CALHM1 controls the Ca^{2+}-dependent MEK, ERK, RSK and MSK signaling cascade in neurons

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Summary

Calcium homeostasis modulator 1 (CALHM1) is a Ca^{2+} channel controlling neuronal excitability and potentially involved in the pathogenesis of Alzheimer's disease (AD). Although strong evidence indicates that CALHM1 is required for neuronal electrical activity, its role in intracellular Ca^{2+} signaling remains unknown. In the present study, we show that in hippocampal HT-22 cells, CALHM1 expression led to a robust and relatively selective activation of the Ca^{2+}-sensing kinases ERK1/2. CALHM1 also triggered activation of MEK1/2, the upstream ERK1/2-activating kinases, and of RSK1/2/3 and MSK1, two downstream effectors of ERK1/2 signaling. CALHM1-mediated activation of ERK1/2 signaling was controlled by the small GTPase Ras. Pharmacological inhibition of CALHM1 permeability using Ruthenium Red, Zn^{2+}, and Gd^{3+}, or expression of the CALHM1 N140A and W114A mutants, which are deficient in mediating Ca^{2+} influx, prevented the effect of CALHM1 on the MEK, ERK, RSK and MSK signaling cascade, demonstrating that CALHM1 controlled this pathway via its channel properties. Importantly, expression of CALHM1 bearing the natural P86L polymorphism, which leads to a partial loss of CALHM1 function and is associated with an earlier age at onset in AD patients, showed reduced activation of ERK1/2, RSK1/2/3, and MSK1. In line with these results obtained in transfected cells, primary cerebral neurons isolated from *Calhm1* knockout mice showed significant impairments in the activation of MEK, ERK, RSK and MSK signaling. The present study identifies a previously uncharacterized mechanism of control of Ca^{2+}-dependent ERK1/2 signaling in neurons, and further establishes CALHM1 as a critical ion channel for neuronal signaling and function.

Key words: CALHM1, Ca^{2+}, ERK1/2, Neuronal signalling, Alzheimer's disease

Introduction

Tissue-specific gene expression profiling (Aguilar et al., 2008; Skrabanek and Campagne, 2001) focusing on genes preferentially expressed in brain regions affected by Alzheimer's disease (AD) identified the novel gene calcium homeostasis modulator 1 (*Calhm1*) as a potential player in AD pathogenesis (Dreses-Werringloer et al., 2008; Lambert et al., 2010). CALHM1 is a highly conserved *N*-glycosylated multipass transmembrane protein, which has no significant homology with any protein of known function. A suggestive similarity with the topology of ionotropic glutamate receptors, however, raised the possibility that CALHM1 might function as a Ca^{2+} channel component (Dreses-Werringloer et al., 2008). The first evidence that CALHM1 could fulfill this role came from the observation that CALHM1 overexpression in different cell lines increases cytoplasmic Ca^{2+} levels by facilitating Ca^{2+} entry into the cells (Dreses-Werringloer et al., 2008; Gallego-Sandín et al., 2011; Moreno-Ortega et al., 2010). Furthermore, electrophysiological studies demonstrated that CALHM1 induces a novel plasma membrane Ca^{2+} selective current (Dreses-Werringloer et al., 2008).

CALHM1-mediated Ca^{2+} influx in cells can be triggered by an experimental protocol that consists of transiently depleting extracellular Ca^{2+} and subsequently, adding-back Ca^{2+} to physiological concentrations to generate a driving force for Ca^{2+} entry (hereafter referred to as 'Ca^{2+} add-back' conditions) (Dreses-Werringloer et al., 2008). A recent study has now demonstrated that CALHM1 is itself a pore-forming ion channel permeable to Ca^{2+}, gated by extracellular Ca^{2+}, and activated by a decrease in extracellular Ca^{2+} levels, a situation that occurs during the Ca^{2+} add-back conditions (Ma et al., 2012). Reductions in Ca^{2+} levels to concentrations within the range of CALHM1 activation (Ma et al., 2012) can be observed in the synaptic cleft during electrical activity in cerebral neurons (Borst and Sakmann, 1999; Rusakov and Fine, 2003; Stanley, 2000; Vassilev et al., 1997), suggesting that CALHM1 activation might have physiological relevance for synaptic activity. Indeed, further investigation revealed that CALHM1 is essential for the control of extracellular Ca^{2+}-dependent excitability in cortical neurons (Ma et al., 2012). These results provide strong support to the notion that CALHM1 is a key Ca^{2+} channel regulator of neuronal electrical activity and motivate further investigation aimed at determining whether CALHM1 controls intracellular Ca^{2+}-mediated signal transduction in neurons.

In the current study, we show that CALHM1 activation upon Ca^{2+} add-back led to a preferential activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), two versatile protein kinases critically involved in intracellular signal transduction during synaptic activity (Adams and Sweatt, 2002; Hardingham and Bading, 2003; Thomas and Huganir, 2004). CALHM1-dependent

activation of ERK1/2 was mediated by Ras and MEK1/2 (dual specificity mitogen-activated protein kinase kinase 1/2) and led to the phosphorylation of RSK1/2/3 (90 kDa ribosomal S6 kinases 1–3) and MSK1 (mitogen- and stress-activated protein kinase 1), two kinase families of downstream effectors of ERK1/2 signaling (Hauge and Frödin, 2006). The effect of CALHM1 on the MEK/ERK/RSK/MSK signaling cascade was blocked by genetic and pharmacological inhibition of CALHM1 permeability. In line with these results obtained in cells overexpressing CALHM1, primary neurons from *Calhm1* knockout (KO) mice displayed an impaired ERK1/2 signaling upon Ca^{2+} add-back conditions. Thus, CALHM1 controls the MEK, ERK, RSK and MSK signaling cascade in neurons via its channel properties.

Results

CALHM1 activates ERK1/2 signaling

Because Ca^{2+} is a universal signaling ion involved in numerous pathways (Berridge, 2012), we asked whether CALHM1 could play a role in intracellular signal transduction. A phospho-kinase panel was utilized as a screening method to determine the effect of CALHM1 activation on the phosphorylation levels of 46 major protein kinases (Fig. 1). CALHM1 transfection in hippocampal HT-22 cells led to the activation of a subset of kinases upon Ca^{2+} add-back, a condition required for CALHM1 activation (Dreses-Werringloer et al., 2008; Ma et al., 2012). The most significant effect was an increase in ERK1/2 phosphorylation with a more than 5-fold activation compared with empty vector-transfected control cells (Fig. 1A,B). MEK1/2, which phosphorylates and thereby activates ERK1/2, was activated as well. Moreover, phosphorylation levels of RSK1/2/3 and MSK1/2, two kinase families downstream from ERK1/2, were also increased (Fig. 1A,B).

The effect on MEK1/2, ERK1/2, RSK1/2/3, and MSK1 was confirmed by western blot (WB) analyses. Time course experiments showed that the effect of CALHM1 expression and activation on MEK1/2, ERK1/2, and RSK1/2/3 phosphorylation was maintained up to 60 minutes after Ca^{2+} add-back with a maximal activation at 10 min (Fig. 2A). Activation of these kinases followed a very congruent pattern indicating a direct correlation between them. Phospho-MSK1 levels exhibited a slightly different kinetic, showing robust activation for only 10 to 20 min after CALHM1 activation (Fig. 2A). Importantly, no effect of CALHM1 expression on MEK1/2, ERK1/2, RSK1/2/3, and MSK1 activation was observed at steady state in the absence of Ca^{2+} add-back (Fig. 2B), suggesting that CALHM1 activation and CALHM1-mediated Ca^{2+} influx are required for the observed effect on the MEK/ERK/RSK/MSK signaling cascade.

ERK1/2 signaling activation by CALHM1 is mediated by Ras

Cellular signaling pathways are seldom linear but often form complex networks. For instance, crosstalk between ERK1/2 signaling and other Ca^{2+}-inducible pathways like cAMP-dependent protein kinase (PKA), protein kinase C (PKC), or Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) has been reported during synaptic plasticity (Adams and Sweatt, 2002). In this context, we asked whether PKA, PKC, or CaMKII mediate the effect of CALHM1 activation on ERK1/2 signaling in transfected HT-22 cells. Fig. 3 shows that pharmacological inhibition of CaMKII (using KN62 and KN93, Fig. 3A), PKC (BisIII and Ro31, Fig. 3B), or PKA (H89 and PKI, Fig. 3C) had no significant effect on the CALHM1-mediated activation of ERK1/2 or RSK1/2/3. A weak inhibitory effect of the PKA inhibitor H89 was, however, observed on CALHM1-mediated ERK1/2 and RSK1/2/3 activation (Fig. 3C). In contrast, inhibition of the small GTPase Ras with farnesyl thiosalicylic acid, and of MEK1/2 with PD98059 fully prevented the CALHM1-mediated activation of ERK1/2, RSK1/2/3, and MSK1 (Fig. 3D). These data show that Ras is the main protein initiator of the MEK/ERK/RSK/MSK signaling cascade by CALHM1 in HT-22 cells.
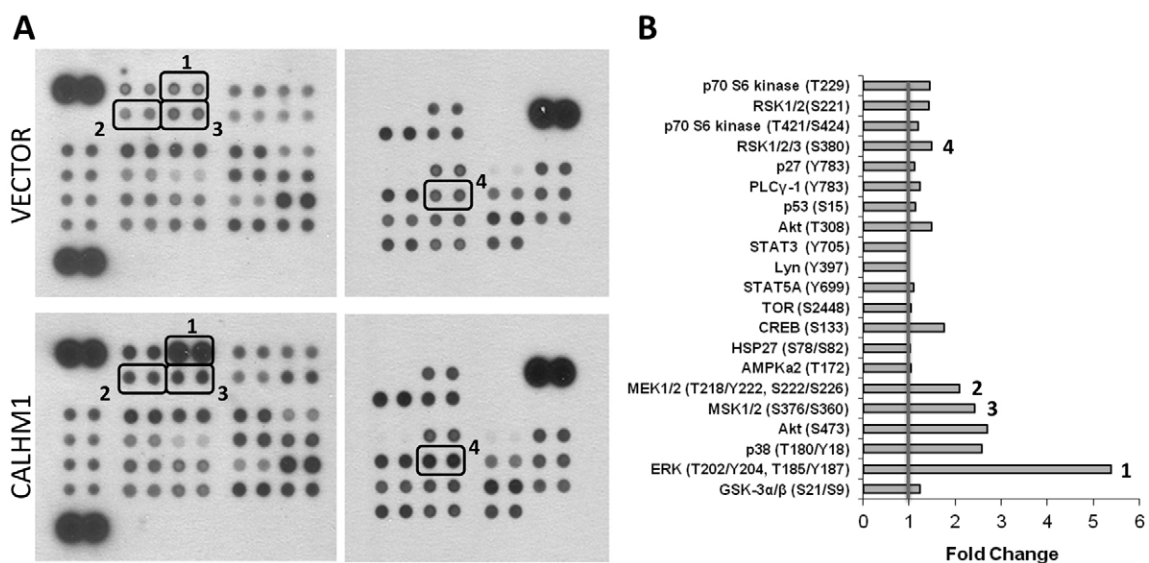
Fig. 1. **Effect of CALHM1 expression on kinase activation.** (A,B) HT-22 cells transiently transfected with CALHM1 or empty vector were challenged with Ca^{2+} add-back conditions for 20 min (see the Materials and methods section). Cell lysates were probed on phospho-protein arrays (A). Densitometric analysis was performed, and results are expressed as fold change compared with empty vector-transfected cells (B).
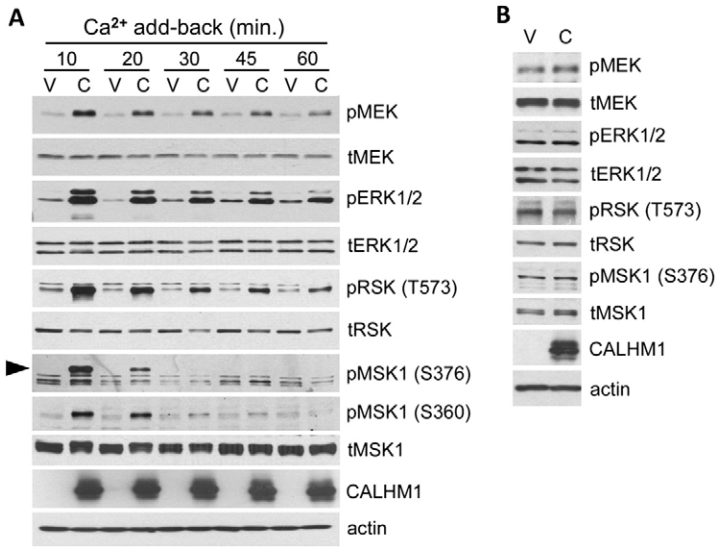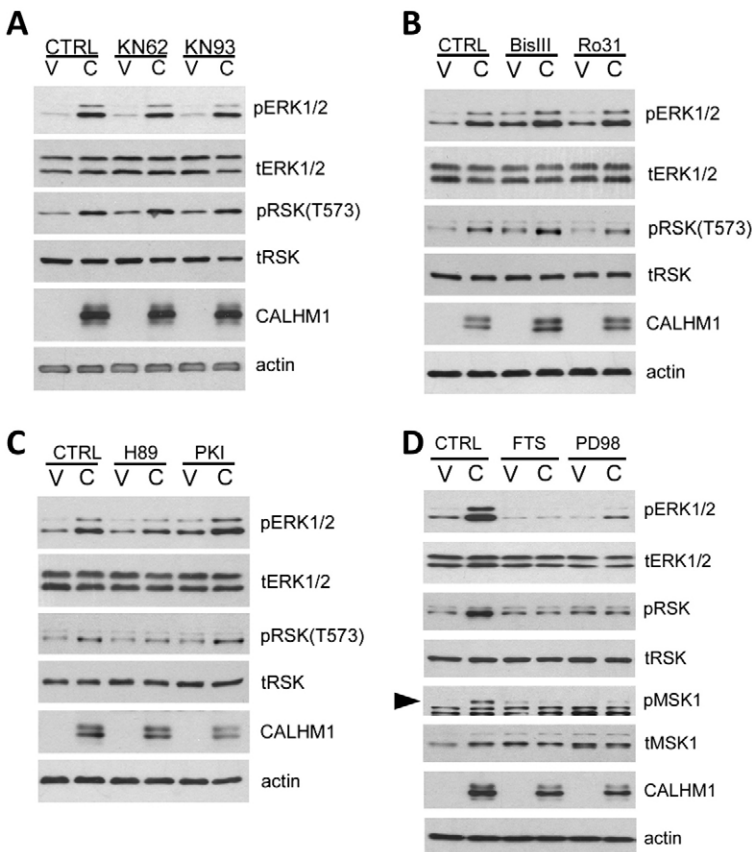
Fig. 2. CALHM1 controls ERK1/2 signaling. (**A,B**) HT-22 cells transiently transfected with CALHM1 or empty vector were challenged (A) or not (B) with Ca^{2+} add-back conditions for the indicated periods of time. Cell extracts were then analyzed by WB for the indicated proteins. C, CALHM1; V, vector. The arrowhead indicates phospho-Ser376-MSK1.

CALHM1 controls ERK1/2 signaling via its channel properties

Because CALHM1 had no effect on ERK1/2 signaling in the absence of Ca^{2+} add-back (Fig. 2B), we then sought to determine whether CALHM1-mediated Ca^{2+} influx is required for the activation of the MEK/ERK/RSK/MSK signaling cascade by CALHM1. Ruthenium Red, a non-selective blocker of several ion channels that completely inhibits CALHM1 currents at 20 µM (Ma et al., 2012), was found to fully prevent CALHM1-mediated Ca^{2+} influx (Fig. 4A) and activation of ERK1/2, RSK1/2/3, and

MSK1 by CALHM1 upon Ca^{2+} add-back (Fig. 4B,D). Similarly, Zn^{2+} and Gd^{3+}, two other inhibitors of CALHM1 permeability (Ma et al., 2012), fully blocked ERK1/2 activation by CALHM1 upon Ca^{2+} add-back (Fig. 4C,D). Moreover, intracellular Ca^{2+} chelation with BAPTA-AM prevented the effect of CALHM1 expression on ERK1/2 phosphorylation, confirming that CALHM1-mediated Ca^{2+} influx controls ERK1/2 activation.

We previously reported that CALHM1 is *N*-glycosylated at residue Asp-140 (Dreses-Werringloer et al., 2008). We found that the substitution of Asp-140 to Ala (N140A), which prevents

Fig. 3. ERK1/2 signaling activation by CALHM1 is mediated by Ras. (**A–D**) HT-22 cells transiently transfected with CALHM1 or empty vector were pre-incubated for 30 min in the presence or absence (CTRL) of 10 µM KN62 or 5 µM KN93 (CaMKII inhibitors); 10 µM bisindolylmaleimide III (BisIII) or 1 µM Ro31-8220 (Ro31) (PKC inhibitors); 20 µM H89 or 0.8 µM PKI (PKA inhibitors); 20 µM PD98059 (PD98, MEK inhibitor) or 50 µM farnesyl thiosalicylic acid (FTS, Ras inhibitor). Cells were then challenged with Ca^{2+} add-back conditions for 10 min and cell extracts were analyzed by WB for the indicated proteins. C, CALHM1; V, vector. The arrowhead indicates phospho-MSK1. Experiments in A–D are representative of three independent experiments.
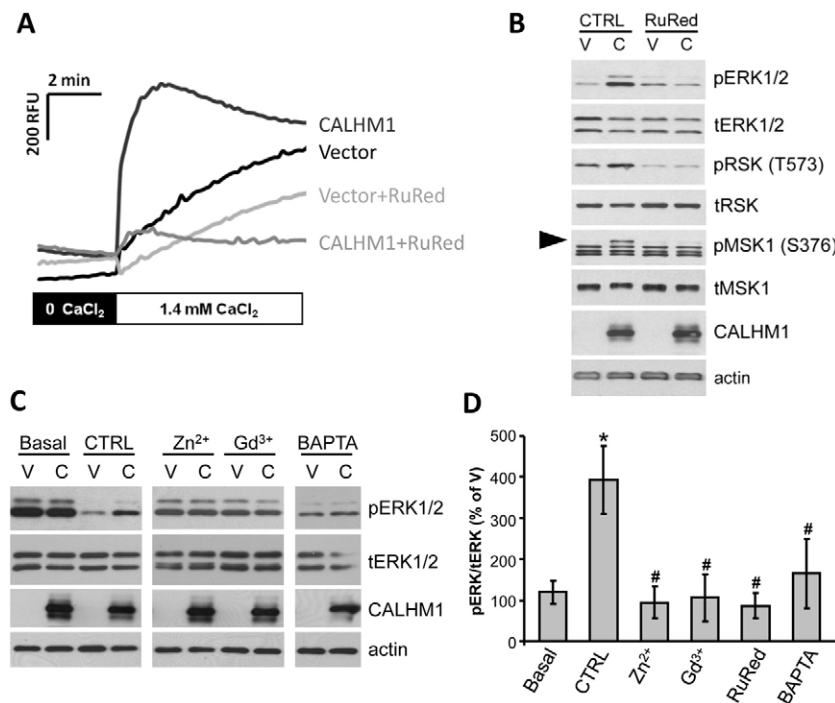
Fig. 4. Effect of CALHM1-mediated Ca^{2+} influx inhibition on ERK1/2 signaling activation by CALHM1. (**A**) Free cytoplasmic Ca^{2+} measurements with Fluo-4 loading and Ca^{2+} add-back in HT-22 cells transiently transfected with CALHM1 or empty vector. Cells pre-incubated in Ca^{2+}-free buffer (0 CaCl$_2$), in either the presence or absence of 20 μM Ruthenium Red (RuRed), were challenged with physiological extracellular Ca^{2+} concentrations (1.4 mM CaCl$_2$) to monitor the progressive restoration of cytoplasmic Ca^{2+} levels. Traces illustrate mean relative fluorescence units (RFU) of 2 independent measurements. (**B,C**) HT-22 cells transiently transfected with CALHM1 or empty vector were pre-incubated in the presence or absence (Basal and CTRL) of 20 μM Ruthenium Red (RuRed), 100 μM Gd^{3+}, 20 μM Zn^{2+}, or 20 μM BAPTA-AM. Cells were then challenged or not (Basal) with Ca^{2+} add-back conditions for 10 min and cell extracts were analyzed by WB for the indicated proteins. C, CALHM1; V, vector. The arrowhead indicates phospho-MSK1. Experiments in B and C are representative of three independent experiments. (**D**) Densitometric analyses and quantification of the phospho-ERK1/2/total ERK1/2 ratio (pERK/tERK) in CALHM1-transfected cells [expressed as a percentage of the ratio in corresponding vector-transfected control conditions (% of V)]. Error bars, s.e.m. [$n=3$; *$P<0.05$ (versus Basal); #$P<0.05$ (versus CTRL); one-way ANOVA Bonferroni post-hoc tests].

CALHM1 N-glycosylation (Dreses-Werringloer et al., 2008), significantly inhibited CALHM1-mediated Ca^{2+} influx upon Ca^{2+} add-back conditions (Fig. 5A–C), indicating that CALHM1 N-glycosylation is required for CALHM1 channel properties. Importantly, N140A-CALHM1 failed to activate ERK1/2, RSK1/2/3, and MSK1 upon Ca^{2+} add-back (Fig. 6A,B).

In its predicted hydrophobic domains (Dreses-Werringloer et al., 2008), CALHM1 should contain residues involved in the formation of the ion pore of the channel. Tryptophan residues are of interest in this context because they have a preference for membrane interfaces (Wimley and White, 1996; Yau et al., 1998) and have functional roles in the pore helix of several channels of the TRPC and Kv families (Gajewski et al., 2011; Owsianik et al., 2006). We found that substitution of the highly conserved Trp-114 residue to Ala (W114A) in the third hydrophobic domain of CALHM1, completely inhibited the effect of CALHM1 expression on Ca^{2+} influx (Fig. 5D–F) and on the activation of ERK1/2, RSK1/2/3, and MSK1 (Fig. 6A,B) upon Ca^{2+} add-back.

Furthermore, expression of the pathogenic P86L-CALHM1 mutant, which affects CALHM1-mediated Ca^{2+} influx (Fig. 5G–I) (Dreses-Werringloer et al., 2008; Ma et al., 2012; Moreno-Ortega et al., 2010), significantly inhibited the activation of ERK1/2 signaling by CALHM1 (Fig. 6A,B). Importantly, the effect of the N140A, W114A, and P86L mutations on Ca^{2+} influx and ERK1/2 signaling activation by CALHM1 could not be explained by changes in CALHM1 expression levels (Fig. 5C,F,I). Together with the observation that CALHM1 controls ERK1/2 signaling only after Ca^{2+} add-back, these results demonstrate the direct association between CALHM1 Ca^{2+} permeability and its control of ERK1/2 signaling.

ERK1/2 signaling is impaired in *Calhm1* KO primary neurons

We then asked whether the MEK/ERK/RSK/MSK signaling cascade is affected in the absence of CALHM1 expression.

CALHM1 expression is relatively restricted to a few tissues and cell types, which include the human brain and mouse brain neurons (Fig. 7A) (Dreses-Werringloer et al., 2008; Ma et al., 2012). ERK1/2 signaling activation upon Ca^{2+} add-back was assessed in primary neurons obtained from *Calhm1* KO mice and WT littermate control mice. Fig. 7B shows that, whereas WT and *Calhm1* KO neurons expressed comparable levels of phosphorylated MEK1/2, ERK1/2, RSK1/2/3, and MSK1 at steady state (basal condition), CALHM1 deficiency led to robust impairments in the activation of the MEK/ERK/RSK/MSK signaling cascade after Ca^{2+} add-back in neurons. Importantly, no significant increase in the activation of the different kinases was observed at any time point of the Ca^{2+} add-back in *Calhm1* KO neurons. In contrast, in WT neurons, phosphorylated MEK1/2, ERK1/2, RSK1/2/3 and MSK1 almost completely recovered to their normal levels after 30 min of Ca^{2+} add-back (Fig. 7B). These data indicate that, in neurons, Ca^{2+}-induced ERK1/2 signaling activation triggered by the Ca^{2+} add-back manipulation is almost exclusively due to CALHM1 expression. Altogether, these results confirm our findings obtained in overexpressing HT-22 cells and demonstrate that CALHM1 is playing a crucial role in the MEK/ERK/RSK/MSK signaling cascade in neurons.

Discussion

Ca^{2+} is a major intracellular messenger in neurons during synaptic activity. The different mechanisms of control of Ca^{2+} homeostasis and Ca^{2+}-dependent signal transduction are thus essential for the formation, maintenance, and plasticity of the cerebral neuronal networks during higher cognitive functions, such as memory (Marambaud et al., 2009; Wiegert and Bading, 2011). Ca^{2+} influx through N-methyl-D-aspartate receptors (NMDARs) and voltage-gated Ca^{2+} channels (VGCCs) represents the main route for Ca^{2+} entry upon synaptic activity, and activation of ERK1/2 is recognized as a central event in
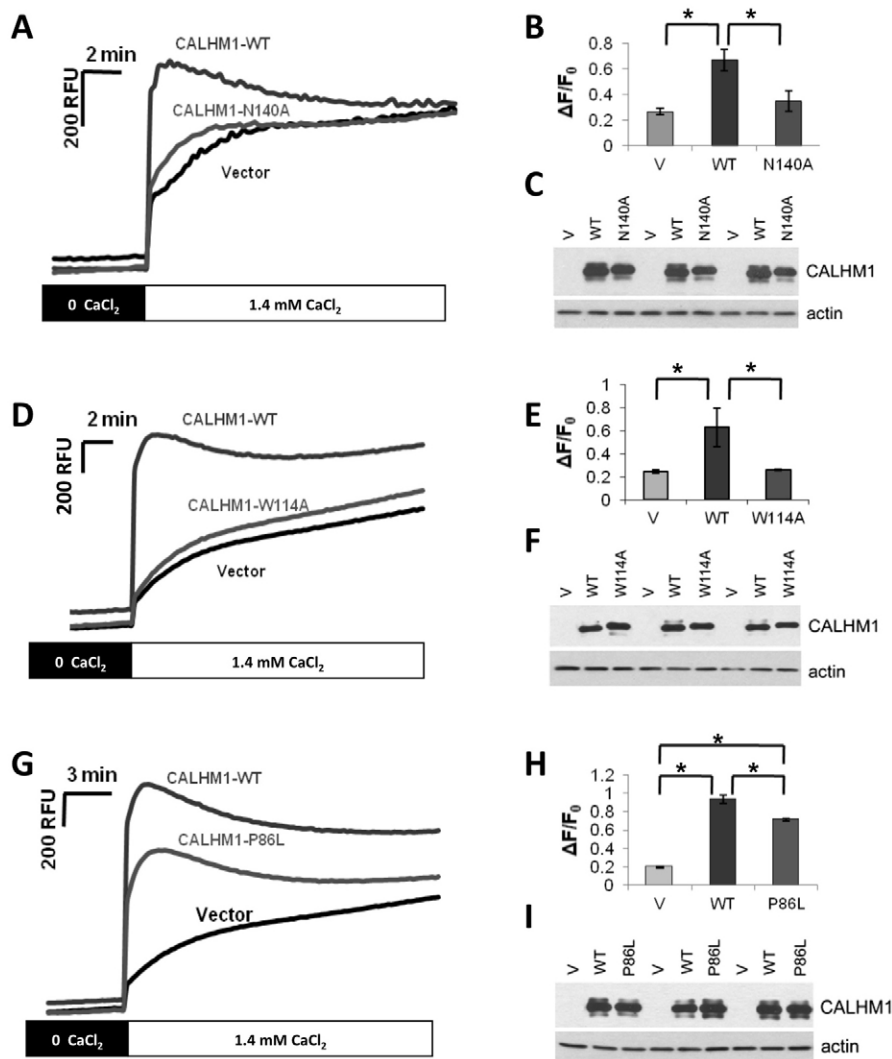
Fig. 5. N140A-, W114A-, and P86L-CALHM1 mutations affect CALHM1-mediated Ca^{2+} influx. (**A,D,G**) Cytoplasmic Ca^{2+} measurements with Fluo-4 loading and Ca^{2+} add-back in HT-22 cells transiently transfected with empty vector, Myc-tagged WT-CALHM1, and the indicated Myc-tagged CALHM1 mutants. Traces illustrate mean relative fluorescence units (RFU) of 3 independent measurements. (**B,E,H**) Peak of Ca^{2+} concentration measurements, as in A, D and G, respectively, expressed as $\Delta F/F_0$. Error bars, s.e.m. (B, $n=6$; E, $n=7$; H, $n=4$; *$P\leq0.0002$; Student's t-test with Bonferroni correction). (**C,F,I**) WB analysis of CALHM1 expression in the corresponding cell lysates. V, vector; WT, WT-CALHM1.

Ca^{2+}-dependent signal transduction during neuronal function (Wiegert and Bading, 2011). Recently, we reported that CALHM1 is another Ca^{2+} channel expressed in neurons required for neuronal excitability (Dreses-Werringloer et al., 2008; Ma et al., 2012). CALHM1 was found to respond to changes in extracellular Ca^{2+} levels (Ma et al., 2012), a situation occurring during synaptic activity, suggesting that CALHM1 might be involved in the global control of activity-dependent Ca^{2+} influx during neuronal function.

In the present study, using CALHM1-transfected hippocampal HT-22 cells and *Calhm1* KO primary cerebral neurons, we show that CALHM1 activation upon Ca^{2+} add-back, a manipulation that induces a transient change in extracellular Ca^{2+} levels and activates CALHM1 Ca^{2+} permeability (Dreses-Werringloer et al., 2008; Ma et al., 2012), led to a robust activation of ERK1/2. Ca^{2+} is a versatile intracellular messenger, which can lead to the activation of several protein effectors that crosstalk with ERK1/2, including Ras, CaMKs, PKA, or PKC. We found that CALHM1 activation in HT-22 cells, however, led to a preferential activation of ERK1/2 signaling by the small GTPase Ras. The implication of Ras in the control of ERK1/2 activation by CALHM1 is in line with reports indicating that Ras activation is the initial step in the control of ERK1/2 by NMDAR- or VGCC-

mediated Ca^{2+} transients during synaptic activity (for a review, see Wiegert and Bading, 2011).

We further demonstrated that CALHM1 acted by controlling Ca^{2+} entry and thus by increasing cytosolic Ca^{2+} levels. Indeed, the effect of CALHM1 on ERK1/2 signaling was completely inhibited by the inorganic dye Ruthenium Red, Zn^{2+}, and Gd^{3+}, which are known to block CALHM1 Ca^{2+} permeability (Ma et al., 2012), or by expression of CALHM1 constructs bearing mutations interfering with CALHM1-mediated Ca^{2+} influx. In the current study, we report for the first time that the *N*-glycosylation-deficient mutant N140A-CALHM1 is impaired in mediating Ca^{2+} influx. *N*-glycosylation can affect many steps of protein maturation, including protein folding, stability, trafficking, and localization, and thus has functional consequences for many receptors and ion channels (Nishizaki, 2003; Roy et al., 2010; Veldhuis et al., 2012; Zhu et al., 2012). We found no significant changes in protein expression or stability of the *N*-glycosylation-deficient mutant N140A-CALHM1 (Fig. 5C) (Dreses-Werringloer et al., 2008), suggesting that other steps of protein maturation or trafficking might be affected.

We also identified the highly conserved Trp-114 residue in the third hydrophobic domain of CALHM1 as critically important for the control of CALHM1-mediated Ca^{2+} influx. Like for the
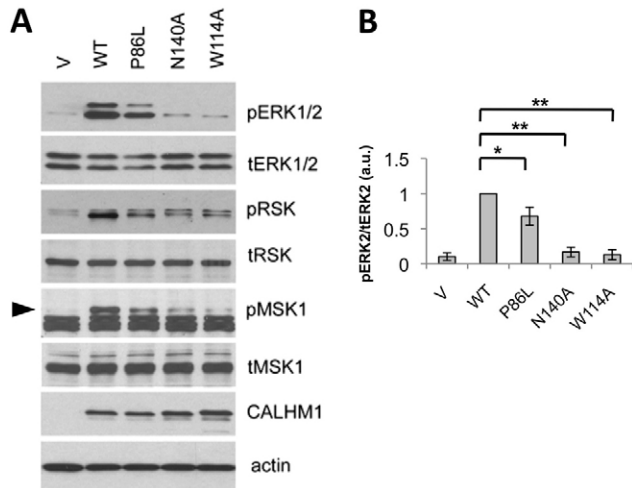
Fig. 6. N140A-, W114A-, and P86L-CALHM1 mutations affect the control of ERK1/2 signaling activation by CALHM1. (**A**) HT-22 cells transiently transfected with empty vector (V), WT-CALHM1 (WT), and the indicated CALHM1 mutants were challenged with Ca^{2+} add-back conditions for 10 min. Cell extracts were then analyzed by WB for the indicated proteins. The arrowhead indicates phospho-MSK1. (**B**) Densitometric analysis and quantification of the ratio phospho-ERK2/total ERK2 (pERK2/tERK2). a.u., arbitrary units. Error bars, s.e.m. ($n=3$; *$P<0.005$; **$P<0.0001$; Student's t-test with Bonferroni correction).

N140A mutation, the effect of W114A on CALHM1 Ca^{2+} influx could not be explained by defects in CALHM1 protein expression or stability (Fig. 5F). Further studies will be required to determine the exact mechanism by which the mutations N140A
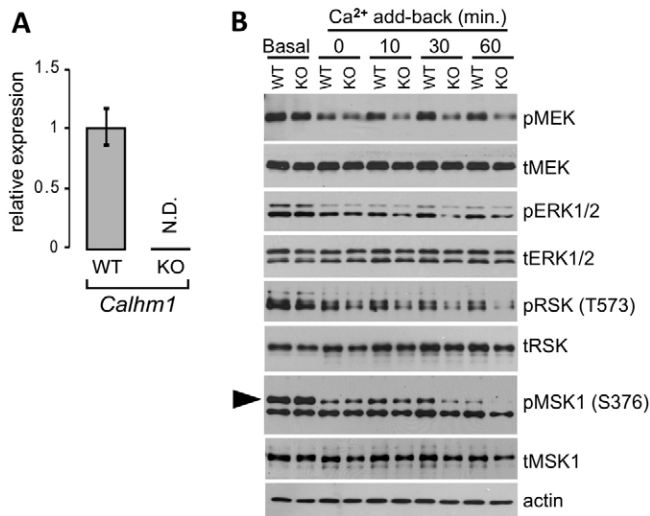
Fig. 7. Primary neurons from *Calhm1* KO mice display impaired ERK1/2 signaling activation. (**A**) Real time PCR analyzing *Calhm1* expression levels in 14 DIV primary neurons from *Calhm1* +/+ (WT) and −/− (KO) mice. *Calhm1* expression was normalized to the reference genes HPRT1, TBP, and POLR2A. N.D., not detected. (**B**) 14 DIV primary neurons isolated from *Calhm1* KO mice and WT littermate controls were challenged or not (Basal) with Ca^{2+} add-back conditions for the indicated periods of time. Cell extracts were then analyzed by WB for the indicated proteins. The arrowhead indicates phospho-MSK1. The figure shows representative results from four independent experiments.

and W114A affect CALHM1 permeability to Ca^{2+} and whether these mutations interfere with intrinsic biophysical properties of the CALHM1 channel. Because Trp residues have functional roles in the formation of the pore helix of several ion channels (Gajewski et al., 2011; Owsianik et al., 2006), it is tempting to speculate, however, that CALHM1 Trp-114 residue is located in the pore domain of the channel where it has important structural functions. Structure and electrophysiological studies will be needed to address this possibility. Nevertheless, these results demonstrate the close relationship between CALHM1 permeability and its control of ERK1/2 signaling. The CALHM1 P86L polymorphism followed a similar pattern, being associated with both a partial loss of CALHM1-mediated Ca^{2+} influx and a decrease in the control of ERK1/2 activation.

Strong evidence indicates that CALHM1 might be involved in the pathogenesis of AD. *In vitro* studies have shown that CALHM1 is a repressor of the accumulation of a main culprit in AD, the amyloid-β (Aβ) peptide (Dreses-Werringloer et al., 2008). Genetic studies have further determined that the natural human polymorphism P86L in CALHM1 is associated with an earlier age at onset in AD patients (Boada et al., 2010; Dreses-Werringloer et al., 2008; Lambert et al., 2010; Minster et al., 2009). Moreover, some studies (Kauwe et al., 2010; Koppel et al., 2011), but not all (Giedraitis et al., 2010), found that Aβ levels in cerebrospinal fluid are elevated in individuals carrying the CALHM1 P86L polymorphism. Together these studies support the notion that CALHM1 is involved in the neurodegenerative process of AD via mechanisms that remain, however, to be clearly identified. The current data indicate that the CALHM1 P86L polymorphism might also act by interfering with the control of CALHM1 on neuronal Ca^{2+} signaling and ERK1/2 activation.

In summary, in the present study we show that CALHM1 is a channel regulator of the MEK/ERK/RSK/MSK signaling cascade in neuronal cells. Because CALHM1 promotes Ca^{2+} influx in neurons and controls neuronal excitability (Dreses-Werringloer et al., 2008; Ma et al., 2012), this channel might be critically involved in ERK1/2-dependent synaptic activity during higher cognitive functions. These results motivate further work aimed at determining whether CALHM1 is involved in learning and memory formation and whether the natural CALHM1 P86L polymorphism influences AD pathogenesis by affecting ERK1/2 signaling during synaptic activity.

Materials and Methods
Cell lines and transfections
HT-22 cells were kindly provided by Dr D. Schubert (Salk Institute, La Jolla, CA) and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, HyClone, Thermo Fisher Scientific Inc., Waltham, MA), 2 mM L-glutamine and penicillin/streptomycin (Life Technologies, Carlsbad, CA) at 37°C under 5% CO_2. Cells were transfected with Lipofectamine 2000 in OptiMEM (Life Technologies) in 6 well plates for WB analysis and phospho-protein arrays or in 12 well plates for intracellular Ca^{2+} measurements. OptiMEM was replaced after ~5 hrs post-transfection with complete DMEM/FBS. Cells were processed 24 hrs after transfection.

Chemicals and antibodies
PD98059, KN62, KN93, H89, myristoylated protein kinase A inhibitor (PKI, 14–22 amide), bisindolylmaleimide III (BisIII), and Ro31-8220 were purchased from Calbiochem (EMD Millipore, Billerica, MA). Farnesyl thiosalicylic acid (FTS) was from Cayman Chemical (Ann Arbor, MI). Ruthenium Red, $ZnCl_2$, $GdCl_3$, and BAPTA-AM were from Sigma-Aldrich (St. Louis, MO). Antibodies directed against total MEK1/2 (tMEK1/2), phospho-MEK1/2 (pMEK1/2, Ser-217/221), tERK1/2, pERK1/2 (Thr-202/Tyr-204), tRSK1/2/3, pRSK (Thr-573), pMSK1 (Ser-376 and Ser-360), and Myc were from Cell Signaling Technology (Danvers,

MA). Anti-actin antibody was from BD Transduction Laboratories (BD Biosciences, San Jose, CA) and anti-tMSK1 antibody was from R&D Systems (Minneapolis, MN). The C-terminal end following the last hydrophobic domain of CALHM1 was subcloned into *Bam*HI and *Xho*I sites of pGEX-5X-1 vector (Amersham, GE Healthcare Biosciences, Pittsburgh, PA). The corresponding peptide expressed in *E. coli* BL21 was used for immunizations to raise a mouse monoclonal antibody directed against the C-terminal domain of CALHM1 (32C2, IgG2a). The antibody was produced using methods described before (Davies, 2000). 32C2 antibody was used to detect untagged CALHM1.

Plasmids and mutagenesis
Myc-tagged WT-, P86L-, and N140A-CALHM1 in pcDNA3.1 vector were described previously (Dreses-Werringloer et al., 2008). W114A-CALHM1 and untagged WT-CALHM1 were created using QuikChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) and confirmed by sequencing.

Intracellular Ca^{2+} measurements
Cytosolic Ca^{2+} levels were measured using Fluo-4 (Fluo-4 NW Ca^{2+} Assay Kit, Life Technologies), as described previously (Dreses-Werringloer et al., 2008). Briefly, HT-22 cells were transfected in 12 well plates with WT-CALHM1, CALHM1 mutants, or empty vector using Lipofectamine 2000, as described above. Cells were loaded with Fluo-4 24 hrs after transfection. Ca^{2+} add-back conditions were performed as described previously (Dreses-Werringloer et al., 2008; Ma et al., 2012). Briefly, cells were incubated for 10 min in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (HBSS), supplemented with 20 mM HEPES buffer, 0.5 mM $MgCl_2$, and 0.4 mM $MgSO_4$. Ca^{2+} was then added back to a final concentration of 1.4 mM. Fluorescence measurement was performed at room temperature with a Tecan Genius Pro plate reader at 485 nm excitation and 535 nm emission. The effect of Ruthenium Red was determined after a preincubation of 10 min. Cells were washed after completing the assay and analyzed by WB.

Phospho-protein array
Array was performed as described before (Vingtdeux et al., 2010; Vingtdeux et al., 2011). HT-22 cells were transfected with CALHM1 cDNA or control empty vector for 24 hrs. Cells were then subjected to Ca^{2+} add-back conditions at 37℃, as described above. Cells were harvested 20 min after Ca^{2+} add-back. Cell lysates (300 µg of total protein) were applied to the phospho-protein arrays following the manufacturer's instruction (Proteome Profiler Human Phosphokinase Array kit, R&D Systems). Blots were scanned and phosphorylation levels were analyzed by densitometric quantification.

WB analyses
Cell extracts were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with the antibodies listed above and analyzed by enhanced chemiluminescence detection.

Primary neuronal cultures
All animal experiments were performed according to procedures approved by the Feinstein Institute for Medical Research Institutional Animal Care and Use Committee. The generation of the *Calhm1*^{+/−} (heterozygous KO) founder mice was outsourced to genOway (Lyon, France) (Ma et al., 2012). The full description and characterization of the *Calhm1* KO mice will be provided in a subsequent manuscript. Primary neurons were prepared as described previously (Vingtdeux et al., 2010). Briefly, *Calhm1* KO and WT littermate control female mice were killed at 17.5 days of gestation. Forebrains were dissected in ice-cold HBSS (Invitrogen, Life Technologies) containing 0.5% w/v D-glucose (Sigma) and 25 mM HEPES (Invitrogen) under a dissection microscope. Dissociation was carried out mechanically in ice-cold dissection medium containing 0.01% papain (Worthington Biochemical Corporation, Lakewood, NJ), 0.1% w/v dispase (Roche Applied Science, Indianapolis, IN), and 0.01% DNase (Worthington Biochemical Corporation) and by incubation at 37℃ twice for 15 min. Cells were then spun down at 220 *g* for 5 min at 4℃; resuspended in Neurobasal medium containing 2% B27, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM Glutamax (Invitrogen); filtered through a 40-µm cell strainer (Thermo Fisher Scientific Inc.); counted; and plated on poly-L-ornithine- and laminin-coated plates at a density of 10^6 cells/well. Culture medium was completely replaced after 16–20 hours, and new medium (30% of starting volume) was added every 3 days until neurons were processed for analysis.

Semi-quantitative PCR
Expression levels of CALHM1 in 14 DIV primary neurons of *Calhm1* +/+ and −/− mice were determined by semi-quantitative PCR. Levels were normalized to the reference genes HPRT1, TBP, and POLR2A. Total RNA from neurons was extracted using RNeasy Mini Kit. DNase-treated RNA was subjected to reverse transcription with VILO cDNA kit (Invitrogen) or MMLV-RT with random hexamers as primers (Invitrogen). Semi-quantitative PCR was performed using TaqMan assays on ABI 7900 HT (Applied Biosystems, Life Technologies).

Author contributions
U.D.-W., V.V., H.Z., P.C., and P.M. performed the experiments. U.D.-W., V.V., P.D., and P.M. designed experiments. U.D.-W., V.V., and P.M. analyzed data and wrote the manuscript. The authors have no conflicts of interest.

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