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Supporting information for article:

X-ray diffraction reveals blunt-force loading threshold for nanoscopic structural change in ex vivo neuronal tissues

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S1. SI Methods

S1.1. X-ray diffraction setup

X-ray diffraction experiments were carried out at the Biophysics Collaborative Access Team (BioCAT) at the Advanced Photon Sources, Argonne National Laboratory, Chicago IL (Fig S1). Freshly extracted optic nerves were each mounted between two thin sheets of mica (<50μm thick), sealed with epoxy to keep dehydration minimal, during the course of scanning. All XRD experiments were conducted at room temperature. Custom sliding sample frames were 3D printed using poly lactic acid material for sheet attachment. Each frame was then slid into a frame holder, mounted on a horizontal arm connected to a stepper motorized sample stage, capable of moving the sample horizontally and vertically at sub-millimeter steps. The CRL X-ray focusing optics were set up to deliver a focused beam, at an energy of 12 keV (1.033Å wavelength) at pre-determined sample positions, and the size of the beam was setup between 50 to 100μm (width and height). Sample motor positions, for the first and last points of regions of interest were determined using readouts on the control interface. An evacuated flight tube of 45cm was used to direct the diffracted x-rays to a marCCD detector (pixel size 79μm). An X-ray scanning software package (developed by BioCAT) was used to move the sample, by a step size of 0.1 to 0.5 mm, to collect diffraction patterns from adjacent points on the samples. Sliver behenate diffraction rings (principle repeat of 58.3Å) were used to calibrate the experimental setup for data analysis. The exposure time was 1-3s. All data analysis was performed using Fit2D (Hammersley, 1997) and DPDAK (Benecke et al., 2014). In-house python scripts were developed for Gaussian peak fitting.

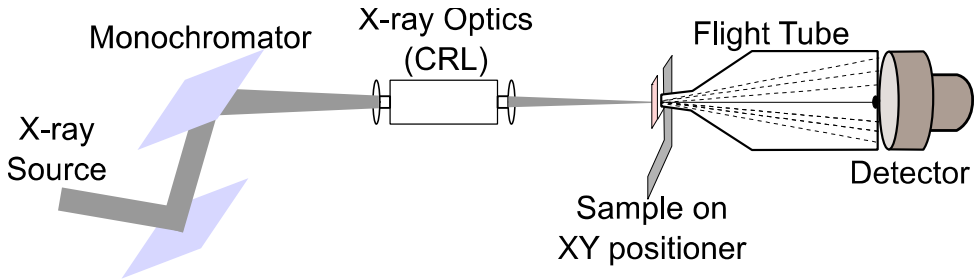

Figure S1 Illustration of the experimental set up for measurement of the fiber diffraction pattern from a sample of optic nerve.

S1.2. Source animals and samples

As described in Materials and Methods, rat optic nerves were dissected from rats utilized as control animals for other experiments and sacrificed before delivery to our experimental facilities. A total of 8 rats were used for the experiments reported here. More details on source animals and sample groups are shown in Table S1.

S2. SI Results and Discussion

S2.1. Gaussian peak fitting and detection of the center of diffraction spot

To avoid discrepancies in selecting the centers of diffraction peaks, Gaussian peaks were fit to data, after local background subtraction, to enable a uniform method of detection. As shown in Figure S2, the positions of these peaks were extracted to calculate averages and perform analyses reported in the main text and in the following sections of supplementary information sections.

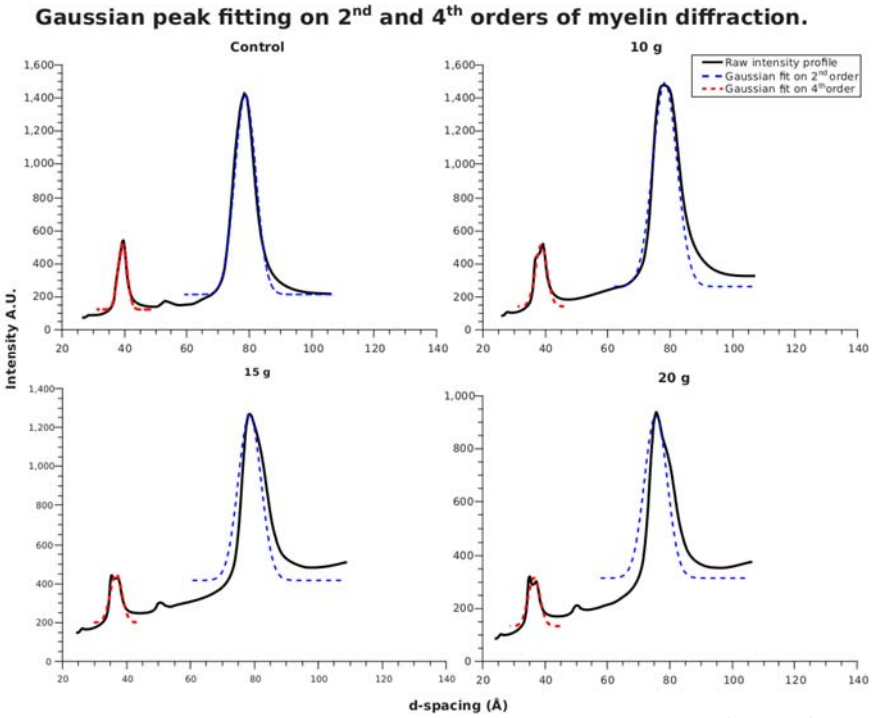

Figure S2 Raw intensity plots and Gaussian peaks fit on myelin 2nd and 4th order peaks from respective sample groups. A split is observed in the 4th order peak, in raw intensity profiles.

A second peak appears in the 4th order diffraction, particularly in impacted (loaded) samples. This may be a direct result of injury where the outer lamellae of the myelin sheath are impacted, giving rise to a collapsed packing structure. This same ‘split’ is not observed in the

2nd order peak, as the inner lamellae might be less prone to impact-mediated damage because of the intra-lamellar water and the stronger internal support offered by the the cytoskeletal components from within the axon. These changes are reported in Figure S3.

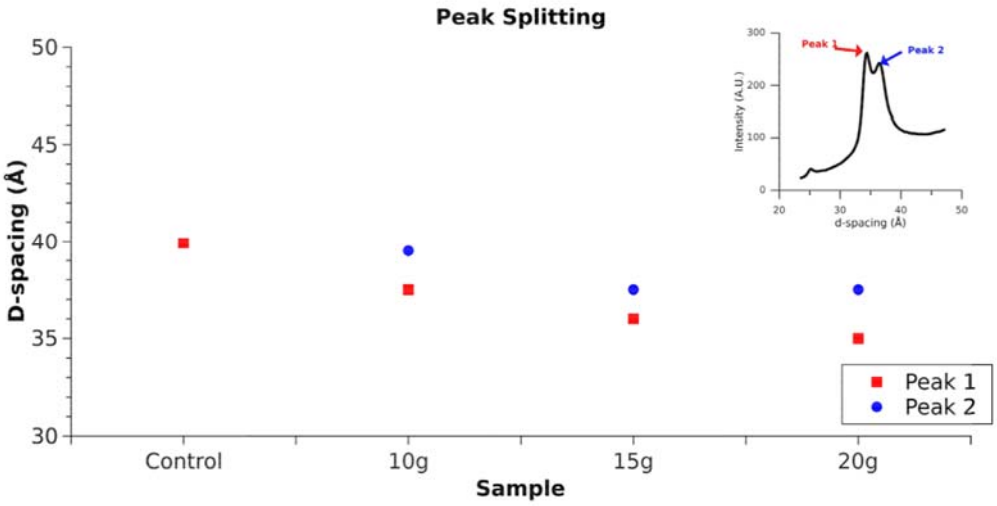

Figure S3 Positions of 'split' peaks observed in myelin 4th order diffraction with impact. Peak 1 and 2 are depicted in the inset. No split is observed in control samples, and the position of the peak gradually decreases (in Å) with impact. A change of ~2.5Å is observed in the position 'Peak 2', in comparison to the control sample in the 20g samples.

S2.2. d-spacing calculations from all optic nerve samples

As described in the Results and Discussion section, an overall change in myelin 2nd order d-spacing (which represents the molecular packing structure of the myelin sheath) was recorded from five samples for each experimental group (Control, 10g, 15g and 20g). From each sample, 20 diffraction patterns were collected and used to calculate the d-spacing. As shown in the plot below (Fig. S4), there is a significant change the average d-spacing between the 15g and 20g sample groups when compared to the control group, alongside an increase in the standard deviation in these groups. Particularly, there is a ~2.8Å change in average d-spacing in the 20g group, from the Control group. This may be a result of a 'permanent' change in molecular packing of myelin, arising from impact, thereby indicating a loading threshold.

The sample numbers, means and standard deviations, by experimental group, are reported in Tables S1 and S2.

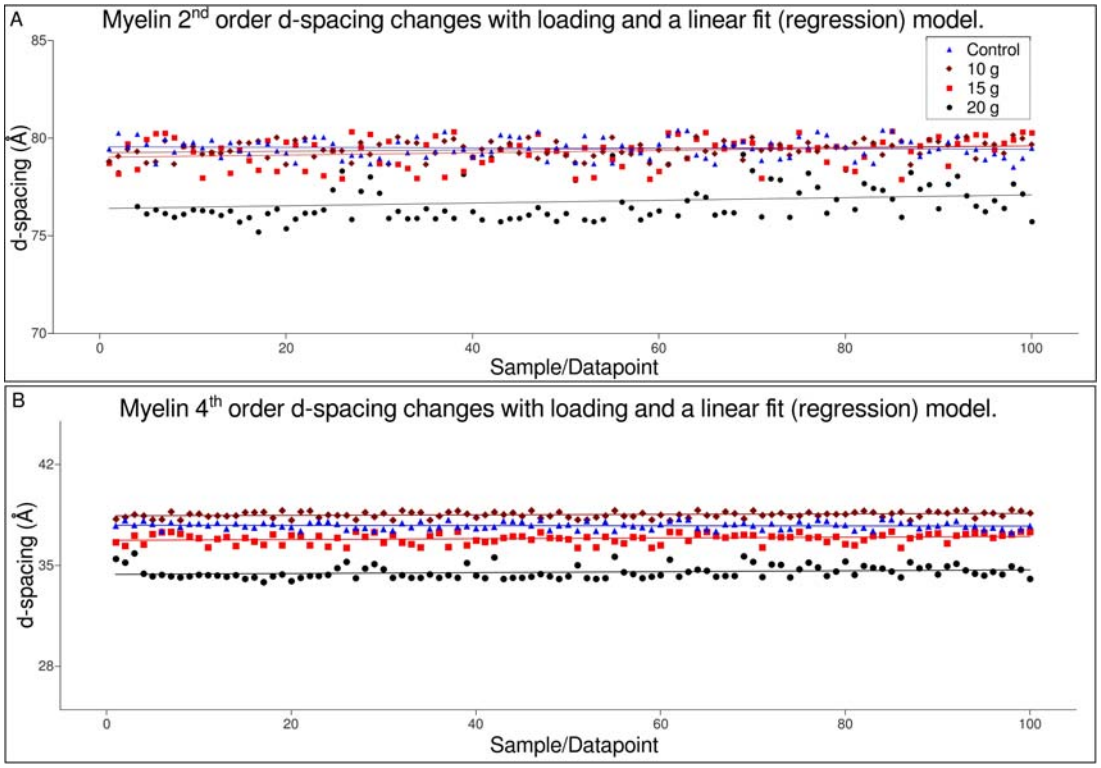

Figure S4 A scatter plot of myelin 2nd order (A) and 4th order (B) d-spacing calculated from the diffraction patterns collected from 5 samples for each experimental group and 20 datapoints per sample. Linear trendlines are used to show the overall changes d-spacings, particularly between the 20g experimental group in comparison to the other groups, indicating a loading threshold between 15g and 20g.

Table S1 Statistics from myelin (2nd order) d-spacings from loaded rat optic nerves.

Source Animal [a]	Sample Group [b]	Mean (Å)	Std. Dev (Å)	No. of Observations
Animals 1,2,3	**Controls**	**79.55**	**0.69**	**100**
	S1	79.62	0.46	20
	S2	79.44	0.51	20
	S3	79.64	0.57	20
	S4	79.69	0.51	20
	S5	79.39	0.54	20
Animals 3,4	**10 g**	**79.90**	**0.33**	**100**
	S1	79.75	0.43	20
	S2	79.84	0.44	20
	S3	80.03	0.19	20
	S4	80.01	0.20	20
	S5	79.89	0.21	20
Animals 4,5,6	**15 g**	**79.50**	**1.73**	**100**
	S1	79.75	1.51	20
	S2	79.51	2.29	20
	S3	79.66	1.63	20
	S4	79.46	1.62	20
	S5	78.93	1.65	20
Animals 6,7	**20 g**	**76.74**	**1.49**	**100**
	S1	76.98	1.11	20
	S2	76.47	1.60	20
	S3	76.97	1.72	20
	S4	76.41	1.55	20
	S5	76.91	1.46	20
Animals 7,8	**Glutaralde-hyde Fixed**	**63.06**	**0.47**	**40**
	S1	63.13	0.46	20
	S2	63	0.5	20

[a] Optic nerves were generally halved and 1-2 samples were collected per half. If a sample was too small or close to the end of the nerve, it was excluded.

[b] The `S' in sample groups (S1, S2, S3 etc.) denotes sample nomenclature from in the sample group.

Table S2 Statistics from myelin (4th order) d-spacings from loaded rat optic nerves.

Source Animal [a]	Sample Group [b]	Mean (Å)	Std. Dev (Å)	No. of Observations
Animals 1,2,3	**Controls**	**37.79**	**0.66**	**100**
	S1	37.42	0.44	20
	S2	37.75	0.48	20
	S3	38.24	0.54	20
	S4	38.15	0.48	20
	S5	38.31	0.51	20
Animals 3,4	**10 g**	**38.75**	**0.32**	**100**
	S1	38.71	0.42	20
	S2	38.83	0.43	20
	S3	38.83	0.18	20
	S4	39.05	0.19	20
	S5	39.07	0.20	20
Animals 4,5,6	**15 g**	**36.77**	**1.60**	**100**
	S1	38.13	1.40	20
	S2	36.99	2.12	20
	S3	36.85	1.51	20
	S4	37.69	1.50	20
	S5	37.35	1.53	20
Animals 6,7	**20 g**	**34.53**	**1.42**	**100**
	S1	35.22	1.05	20
	S2	35.35	1.52	20
	S3	34.25	1.63	20
	S4	34.59	1.47	20
	S5	35.57	1.39	20
Animals 7,8	**Glutaralde-hyde Fixed**	**30.58**	**0.44**	**40**
	S1	30.73	0.43	20
	S2	30.96	0.47	20

[a] Optic nerves were generally halved and 1-2 samples were collected per half. If a sample was too small or close to the end of the nerve, it was excluded.

[b] The `S' in sample groups (S1, S2, S3 etc.) denotes sample nomenclature from in the sample group.

S2.3. Paired T-test on impacted samples against controls

To understand the significance of the detected loading threshold reported in this study, the data from each impact group (10g, 15g and 20g) were used to test the null hypothesis that the difference in average d-spacing from test groups in comparison to control group is not zero. As reported in the table below, the P-values indicate significant change at 15g ($p = 0.01$) and a clear and significant change in the d-spacing from the 20g sample ($p = 7.59E-49$), further verifying the claim of a loading threshold for structural change between 15g and 20g.

Table S3 Results from a paired T-test from the 10,15 and 20g test groups against the Control group.

Samples	Mean (Å)	Std.Dev (Å)	Variance (Å)	Std. Error (Å)	P Value
10g	79.90	0.33	0.18	0.04	0.16
15g	79.50	1.73	0.55	0.07	0.01
20g	76.74	1.49	0.96	0.09	7.58E-49

S2.4. No significant changes in myelin 2nd and 4th order d-spacings with time

To establish the nature, if any, of artifacts from sample preparation, mounting and degradation over time, myelin 2nd and 4th order d-spacing was calculated from samples at different time points and reported here. As shown in Fig. S5, there is no significant change in d-spacing at <1, 3, 6, 9 and 12 hour time points. This validates that the samples were maintained in their near “native” state over time. An overall trendline on all the observations plotted here, shows almost no change, further validating that no changes to the structure were introduced by sample preparation and time before experiments.

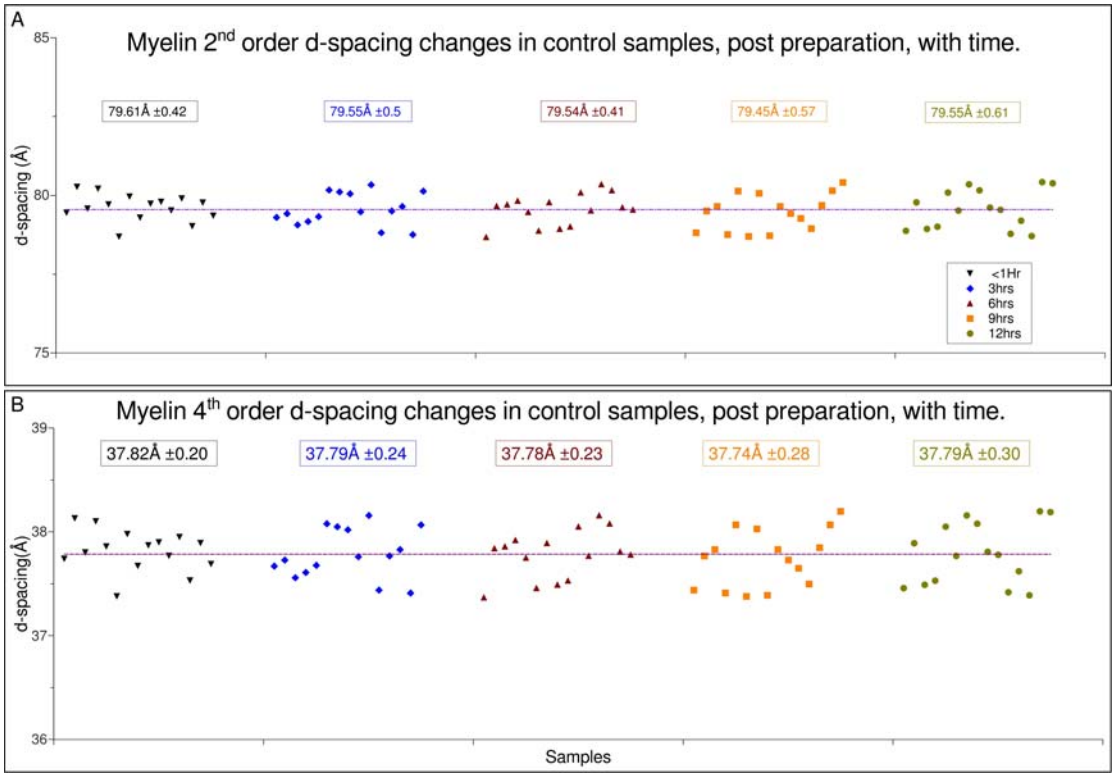

Figure S5 Myelin 2nd order (A) and 4th (B) order d-spacings calculated from samples over time after preparation (impact and sealing between mica sheets). The plot shows no significant changes in d-spacing with time, confirming that no artifacts were recorded as a result of sample degradation, post preparation. The purple dashed line represents the mean calculated using all the points reported here further illustrates this claim. The color matched boxes show the mean and standard deviation of each group.

S2.5. Differences in myelin packing in different source animals

A total of 8 animals were used for the experiments and interpretations reported in this study (see Table S1). It may be possible that different source animals could possess different macroscopic myelin packing. Whether these changes translate to, or are a result of molecular packing, remains to be addressed. However, to record any variations arising from source animals, myelin 2nd order d-spacing for the control group samples from 3 different animals have been compared. As seen in Fig. S6, no significant changes in d-spacing are observed in data collected from different optic nerves from the same animal (contralateral controls; Animal 1, samples 1 and 2) and between different animals, (Animals 1, 2 and 3).

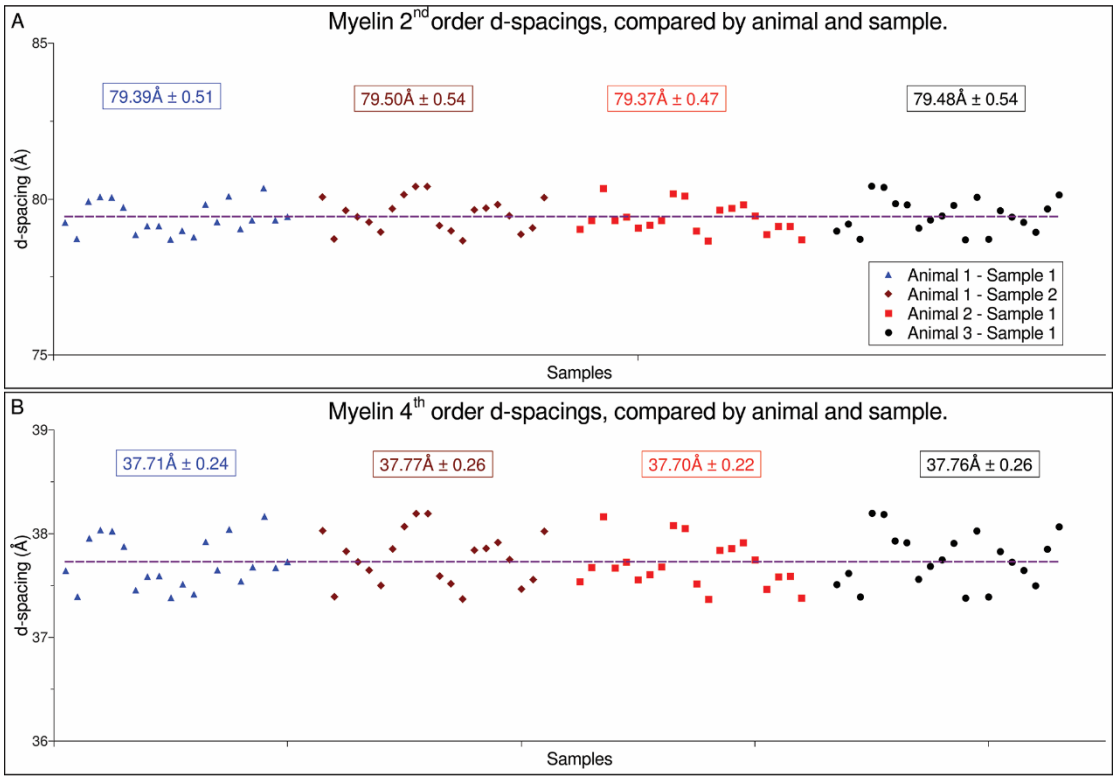

Figure S6 Myelin 2nd order (A) and 4th (B) d-spacings calculated from diffraction patterns from three animals. Samples 1 and 2 from animal are show to demonstrate observations from contralateral control from the same source animal. Data from samples 2 and 3 are used to compare d-spacing from different animals. No significant differences were observed in these observations, as is illustrated by the purple dashed line at the mean calculated from all the datapoints on the plot. The color matched numbers in the box indicate the mean and standard deviation of each test group.