Timothy syndrome is associated with activity-dependent dendritic retraction in rodent and human neurons

Jocelyn F Krey1,4, Sergiu P Pașca1, Aleksandr Shcheglovitov1, Masayuki Yazawa1, Rachel Schwemberger1, Randall Rasmusson2 & Ricardo E Dolmetsch1,3

L-type voltage gated calcium channels have an important role in neuronal development by promoting dendritic growth and arborization. A point mutation in the gene encoding CaV1.2 causes Timothy syndrome, a neurodevelopmental disorder associated with autism spectrum disorders (ASDs). We report that channels with the Timothy syndrome alteration cause activity-dependent dendrite retraction in rat and mouse neurons and in induced pluripotent stem cell (iPSC)-derived neurons from individuals with Timothy syndrome. Dendrite retraction was independent of calcium permeation through the mutant channel, was associated with ectopic activation of RhoA and was inhibited by overexpression of the channel-associated GTPase Gem. These results suggest that CaV1.2 can activate RhoA signaling independently of Ca2+ and provide insights into the cellular basis of Timothy syndrome and other ASDs.

Voltage-gated calcium channels (VGCCs) such as CaV1.2 are essential for coupling electrical events to the activation of intracellular signaling pathways that control the development and function of neurons4. Mutations in VGCC genes have been linked to various neurological and psychiatric disorders, including epilepsy, ataxia, migraine, ASDs and schizophrenia2–6. Although a great deal is understood about how disease-causing mutations change the biophysical properties of these channels7,8, little is known about how these mutations alter neuronal development and cause disease. Unraveling this relationship is critical for understanding how mutations in VGCCs ultimately lead to neuronal dysfunction and regulate neuronal signaling under non-pathological conditions.

L-type voltage-gated calcium channels (LTCs) are characterized by their large single-channel conductance, activation at depolarized potentials and sensitivity to dihydropyridines9. The α1 subunit of the channel contains the voltage sensor and ion pore, and forms a complex with β and δ subunits that regulate channel trafficking and gating9,10. CaV1.2 is the most abundant of the three LTCs in the mammalian brain and is expressed on the cell body, dendrites and growth cones of developing neurons11,12. Timothy syndrome is a multisystem disorder characterized by cardiac arrhythmias, webbing of the fingers and toes, hypoglycemia and autism13. It is caused by a dominant mutation in an alternatively spliced exon of the CACNA1C gene, which encodes the α1 subunit of the L-type VGCC, CaV1.2 (ref. 13). The mutation that causes Timothy syndrome results in a glycine-for-arginine substitution at position 406 in the first of three intracellular loops in CaV1.2 that alters the ability of CaV1.2 to undergo voltage-dependent and calcium-dependent inactivation. This mutation thus presents an excellent opportunity to examine the importance of these processes in neuronal development and function.

A major function of CaV1.2 is to regulate dendritic refinement in response to electrical activity14–16. Dendritic arbors are essential for the processing of information by neurons and have a key role in the formation of the neuronal circuits that underlie cognition16. Precisely how LTCs regulate dendritic development is not well understood. Although LTCs can activate transcription factors such as CREB and CREST that control the expression of genes involved in dendritic arborization15,17, there is considerable evidence that activity-dependent arborization also involves local activation of signaling pathways in dendrites18–20. The RGK (Rad, Rem, Rem2 and Gem/Kir) family of small GTP-binding proteins provides a potential link between CaV1.2 channels and dendritic arborization. Gem and Rem2 are both expressed in neurons21,22 and bind to the β subunit of voltage-gated Ca2+ channels23,24. The function of RGK proteins in neurons is not well understood, but they have been shown to regulate channel function both by altering channel trafficking24–26 and by directly reducing channel activity23,27. The RGK proteins have also been reported to regulate signaling proteins that control dendritic arbors such as Rho-GAP, Gem interacting protein (GMIP)28 and the Rho kinase ROCK1 (ref. 29). Whether the RGK proteins connect voltage-gated Ca2+ channels to the signaling pathways that regulate dendritic morphology is not known.

In this study, we used animal and human models of Timothy syndrome to study the effects of the mutation that causes Timothy syndrome on dendritic arbors both in vitro and in vivo. In vitro we found that expression of CaV1.2 channels with the mutation that causes Timothy syndrome triggered dendrite retraction when these neurons were stimulated electrically, either by bath depolarization or by activation of channelrhodopsin-2. In vivo, we observed changes in the dendritic arbor of neurons in a knock-in mouse model of Timothy syndrome characterized by dendrite retraction.
syndrome. To show that the defects observed in mouse and rat neurons also occur in humans with Timothy syndrome, we generated iPSC-derived neurons from individuals with Timothy syndrome and found that these cells also exhibited activity-dependent dendrite retraction. In studying the mechanism underlying these defects, we discovered that dendrite retraction triggered by the mutation that causes Timothy syndrome was independent of Ca\(^2+\) influx through the channel. Instead, conformational changes in the Cav1.2 channel activated RhoA signaling via the small G protein Gem, leading to dendrite retraction in response to electrical activity. These results identify a new mechanism by which Cav1.2 can regulate signaling cascades in neurons and provide important insights into the pathophysiology of Timothy syndrome and other ASDs.

**RESULTS**

**Dendrite retraction in rat neurons**

Although voltage-gated calcium channels are known to have a key role in dendrite growth in many systems, their importance in regulating dendrite development in cortical neurons has not been established. We first investigated which types of channels are important for controlling dendritic arbors in developing cortical neurons and found that blockers of LTCs completely eliminated dendrite growth in response to depolarization, whereas other channel blockers had no significant effect (P > 0.05; Supplementary Figs. 1a–d). To investigate the effects of the mutation that causes Timothy syndrome on activity-dependent arborization, we introduced plasmids encoding wild-type Cav1.2 channels or Cav1.2 channels with the mutation (G406R) that causes Timothy syndrome (here called TS-Cav1.2 channels) together with YFP into rat cortical neurons. Using anti-Cav1.2 antibodies we found that expression of wild-type Cav1.2 and TS-Cav1.2 channels was similar to that of the endogenous channel, and that both channels were localized at the cell membrane (Supplementary Figs. 2e–f,3).

Then using fluorescence microscopy we measured changes in the dendritic arbor of individual cells over 9 h. We observed no differences in the length of the dendritic arbors in resting neurons expressing wild-type Cav1.2 or TS-Cav1.2 channels. However, activation of LTCs by depolarization caused a pronounced increase in the length and complexity of the dendritic arbor in neurons expressing wild-type channels (Fig. 1a,b). In contrast, depolarization caused a dramatic reduction in dendrite length in neurons containing TS-Cav1.2. This retraction was not due to an increase in apoptosis or necrosis, as we did not observe any differences in terminal deoxynucleotide transferase (TdT)-mediated dUTP-digoxigenin nick-end labeling (TUNEL) or nuclear condensation between TS-Cav1.2–expressing and control neurons (Supplementary Fig. 3).

Dendrite growth is the sum of dynamic extension and retraction events over the course of minutes and hours, and changes in either the number of extension or retraction events or in the amplitude of each event can lead to changes in the total dendrite length. To determine whether the TS-Cav1.2 channel affects either the number or length of dendritic retraction or extension events, we used high-frequency time-lapse imaging to capture these events over 1 h. Dendrites from neurons expressing wild-type Cav1.2 underwent slightly more extension than retraction events, leading to a net growth of the dendritic arbor. In contrast, neurons expressing TS-Cav1.2 exhibited an increase in the number of dendritic retraction events and a decrease in the number of extension events, resulting in an overall decrease in dendrite length (Fig. 1c,d). There was

![Figure 1](image_url)

**Figure 1** TS-Cav1.2 causes activity-dependent dendritic retraction. (a) Representative dendrite tracings of dissociated cortical neurons transfected with constructs encoding both YFP and wild-type (WT) Cav1.2 (left) or TS-Cav1.2 (right) and imaged before (0 h) and after (9 h) stimulation with 67 mM KCl. Two different neurons are illustrated for each condition (top and bottom). (b) Change in total dendrite length after KCl stimulation in individual neurons expressing WT Cav1.2 or TS-Cav1.2 (n ≥ 30 cells per condition; mean ± s.e.m.; **P < 0.001; two-way ANOVA, Bonferroni post-test). (c) Epifluorescence images of representative cortical neurons transfected as in a and imaged every 10 min after depolarization. Arrows mark dendrites that retract. (d) Percentage of dendrites with an increase (extended) or decrease (retracted) in length > 2 µm after 60 min in control or depolarizing (KCl) solutions (n = 4 experiments, n ≥ 25 dendrites/experiment, mean ± s.e.m., **P < 0.05; Student’s t-test). (e) Representative current clamp recording of a hippocampal neuron expressing channelrhodopsin-2 during illumination with 488-nm light. (f) Schematic of the protocol used for illumination and image acquisition (top), and epifluorescence images of a representative hippocampal neuron expressing TS-Cav1.2. (g) Change in total dendritic outgrowth in neurons expressing WT Cav1.2 (n = 52) or TS-Cav1.2 (n = 36) over the time course of stimulation (mean ± s.e.m.; **P < 0.001; Student’s t-test). Scale bars, 50 µm (a,c,f).
no difference in the amplitude of individual retraction or extension events between cells expressing TS-CaV1.2 or wild-type CaV1.2 (Supplementary Fig. 4), suggesting that activation of TS-CaV1.2 caused dendrite retraction by increasing the frequency of retraction events and not the amplitude of each event.

Although membrane depolarization is a robust way of activating LTCs in culture, it is not a physiological stimulus for most neurons. To mimic physiological activation, we introduced channelrhodopsin-2 into rat neurons expressing TS-CaV1.2 or wild-type CaV1.2 and electrically activated the cells by illuminating them with blue light. Patch-clamp recordings showed that neurons fired high-frequency bursts of action potentials for the duration of the illumination (Fig. 1e). We stimulated the cells to generate four 1-s bursts of action potentials every 30 min over 12 h and collected time-lapse images of the dendritic arbor every 30 min (Fig. 1f). Cells expressing TS-CaV1.2 showed a significant decrease ($P < 0.001$) in dendritic outgrowth when compared to cells expressing the wild-type channel (Fig. 1g), confirming that TS-CaV1.2 caused dendrite retraction when neurons were stimulated with physiological patterns of electrical activity.

**Dendritic development defects in mice with Timothy syndrome**

To study the consequences of the mutation that causes Timothy syndrome on neuronal development in vivo, we generated a mouse model of Timothy syndrome\textsuperscript{30}. Using homologous recombination, we introduced a cassette containing a mutation in exon 8 of the mouse gene encoding CaV1.2 with the G406R substitution. Mutations in the homologous exon in humans cause type-2 Timothy syndrome. To determine whether the defects observed in mice and rat neurons are also present in humans, we collected dermal fibroblasts from two individuals with Timothy syndrome and generated iPSC lines by infection with retroviruses containing genes encoding SOX2, OCT3/4, KLF4 and c-Myc\textsuperscript{31}. We differentiated the cells from individuals with Timothy syndrome and control iPSC clones into cortical neurons using a four-stage protocol as previously described\textsuperscript{34} (Fig. 3a). To measure dendrite development in human neurons, we infected the cells with an adenov-associated virus (AAV5) expressing YFP driven by the human synapsin-1 promoter. We then monitored changes in dendrite length in response to depolarization using time-lapse microscopy (Fig. 3b). Neurons derived from Timothy syndrome and control subjects showed similar changes in dendritic length at rest (average change in total dendritic lengths during 30 min of incubation in a 5 mM KCl solution was, for Timothy syndrome samples, 3.67% and for control, 5.62% ± 2.45%; unpaired Student’s $t$-test, $P > 0.05$; Fig. 3g). These findings suggest that the mutation that causes Timothy syndrome has a dramatic effect on dendritic arborization at P14 and causes a net reduction in the basal dendritic complexity of cortical pyramidal neurons.

**Dendrite retraction in human neurons with Timothy syndrome**

Recent advances in somatic cell reprogramming\textsuperscript{31} allow the study of human neurons differentiated in vitro from individuals with genetic neurodevelopmental diseases\textsuperscript{32}. To determine whether the defects observed in mice and rat neurons are also present in humans, we infected the cells with an adenov-associated virus (AAV5) expressing YFP driven by the human synapsin-1 promoter. We then monitored changes in dendrite length in response to depolarization using time-lapse microscopy (Fig. 3b). Neurons derived from Timothy syndrome and control subjects showed similar changes in dendritic length at rest (average change in total dendritic lengths during 30 min of incubation in a 5 mM KCl solution was, for Timothy syndrome samples, 3.67% ± 3.39% and for control, 5.62% ± 2.45%; unpaired Student’s $t$-test, $P > 0.05$, mean ± s.e.m.), but depolarization led to a significant decrease ($P < 0.005$) in dendritic length in Timothy syndrome–affected neurons but caused dendrite growth in control neurons (Fig. 3c–e). Depolarization caused an increase...
We next determined whether excess Ca\(^{2+}\) influx through TS-Ca\(_{V1.2}\) causes dendrite retraction. We measured calcium currents and calcium elevations in neurons expressing TS-Ca\(_{V1.2}\) and wild-type channels. As previously reported\(^{11,13,35}\), we found that the mutation that causes Timothy syndrome decreased voltage-dependent inactivation of the channel and increased the Ca\(^{2+}\) rise triggered by depolarization of the neurons (Fig. 4a,b). To confirm that this increase depended on Ca\(^{2+}\) entering through TS-Ca\(_{V1.2}\), we transfected neurons with wild-type or TS-Ca\(_{V1.2}\) channels containing a T1036Y mutation, which renders the channels resistant to dihydropyridines such as nimodipine\(^{36}\) (Ca\(_{V1.2}\)-DHP or dihydropyridine-insensitive). When these neurons were depolarized in the presence of nimodipine to block endogenous channels, cells expressing the TS-Ca\(_{V1.2}\)-DHP channels had a substantially higher \([\text{Ca}^{2+}]_i\) rise than cells expressing the wild-type Ca\(_{V1.2}\)-DHP channels (Supplementary Fig. 5) indicating that increased Ca\(^{2+}\) influx through TS-Ca\(_{V1.2}\) led to an increase in \([\text{Ca}^{2+}]_i\), in neurons expressing these channels.

To determine whether the increased \([\text{Ca}^{2+}]_i\), caused by TS-Ca\(_{V1.2}\) underlies activity-dependent dendritic retraction, we increased \([\text{Ca}^{2+}]_i\) in wild-type neurons to levels similar to those observed in neurons affected by Timothy syndrome by depolarizing the cells in the presence of elevated extracellular Ca\(^{2+}\) (10 mM \([\text{Ca}^{2+}]_e\)) or the Ca\(_{V1.2}\) agonist BayK 8644 (5 \(\mu\text{M}\)) (Fig. 4c). Although both manipulations increased \([\text{Ca}^{2+}]_i\), to levels similar to those in neurons affected by Timothy syndrome, neither caused dendrite retraction (Fig. 4d), suggesting that in the absence of TS-Ca\(_{V1.2}\) elevating global \([\text{Ca}^{2+}]_i\) is not sufficient to trigger dendrite retraction. To provide additional evidence that dendritic retraction caused by TS-Ca\(_{V1.2}\) was not purely a consequence of elevated \([\text{Ca}^{2+}]_i\), we reduced extracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_e\)) in neurons expressing TS-Ca\(_{V1.2}\) and measured activity-dependent dendritic retraction. Reducing \([\text{Ca}^{2+}]_i\) to 0.2 mM brought the plateau of \([\text{Ca}^{2+}]_i\), in neurons affected by Timothy syndrome to wild-type levels (2 mM \([\text{Ca}^{2+}]_e\); Fig. 4e) but did not prevent dendritic retraction after depolarization (Fig. 4f). In fact, we observed dendritic retraction in neurons affected by Timothy syndrome even in the nominal absence of extracellular Ca\(^{2+}\), strongly suggesting that Ca\(^{2+}\) influx is not required for this process.

Because altering extracellular Ca\(^{2+}\) can affect neuronal signaling in unexpected ways, we tested whether Ca\(^{2+}\) influx was required for...
activity-dependent dendrite retraction in neurons affected by Timothy syndrome using an independent method. We introduced three glutamate-to-glutamine mutations (E363Q, E706Q and E1115Q) in the selectivity filter of TS-CaV1.2 that prevent calcium influx through the channel. The mutations were introduced to the WT 3EQ and mutant pore channel and imaged before and after depolarization. Scale bars, 50 μm. (f) Average change in total dendritic length in individual neurons transfected with WT CaV1.2, TS-CaV1.2 or the pore mutant TS-CaV1.2 3EQ (n ≥ 20 cells per condition; mean ± s.e.m.). (g) Representative dendrite tracings of cortical neurons expressing WT 3EQ or TS-CaV1.2 3EQ pore mutant channels and imaged before and after depolarization. Scale bars, 50 μm. (j) Average change in total dendrite length in individual neurons transfected with WT CaV1.2 or TS-CaV1.2 or their 3EQ pore mutant versions (TS = −36.3 ± 16.7 μm; TS 3EQ = −39.3 ± 18.5 μm, n ≥ 50 cells per condition; mean ± s.e.m.).

Gem is required for activity-dependent dendritic extension

One hypothesis to explain these findings is that voltage-dependent conformational changes in TS-CaV1.2 activate signaling cascades that promote retraction of dendrites in neurons affected by Timothy syndrome. To identify these pathways, we investigated channel-interacting proteins that could be linked to signaling cascades known to alter the cytoskeleton. The RGK proteins are small G proteins that can regulate RhoA signaling and bind to the β subunit of calcium channels31–34,26,28,29. The β subunit binds to a domain of the α subunit that is immediately downstream of the substitution that causes Timothy syndrome in CaV1.2. We therefore explored whether these proteins could be important for Timothy syndrome–dependent dendrite retraction. We first used reverse-transcription (RT)-PCR to determine which transcripts encoding RGK proteins are expressed in cortical neurons, and found transcripts encoding both Gem and Rem2 expressed in these cells (Supplementary Fig. 7a). We next investigated whether either Gem or Rem2 are necessary for activity-dependent...
dendritic arborization by using short hairpin (sh)RNAs to reduce the expression of each protein in developing neurons. We generated three shRNAs to Rem2 and Gem and verified by western blotting that these shRNAs reduced the expression of Rem2 or Gem proteins (Supplementary Fig. 7b–e). Reducing Rem2 expression had no effect on activity-dependent dendritic growth (Fig. 5a), but reducing Gem expression prevented activity-dependent dendritic arborization in a manner that was consistent with the effectiveness of each shRNA.

Figure 5 Reducing Gem expression in cortical neurons prevents activity-induced dendritic arborization. (a) Average change in total dendrite length in dissociated cortical neurons transfected with control shRNA, Rem2 shRNA #1 or Rem2 shRNA #2 after incubation in control or depolarizing conditions (n ≥ 16 cells per condition; mean ± s.e.m.). (b) Representative dendrite tracings of cortical neurons transfected with control shRNA or Gem shRNA #2 and imaged before and after depolarization with 67 mM KCl. Scale bar, 50 μm. (c) Average change in total dendrite length in neurons transfected with control shRNA, Gem shRNA #1 or Gem shRNA #2 in control or depolarizing conditions (n ≥ 20 cells per condition; mean ± s.e.m.; ** P < 0.001 by two-way ANOVA, Bonferroni post-test). (d) Cropped anti-Myc western blot of lysates from HEK 293T cells cotransfected with constructs encoding Myc-Gem or Myc-Gem-R (which contains silent mutations that render it resistant to anti-Myc western blot of lysates from HEK 293T cells cotransfected with constructs encoding Myc-Gem or Myc-Gem-R (which contains silent mutations that render it resistant to Gem shRNA #2) and either control shRNA or Gem shRNA #2. Cells were also transfected with a construct encoding Myc-CFP as a transfection and loading control. Full-length blots are presented in Supplementary Figure 10. (e) Average change in total dendrite length in neurons co-transfected with control shRNA or Gem shRNA #2 and constructs expressing either CFP, Gem, or the shRNA-resistant Gem-R. The amount of activity-dependent dendritic arborization in neurons transfected with Gem-R and Gem shRNA #2 is not significantly different from that in cells expressing control shRNA (Gem-R + control shRNA = 168.9 ± 23.9 μm; Gem-R + shRNA #2 = 141.6 ± 23.8 μm). (n ≥ 20 cells per condition; mean ± s.e.m.; * P < 0.05, ** P < 0.001 by two-way ANOVA, Bonferroni post-test).

Figure 6 Overexpression of Gem prevents dendritic retraction in TS-Cav1.2-expressing neurons and its effects are dependent on association with the Ca3β subunit. (a) Average change in total dendrite length in neurons expressing wild-type (WT) Cav1.2 or TS-Cav1.2 along with either CFP or Gem (n ≥ 18 cells per condition; mean ± s.e.m.). (b) Average change in total dendrite length in neurons expressing CFP or Gem as well as the pore mutant (4EQ) versions of WT Cav1.2 or TS-Cav1.2 channels (n ≥ 17 cells per condition; mean ± s.e.m.). Neurons expressing TS-Cav1.2 4EQ (TS 4EQ) and Gem showed a similar amount of activity-dependent dendritic arborization to neurons expressing WT Cav1.2 4EQ channels (TS 4EQ + Gem = 232.2 ± 38.8 μm; WT 4EQ + CFP = 240.9 ± 36.9 μm; WT 4EQ + Gem = 233.1 ± 38.8 μm). (c) Immunoprecipitation of YFP-Cav1.2 and HA-Cav1.2 with Flag-Myc-Gem in lysates of Neuro2a cells that were transfected with a WT or TS-Cav1.2 α-subunit as well as HA-Cav1.3 and/or Flag-Myc-Gem. Full-length blots are presented in Supplementary Figure 10. (d) Average change in total dendritic length in neurons cotransfected with plasmids expressing WT Cav1.2 or TS-Cav1.2 and Gem containing a R196A or V223A mutation that renders Gem unable to bind to Cav1.2 (n ≥ 10 cells per condition; mean ± s.e.m.). (e) Immunoprecipitation of YFP-Cav1.2 and HA-Cav1.3 with Flag-Myc-Gem in lysates of Neuro2a cells that were transfected with either a WT or TS-Cav1.2 α-subunit as well as HA-Cav1.3 and/or Flag-Myc-Gem. Full-length blots are presented in Supplementary Figure 10. (f) Relative intensity of co-immunoprecipitated TS-Cav1.2 channel bands compared to WT channel bands (mean ± s.e.m.; n = 3).
(Fig. 5b,c). Inhibition of activity-dependent dendritic arborization by Gem shRNA #2 was specific because expression of a mutant Gem that is resistant to the shRNA could reverse this effect (Fig. 5d,e). These results suggest that Gem is necessary for activity-dependent dendritic growth in cortical neurons.

We next asked whether Gem overexpression could prevent TS-CaV1.2-mediated dendritic retraction. We introduced a Gem expression plasmid into wild-type or TS-CaV1.2–expressing neurons and measured dendritic arborization. We found that exogenous expression of Gem had no effect on dendritic arborization in wild-type neurons but completely prevented dendritic retraction in TS-CaV1.2–expressing neurons (Fig. 6a). Gem overexpression also prevented dendritic retraction triggered by the TS-CaV1.2 4EQ channel, which contains pore mutations that prevent Ca\(^{2+}\) influx (Fig. 6b). This suggests that the ability of Gem to rescue TS-CaV1.2–mediated dendritic retraction was independent of the effects of Gem on intracellular Ca\(^{2+}\) levels.

Overexpression of Gem has been found to inhibit RhoA signaling and induce neurite outgrowth in several cell lines\(^{6,28,29}\). To determine whether Gem acts as a global inhibitor of RhoA in neurons or whether its activity is dependent on a direct interaction with CaV1.2, we generated Gem mutants that do not bind to the β subunit\(^{22}\). Gem mutants bearing either an R196A or V223A mutation no longer immunoprecipitated with the β subunit (Fig. 6c) but preserved their ability to increase neurite extension in a neuroblastoma cell line, suggesting that they can still inhibit RhoA (Supplementary Fig. 8).

We expressed the Gem R196A or V223A mutants in TS-CaV1.2–expressing neurons and measured dendritic retraction upon depolarization. Overexpression of either Gem mutant did not prevent TS-CaV1.2–mediated dendritic retraction (Fig. 6d). This suggests that interaction between the β subunit and Gem is required for Gem to prevent the dendritic retraction caused by TS-CaV1.2.

Gem also has been shown to inhibit CaV1.2 activity by interfering with the trafficking of the channel to the cell membrane\(^{24}\). We therefore explored the hypothesis that Gem expression prevented retraction by reducing the membrane expression of TS-CaV1.2. We transfected neurons with constructs encoding either wild-type or TS-CaV1.2 channels and support the hypothesis that TS-CaV1.2 channels cause dendritic retraction by reducing the membrane expression of TS-CaV1.2. We transfected neurons with constructs encoding either wild-type or TS-CaV1.2 channels and support the hypothesis that TS-CaV1.2 channels cause dendritic retraction by reducing the membrane expression of TS-CaV1.2. We transfected neurons with constructs encoding either wild-type or TS-CaV1.2 channels and support the hypothesis that TS-CaV1.2 channels cause dendritic retraction by reducing the membrane expression of TS-CaV1.2. We transfected neurons with constructs encoding either wild-type or TS-CaV1.2 channels and support the hypothesis that TS-CaV1.2 channels cause dendritic retraction by reducing the membrane expression of TS-CaV1.2.
RhoA activation is necessary for TS-CaV1.2–dependent retraction
A possible downstream target of TS-CaV1.2 channels is the GTPase RhoA, which mediates dendrite retraction in many cell types and genetic systems.49–51. We therefore investigated whether activation of TS-CaV1.2 activates RhoA in cortical neurons. We measured Ser19 phosphorylation of myosin light chain 2 (MLC2), a biochemical event that is downstream of RhoA activation.42. Under control conditions, cells expressing wild-type CaV1.2 or TS-CaV1.2 showed low levels of phosphorylated MLC2. After depolarization, TS-CaV1.2–expressing neurons exhibited significantly greater levels of phospho-MLC2 than wild-type cells did (P < 0.01; Fig. 7a,b). This indicates that activation of the TS-CaV1.2 channel leads to upregulation of RhoA signaling.

To determine whether TS-CaV1.2 activation depends on Ca2+ influx, we investigated whether the TS-CaV1.2 3EQ channel could increase phospho-MLC2 levels after depolarization. We found that TS-CaV1.2 3EQ channels significantly increased phospho-MLC2 levels (P < 0.05; Fig. 7c) indicating that activation of RhoA was Ca2+-independent.

We next investigated whether activation of RhoA in wild-type neurons can phenocopy the effects of TS-CaV1.2. We found that expression of a constitutively active form of RhoA (RhoV14) is sufficient to cause dendritic retraction (Fig. 7d). To determine whether activation of RhoA is necessary to cause dendrite retraction in TS-CaV1.2–expressing neurons, we inhibited RhoA activation either by expression of a dominant negative form of RhoA (RhoN19; Fig. 7e) or application of a cell-permeant RhoA inhibitor (C3 transferase; Fig. 7f). Both RhoN19 expression and treatment with C3 transferase prevented activity-dependent retraction, suggesting that this pathway is required for the effects of the channel. Finally, we investigated whether exogenous expression of Gem prevents RhoA activation in neurons affected by Timothy syndrome (Fig. 7g,h). We expressed Gem in neurons expressing TS-CaV1.2 and found significantly decreased levels of phospho-MLC2 in these cells (P < 0.05), which suggested that Gem prevents ectopic activation of RhoA by TS-CaV1.2. Taken together, these studies support a model by which a conformational change in the TS-CaV1.2 reduces recruitment of Gem, leading to ectopic activation of RhoA. RhoA in turn activates biochemical cascades that result in dendrite pruning and retraction.

DISCUSSION
The mutation that causes Timothy syndrome leads to decreased calcium- and voltage-dependent inactivation of the CaV1.2 channel, but the cellular consequences of this defect are not well understood. Here we showed that TS-CaV1.2 channels caused activity-dependent dendrite retraction in rat, mouse and human neurons. This retraction did not depend on excessive Ca2+ influx through TS-CaV1.2 but was mediated by a Ca2+-independent mechanism. Our studies are consistent with a model in which activated CaV1.2 channels recruit Gem and thereby prevent activation of RhoA, allowing dendrite retraction. TS-CaV1.2.1 does not recruit adequate levels of Gem, leading to ectopic activation of RhoA and dendrite retraction.

These studies provide insights into the pathophysiology of Timothy syndrome and other ASDs. Studies of brains from individuals with ASDs and intellectual disability suggest that impaired dendrite formation is a common feature of many neurodevelopmental disorders. Impaired minicolumn formation has been observed in postmortem brains of individuals with ASDs39, and it has been suggested that individuals with autism have changes in long-range and short-range neural connections.44,45. Furthermore, several studies have identified defects in dendritic arborization in mouse models of disorders such as Rett syndrome and Fragile X syndrome46,47. Our data uncovered a direct cellular link between the mutation that causes Timothy syndrome in LTCs and defects in dendritic remodeling, and could shed light on the role of arborization defects in other developmental disorders.

We also observed activity-dependent dendrite retraction in human neurons derived from individuals with Timothy syndrome. Although many studies in rat and mouse models of ASD have been informative, there are examples where defects in such models could not be confirmed in humans. Our study showed that dendrite retraction occurs in rat, mouse and human neurons. This provides strong evidence that dendrite retraction is a feature of Timothy syndrome and shows that iPSC-derived neurons can recapitulate defects observed in neurons in vivo.

Our study also provides tantalizing information about the mechanism by which TS-CaV1.2 causes dendrite retraction. We found that dendritic retraction was independent of the amount of Ca2+ influx through the channel. This result was unexpected because changes in [Ca2+]i are thought to be central to LTC-mediated signal transduction in neurons. We have three lines of evidence that support this conclusion. First, elevating [Ca2+]i, in wild-type neurons to the same levels observed in Timothy syndrome neurons did not trigger dendrite retraction. Second, reducing extracellular Ca2+ in neurons affected by Timothy syndrome, even to resting levels, did not prevent activity-dependent retraction. Finally, introducing substitutions in TS-CaV1.2 that prevent Ca2+ influx through the channel does not prevent the channel from triggering retraction. These results are consistent with the idea that CaV1.2 channels can activate signaling cascades by voltage-dependent conformational changes48–50, reminiscent of the physical mechanism by which a related channel CaV1.1 causes excitation-contraction coupling in skeletal muscle50.

A possible link between conformational changes in CaV1.2 and activation of RhoA is the RGK protein Gem. Gem directly binds to both the α and β subunits of CaV1.2 channels and can modulate channel gating. We found that Gem overexpression prevented dendritic retraction triggered by TS-CaV1.2 and this effect depended on the ability of Gem to bind to the channel. Gem was also required for dendritic arborization in control cells because reduction of Gem with shRNAs prevented activity-dependent dendrite growth. The mechanism by which Gem controls dendrite growth in response to LTCs activation is unclear. One possibility is that activated CaV1.2 recruits Gem, which in turn causes local inhibition of RhoA, preventing dendrite retraction and allowing dendrite extension. Gem and the other RGK proteins inhibit RhoA signaling by activating RhoA GAPs and by inhibiting the downstream kinase ROCK. By binding weakly to Gem, TS-CaV1.2 does not suppress RhoA activation and leads to dendrite retraction, which dominates over dendrite extension.

Another possibility is that Gem prevents retraction by forcing TS-CaV1.2 into an inactivated state where it no longer activates RhoA. This is consistent with the finding that Gem overexpression decreased LTC activity and Ca2+ influx in neurons by increasing the fraction of CaV1.2 channels in a nonconducting state. Despite its negative effects on Ca2+ influx, Gem expression was required for activity-dependent dendritic arborization, and Gem overexpression did not inhibit dendrite growth. This suggests that Gem is necessary for activating the signaling cascades that promote dendrite extension despite reduced Ca2+ influx across the membrane.

In summary, our studies showed that TS-CaV1.2 caused dendrite retraction in mouse, rat and human neurons and identified a new signaling modality for the CaV1.2 channel. This sets the stage for studies aimed at understanding the underlying mechanism of ASD and intellectual disability in these children. In addition, these results unveil a mode of signaling for LTCs that is independent of Ca2+ and directly links CaV1.2 channels to the regulation of RhoA signaling in the brain.
Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

Acknowledgments

We thank K. Timothy and the individuals with Timothy syndrome who participated in this study. J. Bernstein and J. Hallmayer for recruiting the subjects for this study, and E. Nigh for critical reading of the manuscript. J.F.K. was supported by the National Institutes of Health under Ruth L. Kirschstein National Research Service Award (F11 NR005549-03) from the National Institute of Neurological Disorders and Stroke. Financial support was provided by a US National Institutes of Health Director’s Pioneer Award and a Simons Foundation Grant to R.E.D.; the International Brain Research Organization Outstanding Research Fellowship and the Tashia and John Morgridge Endowed Fellowship to S.P.; a Japan Society of the Promotion for Science Postdoctoral Fellowship for Research Abroad and American Heart Association Western States to M.Y.; and a California Institute for Regenerative Medicine Postdoctoral Fellowship to O.S. We are grateful for funding from B. and F. Horowitz, M. McCafferey, B. and J. Packard, P. Kwan and K. Wang.

Author Contributions

R.E.D. and J.F.K. designed the experiments and wrote the manuscript; J.F.K. performed all of the cellular assays in rodent cells, including the calcium imaging and immunocytochemistry studies, all of the mice breeding and the in vitro studies of dendritic arborization; S.J.P. differentiated iPSCs into neurons and performed the dendritic arborization experiments in human cells; A.S. performed and analyzed the electrophysiology experiments; M.Y. generated and characterized the iPSCs; R.S. contributed to the analysis of dendrites in iPSC-derived neurons; R.R. generated the Timothy syndrome mice.

Competing Financial Interests

The authors declare no competing financial interests.

Published online at http://www.nature.com/doifinder/10.1038/nrn.3307. Reprints and permissions information is available online at http://www.nature.com/ reprints/index.html.

1. Dolmetsch, R. Excitation-transcription coupling: signaling by ion channels to the nucleus. Sci. STKE 2003, PE4 (2003).
2. Tonelli, A. et al. Early onset, non fluctuating spinocerebellar ataxia and a novel missense mutation in CACNA1A gene. J. Neurosci. 241, 13–17 (2006).
3. Wang, K. et al. Common genetic variants on 5p14.1 associate with autism spectrum disorders. Nature 459, 528–533 (2009).
4. Nyegaard, M. et al. CACNA1C (rs1006737) is associated with schizophrenia. Mol. Psychiatry 15, 119–121 (2010).
5. Tottene, A. et al. Familial hemiplegic migraine mutations increase Ca2+ influx through a single human Cav2.1 channel and decrease maximal Cav2.1 current density in neurons. Proc. Natl. Acad. Sci. USA 99, 13284–13289 (2002).
6. Saplwski, i. et al. CACNA1H mutations in autism spectrum disorders. J. Biol. Chem. 280, 41864–41871 (2005).
7. Barrett, C.F. & Tsien, R.W. The Timothy syndrome mutation differentially affects voltage- and calcium-dependent inactivation of CaV1.2 L-type calcium channels. Proc. Natl. Acad. Sci. USA 102, 8089–8096 (2005).
8. Dolmetsch, R.E., Pajvani, U., Fife, K., Spotts, J.M. & Greenberg, M.E. Signaling through single human CaV2.1 channels and decrease maximal CaV2.1 current density in neurons. Mol. Psychiatry 11, 1657–1662 (2011).
9. Saplwski, i. et al. Severe arrhythmia disorder caused by cardiac L-type calcium channel mutations. Proc. Natl. Acad. Sci. USA 102, 10458–10463 (2005).
10. Lee, T., Winter, C., Marticke, S.S., Lee, A. & Luo, L. Essential roles of Draposhia RHoA in the regulation of neuroblast proliferation and dendritic but not axonal morphogenesis. Neuroreport 25, 307–316 (2010).
11. Svetman, C.A. et al. Extension, retraction and contraction in the formation of a dendritic cell dendrite: distinct roles for Rho GTPases. Eur. J. Immunol. 32, 2074–2083 (2002).
12. Kranenburg, O. et al. Activation of Rhoh by lysophosphatidic acid and Gai12/13 subunits in neuronal cell bodies: induction of neurite retraction. Mol. Biol. Cell 10, 1851–1857 (1999).
13. Totsukawa, G. et al. Distinct roles of ROCK (Rho-kinase) and MLCK in spatial regulation of PLC phosphorylation for assembly of stress fibers and focal adhesions in 3T3 fibroblasts. J. Cell Biol. 150, 797–806 (2000).
14. Casanova, M.F. et al. Minicolumnar abnormalities in autism. Acta Neuropathol. 112, 287–303 (2006).
15. Belmonte, M.K. et al. Autism and abnormal development of brain connectivity. J. Neurosci. 24, 9228–9234 (2004).
16. Geschwind, D.H. & Levitt, P. Autism spectrum disorders: developmental disconnection syndromes. Curr. Opin. Neurobiol. 17, 103–111 (2007).
17. Cohen-Kutner, M., Nachmanni, D. & Atlas, D. CaV2.1 (P/Q channel) interaction with synaptic proteins is essential for depolarization-evoked release. Proc. Natl. Acad. Sci. USA 108, 15432–15437 (2011).
18. Tottene, A. et al. Identification and differential subcellular localization of the neuronal RhoA GTPase and tau: morphological changes induced by gem GTPase in CHO cells are antagonized by tau. J. Biol. Chem. 279, 27272–27277 (2004).
19. Tottene, A.K., Katz, L.C. & Lo, D.C. Neurotrophin regulation of dendritic growth and plasticity by local and global calcium dynamics. Diabetologia 43, 403–409 (2000).
20. Barres, B.A. et al. Selective regulation of neurite extension and synapse formation by the beta but not the alpha isoform of CalDML. Neuron 34, 999–1010 (2002).
21. Wang, K. et al. Activity-dependent regulation of dendritic growth and patterning. Nat. Rev. Neurosci. 3, 803–812 (2002).
or dominant-negative (N19) RhoA N-terminally tagged with CFP, which were then transfected with a CFP vector to generate plasmids encoding constitutively active (V14) RhoA.

Plasmids encoding Myc-Gem or Myc-Rem2 were generated using Gateway technology from rat embryonic day 18 (E18) cortex using the RNA Easy Kit (Qiagen). Sequences are available upon request.

Rem2 (NM_022685) were cloned from cDNA prepared from mRNA isolated with the XL kit (Stratagene) to generate plasmids encoding the Gem R196A and Gem V223A (T668C). All constructs and mutations were verified by full sequencing.

RGK constructs. The rat sequences encoding Gem (US National Center for Biotechnology Information (NCBI) reference sequence NM_001106637) or Rem2 (NM_022685) were cloned from cDNA prepared from mRNA isolated from rat embryonic day 18 (E18) cortex using the RNA Easy Kit (Qiagen). Plasmids encoding Myc-Gem or Myc-Rem2 were generated using Gateway technology (Invitrogen) by inserting the cloned rat Gem or Rem2 sequences into the TOPO sites of the pCR8 entry vector and then transferring the Gem- or Rem2-coding sequence into the destination vector pDEST Flag/Myc-, which contains a CMV promoter and N-terminal Flag and Myc epitope tags in frame with ATIR acceptor sequences. A pDEST Flag-Myc Gem plasmid was generated in the same fashion (except CFP was cloned from the pDNA3-CFP plasmid) and was used as a control in all experiments using the pDEST Flag-Gem or Rem2 plasmids. Site-directed mutagenesis was performed with the QuickChange II polymerase (Stratagene) to create the Gem R196A and Gem V223A mutants. The following were changed in the Gem coding sequence of the pDEST Flag/Myc Gem construct: Gem R196A (CG86G and CG87C), Gem V223A (T668C). All constructs and mutations were verified by full sequencing of the plasmids. All primers and sequences are available upon request.

RGK shRNA constructs. Two 19-mer oligonucleotide sequences targeted to the rat Gem sequence were designed using the Whitehead Institute Bioinformatics and Research Computing siRNA design tool. Two 19-mer oligonucleotide sequences targeted to the rat Rem2 sequence were based on sequences that have been previously published. The oligonucleotide sequences are as follows: Gem shRNA #1 (GATCCATATGACGCTGAC), Gem shRNA #2 (CCATCAGCTAGCTGACCAT), Rem2 shRNA #1 (GAGGTCGAGGAGGACCT AT), Rem2 shRNA #2 (GCAGGTCGATGTCGACCA). Short hairpin oligonucleotides were designed using (Clontech’s siRNA sequence designer) and inserted into RNAi-Ready pSIREN-DNR-DSRed-Express vector (Clontech) by ligating into the BamHI and EcoRI sites. For the control shRNA, a luciferase shRNA annealed oligonucleotide (Clontech, provided with pSIREN vector) was ligated into the pSIREN-DNR-DSRed-Express vector following the same procedure. To generate a plasmid encoding a Gem protein resistant to suppression by Gem shRNA #2, mutagenesis was performed as above on the pDEST Flag/Myc Gem construct. The three silent mutations were introduced directly into the Gem coding sequence with the following nucleotide changes: A384T, G387A and C390T. Mutations were verified by full sequencing and primers and sequences are available upon request.

Co-immunoprecipitation and western blotting. To test effectiveness of shRNAs, HEK 293T cells grown in DMEM with 5% FBS and transfected with standard Ca2+ phosphate procedures. Each well of the 6-well plate was transfected with 2 μg shRNA, 100 ng pDEST-Flag/Gem, Flag-Myc Gem-R or Flag-Myc Rem2 and 100 ng of pDEST-Flag-Myc Gem. Cells were lysed 36–48 h after transfection, and immunoblots were prepared as described previously. Briefly, cells were lysed in 1× SDS buffer, and lysates were boiled for 10 min at 100 °C, and 10 μl of lysates were run on a 10% polyacrylamide gel. Membranes were blotted with an anti-Myc antibody (1:1,000, 4-A6, Upstate Biotechnology). ImageJ (US National Institutes of Health) was used to quantify the intensity of the bands. The intensity of the RGK (Gem, Gem-R or Rem2) band was normalized to the intensity of the Myc-CFP band in the same lane, which was used as a transfection and loading control.

The Gem and β3 co-immunoprecipitation experiments were performed using HEK 293T cells grown in DMEM with 5% FBS and transfected with standard Ca2+ phosphate procedures. Each well of the 6-well plate was transfected with 1 μg pDEST-Flag-Myc Gem, 1 μg HA-β3 or 1 μg of each alone. Cells were lysed 18–24 h after transfection with 300 μl lysis buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1 mM MgCl2 and 0.5% Triton X-100) per well. Protease inhibitor cocktail tablets (Roche Diagnostics) were added to lysis buffer immediately before lysing cells. Cell lysates were incubated for 1 h at 4 °C, centrifuged for 15 min to remove cell debris, and 250 μl of supernatant was incubated with 25 μl of anti-Flag M2 agarose beads (Sigma) for 3–4 h at 4 °C. Agarose beads were rinsed three times for 15 min each in lysis buffer, and then proteins were eluted with 50 μl of 1× SDS buffer, boiled for 8 min at 95 °C and frozen at −80 °C. Elutions were run on 10% polyacrylamide gels using standard western blot protocols and membranes were blotted with either anti-Myc antibody (1:1,000, 4A6, Upstate Biotechnology) or anti-HA 3F10 antibody (1:1,000, Roche).

The γFP-Cav1.2, Gem and β3 co-immunoprecipitation experiments were performed using N2020 ts cells grown in MEME with 10% FBS in 10-cm dishes and transfected using Lipofectamine 2000 (Invitrogen) following manufacturer’s instructions. Each 10-cm plate was transfected with 6 μg pDEST-Flag-Myc Gem, 6 μg HA-β3 and/or 12 μg of γFP-Cav1.2. Cells were lysed 22–24 h after transfection with 1 ml lysis buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1 mM MgCl2, 1% Triton X-100) per plate. Protease inhibitor cocktail tablets (Roche Diagnostics) were added to lysis buffer immediately before lysing cells. Cell lysates were incubated for 1 h at 4 °C, centrifuged for 15 min to remove cell debris, and 900 μl of supernatant was incubated with 40 μl of anti-Flag M2 agarose beads (Sigma) for 3–4 h at 4 °C. Agarose beads were rinsed two times for 15 min each in lysis buffer, then one time for 15 min in 1× TBS and then proteins were eluted with 150 μg/ml 3× Flag peptide for 30 min at 4 °C. Supernatant was then mixed with 2× SDS sample buffer and boiled for 5 min at 85 °C. Elutions (10 μl of each) were run on 12% Bis-Tris gradient gels using standard western blot protocols and membranes were blotted with either anti-GFP rabbit serum (1:1,000, Invitrogen Molecular Probes), anti-Myc antibody (1:1,000, 4A6, Upstate Biotechnology) or anti-HA 3F10 antibody (1:1,000, Roche).

Dissociated cell cultures and transfections. Cortical neurons from E17-E19 Sprague Dawley rats (Rattus norvegicus) were cultured as described previously and plated at 1 × 104 neurons/ml on poly–ornithine– and laminin-coated 24-or 96-well tissue culture plates. Neurons were maintained in Basal Medium Eagle with 5% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine and 1% glucose. Neurons were transfected using Lipofectamine 2000 and assayed 30–48 h after transfection. For the dendritic arborization assay, Ca2+ imaging and phospho-MLC2 assay, neurons were transfected at 2–3 days in vitro (DIV) with a 2:2:0.5 ratio of oligo (pDNA4 Cav1.2, γFP and YFP constructs). Molar ratios have been optimized so that the majority (>70%) of YFP-positive neurons also express the exogenous channel. For dendritic arborization assays using shRNAs targeting Gem, neurons were transfected at 2–3 DIV with a 2:0.5 ratio of psIREN shRNA and pGW1 YFP, and imaged 48–72 h after transfection. The DiRed expression from the pSIREN construct was used to determine cotransfection efficiencies and >90% of cells expressing YFP also expressed the shRNA. For dendritic arborization assays involving Rh0V4 or Rh0N19 expression neurons were transfected with a 1:0.5 ratio of pDNA3 CFP-RhoV4 (or pDNA3 CFP-as control) and pGW1 YFP plasmids or with a 2:2:0.5 ratio of olgio (pDNA4 CaV1.2): β1b: pDNA3 CFP-RhoN19: YFP constructs. The same ratios were used for dendritic arborization assays involving Gem overexpression with pDEST Flag/Myc CFP (control) or pDEST Flag/Myc Gem replacing the Rh0N19 construct. For all experiments ratios were optimized so that the majority (>70%) of YFP-positive neurons also expressed the channel and other transfected constructs.

Dissociated culture immunocytochemistry. Dissociated cortical neurons were plated at 5 × 104 neurons/well on 15-mm cover slips placed in 24-well tissue culture plates. Thirty-six to forty-eight hours after transfection, neurons were fixed in 4% paraformaldehyde and 2% sucrose, permeabilized with 0.25% Triton X-100 and blocked in 3% BSA in phosphate-buffered saline (PBS). Neurons were incubated with anti-Myc antibodies (1:1,000, 4A6, Upstate Biotechnology) or anti-GFP rabbit serum (1:1,000, Roche). Cells were incubated with Alexa Fluor 594 or 488-conjugated secondary antibodies and images were captured using a Nikon TE2000-U inverted microscope (Nikon). Images were analyzed and processed using ImageJ.
anti-Cav1.2 antibodies (1:150, Chemicon AB5156) diluted in 3% BSA followed by incubation with Alexa Fluor 594–conjugated secondary antibodies (1:1,000, Invitrogen) and then counterstained with Hoechst 333258 (Molecular Probes, 1:10,000) for 15 min before mounting. YH-Cav1.2–expressing neurons were stained as outlined previously51. Briefly, neurons were fixed with 4% paraformaldehyde, blocked with 3% BSA, incubated with an anti-HA 3F10 antibody (1:1,000, Roche 12158676001) and treated with a goat anti-rat Alexa Fluor 594 secondary antibody (1:1,000, Molecular Probes). Slides were visualized by epifluorescence microscopy using a cooled charge-coupled device (CCD) camera (Hamamatsu) coupled to an inverted Nikon Eclipse E2000-U microscope and OpenLab 4 software (Improvision).

**Phospho-MLC2 assays.** Twenty-four to thirty-six hours after transfection, neurons were placed in a control Tyrode's solution or depolarized in a 65 mM KC1 Tyrode's solution for 1 h and then fixed with 4% paraformaldehyde in PBS for 10–15 min at room temperature. Neurons were then washed with 1× PBS two times and blocked in 3% BSA with 0.25% Triton X-100 in PBS for 30 min at room temperature. Neurons were then incubated with anti-phospho-myosin light chain 2 (Ser19) antibodies (1:1,000, Cell Signaling Technology) diluted in 3% BSA overnight at 4 °C. Neurons were then incubated with Alexa Fluor 594–conjugated secondary antibodies (1:1,000, Invitrogen) for 1 h and then counterstained with Hoechst 333258 (1:10,000) for 10 min before mounting cover slips on slides. Slides were visualized by epifluorescence microscopy using a cooled CCD camera (Hamamatsu) coupled to an inverted Nikon Eclipse E2000-U microscope and OpenLab 4 software (Improvision). The fluorescence intensity of phospho-MLC2 staining in YFP-positive neurons was quantified using OpenLab 4 software. At least 50 cells were quantified in each condition, and experiments were repeated at least three times. A histogram of intensity values in the control or stimulated conditions was used to determine a threshold value that separated pMLC2-positive cells from background staining. This value was used to quantify the percentage of cells in each condition that were pMLC2-positive.

**Ca2+ imaging.** Cortical neurons were loaded with 1 mM Fura-2-acetoxyethyl ester (Invitrogen) for 30 min at 37 °C in cortical medium and placed in a specialized imaging chamber. Neurons were pre-incubated and imaged in Tyrode's solution (129 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 30 mM glucose, 25 mM Hepes and 0.1% BSA) for 100 ms followed by perfusion with a Tyrode's solution containing elevated potassium (75 mM NaCl, 67 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 30 mM glucose, 25 mM Hepes and 0.1% BSA). Imaging of selected transfected neurons (YFP-positive) was performed at 25 °C on an inverted epifluorescence microscope with cooled CCD camera and automated stage. Time-lapse excitation ratio images were collected and quantified using OpenLab 4 software as described previously56. Values were converted to [Ca2+]i, using standard calibration procedures.

**Electrophysiology.** HEK-293 cells were grown in EMEM (30–2003, ATCC) supplemented with 10% FBS, penicillin G (100 U/mL) and streptomycin (0.1 mg/mL). Cells plated in 35-mm dishes were transiently transfected with pCMV4 CaV1.2 plasmids encoding wild-type or mutant Cav1.2 mRNAs (10 µg, 2.5 µg, 0.25 µg and 0.05 µg), using Lipofectamine 2000. We optimized the number of days in culture, light on–off conditions and light intensity so that ~50% of transfected HEK-293 cells were fluorescent after 4 d in culture (DIOC). After 2–4 d in culture, transfected cells were washed twice with PBS, and transfected cells were transfected with plasmids encoding Cav1.2– or Cav1.2-TS, and YFP at a 1:1:0.1 ratio using Lipofectamine 2000 reagent (Invitrogen) (30 µl). After 15–20 h, medium was changed to neuronal culture medium, in which cells were kept for an additional 20–24 h. This conditioning medium containing viral particles was filtered and immediately used for neuronal transduction.

To perform photostimulation experiments, dissociated hippocampal neurons were initially cultured for 6–7 h in the conditioning medium containing viral particles. Under these experimental conditions, ~80–90% of neurons were fluorescent after 4 d in culture (DIOC). At 3–4 DIC, neurons were transfected with plasmids encoding Cav1.2– or Cav1.2-TS, and YFP at a 1:1:0.1 ratio using Lipofectamine 2000. We optimized the number of days in culture, light intensity and the duration of light exposure to achieve reliable induction of action potentials in ChR2-expressing neurons without compromising survival. Photostimulation experiments were carried out 6 d after transfection (9 DIC) using a Zeiss Observer.Z1 microscope (Carl Zeiss) equipped with automated stage, X-Cite light source (EXFO) and ×10 objective. During each experiment, neurons were kept in modified Tyrode's solution (129 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 30 mM glucose, 25 mM HEPES, pH 7.4) supplemented with 2% BSA at 32–34 °C. All imaging experiments were performed using the Velocity software (Improvision). For image analysis, background correction was performed in Adobe Photoshop SC3 using the ‘Levels’ function so that the brightest and the darkest pixels had the same intensity in all the pictures.

To analyze dendritic arborizations in these experiments, we used the HCA-vision software (CSIRO), which performs a semiautomatic analysis of cell morphology. Parameters for dendrite detection, once set, were kept constant for all the pictures. Statistical analysis was done using Excel (Microsoft) and Prism 5 (GraphPad).

**Dendritic arborization assay and time-lapse imaging.** Thirty-six to forty-eight hours after transfection, neurons were placed in a control Tyrode's solution or stimulated with Tyrode's solution containing 67 mM KCl and time-lapse images of cells were taken over a 9-h stimulation period at 10× magnification, using a high-throughput automated epifluorescence microscope with an environmentally controlled stage (ImageXpress, Molecular Devices). Imaging was performed on duplicate wells, with at least 20 images taken per well. Individual YFP-positive neurons were chosen in a blinded fashion such that the researcher was unaware of the treatment condition (well numbers were randomly recorded using an automated computer program). For each cell, dendrites were traced and measured at the 0 h and 9 h time points using ImageJ and NeuronJ programs58. The change in the number and length of primary dendrites and branch dendrites for each neuron was then measured and calculated. Similar methods were also used to perform time-lapse imaging analysis of neurons placed in a control Tyrode's solution or stimulated with Tyrode's solution containing 67 mM KCl, and imaged every 10 min over 60–80 min.

**Golgi staining of mouse brain and analysis of layer 2/3 dendritic arbors.** CaNav1.2+/m20 mice (Mus musculus) containing the G406R mutation in exon 8A of the CaNav1.2 gene were generated as described previously30. Mice were backcrossed onto a C57BL/6 background and both male and female mice were used for all analyses. Golgi staining on brains from postnatal day 7 or 14 wild-type and CaNav1.2+/m20 littermate mice was performed using the Rapid GolgiStain Kit (FD Neurotechnologies) following the manufacturer's instructions. Briefly, brains from postnatal day 14 mice were removed and immersed in Golgi solution (solution A plus solution B) in the dark for 2 weeks. Brains were then cryoprotected for at least 48 h in solution C, sectioned at 150 µm thickness on a microtome, mounted onto gelatin-coated slides and developed following the manufacturer's protocol. Matched sections containing the frontal cortex were imaged using a ×20 objective and bright-field illumination on a Zeiss Axiosimager M1 microscope with IRM digital camera system. Z-stacks were collected at ×20 across the width of the section and exported to Neurolucida software (MBF Biosciences) for analysis. The basal dendritic arbors of cortical layer 2/3 pyramidal neurons were reconstructed and measured using Neurolucida. All imaging and analysis was done blinded to the genotype of the mice. All experiments on mice or rats were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Stanford University and following US National Institutes of Health guidelines for animal welfare.
iPSC generation and neural differentiation. The iPSC clones were generated using retroviruses from fibroblasts harvested from two individuals with Timothy syndrome and two healthy subjects, as previously reported\textsuperscript{33,34}. Informed written consent was obtained from all human subjects used for this study. Two lines (clones) from one individual with Timothy syndrome (T7643-7 and T7643-32), one line from a second individual with Timothy syndrome (T9862-61) and three lines from two unaffected controls (NH2-6, JM23-16 and JM23-9) were differentiated into neurons and used for live imaging dendrite tracing. All the iPSC lines used in this study were extensively characterized previously\textsuperscript{33,34}. Neural differentiation of the pluripotent stem cells was performed as previously described\textsuperscript{34}. Briefly, cellular aggregates were generated from iPSC colonies by enzymatic dissociation and kept in suspension for 5 d. For neural induction, the cellular aggregates were plated on a polyornithine/laminin-rich substrate. After 10 d, neural rosettes were mechanically isolated and expanded in suspension as neurospheres for an additional 7 d before the initiation of differentiation by replating, FGF2/EGF withdrawal and the addition of BDNF and NT3. The vast majority of neurons obtained with this protocol expressed cortical layer specific markers, as assessed by single cell quantitative PCR\textsuperscript{34}.

Tracing of dendrites in iPSC-derived neurons. Neuronal cultures, after 5 weeks of in vitro differentiation were infected with an adeno-associated virus (AAV5) carrying a YFP reporter gene under the control of the human Synapsin-1 promoter. Neurons expressing YFP under this promoter were imaged after 50–70 d of in vitro differentiation. For live imaging, neurons were grown on 15 mm glass coverslips (Warner Instruments) and placed in a perfusion chamber on the stage of confocal microscope (PerkinElmer) in a 5 mM KCl Tyrode solution containing 2% B27 (Invitrogen). After 30 min of imaging in a 5 mM KCl solution, a 67 mM KCl solution containing 2% B27 was injected in the chamber. Experiments were performed at ~30 °C. Total dendrite length of individual Synapsin-1–expressing neuron, before and after 2 h of depolarization, was traced semi-automatically using the HCA-Vision software (CSIRO). For assessing the number of retraction and extension events, cells were imaged every 5 min with a ×40 objective (Fig. 3d,f).

51. Green, E.M., Barrett, C.F., Bultynck, G., Shamah, S.M. & Dolmetsch, R.E. The tumor suppressor eIF3e mediates calcium-dependent internalization of the L-type calcium channel CaV1.2. Neuron 55, 615–632 (2007).
52. Gomez-Ospina, N., Tsuruta, F., Barreto-Chang, O., Hu, L. & Dolmetsch, R. The C terminus of the L-type voltage-gated calcium channel Ca(V)1.2 encodes a transcription factor. Cell 127, 591–606 (2006).
53. Yuan, B., Latek, R., Hossbach, M., Tuschl, T. & Lewitter, F. siRNA Selection Server: an automated siRNA oligonucleotide prediction server. Nucl. Acids Res. 32, W130–W134 (2004).
54. Paradis, S. et al. An RNAi-based approach identifies molecules required for glutamatergic and GABAergic synapse development. Neuron 53, 217–232 (2007).
55. Xia, Z., Dudek, H., Miranti, C.K. & Greenberg, M.E. Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. J. Neurosci. 16, 5425–5436 (1996).
56. Kaech, S. & Banker, G. Culturing hippocampal neurons. Nat. Protoc. 1, 2406–2415 (2006).
57. Zhang, F. et al. Multimodal fast optical interrogation of neural circuitry. Nature 446, 633–639 (2007).
58. Meijering, E. et al. Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. Cytometry A 58, 167–176 (2004).