Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Information for

Few-layered graphene increases the response of nociceptive neurons to irritant stimuli

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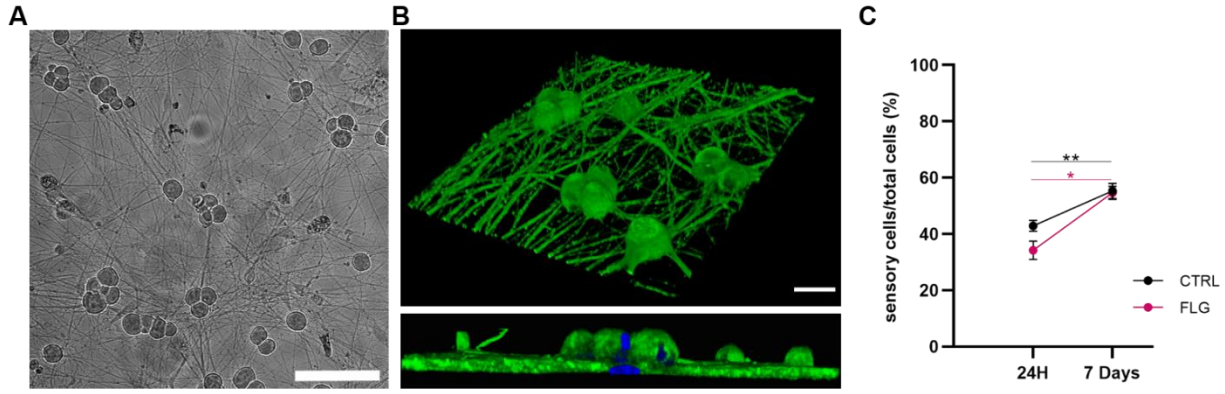

Figure S1. Characterization of DRG sensory neuron cultures. A) 2D bright field (10x magnification, scale bar, 100 µm) of a DRG neurons culture, and **B)** 3D confocal reconstruction of DRG sensory neurons immuno-stained with ß-tubulin-III (63x magnification, scale bar, 20 µm). ß-tubulin-III (green), DAPI (blue). **C)** Number of sensory neurons over total number of cells. Glial cell count decreases over time. Data are expressed as means ± SEM. Statistical analysis: ***p* = 0.0093; **p* = 0.0149, unpaired Student's *t*-test.

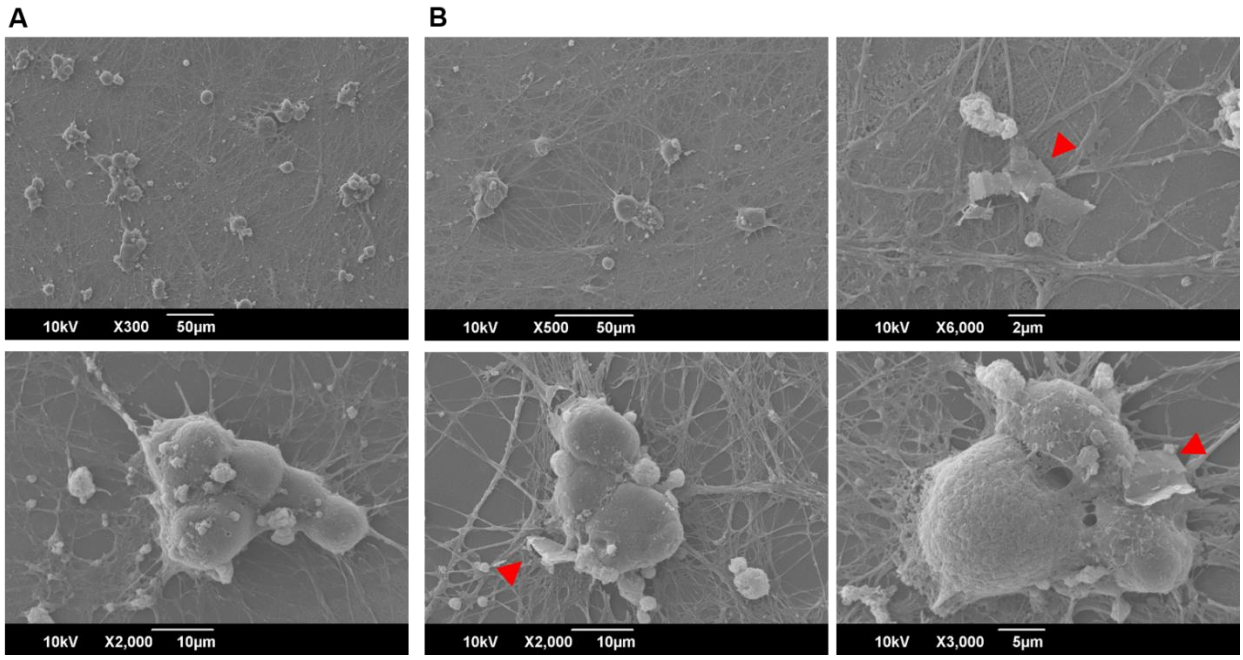

Figure S2. Morphology of DRG sensory neurons exposed to FLG. Representative SEM micrographs of DRG neuron cultures at different magnification. **A)** Untreated control neurons and **B)** neurons treated with FLG for 24 h. Extracellular GRM flakes are highlighted with red arrows.

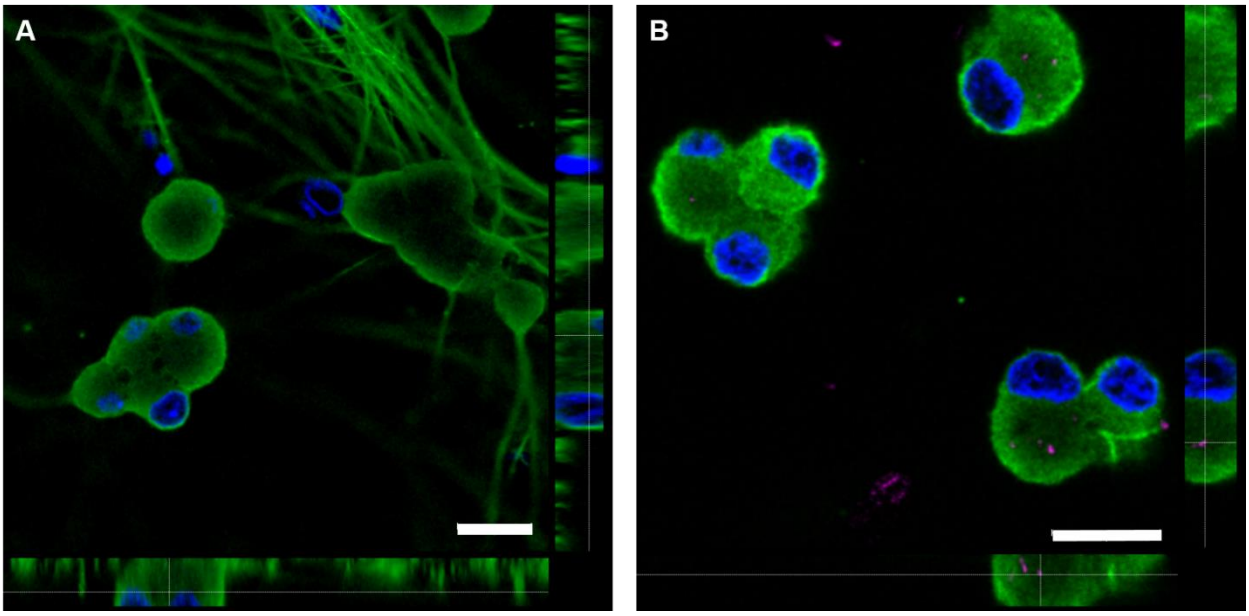

Figure S3. FLG internalization visualized through reflective light at the confocal microscope. **A)** CTRL and **B)** FLG. ß3-tubulin III (green); DAPI (blue); Graphene (Pink). Pictures are taken at one focal plane of a *z*-stack at 63x magnification. Since the sensory cell bodies pop out from the planar plane, not all axons are visible in this picture (see Figure S1). Scale bars, 20 µm.

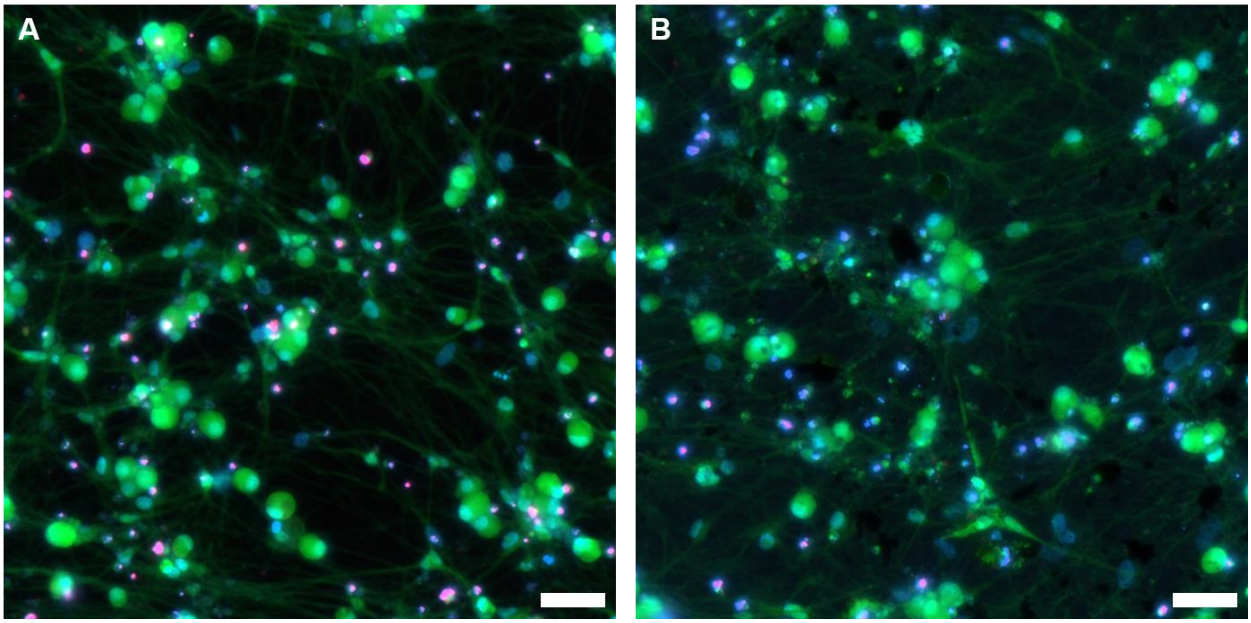

Figure S4. Viability of DRG sensory neurons upon FLG exposure. Calcein-AM (green) and PI (red) staining of DRG neuron cultures at DIV4, exposed for 24 h to 100 µg mL^{-1} of colloidal FLG. Nuclei are visualized by Hoechst staining (blue). **A)** CTRL and **B)** FLG-treated samples at 10x magnification. Scale bars, 50 µm.

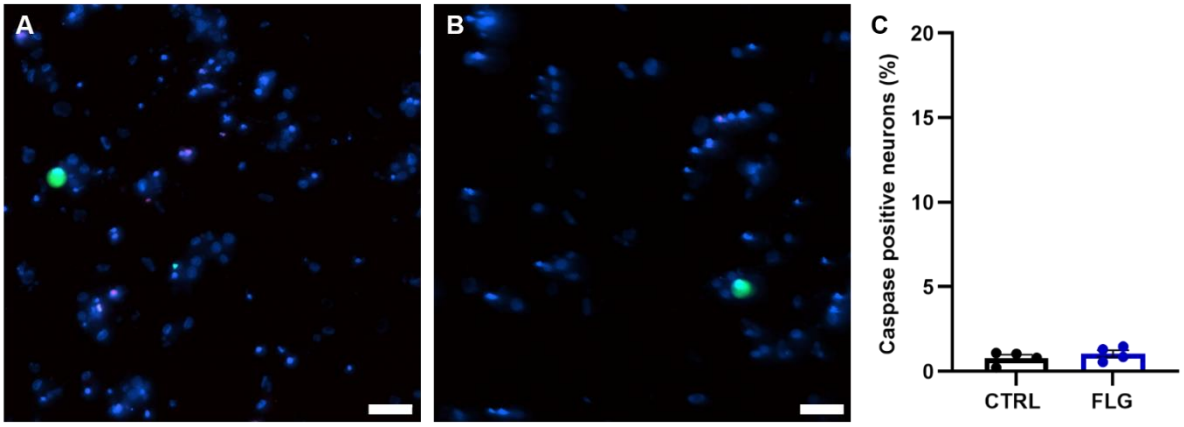

Figure S5. Evaluation of apoptosis cascade activation upon FLG exposure. Caspase 3/7 staining of DRG neuron cultures at DIV4, exposed for 24 h to 100 µg mL^{-1} of colloidal FLG. **A)** CTRL, **B)** FLG and **C)** Percentage of sensory neurons expressing active Caspase over total number of sensory neurons. All data are expressed as means ± SEM; *N= 3* (4 replicates). Statistical analysis was performed using the Student's *t*-test. Caspase (green), DAPI (blue) and PI (red) at 10x magnification. Scale bars, 50 µm.

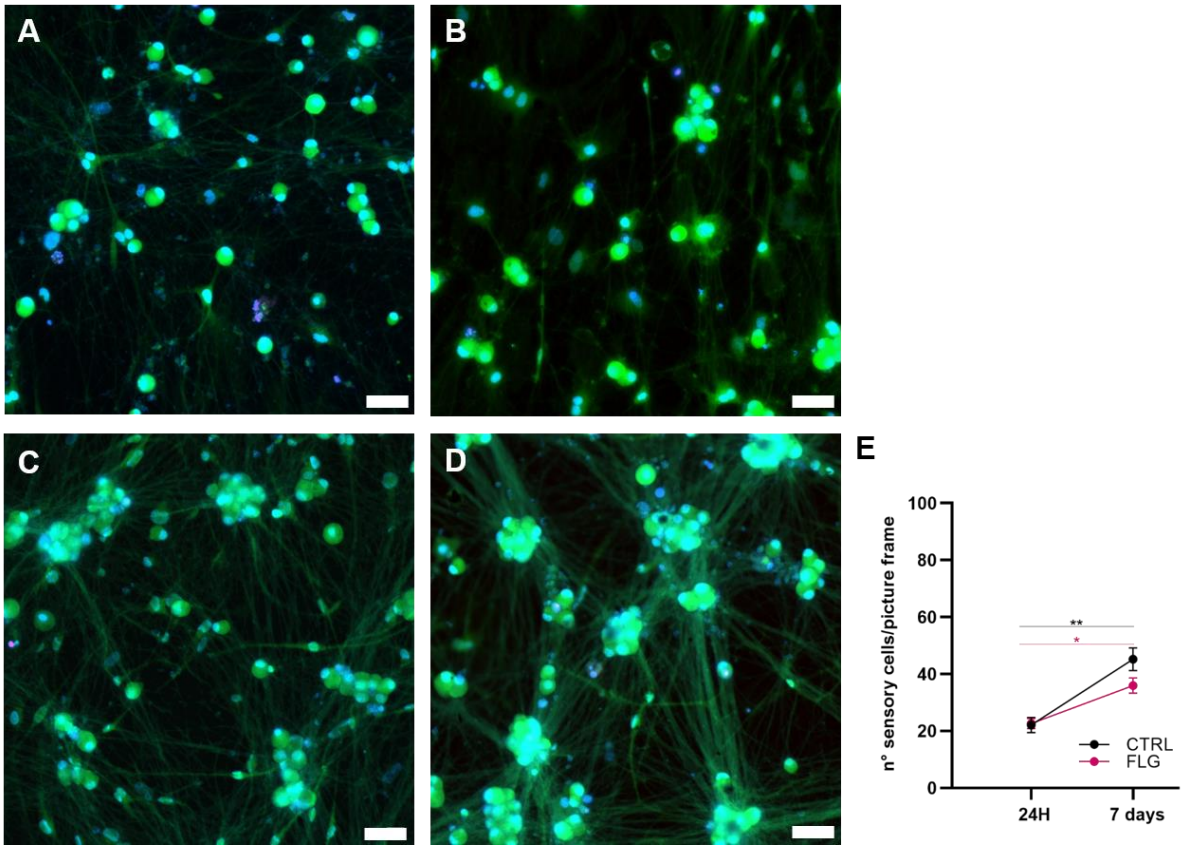

Figure S6. Sensory neuron clustering over time. DRG neurons live stained with Calcein AM (green), DAPI (blue) and PI (red) at 10x magnification from **A)** CTRL and **B)** FLG 10 µg mL^{-1} at DIV5 (24 h treatment); **C)** CTRL and D) FLG at DIV11 (7-day treatment). **E)** Number of sensory neurons per picture frame. All data are expressed as means ± SEM; *N= 3* (3/4 replicates). ***p* = 0.0091; **p* = 0.0149, unpaired Student's *t*-test. Scale bars, 50 µm.

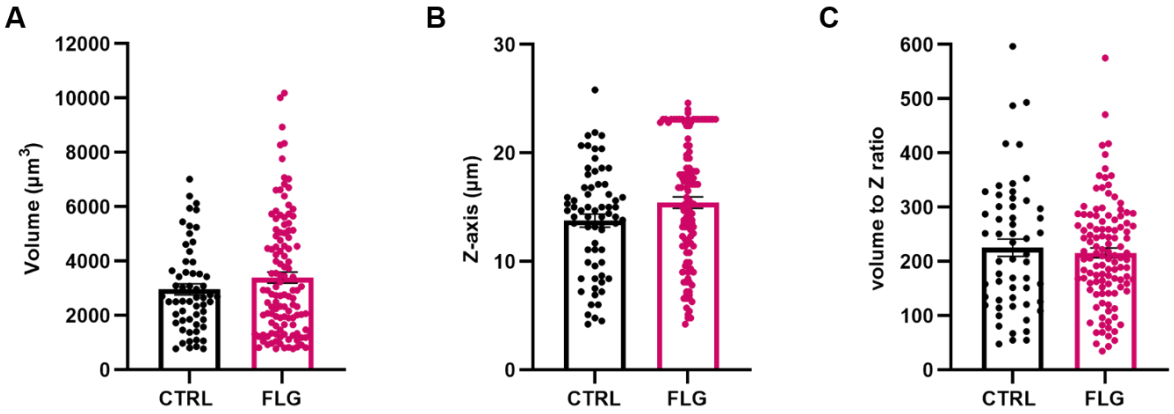

Figure S7. 3D morphological analysis. **A)** Volume of neuron soma, **B)** length of Z-axis and **C)** soma volume/soma height for DRG neuron cultures at DIV4, exposed for 24 h to 10 µg $mL^{-1}$of FLG. Each point represents a single neuron. All data are expressed as means ± SEM, *N=3* (6 different cultures). Unpaired Student's *t*-test/Mann-Whitney *U*-test.

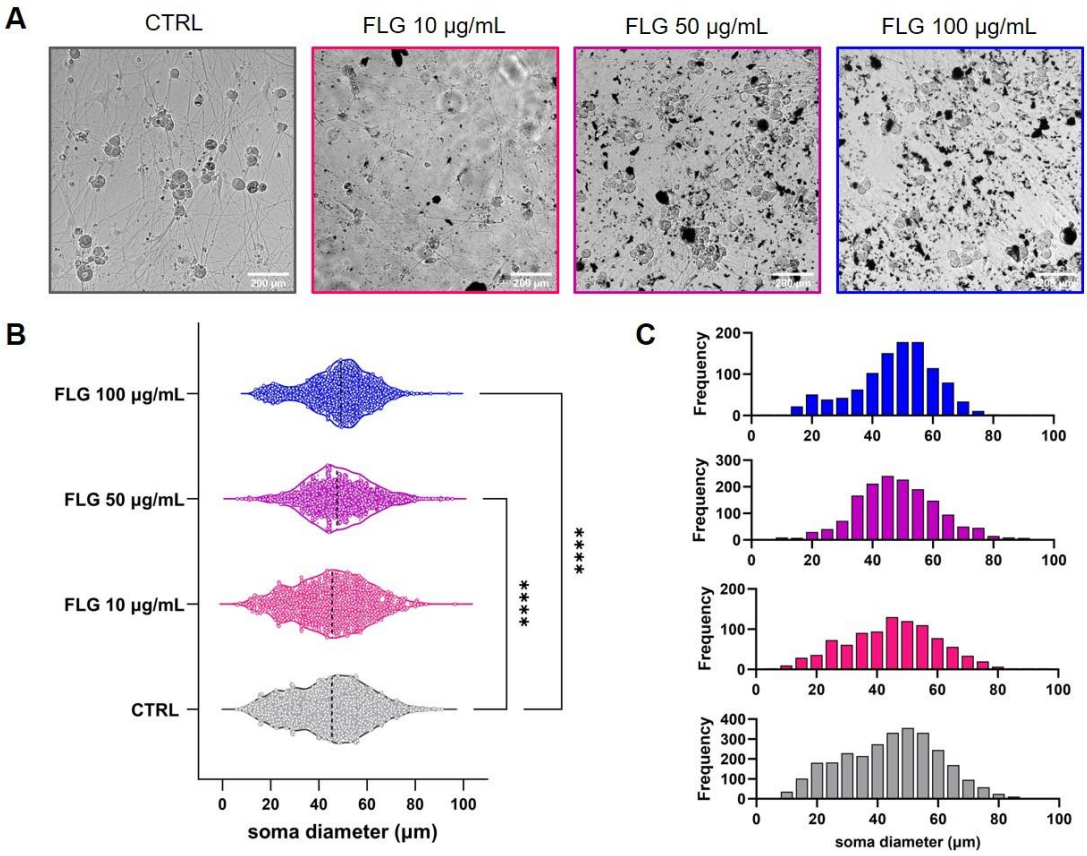

Figure S8. **DRG size distribution analysis. A)** Representative bright field (BF) images used for the 2D size distribution analysis. For increasing concentrations of FLG, dark agglomerates of increasing entities can be noticed. Scale bars: 200 µm. **B)** Violin plots representing the soma diameters when incubated with the different FLG doses (10 µg/mL: 955 cells; 50 µg/mL: 1573 cells; 100 µg/mL: 1074 cells) compared to untreated (CTRL: 2851 cells) conditions. Median value is represented by the black dotted line. Statistical analysis is one-way ANOVA with Tukey's post hoc test, ****=p<0.0001. **C)** Statistical size distributions of the soma diameters when incubated with the different FLG doses compared to untreated (CTRL) conditions. Bin size = 5.

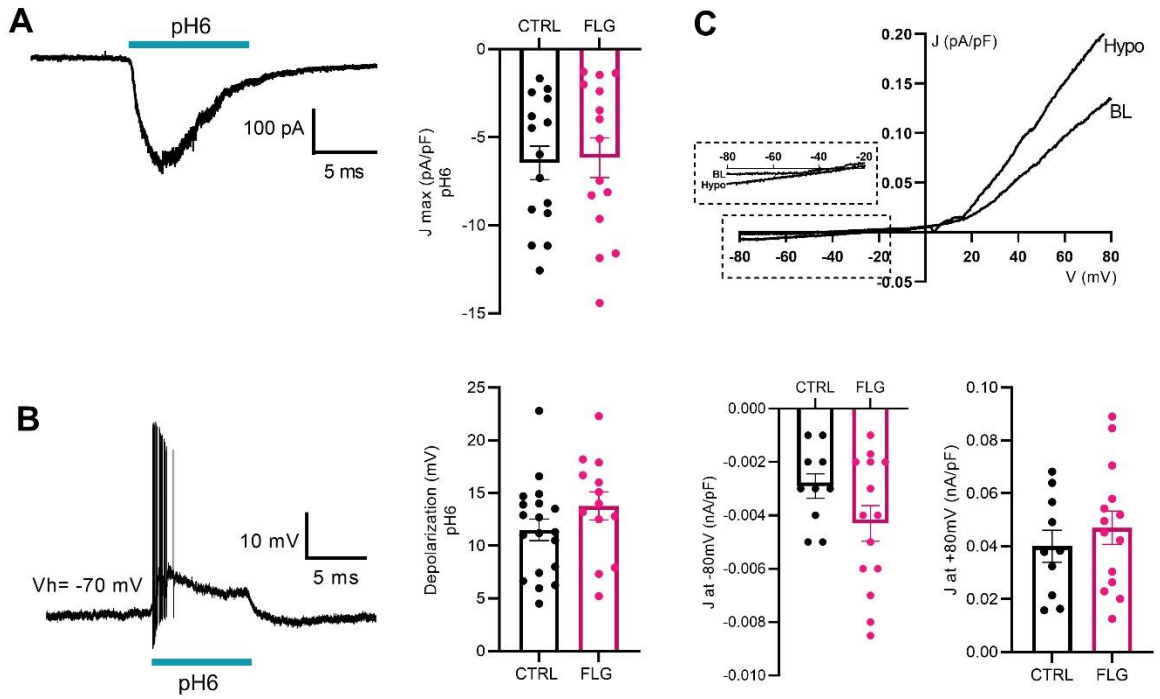

Figure S9. **Response of DRG sensory neurons to pH and hypotonic solution. A)** *Left:* Representative pH 6-elicited inward currents recorded at a holding potential of -70 mV in control and FLG-treated neurons. *Right:* Quantitative evaluation of current density after pH 6 stimulus under both experimental conditions (CTRL, n=15; FLG, n=15). **B)** *Left:* Representative current-clamp recordings of membrane depolarization evoked by pH 6 application in CTRL and FLG-treated neurons. *Right:* Quantitative evaluation of pH 6-dependent depolarization studied under both experimental conditions (CTRL, n=19; FLG, n=13). **C)** *Top:* Representative currents elicited by standard solution (baseline, BL) and hypotonic solution (Hypo) using a voltage ramp protocol. In the inset, magnification of the currents recorded in the -20 to -80 mV range. *Bottom:* Quantitative evaluation of whole-cell currents recorded at 80 mV and -80 mV in CTRL and FLG-treated neurons (CTRL, n=10; FLG, n=14). Data are expressed as means ± SEM. Statistical analysis was performed using the unpaired Student's *t*-test**.**

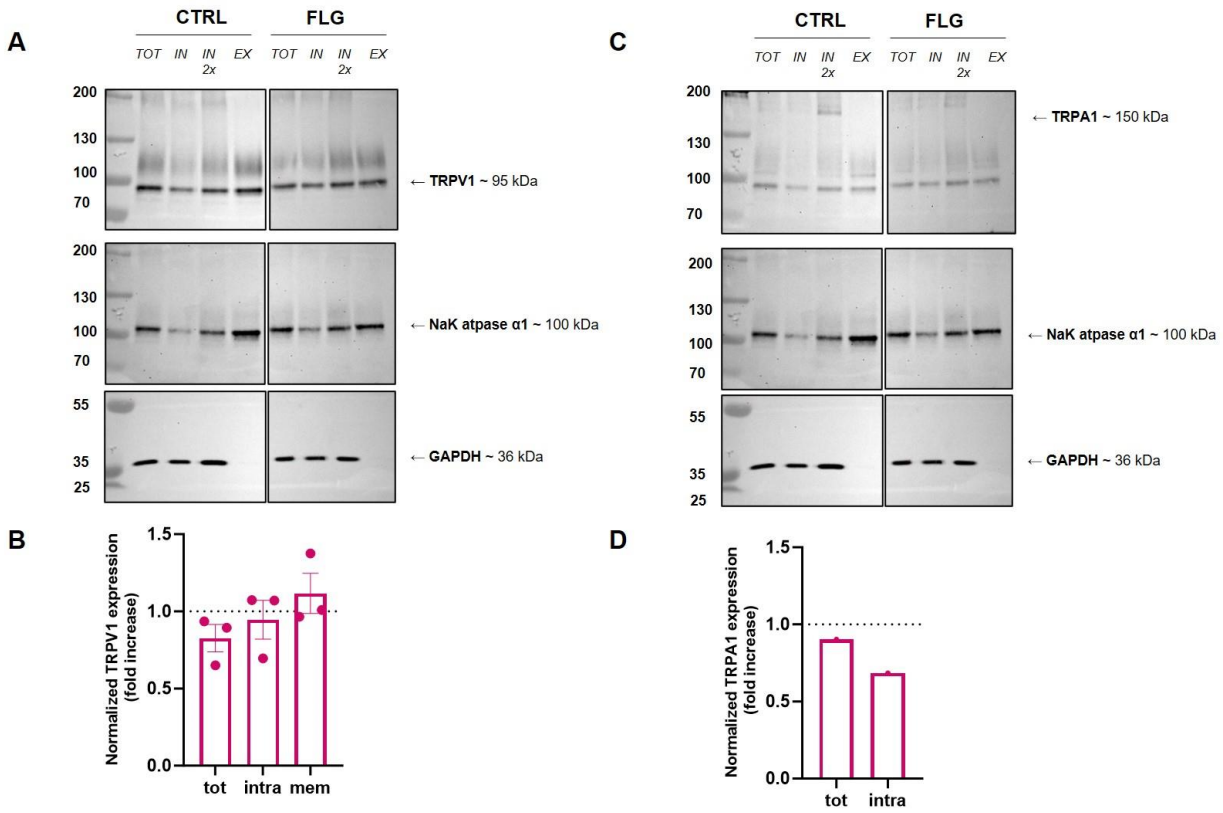

Figure S10. TRPV1 and TRPA1 expression and translocation. Western blotting (WB) was performed on DRG lysates at DIV4, after 24 h exposure to 10 µg mL^{-1} of FLG. Lysine biotinylation was performed to separate membrane and intracellular compartments to quantify the plasma membrane and intracellular fractions of the channels. **A)** Representative WB stained with antibodies to TRPV1 (95 kDa), the intracellular protein GAPDH (36 kDa), and the plasma membrane-associated protein Na^+/K^+ ATPase (100 KDa). **B)** Quantification of TRPV1 expression in FLG-treated (10 µg/mL, 24 h) samples (total, intracellular, and membrane compartments) normalized over untreated control conditions. Data are expressed as means ± SEM, *N*=3. **C)** Representative WB stained with antibodies to TRPA1, GAPDH, and Na^+/K^+ ATPase. The extracellular lane is empty because TRPA1 does not contain lysine residues in the extracellular loops. **D)** Quantification of TRPA1 expression (total, intracellular) normalized over untreated control conditions (N=1).

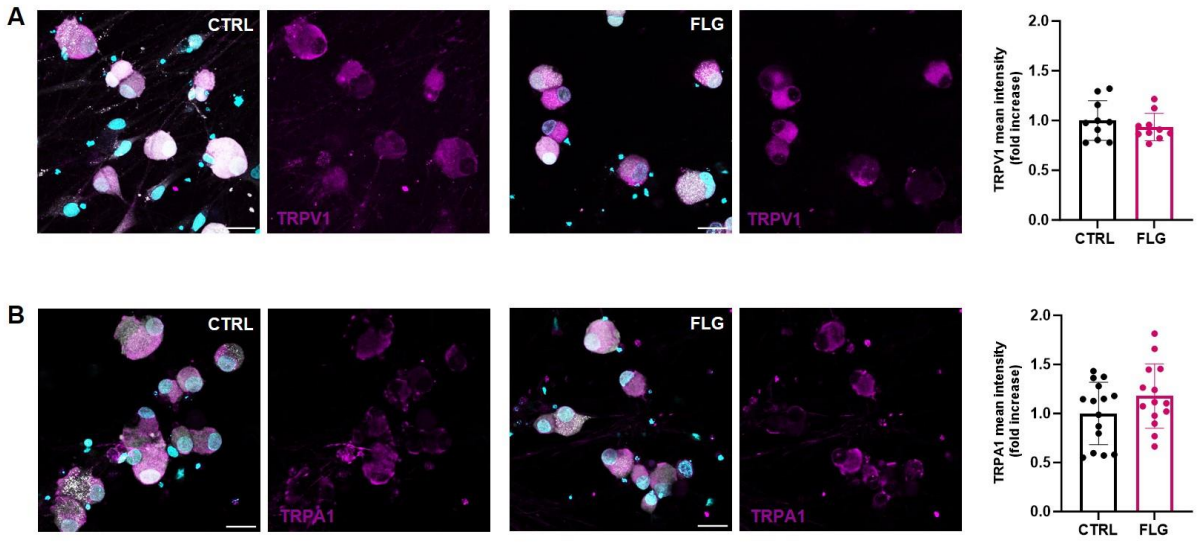

Figure S11. Extracellular TRPV1 and TRPA1. Representative confocal images (left panels) and fluorescence intensity quantification (right panels) of the extracellular exposure of TRPV1 (**A**) and TRPA1 (**B**) labeled by specific antibodies in non-permeabilized control and FLG-treated (10 µg/mL, 24 h) DRG neurons. Nuclei (cyan), intracellular compartment (gray), and channels (purple). Scale bars, 25 µm.