Suppressing DNMT3a Alleviates the Intrinsic Epigenetic Barrier for Optic Nerve Regeneration and Restores Vision in Adult Mice

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ABSTRACT

The limited regenerative potential of the optic nerve in adult mammals presents a major challenge for restoring vision after optic nerve trauma or disease. The mechanisms of this regenerative failure are not fully understood[1,2]. Here, through small-molecule and genetic screening for epigenetic modulators[3], we identify DNA methyltransferase 3a (DNMT3a) as a potent inhibitor of axon regeneration in mouse and human retinal explants. Selective suppression of DNMT3a in retinal ganglion cells (RGCs) by gene targeting or delivery of shRNA leads to robust, full-length regeneration of RGC axons through the optic nerve and restoration of vision in adult mice after nerve crush injury. Genome-wide bisulfite and transcriptome profiling in combination with single nucleus RNA-sequencing of RGCs revealed selective DNA demethylation and reactivation of genetic programs supporting neuronal survival and axonal growth/regeneration by DNMT3a deficiency. This was accompanied by the suppression of gene networks associated with apoptosis and inflammation. Our results identify DNMT3a as the central orchestrator of an RGC-intrinsic mechanism that limits optic nerve regeneration. Suppressing DNMT3a expression in RGCs unlocks the epigenetic switch for optic nerve regeneration and presents a promising therapeutic avenue for effectively reversing vision loss resulted from optic nerve trauma or diseases.

MAIN TEXT

Mature neurons in the central nervous system (CNS) of adult mammals regenerate poorly after injury. Tremendous efforts have been devoted to study the cellular and molecular origins of this regenerative failure, yet no treatment exists for successful stimulation of CNS regeneration in humans. Eye-to-brain circuits, consisting of retinal ganglion cells (RGCs) whose axons form the optic nerve and connect to the central target areas, is an attractive model system for investigation of factors that regulate CNS axon regeneration because of their accessibility and simple anatomy. However, despite decades of research, achieving functional regeneration of the optic nerve has proved to be an elusive goal. Optic nerve regeneration requires a complex sequence of steps controlled by intricate gene networks that mediate crucial processes such as RGC survival, axonal elongation and guidance, and reformation of synapsis and neural circuitry[1,4]. The current prevailing view is that functional regeneration of the optic nerve cannot be achieved by the activation of a single gene or transcription factor[1,2], but rather requires modulation of multiple gene expression or programs.

Embryonic RGCs possess the innate ability to regenerate optic nerve fibers and readily grow their axons even when presented with a hostile or adult brain environment (growth-inhibitory)[5,6]. However, this ability of RGCs is lost perinatally[4-7] at a time coinciding with dynamic changes in epigenetic factors during RGC maturation[8]. We hypothesized that RGC axon growth programs are switched off during maturation through an epigenetic silencing mechanism involving DNA methylation. Indeed, a recent study demonstrated promising results in reprogramming the epigenomes of adult RGCs through the ectopic expression of three transcription factors used to generate stem cells – Oct4, Sox2, and Klf4 (Yamanaka factors)[9]. This resulted in partial restoration of youthful DNA methylation patterns and significant axon regeneration following crush injury. To date, however, no known strategy fully restores optic nerve regeneration with visual recovery following nerve injury in adult mammals. We sought strategies to unleash and completely reverse the epigenetic blockade and restore the regenerative potential of the optic nerve in adult mammals. We show here that DNMT3a is pivotal for postnatal onset of optic nerve regenerative failure, and suppression of this single gene results in reprogramming of RGC transcriptomic landscape to enable unprecedented level of optic nerve regeneration and reversal of vision loss in adult mice.

RGC axon growth enabled by *Dnmt3a* deficiency

Mouse RGCs lose their intrinsic ability to regenerate axons around embryonic day 18 (E18), one day before birth (postnatal day 0; P0)[5]. To assess epigenetic mechanisms responsible for this decline, we examined the expression patterns of DNA methyltransferases (DNMTs) in developing RGCs of mouse pups aged E16, P0, and P10, which is, before, at, and after the time that RGCs lose their ability to regenerate axons. Quantitative polymerase chain reaction (qPCR) of purified RGCs showed that the expression of *Dnmt3a* increased significantly from E16 to P10, which corresponded to the decline of RGCs' axon regenerative capacity (**Fig. 1a**). Other DNMTs, including *Dnmt1* and *Dnmt3b*, did not exhibit this correlation (**Extended Data Fig. 1a**). Immunohistochemistry confirmed this result: expression of DNMT3a was absent in E16 RGCs or retina but intense in P10 retina (**Fig. 1b** and **Extended Data Fig. 1b**), which was especially pronounced in the RGCs as evident by colocalization with RBPMS, an RGC marker (**Fig. 1b**). Moreover, *Dnmt3a* levels were increased in the RGCs two days after optic nerve crush injury (ONC; **Fig. 1c**), while *Dnmt1* and *Dnmt3b* remained unchanged or downregulated (**Extended Data Fig. 1c**). These data suggest a correlation between DNMT3a upregulation and the decrease of optic nerve regenerative capacity in mice.

To ascertain whether DNMTs affect RGC axonal regrowth, we assessed the effects of decitabine, a commercially available pan DNMT inhibitor[10] *in vitro.* The antagonistic effect of decitabine on DNMTs in post-mitotic neurons in the retina and brain of adult mice has been well documented[11-13]. We thus applied decitabine to retinal explant cultures, where the growth of axons mirrors the regenerative capacity of axotomized optic nerve fibers *in vivo*[5,6]. Consistent with the lack of nerve regeneration, retinal explants of adult (> 2 months old) wildtype mice and post-mortem human eyes showed minimal axonal outgrowth as revealed by β-III tubulin immunolabeling. In contrast, both mouse and human retinal explants treated with decitabine exhibited significant increases in the number and length of axonal growth (**Fig. 1d-j**).

We next adopted a genetic approach to determine which DNMT(s) regulate axon growth capacity in postnatal RGCs. Two DNMT families play different roles in methylation: DNMT1 maintains the methylation patterns, whereas the DNMT3 family (especially DNMT3a and DNMT3b) establishes initial methylation patterns[14]. We generated conditional knockout mice carrying RGC-specific allele with abolished catalytic activity of *Dnmt1* (*CKO*^{Dnmt1})[15] or *Dnmt3a* (*CKO*^{Dnmt3a})[16]. *Vglut2-Cre* transgenic mice were previously shown to drive expression of *Cre* recombinase in virtually all RGCs and sporadically cone photoreceptors, but not other retinal cell types[17,18]. We verified this by crossing *Vglut2-Cre* transgenic mice with the *Ai9* mouse line, which expresses the fluorescent reporter tdTomato from the Rosa26 locus in a *Cre*-dependent manner (*R26-tdTomato*). Approximate 90% of RBPMS+ RGCs and a small number of cones[19], but not other retinal cells, were positive for tdTomato (**Extended Data Fig. 1d, e**).

By generating *CKO*^{Dnmt1} and *CKO*^{Dnmt3a}, we noted that adult homozygous *CKO*$^{Dnmt3a-/-}$ mice developed obesity and malocclusion, presumably because *vGlut2* drives *Cre* expression in many CNS neurons. The mice also suffered a high mortality rate from anesthetics during surgical procedures. In contrast, heterozygous *CKO*$^{Dnmt3a+/-}$ mice grew and bred normally without apparent health concerns or retinal structural changes. This prompted us to focus on *heterozygous CKO* mice. We isolated RGCs using magnetic bead-conjugated Thy-1 antibody and detected significant downregulation of *Dnmt3a* mRNA in heterozygous *CKO*$^{Dnmt3a+/-}$ RGCs compared to those from the littermate controls (**Extended Data Fig. 1f**). We noted that in homozygous *CKO*$^{Dnmt3a-/-}$ RGCs, a low level of *Dnmt3a* mRNA was detected, likely because Thy-1 is not exclusively expressed by RGCs in the rodent retinas[20,21]. We confirmed that *Dnmt1* and *Dnmt3a* mRNA levels were downregulated selectively in the RGCs, but not the entire retinas of *CKO*$^{Dnmt1+/-}$ and *CKO*$^{Dnmt3a+/-}$ mice, respectively (note that RGCs comprise ~1% of all

retinal cells; **Extended Data Fig. 1f-i**). We studied axonal growth capacity using retinal explant cultures derived from adult *CKO*$^{Dnmt1+/-}$ and *CKO*$^{Dnmt3a+/-}$ mice, and littermate controls that carried *Cre* without *floxed* alleles or *floxed* alleles without *Cre* (**Fig. 1k-n**). While the retinal explants of control mice showed minimal axonal growth, those from adult *CKO*^{Dnmt3a} mice exhibited an over 20-fold increase in axon number (**Fig. 1l**) and at least a 2-fold increase in average axon length (**Fig. 1m**) compared to control mice. The retinal explants of C*KO*$^{Dnmt3a+/-}$ and C*KO*$^{Dnmt3a-/-}$ mice revealed similarly increased number and rate of axon regeneration (data not shown), suggesting that the absence of one *Dnmt3a* allele is sufficient to unleash the barrier to axon regeneration. In contrast, retinal explants of *CKO*$^{Dnmt1+/-}$ mice showed no significant improvement in axonal growth compared to controls (**Fig. 1l and m, Extended Data Fig. 1j**). We further verified that *Dnmt3a* deficiency in *CKO*$^{Dnmt3a+/-}$ RGCs did not have significant impact on the expression of *Dnmt1* (**Extended Data Fig. 1k**) or *Dnmt3b* (**Extended Data Fig. 1l**). In agreement with the downregulation of *Dnmt3a*, reduced DNA methylation was detected in *CKO*$^{Dnmt3a+/-}$ RGCs, but not other retinal cell types compared to controls (**Extended Data Fig. 1m, n**). We concluded that *Dnmt3a*, rather than *Dnmt1*, negatively regulates the intrinsic program for RGC axon growth during maturation.

Optic nerve reinnervation and restoration of vision by *Dnmt3a* deficiency

Next, we tested *in vivo* if RGC-specific *Dnmt3a* deficiency restores axons' regenerative capacity in adult RGCs using the optic nerve crush (ONC) injury model. Adult *CKO*$^{Dnmt3a+/-}$ mice, as well as their littermate *Cre* or *floxed* control mice, were subjected to ONC. Axonal regrowth was assessed by labeling RGC axons with cholera toxin B subunit (CTB) two days before mice were sacrificed[9,22]. In control mice, CTB-positive axons were observed only proximal to the crush site (**Fig. 2a**) at all time points examined after ONC, confirming the failure of nerve regeneration. In contrast, numerous RGC axons regenerated across the lesion site for long distances in *CKO*$^{Dnmt3a+/-}$ mice, with many growing more than 3 mm distal to the lesion by 14 days post ONC (**Fig. 2a**). Notably, the number of regenerated axons was so great that in most cases, it was impossible to count the number of regenerating axons in optic nerve sections. We thus quantified axon regeneration by measuring CTB-labeling immunofluorescent intensity at various distances posterior to the crush site. At all distances, we detected at least 3-fold increase in the intensity of CTB-labeling in *CKO*$^{Dnmt3a+/-}$ mice compared to controls; the difference was > 8-fold up to 750 μm distal to the lesion (**Fig. 2b**)**.** Moreover, RGCs of *CKO*$^{Dnmt3a+/-}$ mice displayed a significant increase in survival as evidenced by RBPMS-immunolabeling (50% *vs* 12% at 14 days post-ONC. **Fig. 2c, d**). No signs of RGC proliferation were detected as assessed by EdU

incorporation assays (data not shown), suggesting that the increased RGC number was not due to the birth of new neurons or RGCs.

The regenerating axons continued to grow along the optic nerve over time. As early as 4 weeks following the injury, many regenerating axons grew past the optic chiasm, where a major barrier to RGC axon regeneration and target reinnervation was previously reported[23,24]. Greater fluorescent intensity of labeled axons entered the brain when examined at 8- and 16-weeks post-injury, suggesting continual growth of axons. By 16 weeks post-ONC, a large number of regenerating axons crossed the optic chiasm of the contralateral side (**Fig. 2e, f**). In contrast, no axons were evident in the optic chiasm (**Extended Data Fig. 2a**), optic tract, and brain targets (data not shown) of control mice. Unprecedented numbers of regenerating axons were seen in the optic tract of *CKO*$^{Dnmt3a+/-}$ mice, primarily extending along the side contralateral to the injury and reinnervating the dorsal and ventral lateral geniculate nuclei (LGN) as well as the pretectum (**Fig. 2g** and **Extended Data Fig. 2b**). These findings show that *Dnmt3a* deficiency in RGCs empowers long-distance and robust optic nerve regeneration and reinnervation into the central visual targets of the optic nerve in adult mice.

We asked if optic nerve reinnervation into the brain targets of adult *CKO*$^{Dnmt3a+/-}$ mice led to the restoration of RGC function and vision after ONC (**Fig. 3a**). We assessed mouse visual function by measuring the optomotor response (OMR), which uses the head tracking behavior of mice to determine their spatial vision (e.g., visual acuity)[25]. Normal mice without the injury typically exhibited a visual acuity of 0.4 – 0.5 cycle/degree as measured by OMR. When examined at 2 weeks post ONC, all animals exhibited a complete loss of head tracking behavior in OMR assays, indicative of blindness following nerve injury (**Fig. 3b**). OMR remained absent in control mice throughout the study period (up to 16 weeks post-lesion). In contrast, ~40% of the *CKO*$^{Dnmt3a+/-}$ mice started to show a recovery of OMR by 4 weeks post-injury, a time corresponding to the entry of RGC axons into the brain and reinnervating the central visual targets as documented above. The percentage of *CKO*$^{Dnmt3a+/-}$ mice which had regained OMR increased with time and reached ~70% by 16 weeks post-ONC. Quantification of OMR-based visual acuity reached 0.26 cycle/degree, a recovery of more than 50% of the normal value of visual acuity in mice (**Fig. 3b**).

We performed additional assays to evaluate the RGC and visual functions. First, in a dark/light preference assay, normal mice spent over 80% of their time in a dark environment. Whereas

wildtype mice subjected to ONC showed no preference for a dark environment when examined up to 16 weeks post-injury. In contrast, this response in *CKO*$^{Dnmt3a+/-}$ mice with ONC recovered to the same level as observed in uninjured mice by 16 weeks post injury (**Fig. 3c**), clear indication of a restoration of light perception. Second, we measured visually evoked potentials (VEPs) in the cortex. Control mice showed an absence of or flat VEP N1 wave (with an infinite delay of N1 latency; **Fig. 3d** and **Extended Data Fig. 2c, d**) up to 16 weeks after nerve crush. Whereas, in parallel with the OMR recovery, ~60% of *CKO*$^{Dnmt3a+/-}$ mice regained VEP N1 responses at an amplitude to half of its baseline value by 16 weeks post-injury (**Fig. 3d; Extended Data Fig. 2d**). Of note, there was a significant delay in N1 latency in *CKO*$^{Dnmt3a+/-}$ mice after injury compared to the normal uninjured group (**Extended Data Fig. 2c, d**), potentially due to immature synaptic reconnection or incomplete myelination of the regenerated axons that should impede the speed of electrical signal transmission from the eye to the brain[26,27]. Moreover, significant increases in the amplitude of positive scotopic threshold response (pSTR)[28], a readout of the electrical activity of RGCs, was detected in *CKO*$^{Dnmt3a+/-}$ mice during the period of 4 – 16 weeks post-injury when compared to control mice (**Fig. 3e; Extended Data Fig. 2e**). Together, these results demonstrated reversal of RGC function and vision loss following axon regeneration in a crush-injured optic nerve in adult mice via a single gene manipulation.

Wide-scale transcriptome shifts by various RGC types towards an injury resilient and regenerative status

Because mouse retina contains 45 RGC types that differ dramatically in their survival and regenerative ability following ONC[18], it is important to understand if *Dnmt3a* deficiency promotes axon regeneration of specific RGC types. To address the question, we asked if *Dnmt3a* deficiency shifts the RGC landscape or alters the injury responses (pathophysiological condition) in selective RGC types by profiling the RGC transcriptome using single nuclei RNA sequencing (snRNA-seq). Retinas were collected at 2 days post-injury, when few if any RGCs have died but responses to injury are already apparent[5,29]. RGC nuclei were enriched by fluorescence-activated cell sorting (FACS) with a NeuN antibody and profiled by droplet-based snRNA-seq using the 10X platform[30]. We first examined if the proportions of RGC types was affected by *Dnmt3a* deficiency. In the retinas of both *CKO*$^{Dnmt3a+/-}$ and control mice, we observed 35 RGC clusters, within which 42 of the 45 reported RGC types were identified based on the RGC atlas markers[18,29] (**Fig. 3f-h** and **Extended data Fig. 3a-c**). More than half of the clusters (24/35) were 1:1 matches with the reported atlas RGC types, while the other 11 each contained 2-4

RGC types (**Fig. 3h**). The mixture of RGC types within clusters is likely due to the smaller number of RGCs profiled here compared to the earlier report. We found virtually no differences in the distribution of RGC clusters between *CKO*$^{Dnmt3a+/-}$ and control mice (**Fig. 3g**). Over 90% of RGC clusters showed similar frequencies in *CKO*$^{Dnmt3a+/-}$ and control mice (**Fig. 3f-h** and **Extended Data Fig. 3a-c**). Notably, the frequencies of three αRGC subsets (αON-S/M4|αON-T, αOFF-S and αOFF-T|αON-T), which are the rarest and the most vulnerable and regenerative RGC types[31,32], were slightly increased in *CKO*$^{Dnmt3a+/-}$ mice compared to controls. They collectively represented ~6% of the total RGC population in *CKO*$^{Dnmt3a+/-}$ mice compared to <3% in control mice (**Extended Data Fig. 3c**). Collectively, DNMT3a deficiency does not alter the distribution of retinal cell and RGC profiles or drastically changes the frequencies of the vast majority of RGC types except it somewhat increases the occurrence of rare αRGC subsets.

With this assurance, we assessed differences in gene expression changes after ONC between control and *CKO*$^{Dnmt3a+/-}$ RGCs. In line with the robust optic nerve regeneration, we observed upregulation of the nerve growth-related pathways as classified by Gene Ontology (GO) terms, including optic nerve morphogenesis, axonogenesis and dendrite extension (**Fig. 3i**). In contrast, genes associated with neuron death, axon injury response, and neuroinflammation signals classified by GO terms, were downregulated compared to controls, indicating enhanced resilience to injury. By combining the normalized enrichment scores (NES) of injury response- and growth-regulated gene categories, respectively, in each individual RGC clusters, we noted a consistent pattern of gene expression changes across a broad spectrum of RGC types (**Fig. 3j** and **Extended Data Fig. 3d, 4a, b, 5a, HTML File**), indicating cell type-independent transformation into growth-promoting and injury-resilient states. Surprisingly, comprehensive downregulation of *Pten* expression, a well-established endogenous inhibitor of axon regeneration which deletion promotes selective axon regeneration of αRGCs[33,34], was not observed in the majority of *CKO*$^{Dnmt3a+/-}$ RGC types, except in four clusters (**Extended Data Fig. 5b**). Moreover, only two *CKO*$^{Dnmt3a+/-}$ RGC clusters displayed significantly decreased expression of *Socs3*, another well-known axon regeneration inhibitor[29,34], when compared to controls. These findings suggest that *CKO*$^{Dnmt3a+/-}$ mice switch on a *Pten*- or *SOCS3*-independent axon growth mechanism. Contrary to promoting nerve regeneration in selective RGC types, *Dnmt3a* deficiency triggers a broad-spectrum RGC-transcriptome shift toward a gene profile associated with axon regeneration in nearly all RGC types, including those previously shown to be susceptible to injury (e.g., C35, C25/C34) and those relatively resilient to injury (e.g., αRGCs, M1-RGCs)[18]. Thus, *Dnmt3a* deficiency results in a wide-scale change of transcriptome profiles

in various RGC types toward injury-resilient, less inflammatory, and pro-regenerative states after ONC.

Multifaceted induction of axon regeneration machinery in *Dnmt3a* deficient RGCs

The inability of RGC axons to regenerate is believed to stem from a complex interplay of intrinsic factors and environmental cues, with CNS glial cells releasing axon growth-inhibitory signals and posing a formidable barrier to axon regeneration[1]. To gain deeper insights into the signaling mechanisms through which RGC-specific *Dnmt3a* deficiency disinhibits the development of glial barrier to axonal regrowth, we employed CellChat analysis, a bioinformatic algorithm designed to predict and decipher intercellular cues exchanged by various cell types[35]. Given that DNMT3a dysfunction in *CKO*$^{Dnmt3a+/-}$ mice is RGC-specific, we focused on outgoing signals from RGCs to other cell types identified in our snRNA-seq (**Fig. 3f**). As shown above, CellChat analysis consistently predicted significant downregulation of RGC-derived inflammatory signals in *CKO*$^{Dnmt3a+/-}$ mice compared to controls, primarily associated with three immune activator (**Fig. 4a** and **Extended Data Fig. 6a, b**): colony stimulating factor (CSF)[28], granulin (GRN)[36], and major histocompatibility complex class 1 (MHC-1)[37]. Notably, the chord diagram showed that in control mice, CSF originating from the RGCs signaled to multiple other retinal cell classes, especially immune cells, whereas this RGC signal was drastically diminished in the *CKO*$^{Dnmt3a+/-}$ mice (**Fig. 4b**). *Dnmt3a* deficiency also led to downregulation of axon growth-inhibitory signals, such as Semaphorin 4 (SEMA4)[38], and upregulation of synapse and axon growth-promoting signals, angiopoietin (ANGPT)[39,40] and secreted phosphoprotein 1/osteopontin (SPP1)[41] (**Fig. 4a**). Therefore, RGC-specific *Dnmt3a* deficiency not only induces an intrinsic pro-regenerative program within RGCs but also enables a permissive environment by sending decreased inflammatory signals and increased growth-promoting signals.

To determine how *Dnmt3a* deficiency alters the communication among RGC types, we also compared CellChat signaling among RGCs in *CKO*$^{Dnmt3a+/-}$ and control mice. The results indicated the up-regulation of classical *Wnt*[42] and non-canonical *Wnt*[43], PDGF and FGF signaling[44] by many RGC types in *CKO*$^{Dnmt3a+/-}$ mice compared to controls (**Fig. 4c** and **Extended Data Fig. 7a, b**). The chord diagram of the WNT communication indicated that intrinsically photosensitive RGC types M1a and M2 are the sole major source of WNT signal in controls, while in *CKO*$^{Dnmt3a+/-}$ mice, multiple RGC types upregulate outgoing WNT signaling to other RGCs (**Fig. 4d**). *CKO*$^{Dnmt3a+/-}$ RGCs also exhibited distinctive expression of poliovirus receptor (PVR)[45,46] and visfatin[47,48], two growth and survival-related genes that were absent in

control RGCs. Conversely, the growth factors with pro-inflammatory properties, such as vascular endothelial growth factor (VEGF)[49] and interleukin 4[50,51], were highly expressed only in control RGCs but not in *CKO*$^{Dnmt3a+/-}$ RGCs (**Fig. 4c**). These results strengthen the conclusion that *Dnmt3a* deficiency in the RGC contributes to distinctive pro-regenerative intercellular signaling and suppressed inflammatory responses.

DNA methylation of axon growth gene networks regulated by *Dnmt3a*

DNMT mediates DNA methylation to serve biological functions. To investigate how *Dnmt3a* deficiency altered the landscape of DNA methylation in RGCs, we conducted whole genome bisulfite sequencing (WGBS) of RGCs isolated 2 days post-ONC. Compared to controls, *CKO*$^{Dnmt3a+/-}$ RGCs showed lower CG methylation levels specifically in the differentially methylated regions (DMRs) (**Fig. 4e**), which are the genomic regions show different methylation status between individuals. DMRs are highly regarded as possible functional regions involved in gene transcriptional regulation[52]. In contrast, minimal alteration in genome-wide DNA methylation level was detected comparing the two groups (**Extended Data Fig. 8a, b**). WGBS disclosed 4,836 CG DMRs, including 4,004 hypo-DMRs and 832 hyper-DMRs (**Extended data Fig. 8c**). Annotation of the hypomethylated DMR-related genes identified 48 pathways in the biological processes, which reflect the aforementioned transcriptomic shifts toward a pro-regenerative state in the *CKO*$^{Dnmt3a+/-}$ RGCs. Fourteen of these hypomethylated pathways are linked to axon growth, including axon guidance, axonogenesis and axon development (e.g., *Wnt3, Klf7, Map2*)[38,53,54], and 6 are related to synaptic process (e.g., *UNC13a* and *Nrxn1*)[55,56] (**Fig. 4f**). KEGG pathway analysis of total DMR-related genes again depicted distinctive enrichment in pathways related to nerve growth and synaptic process that include key signaling events such as *Wnt*, *Ras*, and *cAMP*, axon guidance, and dopaminergic and glutamatergic synapse (**Fig. 4g**). The data establish that DNMT3a directly regulates the methylation of gene networks that control axon growth and synaptic process.

To validate that hypomethylation of genes controlling axon growth and synaptic process in *Dnmt3a*-deficient mice results in correspondent gene expression changes, we conducted comprehensive transcriptome profiling by bulk RNA-seq on RGCs isolated two days after ONC. Principal component analysis clearly separated *CKO*$^{Dnmt3a+/-}$ RGCs from the controls (**Extended Data Fig. 8d**). We identified 1,459 genes that are differentially expressed (DEGs) (Fold change > 1.5; padj < 0.05) between *CKO*$^{Dnmt3a+/-}$ and control RGCs (**Extended Data Fig. 8e**). KEGG analyses revealed alignment between upregulated gene pathways in *CKO*$^{Dnmt3a+/-}$ RGCs with

those found to be hypomethylated above, including synaptic signaling and axon guidance (**Fig. 4h**). On the other hand, downregulated DEGs in the *CKO*$^{Dnmt3a+/-}$ RGCs were primarily involved in cell death and immune regulation, such as apoptosis, TNFα and chemokine/cytokine signaling (**Extended Data Fig. 9a**). GSEA and GO enrichment studies confirmed the results of KEGG analysis, showing downregulation of immune response (e.g., T cell and macrophage migration, chemokine-mediated signaling, and microglial activation) and inflammatory gene pathways (e.g., IFNγ, TNFα, and IL6-JAK-STAT3 signaling)[57] with concurrent upregulation of key events regulating axon growth (e.g., *Wnt/β-catenin*) and synaptic assembling and transport (**Extended Data Fig. 9b, c**). This result was congruent with the prediction of CellChat analysis.

We further verified the gene expression pattern changes detected by bulk RNA-seq in *CKO*$^{Dnmt3a+/-}$ and control mice by qPCR. *CKO*$^{Dnmt3a+/-}$ RGCs isolated at 2 days post-injury showed consistent significant differences in the expression of the aforementioned gene pathways, including the upregulation of axon growth-related genes (e.g., *mTOR*, *Spp1*, *Gap43*) and downregulation of growth inhibitors (e.g. *Atf3*) as well as cell apoptotic and inflammatory genes (e.g., *Gas5*, *Casp3*, *Casp8*, *Ripk1*)[1,29,57,58] compared to control RGCs (**Fig. 4i**)**.** Interestingly, these differences were only detected in RGCs isolated post ONC. RGCs taken from uninjured *CKO*$^{Dnmt3a+/-}$ mice showed no significant differences in expression of any of these genes compared to RGCs from uninjured control mice (**Fig. 4i**). The data suggest that the loss of DNMT3a driven by *Vglut2-Cre* has little impact on gene expression of normal RGCs but rather results in reprogramming of RGCs' injury responses toward an injury-resilient and pro-regenerative status post-ONC. Together, these studies demonstrated that *Dnmt3a* deficiency reshaped the injury-induced transcriptomic and intercellular communication landscape of RGCs, contributing to the functional regeneration of the optic nerve.

Therapeutic inhibition of *Dnmt3a* rescued visual function in adult wildtype mice after ONC

To assess the therapeutic potential of *Dnmt3a* inhibition, we conducted AAV treatment experiments in adult mice after ONC (**Fig. 5a**). To downregulate *Dnmt3a* we injected AAV-shRNA intravitreally to adult wildtype mice or AAV-Cre to mice carrying heterozygous floxed *Dnmt3a* allele (*fl/+*) immediately after ONC. Detection of GFP expression revealed wide-spread AAV-infection in RGCs, and qPCR analysis confirmed the downregulation of *Dnmt3a* mRNA levels in RGCs of eyes injected with AAV-shRNA or AAV-Cre compared to AAV-scrambled RNA or AAV-GFP injected eyes (**Extended data fig. 9d, e**). Tracking of the spatial vision with OMR

at multiple timepoints post-injury indicated partial recovery of visual acuity in over 50% of mice received AAV-shRNA treatment starting at 6 weeks post-injury (**Fig. 5a, b**), a two-week delay of recovery than that was seen in *CKO*$^{Dnmt3a+/-}$ mice above. This corresponds to the two week-time period that is required for AAV to reach its peak/plateau of gene expression after intravitreal injection (not shown). In contrast, control mice received injection of AAV-scrambled RNA showed no response in the OMR test up to 12 weeks after injury. By 12 weeks post-ONC, AAV-shRNA-treated mice regained light perception and exhibited no difference in light/dark box assays compared to their uninjured baselines (**Fig. 5c**). Moreover, AAV-shRNA-treated mice demonstrated improved pSTR and N1 amplitude of VEP compared to control mice receiving AAV-scrambled RNA injection, which remained blind without any sign of light perception or VEP response (**Fig. 5d-f**)**.** Similar recovery of visual acuity, light perception, pSTR, and VEP improvement were also observed in *fl/+* mice received AAV-Cre compared to AAV-GFP injected mice (**Fig. 5g-m**)**.** Similarly, delays in VEP N1 latency were observed in both AAV-shRNA-treated wildtype mice and *AAV-Cre*-injected *fl/+* mice, suggesting impaired myelination. These results provide compelling evidence that *Dnmt3a* inhibition holds immense therapeutic potential for the treatment of optic nerve diseases or injury, offering a viable avenue for restoring vision *in vivo*.

Discussion

Here we report restoration of vision after traumatic injury of the optic nerve via suppression of *Dnmt3a*, which can overcome the inability of adult RGCs to regenerate axons. Inhibition of DNMT3a-dependent DNA methylation reactivates an intrinsic axon growth program that is lost in adults, promotes RGC survival, and enables axonal regeneration through the optic nerve to central targets. As regenerating axons reach the brain, spatial vision is recovered. This finding highlights the dominant role played by neuron-intrinsic determinants in driving functional optic nerve regeneration and alleviating environmental obstacles, likely through signaling from RGCs to other cell classes and to each other. Moreover, *Dnmt3a* deficiency facilitates selective demethylation of gene networks and multifaced activation of axon growth machinery across diverse RGC subtypes without apparent neuroinflammation or compromised neuronal survival. Most importantly, therapeutic suppression of *Dnmt3a*, either by AAV-shRNA delivery or other means, holds the potential for clinical translation. This discovery not only offers an exciting avenue for treating injury to optic nerve diseases and injury, but also paves the way for future research in brain and spinal cord regeneration.

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Author contributions

D.F.C and W.L.T. perceived the project, analyzed the data, and wrote and revised the manuscript with input from all co-authors. W.L.T. was involved in all experiments and analyses. K.C. contributed to the optic nerve crush studies and trained W.L.T. in all animals works and analyses. E.K. designed and performed all sequencing data computational analysis. A.M. performed single-nucleus RNA sequencing and W.Y. contributed to single-nucleus data preprocessing. A.A. contributed to the culture studies and assisted with image acquisition. Y.L. and X.W. conducted early small-molecule and genetic screening studies. T.P.G. contributed to qPCR and morphometric analyses. J.R.S and P.B. carefully read and edited the manuscript and supervised single-nucleus RNA sequencing studies.

Competing interests

The other authors declare no competing interests.

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Fig.1

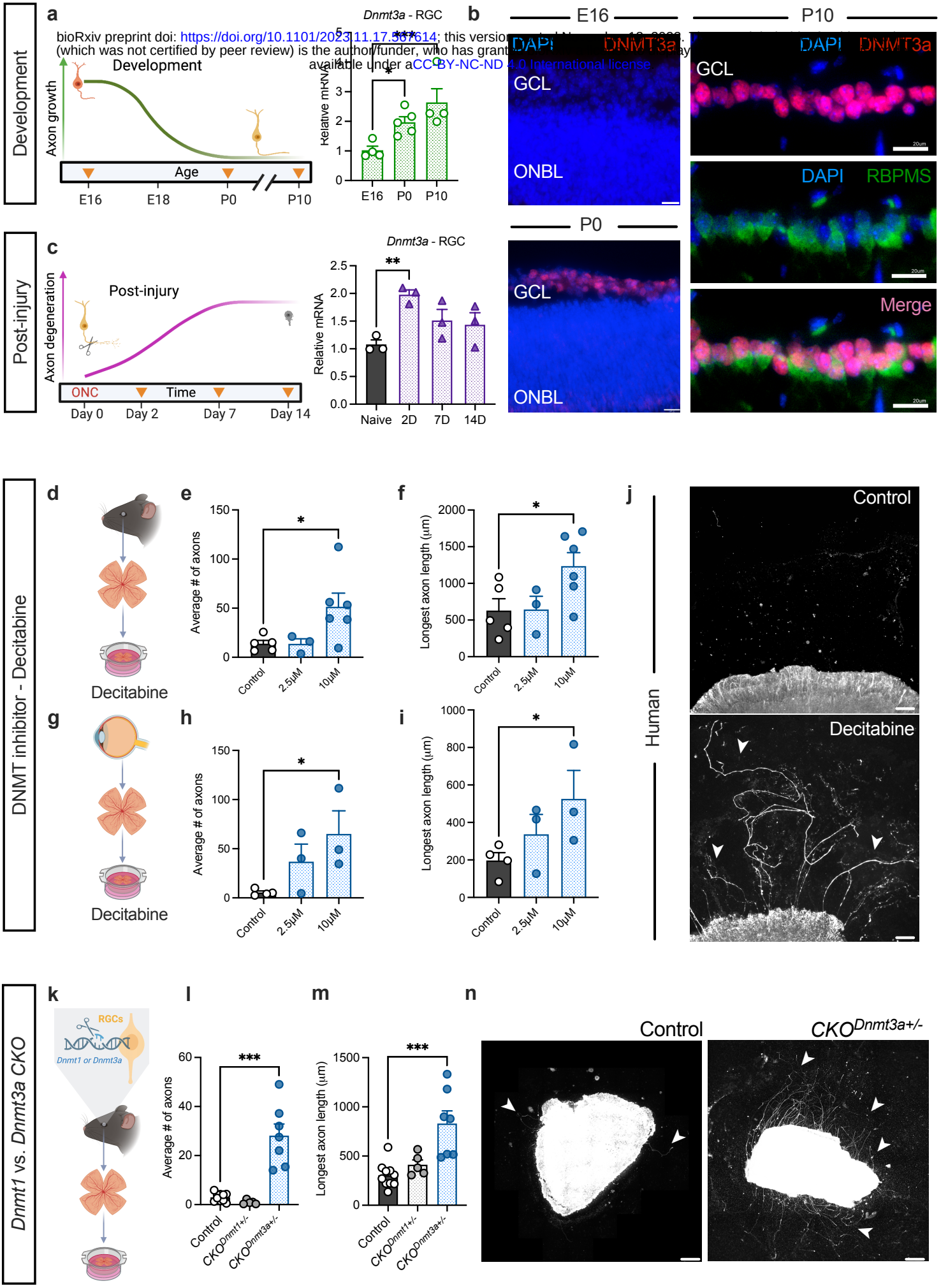


Fig. 2

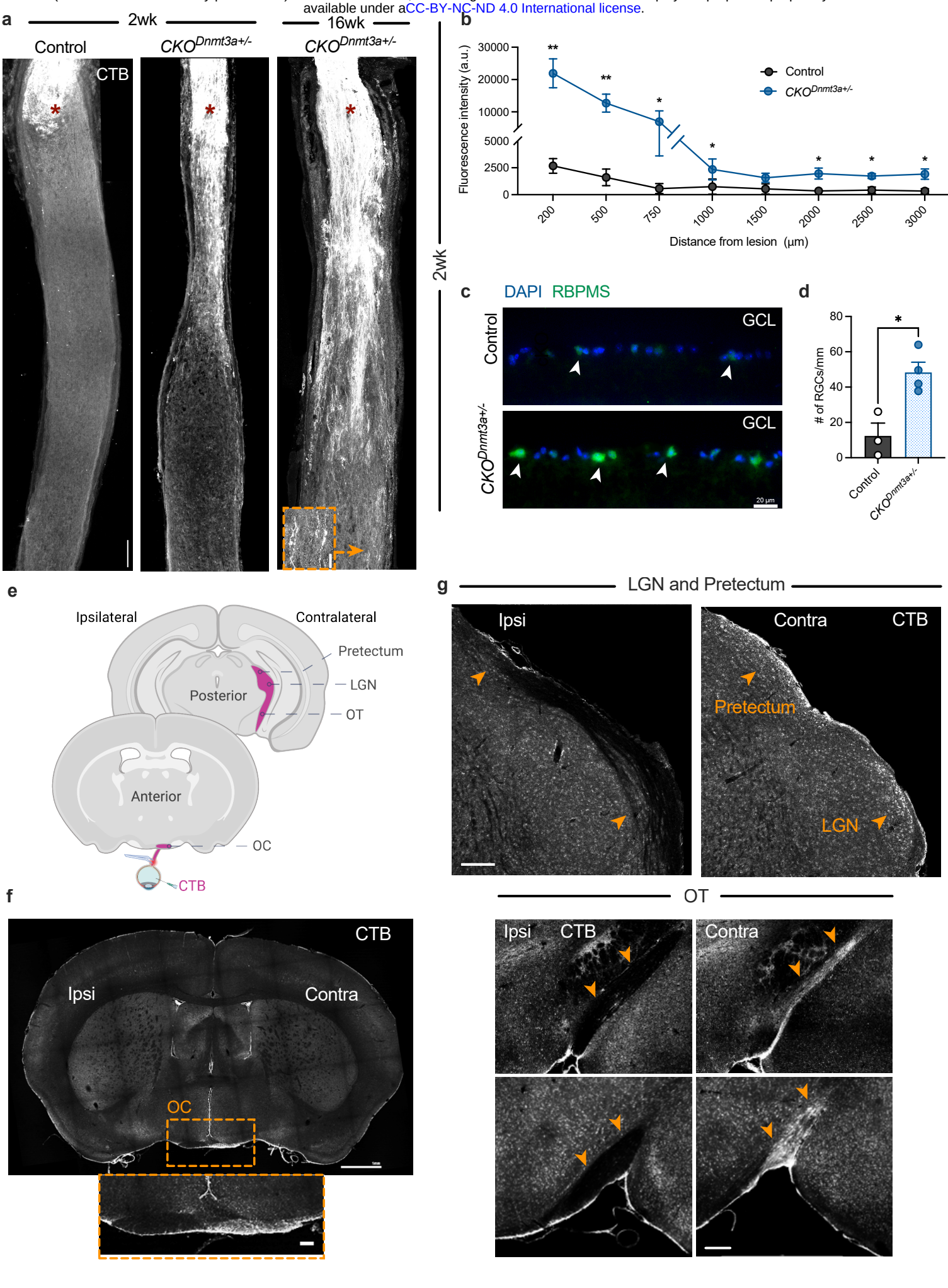


Fig. 3

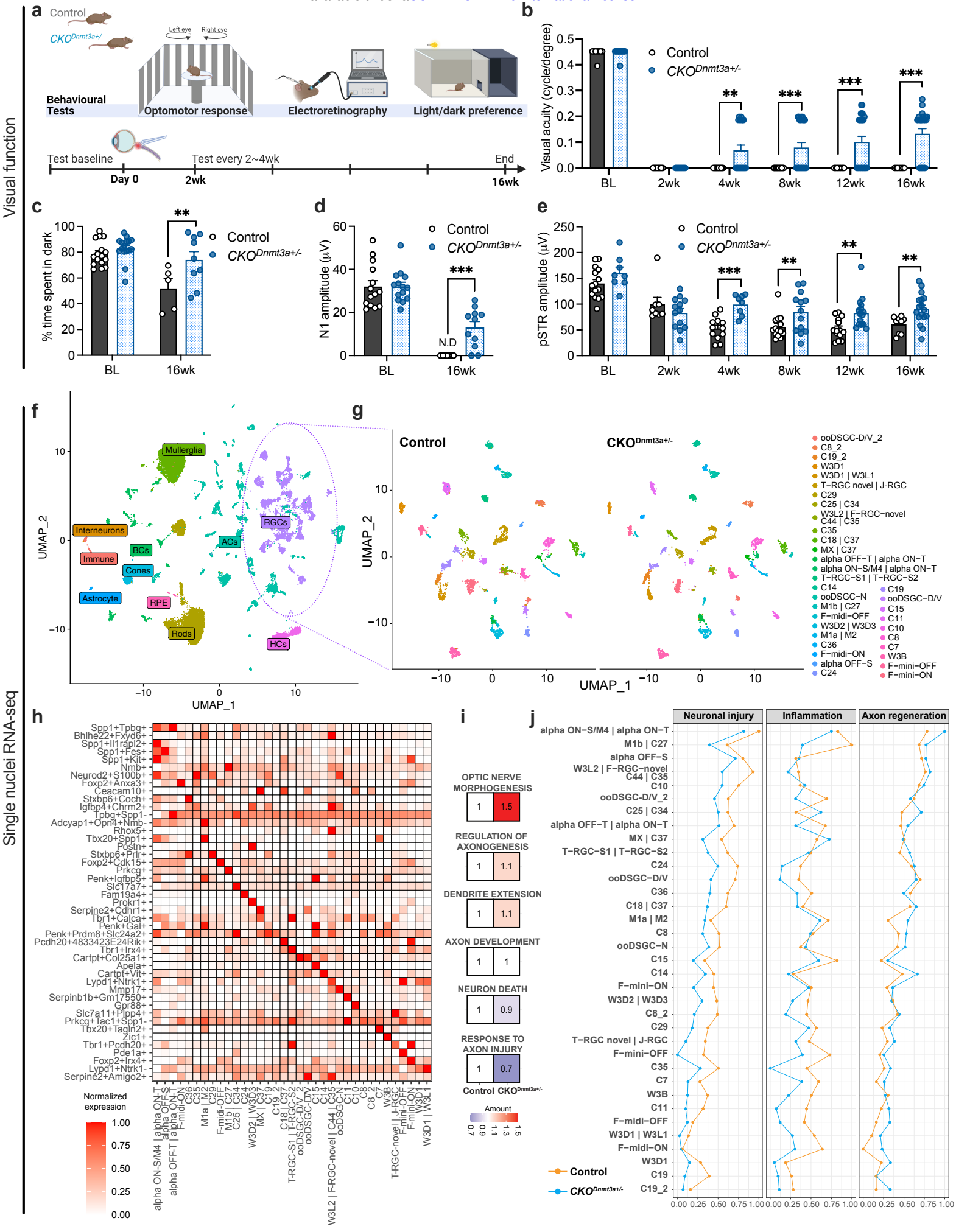


Fig. 4

Single nuclei RNA-seq

Bisulfite-seq and Bulk RNA-seq

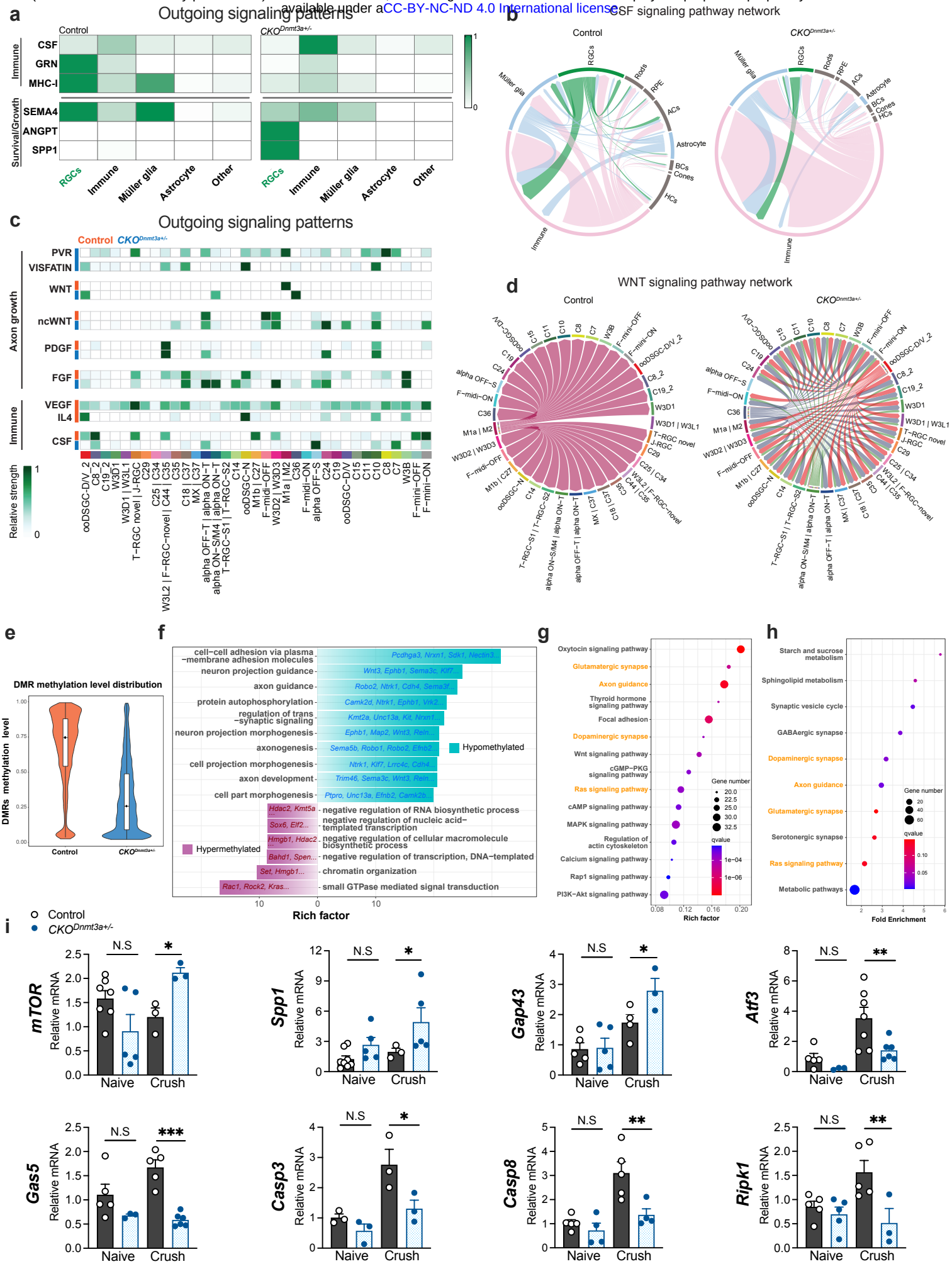
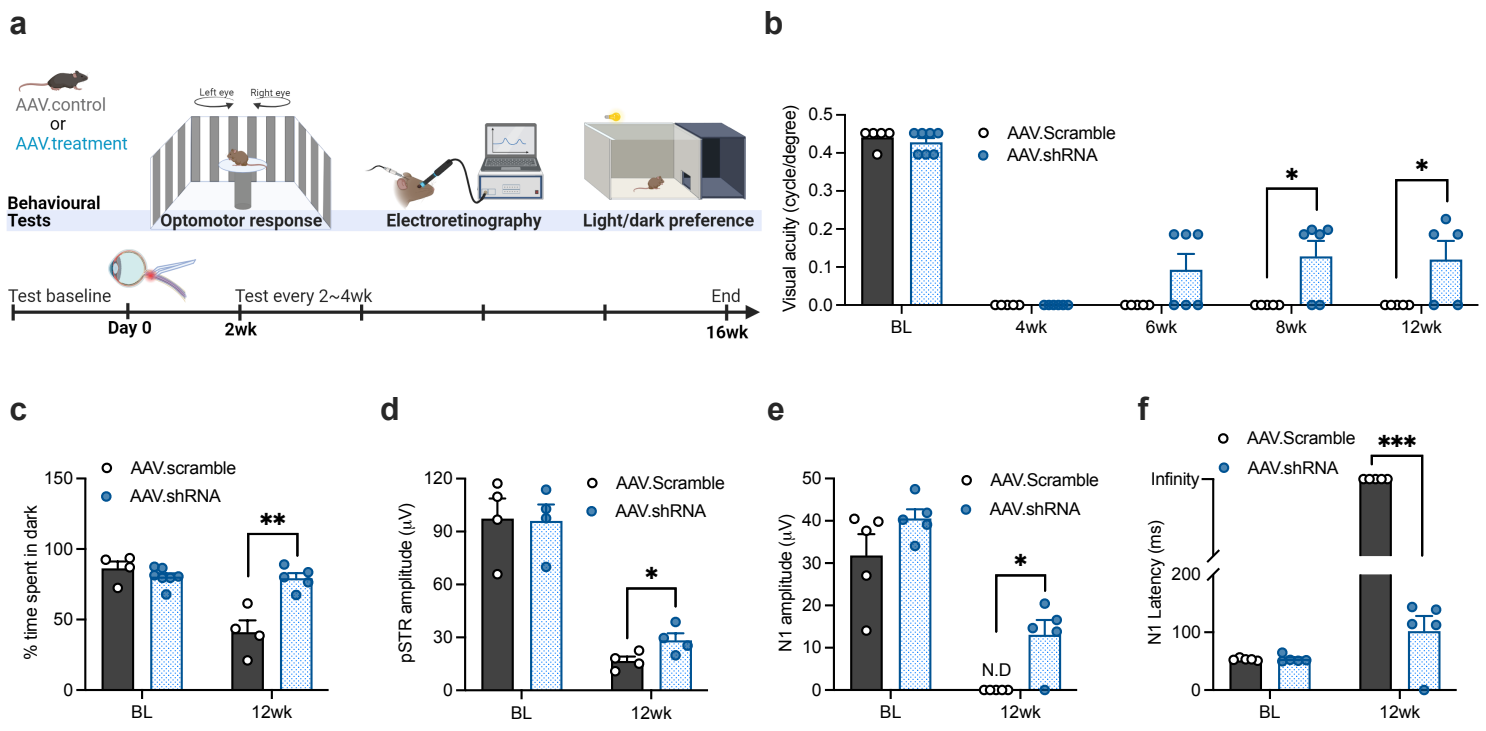


Fig. 5

AAV.Scramble vs. AAV.shRNA in C57BL/6 mice



AAV.GFP vs. AAV.Cre in *Dnmt3a*$^{fl/+}$ mice