

Changes in white matter in mice resulting from low-frequency brain stimulation

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Recent reports have begun to elucidate mechanisms by which learning and experience produce white matter changes in the brain. We previously reported changes in white matter surrounding the anterior cingulate cortex in humans after 2–4 weeks of meditation training. We further found that low-frequency optogenetic stimulation of the anterior cingulate in mice increased time spent in the light in a light/dark box paradigm, suggesting decreased anxiety similar to what is observed following meditation training. Here, we investigated the impact of this stimulation at the cellular level. We found that laser stimulation in the range of 1–8 Hz results in changes to subcortical white matter projection fibers in the corpus callosum. Specifically, stimulation resulted in increased oligodendrocyte proliferation, accompanied by a decrease in the g-ratio within the corpus callosum underlying the anterior cingulate cortex. These results suggest that low-frequency stimulation can result in activity-dependent remodeling of myelin, giving rise to enhanced connectivity and altered behavior.

anterior cingulate cortex | electron microscopy | meditation | mouse | myelination

A variety of training methods have shown white matter change in humans using diffusion tensor imaging (DTI) (1, 2). These include training in working memory, juggling, and meditation (2, 3). Our human studies reported that 2–4 wk (30 min per day) of integrated body mind training (IBMT) resulted in white matter changes as measured by DTI around the anterior cingulate cortex (ACC) compared with a control group practicing relaxation (4, 5). IBMT, a form of mindfulness meditation, is associated with improvements in memory and attention and reductions in self-reported levels of negative affect (6).

We hypothesized (7) that the white matter changes might be due to increases in midfrontal theta oscillations (4–8 Hz) found in our study (8) and other research (9). Moreover, human studies have provided evidence that increases in theta current density are correlated with increased metabolism in the ACC (10). When the ACC receives theta stimulation that is in phase with stimulation of a lateral frontal area, there is an improvement in measures of executive function (11). Based on these findings, we wanted to explore the potential causal relationships between increased midfrontal theta and white matter change.

Depending on factors such as age and brain region, a given fraction of axons within white matter tracts are insulated by myelin. Myelination is thought to be critical for cognition because it is an effective mechanism for optimizing conduction velocity and coordinating spike-timing between distant brain regions (reviewed in refs. 12–14). It has been shown that adult-born oligodendrocytes contribute to remodeling of myelin and their activity can be modulated by both intrinsic and extrinsic factors (2). Specifically, studies in animal models show that proliferation, differentiation, and synthesis of myelin by oligodendrocyte progenitor cells (OPCs) are regulated by neural activity in both the developing and adult brain (13, 15–19). These results demonstrate that neuronal activity is sensed by oligodendrocytes and instructs the selective myelination of an active circuit.

There is mounting evidence from studies in rodents that natural experience can modulate myelination by oligodendrocytes in the adult, allowing the potential for improvement of neural circuit function. Learning a new skill can increase white matter and oligodendrocyte proliferation in regions of the brain engaged by the learned task (20–22). Conversely, social isolation can result in decreased myelination and impaired cognitive function (19, 23, 24). Furthermore, it has been shown that visual deprivation not only shortens the length of myelin internodes, but also results in reduced nerve conduction velocity in the optic nerve (25).

In an effort to elucidate the mechanisms involved in the regulation of myelination, a recent study using optogenetic stimulation of layer 5 pyramidal cells in the mouse motor cortex reported an increase in oligodendrocyte proliferation and differentiation within both the motor cortex and the subcortical projections of the corpus callosum (16). These changes were associated with myelin remodeling and improved behavioral performance. Another study (26) used an implanted electrode array to examine the effect of stimulation frequency (5, 25, or 300 Hz) on OPC proliferation and differentiation in the corpus callosum and effects were dependent upon the stimulation paradigm: 5-Hz stimulation showed the greatest effect on the differentiation of OPCs, whereas 25- and 300-Hz stimulation had the greatest effects on proliferation.

We previously reported the behavioral effects of rhythmic stimulation or suppression of ACC activity in mice (27). Using light-activated channels [both Channelrhodopsin-2 (ChR2) and Arch-aerhodopsin (Arch)], we drove periodic activation or inhibition of fast-spiking inhibitory interneurons at three different frequencies (1, 8, and 40 Hz) to increase or decrease the activity of ACC output neurons. We reported that mice receiving 1- and 8-Hz stimulation, which rhythmically increased ACC spike output, exhibited greater exploratory activity in the light area of a light/dark box compared

Significance

Meditation has been shown to modify brain connections. However, the cellular mechanisms by which this occurs are not known. We hypothesized that changes in white matter found following meditation may be due to increased rhythmicity observed in frontal areas in the cortex. The current study in mice tested this directly by rhythmically stimulating cells in the frontal midline. We found that such stimulation caused an increase in connectivity due to changes in the axons in the corpus callosum, which transmit impulses to and from the frontal midline. This work provides a plausible but not proven mechanism through which a mental activity such as meditation can improve brain connectivity.

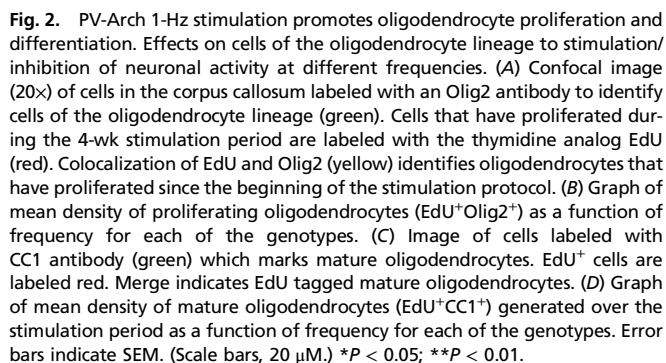
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A two-way ANOVA yielded only an effect of stimulation condition [$F_{(3, 84)} = 4.3$; $P = 0.0073$]. A Tukey multiple-comparison analysis showed no significant increase in EdU+CC1⁺ cells following low-frequency stimulation overall, although a trend was seen in the 1-Hz group compared with nonstimulated controls (1 Hz: $P = 0.0785$; 8 Hz: $P = 0.9995$). The Tukey test estimates the variance using all of the genotypes and stimulation conditions. If we compare each stimulation condition separately to nonstimulated con-

A decrease in g-ratio could be accounted for by either an increase in myelin thickness or a decrease in axon caliber. Fig. 3 *D* and *E* show measures of myelin thickness and axon diameter separately. Comparing the low-frequency stimulated group to the nonstimulated group, there was a small increase in myelin thickness (0.16 ± 0.003 vs. 0.17 ± 0.002) (Fig. 3*D*) as well as a decrease in the fraction of large caliber axons (0.50 ± 0.03 vs. 0.45 ± 0.013) (Fig. 3*E* and *F*). Although there appears to be a contribution of each factor to the change in g-ratio, neither factor alone reached statistical significance for the stimulated group compared with the no-laser control ($P = 0.0598$ for myelin thickness and $P = 0.1354$ for axon diameter). To test whether the reduced fraction of larger diameter axons following stimulation might reflect de novo myelination of small fibers that were unmyelinated before stimulation, we counted the number of myelinated vs. unmyelinated fibers of at least 100-nm diameter in the corpus callosum. No significant increase in de novo myelination was apparent in the stimulated group over the control in the percentage of myelinated axons [stimulated animals ($54.8 \pm 3.1\%$) vs. nonstimulated controls ($55.9 \pm 4.42\%$)].

Table 1. Oligodendrocyte proliferation and differentiation data

	No laser			1 Hz			8 Hz			40 Hz		
	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n
Olig2 or CC1												
Olig2												
Arch	145.7	10.7	8	222.9	17.3	6	138.9	17.9	5	89.2	15.0	5
ChR2	161.9	20.7	3	102.5	17.2	3	116.7	11.5	3	94.6	26.4	3
PV	152.9	13.9	3	164.3	19.5	3	169.4	12.9	3	115.6	20.8	3
CC1												
Arch	66.4	5.5	8	89.1	9.3	6	67.5	9.0	5	38.8	7.2	5
ChR2	57.1	7.6	3	65.3	16.9	3	62.2	9.6	3	44.7	6.6	3
PV	51.9	4.6	3	62.2	7.8	3	54.8	9.2	3	51.3	8.9	3

Correlation of Myelination Data with Behavior. Previously, we presented data showing that low-frequency stimulation at 1 or 8 Hz increased the time animals spent exploring the illuminated half of the light-dark box. In Fig. 4, we show increased time spent in the light is correlated with a decrease in g-ratio calculated from the EM data. Given the small number of experimental animals, we used a nonparametric comparison of the g-ratio and the amount of time spent in the light (Kendall's τ of -0.501 and $P = 0.04$). It is clear that this relationship is driven by the higher g-ratios in the nonstimulated control group, rather than individual variability within the stimulated groups. Although the figure suggests that a reduction in g-ratio is related to reduced anxiety and greater exploration in poststimulation behavior, the largest effect is driven by whether the animals were stimulated or not.

Discussion

Our results can be summarized as follows: (i) Optogenetic stimulation of the mouse ACC in the range of 1–8 Hz results in changes to white matter in the corpus callosum. (ii) This low-frequency stimulation results in increased oligodendrocyte proliferation, accompanied by a decrease in the g-ratio within the corpus callosum underlying the ACC. (iii) These results suggest that low-frequency stimulation can result in activity-dependent remodeling of myelin, which could lead to enhanced connectivity and altered behavior. We discuss each of these points below.

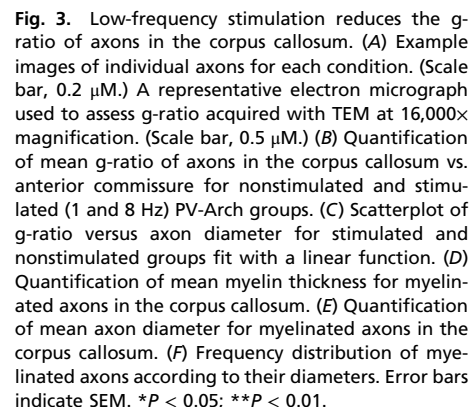
Changes in Oligodendrocytes and Axonal Structure. We found a significant increase in EdU⁺Olig2⁺ oligodendrocytes following 1-Hz stimulation in the PV-Arch group in comparison with nonstimulated controls. However, the increase in EdU⁺CC1⁺ cells, indicating numbers of newly born oligodendrocytes that are mature and capable of myelination, was less robust. It is possible that this is due to the small number of mice involved in the critical conditions, but we also speculate on possible biological explanations for this result, some of which are outlined in Fig. 5. It is conceivable that different aspects of oligodendrocyte maturation are regulated by different factors, and 1-Hz stimulation specifically regulates those related to proliferation. Another possible explanation is that 1-Hz, and possibly 8-Hz stimulation could have effects on the myelinating activity of postmitotic oligodendrocytes, which would not have been labeled with EdU, and thus would not have been identified in our analysis. This alternative could account for why both 1- and 8-Hz stimulation were able to affect the g-ratio and why both also affect behavior. It is also possible that the oligodendrocyte precursors generated by 1-Hz stimulation might not have differentiated into mature myelinating oligodendrocytes. There is evidence that oligodendrocyte precursors can perform metabolic support functions for axons and help maintain homeostasis at synapses rather than becoming myelinating oligodendrocytes (30–34). Finally, oligodendrocyte proliferation could have been activated by inflammation or injury associated with the stimulation protocol (35–38). However, this explanation seems unlikely as: (i) we saw no evidence of apoptosis in the stimulated area at the time of euthanasia (39), (ii) no

behavioral deficits were noted during any of the paradigms tested in our previous study, and (iii) the effects were seen specifically with the PV-Arch mice, while both PV-Arch and PV-ChR2 animals would have experienced localized heating of the tissue with light delivery.

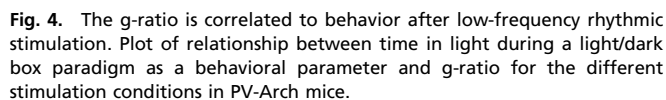
Both axon diameter and myelin thickness contribute to neuronal conduction velocity (13, 14), and even small changes in either parameter can have a meaningful impact on transmission speed. The g-ratio is a quantitative measure of optimal axonal myelination and is calculated by dividing the axon diameter by the diameter of the fiber (axon plus myelin). We found that both 1- and 8-Hz stimulation of PV-Arch animals were essentially equivalent in producing a decrease in g-ratio relative to nonstimulated controls in the corpus callosum, which contains axons from the ACC, but not in the more distally located anterior commissure, which does not contain axons from the ACC. Nonstimulated control values for the corpus callosum and anterior commissure are in agreement with previously published values (16, 29, 40). Both 1- and 8-Hz stimulation resulted in a decreased g-ratio, although only 1-Hz stimulation appreciably increased oligodendrocyte proliferation. It is possible that 1-Hz stimulation drives oligodendrocytes to divide, whereas 8-Hz stimulation drives myelination. Although there is evidence that myelin remodeling can occur independently of OPC proliferation (41), it is unclear to what extent mature OLs can modulate their myelination (37, 38).

Changes in myelination or axon diameter alter the g-ratio and can result in a reduction of conduction velocity. Given that the theoretical value for the optimal g-ratio has been calculated to be 0.6 (40, 42), it would be interesting to examine changes in conduction velocity resulting from the observed reduction in g-ratio seen here. However, communication between brain regions does not depend specifically on the speed of transmission, but on the synchronization of inputs. Thus, optimal connectivity may not require optimal velocity. Unfortunately, we were unable to follow individual axons to determine which ones were directly affected by the optogenetic stimulation. Furthermore, in our experiments we were not able to examine potential changes in the pattern of myelination along the length of an axon. The length of myelinated segments and the spacing between them (internodes) are also factors that can contribute to changes in conduction velocity (43–45). Investigating the contribution of each of these factors to stimulation-induced changes in white matter should be topics for future study.

The reduction in g-ratio in our experiments appears to be due to a combination of both increased myelination and a reduction in myelinated axon caliber in stimulated mice compared with controls. One possibility is that the stimulation protocol resulted in de novo myelination of small nonmyelinated fibers, which would shift the population to smaller diameters overall. However, we did not see a change in the overall percentage of myelinated fibers in the corpus callosum. There is evidence that axon diameters scale by firing frequency (46), suggesting that perhaps axons could not only increase diameter in response to firing rate, but could also decrease. However, we are not aware of similar studies describing a reduction in axon diameter with stimulation or learning.



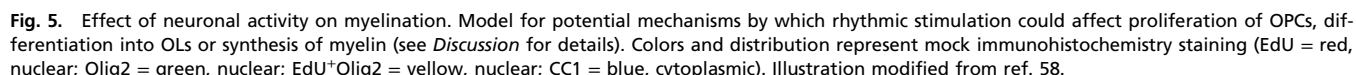
IBMT. Exploratory behavior was increased most by 8-Hz stimulation, but overlap in EdU and Olig2 labeling was greater for 1-Hz stimulation. Furthermore, 1- and 8-Hz stimulation



*Indicates SD due to small number.

The prospect that different stimulation frequencies could underlie distinct changes at the ultrastructural, cellular, and behavioral levels warrants further investigation. Indeed, different stimulation frequencies are known to be effective for different aspects of behavior and physiology. Nagy et al. (26) demonstrated that electrical stimulation of activity at different frequencies in the corpus callosum had distinct effects, with low-frequency stimulation promoting oligodendrocyte differentiation and high-frequency stimulation driving proliferation. Activity-dependent regulation of oligodendrocyte maturation or activity could also vary depending on brain region, cell-type, frequency of stimulation, synchrony of firing with respect to other axons, duration of stimulation, or gender of the animal. There is still much work to be done to characterize how cells of the oligodendrocyte lineage respond to different patterns of neuronal activity.

Although the stimulated 1- and 8-Hz groups showed only about a 10% decrease in g-ratio and a small increase in myelination, this may have been sufficient to reduce anxiety and increase the time mice spent in the light. Increased FA found in our meditation



studies in pathways related to the ACC was about 10% above its initial value (5). These studies suggest that the changes in white matter due to experience could be similar in quality and magnitude in humans and mice.

There is evidence that rhythmic stimulation in the theta range delivered to frontal areas can modulate behavioral performance in humans. One recent study found that 6-Hz transcranial alternating current stimulation over midline and the lateral frontal cortex improved executive function when synchronized, and reduced executive function when desynchronized (11). Many researchers have reported improved learning and performance with direct current frontal stimulation (54), although these results are mixed (55). It is also possible that such stimulation works in whole or part through stimulating an intrinsic rhythm (56).

Based on the findings in mice, we speculate that inducing low-frequency rhythmic activity in humans over multiple sessions by ACC stimulation might be sufficient to alter connectivity and perhaps produce similar white matter changes found with meditation. Inducing such a rhythm less invasively in humans, whether by electrical stimulation, sensory stimulation, or neurofeedback, might work to change white matter in different brain areas and thus serve to improve functionality. The effectiveness of such stimulation might also be enhanced if accompanied by a task activating the same brain areas. If true, it might be possible to improve or restore white matter in any area of the brain for which appropriate electrodes and tasks could be designed. These ideas are preliminary, but they support the need for more research to understand the effects of brain stimulation on behavior and the underlying biological mechanisms.

Methods

All studies were conducted with approved protocols from the University of Oregon Institutional Animal Care and Use Committee, in compliance with National Institutes of Health guidelines for the care and use of experimental animals.

Mice. In the present study, we assessed the effects of rhythmic suppression or stimulation of cortical activity in mice expressing the light-gated ion channel ChR2 or the proton pump Arch in PV⁺ inhibitory interneurons. Parents were homozygous for Pvalb-IRES-Cre (PV, 008069; The Jackson Laboratory), Rosa-CAG-LSL-ChR2(H134R)-EYFP-WPRE (ChR2, 012569; The Jackson Laboratory), or Rosa-CAG-LSL-Arch-GFP-WPRE (Arch, 012735; The Jackson Laboratory). Optogenetic suppression of the ACC was performed using offspring heterozygous for both PV and ChR2 (PV-ChR2). Optogenetic stimulation was performed using offspring heterozygous for both PV and Arch (PV-Arch). Mice homozygous for Pvalb-IRES-Cre (PV-PV) were utilized to control for nonspecific effects of pulsed light delivery. Data were collected from both male and female mice, aged 8–12 wk at the time of surgery. A total of 26 PV-Arch, 16 PV-ChR2, and 10 PV-PV mice were included in immunohistochemistry experiments. A total of 10 PV-Arch mice were included in EM work.

Surgery. To study the behavioral effects of rhythmic cortical activity manipulations, mice were implanted with a 2×2 array of 200- μm diameter optic fibers overlying each hemisphere of the ACC. To determine whether these phasic manipulations of cortical activity altered baseline power of intrinsic rhythms, identical arrays were modified to include a single Teflon-coated stainless steel local field potential recording electrode in each hemisphere between each pair of optic fibers. For recording single-neuron activity, a four-tetrode array with a single 200- μm optic fiber was implanted in the ACC. Mice were administered carprofen (5.0 mg/kg) postoperatively to minimize discomfort and allowed 7 d of postoperative recovery.

Stimulus Delivery. Mice from all three genetic backgrounds were randomly assigned to a 1-Hz (200 ms pulses), 8-Hz (5 ms pulses), 40-Hz (5 ms pulses), or no-laser condition. Mice were given 20 sessions of light pulses (30 min/d, 5 d/wk over 4 wk). For PV-Arch mice, 520-nm wavelength modules were set to 9.5 mW. For PV-ChR2 mice, 445-nm wavelength modules were set to 6.3 mW. These intensities were selected to produce an effective spread through a volume of tissue ~1.5 mm in diameter, thus restricting the direct effect predominantly to the ACC.

Immunohistochemistry. Mice were anesthetized with Euthazol and perfused first with phosphate buffer (PB, pH 7.4) containing heparin (10 units/mL; Sagent Pharmaceuticals) and then with 4% paraformaldehyde (PFA; Electron Microscopy

Sciences, EMS) in 0.1 M PB. Brains were removed, immersed in fresh 4% PFA and stored at 4 °C overnight. Tissue was cryo-protected in 30% (wt/vol) sucrose in 0.1 M PB for 48 h. All brains were embedded in optimal cutting temperature compound and coronal cryo-sections (30-μm thickness) were collected into cryo-protectant medium [30% (wt/vol) sucrose, 1% (wt/vol) polyvinyl-pyrrolidone, 30% (vol/vol) ethylene glycol in 0.1 M PB] and stored at -20 °C before staining. Immunohistochemistry was performed on floating sections.

For immunolabeling, sections were washed with 0.1 M PB and permeabilized with blocking solution containing 0.3% Triton-X100 and 6% Normal Donkey serum (NDS; Sigma) before incubating overnight with rabbit anti-MBP (1:5,000; Abcam), mouse monoclonal CC1 (1:500; against adenomatous polyposis antigen; Calbiochem), or rabbit anti-OLIG2 (1:500; Millipore). Sections were washed three times with 0.1 M PB before adding AlexaFluor 657-conjugated anti-rabbit IgG or AlexaFluor 488-conjugated anti-mouse IgG (1:5,000; Invitrogen) in blocking solution. Floating sections were transferred onto gelatin-coated glass slides and mounted using Fluoromount-G (Southern Biotech). Sections were costained with rabbit anti-Caspase3 (1:500; Cell Signaling Technology) to quantify cell death.

EdU Labeling and Detection. EdU (Invitrogen) is a thymidine analog that is incorporated into the DNA of cells as they undergo DNA replication. EdU was administered to mice via the drinking water (0.2 mg/mL) that was available ad libitum and replaced every day. To determine the identity of newly born cells, 30- μ m floating sections were first processed and stained as above to detect CC1 or Olig2 expression and developed immediately for EdU labeling. The sections were washed with PBS/3% BSA (wt/vol) followed by a 20-min incubation in PBS/0.5% Triton-X100 (vol/vol). The slices were again washed in PBS/3% BSA before EdU detection with the Click-iT EdU Alexa Fluor-555 Imaging kit (Invitrogen). Each slice was immersed in 120 μ L of Click-iT developing mixture and incubated for 40 min at room temperature in the dark, according to the instructions of the manufacturer. Slices were washed once with PBS/3% BSA, twice with PBS and mounted under coverslips in fluorescence mounting medium (Fluoromount-G; Southern Biotech).

Microscopy and Cell Counts. With experimenters blinded to sample identity and condition, protein colocalization (multiple fluorochromes) was assessed in the confocal microscope. At least three mice were examined for each transgenic genotype. From each mouse, 30- μ m coronal brain sections were immunolabeled to count Olig2/EdU⁺ or CC1/EdU⁺ double positive cells. All images were collected on an Olympus confocal microscope as z-stacks with 5- μ m spacing, using standard excitation and emission filters for DAPI, FITC (Alexa Fluor-488), Texas Red (Alexa Fluor- 555), and Far Red (Alexa Fluor-647). For quantification, four or more low-magnification (20 \times objective) fields were collected per animal within each anatomical region of interest. Cells were viewed and counted using NIH ImageJ software (<https://imagej.nih.gov/ij/>).

Electron Microscopy. Four weeks after optogenetic stimulation (as above), mice were anesthetized with Euthazol and perfused with PB containing heparin followed by 2% paraformaldehyde (PFA; EMS), 2.5% glutaraldehyde (EMS), and 2 mM CaCl_2 in 0.1 M sodium cacodylate buffer, pH 7.4. After the brain was removed, a clean blade was used to resect a bilateral section containing either the anterior commissure or the corpus callosum below the ACC using an acrylic adult rodent brain matrix (World Precision Instruments). Following fixation overnight at 4 °C, the tissue was washed 3× for 10 min in 0.1 M sodium cacodylate buffer containing 2 mM CaCl_2 at 4 °C. The samples were postfixed with 1% osmium tetroxide (EMS), 1.5% potassium ferrocyanide (EMS) in 0.1 M sodium cacodylate buffer for 1–2 h at 4 °C. They were washed three times with ultrafiltered water (EMS), and *en bloc*-stained with 2% uranyl acetate (EMS), for 1 h at 4 °C in the dark. Samples were washed twice with ultrafiltered water and allowed to equilibrate to room temperature. The samples were infiltrated and embedded in graded acetonitrile (50, 70, 90, and 100%) at room temperature according to the schedule of Edwards et al. (57). Samples were infiltrated with EMBED-812 resin (EMS) mixed 1:1 with acetonitrile for 1 h, followed by 1:1 EMBED-812:acetonitrile for 18 h. The samples were next placed into EMBED-812 for 3 × 1 h, then placed into silicone-embedding molds filled with fresh resin, which were then baked in a 60 °C oven for 48 h. Sections of 75- and 100-nm thickness were cut on a Reichert-Jung Ultracut E (Reichert-Jung) using a glass or an Ultra Diamond knife (EMS) and mounted on Formvar/carbon-coated slot grids (EMS) or 300-mesh Cu grids (EMS). Grids were contrast stained for 30 min in 2% uranyl acetate. Samples were imaged using an FEI Tecnai G2 Spirit TEM at 120 kV, and images were collected using an FEI Eagle 4k CCD camera at 16K magnification.

Statistics. We performed and analyzed all experiments blind to genotype and stimulation condition. Graphical data analyzed and presented using GraphPad Prism (v7). Group mean differences were analyzed using two-way ANOVA with Tukey or Tukey–Kramer post hoc tests to further examine pairwise differences for the EdU data. EM data were analyzed using a one-way ANOVA with Dunnett's test for multiple comparisons. In some instances, unpaired two-tailed Student's *t* tests were also reported. The relationship between behavioral data [from Weible et al. (27)] and g-ratio data were

calculated using Kendall- τ b. Data are presented as mean \pm SEM, unless otherwise indicated.

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