

1 **Title: Age-dependent dormant resident progenitors are stimulated by injury**  
2 **to regenerate Purkinje neurons**

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19

20 **One sentence summary:** Injury induces a dormant progenitor population  
21 present at birth to regenerate cerebellar neurons in a time-dependent manner.

22

23

24 **Abstract**

25

26 Outside of the neurogenic niches of the brain, postmitotic neurons have not been  
27 found to undergo efficient regeneration. Here we demonstrate that Purkinje cells  
28 (PCs), which are born at midgestation and are crucial for both development and  
29 function of cerebellar circuits, are rapidly and fully regenerated following their  
30 ablation at birth. New PCs are produced by a previously unidentified progenitor  
31 population and support normal cerebellum development. The number of PC  
32 progenitors and their regenerative capacity, however, diminish soon after birth,  
33 and consequently PCs are poorly replenished when ablated at postnatal day 5.  
34 Nevertheless, the PC-depleted cerebella reach a normal size by increasing cell  
35 size, but scaling of neuron types is disrupted and cerebellar function is impaired.  
36 Our findings thus provide a new paradigm in the field of neuron regeneration by  
37 identifying a unipotent neural progenitor that buffers against perinatal brain injury  
38 in a stage-dependent process.

39

## 40 **Introduction**

41 Most neurons in the brain are generated at specific developmental time points,  
42 and once a neuron is postmitotic regeneration following injury is limited, except  
43 for in two forebrain regions that maintain neurogenesis (Chaker, Codega, &  
44 Doetsch, 2016). In the context of injury, adult forebrain neurons undergo limited  
45 recovery that involves either reactive gliosis (Buffo et al., 2008; Robel, Berninger,  
46 & Gotz, 2011; Sirko et al., 2013) or migration of neural stem cells from the  
47 neurogenic niches (Benner et al., 2013; Llorens-Bobadilla et al., 2015; Lopez-  
48 Juarez et al., 2013; Marti-Fabregas et al., 2010). The cerebellum (CB) of the  
49 hindbrain has a complex folded structure that houses the majority of neurons in  
50 the brain and is essential for balance and motor coordination, as well as higher  
51 order reasoning via circuits it forms throughout the forebrain (Fatemi et al., 2012;  
52 Steinlin, 2007; Tavano et al., 2007; Tsai et al., 2012; Wagner, Kim, Savall,  
53 Schnitzer, & Luo, 2017). The postnatal developing mouse CB maintains two  
54 neurogenic progenitor pools for two weeks. Interestingly, the proliferating granule  
55 cell progenitors were recently found to be replenished following injury by adaptive  
56 reprogramming of the second Nestin-expressing glial progenitors (Wojcinski et al.,  
57 2017). However, once a neurogenic process has ended, the degree to which  
58 post mitotic neurons can undergo regeneration is poorly understood.

59

60 Purkinje cells (PC) are born by embryonic day (E) 13.5 in the mouse and during  
61 weeks 10-11 in humans (Rakic & Sidman, 1970; V. Y. Wang & Zoghbi, 2001).  
62 After exiting the cell cycle in the ventricular zone, PCs express FOXP2 and

63 migrate to form a PC layer (PCL) under the cerebellar surface by E17.5, and turn  
64 on Calbindin1 (CALB1) as they mature. PCs play a central role in CB  
65 development by being the main source of sonic hedgehog (SHH), which is  
66 required for proliferation of granule cell progenitors and Nestin-expressing  
67 progenitors (Corrales, Blaess, Mahoney, & Joyner, 2006; Fleming et al., 2013;  
68 Lewis, Gritti-Linde, Smeyne, Kottmann, & McMahon, 2004). PCs also are key for  
69 CB function by integrating the inputs that converge on the cerebellar cortex  
70 (Sillitoe & Joyner, 2007). Hence, PC loss is linked to cerebellar motor behavior  
71 syndromes and has also been implicated in autism (Fatemi et al., 2012; Tsai et  
72 al., 2012; S. S. Wang, Kloth, & Badura, 2014). It is thus essential to determine  
73 the regenerative potential of PCs around birth.

74

## 75 **Results and Discussion**

76 To ablate and track PCs, the diphtheria toxin receptor (DTR) and a lineage  
77 tracer, TdTomato (TdT), were expressed in PCs (*Pcp2*<sup>Cre/+</sup>; *R26*<sup>LSL-DTR/LSL-TdT</sup> or  
78 *PC-DTR* mice; LSL=lox-stop-lox). At postnatal day (P) 1, 53.1 ± 22.6% of PCs  
79 (n=5 mice) expressed TdT, and all TdT+ cells expressed CALB1 and DTR  
80 (Figure 1\_Supplement 1). Strikingly, when DT was injected at P1 into *PC-DTR*  
81 pups (P1-*PC-DTR*), nearly all TdT+ PCs formed an ectopic inner layer by 1 day  
82 post injection (dpi) (Figure 1A-M). The ectopic layer was absent by P8 (Figure  
83 1K), and TdT+ cells in the ectopic layer were TUNEL positive starting at P3 with  
84 a peak at P5, indicating that almost all DTR-expressing TdT+ cells became  
85 misplaced, died and were cleared within 5-7 dpi of DT (Figure 1N,O).

86 Unexpectedly, although the number of CALB1+ PCs in the PCL of P1-*PC-DTR*  
87 mice was significantly reduced at P2 compared to non-injected controls (No DT),  
88 it was not reduced at P3 and later stages (Figure 1P). Furthermore, the total  
89 number of PCs (ectopic layer + PCL) was significantly greater in DT-injected  
90 cerebella than in No DT controls at P2 and P3, and the total number of PCs was  
91 down to normal levels at P5, overlapping with the time of clearance of the ectopic  
92 layer (Figure 1P). The number of TdT+ cells in the PCL remained significantly  
93 lower in P1-*PC-DTR* brains at P30 compared to No DT controls (Figure 1Q).  
94 Consistent with the rapid recovery of PC numbers in the PCL, no significant  
95 decrease in the area of the CB on sections was observed at between P1.5 and  
96 P30 (Figure 1R-T Figure1\_supplement 2), or in the thickness of the outer  
97 (proliferating) and inner (differentiating) external granule cell layers  
98 (Figure1\_supplement 3). In summary, we uncovered that the CB can rapidly  
99 recover (within 24h) from the loss of ~50% of PCs at P1, by producing new PCs  
100 and resuming normal growth.

101

102 In order to document the rapid production of new PCs after ablation, we tested  
103 whether PCs that had recently undergone cell division could be detected at P3.  
104 P1-*PC-DTR* mice were divided into 4 groups; each group receiving three  
105 injections of BrdU (2h apart) during 4-26h after DT-injection (Figure 2A). Indeed,  
106 BrdU+ cells were observed in the PCL of all groups (Figure 2B), and apart from  
107 astrocytes and microglia (Figure2\_supplement 1), all were PCs (FoxP2+ and  
108 CALB1+), with the greatest incorporation being between 10-20h after DT (Figure

109 2B,E,F). Importantly, no BrdU incorporation was observed in PCs in No DT mice  
110 (Figure 2C,D). Furthermore, a lack of BrdU incorporation in the ectopic layer  
111 confirms that the labeling is not due to DNA damage induced by DT-mediated  
112 cell death (Figure 2E,F). These results reveal that a progenitor capable of  
113 proliferating produces the new PCs after ablation at P1.

114

115 Based on the rapid response to ablation, we hypothesized that local progenitors  
116 in the PCL are responsible for the PC replenishment. The population of Nestin-  
117 expressing progenitors in the PCL was a candidate, as they display plasticity  
118 upon ablation of granule cell precursors in newborn mice (Wojcinski et al., 2017).  
119 Furthermore a putative rare Nestin+ cell in the adult CB was recently described  
120 to produce new neurons in response to exercise (Ahlfeld et al., 2017). However,  
121 when we tested the contribution of Nestin-expressing progenitors to PC  
122 regeneration using a *Nes-CFP* reporter allele that transiently maintains CFP  
123 protein after differentiation, no CFP+ cells were found to co-express FOXP2 or  
124 CALB1 at 12h and 2 days post DT injection in P1-PC-DTR mice and in No DT  
125 controls (Figure2\_supplement 2). These results suggested that another  
126 progenitor population mediates regeneration following PC depletion.

127

128 We next examined whether a progenitor exist after birth that express early  
129 (FOXP2) but not late (CALB1) PC markers. Indeed, at P1 we detected cells in  
130 the PCL that expressed FOXP2 but showed only low or no CALB1 expression  
131 (named FEPs for FOXP2-expressing progenitors; Figure 3A-B). Furthermore,

132 temporal analyses revealed a steady decrease in the number of FEPs from P1  
133 ( $74.33 \pm 5.69$ /midline sagittal section) to P5 ( $28.66 \pm 7.51$ /midline sagittal section,  
134 Figure 3A,C), revealing the progenitors are a transient population. Interestingly,  
135 the few FEPs present at P5 were specifically enriched in the central and nodular  
136 zones of the CB, which are developmentally delayed at P5 (Legue, Riedel, &  
137 Joyner, 2015; Sudarov & Joyner, 2007)(Figure 3A). In addition, FEP numbers  
138 significantly increased 12 hours after DT injection in P1-*PC-DTR* mice ( $1.90 \pm$   
139  $0.05$ -fold, Figure 3D), indicating they expand upon injury as the expansion  
140 correlates with the highest BrdU incorporation time window we observed after  
141 injury (Figure 2B). In addition, using *FoxP2*<sup>Fipo/+</sup>; *R26*<sup>FSF-TdT/+</sup> (FSF=frt-stop-frt)  
142 mice in which all PCs and FEPs express TdT at P1 (Figure3\_supplement 1), an  
143 increase in transiently fate mapped TdT+ FEPs was observed 12 hours after DT  
144 injection at P1 ( $1.86 \pm 0.46$ -fold, n=3, Figure3\_supplement 2). Surprisingly, at P5  
145 the number of FEPs was significantly lower in P1-*PC-DTR* animals than in No DT  
146 mice (Figure 3D), possibly reflecting an exhaustion of the progenitor population  
147 by production of new PCs.

148

149 To confirm that FEPs undergo proliferation upon PC depletion, we injected BrdU  
150 10-14h after DT and collected cerebella 1h (~P1.5) later. All BrdU+ PCs in the  
151 PCL of P1-*PC-DTR* mice expressed FOXP2, but only  $45.5 \pm 1.1\%$  expressed  
152 CALB1 (Figure 3E, Figure3\_supplement 3). In addition, Ki67+ FoxP2+ cells were  
153 detected at P1.5 in the PCL of P1-*PC-DTR* pups (Figure3\_supplement 3C),  
154 confirming the presence of proliferative FEPs following PC ablation. Furthermore,

155 the total number of BrdU+ PCs in the PCL was the same at P1.5 ( $38.7 \pm$   
156  $9.1/\text{section}$ ,  $n=3$ ) and at P3 ( $40.3 \pm 19.0/\text{section}$ ,  $n=3$ ). Collectively, our data  
157 argues that the recovery of PCs in P1-PC-DTR mice is mediated by a previously  
158 unrecognized dormant (Ki67- and not labeled with BrdU) and age-dependent  
159 progenitor population (FEPs) that proliferates and differentiates into PCs in  
160 response to injury.

161

162 Given that the population of FEPs is greatly reduced by P5 (Figure 3C), PCs  
163 should not be efficiently replaced when ablated at P5. Indeed, when DT was  
164 injected at P5 (P5-PC-DTR mice) (Figure 4\_supplement 1A), the numbers of PCs  
165 were significantly reduced by P12 compared to No DT controls (Figure  
166 4\_supplement 1B-I, R). TdT+ PCs were TUNEL+ by P8 (Figure 4\_supplement  
167 1J-K) and the majority of TdT+ cells were cleared from the PCL by P12 (Figure  
168 4\_supplement 1G, P and S). Furthermore, PCs had abnormal dendrites at P8  
169 and P12 (Figure 4\_supplement 1B-G and L-P) and some PCs had misplaced  
170 somas at P30 (Figure 4\_supplement 1N-R). Interestingly, the reduction in PCs  
171 numbers observed by P12 was maintained at P30 (Figure 4\_supplement 1R),  
172 such that the number of PCs was reduced by  $32.4 \pm 6.5\%$ . In summary, there is  
173 little replenishment of PCs when they are ablated at P5 (Figure 4A).

174

175 We next tested whether the rare FEPs at P5 (Figure 3A,C) can still proliferate  
176 upon PC depletion. Unlike in P1-PC-DTR mice, very few BrdU+ FEPs were  
177 detected in P5-PC-DTR cerebella injected with BrdU at 10-14h post DT-injection



178 at both 1h ( $5.55 \pm 0.51$ / midline sagittal section, n=3) and 1.5 days ( $6.22 \pm 1.07$ /  
179 midline sagittal section, n=3, Figure 4B) post BrdU-injection and the few BrdU+  
180 FEPs were concentrated in the central and the nodular zones enriched for FEPs  
181 at P5 (Figure 4\_supplement 2). Interestingly, compared to P1-*PC-DTR* mice in  
182 which  $52.29 \pm 0.09$  % (n=3) of FEPs incorporated BrdU, only  $20.55 \pm 0.07$  %  
183 (n=3) incorporated BrdU in P5-*PC-DTR* animals. Overall, these results  
184 demonstrate that replenishment of PCs is not efficient at P5 because with age,  
185 FEPs both diminish in number and in their ability to proliferate in response to PC  
186 depletion.

187

188 We next examined whether the depletion of PCs in P5-*PC-DTR* mice had an  
189 effect on CB development. Indeed, the area of CB sections was significantly  
190 reduced at P12 but not P8 (Figure 4C), and the thickness of the external granule  
191 cell layer was significantly reduced in P5-*PC-DTR* mice at P8, but by P12 the  
192 decrease was diminished (Figure4\_supplement 4A-E). However, despite the lack  
193 of recovery of PC numbers the reduction in CB size became less pronounced  
194 with age and by P30 the area of the CB was normal (Figure 4C,  
195 Figure4\_supplement 3A-I). As a consequence there was a reduction in PC  
196 density compared to No DT or to P1-*PC-DTR* mice (Figure4\_supplement 3J,  
197 Figure4\_supplement 2N,Q). The density of granule cells also was lower  
198 compared to No DT and P1-*PC-DTR* P30 mice (Figure4\_supplement 4F).  
199 Interestingly, PCs in P5-*PC-DTR* mice had a larger soma (Figure 4D) and longer  
200 primary and secondary dendrites (Figure 4E) compared to No DT or P1-*PC-DTR*

201 mice, a cellular phenotype observed in some mouse mutants with PC loss  
202 (Castagna, Merighi, & Lossi, 2016). Furthermore, compared to controls, the  
203 percentage of PCs that survived in *P5-PC-DTR* animals (~66% of No DT  
204 controls) did not match the percentage of granule cells that were produced  
205 (~81% of No DT controls). Thus, the ratio of PCs to granule cells that is important  
206 for proper CB circuitry formation is disrupted in *P5-PC-DTR* animals because  
207 granule cells are over-produced. These results reveal that independent of  
208 producing new PCs following their ablation, there are mechanisms of cell and  
209 organ size regulation that ensure recovery of CB size.

210

211 Finally, we tested whether P30 *P1-PC-DTR* or *P5-PC-DTR* mice recover motor  
212 function following recovery of PC numbers and CB size or only size, respectively.  
213 Remarkably *P1-PC-DTR* animals had no significant changes in their motor  
214 function compared to controls (Figure 4F-J). In contrast, compared to both No DT  
215 and *P1-PC-DTR* mice, *P5-PC-DTR* mice showed a significant reduction in their  
216 latency to fall from the rotarod and had a shorter stride (Figure 4F-G and I-J) with  
217 no change in grip strength (Figure 4H), demonstrating a motor behavior deficit.  
218 Thus, rapid production of new PCs by FEPs enables functional recovery  
219 following depletion of PCs at P1. Furthermore, achieving correct cell numbers  
220 and/or proportions are more important than maintaining CB size for functional  
221 recovery after injury in *P5-PC-DTR* mice.

222

223 In summary, we discovered a new regenerative process in the developing CB  
224 involving a previously unidentified and normally dormant progenitor population  
225 (FEPs) that acts as a developmental back-up population to buffer against early  
226 postnatal loss of postmitotic neurons. Proliferation of FEPs is stimulated by  
227 ablation of PCs at P1 and importantly the response is rapid (~24h), ensuring  
228 other components of the developing CB that are dependent on PCs for their  
229 expansion or differentiation are not compromised. However, FEPs decrease in  
230 number and capacity to regenerate during the first postnatal week, and  
231 consequently PCs are poorly replenished when ablated at P5. The cerebella of  
232 *P5-PC-DTR* mice nevertheless try to adapt by attaining near normal dimensions  
233 through a mechanism that includes increasing cell size (Figure4\_supplement 5).  
234 The CB therefore has multiple mechanisms for regulating organ size following  
235 perinatal injury that depend on the precise stage of development. Furthermore,  
236 the motor deficits seen in *P5-PC-DTR* mice highlight the importance of  
237 maintaining the correct number of PCs during development, not just organ size.

238

239 The regenerative processes previously described in neuronal tissues involve  
240 adaptive reprogramming of cells that are either actively proliferating or retain  
241 proliferative capacity and also have cell fate plasticity (Benner et al., 2013; Buffo  
242 et al., 2008; Jinnou et al., 2018; Lin et al., 2017; Llorens-Bobadilla et al., 2015;  
243 Lopez-Juarez et al., 2013; Marti-Fabregas et al., 2010; Robel et al., 2011;  
244 Samanta et al., 2015; Sirko et al., 2013; Wojcinski et al., 2017). Here we  
245 identified a distinct regenerative process that involves a local, unipotent and

246 dormant progenitor. Unlike Nestin-expressing progenitors of the CB or astrocytes  
247 and neural stem cells in the forebrain that produce neurons upon injury, FEPs do  
248 not require reprogramming and cell fate plasticity as they instead maintain their  
249 lineage and proliferate and mature upon injury. An important question raised by  
250 our study is whether regeneration of postmitotic neurons by age-dependent  
251 unipotent progenitors is unique to the CB, where protracted development might  
252 provide a conducive milieu, or whether all brain regions retain similar progenitors  
253 after each neuron subtype is generated. Furthermore, understanding the  
254 mechanisms of PC regeneration in newborn mice should provide insights that  
255 could enable regeneration in the adult brain.

256

257

## 258 **Acknowledgements**

259

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267

## 268 **Materials and Methods**

### 269 *Animals*

270 All the experiments were performed according to protocols approved by the  
271 Memorial Sloan Kettering Cancer Center's Institutional Animal Care and Use

272 Committee (IACUC). Animals were given access to food and water *ad libitum*  
273 and were housed on a 12-hour light/dark cycle.

274 The following mouse lines were used for these experiments: *Pcp2<sup>Cre</sup>* (Zhang et  
275 al., 2004), *Nestin-CFP*(Mignone, Kukekov, Chiang, Steindler, & Enikolopov,  
276 2004; Wojcinski et al., 2017), *FoxP2<sup>Fipo</sup>*(Bikoff et al., 2016), *Rosa26<sup>LSL-DTR</sup>* (Stock  
277 no: 007900, The Jackson Laboratories)(Buch et al., 2005), *Rosa26<sup>LSL-TdT</sup>* (*ai14*,  
278 Stock no: 007909, The Jackson Laboratories)(Madisen et al., 2010), *Rosa26<sup>FRT-</sup>*  
279 *STOP-FRT-TdT* derived from *ai65* (Stock no: 021875, The Jackson  
280 Laboratories)(Madisen et al., 2015). Both sexes were used for analyses and no  
281 randomization was used. Exclusion criteria for experimental data points were  
282 sickness or death of animals during the testing period. No randomization was  
283 used and masking was used only for the behavior studies where the  
284 experimenter was blind to the genotypes.

285 Diphtheria toxin (30 µg/g of mouse; List Biological Laboratories, INC) was  
286 injected subcutaneously either at postnatal day (P) 1 or P5 and the brains were  
287 collected at various ages (Fig. 1a and Fig. 5a). Mice not given DT (No DT mice)  
288 were *Pcp2<sup>Cre/+</sup>*; *R26<sup>DTR/LSL-TdT</sup>* littermates and injected with the same volume of  
289 vehicle (PBS).

290 BrdU (50 µg/g of mouse; Sigma) was injected subcutaneously.

291

### 292 *Tissue Preparation and Histological Analysis*

293 For P5 and younger animals, brains were dissected and fixed in 4%  
294 paraformaldehyde (PFA) for 24-48 hours (h) at 4°C. Animals older than P5 were

295 anesthetized using intraperitoneal injection of a Ketamine (100 mg/kg) and  
296 Xylaxine (10 mg/kg) cocktail. Once full anesthesia was achieved, animals were  
297 systemically perfused with ice-cold PBS, followed by 4% PFA. Brains were  
298 dissected and post-fixed in 4% PFA for 24-48h. Fixed brains were allowed to sink  
299 in 30% Sucrose in PBS solution and then embedded in OCT (Tissue-Tek) for  
300 cryosectioning. 14µm-thick cryosections were obtained using a Leica cryostat  
301 (CM3050S) and mounted on glass slides. Frozen sections were stored at -20°C  
302 for future analysis.

303 For immunofluorescent (IF) analysis, slides were allowed to warm to room  
304 temperature (RT). After washing once with PBS, slides were blocked using 5%  
305 Bovine Serum Albumin (BSA, Sigma) in PBS-T (PBS with 0.1% Triton-X) for 1h  
306 at RT. Slides were then incubated overnight at 4°C with primary antibodies  
307 diluted in blocking buffer. **Figure1\_source data 1.** summarizes the primary  
308 antibodies used in this study. Upon primary antibody incubation, slides were  
309 washed with PBS-T (3x5 minutes), incubated with specific AlexaFluor-conjugated  
310 secondary antibodies (1:500 in blocking buffer, Invitrogen) for 1h at RT and then  
311 washed again with PBS-T (3x5 minutes). Counterstaining was performed using  
312 Hoechst 33258 (Invitrogen) and the slides were mounted with Fluoro-Gel  
313 mounting media (Electron Microscopy Sciences).

314 Haematoxyline and Eosin (H&E) staining was performed to assess cerebellar  
315 cytoarchitecture and measure area (size).

316

317 *Image Acquisition and Analysis*

318 Images were collected either with a DM6000 Leica microscope or Zeiss LSM 880  
319 confocal microscope and processed using ImageJ Software (NIH).  
320 For each quantification, 3 midline parasagittal sections/brain were analyzed and  
321 data was averaged. Cells were counted using the Cell Counter plugin for ImageJ  
322 (NIH). Analyses of PC and FEPs numbers were performed by counting all of the  
323 PCs on a midline parasagittal section. CB area was calculated by defining a  
324 region of interest by outlining the perimeter of the outer edges of the CB, using  
325 ImageJ. EGL thickness was calculated by dividing the area of the EGL by the  
326 length of the EGL in midline sections. IGL density was calculated by counting the  
327 number of nuclei in three 40x fields from lobule 8 in three midline sections and by  
328 dividing the number by the area of the region counted. PC soma size and  
329 dendrite length were calculated using randomly distributed TdT+ PCs from 3  
330 midline sections (>20 cells/section). Soma area is calculated by outlining the  
331 perimeter of the outer edges of each cell. Cells that show primary dendrites were  
332 used for this analysis to ensure that the region where the maximum soma area is  
333 observed is used for the analyses. For dendrite length quantifications, primary  
334 and secondary dendrite length was measured and summed and PCs around the  
335 base of fissures were omitted.

336

### 337 *Behavioral Testing*

338 5-week old animals (No DT: n=17, DT@P1: n=9 and DT@P5: n=11) were used  
339 to assess differences in motor behavior. The same sets of mice were used for all  
340 three tests described below.

341 *Rotarod:* An accelerating rotarod (47650; Ugo Basile) was used for these  
342 experiments. Animals were put on the rod, and allowed to run till the speed  
343 reached to 5 rpm. Then the rod was accelerated from 5 to 40 rpm over the  
344 course of 300 seconds. Recording was stopped at 305 seconds. Time of fall was  
345 recorded for each animal. Analysis was performed 3 times a day on 3  
346 consecutive days. Between each trial, animals were allowed to rest for 10  
347 minutes in their home cage.

348 *Grip Strength:* To test whether any effects observed in the rotarod test were due  
349 to muscle weakness, grip strength measurements were performed using a force  
350 gauge (1027SM Grip Strength Meter with Single Sensor, Columbus Instruments).  
351 Animals were allowed to hold a horizontal grip while being gently pulled away by  
352 the base of their tail. Measurements were performed 5 times with 5 minute  
353 resting periods in between. Force amount was recorded. Data was normalized to  
354 mouse's weight and represented in (Force/gram).

355 *Footprinting Analysis:* Forefeet and hindfeet were painted with red and blue  
356 nontoxic acrylic paint (Crayola), respectively. Animals were allowed to walk on a  
357 strip of paper laid along the floor of a 50 cm long, 10 cm wide custom-made  
358 Plexiglas tunnel with a dark box at the far end. 3 runs/mouse were performed  
359 and the distances between the markings were measured.

360

### 361 *Statistical Analysis*

362 Prism (GraphPad) was used for all statistical analysis. Statistical comparisons  
363 used in this study were Student's two-tailed t-test; One-way and Two-way



364 analysis of variance (ANOVA), followed by post hoc analysis with Tukey's test for  
365 multiple comparisons. Relevant F-statistics and p-values are stated in the figure  
366 legends and the p-values of the relevant post hoc multiple comparisons are  
367 shown in the figures. Summary of all the statistical analysis performed can be  
368 found in **Figure 1\_source data 2**. The statistical significance cutoff was set at  
369  $P < 0.05$ . Population statistics were represented as mean  $\pm$  standard deviation  
370 (SD) of the mean. No statistical methods were used to predetermine the sample  
371 size, but our sample sizes are similar to those generally employed in the field.  
372  $n \geq 3$  mice were used for each experiment and the numbers for each experiment  
373 are stated in the figure legends.

374

## 375 **References**

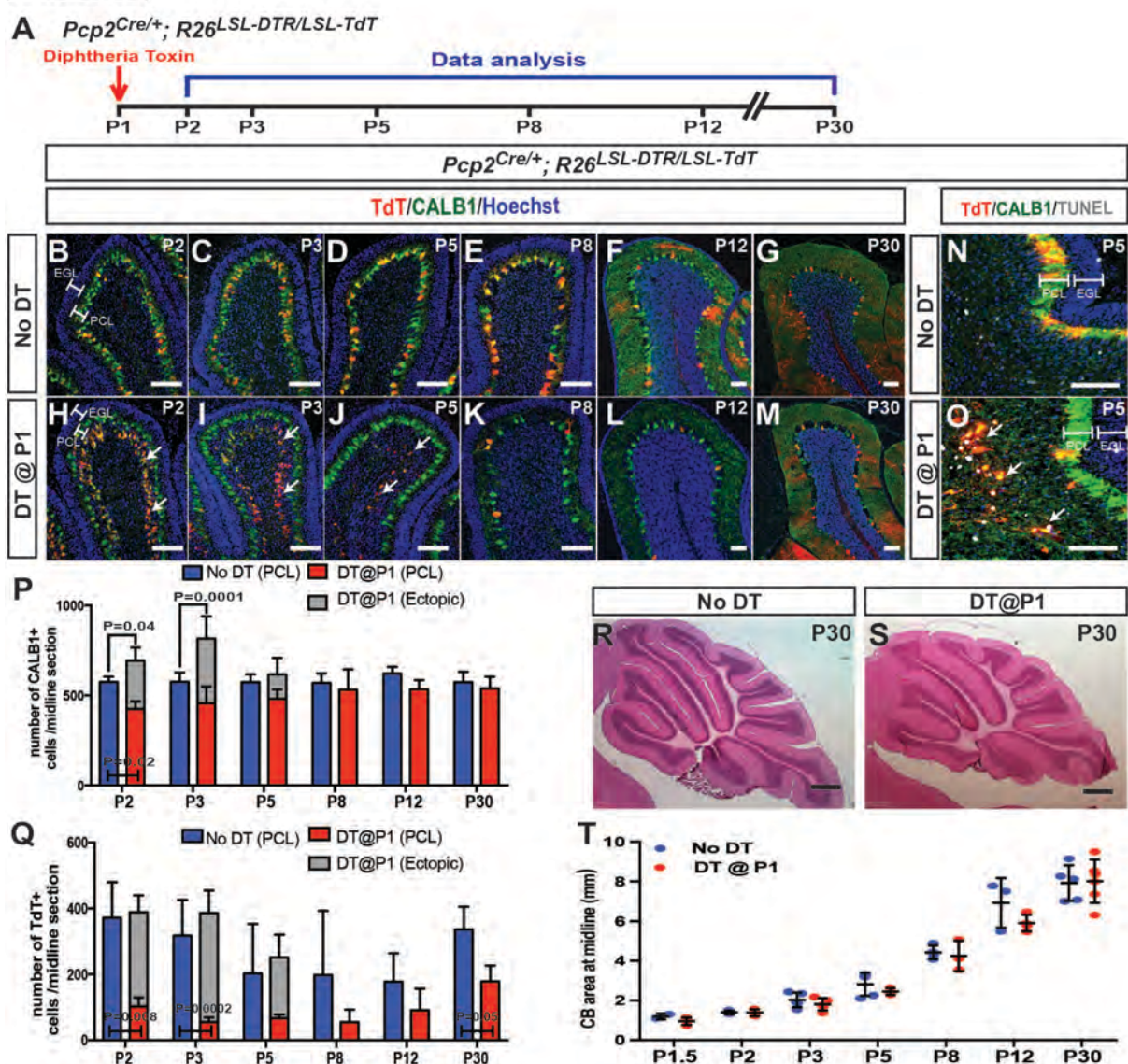
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## Figure 1

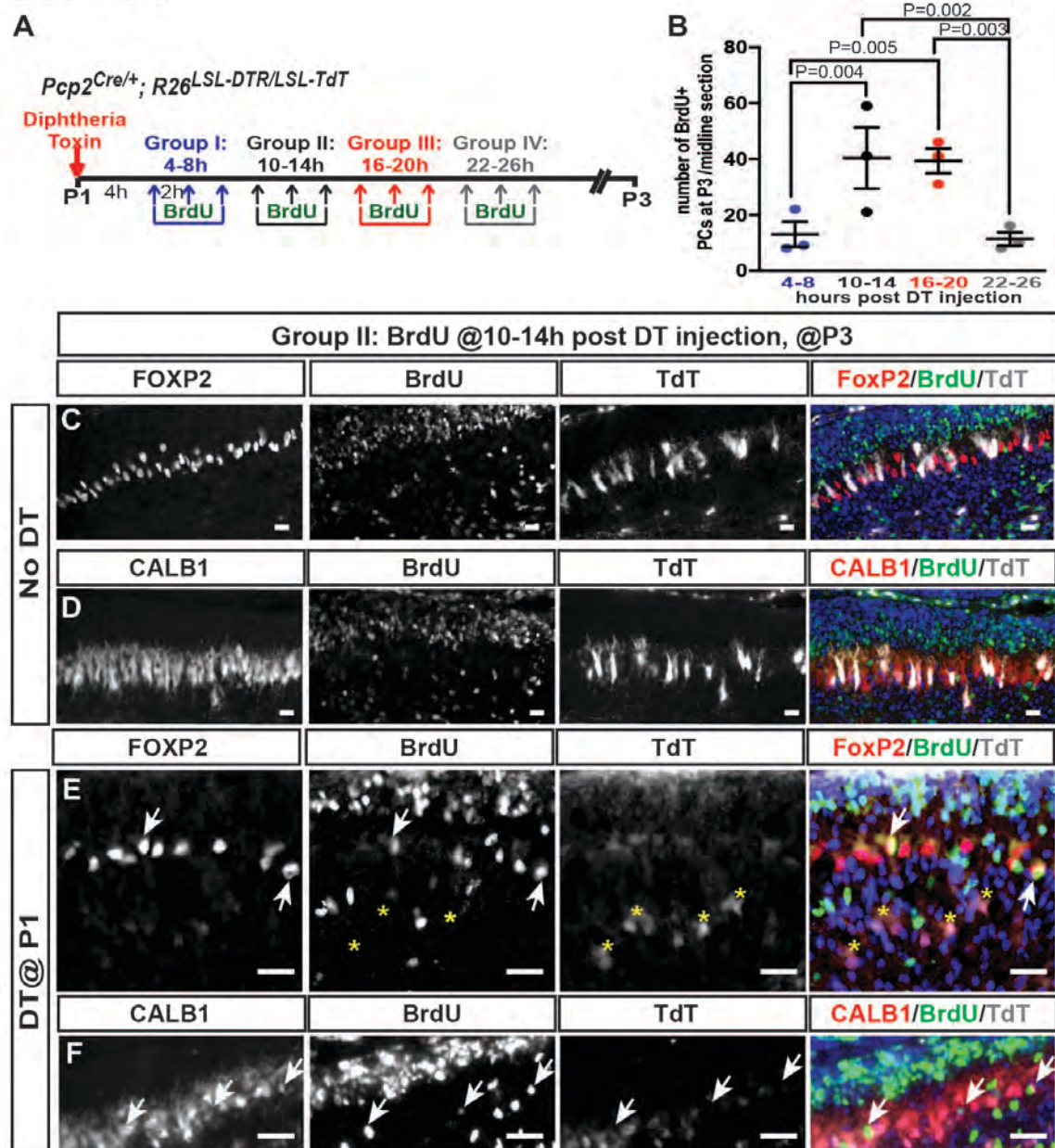


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500 **Figure 1. P1 DT-ablated PCs are replenished and CB size and morphology**  
 501 **appears normal. A.** The experimental plan. **B-M.** IF analysis at the indicated  
 502 ages for TdT and CALB1 in sagittal cerebellar sections of lobule IV-V in No DT  
 503 (b-g) and P1-PC-DTR mice (h-m). **N-O.** Analysis of apoptosis at P5 using  
 504 TUNEL. **P.** Quantification of CALB1+ cells per midline section in PCL (blue or  
 505 red) and ectopic layer (grey) (PCL cells: Two-way ANOVA  $F_{(5,49)}=3.586$ ,  $P=0.008$ ,  
 506 and total number of PCs: Two-way ANOVA  $F_{(5,27)}=4.732$ ,  $P=0.003$ ,  $n \geq 3$

507 animals/condition) **Q.** Quantification of TdT+ cells per section (PCL cells: Two-  
508 way ANOVA  $F_{(5,43)}=7.22$ ,  $P=0.0001$ ). Significant *post hoc* comparisons are  
509 shown. **R-S.** H&E stained midline sagittal sections of cerebella at P30 of No DT  
510 (R) and P1-PC-DTR (S) mice. **T.** Quantification of midline sagittal areas of  
511 cerebella shows no differences upon DT injection ( $P=0.89$ ,  $n \geq 3$  for each age)  
512 Scale bars: 200  $\mu\text{m}$ , (R-S) 500  $\mu\text{m}$ . (EGL: external granule layer, PCL: Purkinje  
513 cell layer)  
514

## Figure 2



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**Figure 2. Progenitors proliferate within 24 hours of DT-injection at P1 in *PC-***

***DTR* mice and produce new PCs. A.** The experimental plan. **B.** Quantification

of the number of BrdU+ PCs (CALB1+) at P3 in P1-*PC-DTR* mice (Two-way

ANOVA  $F_{(3,16)}=6.163$ ,  $P=0.006$ ,  $n=3$  animals/condition). Significant *post hoc*

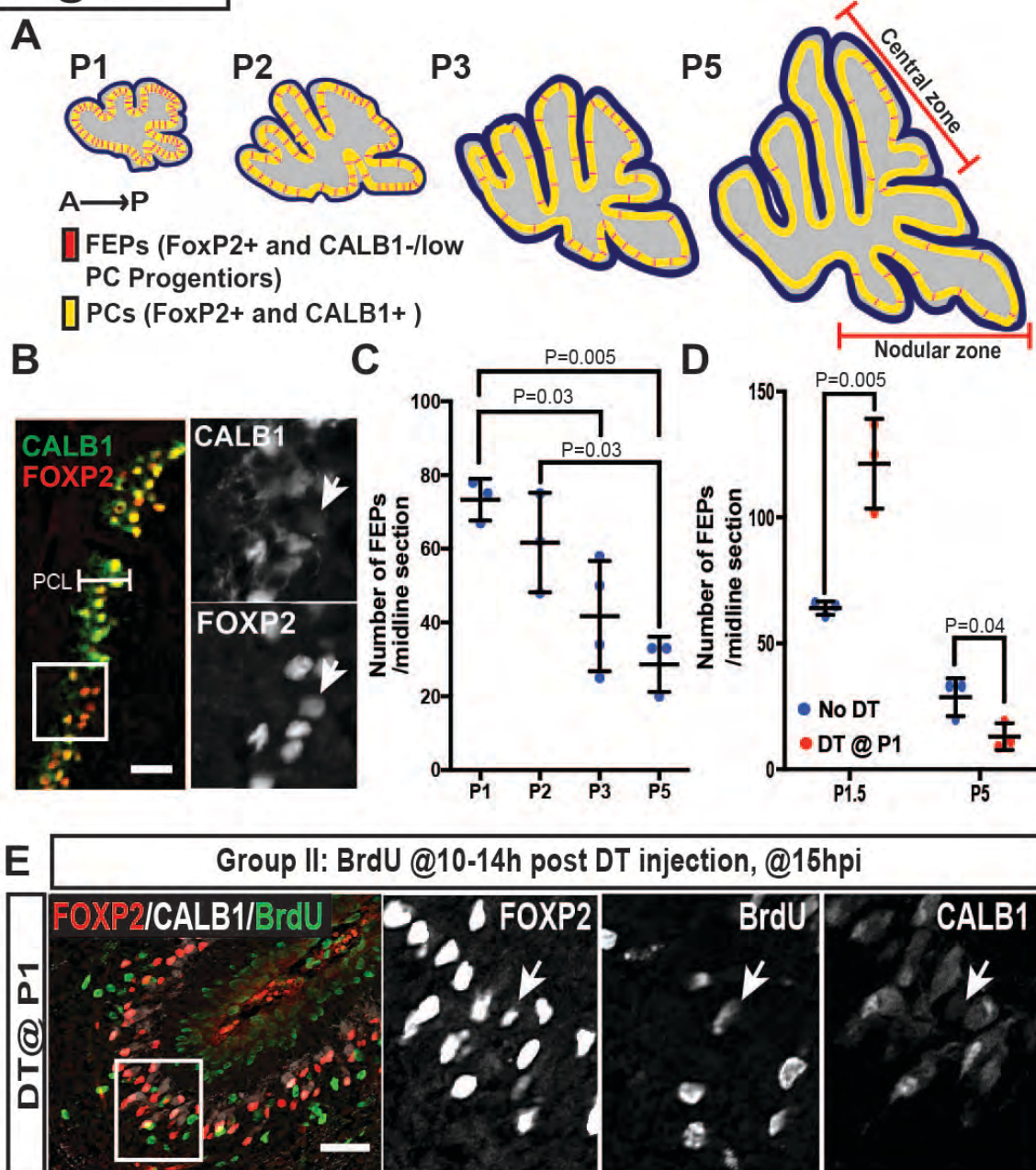
comparisons are designated in the figure. **C-F.** Representative images from BrdU

injection performed 10-14h post DT injection at No DT (C, D) or P1-*PC-DTR* (E,

522 F) or mice P3 cerebella were shown (n=3 animals/condition). IF analysis at P3  
523 from No DT brains showed no BrdU incorporation in PCs, identified by either  
524 FOXP2(C) or CALB1(D). IF analysis of P1-*PC-DTR* animals showing FoxP2+ (E)  
525 and CALB1+ (F) and BrdU+ cells (arrows) at P3. Asterix shows TdT+ cells are  
526 BrdU-. Scale bars: 50  $\mu$ m  
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## Figure 3

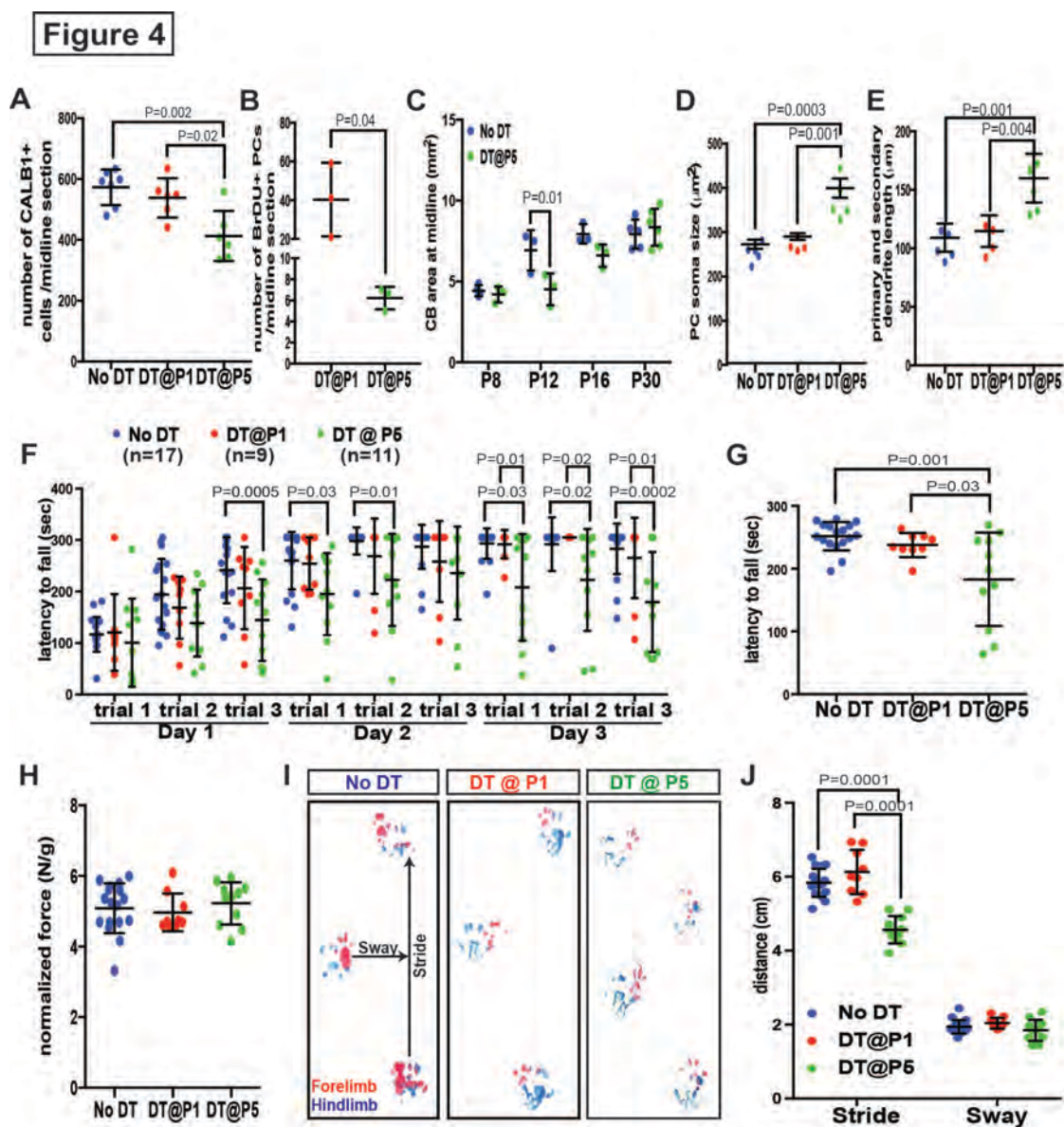


528

529 **Figure 3. Number of FEPs diminishes with age and increases after ablation**  
 530 **of PCs. A.** Schematic representation of the distribution of FEPs (red) in sagittal  
 531 midline sections of P1-5 cerebella (yellow, FoxP2+ and CALB1+ PCs) **B.** IF  
 532 analysis of FEPs (FoxP2+ and CALB1-/low, arrow) at P1.5 in No DT-injected

533 mice. **C.** Quantification of the numbers of FEPs at P1-5 (One-way ANOVA  
534  $F_{(3,9)}=9.074$ ,  $P=0.004$ ,  $n \geq 3$  animals/condition). Significant *post hoc* comparisons  
535 are shown. **D.** Quantification of the numbers of FEPs at P1.5 (Two-tailed t-test,  
536  $P=0.005$ ,  $n=3$ ) and P5 (Two-tailed t-test,  $P=0.04$ ,  $n=3$ ) in No DT and P1-*PC-DTR*  
537 mice. **E.** Arrow shows a BrdU+ FEP (CALB1-/low, FoxP2+) at 15 h post injection  
538 (hpi) in P1-*PC-DTR* mice ( $n=3$ ). Scale bars: 100  $\mu\text{m}$   
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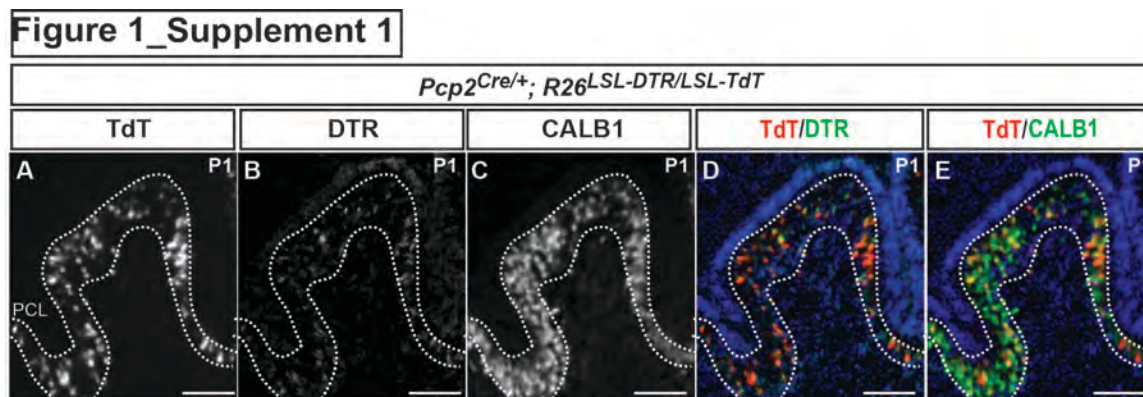
541

542 **Figure 4. Despite the recovery of CB size, PC are poorly replenished and**  
 543 **altered and motor behavior deficits develop when PCs are killed P5 but not**  
 544 **at P1. A.** Number of CALB1+ cells at P30 (One-way ANOVA,  $F_{(2,16)}=9.464$ ,  
 545  $P=0.002$ ,  $n \geq 6$ ). **B.** Number of BrdU+ PCs 2 days post DT-injection at P1- or P5-  
 546 *PC-DTR* mice (Two-tailed t-test,  $P=0.04$ ). **C.** Quantification of CB area in midline  
 547 sagittal sections demonstrates that CB size is smaller at P12 in P5-*PC-DTR* mice

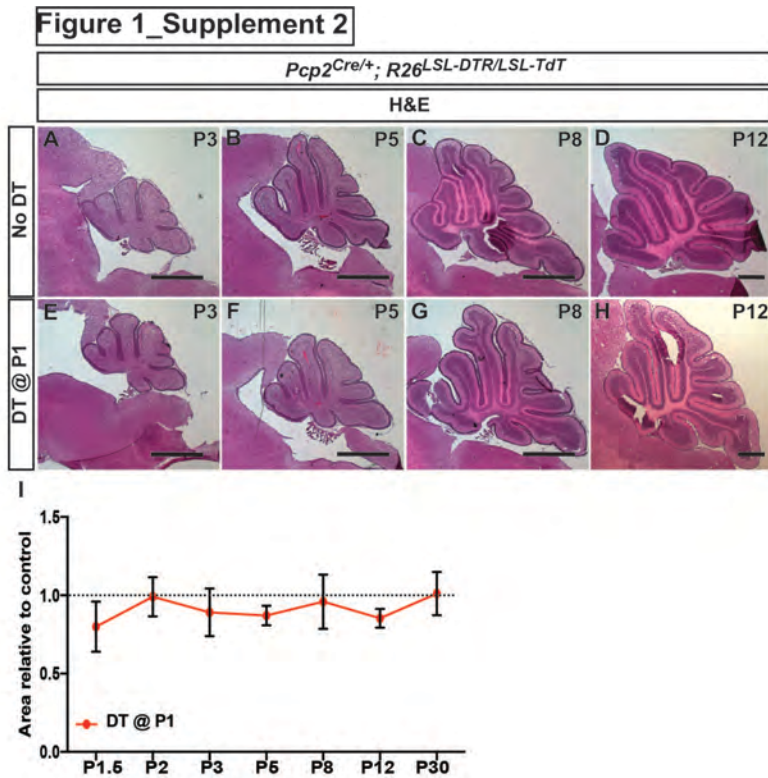
548 but not later (Two-way ANOVA,  $F_{(1,22)}=7.045$ ,  $P=0.01$ ,  $n\geq 3$ ). **D-E.** PC soma size  
549 (D, One-way ANOVA,  $F_{(2,11)}=20.56$ ,  $P=0.0002$ ,  $n\geq 4$ ) and primary and secondary  
550 dendrite lengths (E, One-way ANOVA,  $F_{(2,11)}=14.54$ ,  $P=0.0008$ ,  $n\geq 4$ ) at P30 were  
551 increased in P5-PC-DTR animals compared to No DT and P1-PC-DTR animals.  
552 **F-G.** Latency to fall from rotarod at each trial (F, Two-way ANOVA,  $F_{(2,34)}=8.37$ ,  
553  $P=0.001$ ,  $n\geq 9$ ) and cumulative analysis (G, One-way ANOVA,  $F_{(2,34)}=11.12$ ,  
554  $P=0.0002$ ,  $n\geq 9$ , No DT vs. DT@P1:  $P=0.83$ ). **H.** Analysis of grip strength showed  
555 no change in P1 ( $n=9$ , vs No DT:  $P=0.89$ ) and P5 ( $n=11$ , vs. No DT:  $P=0.84$ , vs.  
556 DT@P1:  $P=0.64$ ) DT-injected mice compared to controls (No DT,  $n=17$ ). **I-J.**  
557 Representative images (I) and quantification (J) of footprint analysis performed  
558 on P1- (vs. No DT: stride:  $P=0.10$  and sway:  $P=0.90$ ) and P5-PC-DTR mice and  
559 controls (Two-way ANOVA,  $F_{(2,133)}=73.45$ ,  $P=0.0001$ ,  $n\geq 9$ ). Significant *post hoc*  
560 comparisons are shown.

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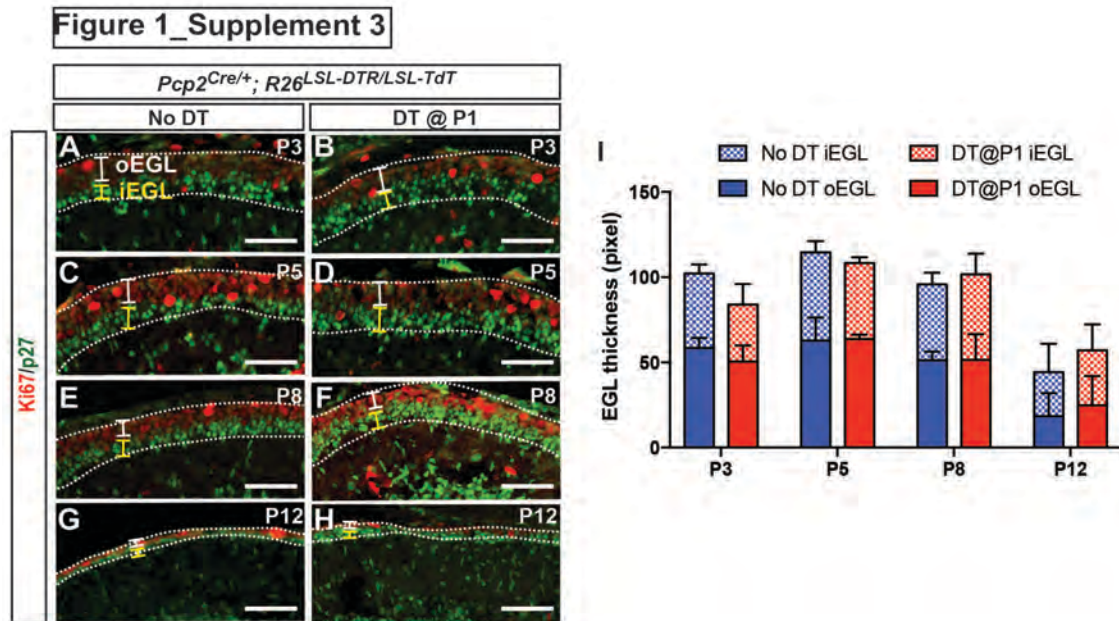
## Supplementary Figures and Tables



**Figure 1\_Supplement 1. Characterization of DTR and TdT expression in PCs of *PC-DTR* mice at P1. A-E.** IF analysis of (A) TdT, (B) DTR, (C) CALB1 and combination shows that all the TdT+ cells express DTR (D) and CALB1 (E). DTR: Diphtheria toxin receptor, PCL: Purkinje cell layer. Scale bar: 100  $\mu$ m

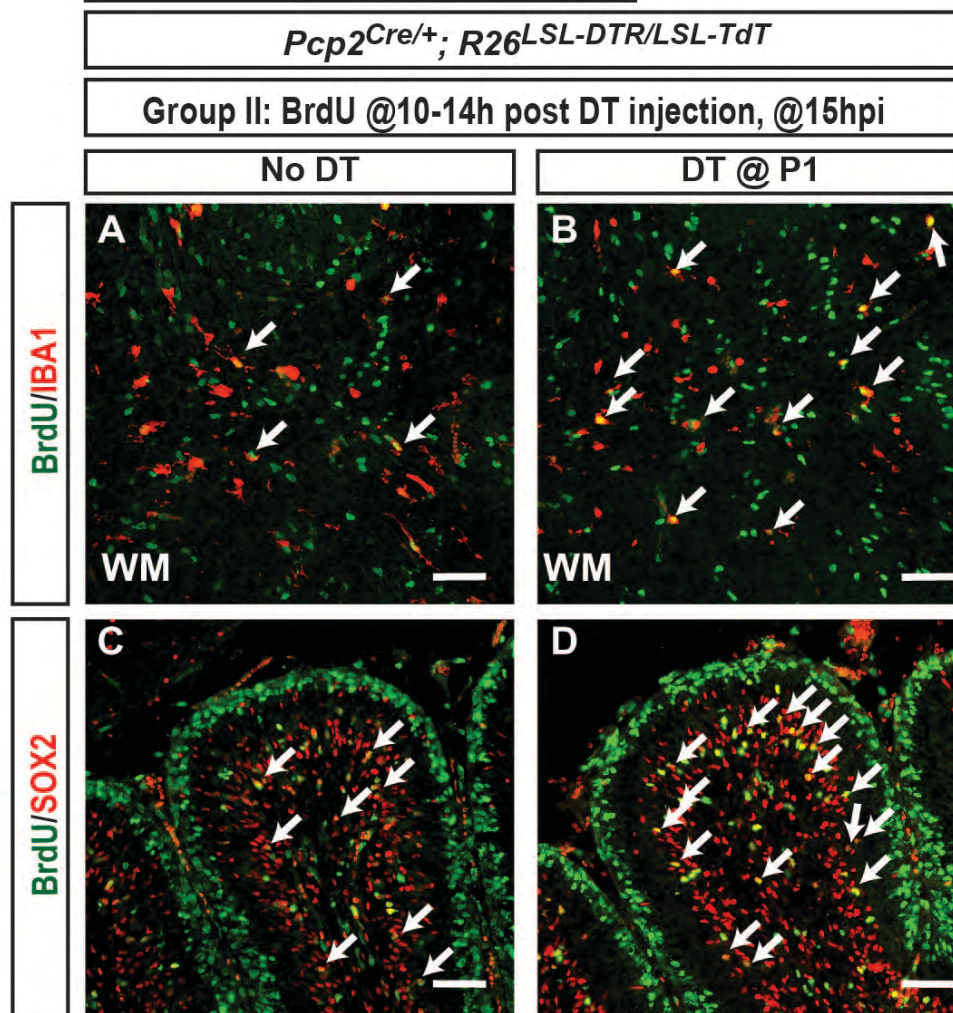


**Figure 1\_Supplement 2. CB size and morphology appears normal following DT-mediated ablation of PCs at P1. A-H.** H&E stained midline sagittal sections of cerebella at the ages indicated for No DT (A-D) and P1-PC-DTR (E-H) mice. **I.** Quantification of midline sagittal areas of cerebella shows no differences upon DT injection ( $n \geq 3$  for each age). Scale bars: 500  $\mu\text{m}$



**Figure 1\_supplement 3. External granule cell layer thickness is not changed after DT-mediated killing of PCs at P1. A-H.** IF analysis of Ki67 (outer EGL, oEGL) and p27 (inner EGL, iEGL) in No DT (A, C, E, G) and P1-PC-DTR (B, D, F, H) animals at the indicated ages. **I.** Quantification of the thickness (area/length) of the oEGL, which contains proliferating granule cell progenitors, and the iEGL, which contains the differentiating granule cells, reveals no significant differences in total EGL area and the ratio of inner and outer EGL areas between No DT and P1-PC-DTR animals ( $n=3$ /condition) ( $P=0.85$ ). EGL: external granule layer. Scale bars: 100  $\mu$ m

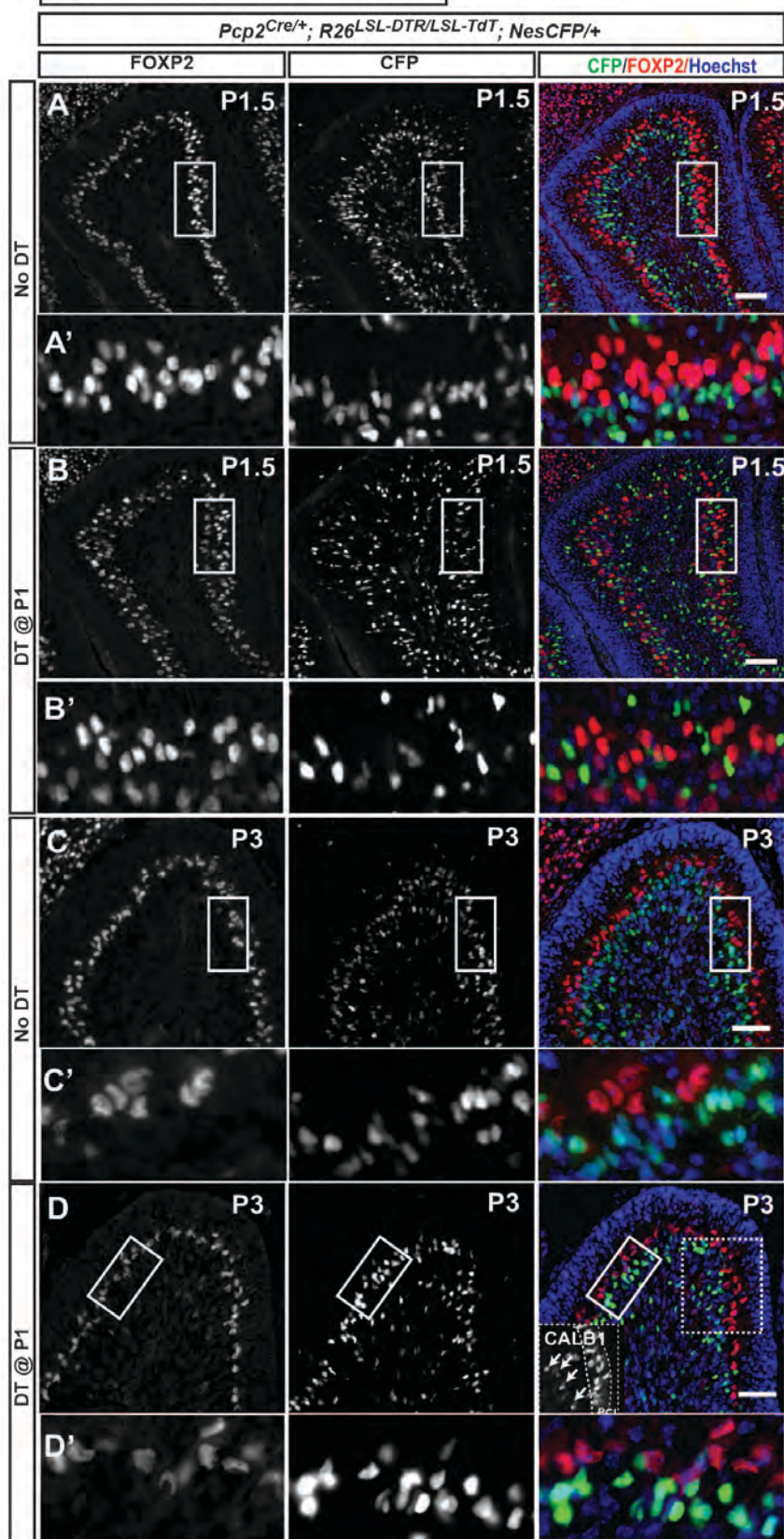
## Figure 2\_Supplement 1



**Figure 2\_supplement 1. Characterization of proliferating cell types that respond to DT-mediated ablation of PCs. A-D.** IF analysis of BrdU+ cells indicates that number of proliferating (A-B) IBA1+ microglia and (C-D) SOX2+ cells (astrocytes and NEPs) increased upon ablation of PCs in P1-*PC-DTR* mice. Arrows show BrdU+ IBA1+ and Sox2+ cells. Scale bars: 100  $\mu$ m



## Figure 2\_Supplement 2



**Figure 2\_supplement 2. NEPs are not responsible for the recovery of PCs following DT-mediated ablation at P1. A-D.** A *Nestin-CFP* reporter was used to transiently track the fate of NEPs and revealed no overlap between FOXP2+ cells and CFP staining 12h (P1.5) (A, A' and B, B') and 2 days (P3) (C, C' and D, D') after PC depletion in P1-*PC-DTR* mice. Note that in B' FOXP2 staining is weaker in the ectopic layer of dying PCs that is forming on the inside of the PCL. Inset in d shows the ectopic CALB1+ cells. NEP: Nestin-expressing progenitors. Scale bars: 100  $\mu$ m

### Figure 3\_Supplement 1

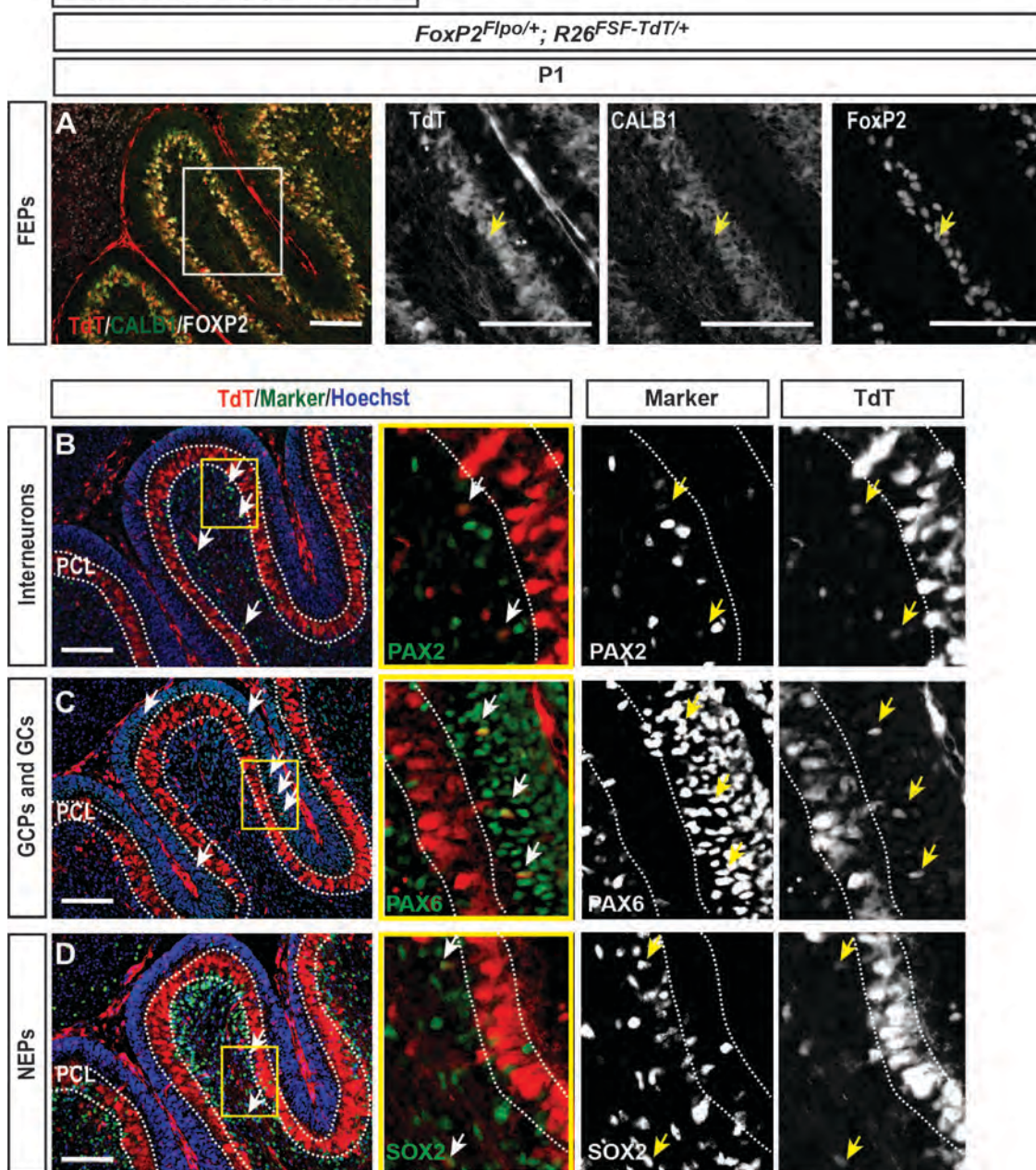


Figure 3\_supplement 1. *FoxP2-TdT* fate mapping at P1 marks FEPs in the PCL as well as other rare cells outside of PCL. *FoxP2<sup>Flpo/+</sup>; R26<sup>FSF-TdT/+</sup>* (*FoxP2-TdT*; FSF=frt-stop-frt) animals were analyzed at P1. **A**. As predicted, all of the FOXP2+ cells in the PCL were labeled with TdT+ and some were CALB1-

/low. Arrow shows a TdT+, FOXP2+ CALB1-/low cell (FEP) in the PCL. **B-D.** *FoxP2-TdT* also marks rare Pax2+ interneurons (B), Pax6+ granule cells (C) and Sox2+ glial cells/progenitors (D), none of which reside in the PCL. These results suggest that *Fipo* allele is unexpectedly expressed transiently in rare embryonic progenitors of other lineages than PCs. Scale bars: 200  $\mu$ m

## Figure 3\_Supplement 2

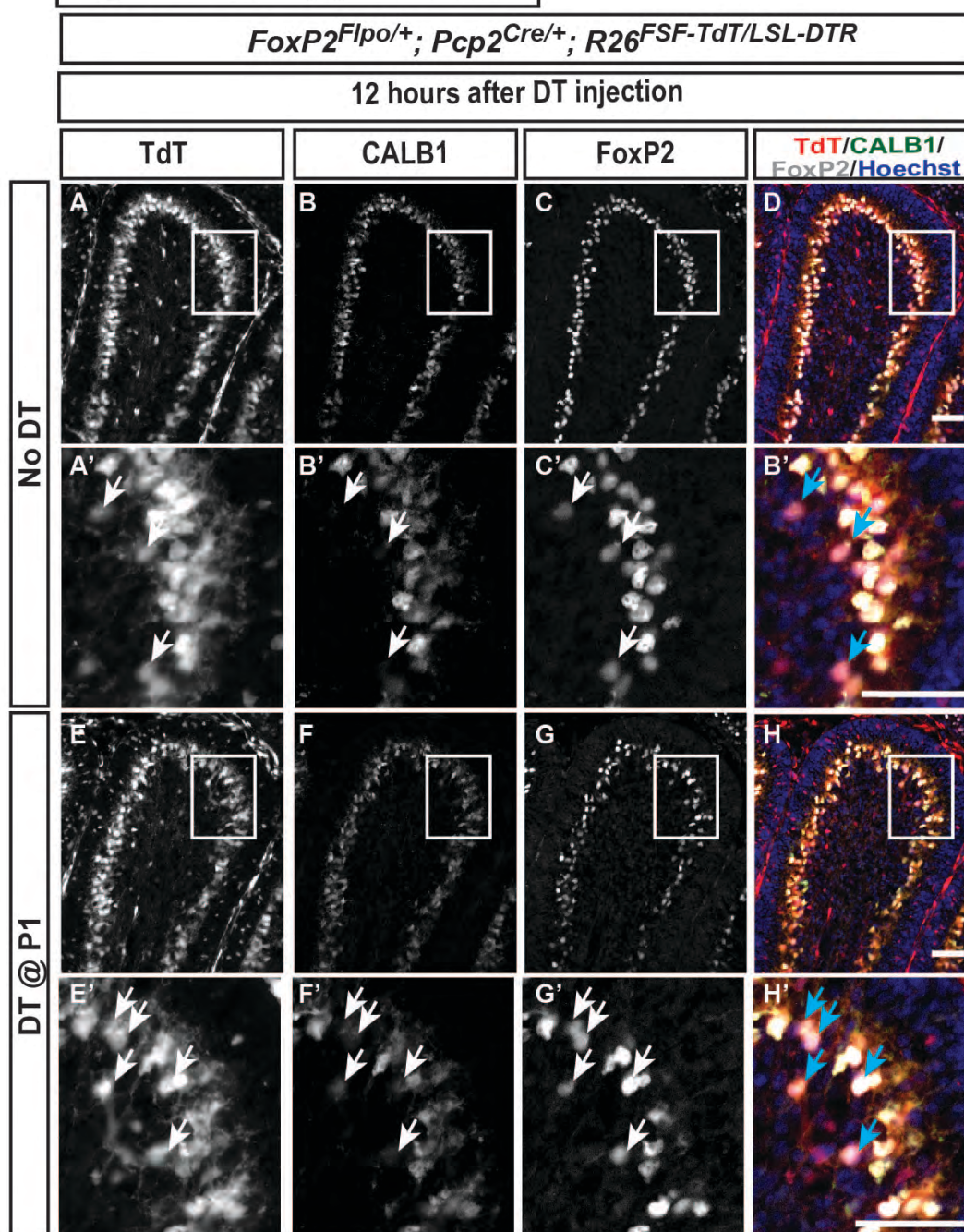
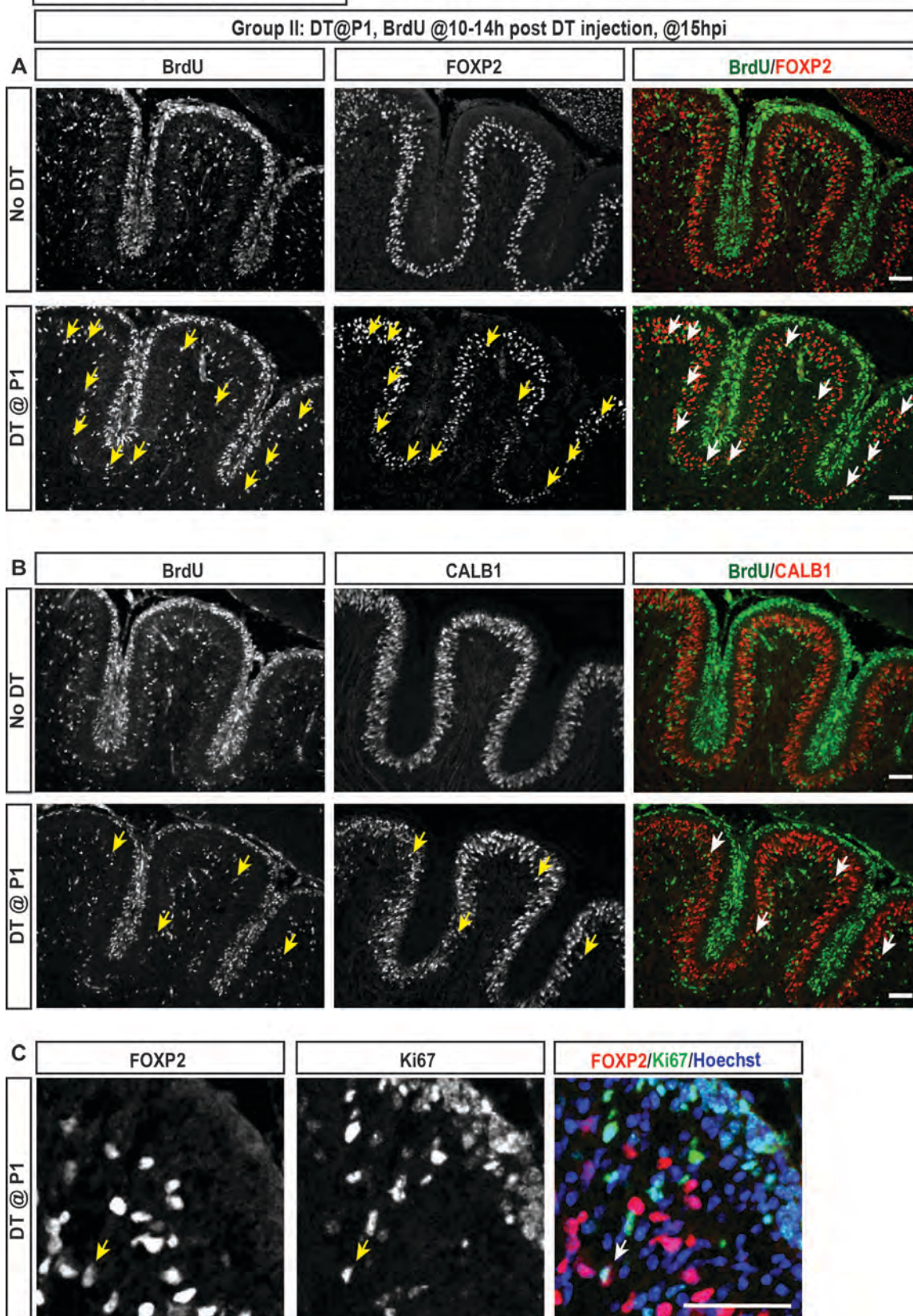


Figure 3\_supplement 2. The number of *FoxP2-TdT*-marked FEPs increases 12 hours after DT injection at P1. A-H. FEPs (TdT+, FOXP2+, CALB1-/low,

arrows in the higher magnified images) are sparsely located in No DT *FoxP2-TdT* pups (A-F) and the number increases 12 hours after DT injection at P1 (E-H).

Scale bars: 50  $\mu$ m

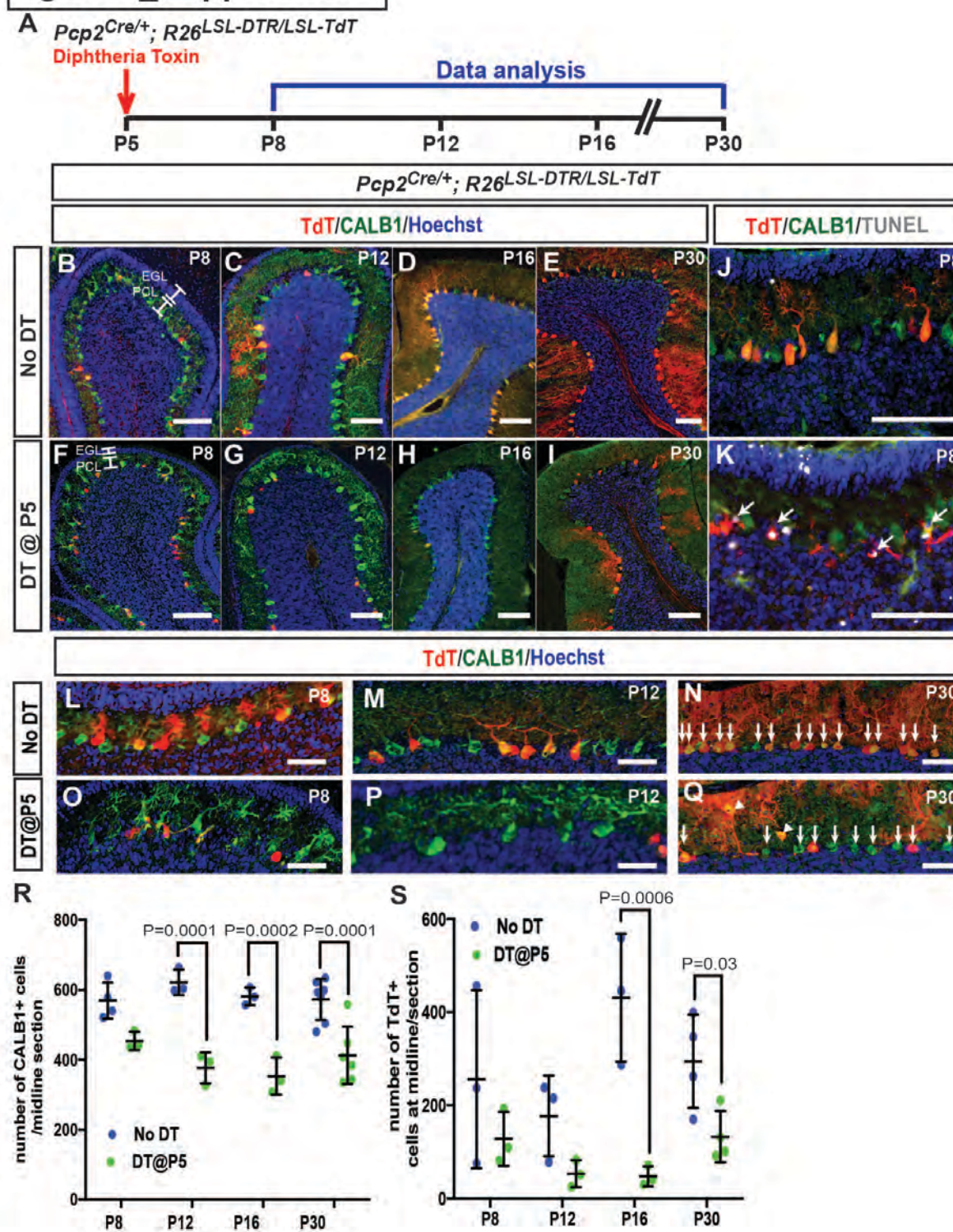
### Figure 3\_Supplement 3



**Figure 3\_supplement 3. IF analysis of PCs at P1.5 (15h post DT injection at P1) shows that FoxP2+ cells proliferate and there are more FoxP2+ cells that incorporate BrdU, compared to CALB1+ cells that are BrdU+. A-B.** Analysis of co-labeling of FoxP2 (A) or CALB1 (B) with BrdU (injected 10-14h post DT) at 15 hpi of DT shows that more FoxP2+ cells incorporate BrdU upon DT injection (lower panels) of P1-*PC-DTR* mice, compared to FoxP2+ CALB1+ cells. Brains of No DT mice did not show any PCs that incorporated BrdU (top panels). **C.** Confocal microscopy reveals FoxP2+ Ki67+ cells 15 hours post DT injection, further confirming proliferation of FOXP2+ cells in P1-*PC-DTR*. Arrows indicate BrdU or Ki67+ PCs that are either FoxP2+ or CALB1+. Scale bars: 100  $\mu\text{m}$

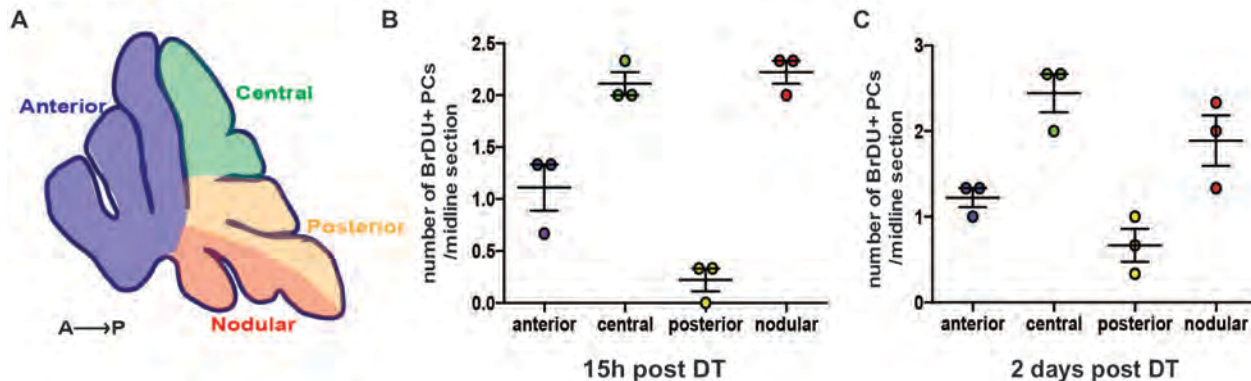


## Figure 4\_Supplement 1



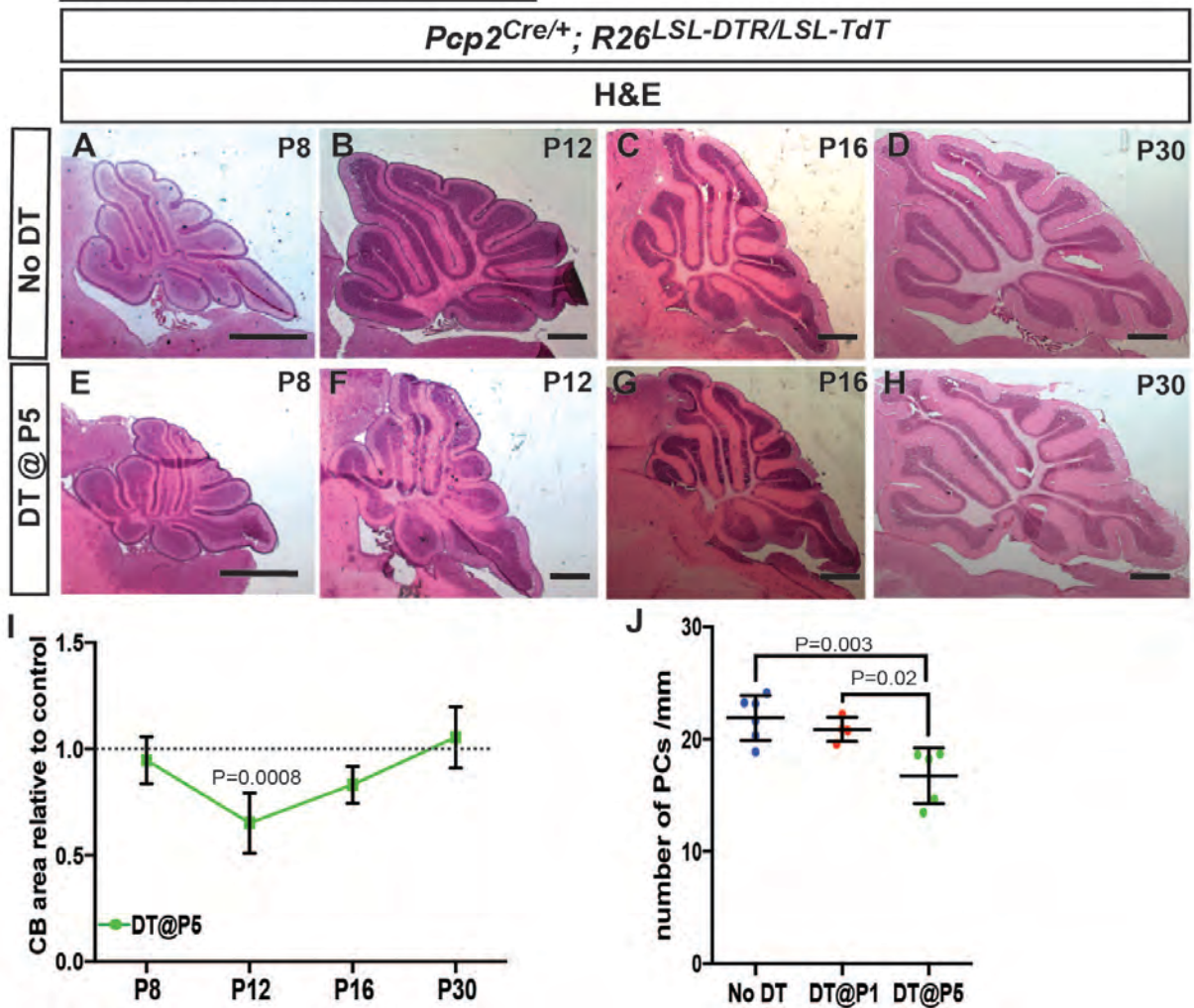
**Figure 4\_supplement 1. PC numbers are reduced upon PC ablation at P5-PC-DTR.** **A.** Schematic representation of the experimental plan. **B-I.** IF analysis of PCs upon ablation at P5 (F, G, H, I) reveals lack of recovery of PC numbers. **J-K.** Analysis of apoptosis by TUNEL reveals TUNEL+ TdT cells (arrows) in PCL of P5-PC-DTR mice (K) but not in No DT mice (J) at P8. **L-Q.** Higher magnification of PCs from P8, P12 and P30 P5-PC-DTR animals and No DT controls (L, M, N) reveal that P5-PC-DTR mice (O, P, Q) have disrupted PC morphology and reduction in their numbers. Arrows show PCs and reduction in their number at P5-PC-DTR mice. Arrowheads: Ectopic PCs. **R.** Quantification of CALB1+ cells shows that PC numbers do not recover from ablation of PCs at P5 (Two-way ANOVA,  $F_{(1,24)}=77.85$ ,  $P=0.0001$ ,  $n\geq 3$ ). **S.** Quantification of the number of TdT+ cells, shows a large variation in recombination efficiency in No DT brains, and an initial decrease in TdT+ cells after DT injection at P5 (Two-way ANOVA,  $F_{(1,18)}=26.29$ ,  $P=0.0001$ ,  $n\geq 3$ ). At P30, P5-PC-DTR brains show decrease in the number of TdT+ cells compared to No DT animals (t-test,  $P=0.03$ ,  $n\geq 4$ ), similar to P1-PC-DTR animals (Fig. 1q). Significant *post hoc* comparisons are shown in the figure. EGL: External granule layer, PCL: Purkinje cell layer. Scale bars: a-k: 100  $\mu\text{m}$ , l-q: 50  $\mu\text{m}$

### Figure 4\_Supplement 2



**Figure 4\_supplement 2. Distribution of BrDU+ PCs in P5-PC-DTR mice at 15 hours and 2 days post injection of DT. A.** Schematic showing the different zones of the CB in a P5 sagittal midline section. **B-C.** Distribution of BrDU+ PCs across different zones analyzed 15h (B) and 2 days (C) in P5-PC-DTR animals reveals that incorporation of BrdU is limited, and most of the cells reside in the central and the nodular zones, correlating with the localization of FEPs (n=3 /condition). No BrdU incorporation was detected in No DT mice at the same ages.

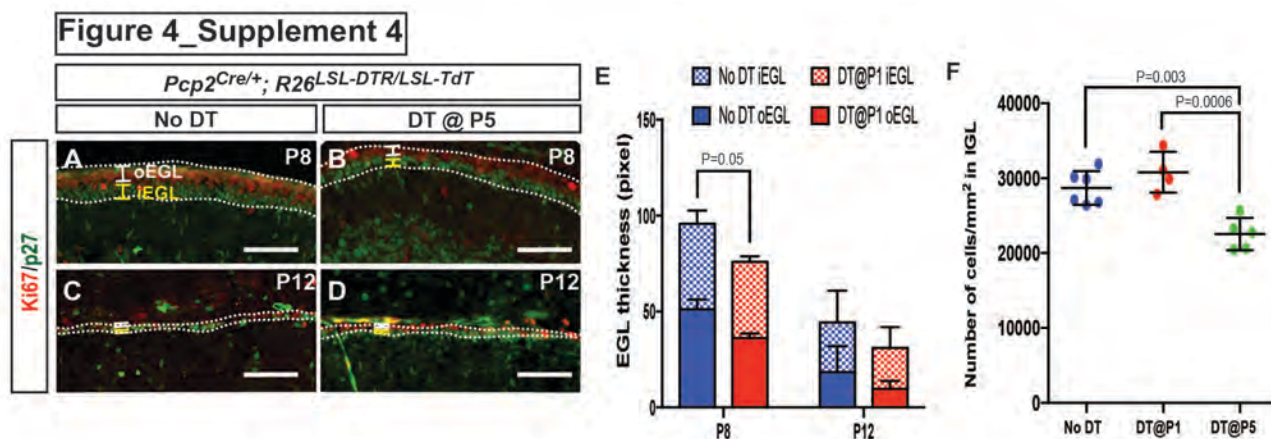
### Figure 4\_Supplement 3



**Figure 4\_supplement 3. Transient decrease in CB size and altered PC characteristics after ablation of PCs at P5. A-H.** H&E staining shows that the area of sagittal CB sections is reduced at P12 after DT injection in P5-PC-DTR mice No DT (F compared to B) and the significant difference is lost at P16 and P30. **I.** Quantification of CB area in midline sagittal sections demonstrates that CB size is smaller at P12. (Two-way ANOVA,  $F_{(1,22)}=7.799$ ,  $P=0.01$ ,  $n\geq 3$ ) **J.** The density of PCs was reduced at P30 in P5-PC-DTR but not in P1-PC-DTR animals, correlating with lack of recovery of PC numbers in parallel with recovery

of CB area in the former mice (One-way ANOVA,  $F_{(2,12)}=9.687$ ,  $P=0.003$ ,  $n \geq 4$ ).

Significant *post hoc* comparisons are shown in the figure. Scale bars: 500  $\mu\text{m}$



**Figure 4\_Supplement 4. Analysis of external granule cell layer thickness after DT injection at P5. A-D.** IF analysis of Ki67 (outer EGL, oEGL) and p27 (inner EGL, iEGL) in No DT (A, C) and P5-*PC-DTR* (B, D) mice. **E.** Quantification shows that both the oEGL and iEGL thicknesses (area/length) were significantly reduced at P8 (Two-tailed t-test,  $P=0.05$ ,  $n=3$ ), but not at P12. **F.** Likely as a consequence of a thinner EGL, granule cell density in the internal granule cell layer (IGL) is reduced only in P5-*PC-DTR* animals, but not in No DT and P1-*PC-DTR* animals (One-way ANOVA,  $F_{(2,12)}=15.73$ ,  $P=0.0004$ ,  $n \geq 4$ ). Significant *post hoc* comparisons are shown in the figure. Scale bars: 100  $\mu\text{m}$

### Figure 4\_Supplement 5

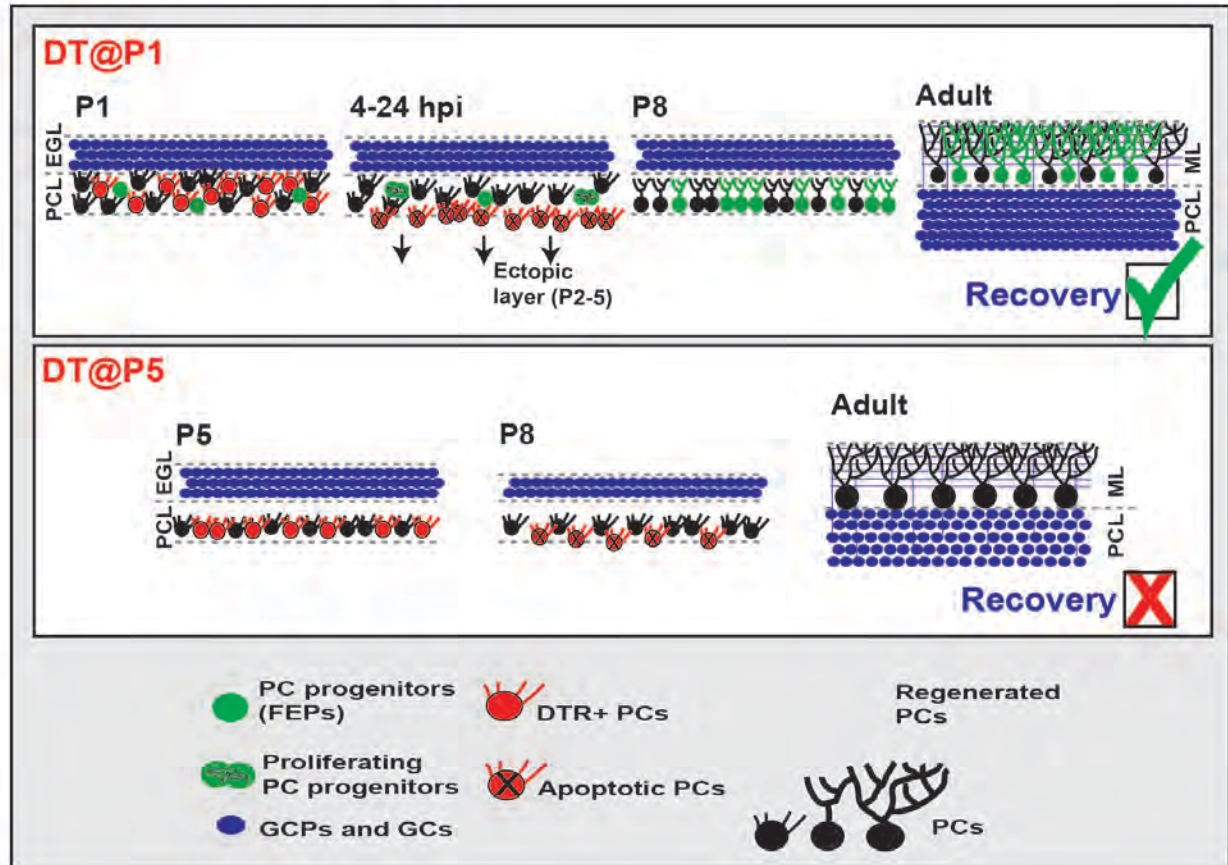


Figure 4\_supplement 5. Graphical summary of the findings.

FEPs: FoxP2-expressing progenitors, EGL: external granule cell layer, PCL: Purkinje cell layer, ML: Molecular Layer, GCP: granule cell progenitors, GC: granule cells.

**Figure 1\_source data 1. Summary of the antibodies used in the study.**

<b>Target</b>	<b>Catalog Number</b>	<b>Company</b>	<b>Dilution</b>
<b>Goat <math>\alpha</math>-FoxP2</b>	EB05226	Everest	1/1000
<b>Rabbit <math>\alpha</math>-Calbindin1</b>	CB38	Swant	1/1000
<b>Mouse <math>\alpha</math>-Calbindin1</b>	300	Swant	1/1000
<b>Goat <math>\alpha</math>-Sox2</b>	AF2018	R&D System	1/200 (adult)- 1/500 (pups)
<b>Rabbit <math>\alpha</math>-Pax2</b>	71600	Invitrogen	1/500
<b>Rabbit <math>\alpha</math>-Pax6</b>	AB2237	Millipore	1/500
<b>Rabbit <math>\alpha</math>-Ki67</b>	RM-9106-S0	Thermo Scientific	1/500
<b>Rat <math>\alpha</math>-BrdU</b>	OBT0030CX	Accurate	1/500
<b>Sheep <math>\alpha</math>-BrdU</b>	ab1893	Abcam	1/500
<b>Mouse <math>\alpha</math>-p27</b>	610241	BD Pharmingen	1/500
<b>Rat <math>\alpha</math>-GFP (CFP)</b>	04404-84	Nacalai Tesque	1/1000
<b>Goat <math>\alpha</math>-hHB-EGF (DTR)</b>	AF231	R&D System	1/500



**Figure 1\_source data 2. Summary of the statistics performed**

Figure	Test performed	P-Value	Multiple comparisons	
Fig. 1p	Two-way ANOVA	PCL cells: $F_{(5,49)}=3.586$ , $P=0.008$ total number of PCs: $F_{(5,27)}=4.732$ , $P=0.003$	P2: No DT (PCL) vs. DT@P1 (PCL)	0.0233
			P2: No DT (PCL) vs. DT@P1 (Ectopic)	<0.0001
			P2: DT@P1 (PCL) vs. DT@P1 (Ectopic)	0.0190
			P3: No DT (PCL) vs. DT@P1 (PCL)	0.0404
			P3: No DT (PCL) vs. DT@P1 (Ectopic)	<0.0001
			P3: DT@P1 (PCL) vs. DT@P1 (Ectopic)	0.0913
			P5: No DT (PCL) vs. DT@P1 (PCL)	0.2274
			P5: No DT (PCL) vs. DT@P1 (Ectopic)	<0.0001
			P5: DT@P1 (PCL) vs. DT@P1 (Ectopic)	<0.0001
			P8: No DT (PCL) vs. DT@P1 (PCL)	0.7404
			P8: No DT (PCL) vs. DT@P1 (Ectopic)	<0.0001
			P8: DT@P1 (PCL) vs. DT@P1 (Ectopic)	<0.0001
			P12: No DT (PCL) vs. DT@P1 (PCL)	0.2148
			P12: No DT (PCL) vs. DT@P1 (Ectopic)	<0.0001
			P12: DT@P1 (PCL) vs. DT@P1 (Ectopic)	<0.0001
			P30: No DT (PCL) vs. DT@P1 (PCL)	0.6314
			P30: No DT (PCL) vs. DT@P1 (Ectopic)	<0.0001
P30: DT@P1 (PCL) vs. DT@P1 (Ectopic)	<0.0001			

			DT@P1 (Ectopic)	
Fig. 1q	Two-way ANOVA	PCL cells: $F_{(5,43)}=7.22$ , $P=0.0001$	P2: No DT (PCL) vs. DT@P1 (PCL)	0.0008
			P2: No DT (PCL) vs. DT@P1 (Ectopic)	0.4328
			P2: DT@P1 (PCL) vs. DT@P1 (Ectopic)	0.0267
			P3: No DT (PCL) vs. DT@P1 (PCL)	0.0002
			P3: No DT (PCL) vs. DT@P1 (Ectopic)	0.9722
			P3: DT@P1 (PCL) vs. DT@P1 (Ectopic)	<0.0001
			P5: No DT (PCL) vs. DT@P1 (PCL)	0.1298
			P5: No DT (PCL) vs. DT@P1 (Ectopic)	0.9649
			P5: DT@P1 (PCL) vs. DT@P1 (Ectopic)	0.2081
			P8: No DT (PCL) vs. DT@P1 (PCL)	0.0772
			P8: No DT (PCL) vs. DT@P1 (Ectopic)	0.0097
			P8: DT@P1 (PCL) vs. DT@P1 (Ectopic)	0.7059
			P12: No DT (PCL) vs. DT@P1 (PCL)	0.3442
			P12: No DT (PCL) vs. DT@P1 (Ectopic)	0.0346
			P12: DT@P1 (PCL) vs. DT@P1 (Ectopic)	0.3106
			P30: No DT (PCL) vs. DT@P1 (PCL)	0.0460
			P30: No DT (PCL) vs. DT@P1 (Ectopic)	<0.0001

			P30: DT@P1 (PCL) vs. DT@P1 (Ectopic)	0.0213
Fig. 1t		$F_{(1, 32)}=3.043$ , $P=0.09$	P1.5 (No DT vs. DT@P1)	0.9997
			P2 (No DT vs. DT@P1)	>0.9999
			P3 (No DT vs. DT@P1)	>0.9999
			P5 (No DT vs. DT@P1)	0.9984
			P8 (No DT vs. DT@P1)	0.9976
			P12 (No DT vs. DT@P1)	0.4230
			P30 (No DT vs. DT@P1)	0.9201
Fig. 2b	Two-way ANOVA	$F_{(3,16)}=6.163$ , $P=0.006$	4-8h post injection vs. 10-14h post injection	0.0037
			4-8h post injection vs. 16-20h post injection	0.0051
			4-8h post injection vs. 22-24h post injection	>0.9999
			10-14h post injection vs. 16-20h post injection	>0.9999
			10-14h post injection vs. 22-24h post injection	0.0022
			16-20h post injection vs. 22-24h post injection	0.0030
Fig. 3c	One-way ANOVA	$F_{(3,9)}=9.074$ , $P=0.004$	P1 vs. P2	0.6249
			P1 vs. P3	0.0259
			P1 vs. P5	0.0050
			P2 vs. P3	0.1830
			P2 vs. P5	0.0293
Fig. 3d	Two-tailed t-test	P1.5: $t(4)=5.523$ , $P=0.005$ P5: $t(4)=2.955$ , $P=0.04$	N/A	
Fig. 4r	Two-way ANOVA	$F_{(1,24)}=77.85$ , $P=0.0001$	P8 (No DT vs. DT@P5)	0.0532
			P12 (No DT vs. DT@P5)	<0.0001
			P16 (No DT vs. DT@P5)	0.0002
			P30 (No DT vs. DT@P5)	0.0001
Fig. 4s	Two-way ANOVA	$F_{(1,18)}=26.29$ , $P=0.0001$	P8 (No DT vs. DT@P5)	0.4207
			P12 (No DT vs. DT@P5)	0.4540
			P16 (No DT vs. DT@P5)	0.0006
			P30 (No DT vs.	0.1210

			DT@P5)	
Fig. 4s	Two-tailed t-test	P30: $t(6)=3.301$ , P=0.03	N/A	
Fig. 4t	Two-tailed t-test	$t(4)=3.301$ , P=0.04	N/A	
Fig. 5e	Two-way ANOVA	$F_{(1,22)}=7.799$ , P=0.01	P8 (No DT vs. DT@P5)	0.9946
			P12 (No DT vs. DT@P5)	0.0121
			P16 (No DT vs. DT@P5)	0.2788
			P30 (No DT vs. DT@P5)	0.8979
Fig. 5f	One-way ANOVA	$F_{(2,12)}=9.687$ , P=0.003	No DT vs. DT@P1	0.7130
			No DT vs. DT@P5	0.0030
			DT@P1 vs. DT@P5	0.0242
Fig. 5k	Two-tailed t-test	P8: $t(6)=2.452$ , P=0.049 P12: $t(6)=0.7087$ , P=0.5	N/A	
Fig. 5l	One-way ANOVA	$F_{(2,12)}=15.73$ , P=0.0004	No DT vs. DT@P1	0.3819
			No DT vs. DT@P5	0.0026
			DT@P1 vs. DT@P5	0.0006
Fig. 5m	One-way ANOVA	$F_{(2,11)}=20.56$ , P=0.0002	No DT vs. DT@P1	0.7328
			No DT vs. DT@P5	0.0003
			DT@P1 vs. DT@P5	0.0013
Fig. 5n	One-way ANOVA	$F_{(2,11)}=14.54$ , P=0.0008	No DT vs. DT@P1	0.8645
			No DT vs. DT@P5	0.0011
			DT@P1 vs. DT@P5	0.0041
Fig. 6a	Two-way ANOVA	$F_{(2,34)}=8.37$ , P=0.001	day1 trial 1: No DT vs. DT@P1	0.9895 0.8013
			No DT vs. DT@P5	0.7792
			DT@P1 vs. DT@P5	
			day1 trial 2: No DT vs. DT@P1	0.6132 0.0773
			No DT vs. DT@P5	0.5734
			DT@P1 vs. DT@P5	
			day1 trial 3: No DT vs. DT@P1	0.4090 0.0005
			No DT vs. DT@P5	0.0941
			DT@P1 vs. DT@P5	
			Day2 trial 1: No DT vs. DT@P1	0.9765 0.0315
			No DT vs. DT@P5	0.1161
			DT@P1 vs. DT@P5	
Day2 trial 2: No DT vs. DT@P1	0.5224 0.0093			
No DT vs. DT@P5	0.2694			
DT@P1 vs. DT@P5				
Day2 trial 3: No DT vs. DT@P1	0.5429 0.1141			
No DT vs. DT@P5	0.7316			
DT@P1 vs. DT@P5				

			Day3 trial 1: No DT vs. DT@P1 No DT vs. DT@P5 DT@P1 vs. DT@P5	0.9993 0.0029 0.0141
			Day3 trial 2: No DT vs. DT@P1 No DT vs. DT@P5 DT@P1 vs. DT@P5	0.8835 0.0192 0.0163
			Day3 trial 3: No DT vs. DT@P1 No DT vs. DT@P5 DT@P1 vs. DT@P5	0.7927 0.0002 0.0117
Fig. 6b	One-way ANOVA	$F_{(2,34)}=8.37$ , $P=0.001$	No DT vs. DT@P1 No DT vs. DT@P5 DT@P1 vs. DT@P5	0.8290 0.0009 0.0280
Fig. 6c	One-way ANOVA	$F_{(2,34)}=0.4181$ , $P=0.66$	No DT vs. DT@P1 No DT vs. DT@P5 DT@P1 vs. DT@P5	0.8869 0.8430 0.6382
Fig. 6d	Two-way ANOVA	No DT vs. DT@P5: $F_{(2,133)}=73.45$ , $P=0.0001$	Stride: No DT vs. DT@P1 No DT vs. DT@P5 DT@P1 vs. DT@P5 Sway: No DT vs. DT@P1 No DT vs. DT@P5 DT@P1 vs. DT@P5	0.0706 <0.0001 <0.0001 0.7319 0.7312 0.3750
Supplementary Fig. 2	Two-way ANOVA	$F_{(1,40)}=4.847$ , $P=0.033$	P1.5 P2 P3 P5 P8 P12 P30	0.6225 >0.9999 0.8429 0.8241 0.9998 0.7582 >0.9999
Supplementary Fig. 3i	Two-way ANOVA	$F_{(1,24)}=0.03658$ , $P=0.8499$	N/A	