Letter

Attenuating the DNA damage response to double strand breaks is neuroprotective

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Summary

DNA double-strand breaks (DSBs) are a feature of many acute and long-term neurological disorders, including neurodegeneration[1,2], following neurotrauma[3] and after stroke[4]. Persistent activation of the DNA damage response (DDR) in response to DSBs contributes to neural dysfunction and pathology as it can force post-mitotic neurons to re-enter the cell cycle leading to senescence or apoptosis[5-8]. Mature, non-dividing neurons may tolerate low levels of DNA damage, in which case muting the DDR might be neuroprotective. Here, we show that attenuating the DDR by targeting the MRN complex, which is responsible for recognition of DSBs, is neuroprotective in three neurodegeneration models in *Drosophila* and prevents Aβ-induced loss of synapses in hippocampal neurons. Attenuating the DDR after optic nerve injury is also neuroprotective to retinal ganglion cells (RGC) and promotes dramatic regeneration of their neurites both *in vitro* and *in vivo*. Dorsal root ganglion neurons similarly regenerate when the DDR is targeted *in vitro* and this strategy also induces a full restoration of lost function after spinal cord injury *in vivo*. We conclude that muting the DDR in the nervous system is neuroprotective in multiple neurological disorders. Our results point to new therapies to maintain or repair the nervous system.

Genetic targeting of the DSB-sensing MRN complex is neuroprotective in Drosophila

A key sensor and early processor of DSBs is the MRN complex, comprising the Mre11, Rad50 and NBS1/Nbn proteins[9]. Association of the MRN complex to DSBs leads to recruitment and activation of the ATM kinase, which coordinates multiple arms of the DDR, including cell-cycle arrest, repair and apoptosis[10]. We targeted the MRN complex by genetically reducing NBS levels by introducing a heterozygous *NBS*$^{-/+}$ null allele into the background of three transgenic *Drosophila* models of neurodegeneration: expression of glutamine-expanded Htt for Huntington's disease (HD)[11]; tandem Aβ_{1-42} oligomers for Alzheimer's disease (AD)[12]; and 0N4R Tau.R406W for frontal temporal dementia with Parkinsonism[13]. In each case, expression of the relevant transgene was restricted to adult neurons to rule out confounding developmental phenotypes.

The ability of flies to climb after being startled is used widely in neurodegeneration studies as a measure of neural output. Climbing ability declines rapidly and correlates with reduced speed of neural transmission[14]. In the HD and AD fly models, the reduction in climbing ability was partially supressed in *NBS*$^{-/+}$ flies. (Fig. 1a and b). Using a tracking system to monitor movement continuously, Tau-expressing flies moved at normal speed when unstimulated (Fig. 1c) but their escape response declined rapidly with age[14]. Again, this was partially suppressed in *NBS*$^{-/+}$ flies (Fig. 1d). Tau expression or phosphorylation was not altered in *NBS*$^{-/+}$ heterozygotes (Extended Data Fig. 1d). These protective effects were not specific to *NBS*$^{-/+}$: depleting *Rad50* was also neuroprotective in Htt-expressing flies

(Extended Data Fig. 1a). These results support the view that attenuating the DDR enhances neuroprotection in these fly models.

AD patients can suffer disrupted circadian behaviour patterns[15] and a similar effect is observed in Aβ-expressing flies[16]. We expressed tandem $A\beta_{1-42}$ specifically in clock neurons under the control of *tim-Gal4* and studied their survival with immunocytochemistry[17]. Aβ-expression induced loss of some Per^{+} neurons but this was partially suppressed in $NBS^{-/+}$ flies (Fig. 1e). Consistent with the loss of clock neurons[17], Aβ-expressing flies exhibited significantly longer free-running circadian locomotor cycles than controls but the normal ~24 h periodicity was restored in $NBS^{-/+}$ flies (Fig. 1e and Extended Data Fig. 1f). We also expressed expanded HttQ128 in the developing eye under the control of *GMR-gal4,* which generates a progressive degeneration evidenced as a widespread loss of pigmentation after 6-7 weeks[11]. In $Rad50^{-/+}$ flies, pigmentation is restored suggesting enhanced cellular survival (Extended Data Fig. 1c).

A common feature of neurodegenerative diseases is the early loss of synapses[18]. Cultured primary hippocampal neurons exposed to $A\beta_{1-42}$ oligomers and stained with anti-γH2AX antibodies revealed the characteristic foci corresponding to DSBs in the nuclei (Extended Data Fig. 1g). Synapsin levels were also significantly reduced 24 h after addition of Aβ oligomers (Fig. 1g) suggesting that synaptic loss might be due to Aβ-induced DSBs triggering the DDR. Mirin, a Mre11 inhibitor, prevented synapsin loss (Fig. 1g), indicating that targeting the MRN complex can also protect mammalian neurons from changes associated with early-stage neurodegeneration.

Mre11 or ATM inhibition prevents apoptosis and stimulates regeneration after neurotrauma

DSB are also a feature of acute neurological disorders[3,4]. DNA strand breaks have been observed in RGC after optic nerve injury[19] and neurons suffering from ischemia display genome fragility and fragmented DNA both *in vitro* and *in vivo*[20]. We used an adult rat primary retinal culture system, enriched in retinal ganglion cells (RGC) where cells are grown in the presence of inhibitory CNS myelin extracts to simulate a post-injury environment[21]. As expected, we detected the presence of DSBs with anti-γH2AX antibodies in the RGC (Fig. 2a and b). The RGC die rapidly by apoptosis and, even if apoptosis is blocked with inhibitors, they fail to regenerate neurites[22]. We treated the RGC with mirin to attenuate the DDR. This blocked RGC apoptosis and dramatically stimulated neurite regrowth. Mirin treatment was significantly more effective than a current positive control treatment: ciliary neurotrophic factor (CNTF) (Fig. 2c and d). Since a key function of the MRN complex is to recruit and activate ATM at DSBs[23], and given that HD-pathology is reduced by targeting ATM[24], we asked whether inhibiting ATM directly would also be neuroprotective to RGC. Treatment with the highly selective

ATM inhibitor, KU-60019, had similarly dramatic effects on RGC survival and neurite outgrowth as mirin (Fig. 2c-f).

We extended our *in vitro* findings to an *in vivo* optic nerve crush injury paradigm. We observed low levels of γH2AX in intact control rat retinae *in vivo* (Fig. 3a and b). However, high levels of γH2AX were observed within 1 and at 24 days after optic nerve crush injury, suggesting rapid activation of DSBs and persistence of high levels for the duration of the experiment i.e. 24 days. Thus, the DDR pathway was sustainably activated in the retina post-injury. We used a pre-optimised dosing regime for mirin or KU-60019 for 24 days after optic nerve crush injury (see Methods) to attenuate the DDR and saw unprecedented RGC survival of 93% and 91%, respectively, when compared to intact controls (Fig. 3c and d). Both mirin and KU-60019 also promoted significant numbers of RGC to regenerate their axons, which emerged from the lesion site and grew for long distances (Fig. 3e and f). The numbers of regenerating axons we see are, to the best of our knowledge, unprecendented when compared to known treatments and the axons also regenerate longer distances into the distal optic nerve than has been seen previously[25-27].

Finally, to confirm that mirin and KU-60019 treatments did in fact attentuate the DDR as expected, we quantified γH2AX levels i.e. phosphorylation on Ser139, which is dependent on ATM activity. In RGC *in vitro*, pSer139 activity was approximately 50% of vehicle controls and *in vivo* in the retina, was almost completely abolished compared to vehicle-treated eyes, demonstrating effective suppression of the DDR (Fig. 3g and h).

Mre11 or ATM inhibitors restore function after spinal cord injury in vivo

DSBs are also generated in spinal neurons after SCI[3] and there is an urgent need for effective therapies to treat SCI patients. Initially, we tested out DDR attenuation strategy using a similar *in vitro* culture model to the retina in which dorsal root ganglion neurons (DRGN) containing DSBs (Extended Data Fig. 3a) are cultured from adult rats and grown in the presence of inhibitory CME. Again, mirin and KU-60019 treatment stimulated significant DRGN survival and promoted neurite outgrowth (Extended Data Fig. 3b-e).

To generate a spinal cord injury *in vivo,* we surgically injured the ascending long tract axons of the dorsal funiculus in adult rats by dorsal column (DC) crush injury[28] and mirin or KU-60019 inhibitors were administered through an intrathecal catheter. DSBs formed in DRGN in all treatment groups after injury, as predicted from previous studies[3] (Extended Data Fig. 2a) and quantification again revealed reduced levels of γH2AX phosphorylation following mirin or KU-60019 treatment, indicating partial suppression of the DDR (Extended Data Fig. 2a-e). We employed electrophysiology and simple functional tests[28] to quantify recovery from the injury after mirin or KU-60019 treatment. In vehicle-

treated rats, the normal compound action potential (CAP) trace across the lesion site was abolished, whilst treatment with either mirin and KU-60019 restored 50-55% of the CAP trace observed in sham-treated animals (Fig. 4a and b). The CAP area was also significantly improved in mirin and KU60019-treated rats compared to vehicle groups (Fig. 4c). The improved electrophysiological function translated into very dramatic improvements in sensory and locomotor function. We used a tape sensing-and-removal test and a ladder-crossing test to quantify recovery of sensory and motor function, respectively[28]. In sham-treated animals, there is a small initial decline in mean sensing time (Fig. 4d) and mean ratio (Fig. 4e) due to the effects of anaesthetic but these are restored to baseline levels within 3 weeks. Vehicle-treated groups displayed significantly impaired sensory and motor function that fails to return to baseline levels even after 6 weeks. In contrast, mirin or KU-60019-treated groups both showed dramatically improved sensing times and ladder-crossing 2 d after surgery and after 3 weeks were indistinguishable from the sham-treated control groups. These results demonstrate that either Mre11 or ATM inhibition is able to promote functional recovery after SCI *in vivo.* As with optic nerve regeneration, we believe this functional recovery to be to unprecedented levels.

Targeting DSB signalling as a therapy for neurological disorders

If DSBs accumulate in neurological conditions, attenuating the DDR as a therapeutic strategy logically should enhance disease rather than suppress it. Here, we demonstrate the opposite is true: inhibiting apical components of the DDR to mute the response to DSBs is beneficial in models of neurodegenation and results in unprecendent survival, axon regeneration and recovery of function after neurotrauma. Our findings suggest the effects of DDR activation is more deleterious to post-mitotic neurons than the DNA damage itself. Given the ubiquity of DSBs, our strategy gives new options to protect the nervous system and promote repair for numerous currently untreatable neurological conditions.

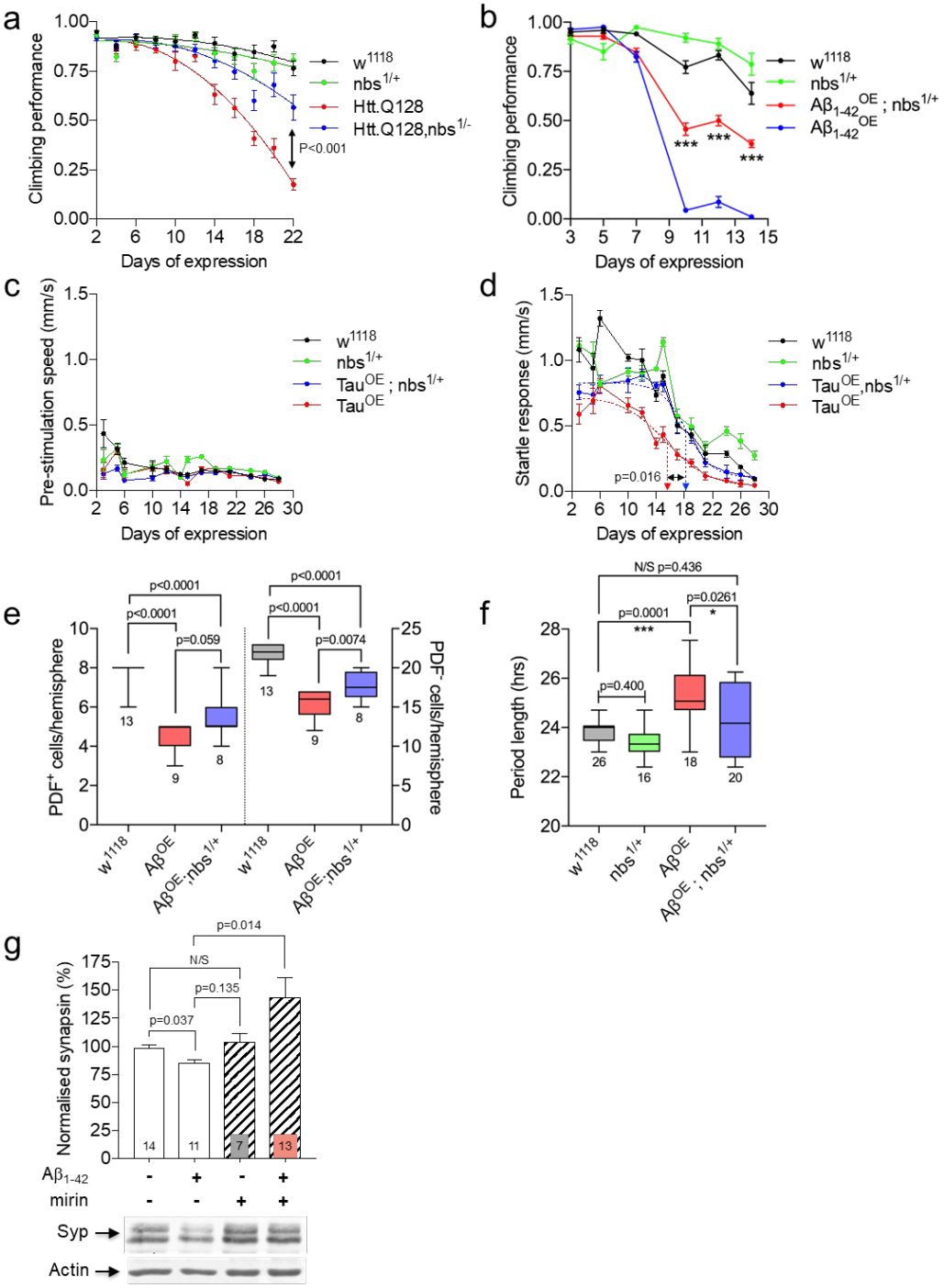


Fig. 1. Genetic targeting of the MRN complex supports the nervous system in Drosophila

a and **b**, Flies were startled by tapping to the base of a vial and the negative geotaxis climbing response quantified as a measure of neural output. Flies expressing **a** Htt.Q128 or **b** $A\beta_{1-42}$ in adult neurons show a rapid decline in climbing ability which is partially suppressed in flies heterozygous for a null allele of *NBS*. Statistical comparisons in **a** by extra sum-of-squares F-test; in **b** by 2-way ANOVA, *** indicates p<0.001. **c**, Flies expressing Tau.R406W in adult neurons move at normal speed under non-stimulated conditions. **d**, When stimulated by vibration, Tau-expressing flies show a rapid decline in the startle response which is partially suppressed in *NBS*$^{-/+}$ flies. Dashed arrows indicate days of expression

required for 50% decline in response determined from fitted lines; comparison by 2-tailed t-test. **e**, Expression of $A\beta_{1-42}$ in clock neurons leads to loss of Per^{+} cells. In *NBS*$^{-/+}$ flies, loss of cells is partially prevented (Kruskal-Wallis with Dunn's post-hoc test; p values and n are indicated). **f**, The free-running circadian locomotor cycle of control flies is ~24 h but is considerably lengthened when $A\beta_{1-42}$ is expressed in clock neurons indicating weaker rhythmicity in the circadian circuitry. The increase is suppressed in *NBS*$^{-/+}$ flies (CLEAN spectral analysis; comparisons by ANOVA with Tukey's post-hoc test; p values and n are indicated). **g**, Quantification of synapsin levels in hippocampal neurons by Western blot. Exposure to $A\beta_{1-42}$ oligomers leads to a loss of the pre-synaptic protein, synapsin, which is reversed by the Mre11 inhibitor, mirin (mean ± SEM; ANOVA with Tukey's post-hoc test; p values and n are indicated).

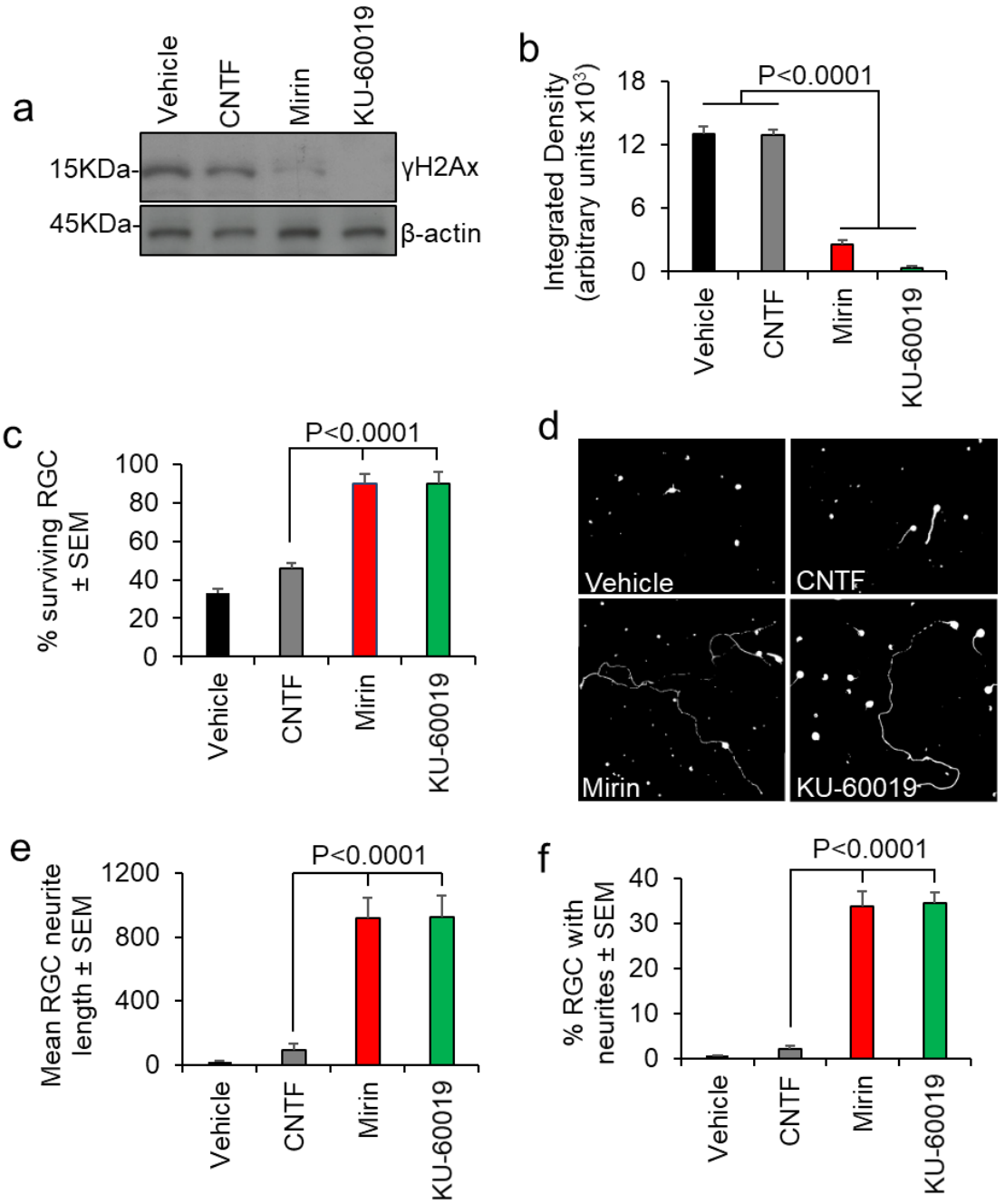


Fig 2. Inhibition Mre11 prevents RGC apoptosis and stimulates neurite outgrowth in the presence of inhibitory CNS myelin extracts. a, Western blot and **b** subsequent densitometry to show attenuated levels of anti-γH2AX after treatment with mirin and KU-60019. **c**, Mirin or KU-60019 signficantly enahnced RGC survival. **d**, Representative images from RGC treated with vehicle, CNTF (positive control), mirin and KU-60019. Mirin or KU-60019 treatment **e**, increased the mean RGC neurite length and **f**, % RGC with neurites. n = 9 wells/treatment. Comparisons in **b**, **c**, **e** and **f** by ANOVA with Dunnett's post-hoc test.

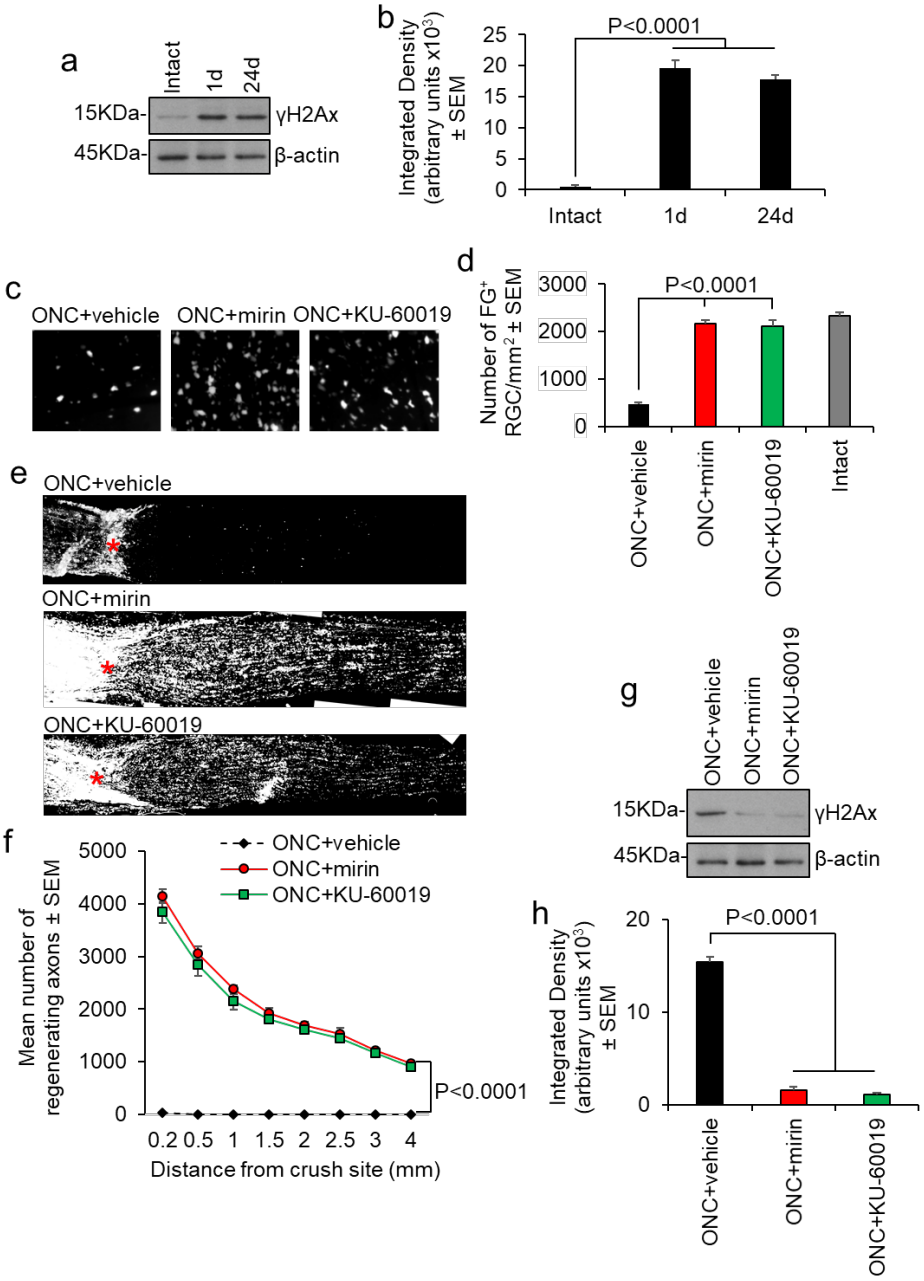


Fig 3. Inhibition Mre11 prevents RGC apoptosis and stimulates axon regeneration after optic nerve crush injury. a, Western blot and **b** subsequent densitometry to show phosphorylation of H2Ax as a marker of DNA damage after optic nerve crush injury (n=12 retinae). **c**, Western blot and **d** densitometry to show that mirin and KU-60019 signficantly suppress optic nerve crush injury-induced γH2Ax levels (n = 12 retinae). **e**, Representative images and **f** quantification of FluroGold backfilled RGC in retinal wholemounts to demsontrate that mirin and KU-60019 signficantly enhanced RGC survival at 24 days after optic nerve injury. **g**, representative images and **h**, quantification to show that mirin and KU-60019 signficantly enhanced RGC axon regeneration as detected by GAP43 immunoreactivity (n=18 nerves). Comparisons in **b**, **d**, **f** and **h** by ANOVA with Dunnett's post-hoc test

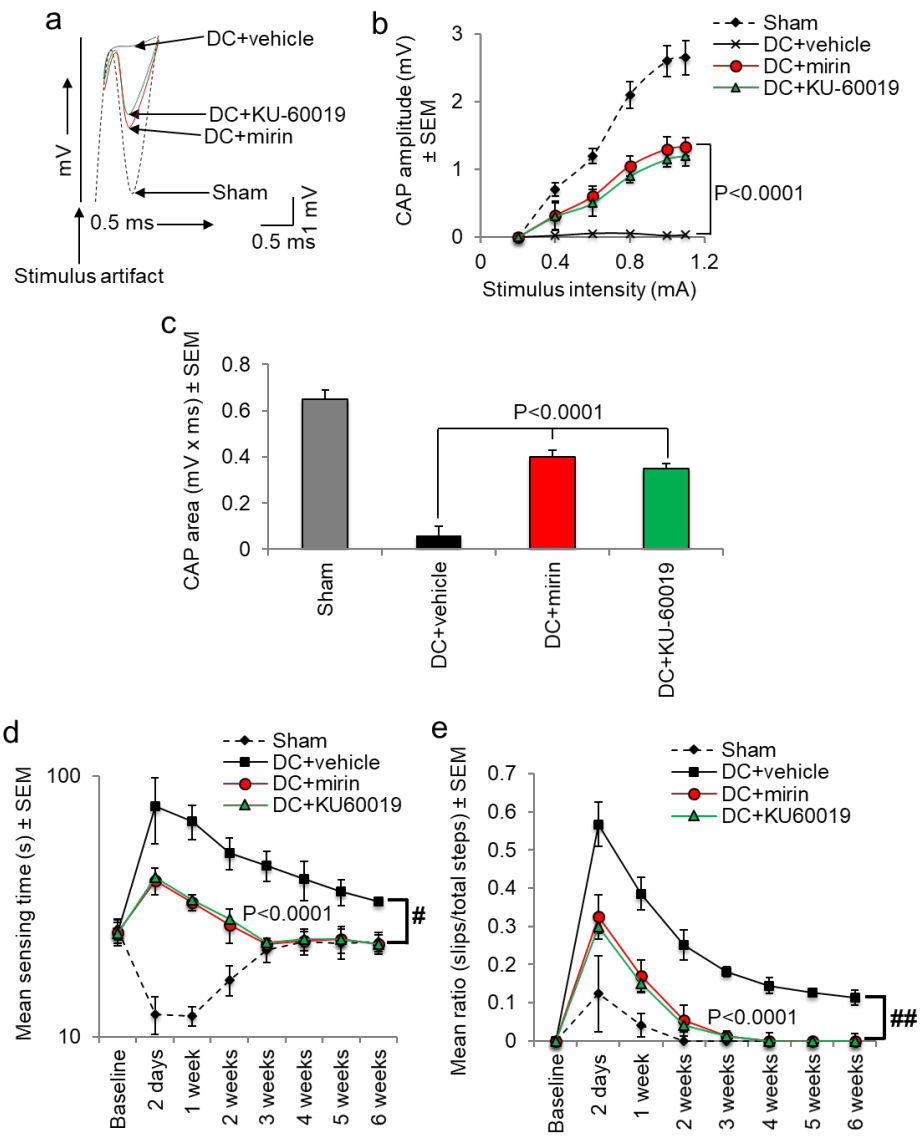
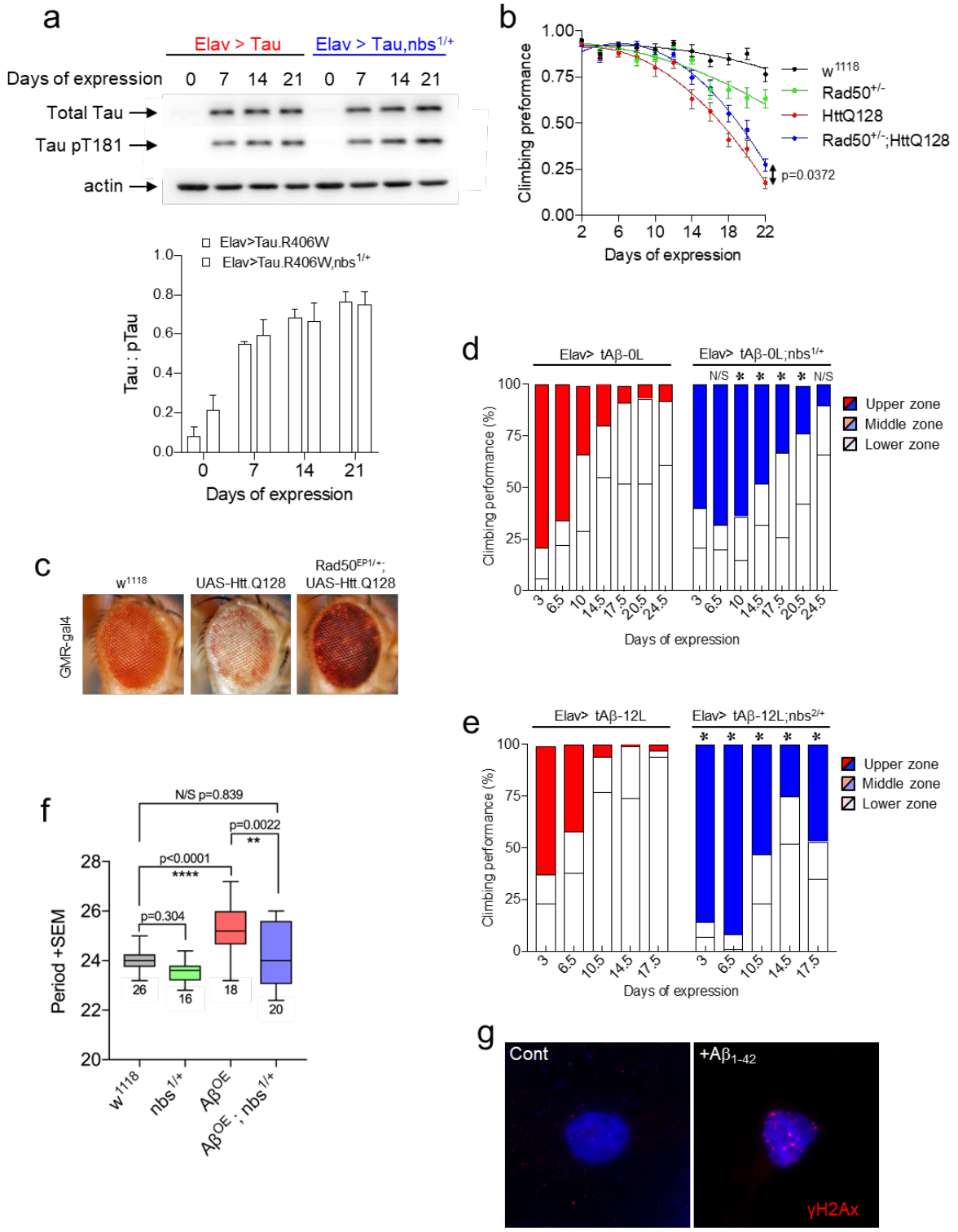
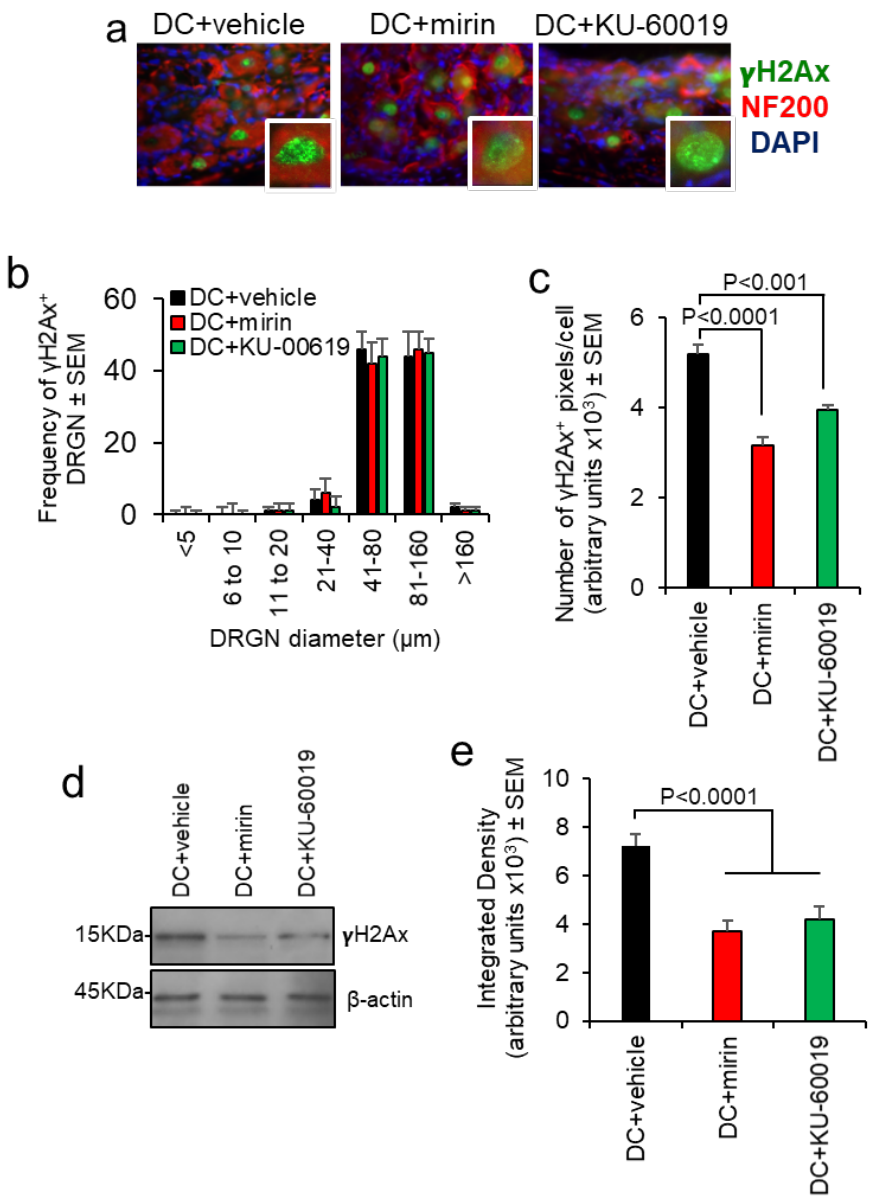


Figure 4. Intrathecal injection of Mre11 and ATM inhibitors promotes a full functional repair after spinal cord injury. **a**, Superimposed CAP traces from representative sham controls, DC+vehicle, DC+mirin and DC+KU-60019-treated rats. **b**, Negative CAP amplitudes were significantly attenuated in DC+vehicle-treated rats but were significantly improved in DC + mirin and DC + KU-60019-treated rats (P<0.0001, ANOVA). **c**, Mean CAP area was significantly attenuated in DC + vehicle-treated rats but improved significantly in DC + mirin and DC + KU-60019-treated rats (P<0.0001, ANOVA). **d**, Mean tape sensing and removal time are restored to normal 3 weeks after treatment with mirin and KU-60019 (P<0.0001, independent sample t-test) whilst a significant deficit remains in DC+vehicle-treated rats (# = P<0.00012, generalised linear mixed models over whole 6 weeks). **e**, Mean error ratio to show the number of slips *versus* total number of steps in the horizontal ladder walking test also returns to normal 3 weeks after treatment with mirin and KU-60019 (P<0.0001, independent sample t-test), with a deficit remaining in DC+vehicle-treated rats (## = P<0.00011, linear mixed models over whole 6 weeks). n=18 rats/treatment/test.

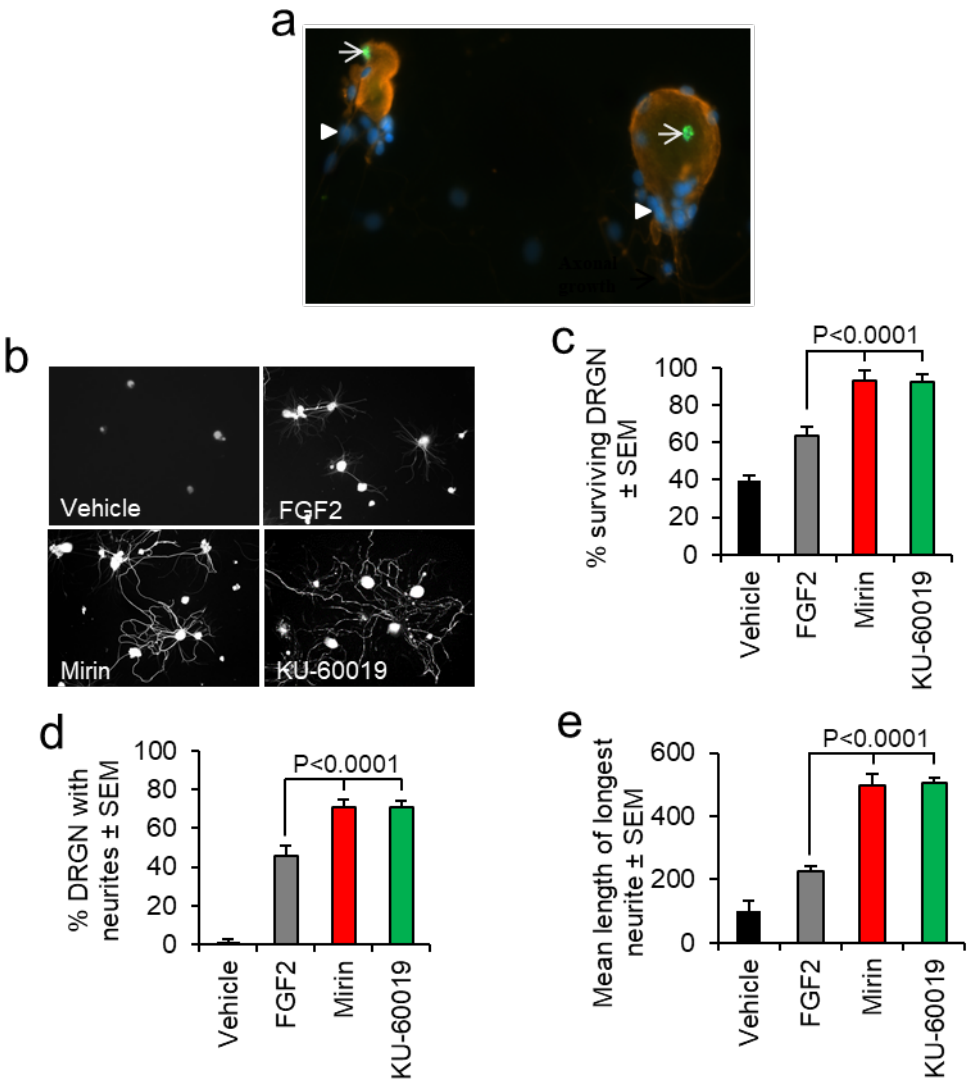


Extended Data Fig. 1. Genetic depletion of the MRN complex is neuroprotective in Drosophila neurodegeneration models. a, Quantification of Tau.R406W expression in the heads of flies by Western blot. Flies were developed at 18 °C to inhibit expression of Tau then decapitated 0,7,14 or 21 d after shifting to 29 °C. Tau proteins levels are not altered in *NBS* heterozygotes. Phosphorylation of Tau at Thr181 is also unchanged. Quantification of Tau levels normalised to actin and the Tau:pTau ratio is shown below (n=3; mean ± s.e.m; ANOVA with Dunn's post-hoc test). **b**, Negative geotaxis

climbing assay. Flies expressing Htt.Q128 show premature decline in climbing ability. This is partially suppressed in flies heterozygous for a null *Rad50*^{EP1} allele (p=0.0372) despite *Rad50* heterozygous flies already showing reduced climbing ability vs. the *w*^{1118} control (p=0.0007). Lines were fitted using non-linear regression and compared by extra sum-of-squares F-test. **c**, Expression of Htt.Q128 in the eye leads to loss of pigment-containing cells in the retina after 7 weeks. Pigment loss is largely prevented in flies *Rad50*$^{-/+}$ flies. **d** and **e** Negative geotaxis climbing assays of flies expressing two forms of tandem Aβ_{1-42} expression with no flexible linker **c** or a 12-amino acid flexible linker **d** in adult neurons [12]. Flies climbed in vials demarked into 3 zones. Aβ_{1-42} expressing (red) flies were compared to Aβ_{1-42}; nbs$^{1/+}$ flies (blue) on the days indicated after shifting to 29 °C to induce expression. Comparison was by χ^2 test (* indicates p<0.05). **f**, Circadian periodicity analysed by cosinor analysis shows similar results to the CLEAN spectral analysis shown in Fig. 1f[29]. Comparison by ANOVA with Tukey's post-hoc test; p values and n are indicated. **g**, Representative images of 18-21 DIV rat hippocampal neuron nuclei fixed and stained to visualise DSBs with anti-pH2AX (red). DAPI was used to visualise DNA (blue). Exposure of neurons to Aβ_{1-42} oligomers for 24 h induces DSBs.



Extended Data Fig. 2. Inhibition of Mre11 and ATM reduces the number of DRGN with DSBs and the levels of phospho-γH2AX after spinal cord injury *in vivo*. a, Representative images from DC + vehicle, DC + mirin and DC + KU-60019-treated sections of rat DRGN (n=18 rats/group) demonstrating phospho-H2Ax (green) localisation in the nucleus of DRGN (red). Images are counterstaining with DAPI (blue) to demarcate the nucleus. **b**, Quantification of the frequency of phospho-H2Ax$^+$ DRGN sorted by different soma size. **c**, Quantification of the mean pixel intensity/DRGN in DC + vehicle, DC + mirin and DC + KU-60019-treated DRGN. **d**, Western blot and **e**, subsequent densitometry to demonstrate reduction of phospho-H2Ax protein levels in DRGN after treatment with mirin and ATM inhibitors. Comparisons in **c** and **e** by ANOVA with Dunnett's post-hoc test.



Extended Data Fig 3. Inhibition Mre11 prevents neuronal apoptosis and stimulates neurite outgrowth in the presence of inhibitory CME. a, Adult DRGN stained with anti-γH2AX antibodies to demonstrate DSB's (arrows) in DRGN *in vitro*, whilst satellite glial cells did not possess DSB's (arrowheads). **b**, Representative images from DRGN treated with vehicle, FGF2 (positive control), mirin and KU-60019. **c-e**, Mirin or KU-60019 treatment enhances **c,** DRGN survival, **d,** % DRGN with neurites and **e,** increased the mean length of the longest neurite. Comparison in (c-e) by ANOVA with Dunnett's post-hoc test, n=9 wells (containing 180 DRGN)/condition.

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Data and materials availability All data is available in the main text or the Extended Data.