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SHORT REPORT

Figures and figure supplements

The intellectual disability gene Kirrel3 regulates target-specific mossy fiber synapse development in the hippocampus

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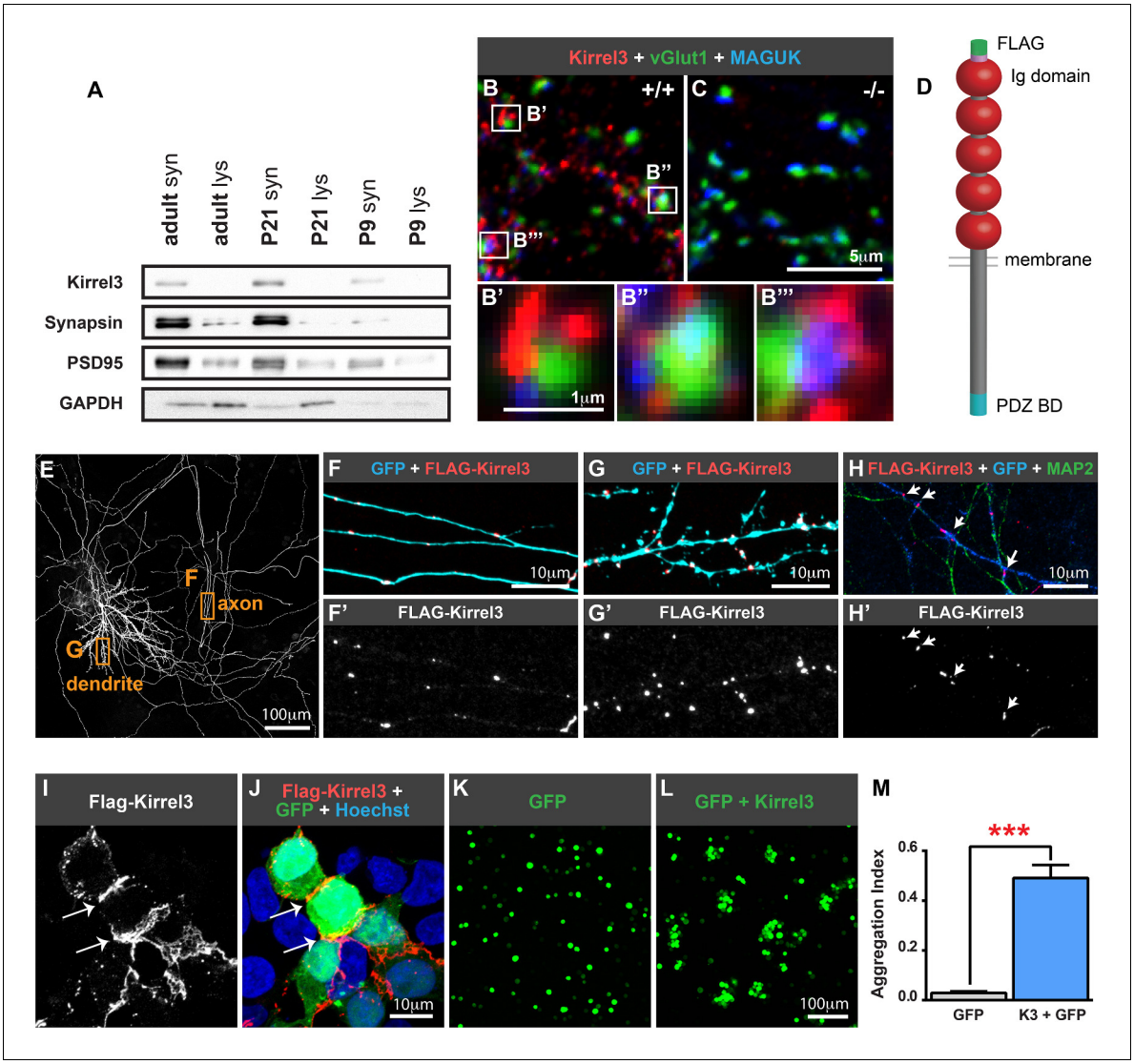

Figure 1. Kirrel3 is a synaptic molecule that mediates homophilic, trans-cellular adhesion. (**A**) Synaptosomes from mouse hippocampi from P9, P21, and adult (P55) were immunoblotted for indicated proteins. lys; lysate. syn; synaptosome. 2 μg protein per lane. (**B**, **C**) 14 days in vitro (DIV) cultured hippocampal neurons immunostained with antibodies against Kirrel3 (red), vGlut1 (green), and MAGUK proteins (blue). Boxed regions in **B** are magnified below in **B′**, **B″**, and **B‴**. Neurons from Kirrel3 knockout mice have no Kirrel3 signal (**C**). (**D**) Diagram of Kirrel3 protein and location of inserted FLAG tag. Ig; immunoglobulin. (**E**–**H**) Cultured hippocampal neurons were co-transfected with FLAG-Kirrel3 and GFP and immunostained for indicated proteins. Anti-FLAG antibodies were added prior to fixation to label only surface Kirrel3. Note surface Kirrel3 is seen as puncta on axons and dendrites after synapse formation in 14DIV neurons (**E**–**G**) and prior to synapse formation in 4DIV neurons (**H**). **H** shows that surface Kirrel3 also clusters at axon–dendrite crossings. Boxed regions in **E** are shown magnified in **F** and **G**. FLAG signal alone is shown in lower panels **F′**, **G′**, and **H′**. (**I**, **J**) Kirrel3 clusters at cell junctions. 293HEK cells were co-transfected with GFP and FLAG-Kirrel3, immunostained for GFP and FLAG, and nuclei labeled with Hoechst. (**K**–**M**) CHO cells transfected with either GFP control or GFP and Kirrel3 were tested for adhesion. Only cells expressing Kirrel3 formed aggregates. Aggregation index was calculated by dividing the total GFP fluorescence in cell aggregates by the total GFP fluorescence in the well. Mean ± SEM are shown, n = 3, *** indicates p = 0.001 by two-tailed t-test.

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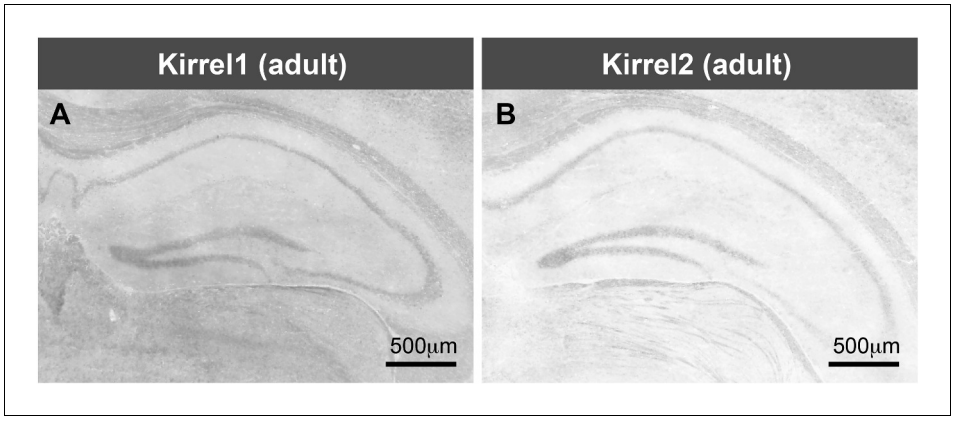

Figure 1—figure supplement 1. Kirrel1 and 2 are not expressed in the hippocampus. (**A**, **B**) In situ hybridizations for Kirrel1 and Kirrel2 mRNA indicate little to no hippocampal expression. This is in agreement with the Allen Brain Atlas. **A**, **B** are tiled images.

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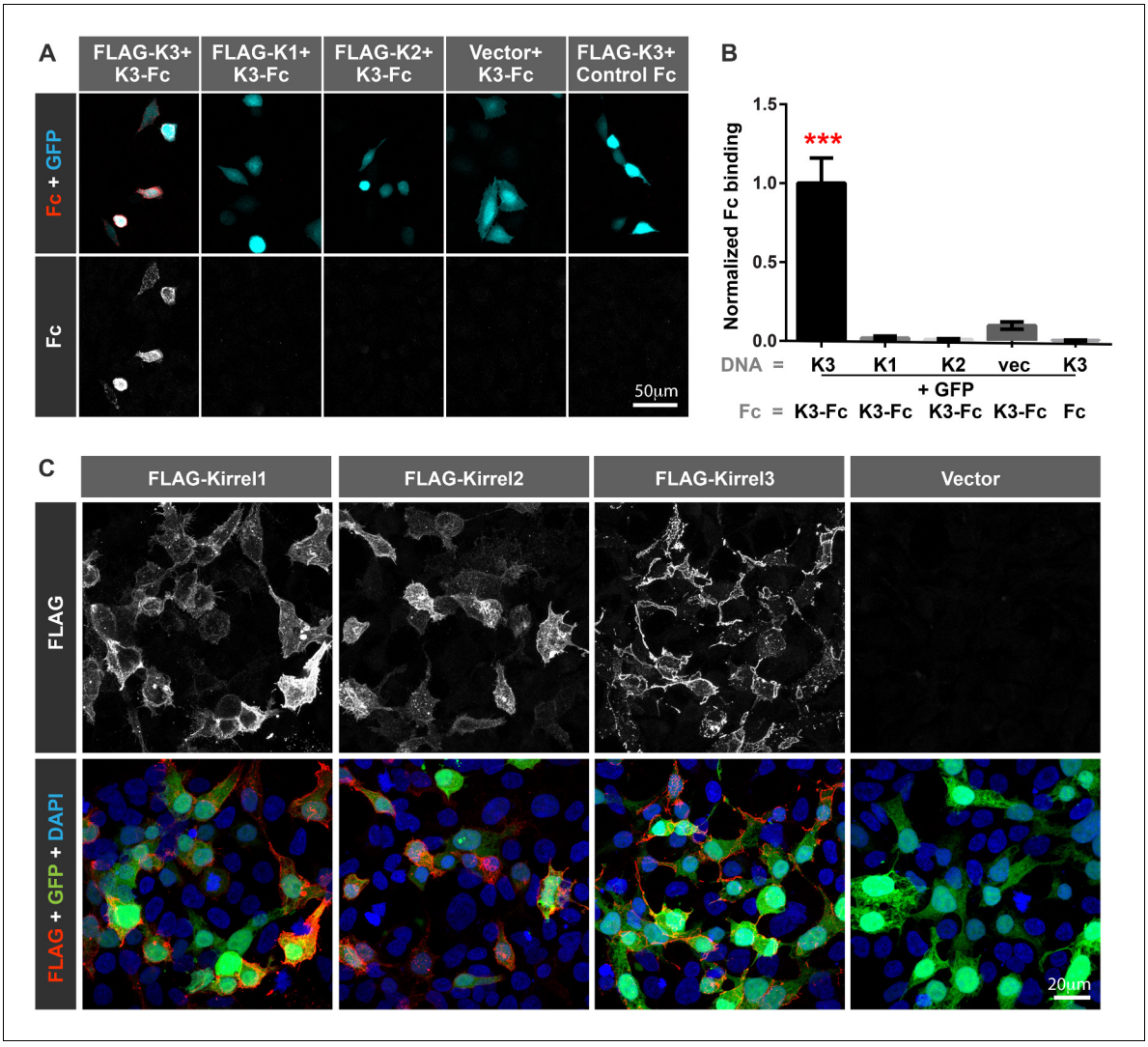

Figure 1—figure supplement 2. Kirrel3 undergoes homophilic binding in cells. (**A**) HEK293 cells were co-transfected with GFP (cyan) and full-length FLAG-tagged Kirrel cDNAs. Cells were then live labeled with soluble F_c constructs (red). This shows the Kirrel3 extracellular domain does not interact with Kirrel1 or Kirrel2 and the FLAG tag does not interfere with homophilic binding. (**B**) F_c binding was quantified by analyzing the percent area of GFP co-stained with F_c. The number of cells per condition from three independent experiments is K3 (96), K1 (53), K2 (70), vec (84), K3 + F_c (48). ***indicates that K3-F_c binds K3 significantly ($p < 0.0001$) more than any other condition as determined by ANOVA and pairwise post-tests. Mean ± SEM are shown. Kirrel1, 2, 3 is abbreviated K1, 2, 3. (**C**) HEK293 cells were co-transfected with GFP (cyan) plus full-length FLAG-tagged Kirrel cDNAs as done in **A** and **B**. Cells were live labeled with anti-FLAG antibodies to confirm each Kirrel protein is properly expressed on the cell surface.

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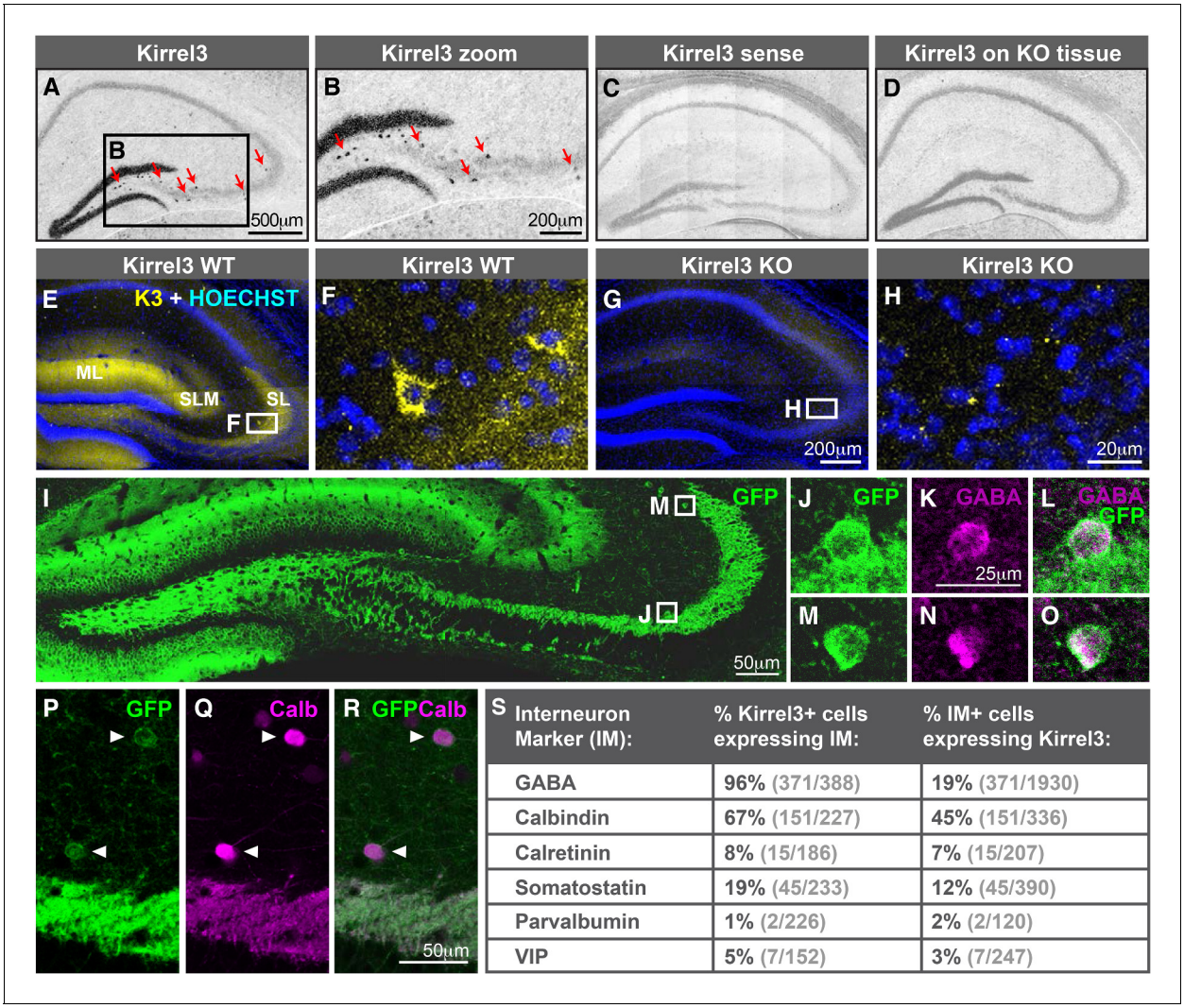

Interneuron Marker (IM):	% Kirrel3+ cells expressing IM:	% IM+ cells expressing Kirrel3:
GABA	96% (371/388)	19% (371/1930)
Calbindin	67% (151/227)	45% (151/336)
Calretinin	8% (15/186)	7% (15/207)
Somatostatin	19% (45/233)	12% (45/390)
Parvalbumin	1% (2/226)	2% (2/120)
VIP	5% (7/152)	3% (7/247)

Figure 2. Hippocampal DG and GABA neurons express Kirrel3. (**A**–**D**) In situ hybridizations for Kirrel3 mRNA on adult P60–P70 hippocampal sections from WT (**A**–**C**) and KO (**D**) mice. A negative control sense probe on WT tissue is shown in **C**. Red arrows in boxed region of **A** point to scattered Kirrel3-expressing cells shown magnified in (**B**). (**E**–**H**) Hippocampal sections from Kirrel3 WT (**E**, **F**) and KO (**G**, **H**) mice were immunostained with anti-Kirrel3 antibodies (yellow) and Hoechst (blue). **F** and **H** are magnified images of boxed regions in **E** and **G**. (**I**–**O**) P14 Kirrel3 KO mice with farnesylated GFP inserted in the Kirrel3 locus were immunostained with anti-GFP antibodies to identify Kirrel3-expressing cells (green). Dentate granule (DG) dendrites and their mossy fiber (MF) axons are brightly labeled (**I**) as well as GABA-expressing cells (magenta) in area CA3. (**P**–**R**) P14 Kirrel3 KO mice were immunostained for GFP (green) and calbindin (Calb, magenta). (**S**) Analysis of Kirrel3-positive cells in P14 Kirrel3 KO mice co-expressing interneuron markers. Abbreviations: wild-type, WT; knockout, KO; molecular layer, ML; stratum lucidum, SL. Stratum lacunosum-moleculare, SLM. Images in **A**–**D**, **E**, **G**, and **I** are tiled.

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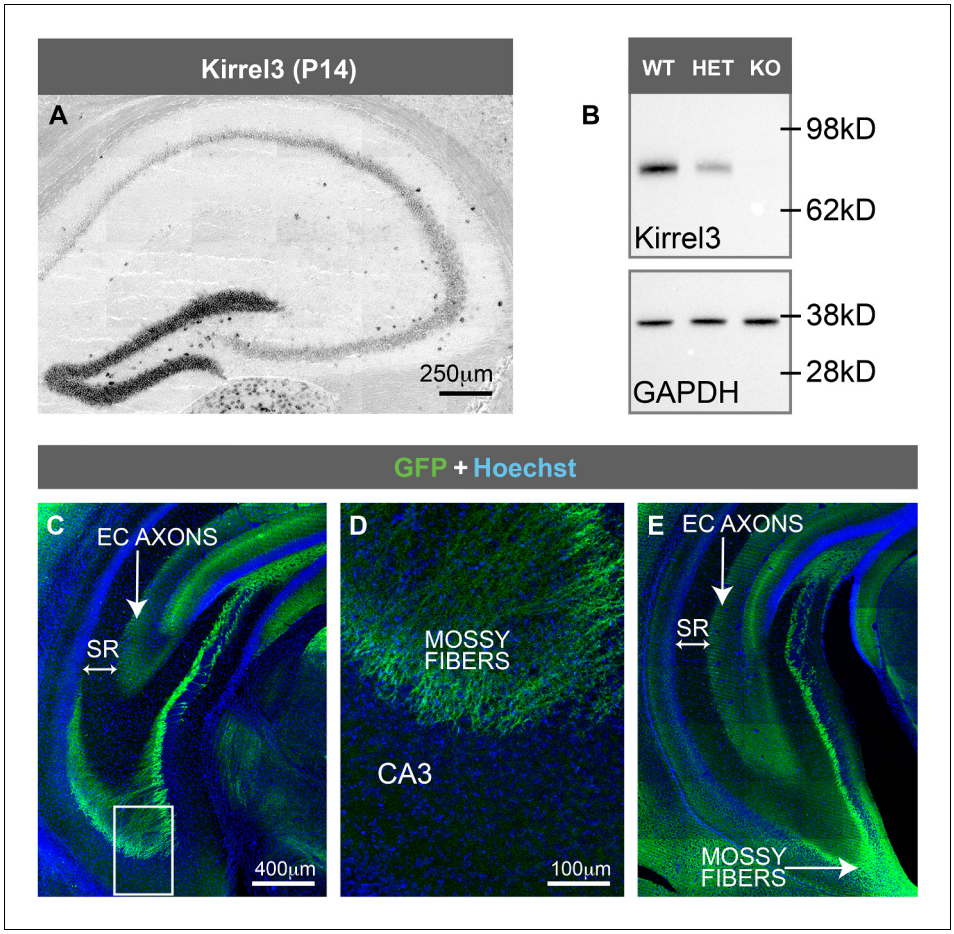

Figure 2—figure supplement 1. Kirrel3 is not expressed by CA3 neurons. (**A**) In situ hybridization for Kirrel3 mRNA at P14 shows the same expression pattern as the adult brain shown in ***Figure 2A***. (**B**) Western blot of hippocampal lysates from adult Kirrel3 wild-type (WT), heterozygous (HET), and knockout (KO) mice indicates Kirrel3 protein is absent in knockout mice as expected. (**C**–**E**) P25 Kirrel3 KO mice with farnesylated GFP inserted in the Kirrel3 locus were immunostained with anti-GFP antibodies to identify Kirrel3-expressing cells. Shown here are tiled confocal images of coronal sections through more ventral hippocampal sections compared to ***Figure 2E***. Boxed region in **C** is magnified in **D**. Note that GFP is not present in CA3 neuron cell bodies or their axons that reside in the stratum radiatum (SR) layer. Thus, throughout the hippocampus, Kirrel3 is not expressed at detectable levels in CA3 neurons. EC; entorhinal cortex. Images **A**, **C**, and **E** are tiled.

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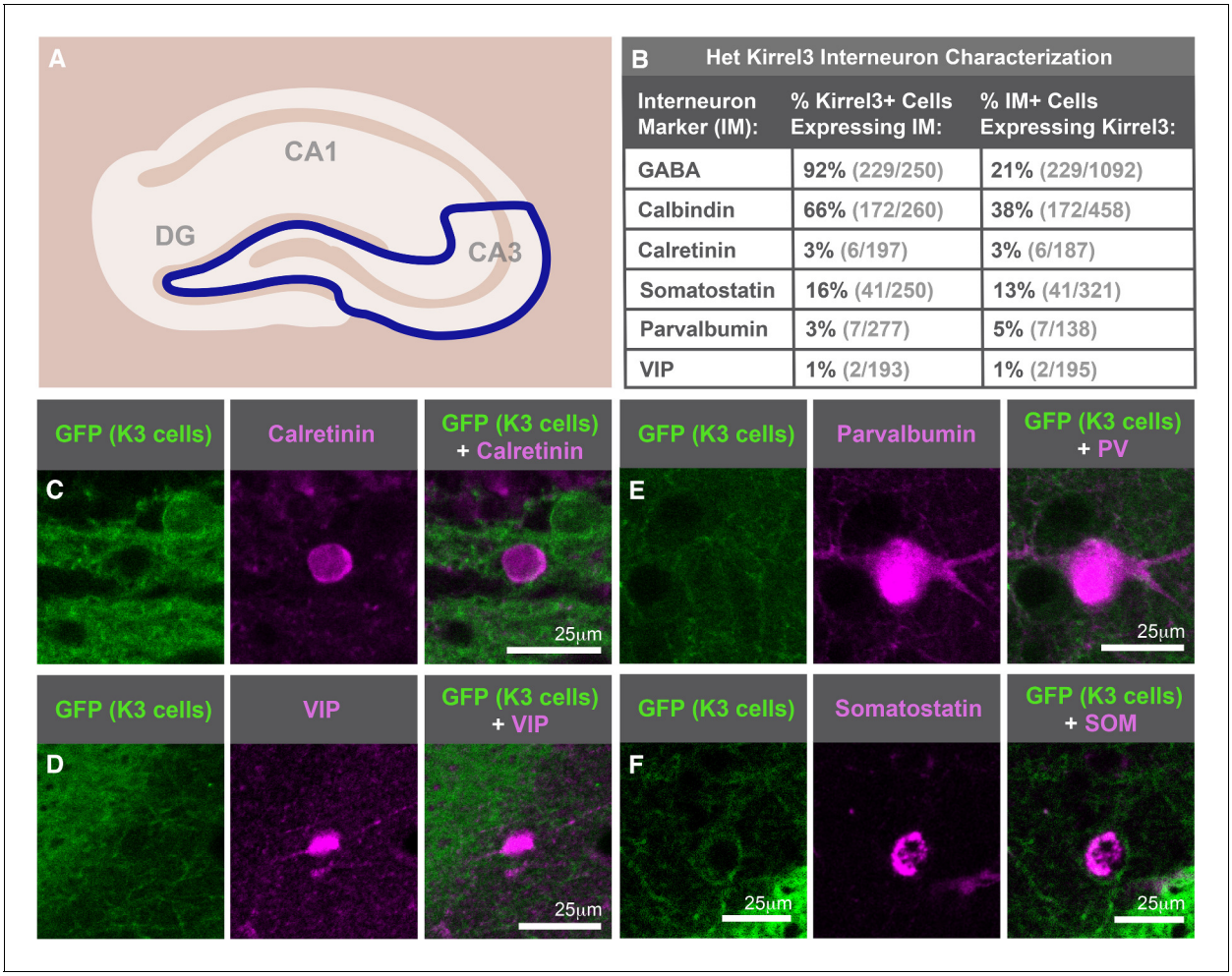

Figure 2—figure supplement 2. Kirrel3 is expressed by mainly calbindin-positive GABA neurons. (**A**) Diagram indicating hippocampal region imaged and analyzed is represented inside the blue line. (**B**) Interneuron analysis of animals heterozygous for Kirrel3 shows little difference from knockout analysis shown in ***Figure 2S***. (**C**–**F**) Sample images of interneuron staining to show each interneuron antibody worked for IHC despite having little overlap with Kirrel3. Mice are immunostained for GFP (green) to mark K3-expressing neurons and interneuron markers (magenta) in tissue.
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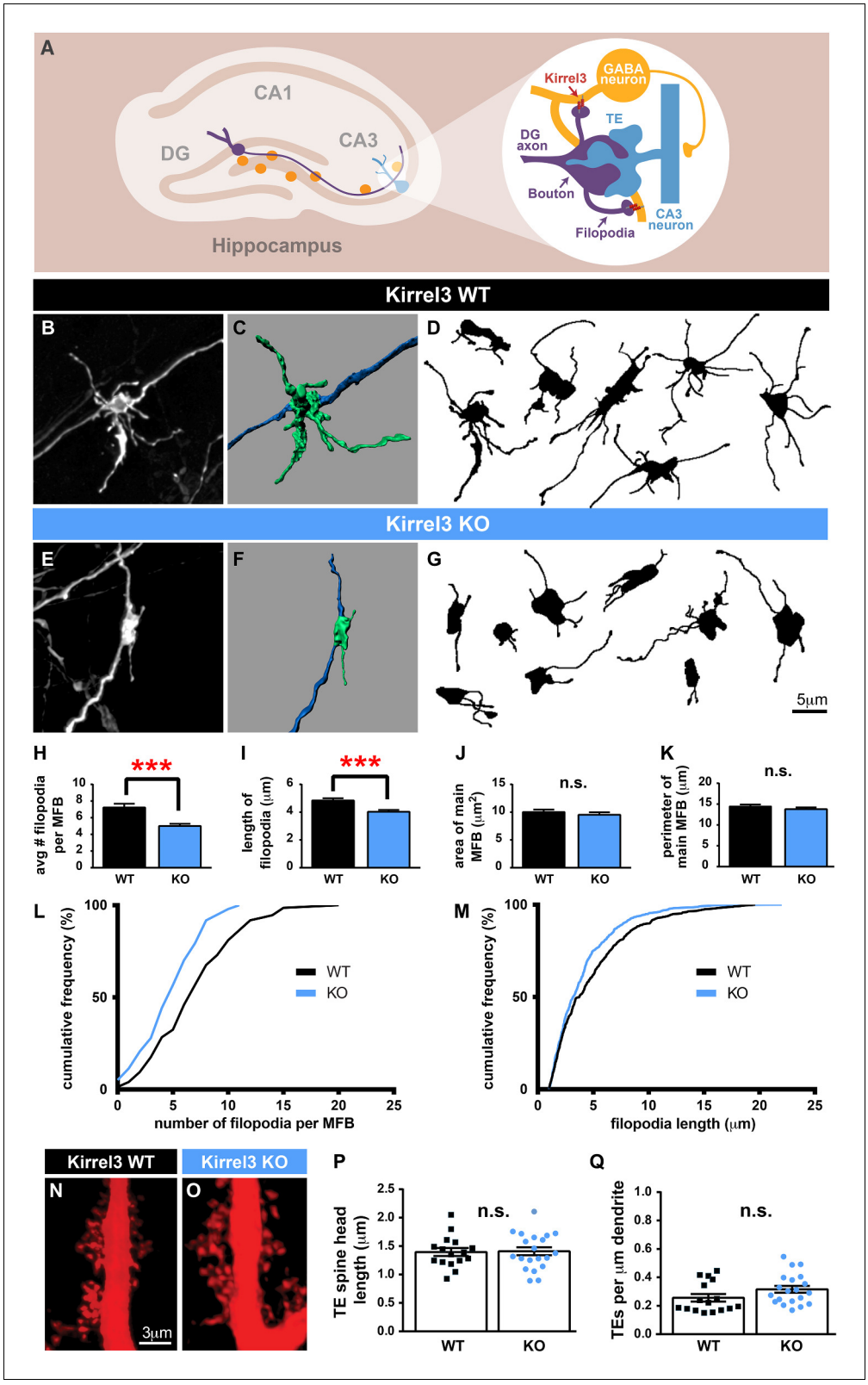

Figure 3. Kirrel3 regulates MF synapse form and function during development. (**A**) MF synapse diagram. TE; thorny excrescence. (**B**, **E**) DiI-labeled MF synapses from P14 Kirrel3 WT and KO mice. (**C**, **F**) 3D renderings of synapses in (**B**, **E**). (**D**, **G**) Tracings of representative DiI-labeled MF synapses. (**H**–**M**) MF synapse morphology quantification. The number of filopodia per MF bouton (**H**, **L**) and filopodia length (**I**, **M**) are reduced in Kirrel3 KO

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mice. **L** and **M** are cumulative histograms of data shown in **H** and **I**, respectively. Area (**J**) and perimeter (**K**) of the main MF bouton are unaffected by genotype. n = 74 WT and 97 KO MF synapses from four mice of each genotype. Two-tailed t-tests: in **H**, *** = $p < 0.001$ and in **I**, *** = $p = 0.0001$. (**N**, **O**) Examples of P21 CA3 TE spines labeled by iontophoresis. (**P**, **Q**) Quantification of P21 spine morphology. No significant differences as determined by two-tailed t-tests. n = 16 WT neurons from four animals and 20 KO neurons from three animals. All bar graphs show mean ± SEM.

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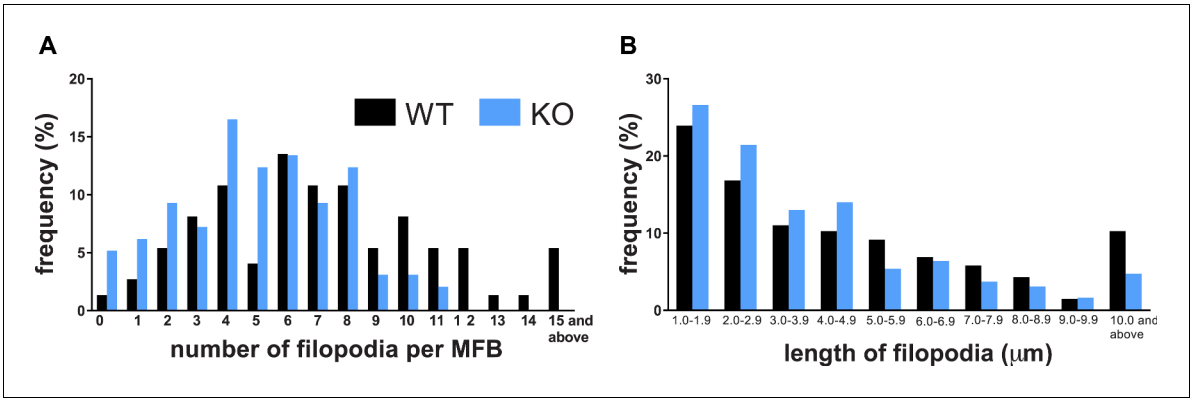

Figure 3—figure supplement 1. Kirrel3 is required for normal development of MF filopodia. (**A**, **B**) Histograms for the number (**A**) and length (**B**) of MF filopodia in each genotype. This is the same P14 data plotted in main ***Figure 3H–K*** but here the entire range of values for all data is shown.
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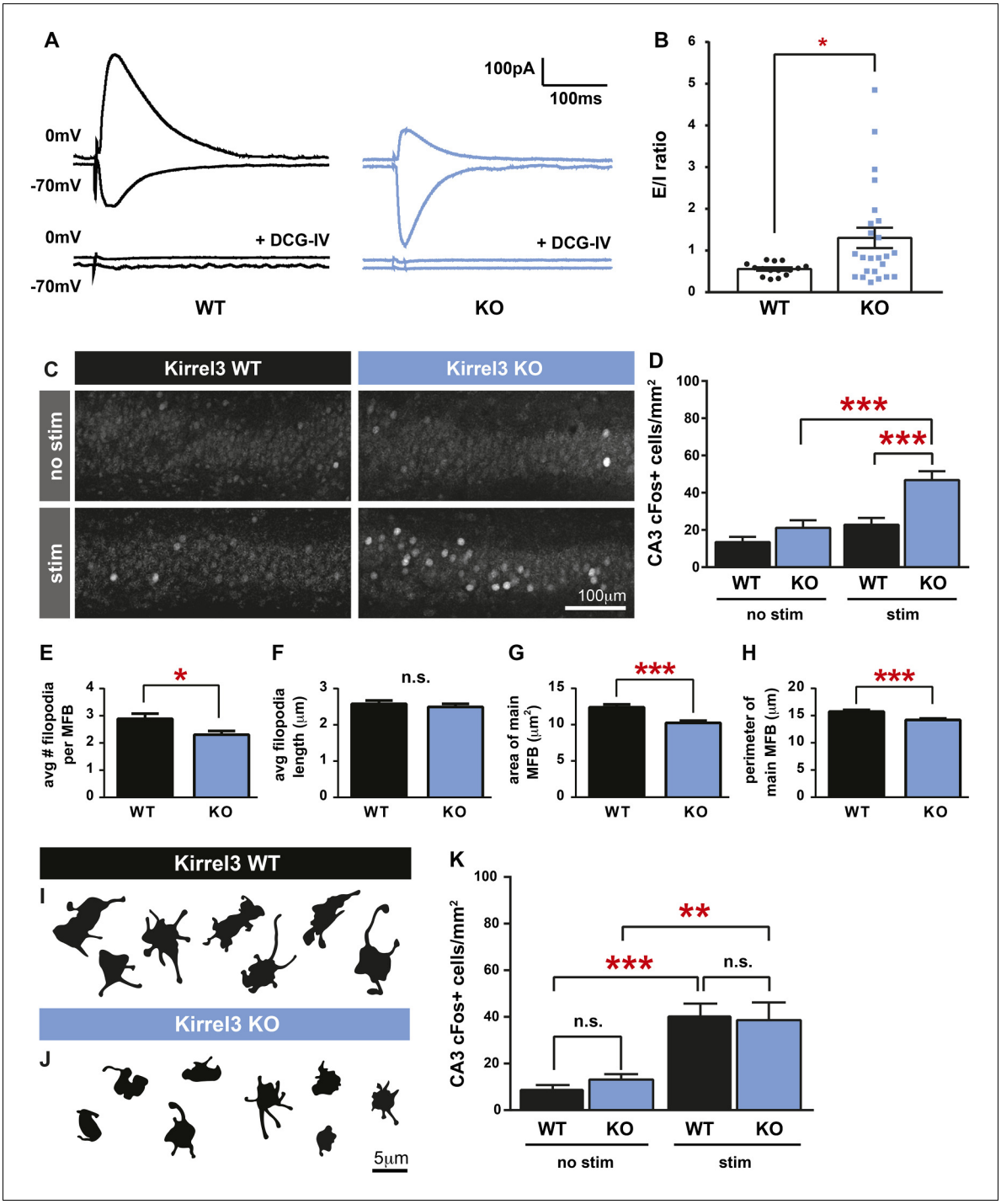

Figure 4. Kirrel3 regulates the activity of CA3 neurons during development. (**A**) Evoked responses at −70 and 0 mV of a CA3 neuron after stimulation of the MF pathway in Kirrel3 WT and KO mice. Lower traces show responses of the same cells after perfusion of 0.5 μm DCG-IV. (**B**) Average excitatory/inhibitory (E/I) ratio for CA3 neurons recorded from P14–P16 WT and KO mice. n = 15 cells from five WT animals and 24 cells from five KO animals. p = 0.02 with unpaired t-test. (**C**) Examples of anti-cFos staining in CA3 neurons from P14 mice. Note increased cell staining in Kirrel3 KO mice after 25 min stimulation (stim) in a novel, enriched environment. (**D**) Quantification of cFos-positive CA3 neurons at P14. n = 14 (WT no stim), 15 (WT stim), 15 (KO no stim), and 15 (KO stim) sections from three mice per condition. Two-way ANOVA indicates there is a significant difference among condition (no stim vs stim) and genotype. p values from post-tests are 0.0001 (KO no stim vs KO stim) and 0.0004 (WT stim vs KO stim). (**E**–**H**) Quantification of MF synapse structure in adult mice. n = 115 WT and 131 KO synapses from three mice per genotype. Two-tailed t-tests indicate p = 0.01 (**E**), p < 0.0001 (**G**), and p = 0.002 (**H**). (**I**, **J**) Tracings of representative DiI-labeled MF synapses from adult (P60–P75) Kirrel3 WT and KO mice. (**K**) Quantification of cFos-positive cells in area CA3 of adult mice. n = 14 (WT no stim), 14 (WT stim), 16 (KO no stim), and 16 (KO stim) sections from three different mice per condition.

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Figure 4 continued

Two-way ANOVA indicates there is a significant difference among condition (no stim vs stim) but not genotype. p values from post-tests are 0.0005 (WT no stim vs WT stim) and 0.003 (KO no stim vs KO stim). All graphs show mean ± SEM.

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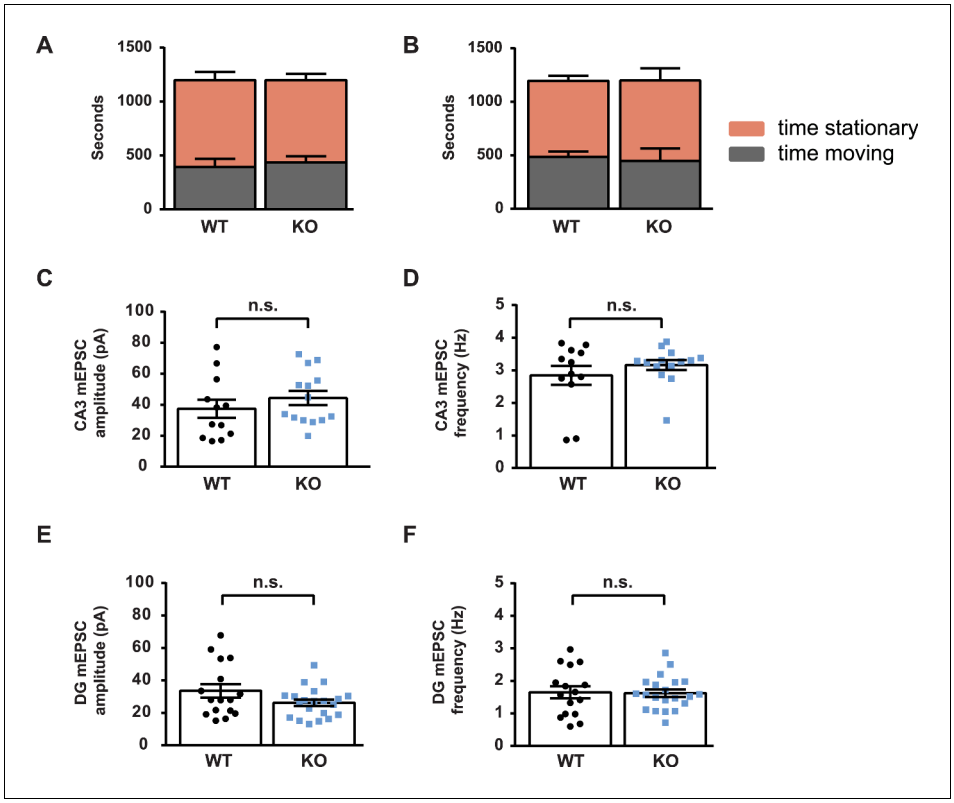

Figure 4—figure supplement 1. Spontaneous mEPSC activity of CA3 and DG neurons is normal in the absence of Kirrel3. (**A**, **B**) Graphs depicting time spent in motion (gray) and stationary (orange) of P14 (**A**) and adult P60–P75 (**B**) Kirrel3 WT and KO mice during cFos environmental stimulation. No significant differences by two-tailed t-test. Mean ± SEM is shown. n = 3 mice of each age and genotype. (**C**, **D**) Miniature excitatory postsynaptic current (mEPSC) amplitudes and frequencies from P17–P21 CA3 neurons. n = 12 cells from two WT animals and 14 cells from five KO animals. No significant differences by unpaired t-tests. (**E**, **F**) mEPSC amplitudes and frequencies from P17–P21 DG neurons. n = 16 cells from three WT animals and 21 cells from three KO animals. No significant differences by unpaired t-tests. All bar graphs show mean ± SEM.

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