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6 Expression and role of Galectin-3 in the postnatal development of the cerebellum

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25

26 **Abstract**

27 Many proteins initially identified in the immune system play roles in neurogenesis, neuronal
28 migration, axon guidance, synaptic plasticity and other processes related to the formation and
29 refinement of neural circuits. Although the function of the immune-related protein Galectin-3
30 (LGALS3) has been extensively studied in the regulation of inflammation, cancer and
31 microglia activation, little is known about its role in the development of the brain. In this
32 study, we identified that LGALS3 is expressed in the developing postnatal cerebellum. More
33 precisely, LGALS3 is expressed by cells in meninges and in the choroid plexus, and in
34 subpopulations of astrocytes and of microglial cells in the cerebellar cortex. Analysis of
35 *Lgals3* knockout mice showed that *Lgals3* is dispensable for the development of cerebellar
36 cytoarchitecture and Purkinje cell excitatory synaptogenesis in the mouse.

37

38 **Introduction**

39 Many proteins initially identified in the immune system are also expressed in the developing
40 and adult central nervous system (CNS) and contribute to a variety of processes during the
41 formation and refinement of neural circuits. Immune system molecules play roles in
42 neurogenesis, neuronal migration, axon guidance and synaptic plasticity (Reviewed in [1,2]).
43 For example, the complement system proteins C1Q and C3, and the transmembrane major
44 histocompatibility complex molecule class I (MHC-I) participate in the promotion of synapse
45 elimination in the developing retinogeniculate pathway and in the vertebrate neuromuscular
46 junction [3,4]. C1Q-related proteins such as the cerebellins and the C1Q-like proteins are
47 known to be essential for synaptogenesis [5–8]. All these immune and immune-related
48 proteins are expressed in neurons [3–8].

49 Galectins are a class of secreted lectins that play roles in various organs including the immune
50 system. Galectins have been involved in inflammation, tumorigenesis, cancer, cell growth and
51 metastasis [9–12]. They regulate cell migration, adhesion to the extracellular matrix, and cell-
52 survival depending on their intra- or extracellular location [13]. The mammalian galectin
53 family of soluble lectins is composed of fifteen members, all of which share a homologous
54 carbohydrate recognition domain (CRD) that typically binds β -galactoside residues [14].
55 There are three architectural types of galectins: proto, tandem-repeat and chimera. The proto-
56 galectins (Galectin-1, 2, 5, 7, -10, -11, -13, -14 and -15) present two homologous CRD
57 (homodimers); the tandem-repeat-galectins (Galectin-4, 6, 8, -9 and -12) are composed by
58 two distinct CRD (heterodimers). Both proto and tandem-repeat galectins have only
59 carbohydrates as ligands. The pleiotropic LGALS3 is the only chimera type of galectins, and
60 is particular in that in addition to the CRD domain, it contains a domain that enables its
61 interaction with non-carbohydrate ligands [15] and the formation of pentamers and
62 heterogeneous complexes with multivalent carbohydrates [16]. LGALS3 has been shown to
63 interact with a broad collection of partners and to play roles at different time points during the
64 life of the cell, including growth, adhesion, differentiation, cell-cycle and apoptosis [15].
65 Finally LGALS3 is involved in various pathologies such as cancer, inflammation and heart
66 disease [17–19].
67 The role of LGALS3 in the central nervous system remain poorly understood. LGALS3 has
68 been proposed to play a role in brain pathology. Its expression is increased in microglial cells
69 upon various neuroinflammatory stimuli as, for instance, after ischemic injury [20,21].
70 LGALS3 binds the toll-like receptor 4 (TLR4) and promotes activation of microglia, a
71 function that might promote the inflammatory response and neuronal death after ischemia
72 [18]. The expression of LGALS3 also increases in reactive astrocytes in the injured cerebral
73 cortex [19]. The role of LGALS3 in normal brain development and function has little been

74 explored. LGALS3 is expressed in the subventricular zone, precisely in astrocytes around
75 neuroblasts, and loss of LGALS3 reduces neuronal migration from the subventricular zone to
76 the olfactory bulb *in vivo* [22]. LGALS3 plays also a role in oligodendrocyte differentiation
77 and contributes to myelin function [23]. It has been shown that LGALS3 is not expressed in
78 neurons in the olfactory bulb [22] or that it is only expressed in neurons under conditions of
79 acute brain inflammation in the hippocampus [18] or in the injured cerebral cortex [19].
80 However, recently, LGALS3 has been reported to be expressed in hippocampal neurons and
81 to play a role in memory formation through its interaction with integrin $\alpha 3$ [24]. LGALS3 can
82 interact with the cell adhesion molecule integrin $\alpha 3\beta 1$ [25], and integrin $\beta 1$ induces the
83 expression of LGALS3 *in vitro* [26]. While integrins have been involved in synaptogenesis
84 and synapse modulation, the role of LGALS3 in these processes has not been explored so far.
85 In this study, we investigated the expression and the role of *Lgals3* during development of the
86 olivocerebellar network in the mouse. The connectivity and physiology of this neuronal
87 network, and its development, have been well described, making it an ideal model to study
88 the molecular mechanisms regulating the development of neuronal networks. Our data show
89 that while LGALS3 is expressed during postnatal development in the olivocerebellar network,
90 it is mainly expressed by glial cells, and it is dispensable for neuronal development and
91 synaptogenesis in this structure.

92

93 **Material and methods**

94 **Ethics Statement**

95 All animal protocols were approved by the *Comité Régional d’Ethique en Experimentation*
96 *Animale* (# 00057.01).

97

98 **Animals**

99 *Lgals3*^{+/+} wild-type and *Lgals3*^{-/-} knockout littermates were obtained by breeding
100 heterozygotes (129Sv background) and were kindly provided by Dr. Françoise Poirier [27].
101 *CX₃CR1*^{eGFP/eGFP} mice were kindly provided by Dr. Etienne Audinat and Prof. S. Jung [28].
102

103 **Antibodies**

104 The following primary antibodies were used: mouse monoclonal anti-CABP (1:1000; swant,
105 Cat#300), rabbit polyclonal anti-CABP (1:1000; swant, Cat#9.03), mouse monoclonal anti-
106 GFAP (1:500; millipore, Cat#MAB360), rabbit polyclonal anti-GLUR δ 1/2 (1:1000;
107 millipore, Cat#AB2285), goat polyclonal anti-LGALS3 (1:200; R&D Systems, Cat#AF1197),
108 mouse monoclonal anti-OLIG2 (1:500; millipore, Cat#MABN50), guinea pig polyclonal anti-
109 VGLUT1 (1:5000; millipore, Cat#AB5905) and guinea pig polyclonal anti-VGLUT2
110 (1:5000; millipore, Cat#AB2251). The following secondary antibodies were used: donkey
111 polyclonal anti-goat Alexa Fluor 568 (1:1000; invitrogen, Cat#A11057), donkey polyclonal
112 anti-mouse Alexa Fluor 488 (1:1000; invitrogen, Cat#R37114), donkey polyclonal anti-rabbit
113 Alexa Fluor 488 (1:1000; invitrogen, Cat#A21206) and goat polyclonal anti-guinea pig Alexa
114 Fluor 594 (1:1000; Invitrogen, Cat#A11076).

115

116 **RTqPCR**

117 RNA samples were obtained from cerebellar and brainstem tissues using the RNeasy Mini kit
118 (QIAGEN, Hilden, Germany) and cDNA amplified using the SuperScript® VILO™ cDNA
119 Synthesis kit (life technologies, Paisley, UK) according to manufacturer's instructions.
120 Quantitative PCR was performed using the TaqMan Universal Master Mix II with UNG

121 (Applied Biosystems, Courboeuf, France) and the following TaqMan probes: *Rpl13a*
122 (#4331182_Mm01612986_gH) and *Lgals3* (#4331182_Mm00802901_m1).

123

124 **Immunohistochemistry**

125 Immunostaining was performed on 30µm-thick parasagittal sections obtained using a freezing
126 microtome from brains of mice perfused with 4% paraformaldehyde in phosphate buffered
127 saline (PBS) solution. Sections were washed three times for five minutes in PBS, then
128 blocked with PBS 4% donkey serum (DS; abcam, Cat#ab7475) for 30 minutes. The primary
129 antibodies were diluted in PBS, 1% DS, 1% Triton X-100 (Tx; sigma-aldrich, Cat#x100). The
130 sections were incubated in the primary antibody solution overnight at 4°C and then washed
131 three times for five minutes in PBS 1%Tx. Sections were incubated in the secondary
132 antibody, diluted in PBS 1%DS 1%Tx solution, for 1h at room temperature. The sections
133 were then incubated for 15 minutes with the nuclear marker Hoechst 33342 (sigma-aldrich,
134 Cat#H6024), followed by three washes for five minutes in PBS 1%Tx and recovered in PBS.
135 The sections were mounted with Prolong Gold (invitrogen; Cat#P36960).

136

137 **Image acquisition and analysis**

138 Imaging was performed using confocal microscopy (SP5, leica). The pinhole aperture was set
139 to 1 Airy Unit and a z-step of 500 nm was used. The software ImageJ was used to measure
140 the area of the cerebellum from images of staining obtained with the nuclear marker Hoechst,
141 and the area and the length of the molecular layer using images of the anti-CABP staining.

142

143 **Statistical Analysis**

144 Data generated with ImageJ were imported in GraphPad Prism for statistical analysis. Values
145 are given as mean \pm s.e.m. Normality was assessed using the Kolmogorov-Smirnov normality
146 test. Differences between two groups were tested using the two-tailed Student's t-test. Groups
147 were considered significantly different when at least a 95% confidence level ($P < 0.05$) was
148 obtained.

149

150 **Results**

151 **Lgals3 mRNA is expressed in the cerebellum and brainstem** 152 **during postnatal development.**

153 The expression level of *Lgals3* mRNA in the cerebellum was measured using quantitative
154 RT-PCR at different time points during late embryonic and postnatal development. The
155 expression of *Lgals3* mRNA is developmentally regulated in the cerebellum (Fig 1A). It
156 peaks at P14 when it quadruples compared to the levels detected at E17 and P0 and returns to
157 the levels detected during the first postnatal week by P21, a stage when neurogenesis,
158 differentiation and synaptogenesis in the cerebellum is to a large extent complete [29]. This
159 developmentally regulated expression profile is similar to the mRNA expression profile of the
160 adhesion G protein coupled receptor BAI3, that was recently shown to regulate Purkinje cell
161 synaptogenesis [5,7]. This pattern thus suggested a role for LGALS3 during cerebellar
162 Purkinje cell development and excitatory synaptogenesis.

163

164 **Fig 1. The cerebellar architecture is not affected in *Lgals3* null mice.** (A) *Lgals3* mRNA
165 expression relative to Rpl13a measured by RTqPCR in the cerebellum during development.
166 E17: embryonic day 17. P0, P7, P14, and P21: postnatal day 0, 7, 14, and 21. (B) *Lgals3*
167 knockout (KO) mice present no defects in cerebellar morphology. Parasagittal cerebellar

168 slices of wild-type (WT) and *Lgals3* KO adult mice were stained using the nuclear marker
169 Hoechst.

170

171 **Cerebellar architecture and excitatory synaptogenesis are not**
172 **affected by *Lgals3* invalidation.**

173 To study the role of LGALS3 in the development of the cerebellum, we analyzed mice
174 invalidated for the *Lgals3* gene [27]. The general architecture of the cerebellum, the different
175 layers and folia, was normal in those mice (Fig 1B). Quantitative analysis showed a slight
176 decrease (less than 10%) in the mean area of the cerebellum and in the mean area and length
177 of the molecular layer in adult *Lgals3* knockout (KO) mice when compared to *Lgals3* wild-
178 type (WT) mice (mean cerebellar area: $10.39 \times 10^6 \pm 1.72 \times 10^5 \mu\text{m}^2$ versus $9.62 \times 10^6 \pm 2.34 \times 10^5$
179 μm^2 , $P=0.0235$; mean molecular layer area: $4.9 \times 10^6 \pm 0.81 \times 10^5 \mu\text{m}^2$ versus $4.42 \times 10^6 \pm 0.72 \times 10^5$
180 μm^2 , $P=0.0015$; mean molecular layer length: $115.95 \pm 1.65 \mu\text{m}$ versus $105.5 \pm 1.53 \mu\text{m}$,
181 $P=0.000082$; for WT and KO six months-old mice, respectively; Student's t-test; $n=3$ animals
182 per genotype). These small differences were associated with a decrease in body weight in
183 adult KO (mean body weight \pm s.e.m.: 26.033 ± 0.07 g for WT, 24.003 ± 0.66 g for KO; $n=5$
184 animals per genotype, $P=0.027$, Student's t-test). These results show that lack of LGALS3
185 does not have a major effect on neurogenesis and neuronal morphogenesis in the cerebellum.
186 *Lgals3* expression peaks at postnatal day 14 (Fig 1A), a period of intense synaptogenesis in
187 the cerebellar cortex, and LGALS3 potentially interacts with integrin $\beta 1$ that is present at the
188 Climbing fiber (CF)-Purkinje cell synapse [25,30]. To analyze the possible role of LGALS3
189 in excitatory synapse formation we used immunolabeling with the pre-synaptic marker
190 VGLUT2, to label the CF presynaptic boutons, and with the pre-synaptic marker VGLUT1,
191 specific for the parallel fiber (PF) presynaptic boutons in the cerebellar cortex. At P15,
192 VGLUT2 clusters are found on the somato-dendritic region of Purkinje cells, in particular on

193 proximal dendrites both in *Lgals3* KO and *Lgals3* WT mice (Fig 2A). Smaller VGLUT2
194 clusters corresponding to maturing PF-Purkinje cell synapses [31] are found in distal
195 dendrites, in particular in the upper part of the molecular layer, in both genotypes. In adult
196 mice, when CF and PF synapses are mature, VGLUT2 clusters are only found on the Purkinje
197 cells proximal dendrites and extend to about 4/5th of the molecular layer height both in *Lgals3*
198 KO and WT cerebella (Fig 2B). The pattern of VGLUT1 immunostaining is similar in *Lgals3*
199 KO mice and control littermates, with a sparse staining of distal dendrites at P15 (Fig 2C) and
200 a dense staining non-overlapping with the VGLUT2 clusters at P65 (Fig 2D). These
201 qualitative data show that both PF and CF excitatory synaptogenesis in cerebellar Purkinje
202 cells is not affected by *Lgals3* invalidation.

203

204 **Fig 2. No changes in excitatory Purkinje cell synapses in *Lgals3* knockout mice.** (A and
205 B) Immunostaining for the Purkinje cell marker CABP (green) and the presynaptic marker
206 VGLUT2 (red) was performed on sections from *Lgals3* WT and KO mice at P15 (A) and at
207 P65 (B). (C and D) Immunostaining for the Purkinje cell marker CABP (green) and the
208 presynaptic marker VGLUT1 (red) specific for mature parallel fiber synapses was performed
209 on sections from *Lgals3* WT and KO mice at P15 (C) and at P65 (D). Scale bar: 25 μ m. Data
210 are representative of three independent experiments.

211

212 **LGALS3 is expressed in glia in the cerebellum**

213 The lack of effect on cerebellar morphology and synaptogenesis of *Lgals3* invalidation raised
214 the question as to what type of cells express the LGALS3 protein during postnatal
215 development. Using a LGALS3 antibody, we localized LGALS3 expression in the cerebellum
216 and brainstem during postnatal development. The specificity of the antibody was confirmed
217 by the lack of immunoreactivity in cerebellar sections from *Lgals3* KO mice (n=3

218 independent experiments; Fig 3A). At P15, the highest expression of LGALS3 was found in
219 the choroid plexus located above the cerebellum, and in the ependymal cells and meningeal
220 cells lining the cerebral aqueduct and the 4th ventricle. In the cerebellar cortex, the white
221 matter is strongly stained, while rare scattered cells in the grey matter also display some
222 LGALS3 expression. Despite a more than two-fold decrease in *Lgals3* mRNA levels in the
223 cerebellum between P14 and P21 (Fig 1A), the pattern of LGALS3 immunostaining was
224 similar at P15 and P22 (Fig 3B and C respectively). At P15, Purkinje cells display a staining
225 that is barely distinguishable from background, and that completely disappears at P21. In the
226 brainstem, LGALS3 immunostaining was mainly found in the white matter underneath the 4th
227 ventricle, between the pons (medial vestibular nucleus) and the medulla (pontine central grey)
228 at both P15 and P22 (Figs 3B-C).

229

230 **Fig 3. LGALS3 expression in the cerebellum and brainstem during postnatal**
231 **development.** (A) Immunohistochemistry for LGALS3 show the specificity of the LGALS3
232 antibody. WT: wild-type mice; KO: knockout mice. Positive cells in the molecular layer are
233 marked with a white arrowhead, scale bar 50µm. (B) Immunohistochemistry for the Purkinje
234 cell marker CABP (green) and LGALS3 (red) on parasagittal cerebellar sections from P15
235 and P22 wild-type mice. Scale bar=100µm. Data are representative of three independent
236 experiments. Legends: 4V, 4th ventricle; chp, choroid plexus; pcg, pontine central gray and
237 mv, medial vestibular nucleus.

238

239 The cerebellar white matter contains both oligodendrocytes and astrocytes. Previous studies
240 have detected LGALS3 expression in Schwann cells of the peripheral nervous system [32]
241 and in oligodendrocytes at different stages of maturation [23]. Co-immunostaining
242 experiments using an antibody against the oligodendrocyte specific marker OLIG-2 show

243 some LGALS3 expression in OLIG-2 positive oligodendrocytes (Fig 4A), while co-
244 immunostaining experiments with an anti-GFAP antibody to label astrocytes showed
245 extensive colocalization of LGALS3 and GFAP in cells of the cerebellar white matter (Fig
246 4B). In the grey matter of the cerebellum, the morphology of scattered LGALS3 positive cells
247 was reminiscent of microglial cells. Immunolabeling of cerebellar sections from a
248 $CX_3CR1^{eGFP/eGFP}$ mouse line that expresses soluble GFP in microglial cells [28], revealed
249 double labeled cells and confirmed the microglial identity of LGALS3 positive cells in the
250 molecular layer (Fig 4C). Overall our study shows that LGALS3 is expressed in non-neuronal
251 cells in the cerebellum and brainstem during postnatal development, in particular
252 subpopulations of astrocytes and microglial cells.

253

254 **Fig 4. LGALS3 protein expression in meningeal cells and in glial cells of the cerebellar**
255 **cortex of the adult mice.** (A) LGALS3 expression in oligodendrocytes in the cerebellar white
256 matter. Co-immunostaining of parasagittal cerebellar slices using anti-LGALS3 (red),
257 oligodendrocyte marker anti-OLIG-2 (green) and the nuclear marker Hoechst (blue). (B)
258 LGALS3 expression in astrocytes in the cerebellar white matter. Co-immunostaining of
259 parasagittal cerebellar slices using anti-LGALS3 (red), astrocyte marker anti-GFAP (green)
260 and the nuclear marker Hoechst (blue). (C) LGALS3 expression in some microglial cells of
261 the molecular layer. Immunostaining anti-LGALS3 (red) in parasagittal sections of mice
262 $CX_3CR1^{eGFP/eGFP}$, microglial CX_3CR1 -GFP (green) and the nuclear marker Hoechst (blue).
263 Scale bar 50 μ m and 20 μ m for the magnification. Data are representative of three independent
264 experiments. Legends: 4V, 4th ventricle; GL, granular layer; ML, molecular layer and WM,
265 white matter.

266

267 Discussion

268 The role of LGALS3 in the development of the CNS remains poorly studied. We show that
269 during postnatal development, LGALS3 is expressed in the choroid plexus in the fourth
270 ventricle, as well as in a subset of glial cells in the cerebellar cortex. This expression is
271 dispensable for the development of the normal cytoarchitecture of the cerebellum and
272 excitatory synaptogenesis in Purkinje cells.

273 LGALS3 expression has been described previously in pathological conditions in the
274 mammalian brain, in particular after ischemia [20,21]. LGALS3 is expressed by a subset of
275 proliferating astrocytes that contribute to reactive gliosis [19]. In the normal brain, high
276 expression of LGALS3 has been found in the subventricular zone, rostral migratory stream
277 and olfactory bulb [22] and in the subependymal zone of the lateral ventricles [19]. Our
278 results are in concordance, showing high expression of LGALS3 in the choroid plexus
279 beneath the cerebellum and in the subependymal zone of the fourth ventricle. In the brain
280 parenchyma, some studies have reported neuronal expression of LGALS3 in the hippocampus
281 and cerebellum [24,33,34], while others did not detect any neuronal expression [19,35]. In the
282 cerebellum in particular, low levels of mRNA expression were reported in Purkinje cells and
283 granule cells in an analysis of Allen Brain Atlas data by John and Mishra [33]. Our results
284 showed very low to undetectable expression of LGALS3 in cerebellar neurons, in agreement
285 with previous results [35,36]. However high levels of LGALS3 were found in subsets of
286 astrocytes and subsets of microglia in the cerebellar cortex. This is in accordance with
287 previous results showing expression of LGALS3 by microglia [18] and astrocytes *in vitro*
288 [23]. LGALS3 has been involved in myelination [23]. Our results show some expression of
289 LGALS3 in oligodendrocytes of the cerebellar white matter during postnatal development.

290 Together with its expression in astrocytes of the white matter, this suggests an implication of
291 LGALS3 in the crosstalk between astrocytes and oligodendrocytes during myelination
292 (reviewed in [37]).

293 Several reasons suggested a role for *Lgals3* during synaptogenesis. First, LGALS3 is a
294 secreted and immune-related protein with a developmentally regulated mRNA expression
295 profile that coincides with the timing of excitatory synaptogenesis in the cerebellum. These
296 characteristics are shared by C1QL1 and CBLN1, two major synaptogenic proteins of the CF-
297 Purkinje cell and PF-Purkinje cell synapses respectively [5–8][5–8]. Second, LGALS3
298 promotes neural cell adhesion and neurite outgrowth in cultured neurons [38]. Third it has
299 been shown that LGALS3 interacts functionally with integrins, adhesion proteins known to
300 play roles in synaptogenesis and synapse modulation. Integrin $\alpha3\beta1$ is necessary for the
301 organization of the cerebellar excitatory synapse CF-Purkinje cell [30], LGALS3 can interact
302 with integrin $\alpha3\beta1$ [25], and integrin $\beta1$ induces the expression of LGALS3 in culture [26].
303 Our analysis of the *Lgals3* knockout mouse did not find any major perturbation of Purkinje
304 cell excitatory synaptogenesis in the absence of LGALS3. Our data thus indicate that either
305 LGALS3 plays no role in synaptogenesis, or that other proteins play redundant functions.
306 Our results do not exclude a role for LGALS3 in regulating synapse maturation and function.
307 In particular, microglia have been involved in synapse elimination during development of the
308 visual system [3], and LGALS3 promotes phagocytosis of PC12 cells by microglia [39].
309 Activity-dependent synapse refinement of the CF-Purkinje cell synapse happens largely
310 during the first three postnatal weeks [29]. Given LGALS3 expression in microglial cells in
311 the cerebellar cortex during this period, it would be interesting to test whether LGALS3 is
312 involved in climbing fiber synapse elimination, in particular, through its interaction with
313 integrin $\alpha3\beta1$, a known regulator of CF synaptogenesis.

314

315 **Author contributions**

316 F.S. and I.G.C. designed the experiments and wrote the manuscript. I.G.C. performed the
317 experiments.

318

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327

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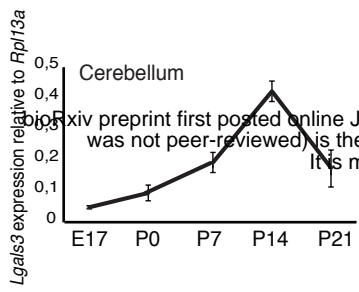
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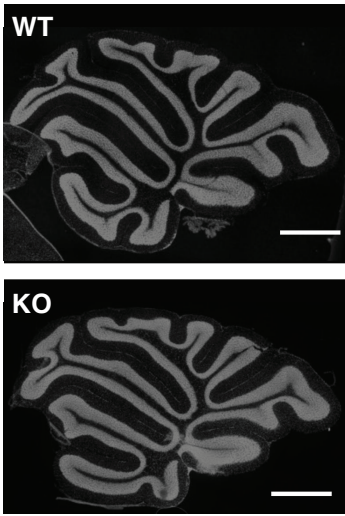
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B**Figure 1**

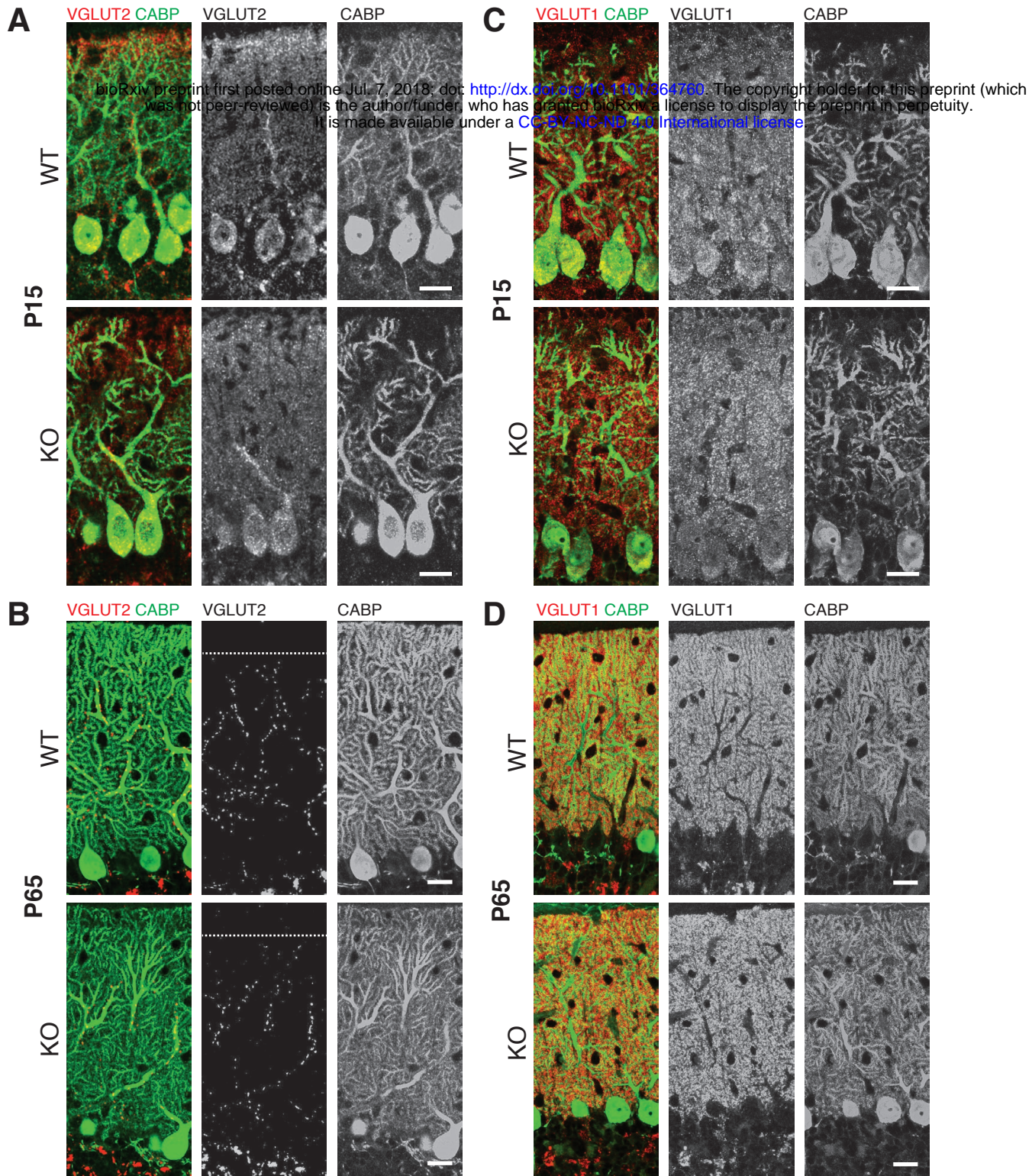


Figure 2

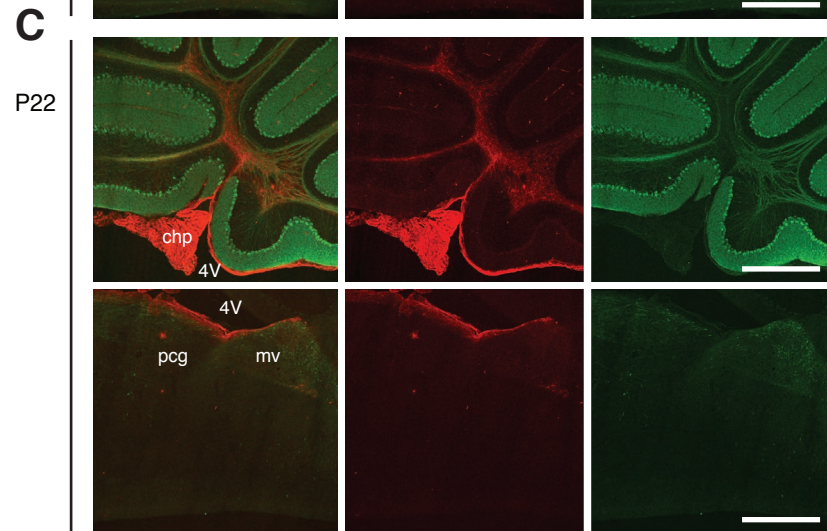
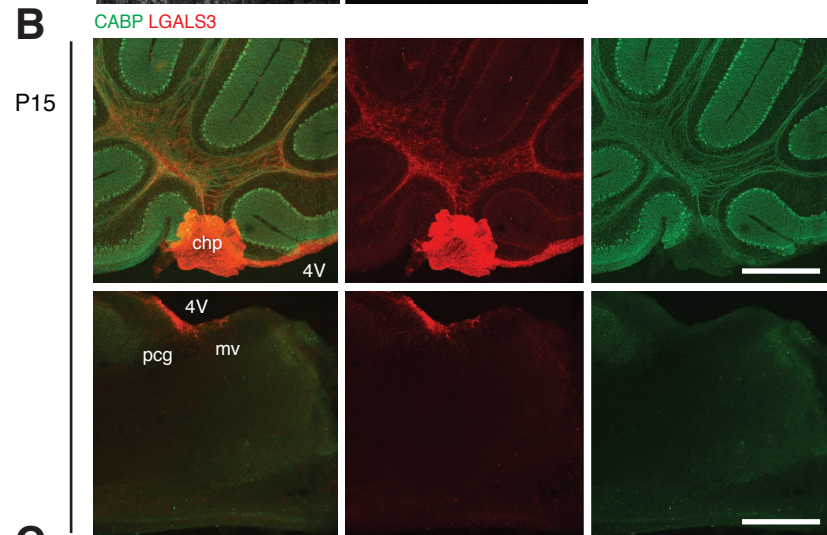
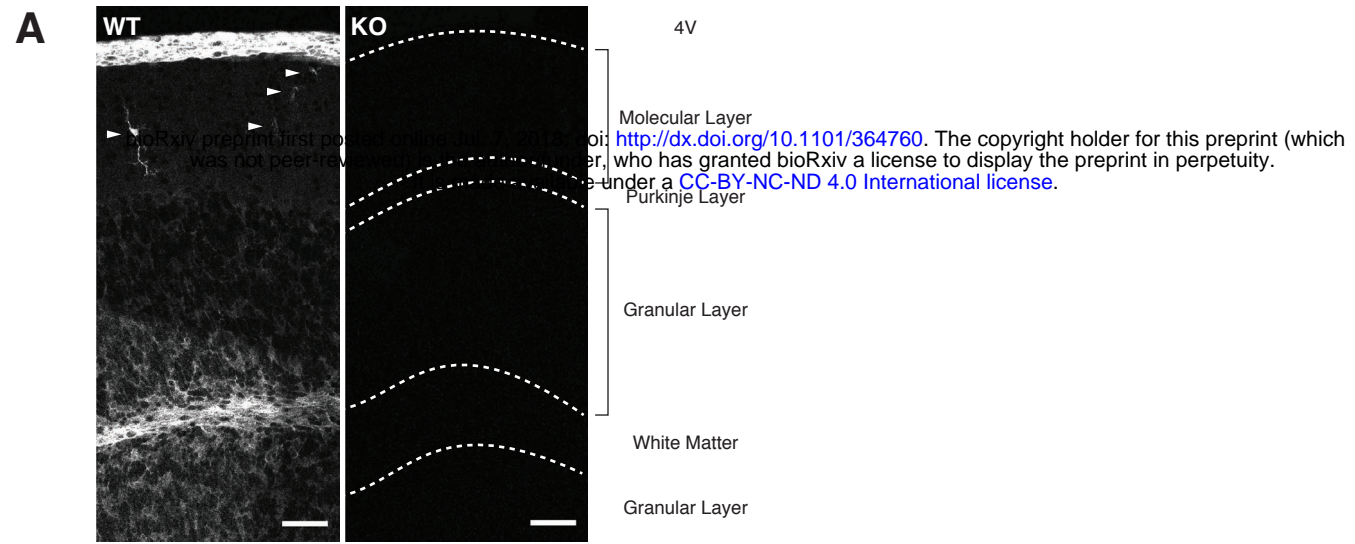


Figure 3

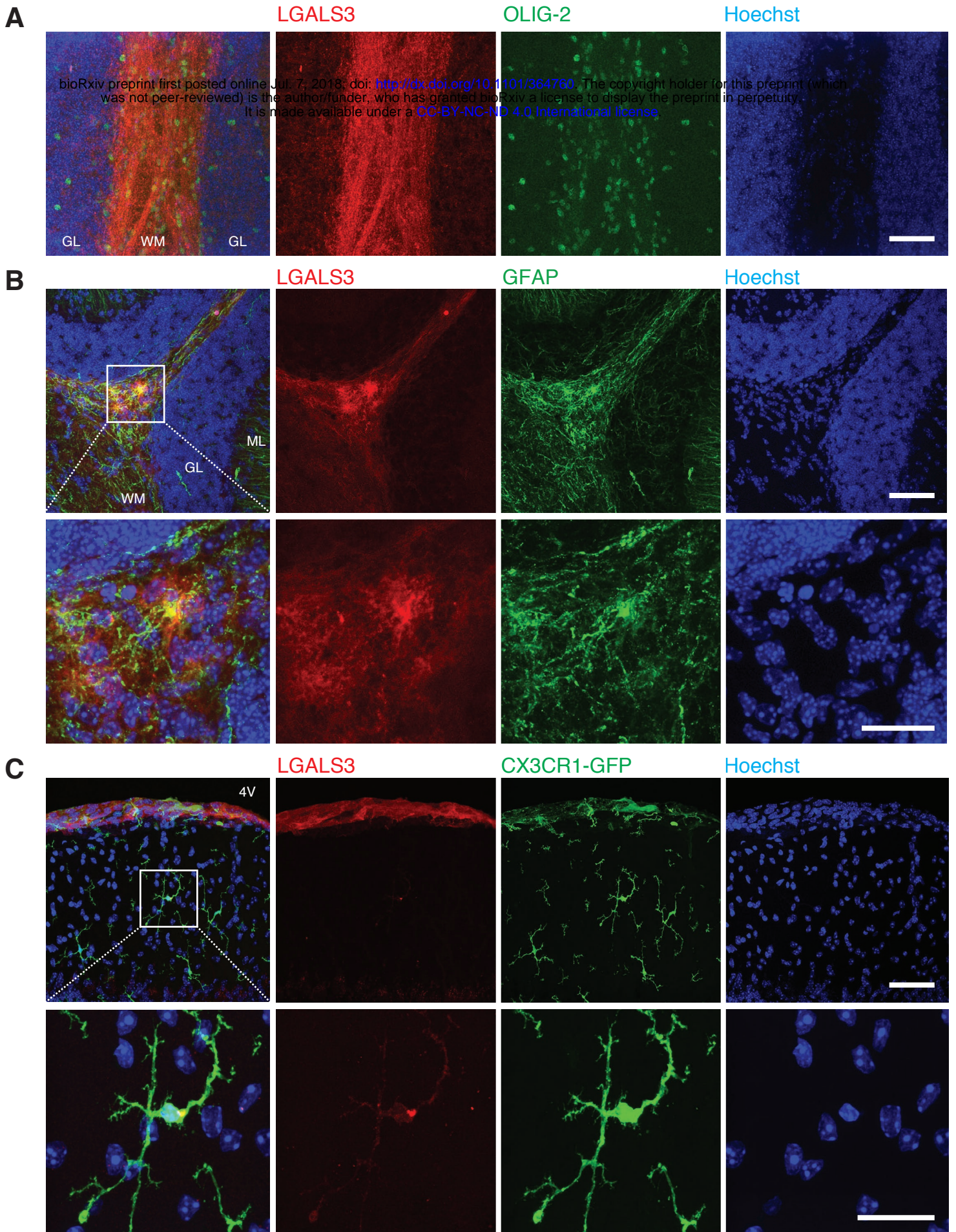


Figure 4