



Citation for published version:

Ferguson, R, Serafeimidou-Pouliou, E & Subramanian, V 2016, 'Dynamic expression of the mouse orthologue of the human amyotrophic lateral sclerosis associated gene *C9orf72* during central nervous system development and neuronal differentiation', *Journal Of Anatomy*, vol. 229, no. 6, pp. 871-891. <https://doi.org/10.1111/joa.12526>

DOI:

[10.1111/joa.12526](https://doi.org/10.1111/joa.12526)

Publication date:

2016

Document Version

Peer reviewed version

[Link to publication](#)

This is the peer reviewed version of the following article: Ferguson, R. , Serafeimidou-Pouliou, E. and Subramanian, V. (2016), Dynamic expression of the mouse orthologue of the human amyotrophic lateral sclerosis associated gene *C9orf72* during central nervous system development and neuronal differentiation. *J. Anat.*, 229: 871-891, which has been published in final form at <https://doi.org/10.1111/joa.12526>. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving.

University of Bath

Alternative formats

If you require this document in an alternative format, please contact:
openaccess@bath.ac.uk

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

1

2 **Dynamic expression of the mouse orthologue of the human amyotrophic lateral**3 **sclerosis associated gene *C9orf72* during central nervous system**4 **development and neuronal differentiation**

5

6

7 **Ross Ferguson, Eleni Serafeimidou-Pouliou and Vasanta Subramanian***

8 Department of Biology and Biochemistry, University of Bath, Bath, United Kingdom.

9

10 * Corresponding author

11 Email: bssvss@bath.ac.uk (VS)

12

13 **RUNNING TITLE:** Expression of *C9orf72* during mouse brain and spinal cord

14 development

15 ABSTRACT

16 The hexanucleotide repeat in the first intron of the *C9orf72* gene is the most significant
17 cause of amyotrophic lateral sclerosis as well as some forms of fronto-temporal
18 dementia. The *C9orf72* protein has been previously reported to be expressed in post-
19 mortem human brain as well as in late embryonic and some postnatal stages in mice.
20 Herein, we present a detailed study of the distribution of *C9orf72* protein in the
21 embryonic, postnatal and adult mouse brain, spinal cord as well as during the
22 differentiation of P19 embryonal carcinoma cells to neurons including motor neurons.
23 We show that the expression levels of the *C9orf72* transcripts in the developing and
24 adult mouse brain as well as in differentiating neurons, is dynamic. Besides the strong
25 expression in the cerebellum and motor cortex reported previously, we show for the
26 first time that *C9orf72* is expressed strongly in the olfactory bulb and also in the
27 hippocampus. Our immunostaining data also reveals a hitherto unreported switch in
28 the cellular distribution of *C9orf72* from a predominantly cytoplasmic to a nucleo-
29 cytoplasmic distribution during corticogenesis. This switch in distribution was also
30 observed during differentiation of the pluripotent embryonal carcinoma P19 cell line to
31 mature neurons. Our findings have implications in interpreting the pathophysiology
32 caused by the repeat expansions in *C9orf72* in mouse models.

33

34 KEY WORDS

35 *C9orf72*, expression, brain development, spinal cord, neuronal differentiation

36

37

38 INTRODUCTION

39 Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are two
40 neurodegenerative diseases that have overlapping genetic causes and pathological
41 features (Ling et al., 2013). In ALS, upper and lower motor neurons (MNs) die rapidly
42 and progressively with fatal consequences. In FTD, which is the second most
43 common form of dementia, the frontal and temporal lobes of the brain are affected
44 (Van Langenhove et al., 2012). At least 15% of patients diagnosed with either disease
45 have been shown to have symptoms characteristic of the other (Ringholz et al., 2005;
46 Wheaton et al., 2007). Common cellular pathologies include aberrant RNA processing
47 and protein homeostasis (Ling et al., 2013). Genetic causes underlying these diseases
48 have been identified within these pathways, such as mutations in *ubiquilin-2*, *FUS*,
49 *TDP-43* and *C9orf72* (DeJesus-Hernandez et al., 2011; Deng et al., 2011; Kabashi et
50 al., 2008; Kwiatkowski et al., 2009; Renton et al., 2011).

51 Mutations in *C9orf72* was first identified by DeJesus-Hernandez et al and
52 Renton et al in 2011 in ALS and ALS-FTD patients.). The mutation in *C9orf72* takes
53 the form of a G₄C₂ hexanucleotide repeat expansion (HRE) in the first intron of the
54 *C9orf72* gene. Typically, up to 20 repeats are found in the normal allele while
55 pathological alleles carry many additional repeats, ranging from ~30 to thousands. The
56 HRE has multiple consequences within the cell. The three main pathological
57 manifestations of the expansion are the appearance of RNA foci, formation of
58 dipeptide proteins and reduced levels of the *C9orf72* protein. The stable G-quadruplex
59 formed by the HRE DNA and RNA result in RNA foci which have been shown to
60 sequester factors involved transcription and splicing leading to dysfunction (Fratta et
61 al., 2012; Lee et al., 2013). Repeat associated non-ATG translation results in the
62 synthesis of dipeptide repeats (DPR) which have been shown to form intracellular

63 inclusions. These aggregates are suggested to be cytotoxic and are found in
64 degeneration-associated neuronal subtypes (Schludi et al., 2015), as are RNA foci.
65 Other reports find no evidence for the cytotoxicity of the DPRs (Mackenzie et al.,
66 2015). However, both DPRs and RNA foci are not necessarily co-incidental (Zu et al.,
67 2013) indicating both mechanisms may contribute separately to the aetiology of the
68 disease. Further evidence for this can be seen in the spinal cord of ALS subjects which
69 contain RNA foci but rarely DPRs (Cooper-Knock et al., 2015; Gomez-Deza et al.,
70 2015). Reduced *C9orf72* expression has been found in ALS-FTD patients by multiple
71 groups, however, conditional ablation of *C9orf72* in mouse does not lead to a
72 pathological phenotype arguing against haplo-insufficiency as a major contributor to
73 pathophysiology (Koppers et al., 2015).

74 FTLD , a form of FTD is best characterised by degeneration of the frontal and
75 temporal lobes. However, MRI studies have also identified atrophy in the
76 hippocampus, entorhinal cortex and cerebellum in FTLD (Hartikainen et al., 2012;
77 Laakso et al., 2000; Tan et al., 2014). In addition to characteristic abnormalities in the
78 corticospinal and cerebellar white matter (Bede et al., 2014), *C9orf72* ALS-FTD
79 patients also show increased atrophy in the anterior temporal, parietal and occipital
80 lobes, as well as the cerebellum, when compared to phenotypes caused by mutations
81 in other FTD causative genes (Bede et al., 2013; Whitwell et al., 2012).

82 The *C9orf72* protein has been detected in the cortex and cerebellum in human
83 post mortem brain lysates. Besides these two regions, a few studies have looked at
84 other regions of the human brain (Gijssels et al., 2012; Hsiung et al., 2012; Stewart
85 et al., 2012; Waite et al., 2014; Xiao et al., 2015). Both Gijssels et al., (Gijssels et
86 al., 2012) and Waite et al., (Waite et al., 2014) have shown decreased levels of
87 *C9orf72* in ALS-FTD brains. Using isoform specific antibodies, Xiao et al., (Xiao et al.,

88 2015) have shown the strongest expression of the C9orf72 human long isoform is in
89 the motor cortex and cerebellum, while the short isoform is detectable in the motor,
90 frontal and temporal cortices with strongest expression in the cerebellum. Interestingly
91 they also show the long isoform is present at reduced levels in ALS-FTD brains with
92 *C9orf72* HRE but not in ALS-FTD brains carrying mutations in other ALS causative
93 genes, and the converse for the short human C9orf72 isoform.

94 If the mouse is to serve as a model for ALS-FTD, it is important to ascertain
95 whether the expression domains of the mouse orthologue of C9orf72 are equivalent
96 to those found in the human brain. In mice with a *lacZ* knock-in at the endogenous
97 *C9orf72* locus, the mouse orthologue of *C9orf72* (3110043O21Rik, referred to as
98 *mC9orf72*) was reported to be expressed throughout the adult mouse brain (Suzuki
99 et al., 2013). *LacZ* expression was undetectable in pre-natal stages which is most
100 likely to be due the low detection sensitivity of the reporter. Atkinson et al., (Atkinson
101 et al., 2015) studied *mC9orf72* expression in the mouse cortex at E18, P1, P14, P28
102 and P56 by immunohistochemistry and observed changes in distribution during neurite
103 outgrowth and synaptogenesis throughout the cortex and hippocampus. Although
104 expression of *mC9orf72* has been demonstrated in the mouse brain it remains unclear
105 whether the protein is present in domains similar to human C9orf72 and regions
106 affected in ALS-FTD.

107 We sought to study in detail the distribution C9orf72 in the mouse brain together
108 with neuronal and brain region specific markers, and whether its distribution was
109 regulated both temporally and spatially during embryonic and postnatal brain
110 development as well as during neuronal differentiation of pluripotent stem cells.

111 Herein, we show that *mC9orf72* distribution in the developing mouse brain in
112 embryonic (E12.5 to E18.5) and post-natal (up to P35) stages is dynamic in location

113 and intracellular distribution. We also describe changes in the distribution of mC9orf72
114 during the development of the brain, with notable changes seen in the olfactory bulb,
115 cerebral cortex, hippocampus and cerebellum. Using mixed cultures of cortical
116 neurons and astrocytes from P0 cortices we stained for the expression of C9orf72 and
117 cortical layer as well as astrocyte markers and show that C9orf72 is predominantly in
118 the neuronal cells and virtually undetectable in the astrocytes. Cortical neurons from
119 all the cortical layers that we identified by staining for layer specific markers expressed
120 C9orf72. We detected a distinct switch in the distribution of C9orf72 from a
121 predominantly cytoplasmic to a nucleo-cytoplasmic distribution during corticogenesis.
122 This switch in distribution was also observed during differentiation of pluripotent stem
123 cells to mature neurons.

124

125 **METHODS**

126 **Cell Culture and differentiation**

127 Embryonic Carcinoma P19 cells (Passage 8-9) were cultured in P19 growth medium
128 containing α -MEM (Gibco Life technologies) supplemented with 10% FCS (Biosera)
129 and 1% NEAA (Gibco Life technologies) at 37°C with 5% CO₂. When cells reached
130 confluency of 70%, approximately every 2 days, they were passaged using 0.05%
131 trypsin/EDTA (Gibco Life technologies). Embryonic bodies (EBs) were made from P19
132 cells with the following method, cells were trypsinised using 0.05% trypsin/EDTA and
133 were seeded in 90mm bacteriological dishes at a density of 2.5 x 10⁵/ml were they
134 spontaneously formed aggregates. The medium used for the EBs was α -MEM
135 supplemented with 10% KOSR (Gibco Life technologies) and 1% NEAA. In order to
136 differentiate the EBs into motor neurons we added 0.5 μ M Retinoic Acid (Sigma) after
137 24 hours and after 48 hours they were plated onto matrigel (BD) coated coverslips.
138 For coating the coverslips we added 200 μ l of matrigel per coverslip and after 2 hours
139 at room temperature matrigel was aspirated and the EBs were added. The EBs were
140 seeded using differentiation medium containing α -MEM, 1% NEAA, 1% KOSR and
141 0.5 μ M Retinoic Acid in order to promote differentiation of the EC cells. The
142 differentiation medium was changed every other day for 6 days and on the 6th day the
143 it was changed to neurobasal media (Gibco Life technologies), 1x B27 (Life
144 Technologies), 1% KOSR and 1% glutamax (Life technologies). We continued
145 changing the medium every 2 days in order to sustain the neuronal culture.

146

147 **Western Blot**

148 CD1 mouse brains (P10) were dissected into cerebral cortex and cerebellum, minced
149 and then snap frozen in liquid nitrogen. Tissue from two mice were thawed with the

150 addition of 2% SDS in 60 mM Tris-Cl pH 6.8, pooled and homogenised by Dounce
151 homogeniser. Lysates were sheared through 21G and 30G needles (BD) then
152 centrifuged at 13k in a benchtop microfuge. Supernatants were stored at -70°C.
153 Protein concentration was determined by BCA microplate assay following
154 manufacturer's instructions (Pierce). Each sample (10µg of protein) was mixed with
155 loading buffer (to final concentrations of 2% SDS, 10% glycerol, 50mM DTT in 60 mM
156 Tris-Cl pH 6.8), boiled for five minutes and proteins resolved on a 10% polyacrylamide
157 gel. Proteins were transferred to 0.45µm PVDF at 50V for 90min in transfer buffer
158 (25mM Tris, 192mM glycine, pH 8.3, 20% methanol).

159 Blots were blocked in 5% Marvel in PBS for an hour then incubated overnight
160 in primary antibody (1:1000) diluted in 5% Marvel in PBS at 4°C. After four 5min
161 washes in PBS with 0.1% Tween20 (PBSTw) the blots were incubated with
162 appropriate Horseradish peroxidase conjugated secondary antibody (1:10000,
163 Abcam) diluted in 5% Marvel in PBS. After further 4x5min washes in PBSTw, blots
164 were incubated with ECL reagent and exposed to film (Amersham hyperfilm). Blots
165 were imaged on a Fusion SL (Vilber Lourmat) using the associated Fusion-Capt
166 software.

167

168 **qRT-PCR**

169 P19 were lysed in Trizol (Invitrogen) for RNA at indicated time points throughout two
170 independent differentiation time courses on matrigel in the presence of RA. Tissue
171 was dissected from CD1 embryos to isolate heads at E12.5, whole brains at E13.5,
172 E14.5 and E16.5, fore and mid/hind brain at E18.5. Brains was also dissected from
173 postnatal mice at P5, P10 and P15 and split into cerebrum and cerebellum. Tissue
174 was snap frozen in trizol and pooled from four individuals from the same litter. Tissue

175 in trizol was thawed and disrupted by Dounce homogeniser and RNA prepared
176 following standard trizol method. RNA was DNased and reverse transcribed using
177 OligodT primers. qPCR was performed using primers specific to transcripts 1, 2 and 7
178 and normalised to Actb. Reactions were carried out in a BioRad iQ5 cycler. Dynamic
179 well factors were collected for 2min 30sec, then forty cycles at 60°C and 95°C for 20s
180 each followed by a melt curve. Expression levels were determined relative to Actb from
181 baseline subtracted curves and corrected using primer efficiencies determined
182 previously from serial dilutions of PCR product. See Table 1 for primer sequences

183

184 **Immunohistochemistry**

185 For isolating embryonic stages, pregnant dams were killed by cervical dislocation prior
186 to dissection. E12.5 and E14.5 embryos were fixed whole. E16.5 embryos were
187 decapitated prior to fixation. Brains were dissected from E18.5 through post-natal
188 stages and bisected prior to fixation. Dissected embryos and brains were fixed
189 overnight in cold 4% PFA, washed twice for 1h with PBS then cryo-protected in 20%
190 sucrose before embedding in OCT. Frozen blocks were sectioned by Leica cryostat
191 and air dried for 1h before storage at -20°C.

192 Frozen sections were incubated in PBS twice to remove OCT prior to antigen
193 retrieval in boiling 10mM Sodium Citrate Buffer, pH 6.0 with 0.05% Tween 20 for
194 20min. Sections were allowed to cool for 20min, washed twice with PBS and blocked
195 with 0.1% gelatin in PBS supplemented with 0.5% Triton X-100 and 1% FBS for 1 hour
196 at room temperature.

197 Cells on coverslips were washed with PBS and fixed with 4% PFA for 15min.
198 After two PBS washes cells were dehydrated with serial ethanol washes. Prior to
199 immunostaining cells were rehydrated, washed with PBS and blocked as for frozen

200 sections.Both cells on coverslips and sections were incubated with appropriate
201 primary antibody at 4°C overnight. The sections and fixed cells were then washed
202 four times with 0.1% Triton X-100 in PBS and then incubated for 1 hour with the
203 appropriate secondary antibody together with 4',6-diamidino-2-phenylindole (DAPI)
204 (Invitrogen) to stain cell nuclei. See Table 2 for list antibodies used. Stained sections
205 and cells on coverslips were mounted using Mowiol. Imaging was done with a Leica
206 DMRB5500 epifluorescent microscope. Images were captured and deconvoluted
207 where required using LAS AF (Leica) and figures composed in Photoshop CS3
208 (Adobe). Brain anatomy was identified using the Allan Brain Atlas ([http://mouse.brain-
209 map.org/](http://mouse.brain-map.org/)).

210

211 **RESULTS**

212 **Antibody characterisation**

213 Several commercially antibodies are available for researchers seeking to detect
214 C9orf72 protein. Since several reports comment about their general unsuitability
215 (Waite et al., 2014; Xiao et al., 2015), we proceeded to characterise the specificity of
216 two popular C9orf72 antibodies both by western blot using purified recombinant
217 human C9orf72 protein and also compared them by immunostaining adjacent sections
218 of adult mouse brain.

219 The mouse C9orf72 ortholog (3110043O21Rik) encodes three transcripts (1, 2
220 & 7) producing different protein isoforms, unlike the human C9orf72 which only
221 produces two (1 & 3) transcripts. Transcript 1 of both mouse and human code for a
222 481aa protein which have 98% homology at the amino acid level. Mouse transcript 7
223 encodes only the first 420aa of transcript 1 (Supplementary Material, Fig. S1A). The
224 second protein coding transcript is substantially different from the first, but also differs

225 dramatically between the two species. Human C9orf72 transcript 2 contains only the
226 first 222aa of transcript 1 while mouse C9orf72 transcript 2 encodes only the last
227 317aa of the homologous mouse transcript 1.

228 We found that the ProteinTech and SantaCruz antibodies detect both the
229 481aa and 222aa human C9orf72 isoforms. The ProteinTech epitope however, is
230 present only in the 481aa and 420aa isoform (transcript 1 &7) of mouse C9orf72 while
231 the SantaCruz antibody detected all three mouse isoforms (Supplementary Material,
232 Fig. S1A).

233 In Western blots both antibodies recognized the pure recombinant human
234 C9orf72 long isoform (kind gift from S Iyer and R Acharya , University of Bath). The
235 SantaCruz C9orf72 antibody produces a more intense signal as compared to the
236 ProteinTech antibody when used at the same concentration at similar exposure times
237 in western blots (Supplementary Material, Fig. S1B, ii and iv compared to i and iii).
238 Additional weak bands seen with the SantaCruz are absent in the blots probed with
239 the ProteinTech antibody. These additional bands could represent degraded C9orf72
240 protein at lower masses and multimers at higher (Supplementary Material, Fig. S1B,
241 II and IV) as we have used C9orf72 protein which has been stored frozen after
242 purification for our western blot analysis. Such additional bands are frequently seen
243 upon storage of the purified C9orf72 protein (S Iyer and K Acharya, personal
244 communication- see lines 363-368 for confirmation).

245 Comparison between adjacent sections of adult mouse brain immunostained
246 using both antibodies however, showed very little observable difference
247 (Representative areas are shown in Supplementary Material, Fig. S2; A: cerebellum,
248 B: hind brain and C: cortex). Since in Western blots the Santa Cruz antibody gave

249 stronger signal, all our immunostaining as well as further Western blot studies were
250 carried out using the Santa Cruz C9orf72 antibody.

251

252 **C9orf72 isoform expression in the cerebral cortex and cerebellum**

253 Protein extracts from postnatal cerebellum and cortex were analysed for expression
254 using the SantaCruz anti-C9orf72 antibody. The dominant isoform in the brain
255 appears to be that derived from transcript 1 (481aa, 54kDa) while the isoforms
256 encoded by transcript 7 (420aa, 47kDa) and transcript 2 (222aa, 36kDa) in both the
257 cortex and cerebellum (Fig. S1C) were significantly lower. The cerebellum showed
258 higher levels of C9orf72 isoform 1 when compared to the cortex.

259 Crude lysates of bacteria expressing recombinant C9orf72 isoform 1, loaded
260 on the PAGE gel directly without purification identified a single band corresponding to
261 C9orf72 upon immunoblotting (FigS1C). We did not observe any additional non-
262 specific bands or cross-reactivity with any bacterial proteins in these samples. This
263 suggests that the additional bands seen with the frozen pure recombinant C9orf72 are
264 degradation or aggregation products rather than non-specific bands.

265

266 **C9orf72 expression in the developing mouse cerebral cortex**

267 Cortical neurogenesis occurs in the last eight days of gestation in the developing
268 mouse. The neural progenitors found in the ventricular zone at E12.5 progressively
269 mature, divide and migrate radially outwards from the cerebral ventricle. By E16.5 this
270 has resulted in the formation of the distinct sub-ventricular zone, sub-plate and cortical
271 plate proper. As cortical neurons are born they migrate outwards through the pre-
272 existing cortical plate neurons resulting in an inside out pattern of early-born deep
273 layers and late-born superficial layers. Concomitantly the sizes of the ventricular and

274 sub-ventricular progenitor pools progressively diminish by E18.5 (Dehay and
275 Kennedy, 2007). We investigated the pattern of expression of C9orf72 in the
276 developing mouse cortex from E12.5 though to E18.5. At early embryonic stages
277 (E12.5-16.5) we studied the expression of C9orf72 in combination with nestin, the
278 neural progenitor marker and with the 160kd neurofilament marker. At later embryonic
279 stages (E16.5-18.5) we studied the co-localisation of C9orf72 with well characterised
280 markers for each cortical layer.

281 In the E12.5 developing mouse cerebral cortex (Fig. 1A-D) expression is seen
282 in regions adjacent to the ventricles both in the ventral and dorsal pallium (Fig. 1A).
283 C9orf72 expression is seen in radially organized cells that co-express the neuronal
284 progenitor marker nestin. This is seen specifically at the most medial zones in coronal
285 sections both dorsal (Fig. 1B) and ventral (Fig. 1C), but is strongest at the dorsal side.
286 C9orf72 is also detectable in tangentially orientated cells at the superficial surface of
287 the dorsal pallium (Fig. 1A-B, dotted lines). C9orf72 staining appears to be specific to
288 neural tissue, as observed at the interface between the strongly stained pre-optic area
289 and adjacent non-neural tissue (Fig. 1D). Staining appears most intense in developing
290 forebrain structures, but is does not appear in the sub-pallium or pre-thalamic regions.

291 The expression of C9orf72 no longer appears concentrated at the ventricular
292 side of the dorsal pallium at E14.5 but staining more strongly at the superficial surface
293 of both dorsal pallium (Fig. 1E) and olfactory bulb (Fig. 1F). This pattern of staining is
294 not observed in the more anterior medial pallium or midbrain (Fig. 1G). Stronger
295 expression is seen within the superficial stratum of the habenula and the pre-thalamic
296 structures in areas of reduced cell density directly adjacent to the convolution
297 separating it from the pallium (Fig. 1H, closed arrows). C9orf72 expression at E14.5
298 is observed in the superficial zones where the hypothalamus and hindbrain abut (Fig.

299 1I, open and closed arrows). Expression is also in the axonal tracts which are
300 neurofilament positive in the medulla (Fig. 1J, dotted lines) and entering the spinal
301 cord (Fig. 1K, dotted lines). Neurofilament positive spinal ganglia can also be seen co
302 expressing C9orf72 (Fig. 1L).

303 As development proceeds to E16.5, (Fig. 2A), increased expression is no
304 longer seen around the ventricles, but remains around the tangentially orientated cells
305 at the pial surface similar to that observed at E14.5. A second zone of increased
306 expression is present below the cortical plate mid-pallium in the intermediate zone
307 between the ventricular zone and the cortical plate. This second zone does not overlap
308 with markers such as Tbr2 in the subventricular zone (SVZ) or Nurr1 in the subplate
309 (SP). This pattern of staining is not seen in the subiculum, sub-pallium, thalamus or
310 developing hippocampus.

311 C9orf72 expression extends through the entire rostro-caudal axis of the pallium
312 at E18.5 (Fig. 2B). Expression of late cortical markers such as SatB2 and Ctip2
313 indicates specification of the upper layers of the cortex in addition to the lower layers
314 present at E16.5 as shown by the staining for Tbr1. Increased expression of C9orf72
315 persists in both the intermediate zone and superficial marginal zone of C9orf72. The
316 expression domains of cortical layer markers confirms that the internal band of
317 C9orf72 is not part of the cortical plate as it is below the deep layer marker Tbr1 and
318 is not in the Tbr2 positive SVZ. At this stage increased C9orf72 expression can also
319 be seen in the superficial region of the midbrain tectum in similar to expression in the
320 cortical marginal zone.

321 The intermediate zone of the cortex dramatically decreases in size at P5 and
322 P10 accompanied by the loss of C9orf72 expression. A gradient of C9orf72 expression
323 can be seen extending from the marginal zone inwards particularly at early postnatal

324 stages (P5 and P10 data not shown) than during embryonic. At P35 (Fig. 2C), the
325 cortex layering is complete. C9orf72 expression is detected throughout the adult cortex
326 but is strongest in layer I with a gradual decrease in intensity through to the base of
327 layer II/III. Further bands of increased C9orf72 expression can be found throughout
328 layer IV and VI. C9orf72 also appears to have transitioned from being predominantly
329 cytoplasmic to a more even nucleo-cytoplasmic distribution.

330 In summary, we find C9orf72 expression throughout the developing cortex,
331 strongest closer to the ventricle and superficially at E12.5 and E14.5, the intermediate
332 and superficial marginal zones at E16.5 and E18.5, and then superficially in layers I,
333 IV and VI in P35 mice. While predominantly cytoplasmic during embryonic stages,
334 C9orf72 takes on a nucleocytoplasmic distribution post-natally (Table 3).

335

336 **C9orf72 expression in cortical neuronal cultures**

337 We also studied the expression of C9orf72 in the cortical neurons and astrocytes
338 isolated from P0 cortex and co-expression with transcription factor markers for cortical
339 layers as well as the astrocyte marker GFAP. The primary cortical neurons in culture
340 stained positive for neurofilament and the synaptic vesicle marker SV2. All the cortical
341 neurons showed robust C9orf72 expression in neurofilament positive axons while
342 GFAP⁺ astrocytes express barely detectable levels in comparison (Fig. 3A, arrows).
343 We observed no differences in expression or localisation of C9orf72 in cortical neurons
344 from the different cortical layers as seen by co-staining for calbindin, SatB2 or Ctip2
345 (Fig. 3B).

346

347 **C9orf72 expression in the developing cerebellum**

348 The anlage of the cerebellum emerges at at E11.5, however, much of the development
349 -ie expansion and organisation occurs postnatally until P15. By E18.5, granule cells
350 precursor cells have migrated tangentially from the rhombic lip to form the external
351 granule layer (EGL). The Purkinje cells (PC) are born and migrate radially from the
352 ventricular zone to form the Purkinje cell layer (PCL). As the development of the
353 cerebellum progresses, granule cells from the ECL descend radially to below the PCL
354 to form the internal granule cell layer (IGL). By P15 the thickness of the EGL has
355 substantially reduced, along with the loss of proliferative precursor cells while the IGL
356 has increased in size. The thickness of the molecular layer between the PCL and EGL
357 also increases and contains the extensive PC dendritic arborisation and granule cell
358 parallel fibres or climbing fibres. The expansion of the cerebellum also results in its
359 foliation and characteristic lobes (Martinez et al., 2013).

360 Expression of C9orf72 can be seen between the developing external granule
361 layer (EGL) of the cerebellum at E18.5 and the internal neurofilament positive areas
362 (Fig. 4A). This band of C9orf72 expression is expanded at P5 and overlaps with the
363 calbindin positive Purkinje cells in the molecular layer (ML) between the EGL and
364 internal granule layer (IGL, more defined at this stage), but staining remains stronger
365 on the interior edge of the EGL. Both the IGL and ML thicken by P10 when Purkinje
366 cells have taken on their characteristic positioning on the superficial side of the IGL
367 (Fig. 4A, pc). This pattern is consistent throughout the lobes and is maintained in the
368 fully developed cerebellum at P35. The C9orf72 staining is strongest in around the
369 cells directly adjacent to proliferating PCNA positive EGL granule cells (Fig. 4B). This
370 persists up to P10 and although by P35 the proliferation and migration in the EGL has
371 ceased the expression of C9orf72 remains strongest at the most superficial part of the
372 molecular layer.

373 C9orf72 staining does not associate with the tangential GFAP positive glial
374 fibres extending through the ML at any stage, or with the calbindin positive Purkinje
375 cell dendrites at E18.5 to P10. At P35 it does appear to be strongest towards the ends
376 of Purkinje dendrites and does appear to partially overlap with the most superficial
377 synapses stained by SV2 from E18.5 to P35. Additionally it does not appear
378 associated with Tbr2 positive granule cell parallel fibres or proliferating EGL which is
379 strongly positive for PCNA (Fig. 4B).

380 Closer inspection of the cerebellar molecular layer at P35 showed no overlap
381 or association between C9orf72 and GFAP positive glial fibres (Fig. 5A). C9orf72 can
382 however be seen in the cell body of the Purkinje cells (Fig. 5A, arrows) and also
383 clustering along the length of calbindin positive Purkinje cell dendrites, both proximal
384 to the cell body and at distal tips. As one would expect from this, no co-localisation is
385 seen with the post-synaptic marker PSD95, but overlap can be seen between C9orf72
386 and the synaptic vesicle marker SV2 (Fig. 5B).

387 To summarise, cerebellar C9orf72 expression is strongest in the regions
388 underlying the EGL at E18.5. Expression at P5 and P10 overlaps with the PCL and
389 ML which is adjacent to the EGL. This distribution pattern is maintained through to P35
390 when very strong C9orf72 expression is seen in the soma and nuclei of PCs as well
391 as the pre-synaptic terminals in the ML (Table 3).

392

393 **C9orf72 expression in the developing olfactory bulb**

394 The olfactory bulb (OB) can first be identified at E12.5 as a protrusion of the rostral
395 telencephalon. After neurogenesis between E11-13, mitral and tufted cells (M/T cells)
396 migrate radially into the OB then orientate tangentially prior to dendritic arborisation to
397 form the external plexiform layer (EPL) between E14 and E16. From E17 onwards

398 these dendrites begin to associate with proto-glomerular structures. Olfactory
399 glomeruli found in the superficial adult olfactory bulb where sensory neuron axons from
400 the olfactory epithelium form synapses. Considerable remodelling and pruning of the
401 M/T cell dendrites occurs post-natally alongside further glomerulogenesis (Treloar et
402 al., 2010).

403 The olfactory bulb showed distinct and high levels of C9orf72 expression. At
404 E16.5 and E18.5, strong expression of C9orf72 is seen in the superficial stratum of the
405 olfactory bulb compared to the deeper layers (Fig. 6A). By P5 the olfactory bulb has
406 delineated into distinct regions and strong expression is seen in the olfactory glomeruli,
407 while individual C9orf72 expressing cells can be identified in the external and internal
408 plexiform areas (Fig. 6Bii and iii). Staining in the olfactory glomeruli at the superficial
409 edge of the olfactory bulb increases between P5 and P35 (Fig. 6Bi, glm). C9orf72
410 staining in the olfactory glomeruli at P35 does not overlap with the presynaptic marker
411 PSD95 (Fig. 6C, inset). C9orf72 expression in the external plexiform area is spread
412 throughout the cells at P5, predominantly at the internal edge of the mitral cell layer
413 (mcl) and in the molecular layer (ml) (Fig. 6Bii). By P10, the diffuse cytoplasmic
414 staining has transitioned to a much more defined nucleo-cytoplasmic staining as is
415 seen in the developing cortex. Strong nucleo-cytoplasmic staining is found in the
416 internal plexiform area from P5 through to P35 and continues to remain at the same
417 intensity as the olfactory bulb expands (Fig. 6Biii). See Table 3 for summary of
418 expression pattern.

419

420 **C9orf72 expression in the hippocampus**

421 The hippocampus forms from the dorso-medial telencephalon. The CA fields form
422 sequentially from the outside in; first the CA3 field is defined at the dentate pole at

423 E14.5, then CA1 at the subicular pole from E15.5 then finally between them CA2 takes
424 on its identity post-natally. The dentate gyrus granule cell layer becomes visible by
425 E18.5 with stratification of the different cell-types finalising by P1-2. We studied
426 C9orf72 expression in the P35 adult hippocampus (Khalaf-Nazzal and Francis, 2013).

427 C9orf72 is not detectable in the nuclei of cells in the granular layer of the dentate
428 gyrus or the CA fields (Fig. 7A, dg and CA1-3). Strong cytoplasmic expression is seen
429 extending from the hilus through to the CA3 stratum radiatum (Fig. 7Bi-iv). In the CA3
430 stratum radiatum, C9orf72 can be seen distributed radially from the granular layer
431 indicating association with the CA3 neuron dendrites (Fig. 7Biv). This distribution
432 extends further round to CA2 (Fig. 7Bv) but is not present in CA1 (Fig. 7Bvi) or in the
433 subiculum. C9orf72 staining also appears to be stronger in the efferent side of CA2 in
434 the stratum oriens adjacent to the granular layer (Fig. 7Bv). This can also be seen to
435 a lesser degree in CA1 (Fig. 7Bvi). See Table 3 for summary of expression pattern.

436

437 **Expression of C9orf72 in the developing spinal cord**

438 The anterior-posterior patterning of the neural tube leads to the specification of the
439 pro- mes- and rhomb-encephalon (the early structures which form the fore- mid- and
440 hind-brain) and the spinal cord. In addition, dorso-ventral patterning occurs between
441 E9.5-11 in response to BMP/Shh signalling resulting in progenitor domains that will
442 generate ventral motoneurons, dorsal sensory neurons and interneurons (Lu et al.,
443 2015). Migration and differentiation of the neural crest derived sensory neurons to the
444 dorsal root ganglia occurs from E9.5 with migration reduced by E11 after which
445 subtype specification continues to occur (Marmigère and Ernfors, 2007).

446 We analysed the expression of C9orf72 expression in the spinal cord and dorsal
447 root ganglia (DRG) in mid-thoracic sections of mouse embryos. We stained for

448 neurofilament to identify axons and Islet1/2 to identify post-mitotic motor neurons and
449 Islet1⁺ dorsal root ganglion neurons (Sun et al., 2008).

450 Expression of C9orf72 is seen throughout the spinal cord but is most intense in
451 the transverse fibres of the corticospinal and spinothalamic tracts in the lateral and
452 ventral funiculus at E12.5 (Fig. 8B). C9orf72 expression is similar to the surrounding
453 tissue where Islet1/2⁺ motor neurons are found in the ventral horn (Fig. 9A) or in the
454 more lateral pools (Fig. 9B) at E12.5. Increased expression of C9orf72 is observed in
455 Islet1⁺ motor neurons at E14.5 and E16.5 (Fig. 9A). Strong C9orf72 expression can
456 also be seen in Islet1⁺ neuronal cell bodies in the dorsal root ganglia at E12.5 with
457 marked increases in staining intensity at E14.5 continuing until E16.5 (Fig. 9B).

458 The intense C9orf72 staining found in the funiculus co-stains with neurofilament
459 in the descending tracts, particularly at the outermost edge of the spinal cord. At E12.5
460 staining appears strongest at the ventral funiculus (Fig. S3A) while it appears evenly
461 distributed between lateral and ventral tracts at E14.5 and E16.5 (**Fig. S3B and 8B,C**).
462 Additionally at E14.5 and E16.5 stronger C9orf72 staining can be found in the dorsal
463 corticospinal tracts (Fig. 8B,C, arrows). See Table 3 for a summary of the expression
464 pattern.

465

466 **C9orf72 expression during *in vitro* neuronal differentiation from stem cells**

467 In order to better observe the changes in distribution and expression of C9orf72 during
468 cell differentiation, we monitored the expression of C9orf72 during *in vitro*
469 differentiation of P19 embryonal carcinoma cell line to mature motor neurons.

470 Undifferentiated P19 grow in nests and C9orf72 is seen uniformly in all cells of
471 the colony (Fig. 10A). C9orf72 expression is predominantly and strongly cytoplasmic

472 in a granular punctate manner in the undifferentiated P19 cells, (Fig. 10Ai). After eight
473 days of differentiation to neurons, C9orf72 is present in both the nucleus and
474 cytoplasm in the soma in a highly speckled pattern, and can very clearly be seen
475 concentrated at the membranes in neurite extensions (Fig. 10Aii and iii). To identify
476 when this transition in cellular distribution occurs, we looked at intervening time points
477 and co-immunostained with markers characteristic of the different stages of
478 differentiation. Early speckled cytoplasmic staining is found on day two and day four
479 (Fig. 10B-D, white arrows). A small proportion of cells on day four have begun to show
480 the later speckled C9orf72 distributed more evenly between nucleus and cytoplasm
481 (Fig. 10B-D, black arrows). By day eight the majority of cells exhibit this nucleo-
482 cytoplasmic staining in the cell body.

483 Expression of nestin, an early neuronal precursor marker, starts off uniformly
484 expressed in all cells on day two (Fig. 10B). By day seven it has increased in intensity,
485 processes have begun to extend and C9orf72 can be seen in these processes. By day
486 eight, nestin positive processes have been replaced by neurofilament and nucleo-
487 cytoplasmic staining is seen in the nestin negative cells with neuronal morphology.
488 Underlying these cells, nestin positive but C9orf72 negative epithelial-like cells can be
489 seen (Fig. 10B, asterisk), mirroring the pattern of C9orf72 seen with neurons and glia
490 in the brain.

491 Expression of neurofilament indicating mature neurons and the frequency of
492 C9orf72 positive neurites, increases between day six and eight and cell bodies
493 associated with neurofilament positive neurites have nucleo-cytoplasmic C9orf72
494 staining rather than strongly cytoplasmic alone (Fig. 10C, asterisks). A later marker of
495 neuronal maturity, the synaptic vesicle marker SV2, is weakly expressed on day four
496 but can be seen expressed robustly on day eight, distributed throughout neurites and

497 neuronal cell bodies (Fig. 10D). Again SV2 is present in neuronal cells which stain
498 positive for the speckled nucleo-cytoplasmic C9orf72 distribution. The C9orf72
499 staining in the neurites appear stronger where SV2 is being expressed (Fig. 10D,
500 asterisk). Around 20% of the neurons generated by day eight are motor neurons and
501 stain positive for the transcription factors Islet1/2 (Fig. 10E). P19 derived motor
502 neurons display the same intracellular distribution of C9orf72 as other P19 derived
503 neurons (Fig. 10F).

504 **Quantitation of C9orf72 transcript specific expression during development and**
505 ***in vitro* differentiation**

506 We also quantified the levels of the different mouse C9orf72 transcripts during
507 embryonic and postnatal mouse developments as well as in adult brain to support the
508 immunohistochemistry data. C9orf72 transcript 1 shows a steady increase in levels
509 throughout the embryonic stages to E18.5 when a clear change in expression levels
510 can be seen with lower levels in mid/hind brain (Fig. 11A). During postnatal stages
511 expression of C9orf72 transcript 1 appears to reach steady levels between P5 and
512 P15 in the cerebrum while a rapid increase in expression levels can be seen in the
513 cerebellum from P5 onwards. C9orf72 transcript 2 also appears to steadily increase
514 during development. During postnatal stages, C9orf72 transcript 2 appears to increase
515 in expression in both the cerebrum and cerebellum concurrently to similar levels by
516 P15. C9orf72 transcript 2 is initially present at levels below that of C9orf72 transcript
517 1 and 7 but reaches similar levels at least in the cerebrum by P15. The expression
518 levels of C9orf72 transcript 7 expression at all stages appears to be between that of
519 transcript 1 and 2. Its expression follows a pattern more similar to C9orf72 transcript
520 2 with a concurrent increase in expression in both cerebrum and cerebellar tissue from
521 P5.

522 Analysis of expression of the three *C9orf72* transcripts during neuronal
523 differentiation of pluripotent P19 cells showed a steady increase in expression over
524 the time course of the differentiation similar to the steady increase in levels seen by
525 immunostaining (Fig. 11B). There is no sudden peak during the time course as seen
526 during the developmental series from post-natal stages. By day sixteen, expression
527 of *C9orf72* transcript 1 and 2 peaked at levels but below that of post-natal mouse
528 brains. *C9orf72* transcript 2 levels appear higher than in embryonic brains. *C9orf72*
529 transcript 7 appears to peak midway through the differentiation and maintain a steady
530 level until day 16 where it appears to have dropped dramatically.

531

532 **DISCUSSION**

533

534 *C9orf72* is linked to neurodegenerative diseases, and the hexanucleotide repeat
535 expansions in the first intron of this gene have been shown to cause neuronal cell
536 death but very little is known about the distribution, cellular function or normal role for
537 the *C9orf72* gene product in the adult brain or during neuronal differentiation. We
538 have observed substantial region specific differences in expression domains in the
539 developing and adult brain, such as increased levels in particular cortical layers. We,
540 like Atkinson et al., (Atkinson et al., 2015), show that C9orf72 protein is detected
541 throughout the mouse brain in a punctate speckled manner. In addition we have
542 analysed the distribution of C9orf72 in early embryonic stages. We also show hitherto
543 unreported notable expression in the olfactory bulb, hippocampus and cerebellum in
544 addition to the cortex which is traditionally associated with FTD. We show in addition,
545 that novel distinct changes occur both in the domains of expression of C9orf72 as well
546 as in the intracellular localisation. This is not an artefact of tissue processing as we

547 also demonstrate this switch in distribution using an *in vitro* model of neuronal
548 differentiation.

549 In contrast to the lacZ knock-in mouse used by Suzuki et al., (Suzuki et al.,
550 2013) where lacZ appeared more frequently in the projection neurons of Layer V and
551 Layer II/III, we found that in the adult cortex increased C9orf72 staining in layers I, IV
552 and VI. This may be due to the lacZ expression highlighting the cell body of the
553 C9orf72 positive neuron while the immunostaining we observed highlights synapses
554 clustered around dendritic processes. This is particularly apparent in the staining seen
555 in layer I, a molecular layer consisting of large numbers of apical dendrites from
556 pyramidal cells in lower layers. During development C9orf72 can be seen as a distinct
557 layer in the intermediate zone where neuronal precursors are migrating from the
558 proliferating progenitors in the ventricular zone through to the nascent cortical plate.

559 The involvement of the cerebellum in FTD is unclear and patients do not show
560 classic signs of cerebellar defects such as ataxia. Despite this, atrophy in the
561 cerebellum has been identified in *C9orf72* ALS-FTD patients (Bocchetta et al., 2016;
562 Hornberger, 2012; Tan et al., 2014) and p62 positive inclusion bodies have been
563 identified in the cerebellum as well as the cortex in FTD (King et al., 2009). These
564 inclusions are present in patients with *C9orf72* HREs and those found in the cortex,
565 hippocampus and cerebellum have been shown to contain dipeptide repeat proteins
566 (Davidson et al., 2014; Mann et al., 2013; May et al., 2014). However in contrast to the
567 strong localisation of *C9orf72* seen by us in the molecular layer of the cerebellum,
568 DPR inclusions are typically found in the cell bodies on the internal granule layer which
569 are the source of the projections into the molecular layer.

570 In the hippocampus, the strongest expression of C9orf72 is within the granule
571 cells of the dentate gyrus and pyramidal cells of CA2/3 where DPR deposits are found

572 in human patients but not in the CA1. (Davidson et al., 2014; Mann et al., 2013; May
573 et al., 2014). Therefore expression of the DPR from the HRE may be linked to overall
574 expression levels of the *C9orf72* gene or expression of a particular transcript. In
575 agreement with our observation that transcript 1 is dramatically upregulated during
576 post-natal development of the cerebellum and increased levels of C9orf72 protein in
577 the expanding cerebellar molecular layer, Atkinson et al., (Atkinson et al., 2015) show
578 the transcript 1 55kDa isoform is highly enriched in synaptosome preparations.

579 Olfactory defects and impaired sense of smell have been identified in some
580 cases of FTD although this is more commonly associated with Alzheimer's and
581 Parkinson's disease (AD and PD) (Heyanka et al., 2014; Luzzi et al., 2007; Orasji et
582 al., 2016; Pardini et al., 2009). The olfactory glomeruli is a structure dense with
583 synapses and is the first level of synaptic processing in the olfactory system (Hamilton
584 et al., 2005). As with the molecular layers of the cortex and cerebellum, C9orf72
585 staining is particularly intense in this region. C9orf72 can be also be seen in the cells
586 of the internal and external plexiform areas.

587 Suzuki et al (2013) did not observe C9orf72 expression in astrocytes and
588 microglia. In mixed cultures of cortical neurons and astrocytes we found that C9orf72
589 expression was virtually undetectable in astrocytes but strongly expressed in the
590 neurons. Similarly in the spinal cord we observed expression in spinal motor neurons
591 as well as the DRGs.

592 C9orf72 is predicted to be a DENN-domain protein (differentially expressed in
593 normal and neoplastic cells) (Levine et al., 2013; Zhang et al., 2012). This family of
594 proteins are Rab guanine nucleotide exchange factors (RabGEFs) which together with
595 Rab family GTPases are involved in membrane and vesicular trafficking (Marat et al.,
596 2011; Stenmark, 2009). In the mouse brain and in P19 derived neurons, we observed

597 that C9orf72 expression and localisation was closely linked to the synaptic vesicle
598 marker SV2 and dense pre-synaptic areas. Consistent with this, the densest C9orf72
599 staining and SV2 overlap was seen in the molecular layer of the adult mouse
600 cerebellum where granule cell axons form synapses with Purkinje dendrites.

601 Xiao et al., (Xiao et al., 2015) using isoform specific antibody, described the
602 intracellular localisation of the long C9orf72 isoform in Purkinje cells as diffuse in the
603 cytoplasm with distinct speckles within the cell body. As of now there is no description
604 of the distribution of the long human C9orf72 isoform for other areas of the human
605 brain. Interestingly, Xiao et al., (Xiao et al., 2015) show that the short human isoform
606 has a very distinct association with the nuclear membrane in Purkinje cells. While the
607 human short isoform is the first 222aa of the human long isoform, the mouse short
608 isoform comprises the last 317aa of transcript 1. Because of this it is not possible to
609 predict what the role of the shorter mouse isoform may be in comparison to human
610 without further characterisation.

611 We also show a distinct switch in intracellular localisation of C9orf72 from a
612 predominantly cytoplasmic distribution predominantly in early developmental stages,
613 to a marked increase in nuclear C9orf72 in addition to the cytoplasmic expression as
614 neurons mature in the brain. We see this switch distinctly in the developing cortex and
615 the external plexiform area of the olfactory bulb, and it is recapitulated *in vitro* during
616 P19 differentiation to mature neurons. A few previous studies have shown C9orf72
617 distribution in mature neurons, however, we are the first to demonstrate the early
618 cytoplasmic distribution followed by a switch to nuclear and cytoplasmic distribution in
619 mature neurons. Nuclear import of C9orf72 is not well characterised and the protein is
620 not predicted to contain an NLS however Xiao et al., (Xiao et al., 2015) have shown
621 association of both human C9orf72 isoforms with importin- β 1, lamin B and Ran-

622 GTPase by immunostaining and immunoprecipitation. What triggers this shift in
623 distribution is unclear. Whether or not this difference in C9orf72 distribution has an
624 effect on the role of the protein during neurogenesis is unknown. The Alzheimer's
625 disease associated proteins presenilin and ApoE have been shown to have a role in
626 adult hippocampal neurogenesis (Chevallier et al., 2005; Kiyota et al., 2015; Mu and
627 Gage, 2011) as does the FTD-associated progranulin (Arrant et al., 2015).

628 In order to better characterise the sub-cellular localisation *in vitro* and *in vivo*
629 epitope tag knock-in approach will be informative. In this approach normal C9orf72
630 expression would be unaffected, potentially yielding clearer results over the
631 heterozygous knock-out required in *LacZ* gene-trap methods (Suzuki et al., 2013).

632 Our study is the first to provide a detailed characterisation of the expression
633 domains and localisation of C9orf72 protein within the tissues of the mouse brain, from
634 early embryonic stages through to mature adult. We also confirm others descriptions
635 of intracellular distribution in mature cells and show a novel C9orf72 distribution within
636 neuronal precursors. The correlation between C9orf72 expression domains and FTD-
637 affected areas highlights that understanding the role of C9orf72 during normal
638 development and cellular function will yield insight into FTD-ALS pathology.

639 **ACKNOWLEDGEMENTS**

640 We would like to acknowledge the Developmental Studies Hybridoma Bank for the
641 2H3, Rat401 and 40.2D6 antibodies. We thank Ravi Acharya and Shalini Iyer for
642 providing the recombinant purified C9orf72 protein and the crude bacterial lysates with
643 C9orf72 used to verify the antibodies to C9orf72. The authors declare no Conflict of
644 Interests.

645 **AUTHOR CONTRIBUTIONS**

646 RF and ESP carried out the experimental work and data analysis, RF wrote the
 647 manuscript and participated in the design of the study. VS conceived and designed
 648 the study, carried out the data analysis and wrote the manuscript.

649

650 **REFERENCES**

- 651 Arrant, A.E., Patel, A.R., Roberson, E.D., 2015. Effects of Exercise on Progranulin Levels and Gliosis in
 652 Progranulin-Insufficient Mice. *eNeuro* 2. doi:10.1523/ENEURO.0061-14.2015
- 653 Atkinson, R.A.K., Fernandez-Martos, C.M., Atkin, J.D., Vickers, J.C., King, A.E., 2015. C9ORF72
 654 expression and cellular localization over mouse development. *Acta Neuropathol. Commun.*
 655 3, 59. doi:10.1186/s40478-015-0238-7
- 656 Bede, P., Bokde, A.L.W., Byrne, S., Elamin, M., McLaughlin, R.L., Kenna, K., Fagan, A.J., Pender, N.,
 657 Bradley, D.G., Hardiman, O., 2013. Multiparametric MRI study of ALS stratified for the
 658 C9orf72 genotype. *Neurology* 81, 361–369. doi:10.1212/WNL.0b013e31829c5eee
- 659 Bede, P., Elamin, M., Byrne, S., McLaughlin, R.L., Kenna, K., Vajda, A., Fagan, A., Bradley, D.G.,
 660 Hardiman, O., 2014. Patterns of cerebral and cerebellar white matter degeneration in ALS. *J.*
 661 *Neurol. Neurosurg. Psychiatry* jnnp-2014-308172. doi:10.1136/jnnp-2014-308172
- 662 Bocchetta, M., Cardoso, M.J., Cash, D.M., Ourselin, S., Warren, J.D., Rohrer, J.D., 2016. Patterns of
 663 regional cerebellar atrophy in genetic frontotemporal dementia. *NeuroImage Clin.* 11, 287–
 664 290. doi:10.1016/j.nicl.2016.02.008
- 665 Chevallier, N.L., Soriano, S., Kang, D.E., Masliah, E., Hu, G., Koo, E.H., 2005. Perturbed neurogenesis
 666 in the adult hippocampus associated with presenilin-1 A246E mutation. *Am. J. Pathol.* 167,
 667 151–159. doi:10.1016/S0002-9440(10)62962-8
- 668 Cooper-Knock, J., Higginbottom, A., Stopford, M.J., Highley, J.R., Ince, P.G., Wharton, S.B., Pickering-
 669 Brown, S., Kirby, J., Hautbergue, G.M., Shaw, P.J., 2015. Antisense RNA foci in the motor
 670 neurons of C9ORF72-ALS patients are associated with TDP-43 proteinopathy. *Acta*
 671 *Neuropathol. (Berl.)* 130, 63–75. doi:10.1007/s00401-015-1429-9
- 672 Davidson, Y.S., Barker, H., Robinson, A.C., Thompson, J.C., Harris, J., Troakes, C., Smith, B., Al-Saraj,
 673 S., Shaw, C., Rollinson, S., Masuda-Suzukake, M., Hasegawa, M., Pickering-Brown, S.,
 674 Snowden, J.S., Mann, D.M., 2014. Brain distribution of dipeptide repeat proteins in
 675 frontotemporal lobar degeneration and motor neurone disease associated with expansions
 676 in C9ORF72. *Acta Neuropathol. Commun.* 2, 70. doi:10.1186/2051-5960-2-70
- 677 Dehay, C., Kennedy, H., 2007. Cell-cycle control and cortical development. *Nat. Rev. Neurosci.* 8,
 678 438–450. doi:10.1038/nrn2097
- 679 DeJesus-Hernandez, M., Mackenzie, I.R., Boeve, B.F., Boxer, A.L., Baker, M., Rutherford, N.J.,
 680 Nicholson, A.M., Finch, N.A., Flynn, H., Adamson, J., Kouri, N., Wojtas, A., Sengdy, P., Hsiung,
 681 G.-Y.R., Karydas, A., Seeley, W.W., Josephs, K.A., Coppola, G., Geschwind, D.H., Wszolek, Z.K.,
 682 Feldman, H., Knopman, D.S., Petersen, R.C., Miller, B.L., Dickson, D.W., Boylan, K.B., Graff-
 683 Radford, N.R., Rademakers, R., 2011. Expanded GGGGCC hexanucleotide repeat in
 684 noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. *Neuron* 72, 245–
 685 256. doi:10.1016/j.neuron.2011.09.011
- 686 Deng, H.-X., Chen, W., Hong, S.-T., Boycott, K.M., Gorrie, G.H., Siddique, N., Yang, Y., Fecto, F., Shi, Y.,
 687 Zhai, H., Jiang, H., Hirano, M., Rampersaud, E., Jansen, G.H., Donkervoort, S., Bigio, E.H.,
 688 Brooks, B.R., Ajroud, K., Sufit, R.L., Haines, J.L., Mugnaini, E., Pericak-Vance, M.A., Siddique,
 689 T., 2011. Mutations in UBQLN2 cause dominant X-linked juvenile and adult-onset ALS and
 690 ALS/dementia. *Nature* 477, 211–215. doi:10.1038/nature10353

- 691 Fratta, P., Mizielińska, S., Nicoll, A.J., Zloh, M., Fisher, E.M.C., Parkinson, G., Isaacs, A.M., 2012.
692 C9orf72 hexanucleotide repeat associated with amyotrophic lateral sclerosis and
693 frontotemporal dementia forms RNA G-quadruplexes. *Sci. Rep.* 2. doi:10.1038/srep01016
- 694 Gijssels, I., Van Langenhove, T., van der Zee, J., Sleegers, K., Philtjens, S., Kleinberger, G., Janssens,
695 J., Bettens, K., Van Cauwenberghe, C., Pereson, S., Engelborghs, S., Sieben, A., De Jonghe, P.,
696 Vandenberghe, R., Santens, P., De Bleecker, J., Maes, G., Bäumer, V., Dillen, L., Joris, G., Cuijt,
697 I., Corsmit, E., Elinck, E., Van Dongen, J., Vermeulen, S., Van den Broeck, M., Vaerenberg, C.,
698 Mattheijssens, M., Peeters, K., Robberecht, W., Cras, P., Martin, J.-J., De Deyn, P.P., Cruts,
699 M., Van Broeckhoven, C., 2012. A C9orf72 promoter repeat expansion in a Flanders-Belgian
700 cohort with disorders of the frontotemporal lobar degeneration-amyotrophic lateral
701 sclerosis spectrum: a gene identification study. *Lancet Neurol.* 11, 54–65.
702 doi:10.1016/S1474-4422(11)70261-7
- 703 Gomez-Deza, J., Lee, Y., Troakes, C., Nolan, M., Al-Sarraj, S., Gallo, J.-M., Shaw, C.E., 2015. Dipeptide
704 repeat protein inclusions are rare in the spinal cord and almost absent from motor neurons
705 in C9ORF72 mutant amyotrophic lateral sclerosis and are unlikely to cause their
706 degeneration. *Acta Neuropathol. Commun.* 3. doi:10.1186/s40478-015-0218-y
- 707 Hamilton, K.A., Heinbockel, T., Ennis, M., Szabó, G., Erdélyi, F., Hayar, A., 2005. Properties of external
708 plexiform layer interneurons in mouse olfactory bulb slices. *Neuroscience* 133, 819–829.
709 doi:10.1016/j.neuroscience.2005.03.008
- 710 Hartikainen, P., Räsänen, J., Julkunen, V., Niskanen, E., Hallikainen, M., Kivipelto, M., Vanninen, R.,
711 Remes, A.M., Soininen, H., 2012. Cortical thickness in frontotemporal dementia, mild
712 cognitive impairment, and Alzheimer's disease. *J. Alzheimers Dis. JAD* 30, 857–874.
713 doi:10.3233/JAD-2012-112060
- 714 Heyanka, D.J., Golden, C.J., McCue, R.B., Scarisbrick, D.M., Linck, J.F., Zlatkin, N.I., 2014. Olfactory
715 deficits in frontotemporal dementia as measured by the Alberta Smell Test. *Appl.*
716 *Neuropsychol. Adult* 21, 176–182. doi:10.1080/09084282.2013.782031
- 717 Hornberger, M., 2012. Assessment of psychiatric changes in C9ORF72 frontotemporal dementia.
718 *Alzheimers Res. Ther.* 4, 49. doi:10.1186/alzrt152
- 719 Hsiung, G.-Y.R., DeJesus-Hernandez, M., Feldman, H.H., Sengdy, P., Bouchard-Kerr, P., Dwosh, E.,
720 Butler, R., Leung, B., Fok, A., Rutherford, N.J., Baker, M., Rademakers, R., Mackenzie, I.R.A.,
721 2012. Clinical and pathological features of familial frontotemporal dementia caused by
722 C9ORF72 mutation on chromosome 9p. *Brain* 135, 709–722. doi:10.1093/brain/awr354
- 723 Kabashi, E., Valdmanis, P.N., Dion, P., Spiegelman, D., McConkey, B.J., Vande Velde, C., Bouchard, J.-
724 P., Lacomblez, L., Pochigaeva, K., Salachas, F., Pradat, P.-F., Camu, W., Meininger, V., Dupre,
725 N., Rouleau, G.A., 2008. TARDBP mutations in individuals with sporadic and familial
726 amyotrophic lateral sclerosis. *Nat. Genet.* 40, 572–574. doi:10.1038/ng.132
- 727 Khalaf-Nazzal, R., Francis, F., 2013. Hippocampal development – Old and new findings. *Neuroscience*
728 248, 225–242. doi:10.1016/j.neuroscience.2013.05.061
- 729 King, A., Al-Sarraj, S., Shaw, C., 2009. Frontotemporal lobar degeneration with ubiquitinated tau-
730 negative inclusions and additional alpha-synuclein pathology but also unusual cerebellar
731 ubiquitinated p62-positive, TDP-43-negative inclusions. *Neuropathol. Off. J. Jpn. Soc.*
732 *Neuropathol.* 29, 466–471. doi:10.1111/j.1440-1789.2008.00966.x
- 733 Kiyota, T., Morrison, C.M., Tu, G., Dyavarshetty, B., Weir, R.A., Zhang, G., Xiong, H., Gendelman, H.E.,
734 2015. Presenilin-1 familial Alzheimer's disease mutation alters hippocampal neurogenesis
735 and memory function in CCL2 null mice. *Brain. Behav. Immun.* 49, 311–321.
736 doi:10.1016/j.bbi.2015.06.014
- 737 Koppers, M., Blokhuis, A.M., Westeneng, H.-J., Terpstra, M.L., Zundel, C.A.C., Vieira de Sá, R.,
738 Schellevis, R.D., Waite, A.J., Blake, D.J., Veldink, J.H., van den Berg, L.H., Pasterkamp, R.J.,
739 2015. C9orf72 ablation in mice does not cause motor neuron degeneration or motor deficits.
740 *Ann. Neurol.* 78, 426–438. doi:10.1002/ana.24453

- 741 Kwiatkowski, T.J., Bosco, D.A., Leclerc, A.L., Tamrazian, E., Vanderburg, C.R., Russ, C., Davis, A.,
742 Gilchrist, J., Kasarskis, E.J., Munsat, T., Valdmanis, P., Rouleau, G.A., Hosler, B.A., Cortelli, P.,
743 de Jong, P.J., Yoshinaga, Y., Haines, J.L., Pericak-Vance, M.A., Yan, J., Ticozzi, N., Siddique, T.,
744 McKenna-Yasek, D., Sapp, P.C., Horvitz, H.R., Landers, J.E., Brown, R.H., 2009. Mutations in
745 the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. *Science*
746 323, 1205–1208. doi:10.1126/science.1166066
- 747 Laakso, M.P., Frisoni, G.B., Könönen, M., Mikkonen, M., Beltramello, A., Geroldi, C., Bianchetti, A.,
748 Trabucchi, M., Soininen, H., Aronen, H.J., 2000. Hippocampus and entorhinal cortex in
749 frontotemporal dementia and Alzheimer's disease: a morphometric MRI study. *Biol.*
750 *Psychiatry* 47, 1056–1063.
- 751 Lee, Y.-B., Chen, H.-J., Peres, J.N., Gomez-Deza, J., Attig, J., Štalekar, M., Troakes, C., Nishimura, A.L.,
752 Scotter, E.L., Vance, C., Adachi, Y., Sardone, V., Miller, J.W., Smith, B.N., Gallo, J.-M., Ule, J.,
753 Hirth, F., Rogelj, B., Houart, C., Shaw, C.E., 2013. Hexanucleotide Repeats in ALS/FTD Form
754 Length-Dependent RNA Foci, Sequester RNA Binding Proteins, and Are Neurotoxic. *Cell Rep.*
755 5, 1178–1186. doi:10.1016/j.celrep.2013.10.049
- 756 Levine, T.P., Daniels, R.D., Gatta, A.T., Wong, L.H., Hayes, M.J., 2013. The product of C9orf72, a gene
757 strongly implicated in neurodegeneration, is structurally related to DENN Rab-GEFs.
758 *Bioinformatics* 29, 499–503. doi:10.1093/bioinformatics/bts725
- 759 Ling, S.-C., Polymenidou, M., Cleveland, D.W., 2013. Converging mechanisms in ALS and FTD:
760 Disrupted RNA and protein homeostasis. *Neuron* 79, 416–438.
761 doi:10.1016/j.neuron.2013.07.033
- 762 Lu, D.C., Niu, T., Alaynick, W.A., 2015. Molecular and cellular development of spinal cord locomotor
763 circuitry. *Front. Mol. Neurosci.* 25. doi:10.3389/fnmol.2015.00025
- 764 Luzzi, S., Snowden, J.S., Neary, D., Coccia, M., Provinciali, L., Lambon Ralph, M.A., 2007. Distinct
765 patterns of olfactory impairment in Alzheimer's disease, semantic dementia, frontotemporal
766 dementia, and corticobasal degeneration. *Neuropsychologia* 45, 1823–1831.
767 doi:10.1016/j.neuropsychologia.2006.12.008
- 768 Mackenzie, I.R.A., Frick, P., Grässer, F.A., Gendron, T.F., Petrucelli, L., Cashman, N.R., Edbauer, D.,
769 Kremmer, E., Prudlo, J., Troost, D., Neumann, M., 2015. Quantitative analysis and clinico-
770 pathological correlations of different dipeptide repeat protein pathologies in C9ORF72. *Acta*
771 *Neuropathol. (Berl.)* 130, 845–861. doi:10.1007/s00401-015-1476-2
- 772 Mann, D.M., Rollinson, S., Robinson, A., Bennion Callister, J., Thompson, J.C., Snowden, J.S.,
773 Gendron, T., Petrucelli, L., Masuda-Suzukake, M., Hasegawa, M., Davidson, Y., Pickering-
774 Brown, S., 2013. Dipeptide repeat proteins are present in the p62 positive inclusions in
775 patients with frontotemporal lobar degeneration and motor neurone disease associated
776 with expansions in C9ORF72. *Acta Neuropathol. Commun.* 1, 68. doi:10.1186/2051-5960-1-
777 68
- 778 Marat, A.L., Dokainish, H., McPherson, P.S., 2011. DENN Domain Proteins: Regulators of Rab
779 GTPases. *J. Biol. Chem.* 286, 13791–13800. doi:10.1074/jbc.R110.217067
- 780 Marmigère, F., Ernfors, P., 2007. Specification and connectivity of neuronal subtypes in the sensory
781 lineage. *Nat. Rev. Neurosci.* 8, 114–127. doi:10.1038/nrn2057
- 782 Martinez, S., Andreu, A., Mecklenburg, N., Echevarria, D., 2013. Cellular and molecular basis of
783 cerebellar development. *Front. Neuroanat.* 7, 18. doi:10.3389/fnana.2013.00018
- 784 May, S., Hornburg, D., Schludi, M.H., Arzberger, T., Rentzsch, K., Schwenk, B.M., Grässer, F.A., Mori,
785 K., Kremmer, E., Banzhaf-Strathmann, J., Mann, M., Meissner, F., Edbauer, D., 2014. C9orf72
786 FTLD/ALS-associated Gly-Ala dipeptide repeat proteins cause neuronal toxicity and Unc119
787 sequestration. *Acta Neuropathol. (Berl.)* 128, 485–503. doi:10.1007/s00401-014-1329-4
- 788 Mu, Y., Gage, F.H., 2011. Adult hippocampal neurogenesis and its role in Alzheimer's disease. *Mol.*
789 *Neurodegener.* 6, 85. doi:10.1186/1750-1326-6-85

- 790 Orasji, S.S.S., Mulder, J.L., de Bruijn, S.F.T.M., Wirtz, P.W., 2016. Olfactory dysfunction in behavioral
791 variant frontotemporal dementia. *Clin. Neurol. Neurosurg.* 141, 106–110.
792 doi:10.1016/j.clineuro.2016.01.003
- 793 Pardini, M., Huey, E.D., Cavanagh, A.L., Grafman, J., 2009. Olfactory function in corticobasal
794 syndrome and frontotemporal dementia. *Arch. Neurol.* 66, 92–96.
795 doi:10.1001/archneurol.2008.521
- 796 Renton, A.E., Majounie, E., Waite, A., Simón-Sánchez, J., Rollinson, S., Gibbs, J.R., Schymick, J.C.,
797 Laaksovirta, H., van Swieten, J.C., Myllykangas, L., Kalimo, H., Paetau, A., Abramzon, Y.,
798 Remes, A.M., Kaganovich, A., Scholz, S.W., Duckworth, J., Ding, J., Harmer, D.W., Hernandez,
799 D.G., Johnson, J.O., Mok, K., Ryten, M., Trabzuni, D., Guerreiro, R.J., Orrell, R.W., Neal, J.,
800 Murray, A., Pearson, J., Jansen, I.E., Sondervan, D., Seelaar, H., Blake, D., Young, K., Halliwell,
801 N., Callister, J.B., Toulson, G., Richardson, A., Gerhard, A., Snowden, J., Mann, D., Neary, D.,
802 Nalls, M.A., Peuralinna, T., Jansson, L., Isoviita, V.-M., Kaivorinne, A.-L., Hölttä-Vuori, M.,
803 Ikonen, E., Sulkava, R., Benatar, M., Wu, J., Chiò, A., Restagno, G., Borghero, G., Sabatelli,
804 M., ITALSGEN Consortium, Heckerman, D., Rogaeva, E., Zinman, L., Rothstein, J.D., Sendtner,
805 M., Drepper, C., Eichler, E.E., Alkan, C., Abdullaev, Z., Pack, S.D., Dutra, A., Pak, E., Hardy, J.,
806 Singleton, A., Williams, N.M., Heutink, P., Pickering-Brown, S., Morris, H.R., Tienari, P.J.,
807 Traynor, B.J., 2011. A hexanucleotide repeat expansion in C9ORF72 is the cause of
808 chromosome 9p21-linked ALS-FTD. *Neuron* 72, 257–268. doi:10.1016/j.neuron.2011.09.010
- 809 Ringholz, G.M., Appel, S.H., Bradshaw, M., Cooke, N.A., Mosnik, D.M., Schulz, P.E., 2005. Prevalence
810 and patterns of cognitive impairment in sporadic ALS. *Neurology* 65, 586–590.
811 doi:10.1212/01.wnl.0000172911.39167.b6
- 812 Schludi, M.H., May, S., Grässer, F.A., Rentzsch, K., Kremmer, E., Küpper, C., Klopstock, T., German
813 Consortium for Frontotemporal Lobar Degeneration, Bavarian Brain Banking Alliance,
814 Arzberger, T., Edbauer, D., 2015. Distribution of dipeptide repeat proteins in cellular models
815 and C9orf72 mutation cases suggests link to transcriptional silencing. *Acta Neuropathol.*
816 (Berl.) 130, 537–555. doi:10.1007/s00401-015-1450-z
- 817 Stenmark, H., 2009. Rab GTPases as coordinators of vesicle traffic. *Nat. Rev. Mol. Cell Biol.* 10, 513–
818 525. doi:10.1038/nrm2728
- 819 Stewart, H., Rutherford, N.J., Briemberg, H., Krieger, C., Cashman, N., Fabros, M., Baker, M., Fok, A.,
820 DeJesus-Hernandez, M., Eisen, A., Rademakers, R., Mackenzie, I.R.A., 2012. Clinical and
821 pathological features of amyotrophic lateral sclerosis caused by mutation in the C9ORF72
822 gene on chromosome 9p. *Acta Neuropathol.* (Berl.) 123, 409–417. doi:10.1007/s00401-011-
823 0937-5
- 824 Sun, Y., Dykes, I.M., Liang, X., Eng, S.R., Evans, S.M., Turner, E.E., 2008. A central role for Islet1 in
825 sensory neuron development linking sensory and spinal gene regulatory programs. *Nat.*
826 *Neurosci.* 11, 1283–1293. doi:10.1038/nn.2209
- 827 Suzuki, N., Maroof, A.M., Merkle, F.T., Koszka, K., Intoh, A., Armstrong, I., Moccia, R., Davis-
828 Dusenbery, B.N., Eggan, K., 2013. The mouse C9ORF72 ortholog is enriched in neurons
829 known to degenerate in ALS and FTD. *Nat. Neurosci.* 16, 1725–1727. doi:10.1038/nn.3566
- 830 Tan, R.H., Devenney, E., Dobson-Stone, C., Kwok, J.B., Hodges, J.R., Kiernan, M.C., Halliday, G.M.,
831 Hornberger, M., 2014. Cerebellar Integrity in the Amyotrophic Lateral Sclerosis -
832 Frontotemporal Dementia Continuum. *PLOS ONE* 9, e105632.
833 doi:10.1371/journal.pone.0105632
- 834 Treloar, H.B., Miller, A.M., Ray, A., Greer, C.A., 2010. Development of the Olfactory System, in:
835 Menini, A. (Ed.), *The Neurobiology of Olfaction*, Frontiers in Neuroscience. CRC Press/Taylor
836 & Francis, Boca Raton (FL).
- 837 Van Langenhove, T., van der Zee, J., Van Broeckhoven, C., 2012. The molecular basis of the
838 frontotemporal lobar degeneration-amyotrophic lateral sclerosis spectrum. *Ann. Med.* 44,
839 817–828. doi:10.3109/07853890.2012.665471

- 840 Waite, A.J., Bäumer, D., East, S., Neal, J., Morris, H.R., Ansorge, O., Blake, D.J., 2014. Reduced
841 C9orf72 protein levels in frontal cortex of amyotrophic lateral sclerosis and frontotemporal
842 degeneration brain with the C9ORF72 hexanucleotide repeat expansion. *Neurobiol. Aging*
843 35, 1779.e5-1779.e13. doi:10.1016/j.neurobiolaging.2014.01.016
- 844 Wheaton, M.W., Salamone, A.R., Mosnik, D.M., McDonald, R.O., Appel, S.H., Schmolck, H.I.,
845 Ringholz, G.M., Schulz, P.E., 2007. Cognitive impairment in familial ALS. *Neurology* 69, 1411–
846 1417. doi:10.1212/01.wnl.0000277422.11236.2c
- 847 Whitwell, J.L., Weigand, S.D., Boeve, B.F., Senjem, M.L., Gunter, J.L., DeJesus-Hernandez, M.,
848 Rutherford, N.J., Baker, M., Knopman, D.S., Wszolek, Z.K., Parisi, J.E., Dickson, D.W.,
849 Petersen, R.C., Rademakers, R., Jack, C.R., Josephs, K.A., 2012. Neuroimaging signatures of
850 frontotemporal dementia genetics: C9ORF72, tau, progranulin and sporadics. *Brain* 135,
851 794–806. doi:10.1093/brain/aws001
- 852 Xiao, S., MacNair, L., McGoldrick, P., McKeever, P.M., McLean, J.R., Zhang, M., Keith, J., Zinman, L.,
853 Rogaeva, E., Robertson, J., 2015. Isoform-specific antibodies reveal distinct subcellular
854 localizations of C9orf72 in amyotrophic lateral sclerosis. *Ann. Neurol.* 78, 568–583.
855 doi:10.1002/ana.24469
- 856 Zhang, D., Iyer, L.M., He, F., Aravind, L., 2012. Discovery of novel DENN proteins: implications for the
857 evolution of eukaryotic intracellular membrane structures and human disease. *Bioinforma.*
858 *Comput. Biol.* 3, 283. doi:10.3389/fgene.2012.00283
- 859 Zu, T., Liu, Y., Bañez-Coronel, M., Reid, T., Pletnikova, O., Lewis, J., Miller, T.M., Harms, M.B.,
860 Falchook, A.E., Subramony, S.H., Ostrow, L.W., Rothstein, J.D., Troncoso, J.C., Ranum, L.P.W.,
861 2013. RAN proteins and RNA foci from antisense transcripts in C9ORF72 ALS and
862 frontotemporal dementia. *Proc. Natl. Acad. Sci. U. S. A.* 110, E4968-4977.
863 doi:10.1073/pnas.1315438110
- 864

865

866

867

868

869

870

871

872

873

874

875 **TABLES****Table 1 – qRT-PCR Primers**

Transcript (Accession)	Primer Name	Sequence 5'-3'	Product (bp)
Transcript 1 (3110043O21Rik- 001)	mC9T1 F	AGCGGCGAGTGGCTATTG	162
	mC9T1 R	CAAAGGTAGCCGCCAACAAG	
Transcript 2 (3110043O21Rik- 002)	mC9T2 F	GGCGAGTGGGAAAGACAAGA	104
	mC9T2 R	ACTTCCCCAGTAAGCATGGG	
Transcript 7 (3110043O21Rik- 007)	mC9T7 F	AGTGAAAGCCTTCCTGGATCA	190
	mC9T7 R	GGGAACGAACAGCAACTGTC	
ActB (NM_001101.3)	mActB-F	ACCATGGATGATGATATCGC	281
	mActB-R	TCATTGTAGAAGGTGTGGTG	

876

877

878

879

880

881

882

883

Table 2 – Antibodies used in this study

Target	Dilution	Supplier	Cat#
C9orf72	1:500	ProteinTech	22637-1-AP
C9orf72	1:500	SantaCruz	SC138763
Nestin	1:50	DSHB	Rat 401
Neurofilament	1:5	DSHB	2H3
Pax6	1:500	Covance	PRB-278P
Tbr2	1:500	Abcam	Ab23345
Nurr1	1:100	R&D	AF2156
Tbr1	1:500	Abcam	Ab31940
Satb2	1:100	Abcam	Ab51502
Ctip2	1:500	Abcam	Ab18465
Cux1	1:50	SantaCruz	SC13024
GFAP	1:500	Sigma	A2052
Calbindin	1:4000	Swant	300
SV2	1:5	DSHB	SV2
PCNA	1:2000	Cell Signalling	2586
PSD95	1:200	Neuro-mAb	K28/43
Islet1/2	1:5	DSHB	40.2D6
Alexa Fluor 488, Goat anti-mouse	1:1000	Invitrogen	A-11001

Alexa Fluor 488, Goat anti-rabbit	1:1000	Invitrogen	A-11035
Alexa Fluor 568, Goat anti-mouse	1:1000	Invitrogen	A-11004
Alexa Fluor 594, Goat anti-rabbit	1:1000	Invitrogen	A-11012
Alexa Fluor 488, Goat anti-rat	1:1000	Invitrogen	A-11006

884

885

886

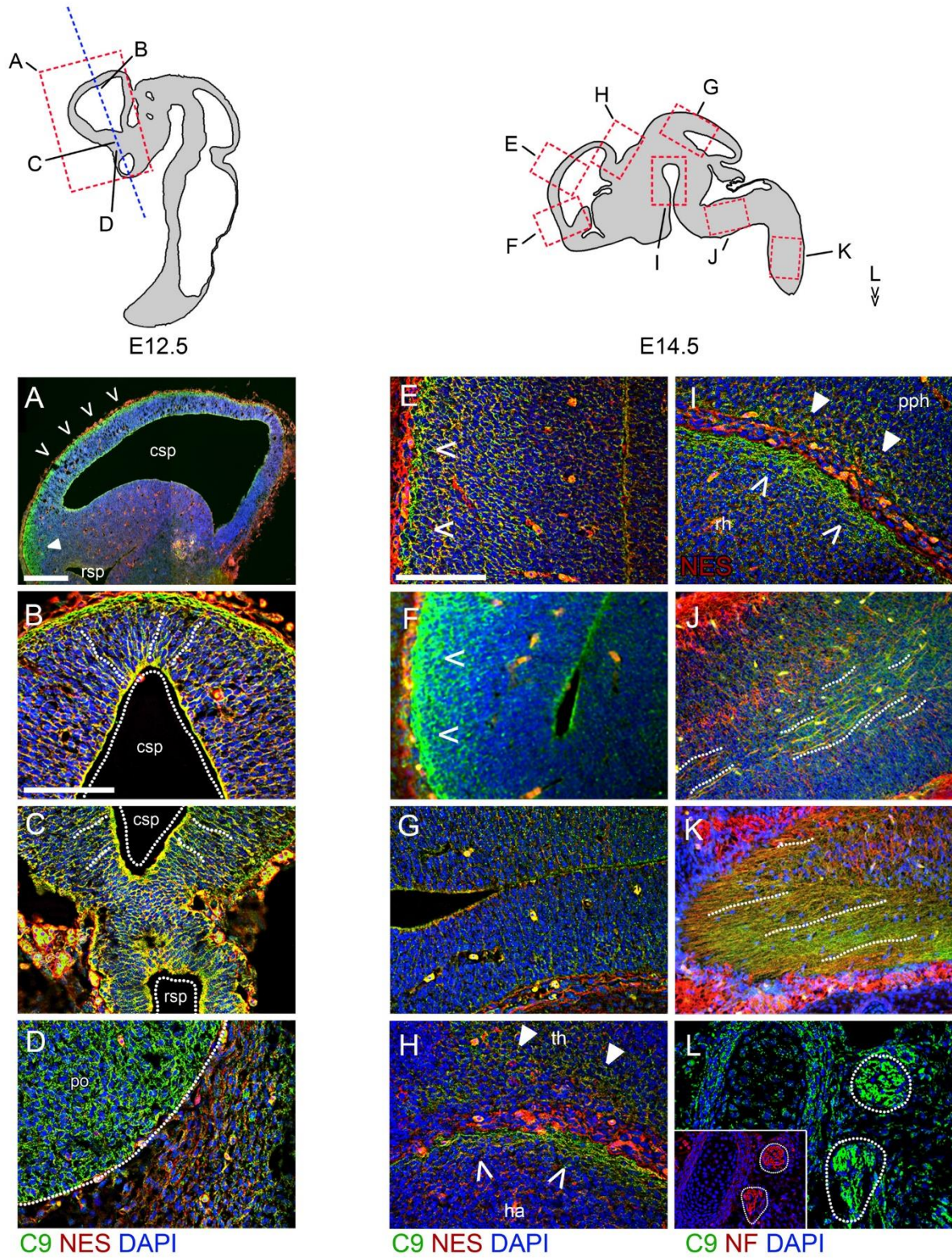
887

888

889

890

891 **FIGURES AND LEGENDS**



892

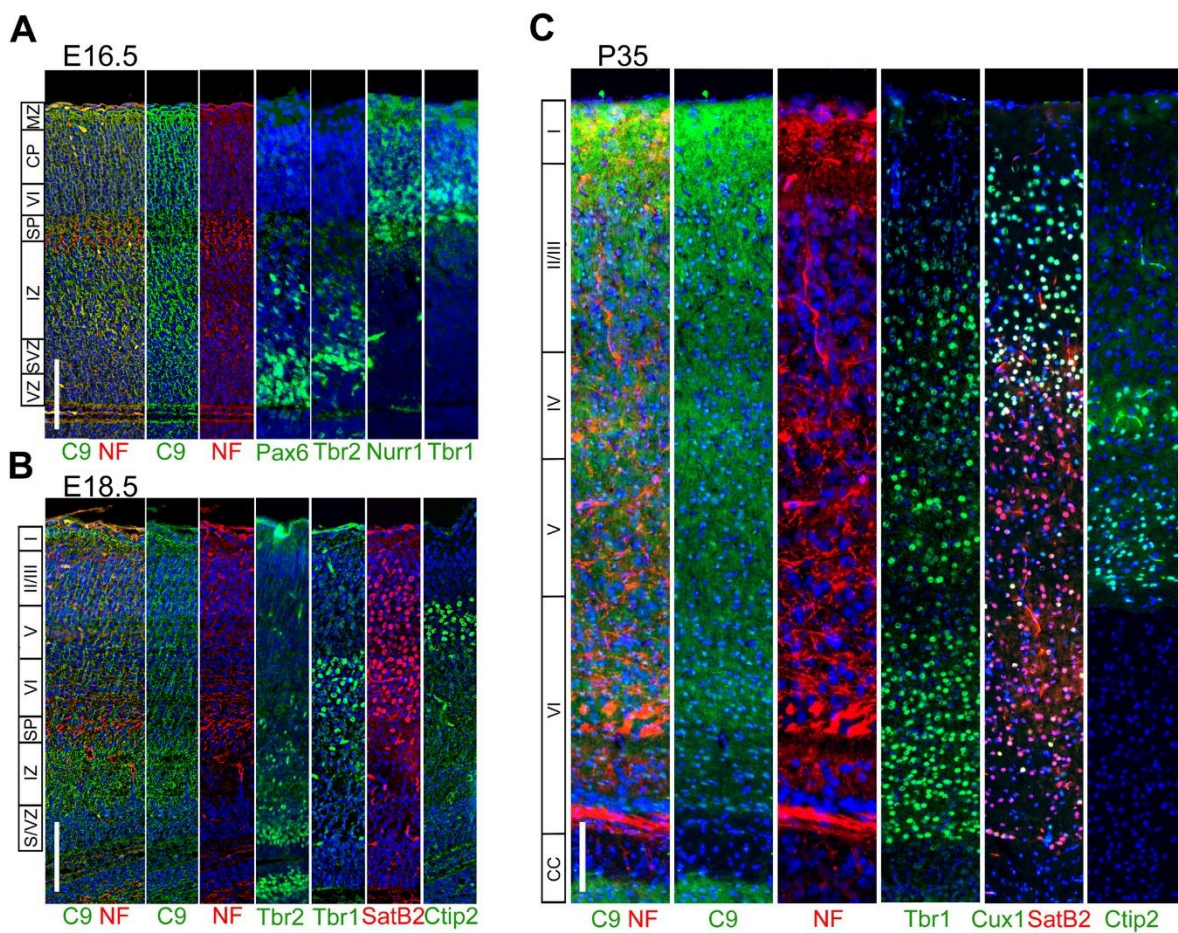
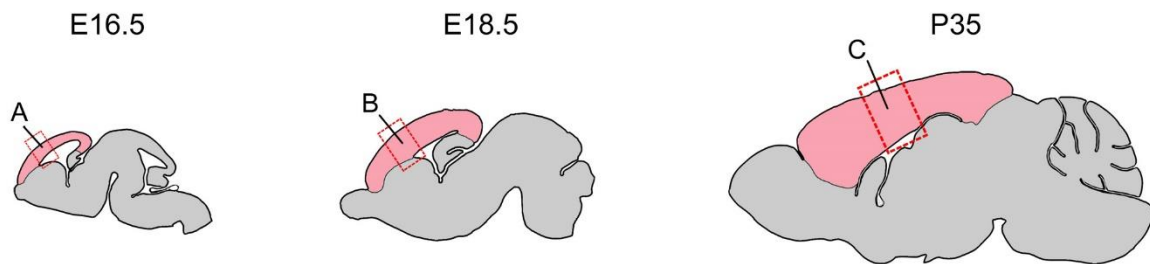
893

894

895 **Figure 1 – C9orf72 is present in the early embryonic brain and spinal cord**

896 Immunostaining for C9orf72 and nestin in E12.5 embryos (A-D) in sagittal (A) and
897 coronal (B-D) orientations. Strong C9orf72 staining can be seen in the dorsal pallium
898 of the telecephalon (A), in the superficial layer of the rostral pallium (B) as well as in
899 regions adjacent to the caudal secondary proencephalic ventricle (csp) in a radial
900 arrangement spanning outwards (dotted lines). A similar pattern is seen in the caudal
901 subpallium (C) adjacent to the rostral secondary proencephalic ventricle (rsp). C9orf72
902 is strongest in neuronal tissues, as shown by expression in the pre-optic area (D, po)
903 adjacent to non-neuronal tissue. Immunostaining for C9orf72 and nestin (E-I) or
904 neurofilament (J-L) in E14.5 brain. Expression seen is strongest in the superficial
905 layers of the caudal rostral pallium (E) and pre-olfactory pallium (F). This pattern of
906 expression is not seen in the caudal midbrain tectum (G). Strong staining again is
907 found in the superficial layer of the hippocampal allocortex (H, ha, open arrows) and
908 spread in deeper layers of the adjacent thalamic tissue (th, closed arrows). Strong
909 staining is also seen in the superficial layer of the rostral hypothalamus (rh) and
910 prepontine hindbrain (pph) (I). C9orf72 can be found aligned with neurofilament
911 positive tracts in the medulla (J) and spinal cord (K). C9orf72 and neurofilament are
912 found together in the spinal ganglia (L). Scale bars 100µm.

913



914

915

916

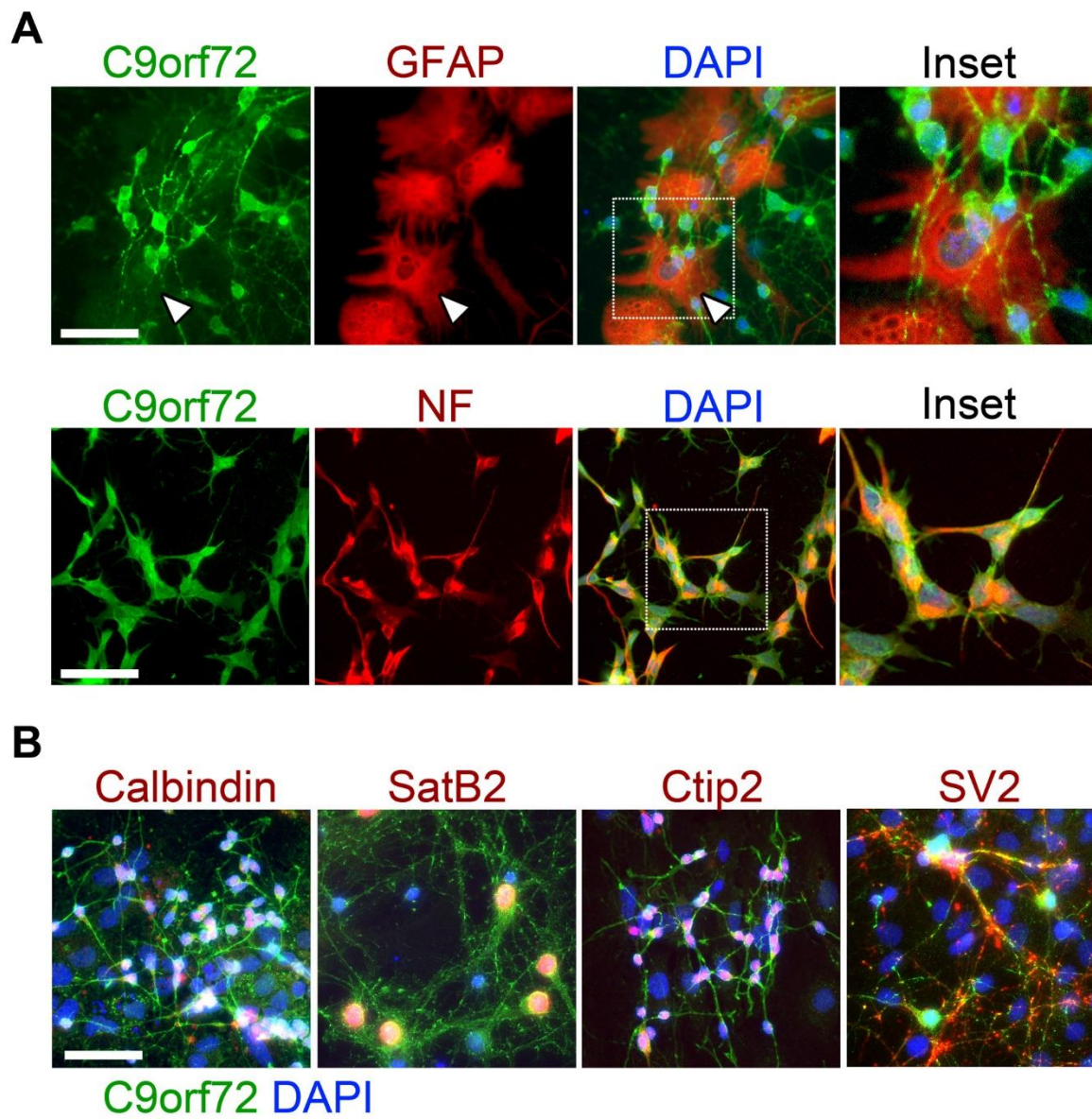
917

918

919

920 **Figure 2 – Layer specific C9orf72 distribution changes in the developing and**
921 **adult cortex**

922 Immunostaining for C9orf72, neurofilament and layer specific markers in adjacent
923 sections of the brain. At E16.5 (A) the cortex is divided into the Pax6⁺ ventricular zone
924 (vz), Tbr2⁺ subventricular zone (svz), intermediate zone (iz), Nurr1⁺ subplate (SP),
925 Tbr1⁺ layer VI/cortical plate (CP) and finally the marginal zone (mz) at the pial surface.
926 C9orf72 expression appears throughout but is strongest in the marginal and
927 intermediate zones. At E18.5 (B), further layers have formed in the cortical plate
928 (layers II/II-VI, marked by SatB2 and Ctjp2). Strong expression continues in the
929 marginal zone and in a narrower region of the intermediate zone. By P35 all the layers
930 of the cortex have formed (C) and the intermediate zone is absent. C9orf72 staining
931 remains strongest in layer I at the pial surface and is also seen strongly in upper
932 portions of layer II, throughout layer IV and the middle of layer VI. C9orf72 has also
933 switched from a mainly cytoplasmic distribution at embryonic stages to a more uniform
934 distribution with staining seen throughout the cytoplasm and nucleus. Scale bars
935 100µm.



936

937

938

939

940

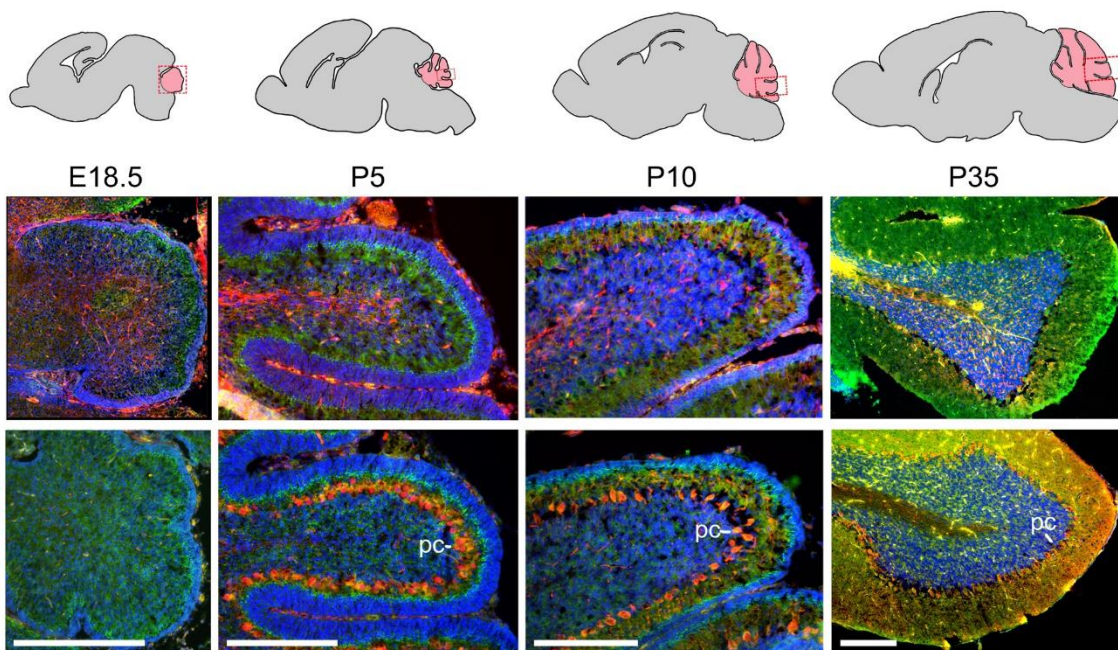
941

942

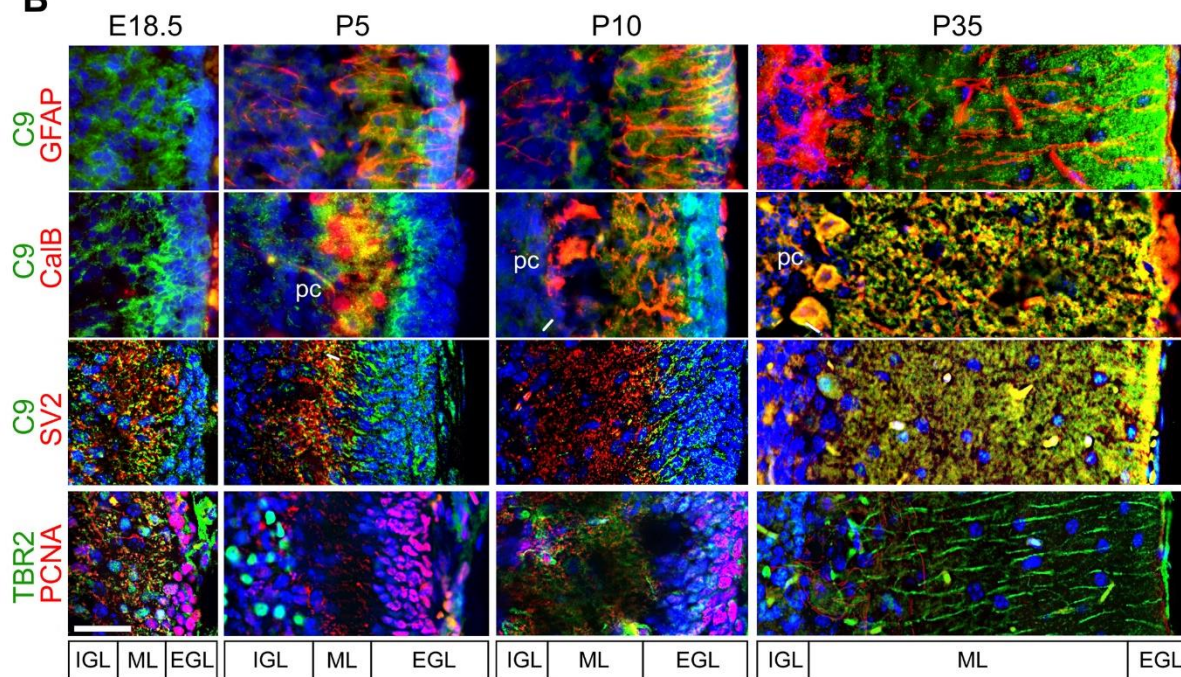
943 **Figure 3 – C9orf72 is highly expressed in primary cortical neurons in**
944 **comparison to glia**

945 Strong expression of C9orf72 is seen in the cell body and neurites of neurofilament
946 positive cultured primary cortical neurons isolated at P0 (A). C9orf72 expression in
947 GFAP positive glial cells is largely undetectable (A, arrows). C9orf72 expression is
948 found consistently in all cortical neuron subtypes identified (B). Scale bars 50µm.

A



B



949

950

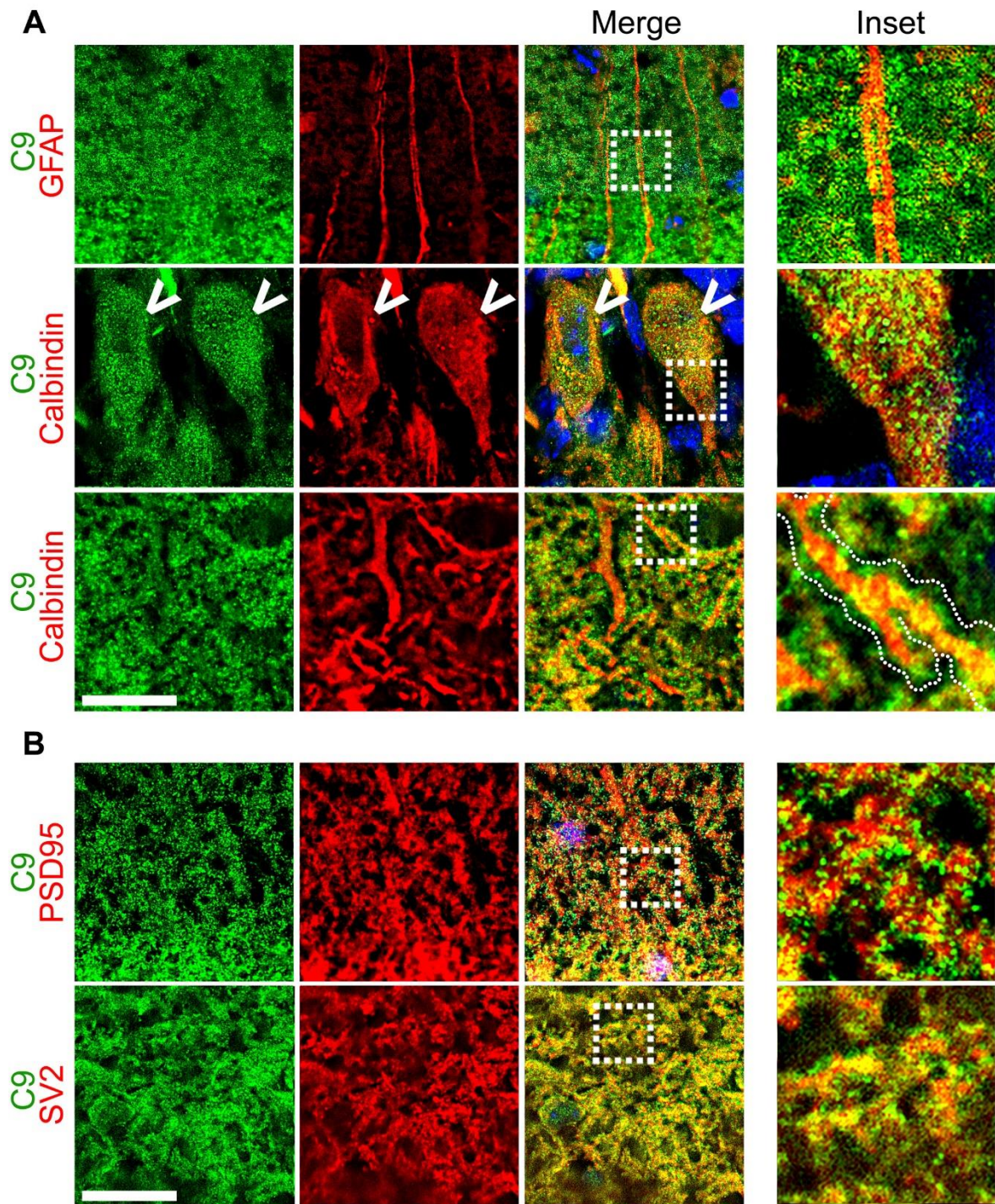
951

952

953 **Figure 4 – C9orf72 is strongly expressed in the molecular layer of the developing**
954 **and adult cerebellum**

955 Expression of C9orf72 in the developing cerebellum from E18.5 to P35 (A). C9orf72
956 staining is not seen in the neurofilament positive internal structures at any stage, but
957 is found strongly at the inner edge of the developing external granule layer (EGL) at
958 E18.5 which then goes onto to expand as the molecular layer (ML) expands, between
959 the EGL and calbindin (CalB) positive Purkinje cell bodies (pc) adjacent to the forming
960 inner granule layer (IGL). Scale bars 500µm.

961 Throughout cerebellar development (B) C9orf72 expression in the molecular layer is
962 not associated with Bergmann glia (GFAP) or proliferating granule cells (PCNA) but
963 does associate with Purkinje dendrites (CalB) and synapses (SV2). C9orf72 appear
964 strongest at the most superficial part of the molecular layer. Scale bars 50µm.



965

966

967

968

969

970 **Figure 5 – C9orf72 in the adult cerebellar molecular layer is pre-synaptic**

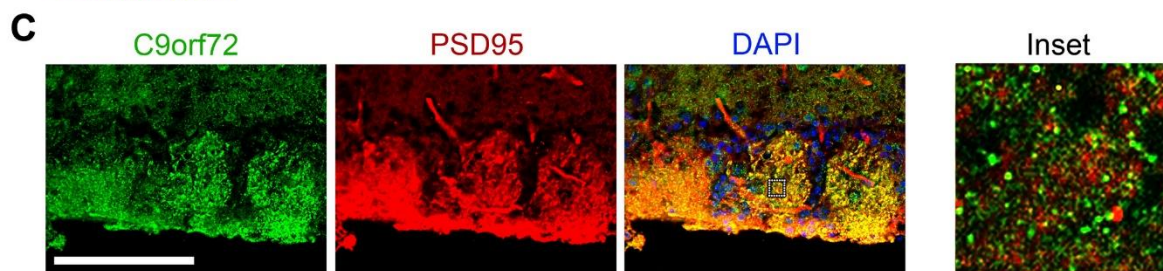
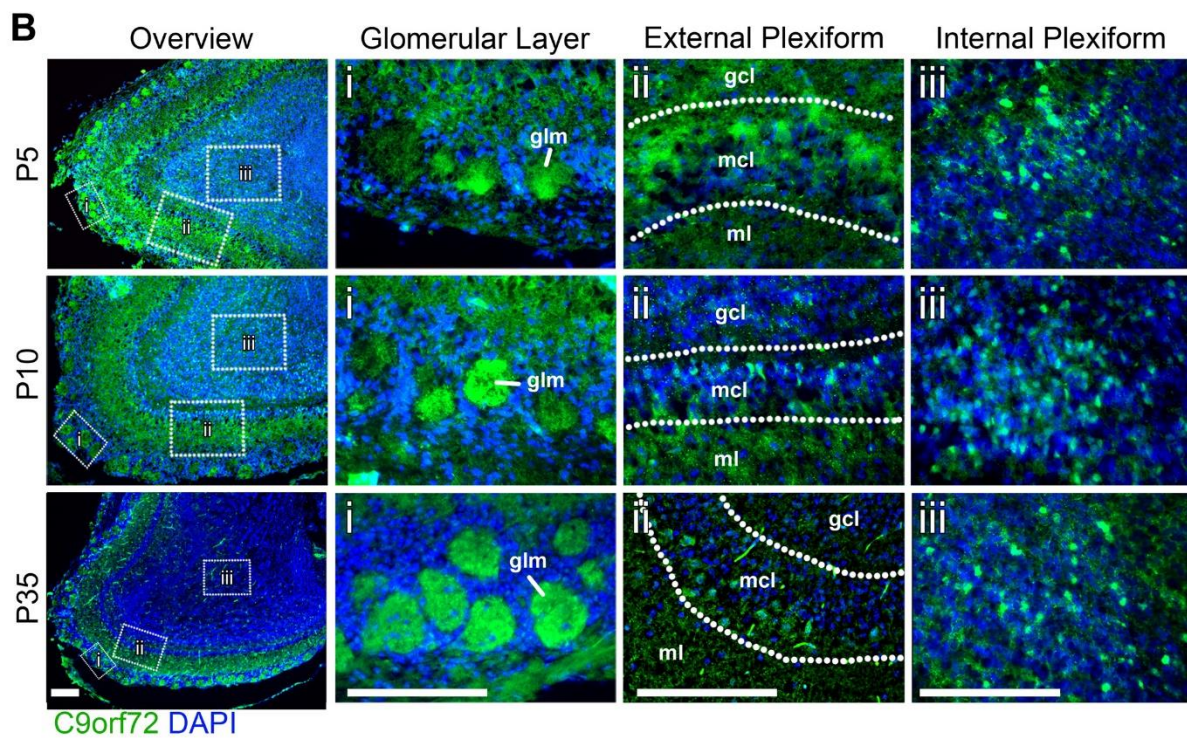
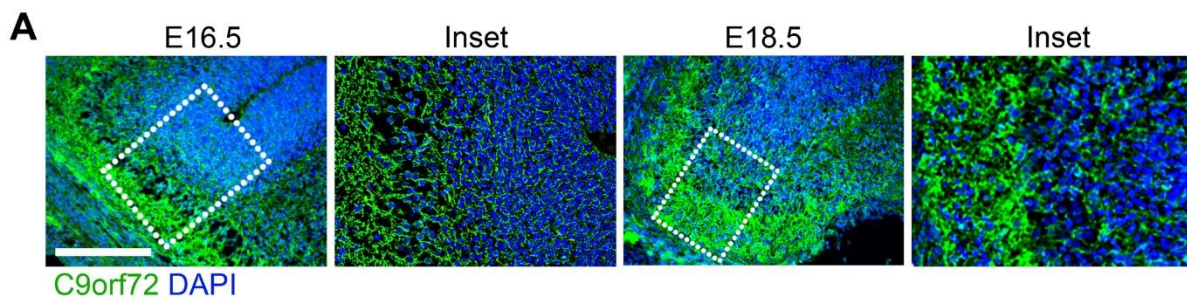
971 The molecular layer in the adult cerebellum (p35) shows no association of C9orf72
972 with Bergman glia fibres (GFAP), but clustered staining can be found directly adjacent
973 to calbindin positive Purkinje dendrites (A, inset, dotted outline). C9orf72 expression
974 can also be seen within the Purkinje cell body (arrow). C9orf72 shows no overlap with
975 the post-synaptic marker PSD95 but co-localises with the synaptic vesicle marker SV2
976 (B). Scale bars 20µm.

977

978

979

980



981

982

983

984

985

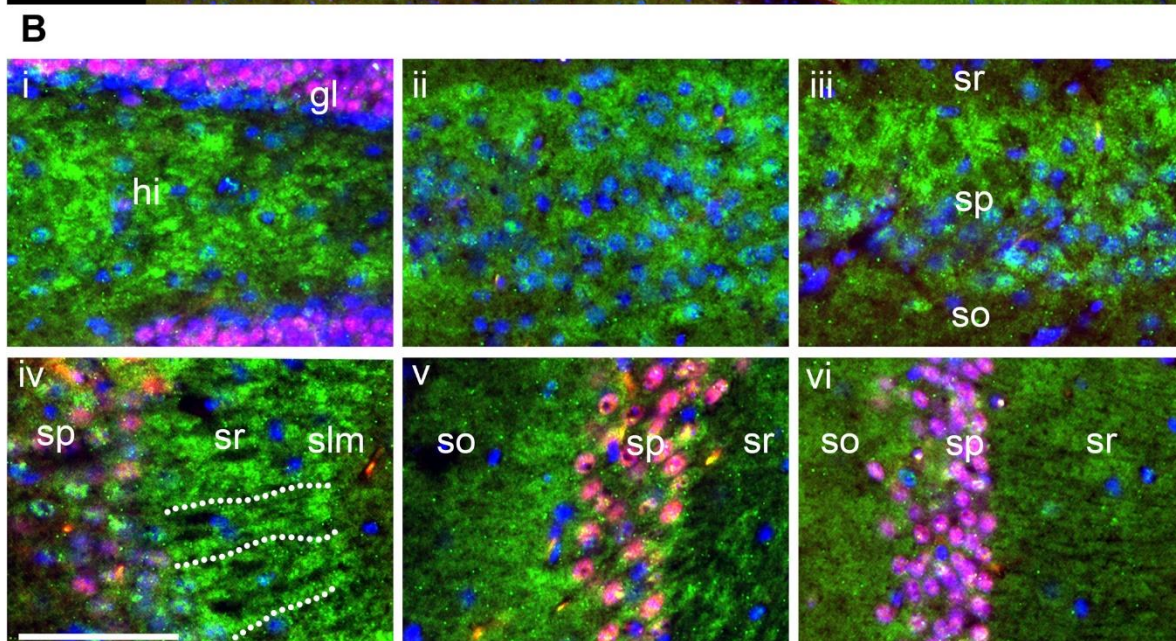
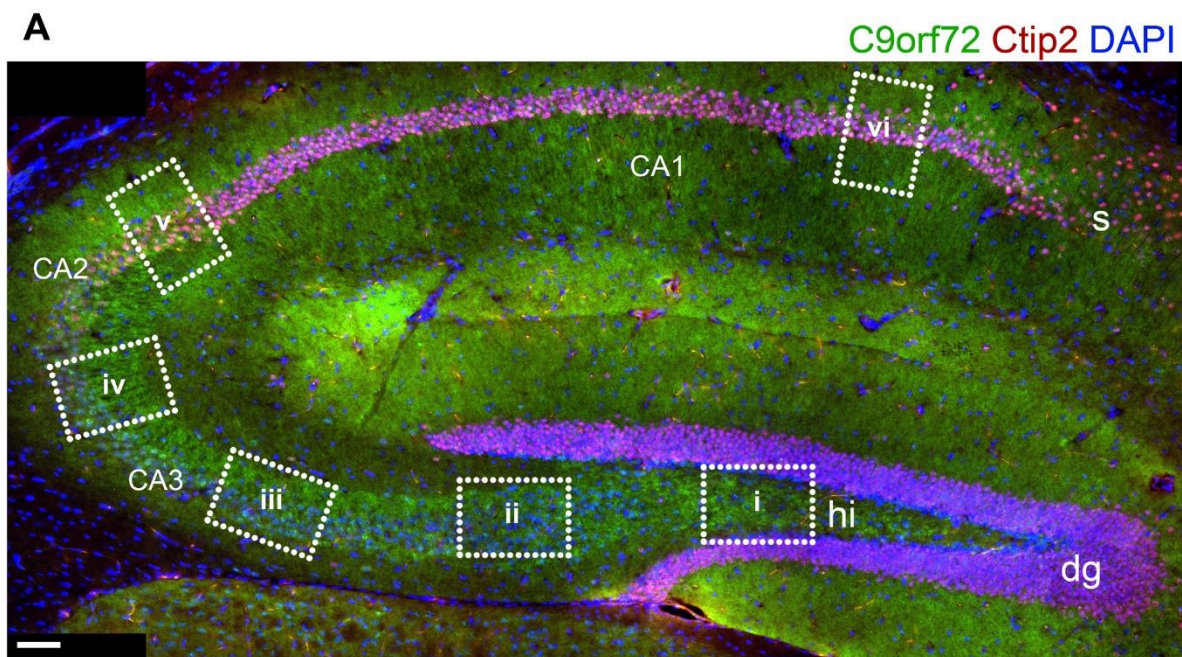
986

987 **Figure 6 – C9orf72 is strongly expressed in the olfactory bulb**

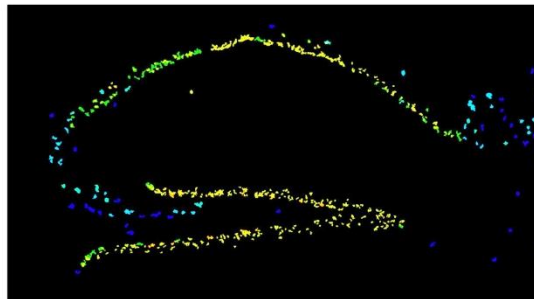
988 Between E16.5 and E18.5 expression is seen in the superficial layer of the olfactory
989 bulb (A) scale bars 200µm.

990 In post-natal mice from P5 onwards, C9orf72 expression is found in in three distinct
991 olfactory layers (B); the glomerular (i), internal (ii) and external plexiform areas (iii).

992 Between P5 and P10 the distribution of C9orf72 in the mitral cell layer of the external
993 plexiform area (mcl) changes from a predominantly cytoplasmic to one with increased
994 nuclear staining. C9orf72 staining in the olfactory glomeruli (glm) becomes more
995 distinct as the size and frequency of glomeruli increases with age. C9orf72 in the
996 glomeruli (C) is strongly co-incident with PSD95 but without overlap similar to the
997 cerebellum . Scale bars 250µm.



C

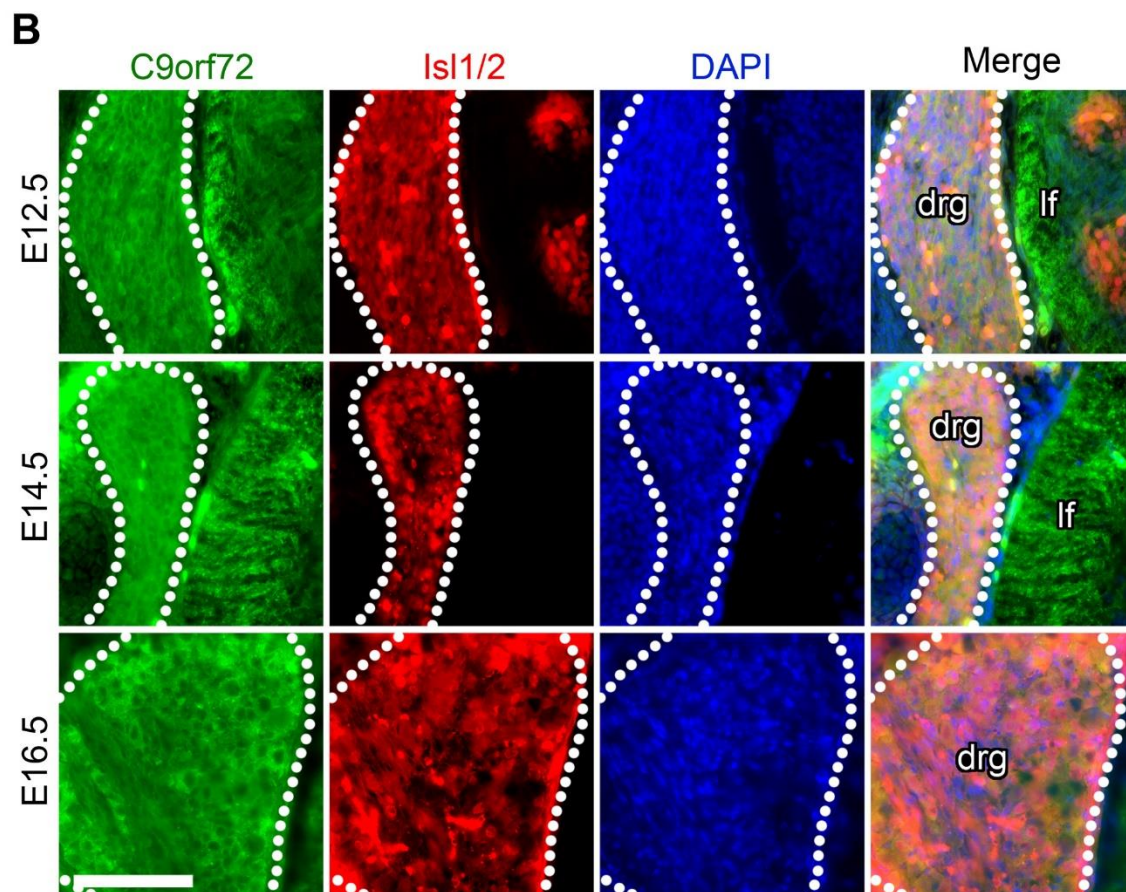
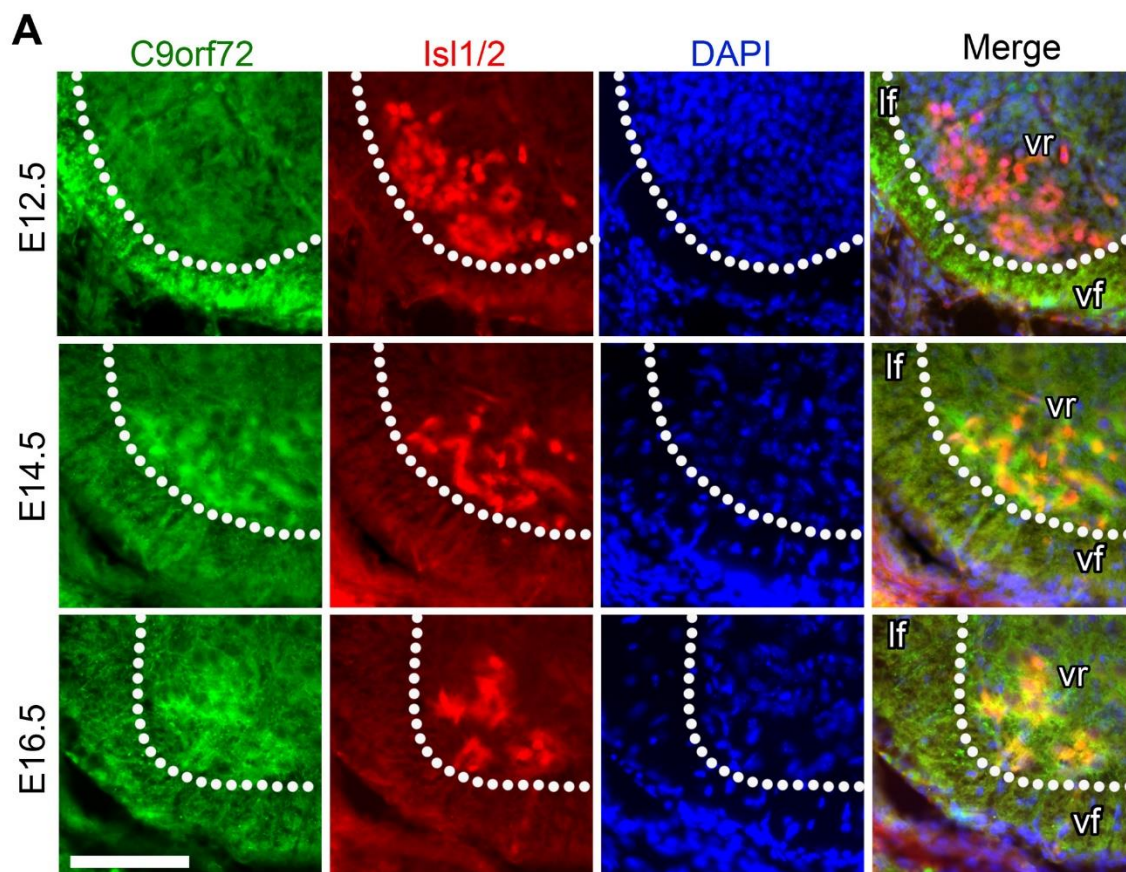


998

999

1000 **Figure 7 – C9orf72 in the hippocampus is found in areas associated with the**
1001 **mossy fibre tracts**

1002 An intense band of C9orf72 can be seen extending from the hilus (hi) of the dentate
1003 gyrus (dg) to the stratum radiatum (sr) of the CA3 field and part of CA2 (A). It does not
1004 appear as tracts between the granule layer (gl) and CA3sr (B, i-iii), but can be seen
1005 organised radially from the CA3 stratum pyramidale (sp) (B, iv). Further C9orf72
1006 staining can be seen at the efferent stratum oriens (so) adjacent to the CA2 sp (B, v).
1007 C9orf72 staining is not as distinct in the CA1 or the subiculum (s) (B, vi). Allen Brain
1008 Atlas expression data (C) shows strong expression in the dg but not CA3 granule
1009 layers. Scale bars 100µm.

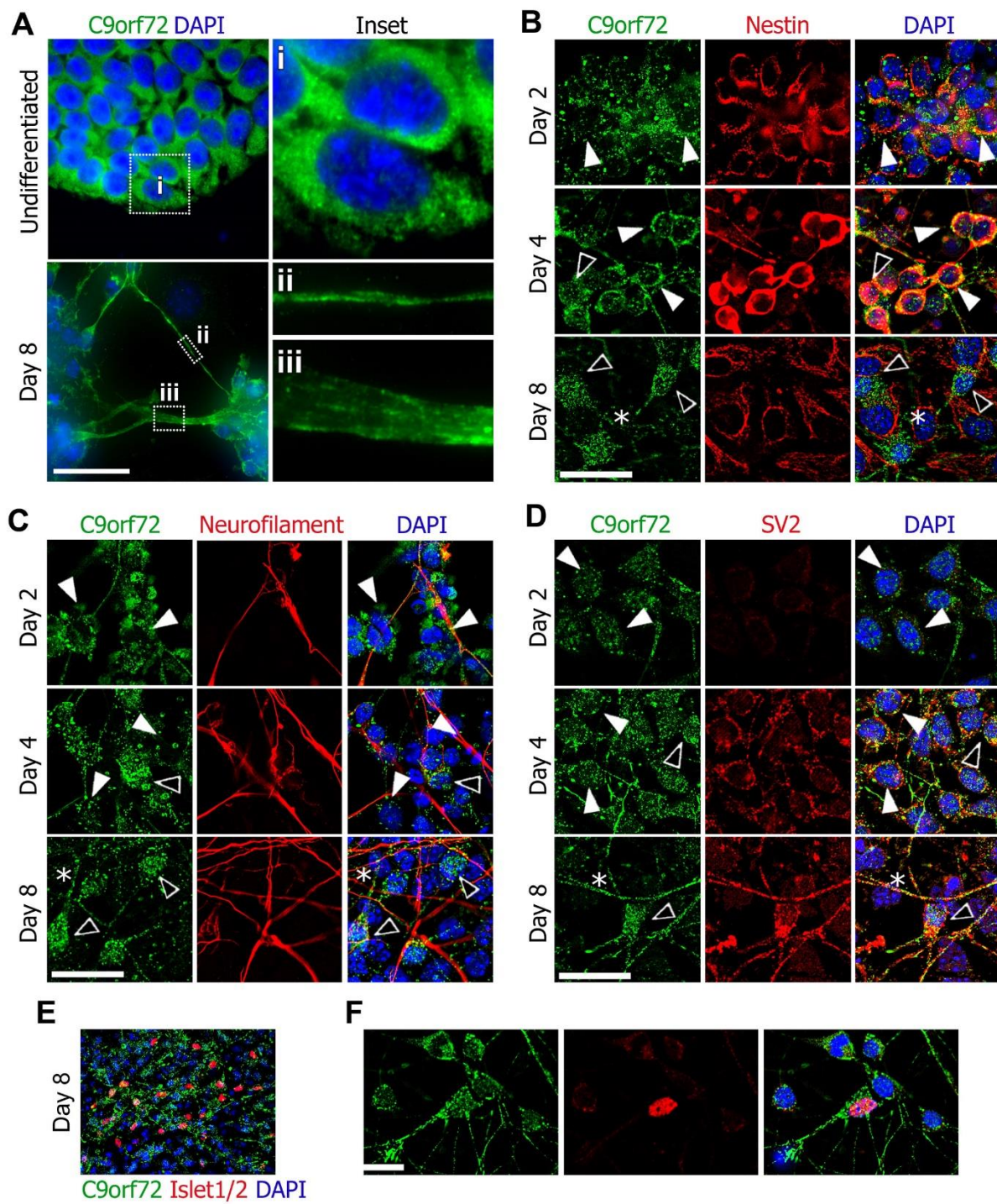


1011 **Figure 8 - Overview of C9orf72 expression in the developing spinal cord**

1012 Transverse sections of mid-thoracic spinal cord from the indicated embryonic stages.
1013 Comparison of adjacent sections immunostained with either Isl1/2 or Neurofilament
1014 (NF) and C9orf72, counterstained with DAPI at E12.5 (A), E14.5 (B) and E16.5 (C).
1015 Motor pools highlighted by dashed boxes. Ventral spinal cord (i) shown in detail in
1016 figures 8A and S4A. Dorsal root ganglion shown in detail in figures 8B and S4B. Arrows
1017 indicate dorsal corticospinal tracts. Scale bars 100µm.

1018 Transverse sections of mid-thoracic region of indicated embryonic stages, stained with
1019 anti-C9orf72 and anti-Islet1/2 (Isl1/2) antibodies , counterstained with DAPI.
1020 C9orf72 expression in the ventral spinal cord is strongest in the transverse tracts of
1021 the lateral (lf) and ventral funiculus (vf) at E12.5. Increased expression can be seen in
1022 the motor neuron pool in the ventral root (vr) from E14.5 to E16.5 (A). C9orf72
1023 expression is also found in the dorsal root ganglia with expression increasing from
1024 E12.5 to E16.5. Scale bars 100µm.

1025



1026

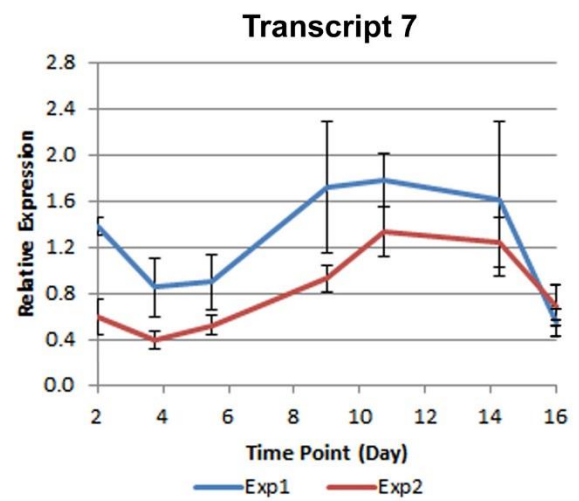
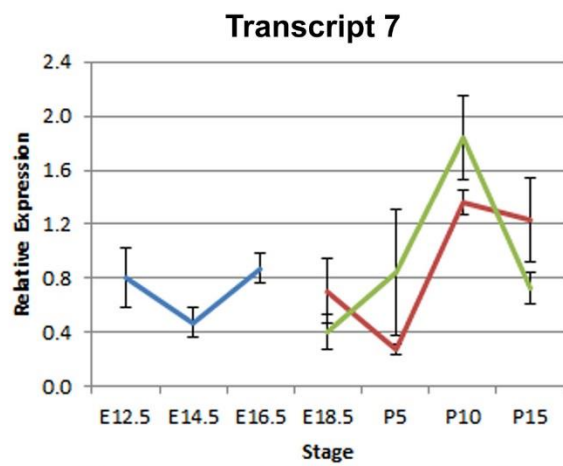
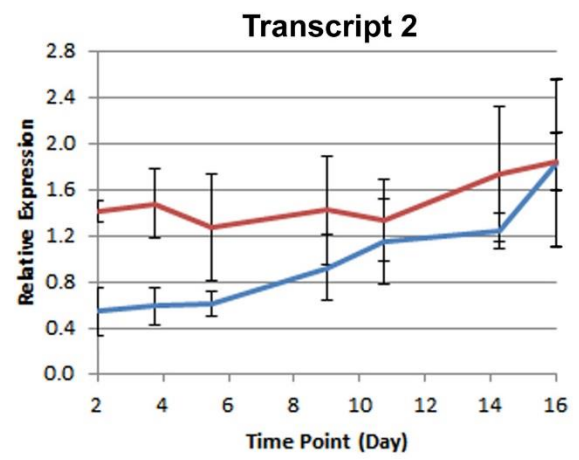
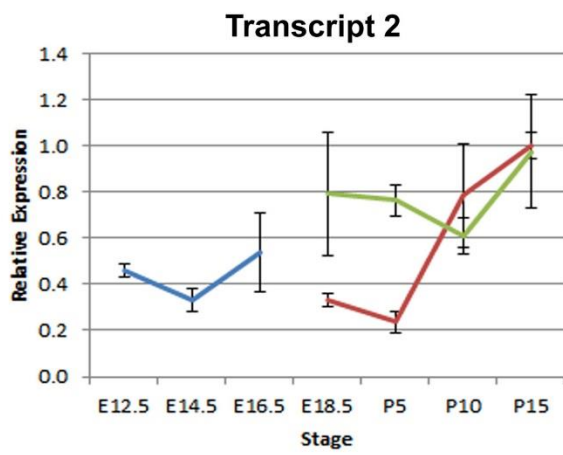
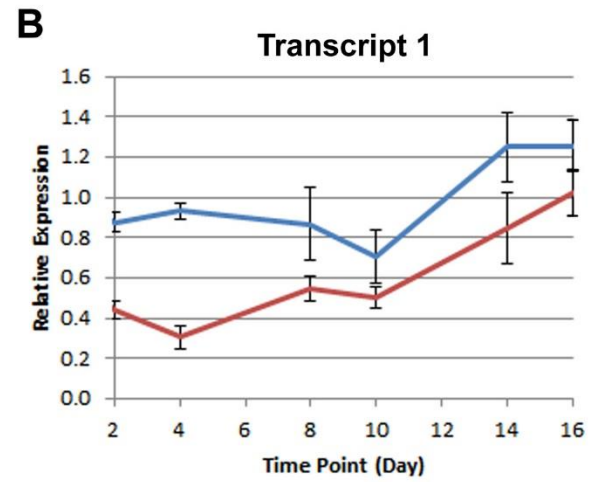
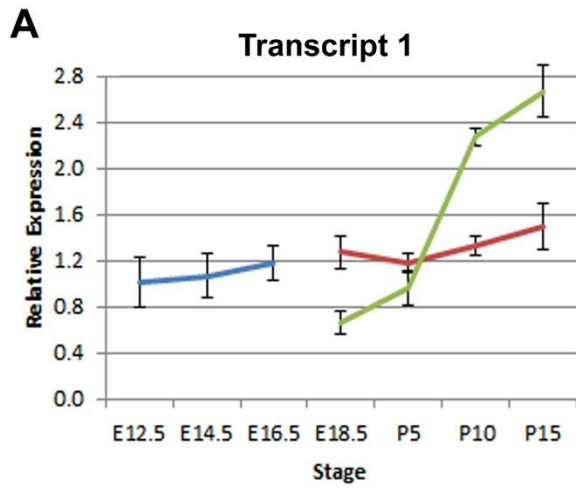
1027

1028

1029

1030 **Figure 9 – Neurons differentiated from pluripotent P19 cell line including motor**
1031 **neurons show a nucleo-cytoplasmic change in C9orf72 distribution during**
1032 **differentiation**

1033 Undifferentiated P19 cells show a predominantly cytoplasmic distribution of C9orf72
1034 but after differentiation to neurons, distribution is more nuclear and C9orf72 can also
1035 be seen on the cell membranes in neurites (A). The early cytoplasmic distribution of
1036 C9orf72 is found in nestin positive neuronal precursors seen on days two and four
1037 while nestin negative mature neurons display increased nuclear C9orf72 (B). C9orf72
1038 is found weakly within nestin positive processes (B, asterisk). Neurofilament
1039 expression follows nestin and as expected, increased nuclear C9orf72 is seen in
1040 mature neurofilament positive neurons (C). C9orf72 expression also seems to be
1041 found at higher levels in neurofilament positive neurites (C, asterisk). The presence of
1042 synaptic vesicle marker SV2 indicates mature axons (D), the strongest C9orf72
1043 staining is found in SV2 positive neurites (D, asterisk). The motor neuron (MN) marker
1044 Islet1/2 expression can be seen on day eight (E) and C9orf72 expression in MNs is
1045 similar to other neurons present in the culture (F). Scale bars 25µm.



— Whole Brain — Cerebrum — Cerebellum

— Exp1 — Exp2

1046

1047

1048

1049

1050 **Figure 10 – C9orf72 expression levels increase throughout development and**
1051 **differentiation in the CNS**

1052 Expression levels of mC9ORF79 transcripts relative to Actb determined by qRT-PCR.
1053 (A) C9orf72 expression in a developmental series of mouse brains; E12.5 head; E14.5
1054 and E16.5 brains; E18.5 fore and mid/hind brain; P5, 10 and 15 cerebrum and
1055 cerebellum. RNA from four siblings pooled in duplicate. Levels of all three transcripts
1056 increase over development, particularly transcript 1 in the cerebellum. (B) A time
1057 course of C9orf72 expression in P19 embryonal carcinoma cells differentiated on
1058 matrigel as described in Methods at the indicated time points. As with transcripts in
1059 brain tissue, all three C9orf72 transcripts appear to steadily rise during differentiation.
1060 RNA from two independent experiments and all reactions performed in triplicate.

1061

A

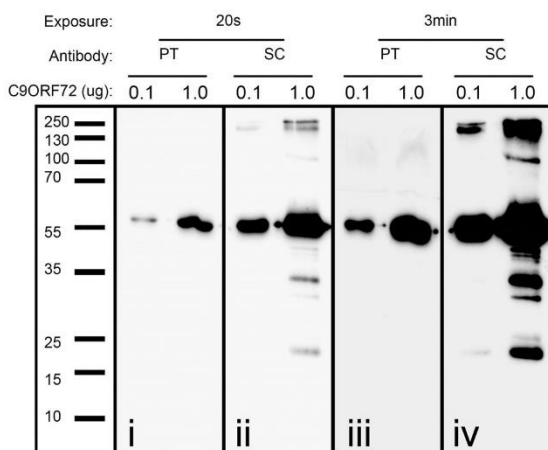
```

Mouse_001_481aa  MSTICPPSPAVAKTEIALSGESPLLAATFAYWNNILGPRVRHIWAPKTDQVLLSDGEITFLANHTLNGEILRNAESGAI DVKFFVLSKSGVIIIVSLIFDGNWNGDRSTYGLSIIILPQTE
Mouse_002_317aa  MSTICPPSPAVAKTEIALSGESPLLAATFAYWNNILGPRVRHIWAPKTDQVLLSDGEITFLANHTLNGEILRNAESGAI DVKFFVLSKSGVIIIVSLIFDGNWNGDRSTYGLSIIILPQTE
Mouse_007_420aa  MSTICPPSPAVAKTEIALSGESPLLAATFAYWNNILGPRVRHIWAPKTDQVLLSDGEITFLANHTLNGEILRNAESGAI DVKFFVLSKSGVIIIVSLIFDGNWNGDRSTYGLSIIILPQTE
Human_001_481aa  MSTICPPSPAVAKTEIALSGESPLLAATFAYWNNILGPRVRHIWAPKTDQVLLSDGEITFLANHTLNGEILRNAESGAI DVKFFVLSKSGVIIIVSLIFDGNWNGDRSTYGLSIIILPQTE
Human_003_222aa  MSTICPPSPAVAKTEIALSGESPLLAATFAYWNNILGPRVRHIWAPKTDQVLLSDGEITFLANHTLNGEILRNAESGAI DVKFFVLSKSGVIIIVSLIFDGNWNGDRSTYGLSIIILPQTE
ProteinTech_antigen  MSTICPPSPAVAKTEIALSGESPLLAATFAYWNNILGPRVRHIWAPKTDQVLLSDGEITFLANHTLNGEILRNAESGAI DVKFFVLSKSGVIIIVSLIFDGNWNGDRSTYGLSIIILPQTE
SantaCruz_antigen  MSTICPPSPAVAKTEIALSGESPLLAATFAYWNNILGPRVRHIWAPKTDQVLLSDGEITFLANHTLNGEILRNAESGAI DVKFFVLSKSGVIIIVSLIFDGNWNGDRSTYGLSIIILPQTE

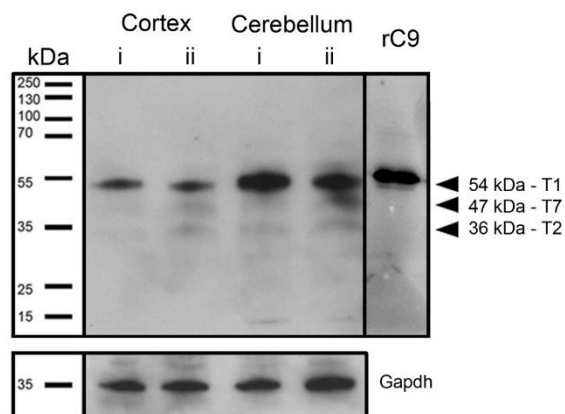
Mouse_001_481aa  LSFYLP LHRVCDRLTHIIRKGRIMMHERQENVQKIVLEGERMEDQGQSIIPMLTGEVIVMELLASMKSHSVPEEIDDIADTVLNDDDIGDSCHEGFLNNAISSHLQTCGCSVVVGS...
Mouse_002_317aa  LSFYLP LHRVCDRLTHIIRKGRIMMHERQENVQKIVLEGERMEDQGQSIIPMLTGEVIVMELLASMKSHSVPEEIDDIADTVLNDDDIGDSCHEGFLNNAISSHLQTCGCSVVVGS...
Mouse_007_420aa  LSFYLP LHRVCDRLTHIIRKGRIMMHERQENVQKIVLEGERMEDQGQSIIPMLTGEVIVMELLASMKSHSVPEEIDDIADTVLNDDDIGDSCHEGFLNNAISSHLQTCGCSVVVGS...
Human_001_481aa  LSFYLP LHRVCDRLTHIIRKGRIMMHERQENVQKIVLEGERMEDQGQSIIPMLTGEVIVMELLASMKSHSVPEEIDDIADTVLNDDDIGDSCHEGFLNNAISSHLQTCGCSVVVGS...
Human_003_222aa  LSFYLP LHRVCDRLTHIIRKGRIMMHERQENVQKIVLEGERMEDQGQSIIPMLTGEVIVMELLASMKSHSVPEEIDDIADTVLNDDDIGDSCHEGFLNNAISSHLQTCGCSVVVGS...
ProteinTech_antigen  LSFYLP LHRVCDRLTHIIRKGRIMMHERQENVQKIVLEGERMEDQGQSIIPMLTGEVIVMELLASMKSHSVPEEIDDIADTVLNDDDIGDSCHEGFLNNAISSHLQTCGCSVVVGS...
SantaCruz_antigen  LSFYLP LHRVCDRLTHIIRKGRIMMHERQENVQKIVLEGERMEDQGQSIIPMLTGEVIVMELLASMKSHSVPEEIDDIADTVLNDDDIGDSCHEGFLNNAISSHLQTCGCSVVVGS...

```

B



C

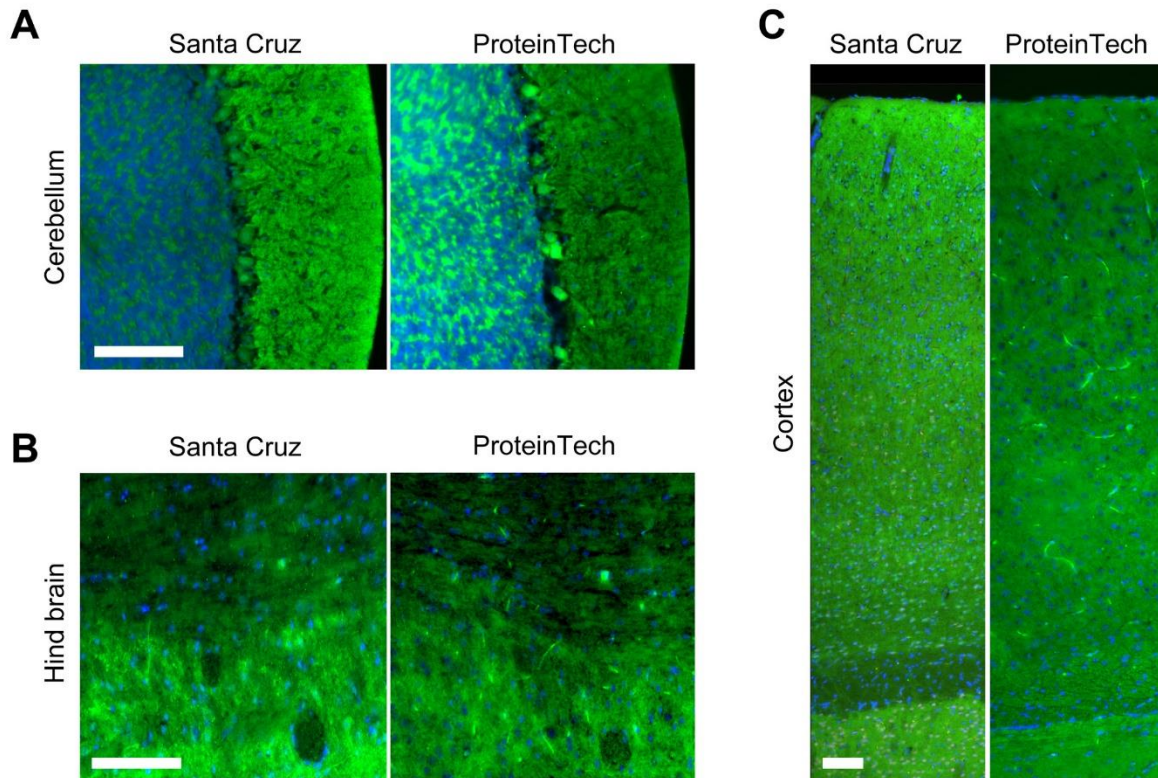


1062

1063 **Supplementary Figure 1 – Comparison of two C9orf72 antibodies by Western** 1064 **blot**

1065 ClustalO alignment of mouse and human C9orf72 protein isoforms showing antibody
1066 epitopes (A). Western blot for purified recombinant human C9orf72 using either
1067 ProteinTech (PT) or SantaCruz (SC) primary antibody showing a strong band at 55kDa
1068 for His-tagged C9orf72 isoform 1 (B). Western blot of P10 mouse cortex and
1069 cerebellum using SantaCruz primary antibody shows isoform 1 is the predominant
1070 form (C). Each lane represents tissue pooled from two mice. Gapdh used as loading
1071 control. Unpurified bacterial lysate of recombinant human his-tagged C9orf72 isoform
1072 1 used as a positive control (rC9).

1073

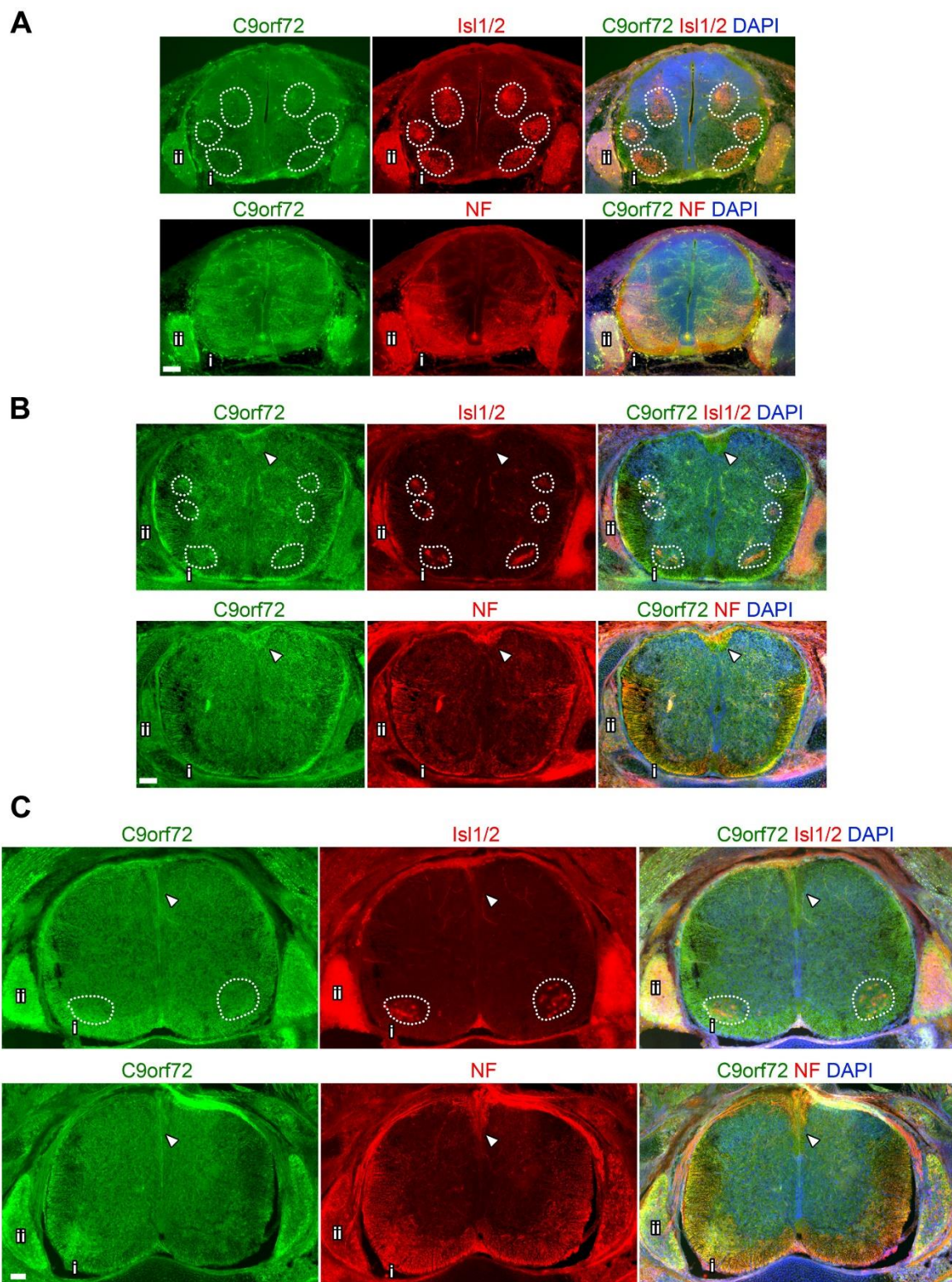


1074

1075 **Supplementary Figure 2 – Comparison of two C9orf72 antibodies by**
1076 **immunostaining on brain sections**

1077 Comparison of adjacent sections of mouse brain immunostained with either
1078 ProteinTech or SantaCruz anti C9orf72 antibody and counterstained with DAPI in the
1079 cerebellum (C), hind brain (D) and cortex (E).

1080



1081

1082

1083

1084 **Supplementary Figure 3 – C9orf72 expression in the spinal cord is associated**
1085 **strongly with neurofilament**

1086 Transverse sections of mid-thoracic spinal cord from the indicated embryonic stages.
1087 Stained with C9orf72 and neurofilament (NF), counterstained with DAPI. C9orf72
1088 expression in the ventral spinal cord is strongest in the transverse tracts of the lateral
1089 (lf) and ventral funiculus (vf) at E12.5 (A). C9orf72 expression is also found with
1090 associated neurofilament in the dorsal root ganglia with expression increasing from
1091 E12.5 to E16.5. Scale bars 100µm.

1092

1093

