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Mature oligodendrocytes bordering lesions limit demyelination and favor myelin repair via heparan sulphate production

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Impact statement: Heparan sulfate synthesis by mature oligodendrocytes creates a protective and permissive environment controlling microglia and oligodendrocyte progenitors reactivation during remyelination.**Competing interests:** No competing interests declared**Author contributions:**

Magali Macchi: Conceptualization; Formal analysis; Investigation; Methodology; Writing - original draft Karine Magalon: Conceptualization; Formal analysis; Investigation; Methodology; Writing - review and editing Céline Zimmer: Conceptualization; Formal analysis; Writing - original draft Elista Peeva: Formal analysis Bilal El Waly: Formal analysis Béatrice Brousse: Formal analysis Sarah Jaekel: Formal analysis Kay Grobe: Formal analysis; Validation; Writing - original draft Friedemann Kiefer: Resources Anna Williams: Formal analysis; Supervision; Investigation; Methodology; Writing - original draft; Writing - review and editing Myriam Cayre: Conceptualization; Formal analysis; Supervision; Validation; Investigation; Methodology; Writing - original draft; Writing - review and editing Pascale Durbec: Conceptualization; Supervision; Funding acquisition; Validation; Methodology; Project administration

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1 **Mature oligodendrocytes bordering lesions limit demyelination and favor**
2 **myelin repair via heparan sulphate production**

3

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26

27 **Abstract**

28 Myelin destruction is followed by resident glia activation and mobilization of endogenous
29 progenitors (OPC) which participate in myelin repair. Here we show that in response to
30 demyelination, mature oligodendrocytes (OLG) bordering the lesion express Ndst1, a key
31 enzyme for heparan sulfates (HS) synthesis. Ndst1+ OLG form a belt that demarcates
32 lesioned from intact white matter. Mice with selective inactivation of Ndst1 in the OLG
33 lineage display increased lesion size, sustained microglia and OPC reactivity. HS production
34 around the lesion allows Sonic hedgehog (Shh) binding and favors the local enrichment of
35 this morphogen involved in myelin regeneration. In MS patients, Ndst1 is also found
36 overexpressed in oligodendroglia and the number of Ndst1-expressing oligodendroglia is
37 inversely correlated with lesion size and positively correlated with remyelination potential.
38 Our study suggests that mature OLG surrounding demyelinated lesions are not passive
39 witnesses but contribute to protection and regeneration by producing HS.

40

41 INTRODUCTION

42 In multiple sclerosis (MS), OLG are the target of inflammatory and immune attacks
43 and their death results in multiple focal demyelinated lesions in the CNS. Myelin loss remains
44 in defined areas, rather than expanding to involve all of the white matter, and the mechanism
45 by which demyelinated lesions stop expanding is not understood. Some of these lesions may
46 stop expanding as inflammation is controlled and lesions are remyelinated. Remyelination
47 involves OPC recruitment to the lesion, differentiation into myelin forming cells and
48 remyelination of denuded axons, and the success of this depends on environmental context,
49 including secreted factors from neighboring cells [1, 2]. This repair process, which occurs
50 spontaneously in MS patients, is highly variable between patients and between lesions [2].
51 Regenerative failure is mainly attributed to defects in OPC recruitment towards the
52 demyelinated areas [3] and/or to their incapacity to differentiate into myelinating OLG at the
53 lesion site [1, 4-6].

54 Multiple factors are involved in this regenerative process including those produced by
55 reactive astrocytes or microglia and macrophages [7]. These contribute to myelin destruction,
56 but also to myelin debris removal and beneficial effects by secreting factors that directly or
57 indirectly support remyelination [8-13]. Interestingly oligodendrocyte lineage cells also
58 produce factors that modulate remyelination, such as the morphogen shh which is produced
59 by OLG and OPC at the onset of demyelination in lysophosphatidyl choline (LPC)-induced
60 lesions [14]. In this context, blocking Shh activity induces an increase in lesion size and a
61 block in OPC proliferation and differentiation, and conversely Shh overexpression leads to the
62 attenuation of the lesion extent and promotes oligodendrogenesis [14]. Identifying such actors
63 involved in myelin damage and remyelination is needed for the design of future protective
64 and regenerative therapies.

65 The presence of a multitude of signals regulating specific steps of remyelination raises
66 the hypothesis that key factors may be necessary to integrate all these cues. One of these key
67 factors may be HS proteoglycans (HSPG), as there is now compelling evidence that HSPGs
68 play a critical role in regulating spatiotemporal coordination of signals in the extracellular
69 microenvironment of many tissues during brain development and in adulthood [15]. HS
70 chains consist of linear repeated disaccharide units of N-acetyl glucosamine and glucuronic
71 acid which are synthesized on proteoglycan core proteins. *Ndst* enzymes perform the first step
72 of these sugar modifications thus specifying the functional properties of HSPGs [16-18].
73 Among the four known *Ndst* enzymes, *Ndst1* appears as the key enzyme for addition of N-
74 sulfated motifs to HS chains in brain during development, as shown by limited functional
75 redundancy mediated by other *Ndst* enzymes (2-4) in *Ndst1* KO mice [19, 20]. During
76 development, HS proteoglycans provide an important signaling scaffold allowing spatial
77 concentration or trapping of numerous molecules such as morphogens and growth factors [21]
78 and the control of receptor activity [21-24]. Following CNS injury, HSPGs are known to play
79 a pivotal role in post-lesional plasticity and regeneration [25] [26]. Some HS proteoglycans
80 are over-expressed by reactive astrocytes in injured mouse brain and provide positive [25] or
81 negative [26] environmental support for axon regenerative responses. In vitro, HS
82 proteoglycans can prevent OLG differentiation, maintaining OPC in an immature proliferative
83 phenotype by acting as a FGF-2 co-receptor [27, 28]. Therefore, we hypothesized that HS
84 proteoglycans play an organizing role in controlling myelin damage and repair.

85 Here we show that mature OLG bordering a demyelinated lesion limit lesion extension
86 and influence OPC mobilization via HS production. Using a model of acute focal
87 demyelination of the corpus callosum in mice, we show that *Ndst1* expression is induced in
88 OLG around the lesion throughout the phases of demyelination and remyelination. *Ndst1*
89 expression and subsequent HS accumulation mostly accumulate at the margin of the lesion,

90 delimiting the lesion from the intact corpus callosum during demyelination. To evaluate the
91 relevance of *Ndst1* induction for lesion formation and repair, we exposed genetically modified
92 mice with selective deletion of *Ndst1* in oligodendroglia to focal demyelination of the corpus
93 callosum. Lack of *Ndst1* in OLG resulted in an increased lesion size, and a sustained OPC and
94 microglia/macrophage activation at the early stage of remyelination. HS enrichment correlates
95 with and is necessary for the binding around the lesion site of the morphogen Shh, suggesting
96 that *Ndst1* expression and HS secretion by OLG enhances Shh signaling after demyelination,
97 thus favoring remyelination [14, 29]. Furthermore, NDST1 expression in OLG was also
98 increased in human postmortem tissues from multiple sclerosis patients. This increased
99 density of NDST1+ OLG in lesions was inversely correlated with the size of the lesion and
100 positively correlated with remyelination.

101

102 **RESULTS**

103 **Demyelination triggers *Ndst1* up-regulation by OLG and creates a transient N-** 104 **sulfated belt around the lesion**

105 To identify candidates that could regulate interactions between progenitors and the injured
106 environment, a microarray analysis was performed to compare gene expression in purified
107 oligodendroglia from adult healthy and demyelinated animals [30]. One of the most robustly
108 and significantly up-regulated genes after demyelination was *Ndst1*, a key enzyme of HS
109 proteoglycan synthesis (fold increase of 48.9 and 14.0 in two different trials; $p \leq 0.001$;
110 microarray data are available at GEO with accession number GSE47486). This up-regulation
111 of *Ndst1* was confirmed *in vivo* at 21 days in mice exposed to EAE by in situ hybridization
112 combined with Olig2 labeling, a pan OLG marker. While *Ndst1* was not detected in the
113 corpus callosum of control brains (Figure 1-figure supplement 1A), it was highly expressed

114 by the Olig2⁺ population after EAE in the corpus callosum (Figure 1-figure supplement 1B-
115 C) in close proximity to lesion sites (Figure 1-figure supplement 1C).

116 To characterize the up-regulation of *Ndst1* after demyelination, we used LPC to trigger focal
117 demyelination lesions in the mouse corpus callosum (Figure 1A). In this model,
118 demyelination is not T cell driven, and demyelination and remyelination proceed in a
119 stereotypic sequence: demyelination occurs within few days, endogenous progenitor
120 mobilization peaks at 8 dpi and is followed by OPC differentiation [8]. Production of new
121 myelin is then observed after 2 weeks. Demyelination of the corpus callosum is clearly visible
122 after LPC injection in a reporter mouse where myelin fluoresces green (*plp-GFP*) [31, 32] by
123 the total loss of GFP signal around the injection site (Figure 1-figure supplement 2). The
124 lesion is also characterized by a strong increase in cell density (due to glia proliferation and to
125 microglia/macrophage infiltration) observed by Hoechst staining (Figure 1-figure supplement
126 2) that strictly coincides with loss of GFP fluorescence.

127 We first evaluated *Ndst1* expression levels by performing RT-qPCR analysis using the corpus
128 callosum of healthy or demyelinated mice on the ipsi- and contralateral sides to the LPC
129 injection, 8 days post injection (dpi). We quantified a mean 41% increase in the *Ndst1*
130 expression level in the demyelinated corpus callosum compared to healthy corpus callosum
131 (Figure 1B; p=0.05). *Ndst1* transcripts were found up-regulated in demyelinated corpus
132 callosum by in situ hybridization at 5 dpi during demyelination (Figure 1C-F), 8 dpi (Figure
133 1G), and 14 dpi (Figure 1H). At days 5 and 8 dpi, *Ndst1* expressing cells delimited a belt
134 around the lesion site. Weak staining was observed distal to the lesion, in the contralateral
135 side of the corpus callosum and at the core of the lesion (Figure 1C-D). Thus, the induction of
136 demyelination in the corpus callosum triggers *Ndst1* up-regulation, and this change is
137 sustained throughout the phases of demyelination and remyelination.

138 Since *Ndst1* catalyzes a mandatory step in the synthesis of HS chains, its expression is
139 likely to reflect the distribution of HS. We thus examined the outcome of *Ndst1* activity by
140 analyzing the distribution of N-sulfated motifs after demyelination. To do so, we used the
141 anti-HS antibody 10E4 [19, 33], which exclusively detects the N-sulfated motifs produced by
142 *Ndst1* activity [20]. While no staining was detected in the contralateral corpus callosum
143 (Figure 2A), N-sulfated HS positive puncta formed a belt around the demyelination lesion
144 (Figure 2B-C), similar to the profile of *Ndst1*-induction. Furthermore, as with *Ndst1*
145 expression, no HS staining was detected within the lesion (Figure 2B-C). This staining is
146 specific as it was absent after treatment with Heparinase, an enzyme that digests N-sulfated
147 motifs on the HS chains [34] (Figure 2D). This correlation between *Ndst1* up-regulation and
148 the presence of a highly N-sulfated microenvironment indicates the functional activity of
149 *Ndst1* after LPC-induced demyelination in the corpus callosum.

150 The phenotype of *Ndst1* expressing cells around the demyelinated lesion was
151 examined at 5 dpi, using co-staining for several markers combined with *Ndst1* in situ
152 hybridization. We found that *Ndst1* expressing cells are immunopositive for Olig2 (Figure
153 3A) and *Plp*⁺ using double in situ hybridization (Figure 3B). We quantified that $98.0\pm 1.5\%$
154 *Ndst1*⁺ cells express Olig2 indicating that virtually all *Ndst1* cells belong to the
155 oligodendroglia lineage. We found that *Ndst1* expressing cells are immunopositive for Olig2,
156 PDGFR α and CC1 (Figure 3A,C,D) and *Plp*⁺ using double in situ hybridization (Figure 3B)
157 At 5 dpi, $97.5\pm 1.7\%$ of *Ndst1* expressing cells were co-labelled with CC1, a mature OLG
158 marker (Figure 3D), while only $0.8\pm 0.3\%$ co-expressed the OPC marker PDGFR α (Figure
159 3C).

160 Of note, in the belt delimited by *Ndst1* staining surrounding the lesion, only half of
161 the Olig2⁺ cells expressed *Ndst1* ($45.8\pm 3.4\%$). This may indicate that not all stages of
162 oligodendroglial maturation or not all oligodendrocytes respond equally to the lesion. We

163 observed that, $8.5 \pm 3.2\%$ of PDGFR α + cells and $48.9 \pm 8.5\%$ of CC1+ cells surrounding the
164 lesion co-labelled with *Ndst1*. Our data indicate that the majority of *Ndst1*+ cells around the
165 lesion are mature OLG which are more prone than OPC to activate *Ndst1* expression in
166 response to demyelination

167

168 **Deletion of *Ndst1* in the Olig2+ population transiently worsens the extent of**
169 **demyelination and modifies OPC reactivity after LPC injection.**

170

171 To test if *Ndst1* activity in oligodendroglia controls demyelination and/or remyelination, we
172 generated transgenic mice with a conditional deletion of *Ndst1* in Olig2+ cells, by breeding
173 *Olig2-Cre*^{+/-} mice and *Ndst1*^{Flox/Flox} mice [19, 35]. The efficiency of inactivation of *Ndst1*
174 expression in Olig2 cells was monitored by in situ hybridization in the context of LPC-
175 induced demyelinating lesion, revealing a drastic decrease in *Ndst1* expression in lesioned
176 mutants compared to control (Figure 4-figure supplement 1A-D). As revealed by
177 immunostaining using the anti-HS antibody, a strong reduction in N-sulfated HS positive
178 puncta around the demyelination lesion was also observed in mutant compared to control
179 (Figure 4-figure supplement 1G-H). In healthy mice, quantitative analysis of myelin content
180 (Figure 4-figure supplement 2A-C; p=0.2), astrocyte density (Figure 4-figure supplement 2D-
181 F; p=0.4) and oligodendroglial lineage cell density (Figure 4-figure supplement 2G-L; p=0.6,
182 0.2 and 0.2 for Olig2, CC1 and PDGFR α cell density respectively) revealed no difference
183 between control (*Olig2-Cre*^{+/-}) and mutant (*Olig2-Cre*^{+/-}; *Ndst1*^{Flox/Flox}) adult mice, thus
184 indicating that conditional deletion of *Ndst1* in the Olig2+ cell population does not interfere
185 with brain development and with subsequent myelin maturation.

186 We first performed LPC-induced demyelination of the corpus callosum in control and
187 mutant mice and measured the size of the lesion at 4, 8 and 14 dpi. As before, lesions were

188 identified based on the high density of nuclei at the injection point (Figure 1-figure
189 supplement 2). While no difference was detected at 4 dpi (0.226 ± 0.036 vs. 0.159 ± 0.033 mm³
190 in control and mutant mice, respectively; $p=0.23$), a significant two-fold increase in lesion
191 size was observed in mutant compared to control mice at 8 dpi (0.199 ± 0.032 vs. 0.097 ± 0.022
192 mm³, $p=0.023$) (Figure 4A-C). During the remyelination phase (between 8 and 14 dpi), the
193 lesion area decreased in both groups reaching comparable sizes at 14 dpi (0.033 ± 0.02 vs.
194 0.028 ± 0.012 mm³ in control and mutant mice, respectively; $p=0.97$) (Figure 4C).

195 We examined how these changes in the local environment in these mutant mice affect
196 OPC mobilization during remyelination by analyzing Olig2+ cells density (Figure 4D-F),
197 maturation status (Figure 4G-I) and proliferation (Figure 4J-O). In accordance with
198 demyelination, there was a marked decrease in Olig2+ cell density within the demyelinated
199 area compared to healthy corpus callosum in both groups at 4 dpi ($55.5\pm 3.3\%$ and $57.3\pm 3.9\%$
200 decrease in control and mutant mice respectively, $p=0.03$ and $p=0.001$) (Figure 4F), reflecting
201 the loss of oligodendrocytes. We observe that in both conditions the density of Olig2+ cells
202 returned to uninjected control values at 14 dpi. Quantification of mean cell densities of mature
203 OLG (CC1+) (Figure 4G-I) within the lesion throughout the time course revealed no
204 significant difference between the two groups indicating that cell differentiation is not
205 affected by *Ndst1* inactivation.

206 We observed that the percentage of Ki67+ proliferating cells within the lesion area
207 was two-fold increased in *Ndst1* mutant mice compared to control mice at 8 dpi ($217.8\pm 42.8\%$
208 vs. $100\pm 13.5\%$ in mutant and control mice, respectively, $p=0.037$) (Figure 4J-K). Some of
209 these proliferative cells are OPC since they co-express Olig2 and Ki67 (Figure 4M-O). At 4
210 dpi, the percentage of proliferating Olig2+ cells was lower (but not significantly different) in
211 mutant mice compared to control ($23\pm 3.6\%$ vs. $17\pm 2.6\%$ of Ki67+/Olig2+ cells in control and
212 mutant mice, respectively, $p=0.29$) (Figure 4O). At 8 dpi, the percentage of proliferating

213 Olig2+ cells was significantly increased in mutant mice ($7.6\pm 0.8\%$ vs $3.2\pm 0.5\%$ in mutant and
214 control mice, respectively, $p=0.0004$) indicating a prolongation of OPC reactivity during the
215 repair phase. These data show that OPC reactivity is altered in absence of *Ndst1* at the onset
216 of remyelination (8dpi). To note, no significant difference in cell death was detectable in the
217 lesion between the two groups at 4 dpi (341.7 ± 4.5 and 357 ± 111.1 caspase3+ cells per mm^2 in
218 control and mutant mice, respectively, $p=0.28$).

219 Together, these results suggest that *Ndst1* expression in the Olig2+ population has no
220 effect on initial demyelination (equivalent lesion size at 4 dpi) but protects the lesion from
221 enlarging and participates in the control of OPC mobilization.

222

223 **Deletion of *Ndst1* in the Olig2+ population modulates microglia/macrophage activation.**

224 While the total number of proliferating cells within the lesion area was strongly increased in
225 *Ndst1* mutant mice compared to control mice at 8 dpi (Figure 4J-L), the percentage of OPC
226 among these cells represent only 7.6% in the mutant. These data suggest that *Ndst1* loss in the
227 Olig2 population indirectly modulates proliferation of surrounding cell types in the context of
228 a demyelinating lesion. To address this, we evaluated the proliferation and activation states of
229 the macrophage/microglia participating in demyelination-remyelination in this acute
230 demyelination model. We found a robust increase in proliferation of CD68+ cells in mutant
231 compared to control mice at 8 dpi (166.4 ± 24.3 vs. 79.3 ± 20.6 CD68+/Ki67+ cells per mm^2 in
232 mutant and control mice, respectively, $p=0.026$) (Figure 5A-C). Upon CNS insult,
233 microglia/macrophages are quickly activated, changing their shape from ramified to
234 rhomboid. Rhomboid versus ramified polarization of total or activated microglia/macrophages
235 was examined using respectively Iba1 (Figure 5D-E) and CD68 (Figure 5F-H)
236 immunostaining. We observed a switch of the microglia/macrophage polarization among the
237 whole Iba1 and CD68 population in favor of the rhomboid phenotype in mutant mice

238 compared to control at 8 dpi. This effect was quantified for activated microglia (ratio of
239 rhomboid /ramified CD68+ cells of 0.28 ± 0.05 in control vs. 0.66 ± 0.1 in mutant mice,
240 $p=0.038$) (Figure 5H). While the activation phenotype tended to decrease between 4 and 8 dpi
241 in control mice, it tended to increase in mutant animals. We then evaluated the expression
242 level of Cox2, a marker of pro-inflammatory (M1) microglia/macrophage [36] and observed a
243 significant 77% increase in the number of Cox2+ microglial cells in mutant mice compared to
244 control ($p=0.01$), indicating a delay in the pro-inflammatory (M1) to pro-regenerative (M2)
245 switch in the absence of *Ndst1* in oligodendroglia (Figure 5I-K). These results demonstrate
246 that *Ndst1* deletion in the Olig2 population is sufficient to enhance microglia/macrophage
247 proliferation and activation at the lesion site at the onset of remyelination.

248

249 **Shh binds to HS around the focal LPC-induced demyelinated lesion in the corpus** 250 **callosum**

251 As previously mentioned, HSPGs form a scaffold that shapes the distribution and activity of
252 numerous growth factors and morphogens during development and provide environmental
253 support for regenerative responses following CNS injury. Among several known HSPG-
254 binding morphogens, Shh was previously identified as a positive regulator for myelin repair
255 [14, 29]. In order to determine whether lesion-induced HS enrichment around the lesion site
256 could influence Shh distribution (hence signaling), we used an Alkaline Phosphatase (AP)
257 tagged version of Shh (AP-SHH) to directly assay its binding capacity in demyelinating
258 context. Knowing that the CW sequence serves as a major HS-binding site for Shh [37, 38],
259 we also used AP-SHH recombinant proteins deleted for the CW sequence (AP-SHH-
260 CWdeleted), as a control. Probes were incubated on fresh brain sections obtained 4 days after
261 LPC injections. AP-Shh binding was observed in the cortex in healthy conditions (data not
262 shown) and after lesion (Figure 6B,B'). While no AP-Shh binding was observed in healthy or

263 uninjured contralateral corpus callosum, AP-Shh binding delimited a clear belt surrounding
264 the lesion site in the corpus callosum after LPC injection (Figure 6B-B'). In contrast, AP-Shh-
265 CWdeleted did not bind around the same lesion on adjacent sections (Figure 6C-D). As AP-
266 Shh binding depends on the integrity of its HS-binding motif, this indicates that endogenous
267 Shh localization and concentration may be controlled by HS production by peri-lesional OLG.
268 In order to assess whether SHH signaling was indeed reduced in *Ndst1* mutant mice compared
269 to control mice, we quantified *Ptch1* (the main SHH receptor) expression around LPC-
270 induced demyelination lesions at 8 dpi using RNAscope (Figure 6E-G). *Ndst1* mutant mice
271 exhibited 38% decrease in *Ptch1* expression compared to control mice (8.6 ± 0.7 vs 13.8 ± 3.0
272 dots/cells in mutant and control mice respectively, n=5 mice/group) although it did not quite
273 reach significance ($p=0.07$) (Figure 6E). Altogether these results suggest that lack of NDST1
274 in OLG lineage attenuates SHH signaling following demyelination insult.

275

276 **NDST1 is expressed by oligodendroglia in multiple sclerosis lesions and correlates** 277 **with lesion size and remyelination**

278 To examine the relevance of our findings for multiple sclerosis (MS) pathophysiology, we
279 examined *Ndst1* expression in MS tissue. We first probed the snRNAseq data provided by
280 Jakel et al work [39], and observed that few cells express *Ndst1* in both control and MS
281 tissues but when the oligodendrocytes that express *Ndst1* are compared, there is a trend to
282 increased expression in MS tissue (Figure 7-figure supplement1). Because such approach
283 identifies around 15% only of nuclear RNA, we then directly examined the expression pattern
284 of NDST1 protein in MS patient brain sections. Normal appearing white matter (WM),
285 remyelinating, active, chronic active or chronic inactive lesions were analyzed. While NDST1
286 staining was very weak in control WM (without MS), we observed a significant increase of
287 NDST1 labeling in MS patients WM (Figure 7A-B). Comparison of healthy control, MS

288 normal appearing WM and MS lesions showed that there is a significant increase of NDST1
289 staining in multiple sclerosis lesions vs. control ($p=0.0014$) (Figure 7C). Comparison of each
290 MS lesion with its surrounding normal appearing WM using a paired t test, revealed that there
291 is significantly more NDST1 labeling in MS lesions compared to their surrounding normal
292 appearing WM (paired two-tailed t test, $t_9=3.39$, $p=0.0095$). However, NDST1+ cells were
293 distributed evenly throughout the lesion, rather than forming a delimiting band (Figure 7-
294 figure supplement 2D).

295 We then performed double-labeling immunohistochemistry to characterize NDST1-positive
296 cells in various types of lesions and normal appearing WM, using OLIG2 for oligodendroglia
297 (Figure 7D), GFAP for astrocytes (Figure 7E), NEUN for neurons (Figure 7F) and IBA1 for
298 microglia/macrophages (Figure 7G). Quantitative analysis showed that the majority of
299 NDST1 cells were oligodendroglia in all types of lesions (remyelinated, active, chronic active,
300 chronic inactive) and in normal appearing WM (Figure 7H).

301 The vast majority of OLIG2+ cells in the lesions expressed NDST1, with a gradual reduction
302 in the proportion of OLIG2+NDST1+ cells as lesions become more chronic, and with
303 significantly fewer in control tissue (Figure 7I; $p=0.016$). The number of NDST1+
304 oligodendroglia in each lesion was inversely correlated with the lesion's size (Figure 7J). As
305 blocks of MS tissue contained multiple lesions and sometimes we had multiple blocks from
306 the same patient (see Table1), we gave each patient an overall remyelination ability score
307 corresponding to how many lesions in the blocks from that patient were remyelinated, or
308 likely to remyelinate if the patient had survived. Here, we were aiming to see whether patients
309 considered being "good remyelimators" using this score express more NDST1. A lesion was
310 given a score of 3 points (complete remyelination), 2 points (active - likely to remyelinate), 1
311 point (chronic active less likely to remyelinate) and 0 points (chronic inactive -unlikely to
312 remyelinate). The total score of all the lesions per patient was then divided by the number of

313 lesions per patient, to allow comparisons. We showed that NDST1+ cell density positively
314 correlated with patient's score of remyelination ability (Figure 7K). These data reveal that MS
315 tissues with a higher repair potential (containing most active and remyelinated lesions)
316 display a high number of NDST1+ cells therefore suggesting that higher numbers of NDST1+
317 cells in a lesion may provide a positive environmental support for myelin repair.

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323 **DISCUSSION**

324 In this study, we investigated the role of *Ndst1* and HS after demyelination. Using LPC-
325 induced demyelination of the corpus callosum in mouse, we showed for the first time that
326 *Ndst1*-dependent N-sulfate sugar modifications occur at the onset of demyelination and
327 during remyelination. These modifications limit the extension of demyelination and create a
328 permissive substrate enhancing remyelination. First, we show that *Ndst1* is almost exclusively
329 expressed by oligodendroglia present at the margin of the lesion delimiting the lesion from the
330 intact corpus callosum. Second, we found that the conditional deletion of *Ndst1* in the *Olig2*
331 population concomitantly triggers an enlargement of the LPC-induced demyelinated area,
332 alters OPC mobilization and favors the pro-inflammatory (M1) phenotype in microglia. We
333 propose that these effects could be mediated through HS dependent binding of the morphogen
334 *Shh* which has been shown to be a positive regulator of myelin repair through increased
335 oligodendrogenesis and microglial regulation [14, 29]. Finally, using MS brain tissue
336 samples, we show that NDST1 is up-regulated, especially within lesions and that the density
337 of NDST1+ cells in these lesions is negatively correlated to lesion size, and positively
338 correlated to the patient's potential remyelination ability. Our data suggest that *Ndst1*/HS
339 expressed by oligodendrocytes around the lesion create a protective and permissive
340 environment playing a positive role in myelin repair.

341

342 HS and chondroitin sulfates are the two main classes of sulfated proteoglycans constituting
343 the extracellular space. Chondroitin sulfates are strongly expressed by astrocytes and
344 microglia providing a hostile environment impeding regeneration and remyelination following
345 brain injury [40-43]. Enzymatic degradation of chondroitin sulfate proteoglycans using
346 chondroitinase ABC treatment *in vivo* promotes OPC mobilization and remyelination [43]. In
347 *vitro*, chondroitin sulfates reduce OPC maturation [44] and *in vivo* the use of a CSPG

348 synthesis inhibitor promotes OPC maturation and accelerates remyelination following focal
349 demyelination in mice [45]. A recent study has shown that Surfen, a proteoglycan binding
350 agent, reduces inflammation and delays remyelination. [46]. Our study provides evidence for
351 a beneficial effect of HS proteoglycans (and upstream enzyme *Ndst1*) on limiting
352 demyelinated lesion size and promoting remyelination. Here we propose that HS on mature
353 OLG changes the amount/stability of soluble factors, and thus the recruitment and
354 proliferation of surrounding cells (such as OPC or microglia). Thus, OLG modify their
355 environment which in turn regulates the behavior of cells in the lesioned CC including
356 microglia and OPC. Therefore, two classes of sulfated proteoglycans have opposite effects on
357 myelin repair; one being detrimental (chondroitin sulfates), and the other (HS) having a
358 beneficial effect. Interestingly, both are found at the margin of the lesion, but while the
359 chondroitin sulfates are secreted by astrocytes and microglia, HS are expressed by
360 oligodendroglia. Our data show that the majority of *Ndst1*⁺ cells at the margin of the LPC-
361 induced lesion are mature CC1⁺ OLG revealing that mature OLG around a demyelination
362 lesion, respond to post lesional cues. Interesting, recent data have shown that pre-existing
363 mature OLG can also participate to myelin regeneration in human and rodent by forming new
364 myelin sheaths further indicating that mature OLG have an active role in myelin repair [47,
365 48].

366

367 This OLG response to nearby demyelination appears propitious to regeneration by restricting
368 the lesion spread, favoring OPC mobilization and modulating microglia response.

369 Macrophages/microglia participate to myelin repair through myelin debris clearance and the
370 secretion of regenerative factors that altogether promote the recruitment of OPC, their
371 proliferation and differentiation into mature myelin-forming cells. Activated microglia also
372 produces various pro-inflammatory mediators (cytokines, chemokines...) that may affect

373 OPC since they express a battery of receptors [49]. Recent studies have shown that efficient
374 remyelination required the dynamic regulation of functional microglia phenotype [7, 50].
375 Microglia respond to demyelination with initial pro-inflammatory phenotype (M1) followed
376 later by pro-regenerative phenotype (M2) which actively contributes to myelin repair [7, 51].
377 Interestingly, this transition from pro-inflammatory to pro-regenerative phenotype is a rate-
378 limiting step in the repair process since intra-lesional depletion of pro-regenerative microglia
379 blocks oligodendrocyte differentiation and delays the regenerative process [7]. In our study,
380 lack of Ndst1/HS from Olig2+ cells in transgenic mice leads to increased microglial
381 proliferation, a strongly activated rhomboid polarization of CD68-expressing cells and an
382 increased density of pro-inflammatory (M1) microglia at an early stage of the remyelination
383 phase (8 dpi). Altogether these effects may contribute to enlargement of the demyelinated
384 lesion and delayed myelin repair. This non-cell autonomous effect observed on microglia
385 activation could in turn disturb OPC mobilization and thus modify the repair process [7].

386

387 The beneficial action of HS may be related to their ability to bind numerous growth factors
388 and morphogens, as observed during development [23]. HS can act as co-receptors for these
389 ligands or are involved in the stabilization and/or local concentration of ligands in the
390 extracellular space, which modulates cell signaling. Ndst1 global knock out mice display
391 developmental defects that mainly resemble those found in embryos deficient for Shh or FGFs
392 [52]. FGF and Shh implication in myelin regeneration and glial reactivation have been
393 extensively examined in mouse models of demyelination (for review, see [8]). A role of both
394 factors in remyelination was first inferred by correlating their spatial and temporal
395 upregulation after demyelination particularly in LPC-induced demyelination [14, 53-55]
396 Concerning FGF, conflicting results have been published on its activity [56-61]. A recent
397 report has addressed this issue using the simultaneous ablation of both FGFR1 and FGFR2

398 specifically in cells from the oligodendrocyte lineage [62]. This study revealed that FGF
399 signaling is not required for myelin regeneration in acute models of demyelination including
400 LPC-induced demyelination of the spinal cord and cuprizone intoxication [62]. Overall, in all
401 these analyses the phenotypes observed after LPC-induced demyelination never recapitulate
402 (even partially) the phenotype observed in the present report using *Olig2-Cre; Ndst1^{Flox/Flox}*
403 mice. This suggests that FGF is probably not the main ligand regulated by HS activity during
404 myelin repair in this model.

405 By contrast, blocking Shh activity leads to an increase in demyelinated lesion size and an
406 altered OPC mobilization after LPC-induced demyelination [14] similar to what we observe
407 in *Olig2-Cre; Ndst1^{Flox/Flox}* mice. However, these effects persist at later time points at the end
408 of remyelination, and OPC maturation is also inhibited after Shh inactivation [14]. Of note,
409 recent *in vitro* findings show that the proteolytic processing of Shh required for signaling
410 pathway activation [63] is finely regulated by HS chains [64], perhaps influencing Shh
411 concentration and/or spreading. Here we show that Shh binds to HS around demyelinated
412 lesions in mouse. Thus, we propose that HS removal may delay the local accumulation around
413 the demyelinated lesion of Shh produced by OLG [14] and/or delay Shh activation and
414 spreading, leading to a reduction in signaling as suggested by reduced *Ptch1* expression at
415 early time-points. Later, the sustained production of Shh may overtake the absence of HS,
416 leading to efficient recovery. Interestingly, Ferent et al. have shown that the main Shh
417 responding cells (cells expressing *Gli1* and/or *Smo*) after LPC-induced demyelination of the
418 corpus callosum are OLG and microglia [14]. These observations further support the idea that
419 sustained microglia and OPC activation in the absence of HS are at least in part due to altered
420 Shh signaling.

421 Finally, we can confirm that NDST1 is also over-expressed in human MS brain samples,
422 mostly in *Olig2*-positive oligodendroglia. High levels of HS proteoglycans and chondroitin

423 sulfate proteoglycans have been previously associated with inflammatory CNS diseases such
424 as MS [65-68]. Here, we show that NDST1 is upregulated within and surrounding MS lesions
425 in post mortem tissue compared to control. NDST1 expression is significantly higher in MS
426 lesions compared to surrounding normal appearing white matter (NAWM), irrespective of
427 lesion type (remyelinated, active, chronic active or chronic inactive). The distribution of the
428 NDST+ cells was different from the mouse model, in that we saw no surrounding band of
429 positive cells, but instead the entire lesions contained positive cells, though the majority of
430 these cells were OLIG2+ oligodendroglia. This difference may be related to the timing of
431 examination of the tissue after the lesion onset, which is later in the human tissue, and
432 secondary to poorer repair in humans. In MS lesions, a large proportion of these OLIG2+
433 cells express NDST1, suggesting that at least some of these are mature, but we were unable to
434 distinguish mature and immature oligodendroglia in this tissue (due to limitations in double-
435 labelling using effective antibodies in human tissues). However, these observations still
436 concur with our mouse data indicating that oligodendroglia respond to neighboring
437 demyelination. Also consistent with our results showing increased lesion size in *Olig2-Cre+/-*
438 ; *Ndst1*^{Flox/Flox} mice, in human samples we observed an inverse correlation between lesion
439 size and density of NDST1-expressing Olig2+ cells. NDST1 cell density also positively
440 correlates with a pathological score of potential remyelination ability in patients.

441 Overall, our results in mouse and human tissues suggest that NDST1/HS levels are an
442 indicator of oligodendroglial reactivity after demyelination, and are involved in both limiting
443 the size of the lesion and creating a permissive environment for myelin regeneration.
444 Furthermore, this study shows for the first time that mature oligodendrocytes around lesion
445 are active players during demyelination/remyelination by producing HS and thus modifying
446 the local environment. This study will help improve understanding the neuropathology of MS

447 in both limitation of damage and promotion of remyelination, which may in the future help
 448 target pharmacological approaches to potentiate myelin repair.

449

450 **Acknowledgements**

451 We are grateful to E. Traiffort, F Helmbacher and C. Bertet for critical reading of the
 452 manuscript.

453

454 **MATERIALS AND METHODS**

455

Key Resources Table				
Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Genetic reagent (<i>M.musculus</i>)	<i>Olig2^{Cre}</i>	PMID:18046410		B6D2F1J/Rj genetic background
Genetic reagent (<i>M.musculus</i>)	<i>Ndst1^{flx/flx}</i>	PMID:16020517		Dr. Kay Grobe (University of Münster, Münster, Germany)
Genetic reagent (<i>M.musculus</i>)	<i>Pip^{gfp}</i>	PMID:15906234 PMID:11756747		Dr. Bernard Zalc (University of Sorbonne, Paris, France)
Biological sample (<i>H. Sapiens</i>)	Brain tissue from 9 MS patients	UK Multiple Sclerosis Tissue Bank (MREC/02/2/39)		Postmortem unfixed frozen
Biological sample (<i>H.</i>	Brain tissue from 4 Control	UK Multiple Sclerosis Tissue		Postmortem unfixed frozen

<i>Sapiens</i>)	patients	Bank (MREC/02/2/39)		
Cell line (<i>H. Sapiens</i>)	293T HEK	ATCC	CRL3216	
Transfected construct (<i>M.musculus</i>)	pWiz-AP-SHH	PMID: 16020517		Production of AP-tagged SHH recombinant protein
Transfected construct (<i>M.musculus</i>)	pWiz-AP-SHH-CWdeleted	PMID: 11959830		Production of AP-tagged deleted SHH recombinant protein
Antibody	Rabbit polyclonal anti-OLIG2	Millipore	AB9610	IF (1/1000)
Antibody	Rabbit polyclonal anti-OLIG2	Sigma-Aldrich	HPA003254	IF (1/100)
Antibody	Mouse monoclonal anti-APC (clone CC1)	Calbiochem	OP-80	IF (1/400)
Antibody	Rat monoclonal anti-PDGFR α (clone APA5)	Millipore	CBL1366	IF (1/250)
Antibody	Mouse monoclonal anti-MBP	Millipore	MAB384	IF (1/500)
Antibody	Mouse monoclonal anti-Ki67	BD Pharmingen	556003	IF (1/500)

Antibody	Rabbit polyclonal anti-Caspase 3	Cell Signalling	9661	IF (1/200)
Antibody	Rabbit polyclonal anti-GFAP	Dako	Z0334	IF (1/400)
Antibody	Goat polyclonal anti-IBA1	Abcam	Ab5076	IF (1/500)
Antibody	Rabbit polyclonal anti-IBA1	Wako Chemicals	019-19741	IF (1/500)
Antibody	Rat monoclonal Anti-CD68	Abcam	Ab53444	IF (1/400)
Antibody	Rabbit polyclonal anti-COX2	Abcam	Ab15191	IF (1/400)
Antibody	Mouse monoclonal IgM anti-N-sulfated motifs on HS chains (clone10E4)	Amsbio	370255-1	IF (1/500)
Antibody	Mouse monoclonal anti-NDST1	Abcam	ab55296	IF (1/50)
Antibody	Rabbit polyclonal anti-NeuN	Abcam	Ab104225	IF (1/500)
Sequence-based reagent	<i>Ndst1_F</i>	Eurofins Genomics	RT-qPCR primers	gctggacaagatc atcaatgg

Sequence-based reagent	<i>Ndst1_R</i>	Eurofins Genomics	RT-qPCR primers	acacagtacttctacgactatcc
Sequence-based reagent	<i>Gapdh_F</i>	Eurofins Genomics	RT-qPCR primers	gggttcctataaatacggactgc
Sequence-based reagent	<i>Gapdh_R</i>	Eurofins Genomics	RT-qPCR primers	ctggcactgcacaagaagat
Sequence-based reagent	<i>plp/dm20</i>	PMID:9373029		Probe for ISH
Sequence-based reagent	<i>Ndst1</i>	PMID:16020517		Probe for ISH
Sequence-based reagent	<i>Ptch1</i>	Advanced Cell Diagnostics	402811-C2	Probe for RNAScope
Peptide, Recombinant Protein	Human NDST1	Abcam	ab116875	
Commercial assay or kit	RNAScope Multiplex Fluorescent kit	Advanced Cell Diagnostics	323133	
Commercial assay or kit	DAB Peroxidase (HRP) Substrate Kit (with Nickel)	Vector Laboratories	SK-4100	
Commercial assay or kit	VECTOR Blue AP Substrate Kit	Vector Laboratories	SK-5300	
Commercial assay or kit	ImmPRESS™-AP Anti-Rabbit IgG Polymer Detection Kit	Vector Laboratories	MP-5401	

Commercial assay or kit	ImmPRESS™ HRP Anti-Mouse IgG Polymer Detection Kit	Vector Laboratories	MP-7402	
Chemical compound, drug	Lysolecithin	Sigma-Aldrich-Merck	L1381	
Chemical compound, drug	Heparinase	Amsbio	100700	
Chemical compound, drug	Lipofectamine 2000	Invitrogen	11668-030	
Chemical compound, drug	Vector Bloxall	Vector Laboratories	SP-6000	
Software, algorithm	ImageJ	https://imagej.nih.gov/ij/		
Software, algorithm	Zen 2 lite	Zeiss		
Software, algorithm	GraphPad Prism	https://graphpad.com		

456

457

458 **Animals and treatments**

459 All experimental and surgical protocols were performed following the guidelines established
460 by the French Ministry of Agriculture (Animal Rights Division). The architecture and
461 functioning rules of our animal house, as well as our experimental procedures have been
462 approved by the “Direction Départementale des Services Vétérinaires” and the ethic

463 committee (ID numbers F1305521 and 2016071112151400 for animal house and research
464 project,
465 respectively).. Surgery and perfusions were performed under ketamine (100 mg/kg, MERIAL,
466 Lyon, France))/xylazine (10 mg/kg, BAYER, Puteaux, France) anesthesia. C57BL/6 wild-
467 type and transgenic mice were successively used to characterize post-lesional expression of
468 *Ndst1* and HS after demyelination and to investigate the impact of conditional deletion of
469 *Ndst1* in the Olig2-positive cell population. Heterozygous *Olig2-Cre*^{+/-} (from B6D2F1J/Rj
470 genetic background) [35] and double transgenic *Olig2-Cre*^{+/-}; *Ndst1*^{Flox/Flox} mice [19, 35]
471 will be referred below as control and mutant mice, respectively. Mice expressing GFP under
472 the control of the proteolipid protein (*plp*, a protein largely present in myelin) promoter were
473 used in some experiments to better observe demyelination lesions (called thereafter *plpGFP*
474 mice). Animals were housed under standard conditions with enrichment and access to water
475 and food *ad libitum* on a normal 12 h light/dark cycle.

476

477 **Human postmortem samples**

478 Postmortem unfixed frozen tissues were obtained from the UK Multiple Sclerosis Tissue
479 Bank via a UK prospective donor scheme with full ethical approval (MREC/02/2/39). Luxol
480 fast blue (LFB) (staining myelin; Figure7 -figure supplement 2C) and Oil Red O (staining
481 lipids phagocytosed by macrophages) were performed to characterize and classify the lesion
482 types [3]. Active lesions have indistinct borders on LFB and lipid-laden
483 macrophages/microglia. Chronic active lesions have a ring of lipid-laden
484 macrophages/microglia and a core with few immune cells. Chronic inactive lesions have a
485 distinct border on LFB and few immune cells. Finally, shadow plaques, thought to represent
486 remyelination, have less intense staining on LFB. This classification was done by two
487 independent researchers for a previous publication [3]. In this study, we used active (n=7),

488 chronic active (n=4), chronic inactive (n=14) and remyelinated (shadow) MS plaques (n=21)
489 from 14 blocks of brain tissue from 9 MS patients and 4 blocks of brain tissue from 4 controls
490 with no neurological disease (Table 1)

491

492 **Focal demyelination in the corpus callosum and tissue processing**

493 Focal demyelination was performed by stereotactic injection of Lysolecithin (LPC) (SIGMA-
494 ALDRICH, St Louis, USA) as described previously [30, 69]. The corpus callosum from
495 healthy or demyelinated mice, from the ipsilateral and contralateral side to the LPC-induced
496 lesion, were dissected 7 days post injection (dpi) from 1 mm thick coronal slices in cold
497 Hank's Balanced Salt Solution (GIBCO by life technologie, Paisley, UK) and processed for
498 RT-qPCR analysis (*Ndst1* primers : exon 6 (forward, 5'-gctggacaagatcatcaatgg-3') and exon 7
499 (reverse, 5'-acacagtacttctacgactatcc-3'); for *Gapdh* : exon1 (forward, 5'-
500 gggttctataaatacggactgc-3') and exon2 (reverse 5'-ctggcactgcacaagaagat-3'). Primers from
501 EUROFINS GENOMICS, Ebersberg, GERMANY). For histological analysis, mice were
502 anesthetized and perfused with ice-cold 4% paraformaldehyde (FISHER SCIENTIFIC,
503 Loughborough Leics, UK) in PBS (GIBCO by life technologie, Paisley, UK). Brains were
504 post-fixed overnight in 4% paraformaldehyde in PBS and cut on a vibratome (Leica) in 4
505 series of coronal sections (50 µm thick) for immunofluorescence, or cryopreserved and cut
506 with cryostat (20 µm thick) for in situ hybridization.

507

508 **In situ hybridization and immunohistochemistry.**

509 In situ hybridization was performed using *plp/dm20* [70] and *Ndst1* probes [19] as described
510 in [71]. RNAscope Multiplex Fluorescent kit (323133; Advanced Cell Diagnostics) was used
511 to detect *Ptch1* mouse mRNA. Briefly cryosections were baked for 30 min at 60°C and
512 dehydrated, incubated for 30min at 40°C with protease III before incubation with RNAScope

513 probe Ptch1 (402811-C2) for 2 hrs at 40°C. Immunohistochemistry was performed as
514 described in [30]. The following primary antibodies were used: rabbit anti-Olig2 (AB9610;
515 1/1000, Millipore, USA), mouse anti-APC (CC1) (1/400; Calbiochem, USA), and rat anti-
516 PDGFR α (CBL1366; 1/250; Millipore, USA) for oligodendroglial lineage cells; anti-MBP
517 (mouse, 1/500, Chemicon, Millipore S.A.) for myelin sheaths; mouse anti-Ki67 (556003;
518 1/500; BD Pharmingen) for proliferating cells; rabbit anti-caspase 3 (9661; 1/200; Cell
519 Signaling) for apoptotic cells, rabbit anti-GFAP (1/400) for astrocytes; goat anti-Iba1 (1/500,
520 Abcam) for microglia and macrophages; rat anti-CD68 (1/400, Abcam) for activated
521 microglia and macrophages; rabbit anti-Cox2 (1/400, Abcam) for proinflammatory M1
522 microglia/macrophage; mouse IgM anti-N-sulfated motifs on HS chains (10E4 antibody,
523 1/500; Seikagaku, Japan). For Olig2 and Ki67 immunofluorescence, antigen unmasking was
524 performed by 20 min incubation in boiling citrate buffer (10mM pH6). For N-sulfated motifs
525 labeling, floating sections from PFA perfused-brain were incubated for 2 h 30 at 37°C in
526 buffer (100 mM Sodium Chloride, 1 mM Calcium Chloride, 50 mM Hepes 5 μ g, BSA pH 7)
527 with or without Heparinase (3.3 mU from Flavobacterium heparinum, Seikagaku Kogyo Co. #
528 100700, Japan) [34] before permeabilization. Secondary antibodies coupled to alexa 488, 555
529 and 647 (1/500, Invitrogen Molecular Probes) were applied for 2 h 30 at RT in a humid
530 chamber. Sections were counterstained with Hoechst 33342 (1/500, Sigma).

531

532 **AP-Shh recombinant protein binding test in mice**

533 Plasmids containing sequences for AP-tagged N-terminal WT or deleted Shh were produced
534 by PCR and ligated into pWIZ vector as described in [19, 38]. Briefly, plasmids were
535 transiently transfected into HEK cells using lipofectamine 2000 (Invitrogen). Transfection
536 proceeded for 3 h. Culture supernatants were collected after 60 h and filtered through 0.45 μ m
537 filters (Corning Incorporated, Durham, USA). Hepes 10mM pH7 was added to increase

538 stability. Shh concentration was then evaluated measuring AP activity in culture supernatants.
539 Preparations from mock-transfected HEK cells were generated and used as vehicle controls.
540 The AP-Shh binding test was performed as described in [38].
541 Fresh frozen brain sections were post-fixed with ice-cooled methanol for 8 min. After rinsing
542 with phosphate-buffered saline containing 4mM MgCl₂ and blocking with 1% Bovine Serum
543 Albumin (SIGMA-ALDRICH, St Louis, MO, USA) 1 h at RT, frozen adjacent sections from
544 healthy or demyelinated C57BL/6 mice were incubated with 5nM of two AP tagged versions
545 of Shh: AP-Shh recombinant proteins carrying the N-terminal CW sequence (AP-SHH), the
546 main HS-binding site for Shh [38] [37], or lacking this motif (AP-SHH-CWdeleted). Sections
547 were then washed with PBS to dissociate any low affinity interaction and endogenous
548 phosphatases were inactivated by heating at 65°C for 2 h. AP was revealed by incubating
549 overnight in NBT (100mg/ml) /BCIP (50mg/ml) in 100mM Tris pH 9,5 with 100mM NaCl
550 and 50mM MgCl₂.

551

552 **Immunohistochemistry on human post-mortem tissue**

553 Tissue was fixed in 4% paraformaldehyde in PBS for 30 min. Endogenous peroxidase and AP
554 activity was blocked by 10 min incubation with Vector Bloxall (Vector, SP-6000 VECTOR
555 LABORATORIES, Burlingame, USA). Slides were blocked with ready-to-use 2.5% normal
556 horse serum from Vector secondary antibody kits for at least 20 min. Primary antibodies were
557 incubated overnight in antibody diluent (Spring Bioscience, ADS-125) at 4°C. Primary
558 antibodies used: mouse anti-NDST1 (1/50; Abcam, ab55296), rabbit anti-NeuN (1/500;
559 Abcam, ab104225), rabbit anti-IBA1 (1/500; Wako chemicals, 019-19741), rabbit anti-Olig2
560 (1/100; Sigma, HPA003254). HS staining with the mouse IgM anti-N-sulfated motifs on HS
561 chains (10E4 antibody, Seikagaku, Japan) did not give any signal on human tissue. NDST1
562 intensity was evaluated after a short exposition (exactly 2 min). All other stainings were fully

563 developed. To ensure antibody specificity, the NDST1 antibody was pre-absorbed with
564 human NDST1 recombinant protein (Abcam, ab116875), and added to tissue sections, with no
565 staining seen (Figure7 -figure supplement 2A-B).

566 Secondary antibodies were incubated at RT for 1h. Staining was developed with a DAB
567 Peroxidase (HRP) Substrate Kit (with Nickel), 3,3'-diaminobenzidine (Vector, SK-4100) and
568 a VECTOR Blue AP Substrate Kit (Vector, SK-5300) as per manufacturer's guidelines.
569 Secondary antibodies used: ImmPRESS™-AP Anti-Rabbit IgG Polymer Detection Kit
570 (Vector, MP-5401) and ImmPRESS™ HRP Anti-Mouse IgG Polymer Detection Kit, made in
571 Horse (Vector, MP-7402). PBS washes were performed between each treatment.

572

573 **Microscopy and quantification**

574 For mouse tissue analysis, imaging was performed with the Apotome system (Zeiss). The
575 demyelinated area and cell counts were evaluated using Zen software (Zeiss).
576 Immunofluorescent or in situ hybridization positive cells were counted in every fourth section
577 through the whole demyelinated lesion per mouse and averaged for each mouse. Cell counts
578 are presented as the mean of at least 3 mice. For RNAscope ISH, each punctate dot signal was
579 counted around lesion (by using ROI and analyze particule Fiji Plugins) and reported to total
580 nuclei number. Lesion size was quantified by measuring the area of high density of nuclei in
581 every fourth section through the whole demyelinated lesion per mouse. In this analysis, high
582 density of nuclei was correlated with myelin loss visualized by MBP staining or by loss of
583 fluorescence in *plpGFP* mice (Figure1 -figure supplement 2).

584 For the human post-mortem tissue analysis, slides were imaged using a ZEISS Axio Scan.Z1
585 slide scanner. One researcher marked out the lesion sites and normal appearing white matter
586 (WM) as areas of interest, while another counted single positive (NDST1+ cells) and double

587 positive cells (NDST+ cells and other brain cell markers combined as above) in these areas of
588 interest (ensuring blinding of counting).

589 Myelin content was evaluated by double blind scoring of images taken from Plp
590 immunostaining on brain sections (3 photos per section and 3 sections per brain). Score of 4
591 was attributed to maximum myelination down to 0 for absence of myelin. The mean score for
592 the control group was considered as 100%.

593

594 **Gene expression profile of demyelinated versus healthy mouse progenitors**

595 This protocol is fully described in Cayre et al. [30]. Briefly, OPC from eight mice induced for
596 experimental autoimmune encephalomyelitis (EAE mice) at the peak of paralytic symptoms
597 and from eight adult healthy mice as controls were purified using magnetic cell sorting
598 (Miltenyi Biotec). This experiment was replicated in an independent similar experiment.
599 cDNAs were prepared and used (250 ng) as template for Cy3 and Cy5, combined and
600 hybridized to Agilent Whole Mouse Genome Oligo Microarrays 4Å~44K. Agilent Feature
601 Extraction Software (FES) determines feature intensities and ratios (including background
602 subtraction and normalization), rejects outliers and calculates statistical confidences (P-
603 values). We obtained a gene list with all normalized Cy5/Cy3 log₁₀ ratios, Cy5/Cy3 fold
604 changes, sequence description and P-values. Microarray data are available at GEO with
605 accession number GSE47486.

606

607 **Statistical analysis**

608 All the presented values in mice are means \pm S.E.M unless otherwise stated. Data were
609 statistically processed with the non-parametric Mann-Whitney test (independent two group
610 comparisons). P<0.05 was considered significant and p<0.01 highly significant. All
611 measurements and subsequent evaluations were performed blind to the experimental group to

612 which the animals belonged. For the human post-mortem tissue analysis, a d'Agostino and
613 Pearson omnibus normality test was used to test whether the data fits a normal distribution
614 and a parametric test were done only if all compared data sets passed the normality test. The
615 NDST1+ cells in control versus multiple sclerosis WM was compared using a two-tailed
616 Mann Whitney U test. Multiple sclerosis lesions and their surrounding WM were compared
617 using a paired two-tailed t test. The absolute numbers of NDST1+ cells and double positive
618 NDST1+ OLIG2+ cells in individual lesions/normal appearing WM were compared by
619 Kruskal-Wallis test. As MS tissue blocks contained more than one lesion, and we had several
620 blocks from the same patients, we gave each patient an overall remyelination ability score
621 corresponding to how many lesions in the blocks from that patient were remyelinated, or
622 likely to remyelinate if the patient had survived. Remyelinated lesions received an arbitrary 3
623 points, active lesions 2 points, chronic active lesions 1 point and chronic inactive lesions 0
624 points. This was divided by the number of lesions counted for each patient, to allow
625 comparisons.

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789

790 **Figure 1. *Ndst1* up-regulation upon LPC-induced demyelination of the corpus callosum.**

791 (A) Scheme showing the site of LPC injection (red point) in the adult corpus callosum and the
 792 location of picture shown in C (red rectangle). (B) *Ndst1* expression levels (RT-qPCR) in the
 793 corpus callosum of healthy or demyelinated mice, contralateral (contra) and ipsilateral (ipsi)
 794 to the lesion site showing the *Ndst1* up-regulation in the ipsilateral side. Tissues from 5 mice
 795 were pooled in each condition. Error bars represent S.E.M. * $p < 0.05$, non-parametric ANOVA
 796 followed by Kruskal-Wallis test (independent two group comparisons). (C-H) *Ndst1* in situ
 797 hybridization performed at 5 (C-F, n=4), 8 (G, n=4) and 14 (H, n=4) dpi illustrating the *Ndst1*
 798 expression pattern at different time points of demyelination (C-F) and remyelination (G-H).

799 (D-E) Enlarged views of the CC in C corresponding to contralateral side (D) and positive
 800 cells at the margin of the demyelinated area at the site of LPC injection (E). CC, corpus
 801 callosum; Cx, cortex; SVZ, sub-ventricular zone; V, ventricle (structures are delineated by
 802 brown dotted lines, lesion with white dotted lines). Scale bars: 50 μm in F, G and H; 20 μm in
 803 D, F, H; 10 μm in D and E Asterisk in G indicates the site of injection since the demyelinated
 804 lesion is no longer visible at 14 dpi.

805

806 **Figure 1-figure supplement 1. *Ndst1* is up-regulated by the Olig2+ cell population in**
807 **close proximity to inflammation sites in corpus callosum, in the experimental**
808 **autoimmune encephalomyelitis mouse model of demyelination.** *Ndst1* is not expressed in
809 control brain (A) (n=2) while it is up-regulated by Olig2+ cells after experimental
810 autoimmune encephalomyelitis induction (B)(n=3) in close proximity to lesions in the corpus
811 callosum (C). Enlarged views correspond to boxed region. CC, corpus callosum. Scale bars:
812 50 μm in A-B; 20 μm in C.

813

814 **Figure 1 Supplement 2. *PlpGFP* mice (n=3) were used to detect demyelinated lesions (A, B,**
815 **D, E).** Demyelination was clearly visible in the corpus callosum around the injection site by
816 the lack of GFP fluorescence (B, E). Hoechst staining shows a high cell density (C, F)
817 correlating with the loss of myelin (A, D). CC, corpus callosum, Cx, cortex, V, ventricle, St,
818 Striatum. Scale bars: 100 μm .

819

820 **Figure 2. N-sulfate-enriched microenvironment forms a belt around the demyelinated**
821 **lesion.** HS (10E4) labeling on the contra- (A) and ipsi- (B-C) lateral side to the lesion
822 illustrates the generation of a N-sulfated microenvironment surrounding the lesion (delimited
823 by white dashed lines) at 5 dpi (n=3). No immunoreactivity was found after Heparinase I
824 treatment (D) thus validating the 10E4 antibody specificity. Scale bars: 20 μm in A, B, D;
825 10 μm in C. CC, corpus callosum; V, ventricle.

826

827 **Figure 3. *Ndst1* expressing cells around the lesion belong to the oligodendroglial lineage.**
828 (A-B) *Ndst1* in situ hybridization successively combined with Olig2 immunostaining (A) or
829 *Plp* in situ hybridization (B) labeling, two OLG markers, illustrating *Ndst1* up-regulation in

830 oligodendroglia lineage cells surrounding the lesion site at 5 dpi (n=3). **(C-D)** Representative
831 images of *Ndst1*/PDGFR α **(C)** and *Ndst1*/CC1 **(D)** co-labeling illustrating that both OPC **(C)**
832 and mature OLG **(D)** up-regulate *Ndst1* after demyelination at 5 dpi (n=4). Inserts in **(A-D)**
833 illustrate boxed regions at high magnification. Scale bars: 20 μ m.

834

835

836 **Figure 4. Deletion of *Ndst1* in Olig2+ cells affects lesion size and OPC mobilization after**
837 **LPC-induced demyelination of the corpus callosum.**

838 **(A-B)** Representative images of the lesion site (delineated by white dashed lines) in the
839 corpus callosum of control **(A)** and mutant **(B)** mice at 8 dpi illustrating the enlargement of
840 the lesion size in mutant mice compared to control mice. **(C)** Quantitative analysis of the
841 lesion size at 4, 8 and 14 dpi (n=8,9,4 control and n=9,12,6 mutant mice respectively). **(D-E)**
842 Oligodendroglia labeled by Olig2 staining within the demyelinated area at 8 dpi **(E)** compared
843 to control mice **(D)**. **(F)** Olig2 mean cell density in healthy (CTL) or demyelinated control and
844 mutant mice at 4, 8, 14 dpi. **(G-H)** Mature OLG co-labeled by Olig2/CC1 within the
845 demyelinated lesion at 8 dpi in control **(G)** and mutant **(H)** mice. **(I)** Quantification of mean
846 cell density of Olig2+/CC1+ cells within the demyelination lesion in healthy (CTL) or
847 demyelinated control and mutant mice at 4, 8, 14 dpi. **(J-K)** Ki67+ immunolabeling shows the
848 proliferation status of cells within the lesion 8dpi in control **(J)** and mutant **(K)** mouse. **(L)**
849 Graph represents the cell proliferation (Ki67+ cells) in mutant relative to control mice at 4
850 and 8 dpi (n=9,12 control and n=8,16 mutant mice respectively). **(M-N)** Co-immunolabelling
851 of Olig2 and Ki67 showing OPC proliferation in control **(M)** and mutant **(N)** mouse 8dpi. **(O)**
852 Quantification of proliferating OPC (Ki67+/olig2+ cells) in lesion sites at 4 and 8 dpi (n=6,11
853 control and n=7,13 mutant mice respectively). Error bars represent S.E.M. *p<0.05,

854 ***p<0.001, non-parametric Mann-Whitney test (independent two group comparisons). Scale
855 bars: 50 μm in A, B, D, E and 10 μm in, G, H, J, K, M and N.

856 **Figure 4- figure supplement 1. *Ndst1* inactivation in oligodendrocyte lineage cells in**
857 ***Olig2-Cre+/-; Ndst1^{Flox/Flox}* mice. (A-B)** Representative images of the lesion site (delineated
858 by white dashed lines) in the corpus callosum of control (A) (n=2) and mutant (B) (n=2) mice
859 at 8 dpi illustrating the enlargement of the lesion size in mutant mice compared to control
860 mice. Olig2 (in red) is used to label oligodendrocyte lineage cells. In situ hybridization
861 revealed a marked reduction in *Ndst1* expression surrounding the lesion site in mice with
862 conditional inactivation in the oligodendroglial lineage cells (B, D, F) compared to control
863 mice (A, C, E). C and D are high magnifications of the squares in A and B respectively. E and
864 F are high magnifications of the squares in C and D respectively. Representative images of
865 10E4 immunostaining at the lesion site (delineated by white dashed lines. 8dpi) in the corpus
866 callosum of control (G) and mutant (H) mice showing a strong reduction of heparan sulfate
867 labeling in absence of *Ndst1* in oligodendrocytes. CC, corpus callosum, V, ventricle, St,
868 Striatum. Scale bars: 100 μm in A-; 20 μm in C-D. 30 μm in G-H

869

870

871 **Figure 4 –figure supplement 2. Myelin content and glial density in adult unlesioned**
872 ***Olig2-Cre+/-; Ndst1^{Flox/Flox}* mice. (A-B)** Representative images of the myelin content in the
873 corpus callosum of control (A) and *Olig2-Cre; Ndst1^{Flox/Flox}* (B) mice. (C) Quantitative
874 analysis of the myelin content by double blind scoring of PLP staining in control (n=3) and
875 mutant mice (n=3). Results are expressed in percentage of the control. (D-E) Astrocyte
876 labeling by GFAP immunofluorescence in the corpus callosum of control (D) (n=2) and
877 mutant (n=3) mouse brain (E). (G-H, J-K) Phenotype of oligodendroglia in the corpus
878 callosum of control (G, J) (n=5) and mutant (H, K) (n=5) mice by triple immunostaining for

879 Olig2/PDGFR α /CC1. **(F, I)** Quantification of mean cell density of astrocytes (GFAP+ cells)
880 (n=2 and 3) **(F)** and oligodendroglia (Olig2+ cells) **(I)** in the corpus callosum of control and
881 mutant mice (n=5 in each group). **(L)** Quantitative analysis of the percentage of Olig2+/CC1+
882 and Olig2+/PDGFR α + in the corpus callosum of control and mutant mice (n=?). No
883 significant difference was observed between the 2 groups using non-parametric Mann-
884 Whitney test (independent two group comparisons). Error bars represent S.E.M. Scale bars:
885 10 μ m.

886

887 **Figure 5. Effect of *Ndst1* deletion on microglia/macrophage activation.**

888 **(A-B)** CD68+/Ki67+ co-immunolabeling shows the proliferation status of activated
889 microglia/macrophages. **(C)** Quantification of proliferating microglia/macrophages
890 (Ki67+/CD68+ cells) in lesion sites at 4 and 8 dpi (n=3,7 control and n=3,7 mutant mice
891 respectively). Iba1 **(D-E)** and CD68 immunolabeling **(F-G)** shows the increase in rhomboid-
892 polarized microglia/macrophages in the demyelinated area of mutant mice at 8 dpi. **(H)**
893 Quantification of the ratio of rhomboid/branched CD68+ cells in lesion sites at 4 and 8 dpi
894 (n=3,4 control and n=3,6 mutant mice respectively) showing a switch of the
895 microglia/macrophage polarization in favor of the rhomboid phenotype in mutant mice at
896 8 dpi. **(I-J)** Cox2 immunolabeling shows an increase in this M1 phenotype marker at 8dpi in
897 mutant mice. **(K)** Quantification of Cox2+ cells in lesion sites at 8dpi (n=4 control and n=5
898 mutant mice). Error bars represent S.E.M. *p \leq 0.05, non-parametric Mann-Whitney test
899 (independent two group comparisons). Scale bars: 50 μ m in I-J and 10 μ m in A-B, D-E, F-G.

900

901

902 **Figure 6. AP-tagged Shh protein binds to HS concentrated around LPC-induced lesions**
903 **in the corpus callosum.** Representative images of adjacent serial coronal sections derived

904 from control mice 4 days after LPC injection and incubated with the fusion proteins AP-Shh-
905 WT (**A-B'**) or AP-Shh-CW in which the CW sequence responsible for HS binding is absent
906 (**C-D**) (n=4). The lesion site is delineated by dashed lines. Staining using B-gal is clearly
907 visible around the lesion after AP-Shh incubation (**B-B'**), while no staining is observed when
908 the AP-Shh-CW deleted protein is used (**D**). These data show that Shh is concentrated around
909 the lesion and that this distribution depends on the integrity of the HS binding motif. (E)
910 Quantification of Ptch1 expression at 8dpi in control and mutant mice reported in number of
911 dots per cell (n=4 control and n=5 mutant mice). (F-G) Illustration of Ptch1 expression in
912 peri-lesional areas in control (F) and mutant (G) mice after labeling as detected by RNAscope
913 technology. CC, corpus callosum; Cx, cortex. Scale bars: 100µm in A-D. 10 µm in F and G.

914

915 **Figure 7. NDST1 is highly expressed in MS tissue and NDST1+OLIG2+ cell density**
916 **negatively correlates with lesion size.** **(A-B)** Representative images of NDST1 staining in
917 control **(A)** and MS **(B)** WM. **(C)** Quantification of NDST1 labeling shows a significant over-
918 expression of NDST1 in MS lesions (n=9) compared to control tissue (n=4) (Kruskal-Wallis
919 test, H=13.09, n=4,9,9, p<0.01, means plus standard deviation). The colors represent paired
920 samples from the same patients. **(D-G)** Representative images of immunostaining against
921 NDST1 successively co-labelled with OLIG2+ for oligodendroglia **(D)**, GFAP+ for astrocytes
922 **(E)**, NEUN+ for neurons **(F)**, and IBA1+ for microglia/macrophages **(G)**. **(H)** Quantification
923 of the proportions of different NDST1+ cell types in normal appearing WM and various MS
924 lesions shows that NDST1 expressing cells are mainly oligodendroglia. **(I)** The proportion of
925 OLIG2+ cells which is NDST1+ is significantly increased in active lesions compared to
926 control (Kruskal-Wallis test, H=13.92, n=7,21,4,14,14 p<0.05). Overall, the majority of
927 OLIG2+ cells are NDST1+ in MS lesions and NAWM while this is not true in control brain
928 tissue. **(J)** The number of oligodendroglia expressing NDST1 is inversely correlated to lesion

929 size. **(K)** NDST1+ cell numbers positively correlate with the remyelination score assigned to
930 each patient, summing all lesions within blocks from the same MS patients (see methods).
931 NAWM, normal appearing white matter; RM, remyelinated lesion; A, active lesion; CA,
932 chronic active lesion; CI, chronic inactive lesion. Scale bars represent 50 μm (A-B) or 10 μm
933 (D-G).

934

935 **Figure 7-figure supplement1. Comparisons of Ndst1 expression levels in control and MS**
936 **brain tissue from all nuclei (A), or just oligodendroglia (B)** showing a tendency to
937 increased levels in MS samples. Data extracted from snRNA seq [37].

938

939 **Figure 7-figure supplement2. NDST1 staining is specific and no lesion belt effect is**
940 **observed in human brain. (A)** Staining with NDST1 antibody in MS WM **(B)** There is no
941 staining of MS WM with NDST1 antibody in in the presence of human recombinant NDST1.
942 **(C)** LFB stain of MS tissue with the lesion delineated in red. **(D)** Representative NDST1+
943 staining in lesion (delineated with red line) shows uniform NDST1+ cell distribution. Scale
944 bars: 100 μm .

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	Patient	Sex	Age (years)	MS type	Disease duration (years)	Time to post mortem (h)	Number of lesions	Active	Chronic active	Chronic inactive	Remyelinating
MS	MS100	M	46	SP	8	7	6	0	0	4	2
	MS121	F	49	SP	14	24	2	1	0	1	0
	MS122	M	44	SP	10	16	2	1	1	0	0
	MS136	M	40	SP	9	10	9	1	0	3	5
	MS154	F	34	SP	21	12	4	2	0	1	1
	MS176	M	37	PP	27	12	7	0	0	2	5
	MS187	F	57	SP	27	13	4	0	0	0	4
	MS207	F	46	SP	25	10	8	0	3	3	2
	MS230	F	42	SP	19	31	4	2	0	0	2
Control	CO14	M	64	-	-	26	-	-	-	-	-
	CO25	M	35	-	-	22	-	-	-	-	-
	CO28	F	60	-	-	13	-	-	-	-	-
	CO39	M	82	-	-	21	-	-	-	-	-
Total							46	7	4	14	21

948 **Table 1: Classification and characteristic of human post-mortem samples.**

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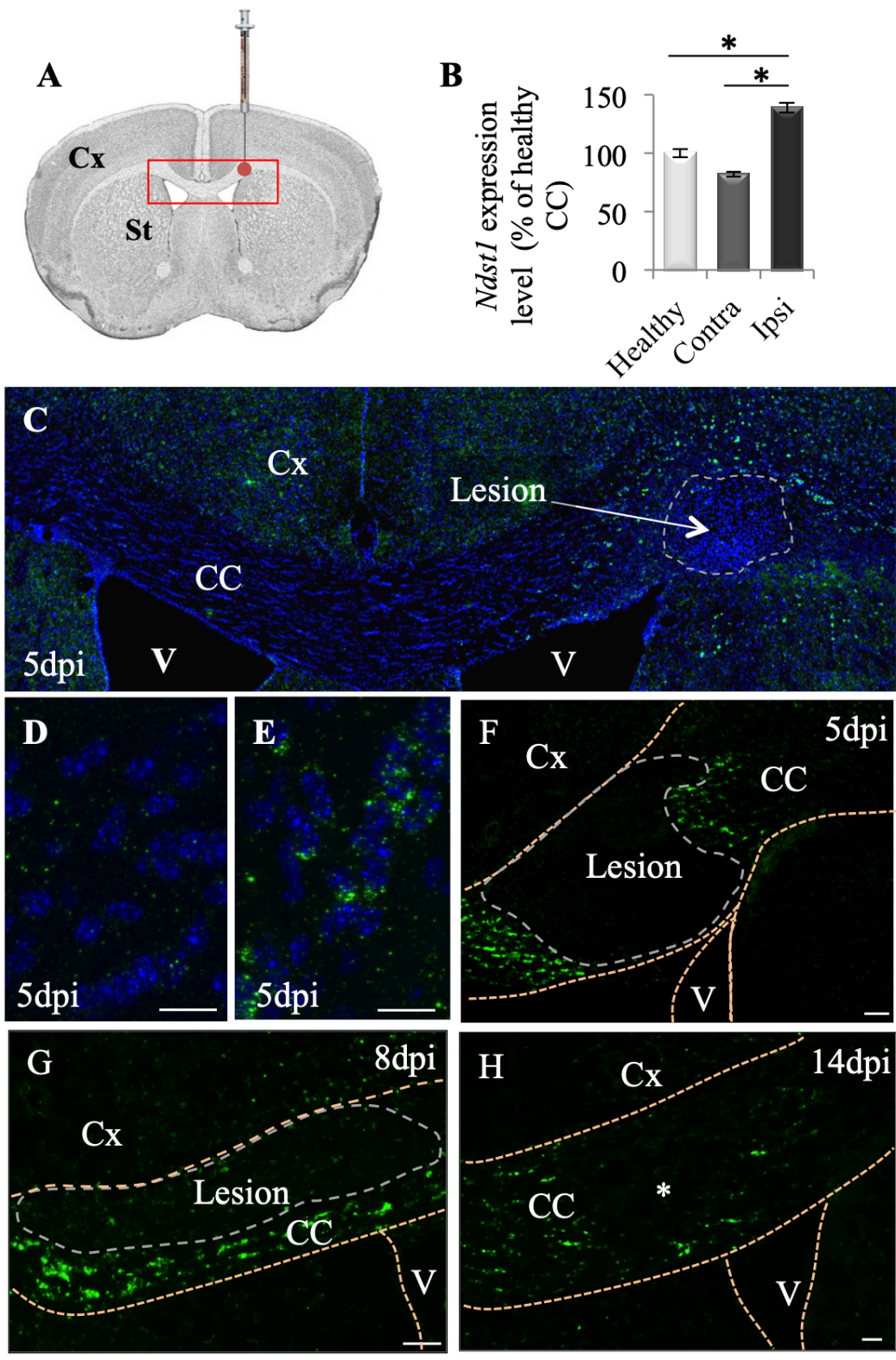


Figure 1

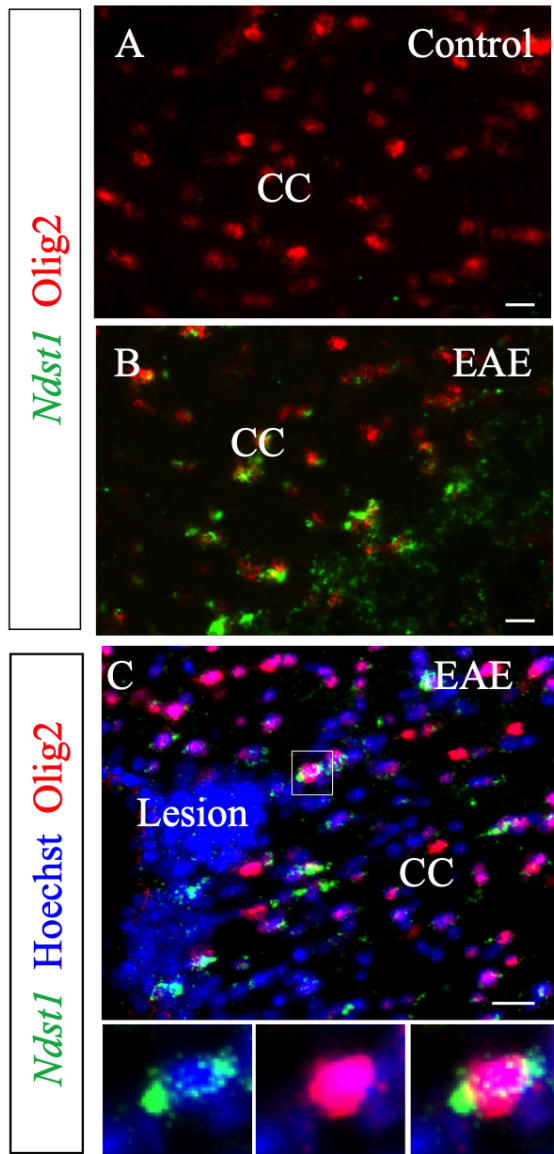


Figure1 supplement1

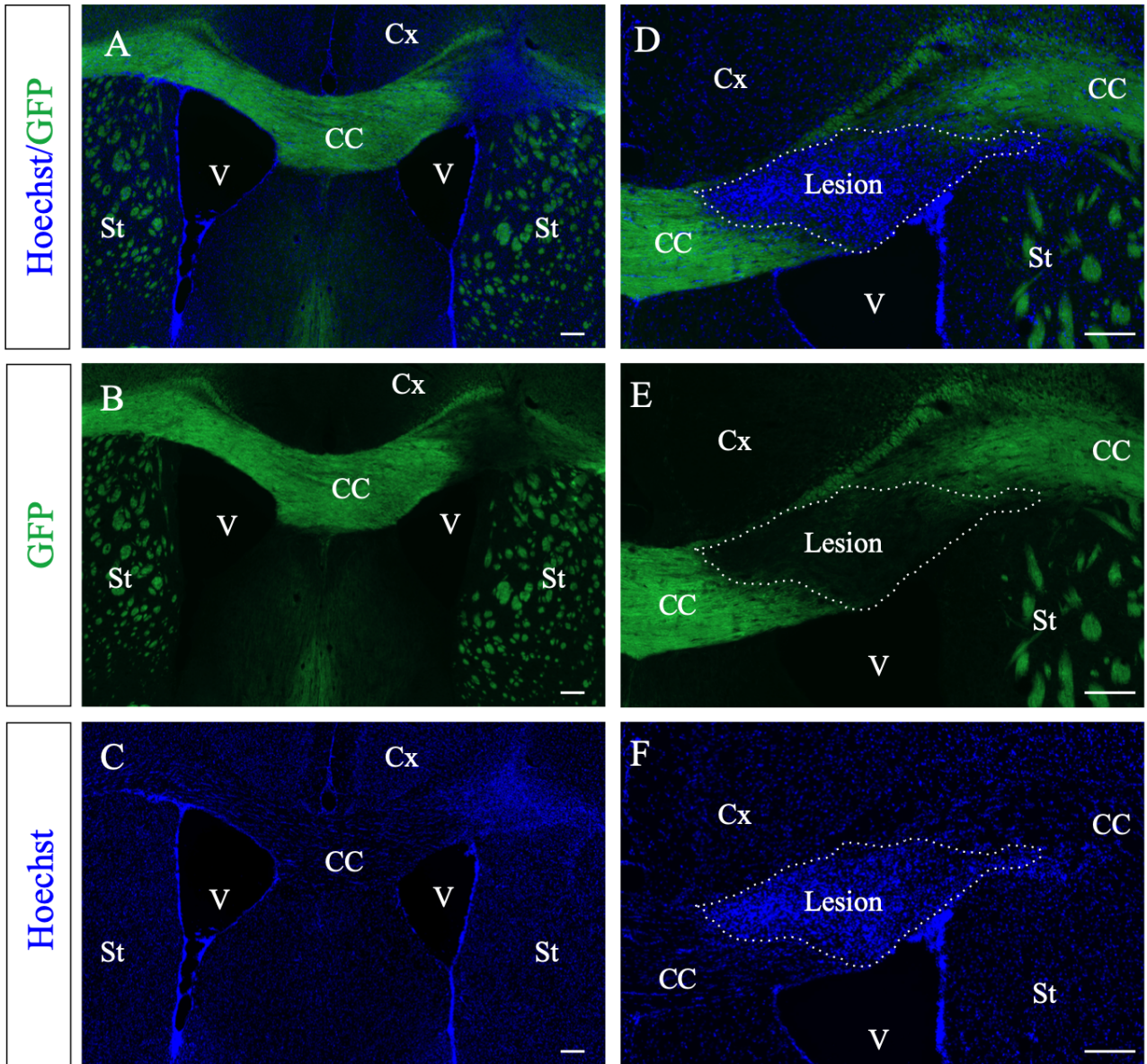


Figure1 Supplement2

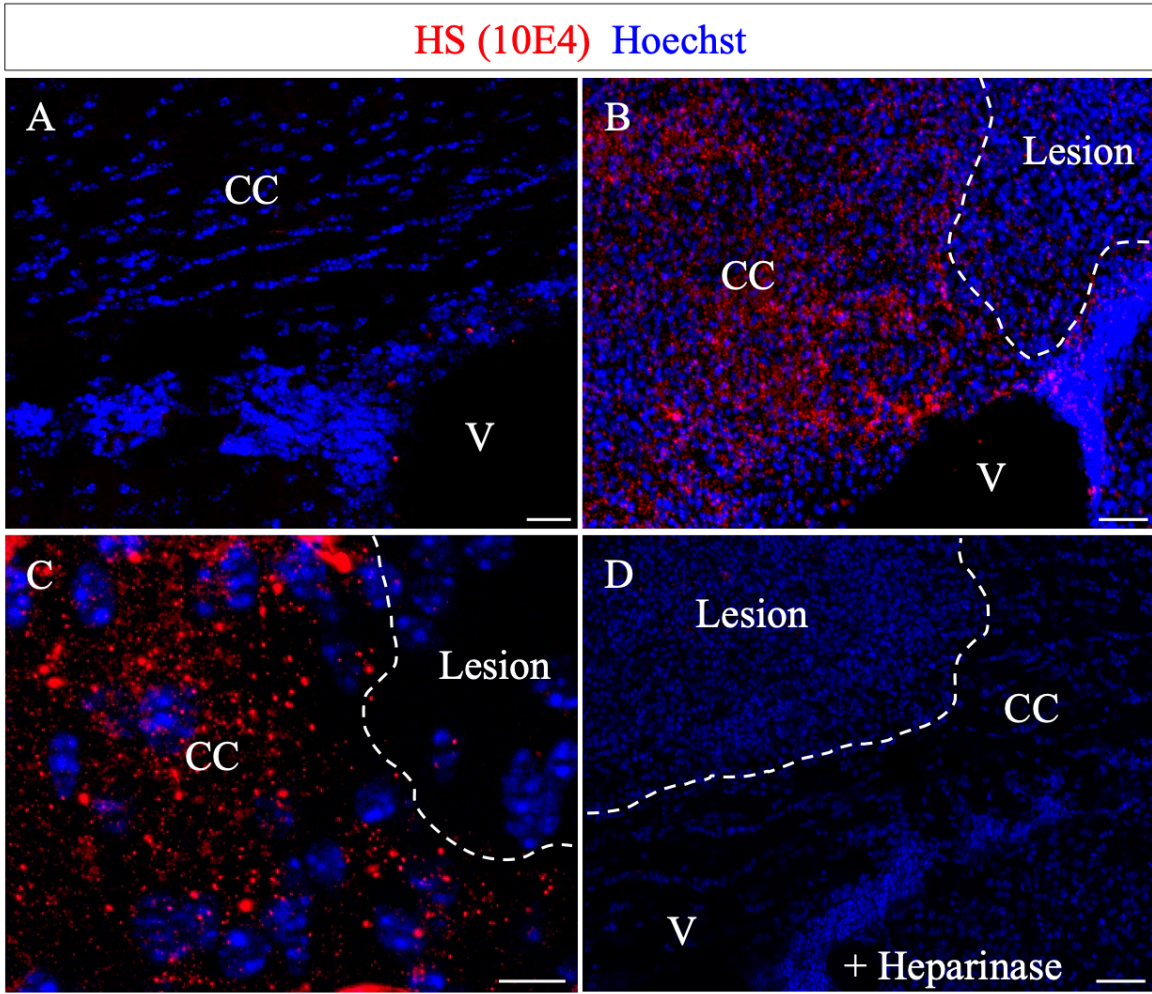


Figure 2

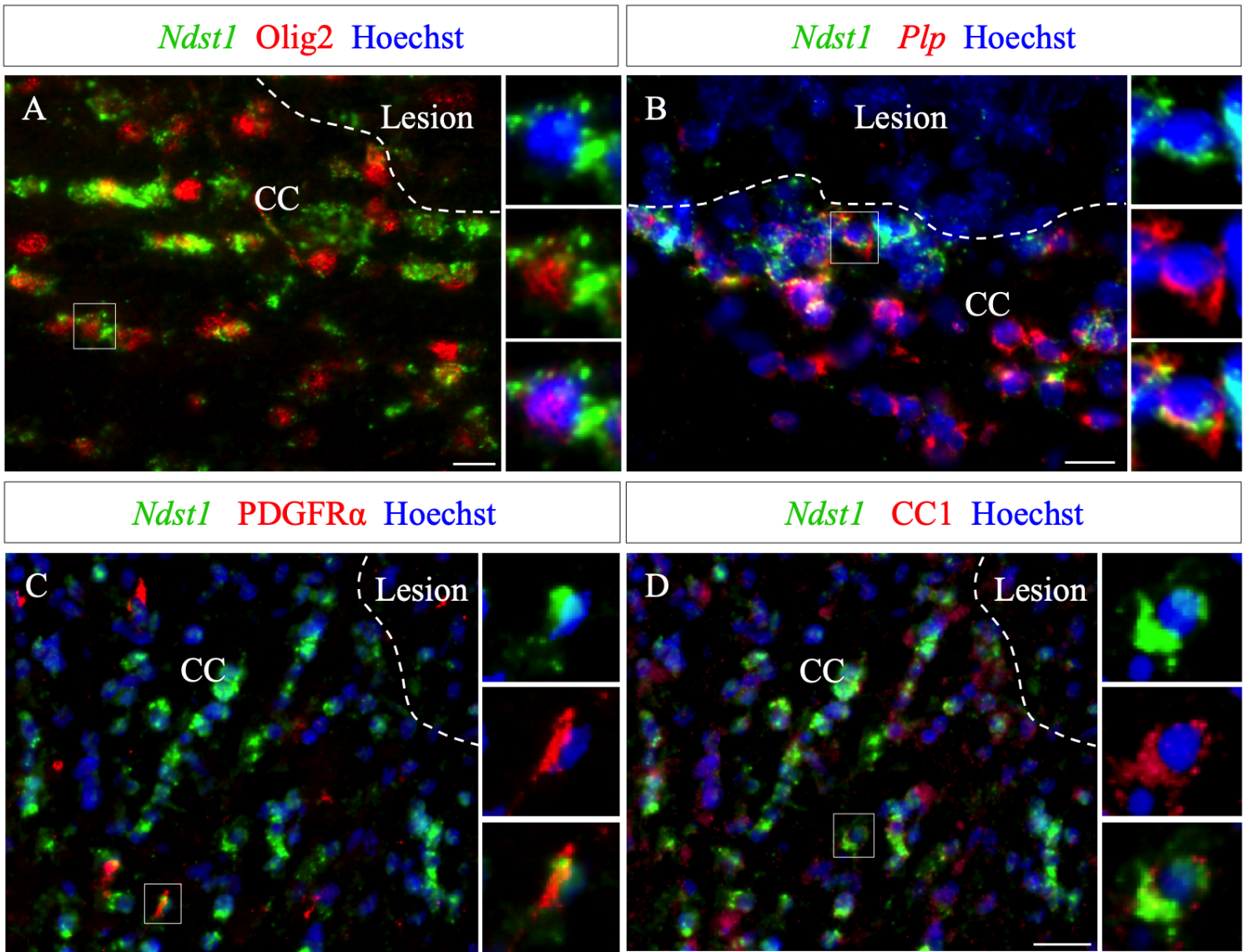


Figure 3

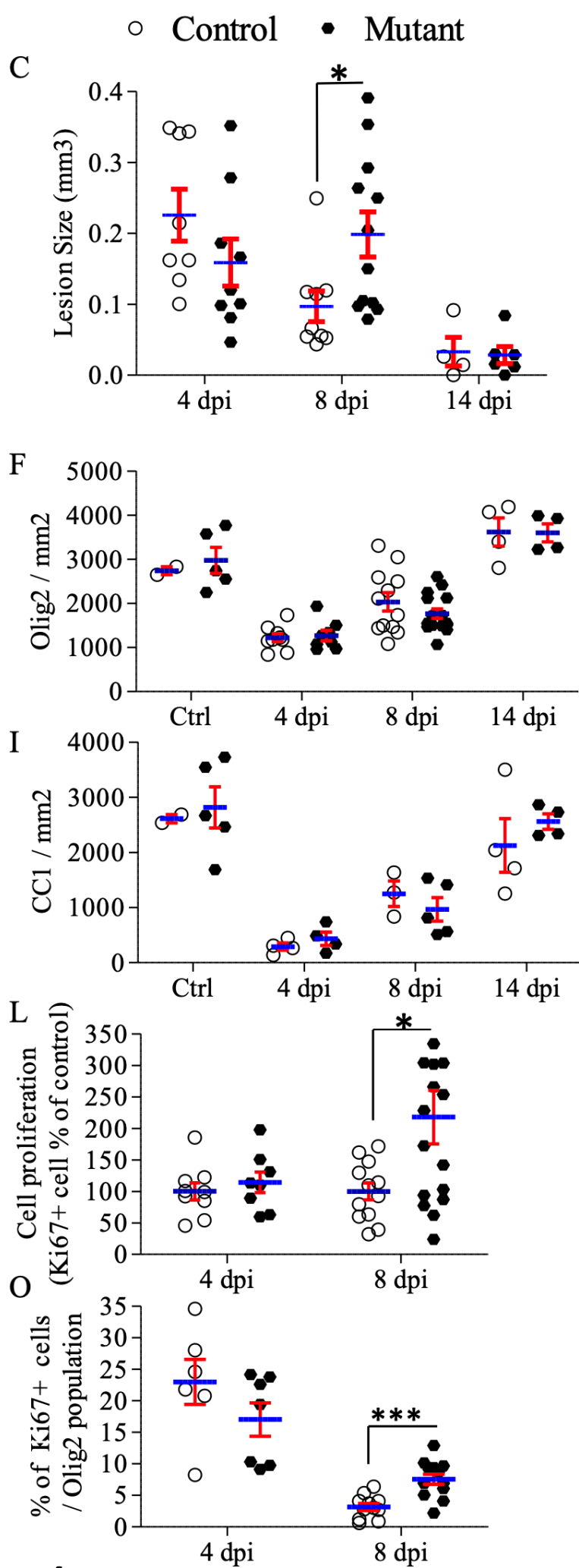
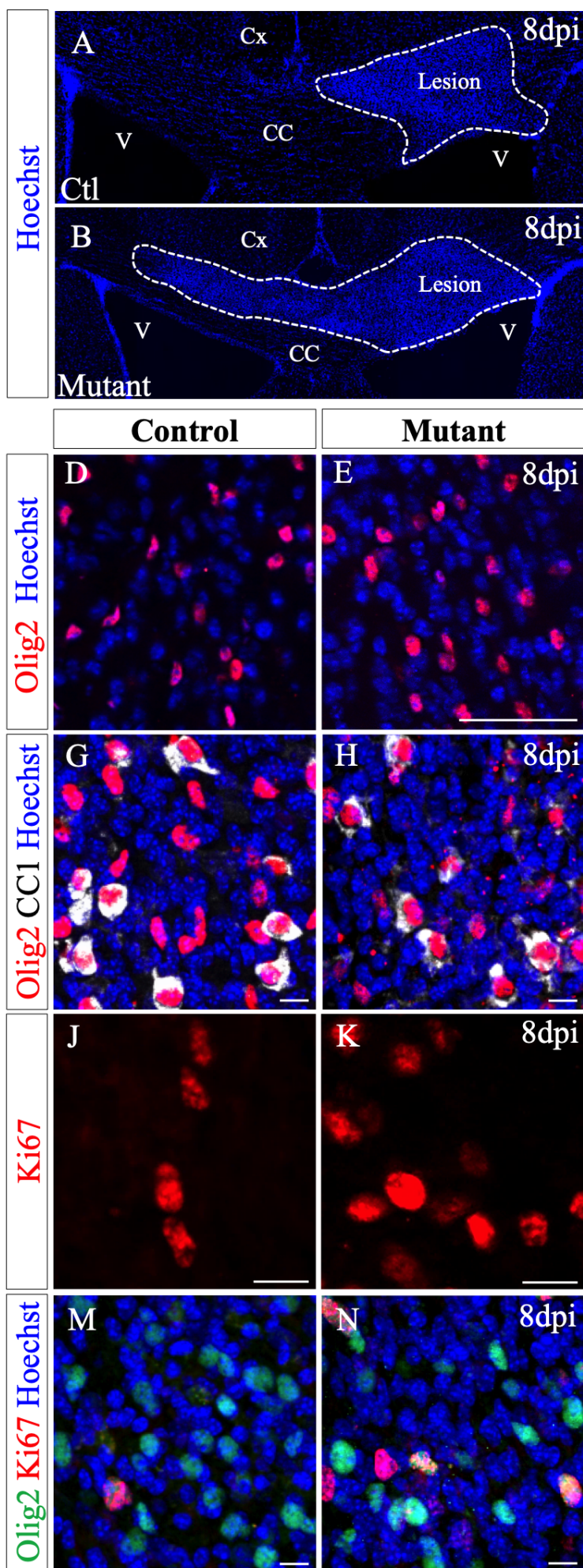


Figure 4

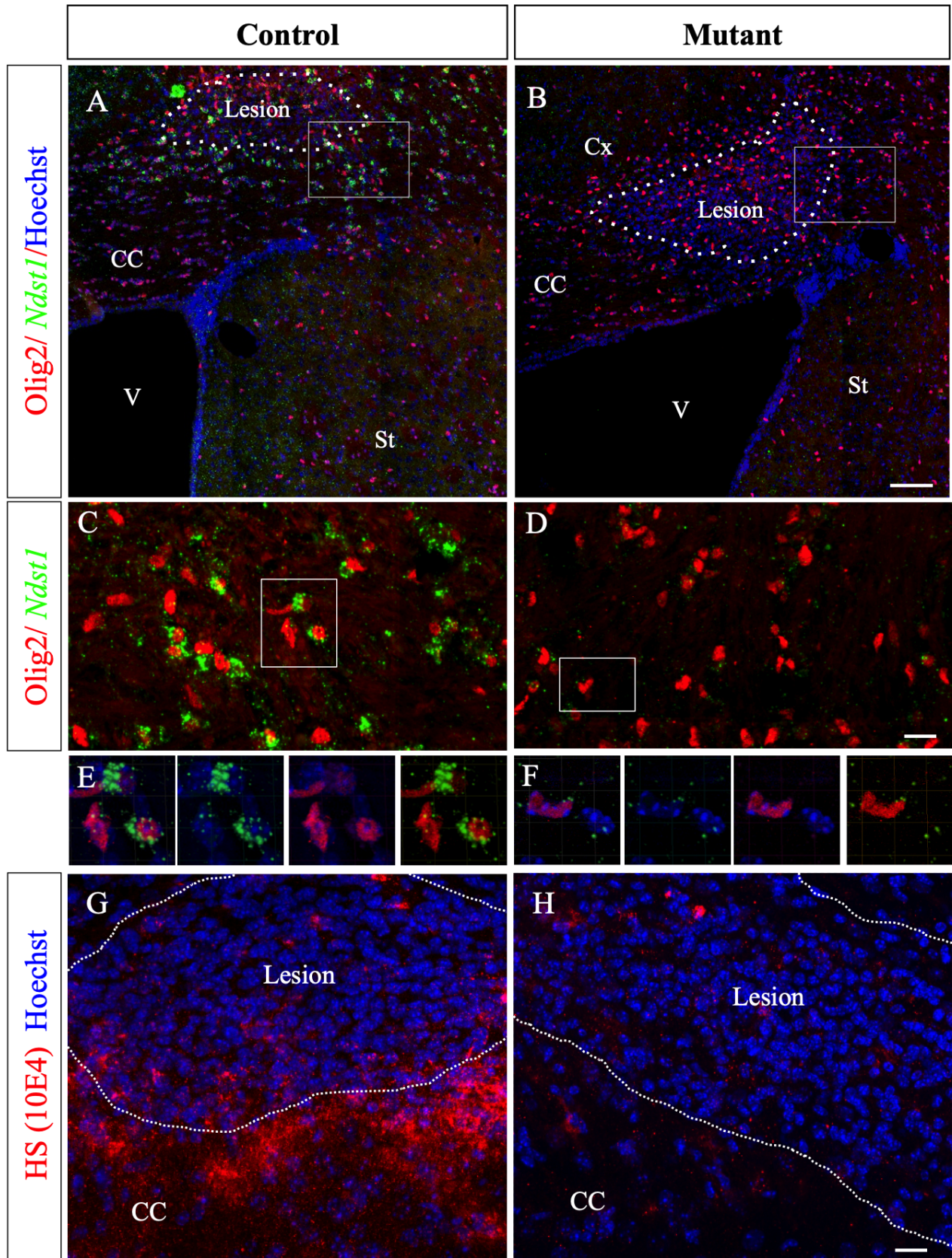


Figure 4 Supplement1

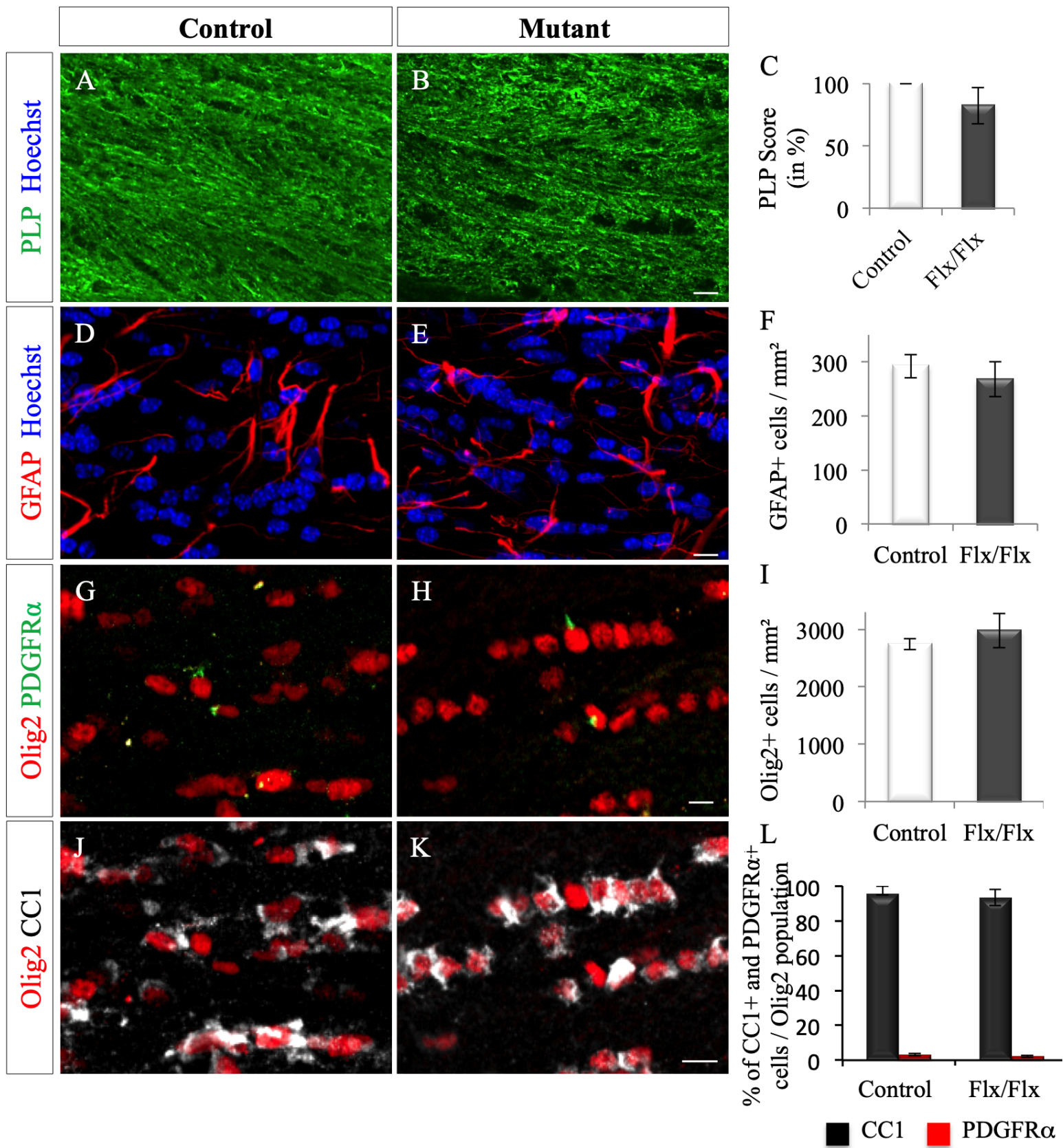


Figure 4 Supplement2

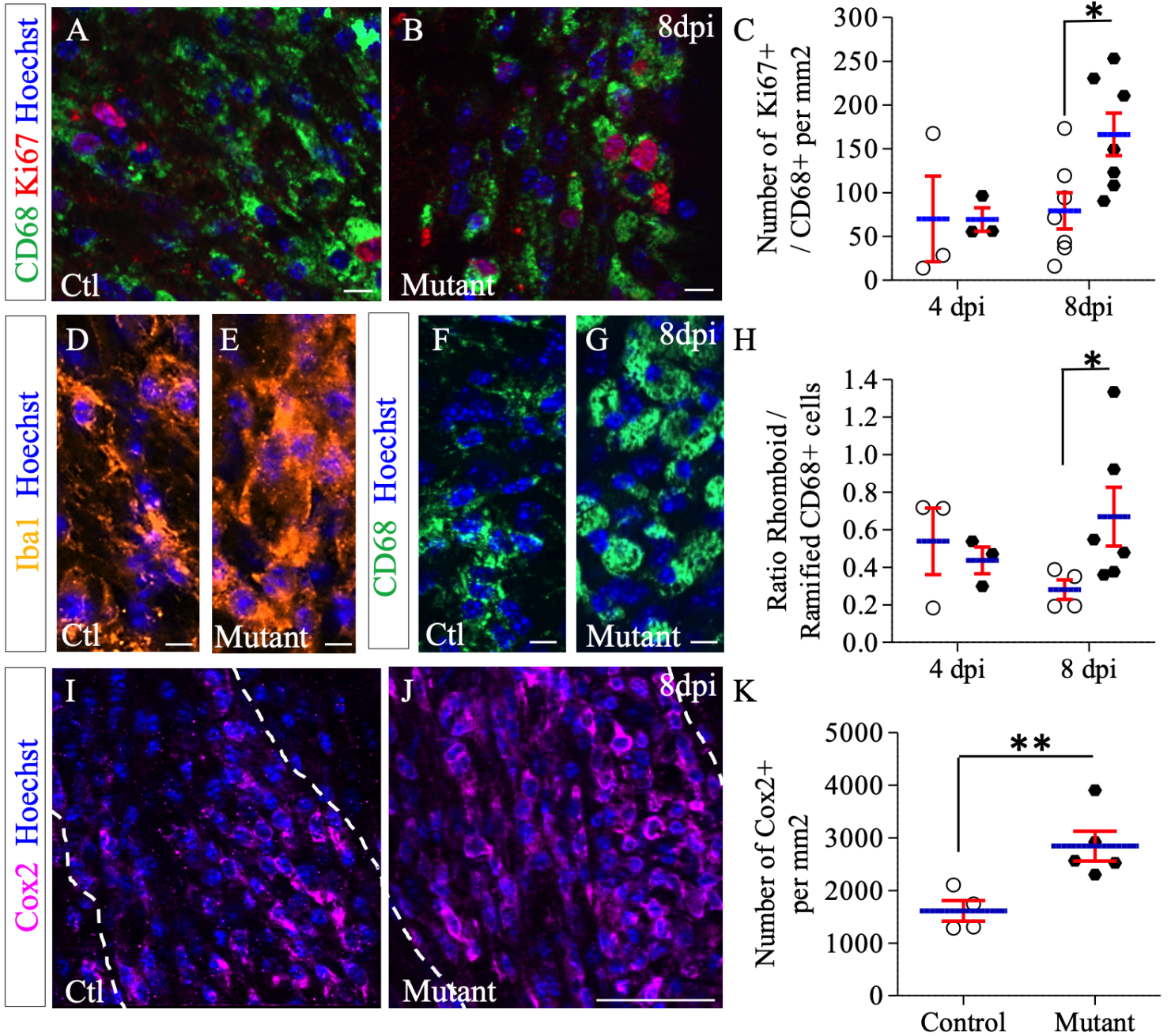


Figure 5

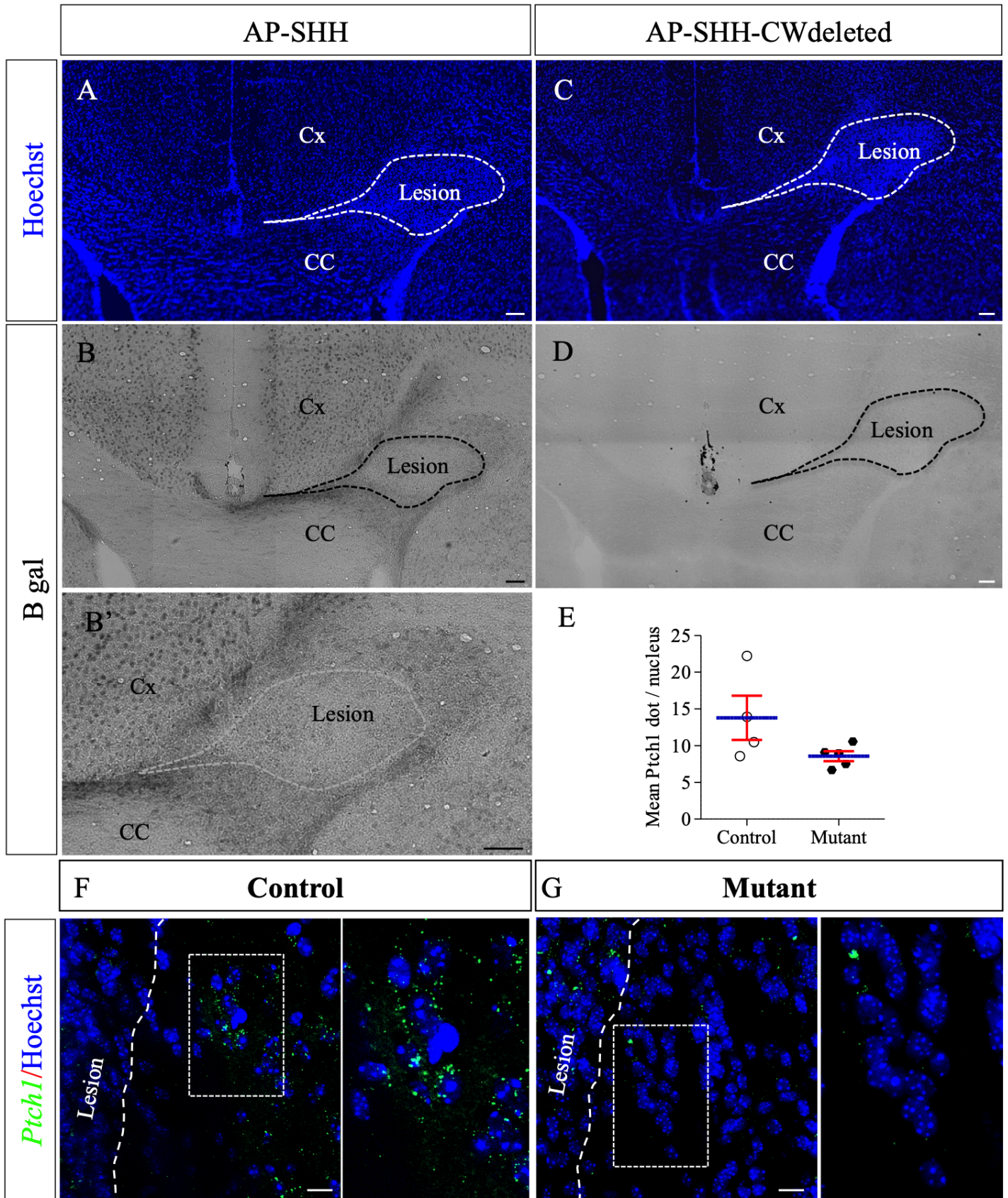
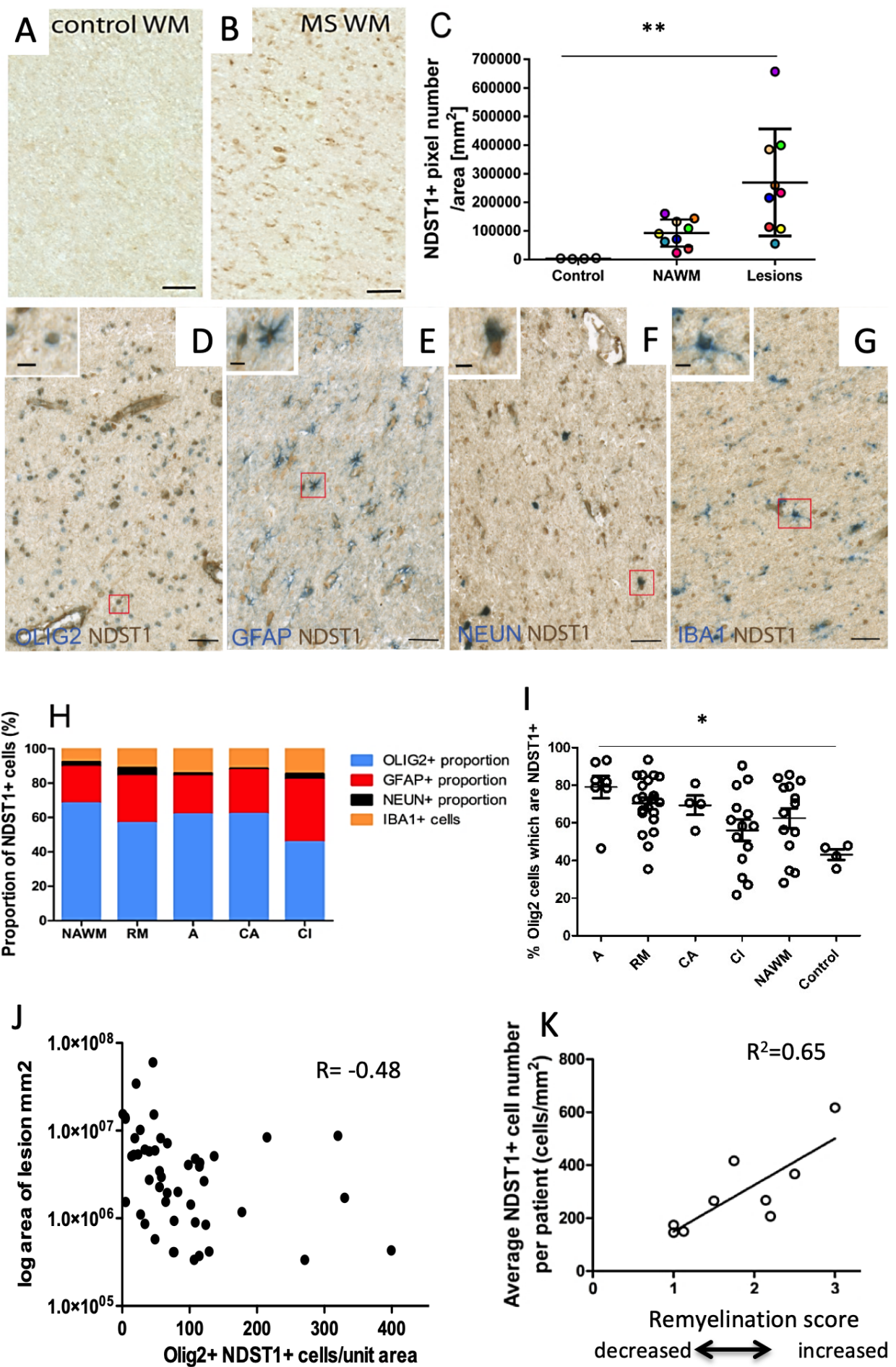


Figure 6



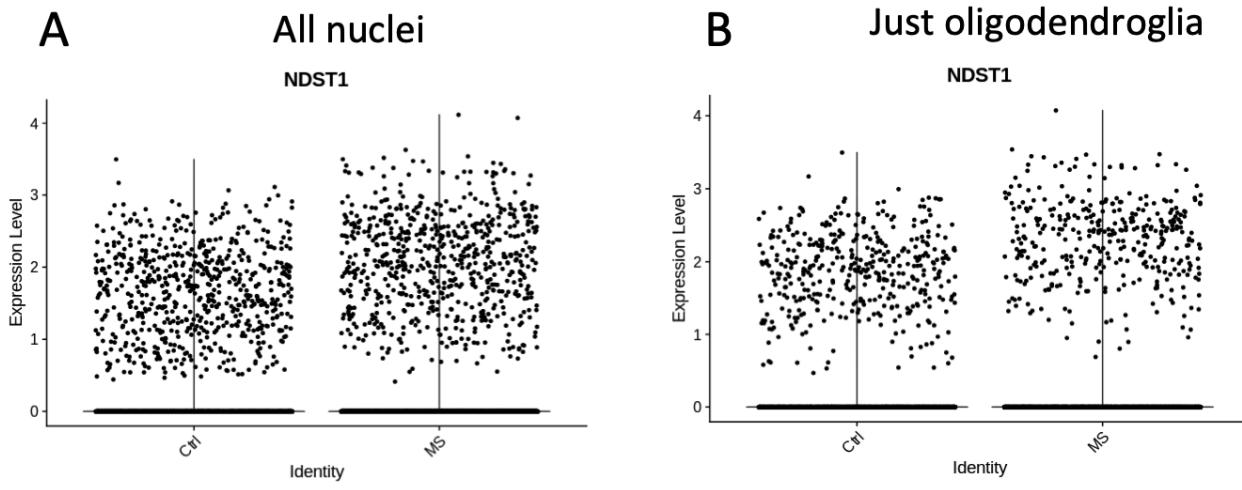
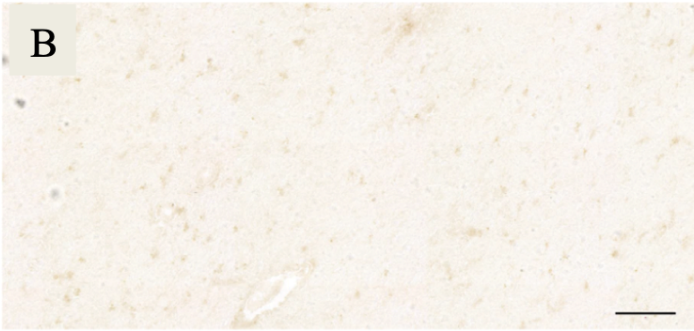


Figure7 Supplement1

NDST1



NDST1+recombinant NDST1 protein



LFB Lesion outline



NDST1 Lesion outline



Figure 7 Supplement2