Granule Cell Dispersion in Human Temporal Lobe Epilepsy: Proteomics investigation of neurodevelopmental migratory pathways

Running title: Granule cell layer in Temporal Lobe Epilepsy

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Abstract

Granule cell dispersion (GCD) is a common pathological feature observed in the hippocampus of patients with Mesial Temporal Lobe Epilepsy (MTLE). Pathomechanisms underlying GCD remain to be elucidated, but one hypothesis proposes aberrant reactivation of neurodevelopmental migratory pathways, possibly triggered by febrile seizures. This study aims to compare the proteomes of basal and dispersed granule cells in the hippocampus of eight MTLE patients with GCD to identify proteins that may mediate GCD in MTLE.

Quantitative proteomics identified 1882 proteins, of which 29% were found in basal granule cells only, 17% in dispersed only and 54% in both samples. Bioinformatics analyses revealed upregulated proteins in dispersed samples were involved in developmental cellular migratory processes, including cytoskeletal remodelling, axon guidance and signalling by Ras homologous (Rho) family of GTPases (P<0.01). The expression of two Rho GTPases, RhoA and Rac1, was subsequently explored in immunohistochemical and *in situ* hybridisation studies involving eighteen MTLE cases with or without GCD, and three normal post mortem cases. In cases with GCD, most dispersed granule cells in the outer-granular and molecular layers have an elongated soma and bipolar processes, with intense RhoA immunolabelling at opposite poles of the cell soma, while most granule cells in the basal granule cell layer were devoid of RhoA. A higher density and percentage of cells expressing RhoA was observed in cases with GCD than without GCD (P<0.004). In GCD cases, the density and percentage of cells expressing RhoA was significantly higher in the inner molecular layer than granule cell layer (P<0.026), supporting proteomic findings. *In situ* hybridisation studies using probes against *RHOA* and *RAC1* mRNAs revealed fine peri- and nuclear puncta in granule cells of all cases. The density of cells expressing *RHOA* mRNAs were significantly higher in the inner molecular layer of cases with GCD than without GCD(P=0.05). In summary, our study has found limited evidence for ongoing adult neurogenesis in the hippocampus of patients with MTLE, but evidence of differential dysmaturation between dispersed and basal granule cells has been demonstrated, and elevated expression of Rho GTPases in dispersed granule cells may contribute to the pathomechanisms underpinning GCD in MTLE.

Introduction

Temporal lobe epilepsy is the most common form of pharmacoresistant epilepsy in adults (Engel, 1998). Up to 80% of patients with mesial form of temporal lobe epilepsy (MTLE) have structural abnormalities in the hippocampus (de Tisi et al., 2011; Blumcke et al., 2017). Over 60% of patients with MTLE and hippocampal pathologies remained seizure-free for at least a year after surgical resection of the hippocampus as treatment for their epilepsy (de Tisi et al., 2011; Engel et al., 2012; Blumcke et al., 2017), suggesting that the hippocampus is the primary site for epileptogenesis.

Hippocampal Sclerosis ILAE Type 1 (HS Type 1) is the most common pathology observed in patients with pharmacoresistant MTLE, and it is primarily characterised by segmental loss of pyramidal neurons in cornu Ammonis subfields CA1, 3, and 4 (Blumcke et al., 2017). Over half of the MTLE patients with HS Type 1 also have abnormalities affecting dentate granule cells (DGCs) including granule cell dispersion (GCD) and mossy fibre sprouting (Wieser, 2004; Blümcke et al., 2009; Thom et al., 2010; Da Costa Neves et al., 2013). In the normal human hippocampus, DGCs are organised into a compact layer of up to ten cells thick or ≤120µm (Houser, 1990; Wieser, 2004). In GCD, ectopic DGCs in clusters or rows disperse to the inner and outer molecular layers of the sclerotic hippocampus, widening the granule cell layer to up to 400 µm in thickness (Blümcke et al., 2009). The presence, severity and extent of GCD along the hippocampal body is highly variable amongst patients with MTLE (Thom et al., 2010; Blumcke et al., 2013) and there is no confirmed grading scheme for GCD in MTLE. The exact cause of GCD in human MTLE is unknown; however, animal models of MTLE with or without hippocampal sclerosis have shown that seizures can displace the migration of newly-generated (Parent et al., 2006; Hester et al., 2013) and mature DGCs to CA4 and molecular layer (Murphy et al., 2011; Ryuta et al., 2012; Chai et al., 2014). Reelin is an extracellular matrix glycoprotein secreted by Cajal-Retzius cells during neurodevelopment to regulate the correct layering of migrating cells (Frotscher, 1998). Low levels of reelin transcript and protein have been reported in the hippocampus of patients with MTLE (Haas et al., 2002; Haas et al., 2010) and animal models of MTLE (Heinrich et al., 2006), possibly as a consequence of elevated methylation at the promotor region of *RELN* (Kobow et al., 2009) or loss of reelin-synthesising neurons in hippocampus (Haas et al., 2002; Orcinha et al., 2016). The loss of reelin in MTLE is believed to lead to 'over-running' of DGCs into the molecular layer. Past studies have shown that pharmacological inhibition of mammalian target of rapamycin (mTOR) pathway can prevent the development of the mossy fibre sprouting (Buckmaster et al., 2009) and reduce the severity of GCD in animal models of MTLE (Lee et al., 2018), suggesting that the mTOR pathway may have a role in the pathomechanisms of these abnormalities. In patients with MTLE, most astroglial cells strongly expressed markers of mTOR signalling activation such as phospho-S6 ribosomal protein in the sclerotic hippocampus, whereas DGCs showed minimal immunohistochemical evidence of mTOR activation (Sha et al., 2012; Sosunov et al., 2012; Liu et al., 2014). Clinicopathological studies reported that the presence of GCD in patients with MTLE was associated with a history of early onset of epilepsy and febrile seizures (<4years) and longer duration of epilepsy (Lurton et al., 1998; Blümcke et al., 2009) suggesting that GCD may be a consequence of seizures or brain trauma acquired during the first decade of life where dentate neurogenesis is still active. Although it is unclear whether the presence of GCD is associated with positive surgical outcomes for patients with pharmacoresistant MTLE based on existing literature (Blümcke et al., 2009; Thom et al., 2010; Da Costa Neves et al., 2013), there is supportive evidence from animal studies to show that ectopic DGCs increase hippocampal excitability by having a lower activation threshold, forming excess dendritic axonal connections and receiving more excitatory and fewer inhibitory synaptic inputs than normal cells (Murphy, Danzer, 2011)(Zhan

115 et al., 2010)(Althaus et al., 2019). In patients with MTLE, GCD is often observed in
116 conjunction with mossy fibre sprouting, where mossy fibres of DGCs form excitatory synaptic
117 contact with apical dendrites and spines of neighbouring DGCs in the molecular layer (Sutula
118 et al., 1989; Cavazos et al., 2003), thus potentially creating an internal, pro-epileptogenic
119 circuit.
120
121 DGCs are functionally important for cognition and memory since they filter the main inputs
122 into the hippocampus, and propagate signals by innervating pyramidal neurons in CA3 and
123 CA1. Electrophysiological *in vivo* animal studies have demonstrated that DGCs normally have
124 low-excitability, and only a small, spatially-defined population of DGCs would fire to allow
125 the execution of fine and spatially-complex activities such as pattern separation, novelty
126 detection and spatial discrimination (Kahn et al., 2019). Stimulated DGCs release vesicles
127 containing glutamate to activate population firing of interconnected CA3 pyramidal cells
128 (Miles et al., 1983; Scharfman et al., 2014). Consequently, many stimulated DGCs would
129 enhance hippocampal excitability, thus increasing the chances of seizures (Overstreet-Wadiche
130 et al., 2006; Hester, Danzer, 2013), and reducing the ability to perform fine, spatial
131 discrimination tasks (Kahn et al., 2019). Silencing DGCs using ontogenetic manipulation can
132 reduce seizure frequency and reverse cognitive impairments in animal models of MTLE
133 (Krook-Magnuson et al., 2015).
134
135 In view of the important role DGCs play in cognition and promoting epileptogenesis, it is
136 important to understand mechanisms and substrates driving abnormal displacement of DGCs
137 in patients with MTLE. The proteome of the human hippocampus has been studied in normal
138 (Edgar et al., 1999a; Föcking et al., 2012; Koopmans et al., 2018), and diseased post mortem
139 human brains, including in the context of schizophrenia (Edgar et al., 1999b), Alzheimer's
140 disease (Edgar et al., 1999b; Sultana et al., 2007; Begcevic et al., 2013; Hondius et al., 2016),
141 and non-neural malignancies (Yang et al., 2004a). In epilepsy, the proteomes of surgically-
142 resected hippocampi from patients with refractory TLE (Czech et al., 2004; Yang et al., 2004b;
143 Yang et al., 2006; Persike et al., 2012; Mériaux et al., 2014; Persike et al., 2018) or temporal
144 cortex (Eun et al., 2004; He et al., 2006; Keren-Aviram et al., 2018) have been studied . Most
145 of these past studies investigated the whole hippocampus rather than specific hippocampal
146 subregions, and information about structural hippocampal pathology was not disclosed in three
147 studies (Czech et al., 2004; Persike et al., 2012; Persike et al., 2018). None of the previous
148 human proteomic studies discussed GCD in their samples. We aimed to investigate the
149 proteomes of DGCs located in the basal and dispersed regions of the granule and molecular
150 layers of patients with HS Type 1 and GCD, and to identify the molecular substrates that
151 mediate GCD. Ectopic DGCs are potential substrates for recurrent excitation, and they may be
152 the key to understanding MTLE with hippocampal sclerosis and GCD, and its comorbidities
153 including cognitive and memory impairments.
154
155 **Materials and methods**
156
157 **Cases**
158 Patients with refractory MTLE who had undergone surgical resection of hippocampus as
159 treatment for their epilepsy between 2005 and 2016 were identified from the records of UCL
160 Epilepsy Society Brain and Tissue Bank (Table 1). All patients provided written informed
161 consent for use of tissue in research studies in accordance with the Declaration of Helsinki, and
162 the study has obtained ethical approval. Eight cases with age at surgery ranged from 20 to 60
163 years were submitted to proteomic analysis. All cases had HS Type 1 with marked GCD
164 pathology as confirmed by an experienced neuropathologist.

165
166 At initial neuropathological assessment, two 5mm-thick blocks from each case were coronally
167 sampled from the middle of the hippocampus (2 cm from anterior tip) to ensure that the dentate
168 gyrus was presented for assessment and subsequent experiments. One block of the sampled
169 hippocampus was snap-frozen in liquid nitrogen, stored in -70 freezers and later retrieved for
170 proteomic studies, while the other block was fixed in 10% neutral-buffered formalin, processed
171 and embedded in paraffin wax for histological staining and immunohistochemistry.
172
173 **Laser Capture Microdissection**
174 Fourteen sections of 14 µm thickness were sectioned from each frozen hippocampal sample,
175 and sections were collected onto polyethylene terephthalate metal frame slides for laser capture
176 microdissection (Leica, Milton Keynes, UK). Two additional sections were collected onto a
177 microscopic slide (VWR International, UK) and stained briefly in 0.1% toluidine blue (pH 4.5)
178 solution for five seconds to visualise the granule cell layer. Laser capture microdissection
179 (LCM 700; Leica, Milton Keynes, UK) was then carried out along the entire length of the GCL
180 of multiple sections per case to capture basal and dispersed DGCs (Figure 2A). Basal samples
181 included DGCs in the granule cell layer closest to CA4, while the dispersed samples included
182 ectopic DGCs in the outer-granular layer and inner and outer molecular layers. A total tissue
183 area of 9±1 mm2 was dissected for each case, and submitted for proteomic analysis.
184
185 **Proteomic and bioinformatics analyses**
186 Samples were denatured using in-solution trypsin digestion and prepared for MSe label-free
187 quantitative proteomics as described previously (Heywood et al., 2013; Manwaring et al., 2013;
188 Liu et al., 2016). Data was processed using ProteinLynx GlobalServer version 2.5. Protein
189 identifications were obtained by searching the UniProt reference human proteomes with the
190 sequence of porcine trypsin (P00761) added. Protein identification from the low/high collision
191 spectra for each sample was processed using a hierarchical approach where more than three
192 fragment ions per peptide, seven fragment ions per protein, and more than two peptides per
193 protein had to be matched. Peptide identification was accepted if they could be established at
194 95% or greater probability. Statistical analyses of group means were performed using t-test in
195 SPSS (v25; IBM, USA) to identify significantly differentially expressed proteins between
196 dispersed and basal samples, or younger and older cohorts (P<0.05). Younger cohort consisted
197 of four cases with an age at surgery ranging from 20 to 34 years, and the older cohort included
198 four cases with an age at surgery ranging from 51 to 60 years. For volcano plots, fold change
199 between groups was transformed using base 2 logarithmic transformation and plotted against
200 negative logarithmic transformation of P-value. List of differentially expressed proteins in
201 Basal, Dispersed, Younger and Older clusters included proteins found only in the respective
202 samples, and proteins that were significantly different between comparison group (fold
203 change>1.5, P<0.05). Enriched lists were analysed using bioinformatics resources, Database
204 for Annotation, Visualisation and Integrated Discovery (DAVID; version 6.8; (Huang da et al.,
205 2009b, 2009a) and Enrichr (Chen et al., 2013; Kuleshov et al., 2016), to obtain information
206 regarding biological, cellular and molecular functions of proteins. Interactions between
207 proteins were investigated using STRING analysis (https://string-db.org/), and proteins in
208 functional pathways were explored in established pathway databases, Kyoto Encyclopaedia of
209 Genes and Genomes (KEGG), Reactome, and Wikipathways. P values and adjusted P values
210 (corrected according to Bonferroni's method and false discovery rate) were calculated by
211 bioinformatics resources. For gene ontology annotation and enriched pathway analyses,
212 stringency of the criteria was set to high, and P value cut-off was set at 0.01 and only pathways
213 with four proteins of interests or more were presented. The dendrogram was constructed using
214 Morpheus (Broad Institute; https://software.broadinstitute.org/morpheus) and included

215 hierarchical clustering based on Pearson correlation coefficient, and average linkage clustering
216 (Meunier et al., 2007).
217
218 Other clinical details, including patients' epilepsy history and psychometry were also reviewed
219 and analysed with proteomic data. Pre-operative MRI sequences, PET, EEG, video-telemetry,
220 and psychometry had been carried out according to the epilepsy surgical protocols at the
221 National Hospital of Neurology and Neurosurgery, and clinical findings were reviewed for
222 each case.
223
224 **Immunohistochemistry**
225 Eighteen cases were included in RhoA, Rac1, and Cdc42 immunohistochemistry and/or *in situ*
226 hybridisation studies. Cases included eight MTLE cases submitted for proteomics, an
227 additional six surgical MTLE with hippocampal sclerosis and GCD, four surgical MTLE with
228 hippocampal sclerosis but no GCD, and three postmortem healthy controls (Table 1).
229
230 5µm-thick formalin-fixed, paraffin-embedded sections from selected cases were examined
231 histologically with Haematoxylin and Eosin, and Luxol Fast Blue stains. Routine automated
232 immunochemistry was performed using antibodies against neuronal nuclei (NeuN), glial
233 fibrillary acidic protein (GFAP), non/phosphorylated neurofilament (SMI32, SMI31), myelin
234 basic protein (SMI94), calbindin, nestin (Table 2). Additional immunohistochemistry using
235 anti-RhoA was performed manually using pretreatment and incubation procedures described
236 in Table 2, and breast carcinoma tissue was used as positive control (Ma et al., 2010). Negative
237 controls where primary antibodies were omitted showed no specific labelling. A number of
238 commercially-available antibodies against RhoA, RhoB and Cdc42 (Santa Cruz
239 Biotechnology, Germany), RhoC and pan-Rac1 (Cell Signalling Biotechnology, UK) were also
240 tested using formalin-fixed, paraffin-embedded human brain tissue, but no specific
241 immunolabelling was obtained after applying a number of different antigen retrieval methods.
242 These antibodies were excluded from further immunohistochemical studies.
243
244 *In situ* **hybridisation**
245 5µm-thick formalin-fixed, paraffin-embedded sections from selected surgical cases were
246 processed for *in situ* hybridisation following supplier's instructions (Wang et al., 2012). Probes
247 against human *RHOA, RAC1, CDC42* and *RELN* mRNAs were used in conjunction with ready-
248 to-use reagents from RNAscope® 2.5 HD Reagent Brown and Duplex kits (Table 2; Bio-
249 Techne, Abingdon, UK). Positive probe control, *UBC*, and negative probe control, *dapB*
250 transcripts, were performed on Hela cell tissue (positive tissue control) and formalin-fixed,
251 paraffin-embedded surgical human brain tissue to ensure specificity of labelling prior to final
252 studies. Labelled sections were counterstained with Gills I haematoxylin solution (VWR, UK)
253 before coverslipped.
254
255 **Image analysis**
256 Labelled sections were assessed qualitatively using a brightfield microscope (Nikon Eclipse
257 80i), and subsequently scanned at 40x magnification using the whole slide scanner,
258 AxioScan.Z1 (Zeiss, Germany) to obtain high-quality digital images for quantitative analysis
259 and data interpretation. All images were viewed and analysed using the image analysis
260 software, QuPath (Bankhead et al., 2017). Criteria for GCD: At presently, there are no strict
261 criteria to evaluate GCD, or to identify the boundaries amongst granule cell layer and inner and
262 outer molecular layers. In this study, the extent of GCD in each case was assessed by measuring
263 the thickness of the granule cell layer along the external and internal limbs on haematoxylin
264 and eosin stained, whole-slide images at 2.8x magnification. The curved region joining the

265 internal and external limbs of GCL was not included. An average of 19 ± 2 measurements
266 (mean ± s.e.m.) with a mean interval of 400 ± 3 µm were made along the entire length of GCL
267 in each case. Each line measurement was taken from the basal granule cell layer closest to CA4
268 to the furthest dispersed DGCs in the outer molecular layer. The length of each line was then
269 divided into three groups: ≤120 µm (no GCD, DGCs within granule cell layer as previously
270 reported in (Blümcke et al., 2009)), between 121 to 215 µm (moderate GCD, scattered DGCs
271 in the inner molecular layer) and ≥216 µm (severe GCD, scattered, clusters or rows of DGCs
272 in the outer molecular layer). The threshold of 215 µm was taken from the longest line
273 measurement to the most dispersed cell minus 120 µm (granule cell layer) to get the thickness
274 of the molecular layer and then divided by two to derive a mid-point to divide the inner and
275 outer molecular layer (Supplementary Material 1). The frequency of lines in each measurement
276 group was used to categorise the extent of GCD in each case.
277
278 Automated quantification of RhoA immunopositive labelling, and RhoA and Rac1-positive
279 puncta from *in situ* hybridisation studies were performed using QuPath. First, granule cell
280 layer, inner and outer molecular layers spanning 120, 215 and 310 µm from the basal granule
281 cell layer were annotated. The software was then trained to recognise haematoxylin-stained
282 nuclei, positive labelled cells and puncta using positive cell and subcellular detection modules
283 (Bankhead et al., 2017). The number of cells with RhoA-positive labelling and RhoA/ Rac1-
284 positive puncta per µm², and the percentage of cells detected with RhoA immunolabelling and
285 RhoA/ Rac1-positive puncta were recorded and compared between MTLE cases with or
286 without GCD, three dentate regions and younger or older cohorts using non-parametric Mann-
287 Whiney or Krusal-Wallis tests in SPSS (IBM, USA; P<0.05). Spearman correlations were
288 performed to examine the relationship between quantitative measures and thickness of granule
289 cell layer, age of onset or age at surgery (P<0.01).
290
291 **Results**

292 *Granule cell pathology observed in cases submitted for proteomics*
293 In MTLE cases with GCD, a thick band of **NeuN**-positive cells was observed in the dentate
294 granule cell layer (Figure 1A). Two morphologies of NeuN-positive DGCs were noted: the
295 basal population of DGCs located closer to CA4 were round and tightly packed with
296 neighbouring DGCs (basal DGCs), and a dispersed population of NeuN-positive DGCs with
297 round or elongated soma with uni- or bipolar processes located in outer-granular and molecular
298 layers of the hippocampus (dispersed DGCs). In case E6, bilamination of granule cell layer
299 was observed, and a gap of approximately 130 µm measured from bottom of the basal layer to
300 the top of the dispersed layer was noted. No marked difference in NeuN-positive
301 immunolabelling was noted between younger and older cohorts. In contrast, the granule cell
302 layer in the hippocampus of four MTLE cases with no GCD (Figure 1F) and healthy post
303 mortem controls contained round, tightly packed NeuN-positive cells, and no immunopositive
304 cells were observed in the molecular layer. Quantitative measures of granule cell layer revealed
305 a significantly thicker granule cell layer in MTLE cases with GCD than cases without GCD or
306 healthy controls (P=0.004; mean±s.e.m., range; *cases with GCD* 133±11 µm, 71-213 µm; *cases*
307 *without GCD* 64±10µm, 34-82 µm; *healthy controls*; 70±4 µm, 63-76 µm).
308
309 **MAP2**-positive cells were observed in the granule cell layer of all MTLE cases (Figure 1B,
310 1G). Numerous MAP2-positive processes were observed in the granule and molecular layer.
311 Random short MAP2-positive processes were observed in the CA4 around a few large densely-
312 labelled MAP2-positive cells. **Calbindin**-positive cells were observed in DGCs scattered
313 throughout the outer-granule cell layer and molecular layer of MTLE cases with GCD (Figure

314   1C). In cases with GCD, calbindin-positive dispersed DGCs had round cell bodies, and
315   occasionally, calbindin-positive cells with long processes extending into the molecular layer
316   were noted. Not all DGCs in the outer-granular layer were calbindin-positive, and majority of
317   basal DGCs in the sclerotic hippocampus were devoid of calbindin immunolabelling. In
318   contrast, calbindin-positive DGCs were often observed in the granule cell layer of cases
319   without GCD (Figure 1H). **ZnT3**-positive processes were observed in between DGCs in
320   granule cell layer and in the molecular layer and CA4 subfield of cases with GCD (Figure 1D).
321   In cases without GCD, ZnT3 immunolabelling was primarily observed in CA4 (Figure 1I).
322   **GFAP** immunolabelling was observed throughout the hippocampus of all cases, with more
323   intense labelling detected in MTLE cases with GCD (Figure 1E) compared to cases without
324   hippocampal sclerosis or GCD (Figure 1J), and healthy controls. DGCs in granule cell layer
325   were not immunopositive for GFAP, but GFAP-positive processes extended between DGCs in
326   the granule cell layer, and individual GFAP-positive cells were observed in molecular layer of
327   cases with GCD. In cases with GCD, very dense matrix of GFAP processes were observed in
328   the CA4. In contrast, distinctive GFAP-positive cells were seen in the CA4 of cases without
329   GCD (Figure 1J) and in healthy postmortem controls.

331   *Proteomic analysis*
332   1882 proteins were identified in the proteomic analysis of eight patients with HS Type 1 and
333   GCD (Table 1, E1-8). 29% of the extracted proteins were observed in the basal samples only,
334   17% in the dispersed samples only, and 54% of the proteins were observed in both samples
335   (Figure 2B). 19% of identifiable proteins were observed in the younger cohort only, 28% in
336   older samples only and the remaining proteins were found in both younger and older samples.
337   Similar changes in protein expression were observed between two cases in the younger cohort
338   (E1 and E2), and amongst three older cases, E5, E6, E8 based on hierarchical clustering (Figure
339   2C).

341   Common neuronal markers such as MAP2, calbindin, and calretinin were detected (Figure 2D).
342   The protein abundance of MAP2, calretinin and calbindin were significantly higher in
343   dispersed and younger samples than basal and older samples. The quantity of MAP2 detected
344   was higher than calbindin and calretinin in all samples as expected based on earlier
345   immunohistochemical studies (Figure 1B-C). Astroglial (GFAP, vimentin, S100-B) and
346   oligodendroglial markers (Myelin Basic Protein, MBP) were also detected in all samples,
347   consistent with immunohistochemical findings (Figure 1E). The expression of GFAP was
348   comparable between basal and dispersed samples and was slightly higher in younger than older
349   samples; of interest, the expression of vimentin, expressed in immature astrocytes, was
350   predominantly observed in basal and younger samples (Figure 2D) in keeping with our
351   previous studies (Liu et al., 2018). The expression of connexion 43, a gap junction protein
352   expressed in astrocytes, was similar to the expression of GFAP. Common microglial-specific
353   proteins including TREM2, LST1, HLA-DRA, SP11, MMP9 were not found in our samples,
354   but complement component 1q subcomponent binding protein (C1QBP), a protein found to be
355   highly expressed in microglia around site of lesion in a recent animal study (Barna et al., 2019),
356   was found to be expressed in both dispersed and basal samples. We did not detect common
357   markers of neural progenitors or neuroblasts including SOX2, PAX6, TBR1, TBR2, and DCX;
358   however, a higher abundance of cell adhesion molecule 2 (CADM2), a synapse-associated
359   protein found in subventricular neurogenic niche (Lee et al., 2012; Frese et al., 2017), as well
360   as a number of cell cycle markers (MCM2, PCNA, Cyclin B3) were found in higher amount in
361   the basal DGCs than dispersed DGCs in cases with GCD (Figure 2D). Neural cell adhesion
362   molecule 1 (NCAM) was also detected in dispersed samples of cases with GCD. Together these
363   findings suggest that apart from DGCs and astroglial cell types, minimal level of microglia and

364 neural progenitor cells were included in our capture. A number of proteins involved in actin
365 and cytoskeleton remodelling including profilin-1 and 2, alpha synuclein, f-actin capping
366 proteins, dihydropyrimidinase-related protein 2 (also known as CRMP2), and synapse proteins
367 such as synaptotagmin-1 and synapsin-2, were identified in dispersed and younger samples at
368 a higher level than in basal and older samples. The detection of serum albumin, carbonic
369 anhydrase 1 and haemoglobin subunits was noted in all samples, possibly due to increased
370 permeability of blood brain barrier in the hippocampus of patients with MTLE.
371
372 Volcano plots in Figure 2E and 2H highlighted the most significantly differentially-expressed
373 proteins between dispersed and basal samples, and between younger and older cohorts
374 respectively. Proteins uniquely expressed in dispersed samples, and proteins significantly
375 displaying over 1.5-fold change in dispersed compared to basal samples (Figure 2E;
376 Supplementary Material 2) were first submitted to gene ontology and pathway bioinformatics
377 analyses (Dispersed cluster, 330 proteins). The top functional annotation clustering based on
378 Gene Ontology, Uniprot keywords and sequences and INTERPRO terms were GTP-binding
379 and activity (enrichment score, 10; $P=3.67 \times 10^{-8}$), cell to cell adhesion (enrichment score, 7;
380 $P=2.61 \times 10^{-6}$), and small GTPase-mediated signal transduction (enrichment score, 7;
381 $P=5.60 \times 10^{-4}$); Figure 2F and Supplementary Material 3). Under the functional clustering of
382 small GTPase-mediated signal transduction, a number of proteins in the Ras homolog (Rho)
383 GTPase family such as RhoA, Rac1, Cdc42 and ARGAP1 and ARGAP35 were identified
384 (Table 3 and Supplementary Material 5). Key pathways associated with differential proteins in
385 the Dispersed cluster were related to cellular migration and actin cytoskeletal remodelling,
386 including signalling by Rho GTPases pathway (R-HSA194315; $P=1.02 \times 10^{-6}$), axon guidance
387 (R-HSA422475; $P=1.25 \times 10^{-6}$), regulation of actin cytoskeleton (K-HSA04810; $P=1.01 \times 10^{-4}$),
388 and vesicle mediated transport (R-HSA5653656, $P=9.29 \times 10^{-7}$; Figure 2I and Supplementary
389 Material 4). Rho GTPases are small GTPases that regulate cytoskeletal dynamics and cell
390 migration, maybe of relevance to abnormal migration of DGCs in GCD. The signalling by Rho
391 GTPases pathway shared common proteins with other pathways related to cytoskeletal
392 dynamics as illustrated in Figure 2I. Proteins detected in basal samples only, and proteins that
393 were significantly overexpressed in basal samples by at least 1.5-fold compared to dispersed
394 samples (Figure 2E and Supplementary Material 2) were subsequently submitted to gene
395 ontology and pathway enrichment analyses (Basal cluster; 555 proteins). The top functional
396 annotation clustering included proteins involved in ribosomes, translation and RNA processing
397 (enrichment score, 18; $P=1.79 \times 10^{-14}$), cell-cell adherens (enrichment score, 10; $P=1.93 \times 10^{-8}$),
398 GTP binding (enrichment score, 9; $P=4.31 \times 10^{-7}$), ubiquitin-protein ligase activity (enrichment
399 score, 4; $P=4.926 \times 10^{-2}$), and regulation of amino acid metabolic processes and proteasome
400 activity (enrichment score, 3; $P=2.07 \times 10^{-2}$; Figure 2G and Supplementary Material 3). The
401 interactions between proteins clustered under ribosomes, translation and RNA processing are
402 shown as network map in Figure 2G. Pathways associated with Basal cluster were
403 predominantly related to metabolism (R-HSA1430728, $P=2.53 \times 10^{-26}$), including amino acid
404 (WP3925, $P=234 \times 10^{-5}$) and selenocysteine metabolisms (H-HSA2408557, $P=1.71 \times 10^{-24}$), and
405 electron transport chain (WP111, $P=4.73 \times 10^{-3}$), and ribosomes (K-HSA03010, $P=5.00 \times 10^{-13}$),
406 translational mechanisms (R-HSA156842, $P=2.90 \times 10^{-24}$), and proteasomal degradation
407 (WP111, $P=4.73 \times 10^{-3}$; Figure 2J and Supplementary Material 4).
408
409
410 ***Immunohistochemical and in situ hybridisation using markers against Rho GTPases***
411 Bioinformatics analyses revealed a number of proteins in the signalling by Rho GTPases
412 pathway either uniquely expressed or upregulated in dispersed samples of MTLE cases with
413 GCD. To further investigate the expression of Rho GTPases, immunohistochemistry and *in*

414 *situ* hybridisation using antibodies and probes against RhoA, Rac1 and Cdc42 protein and
415 mRNAs respectively were performed on surgical, formalin-fixed, paraffin-embedded,
416 hippocampal tissue from cases submitted to proteomics, and additional surgical MTLE cases
417 with or without GCD. Post mortem hippocampal tissue from three healthy donors were also
418 included for qualitative assessment.
419
420 In cases without GCD, majority of DGCs in the granule cell layer were immunonegative for
421 RhoA (Figure 3A-A'). In contrast, cases with GCD had numerous RhoA-immunopositive
422 DGCs in the outer-granule and molecular layer. RhoA-immunopositive DGCs in the granule
423 cell layer generally had a large, round nucleus surrounded by a thin, perinuclear 'ring' of
424 immunopositive labelling, or a 'cone' of localised RhoA immunolabelling at one end of the
425 cell soma, usually facing either towards the molecular layer or CA4 region (Figure 3B-C).
426 Occasionally, RhoA-positive cells in single chain formation near vascular structures were
427 observed (Figure 3C-C'). Most RhoA-positive cells in the molecular layer had an elongated
428 cell soma with protruding uni- or bipolar processes. In a case with a bilaminar granule cell
429 layer (E6), most DGCs in the basal granule cell layer were devoid of RhoA immunolabelling
430 (Figure 3D), while most DGCs in the second granule cell layer had distinct RhoA
431 immunoreactivities in one or bipolar ends of the cell soma (Figure 3D' and D''). Quantitative
432 studies revealed a significantly higher density of RhoA-positive cells and a higher percentage
433 of cells expressing RhoA in the granule and molecular layer of MTLE cases with GCD than
434 without GCD (mean±s.e.m; *GCL,* GCD 44±3%, no GCD 23±6%, P=0.035; *IML,* GCD 56±3%,
435 no GCD 14±3%, P=0.001; *OML* GCD 27±5%, no GCD 10±3%, P=0.008; Figure 3E and data
436 in Supplementary Material 6). In cases with GCD, a higher percentage of cells expressing
437 RhoA were detected in the inner molecular cell layer than the granule cell layer and the outer
438 molecular layer (IML-GCL, P=0.026; IML-OML, P=0.003; Figure 3E). In contrast, the
439 percentage of RhoA-positive cells detected were not significantly different between regions in
440 cases without GCD. No significant difference in density or percentage of cells expressing
441 RhoA was noted between younger and older cohorts. A probable relationship was observed
442 between the percentage of cells expressing RhoA and age of surgery (r_s=0.390, P=0.014; Figure
443 3F), and the mean thickness of granule cell layer (r_s= 0.675, P<0.001; Figure 3G).
444
445 *In situ* hybridisation studies using probes against *RHOA* and *RAC1* mRNA sequences revealed
446 fine nuclear and perinuclear puncta in DGCs in the granule and molecular layers of all cases
447 (Figure 4A-D). *RHOA*-positive and *RAC1*-positive puncta were also observed in the neuropil,
448 likely representing processes of DGC which were not visible in the sections. In cases with
449 GCD, a high number of *RHOA*-positive and *RAC1*-positive puncta clustered at the polar ends
450 of the DGC soma, particularly in DGCs situated in the molecular layer (Figure 4B, D). Some
451 smaller haematoxylin-stained glial cells were associated with none or only a few number of
452 *RHOA*-positive or *RAC1*-positive puncta. Further immunohistochemical and *in situ*
453 hybridisation double-label studies showed that glutamine synthetase-labelled astrocytes did not
454 have *RHOA*-positive or *RAC1*-positive puncta (Supplementary Material 7).
455
456 Quantitative analyses showed that a higher density of cells with *RAC1*-positive puncta than
457 *RHOA* was observed in MTLE with GCD (mean±s.e.m, *RAC1* $2.05 \times 10^{-3} \pm 2.15 \times 10^{-4}$ per μm^2;
458 *RHOA* $1.74 \times 10^{-3} \pm 2.71 \times 10^{-4}$; P=0.017), which was not observed in cases without GCD (*RAC1*
459 $1.72 \times 10^{-3} \pm 2.27 \times 10^{-4}$ per μm^2; *RHOA* $1.25 \times 10^{-3} \pm 1.81 \times 10^{-4}$; P>0.05). In all cases, a
460 significantly higher density of cells with *RHOA*-positive puncta was observed in the granule
461 cell layer than outer molecular layer (GCD, GCL $2.83 \times 10^{-3} \pm 5.61 \times 10^{-4}$ per μm^2, OML $8.97 \times 10^{-4} \pm 1.01 \times 10^{-4}$, P=0.004 (blue bars); no GCD, GCL $2.11 \times 10^{-3} \pm 2.23 \times 10^{-4}$ per μm^2, OML $7.24 \times 10^{-4} \pm 6.94 \times 10^{-5}$, P=0.004 (red bars); Figure 4E and Supplementary Material 6). A significantly

higher density of *RHOA*-positive puncta was observed in the granule cell layer than inner molecular layer in cases without GCD (GCL $2.11 \times 10^{-3} \pm 2.23 \times 10^{-4}$ per μm^2, IML $9.25 \times 10^{-4} \pm 9.15 \times 10^{-5}$, P=0.023), but not in cases with GCD (GCL $2.83 \times 10^{-3} \pm 5.61 \times 10^{-4}$ per μm^2, IML $1.49 \times 10^{-3} \pm 1.84 \times 10^{-4}$, P>0.05), indicating that the level of *RHOA* gene expression was more similar between granule and inner molecular layers in cases with GCD than in cases without GCD. A significantly higher density of *RHOA*-positive puncta was observed in the inner molecular layer of cases with GCD than without GCD (GCL $1.49 \times 10^{-3} \pm 1.84 \times 10^{-4}$ per μm^2 IML; no GCD IML $9.25 \times 10^{-4} \pm 9.15 \times 10^{-5}$, P=0.05). In all cases, the densities of cells with *RAC1*-positive puncta were higher in granule cell layer than the outer molecular layer (GCD, $3.31 \times 10^{-3} \pm 4.47 \times 10^{-4}$ per μm^2 GCL, $1.14 \times 10^{3} \pm 1.24 \times 10^{-4}$ OML, P=0.017; no GCD, $2.92 \times 10^{-3} \pm 3.47 \times 10^{-4}$ per μm^2 GCL, $1.01 \times 10^{-4} \pm 8.99 \times 10^{-5}$ OML, P=0.018, Figure 4G). A significantly higher density of *RAC1*-positive puncta was noted in the granule cell later compared to inner molecular layer in cases without GCD (GCL $2.92 \times 10^{-3} \pm 3.47 \times 10^{-4}$ per μm^2, IML $1.23 \times 10^{-3} \pm 1.62 \times 10^{-4}$, P=0.001), but not in cases with GCD (GCL $3.31 \times 10^{-3} \pm 4.47 \times 10^{-4}$ per μm^2, IML $1.70 \times 10^{-3} \pm 2.06 \times 10^{-4}$, P>0.05). The percentage of cells with *RHOA*-positive and *RAC1*-positive puncta weakly correlate with age of onset (*RHOA*, r_s=0.439, P=0.011, Figure 4F; *RAC1*, r_s=0.294, p=0.049, Figure 4H).

In situ hybridisation using probes against *CDC42* and *RELN* mRNAs were also performed on surgical cases, and labelling was qualitatively assessed as only four cases were investigated. The regional distribution and density of cells with *CDC4*2-positive puncta were similar to *RHOA*-positive and *RAC1*-positive positive labelling. *RELN*-positive puncta were not observed in any of these cases.

Discussion
We investigated the proteome of basal and dispersed DGCs in the dentate gyrus of pharmacoresistant MTLE patients with hippocampal sclerosis to explore the neurodevelopmental pathomechanisms of GCD. We have identified differences in the proteomes between basal and dispersed populations of DGCs in the hippocampus of patients with MTLE and GCD. Specifically, dispersed DGCs in cases with GCD highly expressed mature neuronal markers, MAP2, calbindin and calretinin as well as a number of Rho GTPases and proteins associated with cell migration, cytoskeleton and synapse remodelling. These results were further supported by findings from immunohistochemical and *in situ* hybridisation studies, where a significantly higher density and percentage of cells expressing RhoA mRNA and protein was observed in dentate gyrus of cases with GCD than cases without GCD. The expression of RhoA protein was localised to the opposite ends of uni- or bipolar of DGCs in molecular layer of cases with GCD. Consistently, the mRNAs of *RHOA, RAC1* and *CDC42* were also found in the same intracellular region of DGCs, while *RAC1* mRNA was also detected in the proximal portion of processes. These findings provide evidence supportive of DGCs undergoing neurodevelopment processes relating to cellular migration, which may contribute to the abnormal dispersion of DGCs in patients with MTLE.

Comparison with previous human proteomics studies
The current study reported on 1882 proteins identified from the dentate gyrus of eight patients with refractory MTLE and HS Type I and GCD (or Type II granule cell pathology according to (Blümcke et al., 2009)) using protocols established in our previous proteomic study (Liu et al., 2016). There is no specific marker for dentate granule cells in the human brain (unlike Prox1 or NeuroD in rat brains), but other neuronal markers such as MAP2 and calbindin, which are expressed by granule cells, were detected at high levels in our immunohistochemical and proteomic studies. Our samples did not capture microglial cell contaminants, but astroglial

514 markers (GFAP, S100-B) and myelin basic protein were detected, and this is consistent with
515 the intimate intermingling of radial glial and myelinated axons in the dentate gyrus in MTLE
516 with hippocampal sclerosis.
517
518 Our proteomic studies did not include surgical or post mortem healthy controls as seen in most
519 previous human proteomic studies of MTLE. This is because (i) surgically-resected 'normal'
520 hippocampal tissue from healthy individuals is not available for research, (ii) the process of
521 protein extraction for proteomic analyses is different for surgical and post mortem brain tissue
522 so one standardised protocol cannot be applied to both type of tissue, and (iii) autopsy tissue is
523 likely to be affected by post mortem processes such as rapid protein degradation (especially for
524 tubulins, intermediate filaments, high motility group box protein-1, proapolioprotein,
525 haemoglobin and mutant derivative (He et al., 2006)) which may influence our findings. In
526 view of these arguments, we compared the protein profile of basal and dispersed DGCs as well
527 as between younger (<35 years) and older groups (>50 years) to avoid detection of changes
528 associated with tissue type.
529
530 The differential expression of certain proteins in our study is generally consistent with findings
531 from previous human MTLE studies. There is good agreement amongst human TLE
532 proteomics studies that most proteins related to metabolism are significantly upregulated in
533 epilepsy compared to archival normal brain hippocampal samples (Eun et al., 2004; Yang et
534 al., 2006; Persike et al., 2012; Persike et al., 2018). This may be because the generation of
535 epileptiform activity, or response to such activity, consumes a large amount of energy, and
536 rapid glycolysis and oxidative phosphorylation are necessary to fulfil energy demands in the
537 epileptic human brain (Kovac et al., 2017). Consistently, we found 15% of proteins extracted
538 from our epilepsy samples were involved in metabolic processes, and up to 8% of proteins in
539 the Basal cluster were involved in tricyclic acid cycle and mitochondrial electron chain (Figure
540 2G and Supplementary Material 4).
541
542 A key finding in this study is the identification of proteins in dispersed DGCs of cases with
543 GCD specifically involved in axonal guidance (including beta-spectrin, growth associated
544 protein 43, postsynaptic density protein 95, profilins), regulation of actin cytoskeleton
545 (cytoplasmic FMR1-interacting proteins, WAVE complex proteins), cytoskeletal and synaptic
546 remodelling (DPYL2, alpha-synuclein, synatotagmin 1, synapsin II, and stathmin1), as well as
547 Rho GTPases signalling (RhoA, Cdc42, Rac1, brain-specific angiogenesis inhibitor, Rho
548 GTPases activating proteins; Table 3). These processes are active during neurodevelopment
549 and in neurogenic niches in the adult mammalian brain (Lee et al., 2012; Frese et al., 2017).
550 Our findings are consistent with previous human proteomic studies which have reported
551 increased expression of DPYL2 (also known as collapsing response mediator protein 2,
552 CRMP2), a cytoplasmic phosphoprotein that binds microtubules and promotes neurite
553 outgrowth during neurogenesis and neuronal migration (Inagaki et al., 2001; Fukata et al.,
554 2002), in the hippocampus of MTLE patients compared to controls (Persike et al., 2012; Keren-
555 Aviram et al., 2018; Persike et al., 2018). The CRMP2 antagonist, lacosamide, is an
556 antiepileptic drug often used in patients with drug-resistant epilepsy (Kelemen et al., 2010).
557
558 **GCD and neurogenesis: evidence?**
559 During neurodevelopment, new DGCs migrate from the dentate ventricular zone to the granule
560 cell layer to form an 'outside-in' dentate gyrus, where the outer-granular cells are the oldest
561 (and showing earliest NeuN immunoreactivity), and DGCs located in the inner layer (basal
562 granule cells) are the youngest (Altman et al., 1990; Seress et al., 2001). A secondary dentate
563 matrix is formed at gestation week (GW) 10-11 which will later become the subgranular zone

564 that supports ongoing neurogenesis that continues to adulthood so there is also an outside-in
565 gradient for neurogenesis (Cipriani et al., 2017). Previous studies have reported that the dentate
566 neurogenesis is still active postnatally at one year of age, but proliferative and neurogenic
567 activities decline sharply between 7 to 13 years, and argued to be minimally detected in
568 adulthood (Sorrells et al., 2018) even in the brains of adult patients with MTLE (Blümcke et
569 al., 2001; Cipriani et al., 2017). The level of hippocampal neurogenesis in the normal and
570 diseased adult human brain is still controversial because the detection of neural progenitor cells
571 and new neurons in adult human brains depends on tissue quality and the immunohistochemical
572 protocols employed in studies (Sorrells et al., 2018; Moreno-Jiménez et al., 2019). Considering
573 that the level of neurogenesis is lower in adults than children, it is more plausible that new
574 DGCs born in early childhood contributes to GCD. Although, our current findings did not
575 capture immature neural progenitor cell proteins (SOX1, SOX2, TBR1, TBR2, PAX6), or
576 neuroblast proteins (DCX), even in the youngest MTLE patients with age at surgery of twenty
577 and twenty-two years, we did detect a number of proliferative cell cycle proteins, mini
578 chromosome maintenance protein 2 (MCM2), proliferative cell nuclear antigen (PCNA) and
579 cyclin B3, and immature glial progenitor protein, vimentin, in the basal DGCs of younger
580 MTLE cases and not in the dispersed DGCs, thus providing some supporting evidence of
581 neuroplasticity. Our previous immunohistochemical study found a number of proliferative cells
582 expressing MCM2 in the granule cell layer of patients with MTLE (Thom et al., 2005). In
583 addition, we detected a high abundance of neural cell adhesion molecule 1 in dispersed DGCs
584 of younger MTLE cases. In adult animal neurogenesis studies, calretinin is transiently
585 expressed in newborn DGCs before calbindin (Brandt et al., 2003). Calretinin was detected in
586 the dispersed samples of this study, however as we know marked re-organisation of calretinin
587 neurons and networks occurs in hippocampus of patients with MTLE and hippocampal
588 sclerosis (Thom et al., 2012), calretinin may not be a reliable marker of adult neurogenesis in
589 this context. Calbindin D28K was only detected in dispersed DGCs in our study, which is
590 consistent with previous studies that reported reduced expression of calbindin in basal granule
591 cell layer of patients with MTLE (Maglóczky et al., 1997; Arellano et al., 2004; Ábrahám et
592 al., 2009; Abrahám et al., 2011; Martinian et al., 2012).
593
594 We did not detect reelin, a glycoprotein secreted by Cajal-Retzius cells to regulate the
595 migration of DGCs during neurodevelopment, in our samples. This is confirmed in subsequent
596 *in situ* hybridisation studies using a probe against *RELN* mRNA, where no positive labelling
597 was observed in the hippocampus of MTLE cases with GCD. This finding is consistent with
598 previous studies that have reported low levels of *RELN* mRNA in MTLE patients with
599 hippocampal sclerosis and GCD (Haas et al., 2002; Frotscher et al., 2003; Kobow et al., 2009).
600 In those studies, reelin was detected in the CA1, 3 and 4, and we did not sample those areas.
601
602 **Rho GTPases and mechanisms for migration or GCD**
603 Due to the observation of Rho GTPases in the dispersed DGCs, these proteins have become
604 the focus of further investigation as to their role in pathomechanisms underlying GCD.
605
606 A main finding in this study is the upregulation of proteins involved in the Rho GTPase
607 signalling pathway in dispersed DGCs of patients with MTLE and GCD. Rho GTPases belong
608 to the Rho family of GTPases, which is a subgroup of Ras family of small GTP binding protein
609 (Heasman et al., 2008). Ras homologous member A (RhoA), ras related C3 (Rac1) and cell
610 division cycle 42 (Cdc42) are the three most-studied Rho GTPases, and the activated form of
611 these Rho GTPases bind with a number of effector molecules (Dia, ROCK, myosin light chain,
612 phosphatase and kinase, WAVE, Arp2/3, PAK, LIMK, Cofilin-P, Mec-3, WASP; see
613 Supplemental Material 5) to regulate actin polymerisation, microtubule stabilisation and

actomyosin contractility during neurodevelopment when cells undergo active morphogenesis, and participate in migratory, proliferative, and survival activities (Heasman, Ridley, 2008; Zarco et al., 2019). Data from the Human Brain Transcriptome project has reported continuous gene expression of *RHOA, RAC1* and *CDC42* in the human hippocampus from embryonic and early fetal periods to adulthood (4 post-conceptional weeks to 60 years of age; Supplementary Material 8) (Kang et al., 2011). Similarly, we observed a high number of DGCs expressing *RHOA, RAC1* and *CDC42* mRNAs in the brains of adult MTLE patients with and without GCD, with age at surgery spanning 20 to 60 years. Whether the protein expression of these Rho GTPases is continuously expressed in DGCs from development to adulthood in the normal and MTLE human brain remain to be investigated as there is currently limited information. In this study, we found significantly higher protein expression of RhoA in uni- or bipolar ends of DGCs in cases with GCD than without GCD, particularly in displaced DGCs in the outer-granule and molecular layers. RhoA was absent in most DGCs located in basal granule cell layer of MTLE cases. Considering the established role of RhoA in polarised cell migration in neurogenic niches, it is plausible that RhoA has a role in the mismigration of DGCs in cases with DGCs. The distinct localisation of RhoA to the apices of dispersed DGC soma may be suggestive of proximal cytoplasmic bulging, a characteristic feature of cells undergoing saltatory migratory during neurodevelopment and in neurogenic zones of adult mammalian brains (Schaar et al., 2005; Wang et al., 2019). During saltatory migration, the centrosome of the migrating cell moves forward to form a transient swelling in the proximal leading process and then cell body and nucleus move towards the centrosome then pause and this process is repeated again. Previous *in vitro* morphodynamic studies have found spatiotemporal changes to the expression of RhoA in migrating cells as they undergo saltatory migration (Fritz et al., 2013; Martin et al., 2016; Kaneko et al., 2017). These studies detected RhoA initially at the transient swelling of the leading proximal process where RhoA interacted with effector proteins to regulate f-actin and myosin II in the actomyosin contraction to allow cell soma to translocate forward (Solecki et al., 2009; Ota et al., 2014). As the cell moved forward then stalled, RhoA expression was reduced in the front and gradually increased at the rear of the cell to facilitate retraction of cell soma and back processes, allowing the entire cell to move forward. Thus, RhoA expression may be detected at either or both ends of the cell soma depending on the cell's migratory state. In agreement, we observed varying numbers of DGCs with RhoA accumulation in one or both ends of the cell soma in cases with GCD. Rho GTPase-mediated cell migration is a well-coordinated process during neurodevelopment, tightly regulated by a number of guanine nucleotide exchange factors and dissociation inhibitors, and GTPase-activating proteins to ensure new neurons arrive timely at a specific location (Schmidt et al., 2002). Genetic knockout of Rho GTPase-activating proteins, such as Gimp, in animal studies can lead to appearance of ectopic cells (Ota et al., 2014). In this study, Gimp was not detected in our samples, but other GTPase-activating proteins, such as ARGAP1, ARGAP35, A-kinase anchor protein 13, SLIT-ROBO Rho GTPase-activating protein 3 were detected, and further studies are needed to investigate their cellular expression and role in regulating DGC migration in MTLE. In our study, a number of *RHOA* and *RAC1*-positive puncta was observed in the neuropils of MTLE cases. It is possible that some of these positive puncta may be transcripts localised to the processes of DGCs. Previous studies have demonstrated the protein expression of Rac1 and Rho GTPases-exchange factors at the tip of leading processes of migrating cells (Shinohara et al., 2012; Hikita et al., 2014). It is also plausible that *RHOA* and *RAC1* mRNAs are expressed in surrounding glial cells in the granule and molecular layers, although our initial double labelling experiments did not detect *RHOA* and *RAC1* transcripts in astroglial cells expressing glutamine synthetase.

663 Other modes of cellular migration involving Rho GTPases include 'frog leap' and 'vessel-
664 based' migrations. Using live imaging techniques, a study reported that 50% of the adult-born
665 new DGCs had leading process pointed tangentially in the subgranular zone of the hippocampal
666 granule cell layer, and these cells underwent lateral migration in small clusters coupled by
667 connexion 43 before migrating radially into the deeper granule cell layer (Wang et al., 2019).
668 During this migratory process, the leading cells in the migratory cluster changed repeatedly
669 (akin to leapfrog), hovering forwards and backwards as the cluster moved towards their final
670 destination. Other animal studies have also reported that gap junctions are important for
671 neuronal dispersion in embryonic cortex (Elias et al., 2007; Elias et al., 2010; Yu et al., 2012).
672 In our study, expression of connexin 43 was observed in dispersed DGCs of cases with GCD
673 and we did observe a number of dispersed DGCs in single line formation in outer-granule and
674 molecular layers of cases with GCD, sometimes in close proximity to vascular structures. In
675 microvessel-based migration, DGCs are postulated to migrate tangentially along blood vessels,
676 followed by limited radial migration into the granule cell layer (Sun et al., 2015). In developing
677 animals, it is known that large plexuses of vasculature are developed in the molecular layer and
678 CA4 during postnatal day 0 to 7, while only short bridges of blood vessels are found to extend
679 through the granule cell layer (Pombero et al., 2018).
680
681 **CONCLUSION**
682 In conclusion, we have shown limited evidence to support ongoing adult neurogenesis in the
683 hippocampus of patients with MTLE, but evidence of differential dysmaturation between
684 dispersed and basal DGCs has been shown. We have provided evidence from proteomic and
685 immunohistochemical studies to demonstrate that DGCs contribute to ongoing structural and
686 synaptic changes in the MTLE human brain, and expression of Rho GTPases in these cells may
687 support abnormal cellular migratory activities that are linked to GCD pathology. Further
688 studies are required to assess the possible contribution of DGCs expressing Rho GTPases to
689 seizure generation and cognitive impairments.
690
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693 and Tissue Bank and consented for the use of their valuable tissue in research. We would like
694 to acknowledge Houda Aljibouri for her assistance with optimising conditions for double-label
695 *in situ* hybridisation and immunohistochemical experiments.
696
697 **AUTHORS CONTRIBUTIONS**
698 JL, MT and SMS planned and designed the study. KM and this team performed the proteomics
699 analyses. ND and BA conducted experiments using *in situ* hybridisation and
700 immunohistochemistry respectively. JL performed bioinformatics and quantitative analyses.
701 All authors contributed to the writing and reviewing of the manuscript.
702
703 **CONFLICT OF INTEREST**
704 Authors do not have any conflict of interest.
705
706 **CONTRIBUTION TO FIELD**
707 Epilepsy is a neurological disorder that affects over 600,000 people in the UK and 1% of the
708 population worldwide. Currently, there is no cure for patients with epilepsy. A number of
709 structural abnormalities can be found in the surgical and postmortem brains of patients with
710 epilepsy, and these abnormalities may contribute to seizure generation and cognitive
711 impairments. Understanding the mechanisms and molecular substrates underlying abnormal
712 structural changes in epilepsy may lead to the identification of new targets for therapeutic

713 interventions. The present study explored the protein profiles of granule cells situated in the
714 basal (control) and dispersed hippocampal granule cell layer of eighteen patients with Mesial
715 Temporal Lobe Epilepsy and granule cell pathology. Proteomics and bioinformatics analyses
716 demonstrated that many key Rho GTPases and related proteins were upregulated in displaced
717 granule cells, and further *in situ* hybridisation and immunohistochemical studies showed that
718 mRNAs and proteins of Rho GTPases were spatially located in polar ends of cell body and
719 proximal part of extending processes, resembling Rho GTPases expression in actively
720 migrating cells during neurodevelopment. Findings from our study suggest that hippocampal
721 granule cells may undergo Rho GTPase-mediated migration, possibly contributing to granule
722 cell pathology and hippocampal reorganisation in MTLE.
723

FUNDING

734

Table and figure legends

736

737 **Table 1. Clinical details of cases studied.** Eight cases with Hippocampal Sclerosis Type I
738 (according to the International League Against Epilepsy classification system) and granule cell
739 dispersion were analysed using proteomics (E1-8, in grey). In addition to these cases, another
740 ten surgical cases with MTLE and three post mortem normal cases were included in RhoA and
741 Rac1 immunohistochemical and/or *in situ* hybridisation studies. *In situ* hybridisation studies
742 using probes against *CDC42*, or *RELN* transcripts were performed on four cases (E1P, E122,
743 E1, E3). *Abbreviations:* Carb, carbamazepine; COD, cause of death, Clob clozabam; Dia
744 diazepam, Gaba, Gabapentin; GCD, granule cell dispersion; GG ganglioglioma; HS
745 hippocampal sclerosis; Lam, Lamotrigine; Lev, Levetiracetam; MCD malformation of cortical
746 development; MFS mossy fibre sprouting; OH oligodendroglial hyperplasia in white matter;
747 Ox, Oxcarbazepine; Pheny, Phenytoin; Pheno, Phenobarbitone; Preg, Pregabalin; Prim,
748 Primidone; TS temporal sclerosis, Tem temapazem, Top topirmate; Val, Sodium valproate;
749 Zon, Zonisamide.

750

751 **Table 2 Antibodies and protocols for chromogenic *in situ* hybridisation and**
752 **immunohistochemical studies.** Automated immunohistochemistry was performed using the
753 Bond Max automated immunostainer and reagents (Leica, Milton Keynes, UK). *RNA
754 detection was performed using RNAscope patent assays system following supplier's
755 instruction. *Abbreviations:* aa, amino acids; Cdc42 Cell division control protein 42 homolog;
756 GFAP, glial fibrillary acidic protein; K, thousands; MAP microtubule associated protein;
757 NeuN, neuronal nuclei; ov, overnight, Rac1 Ras-Related C3 Botulinum Toxin Substrate 1;
758 RhoA Transforming protein RhoA or Ras homolog family member A; RT room temperature.
759 Antigen retrieval buffers: ENZ1, Leica Bond enzyme concentrate and diluent; ER1, Leica
760 Bond citrate-based buffer; ER2, Leica Bond EDTA-based buffer; H-3301, Vector's Tris-based
761 buffer pH 9.0; H-3300, Vector's citrate-based buffer pH 6.0; SC, Sodium citrate buffer, pH
762 6.0. Suppliers: Abcam plc., Cambridge, UK; BD Transduction Lab., Oxford, UK; Bio-Techne,

763 Abingdon, UK; DAKO, Cambridgeshire, UK; Millipore, Watford, UK; Sternberger, Maryland,
764 US; Swant, Marly, Switzerland; Thermo Fisher Scientific, Hemel Hempstead, UK; Synaptic
765 systems, Goettingen, Germany.
766
767 **Figure 1 Hippocampal pathology observed in a MTLE patient with Hippocampal**
768 **Sclerosis and Granule Cell Dispersion.** (**A**) NeuN-positive granule cells were observed in the
769 granule cell layer of the hippocampal dentate gyrus. The number of dentate granule cells
770 (DGCs) remained abundant despite marked loss of pyramidal neurons in the cornu Ammonis
771 (CA) 1, 3 and 4 subfields, which are typical features of Hippocampal Sclerosis Type 1 based
772 on classification scheme established by the taskforce of International League against Epilepsy
773 (ILAE)(Blumcke et al., 2013). However, majority of the NeuN-positive DGCs appeared to
774 scatter in rows or clusters to the outer-granular layer and molecular layer (MOL), which is
775 consistent with granule cell dispersion pathology. NeuN-positive pyramidal neurons were still
776 observed in the CA2 region. (**B**) Strong MAP2-positive DGCs were observed in the granule
777 cell layer. MAP2-positive DGCs had immunopositive fibres projected towards the molecular
778 layer, and MAP2-positive labelling appeared to be stronger in the molecular layer than in the
779 basal granule cell layer. MAP2-positive fibres of various length and a small number of MAP2-
780 positive cells were observed in CA4 region. (**C**) Calbindin immunoreactivity was observed in
781 most, but not all, dispersed and elongated DGCs in the granule and molecular layer. In contrast,
782 small, round basal DGCs were immunonegative for calbindin. An intense blush of granular
783 calbindin-positive labelling was also observed in the molecular layer. (**D**) Intense ZnT3-
784 positive labelling was observed in the molecular layer, particularly in the inner molecular layer.
785 This is consistent with mossy fibre sprouting pathology in MTLE. Clusters of ZnT3-positive
786 fibres were observed predominantly in the CA4 region. (**E**) Dense matrix of GFAP-positive
787 fibres was observed in all CA regions. GFAP-positive radially-directed fibres extended
788 between DGCs in the granule cell layer, and GFAP-positive cells were visible in the molecular
789 layer. (F-J) Images showing the immunoreactivities of NeuN, MAP2, calbindin, ZnT3 and
790 GFAP in the hippocampus of a MTLE patient with no remarkable hippocampal pathologies.
791 Scale bars in A left 500 µm, and A right 50 µm.
792
793 **Figure 2 Bioinformatics analyses.** (**A**) An image of a toluidine-stained hippocampal tissue
794 section showing marked granule cell dispersion pathology (top). DGCs were extracted from
795 frozen hippocampal tissue sections during laser microdissection. Extracted DGCs (including
796 nuclei and ~10µm-perinuclear rim as cytoplasm) were divided into either basal or dispersed
797 samples per case based on their location within the dentate gyrus. Basal samples contained
798 DGCs located in the basal layer of the granule cell layer (black outline). These round DGCs
799 were closely situated to neighbouring DGCs, and they were aligned along the border of CA4
800 and granule cell layer. Dispersed samples contained mixture of round and elongated DGCs
801 located ectopically, in rows or clusters in the outer-granular, inner and outer molecular layers
802 (green outline). Individual, small, round nuclei scattered in the dentate gyrus, sometimes in
803 close proximity to DGCs, were likely to glial cells, and therefore these small cells were avoided
804 where possible. (**B**) 1882 proteins were identified from proteomic analyses of basal and
805 dispersed DGCs of eight cases with MTLE, hippocampal sclerosis and GCD. More than 50%
806 of the identifiable proteins were shared between basal and dispersed samples, and between
807 younger (<35 years) and older cohorts (<50 years). Less than one-third of the identifiable
808 proteins were uniquely detected in basal, dispersed, younger and older samples. (**C**) A
809 dendrogram showing hierarchical clustering of all differentially expressed proteins in eight
810 MTLE cases with GCD. The pattern of protein changes between dispersed and basal samples
811 was more similar between cases E1 and E2 (younger cohort) and E5, E6 and E8 (older cohort).
812 (**D**) Heat map showing logarithmic-2 fold change between dispersed and basal samples (D/B),

813   or younger or older cohorts (Y/O) of selected proteins expressed in neuronal (MAP2, calbindin,
814   calretinin), glial (GFAP, S100-B, MBP, connexion 43), vascular (VEGFR), immature cell
815   (vimentin) and proliferative populations (MCM2, MCM4, PCNA, cyclin B3), or are proteins
816   associated with cytoskeletal (profiling-1, alpha synuclein, f-actin capping protein, DPYL2,
817   stathmin) and synapse remodelling (synaptotagmin-1, synapsin-2), mTOR pathway (PS6, PS6
818   kinase), neurogenic niches (NCAM1, CADM2), vascular changes (carbonic anhydrase 1,
819   haemoglobin) and inflammation (C1QBP, HSP70). *Keys:* * or ^ significantly overexpressed
820   proteins (P<0.05), **proteins found in dispersed samples of GCD cases only, ***proteins
821   found in basal samples of GCD cases only, ^^proteins only found in younger cohort only,
822   ^^^proteins found in older cohort only. *Abbreviations:* C1QBP, complement component 1Q
823   subcomponent-binding protein; DPYL2, dihydropyrimidinase-related protein 2; GFAP glial
824   fibrillary acidic protein; HSP70, heat shock proteins; MAP2, microtubule associated protein 2;
825   MBP myelin binding protein; MCM2, minichromosomal maintenance 2; NCAM1, neural cell
826   adhesion molecule 1; PCNA, proliferating cell nuclear antigen; PS6 ribosomal protein S6;
827   VEGFR vascular endothelial growth factor receptors. (**E, H**) Volcano plots showing
828   logarithmic-2 fold change against –log P value for proteins that were expressed in both
829   dispersed and basal samples (E) or in both younger and older cohorts (H). Significantly
830   differentially expressed proteins that showed over 1.5-fold change were highlighted in the plots
831   (P<0.05). (**F, G**) The abundance of each protein was compared between basal and dispersed
832   samples, and between younger and older cohorts. Proteins that were uniquely expressed in one
833   group only, and proteins that were significantly expressed by 1.5-fold than comparative group
834   (fold change>1.5, P<0.05) generated the Dispersed, Basal, Younger and Older clusters. Each
835   cluster was submitted to bioinformatics platforms for Gene Ontology and Pathways analyses.
836   Top functional annotation clusters based on Gene Ontology, Uniprot keywords and sequences
837   and INTERPRO terms for proteins in the Dispersed (F) and Basal clusters (G) were listed
838   (P<0.01; also refer to Supplementary Material 3). The enrichment scores generated by
839   bioinformatics platforms, and the percentage of proteins submitted that were associated with
840   each functional annotation cluster are illustrated. Network maps show the connections between
841   proteins in small GTPase mediated signal transduction (F) and ribosomes annotation clusters
842   (G). (**I, J**) Proteins in the Dispersed and Basal clusters were significantly involved in a number
843   of pathways (P<0.01; also refer to Supplementary Material 4). The size of the circle represents
844   the number of proteins submitted that were involved in the pathways. The thickness of
845   connections refers to the number of proteins that belongs to both connecting pathways.
846
847   **Figure 3 RhoA immunohistochemical studies.** (**A**) Most DGCs were immunonegative for
848   RhoA in the granule cell layer of a MTLE case without GCD (E5P). Occasionally, weak
849   perinuclear RhoA-positive labelling were observed in a few DGCs (**A'**, *higher magnification*
850   *of area outlined in A*). No DGCs were observed in the molecular layer of cases without GCD.
851   (**B-C**) Numerous DGCs with intense RhoA immunolabelling were observed in the granule and
852   molecular cell layer of two MTLE cases with GCD (E14, B; E3, C). In some DGCs, RhoA
853   immunolabelling was detected in one polar end of the DGC soma directed towards the CA4 or
854   molecular layer. A number of RhoA-positive cells in the outer-granular and molecule layer had
855   elongated soma with uni- or bipolar processes (**B'** and **C'**). In bipolar DGCs, RhoA
856   immunolabelling was observed at one or both apices of cell soma. Occasionally, RhoA-positive
857   DGCs were found to align single line formation near blood vessels (C'). In contrast, most basal
858   DGCs closest to the CA4 border had no or minimal RhoA immunolabelling. (**D**) Bilamination
859   of the granule cell layer was observed in a case with MTLE and GCD (E6). In this case, most
860   DGCs in the basal granule cell layer appeared to be devoid of RhoA immunolabelling. In
861   contrast, nearly all DGCs in the outer-granule layer (**D'**) and second granule cell layer
862   expressed RhoA (**D''**) either in one or both ends of the cell. Majority of RhoA-positive cells in

863 the second granule cell layer had stronger and distinct RhoA immunoreactivities in the end
864 directed towards the molecular layer. (**E**) A boxplot showing that the percentage of cells with
865 RhoA-positive labelling in the dentate gyrus of MTLE cases with GCD (blue) or without GCD
866 (red). A significantly higher percentage of cells expressing RhoA was observed in granule and
867 molecular layers of cases with GCD than cases without GCD (GCL, P=0.035; IML, P=0.001;
868 OML, P=0.008; see Supplementary Material 6). In cases with GCD, the percentage of cells
869 with RhoA immunolabelling was significantly higher in the inner molecular layer than the
870 granule cell (P=0.026) and outer molecular layer (P=0.003). (**F-G**) The percentage of cells
871 expressing RhoA in the dentate gyrus weakly correlated with the age at surgery of MTLE
872 patients (r_s=0.390, P=0.014; F) and the mean thickness of granule cell layer (r_s=0.675, P<0.001;
873 G). *Abbreviations:* DG dentate gyrus; GCL granule cell layer; IML, inner molecular layer;
874 OML, outer molecular layer. Scale bars, 50 µm.
875
876 **Figure 4 *RHOA* and *RAC1 in situ* hybridisation studies.** (**A-D**) *RHOA* and *RAC1*-positive
877 puncta (cyan) were observed in the dentate gyrus (DG) of MTLE cases without GCD (A, C)
878 and cases with GCD (B, D). Majority of positive puncta were found in or around haematoxylin-
879 stained nuclei (purple) in the granule and molecular cell layers. In MTLE cases with GCD,
880 most *RHOA* and *RAC1*-positive puncta were localised peri-nuclearly and at opposite ends of
881 each nucleus (B, D). Many *RAC1*-positive puncta were also observed in the proximal portion
882 of uni- or bipolar processes (D). A number of *RHOA* and *RAC1*-positive puncta were also
883 observed in the neuropils. (**E, G**) In cases without GCD, the density of cells with *RHOA and*
884 *RAC1*-positive puncta was higher in the granule cell layer than inner (*RHOA* P=0.023; *RAC1*
885 P=0.001) and outer molecular layer (*RHOA* P=0.004; *RAC1* P=0.018). In cases with GCD,
886 significantly higher densities of cells with *RHOA* and *RAC1*-positive puncta were observed in
887 the granule and outer molecular layers (*RHOA* P=0.004; *RAC1* P=0.017), but not in the inner
888 molecular layer (P>0.05). The density of cells expressing *RHOA* mRNAs in the inner molecular
889 layer was significantly higher in cases with GCD than cases without GCD (P=0.05), but this
890 difference was not observed in granule cell layer or outer molecular layer (P>0.05).   (**F, H**)
891 The percentage of cells with *RHOA* or *RAC1*-positive puncta weakly correlated with age of
892 onset in patients with GCD (*RHOA*, r_s=0.439, P=0.011; *RAC1*, r_s=0.295, P=0.049), but not in
893 patients without GCD. *Abbreviations:* DG dentate gyrus; GCL granule cell layer; IML, inner
894 molecular layer; OML, outer molecular layer. Scale bars, 50 µm.
895
896 **Supplementary Materials**
897 Data is available upon request.
898
899 Supplementary Material 1 Schematic diagram showing basal and dispersed populations of
900 DGCs within the dentate gyrus of MTLE cases with or without GCD.
901
902 Supplementary Material 2 Proteins that were significantly overexpressed in specific clusters
903 as illustrated in Figure 2E and 2H.
904
905 Supplementary Material 3 Top eight functional annotation clusters associated with proteins in
906 Dispersed and Basal clusters. Also refer to Figure 2F and 2G.
907
908 Supplementary Material 4 Pathways associated with proteins in Dispersed and Basal clusters
909 (P<0.01). Also refer to Figure 2I and 2J.
910
911 Supplementary Material 5 Diagram illustrating the role of Rho GTPases and their effector
912 proteins in cytoskeletal dynamics.

913

914 Supplementary Material 6 Quantitative results from immunohistochemical and *in situ*
915 hybridisation studies.

916

917 Supplemental Material 7 Additional findings from *in situ* hybridisation studies.

918

919 Supplemental Material 8 Transcriptome expression of *RHOA, RAC1* and *CDC42* in fetal and
920 adult postmortem human brains extracted from the Human Brain Transcriptome (Kang *et al.,*
921 2011).

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Table 1. Clinical details of cases studied. Eight cases with Hippocampal Sclerosis Type I (according to the International League Against Epilepsy (ILAE) classification system) and granule cell dispersion were analysed using proteomics (E1-8, in grey). In addition to these cases, another ten surgical cases with MTLE and three post mortem normal cases were included in RhoA and Rac1 immunohistochemical and/or *in situ* hybridisation studies. *In situ* hybridisation studies using probes against *CDC42*, or *RELN* transcripts were performed on four cases (E1P, E122, E1, E3). *Abbreviations:* Carb, carbamazepine; COD, cause of death, Clob clozabam; Dia diazepam, Gaba, Gabapentin; GCD, granule cell dispersion; GG ganglioglioma; HS hippocampal sclerosis; Lam, Lamotrigine; Lev, Levetiracetam; MCD malformation of cortical development; MFS mossy fibre sprouting; OH oligodendroglial hyperplasia in white matter; Ox, Oxcarbazepine; Pheny, Phenytoin; Pheno, Phenobarbitone; Preg, Pregabalin; Prim, Primidone; TS temporal sclerosis, Tem temapazem, Top topirmate; Val, Sodium valproate; Zon, Zonisamide.

Age at surgery	Cases	Relevant clinical history under age of 4 years	Age of onset (years)	Antiepileptic medications	Neuro-psychometric findings	DG pathology Laterality (other pathology)	Type of studies Proteomic (n=8) RhoA IHC (n=17) RhoA ISH (n=10) Rac1 ISH (n=15)
Between 20 to 30 years	E1	FS	13	Carb, Lam, Lev, Mid, Val	No remarkable cognitive deficits	HS Type 1. Left. GCD. MFS	All studies
	E20	FS, meningitis	12	Carb	Average intellectual abilities and language functions	HS Type 1. Left. GCD. MFS (TS)	RhoA IHC
	E2	FS	14	Carb, Lev, Ox, Val	Weak verbal and recognition functions	HS Type 1. Right. GCD. MFS (TS)	All studies
	E122	none	14	Carb, Lam, Preg	Weak working and verbal memory	HS Type 1. Right. GCD. MFS.	All except proteomics
	E123	FS	1.5	Carb, Lam, Val	Average intellectual abilities and memory. Weak visuospatial processing abilities	HS Type 3 (OH)	All studies except proteomic
	E16	None	9	Lam, Lev	Average cognition and memory functions	HS Type 3. Right	Rac1-ISH
	E3	Head trauma	11	Carb, Lev, Zon	Weak verbal intelligence and verbal and visual memory	HS Type 1. Left. GCD. MFS	Proteomic, RhoA-IHC
	E12	None	15	Lam, Zon	Weak verbal memory	HS Type 3. Left.	Rac1-ISH
Between 31 to 40 years	E4	None	10	Carb, Lev	Average cognition and memory functions	HS Type 1. Right. GCD. MFS	Proteomic, Rac1-ISH
	E14	B cell non-Hodgkin's lymphoma	8	Carb, Lev, Dia	Weak verbal and working memory Depression. Psychosis.	HS Type 1. Left. GCD. MFS	All except proteomics
	E13	FS	1	Val, Clob, Carb	Average cognition and memory functions.	HS Type 1. Right. GCD. MFS	All except proteomics
Between 41 to 50 years	E5P	None	2	Lev, Carb,	Weak visual memory	HS Type 3. Right.	All except proteomic
	E11	None	8	Clonazepam, Ox	Weak cognition and and visual memory. Depression, Paranoia	HS Type 1. Right. GCD. MFS	All except proteomics
Between 51 to 60 years	E5	Head trauma	16	Carb, Lam, Pheny	Weak verbal intelligence and working memory. Anxiety. Depression	HS Type 1. Right. GCD. MFS	Proteomic
	E6	FS	Late 30s	Carb, Ox, Lev, Lam, Pheno	Weak verbal and non-verbal memory. Psychosis	HS Type 1. Left. GCD. MFS.	All except RhoA-ISH
	E7	n/a	11	Val, Preg, Carb	Weak verbal memory and spatial recognition. Paranoia	HS Type 1. Left. GCD. MFS	All studies

	E1P	FS	22	Carb, Clob	Impaired intellectual ability and visual and verbal memory	HS Type 1. Left. GCD. MFS	All except proteomic
	E8	Head trauma	8	Carb, Gaba, Lam, Prim	Poor verbal memory	HS Type 1. Left. GCD. MFS	All studies
29	PM44	COD: sudden death	n/a	None	None	None	RhoA-IHC, qualitative only
35	PM45	COD: cardiac arrest	n/a	None	None	None	RhoA-IHC, qualitative only
57	PM46	COD: no notes available	n/a	None	None	None	RhoA-IHC, qualitative only

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1311 **Table 2** Table 2 Antibodies and protocols for RNA and immunohistochemical studies.
1312 Automated immunohistochemistry was performed using the Bond Max automated
1313 immunostainer and reagents (Leica, Milton Keynes, UK). *RNA detection was performed
1314 using RNAscope patent assays system following supplier's instruction. Abbreviations: aa,
1315 amino acids; Cdc42 Cell division control protein 42 homolog; GFAP, glial fibrillary acidic
1316 protein; K, thousands; MAP microtubule associated protein; NeuN, neuronal nuclei; ov,
1317 overnight, Rac1 Ras-Related C3 Botulinum Toxin Substrate 1; RhoA Transforming protein
1318 RhoA or Ras homolog family member A; RT room temperature. Antigen retrieval buffers:
1319 ENZ1, Leica Bond enzyme concentrate and diluent; ER1, Leica Bond citrate-based buffer;
1320 ER2, Leica Bond EDTA-based buffer; H-3301, Vector's Tris-based buffer pH 9.0; H-3300,
1321 Vector's citrate-based buffer pH 6.0; SC, Sodium citrate buffer, pH 6.0. Suppliers: Abcam plc.,
1322 Cambridge, UK; BD Transduction Lab., Oxford, UK; Bio-Techne, Abingdon, UK; DAKO,
1323 Cambridgeshire, UK; Millipore, Watford, UK; Sternberger, Maryland, US; Swant, Marly,
1324 Switzerland; Thermo Fisher Scientific, Hemel Hempstead, UK; Synaptic systems, Goettingen,
1325 Germany.
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Antibodies/Probes Clone, Code	Immunogen or target epitope	Labelled cell or protein type	Pre-treatment (mins)	Antibody supplier, dilution, incubation time (mins) and temperature
Anti-NeuN A60, MAB377	Purified cell nuclei from mouse brain	Nuclei of most neurons, some proximal dendrites	ER1 (20)	Millipore; 1:2K, 15, RT
SMI31 801601	Phosphorylated epitope in neurofilament H and M	Neurons	H-3301 (12)	Sternberger; 1:500, ov, 4$_o$C
SMI32 801707	Non-phosphorylated epitope in neurofilament H	Neurons	H-3301 (12)	Sternberger; 1:500, ov, 4$_o$C
Anti-MAP2 HM-2, M4403	Rat microtubule-associated proteins	Neurons	H-3301 (12)	Sigma; 1:1500, ov, 4$_o$C
Anti-Calbindin D-28K, 300	Calbindin D-28k	Calbindin-expressing interneurons	H-3301 (12)	Swant; 1:10k, ov, 4$_o$C
Anti-ZnT3 197-003	Recombinant protein of mouse ZnT3 (aa. 2-75)	Synaptic vesicle located zinc-transporter	H-3301 (12)	Synaptic Systems; 1:10K, ov, 4$_o$C
SMI 94 SMI-94R	70-89 aa of human myelin basic protein	Myelinated fibre in white matter	ENZ1 (10)	DAKO; 1:2000, 20, RT
Anti-Nestin 10C2, AB22035	150 aa recombinant fragment from human nestin conjugated to GST	Immature progenitors, glia, endothelial cells	H-3301 (12)	Abcam; 1:1K, ov, 4$_o$C
Anti-GFAP Z0334	GFAP isolated from cow spinal cord	Astrocytes, some ependymal cells	ENZ1 (10)	DAKO; 1:2.5K, 20, RT
Anti-RhoA OSR00266W	A synthetic peptide from human Transforming protein RhoA conjugated to an immunogenic carrier protein	Small GTPase protein that regulates actin cytoskeleton in the formation of stress fibres, and cell division. Expresses in cytoplasm of cells undergoing structural changes in preparation for migration	Protease (20, 37$_o$C)	Thermo; 1:500, ov, 4$_o$C
RHOA mRNA 416291	NM_001664.2. Probe region from 135 to 1796		Pretreatment 2 and 3*	BioTechne; 120, 40$_o$C
RAC1 mRNA 419851	NM_006908.4 Probe region from 1194 to 2256		Pretreatment 2 and 3*	BioTechne; 120, 40$_o$C
CDC42 mRNA 502651	NM_044472.2 Probe region from 2 to 1091		Pretreatment 2 and 3*	BioTechne; 120, 40oC
RELN mRNA 413051	NM_173054.2 Probe region from 4401 to 5340	Glycoprotein expressed by Cajal-Retzius cells	Pretreatment 2 and 3*	BioTechne; 120, 40oC

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Table 3: Proteins in the Dispersed cluster that were involved in signalling by Rho GTPases pathway (Reactome, R-HSA194315; $P=4.68 \times 10^{-9}$).

Entry ID	Gene	Protein
P60953	CDC42	Cell division control protein 42 homolog
E7EVJ5	CYFIP2	Cytoplasmic FMR1-interacting protein 2
Q7L576	CYFIP1	Cytoplasmic FMR1-interacting protein 1
F8W7L3	A2M	Alpha-2-macroglobulin
Q12802	AKAP13	A-kinase anchor protein 13
H0YE29	ARHGAP1	Rho GTPase-activating protein 1
Q9NRY4	ARHGAP35	Rho GTPase-activating protein 35
I3L4C2	BAIAP2	Brain-specific angiogenesis inhibitor associated protein 2
P11274	BCR	Breakpoint cluster region protein
A0A087X0P0	CENPE	Kinesin-like protein Dispersed
G3XAM7	CTNNA1	Catenin-alpha-1
P78352	DLG4	Disks large homolog 4
Q99880	HIST1H2BL	Histone H2B type 1-L
Q15691	MAPRE1	Microtubule-associated protein RP/EB family member 1
Q9Y2A7	NCKAP1	Nck-associated protein 1
Q9NZQ3	NCKIPSD	NCK-interacting protein with SH3 domain
P35080	PFN2	Profilin-2
P62140	PPP1CB	Serine/threonine-protein phosphatase PP1-beta catalytic
C9J9C1	PPP2R1A	**Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A**
P63000	RAC1	Ras-related C3 botulinum toxin substrate 1
C9JNR4	RHOA	Transforming protein RhoA/ Ras homologous family member A
P62745	RHOB	Rho-related GTP-binding protein RhoB
Q8IXI2	RHOT1	Mitochondrial Rho GTPase 1
P31947	SFN	14-3-3 protein sigma
O43295	SRGAP3	SLIT-ROBO Rho GTPase-activating protein 3
A2IDB2	YWHAH	14-3-3 protein eta