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

Inés González-Calvo¹ & Fekrije Selimi^{1*}

¹ Center for Interdisciplinary Research in Biology (CIRB), Collège de France, CNRS UMR 7241, INSERM U1050, Paris, France.

* Corresponding author
E-mail: fekrije.selimi@college-de-france.fr

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.

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].

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-GLURδ1/2 (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).

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

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

Immunohistochemistry

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

Image acquisition and analysis

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

Statistical Analysis

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

Results

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

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

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

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

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

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

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

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

LGALS3 is expressed in glia in the cerebellum

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

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

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

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

some LGALS3 expression in OLIG-2 positive oligodendrocytes (Fig 4A), while co-immunostaining experiments with an anti-GFAP antibody to label astrocytes showed extensive colocalization of LGALS3 and GFAP in cells of the cerebellar white matter (Fig 4B). In the grey matter of the cerebellum, the morphology of scattered LGALS3 positive cells was reminiscent of microglial cells. Immunolabeling of cerebellar sections from a CX₃CR1^{eGFP/eGFP} mouse line that expresses soluble GFP in microglial cells [28], revealed double labeled cells and confirmed the microglial identity of LGALS3 positive cells in the molecular layer (Fig 4C). Overall our study shows that LGALS3 is expressed in non-neuronal cells in the cerebellum and brainstem during postnatal development, in particular subpopulations of astrocytes and microglial cells.

Fig 4. LGALS3 protein expression in meningeal cells and in glial cells of the cerebellar cortex of the adult mice. (A) LGALS3 expression in oligodendrocytes in the cerebellar white matter. Co-immunostaining of parasagittal cerebellar slices using anti-LGALS3 (red), oligodendrocyte marker anti-OLIG-2 (green) and the nuclear marker Hoechst (blue). (B) LGALS3 expression in astrocytes in the cerebellar white matter. Co-immunostaining of parasagittal cerebellar slices using anti-LGALS3 (red), astrocyte marker anti-GFAP (green) and the nuclear marker Hoechst (blue). (C) LGALS3 expression in some microglial cells of the molecular layer. Immunostaining anti-LGALS3 (red) in parasagittal sections of mice CX₃CR1^{eGFP/eGFP}, microglial CX₃CR1-GFP (green) and the nuclear marker Hoechst (blue). Scale bar 50µm and 20µm for the magnification. Data are representative of three independent experiments. Legends: 4V, 4th ventricle; GL, granular layer; ML, molecular layer and WM, white matter.

Discussion

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

LGALS3 expression has been described previously in pathological conditions in the mammalian brain, in particular after ischemia [20,21]. LGALS3 is expressed by a subset of proliferating astrocytes that contribute to reactive gliosis [19]. In the normal brain, high expression of LGALS3 has been found in the subventricular zone, rostral migratory stream and olfactory bulb [22] and in the subependymal zone of the lateral ventricles [19]. Our results are in concordance, showing high expression of LGALS3 in the choroid plexus beneath the cerebellum and in the subependymal zone of the fourth ventricle. In the brain parenchyma, some studies have reported neuronal expression of LGALS3 in the hippocampus and cerebellum [24,33,34], while others did not detect any neuronal expression [19,35]. In the cerebellum in particular, low levels of mRNA expression were reported in Purkinje cells and granule cells in an analysis of Allen Brain Atlas data by John and Mishra [33]. Our results showed very low to undetectable expression of LGALS3 in cerebellar neurons, in agreement with previous results [35,36]. However high levels of LGALS3 were found in subsets of astrocytes and subsets of microglia in the cerebellar cortex. This is in accordance with previous results showing expression of LGALS3 by microglia [18] and astrocytes *in vitro* [23]. LGALS3 has been involved in myelination [23]. Our results show some expression of LGALS3 in oligodendrocytes of the cerebellar white matter during postnatal development. Together with its expression in astrocytes of the white matter, this suggests an implication of LGALS3 in the crosstalk between astrocytes and oligodendrocytes during myelination (reviewed in [37]).

Several reasons suggested a role for *Lgals3* during synaptogenesis. First, LGALS3 is a secreted and immune-related protein with a developmentally regulated mRNA expression profile that coincides with the timing of excitatory synaptogenesis in the cerebellum. These characteristics are shared by C1QL1 and CBLN1, two major synaptogenic proteins of the CF-Purkinje cell and PF-Purkinje cell synapses respectively [5–8][5–8]. Second, LGALS3 promotes neural cell adhesion and neurite outgrowth in cultured neurons [38]. Third it has been shown that LGALS3 interacts functionally with integrins, adhesion proteins known to play roles in synaptogenesis and synapse modulation. Integrin $\alpha 3\beta 1$ is necessary for the organization of the cerebellar excitatory synapse CF-Purkinje cell [30], LGALS3 can interact with integrin $\alpha 3\beta 1$ [25], and integrin $\beta 1$ induces the expression of LGALS3 in culture [26]. Our analysis of the *Lgals3* knockout mouse did not find any major perturbation of Purkinje cell excitatory synaptogenesis in the absence of LGALS3. Our data thus indicate that either LGALS3 plays no role in synaptogenesis, or that other proteins play redundant functions. Our results do not exclude a role for LGALS3 in regulating synapse maturation and function. In particular, microglia have been involved in synapse elimination during development of the visual system [3], and LGALS3 promotes phagocytosis of PC12 cells by microglia [39]. Activity-dependent synapse refinement of the CF-Purkinje cell synapse happens largely during the first three postnatal weeks [29]. Given LGALS3 expression in microglial cells in the cerebellar cortex during this period, it would be interesting to test whether LGALS3 is involved in climbing fiber synapse elimination, in particular, through its interaction with integrin $\alpha 3\beta 1$, a known regulator of CF synaptogenesis.

Author contributions

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

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319 **Acknowledgments**

320 We thank Dr. F. Poirier for *Lgals3*^{+/+} and *Lgals3*^{-/-} mice, Prof. S. Jung and Dr. E. Audinat for
321 CX₃CR1^{eGFP/eGFP} mice, and Maëva Talleur for the RTqPCR. We also thank the CIRB Imaging
322 Facility.

323 This work has received support under the Investissement d'Avenir program launched by the
324 French government and implemented with the ANR-11-IDEX-0001-02 PSL* Research
325 University (to I.G.C. and F.S.). Funding was also provided by the ATIP AVENIR program (to
326 F.S.) and Fondation pour la Recherche Medicale (DEQ20150331748).

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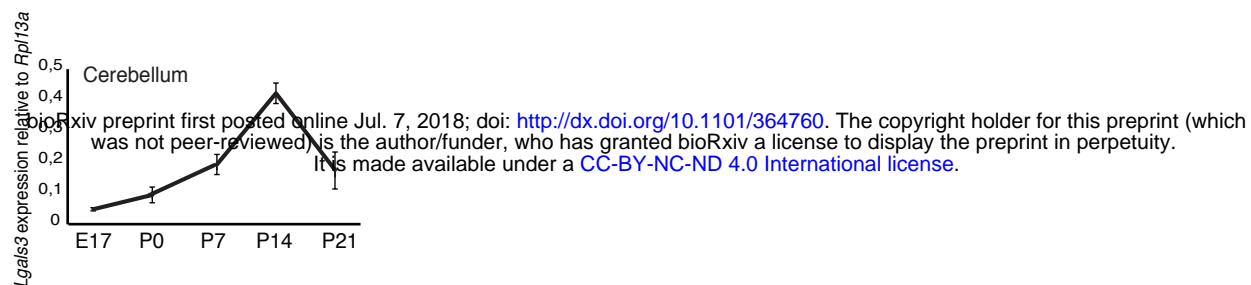
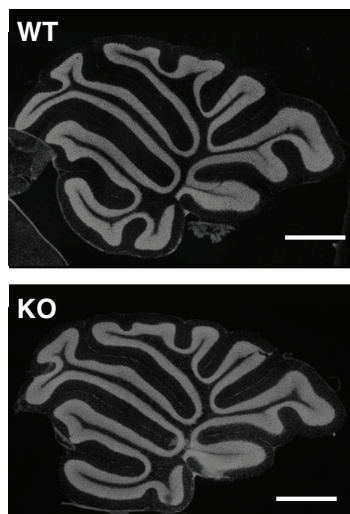
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Figure 1

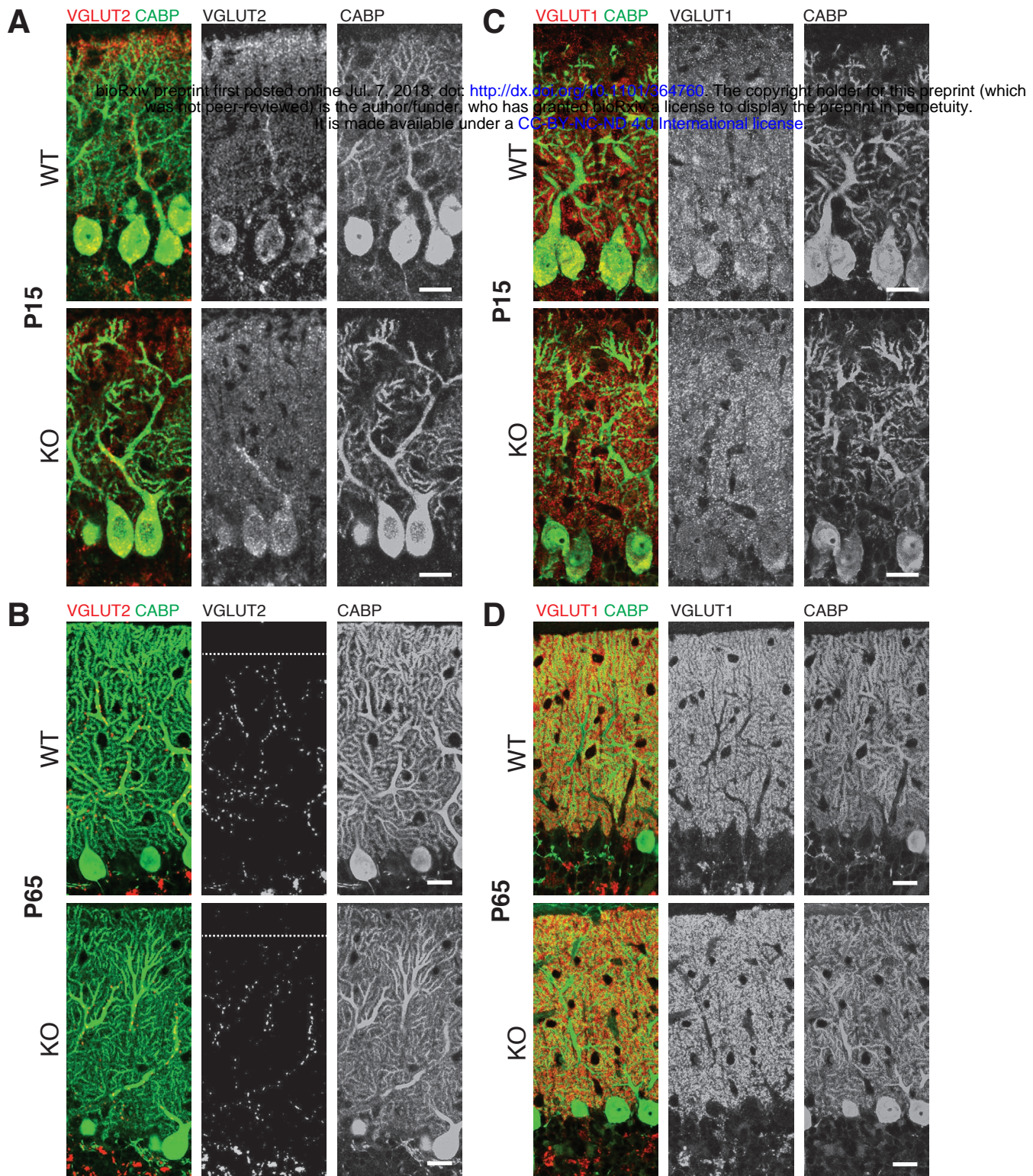


Figure 2

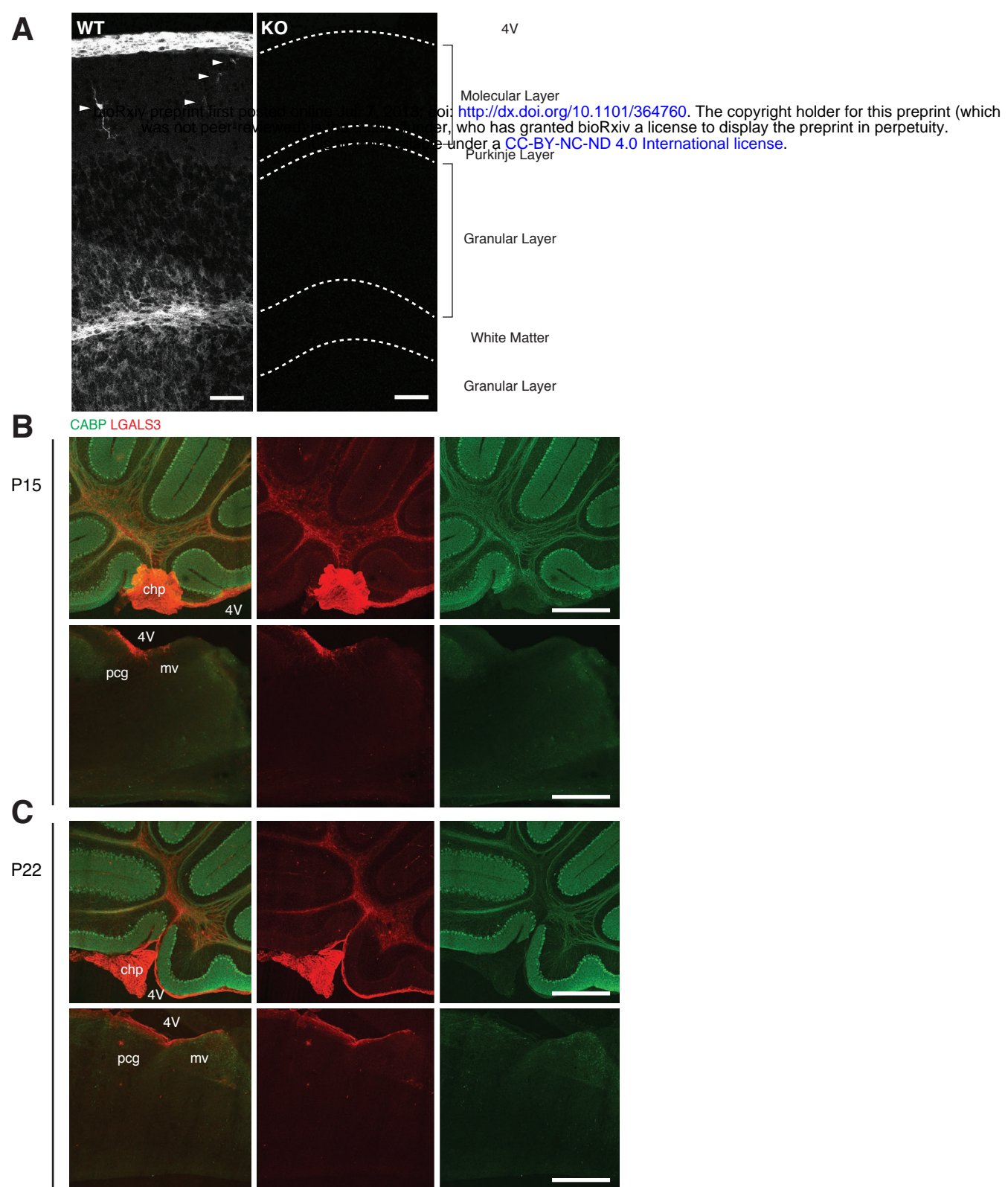


Figure 3

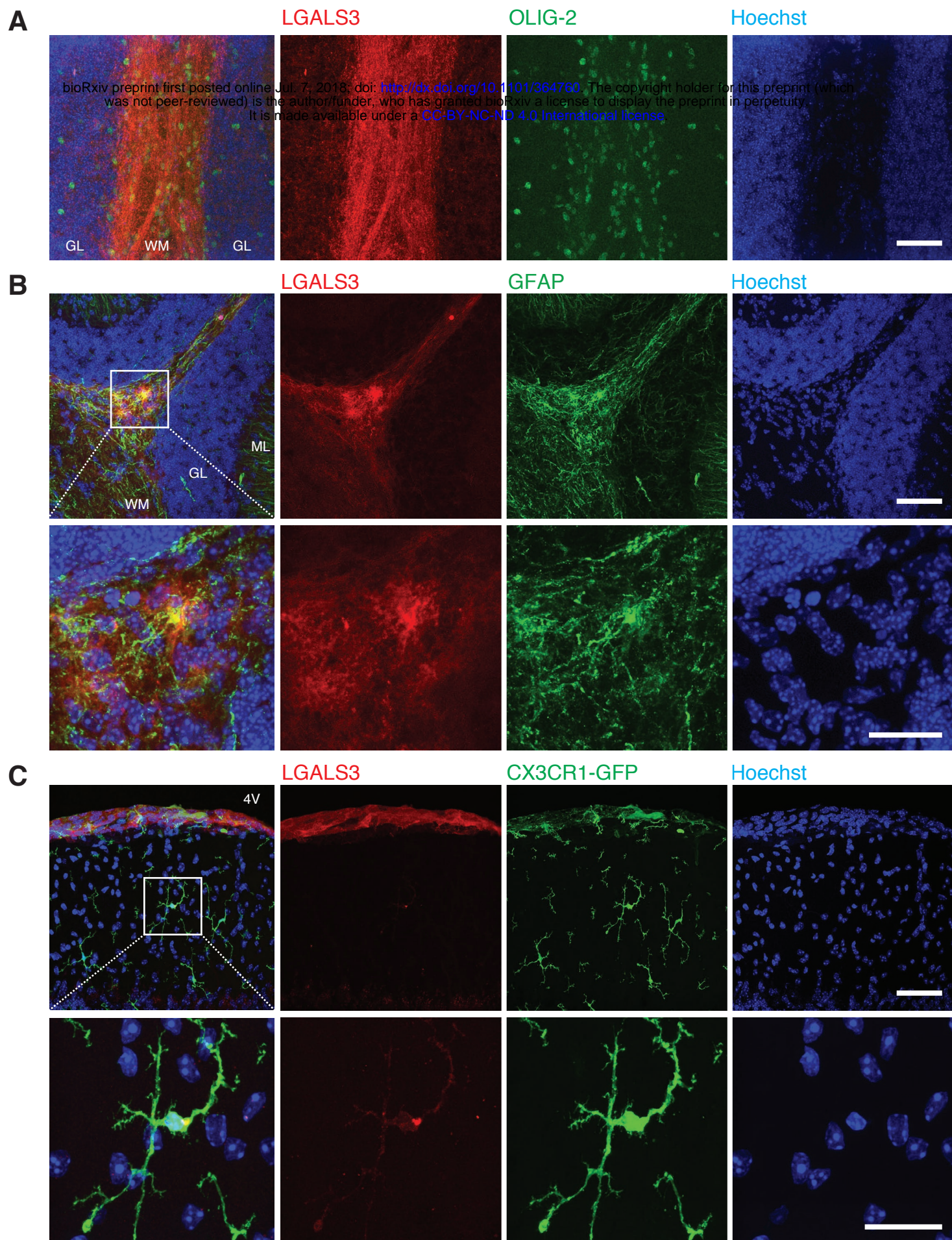


Figure 4