An increase in reactive oxygen species underlies neonatal cerebellum repair

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Abstract

The neonatal mouse cerebellum shows remarkable regenerative potential upon injury at birth, wherein a subset of Nestin-expressing progenitors (NEPs) undergoes adaptive reprogramming to replenish granule cell progenitors that die. Here, we investigate how the microenvironment of the injured cerebellum changes upon injury and contributes to the regenerative potential of normally gliogenic-NEPs and their adaptive reprogramming. Single cell transcriptomic and bulk chromatin accessibility analyses of the NEPs from injured neonatal cerebella compared to controls show a temporary increase in cellular processes involved in responding to reactive oxygen species (ROS), a known damage-associated molecular pattern. Analysis of ROS levels in cerebellar tissue confirm a transient increased one day after injury at postanal day 1, overlapping with the peak cell death in the cerebellum. In a transgenic mouse line that ubiquitously overexpresses human mitochondrial catalase (mCAT), ROS is reduced 1 day after injury to the granule cell progenitors, and we demonstrate that several steps in the regenerative process of NEPs are curtailed leading to reduced cerebellar growth. We also provide evidence that microglia are involved in one step of adaptive reprogramming by regulating NEP replenishment of the granule cell precursors. Collectively, our results highlight that changes in the tissue microenvironment regulate multiple steps in adaptative reprogramming of NEPs upon death of cerebellar granule cell progenitors at birth, highlighting the instructive roles of microenvironmental signals during regeneration of the neonatal brain.

Introduction

The microenvironment surrounding a brain injury and the cellular responses elicited in the remaining cells are key determinants of how efficiently a repair process will unfold. An important factor underlying the effectiveness of regenerative responses to an injury is the plasticity of the stem/progenitor cells in a tissue (Burda and Sofroniew, 2014). The degree to which the microenvironment and specific cell types within it provide pro- or anti-regenerative factors is highly context dependent. The neonatal mouse cerebellum has a remarkable capacity to regenerate cells ablated around birth (Wojcinski et al., 2017, Bayin N. S., 2021, Bayin et al., 2018, Altman and Anderson, 1971). Thus, the cerebellum provides an ideal system to study the roles that signals in the microenvironment play in key steps of the repair process in the brain.

The cerebellum is a folded hindbrain structure that is critical for motor coordination. It also participates in higher order social and cognitive behaviors through its circuit connections with all other brain regions (Badura et al., 2018, Buckner, 2013, Burda and Sofroniew, 2014, Salman and Tsai, 2016, Strick et al., 2009, Tomlinson et al., 2013). Compared to the rest of the brain, the cerebellum has protracted development, as its major growth occurs during the first two weeks after birth in mice and at least six months surrounding birth in humans (Altman and Bayer, 1997, Rakic and Sidman, 1970, Dobbing and Sands, 1973). This timing of the major growth of the cerebellum makes it susceptible to injury around birth. Indeed, cerebellar hypoplasia is the second leading risk factor for autism spectrum disorders and cerebellar injury around birth can have devastating outcomes and significant effects on subsequent quality of life (Tsai et al., 2018, Stoodley et al., 2017, Wang et al., 2014). Therefore, it is critical to better understand the regenerative processes that allow repair of the cerebellum.

All the cell types in the cerebellum are derived from two progenitor zones, the embryonic rhombic lip and the ventricular zone that give rise to the excitatory neurons, or the inhibitory neurons and glia, respectively (Leto et al., 2015, Joyner and Bayin, 2022). During postnatal growth, the rhombic lip-derived granule cell precursors (GCPs) cover the surface of the cerebellum in a structure named the external granule layer (EGL) and continue to proliferate in a sonic hedgehog (SHH) dependent manner for two weeks after birth in mice (Wechsler-Reya and Scott, 1999, McMahon et al., 2003, Corrales et al., 2006). Following their exit from the cell cycle, the granule cells (GC) migrate inwards to form the internal granule layer (IGL). Other SHH-dependent progenitor populations of the neonatal cerebellum are either gliogenic Nestin-Expressing Progenitors (NEPs)

that express SOX2 and generate astroglia (astrocytes and Bergman glia) or neurogenic-NEPs that generate late born interneurons (Bayin N. S., 2021, Cerrato et al., 2018, Parmigiani et al., 2015). Gliogenic-NEPs reside either in the Bergmann glia layer (BgL) intermixed with Purkinje cells and generate Bergmann glia (Bg) and astrocytes, or in the white matter in the center of the lobules (folds) and generate astrocytes. Neurogenic-NEPs are restricted to the white matter and produce interneurons that migrate outwards to the outermost molecular layer (Bayin N. S., 2021, Brown et al., 2020, De Luca et al., 2015). Surprisingly, when the GCPs are killed upon injury soon after birth, the gliogenic-NEPs in the BgL (BgL-NEPs) undergo adaptive reprogramming to generate GCPs and replenish the EGL via a transitory cellular state that involves upregulation of the neurogenic gene *Ascl1* to promote a glial-to-neural fate switch (Wojcinski et al., 2017, Bayin N. S., 2021). Adaptive reprogramming involves multiple sequential stages starting with increased proliferation of BgL-NEPs, then a fate switch to neuronal progenitors, migration to the site of injury (EGL) and acquisition of a GCP identity. The full repertoire of injury-induced signals that initiate and govern adaptive reprogramming remains to be discovered.

In the adult brain, numerous cell types communicate and provide a concerted response to injury, including astrocytes, microglia (macrophages of the brain) and stem cells of the neurogenic niches (Frik et al., 2018). The timelines of the cellular responses of each cell type to injury - cell death, activation of microglia, reactive gliosis, proliferation, scar formation and cellular remodeling - have been delineated for specific adult brain injuries, particularly in the cerebral cortex. For example, upon traumatic brain injury cells release damage-associated molecular patterns (DAMPs), which act as an inflammatory stimulus and activate microglia that can lead to gliosis, eventually causing neurotoxicity and scarring (Donat et al., 2017). However, the cellular composition and microenvironment of the early postnatal brain are very different from the adult. In the neonatal cerebellum, microglia are immature (Li et al., 2019) and are still being generated, and NEPs and GCPs are actively proliferating and producing astroglia and neurons. Therefore, the existing knowledge on how adult brain cells react to injury might not apply to the neonatal cerebellum. For example, in the spinal cord, while neonatal microglia and astrocytes facilitate scarless repair, the same cells in the adult promote scarring upon spinal cord injury in mice (Li et al., 2020). It is thus important to study the microenvironment of the neonatal brain during repair to determine what factors promote or inhibit regeneration.

Dying cells release many factors, including reactive oxygen species (ROS) that activate signaling cascades in neighboring cells. However, little is known about how these signals regulate brain

116 repair, especially during development. The level of ROS during homeostasis is regulated by
117 metabolic processes, and typically is increased following injury (Niethammer, 2016). Furthermore,
118 ROS can directly react with proteins that regulate proliferation, viability, quiescence or
119 differentiation and metabolism (Bigarella et al., 2014, Tan and Suda, 2018). Thus, ROS are
120 considered key signaling molecules that participate in the crosstalk between progenitor cell fate
121 decisions and metabolic switches in a context- and cell type-dependent manner (Bigarella et al.,
122 2014). One significant mechanism by which ROS signaling is implicated during inflammatory
123 responses following an injury is through the activation of microglia, which in turn can lead to more
124 ROS production (Smith et al., 2022). This process is critical as it can potentially promote repair.
125 However, the role of ROS signaling during adaptive reprogramming of NEPs following neonatal
126 cerebellar injury is not known.
127
128 Here we first delineate the sequential changes in the microenvironment upon injury (focused
129 irradiation) to the mouse hindbrain at postnatal day 1 (P1). We then demonstrate a requirement
130 for a transient increase in ROS levels at ~24 hours (hr) post injury for cerebellar regeneration.
131 Single cell RNA-sequencing (scRNA-seq) and bulk ATAC-seq analyses revealed increased ROS
132 signaling compared to controls that peaks 24 hr after injury in NEPs, particularly those in the BgL,
133 demonstrating that ROS is an acute signal associated with the NEP response to GCP death. A
134 functional role of ROS signaling was established using a transgene (*mCAT*) that expresses the
135 human mitochondrial Catalase which can reduce ROS levels broadly. Several key steps in
136 adaptive reprogramming were abrogated in *mCAT*/+ mice leading to reduced replenishment of
137 the EGL and a smaller adult cerebellum. Finally, we show that the density of microglia is reduced
138 at P5 in irradiated *mCAT* mice compared to controls and provide evidence that microglia play a
139 role in the step of replenishing the EGL with BgL-derived GCPs during adaptive reprogramming.
140

141 ## Materials and Methods

142 ### Animals

143 All the mouse experiments were performed according to protocols approved by the Institutional
144 Animal Care and Use Committee of Memorial Sloan Kettering Cancer Center (MSKCC) (protocol
145 no. 07-01-001). Animals were housed on a 12-hour light/dark cycle and given access to food and
146 water ad libitum.
147
148 Two mouse lines were used in this study: *Nes-Cfp* (JAX #034387) (Encinas et al., 2006) and
149 *mCAT* (JAX #016197) (Schriner et al., 2005). Animals were maintained on an outbred Swiss

150 Webster background. Both sexes were used for analyses except for the genomics experiments
151 (scRNA-seq and ATAC-seq) where males were used.
152
153 *EdU administration*
154 5-ethynyl-2'-deoxyuridine (EdU) stock was dissolved in sterile phosphate-buffered saline (PBS)
155 at 10 mg/mL and a dose of 5 μg/g was intraperitoneally injected into animals 1 hour prior to
156 euthanasia.
157
158 *PLX5622 administration*
159 PLX5622 powder was provided by Plexxikon under a Materials Transfer Agreement. PLX5622
160 powder was first diluted in DMSO at 20mM and then diluted 1X in PBS just before intraperitoneal
161 injection into newborn pups. Injections were given every day from P1 to P8 at a dose of 10μg/g
162 of body weight. Control pups were injected with PBS-DMSO vehicle control.
163
164 *Irradiation*
165 P1 mice were anesthetized by hypothermia and given a single dose of ~5Gy γ-irradiation in an X-
166 RAD 225Cx (Precision X-ray) Microirradiator in the MSKCC Small-Animal Imaging Core facility.
167 A 5-mm diameter collimator was used to target the hindbrain from the left side of the animal.
168
169 **Tissue preparation and histology**
170 For immunocytochemistry, animals younger than P8 were sacrificed and then brains were
171 dissected, fixed in 4% paraformaldehyde for 24 hr at 4°C, cryoprotected in 30% sucrose in
172 phosphate-buffered saline (PBS) until they sank and then frozen in Cryo-OCT (Tissue-Tek). Older
173 animals were anesthetized and then perfused with cold PBS followed by 4% paraformaldehyde
174 prior to brain dissection. Frozen brains were cryosectioned sagittally at 14 μm and slides stored
175 at -20°C. Midline cerebellar sections were used for quantification in all downstream analyses.
176
177 For immunofluorescence staining, slides were allowed to warm to room temperature (RT) and
178 washed 3 times in PBS. Then, tissues were blocked for one hour with blocking buffer (5% BSA
179 (w/v) in 1XPBS with 0.1% Triton-X) at RT. Primary antibodies diluted in the blocking buffer were
180 placed on slides for overnight incubation at 4°C. Slides were then washed in PBS with 0.1%
181 Triton-X and incubated with fluorophore-conjugated secondary antibodies diluted in the blocking
182 buffer for 1 hr at RT. Following washes in PBS with 0.1% Triton-X after the secondary antibody
183 incubation, nuclei were counterstained with Hoechst (1:3000) and the slides were mounted using

184 mounting media (Electron Microscopy Sciences). Primary antibodies used are described in Table
185 S1 and secondary antibodies were Alexa Fluor-conjugated secondary antibodies (1:1000).
186
187 EdU was detected using a Click-it EdU (Invitrogen, C10340) assay with Sulfo-Cyanine5 azide
188 (Lumiprobe Corporation, A3330).
189
190 For TUNEL staining, after primary antibody incubation and washes, sections were permeabilized
191 in PBS with 0.5% TritonX-100 for 10 minutes (min) and then pre-incubated in TdT buffer (30mM
192 Tris HCl, 140 mm sodium cacodylate and 1mM $CoCl_2$) for 15 min at RT. Slides were then
193 incubated for 1hr at 37°C in TUNEL reaction solution containing Terminal Transferase and
194 Digoxigenin-11dUTP (Roche). After the TUNEL reaction and washes, slides were incubated with
195 a secondary antibody solution which included a sheep anti-dixogenin-rhodamine (Roche) for the
196 visualization of TUNEL reaction.
197
198 **Image acquisition and analysis**
199 Images were collected with a DM6000 Leica microscope, a NanoZoomer Digital Pathology
200 microscope (Hamamatsu Photonics), or an LSM880 confocal microscope (Zeiss). Images were
201 processed using NDP.view2 software, ImageJ software (NIH, Bethesda, MA, USA) and
202 Photoshop (Adobe).
203
204 **Cell dissociation for FACS and flow cytometry**
205 Cerebella were dissected into ice-cold 1x Hank's Buffered Salt Solution (Gibco) and dissociated
206 using Accutase (Innovative Cell Technologies) at 37°C for 10-15 min. After dissociation, Accutase
207 was diluted with 3x excess volume of neural stem cell media (Neurobasal medium, supplemented
208 with N2, B27 (without vitamin A)), and nonessential amino acids (Life Technologies, Gibco) and
209 cells were filtered using a 40 μm mesh cell strainer. After 5 min of centrifugation in a chilled
210 centrifuge at 500g, the pellet was resuspended in neural stem cell media and strained using
211 strainer cap tubes (Falcon) for downstream experimentation. All centrifugation was performed at
212 4°C and cells were kept on ice when possible.
213
214 **Flow cytometry analysis for ROS and mitochondria mass**
215 For MitoSOX and MitoTracker analyses, cells were incubated for 30 min at 4°C with 5 μM of
216 MitoSOX and 100 μM of MitoTracker (Thermo Fisher Scientific) to assess mitochondrial
217 superoxide production and mitochondrial mass, respectively. Data were collected using a LSR

218 Fortessa flow cytometer (BD) and analyzed using FlowJo software. The gating for the MitoSOX
219 or MitoTracker high populations (top 90%) were performed as previously described (Clutton et
220 al., 2019)
221
222 **Multiplexed sc-RNAseq**
223 *Sample preparation*
224 2-4 *Nes-Cfp/+* cerebella/replicates from male control non-irradiated pups or pups that were
225 irradiated at P1 were collected at P1 (control only), P2, P3 and P5 and dissociated as described
226 above. All conditions were performed in 2 replicates for nonIR and IR conditions, except for P5.
227 Following dissociation, CFP+ cells were immediately sorted on a BD FACS Aria sorter (BD
228 Biosciences) using a 100 μm nozzle. 50,000 Nes-CFP+ cells from each sample were processed
229 for scRNA-seq. Cells were sorted into neural stem cell media.
230
231 *Multiplexing, droplet preparation, sequencing and data processing*
232 The scRNA-Seq of FACS-sorted Nes-CFP+ cell suspensions was performed on a Chromium
233 instrument (10X genomics) following the user guide manual for 3′ v3.1. In brief, 50,000 FACS-
234 sorted cells from each condition were multiplexed using CellPlex reagents (10x Genomics) as
235 described by the manufacturer's protocol. P3 nonIR replicate #2 did not yield sufficient cells after
236 multiplexing. The viability of cells was above 95%, as confirmed with 0.2% (w/v) Trypan Blue
237 staining and barcoded cells were pooled into a single sample in PBS containing 1% bovine serum
238 albumin (BSA) to a final concentration of 700–1,300 cells per µl. 2-3,000 cells were targeted for
239 each sample. Samples were multiplexed together on one lane of a 10X Chromium following
240 the 10x Genomics 3' CellPlex Multiplexing protocol and a total of ~30,000 cells were targeted for
241 droplet formation. Cells were captured in droplets. After the reverse transcription and cell
242 barcoding in droplets, emulsions were broken, and cDNA was purified using Dynabeads MyOne
243 SILANE followed by PCR amplification per the manufacturer's instruction. Final libraries were
244 sequenced on an Illumina NovaSeq S4 platform (R1 – 28 cycles, i7 – 8 cycles, R2 – 90 cycles)
245 by the MSKCC core facility.
246
247 *Data Analysis*
248 Single-cell RNA-seq fastq files were demultiplexed using sharp (https://github.com/hisplan/sharp)
249 and initially mapped to the mouse mm10 reference genome using Cell Ranger v6.0.1 (Zheng et
250 al., 2017). The Cell Ranger BAM files for individual samples were then converted back to fastq
251 files using *bamtofastq* (Cell Ranger v7.0.1), with --reads-per-fastq=1000000000. Reads were then

252 mapped to the GRCm39 mouse reference with Gencode annotations (vM30) using STARsolo
253 v2.7.10a (--soloFeatures Gene Velocyto, --soloType CB_UMI_Simple, --soloCBwhitelist 3M-
254 february-2018.txt, --soloUMIlen 12, --soloCellFilter EmptyDrops_CR, --soloMultiMappers EM)
255 (Kaminow et al., 2021, Frankish et al., 2021).
256
257 The STARsolo output was used to create Seurat (v4.3) objects for each sample with spliced and
258 unspliced read count matrices (Hao et al., 2021). The objects were integrated, by running
259 NormalizeData and FindVariableFeatures (selection.method = "vst", nfeatures = 3000) on each
260 one, then SelectIntegrationFeatures and FindIntegrationAnchors on the list of objects and finally
261 IntegrateData with the anchors. Counts were then normalized with SCTransform. Based on
262 manual analysis, cells were filtered out where number of detected genes was ≤ 1,500, number of
263 detected transcripts was ≥ 40,000 and mitochondrial gene percentage ≥ 5%. To determine cell
264 cycle phases, the Kowalczyk et al. (Kowalczyk et al., 2015) cell cycle markers were used,
265 assuming the gene names, capitalized to the title case, to be orthologous between mouse and
266 human with the CellCycleScoring function. SCTransform was used to regress out the “Cell Cycle
267 difference” score (S score – G2M score). Dimension reduction was performed using RunPCA and
268 RunUMAP (dims = 1:20, n.neighbors = 30). For clustering, FindNeighbors was run using the first
269 20 principal components, then FindClusters with the Leiden algorithm with the default settings
270 (Traag et al., 2019). Clusters were annotated using known markers: GCPs
271 (*Atoh1*+/*Barhl1*+/*Tubb3*+), BgL-NEPs (*Hopx*+), ependymal cells (*Foxj1*+), immature interneurons
272 (*Pax2*+), Neurogenic NEPs (*Ascl1*+), astrocytes (*Slc6a11*+), oligodendrocytes (*Olig1*+),
273 meninges (*Col3a1*+/*Vtn*+/*Dcn*+), microglia (*Ly86*+/*Fcer1g*+).
274
275 The differential gene expression analyses were performed individually on *Hopx*+ gliogenic-NEPs,
276 *Ascl1*+ neurogenic-NEPs, and GCPs following the same computational workflow. First, clusters
277 containing *Hopx*-NEPs (clusters 2, 3, 6, 10), *Ascl1*-NEPs (clusters 5, 8, 11), or GCPs (clusters 1,
278 4, 7, 12, 14) were subsetted from the original data set based on the expression of *Hopx*, *Ascl1*,
279 and *Atoh1*, *Barhl1* and *Rbfox*, respectively. Second, the subsetted cells were divided according
280 to whether the cells were from P2 or P3+P5 and split based on their biological replicate. The split
281 data sets were normalized using NormalizeData with default parameters, and the 3,000 top
282 variable features were computed using FindVariableFeatures with default settings. Re-integration
283 of the data sets was subsequently performed using SelectIntegrationFeatures,
284 FindIntegrationAnchors, and IntegrateData all with default parameters as previously described,
285 except for IntegrateData in the *Ascl1*+ neurogenic-NEP analyses where k.weight = 50 was used.

286 Following re-integration, SCTransform with default parameters was used to normalize
287 mitochondrial read percentage, cell cycle difference score, number of features, and number of
288 counts. Dimension reduction was thereafter performed using RunPCA with default parameters
289 and RunUMAP with default parameters except dims = 1:40, repulsion.strength = 0.1, and min.dist
290 = 0.5. Clustering was subsequently performed using FindNeighbors with default settings except
291 dim = 30 and FindClusters with default settings for resolutions between 0.1 and 3 using the
292 original Louvain algorithm. A final resolution which gave a high silhouette score with a relatively
293 low negative silhouette proportion was selected for individual data sets. To allow downstream
294 DESeq2 analyses, count matrices were constructed by aggregating counts from cells from the
295 same treatment condition and biological replicate using AggregateExpression. The aggregated
296 count matrices were converted into a DESeq2 dataset object using DESeqDataSetFromMatrix,
297 grouping the samples by treatment conditions. Genes with fewer than 10 counts were filtered out.
298 DESeq2 (v1.36.0) was used to perform the differential expression analyses using a negative
299 binomial distribution and default settings (Love et al., 2014). The results were visualized using
300 EnhancedVolcano with a fold change cut-off of ±0.5 and an adjusted p-value threshold of 0.05.
301
302 The GO term analyses were performed on differentially expressed genes from the DESeq2 results
303 filtered with a log2 fold change threshold of ±0.5 and an adjusted p-value threshold of 0.05 for
304 each comparison. The probability weight function was computed using nullp with default
305 parameters and the mm8 mouse genome. The category enrichment testing was performed using
306 goseq with default parameters from the goseq package (v1.48.0).
307
308 **Bulk ATAC-seq**
309 *Sample preparation*
310 FACS-sorted Nes-CFP+ cells (30,000-50,000 per replicate) were isolated from control or
311 irradiated (at P1) P2 cerebella. 2-3 cerebella were pooled for each sample. Cells were
312 immediately processed for nuclei preparation and transposition using the OMNI-ATAC protocol
313 (Corces et al., 2017). Sequencing was performed at the MSKCC genomics core facility using the
314 Illumina NovaSeq S4 platform.
315
316 *Data Processing and Analysis*
317 Raw sequencing reads were 3' trimmed and filtered for quality and adapter content using version
318 0.4.5 of TrimGalore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore), with a
319 quality setting of 15, and running version 1.15 of cutadapt and version 0.11.5 of FastQC. Version

320 2.3.4.1 of bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml) was used to align reads
321 to mouse assembly mm10 and alignments were deduplicated using MarkDuplicates in version
322 2.16.0 of Picard Tools. Enriched regions were discovered using MACS2
323 (https://github.com/taoliu/MACS) with a p-value setting of 0.001, filtered for blacklisted regions
324 (http://mitra.stanford.edu/kundaje/akundaje/release/blacklists/ mm10-
325 mouse/mm10.blacklist.bed.gz), and a peak atlas was created using +/- 250 bp around peak
326 summits. The BEDTools suite (http://bedtools.readthedocs.io) was used to create normalized
327 bigwig files. Version 1.6.1 of featureCounts (http://subread.sourceforge.net) was used to build a
328 raw counts matrix and DESeq2 was used to calculate differential enrichment for all pairwise
329 contrasts. Peak-gene associations were created by assigning all intragenic peaks to that gene,
330 while intergenic peaks were assigned using linear genomic distance to transcription start site.
331 Network analysis was performed using the assigned genes to differential peaks by running
332 enrichplot::cnetplot in R with default parameters. Composite and tornado plots were created using
333 deepTools v3.3.0 by running computeMatrix and plotHeatmap on normalized bigwigs with
334 average signal sampled in 25 bp windows and flanking region defined by the surrounding 2 kb.
335 Motif signatures were obtained using Homer v4.5 (http://homer.ucsd.edu) on differentially
336 enriched peak *regions.*
337
338 **Data and code availability**
339 The scRNA-seq data was submitted to ArrayExpress (Accession E-MTAB-13353). Bulk ATAC-
340 seq data has been submitted to GEO (GSE269342). The code used to carry out the scRNA-seq
341 analysis can be found on GitHub repository: https://github.com/BayinLab/Pakula_et_al_23
342
343 **Quantification and statistical analysis**
344 For detecting TUNEL, IBA1, GFAP, CFP and SOX2, images were captured using a DM600 Leica
345 fluorescent microscope and subsequently quantified on ImageJ Software (NIH). Measurements
346 were conducted on lobules 3, 4 and 5 of midsagittal sections unless indicated in Figure legends.
347 Positive cells were counted and densities were calculated based on BgL length, EGL area, WM
348 area or on the whole cerebellum without the EGL (outside EGL) as indicated in the figures. For
349 the cerebellar section area, images were acquired using a Nanozoomer2.0 HT slide scanner
350 (Hamamatsu). Midsagittal sections were selected and exported for manual analysis using ImageJ
351 software. For EGL thickness, images of lobules 3, 4 and 5 from midsagittal sections were obtained
352 using a DM600 Leica fluorescent microscope and analyzed on ImageJ. EGL thickness was
353 calculated as the EGL area divided by the EGL perimeter.

Prism (GraphPad) was used for all statistical analyses. Statistical tests performed in this study were Welch's two-tailed t-test and Two-way analysis of variance (ANOVA) followed by post hoc analysis with Tukey's multiple comparison tests. A p-value ≤ 0.05 was considered as significant. Results are presented as the mean ± SEM. At least three biological samples and 2 to 3 sections per sample were analyzed for each experiment to ensure reproducibility and the sample sizes are reported in the Results section for significant data. For qualitative analysis, midsagittal sections from at least 4 samples were observed.

Results

scRNA-seq of NEPs during adaptive reprogramming reveals increased cellular stress, ROS signaling and DNA damage

To investigate the molecular changes that NEP subpopulations undergo upon injury to the EGL, in particular an increase in the signaling pathways associated with injury induced cellular stress and ROS, we performed multiplexed scRNA-seq of CFP+ cells isolated by fluorescence-activated cell sorting (FACS) of cerebella from *Nes-Cfp/+* transgenic neonates either irradiated at P1 (IR; P2, P3, P5) or non-irradiated (nonIR; P1, P2, P3, P5) (Figure 1A, Supplementary Figure 1A, B). Following the filtering out of poor quality cells and integration of replicates and the two conditions, the clustering of 11,878 cells (6,978 nonIR and 4,900 IR) was performed (Hao et al., 2021). The analysis revealed the expected three distinct groups of cells: gliogenic-NEPs and astrocytes (*Hopx*+ clusters 2, 3, 6, 10), neurogenic-NEPs (*Ascl1*+ clusters 5, 8, 11) and GCP (*Atoh1*+ clusters 1, 4, 7, 12, 14) that were present at each stage and in both conditions (Figure 1B-E, Supplementary Figure 1C, Table S2). These groups of cells were further subdivided into molecularly distinct clusters based on their cell cycle profiles or developmental stages (Table S2). In addition, oligodendrocyte progenitors (cluster 15), microglia (clusters 17, 21), ependymal cells (clusters 13, 18, 19) and meninges (cluster 16) were detected (Figure 1B, D, Table S2). Cluster 20 represented low-quality cells and was omitted from downstream analyses. As expected, the GCP clusters were enriched in the cells from irradiated mice and at P5 (Supplementary Figure 1C).

Our further analyses focused on changes in the signaling pathways associated with injury induced cellular stress and ROS. We previously showed that a subset of the *Hopx*+ gliogenic-NEPs that are present in the BgL are the ones that undergo adaptive reprograming following GCP death

389 (Wojcinski et al., 2017, Bayin N. S., 2021). We therefore assessed the immediate and later effects
390 of GCP death on *Hopx*+ gliogenic-NEPs by performing differential expression analyses between
391 nonIR and IR gliogenic-NEPs (*Hopx+,* clusters 2, 3, 6, 10) at P2, or at P3 and P5 (P3+5). 24 hr
392 after injury at P1, 132 genes in gliogenic-NEP clusters were significantly upregulated in IR
393 compared to 34 genes that were upregulated in nonIR P2 cells (adjusted p-value≤0.05, Figure
394 1F, Table S3). The significantly increased genes included *Cdkn1a, Phlda3, Ass1* and *Bax,* all of
395 which have been implicated as increased in response to DNA damage and in ROS signaling, as
396 well as in anti-apoptotic functions (Figure 1G) (Masgras et al., 2012, Bensellam et al., 2019, Qiu
397 et al., 2014, Jiang et al., 2008). Indeed, some of the top gene ontology (GO) terms associated
398 with the genes upregulated in gliogenic-NEPs with injury were related to response to irradiation,
399 DNA damage, the P53 pathway and ROS metabolic processes, whereas the top GO terms
400 associated with the genes upregulated in the nonIR cells at P2 were related to metabolic
401 processes (p-value≤0.05, Figure 1H, Table S4). Interestingly, the transcriptional changes
402 observed at P2 were less pronounced at later time points, where although some of the top
403 differentially expressed genes at P2 were still significantly upregulated at P3+5 stages combined
404 (e.g. *Cdkn1a, Phlda3, Ass1, Bax*), the increase in expression levels of these genes upon injury
405 and/or the number of cells expressing them gradually declined after P2 in IR gliogenic-NEPs when
406 compared to their nonIR counterparts (Figure 1G). Genes upregulated in IR P3+5 gliogenic NEPs
407 were associated with similar GO terms to those at P2, such as response to irradiation and the
408 P53 pathway, however, "ROS metabolic processes" was no longer a significantly enriched GO
409 term (p-value≤0.05, Supplementary Figure 2A, F, Table S3-4).
410
411 To assess whether the injury induced transcriptional signatures are specific to gliogenic-NEPs,
412 we performed the same differential expression analysis on nonIR and IR neurogenic-NEPs
413 (*Ascl1+,* clusters 5, 8, 11) and GCPs (*Atoh1+,* clusters 1,4,7,12,14) at P2, or at P3+P5 to identify
414 the immediate and later changes upon injury at birth. Similar to the gliogenic-NEPs, P2 IR
415 neurogenic-NEPs showed significant upregulation of genes associated with GO terms related to
416 stress response and apoptosis following injury, although the ROS related GO term was not
417 prominent (Supplementary Figure 2B, G, Table S3-4). Interestingly, although IR neurogenic-
418 NEPs at P3+5 had only 27 genes that were significantly upregulated following injury (adjusted p-
419 value≤0.05, Table S3, Supplementary Figure 2C), the genes included ones associated with
420 neural stem cells and BgL-NEPs (e.g. *Id1, Apoe, Ednrb*). The latter genes could represent the
421 transitory *Ascl1*+ BgL-NEP population that induces *Ascl1* expression during adaptive
422 reprogramming and would be included in the *Ascl1*+ neurogenic clusters or reflect the delayed

423 neurogenesis of neurogenic-NEPs previously demonstrated (Bayin N. S., 2021). Consistent with
424 the latter, the nonIR P3+5 neurogenic-NEPs showed an increase in mature neuron markers
425 compared to IR cells (adjusted p-value<0.05, Supplementary Figure 2C, H, Tables S3-4).
426
427 In contrast to the gliogenic- and neurogenic-NEP subtypes, P2 IR GCPs showed upregulation of
428 genes enriched in GO terms such as neural differentiation, axonogenesis and nonIR P2 GCPs
429 showed increased expression of genes involved in cell cycle and mitosis (Supplementary Figure
430 2D, I). This result could reflect the death of highly proliferative GCPs after irradiation and sparing
431 of only postmitotic granule cells upon irradiation at P1. P3+5 IR GCPs showed increased
432 expression of genes associated with BgL-NEPs (*Id1* and *Gdf10*, adjusted p-value $\leq$ 0.05) and
433 genes associated with GO terms such as cell cycle and cell fate commitment whereas P3+5 nonIR
434 GCPs showed enrichment for GO terms related to cell migration and neurogenesis (adjusted p-
435 value $\leq$0.05, Supplementary Figure 2D, E, I, J, Tables S3-4). This result could reflect the delayed
436 neurogenesis of GCPs following injury. Interestingly, we did not observe significant enrichment
437 for GO terms associated with cellular stress response in the GCPs that survived the irradiation
438 compared to controls (Table S4). Collectively, these results indicate that the gliogenic- and
439 neurogenic-NEP subtypes transiently upregulate stress response genes upon GCP death, but
440 only gliogenic-NEPs have a strong upregulation of ROS signaling.
441
442 **Injury induces changes in NEP chromatin landscape at P2**
443
444 We next tested whether GCP death at birth induces changes to the chromatin landscape of NEPs
445 that reflect the altered gene expression observed with scRNA-seq, by performing bulk ATAC-seq
446 on FACS-isolated CFP+ cells from P2 control and injured *Nes-Cfp/+* pup cerebella (Figure 1A).
447 P2 was chosen as it is the stage when GCPs contribute the least to the total Nes-CFP+ FACS
448 population and to identify the immediate effects of the injury on NEP chromatin. Analysis of
449 differentially open chromatin showed that injury induces major changes to the chromatin
450 landscape of the NEPs (1168 differentially open regions, adjusted p-value<0.05, Figure 1I. Table
451 S5). Of interest, *Cdkn1a* and *Phlda3*, two genes stimulated by ROS and injury (Bensellam et al.,
452 2019, Masgras et al., 2012) and that were upregulated in gliogenic-NEPs after irradiation (Figure
453 1F-G, Supplementary Figure 2B) exhibited new accessible regions around their gene bodies
454 compared to the nonIR P2 Nes-CFP+ cells (Figure 1J). Known motif analysis in the regions with
455 increased accessibility upon injury showed enrichment for binding motifs of the JUN/AP1
456 transcriptional complex (p-value = 10^{-14}, % of target sequences with motif = 15%, Table S6) which

457 is known to act in response to cellular stress and be activated by ROS. In addition, the DNA
458 binding site for FOXO3, a transcription factor that regulates ROS levels, had increased chromatin
459 accessibility (p-value = 0.001, % of target sequences with motif = 22.41%) (Table S6) (Filosto et
460 al., 2003, Auten and Davis, 2009, Hagenbuchner et al., 2012). Finally, gene network analysis of
461 ATAC-seq data revealed an active transcriptional network involved in regulating cell death and
462 apoptosis (Figure 1K). Furthermore, some of the genes involved in this response, such as *Ppara*,
463 *Egln3*, *Foxo3*, *Jun* and *Nos1ap,* have been implicated as upregulated with increased ROS levels
464 or involved in ROS signaling (Devchand et al., 2004, Kaelin, 2005, Hagenbuchner et al., 2012,
465 Filosto et al., 2003). In summary, our ATAC-seq data analysis along with the scRNA-seq provide
466 strong evidence that irradiation causes increased ROS signaling predominantly in the BgL-NEPs
467 upon GCP death by inducing transcriptional and epigenetic changes within 1 day after injury (P2).
468 In addition, genes related to cell survival and death, cellular stress and DNA damage are
469 upregulated in NEPs shortly upon injury, possibly as a means to overcome the cellular effects of
470 injury and induce adaptive reprogramming of NEPs to replenish the lost cells.
471
472 **Transient increase in cerebellar ROS during apoptosis of granule cell precursors**
473
474 To validate that the transient increase in expression of genes associated with cellular stress and
475 ROS signaling in NEPs is due to an increase in ROS upon cerebellar injury, we quantified ROS
476 levels in whole cerebellum samples using a mitochondrial superoxide indicator (MitoSOX) via flow
477 cytometry. A significant increase in ROS (cells present in the top 90% MitoSOX+ intensity) was
478 observed specifically at P2 (p=0.0005, n≥10) and not at P3 or P5 in IR pups compared to nonIR
479 (Figure 2A, B and Supplementary Figure 3A, B). Furthermore, quantification of mitochondrial
480 mass with MitoTracker revealed a reduction only at P2 (p=0.0005, n≥10) in IR pups (Figure 2C
481 and Supplementary Figure 3C-E). Additionally, quantification of TUNEL staining in midline sagittal
482 sections of the cerebella showed a large increase in cell death in the EGL of injured cerebella at
483 P2 (p=0.0040, n=4), but not at P3 compared to the controls (Figure 2D, E; see also Figure 3K, L).
484 Outside the EGL there was a small increase in cell death after injury that was only significant at
485 P3 (p=0.0379, n≥3) (Figure 2F, G). Thus, a transient increase in ROS in the cerebellum one day
486 after irradiation at P1 correlates with the timing of the major death of GCPs.
487
488 **Altered glial microenvironment following death of granule cell precursors**
489
490 Given the potential importance of glial cells to regenerative cellular responses after injury, we next
491 asked whether the glial microenvironment of the cerebellum changes during early postnatal

492 development in response to irradiation at P1. We first analyzed the astrocyte marker GFAP, since
493 it is generally upregulated in soon after brain injury (Burda and Sofroniew, 2014). Most astrocytes,
494 including the specialized Bg, express GFAP in the adult cerebellum, but the cells are generated
495 by gliogenic-NEPs during the first two weeks after birth. We therefore determined the normal
496 location and timing of initiation of GFAP expression in these cells during postnatal cerebellum
497 development. GFAP was first detected in rare astrocytes at P2 located in the white matter (WM)
498 below the lobules, with strong expression in all WM astrocytes at P5 and later stages (P8 and
499 P12) (Figure 3A-D, observed in n=4 mice/stage). In contrast, GFAP expression in astrocytes in
500 the developing IGL and in the glial processes of Bg that project through the molecular layer (ML)
501 and EGL was not detectable until P5 and was much stronger at P8 and P12 (Figure 3A-D).
502 Interestingly, after injury at birth, GFAP expression was prematurely upregulated in the WM
503 astrocytes below the lobules at P2 and in the remaining astrocytes at P5, including in the fibers
504 of Bg that extend through the ML and EGL, compared to nonIR cerebella (Figure 3A-H, observed
505 in n=4 mice/stage). GFAP expression was similar in all glia in both conditions at P8 and P12.
506 These results reveal that astrocytes and Bg react to EGL injury caused by irradiation at P1 by
507 initiating GFAP expression earlier than normal in the deep WM, and then the IGL and in Bg.
508
509 The macrophages of the brain, microglia, are generated in the early embryo but their main
510 increase in cell number occurs during neonatal development in mice (Hammond et al., 2018). We
511 found that most microglia were located in the WM of the cerebellum, and that the density of IBA1+
512 microglia increased in the WM between P2 (235.1 ± 18.3 cells/mm^2) and P3 (344.3 ± 53.1
513 cells/mm^2) and then was maintained at P5 (350.4 ± 40.2 cells/mm^2) (Fig. 3I-K, Supplementary
514 Figure 3F). Since the area of the cerebellum is increasing between P2 and P5, active microglia
515 production and/or infiltration must continue to occur between P2 and P5. As expected, after
516 irradiation the density of microglia in the injured EGL was significantly increased (~3 fold) at P2
517 but not at P3 or P5 compared to controls (p=0.0331, n=4) (Fig. 3L-N, Supplementary Figure 3G).
518 No difference in the microglial density in the WM was detected between conditions at both time
519 points (Fig. 3I-L; Supplementary Figure 3F). Thus, as expected microglia density was increased
520 in the EGL one day after injury when the maximum GCP cell death occurs.
521
522 **Decreasing ROS impairs cerebellar repair**
523
524 Given the transient increase in ROS signaling in gliogenic-NEPs during peak GCP death and
525 recruitment of microglia to the EGL, and the later astroglial response, we tested whether an
526 increase in ROS is necessary for adaptive reprogramming and cerebellar repair following EGL

527 injury at P1. To reduce the level of ROS we utilized an *mCAT* transgenic mouse line that
528 expresses the human mitochondrial catalase ubiquitously from a *CMV* promoter (Schriner et al.,
529 2005). The transgene is expected to reduce mitochondrial ROS levels in all cells by catalyzing
530 the breakdown of hydrogen peroxide into water and oxygen, hence protecting the cells from
531 oxidative damage. We confirmed that mCAT protein is expressed throughout the cerebellum
532 using immunohistochemical (IHC) staining of cerebellar sections (Figure 4A, B). MitoSOX flow
533 cytometry revealed a significant decrease in ROS in the cerebella of *mCAT/+* mice 1 day after
534 irradiation (P2) ($p=0.0163$, $n\geq8$) and not at P3 or P5 compared to control IR mice and no baseline
535 decrease in ROS at any stage in nonIR mice (Figure 4C and Supplementary Figure 4A, B).
536 Mitochondrial mass, as measured by MitoTracker flow cytometry, was reduced at P2 in *mCAT/+*
537 IR compared to nonIR *mCAT/+* pups with no significant difference observed between *mCAT/+*
538 and control IR mice (Fig. 4D, Supplementary Figure 4C, D). The level of cell death in the EGL
539 and density of IBA1+ microglia in the EGL and elsewhere in the cerebellum were similar between
540 *mCAT/+* and littermate control IR mice at P2 and P3 (Fig. 4E, F, Supplementary Figure 4G, J).
541 There was a slight increase in cell death outside the EGL at P2 in IR *mCAT/+* compared to nonIR
542 *mCAT/+* mice but not compared to IR controls (Supplementary Figure 4F, E). Thus, the *mCAT*
543 transgene counteracts the transient increase in cerebellar ROS following EGL injury but does
544 have a major effect on GCP death or the infiltration of microglia to the injured EGL (Supplementary
545 Figure 4 F-J).
546
547 We next determined the regenerative efficiency of *mCAT/+* mice by analyzing the area of sections
548 of cerebella from nonIR and IR P30 *mCAT/+* mice compared to littermate controls. Strikingly, the
549 cross-sectional area of the medial cerebellum (vermis) of IR *mCAT/+* adult mice was significantly
550 reduced compared to IR controls ($p=0.0040$, $n\geq6$) (Fig. 4G-K). Analysis of cerebellar area across
551 ages (P3, 5, 8 and 12) revealed that the vermis sectional area of the IR *mCAT/+* cerebella was
552 only significantly reduced at P30 compared to IR controls, however at P12 it was reduced in
553 *mCAT/+* IR cerebella compared to *mCAT/+* nonIR mice, whereas it was not significantly different
554 between control IR and control nonIR mice (Fig. 4L, Supplementary Figure 4 K-N). These results
555 indicate that cerebellar growth begins to be reduced at P12 in *mCAT/+* mice following injury. Thus,
556 a reduction in ROS at the time of cell death in the EGL leads to a diminution of cerebellar recovery.
557
558 **Reduced regeneration in *mCAT* mice is associated with reduced adaptive reprogramming**
559 **at P5.**
560

561 Given that a decrease in ROS following EGL injury reduces regeneration of the neonatal
562 cerebellum, we determined whether specific stages of the adaptive reprogramming process are
563 altered in *mCAT*/+ mice compared to controls. First, we analyzed the replenishment of the EGL
564 by BgL-NEPs in vermis lobules 3-5. Interestingly, we found that although the thickness of the EGL
565 in IR mice of both genotypes was similarly reduced compared to nonIR mice at P5 (p=0.0041
566 control and p=0.0005 *mCAT*/+; n≥4) by P8 the control EGL was a similar thickness to the control
567 nonIR whereas the thickness of the *mCAT*/+ IR EGL was significantly reduced compared to
568 *mCAT*/+ nonIR mice (p=0.035, n=4)(Figure 5A, B). A key regenerative process that contributes
569 to the expansion of the EGL following injury is the migration of BgL-NEPs to the EGL. Strikingly,
570 the density of CFP+ cells in the EGL (mainly BgL-derived NEPs) was significantly decreased in
571 *mCAT*/+ IR mice compared to control IR mice at P5 (p=0.0002, n=4) but not P3 (Figure 5C-G,
572 Supplementary Figure 5A). Furthermore, the density of NEPs (CFP+ or SOX2+ cells) in the BgL
573 was significantly decreased at P5 in *mCAT*/+ IR cerebella compared to controls (p=0.0010, n≥5)
574 but not at other stages (Figure 5H, Supplementary Figure 5B-D). These results indicate that BgL-
575 NEPs have a blunted response to EGL injury, and therefore do not fully expand and contribute to
576 the replenishment of GCPs in the EGL after irradiation.
577
578 **Microglia contribute to one aspect of adaptive reprogramming.**
579 Given that several steps in adaptive reprogramming were decreased specifically at P5 in *mCAT*/+
580 IR cerebella, we asked whether microglia/macrophages could be involved in any of the processes.
581 We first determine the density of IBA1+ cells in the EGL and WM of vermis lobules 3-5 at P5 in
582 nonIR and IR mice of both genotypes. As expected, the density of microglia in the EGL was very
583 low in the nonIR control and *mCAT/*+ cerebella (Figure 6A-E). Interestingly, whereas control IR
584 mice had a similar number of IBA1+ cells in the WM as nonIR mice of both genotypes at P5, the
585 *mCAT*/+ IR mice had a lower density of microglia/macrophages in the WM compared to control
586 IR mice at P5 (p=0.0012, n≥3) (Figure 6A-D, F). This result raised the question of whether
587 macrophages/microglia play a role in adaptive reprogramming. We, therefore, tested whether
588 reducing the density of IBA1+ microglia/macrophages after birth would alter adaptive
589 reprogramming at P5 or cerebellar regeneration at later stages. Since macrophages and
590 cerebellar microglia are dependent on colony stimulating factor 1 (CSF1) for their survival, we
591 administered PLX5622, a small molecule inhibitor of CSF receptor 1 (CSFR1), to pups every day
592 from P0-5 (PLX treatment) (Kana et al., 2019, Tan et al., 2021). As expected, IBA1+ cells were
593 significantly decreased in the cerebellum of PLX-treated mice at P5 compared to their controls,
594 both nonIR and IR (p=0.0015 and p=0.0059, n=3 and n=5, respectively) (Figure 6G,

595 Supplementary Figure 6B-E). The thickness of the EGL was not significantly altered at P5 in PLX-
596 treated IR mice compared to IR controls (Figure 6H). Interestingly, similar to *mCAT/+* mice, the
597 density of Nes-CFP+ cells in the EGL was significantly decreased in PLX-treated IR mice
598 compared to IR controls at P5 (p=0.0008, n=5) (Figure 6I-M). In contrast, the density of SOX2+
599 cells in the BgL, corresponding to the gliogenic BgL-NEPs, were unchanged in the PLX-treated
600 and control mice, whether irradiated or not, suggesting that the decrease in expansion of BgL-
601 NEPs caused by ROS is not mediated by microglia (Supplementary Figure 6F). When mice were
602 treated with PLX from P0-8 and allowed to age to P30, we found the cerebellar vermis section
603 area was not decreased in PLX-treated IR mice compared to IR controls (Figure 6N). Thus,
604 reducing the density of IBA1+ microglia/macrophages in neonatal mice reduces the recruitment
605 of Nes-CFP+ cells to the EGL at P5, but does not have a long-term significant impact on
606 regeneration of the cerebellum.
607
608 **Discussion**
609 We demonstrate that a transient increase in ROS signaling after cerebellar injury to the EGL is
610 critical for adaptive reprogramming and full recovery of cerebellar growth. ROS likely acts as an
611 alarm signal shortly after injury and is primarily detected by BgL-NEPs. scRNA-seq at P1-5 and
612 bulk ATAC-seq at P2 of NEPs following targeted irradiation at P1 revealed a rapid increase in
613 transcriptional and epigenetic changes associated with upregulation of ROS and stress related
614 pathways in NEPs that peaked at P2. A transient upregulation in ROS at P2 was confirmed using
615 flow cytometry and found to correlate with the timing of cell death in the EGL one day after
616 irradiation. By reducing mitochondrial ROS levels across all cell types at P2 using a *mCAT*
617 transgene, we uncovered that ROS is required for several steps of adaptive reprogramming of
618 BgL-NEPs. In addition, we found that microglia are reduced in injured *mCAT/+* pups, which is
619 consistent with prior evidence that ROS can trigger immune cell recruitment in other systems (Kim
620 et al., 2010, Mehl et al., 2022). Moreover, temporary depletion of microglia caused a reduction in
621 the number of NEPs that migrate into the EGL at P5 following injury at P1, but no long-term
622 reduction of cerebellar size. Thus, we identified key transcriptomic and epigenomic changes in
623 cerebellar NEPs upon GCP ablation at birth and discovered roles for the tissue microenvironment,
624 especially ROS and a more limited role of microglia during neonatal cerebellum regeneration.
625
626 scRNA-seq analysis of NEPs from nonIR (P1-3, P5) and nonIR (P2, P3, P5) showed an increase
627 in genes associated with stress responses after irradiation in both the gliogenic and neurogenic
628 subpopulations, but not in the GCPs. Furthermore, a predominant increase in ROS signaling was

629 detected only in the gliogenic-NEPs one day after injury (P2). The injury-induced ROS and stress-
630 related gene signatures were not observed in the later P3+5 gliogenic- and neurogenic-NEPs,
631 suggesting that the increase in ROS levels is an early injury induced signal affecting the NEP
632 transcriptome. Our bulk ATAC-seq data generated at P2 revealed that the open chromatin regions
633 in the IR NEPs were enriched for transcription factor binding motifs related to ROS signaling and
634 stress induced transcription factors such as FOXO3 and AP1 (Table S6). Thus, cerebellar injury
635 during development induces transcriptional and epigenomic signatures in the NEPs and ROS
636 signaling could be a key driver of NEP adaptive reprogramming. Our results are in line with other
637 regeneration systems where a temporary increase in ROS is observed upon cell death or injury
638 and is considered to be a DAMP (Niethammer, 2016).
639
640 Interestingly, although both gliogenic- and neurogenic-NEPs showed induction of cellular stress
641 related genes upon injury, upregulation of ROS signaling and related genes was only observed
642 in the *Hopx*-expressing gliogenic NEPs that undergo adaptive reprogramming. Whether the
643 upregulation of ROS signaling primarily in the BgL-NEPs but not in the *Ascl1*+ neurogenic-NEPs
644 is due to BgL-NEPs being in proximity to the dying GCPs after injury or their direct contact due to
645 their radial projections remains to be determined. The ability of BgL-NEPs to respond to GCP
646 death via upregulating ROS signaling and impaired regeneration upon reduction of ROS levels,
647 shows that ROS signaling is involved in triggering adaptive reprogramming upon injury.
648
649 The cellular composition of the neonatal cerebellum is dramatically different from the adult. During
650 the early postnatal period, we found that astrocytes in the WM below the lobules are the first to
651 initiate GFAP expression at P2 and that by P5 all astrocytes express a high level of GFAP. In
652 contrast, Bg express a low level of GFAP at P5 and reach a high level by P8. Interestingly, we
653 found that injury leads to an increase in the level of GFAP expression in each type of astroglia
654 when they first initiate expression, deep WM astrocytes at P2 and Bg (and all astrocytes) at P5.
655 Once adaptive reprogramming is nearing completion (P8), the GFAP levels remain similar in all
656 astroglia in nonIR and IR cerebella. These results suggest that the neonatal astrocytes respond
657 to injury differently than in the adult where GFAP upregulation is observed immediately after injury
658 (Burda and Sofroniew, 2014), since Bg and astrocytes in the lobules have a delayed response to
659 injury with GFAP not being upregulated for several days. Furthermore, at birth the microglia have
660 not fully expanded in number in the cerebellum and previous scRNA-seq showed that the
661 neonatal and adult microglia are transcriptionally distinct (Hammond et al., 2019). Perhaps
662 neonatal microglia are anti-inflammatory and pro-regenerative upon injury in neonates, in contrast

663 to adult microglia where upon traumatic brain injury they can inhibit regeneration (Donat et al.,
664 2017). Collectively, the differences in glial responses to injury in the neonatal cerebellum and
665 adult brain likely contributes to the permissiveness of the neonatal cerebellum to regeneration.
666 Details of the molecular changes that the neonatal cerebellar microglia/macrophages and
667 astrocytes undergo upon GCP injury and their molecular crosstalk with NEPs remain to be
668 determined.
669
670 We demonstrated the significance of ROS activation in the NEP reprogramming process using
671 an *mCAT* transgenic mouse line in which human Catalase (CAT) is expressed in mitochondria, a
672 protein that can lead to a reduction in hydrogen peroxide (H_2O_2) and thus lower ROS. However,
673 in *mCAT* neonatal cerebella we found that the percentage of MitoSOX high cells are comparable
674 to control mice under nonIR conditions. Importantly, however, one day after irradiation at P1 when
675 the percentage of MitoSOX high cells increases in control IR mice, the *mCAT* transgene reduces
676 the percentage of MitoSOX high cells, such that it remains at the baseline nonIR *mCAT* level.
677 Therefore, human CAT expression in mitochondria in this model inhibits the injury-induced
678 increase in ROS levels without affecting the homeostatic production of superoxide. Of possible
679 relevance, in this mouse model the observed change in ROS levels is likely global, impacting all
680 cell types. The specific impact of increased mCAT in BgL-NEPs or microglia on their recruitment
681 and function after an injury remains to be determined with new cell type-specific tools.
682
683 Our experiments depleting microglia using PLX5622 indicate that microglia/macrophages are
684 involved in the regeneration of the EGL following irradiation. Previous studies have demonstrated
685 a dual role of microglia in promoting and inhibiting regenerative processes within the nervous
686 system (Lee et al., 2021, Wang et al., 2020). Our data support the idea that microglia are involved
687 in the adaptive reprogramming of NEPs to GCPs by promoting their replenishment of GCPs in
688 the EGL. While the direct mechanisms remain to be discovered, this finding highlights that the
689 neonatal microglia support regeneration upon injury to the newborn cerebellum. PLX-treatment
690 for 8 days after irradiation did not reduce the later growth of the injured cerebellum. One possibility
691 is that after the cessation of PLX administration, regeneration proceeds normally. Alternatively, it
692 raises the possibility that an additional regenerative process is required in parallel to microglial
693 signaling.
694
695 Collectively, we have delineated the spatiotemporal cellular changes in the cerebellar glial
696 microenvironment upon ablation of GCPs at birth and highlight ROS signaling as a key stimulator

697 of adaptive reprogramming of NEPs. How DAMP-glia-progenitor crosstalk is orchestrated remains
698 to be untangled. Understanding how microenvironmental responses shape repair processes is a
699 crucial first step towards developing strategies to promote regeneration.
700

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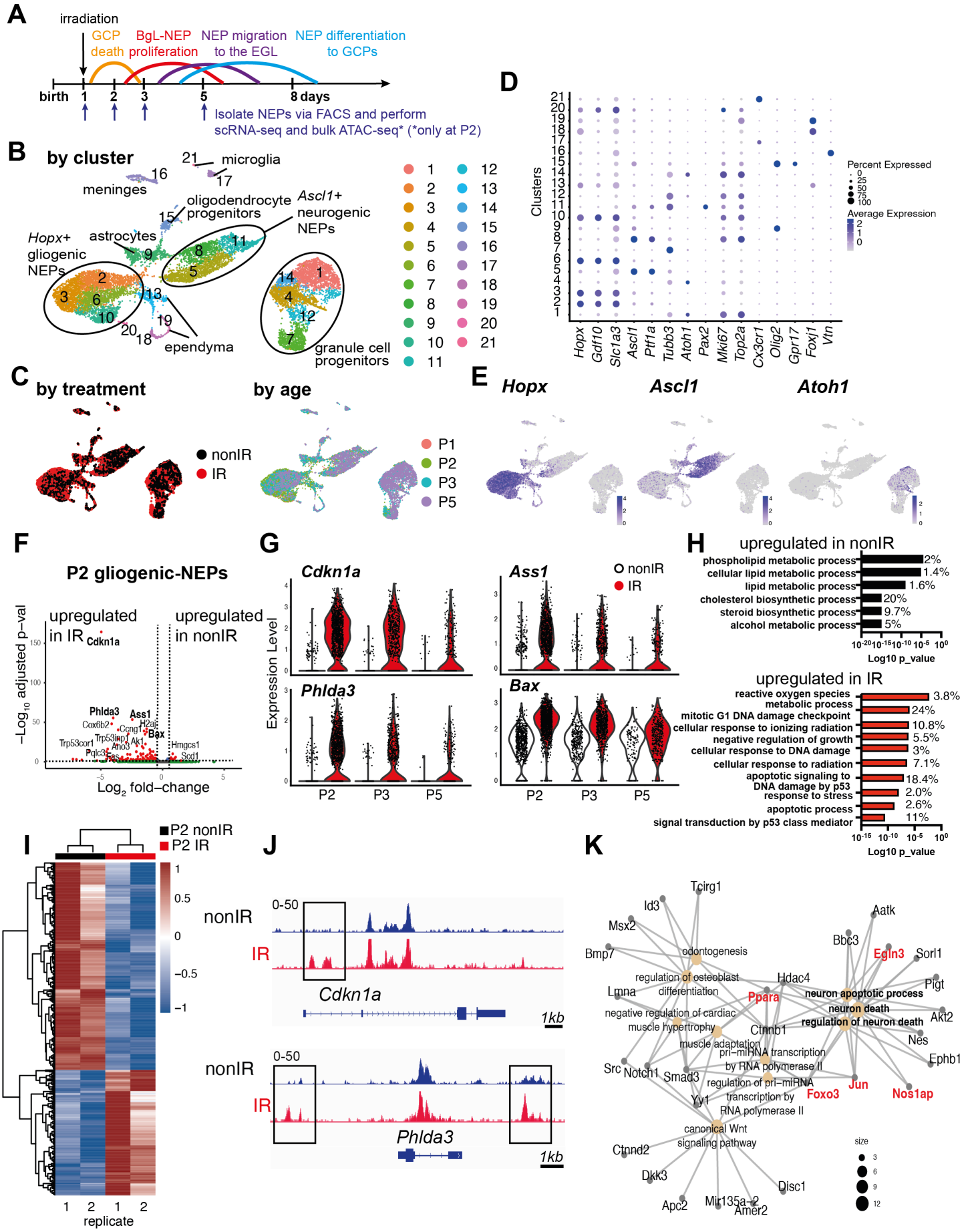

A
irradiation
GCP death
BgL-NEP proliferation
NEP migration to the EGL
NEP differentiation to GCPs
birth 1 2 3 5 8 days
Isolate NEPs via FACS and perform scRNA-seq and bulk ATAC-seq* (*only at P2)
B
by cluster
microglia
meninges
oligodendrocyte progenitors
Ascl1+ neurogenic NEPs
Hopx+ gliogenic NEPs
astrocytes
ependyma
granule cell progenitors
C
by treatment
nonIR
IR
by age
P1
P2
P3
P5
D
Clusters
Percent Expressed
Average Expression
Hopx
Gdf10
Slc1a3
Ascl1
Ptf1a
Tubb3
Atoh1
Pax2
Mki67
Top2a
Cx3cr1
Olig2
Gpr17
Foxj1
Vtn
E
Hopx
Ascl1
Atoh1
F
P2 gliogenic-NEPs
upregulated in IR
upregulated in nonIR
Cdkn1a
Phlda3
Ass1
Bax
Log2 fold-change
−Log10 adjusted p-val
G
Cdkn1a
Ass1
Phlda3
Bax
nonIR
IR
Expression Level
P2
P3
P5
H
upregulated in nonIR
phospholipid metabolic process 2%
cellular lipid metabolic process 1.4%
lipid metabolic process 1.6%
cholesterol biosynthetic process 20%
steroid biosynthetic process 9.7%
alcohol metabolic process 5%
Log10 p_value
upregulated in IR
reactive oxygen species metabolic process 3.8%
mitotic G1 DNA damage checkpoint 24%
cellular response to ionizing radiation 10.8%
negative regulation of growth 5.5%
cellular response to DNA damage 3%
cellular response to radiation 7.1%
apoptotic signaling to DNA damage by p53 18.4%
response to stress 2.0%
apoptotic process 2.6%
signal transduction by p53 class mediator 11%
Log10 p_value
I
P2 nonIR
P2 IR
replicate
J
nonIR
IR
0-50
Cdkn1a
Phlda3
1kb
K
Tcirg1
Id3
Msx2
Bmp7
odontogenesis
regulation of osteoblast differentiation
Lmna
negative regulation of cardiac muscle hypertrophy
muscle adaptation
Hdac4
Ppara
Ctnnb1
Aatk
Bbc3
Egln3
Sorl1
Pigt
neuron apoptotic process
neuron death
regulation of neuron death
Akt2
Nes
Ephb1
Nos1ap
Jun
Foxo3
Src
Notch1
Smad3
Yy1
pri-miRNA transcription by RNA polymerase II
regulation of pri-miRNA transcription by RNA polymerase II
canonical Wnt signaling pathway
Ctnnd2
Dkk3
Apc2
Mir135a-2
Amer2
Disc1
size

Figure 1: Injury induces ROS and cell stress signaling reflected by changes in the transcriptome and chromatin landscape of gliogenic-NEPs.

(A) Schematic summarizing the experimental plan.

(B-C) UMAP projections of 11,878 cells (6,978 nonIR and 4,900 IR) showing cluster annotations (B), treatment (black: nonIR, red: IR) and the age of the samples (red: P1, green: P2, blue: P3, purple: P5) (C).

(D) Dot plot showing the expression levels of key marker genes used for cluster annotation (gliogenic-NEPs: *Hopx, Gdf10, Slc1a3*, neurogenic-NEPs: *Ascl1, Ptf1a*, immature neurons: *Pax2*, GCPs: *Atoh1*, postmitotic neurons: *Tubb3*, microglia: *Cx3cr1*, oligodendrocyte progenitors: *Olig2*, oligodendrocytes: *Gpr17*, Ependymal cells: *Foxj1*).

(E) Feature plots showing *Hopx* (gliogenic-NEPs), *Ascl1* (neurogenic-NEPs) and *Atoh1* (GCPs) expression highlighting the three main populations of interest. Clusters containing *Hopx*-NEPs (clusters 2, 3, 6, 10), *Ascl1*-NEPs (clusters 5, 8, 11), or GCPs (clusters 1, 4, 7, 12, 14) were subsetted from the original data set and were divided according to age (P2 or P3+P5) for the downstream differential expression analyses.

(F) Volcano plot showing differentially expressed genes in the P2 gliogenic-NEPs (red: adjusted p-value$\leq$0.05, $\log_2$fold-change>|1|).

(G) Violin plots showing some of the top differentially expressed genes in P2 gliogenic-NEPs and how their expression changes over time with respect to their expression in control cells**.**

(H) Top GO terms associated with differentially expressed genes in P2 gliogenic-NEPs that were either upregulated in nonIR (top panel) or IR (bottom panel) cells (adjusted p-value$\leq$0.05, Table S3).

(I) Heatmap showing differentially open chromatin regions in P2 nonIR and IR NEPs, identified by bulk ATAC-seq (1168 differentially open regions, adjusted p-value<0.05, Table S4).

(J) Tracks highlighting the injury-induced open chromatin regions around *Cdkn1a* and *Phlda3,* the top differentially expressed genes identified in (F).

(K) Gene network analysis of ATAC-seq data revealed an active transcriptional network involved in regulating cell death and apoptosis. Genes colored in red (*Ppara*, *Egln3*, *Foxo3*, *Jun* and *Nos1ap)* have been implicated as upregulated with increased ROS levels or involved in ROS signaling.

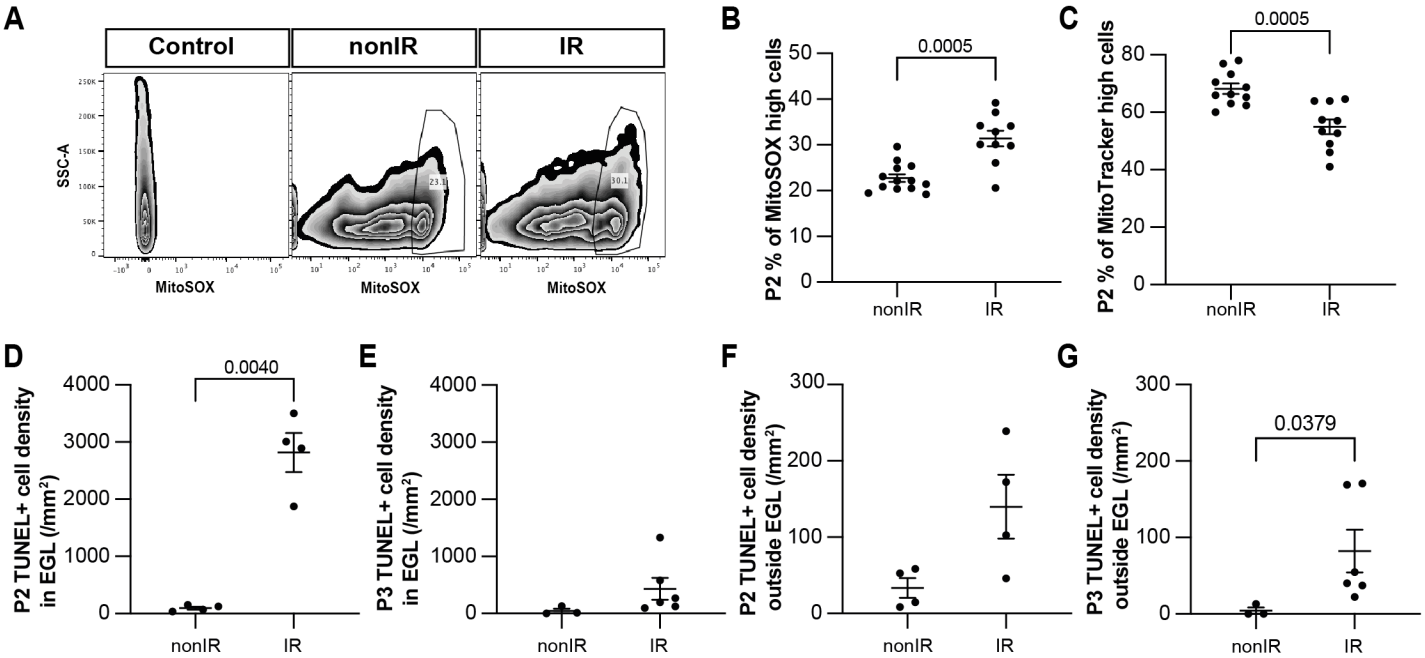


Figure 2: Cerebellar injury at P1 results in increased superoxide production, a reduction in mitochondria and increased cell death in the EGL that peaks 24h after injury.

(A) Examples of flow cytometry analysis of mitochondrial ROS at P2 from nonIR and IR cerebella using MitoSOX dye. Gating determined the top 90% MitoSOX signal (MitoSOX high cells).

(B, C) Quantification of MitoSOX high (B) and MitoTtracker high (C) expression in nonIR and IR cerebella at P2.

(D, E) Quantification of TUNEL+ cell density in the EGL at P2 (D) and P3 (E) in lobules 3-5 of nonIR and IR mice.

(F, G) Quantification of TUNEL+ cell density outside the EGL at P2 (F) and P3 (G) in lobules 3-5 of nonIR and IR mice.

EGL, External granular layer; SSC, side scatter; P, postnatal day; nonIR, non-irradiated; IR, irradiated. All statistical significance was determined using an unpaired t-test and data are represented as mean ± SEM.

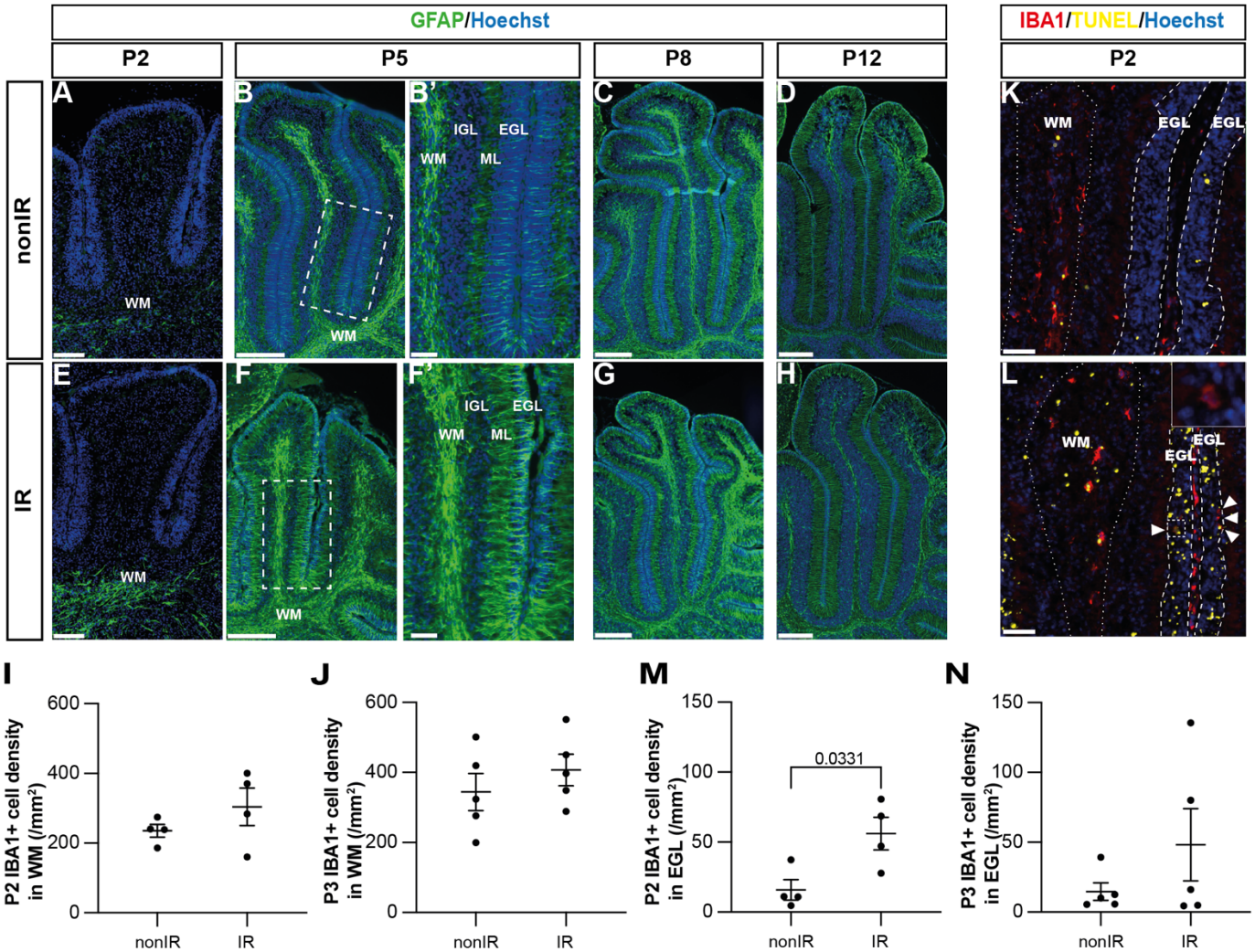


Figure 3: Cerebellar injury at P1 induces transient microglial recruitment to the EGL and prolonged astroglial microenvironment changes in the cerebellum.

(A-H) Immunohistochemical (IHC) staining of medial sagittal cerebellar sections for GFAP (green) in lobules 4/5 of nonIR and IR cerebellum at the stages indicated. Nuclei were counterstained with Hoechst. (B') and (F') show high-power images of white dashed line boxes in (B) and (F), respectively.

(I, J) Quantification of IBA1+ cell density in the WM at P2 (I) and P3 (J) in lobules 3-5 of nonIR and IR mice.

(K, L) IHC staining of medial sagittal cerebellar sections for IBA1 and TUNEL in lobule 3 of nonIR and IR cerebellum at P2. Nuclei were counterstained with Hoechst. White matter (WM) and external granular layer (EGL) are delineated by white dotted lines and dashed lines, respectively. High-power image in (L) of the area indicated by the white dashed line represents an IBA1+ cell present in the EGL. White arrowheads indicate additional IBA1+ cells in the EGL.

(M, N) Quantification of IBA1+ cell density in the EGL at P2 (M) and P3 (N) in lobules 3-5 of nonIR and IR mice. EGL, External granular layer; WM, White matter; P, postnatal day; nonIR, non-irradiated; IR, irradiated. Scale bar: A and E 100µm, B, C, D, E, F, G and H: 250µm, B' and F': 50µm, I and J: 50µm. All statistical significance was determined using an unpaired t-test and data are represented as mean ± SEM.

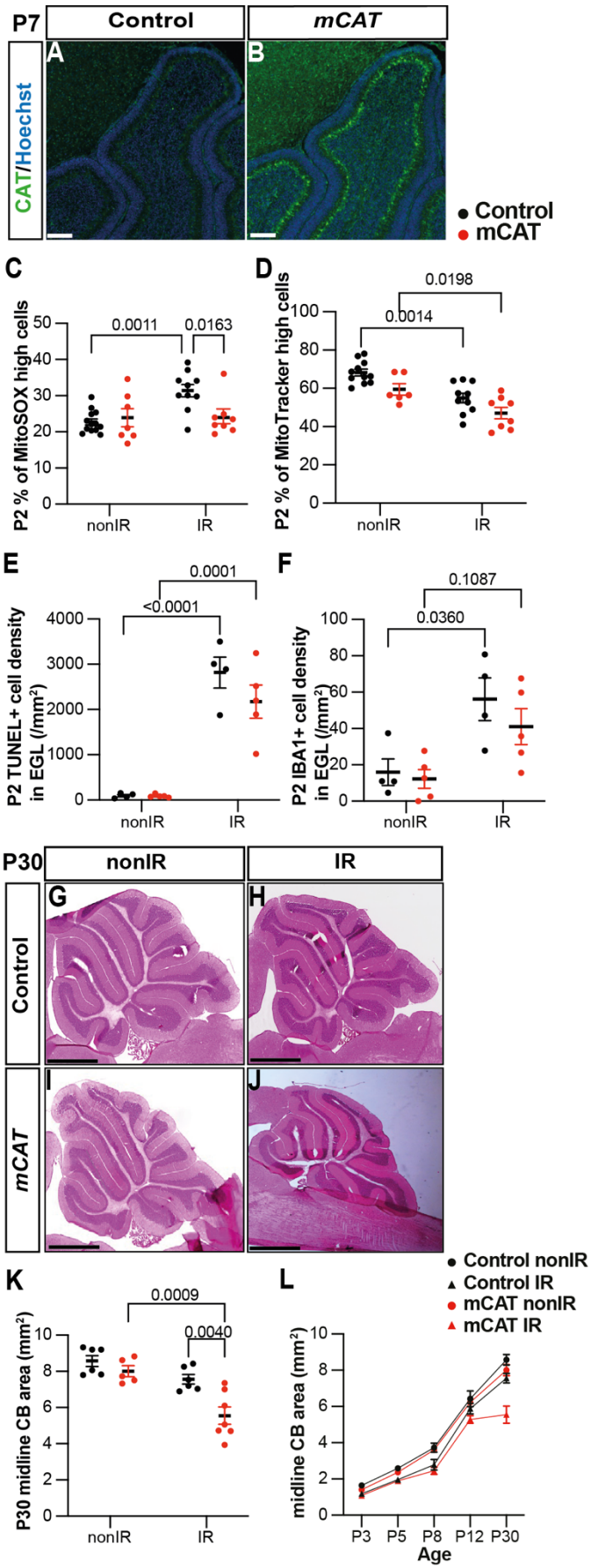


Figure 4: Reduction of ROS impairs adaptive reprogramming and cerebellar repair.

(A, B) IHC staining of medial sagittal cerebellar sections for human catalase in control (A) AND *mCAT/+* mice (B) at P7. Nuclei were counterstained with Hoechst (blue). Similar staining was seen in four *mCAT/+* mice.

(C) Quantification of MitoSOX high expression at P2 in control and *mCAT/+* cerebella, with and without irradiation at P1 (Two-way ANOVA, $F_{(1,34)}$=6.768, p=0.0136).

(D) Quantification of MitoTracker high expression at P2 in control and *mCAT/+* cerebella, with and without irradiation at P1 (Two-way ANOVA, $F_{(1,31)}$=25.06, p<0.0001).

(E) Quantification of TUNEL+ cell density in the EGL at P2 in control and *mCAT/+* cerebella, with and without irradiation at P1 (Two-way ANOVA, $F_{(1,14)}$=87.56, p<0.0001).

(F) Quantification of IBA1+ cell density in the EGL at P2 in control and *mCAT/+* cerebella, with and without irradiation at P1 (Two-way ANOVA, F(1,14)=15.58, p=0.0015).

(G-J) Hematoxylin and eosin staining on mid-sagittal sections of P30 control and *mCAT/+* cerebellum with or without irradiation.

(K) Quantification of P30 cerebellar mid-sagittal section area in controls and *mCAT/+* nonIR and IR mice (Two-way ANOVA, $F_{(1,20)}$=11.82, p=0.0026).

(L) Graph showing the average area of mid-sagittal cerebellar sections at P3, P5, P8, P12 and P30 in control and *mCAT/+* non-irradiated and irradiated mice. Detailed statistics are shown in Supplementary Figure 4.

EGL, External granular layer; P, postnatal day; nonIR, non-irradiated; IR, irradiated. Scale bar: A and B: 100µm, F-I: 1mm. Significant *Tukey's post hoc* multiple comparison tests are shown in the figures and data are represented as mean ± SEM.

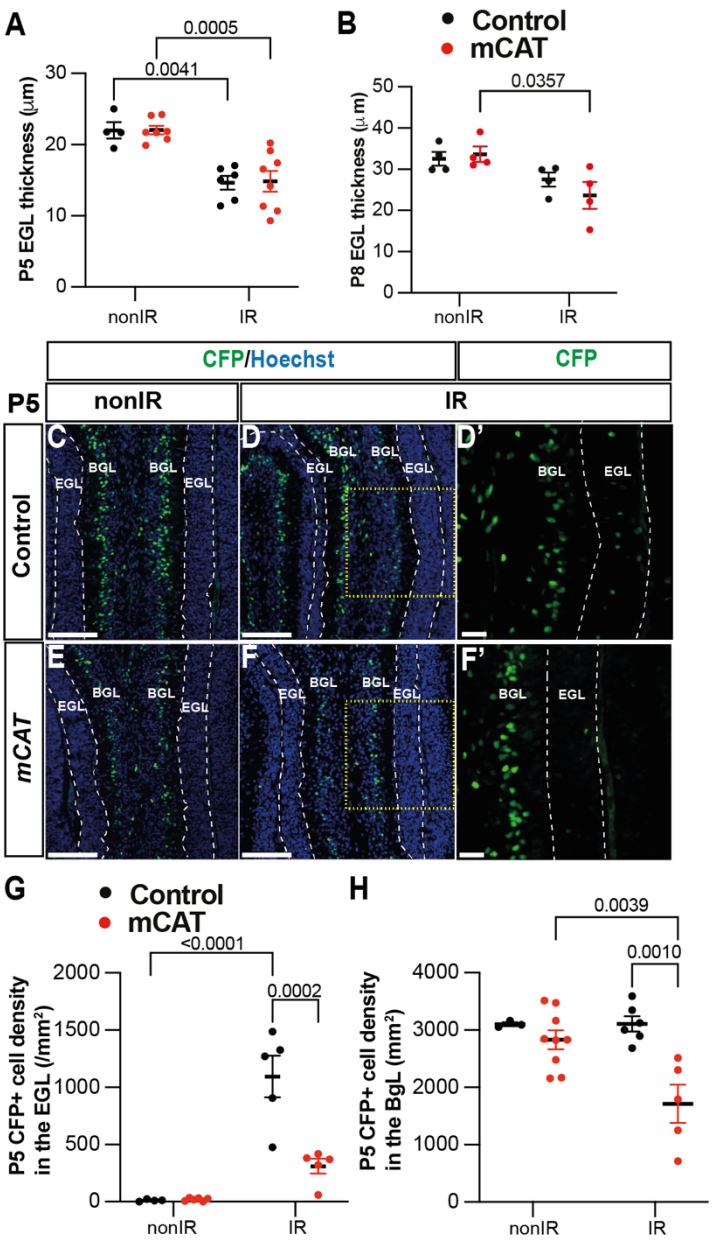


Figure 5: Reduced ROS impairs expansion of BgL-NEPs and their recruitment to the EGL after injury.

(A, B) Quantification of EGL thickness at P5 (Two-way ANOVA, $F_{(1,21)}=36.64$, $p<0.0001$) (A) and P8 (Two-way ANOVA, $F_{(1,12)}=11.34$, $p=0.0056$)(B) in lobules 3-5 of *Nes-Cfp/+* control and *Nes-Cfp/+; mCAT/+* mutant mice with and without irradiation at P1

(C-F) IHC staining of medial sagittal cerebellar sections showing expression of CFP (green) in the lobules 4/5 of *Nes-Cfp/+* control and *Nes-Cfp/+; mCAT/+* mutant mice at P5. Nuclei were counterstained with Hoechst (blue). (D') and (F') show a high-power images of the yellow boxed area in the single channel CFP. EGL is delineated by the dashed white lines.

(G, H) Quantification of CFP+ cell density in the EGL (Two-way ANOVA, $F_{(1,19)}=5.192$, $p=0.0359$) (G) and BgL (Two-way ANOVA, $F_{(1,17)}=6.191$, $p=0.0223$) (H) at P5 in *Nes-Cfp/+* control or *Nes-Cfp/+; mCAT/+* mutant non-irradiated and irradiated mice.

EGL, External granular layer; BgL, Bergmann glia Layer; P, postnatal day; nonIR, non-irradiated; IR, irradiated. Scale bar: D-F: 100µm. Significant *Tukey's post hoc* multiple comparison tests are shown in the figures and data are represented as mean ± SEM.

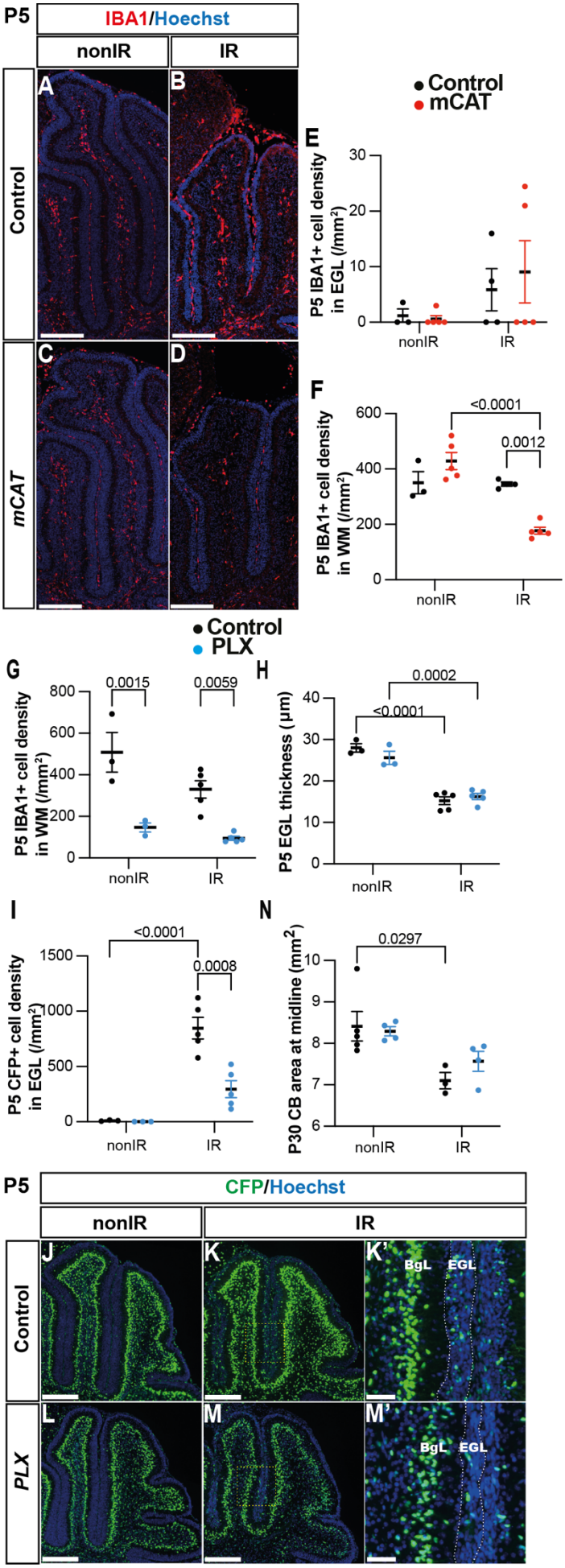


Figure 6: Microglia promote recruitment of NEPs to the EGL during cerebellar adaptive reprogramming after injury.

(A-D) IHC staining of medial sagittal cerebellar sections for IBA1 (red) in control and *mCAT/+* mice at P5. Nuclei were counterstained with Hoechst (blue).

(E, F) Quantification of IBA1+ cell density in the external granular layer (E) and white matter (Two-way ANOVA, $F_{(1,13)}$=24.74, p=0.0003) (F) at P5 on midsagittal sections of lobules 3-5 in the cerebellum of control and *mCAT/+* animals, with or without irradiation.

(G-J) IHC staining of medial sagittal cerebellar sections at P5 for CFP (green) in lobules 4/5 of *Nes-Cfp/+* mice treated with PLX5622 or control DMSO with or without irradiation. Nuclei were counterstained with Hoechst (blue). (H') and (J') show a high-power image of area indicated by yellow boxes. EGL is delineated by the white dashed lines.

(K) Quantification of IBA1+ cell density in the white matter at P5 on mid-sagittal sections in lobules 3-5 of *Nes-Cfp/+* mice treated with PLX5622 or control DMSO, with or without irradiation (Two-way ANOVA, $F_{(1,12)}$=42.40, p<0.001).

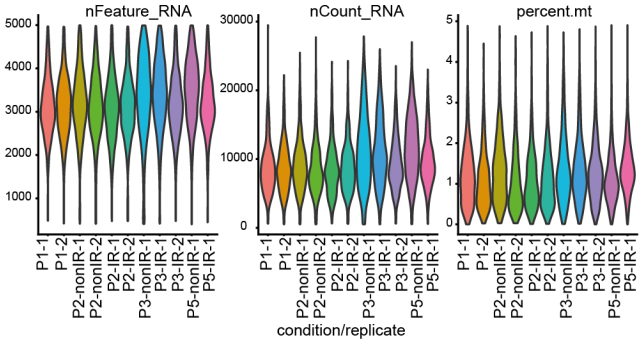
(L) Quantification of EGL thickness at P5 in lobules 3-5 of *Nes-Cfp/+* mice treated with PLX5622 or control DMSO with or without irradiation (Two-way ANOVA, $F_{(1,12)}$=109.5, p<0.001).

(M) Quantification of CFP+ cells density in the EGL at P5 on mid-sagittal sections in lobules 3-5 of *Nes-Cfp/+* mice treated with PLX5622 or control DMSO with or without irradiation (Two-way ANOVA, $F_{(1,12)}$=10.62, p=0.0068).

(N) Measurement of cerebellar mid-sagittal section area at P30 in controls or mice treated with PLX, with or without irradiation at P1 (Two-way ANOVA, $F_{(1,12)}$=13.29, p=0.0034).

EGL, External granular layer; WM, White matter; P, postnatal day; nonIR, non-irradiated; IR, irradiated. Scale bar: A-D and G-J: 250 µm. Significant *Tukey's post hoc* multiple comparison tests are shown in the figures and data are represented as mean ± SEM.

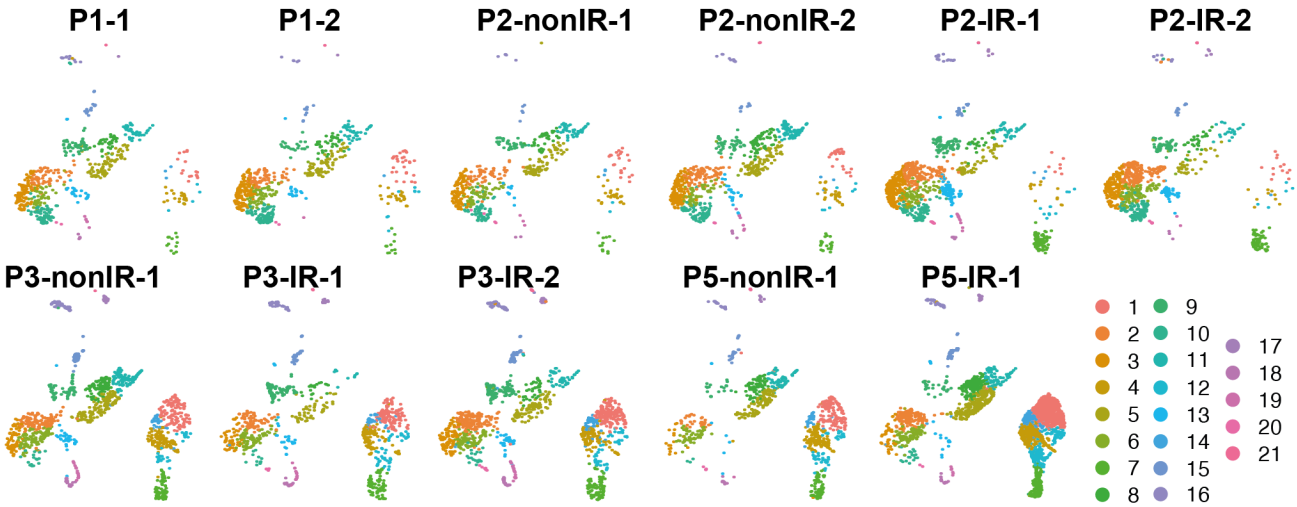
A



B

	P1	P2		P3		P5	
	NON-IR	NON-IR	IR	NON-IR	IR	NON-IR	IR
Replicate 1	598	564	1085	1293	896	866	2407
Replicate 2	643	826	880	-	1330	-	-
Total	1241	1390	1965	1293	2226	866	2407

C

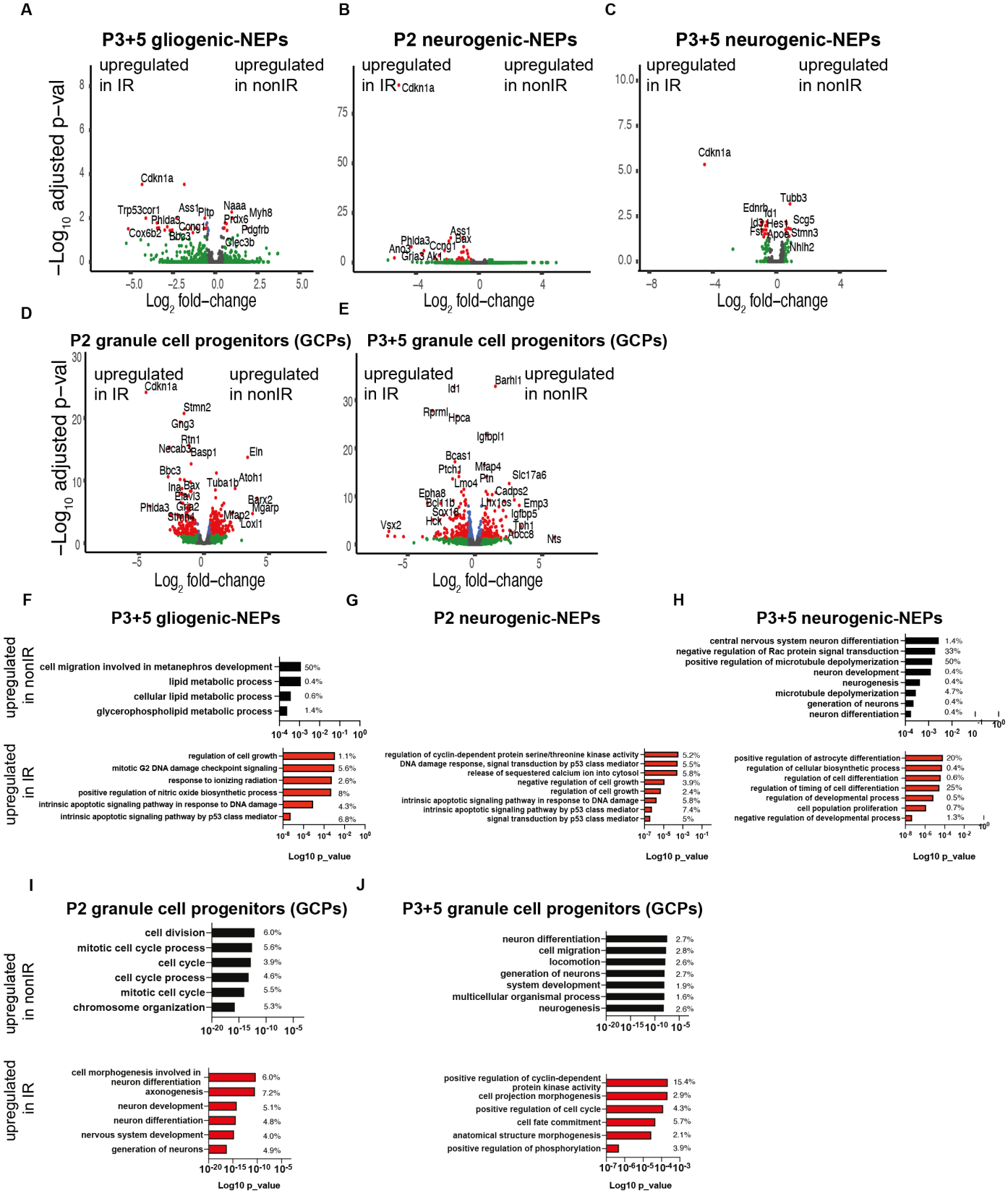


Supplementary Figure 1. scRNA-seq quality metrics and number of cells sequenced in each condition and biological replicate.

(A) Violin plots showing the number of features, RNA and percent mitochondrial RNA count across the biological replicates of the scRNA-seq data set after filtering the bad quality cells (cells were filtered out where number of detected genes was ≤ 1500, the number of detected transcripts was ≥ 40,000 and mitochondrial gene percentage ≥ 5%).

(B) Number of cells from each replicate and condition used for downstream analyses after filtering.

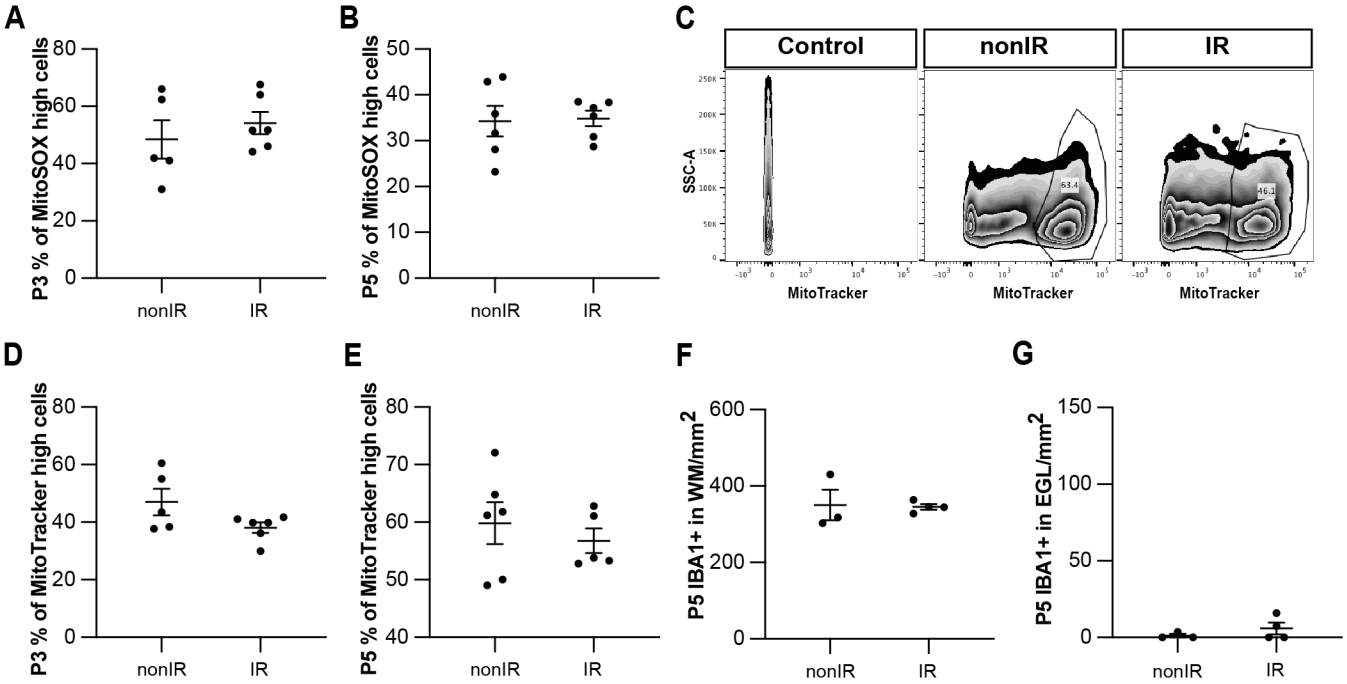
(C) UMAPs showing the distribution of cells across different clusters based on the samples.



Supplementary Figure 2: Injury induces distinct transcriptional changes in NEP subtypes and GCPs during adaptive reprograming.

(A-E) Volcano plot showing differentially expressed genes in the P3+5 gliogenic-NEPs (A), P2 and P3+5 neurogenic NEPs (B, C) and P2 or P3+5 GCPs (D, E) (red: adjusted p-value≤0.05, $\log_2$fold-change=1, Table S2).

(F-J) Top GO terms associated with differentially expressed genes in the P3+5 gliogenic-NEPs (F), P2 or P3+5 neurogenic NEPs (G, H) and P2 or P3+5 GCPs (I, J) that were either upregulated in nonIR (top panel) or IR (bottom panel) (p-value≤0.05).



Supplementary Figure 3: Irradiation of cerebella at P1 results in increased superoxide production and cell death and recruitment of microglia to the EGL that peaks at 24h.

(A, B) Quantification of high MitoSOX expression in nonIR and IR cerebella at P3 (A) and P5 (B).

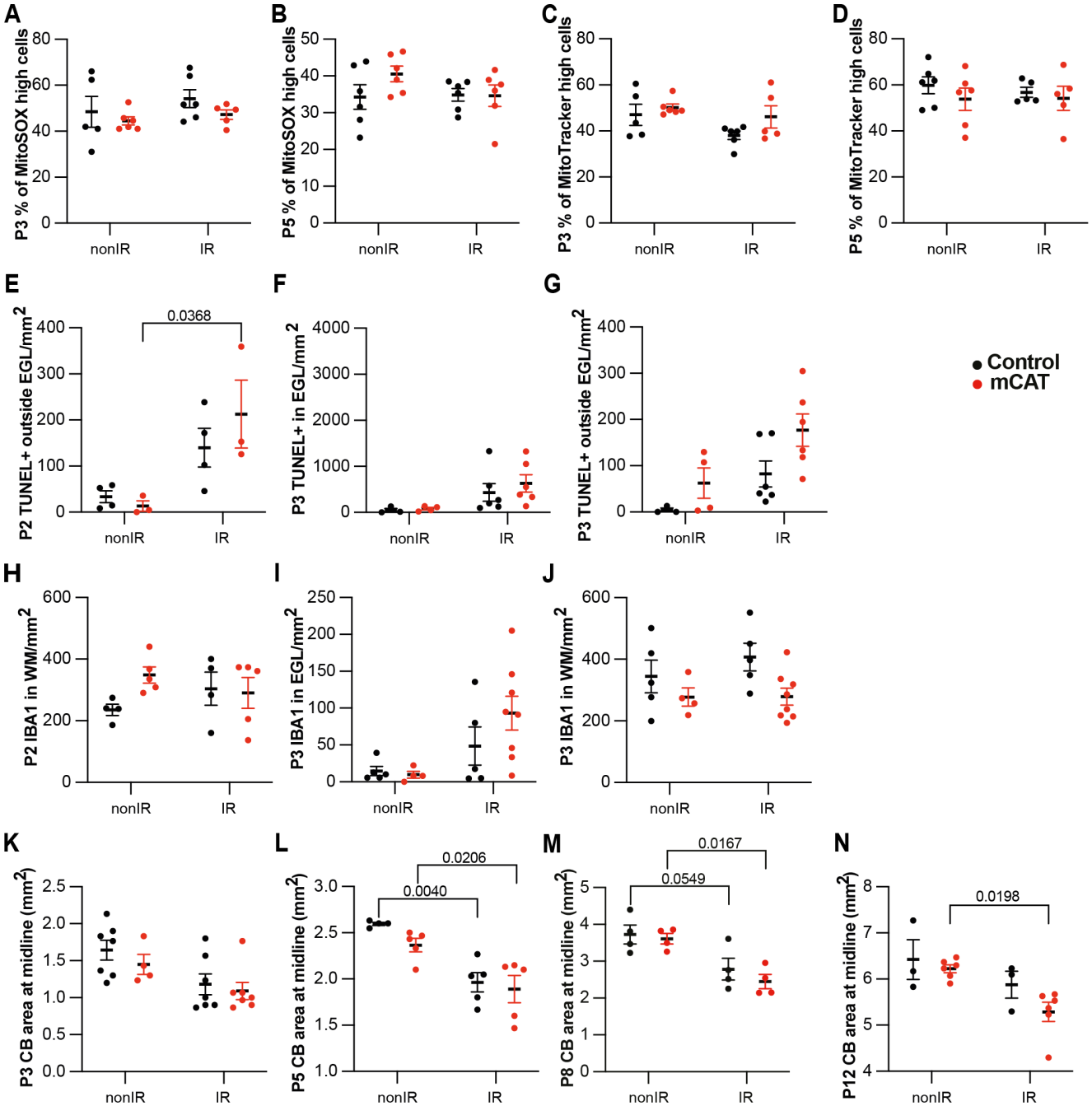
(C) Examples of flow cytometry analysis of mitochondria at P2 from nonIR and IR cerebella using MitoTracker dye. Gating determined the top 90% MitoTracker signal (MitoTracker high cells).

(D, E) Quantification of MitoTracker high expression in nonIR and IR cerebella at P3 (D) and P5 (E).

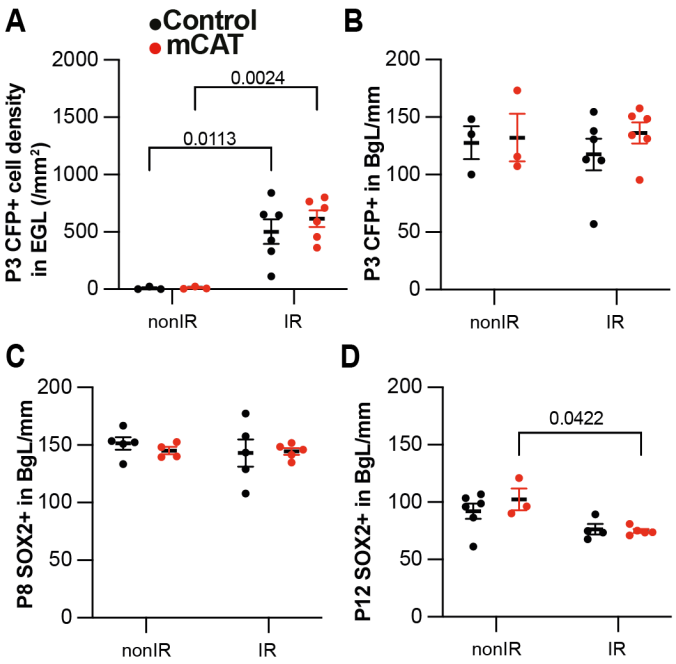
(F) Quantification of IBA1+ cell density in the WM at P5 in lobules 3-5 of nonIR and IR mice.

(G) Quantification of IBA1+ cell density in the EGL at P5 in lobules 3-5 of nonIR and IR mice.

EGL, External granular layer; WM, White matter; P, postnatal day; nonIR, non-irradiated; IR, irradiated. All statistical significance was determined using an unpaired t-test and data are represented as mean ± SEM.



Supplementary Figure 4: Reduction of ROS impairs adaptive reprogramming and cerebellar repair.
(A, B) Quantification of high MitoSOX expression at P3 (A) and P5 (B) in control and *mCAT/+* cerebella, with and without irradiation at P1.
(C, D) Quantification of MitoTracker high expression at P3 (C) and P5 (D) in control and *mCAT/+* cerebella, with and without irradiation at P1.
(E-G) Quantification of TUNEL+ cell density outside EGL at P2 (Two-way ANOVA, $F_{(1,10)}=14.20$, $p=0.0037$) (E), at P3 (G), and in the EGL at P3 (F) in lobules 3-5 of nonIR and IR mice.
(H-J) Quantification of IBA1+ cell density in WM at P2 (H), at P3 (J), and in the EGL at P3 (I) in lobules 3-5 of nonIR and IR mice.
(**K-N)** Quantification of cerebellar midsagittal section area in controls and *mCAT/+* nonIR and IR mice at P3 (K), P5 (Two-way ANOVA, $F_{(1,15)}=28.52$, $p<0.001$) (L), P8 (Two-way ANOVA, $F_{(1,12)}=21.21$, $p=0.0006$) (M) and P12 (Two-way ANOVA, $F_{(1,14)}=9.682$, $p=0.0077$) (N).
EGL, External granular layer; WM, White matter; P, postnatal day; nonIR, non-irradiated; IR, irradiated. Significant *Tukey's post hoc* multiple comparison tests are shown in the figures and data are represented as mean ± SEM.



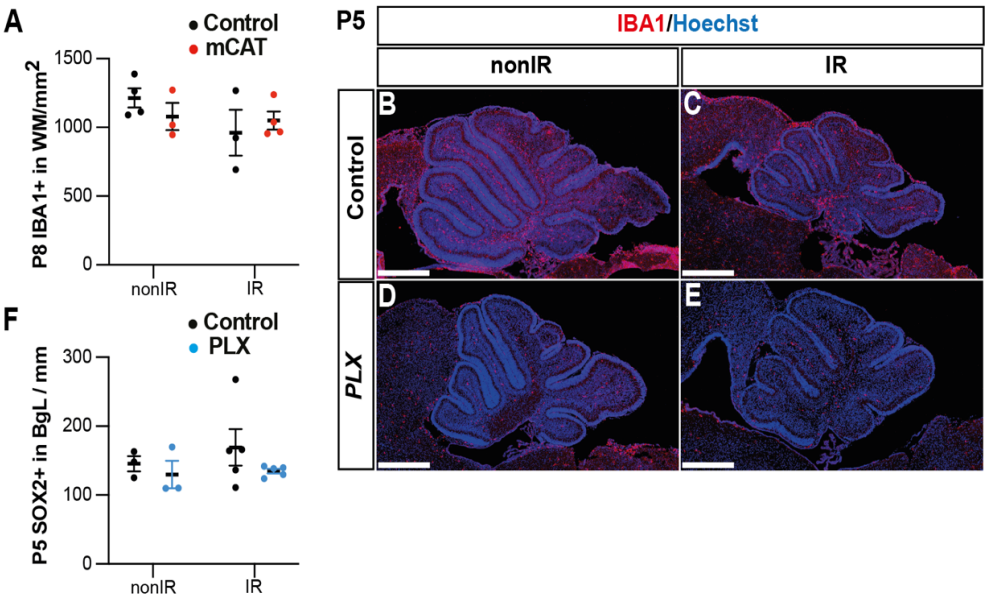
Supplementary Figure 5: Reduced ROS impairs expansion of BgL-NEPs and their migration to the EGL after injury.

(A) Quantification of CFP+ cell density in the EGL at P3 in *Nes-Cfp/+* control or *Nes-Cfp/+ mCAT* mutant nonIR and IR mice. (Two-way ANOVA, $F_{(1,14)}=33.77$, $p<0.0001$).

(B) Quantification of CFP+ cell normalized on BgL length at P5 in *Nes-Cfp/+* control or *Nes-Cfp/+ mCAT* mutant nonIR and IR mice.

(C, D) Quantification of SOX2+ NEP cell density on BgL length at P8 (C) and P12 (Two-way ANOVA, $F_{(1,12)}=12.50$, $p=0.0033$) (D) in control or *mCAT* mutant nonIR and IR mice.

EGL, External granular layer; BgL, Bergmann glia Layer; P, postnatal day; nonIR, non-irradiated; IR, irradiated. Significant *Tukey's post hoc* multiple comparison tests are shown in the figures and data are represented as mean ± SEM.



Supplementary Figure 6: Microglia promote recruitment of NEPs to the EGL during cerebellar adaptive reprogramming after injury.

(A) Quantification of IBA1+ cell density in the WM at P8 in control or *mCAT* mutant nonIR and IR mice.

(B-E) Immunostaining of medial sagittal cerebellar sections at P5 showing expression of IBA1 (red) in mice treated with PLX5622 or control DMSO, with or without irradiation.

(F) Quantification of SOX2+ NEP cell density in the BgL at P5 in control or PLX treated nonIR and IR mice.

BgL, Bergmann glia Layer; WM, White Matter; P, postnatal day; nonIR, non-irradiated; IR, irradiated. Scale bar: 500 µm. Data are represented as mean ± SEM.

Supplementary Tables

Antigen	Species	Concentration	References	Source
Catalase	Rabbit	1 to 100	01-05-030000	Athens Research & Technology
GFAP	Chicken	1 to 500	ab4674	Abcam
GFP	Rat	1 to 1000	440484	Nacalai Tesque
IBA1	Rabbit	1 to 500	019-19741	Wako Chemicals
SOX2	Goat	1 to 500	AF2018	R&D System

Table S1: List of antibodies and related information.

Table S2. Marker genes expressed by cluster in scRNA-seq dataset (irradiated at P1 (IR; P2, P3, P5) or non-irradiated (nonIR; P1, P2, P3, P5). pct1: % cells in a cluster that express the gene; pct2: % cells that express the gene outside the given cluster.

Table S3. Pseudobulk differential expression analysis between nonIR and IR gliogenic-NEPs (*Hopx+,* clusters 2, 3, 6, 10), neurogenic-NEPs (*Ascl1+,* clusters 5, 8, 11) and GCPs (*Atoh1+,* clusters 1,4,7,12,14) at P2, or at P3 and P5 (P3+5).

Table S4. GO Term analyses of differentially expressed genes (Table S3) of nonIR and IR gliogenic-NEPs (*Hopx+,* clusters 2, 3, 6, 10), neurogenic-NEPs (*Ascl1+,* clusters 5, 8, 11) and GCPs (*Atoh1+,* clusters 1,4,7,12,14) at P2, or at P3 and P5 (P3+5).

Table S5. Differentially open peaks at P2 identified by bulk ATAC-seq from nonIR and IR NEPs.

Table S6. Motif analysis of regions with increased accessibility in IR NEPs compared to the nonIR at P2.