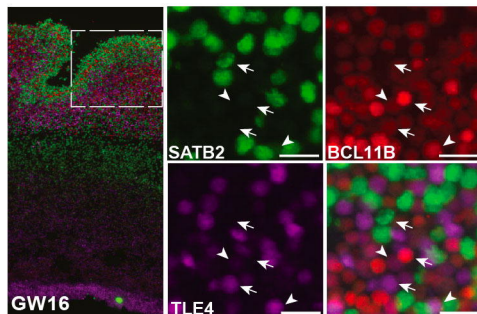
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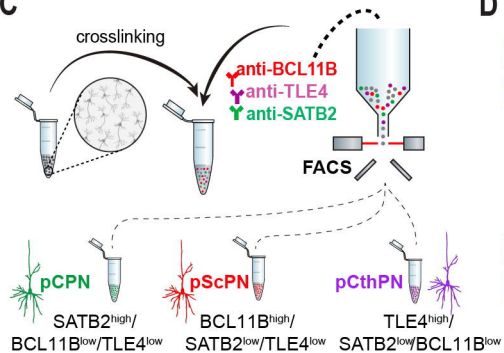
A Human Fetal Cortical Neurons



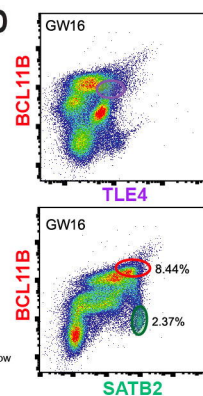
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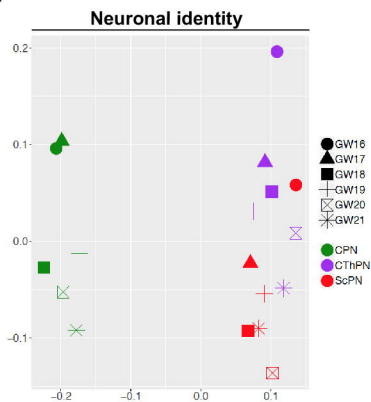
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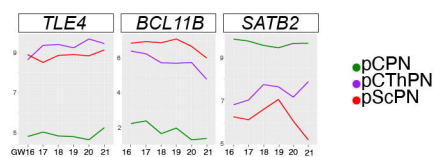
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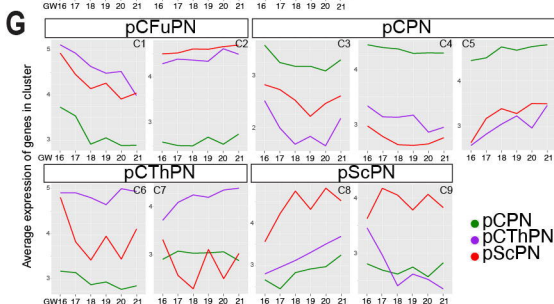
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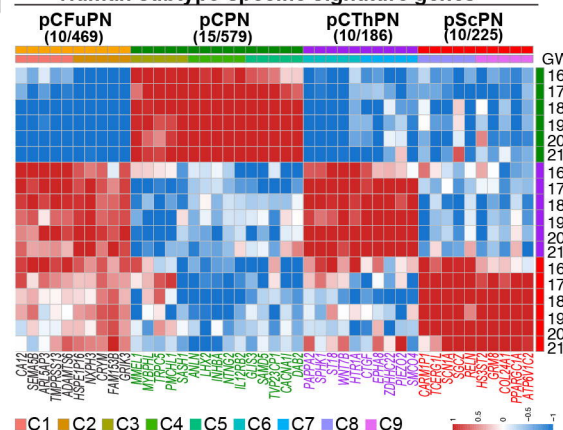
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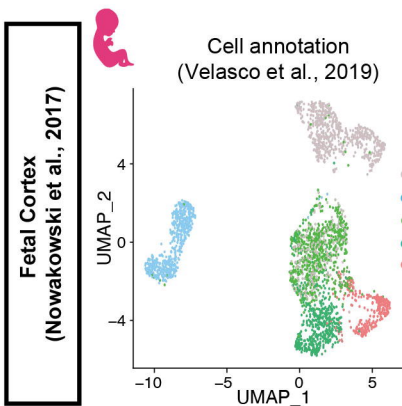
G



H Human subtype-specific signature genes

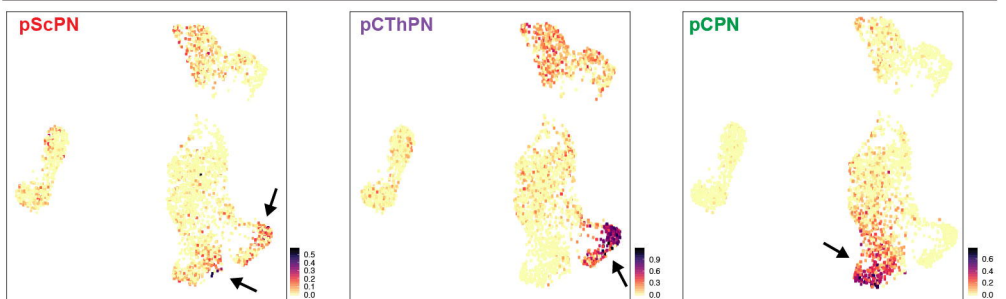


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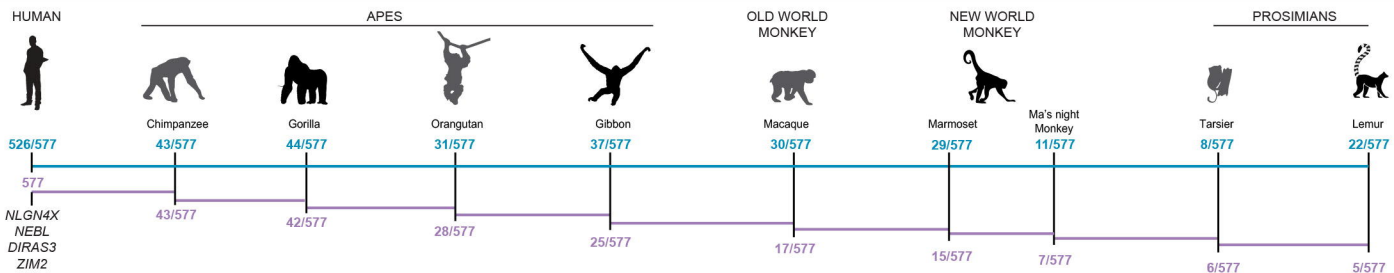
J

Subtype-specific signature gene module expression score



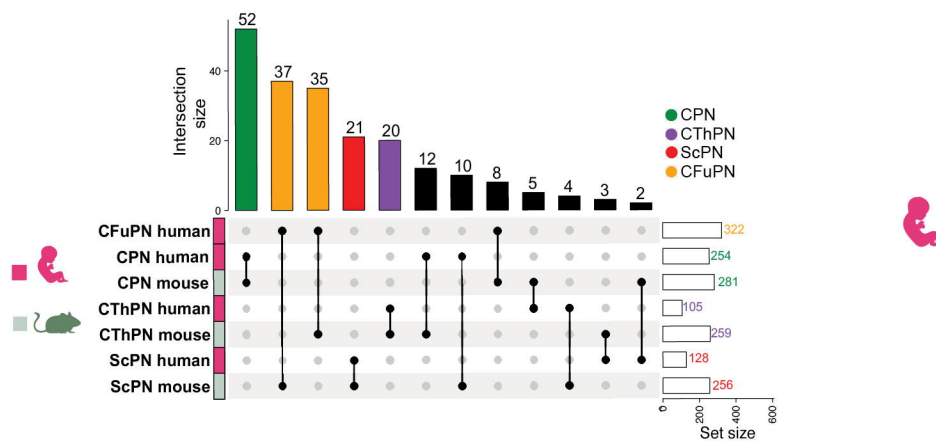
A

Conservation of human-specific signature genes in primates



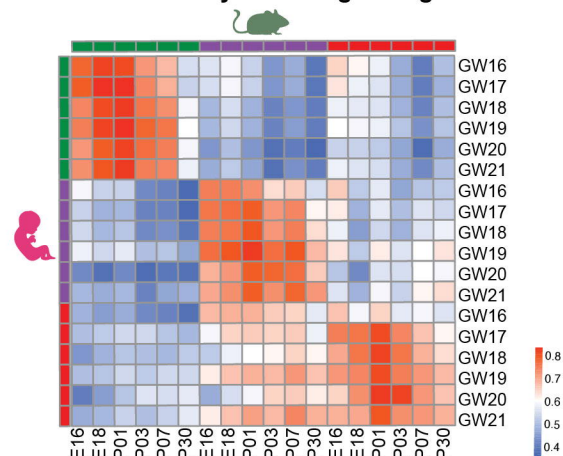
B

Conservation of PN subtype-specific signature in human and mouse

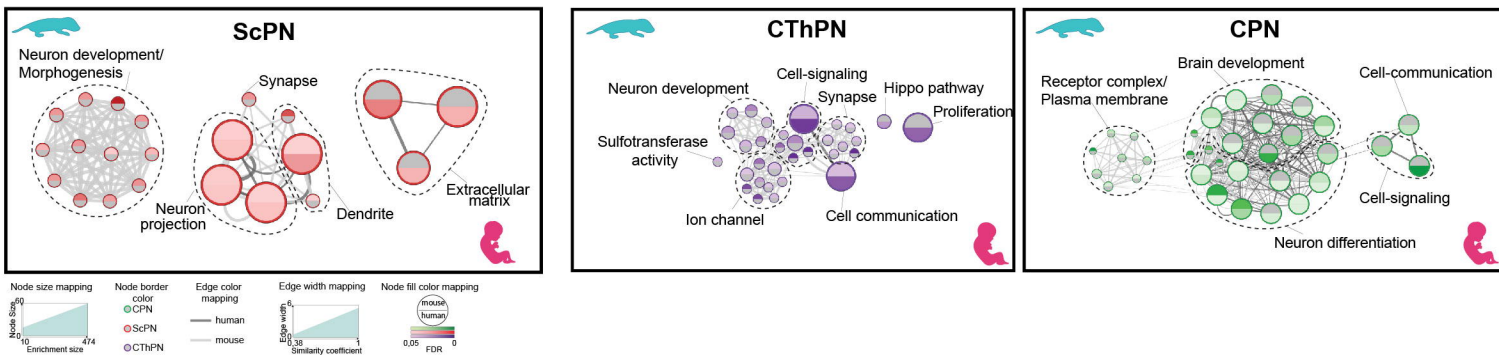


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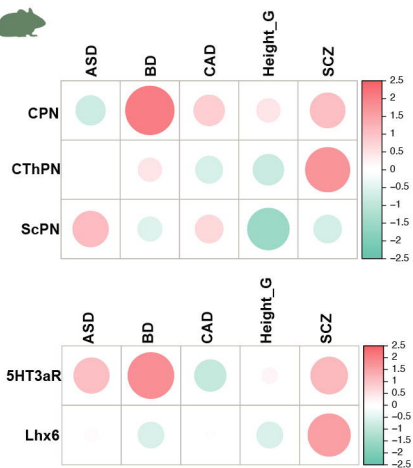
Correlation analysis among PN signatures



D

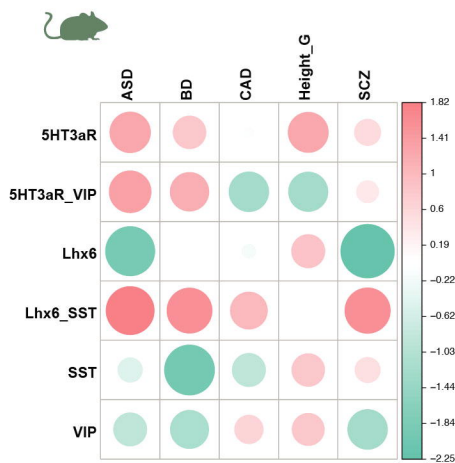


A

Cortical neuron developmental signatures
E16.5 → P30

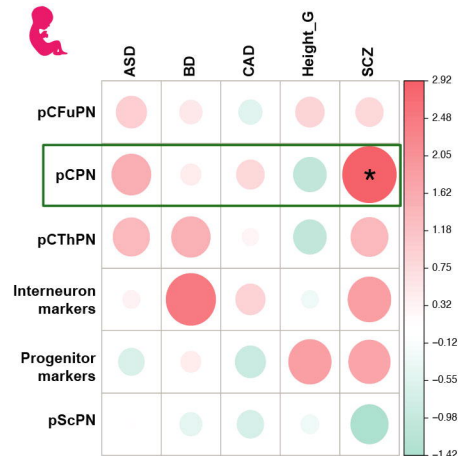
B

Interneuron signatures at P30

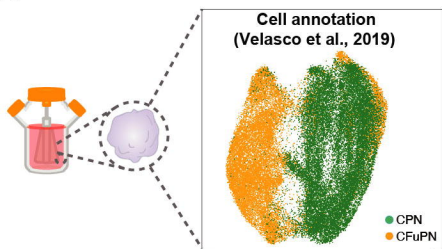


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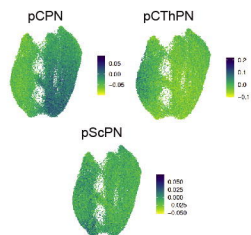
Fetal cortical neuron signature genes



D

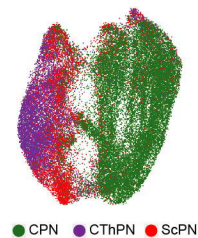


E

Subtype-specific signature gene
module expression score

F

Novel annotation



G

Cerebral Organoids

