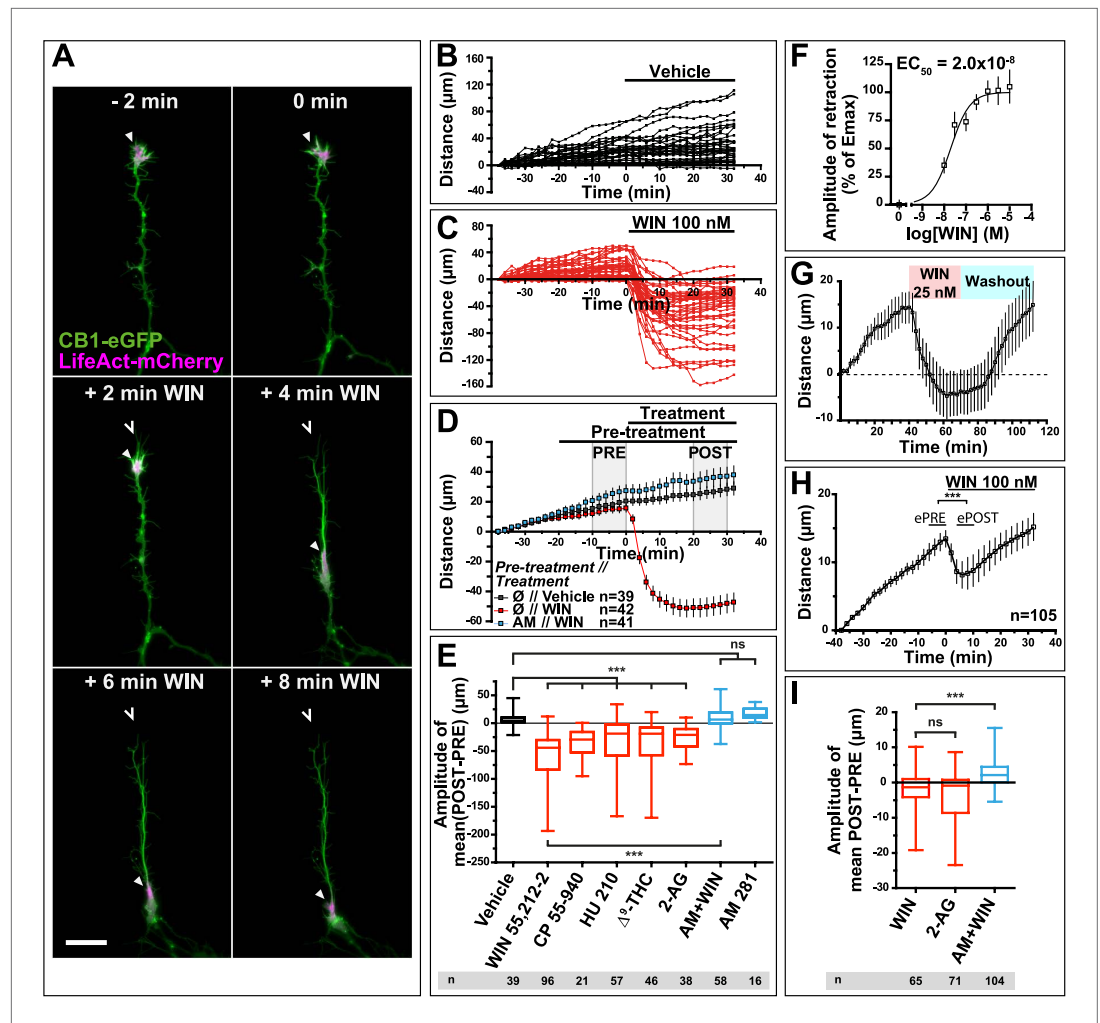


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## Figures and figure supplements

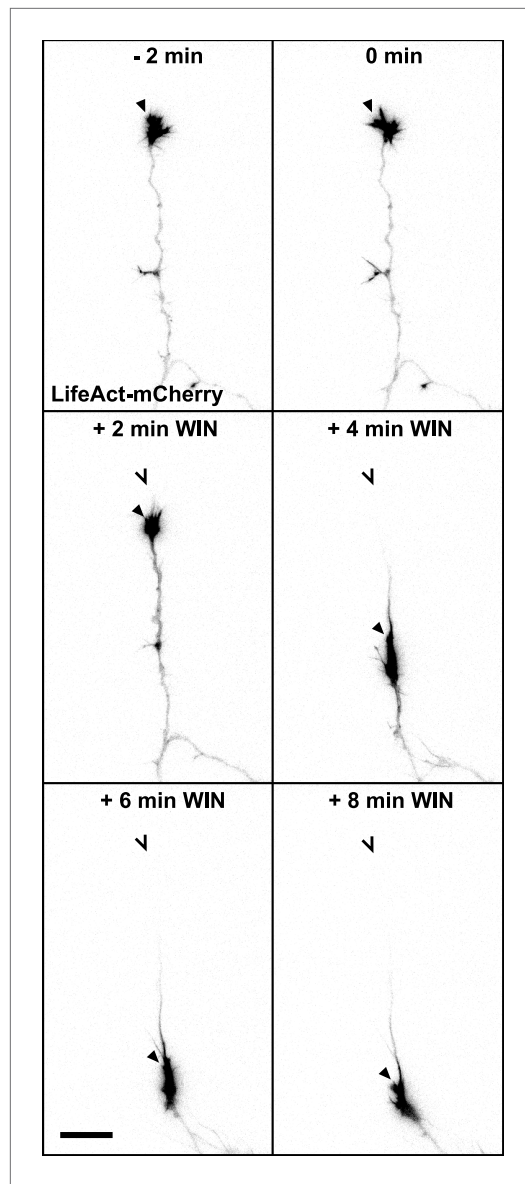
Cannabinoid-induced actomyosin contractility shapes neuronal morphology and growth

**Alexandre B Roland, et al.**



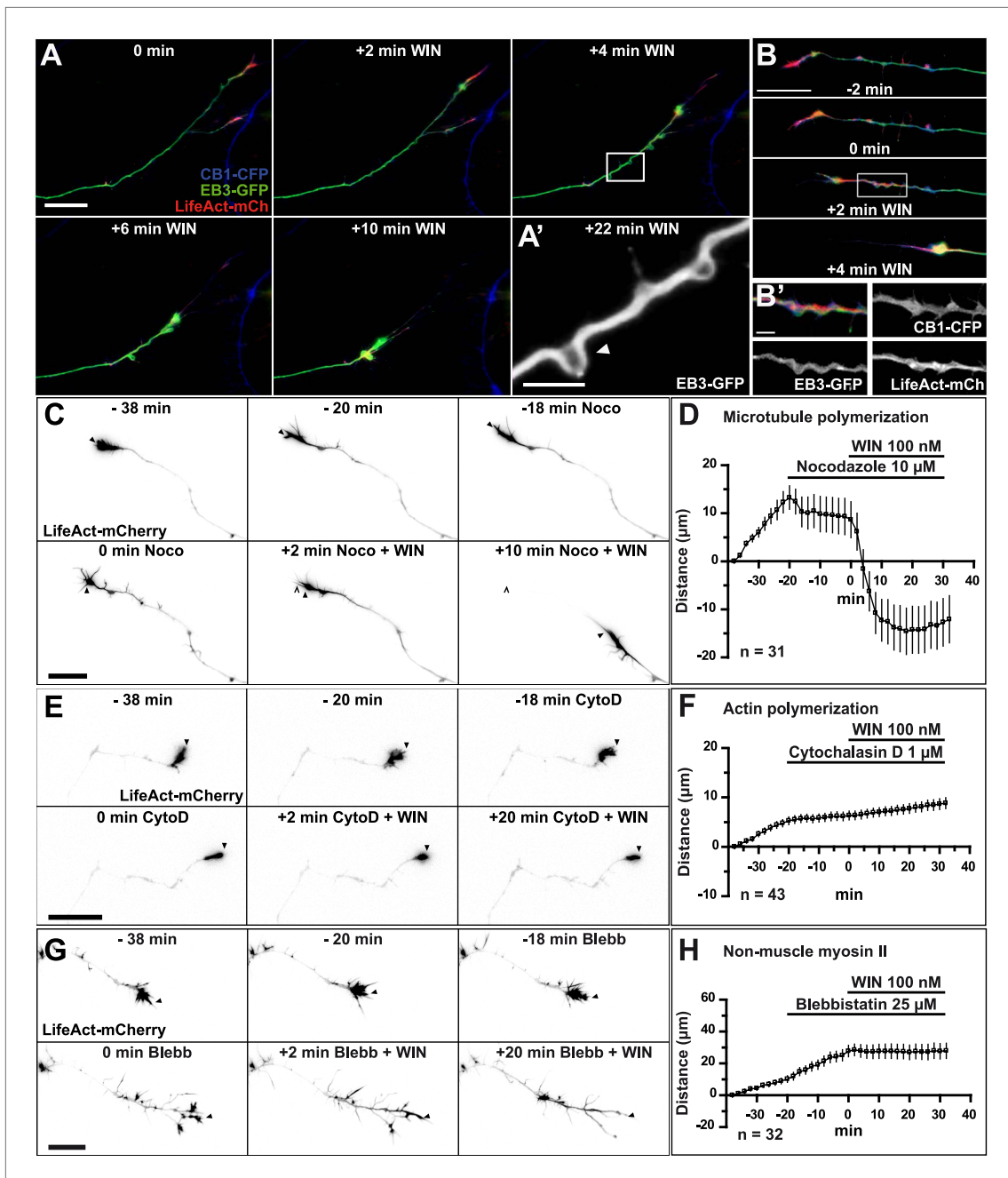
**Figure 1.** CB1R activation induces retraction of actin-rich growth cones. Cultured DIV8 hippocampal neurons co-expressing Flag-CB1R-eGFP and LifeAct-mCherry on (A–G) and LifeAct-mCherry only on (H and I). (A) Treatment with CB1R agonist WIN55,212-2 (WIN, 100 nM, added at 0 min) induces rapid retraction of the F-actin-rich domain (arrowheads). Open arrowheads: growth cone position at 0 min. (B) Progression of individual growth cones in control conditions. (C) WIN-induced retraction of individual growth cones. (D) Mean values of growth cone progression in control condition or after treatment with WIN with or without pre-treatment with the CB1R-specific antagonist AM281 (AM, 1  $\mu$ M). WIN-induced growth cone retraction is effectively abolished by AM. (E) Amplitudes of growth cone retraction induced by different exo- and endocannabinoids, calculated as the net difference of mean growth cone position in the pre-treatment (PRE on D) and post-treatment (POST on D) time intervals from at least three independent experiments. (F) Concentration-response curve of WIN-induced retraction, 9 to 27 neurons per concentration from two independent experiments expressed as percentage of maximal retraction,  $E_{max} = 52.2$   $\mu$ m. (G) WIN-induced retraction (25 nM at 40 min) is fully reversible after WIN-washout (at 70 min),  $n = 9$ . (H) Mean values of growth cone retraction downstream of endogenous CB1R activation, from four pooled independent experiments, outliers were removed in accordance with the Grubb's test. (I) Amplitudes of growth cone retraction downstream of endogenous CB1R activation after treatment with WIN (100 nM), 2-AG (1  $\mu$ M), or with WIN (100 nM) after pre-treatment with the CB1R-specific antagonist AM281 (AM, 1  $\mu$ M). WIN-induced growth cone retraction is effectively abolished by AM. Values in D, F, G, and H are mean  $\pm$  SEM; values in E and I are presented as boxplots;  $n.s = p > 0.05$ ,  $***p < 0.001$ , calculated using Kruskal–Wallis one-way ANOVA followed by Dunn's post-tests on (E and I) and paired t-test on (H). Scale bar: 20  $\mu$ m.

DOI: [10.7554/eLife.03159.003](https://doi.org/10.7554/eLife.03159.003)



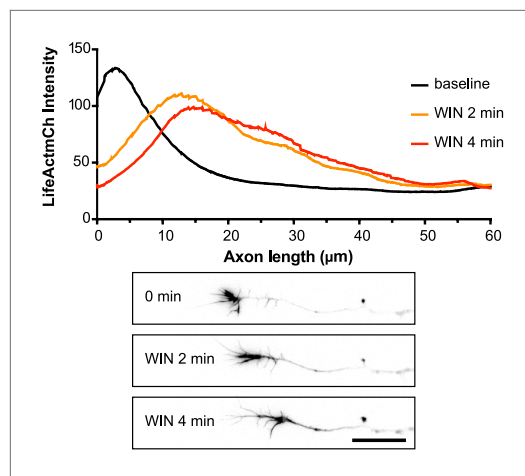
**Figure 1—figure supplement 1.** mCherry-LifeAct label (red channel) from **Figure 1A**. Scale bar: 20  $\mu$ m.

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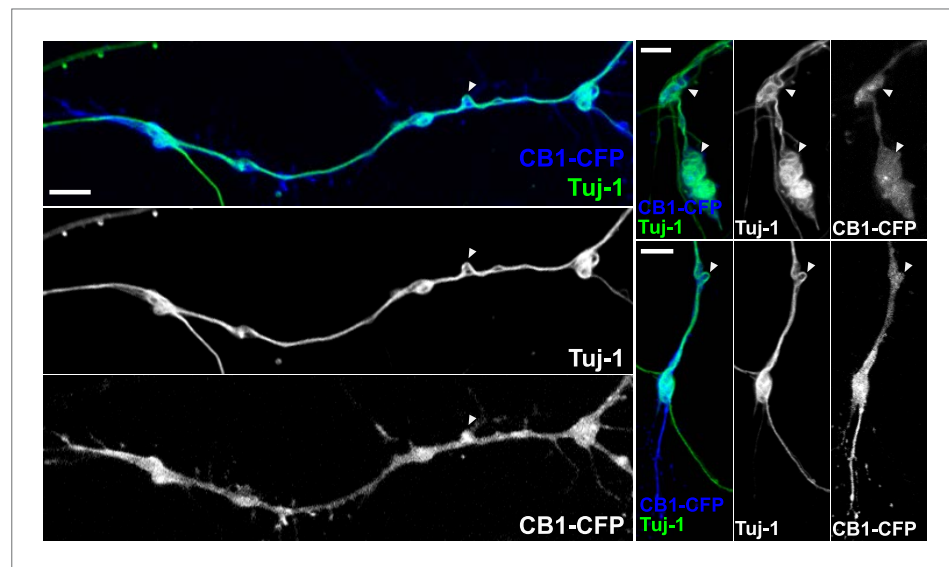


**Figure 2.** CB1R-induced retraction is mediated by non-muscle myosin II dependent actomyosin contraction. Cultured hippocampal neurons co-expressing Flag-CB1R-eCFP, LifeAct-mCherry, and EB3-eGFP at DIV6 were treated by WIN (100 nM) at 0 min. **(A)** Microtubules (MT) bend and form small loops (arrowhead on **A'**) in the first 4 min **(B)** F-actin is reorganized from the growth cone tips and isolated patches to homogenous cable-like distribution in distal axonal shaft. **(C–H)** Pre-treatment with: **(C and D)** MT polymerization inhibitor nocodazole (10  $\mu\text{M}$ ), **(E and F)** actin polymerization inhibitor cytochalasin D (1  $\mu\text{M}$ ), **(G and H)** Non-muscle myosin II-inhibitor blebbistatin (25  $\mu\text{M}$ ). Scale bars: 5  $\mu\text{m}$  on **(A')** and **(B')**, 20  $\mu\text{m}$  elsewhere.

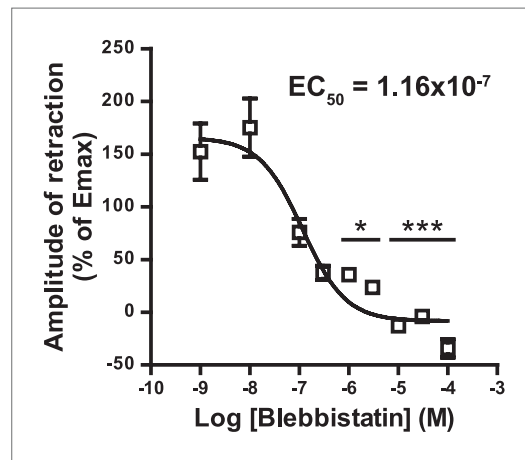
DOI: 10.7554/eLife.03159.005



**Figure 2—figure supplement 1.** Averaged F-actin relocalization in the distal 60  $\mu\text{m}$  in growth cones in the first 4 min after WIN treatment in five randomly chosen neurons from **Figure 1C**. Black, orange, and red curves represent the mean intensity of LifeAct-mCherry labeling at baseline, at 2 min and 4 min after addition of WIN (100 nM), respectively. Scale bar: 20  $\mu\text{m}$ . DOI: [10.7554/eLife.03159.006](https://doi.org/10.7554/eLife.03159.006)

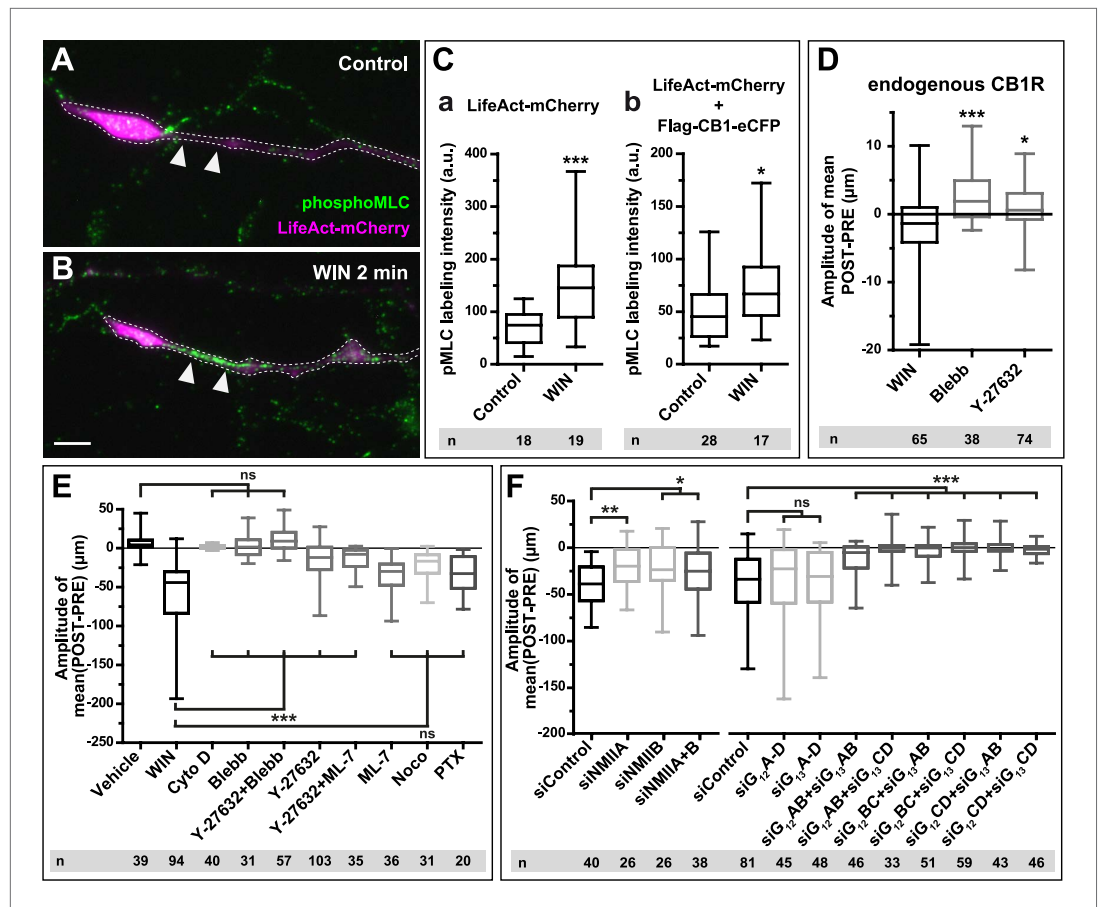


**Figure 2—figure supplement 2.** CB1R-induced periodic microtubule bends are not due to EB3-eGFP and LifeAct-mCherry expression. Cultured hippocampal neurons were transfected at DIV6 with CB1R-eCFP and treated after 24 hr with 100 nM WIN55,212-2 for 10 min before fixation. Presence of periodic bends is shown by immunolabeling microtubules with anti-Tuj1 antibody. Scale bar: 5  $\mu\text{m}$ . DOI: [10.7554/eLife.03159.007](https://doi.org/10.7554/eLife.03159.007)



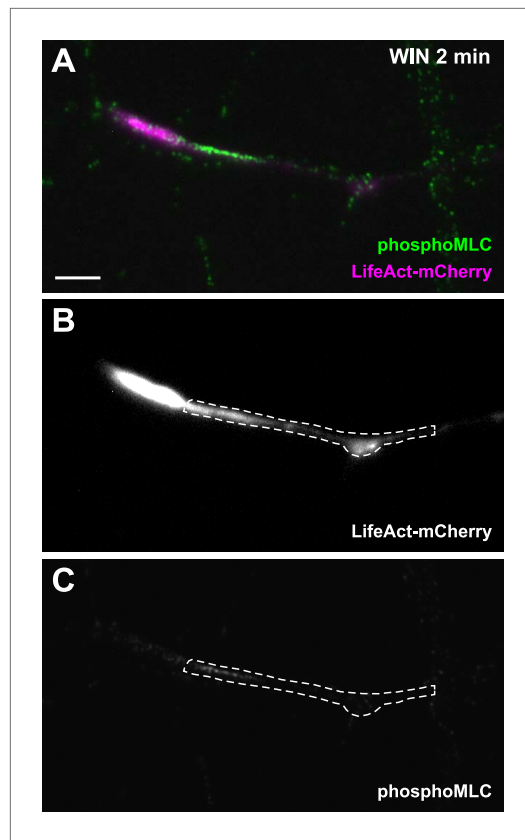
**Figure 2—figure supplement 3.** Concentration-response curve for the blebbistatin effect on the growth cone retraction assay after treatment with WIN (100 nM). \* $p < 0.05$ ; \*\*\* $p < 0.001$  calculated using Kruskal–Wallis one-way ANOVA followed by Dunn's post-tests on (I) and using one-way ANOVA followed by Newman–Keuls post-tests. Scale bar: 20  $\mu\text{m}$  on **A**, **B**, and **B'** and 5  $\mu\text{m}$  on **C**.

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**Figure 3.** CB1Rs activate non-muscle myosin II through heterotrimeric  $G_{12}/G_{13}$  proteins, Rho GTPase, and ROCK. Cultured hippocampal neurons at DIV6 co-expressing a combination of LifeAct-mCherry, Flag-CB1R-eCFP, and EB3-eGFP as indicated and treated by WIN (100 nM) at 0 min. (A–B) Representative LifeAct-mCherry expressing growth cones (delimited with a dotted line) at 2 min after treatment with vehicle (A) or WIN (100 nM, B), labeled with a phospho-Myosin Light Chain (phosphoMLC) antibody. Arrowheads show the distal axon adjacent to the F-actin-rich growth cone where WIN induces rapid and strong upregulation of myosin light chain phosphorylation. (C) pMLC labeling intensity at the distal 50–60  $\mu\text{m}$  of the axon, adjacent to the actin-rich growth cone, from neurons expressing LifeAct-mCherry (A) or co-expressing LifeAct-mCherry and Flag-CB1R-eCFP (B). The region-of-interest used to measure pMLC labeling intensity is delimited with a dotted line on a representative growth cone on **Figure 3—figure supplement 1**. (D) Amplitude of 100 nM WIN-induced growth cone retraction in neurons co-expressing LifeAct-mCherry and EB3-eGFP pre-treated with 25  $\mu\text{M}$  blebbistatin or 10  $\mu\text{M}$  Y-27632. (E) Amplitude of 100 nM WIN-induced growth cone retraction in neurons co-expressing LifeAct-mCherry, EB3-eGFP, and Flag-CB1R-eCFP pre-treated with: 1  $\mu\text{M}$  cytochalasin D; 25  $\mu\text{M}$  blebbistatin; 25  $\mu\text{M}$  blebbistatin + 10  $\mu\text{M}$  Y-27632; 10  $\mu\text{M}$  Y-27632; 30  $\mu\text{M}$  ML-7 + 10  $\mu\text{M}$  Y-27632; 30  $\mu\text{M}$  ML-7; 10  $\mu\text{M}$  nocodazole; 100 ng/ $\mu\text{l}$  PTX. (F) Effect of siRNA-mediated knock-down of endogenous myosin IIA, IIB or of endogenous  $G_{12}/G_{13}$  proteins on growth cone-retraction induced by 100 nM WIN in neurons co-expressing the three constructs, as compared to control (luciferase) siRNA. Results are pooled from at least two independent experiments, and outliers were removed in accordance with Grubb's test. Results are expressed as boxplots. n.s.  $p > 0.05$ ; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  calculated using Student's t-test on (C), Kruskal–Wallis one-way ANOVA followed by Dunn's post-tests on (D) and (E), and using one-way ANOVA followed by Newman–Keuls post-tests on (F). Scale bar: 10  $\mu\text{m}$ .

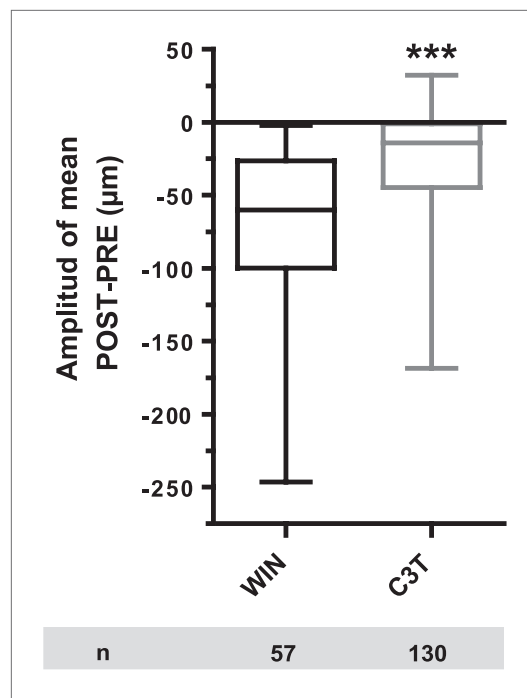
DOI: [10.7554/eLife.03159.010](https://doi.org/10.7554/eLife.03159.010)



**Figure 3—figure supplement 1.** Another representative LifeAct-mCherry expressing growth cone 2 min after treatment with WIN (100 nM), labeled with the phosphoMLC antibody, similarly to **Figure 3B**. The region of interest used for the quantification of phosphoMLC labeling intensity, approximately 50–60  $\mu\text{m}$  on each image, is marked with dotted lines. This region was delimited using the LifeAct-mCherry image (**B**) and quantified on the raw pMLC image (**C**). Scale bar: 10  $\mu\text{m}$ .

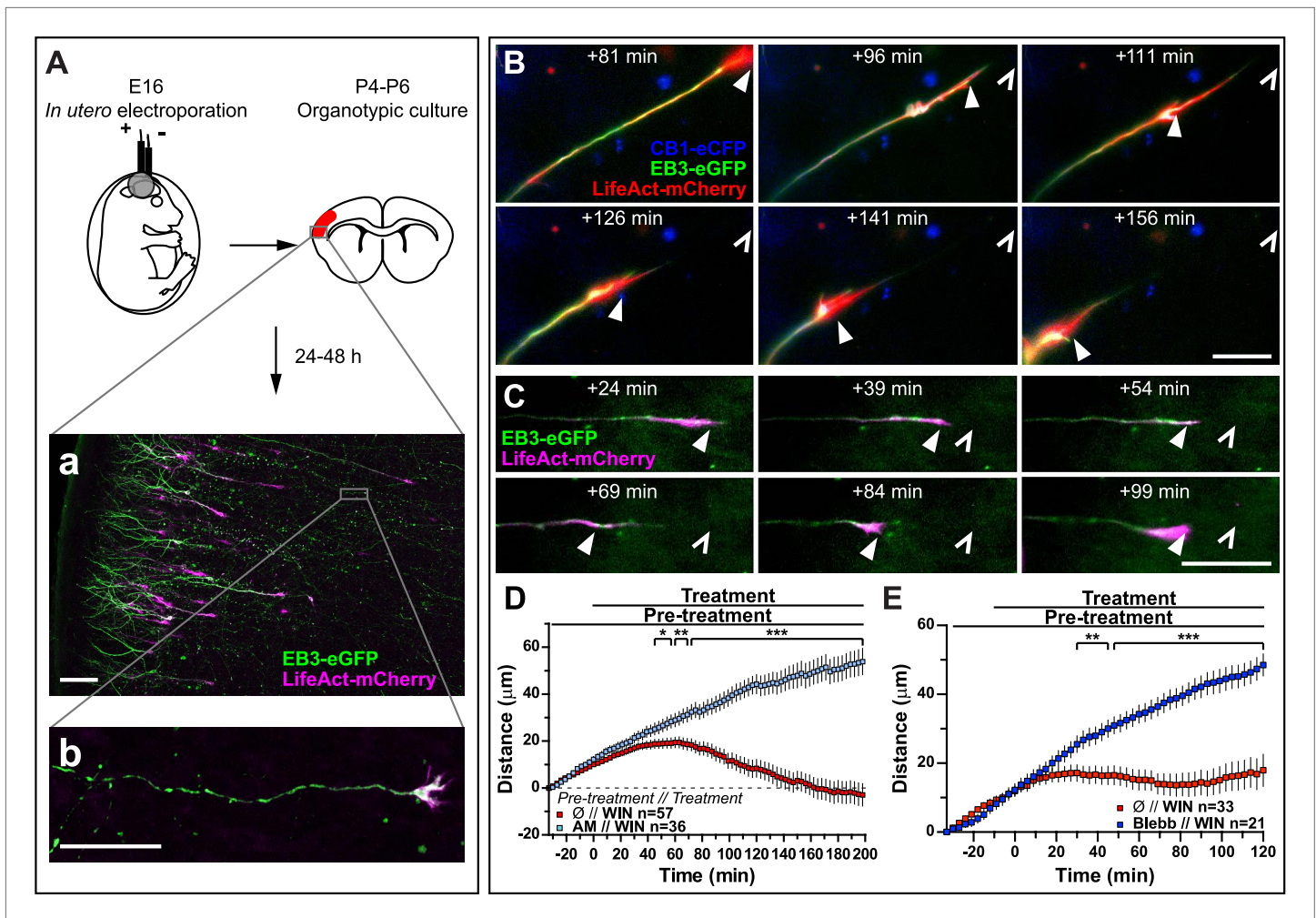
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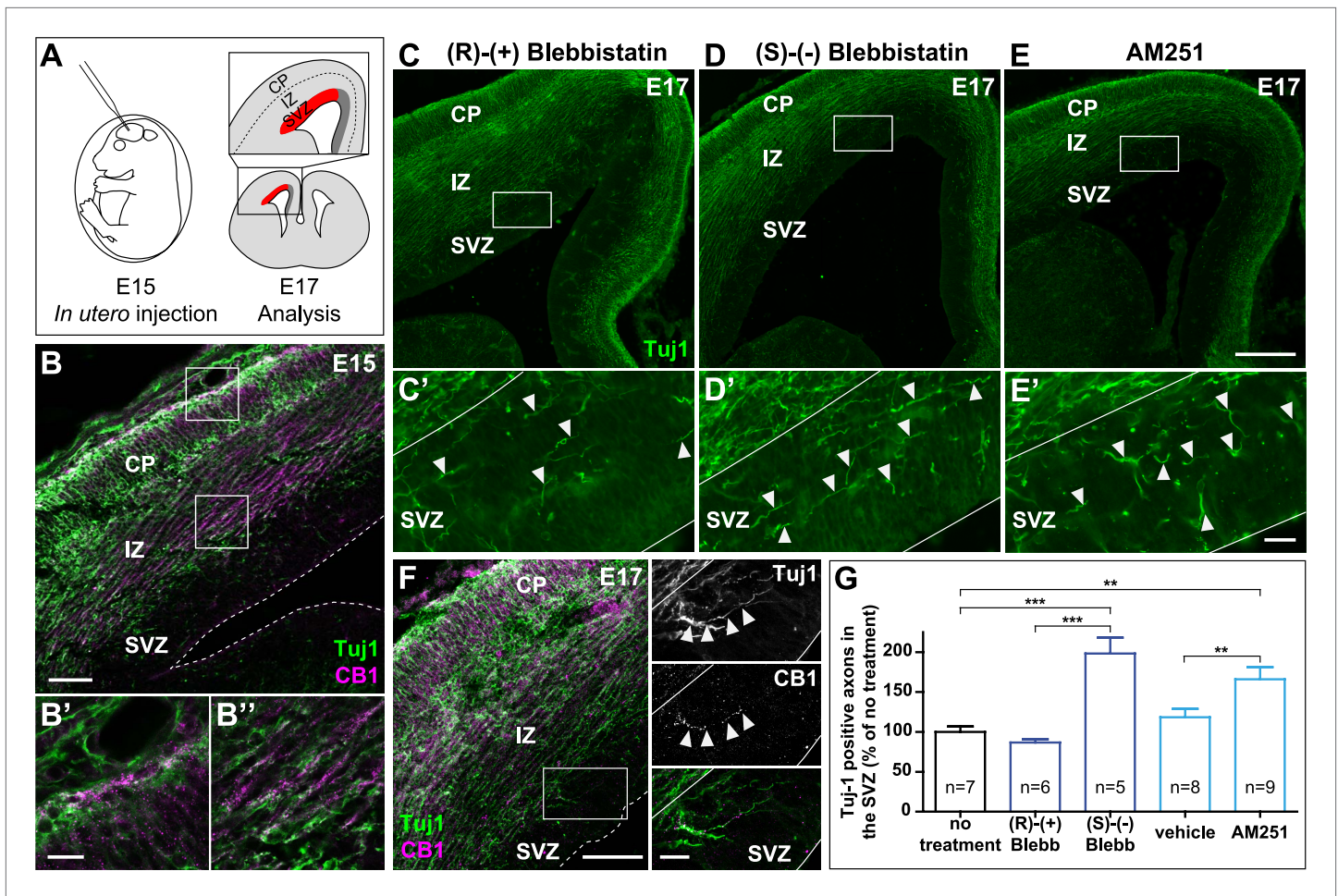
**Figure 3—figure supplement 2.** Amplitude of 100 nM WIN-induced growth cone retraction in neurons co-expressing LifeAct-mCherry, EB3-eGFP, and Flag-CB1R-eCFP with (C3T) or without (WIN) pre-treatment with 1 µg/ml C3T. Results are pooled from two independent experiments and outliers were removed in accordance with Grubb's test. Results are expressed as boxplots. \*\*\* $p < 0.001$  calculated using Student's t-test.

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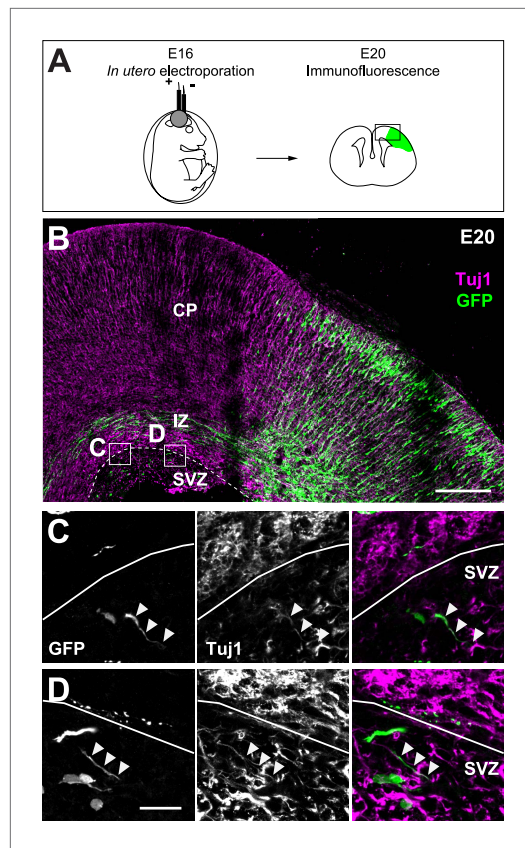
**Figure 4.** Activation of exogenous or endogenous CB1Rs modifies growth cone dynamics ex vivo. Progression of dynamic, F-actin-rich corticofugal growth cones from organotypic slices cultured for 24 to 48 hr, prepared from P4-6 rat brains, previously electroporated in utero at E16 to express EB3-eGFP, LifeAct-mCherry, with or without Flag-CB1R-eCFP, was followed by time-lapse imaging. **(A)** Experimental design and illustration of a typical transfected cortical area **(A)** and of a typical labeled growing axon **(B)**. For the illustration, the organotypic section was fixed and EB3-eGFP signal was enhanced by incubation with an anti-GFP antibody. **(B–E)** Response to CB1R agonist WIN (1  $\mu\text{M}$ , added at 0 min). The F-actin-rich growth cone is indicated by arrowheads. Open arrowheads indicate growth cone position at 0 min **(B, D)** WIN-induced retraction in growth cones expressing EB3-eGFP, LifeAct-mCherry, and Flag-CB1R-eCFP is abolished by pre-treatment with 5  $\mu\text{M}$  CB1R-specific antagonist AM281. **(C, E)** WIN-induced retraction in growth cones expressing EB3-eGFP and LifeAct-mCherry is abolished by pre-treatment with blebbistatin (25  $\mu\text{M}$ ). Results are pooled from at least two independent experiments and are expressed as mean  $\pm$  SEM. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ , calculated using Student's t-test. Scale bar: 100  $\mu\text{m}$  on **A**, 20  $\mu\text{m}$  elsewhere.

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**Figure 5.** Actomyosin contractility is required for the correct targeting of CB1R expressing corticofugal axons. **(A)** Experimental design. Left: in utero intracerebroventricular injection of E15 rat embryos. Right: analysis of axons in the lateral sub-ventricular zone (SVZ, red). **(B)** E15 corticofugal axons starting from the cortical plate (CP) and progressing through the intermediate zone (IZ) highly co-express Tuj-1 (green) and CB1R (magenta) and mostly avoid the SVZ. **(C–G)** In embryos injected with 1  $\mu$ l of the active NM II-ATPase inhibitor (S)-(-)-blebbistatin (250  $\mu$ M) **(D, D')**, or with AM251 (1 mM) **(E, E')**, but not with the inactive (R)-(+)-stereoisomer (250  $\mu$ M) or the vehicle of AM251 (DMSO 2.8%) **(C–C')**, there is a significant increase of mistargeted corticofugal axons in the lateral SVZ (arrowheads, **G**). **(F)** Expression of endogenous CB1Rs in a representative Tuj1 positive axon invading the SVZ (arrowheads) from an embryo treated with active (S)-(-)-blebbistatin. Results are pooled from three independent experiments and are expressed as mean  $\pm$  SEM, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001 calculated using one-way ANOVA followed by Newman–Keuls post-tests. Scale bars: 100  $\mu$ m on **B** and **F** (left), 250  $\mu$ m on **C, D,** and **E** and 25  $\mu$ m on **B', B'', C', D', E',** and **F** (right).

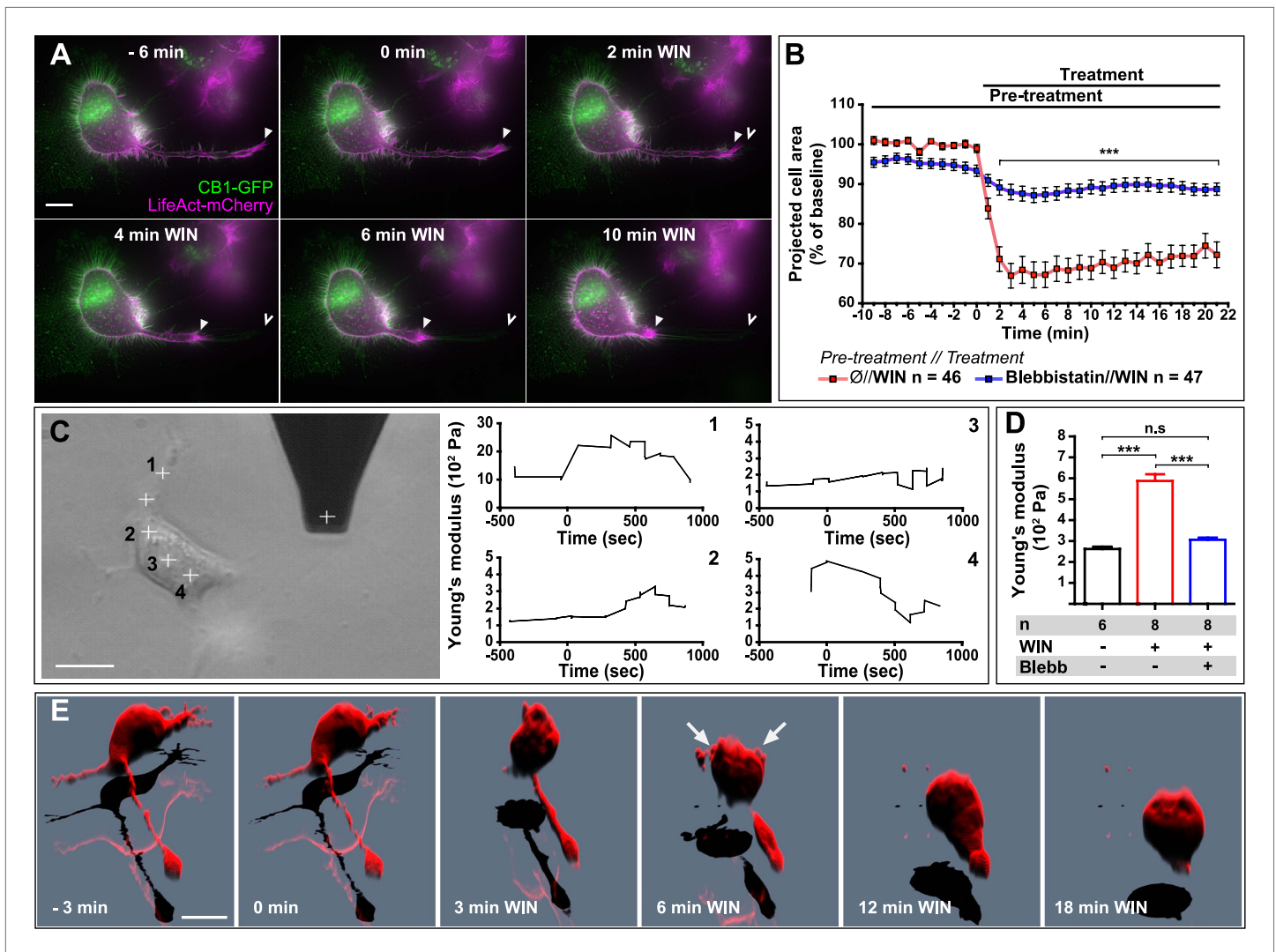
DOI: [10.7554/eLife.03159.018](https://doi.org/10.7554/eLife.03159.018)



**Figure 5—figure supplement 1.** Corticofugal origin of Tuj1-expressing axons in the SVZ. **(A)** Experimental design. Left: in utero transfection of cortical progenitors of E16 rat embryos with GFP. Right: analysis of GFP-expressing corticofugal axons of transfected and radially migrated neurons (green) at E20. **(B)** Corticofugal axons starting from the cortical plate (CP) and progressing through the intermediate zone (IZ) co-express Tuj-1 (magenta) and GFP (green) and mostly avoid the SVZ. **(C and D)** Examples of the Tuj1 positive rare axons in the SVZ (arrowheads) that express GFP, suggesting their corticofugal origin. Scale bars: 200  $\mu\text{m}$  on **B** and 20  $\mu\text{m}$  on **C and D**.

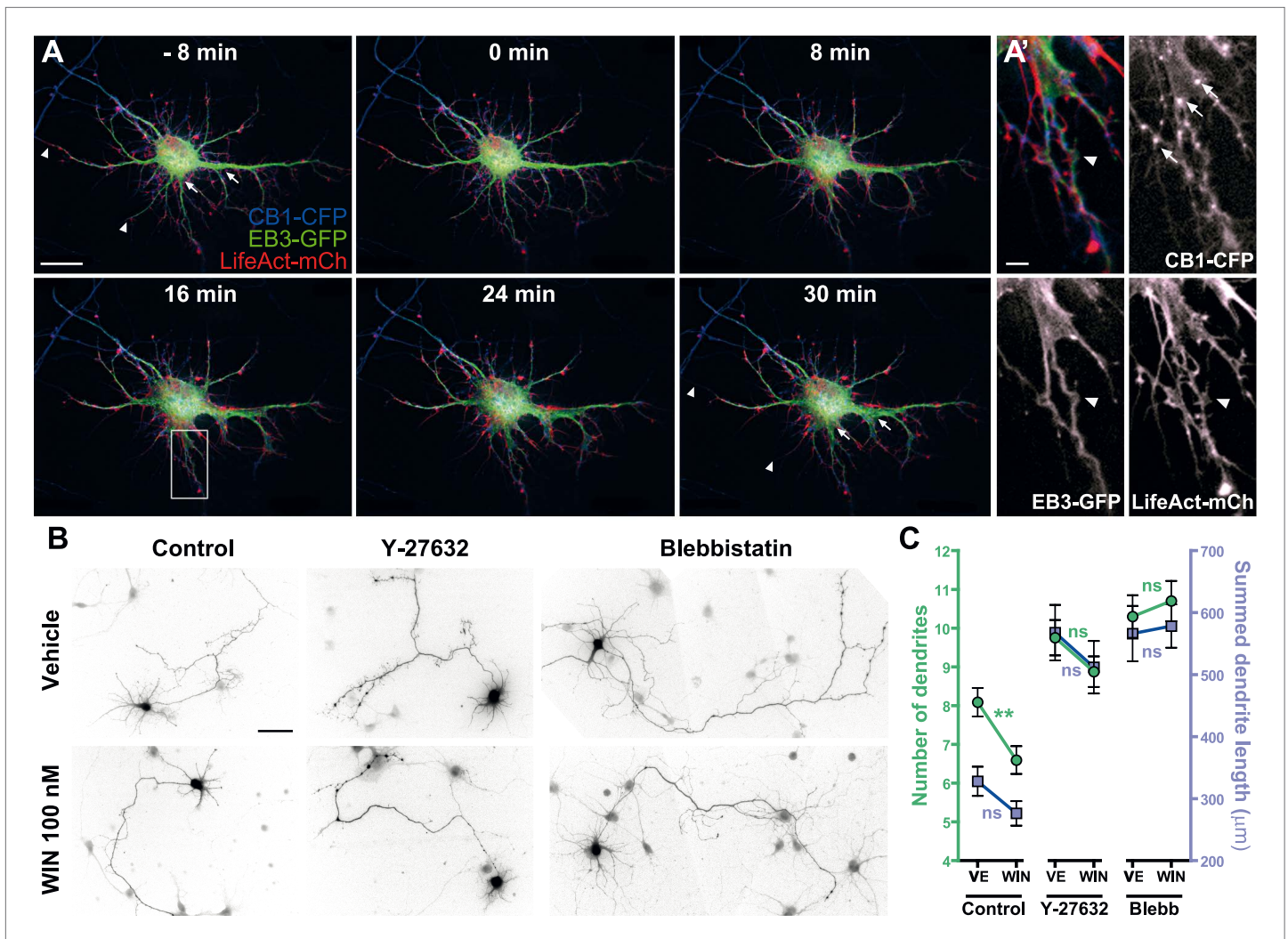
DOI: [10.7554/eLife.03159.019](https://doi.org/10.7554/eLife.03159.019)





**Figure 6.** CB1R-induced actomyosin contraction results in neurite retraction and transiently increased cell stiffness in Neuro2A cells. **(A and B)** Cells expressing Flag-CB1R-eGFP and LifeAct-mCherry. F-actin accumulates in the extremity and shaft of neurites (arrowheads). Agonist WIN (100 nM) induces retraction of neurites. Open arrowheads: neurite tip at 0 min. **(B)** Blebbistatin (25  $\mu$ M) significantly reduces 100 nM WIN-induced cell rounding. Results are expressed as mean  $\pm$  SEM. **(C)** Phase-contrast image of a Neuro2A cell and the AFM cantilever. Stiffness response to 100 nM WIN at different cell locations (crosses). Subsequent measurements were focused on the cell bodies, corresponding to positions 2 and 3. **(D)** Blebbistatin (25  $\mu$ M) significantly reduces 100 nM WIN-induced increase of cell stiffness. Results are pooled from at least three independent experiments and are expressed as mean stiffness between 2 and 8 min after stimulation  $\pm$ SEM. **(E)** 3D reconstruction shows neurite retraction, cell rounding, and transitory blebbing (arrows) following WIN treatment (100 nM). n.s  $p > 0.05$ ; \*\*\* $p < 0.001$ , calculated using Student's t-test on B and using one-way ANOVA followed by Newman-Keuls post-tests on **(D)**. Scale bars: 10  $\mu$ m on **(A)** and **(E)**, 15  $\mu$ m on **(C)**.

DOI: 10.7554/eLife.03159.020



**Figure 7.** Acute and chronic effects of CB1R-mediated actomyosin contraction on somatodendritic morphology. **(A)** Cultured hippocampal neurons expressing CB1R-eCFP, LifeAct-mCherry, and EB3-eGFP at DIV8. Application of 100 nM WIN results in rapid and significant reorganization of somatodendritic morphology, characterized by retraction of distal dendritic parts (arrowheads), and broadening of the proximal part of dendrites (arrows). **(A')** In dendrites, characteristic microtubule bending (arrowheads) and appearance of straight cable-like F-actin bundles (arrowheads) are accompanied by CB1R endocytosis after agonist activation (arrows). **(B and C)** Chronic inhibition of ROCK or NM II abolishes CB1R-activation induced changes structure of the cultured hippocampal neurons expressing Flag-CB1R-eGFP and the structural marker DsRed2 at DIV4. Cells were fixed at 24 hr after treatment with inhibitors of ROCK (Y-27632, 10  $\mu$ M) or NM II (blebbistatin, 25  $\mu$ M) in the presence of vehicle (VE) or CB1R agonist WIN (100 nM). A representative cell is shown for each condition. **(C)** Results are pooled from at least two independent experiments and are expressed as mean  $\pm$  SEM. n.s  $p > 0.05$ ; \*\* $p < 0.01$ , calculated using one-way ANOVA followed by Newman-Keuls post-tests. Scale bars: 20  $\mu$ m on **(A)**, 5  $\mu$ m on **(A')**, and 50  $\mu$ m on **(B)**.

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