

SUPPLEMENTAL FIGURE LEGENDS

Figure S1: Localization of JIP3, GSK3 β , and DCX in cerebellar granule neurons

(A) Granule neurons transfected with the GFP-JIP3 expression plasmid together with DsRed were fixed and subjected to immunocytochemistry using the GFP and DsRed antibodies. GFP-JIP3 was expressed throughout the cytoplasm, but was enriched in the distal portion of the axon. Scale bar is 50 microns.

(B) Granule neurons in low density cultures were co-stained for JIP3 and Tuj1 or JIP3 and MAP2. JIP3 expression was enriched in the distal portion of the axon. Scale bar is 50 microns.

(C) Granule neurons transfected with the GFP-GSK3 β expression plasmid together with DsRed were fixed and subjected to immunocytochemistry using the GFP and DsRed antibodies. GFP-GSK3 β was expressed throughout the cytoplasm, including the axon. Scale bar is 50 microns.

(D) Granule neurons in low density cultures were co-stained for GSK3 β and JIP3. GSK3 β was expressed throughout the cytoplasm, including the axon. Scale bar is 50 microns.

(E) Granule neurons in low density cultures were co-stained for DCX and Tuj1 or DCX and MAP2. DCX expression was enriched in the distal portion of the axon and dendrites. Scale bar is 50 microns.

Figure S2: JIP3 knockdown reduces axon length

(A) Quantification of total axon length and primary axon length of granule neurons transfected with the U6/JIP3i or control U6 plasmid together with the GFP expression plasmid, analyzed as in Figure 1B. JIP3 knockdown significantly decreased both total axon length (t-test, $p < 0.001$) and primary axon length (t-test, $p < 0.001$). A total of 222 neurons were measured.

(B) Quantification of total dendrite length for same neurons as in (A). JIP3 knockdown did not significantly alter total dendrite length. A total of 222 neurons were measured.

Figure S3: JIP3 inhibits axon branching and self-contact

(A) Lysates of 293T cells transfected with the FLAG-JIP3 expression plasmid and the control pU6-puro RNAi plasmid or the pU6-puro/JIP3i RNAi plasmid, encoding shRNAs targeting a region of JIP3 distinct from those targeted by the U6/JIP3i and the pmMKO.1/JIP3i RNAi plasmids, were immunoblotted using the FLAG and HSP60 antibodies.

(B) Granule neurons transfected with the pU6-puro/JIP3i or control pU6-puro RNAi plasmid together with the GFP expression plasmid were analyzed as in Figure 1B. Scale bars are 50 microns.

(C) Quantification of axon branching of granule neurons treated as in (B). JIP3 knockdown significantly increased both the number of axonal tips per hundred microns (t-test, $p < 0.001$) and the number of axonal self-contact points per hundred microns (t-test, $p < 0.001$). A total of 156 neurons were measured.

Figure S4: JIP3 does not appear to regulate the stability of GSK3 β protein

Lysates of granule neurons nucleofected with the U6/JIP3i or control U6 RNAi plasmid and treated with 10 μ M MG132 or the vector control DMSO for sixteen hours prior to lysis were immunoblotted with the JIP3, GSK3, and HSP90 antibodies. Inhibition of the proteasome by MG132 had little or no effect on the JIP3 knockdown-induced decrease in GSK3 β protein levels in neurons. HSP90 served as the loading control.

Figure S5: Axon branches in primary granule neurons are generated by backbranching

(A) Granule neurons transfected with the U6/JIP3i, U6/GSK3 β i2, or control U6 RNAi plasmid together with a GFP expression plasmid were subjected to time lapse imaging, with images being collected at fifteen minute intervals for a period of two days beginning twenty-four hours after transfection. The majority of axon branches in all three conditions were generated via backbranching. Panels show representative images of branch formation events for each condition. Arrows indicate sites of branch formation. All times listed are relative to the zero minute time point in each time series. Scale bar is 20 microns.

(B) Quantification of granule neurons treated as in (A). Axon branches formed over an image series consisting of 112 time points spanning a period of twenty-eight hours were tracked. For each neuron, the percent of total axon branches generated via backbranching events was calculated. Bars on the graph show averages taken across all neurons in a condition. A total of 41 neurons were measured.

Figure S6: High salt washes effectively remove priming kinase from substrate beads prior to GSK3 β kinase reaction

Bacterially-purified GST-DCX bound to glutathione beads was treated with FLAG-JNK1 purified from 293T cells, as in the sequential *in vitro* kinase assays shown in Figure 5A. Following high salt washes, no JNK1 was detected on the substrate beads, as determined by immunoblotting with the JNK1 antibody. The first lane was loaded with purified JNK1 at an amount added to each GST-DCX sample. Samples were also immunoblotted with the DCX antibody to confirm the presence of the substrate.

Figure S7: DCX overexpression overcomes JIP3 knockdown- and GSK3 β knockdown-induced axon branching and self-contact

(A) Granule neurons transfected with the DCX-IRES-GFP or control vector expression plasmid and the U6/JIP3i or control U6 RNAi plasmid were fixed three days after transfection and subjected to immunocytochemistry using the GFP antibody. For each representative image, boxed regions in the left panel highlight the distal axon, which is shown at higher magnification in the right panel. Scale bar is 50 microns in the whole cell image and 10 microns in the higher magnification panel. DCX overexpression suppressed JIP3 knockdown-induced axon branching and self-contact.

(B) Quantification of axon branching of granule neurons treated as in (A). In the vector background, knockdown of JIP3 significantly increased the number of axonal tips and self-contact points per hundred microns (ANOVA followed by Fisher's PLSD test, $p < 0.001$ for both). In the background of JIP3 knockdown, overexpression of DCX as compared to the vector control significantly decreased the number of axon tips and self-contact points per hundred microns (ANOVA followed by Fisher's PLSD test, $p < 0.001$ for both). A total of 238 neurons were measured.

(C) Granule neurons transfected with the DCX-IRES-GFP or control vector expression plasmid and the U6/GSK3 β i2 or control U6 RNAi plasmid were fixed three days after transfection and subjected to immunohistochemistry using the GFP antibody. For each representative image, boxed regions in the left panel highlight the distal axon, which is shown at higher magnification in the right panel. Scale bar is 50 microns in the whole cell image and 10 microns in the higher magnification panel. DCX overexpression overcame GSK3 β knockdown-induced axon branching and self-contact.

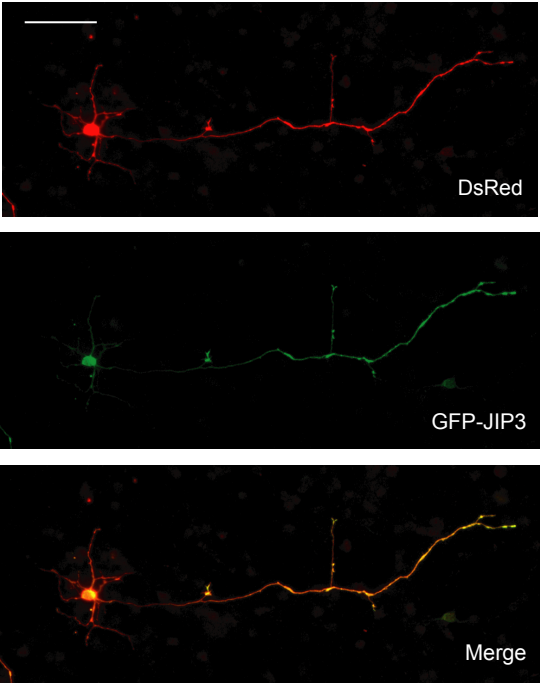
(D) Quantification of axon branching of granule neurons treated as in (C). In the vector background, knockdown of GSK3 β significantly increased the number of axonal tips and self-contact points per hundred microns (ANOVA followed by Fisher's PLSD test, $p < 0.001$ for both). In the background of GSK3 β knockdown, overexpression of DCX as compared to the vector control significantly decreased the number of axonal tips and self-contact points per hundred microns (ANOVA followed by Fisher's PLSD test, $p < 0.001$ for both). A total of 220 neurons were measured.

Figure S8: JNK does not appear to regulate GSK3 β protein levels

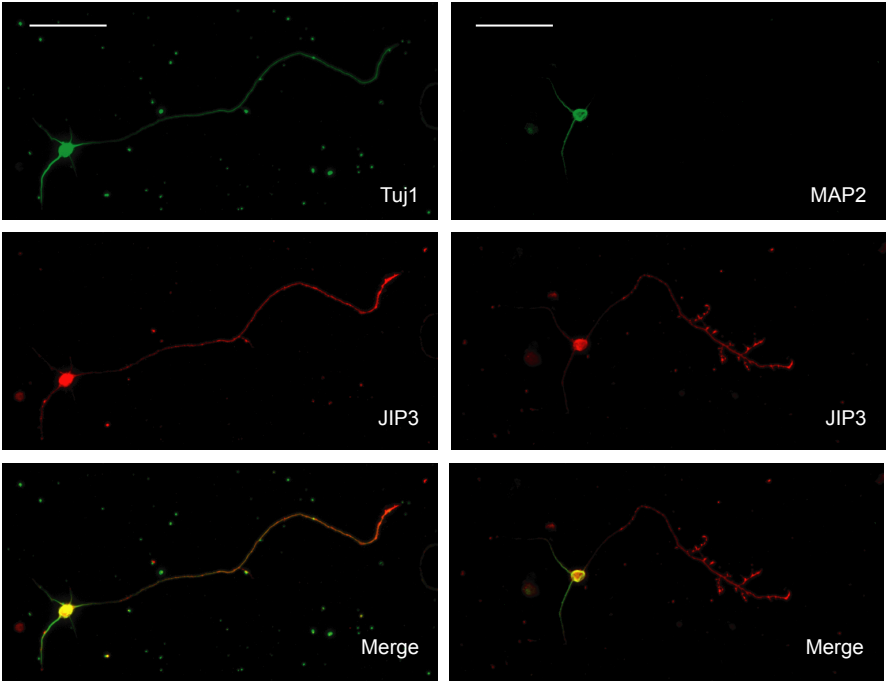
Lysates of cerebellar granule neurons treated for twenty-four hours with SP600125, a selective inhibitor of JNK, at the indicated concentrations or the vehicle control DMSO were immunoblotted using the GSK3 and 14-3-3 β antibodies. Inhibition of JNK had little or no effect on GSK3 β protein levels in neurons. 14-3-3 β served as the loading control.

Figure S1

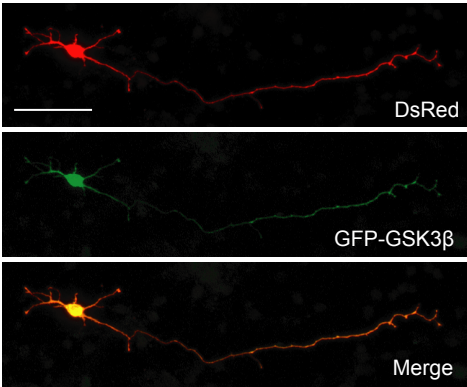
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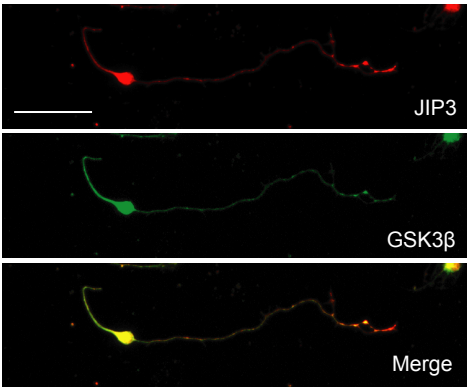
B



C



D



E

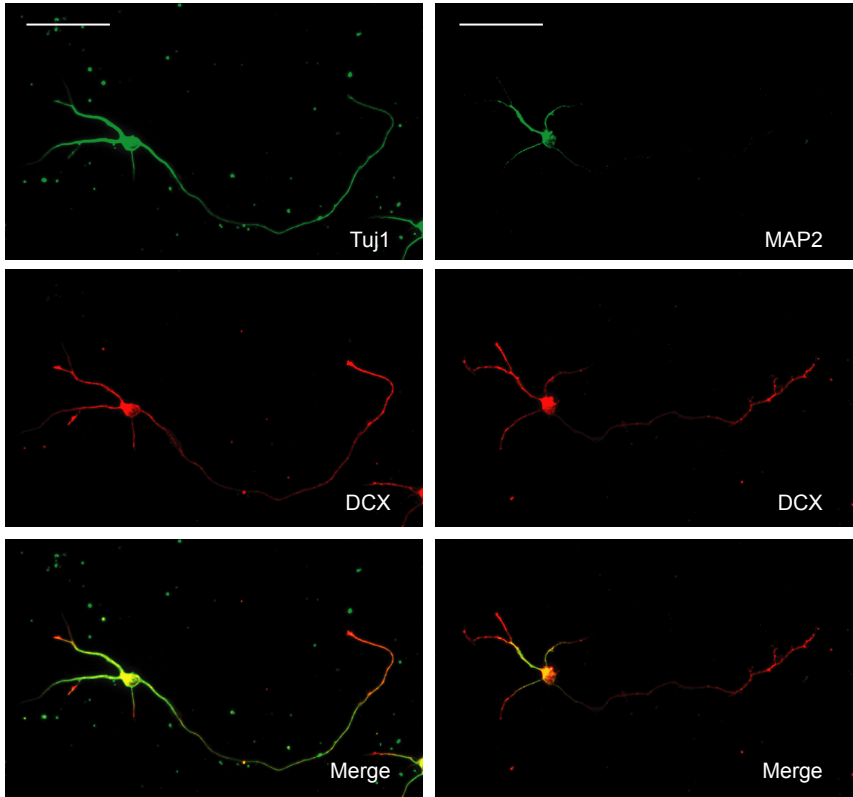
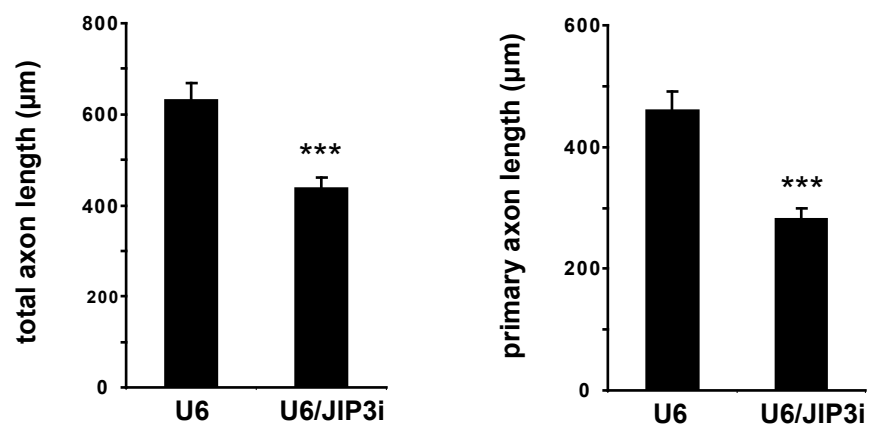


Figure S2

A



B

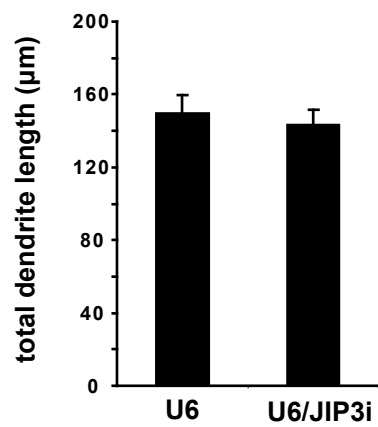


Figure S3

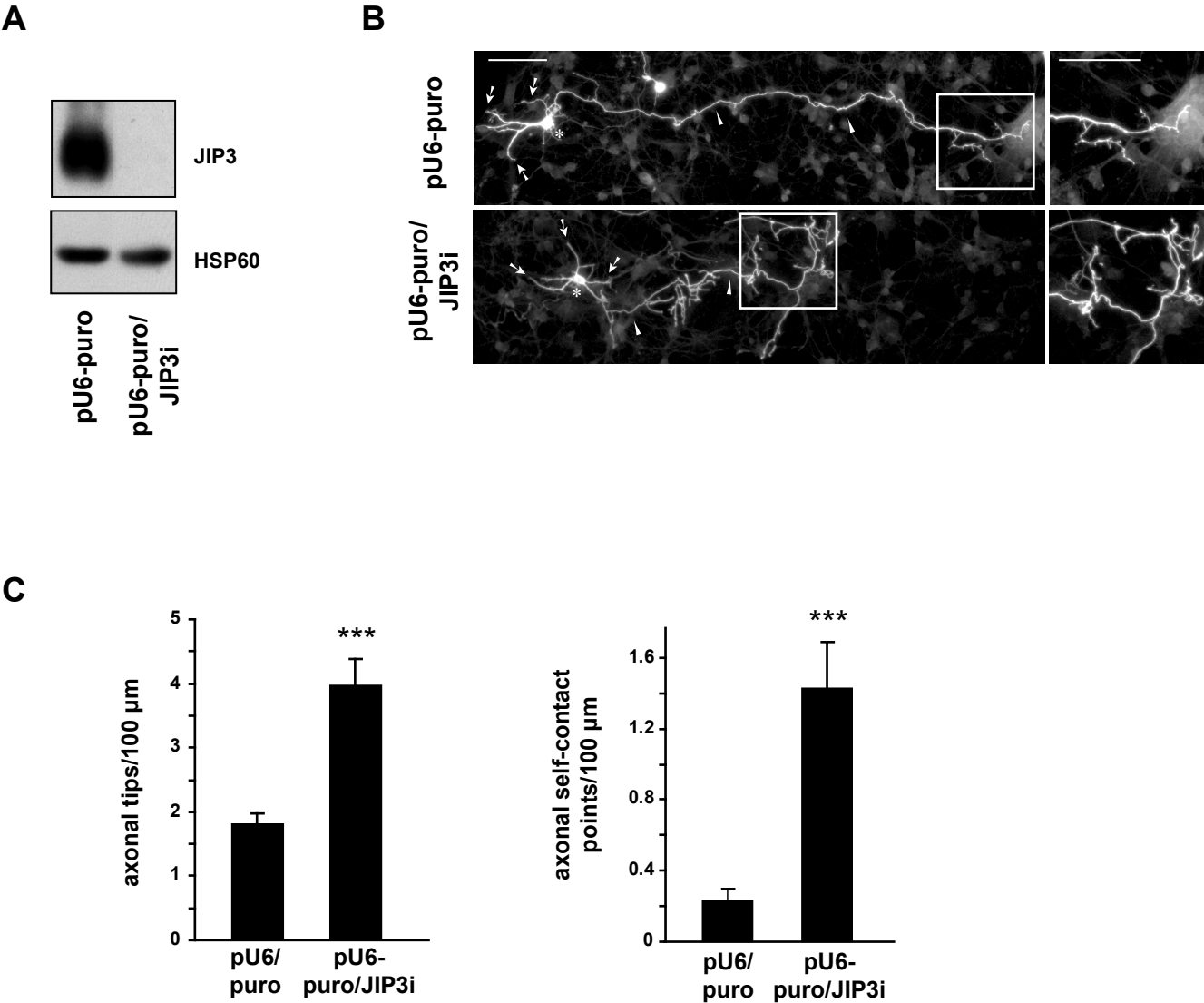
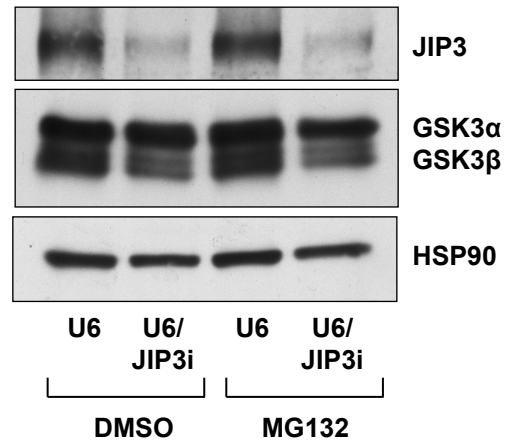
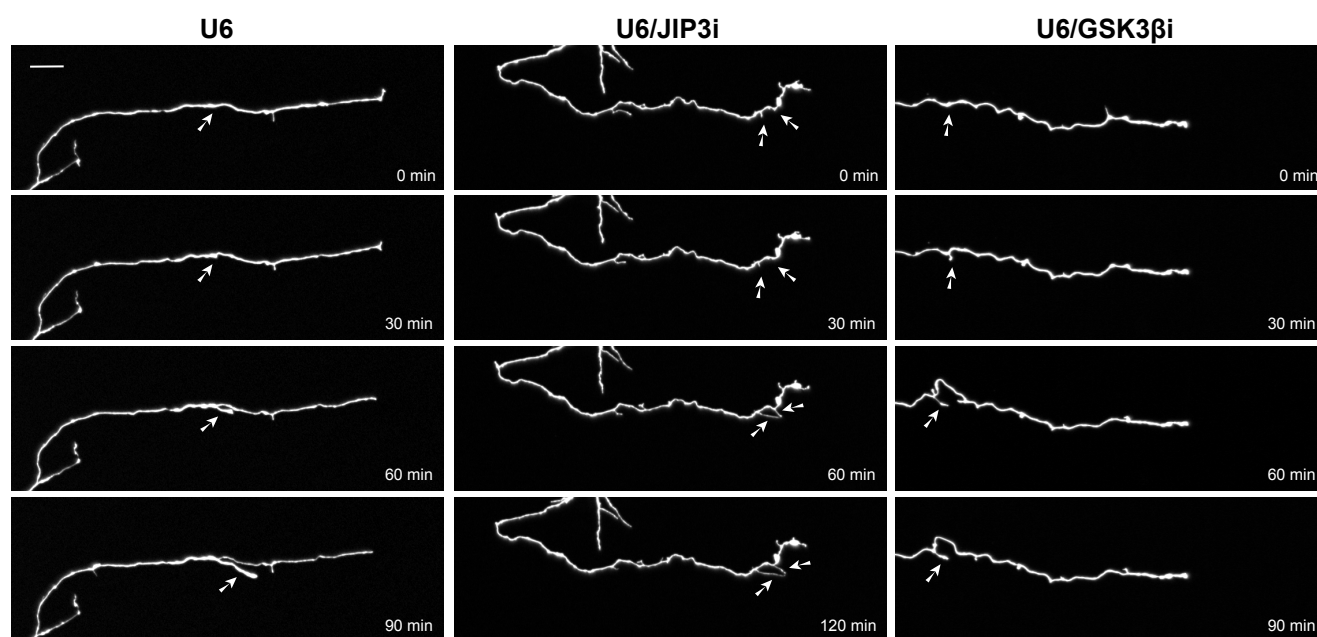


Figure S4



A



B

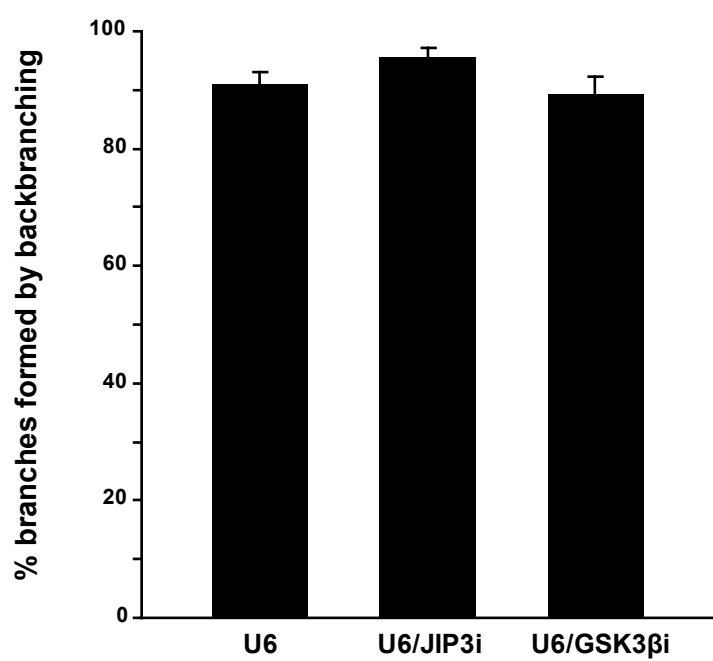
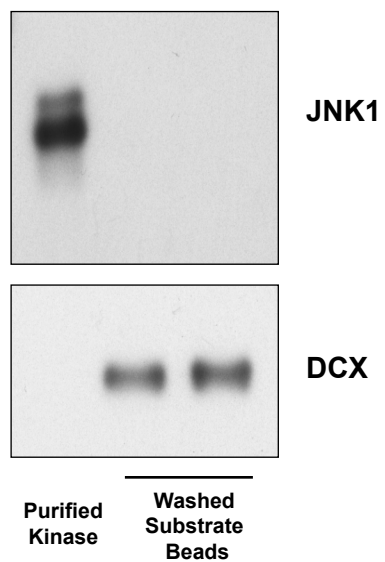
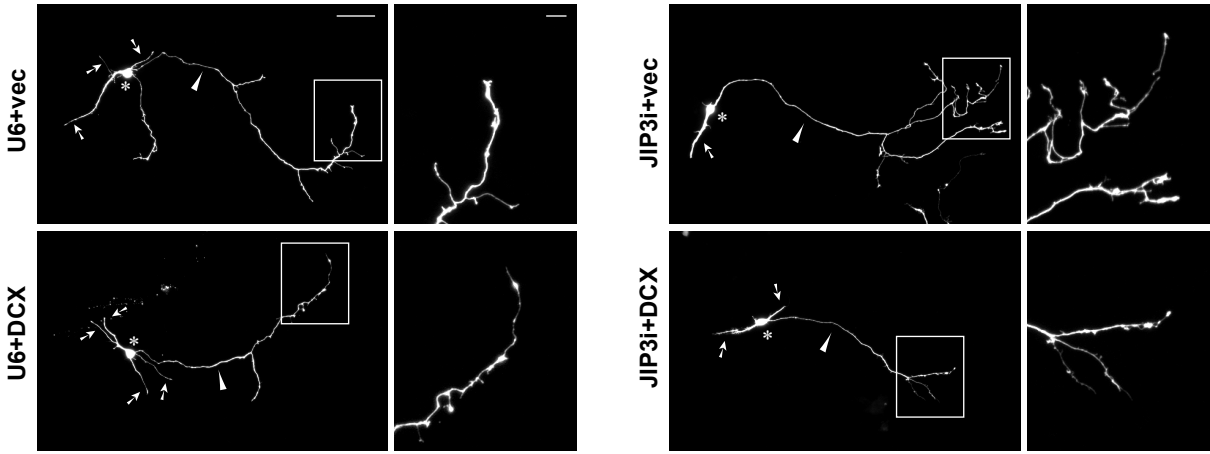


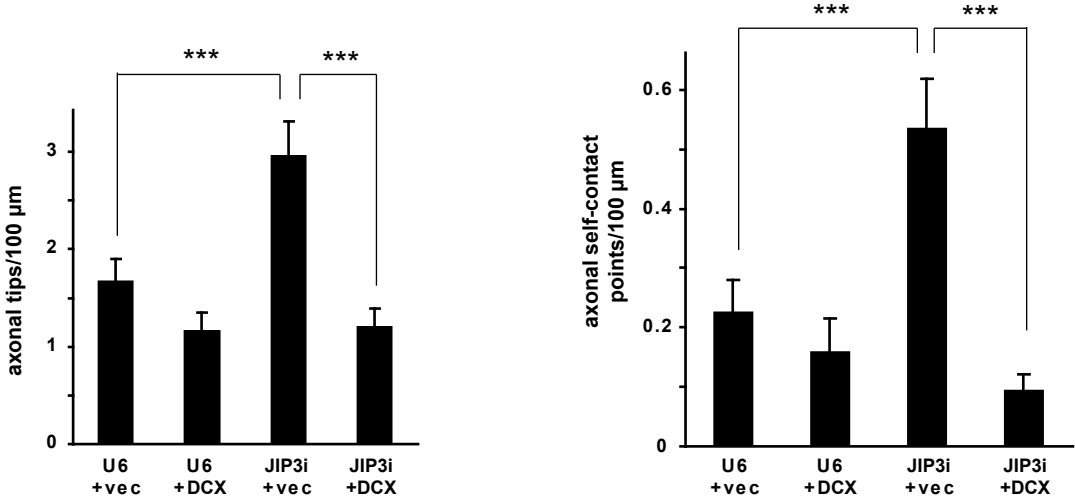
Figure S6



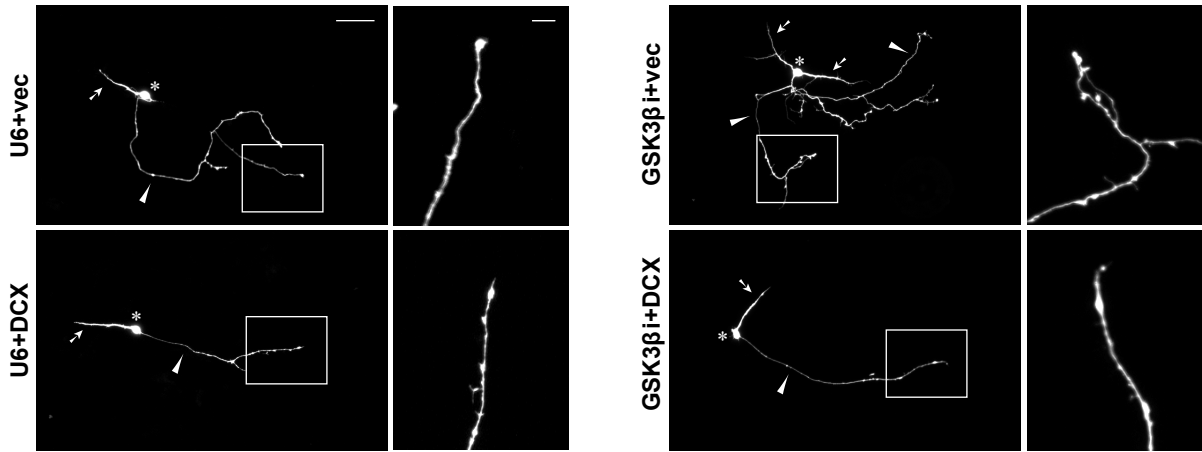
A



B



C



D

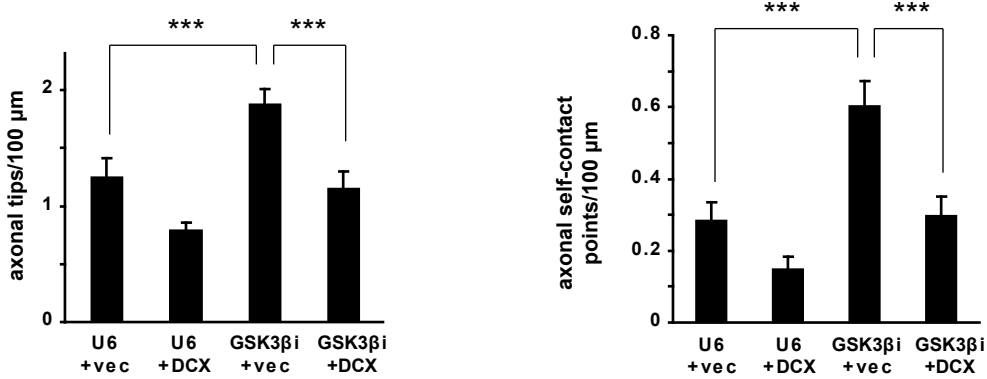


Figure S8

