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Nuclear Calcium Signaling Induces Expression of the Synaptic Organizers *Lrrtm1* and *Lrrtm2*[*]

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Background: Lrrtms are synaptic organizers and can induce presynaptic differentiation.
Results: *Lrrtm1* and *Lrrtm2* mRNA expression is controlled by synaptic activity and nuclear calcium signaling.
Conclusion: The results define a pathway for the transcriptional regulation of two synaptic organizers.
Significance: Nuclear calcium controls the expression of synaptogenic proteins.

Calcium transients in the cell nucleus evoked by synaptic activity in hippocampal neurons function as a signaling end point in synapse-to-nucleus communication. As an important regulator of neuronal gene expression, nuclear calcium is involved in the conversion of synaptic stimuli into functional and structural changes of neurons. Here we identify two synaptic organizers, *Lrrtm1* and *Lrrtm2*, as targets of nuclear calcium signaling. Expression of both *Lrrtm1* and *Lrrtm2* increased in a synaptic NMDA receptor- and nuclear calcium-dependent manner in hippocampal neurons within 2–4 h after the induction of action potential bursting. Induction of *Lrrtm1* and *Lrrtm2* occurred independently of the need for new protein synthesis and required calcium/calmodulin-dependent protein kinases and the nuclear calcium signaling target CREB-binding protein. Analysis of reporter gene constructs revealed a functional cAMP response element in the proximal promoter of *Lrrtm2*, indicating that at least *Lrrtm2* is regulated by the classical nuclear Ca^{2+}/calmodulin-dependent protein kinase IV-CREB/CREB-binding protein pathway. These results suggest that one mechanism by which nuclear calcium signaling controls neuronal network function is by regulating the expression of *Lrrtm1* and *Lrrtm2*.

The ability of neurons to undergo structural and functional changes in response to synaptic stimulation is not only important for brain development but also forms the basis for virtually all adaptive processes in the adult nervous system, including information storage, memory formation, the buildup of neuroprotection, and the development of chronic pain (1–8). Gene programs activated by synaptic stimuli are required for adaptations to be persistent (4, 9). The transduction of signals from the synapse to the nucleus is primarily mediated by calcium signals (10). In particular, calcium transients in the cell nucleus function as signaling end points in synapse-to-nucleus communication (11). Nuclear calcium has emerged as one of the most potent regulators of neuronal gene expression (2, 11). It acts primarily via the nuclear calcium/calmodulin (CaM)-dependent[2] protein kinases II and IV to control the activity and/or localization of transcriptional regulators, including CREB, CBP, MeCP2, Fox03a, and class IIa histone deacetylases (12–16). Inhibition of nuclear calcium signaling compromises the ability of neurons to increase their neuroprotective activity in response to synaptic activity (2). It also blocks the conversion of memories from labile to persistent forms and attenuates the development of chronic pain (3, 17). In the context of acquired neuroprotection, a nuclear calcium-regulated core gene program has been identified that mediates this process (2). The picture of how activity-driven gene transcription contributes to synaptic plasticity and cognitive functions is less complete, although several memory-relevant, nuclear calcium-regulated genes have been described, including VEGFD, Arc, Dnmt3a2, Homer1, Npas4, and nr4a1 (2, 18–25). In recent years, synaptic cell adhesion molecules have attracted much attention as possible master regulators of synapse function and plasticity. Two of them are leucine-rich repeat transmembrane neuronal 1 and 2 (Lrrtm1 and Lrrtm2). These molecules have been identified as synaptogenic proteins (26) that contribute to the regulation of synapse density and synaptic transmission (27–29). Here we show that *Lrrtm1* and *Lrrtm2* are targets of nuclear calcium signaling in hippocampal neurons.

EXPERIMENTAL PROCEDURES

Mouse Cell Culture—Hippocampal neurons from newborn C57Black mice were plated on poly-D-lysine/laminin-coated (Sigma) culture dishes (diameter, 35 mm) at a density of ~400,000 cells/1 ml Neurobasal medium (Invitrogen) containing 1% rat serum and B27 (Invitrogen). For inhibition of glial cell growth, cytosine-1-β-D-arabinofuranose (2.7 μM, Sigma) was added to the culture medium at day *in vitro* 3. At day *in vitro* 8, the medium was changed to transfection medium containing salt-glucose-glycine solution (114 mM NaCl, 26.1 mM

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[2] The abbreviations used are: CaM, calmodulin; CREB, cAMP response element-binding protein; CBP, CREB-binding protein; ANOVA, analysis of variance; ab, antibody; NLS, nuclear localization signal; rAAV, recombinant adeno-associated virus; AP, action potential; CaMK, calmodulin-dependent protein kinase.

$NaHCO_3$, 5.3 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES (pH 7.4), 1 mM glycine, 30 mM glucose, 0.5 mM sodium pyruvate, and 0.001% phenol red) (30) and minimum Eagle's medium (with Earle's salt and without L-glutamine) (Invitrogen, 9:1, vol: vol) supplemented with insulin-transferrin-sodium selenite media supplement (6.3–5.7–7.5 μg/ml, Sigma), and penicillin/streptomycin solution (1:200, Sigma) (30). Following the medium change on day *in vitro* 8, half of the medium was changed every second day to provide a continuous supply of growth and trophic factors.

Pharmacological Treatments, RNA Isolation, and Quantitative PCR—Pharmacological treatments were done after a culturing period of 10–12 days *in vitro* during which hippocampal neurons expressed functional glutamate receptors (NMDA/AMPA/kainate) and developed a rich network of synaptic contacts (31, 32). Action potential bursting in hippocampal neurons was induced at days *in vitro* 10–12 by supplementing the medium with the $GABA_A$ receptor antagonist bicuculline (50 μM, Alexis) for 1–16 h (33). For the pharmacological inhibitor experiments, neurons were treated for 2–4 h with bicuculline, either with or without a 45-min pretreatment with the pharmacological inhibitors MK801 (10 μM, Sigma), KN62 (5 μM, Calbiochem), and anisomycin (20 μg/ml, Applichem). Cells were harvested in RNeasy lysis buffer (Qiagen), and RNA was isolated using an RNeasy mini kit (Qiagen) according to the instructions of the manufacturer, with additional on-column DNase digestion during RNA purification. cDNA was synthesized from 1 μg of total RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems) according to the instructions of the manufacturer.

Quantitative RT-PCR was done on an ABI7300 thermal cycler using universal quantitative PCR master mix with TaqMan gene expression assays (Applied Biosystems) for the following genes: *Gusb* (Mm00446953_m1), *c-fos* (Mm00487425_m1), *Atf3* (Mm00476032_m1), *Lrrtm1* (Mm00551337_g1), and *Lrrtm2* (Mm00997210_g1). The expression levels of the target genes were normalized to the relative ratio of the expression of the housekeeping gene Gusb. For analyses of statistical significance, one-way analysis of variance (ANOVA) was performed, followed by Tukey post hoc analysis. The data represent mean values ± S.E. from at least three independent experiments, except for the results obtained for *Atf3* shown in Fig. 1*D*, which were log-transformed and autoscaled. Mean ± SD was calculated (34). For graphical representation, the data were back-transformed to the original scale. Error bars represent upper and lower limits back-transformed as mean ± S.D. Data from three independent experiments are shown.

Immunoblot Analysis—For immunoblot analysis, cells were harvested in standard cell lysis buffer and stored at −20 °C. Gel electrophoresis and immunoblotting of protein samples were done using standard procedures. HRP-based secondary antibodies were used, and signals were detected on film (GE Healthcare) by chemiluminescence. Antibodies (ab) to the following proteins were used: α-Lrrtm2 (sheep polyclonal ab, 1:1000, R&D Systems), α-tubulin (mouse monoclonal ab, 1:500,000, Sigma), HRP-conjugated α-sheep (donkey polyclonal ab, 1:5000, Jackson ImmunoResearch Laboratories), and HRP-conjugated α-mouse (goat monoclonal ab, Sigma). Immunoblot signals were quantified using ImageJ software. The Lrrtm2 signals were normalized to that of tubulin. Three independent experiments were performed. Data represent mean values ± S.E.

Calcium Imaging—Calcium imaging using Fluo-3 or a recombinant calcium sensor (35–37) was done with mouse hippocampal neurons plated on poly-D-lysine/laminin-coated coverslips. After a culturing period of 10–12 days *in vitro*, neurons were loaded with Fluo-3 (Invitrogen) for 45 min in CO_2-independent salt-glucose-glycine solution (SGG_{ind}) (140.1 mM NaCl, 5.3 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES (pH 7.4), 1 mM glycine, 30 mM glucose, and 0.5 mM sodium pyruvate) (30). Cells were washed with SGG_{ind}, and images were acquired with a Leica SP2 confocal microscope. To calibrate the fluorescence signal (F), Fluo-3 was saturated by adding 50 μM ionomycin (F_{max}) (Sigma-Aldrich) and then quenched with $MnCl_2$ (F_{min}). $[Ca^{2+}]$ was expressed as a function of the Fluo-3 fluorescence $K_d \times ((F - F_{min})/(F_{max} - F))$ (36). As a recombinant calcium sensor we used GCaMP6F (35), which we targeted to the cell nucleus by means of fusion to a nuclear localization signal (NLS) (GCaMP6F-NLS).[3] Neurons were infected on day *in vitro* 4 with a recombinant adeno-associated virus (rAAV) containing an expression cassette for GCaMP6F-NLS and were imaged on days *in vitro* 10–12. The data for GCaMP6F-NLS are presented as $\Delta F/F0 = (F - F_0)/F_0$ (37), where F represents the average background-subtracted emission fluorescence intensity in a region of interest, and F_0 represents the baseline fluorescence measured prior to the stimulation. Immunostaining of the rAAV-GCaMP6F-NLS-infected neurons was done on days *in vitro* 10–12 using an antibody to GFP (mouse monoclonal ab, Invitrogen) and Hoechst 33258 for nuclear counterstaining.

Luciferase Reporter Gene Assay—A 356-bp-long sequence of the *Lrrtm2* promoter region was amplified from mouse genomic DNA with PyroStart Fast PCR Master Mix (Fermentas) using the primers 5′-CTCGAGAGCTCTCACACGCATTAGAA-3′ (sense) and 5′-AGATCTCAGCATGAGTGCATTTACTG-3′ (antisense) and cloned into pGL4.10[luc2] (Promega) in front of the firefly luciferase coding sequence ($Lrrtm2^{WT}$-Fluc). The CRE sites were mutated from CGTCA to CcaCA and from TGACGTCA to TGtgGTCA ($Lrrtm2^{\Delta CRE}$-Fluc) by overlap extension PCR using Phusion high-fidelity DNA polymerase (New England Biolabs) and the following primers: sense, 5′-ACAAAAGACACcaCACCCGGTGtgGTCAGC-3′; antisense, 5′-GCTGACcaCACCGGGTGtgGTGTCTTTTGT-3′. The correct sequence of the promoter regions was verified by DNA sequencing.

Reporter Gene Studies—Rat hippocampal neurons from newborn Sprague-Dawley rats (Charles River Laboratories) were plated on poly-D-lysine/laminin-coated culture dishes at a density of ~400,000 cells/1 ml Neurobasal medium containing 1% rat serum and B27 (Invitrogen). Transfection was done on day *in vitro* 10 with $Lrrtm2^{WT}$-Fluc and $Lrrtm2^{\Delta CRE}$-Fluc, respectively, using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. pGL4.29[luc2P/CRE/Hygro] (Promega), a plasmid containing a CRE site as reporter gene-

[3] H. E. Freitag and H. Bading, unpublished data.

driving element, was included as a positive control. For normalization of the luciferase assays, pGL4.83[hRlucP/Puro] (Promega) containing the promoter of human *EF1α* (elongation factor-1α, a gift from P. Pruunsild and M. Sepp (38)) was used at a ratio of 10:1 (promoter:normalizer). Transfection was performed using 1 μg of plasmid DNA plus 1 μl of Lipofectamine 2000 in a final volume of 250 μl of Neurobasal medium. The neurons were pretreated with 2-amino-5-phosphonovaleric acid (20 μM, Biotrend) overnight and stimulated on day *in vitro* 11 with bicuculline (50 μM) and 4-aminopyridine (250 μM, Sigma) for 8 h.

The luciferase assay was performed using the Dual-Glo Luciferase assay system (Promega) according to the instructions of the manufacturer. Chemiluminescence was measured by a GloMax luminometer and software (Promega and Turner Biosystems, respectively). Induction values were considered when the positive control reached ≥100-fold relative luciferase units. For presentation of the relative luciferase activity data, the background signals from untransfected neurons were subtracted from signals obtained from transfected cells. For analyses of statistical significance, Student's *t* tests were performed (two-sample, assuming equal variances). Data represent mean values ± S.E. from four independent experiments, each performed in triplicate.

Virus Production and Infection—The procedure of rAAV production has been described previously (39). In brief, human embryonic kidney 293 cells were transfected with the rAAV expression plasmid and the adeno helper plasmids 179 and 180 for AAV capsid 1 protein expression and 181 for AAV capsid 2 protein expression by standard calcium phosphate transfection (39). 72 h after transfection, cells were harvested, and the virus was purified using HiTrap heparin columns (GE Healthcare).

Cultured mouse hippocampal neurons were infected on days *in vitro* 3–4 by addition of the virus to the medium. The following rAAVs were used: rAAV-*shLrrtm2*, rAAV-*Lrrtm2-OE*, rAAV-*mCherry-NLS*, rAAV-*CaMBP4-mCherry*, rAAV-*E1A*, and rAAV-*E1AΔCR1*. The sequence of the shRNA targeting *Lrrtm2* mRNA was as follows: 5′–TGCTATTCTACTGCGACTC-3′ (27).

Data for CaMBP4 and E1A are represented as mean values ± S.E. from at least three independent experiments. For analyses of statistical significance, one-way ANOVA was performed. For post hoc comparison, Tukey or Fisher's least significant difference test were applied where appropriate.

Chromatin Immunoprecipitation—Chromatin immunoprecipitation was done using Magna ChIP chromatin immunoprecipitation kit (Millipore) according to the instructions of the manufacturer. In brief, three dishes (diameter, 6 cm) of cultured mouse hippocampal neurons, days *in vitro* 11–12, were treated with bicuculline (50 μM) for 0.5–4 h and then fixed by addition of freshly prepared paraformaldehyde (1%). Cells were harvested and lysed in a total volume of 300 μl of nuclear lysis buffer (Millipore). Chromatin was sheared to fragments of 200–1000 bp by applying eight pulses of 5-s duration and 20% power output using a Branson digital sonifier. 50 μl of sonicated DNA per immunoprecipitation were incubated with 2 μg of α-CREB and magnetic protein A/G beads (Millipore) overnight at 4 °C. Protein-DNA complexes were eluted in 100 μl of elution buffer (Millipore), and DNA was purified using QIAquick PCR purification kit (Qiagen). The following antibodies were used: α-CREB (rabbit monoclonal ab, Cell Signaling Technology) and rabbit IgG (Santa Cruz Biotechnology).

Purified DNA was diluted 1:10 for further analysis. The immunoprecipitated DNA was analyzed by quantitative PCR using Power SYBR Green PCR Master Mix (Applied Biosystems) and the following promoter-specific primers with an annealing temperature of 55 °C: p*c-fos*, 5′-AGATGTATGCCAAGACGGGGG-3′ (sense) and 5′-CAGTCGCGGTTGGAGTAGTAG-3′ (antisense); p*Mef2c*, 5′-CACTTGAGCACACGCGTACA-3′ (sense) and 5′-ACCCACACAGAACCTTCAAAGTC-3′(antisense); p*Lrrtm1*, 5′-TCGAGCCCCGAGTTTGGAGTT-3′ (sense) and 5′-TGCTTCTCGCCTTCCTGCCT-3′ (antisense); and p*Lrrtm2*, 5′-CGCCCCTGACACTGTTACAA-3′ (sense) and 5′-CCGAGAAACGGCACAAGAAT-3′ (antisense).

Four independent experiments were performed, and each sample was measured in triplicate with primers detecting the gene-specific regions. The amount of immunoprecipitated DNA is presented as percent of input and -fold enrichment over IgG. Statistical significance was assessed by Student's *t* test. The data represent mean values ± S.E.

RESULTS

Activity-dependent Expression of Lrrtm1 and Lrrtm2—The Lrrtm family has been identified recently as a group of proteins that can induce synaptic differentiation (26). Their mRNA levels have been shown to be regulated developmentally, with increasing expression during embryogenesis and a peak level at the day of birth that persists into adulthood (40). Because mechanisms that are important during embryonic development may function in a similar fashion in neuronal activity-induced plasticity in the adult nervous system, we investigated whether *Lrrtm1* and *Lrrtm2*, the two most studied members of the Lrrtm family, are regulated by neuronal activity. To study their activity-induced expression, a network of cultured hippocampal neurons was exposed to the $GABA_A$ receptor antagonist bicuculline. GABAergic interneurons, which represent about 10% of the neuronal population, impose a tonic inhibition onto the neuronal network (33). Removal of $GABA_A$ergic inhibition with bicuculline leads to action potential (AP) bursting (33), which stimulates calcium entry through synaptic NMDA receptors, induces nuclear calcium-dependent transcription, and activates a variety of gene programs (2, 13, 41, 42). A time course analysis revealed peak expression levels of *Lrrtm1* and *Lrrtm2* mRNAs 4 and 2 h, respectively, after the induction of AP bursting (Fig. 1*A*). The induction detected using quantitative reverse transcriptase PCR was about 2-fold for both *Lrrtm1* and *Lrrtm2*. We next aimed at analyzing Lrrtm1/2 protein expression using immunoblot analyses. Because of the lack of suitable antibodies to Lrrtm1, we focused our efforts on Lrrtm2. Of a panel of different commercially available antibodies, only one sheep polyclonal antibody was useful. Although this antibody detected several bands in the immunoblots, by using RNAi-mediated knockdown of Lrrtm2 as well as Lrrtm2 overexpression we were able to unambiguously identify the right band (Fig. 1*B*, *left panel*; the *arrow* indicates the Lrrtm2-specific signal). Quantitative assessment of

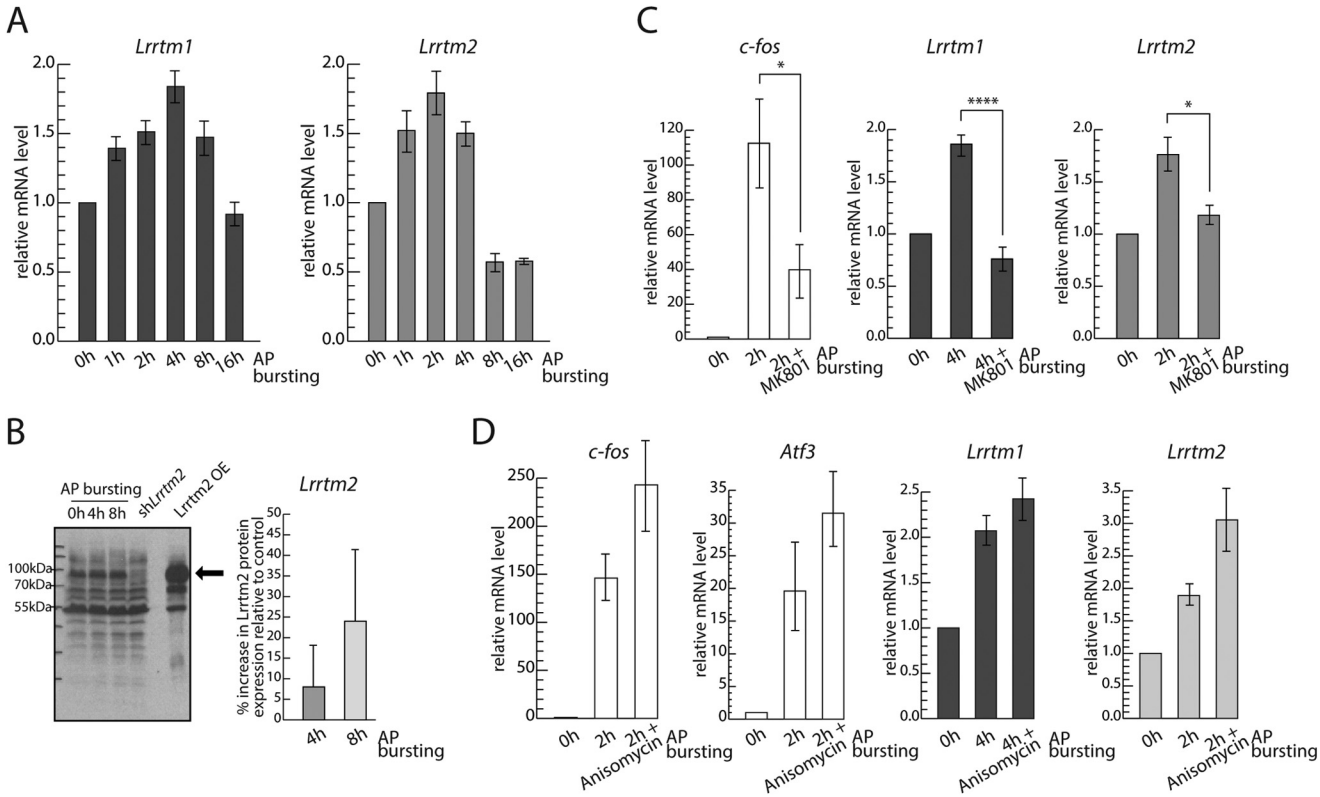

FIGURE 1. **Synaptic activity-dependent regulation of *Lrrtm1* and *Lrrtm2* expression.** *A*, primary mouse hippocampal neurons were treated with bicuculline (50 μM) for the indicated times, and endogenous *Lrrtm1* and *Lrrtm2* mRNA levels were measured by quantitative RT-PCR. *B*, *left panel,* immunoblot analysis of expression of endogenous Lrrtm2 protein in mouse hippocampal neurons. Unstimulated neurons or neurons stimulated with bicuculline are shown (*lanes 1*-3). For overexpression of Lrrtm2 protein (*Lrrtm2 OE*) and for RNAi-mediated knockdown of *Lrrtm2* expression (*shLrrtm2*), neurons were infected with rAAV-*Lrrtm2-OE* and rAAV-*shLrrtm2*, respectively. The location of the signal corresponding to Lrrtm2 is indicated with an *arrow*. *Right panel*, quantitative analysis of Lrrtm2 immunoblot analyses. *C* and *D*, analysis of *Lrrtm1*, *Lrrtm2*, *c-fos*, and *Atf3* mRNA expression in untreated mouse hippocampal neurons and in mouse hippocampal neurons after treatment with bicuculline for the indicated time in the presence or absence of MK801 (10 μM) (*C*) or anisomycin (20 μg/ml) (*D*). Treatment of the neurons with dimethyl sulfoxide, which served as the solvent for the pharmacological compounds, for 2 or 16 h or treatment of the neurons with MK801 or KN62 for 2.5 h (see Fig. 2) did not cause a significant change in the basal mRNA levels of c-*fos*, *Lrrtm1*, and *Lrrtm2* (data not shown). Treatment of the neurons with anisomycin for 2.5 h led to a small increase in the basal expression levels of mRNA levels of c-*fos*, *Lrrtm1*, and *Lrrtm2* (data not shown). The data in *A*, *C*, and *D* were obtained from at least three independent experiments with duplicate measurements and normalized to *Gusb* expression. Data are mean ± S.E. (*Atf3* in *D*, mean ± S.D.). Differences between groups were detected using one-way ANOVA followed by Tukey post hoc test. Significance was evaluated at a probability of 5% or less. *, $p < 0.05$; ****, $p < 0.00005$.

several immunoblot experiments revealed a small reproducible increase of about 25% in Lrrtm2 protein expression 8 h after the onset of AP bursting, which, however, in the statistical analysis reached a *p* value of only 0.25 (Fig. 1*B*, *right panel*).

Similar to *c-fos* and *Atf3*, two typical neuronal activity- and calcium-regulated immediate-early genes (2, 30, 43) analyzed in parallel, the induction of *Lrrtm1* and *Lrrtm2* mRNA by synaptic activity was dependent on NMDA receptor activation and blocked by the non-competitive NMDA receptor antagonist MK 801 (Fig. 1*C*). Furthermore, the regulation of *Lrrtm1* and *Lrrtm2* expression resembles that of classical immediate-early genes inasmuch as their induction by neuronal activity was not inhibited by the protein synthesis inhibitor anisomycin and, therefore, occurs independently of ongoing protein synthesis (Fig. 1*D*).

Signaling Pathways Involved in Activity-induced Expression of Lrrtm1 and Lrrtm2—We next investigated the role of CaM kinases in the activity-induced up-regulation of *Lrrtm1* and *Lrrtm2*. We found that blockade of the CaM kinases using KN62 completely abolished the activity-induced increase in *Lrrtm1* and *Lrrtm2* mRNA levels (Fig. 2*A*). Because there is a known inhibitory effect of KN62 on voltage-gated calcium channels that might affect the generation of AP bursting (44, 45), we performed calcium imaging experiments to ensure that KN62 application did not interfere with activity-induced calcium influx into the neuron. We found that, in the hippocampal culture system, KN62, when used at a concentration of 5 μM, did not compromise bicuculline-induced calcium transients (Fig. 2*B* and Ref. 14). Therefore, the inhibition of the bicuculline-induced increase in *Lrrtm1* and *Lrrtm2* mRNA levels by KN62 reflects the requirement for CaM kinases in activity-dependent regulation of these genes.

Nuclear Calcium Signaling Regulates the Induction of Lrrtm1 and Lrrtm2—We next investigated the possible role of nuclear calcium-CaM kinase signaling in the activity-dependent regulation of *Lrrtm1* and *Lrrtm2*. Nuclear calcium is a known activator of nuclear-localized CaM kinases such as CaMK II and CaMK IV, both of which are important factors in mediating activity-induced genomic responses (2, 14, 46–48). To study the function of nuclear calcium signaling in the regulation of *Lrrtm1* and *Lrrtm2*, we used CaMBP4, a nuclear protein that contains four repeats of the M13 calmodulin binding peptide from myosin light chain kinase. It binds to and inactivates the nuclear calcium-CaM complex (49). CaMBP4 has been used

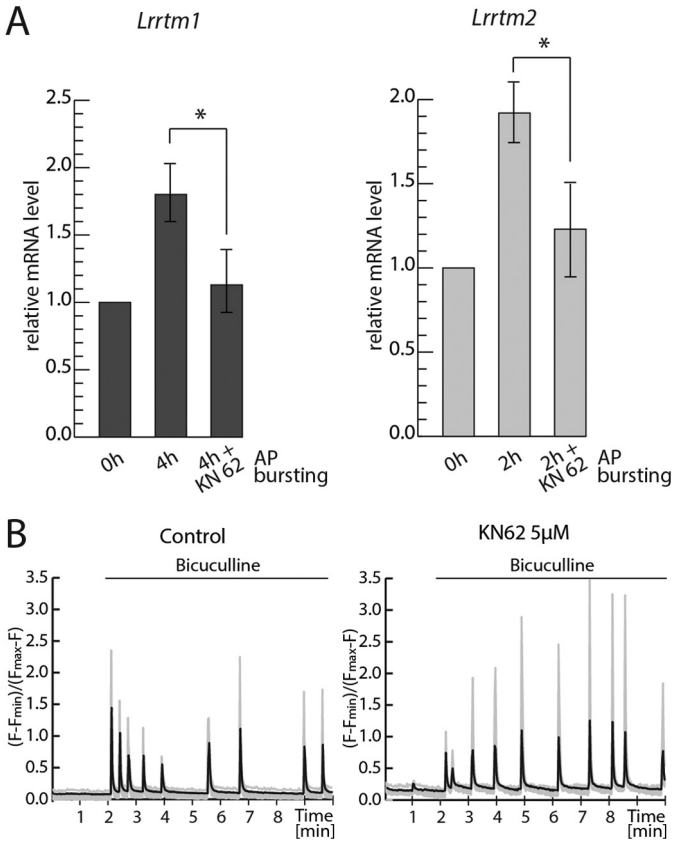

FIGURE 2. **Role of CaMKs in activity-dependent regulation of *Lrrtm1* and *Lrrtm2* expression and Fluo calcium imaging.** *A*, analysis of *Lrrtm1* and *Lrrtm2* mRNA expression in untreated mouse hippocampal neurons and in mouse hippocampal neurons treated with bicuculline for the indicated times in the presence or absence of KN62 (5 μM). Data were obtained from at least four independent experiments with duplicate measurements and normalized to *Gusb* expression. *Lrrtm1* and *Lrrtm2* data are mean ± S.E. Statistical significance was assessed by one-way ANOVA followed by Tukey post hoc analysis. *, $p < 0.05$. *B*, Fluo-3 calcium imaging of mouse hippocampal neurons stimulated with bicuculline in the absence (*left panel*) or presence (*right panel*) of KN62 (see "Experimental Procedures" for details). Preincubation with KN62 was for 45 min. *Gray lines* represent individual cells. The mean signal is depicted as a *black line*. Representative traces from one of three independent experiments are shown.

previously to identify nuclear calcium-regulated genes that are important for neuroprotection, memory consolidation, and the development of chronic pain (2, 3, 18, 50, 51).

Primary mouse hippocampal neurons were infected with an rAAV containing an expression cassette for *CaMBP4* (rAAV-*CaMBP4-mCherry*) or for mCherry-NLS as a control (rAAV-*mCherry-NLS*). We found that the AP bursting-induced increase in mRNA expression of both *Lrrtm1* and *Lrrtm2* observed in uninfected and control-infected cultures was abolished in cultures infected with rAAV-*CaMBP4-mCherry* (Fig. 3*A*). CaMBP4 also blocked the activity-dependent induction of *c-fos* and *Atf3*, two known nuclear calcium-regulated genes (2, 50) that we analyzed in parallel (Fig. 3*A*). These results indicate that nuclear calcium signaling is required for the synaptic activity-dependent regulation of *Lrrtm1* and *Lrrtm2*.

To confirm that AP bursting does induce calcium transients that invade the cell nucleus, we carried out calcium imaging experiments using the nuclearly targeted recombinant calcium sensor GCaMP6F-NLS. We found that AP bursting triggered by bicuculline treatment gave rise to robust increases in nuclear calcium concentration (Fig. 3*B*). Immunocytochemical analysis revealed colocalization of GCaMP6F-NLS with the nuclear stain Hoechst 33258, confirming the nuclear localization of the calcium sensor (Fig. 3*C*).

Lrrtm1 and Lrrtm2 Expression and CREB—To identify putative binding sites of transcription factors that are activated downstream of nuclear calcium signaling, we performed an online database search of a 2000-bp-long upstream region of *Lrrtm1* and *Lrrtm2* genes using the Transcription Element Search System to recognize transcription regulatory elements. The search retrieved two CREs in the immediate vicinity of the transcription start site of *Lrrtm2*. Because CRE functions as a nuclear calcium response element (52), its presence in the *Lrrtm2* promoter might confer nuclear calcium responsiveness to this gene. The CRE is bound by CREB, a transcription factor that, together with CBP, forms a prototypical nuclear calcium-controlled transcription-regulating complex (12, 13, 53). To study the role of the CREs in the promoter of *Lrrtm2*, we constructed two *Lrrtm2* promoter-containing reporter plasmids. The wild-type reporter (p*Lrrtm2*^{WT}*-FLuc*) consists of a *firefly luciferase* (*FLuc*) reporter gene driven by a 356-bp-long sequence of the mouse *Lrrtm2* promoter region harboring a TATA box as well as a half and a full CRE site (Fig. 4*A*, *top panel*). Mutations were introduced into both CREs to generate a reporter construct that lacks the binding sites for CREB (p*Lrrtm2*$^{\Delta CRE}$*-FLuc*) (Fig. 4*A*, *bottom panel*). These constructs were transfected into hippocampal neurons and tested for their regulation by neuronal activity. To reduce the basal level of reporter gene expression, the neurons were kept in a medium containing 20 μM 2-amino-5-phosphonovaleric acid for 16 h prior to AP bursting, which was induced by bicuculline treatment in the presence of 4-aminopyridine, a weak potassium channel blocker that increases the bursting frequency (54). We found that bicuculline/4-aminopyridine treatment lead to a small but significant 1.5-fold induction of the firefly reporter signal generated by the wild-type promoter, p*Lrrtm2*^{WT}*-FLuc* (Fig. 4*B*). This induction was not observed with the firefly reporter containing the mutant promoter, p*Lrrtm2*$^{\Delta CRE}$*-FLuc*, which, compared with the wild type, *Lrrtm2*^{WT}*-Fluc*, also showed lower basal expression levels (Fig. 4*B*). These results indicate that the CRE present in the *Lrrtm2* promoter is functionally relevant for both the basal and activity-induced regulation of *Lrrtm2* expression.

We next used chromatin immunoprecipitation experiments to investigate whether CREB binds to the genomic regions adjacent to the transcription start sites of *Lrrtm1* and *Lrrtm2*. Binding of endogenous CREB to the *Lrrtm1* and *Lrrtm2* regulatory regions was detected by immunoprecipitation of sheared chromatin from cultured hippocampal neurons using an antibody to CREB. The amount of immunoprecipitated DNA was measured by quantitative PCR with primers specific for the regulatory regions of *Lrrtm1* (p*Lrrtm1*) and *Lrrtm2* (p*Lrrtm2*). We observed a robust enrichment of the regulatory region of *Lrrtm2* but not of *Lrrtm1* (Fig. 4*C*). *c-fos*, a gene known to be regulated by CREB (55, 56), was analyzed in parallel and served as a positive control (p*c-fos*). As expected, the promoter of *c-fos* was enriched in the immunoprecipitated DNA (Fig. 4*C*). In

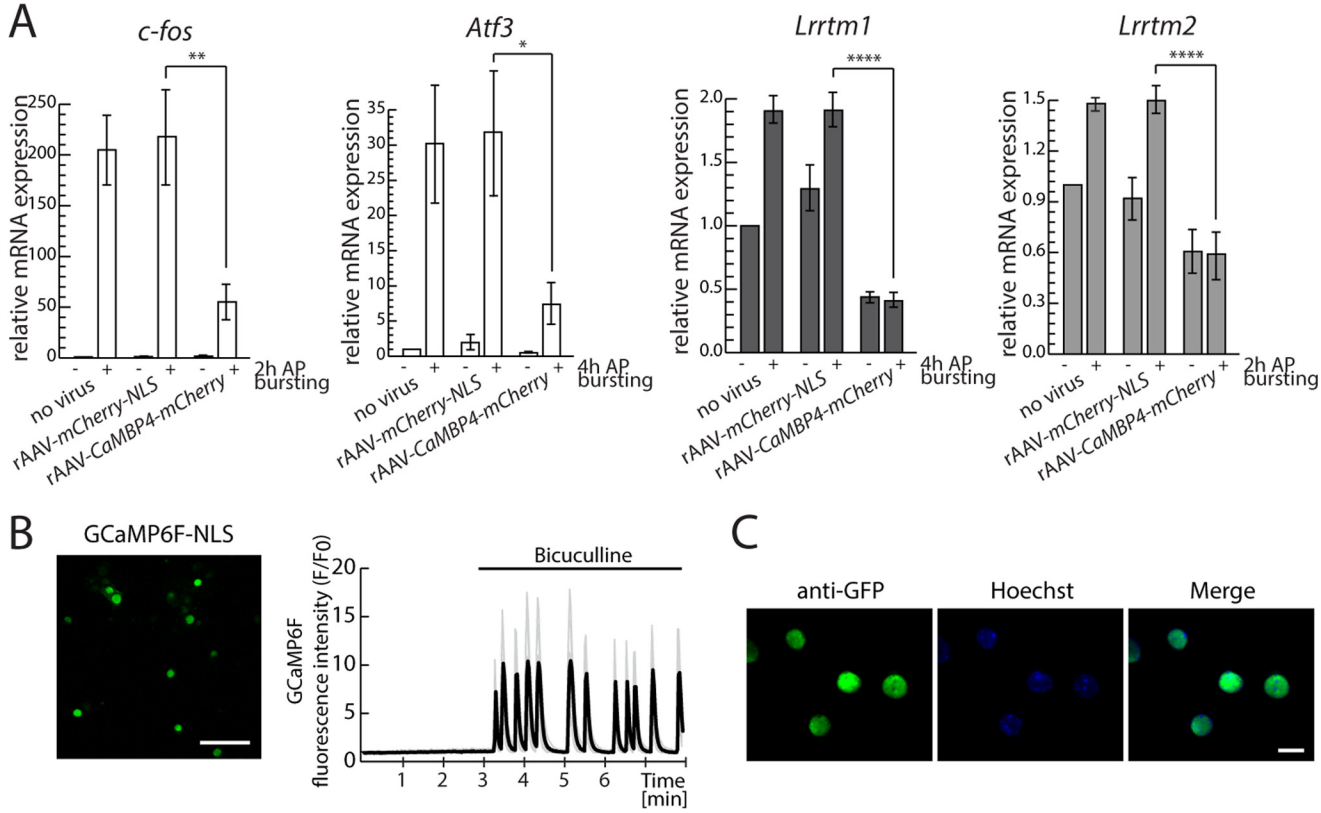

FIGURE 3. **Role of nuclear calcium signaling in the activity-dependent regulation of *Lrrtm1* and *Lrrtm2* expression and imaging of nuclear calcium using GCaMP6F-NLS.** *A*, analysis of *Lrrtm1*, *Lrrtm2*, *c-fos*, and *Atf3* mRNA expression in uninfected mouse hippocampal neurons and in mouse hippocampal neurons infected with rAAV-*CaMBP4-mCherry* or rAAV-*mCherry-NLS*. Neurons were treated with bicuculline (50 μM) for the indicated times to induce AP busting or were left unstimulated. Data represent means ± S.E. from at least three independent experiments with duplicate measurements and are normalized to *Gusb*. Statistical significance was assessed by one-way ANOVA followed by Tukey post hoc analysis. *, $p < 0.05$, **, $p < 0.005$; ****, $p < 0.00005$). *B*, calcium imaging of mouse hippocampal neurons infected with rAAV-*GCaMP6F-NLS*. GCaMP6F-NLS-derived signals were measured before and after stimulation of the neurons with bicuculline (50 μM) (*right panel*) and expressed as ΔF/F0 (see "Experimental Procedures" for details). *Gray lines* represent individual cells. The mean signal is depicted as a *black line*. *Left panel*, projection of several GCaMP6F-NLS fluorescence images taken after exposure of the neurons to bicuculline (50 μM). *Scale bar* = 50 μm. *C*, immunocytochemical detection of GCaMP6F-NLS in mouse hippocampal neurons infected with rAAV-*GCaMP6F-NLS* using an antibody to GFP. Neurons were counterstained with Hoechst 33258 to mark the location of the cell nucleus. *Scale bar* = 10 μm.

contrast, no significant enrichment could be measured for the promoter of *Mef2c*, which was used as a negative control (Fig. 4*C*, p*Mef2c*). These results indicate that CREB binds to the analyzed regulatory region of *Lrrtm2* but not to that of *Lrrtm1*.

The Role of CBP in Lrrtm1 and Lrrtm2 Expression—An online database search using the Transcription Element Search System retrieved a list of possible binding sites for transcription factors in the promoter regions of *Lrrtm1* and *Lrrtm2,* including activator protein 1 complex (AP1), CREB, specificity protein 1 (SP1), upstream stimulatory factor, and nuclear factor of activated T cells. CBP is a transcriptional coactivator that interacts with a variety of transcription factors (57), including factors with putative binding sites in the *Lrrtm1* and *Lrrtm2* promoters. Also, like the expression of *Lrrtm1* and *Lrrtm2*, nuclear calcium and CaM kinases control CBP activity (12). Therefore, we investigated the role of CBP in the regulation of *Lrrtm1*, *Lrrtm2*, and the control gene *c-fos* using an rAAV containing an expression cassette for the adenovirus protein E1A. E1A binds to CBP via its amino terminus-conserved region 1 (CR1) and disrupts CBP function (58, 59). Mouse hippocampal neurons were infected with rAAV-*E1A* or with rAAV-*E1AΔCR1*, a control virus expressing E1A that lacks CR1 and fails to interact with CBP (58, 59). To induce AP bursting, the cultures were exposed to bicuculline, which induced an about 2-fold increase of *Lrrtm1* and *Lrrtm2* mRNA levels after 4 and 2 h, respectively, in uninfected cultures. This increase was reduced in neurons infected with rAAV-*E1A* but not in neurons infected with the control virus, rAAV-*E1AΔCR1* (Fig. 5). These results suggest a key role for CBP in the synaptic activity-dependent regulation of *Lrrtm1* and *Lrrtm2* expression.

DISCUSSION

This study uncovered the neuronal activity-dependent regulation of two synaptic cell adhesion molecule genes, *Lrrtm1* and *Lrrtm2.* Both *Lrrtm1* and *Lrrtm2* are immediate-early genes whose synaptic activity-dependent increase in mRNA expression requires nuclear calcium signaling, the activation of CaM kinases, and CBP function.

Regulation of Genomic Responses through the Signaling Cascade of Neuronal Activity-Nuclear Calcium-CREB/CBP—Nuclear calcium-CaMKIV-CREB/CBP is a major signaling pathway in gene regulation by synaptic activity (11). Other pathways include the ERK-MAP kinase cascade, p38 MAP kinases, and calcineurin/nuclear factor of activated T-cells signaling (11). CREB is involved in many functions of the central nervous system, particularly in neuronal survival, memory formation,

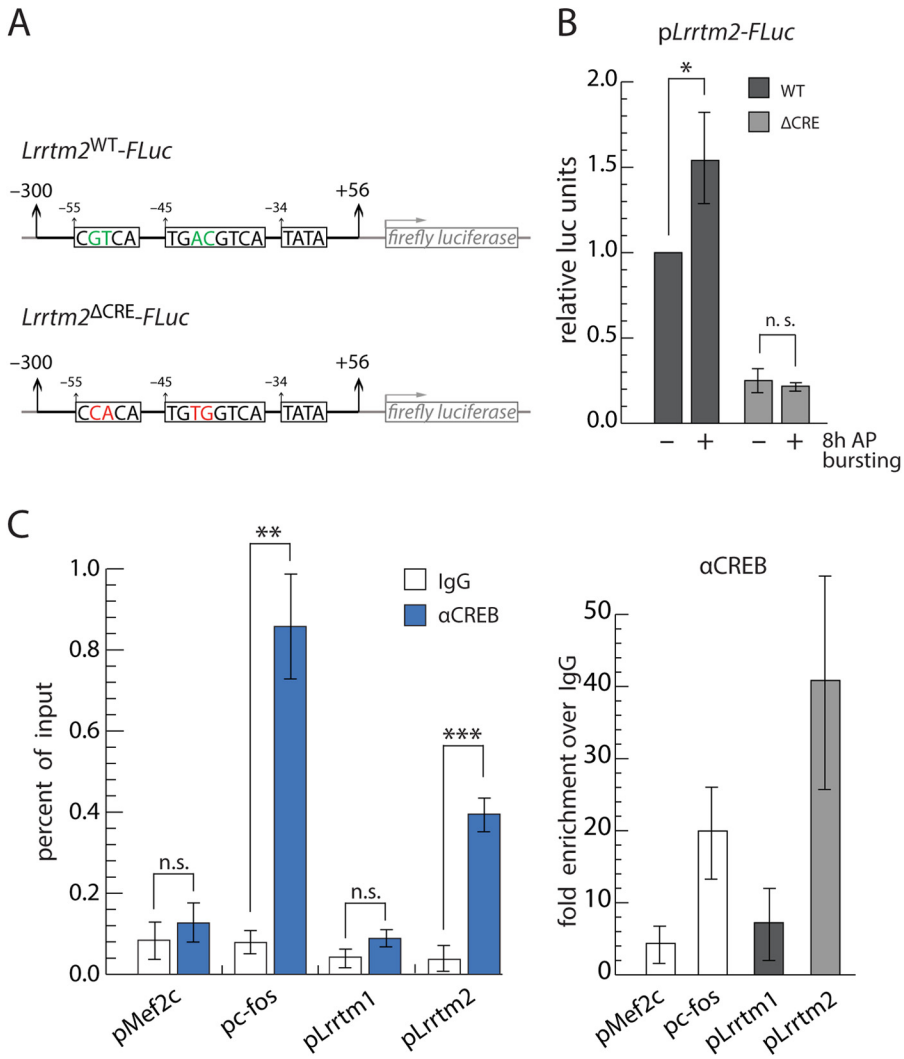

FIGURE 4. **Analysis of Lrrtm2 reporter gene expression and ChIP analysis of CREB binding to the *Lrrtm2* promoter.** *A* and *B*, primary rat hippocampal neurons were transfected with wild-type (p*Lrrtm2*^{WT}-FLuc) or mutant (p*Lrrtm2*$^{\Delta CRE}$-FLuc) pGL4.10-based firefly luciferase (*FLuc*) reporter constructs (shown schematically in *A*; *green letters* indicate the wild-type sequence, and *red letters* indicate the mutant sequence) alongside an *EF1*α promoter-dependent humanized *Renilla* luciferase (*hRLuc*) construct. Following incubation with 2-amino-5-phosphonovaleric acid (20 μM) overnight, the neurons were stimulated with bicuculline (50 μM) plus 4-aminopyridine (250 μM) for 8 h. Luciferase activities were measured and represented as -fold change in expression relative to the *Lrrtm2* wild-type reporter gene construct (*B*). Data represent mean ± S.E. from four independent experiments with triplicate measurements normalized to hRluc activities. Statistical significance was assessed by Student's *t* test. *, $p < 0.05$; *n.s.*, not significant. *C*, *left panel*, ChIP analysis of CREB binding to the promoter of *Lrrtm1* and *Lrrtm2* in mouse hippocampal neurons. The binding was detected with anti-CREB antibodies in cell lysates from neurons treated with bicuculline (50 μM) for 30 min. The data are represented as percent of input DNA determined by quantitative PCR using promoter-specific primers. *Right panel*, representation of the data as -fold enrichment of anti-CREB immunoprecipitated DNA over the IgG control for the indicated genes. The data represent mean ± S.E. from four independent experiments measured in triplicates and normalized to the levels of the respective target in the input DNA. Statistical significance was assessed by Student's *t* test. **, $p < 0.005$, ***, $p < 0.0005$.

addiction, and neurogenesis (2, 50, 60–63). In this study, we identified *Lrrtm2* as a direct CREB target. Lrrtm2 plays a role in stabilizing AMPA receptors in the postsynaptic membrane during the maintenance of long-term potentiation (LTP) (64) and may, therefore, be part of a nuclear calcium-CREB/CBP-regulated gene program required for the long-term implementation of changes in synaptic efficacy.

CBP in Lrrtm1 and Lrrtm2 Regulation—Our finding that the activity-dependent expression of both *Lrrtm1* and *Lrrtm2* requires CBP function may be relevant for Rubinstein-Taybi syndrome, which is caused by mutations in the *CBP* gene (65). It is conceivable that a disruption of the "CBP-Lrrtm axis" and possible alterations in the Lrrtm1/2-dependent maintenance of LTP (64) may contribute to the learning difficulties associated with this disease (66). Consistent with this idea is the finding that, in humans, a microdeletion affecting *LRRTM2* is associated with mild cognitive impairment and developmental delay (67) that resemble the neuropsychiatric deficits found in Rubinstein-Taybi syndrome (66). Whether or not the expression levels of *Lrrtm1* and *Lrrtm2* are altered in Rubinstein-Taybi syndrome remains to be investigated.

Differences in Lrrtm1 and Lrrtm2 Regulation—Although both *Lrrtm1* and *Lrrtm2* are subject to regulation by synaptic activity, NMDA receptors, CaMK signaling, and CBP, their mRNA expression profiles follow different kinetics after stimulation, and only *Lrrtm2* appears to be a direct target of CREB. This suggests the involvement of different transcription factors in the control of *Lrrtm1* and *Lrrtm2* expression. Their func-

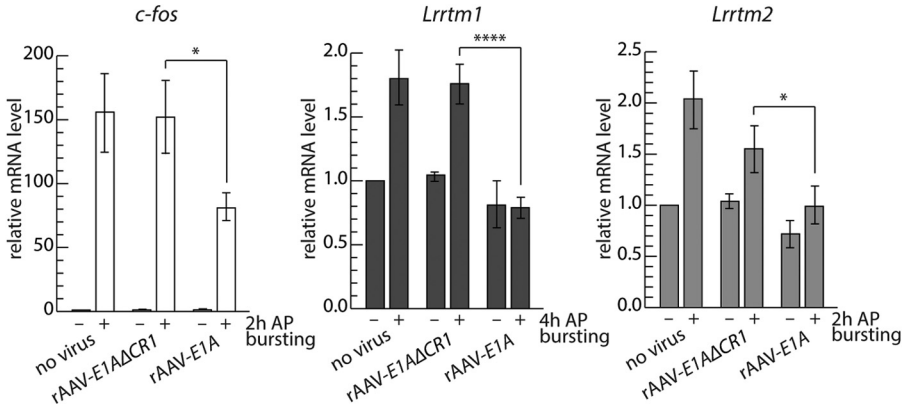

FIGURE 5. **Role of CBP in activity-dependent regulation of *Lrrtm1* and *Lrrtm2* expression.** Analysis of *Lrrtm1*, *Lrrtm2*, and *c-fos* mRNA expression in uninfected mouse hippocampal neurons and in mouse hippocampal neurons infected with rAAV-*E1A* or rAAV-*E1A*Δ*CR1*. Neurons were treated with bicuculline (50 μM) for the indicated times to induce AP busting or were left unstimulated. Data represent mean ± S.E. from at least three independent experiments with duplicate measurements and are normalized to *Gusb*. Statistical significance was assessed by one-way ANOVA followed by Fisher's least significant difference post hoc analysis. *, $p < 0.05$; ****, $p < 0.00005$.

tional properties, however, are similar and include binding to neurexin, a synaptogenic activity, and a role in LTP (26, 64). Mutations and polymorphisms are found more frequently for *Lrrtm1*, and, in humans, are associated with handedness and schizophrenia (68, 69). Only one case of *LRRTM2* mutation has been reported in humans, which caused a mild cognitive impairment and developmental delay (67).

Possible Function of Activity-dependent Regulation of Lrrtm1 and Lrrtm2—The functional significance of the activity-dependent regulation of *Lrrtm1* and *Lrrtm2* still remains to be demonstrated. Given the synaptic localization of Lrrtm1 and Lrrtm2, changes in their expression levels provide a means of returning signals and information from the nucleus back to the synapses. This process may be relevant for the maintenance of synapses. Synaptic activity induces proteolytic cleavage of postsynaptic neuroligin 1, which destabilizes the interaction with its presynaptic binding partner neurexin 1, leading to a reduction of synaptic transmission (70, 71). The cleavage of neuroligin 1 is triggered by the activation of NMDA receptors and requires CaM kinase activity. The same NMDA receptor-CaM kinase signaling pathway is also responsible for the induction of *Lrrtm1* and *Lrrtm2* expression upon synaptic activity. Given that Lrrtm1 and Lrrtm2 can also bind neurexin, they may substitute for neuroligin 1 in the stabilization of synaptic contacts until a new neuroligin-neurexin complex is formed. Therefore, the activity-dependent regulation of *Lrrtm1* and *Lrrtm2* expression may help maintain the structural integrity of the synapse.

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