TITLE

Identifying developing interneurons as a potential target for multiple genetic autism risk factors in human and rodent forebrain.

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ABSTRACT

Autism spectrum condition or ‘autism’ is associated with numerous monogenic and polygenic genetic risk factors including the polygenic *16p11.2* microdeletion. A central question is what neural cells are affected. To systematically investigate we analysed single cell transcriptomes from gestational week (GW) 8-26 human foetal prefrontal cortex and identified a subset of interneurons (INs) first appearing at GW23 with enriched expression of a disproportionately large fraction of risk factor transcripts. This suggests the hypothesis that these INs are disproportionately vulnerable to mutations causing autism. We investigated this in a rat model of the *16p11.2* microdeletion. We found no change in the numbers or position of either excitatory or inhibitory neurons in the somatosensory cortex or CA1 of *16p11.2*$^{+/-}$ rats but found that CA1 Sst INs were hyperexcitable with an enlarged axon initial segment, which was not the case for CA1

pyramidal cells. This study prompts deeper investigation of IN development as a convergent target for autism genetic risk factors.

KEYWORDS

Development, telencephalon, autism, genetics, *16p11.2*, GABAergic, human, rat, electrophysiology, AIS.

Contributions and acknowledgements: YY and IQ performed bioinformatic analysis of human data, SAB, JMC, and AS performed rat experiments and analysis, ZK and OD contributed to linear modelling, SM and YH provided the *16p11.2* microdeletion rats, PCK and DJP contributed to design, TP designed the study and wrote the paper. This work was funded by the Simons Initiative for the Developing Brain (SFARI - 529085) and Biotechnology and Biological Sciences Research Council (BB/M00693X/1).

INTRODUCTION

Autism spectrum conditions (ASC – referred to here as 'autism') describe several symptoms and behaviours which affect the way in which a group of people understand and react to the world around them (Mental Health Foundation) and may co-occur with other conditions including epilepsy and intellectual disability (ID). Recent efforts to understand the genetic landscape of autism identified hundreds of genetic risk factors predisposing to autism including *de novo* single gene mutation (5-10%), copy number variations (CNVs) and chromosome anomalies (5%), and inherited single gene mutations (3%), although most cases (80-85%) have no known genetic cause.

Genetic risk factors can be either 'monogenic' where a single gene mutation is sufficient to predispose to autism or 'polygenic' where one mutation directly affects several genes simultaneously. CNVs are an example of the latter where chromosomal microduplication or microdeletion affect the gene dosage of multiple genes. The Simons Foundation Autism Research Initiative (SFARI) curates a list of ~1000 monogenic genetic risk factors of which 83 are categorised as the highest ranking (Categories 1 'high confidence' and 2 'strong candidates') indicating a robust

association between mutation in these genes and autism. SFARI lists 2240 CNVs associated with autism of which *16p11.2* microdeletion and microduplication are among the most frequent accounting for approximately 1% of cases. The *16p11.2* CNV comprises 27 protein coding genes so is a polygenic risk factor although it is currently unknown the extent to which each of the *16p11.2* genes are individual risk factors.

Autism manifests in early infancy and then persists into later life. A number of lines of evidence suggest that events during brain development *in utero* contribute to the subsequent development of symptoms (Packer, 2016). The developing cerebral cortex is comprised of three neuronal cardinal cell classes (progenitors, excitatory neurons, and inhibitory neurons) and three non-neuronal cardinal cell classes (astrocytes, oligodendrocyte precursors, and microglia). One important factor for cerebral cortex function is the balance between glutamatergic, excitatory principal neurons, which originate from progenitors located in the ventricular zone of the cerebral cortex, and inhibitory GABAergic interneurons (INs) which originate from progenitors located in the ganglionic eminences and then migrate into the cortex and integrate within functional circuits of the cortical plate (Hansen et al., 2013; Ma et al., 2013). Changes in the number, position, anatomy, or electrophysiology of inhibitory or excitatory neurons may perturb the excitatory/inhibitory balance (the E/I balance) and is hypothesised to be a convergent mechanism in autism and its co-occuring conditions (Bozzi et al., 2018; Nelson and Valakh, 2015; Puts et al., 2017; Rapanelli et al., 2017; Robertson et al., 2016) (Antoine et al., 2019)

The aim of the current study is to systematically identify types of cell in developing human cerebral cortex that are potentially vulnerable to autism risk factors using a single cell mRNA sequencing (scRNA-seq) dataset acquired from developing human foetal cortex spanning gestational weeks (GW) 8 to 26 (Zhong et al., 2018). While we found that some autism associated transcripts are differentially expressed between the cardinal cell classes our most striking finding was that a subset of differentiating INs first appearing at GW23 exhibited enriched expression of a strikingly high proportion of risk transcripts. This molecular analysis suggests the hypothesis that a large number of monogenic risk factors and the polygenic *16p11.2* microdeletion selectively target IN development resulting in IN phenotypes postnatally that contribute to autism and its comorbid conditions. We support this hypothesis using a *16p11.2*

94 microdeletion rat model where we identified hypersensitive electrophysiology and
95 enlarged axon initial segment (AIS) phenotypes in somatostatin (Sst) expressing
96 hippocampal INs.

97

98 **METHODS.**

99 **Datasets**

100 Three published cortical transcriptome datasets were used in this study to explore the
101 gene expression pattern of autism-associated genes during cortical development.

102 The raw gene expression matrix in the scRNA-seq data of human foetal PFC was
103 obtained from the Gene Expression Omnibus (GEO) under the accession number
104 GSE104276, then the data was normalized as the original paper described (Zhong et
105 al., 2018). We used the authors' original classification of six cardinal cell classes (NPC,
106 ExN, IN, OPC, Astrocyte and Microglia).

107 The expression matrix of genes in the adult human cortical single nuclei RNA-seq data
108 were downloaded under the accession number of GSE97930 (Lake et al., 2018). In
109 the dataset from Lake et al., only cells that identified as "INs" were used for further
110 analysis. The original eight interneuron clusters were grouped based on the
111 expression pattern of marker genes (In1/2/3 as VIP, In4 as NG, In6 as PV, In7/8 as
112 SST, Figure 2B,C in Lake et al., 2018).

113 For the mouse scRNA-seq datasets at of Dlx6a-cre fate-mapped cortical inhibitory
114 neurons, the pre-processed Seurat objects were downloaded from the author's share
115 link (https://www.dropbox.com/s/qe2carqnf9eu4sd/Filtered_Mayer-et-
116 al.Rda.zip?dl=0) (Mayer et al., 2018). We used the authors' original classification of
117 seven IN cell types (Sst, Pvalb, Vip, Id2, Nos1, Th and Igfbp6).

118 All three datasets were converted into Seurat objects by R package Seurat (version
119 2.3.0) for further analysis. In detail, in the dataset from Zhong et al., raw read counts
120 were normalized based on the original paper described. Any cells with less than 1000
121 genes expressed were removed, and any gene expressed by less than 3 cells at less
122 than 1 normalized expression value was removed. Pseudogenes, miRNA, rRNA,
123 mitochondrial associated and ribosome related genes were excluded from further

124 analysis. The filtered gene expression matrix and the classification of the cardinal cell
125 classes were used to create a Seurat object. We also create a Seurat object for the
126 dataset from Lake et al. using the same procedure. The pre-processed Seurat object
127 from Mayer et al was not changed. The scRNA-Seq data was also analyzed with
128 BBrowser version 2.2.44 (SingleCell).

129 **Lists of autism risk genes**

130 Monogenic autism associated genes were downloaded from the SFARI database
131 (released May 2019) (https://gene.sfari.org/database/human-gene/) and the 83
132 highest ranking (SFARI 1+2) were analysed as these genes are significant statistically
133 in genome-wide studies between cases and controls. Besides these monogenetic
134 genes, the copy number variance (CNV) of genetic loci (CNV genes), either deletions
135 or duplications, are also linked to autism. We selected the 27 protein coding genes at
136 the *16p11.2* locus since both duplication and deletion of these genes has been linked
137 to significantly increased incidence of autism representing a potentially polygenic
138 cause of autism.

139 **Clustering and visualization of cell types**

140 The identification of six cardinal cell classes were obtained from the original paper and
141 re-plotted in a two-dimensional space of t-Distributed Stochastic Neighbor Embedding
142 (tSNE). In details, the highly variable genes (HVGs) were identified using Seurat
143 function FindVariableGenes. The mean of logged expression values was plotted
144 against variance to mean expression level ratio (VMR) for each gene. Genes with log
145 transformed mean expression level between 1 and 8, and VMR lower than 1.2 were
146 considered as highly variable genes. Then principal component analysis (PCA) was
147 performed with RunPCA function in Seurat using HVGs to analyze all the cells.
148 Following the PCA, we conducted JACKSTRAW analysis with 100 iterations to identify
149 statistically significant (p value < 0.01) principal components (PCs) that were driving
150 systematic variation. We used tSNE to present data in two-dimensional coordinates,
151 generated by RunTSNE function in Seurat, and the first 7 significant PCs identified by
152 JACKSTRAW analysis were used as input to RunTSNE function. Perplexity was set
153 to 20. t-SNE plot and the violin plot were generated using R package ggplot2.

154 We further clustered the three cardinal cell classes (NPC, ExN and IN) from the foetal
155 cortical dataset. Due to the different number of cells and the variant gene expression

156 pattern in each cardinal cell class, the HVGs were identified using the same method
157 but with the different parameters. For the cells in NPC, genes with log transformed
158 mean expression level between 0.5 and 8, and VMR lower than 1.2 were considered
159 as highly variable genes. For the cells in ExN and IN classes, genes with log
160 transformed mean expression level between 1 and 10, and VMR lower than 0.5 were
161 considered as highly variable genes. Then the statistically significant PCs were
162 calculated by JACKSTRAW analysis and used as input to get tSNE coordinates.
163 Clustering was done with Luvain Jaccard algorithm using t-SNE coordinates by
164 FindClusters function from Seurat. The resolution parameters used to IDENTIFY
165 clusters within the three cardinal cell classes were: NPC, resolution = 1; ExN,
166 resolution = 0.1; and IN, resolution = 0.5. Other parameters that we left at default.

167 **Identification of differential expressed genes**

168 All differential expression (DE) analyses were conducted using Seurat function
169 *FindAllMarkers*. In brief, we took one group of cells and compared it with the rest of
170 the cells, using Wilcoxon rank sum test. For any given comparison we only considered
171 genes that were expressed by at least 33% of cells in either population. Genes that
172 exhibit p values under 0.05, as well as log fold change over 0.33 were considered
173 significant. All heatmaps of DE analysis were plotted using R package pheatmap
174 (Figure 1C, Figure 3A and 3B, and Figure 5D).

175 **MetaNeighbor analysis**

176 MetaNeighbor analysis was performed using the R function MetaNeighbor with default
177 settings (Crow et al., 2018). The AUROC (Area under the Receiver Operating
178 Characteristic) scores produced by MetaNeighbor analysis indicate the degree of
179 correlation between cell clusters. Three gene lists were used as input to do
180 MetaNeighbor analysis among the 21 clusters of human foetal dataset: Highly variable
181 genes (HVGs) identified as significant differentialy expressed genes (DEGs) between
182 the clusters (Figure S2A); monogenic autism risk genes (Figure S2B); and*16p11.2*
183 genes (Figure S2C). The results from the MetaNeighbor analysis were plotted as a
184 heatmap using the gplots function heatmap. For a given gene set each pairwise
185 comparison between cell clusters is given an AUROC score ranging from 1.0 (red on
186 the heatmap) indicating that cells were highly probable to be of the same type to 0.0
187 (blue on the heatmap) indicating that it was highly improbable that the cells were of

188 the same type. A score on 0.5 (yellow on the heatmap) indicates that the gene set
189 used was unable to distinguish between the cells better than by chance.

190 **Projection based on multiple datasets**

191 We conducted canonical correlation analysis (CCA) and k-nearest neighbors analysis
192 (KNN) as we previous described to classify the cell types of foetal INs based on the
193 cell type features in the adult transcriptomics datasets (Mi et al., 2018). Briefly, we first
194 performed random forest analysis within HVGs to do feature selection for both foetal
195 and adult human cortical INs. Then we selected the shared HVGs between two
196 datasets that best represented the feature of IN cell types. The HVGs were used as
197 input gene list to RunCCA function, and the first 4 dimensions were used as input to
198 AlignSubspace function. The aligned projection vectors were used as input to do
199 dimensional reduction by RunTSNE function. Perplexity was set to 40. We used the
200 two t-SNE coordinates for adult cells to conduct KNN and re-assign foetal IN identities
201 using the knn.cv function from R package FNN. A foetal IN was assigned the identity
202 represented by the majority, and at least 5, of its closest 30 neighbours; in case of ties,
203 the cell remains unassigned. t-SNE plots, and the bar plots were generated using R
204 package ggplot2.

205 **Gene ontology analysis**

206 The resulting gene list, ordered by sign-adjusted P value, was the input for gene set
207 enrichment analysis to test for enriched gene ontology (GO) terms using the
208 clusterProfiler package version 3.4.4 with default settings. GO term analysis was
209 performed on three categories (Biological process. Molecular function. Cellular
210 component), and gene sets with a BH adjusted $P < 0.05$ were considered to be
211 significantly enriched. The top three significant GO terms in each category were
212 plotted by R package ggplot2.

213 **Animals**

214 All rats were bred in-house according to Home Office UK legislation and licenses
215 approved by the University of Edinburgh Ethical Review Committees and Home Office.
216 Animal husbandry was in accordance with UK Animals (Scientific Procedures) Act of
217 1986 regulations. Rat 16p11.2 DEL rat model (*16p11.2*$^{+/-}$) was generated by
218 CRISPR/Cas9 genome editing of the Sprague Dawley line (Qiu et al., 2019). Rats

were maintained on the Sprague Dawley background. P21 rat tissue was fixed by transcardial perfusion with 4% paraformaldehyde in PBS, brains were then dissected and immersed in 4% paraformaldehyde in PBS overnight at 4°C.

In Situ Hybridisation and Immunofluorescence labelling

Brains were cryoprotected in 30% sucrose in PBS, embedded in OCT and sectioned at a thickness of 10µm using a cryostat (Leica, CM3050 S). Frozen sections were then mounted on SuperFrost Plus™ slides (Thermo Fisher). *Gad1* In situ hybridisation on frozen sections was performed as previously described (Wallace and Raff, 1999). NeuN Immunofluorescence was performed following in situ hybridisation as described previously (Clegg et al., 2014) with rabbit anti-NeuN (1/300, Abcam) Secondary antibodies used were donkey anti-goat Alexa Fluor 488 and donkey anti-rabbit Alexa Fluor 568 (both used 1/200 and from Life Technologies). Tissue was counterstained using DAPI (1/1000, Life Technologies).

Axon initial segment (AIS) labelling, was performed as previously described (Oliveira et al., 2020). Briefly, rats were perfused as described above, then post-fixed for 1 hour at room temperature with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were then transferred to 0.1 M phosphate buffered saline (PBS) and 60 µm thick coronal slices containing the CA1 region of hippocampus were cut on an oscillating blade vibratome (Leica VT1000, Leica, Germany) and transferred to PBS. Briefly, sections were rinsed in PBS then transferred to a blocking solution containing 10% normal goat serum, 0.3% Triton X-100 and 0.05% NaN_3 diluted in PBS for 1 hour. Primary antibodies raised against AnkyrinG (1:1000; 75-146, NeuroMab, USA) and somatostatin (Somatostatin-14, T-4102.0400; 1:1000, Peninsula Labs, USA) were applied in PBS containing 5% normal goat serum, 0.3% Triton X-100 and 0.05% NaN_3 for 24-72 hours at 4 °C. Slices were washed with PBS and then secondary antibodies applied (Goat anti-rabbit AlexaFluor 488 and Goat anti-mouse AlexaFluor 633, Invitrogen, UK, both 1:500) in PBS with 3% normal goat serum, 0.1% Triton X-100 and 0.05% NaN3 added, overnight at 4°C. Sections were then washed with PBS, desalted in PB, and mounted on glass slides with Vectashield® mounting medium (Vector Labs, UK). Confocal image stacks of either the *str. pyramidale* or *str. oriens/*alveus border were acquired on a Zeiss LSM800 laser scanning microscope equipped with a 63x (1.4 NA) objective lens at 1024x1024 resolution (step size of 0.25 µm). Individual AIS

were measured offline using ImageJ as segmented lines covering the full extent of AnkyrinG labelling observed. As in SSt INs the AIS often emerges from a proximal dendrite, they were only identified where they emerged from a clearly fluorescent labelled dendrite. A minimum of 25 AIS were measured from each rat.

For identification of somatostatin INs, slices were fixed following whole-cell patch-clamp recording (see below) and fixed overnight in 4% PFA in 0.1 M PB. Immunofluorescent labelling was performed according to the same protocol as above, but excluding the AnkyrinG antibody. Secondary antibodies (goat anti-rabbit AlexaFluor488, 1:500, Invitrogen, Dunfermline, UK) were applied with the added inclusion of fluorescent-conjugated streptavidin (Streptavidin AlexaFluor 633, 1:500, Invitrogen, Dunfermline, UK) to visualise recorded neurons.

Imaging

All fluorescence images were acquired using either a Leica AF6000 epifluorescence microscope coupled to a Leica DFC360 digital camera running Leica LAS-X software, or a Nikon Ti: E Inverted confocal microscope running Nikon NIS-Elements Confocal software.

NeuN/Gad1 Cell Quantification

Gad1$^+$ and NeuN$^+$ cells within the cortex were quantified by counting *Gad1*$^+$ (red) and NeuN$^+$ cells (green) within a 200µm wide column spanning the somatosensory cortex (indicated region, Figure 5A). *Gad1*$^+$ and NeuN$^+$ cell position was quantified by counting cells in 10 adjacent counting bins within the same 200µm wide column spanning the somatosensory cortex.

Gad1$^+$ and NeuN$^+$ cells within the hippocampus were quantified by counting cells within the *str. oriens* and *str. pyramidale* of the CA1 region (indicated region, Figure 5A). To control for the varying size of the counting area *Gad1*$^+$ cell number was expressed as *Gad1*$^+$ cells per length (mm) of the CA1 region, length was measured along the centre of the *str. pyramidale*. Gad1$^+$ cells were classified as belonging to *str. pyramidale* if in contact with NeuN$^+$;*Gad1*$^-$ pyramidal cells, all other hippocampal

281 *Gad1*$^{+}$ cells superficial to this layer were classified as belonging to *str. oriens.* All
282 measurements and quantification was performed using FIJI software.

283

284 ***In vitro* slice electrophysiology***:*

285 Acute rat brain slices were prepared as previously described (Oliveira et al., 2021).
286 Briefly, rats were decapitated without anaesthesia and the brain rapidly dissected into
287 ice-cold sucrose-modified artificial cerebrospinal fluid (ACSF; in mM: 87 NaCl, 2.5 KCl,
288 25 $NaHCO_3$, 1.25 NaH_2PO_4, 25 glucose, 75 sucrose, 7 $MgCl_2$, 0.5 $CaCl_2$), which was
289 saturated with carbogen (95 % O2/5 % CO2). 400 µm horizontal brain slices were cut
290 on a vibratome (VT1200S, Leica, Germany) and transferred to sucrose-ACSF at 35°C
291 for 30 min and then room temperature until needed.

292 For whole-cell patch-clamp recordings slices were transferred to a submerged
293 recording chamber flowing with pre-warmed ACSF (in mM: 125 NaCl, 2.5 KCl, 25
294 $NaHCO_3$, 1.25 NaH_2PO_4, 25 glucose, 1 $MgCl_2$, 2 $CaCl_2$), bubbled with carbogen, and
295 perfused a rate of 4-6 $mL.min^{-1}$ at 30± 1 °C). Slices were viewed under infrared
296 differential inference contrast microscopy with a digital camera (SciCamPro,
297 Scientifica, UK) mounted on an upright microscope (SliceScope, Scientifica, UK) with
298 40x water-immersion objective lens (1.0 N.A., Olympus, Japan). Recording pipettes
299 were pulled from borosilicate glass capillaries (1.7 mm outer/1mm inner diameter,
300 Harvard Apparatus, UK) on a horizontal electrode puller (P-97, Sutter Instruments, CA,
301 USA), which when filled with a K-gluconate based internal solution (in mM 142 K-
302 gluconate, 4 KCl, 0.5 EGTA, 10 HEPES, 2 $MgCl_2$, 2 Na_2ATP, 0.3 Na_2GTP, 1
303 Na_2Phosphocreatine, 2.7 Biocytin, pH=7.4, 290-310 mOsm) which resulted in a 3-
304 5 MΩ tip resistance. Cells were rejected if: they were more depolarised than -50 mV,
305 had series resistance >30 MΩ, or the series resistance changed by more than 20%
306 during the recording. Recordings were performed with a MultiClamp 700B (Molecular
307 Devices, CA, USA) amplifier and filtered online at 10 kHz with the built-in 4-pole Bessel
308 filter and digitized at 20 kHz (Digidata1550B, Molecular Devices, CA, USA).)

309 Cells were identified either as CA1 pyramidal cells (CA1 PCs) with having large, ovoid
310 somata located in *str. pyramidale* and an apical dendrite entering *str. radiatum* or
311 somatostatin INs having bipolar, horizontally oriented somata at the *str. oriens*/alveus
312 border. All intrinsic membrane properties were measured in current-clamp. Passive

313 membrane properties, included resting membrane potential, membrane time constant,
314 and input resistance, were measured from hyperpolarising steps (-10 pA, 500 ms
315 duration), from resting membrane potential. Active properties were determined from a
316 series of hyper- to depolarising current steps (-500 to +500 pA, 500 ms) from a holding
317 potential of -70mV, maintained with a bias current injection. All AP properties were
318 determined from the first AP elicited above rheobase. Spontaneous EPSCs were
319 measured in voltage-clamp from a holding potential of -70mV and detected offline
320 based on a triexponential curve fit and a threshold of 3*SD of the baseline noise.
321 Traces were collected in pCLAMP 9 (Molecular Devices, CA, USA) and stored on a
322 desktop computer. Analysis of electrophysiological data was performed offline Stimfit
323 (Guzman, Schlögl, and Schmidt-Hieber 2014), blind to both genotype. All data from
324 somatostatin INs is shown only for those cells where clear immunofluorescent labelling
325 was detected at the level of the soma.

326

327 **RESULTS.**

328 **Differential expression of autism risk genes among foetal cortical cardinal cell**
329 **classes**

330 A general supposition is that functional disruption of a gene will more likely affect the
331 cells expressing high levels of it's transcript. Based on such a principle, a cell type
332 expressing high levels of an autism associated transcript is regarded as potentially
333 vulnerable to genetic mutation in that gene with the resulting cellular phenotype
334 contributing to the development of autism. Accordingly, we have calculated differential
335 expression of autism associated transcripts among cell types in foetal human cerebral
336 cortex to identify cells potentially vulnerable to autism genetic risk factors during brain
337 development.

338 We started with a scRNA-seq dataset comprising 2306 cells taken from human foetal
339 pre-frontal cortex spanning gestational weeks (GW) 8 to 26 (Zhong et al., 2018). Six
340 cardinal cell types were identified in the authors' original classification, including neural
341 progenitor cells (NPCs), excitatory neurons (ExN), interneurons (IN), oligodendrocyte
342 precursor cells (OPCs), astrocytes, and microglia (Fig. 1A,B and Fig. S1A,B).
343 Differentially expressed genes (DEGs) were calculated across these six cardinal cell
344 classes. Based on the DEGs, we find that the six cell classes showed distinct cardinal

Figure 1

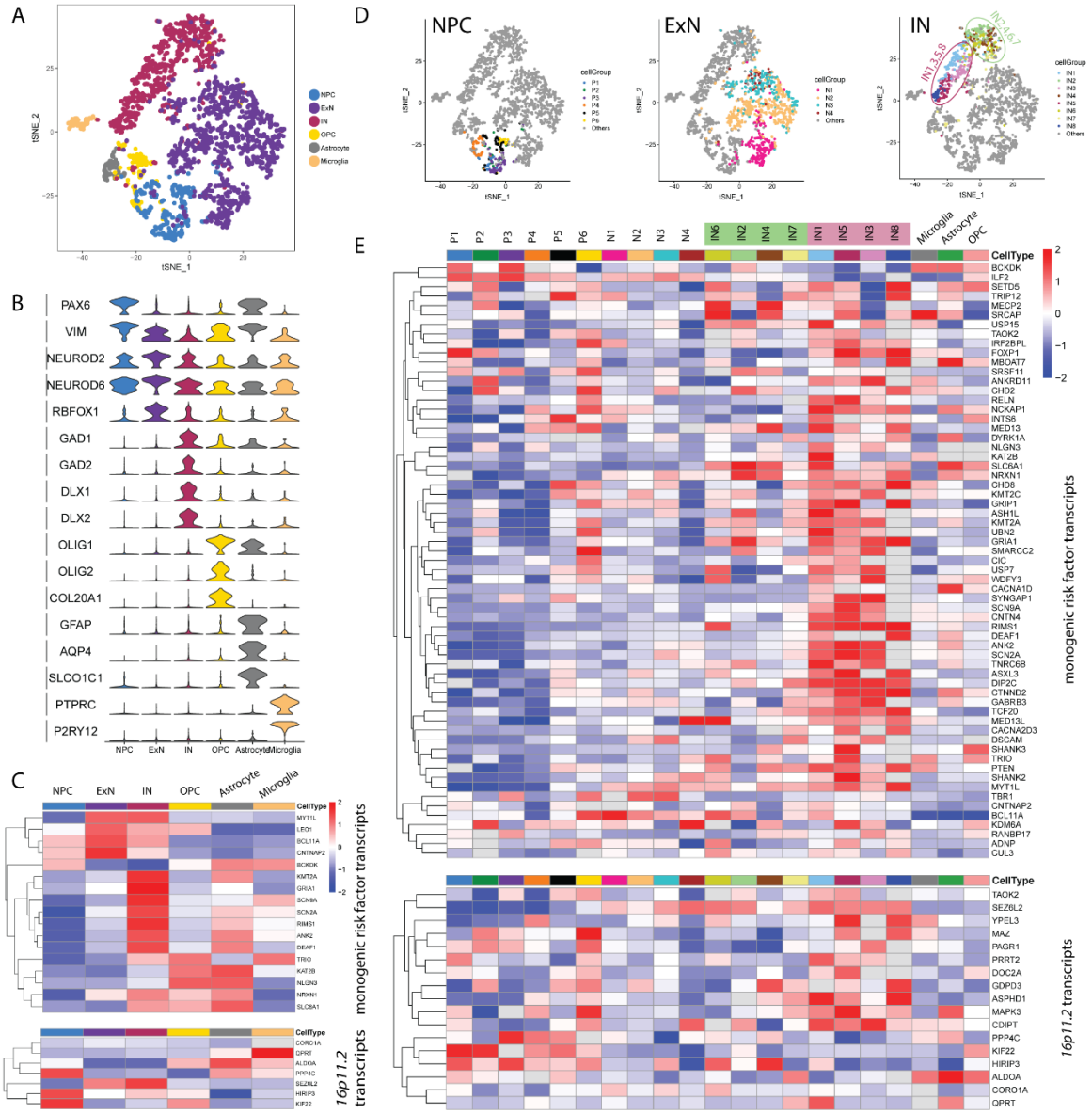


Figure 1: (A-C) Differentially expression of autism risk factor transcripts among foetal cortical cardinal cell classes. (A) *t*-SNE plot showing the cardinal cell classes identified in the dataset. (B) Violin plot illustrating the expression pattern of marker genes among the six cardinal cell classes. (C) Heatmap illustrating the expression pattern of significantly differentially expressed autism risk factor transcripts across cardinal cell classes (Wilcox test, adjust $p < 0.05$, log (fold change) > 0.3). Top: monogenic autism risk factor transcripts; Bottom: *16p11.2* transcripts. (D,E) Unsupervised clustering within the cardinal classes and similarity comparison between cell clusters. (D) Unsupervised clustering subdividing the cardinal classes into 21 different cell clusters. OPC, astrocytes, and microglia were not further clustered. (E) Heatmap illustrating the

356 expression pattern of differentially expressed autism risk factor transcripts across 21
357 cell clusters (Wilcox test, adjust $p < 0.05$, log (fold change) > 0.3) for differentially
358 expressed monogenic autism risk factor transcripts (top panel) and differentially
359 expressed *16p11.2* transcripts (bottom panel).

360

361 class aggregation and specific gene expression profiles associated with neural
362 progenitor cells (NPC), excitatory neurons (ExN), inhibitory neurons (IN),
363 oligodendrocyte precursors (OPC), astrocytes, and microglia. A list of well-known cell
364 class markers that are included in the DEGs was used to illustrate the classification
365 across six cardinal cell classes (Nowakowski et al., 2017, Pollen et al., 2014, Camp et
366 al., 2015) (Fig. 1B). The markers used to identify different cardinal cell classes were:
367 *PAX6*, *HES2* and *VIM* (NPCs); *NEUROD2*, *NEUROD6* and *RBFOX1* (ExNs); *GAD1*,
368 *GAD2*, *DLX1* and *DLX2* (INs); *OLIG1*, *OLIG2* and *COL20A1* (OPCs); *GFAP*, *AQP4*
369 and *SLCO1C1* (astrocytes); *PTPRC* and *P2RY12* (microglia). The expression pattern
370 of these marker genes show that the cardinal cell classes were correctly represented
371 in our analysis (Fig 1B).

372 Then we identified the expression pattern of autism risk factor transcripts across the
373 cardinal cell classes and found that 17/83 high confidence and strong candidate
374 monogenic risk factor transcripts and 7/27 *16p11.2* transcripts were significantly
375 differentially expressed between cardinal cells classes (Fig 1C). A heatmap of
376 expression of the monogenic autism risk factor transcripts (Fig 1C – top) and the
377 *16p11.2* transcripts (Fig 1C – bottom) shows expression of each autism risk factor
378 transcript (rows) in each of the six cardinal cell classes (columns). Transcript levels
379 with expression greater than average across the cardinal classes are shown in red,
380 while transcripts with lower than average expression are shown in blue. There was no
381 obvious pattern to suggest that any cardinal class was particularly vulnerable to a large
382 proportion of either monogenic autism genetic risk factors.or the *16p11.2*
383 microdeletion.

384

385

386 **Identification of human foetal INs potentially disproportionately vulnerable to**
387 **genetic autism risk factors.**

388 The single-cell approach allows us to investigate the variability of highly expressed
389 genes among molecularly defined cell subpopulations and identify cells within cardinal
390 classes which may be vulnerable to genetic autism risk factors. Based on
391 unsupervised clustering, we subdivided the cardinal classes into 21 different cell
392 clusters (Fig. 1D): 6 for NPCs (P1-6); 4 for ExNs (N1-4); and 8 for INs (IN1-8). The
393 non-neuronal cardinal cell classes (OPC, astrocytes, and microglia) contained small
394 numbers of tightly clustered cells and were not further subdivided.

395 We clustered the 21 clusters according to transcriptomic similarity using
396 MetaNeighbour analysis with ~2000 highly variable genes (Fig. S2A) and used this
397 ordering to generate a heatmap of the 62/83 monogenic risk factor transcripts (Fig.
398 1E, top) and 17/27 *16p11.2* transcripts genes (Fig. 1E, bottom) that were significantly
399 differentially expressed between clusters. Violin plots of all autism risk transcripts
400 (including those not differentially expressed between clusters) for the 83 monogenic
401 autism risk transcripts (Fig. S3A) and 27 *16p11.2* transcripts (Fig. S3B) show the
402 expression profile in each cluster.

403 Of the differentially expressed monogenic risk factor transcripts (Fig 1E, top) a few
404 were enriched in progenitor cells, (for example *IRF26PL*, *BCL11A*, and *CHD2*), with
405 fewer transcripts enriched in excitatory neurons (for example *TBR1*). Other transcripts
406 were expressed across many clusters (for example *KMT2A, ILF2, SMARCC2,*
407 *SRSF11, UPF3B, TNRC6B*), while others showed relatively low expression across cell
408 types (for example *GRIN2B, MAGEL2, and MET*). A striking feature of the heatmap
409 was a preponderance of relatively high gene expression (red shading) in the IN cell-
410 types (for example *SCN2A*, *SCN9A, DEAF1, SHANK2, RIMS1, GRIP1, SYNGAP1*),
411 which was most apparent for the IN subgroups IN1,3,5,8 (purple highlight in Fig. 2E
412 and circle in Fig. 1D) compared to IN2,4,6 7 (green highlight in Fig. 2E and circle in
413 Fig. 1D) and other clusters.

414 We observed a similar pattern for *16p11.2* transcript expression (Fig 1E, bottom) with
415 a preponderance of high expression in IN clusters1,3,5,8, indicating specifically
416 enriched expression of subset of *16p11.2* transcripts (for example *SEZL6L2*, *PRRT2*,
417 *QPRT*, and *YPEL3*) with the next highest number of enriched transcripts in progenitor

418 clusters (for example *KIFF22* and *PPC4C*). Other transcripts are expressed both in
419 progenitors and INs but much less in excitatory neurons (for example *MAPK3*), and
420 others broadly across all cell clusters (for example *TMEM219*) or at very low levels in
421 any cluster (*ASPHD1*, *C16orf54*, *C16orf92*, *SPN*, *TBX6*, *ZG16*).

422 To investigate how well the expression of autism risk factor transcripts defined the cell
423 clusters more systematically we used MetaNeighbor analysis which reports on how
424 similar cells are to each other based on expression of a given input gene set using
425 AUROC scores (Crow et al., 2018). From this, we generated a pairwise comparison
426 matrix between the 21 cell clusters (Fig. S2). Performing MetaNeighbor with the same
427 ~2000 DEGs used to perform hierarchical clustering (Fig. S2A) we confirmed that cells
428 in each cardinal class were generally more similar within class (red on heatmap) and
429 less similar (blue on heatmap) between cardinal classes. Nevertheless, some neurons
430 (N1 and N2) were quite similar to progenitors (P1-P6) likely indicating that they
431 represented relatively immature excitatory neurons that retained some progenitor
432 identity. Within the INs there was a clear divide between IN1,3,5,8 (red box in Fig.
433 S2A-C) and IN2,4,6 (green box in Fig. S2A-C) cluster groups, with highest similarity
434 within group and low similarity between groups. Next, we performed the same analysis
435 for the 83 SFARI monogenic autism risk factor transcripts (Fig. S2B) and found that
436 strongest similarity was retained within the progenitor group P1-P6 and the IN group
437 IN1,3,5,8. A similar pattern emerged when we used the 27 *16p11.2* transcripts as the
438 gene-set (Fig. S2C), although here the strongest similarity was within the progenitor
439 group P1-6 and between IN5 and IN8. These results indicate that the immature INs
440 can be robustly distinguished from other cells in the developing brain by their
441 expression pattern of autism risk factor transcripts.

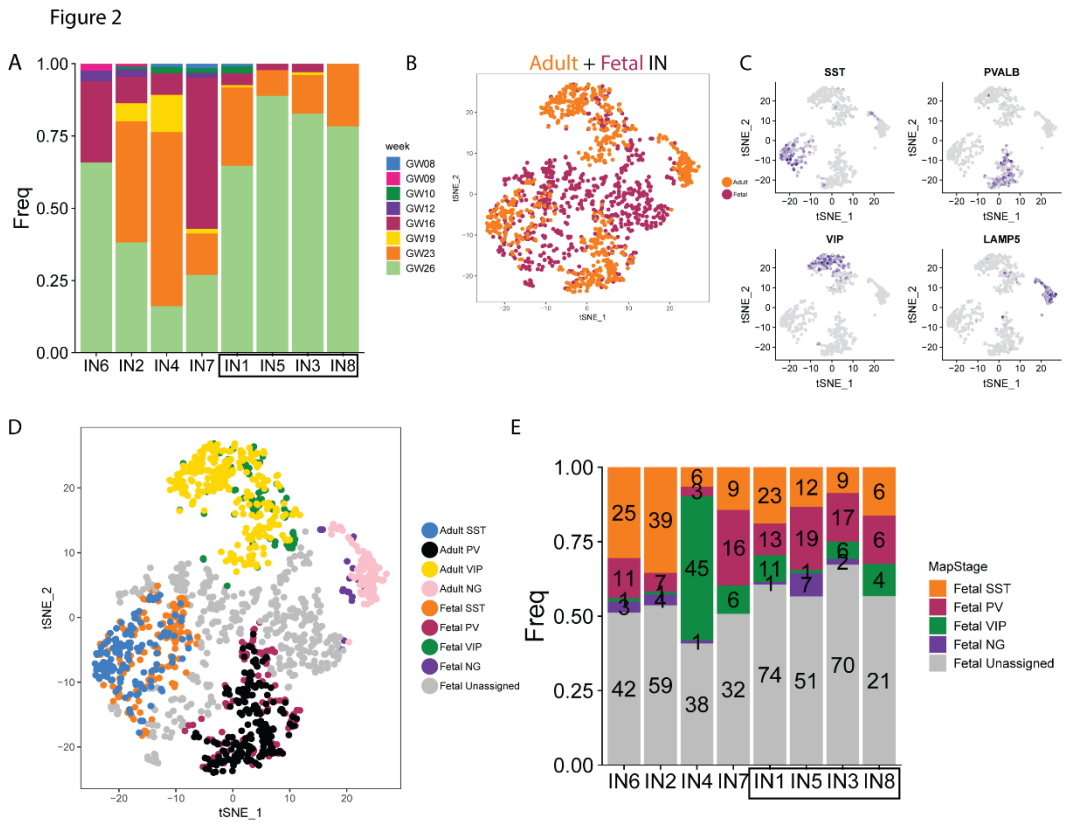
442 We conclude from the gene expression analysis that a subset of developing INs,
443 IN1,3,5,8, in GW8-26 human foetal cerebral cortex express a high proportion of autism
444 associated transcripts at higher levels than other cells. This suggests that IN1,3,5,8
445 are vulnerable, and in some instances selectively vulnerable, to a large number of
446 independent monogenic genetic autism risk factor and *16p11.2* microdeletion during
447 cerebral cortex development.

448

449

450 **Properties of human foetal INs potentially vulnerable to autism risk factors.**

451 Having identified IN1,5,3,8 as a potentially important class of INs targeted by genetic
452 autism risk factors we next examined their developmental and transcriptional
453 properties. We found that very few IN1,3,5,8 INs were present during the earlier stages
454 (GW8-19) of cerebral cortex development, typically appearing from GW23 and with
455 the vast majority of IN1,5,3,8 INs present at GW26 (Fig. 2A). On the other hand,
456 IN2,4,6,7 clusters contained higher proportions of cells from earlier stages (Fig. 2A),
457 suggesting that IN1,3,5,8 might represent a more mature state than the rest of IN
458 clusters in our dataset. INs have reached the cortex in substantial numbers by GW16
459 (Fig. S1B) indicating that IN1,3,5,8 cells correspond to a stage of the developmental
460 trajectory after tangentially migrating INs enter the cortex (Hansen et al., 2013; Ma et
461 al., 2013).



462

463 **Figure 2: (A)** Bar plot depicting the proportion of sample ages in each IN cluster. **(B)**
464 Canonical correspondence analysis (CCA) integrating the foetal and adult human
465 scRNA-seq data. **(C)** CCA-KNN analysis in t-SNE space provides a method to
466 categorise immature INs into SST, PV, VIP and Neuroglia form (NG) classes

467 according to their transcriptomic similarity to mature neurons from human adult cortex.
468 **(D)** Gradient plots showing gene expression pattern of marker genes of IN lineages in
469 t-SNE space. **(E)** Bar plot depicting the number and proportion of IN cell types in each
470 IN cluster.

471 We used canonical correlation analysis (CCA-KNN) to integrate foetal (Zhong et al.,
472 2018) and mature (Lake et al., 2018) human IN scRNAseq datasets to identify mature
473 cell types corresponding to developing IN1,3,5,8 clusters. We first reduced the
474 dimensionality of both datasets (adult IN cells = orange; foetal IN cells = blue) onto the
475 same two-dimensional space using *t*-SNE (Fig. 2B), which allowed the identification
476 of 4 major cell types of adult INs based on the expression of variable genes shared
477 between both datasets. We then assigned identities to adult based on expression of
478 markers for PV, SST, VIP and neurogliaform cells illustrated by gradient plots of *SST*,
479 *PVALB*, *VIP* and/or *LAMP5* transcripts to identify defined classes of cortical INs (Fig.
480 2C). This then allowed us to assign foetal INs to each of these cell types based on
481 transcriptional similarity to the mature INs (Fig. 2D). Of the IN1,3,5,8 cells classified in
482 this manner we found that they were not homogenous, but rather consisted of PV,
483 SST, and VIP cell types (Fig. 2E). A parsimonious interpretation is that the foetal
484 IN1,5,3,8 are cells destined to become several categories of mature IN cell types,
485 although this awaits further investigation as assignation of cell lineage from scRNA-
486 seq data is ambiguous.

487 To gain further insight into the identity and developmental cell state represented by
488 IN1,3,5,8, we first performed differential expression analysis with respect to other INs
489 (Fig. 3A) and used the genes enriched in IN1,3,5,8 with a log fold change higher than
490 3 (1623 genes FDR < 0.001) to test for Gene Ontology (GO, biological process)
491 enrichment. We found that within the top 30 GO terms (ordered according to Fold
492 Enrichment), 9 categories were related to synaptic plasticity, synaptic maturation and
493 synaptic transmission and 6 categories were related to neuron projection and growth
494 (Fig. 3B), with fold enrichments ranging from 6 to 10, suggesting that IN1,3,5,8 cells
495 show earlier maturation of neurites and synapses than other INs. The Gene Ontology
496 term “Regulation of Synaptic Plasticity” contains 192 genes, from which 54 are
497 differentially expressed in IN1,3,5,8. A closer inspection of the expression pattern in
498 the t-SNE space showed that many of these genes followed a general expression level
499 gradient trend with its maximum levels in INs corresponding to IN5 and IN8 (*MEF2C*,

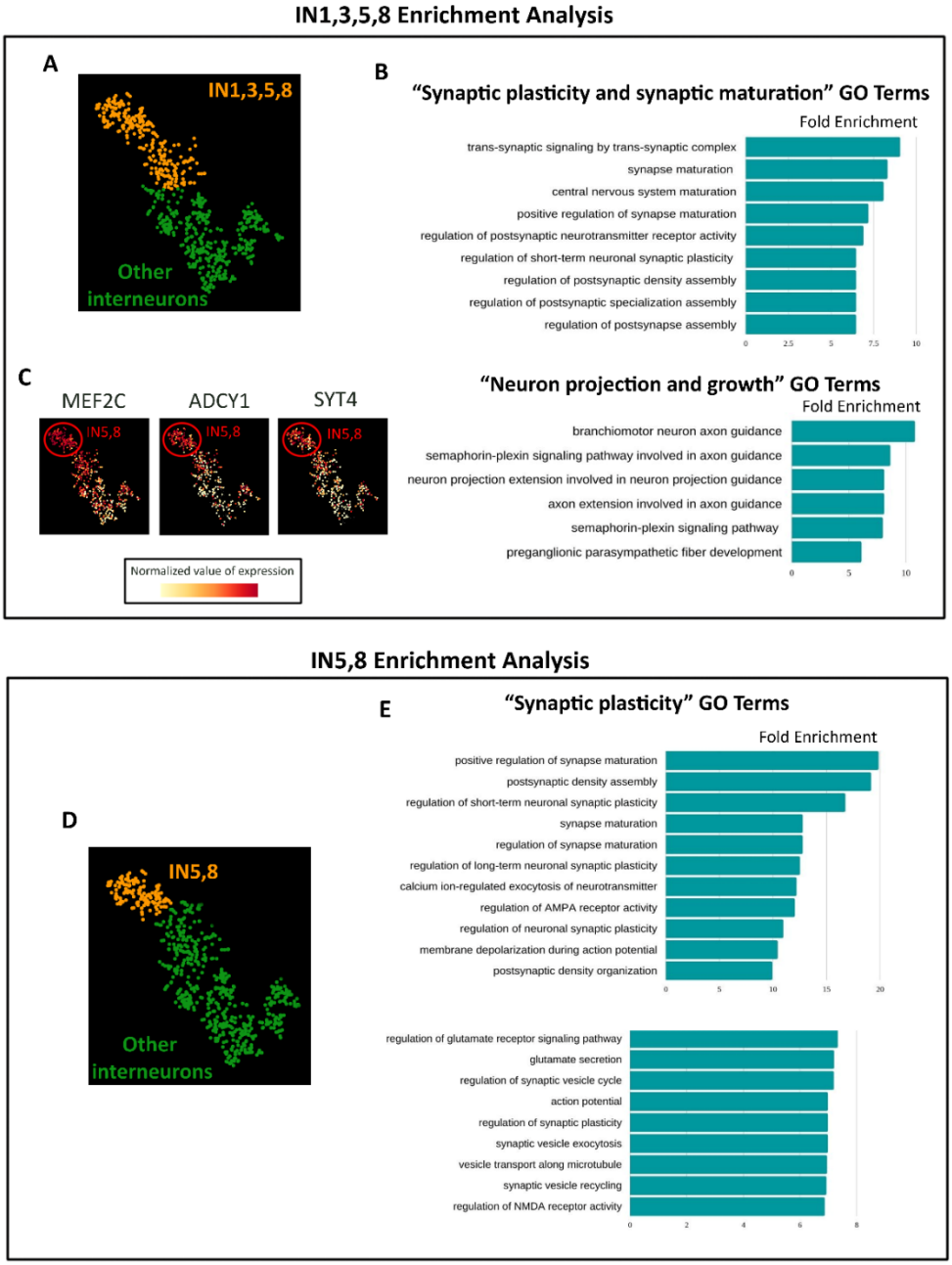


Figure 3. Characterisation of INs by gene ontology analysis (A-C) Gene ontology (GO) analysis in IN1,3,5,8 (orange in A) versus other INs (green in A) reveals enrichment of GO terms associated with (B) synaptic maturation and plasticity and axon extension and guidance. (C) gradient plots of MEF2C, ADCY1, and SYT4 showing that these transcripts are expressed in a gradient across INs with highest expression in IN5,8. (D,E) Gene ontology (GO) analysis in IN5,8 (orange in D) versus other INs (green in D) reveals enrichment of GO terms associated with (E) synaptic maturation and plasticity.

509 *ADCY1*, and *SYT4* shown as examples in Fig. 3C). This suggested that the INs in the
510 dataset must be ordered in the t-SNE space according to a gradient of synapse
511 formation, with IN5,8 being the higher extreme of this axis. To confirm this, we
512 performed differential expression analysis between IN5,8 IN cells versus all other INs
513 (Fig. 3D) and found a high enrichment of synaptic plasticity-related terms but this time
514 showing fold enrichments ranging from 7 to 20 (Fig. 3E), almost doubling the values
515 of the previous comparison.

516 Finally, to gain further insight into IN5,8 neuronal identity, we compared IN5,8 cluster
517 versus all other neurons (including both excitatory and inhibitory, Fig. S4A).
518 Interestingly, enriched functional terms were mainly related to synaptic plasticity,
519 learning and social behaviour (Fig. S4B). Visual inspection of gradient plots in the t-
520 SNE space confirmed that many of the genes linked to synaptic plasticity and
521 maturation are selectively expressed in IN5,8 (Fig. S4C). Together this analysis
522 suggests that IN1,3,5,8 are relatively differentiated INs elaborating processes and
523 forming synapses.

524 This raises the possibility that by targeting the stage of the IN developmental trajectory
525 represented by IN1,3,5,8, multiple genetic autism risk factors perturb the development
526 of the physiological properties of foetal INs. The remaining interneurons IN2,4,6,7
527 appear less vulnerable and may represent an earlier stage in the IN developmental
528 trajectory or correspond to different IN lineages. Either way our analysis suggests
529 genetic autism risk factors impact on many INs during foetal development and may
530 affect their function into postnatal life.

531

532 **Conservation of potentially vulnerable INs between humans and rodents.**

533 These analyses of human foetal neurons suggest the testable hypothesis that a large
534 proportion of autism related genes selectively regulate IN development in the human
535 foetal cortex. A prediction of this hypothesis is that there will be IN phenotypes initiated
536 during human brain development *in utero* that persist into postnatal life and predispose
537 to autism and its comorbid conditions. Such investigation is currently not possible,
538 however, rodent models provide a complementary means to test cellular vulnerability
539 to autism genetic risk factors under physiological conditions. As such, we next

confirmed that developing rodent brain contains INs with similar molecular properties to the potentially vulnerable human foetal INs IN1,3,5,8 identified above.

Figure 4

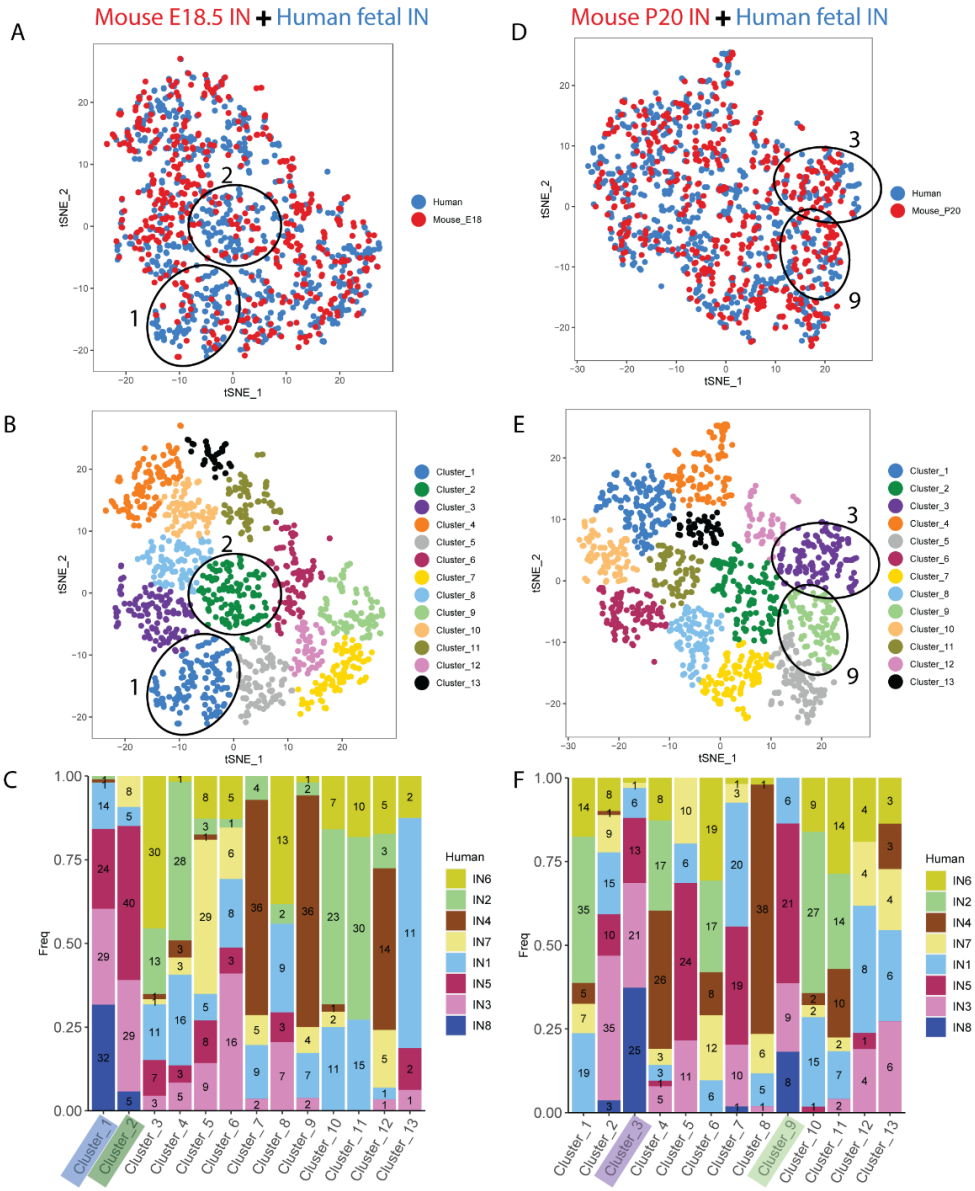


Figure 4: Identifying transcriptomic correlates between developing human and mouse INs at (A-C) E18.5 and (D-F) P20. (A,D) CCA integration of mouse (red) and human (blue) INs in tSNE space. (B,E) JACCARD clustering into 13 mixed clusters. (C,F) Distribution of human IN1-8 INs in each of the mixed clusters with numbers of cells shown within each bar. The mixed clusters 1&2 for E18.5 (A-C) and 3&9 for P20 (D-F) that are most enriched for human IN1,3,5,8 cells are indicated on each panel.

549 We identified two mouse scRNAseq data sets comprising FACS sorted cortical INs at
550 embryonic day (E) 18.5 and postnatal (P) day 20, when INs are differentiating and
551 forming circuits (Mayer et al., 2018). For each mouse developmental stage we used
552 CCA-KNN to integrate the mouse and human INs into the same tSNE space (Fig. 4
553 A,D) to allow us to identify mixed clusters of transcriptomically similar mouse and
554 human INs (Fig. 4B,E). For each mouse developmental stage, we examined how the
555 human IN cell types IN1-8 were distributed between the mixed mouse+human clusters
556 (Fig. 4C,F). This analysis revealed that E18.5 clusters 1 and 2 (indicated in Fig 4A-C)
557 and P20 clusters 3 and 9 (indicated in Fig 4D-F) contained the greatest enrichment of
558 human IN1,3,5,8 cells. Critically, these clusters contained comparable numbers of
559 mouse and human cells indicating that the developing and postnatal mouse possesses
560 INs molecularly similar to human IN1,3,5,8 cells. These findings suggest that INs we
561 have identified as potentially vulnerable to genetic autism risk factors are shared
562 between humans and rodents allowing us to investigate them under physiological
563 conditions using rodent models.

564

565 **Changes to IN function in the rat model of *16p11.2* microdeletion.**

566 The *16p11.2* microdeletion causes *16p11.2* transcript levels to be reduced by about
567 50% in humans and rodent models (Tai et al., 2016) (Pucilowska et al., 2015) (Horev
568 et al., 2011) (Blumenthal et al., 2014). As multiple *16p11.2* transcripts are normally
569 highly enriched in developing INs (Fig. 1), we hypothesised that their simultaneous
570 reduced expression following *16p11.2* microdeletion may synergistically impact IN
571 development with post-natal consequences on IN phenotypes. We next set out to test
572 this hypothesis by performing electrophysiological and anatomical interrogation of the
573 rat *16p11.2* microdeletion model (*16p11.2*$^{+/-}$ rats).

574 As *16p11.2* transcripts are expressed in the ganglionic eminences, from which cortical
575 IN progenitors reside (Morson et al., 2021), we first asked if the numbers of inhibitory
576 and/or excitatory neurons populating the cortex post-natally was different in the
577 *16p11.2*$^{+/-}$ rats. To investigate this, we counted these cardinal cell classes in WT and
578 *16p11.2*$^{+/-}$ rats at postnatal day (P) 21, an age by which INs have migrated into the
579 cortex and assumed their final laminar positions forming circuits with resident
580 excitatory neurons. We combined immunostaining for the pan-neuron-specific marker

581 NeuN and *in situ* hybridization for the IN marker *Gad1* to identify NeuN^{+};*Gad1*$^{-}$
582 excitatory neurons and NeuN^{+};*Gad1*$^{+}$ INs in the cortex (Fig. 5A). Quantification within
583 a 200µm wide column spanning the somatosensory cortex and hippocampus (shown
584 figure 5A) shows no significant difference between WT and *16p11.2*$^{+/-}$ rats in total IN
585 number ($t_{(8)}$ = 0.27, p = 0.80 t test, Figure S5A) or in the proportion of the neuronal
586 population identified as inhibitory or excitatory ($t_{(8)}$ = 0.15, p = 0.89 t test, Fig. S5B). To
587 assess whether the cortical laminar position of INs was altered in *16p11.2*$^{+/-}$ rats we
588 quantified IN and excitatory neuron number in 10 adjacent counting areas spanning
589 the somatosensory cortex. We found no significant change in the distribution of INs
590 across the cortex (Figure 5B), nor did we see any change in the inhibitory/excitatory
591 proportion in any counting area (Fig. S5B). Next, we examined IN number and position
592 in the CA1 region of the hippocampus. Total combined IN number within the *str. oriens*
593 (SO) and *st. pyramidale* (SP) of CA1 was unchanged between WT and *16p11.2*$^{+/-}$ rats
594 ($t_{(8)}$ = -0.09, p = 0.93 t test, Fig. S5A). Total IN number within the SO and SP was also
595 unchanged indicating that the position of INs within the hippocampus is unaffected in
596 *16p11.2*$^{+/-}$ rats (Figure 5C). These data indicate that the *16p11.2* microdeletion does
597 not have a major impact on number or distribution of inhibitory or excitatory neurons
598 in the cerebral cortex or CA1 INs post-natally.

599 Our bioinformatic analysis indicated that human foetal IN1,3,5,8 cells contribute to
600 mature somatostatin (SST) INs (Fig 2E). As such, we next asked whether the *16p11.2*
601 microdeletion might have an effect on SSt IN development to alter their physiological
602 properties. To test this, we performed whole-cell patch-clamp recordings from
603 identified SSt INs and local pyramidal cells (PCs) in the CA1 region at P21.

604 We performed recordings from identified INs that expressed SSt in CA1 of the
605 hippocampus, which also show expression of PV in parvalbumin (PV) in 50% of cells
606 (Booker and Vida, 2018). The recorded Sst INs predominantly had horizontally
607 oriented dendrites in *str. oriens,* which when present had an axon extending into *str.*
608 *lacunosum-moleculare* (Booker and Vida, 2018). In the present study, we recorded

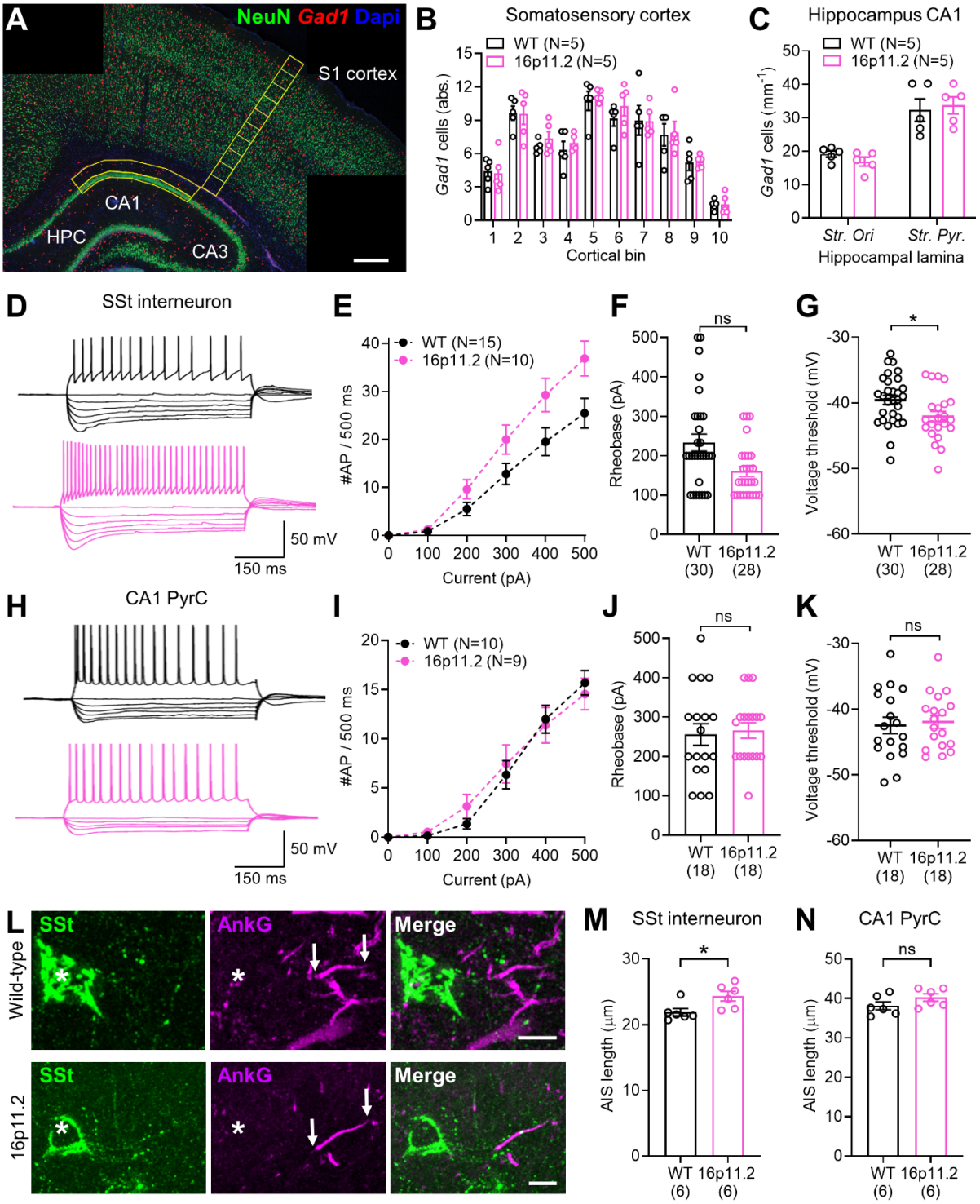


Figure 5. Selective hyperexcitability of SSt INs in *16p11.2*$^{+/-}$ rat hippocampus, but with no change in IN number. (A) Overview micrograph of *Gad1* mRNA and NeuN protein expression in the rat hippocampus and cortex at P21. Gad1 expressing INs (red) and NeuN expressing, Gad1 negative excitatory neurons (green) can be observed in the cortex and hippocampus. Scale bars 400µm **(B)** Quantification of *Gad1*-positive IN (IN) number through the somatosensory cortex in WT (black, n=5)

616 and 16p11.2$^{+/-}$ (pink, n=5) rats. Counting areas indicated yellow in A with cortical bins
617 numbered from 1 at the ventricular edge to 10 at the pial surface. **(C)** Quantification of
618 the combined total number of *Gad1*-positive neurons in the s*tr.oriens* (*Str. ori*) and s*tr.*
619 *pyramidale* (*Str pyr.*) of the CA1 region of the hippocampus in WT (N=5) and 16p11.2$^{+/-}$
620 (N=5)rats. Counting area indicated in **A**. (**D**) Representative action potential discharge
621 in response to hyper- to depolarising current steps in putative SSt-INs, from the *str.*
622 *oriens* of CA1 from WT (top) and 16p11.2$^{+/-}$ rats (bottom). **(E)** Summary current-
623 frequency plots from identified SSt INs from WT (N=15 rats) and *16p11.2*$^{+/-}$ (N=10
624 rats). (**F**) Quantification of rheobase current in identified SSt-INs from WT (n=30 cells)
625 and *16p11.2*$^{+/-}$ (n=28 cells) rats. (**G**) Measurement of the voltage threshold of the first
626 action potential elicited at rheobase for the same cells in **F**. (**H-K**) the same analysis
627 performed in CA1 pyramidal cells from WT (N=10 rats, n=18 cells) and *16p11.2*$^{+/-}$ (N=9
628 rats, n=18 cells). (**L**) Representative micrographs showing immunohistochemical
629 labelling for SSt (green), the AIS marker AnkyrinG (AnkG, magenta), and their overlap
630 (merge). The SSt soma is indicated with an asterisk (*) and the start and end of the
631 AIS localised to that IN indicated (arrows). Scale bar: 10 µm. (**M**) Quantification of the
632 AIS length of SSt INs from WT (N=6 rats, n= 162 AIS) and *16p11.2*$^{+/-}$ (N=6 rats, n=151
633 AIS). (**N**) Quantification of AIS lengths of putative CA1 pyramidal cells from WT (N=6
634 rats, n= 150 AIS) and *16p11.2*$^{+/-}$ (N=6 rats, n=155 AIS) rats. Statistics shown: ns –
635 p>0.05, * - p<0.05, from Linear Mixed Effects modelling.

636

637 from 30 WT (from 15 rats) and 28 *16p11.2*$^{+/-}$ (10 rats) SSt INs, which all displayed clear
638 immunoreactivity for the SSt neuropeptide at the somata. In response to
639 depolarisingcurrent injections (0-500 pA, 100 pA steps, Fig. 5D), SSt INs generally
640 responded with high action potential discharge rates, which had a peak action potential
641 discharge of 27.4 ± 2.5 action potentials/500 ms (Fig. 5E). We found that SSt INs from
642 the *16p11.2*$^{+/-}$showed elevated action potential discharge, compared to WT controls
643 ($F_{(5,\ 58)}$=5.10,P=0.0006 for interaction of genotype and current, N=15 WT and 10
644 *16p11.2*$^{+/-}$ rats, Fig. 5E), indicating cellular hyperexcitability. Comparison of the
645 intrinsic physiology of SSt INs revealed a general 17% reduction of rheobase current
646 in 16p11.2$^{+/-}$ compared to WT rats, albeit not significantly so (p=0.1087 LME, Fig. 5F),
647 this was accompanied by a 6% hyperpolarisation of the voltage threshold (p=0.0279
648 LME, Fig. 5G). All other physiological parameters were similar between genotypes

649 (Supplementary Table S1). To confirm that SSt IN intrinsic excitability changes were
650 not a result of compensation mechanism to altered synaptic input from local CA1 PCs
651 (Booker et al., 2020), we asked whether the spontaneous excitatory postsynaptic
652 currents (EPSC) they receive were different between genotypes. We saw no change
653 in either the spontaneous EPSC amplitude (p=0.4495, LME) or frequency (p=0.2131,
654 LME), implying typical circuit integration of SSt INs to the local network.

655 We confirmed that the effect on intrinsic cell excitability were restricted to SSt INs, by
656 performing recordings from local ExNs – the CA1 PCs. CA1 PCs were identified on
657 the basis of having a somata located in *str. pyramidale,* with a single large-calibre
658 apical dendrite entering *str. radiatum* as observed under IR-DIC. We obtained
659 recordings from 18 putative CA1 PCs per group from 10 WT and 9 *16p11.2*$^{+/-}$ rats. In
660 response to depolarising current injections (0-500 pA, 100 pA steps, Fig. 5H), we
661 observed no change in the number of action potentials generated by the recorded CA1
662 PCs in the *16p11.2*$^{+/-}$ rats compared to WT ($F_{(5, 85)}$=0.9185, P=0.4731, 2-way ANOVA
663 for current/genotype interaction, Fig. 5I). CA1 PCs typically had lower peak action
664 potential discharge rates than for SSt INs (WT: p=3.29x10^{-5}, *16p11.2*$^{+/-}$: p=1.63x10^{-6};
665 LME). Consistent with this lack of altered action potential discharge, we found no
666 change in CA1 PC rheobase current (p=0.7098, LME, Fig. 5J), action potential
667 threshold (p=0.4116, LME, Fig. 5K), or any other parameter tested (Supplementary
668 Table S1). These data strongly suggest that excitatory CA1 PCs are physiologically
669 typical in the *16p11.2* rat model of ASD.

670 We have recently shown that changes to cell excitability effected the voltage threshold
671 and action potential discharge in genetic models of intellectual disability can result
672 from changes to the structure of the axon initial segment (AIS) (Booker et al., 2020).
673 To determine if the changes in SSt IN excitability arise from changes to AIS structure
674 we next performed immunolabelling of perfusion fixed tissue from the hippocampus of
675 WT (N=5) and *16p11.2*$^{+/-}$ (N=5) rats. Immunofluorescent double labelling with
676 AnkyrinG reliably labelled the AIS of all neurons in the CA1 region, which could be
677 identified emerging from the soma, or more often the proximal dendrites of
678 immunolabelled SSt INs (Fig. 5L). Comparison of AIS lengths on SSt INs revealed an
679 11% longer AIS in 16p11.2$^{+/-}$ rats compared to WT (p=0.0064, LME, Fig. 5M). There
680 was no change in the lengths of putative CA1 PC AISs between genotypes (p=0.0962,
681 LME, Fig. 5N).

682 Together, consistent with the hypothesis that the *16p11.2* microdeletion selectively
683 targets INs, these data show a preferential increase of SSt IN excitability in the
684 *16p11.2* rat autism model, with no changes observed in the local excitatory principal
685 cells. This increased cellular excitability coincided with selective alteration to the length
686 of the AIS, corresponding to changes in voltage threshold. Together, these changes
687 could potentially lead to an aberrant network activity and gating of information flow
688 through hippocampal circuits.

689

690 **DISCUSSION.**

691 This study reveals that molecularly defined classes of INs in the foetal human cerebral
692 cortex display enriched expression of multiple gene transcripts associated with autism.
693 This result is striking, suggesting the testable hypothesis that some INs are
694 disproportionately vulnerable to autism genetic risk factors. Within INs as a whole the
695 most autism associated transcripts are enriched in a subset we described as
696 'IN1,3,5,8' suggesting that these cells may represent a convergent cellular target
697 underpinning genetic predisposition of the developing brain to autism and its co-
698 occuring conditions during post-natal life. This poses the question of what is the
699 identity of these cells? Our data-set was acquired from dissected human foetal cortex,
700 which includes INs that are migrating tangentially through and in the cerebral cortex,
701 but not INs undergoing neurogenesis or early migration in the ganglionic eminences
702 (Hansen et al., 2013; Ma et al., 2013). We examined human foetal cortical cells
703 spanning the interval GW8-26 and found IN1,3,5,8 cells are not present in the cerebral
704 cortex before GW23 and then increase in numbers to GW26. As many INs have
705 migrated into the cortex well before GW23 this suggests that IN1,3,5,8 represent a
706 relatively differentiated stage on the IN developmental trajectory. This is consistent
707 with the enrichment of GO terms relating to synapse maturation and neurite formation
708 in these cells. Transcriptomic similarity between foetal IN1,3,5,8 and adult PV, SST,
709 and VIP INs suggests these cells are destined to form a variety of IN cell types and
710 that changes in their developmental trajectory caused by autism causing mutations
711 may have far reaching consequences for the formation on inhibitory circuitry in the
712 post-natal brain.

713 Although the most striking enrichment of autism associated transcripts was observed
714 in INs we also saw enrichment of some transcripts in other cardinal cell classes.
715 Progenitor cells had a much smaller number of enriched autism associated transcripts
716 than INs and most of these (eg *ADNF*, *ZNF462*, *PHIP*, *HNRNPU*, *RPS6KA3* for
717 monogenic and *PAGR1*, *HIRIP3*, *KIF22*, and *PP4C* for *16p11.2* transcripts). This
718 suggests that progenitor cells in the cerebral cortex may also be vulnerable to a subset
719 of autism causing mutations. Progenitors in the developing neocortex are destined to
720 differentiate into excitatory pyramidal neurons and non-neuronal cell-types (eg
721 astrocytes) suggesting that mutations in these progenitor enriched genes may
722 dysregulate their production or function. This study also prompts future investigation
723 into the expression of autism associated transcripts in IN progenitors located in the
724 ganglionic eminences and the consequence of mutation for IN neurogenesis in
725 humans although our findings in the rat *16p11.2* microdeletion rat model suggest that
726 gross IN output is not affected in this context in rodents.

727 We found that large numbers of monogenic autism risk factor transcripts are highly
728 expressed in IN1,3,5,8 INs, suggesting that their mutation may contribute to the
729 aetiology of autism via alterations to IN development. This hypothesis remains to be
730 tested for the majority of genes. However, for *ARID1B*, *DYRK1A*, *MECP2*, and
731 *CNTNAP2* there is already evidence that monogenic mutation causes abnormal
732 numbers or physiological properties of INs in rodent models (Gao et al., 2018; Jung et
733 al., 2017; Penagarikano et al., 2011; Souchet et al., 2019; Tomassy et al., 2014; Vogt
734 et al., 2018). We also found *KCTD13*, *MAPK3*, and *MVP* transcripts expressed from
735 the *16p11.2* locus are enriched in IN1,3,5,8. *KCTD13* modulates synaptic transmission
736 by suppressing RHOA signalling via interaction with the ubiquitin ligase *CUL3*, itself
737 an autism risk factor (Escamilla et al., 2017; Willsey et al., 2013). *CUL3* is co-
738 expressed with *KCTD13* in IN1,3,5,8 cells suggesting a molecular mechanism for the
739 *16p11.2* microdeletion to impact on cellular and synaptic function via perturbed RHOA
740 signalling. Interestingly, the inhibition of RHOA pathway has been prosposed as a
741 treatment to restore cognition in *16p11.2* mouse models (Martin Lorenzo et al., 2021).
742 *MAPK3* and *MVP* are both implicated in ERK signalling which impacts diverse cellular
743 processes including cell proliferation, migration, and synaptic function. Indeed, a
744 mouse model of *16p11.2* microdeletion shows elevated ERK signalling leading to

745 perturbed cortical development and autism-like phenotypes (Pucilowska et al., 2018;
746 Pucilowska et al., 2015), although the involvement of INs was not tested.

747 Our analysis of the *16p11.2* microdeletion rat indicates that neither ExN or IN number
748 and location were altered in the cerebral cortex or hippocampal region CA1, so it
749 seems unlikely that a numerical excitation/inhibition imbalance is present in the
750 *16p11.2*$^{+/-}$ rat model. However, whole-cell patch-clamp recordings in CA1 revealed
751 intrinsic hyperexcitability of SSt INs, coincident with increases AIS length. No effect
752 was observed in CA1 excitatory neurons. This suggests a mechanism by which the
753 *16p11.2* microdeletion perturbs the E/I balance by selectively altering the intrinsic
754 excitability of INs. Although IN hyperexcitability may lead to greater inhibition within
755 cortical circuits and tilt the E/I balance towards inhibition, the complexity of such
756 circuits interaction makes prediction of the consequences to gross activity difficult.
757 However, SSt INs themselves possess both direct inhibitory and disinhibitory
758 mechanisms within the prototypical CA1 circuit, leading to alterations to synaptic
759 plasticity when measured at the circuit level (Leao et al., 2012). As such, the outcome
760 of greater SSt IN activity may directly lead to altered cognition observed in ASC/ID.
761 Furthermore, a shift in the E/I balance has been identified in the somatosensory cortex
762 of ASC mouse models (including *16p11.2*$^{+/-}$ mice), attributed to homeostatic regulation
763 of IN function (Antoine et al., 2019). Our combined bioinformatic and physiological
764 approach diverges from this view, suggesting that SSt INs in *16p11.2* microdeletion
765 are genetically cued to perturbation from early in their developmental trajectory. A
766 deeper understanding of the functional consequences of SSt hyperexcitability on the
767 E/I balance in cortical circuits and their homeostasis requires further investigation.

768 To conclude, our bioinformatic analysis of developing human foetal cerebral cortex
769 single cell transcriptomes suggests that developing INs are disproportionately
770 vulnerable to genetic autism risk factors, which is supported by physiological
771 correlates in a *16p11.2* microdeletion rat model. This study paves the way for more in
772 depth investigations of how polygenic and monogenic autism risk factors impact on IN
773 development and function.

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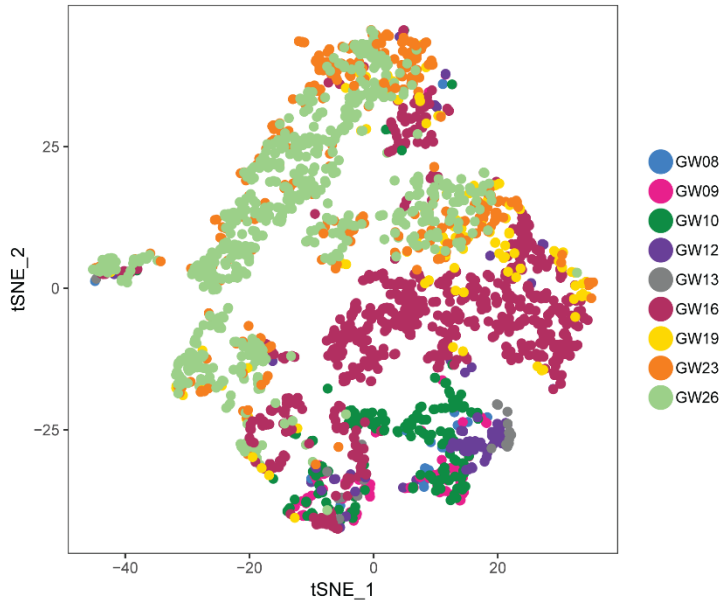
905 **SUPPLEMANTARY FIGURES**

Figure S1

A



B

Cell class	PCW	# of cells	Cell class	PCW	# of cells	Cell class	PCW	# of cells
	GW08	3		GW08	15		GW08	3
	GW09	50		GW09	35		GW09	3
	GW10	83		GW10	93		GW10	7
	GW12	12		GW12	63		GW12	7
NPCs	GW13	7	**ExNs**	GW13	16	**INs**	GW13	0
	GW16	123		GW16	545		GW16	83
	GW19	5		GW19	82		GW19	22
	GW23	2		GW23	89		GW23	174
	GW26	4		GW26	118		GW26	402
	Total	**289**		**Total**	**1056**		**Total**	**701**
Cell class	PCW	# of cells	Cell class	PCW	# of cells	Cell class	PCW	# of cells
	GW08	0		GW08	0		GW08	2
	GW09	0		GW09	0		GW09	0
	GW10	0		GW10	0		GW10	1
	GW12	2		GW12	0		GW12	1
OPCs	GW13	0	**AST**	GW13	0	**MIC**	GW13	1
	GW16	14		GW16	0		GW16	11
	GW19	4		GW19	6		GW19	1
	GW23	25		GW23	7		GW23	10
	GW26	72		GW26	63		GW26	40
	Total	**117**		**Total**	**76**		**Total**	**67**

B

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907 **Figure S1 (A)** *t*-SNE plot showing the distribution of foetal stages **(B)** table showing
908 numbers of cells of each cardinal class at each foetal stage.

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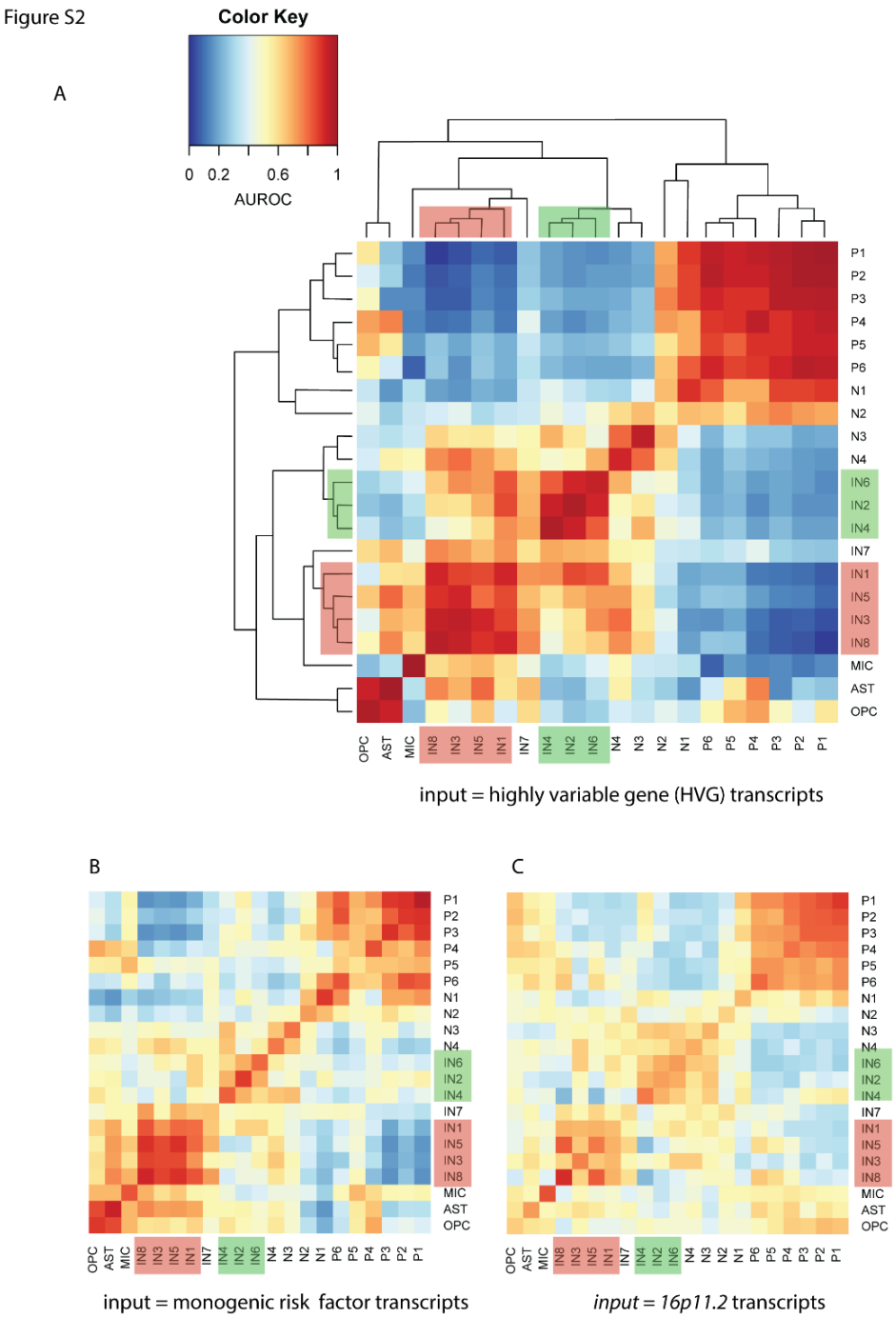

Figure S2 Pairwise comparison of the cluster similarity calculated by MetaNeighbor between the 21 cell clusters. AUROC scores represented as a heatmap where high similarity between clusters is coloured red and low similarity blue. Three plots are shown using different input gene sets **(A)** ~2000 highly variable gene transcripts between clusters. **(B)** the 83 high confidence and strong candidate (SFARI lists 1 and 2) monogenic autism risk factor transcripts. **(C)** the 27 *16p11.2* transcripts.

Figure S3

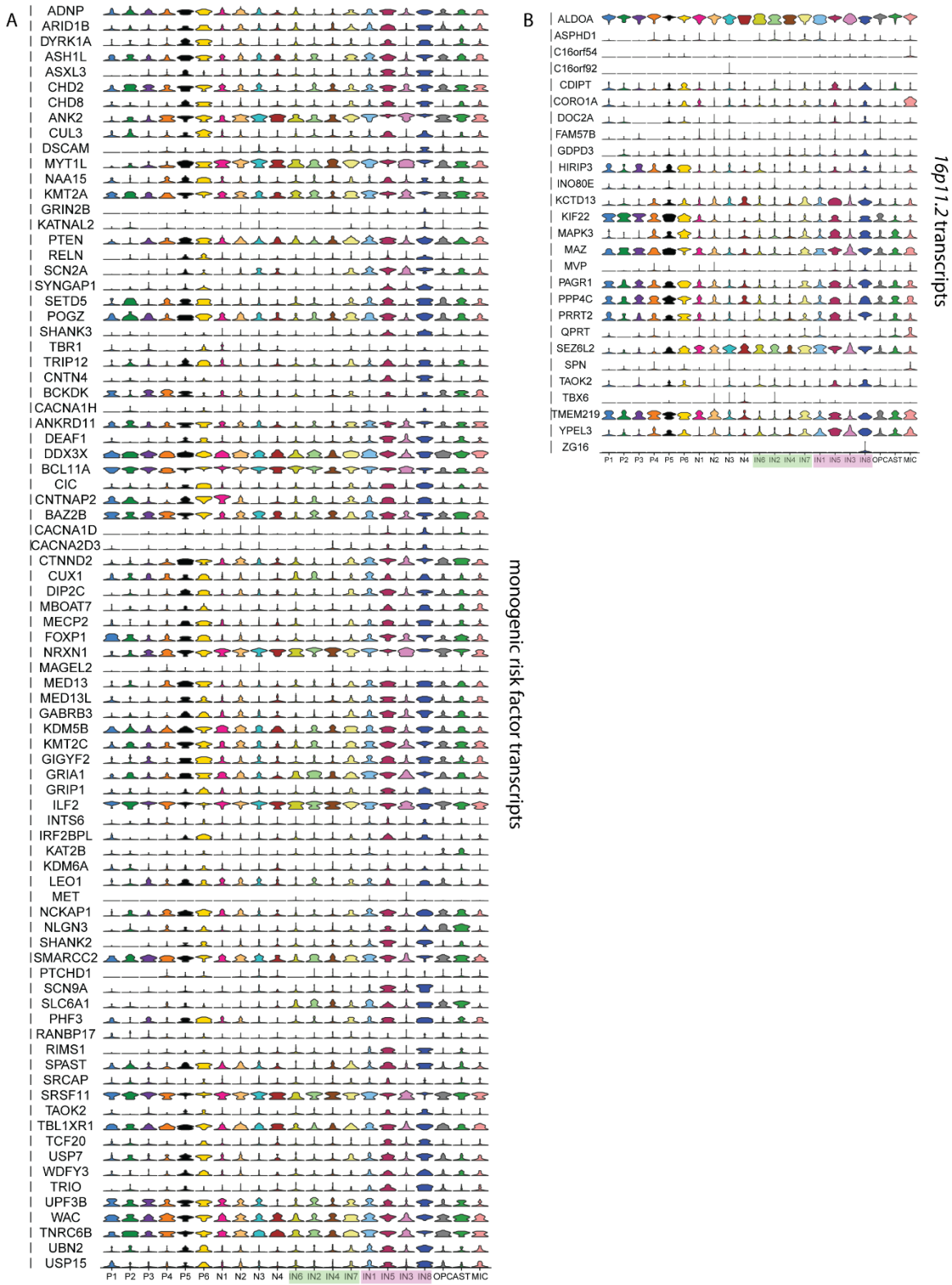

Figure S3 Violin plots showing transcript levels in the 21 different clusters for **(A)** the 83 high confidence and strong candidate (SFARI lists 1 and 2) monogenic autism risk transcripts and **(B)** the 27 *16p11.2* transcripts.

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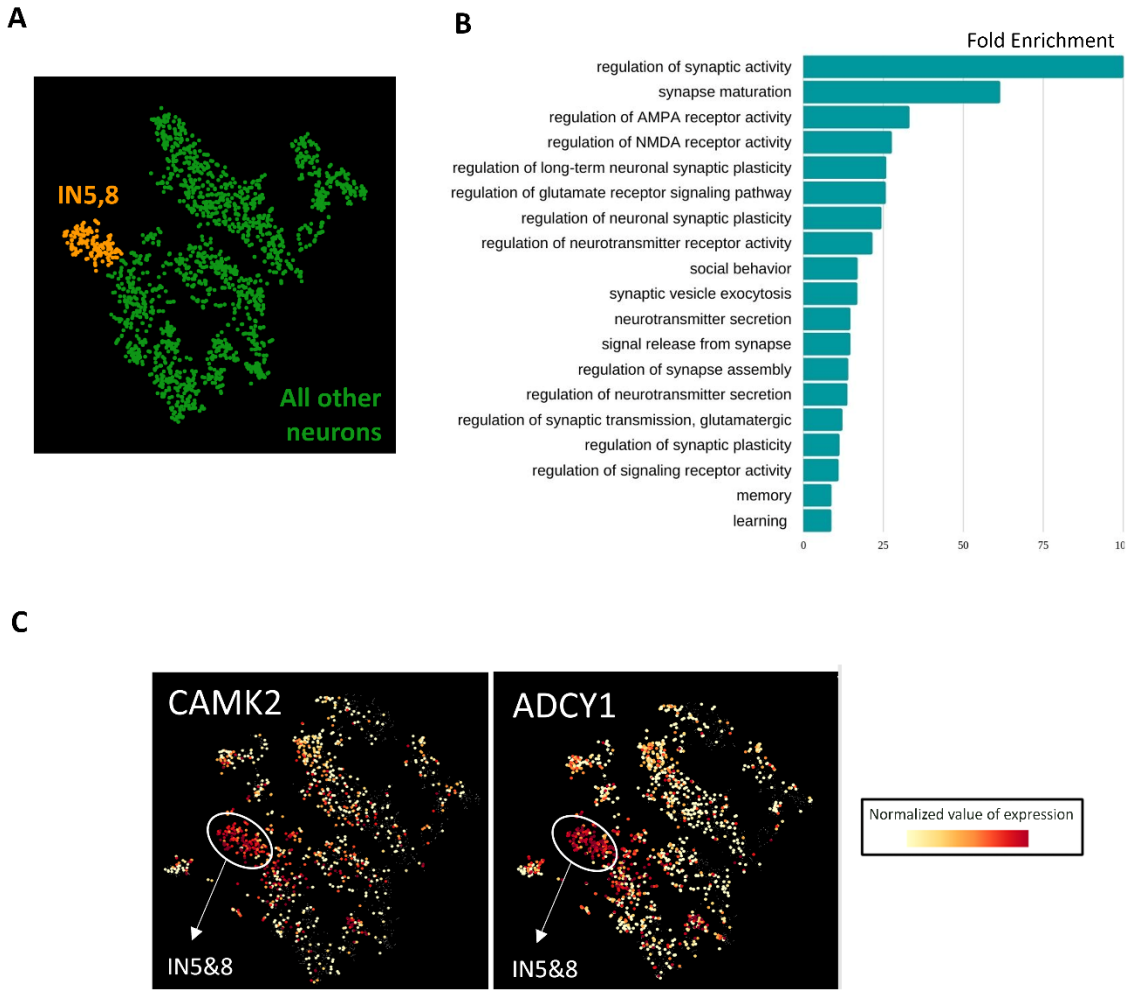

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923 **Figure S4**. Characterisation of INs by gene ontology analysis. Gene ontology (GO)
924 analysis in IN5,8 (orange in A) versus all other cells (green in A) reveals enrichment
925 of GO terms associated with (B) synaptic activity, maturation, and plasticity. (C)
926 gradient plots of CAMK2, ADCY1 showing that these transcripts are most highly
927 expressed in IN5,8.

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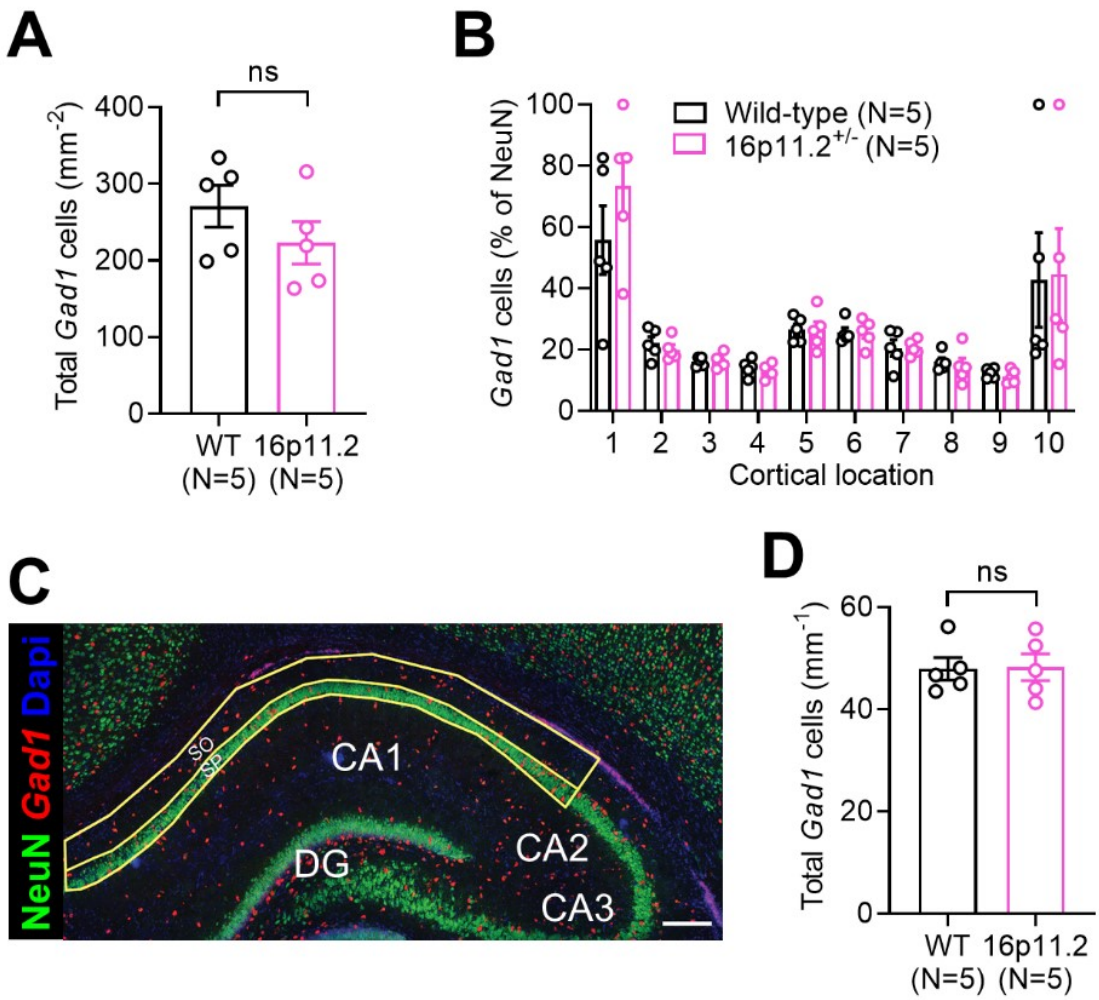

Figure S5: No change in total IN number in somatosensory cortex or hippocampus of the *16p11.2*$^{+/-}$ rat. (A) Based on the expression of *Gad1* mRNA, quantification of the total number of INs across the whole somatosensory column from WT (N=6) and *16p11.2*$^{+/-}$ (N=6) rats. (**B**) No change in the relative ratio of *Gad1*-positive cells to total neurons (NeuN) was observed within the cortical column. (**C**) Expanded view of CA1 of the hippocampus from the same image as in Figure 5A, showing Gad1 in situ hybridisation (red), NeuN immunolabelling (green) and DAPI nuclei (blue). Regions used for cell counts in *str. pyramidale* (SP) and *str. oriens* (SO) are delineated with yellow lines. Scale bar: 200 µm. (**D**) Total number of *Gad1*-positive neurons measured in CA1 from WT (N=5) and *16p11.2*$^{+/-}$ (N=5) rats. Statistics shown: ns – p>0.05 from Student's 2-tailed t-test.

946 **SUPPLEMANTARY TABLES**

947 **Table S1**

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	SSt INs			CA1 pyramidal cells		
Parameter tested	Wild-type	16p11.2$^{+/-}$	*p*	Wild-type	16p11.2$^{+/-}$	*p*
n (N)	30 (15)	18 (13)		18 (10)	18(9)	
Resting membrane potential (mV)	-56.6 ± 1.5	-60.9 ± 1.8	0.25	-64.6 ± 1.9	-65.3 ± 1.1	0.88
Input resistance (MΩ)	204 ± 22	220 ± 26	0.65	113 ± 15	105 ± 18	0.73
Membrane time-constant (ms)	34.5 ± 5.3	34.9 ± 6.3	0.72	24.2 ± 1.7	23.0 ± 2.3	0.74
Membrane capacitance (pF)	162 ± 11	152 ± 16	0.30	239 ± 17	245 ± 16	0.97
Voltage sag (mV)	18.2 ± 1.7	17.9 ± 2.0	0.50	8.4 ± 0.6	9.4 ± 1.2	0.43
Sag (% of maximum)	32.9 ± 2.2	36.0 ± 3.7	0.76	23.9 ± 1.6	25.1 ± 1.5	0.65
Rheobase (pA)	233 ± 22	194 ± 28	0.11	256 ± 28	266 ± 20	0.71
Voltage threshold (mV)	-39.6 ± 0.7	-42.1 ± 0.9	**0.03**	-42.4 ± 1.3	-41.9 ± 1.0	0.41
AP amplitude (mV)	98.3 ± 3.2	97.7 ± 3.3	0.64	122.5 ± 1.8	119.8 ± 2.9	0.61
AP 20-80% rise-time (ms)	0.17 ± 0.01	0.16 ± 0.01	0.38	0.15 ± 0.01	0.16 ± 0.01	0.87
AP half-height duration (ms)	0.62 ± 0.02	0.61 ± 0.04	0.81	1.00 ± 0.02	0.98 ± 0.03	0.60
AP max. rise-rate (mV.ms^{-1})	276 ± 18	310 ± 22	0.48	455 ± 19	443 ± 29	0.96
AP max. decay-rate (mV.ms^{-1})	124 ± 7	139 ± 13	0.53	77 ± 1	79 ± 4	0.64
Peak firing (Hz)	54.8 ± 5.0	71.6 ± 7.9	**0.02**	30.3 ± 3.1	31.4 ± 1.8	0.70

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