Cellular/Molecular

Activity-Dependent Nr4a2 Induction Modulates Synaptic Expression of AMPA Receptors and Plasticity via a Ca^{2+}/CRTC1/CREB Pathway

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Transcription factors have a pivotal role in synaptic plasticity and the associated modification of neuronal networks required for memory formation and consolidation. The nuclear receptors subfamily 4 group A (Nr4a) have emerged as possible modulators of hippocampal synaptic plasticity and cognitive functions. However, the molecular and cellular mechanisms underlying Nr4a2-mediated hippocampal synaptic plasticity are not completely known. Here, we report that neuronal activity enhances Nr4a2 expression and function in cultured mouse hippocampal neurons (both sexes) by an ionotropic glutamate receptor/Ca^{2+}/cAMP response element-binding protein/CREB-regulated transcription factor 1 (iGluR/Ca^{2+}/CREB/CRTC1) pathway. Nr4a2 activation mediates BDNF production and increases expression of iGluRs, thereby affecting LTD at CA3-CA1 synapses in acute mouse hippocampal slices (both sexes). Together, our results indicate that the iGluR/Ca^{2+}/CREB/CRTC1 pathway mediates activity-dependent expression of Nr4a2, which is involved in glutamatergic synaptic plasticity by increasing BDNF and synaptic GluA1-AMPARs. Therefore, Nr4a2 activation could be a therapeutic approach for brain disorders associated with dysregulated synaptic plasticity.

Key words: **glutamate receptors; hippocampus; LTD; neuronal activity; Nr4a2; synaptic plasticity**

Significance Statement

A major factor that regulates fast excitatory synaptic transmission and plasticity is the modulation of synaptic AMPARs. However, despite decades of research, the underlying mechanisms of this modulation remain poorly understood. Our study identified a molecular pathway that links neuronal activity with AMPAR modulation and hippocampal synaptic plasticity through the activation of Nr4a2, a member of the nuclear receptor subfamily 4. Since several compounds have been described to activate Nr4a2, our study not only provides mechanistic insights into the molecular pathways related to hippocampal synaptic plasticity and learning, but also identifies Nr4a2 as a potential therapeutic target for pathologic conditions associated with dysregulation of glutamatergic synaptic function.

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Introduction

The capability of the brain to adapt to environmental stimuli requires dynamic changes in neuronal circuits. By strengthening or weakening neuronal connections, experience-mediated modifications to synaptic transmission and plasticity can support brain functions, such as learning and memory (Bocchio et al., 2017; Lisman et al., 2018). Understanding the cellular and molecular mechanisms underlying synaptic transmission and plasticity is critical to advancing our knowledge of normal brain function. Furthermore, the identification of aberrations in synaptic transmission and plasticity associated with cognitive impairments, as seen in Alzheimer's disease (Selkoe, 2002; Forner et al., 2017), may uncover novel therapeutic targets.

A main event supporting dynamic changes in excitatory synaptic strength is the modulation of the number, subunit composition,

and properties of postsynaptic ionotropic glutamate receptors, specifically AMPARs (Kessels and Malinow, 2009; Huganir and Nicoll, 2013; Diering and Huganir, 2018). Moreover, long-lasting changes to neurotransmission associated with synaptic plasticity require *de novo* gene expression controlled by transcription factors (Alberini, 2009; Benito and Barco, 2015). Several transcription factors are needed for synaptic plasticity processes, such as LTP and LTD (Abraham et al., 1993; Albensi and Mattson, 2000; Barco et al., 2002; Bozon et al., 2002). Meanwhile, alterations in gene-expression programs controlled by transcription factors have been linked to synaptic dysfunction underlying several neurologic disorders (Parra-Damas et al., 2014; Tindi et al., 2015). Among these transcription factors, CREB protein is implicated in regulating hippocampal synaptic plasticity (Impey et al., 1996; Barco et al., 2002). It is known that CREB impacts the expression of hundreds of neuronal genes, and specific CREB-mediated transcriptional programs seem to depend on the association to different CREB coactivators (Saura and Cardinaux, 2017). The CREB-regulated transcription coactivator 1 (CRTC1) interacts with CREB in response to neuronal activity (Ch'ng et al., 2012) and is involved in the activation of a transcriptional program related to hippocampal synaptic plasticity and learning (Kovács et al., 2007; Sekeres et al., 2012; Uchida et al., 2017). However, the impact of CRTC1-mediated signaling cascades on hippocampal synaptic plasticity remains poorly understood. Given their potential role in synaptic plasticity and learning, the Nr4a family of orphan nuclear receptors have received attention as an important CREB/CRTC1 regulated gene family. For instance, general genetic blockade of the Nr4a family in the hippocampus resulted in LTP impairment and produced severe deficits in hippocampal-dependent contextual fear memory (Hawk et al., 2012; Bridi and Abel, 2013). The molecular mechanisms regulated by this transcription factor family during learning and memory consolidation in the hippocampus are unknown. Nr4a1 was shown to regulate gene transcription leading to hippocampal spine loss (Chen et al., 2014); and recently, its relevance in hippocampal long-term memory processes was associated with downstream regulation of several chaperone genes, such as *Hspa5* (Chatterjee et al., 2022). More evidence has linked Nr4a2 to hippocampal learning and memory processes. For example, Nr4a2 expression was induced in the hippocampus after active avoidance conditioning (Aldavert-Vera et al., 2013). Also, Nr4a2 failed to be induced by an object location memory test in cognitively impaired aged rats, whereas overexpression of Nr4a2 ameliorated this age-related impairment (Kwapis et al., 2019). In addition, pharmacological activation of Nr4a2 enhanced contextual fear learning, increased hippocampal LTP duration (Bridi et al., 2017), and rescued spatial object recognition deficits observed in aged mice (Chatterjee et al., 2020). Despite the evidence that Nr4a2 plays an important role in hippocampal synaptic plasticity and learning, the precise molecular and cellular mechanisms by which Nr4a2 regulates these processes remain unknown.

Here, we sought to elucidate how activity-dependent induction of Nr4a2 modulates hippocampal synaptic plasticity, which may provide important mechanistic insights on the cognitive deficits associated with aging or several brain disorders. We found that Nr4a2 activation in cultured hippocampal neurons relies on an iGluR/Ca^{2+}/CREB/CRTC1 pathway. Moreover, we have discovered that Nr4a2 modulates hippocampal LTD by increasing synaptic AMPARs in a BDNF-dependent manner. To our knowledge, this finding reveals for the first time a molecular pathway underlying the functional role of Nr4a2 in hippocampal synaptic plasticity.

Materials and Methods

Animals. C57Bl-6J/RccHsd mice and Sprague Dawley rats were handled and used under institutional and national regulation approved by the Animal and Human Ethical Committee of the Universitat Autònoma de Barcelona (CEEAH 2896, DMAH 8787) following European Union regulation (2010/63/EU) and by the Albert Einstein College of Medicine Institutional Animal Care and Use Committee in accordance with the National Institute of Health guidelines. All animals were group-housed under standard laboratory conditions (food and water *ad libitum*, 22 ± 2°C, 12 h light:dark cycle).

We have used C57Bl-6J/RccHsd mice (primary neuronal cultures, AAV manipulations, acute hippocampal slices for electrophysiology experiments) and Sprague Dawley rats (acute hippocampal slices for electrophysiological experiments). Both male and female animals were used in all the experiments.

Primary neuronal cultures. Primary hippocampal neurons were prepared from C57Bl-6J/RccHsd mice pups from postnatal day 0-2 (P0-P2). Hippocampal cells were mechanically and enzymatically dissociated with papain (Sigma). Neurons were plated in Neurobasal-A medium supplemented with 2% B27 (Fisher Scientific). Half of medium was replaced 1 d later, and then every 6–7 d. The mitotic inhibitor fluorodeoxyuridine (Sigma) was added at 3 DIV to inhibit proliferation of non-neuronal cells.

Primary cortical neurons were prepared from C57Bl-6J/RccHsd embryos of 15 d (E15). Cortical cells were mechanically and enzymatically dissociated with trypsin (Sigma). Neurons were plated in DMEM supplemented with 10% heat inactivated FBS, 1× penicillin/streptomycin, and 30 mM glucose. Three hours after seeding, medium was replaced with Neurobasal medium supplemented with 2% B27, 1× penicillin/streptomycin, and 1× glutaMAX-I. Half of the medium was replaced every 4–5 d.

Neurons were plated at specified density (70,000 cells/ml for immunocytochemistry, 125,000 cells/ml for luciferase assay, 250,000 cells/ml for molecular and biochemical assays) in poly-D-lysine-pretreated plates (24-well plates for immunocytochemistry and luciferase assays, 12-well plates for molecular and biochemical assays, 60-mm-diameter plates for chromatin immunoprecipitation [ChIP]). Cultures were maintained in a humidifier incubator at 37°C with 5% CO_2. Experiments were performed at mature hippocampal neuronal cultures (18-21 DIV) or mature cortical neuronal cultures (13-15 DIV) unless otherwise indicated.

Hippocampal slice preparation. Acute transverse hippocampal slices were prepared from P17 to P27 Sprague Dawley rats (400 μM) and P49 to P57 C57Bl/6J mice (300 μM) (Charles River Labs) using a VT1200 Leica vibratome. The cutting solution for rat hippocampi contained the following (in mM): 215 sucrose, 2.5 KCl, 26 $NaHCO_3$, 1.6 NaH_2PO_4, 1 $CaCl_2$, 4 $MgCl_2$, 4 $MgSO_4$, and 20 D-glucose. Mouse slices were prepared using an NMDG-based cutting solution containing the following (in mM): 93 N-methyl-D-glucamine, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 D-glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 $CaCl_2$, 10 $MgCl_2$. The ACSF recording solution contained the following (in mM): 124 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1 NaH_2PO_4, 2.5 $CaCl_2$, 1.3 $MgSO_4$, and 10 D-glucose. After ice-cold cutting, rat slices recovered at 34°C in 50% cutting solution, 50% ACSF for 30 min and then at room temperature (RT) for >1 h in ACSF. Mouse slices were transferred directly to ACSF at 34°C in warm water bath and then incubated at RT for at least 1 h. All solutions were equilibrated with 95% O_2 and 5% CO_2 (pH 7.4).

Immunoblotting. Primary neuronal cultures were homogenized in RIPA-lysis buffer containing 50 mM Tris hydrochloride (Tris-HCl), pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 1% Nonidet P-40, 0.1% SDS, 1 mM Na_3VO_4, 50 mM NaF, and 1 mM PMSF supplemented with a cocktail of protease and phosphatase inhibitors (Sigma). Protein concentration was determined using the Pierce BCA protein assay kit (Fisher Scientific). Equal amounts of protein were separated on 7.5%-

12% SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare). Blots were blocked 1 h with 10% dry milk, 0.1% BSA pH 7.4 in PBS or Tris-HCl buffered saline (TBS) and incubated at 4°C overnight with primary antibodies. After incubation with appropriate HRP-conjugated secondary antibodies (HRP-linked anti-mouse or anti-rabbit IgG; BD Biosciences) at RT for 1 h, blots were developed using ECL Western blotting Detection Reagents (GE Healthcare). Blots densitometry was performed using ImageJ (National Institutes of Health), and protein levels were corrected for corresponding loading control. For each blot, values were normalized to basal or bicuculline conditions (assigned with an arbitrary value of 1), as indicated in the figure legends.

Immunocytochemistry. Cultured hippocampal neurons were fixed in a solution containing 4% PFA and 4% sucrose in PBS for 15 min at 4°C, then permeabilized with 0.1% Triton X-100 20 min at RT and blocked in 2% normal goat serum (Sigma) in PBS (blocking solution) 45 min at 37°C. Fixed neurons were stained in blocking solution with anti-CRTC1 (1:300, Cell Signaling) primary antibody 1 h at 37°C, and detected with Alexa-568 secondary antibody (1:500, Fisher Scientific) and Hoechst 33258 (1:10,000; Fisher Scientific) to visualize the nuclear area. All images (40×) were acquired with a Zeiss Axio Examiner D1 LSM700 laser scanning microscope (Carl Zeiss Microscopy) using the same settings for photomultiplier voltage, gain, and offset. Images were analyzed with ImageJ software. Nuclear CRTC1 staining intensity was measured using the maximal projections of *Z*-stacked images (10-15 stacks of 0.5 μm/section). Integrated density values obtained were indicated as arbitrary units (AU).

RNA extraction, cDNA synthesis, and qRT-PCR. Total RNA was extracted using the RNeasy Mini kit according to supplier's recommendations. RNA concentration was determined using the NanoDrop assay (Fisher Scientific), and RNA integrity was assessed with the Agilent RNA 6000 Nano kit on an Agilent 2100 BioAnalyzer (Agilent Technologies); 500 ng of RNA was used for reverse transcription with oligo(dT) primers and SuperScriptIV reverse transcriptase (Fisher Scientific). qRT-PCR was performed in triplicate using the Power SYBR Green PCR Master Mix (Fisher Scientific) in the Applied Biosystems 7500 Fast Real-Time PCR system (Fisher Scientific). Fold change was calculated with the ΔΔCt-method and normalized to the geometric mean of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and peptidylpolyl isomerase A (*Ppia*) gene expression. The sequences of specific primers were as follows: *Nr4a2* (forward) 5′-CTA TTC CAG GTT CCA GGC A-3′; (reverse) 5′-TGT TGG GTA TCA TCT CCA CTC-3′; *Bdnf* exon IV (forward) 5′-CTT CTT TGC TGC AGA ACA GG-3′; (reverse) 5′-CTT CTC ACC TGG TGG AAC TT-3′ *Gapdh* (forward) 5′-AAT TCA ACG GCA CAG TCA AGG C-3′; (reverse) 5′-TAC TCA GCA CCG GCC TCA CC-3′; *Ppia* (forward) 5′-GAC TGA ATG GCT GGA TGG-3′; (reverse) 5′-GGA AAT GGT GAT CTT CTT GCT-3′.

ChIP assay. ChIP was performed as previously described (España et al., 2010). Briefly, cultured neurons were cross-linked with 1% formaldehyde for 10 min at RT. Crosslinking was stopped with 0.125 M glycine. Cells were lysed and sonicated (Bioruptor Plus, Diagenode) in SDS-lysis buffer containing 50 mM Tris-HCl, pH 8.1, 100 mM NaCl, 5 mM EDTA, 1% SDS, 0.1% Na deoxycholate, and a cocktail of protease and phosphatase inhibitors (Sigma). ChIP was performed overnight in ChIP dilution buffer containing 16.7 mM Tri-HCl, pH 8.1, 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100, 0.01% SDS, 0.1% Na deoxycholate with or without rabbit anti-CRTC1 and CREB antibodies (Cell Signaling). Input and immunoprecipitated DNA were decrosslinked and amplified by qRT-PCR using specific primers. *Nr4a2* (forward) 5′-TAC CAA GGT GAA CCG TTC C-3′; (reverse) 5′-GCC AAC ATG CAC CTA AAG TC-3′; *Nr4a3* irrelevant region (forward) 5′-TCA GTC TTT GCC AGC AGG T-3′; (reverse) 5′-GCT CAG AAG CCA GTT GAC AC-3′.

Plasmid construction. Complementary oligonucleotides for mouse CREB and CRTC1 shRNA were as follows: sh-CRTC1 forward: 5′-gatc cccGCAGCGTGACAATCGACCTATttcaagagaATAGGTCGATTGTCAC GCTGCttttt-3′. sh-CRTC1 reverse: 5′-agctaaaaaGCAGCGTGACAATC GACCTATtctcttgaaATAGGTCGATTGTCACGCTGCggg-3′. sh-CREB forward: 5′-gatccccCTGAAGAAGCAGCACGAAAttcaagagaCTGAAG AAGCAGCACGAAAtctcttgaa-3′. sh-CREB reverse: 5′-agctaaaaaCTGA AGAAGCAGCACGAAAtctcttgaaTTTCGTGCTGCTTCTTCAGggg-3′. shCRTC1 and shCREB constructions were generated as previously described (España et al., 2010; Barneda-Zahonero et al., 2012). Briefly, oligonucleotides were cloned into BglII/HindIII sites of the pSUPER.retro.puro plasmid (OligoEngine). Lentiviral vectors were obtained by digesting EcoRI-ClaI sites from pSUPER-Sh to generate the sequence H1-shRNA that was inserted into pLVHTM vector (from Didier Trono, Addgene).shNr4a2 construction was generated using the Fisher Scientific RNAi designer web tool using specific oligonucleotides against mouse *Nr4a2*, as follows: sh-Nr4a2 forward: 5′-GAT CCC CGG GCA CAA GTA TCA GTA CAT TGG AAT TCA AGA GAT TCC AAT GTA CTG ATA CTT GTG CCC TTT TTG-3′; Sh-Nr4a2 reverse: 5′-CTA GCA AAA AGG GCA CAA GTA TCA GTA CAT TGG AAT CTC TTG AAT TCC AAT GTA CTG ATA CTT GTG CCC GGG-3′. Oligonucleotides were provided by Fisher Scientific and cloned between NheI and Bam1 sites of a modified version of the FUGW vector backbone. The vector included the HIV-1 flap sequence, H1-shRNA-expressing promoter, the human polyubiquitin promoter-c, and a WRE element. This vector was kindly provided by Robert C. Malenka.

Transfection and lentiviral vectors production. HEK293T cells were grown in DMEM supplemented with 10% FBS (heat inactivated), 1× penicillin/streptomycin, and 30 mM glucose. At 70% of confluence, HEK293T cells were transfected using the CalPhos Mammalian Transfection kit (Clontech) with the four-plasmid system containing 20 μg DNA of Nr4a2-specific shRNA, and 10 μg DNA each of pVSVG, pREV, and pRRE (kindly provided by Robert C. Malenka), or by triple transfection of 20 μg of CREB or CRTC1-specific shRNA, and 10 μg each of psPax2 and pMD2.G (from Didier Trono, Addgene). Control viruses (empty) were prepared without the specific shRNAs. Infectious particles were purified from media after collecting culture media every 12 h and centrifugation at 25,000 rpm for 90 min at 4°C. Lentiviral particles were resuspended in 100 μl cold PBS and left at 4°C overnight before being aliquot and stored at −80°C. Biological titers of the viral preparations expressed as a number of transducing units per milliliter were assessed in HEK293T cells by checking the percentage of GFP-positive cells by flow cytometry (Cytomics FC 500, Beckman Coulter) in serial dilutions.

Transduction of primary hippocampal cultures. At 7 DIV, hippocampal neurons were transduced with lentiviral vectors (1-2 transducing units/cell) containing an shRNA against CREB, CRTC1, or Nr4a2 using the CalPhos Mammalian Transfection kit (Clontech) according to the supplier's recommendations.

Luciferase reporter assay. pGL3-Nr4a2 promoter was generated as previously described (Barneda-Zahonero et al., 2012). Hippocampal neurons were transduced with the luciferase reporter plasmid pRL-TK, pGL3basic (Promega), and pGL3-Nr4a2promoter using the CalPhos Mammalian Transfection kit (Clontech). After 24 h of transfection, cells were lysed and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) according to the supplier's recommendations. Relative units were measured in a Varioskan microplate luminometer.

Luminex assay. Culture media to analyze extracellular BDNF was kept aliquoted at −80°C until needed. A Magnetic Milliplex kit (Merck-Millipore) containing antibodies to detect BDNF released by cultured hippocampal neurons was used following the manufacturer instructions. Quantification was performed with a Magpix-Luminex 200 analytical test instrument (Fisher Scientific) and xPONENT 4.2 software (ThermoFisher Scientific).

AAV production and stereotaxic injections. Adeno-associated vectors, AAV2/10.H1.scramble.RSV.GFP (sense 5′-CCUAAGGUUAAGUCGCC CUCG-3′; antisense 5′-CGAGGGCGACUUAACUUAGG-5′) and AAV2/10.H1.shNr4a2.RSV.GFP (sense 5′-GGACAAGCAUGUGAUUCUAGGU UGA-3′; antisense 5′-UCAACCUAGAAUCACAUGCUUGUCC-3′) were generated at the Viral Vector Production Unit at UAB by triple transfection in HEK 293-AAV cells (Stratagene) with branched polyethylenemine (Sigma-Aldrich) and purified by iodixanol gradients, after benzonase treatment. For AAV vector injections, mice were placed in a stereotaxic frame (Kopf), anesthetized with isoflurane (up to 3% for induction and 0.5%-1.5% for maintenance), and AAV vectors were injected (1 μl; 6×10^{12} gc/ml;

0.15 μl/min) bilaterally in C57BI/6 P17 mice into the dorsal CA1 region (2.18 mm posterior to bregma, 1.75 lateral to bregma, 1.6 ventral from dural surface, according to Paxinos and Franklin mouse brain atlas) using a beveled syringe needle (Hamilton). Both male and female mice were used with similar ratio for the two types of viruses. Slices for electrophysiology were prepared from NMDG-perfused injected animals 3-4 weeks after injection when we observe that at least 90% of the CA1 neurons transfected with AAV-shNr4a2 did not express Nr4a2 ($GFP^+/Nr4a2^-$).

Electrophysiology. All experiments, except when indicated, were performed at 26.5 ± 1°C in a submersion-type recording chamber perfused at ~2 ml/min with ACSF.

Extracellular field potentials (fEPSPs) were recorded with a patch-type pipette filled with 1 M NaCl and placed in the stratum radiatum (50-100 μm from CA1 pyramidal neurons somas).

EPSCs and miniature EPSCs (mEPSCs) were recorded from CA1 pyramidal neurons voltage-clamped at −60 mV using borosilicate glass pipettes (3-4 MΩ) containing the following (in mM): 131 Cs-gluconate, 8 NaCl, 1 $CaCl_2$, 10 EGTA, 10 glucose, 10 HEPEs, pH 7.3, 292 mmol/kg osmolality. AMPA/NMDA ratios were determined by recording evoked AMPAR-mediated EPSCs at −60 mV and NMDAR-mediated EPSCs at 40 mV in the presence of 10 μM NBQX and 100 μM picrotoxin. AMPA/NMDA experiments were performed in the presence of 100 μM picrotoxin (PTX) to block fast inhibitory transmission. mEPSCs were detected at 32°C with ACSF supplemented with 100 μM PTX and 0.5 μM TTX. The series resistance (8-16 MΩ) was monitored continuously. Recordings showing >15% change in series resistance were excluded from analysis.

Synaptic responses were evoked at 20 s intervals by stimulating Schaffer collaterals with a monopolar glass pipette stimulator filled with ACSF and positioned ~100 μm away from the recording pipette elicited at 20 s intervals. Stimulation was adjusted to obtain comparable magnitude of synaptic responses across experiments (~0.7 mV). Paired-pulse ratio (PPR) was studied by delivering two stimuli at various interstimulus intervals (10–500 ms) and measuring the ratio of slopes of the second and first fEPSP. LTD was induced after 20 min of stable baseline by low-frequency stimulation (LFS) of 900 pulses at 1 Hz. The magnitude of LTD was determined by comparing baseline-averaged responses before induction with the last 10 min of the experiment.

Output signals from field and whole-cell recordings were acquired at 5 kHz, filtered at 2.4 kHz, and analyzed using custom-made software written in Igor Pro 4.09A (Wavemetrics). All experiments were performed using a Multiclamp 700A amplifier (Molecular Devices).

Statistical analysis. Statistical analysis was performed using the GraphPad Prism version 6.01 (GraphPad Software) or OriginPro 7.0 (OriginLab) software. We performed either unpaired Student's *t* tests or ANOVA followed by appropriate between-group comparisons according to each analysis requirements. Data are shown as mean ± SEM or SD. For electrophysiological recordings, unless otherwise stated, data are mean ± SEM, and illustrated traces are average of 20-31 responses. Statistically significant difference was set at $p < 0.05$ and is indicated in the figure legends. Actual *p* values are reported in Results.

Results

The iGluR-Ca^{2+}/CREB/CRTC1 pathway mediates activity-dependent regulation of Nr4a2 in mature hippocampal neurons

As other orphan nuclear receptors, Nr4a2 is widely accepted to be activated in a ligand-independent manner (Maxwell and Muscat, 2006). Thus, regulation of Nr4a2 activity is highly dependent on its cellular protein levels. Prior studies in PC12 cells and cultured cerebellar granule cells demonstrated that extracellular KCl-mediated broad depolarization induced Nr4a2 (Law et al., 1992; Barneda-Zahonero et al., 2012). However, how neuronal activity activates Nr4a2 is largely unknown. To investigate activity-dependent induction of Nr4a2 in mature hippocampal neuronal cultures, we blocked inhibitory transmission by applying the $GABA_A$ receptor antagonist bicuculline during the time indicated in the figures. As shown in Figure 1*A*, bicuculline treatment significantly elevated *Nr4a2* promoter activity after 15 min (2.22 ± 0.48-fold increase vs basal; $p = 0.0158$) as determined by a transcriptional luciferase assay. The increase in *Nr4a2* promoter activity resulted in a significant upregulation of *Nr4a2* mRNA (Fig. 1*B*) and protein levels (Fig. 1*C*). Nr4a2 protein levels in mature hippocampal cultures were dramatically enhanced after 1 h of bicuculline treatment (13.53 ± 4.07-fold increase vs basal; $p = 0.0247$) and reached the highest levels after 4 h (37.19 ± 1.27-fold increase vs basal; $p < 0.0001$). A decline to basal protein levels was observed after 12 h, and this decrease continued up to 24 h of bicuculline treatment. The proteasome inhibitor MG-132 prevented reductions in Nr4a2 protein levels and elevated Nr4a2 basal levels indicating constitutive proteasomal regulation of Nr4a2 protein (Fig. 1*E*). A similar time course of *Nr4a2* induction was observed with the voltage-dependent K^+ channel blocker 4-aminopyridine, which increases neuronal activity by prolonging action potential duration (Fig. 1*D*). The above results suggest that neuronal activity could elicit calcium-dependent signaling mechanisms that may impact gene expression as previously reported (Flavell and Greenberg, 2008; Hagenston and Bading, 2011). To explore the role of calcium in activity-dependent upregulation of Nr4a2 levels, we incubated hippocampal cultures with the permeable calcium chelator BAPTA-AM. This manipulation significantly attenuated Nr4a2 induction (Fig. 1*F*; ~60% decrease vs bicuculline treatment; $p = 0.0003$), implicating calcium elevation as a trigger of Nr4a2 activation. iGluRs and L-type voltage-dependent calcium channels (VDCCs) are major routes for postsynaptic calcium influx. L-type VDCC blockade with nifedipine had no effect on the bicuculline-mediated increase in Nr4a2 levels. In contrast, iGluR antagonists targeting NMDARs and AMPARs, MK-801 and NBQX, respectively, significantly reduced Nr4a2 levels (Fig. 1*G*; 60% MK-801 and 45% NBQX vs bicuculline; $p < 0.0001$ in both). Together, these observations strongly suggest that iGluRs-mediated calcium influx supports activity-dependent *Nr4a2* induction in mature cultured hippocampal neurons. We do not know whether the reduction observed in the presence of NBQX was because of a reduction of AMPAR-mediated calcium influx or to a decrease in NMDAR activation because of AMPAR blockade since bicuculine did not increase Nr4a2 levels when experiments were done in the absence of Mg^{2+}.

We previously found that CREB and CRTC1 mediate activity-dependent *Nr4a2* expression (Barneda-Zahonero et al., 2012; Parra-Damas et al., 2014). To test whether this mechanism could account for our findings, we first determined the potential interaction of CREB and the *Nr4a2* promoter using ChIP analysis. Our results revealed that CREB is bound to the Nr4a2 promoter in control conditions, and this binding is not altered by neuronal activity following bicuculline application (Fig. 2*A*), although we detected an increase in CREB phosphorylation (Fig. 3*A*). On the other hand, both CRTC1 binding to the *Nr4a2* promoter (Fig. 2*A*; vehicle: 3.08 ± 1.52; bicuculline: 8.00 ± 2.05; $p = 0.0015$) and nuclear CRTC1 levels (Fig. 3*B*) increased in an activity-dependent manner. These data suggested that CREB and CRTC1 could be involved in activity-dependent Nr4a2 activation in hippocampal neurons. To confirm the functional impact of CREB and CRTC1 on *Nr4a2* gene expression, we generated lentiviruses to knock down these proteins. Both shCREB and shCRTC1 RNAs lentiviruses significantly reduced the bicuculline-mediated elevation in *Nr4a2* mRNA and protein levels (Fig. 2*B*,*C*). CRTC1 activation relies on its dephosphorylation by calcineurin. Accordingly, pharmacological inhibition of calcineurin with FK-506 dramatically

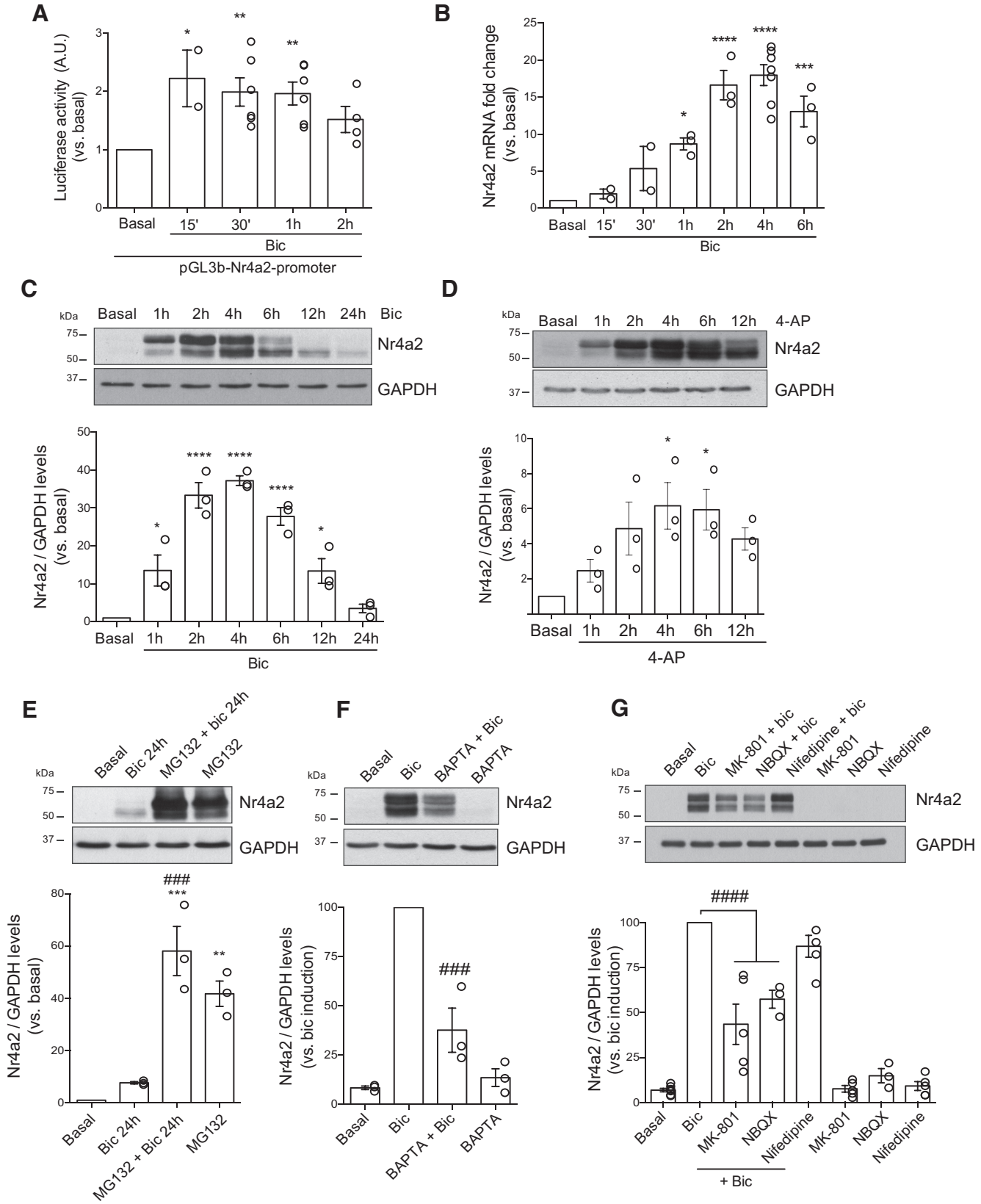

Figure 1. Activity-dependent induction of Nr4a2 in hippocampal neurons is dependent on Ca^{2+} influx through iGluRs. ***A***, *Nr4a2* promoter activity measured by luciferase assay after bicuculline (bic; 50 μM) treatment in DIV18-DIV21 primary cultures. ***B***, Time course of *Nr4a2* mRNA and (***C***) protein levels after bic treatment. ***D***, Time course of Nr4a2 protein levels after 4-aminopyridine (4-AP; 50 μM) treatment. ***E***, Nr4a2 protein levels in the presence of the proteasome inhibitor MG-132 (10 μM). ***F***, Nr4a2 protein levels after bic treatment in BAPTA-AM (20 μM) conditions. ***G***, Nr4a2 protein levels after bic application in the presence of the NMDAR antagonist MK-801 (10 μM), AMPAR antagonist NBQX (10 μM), and L-type VDDC blocker nifedipine (10 μM). $n \geq 3$ independent hippocampal cultures. Data are mean ± SEM. Values were normalized versus basal (***A-D***) or bicuculline (***E,F***). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; one-way ANOVA followed by Bonferroni *post hoc* test (* vs basal, # vs bic).

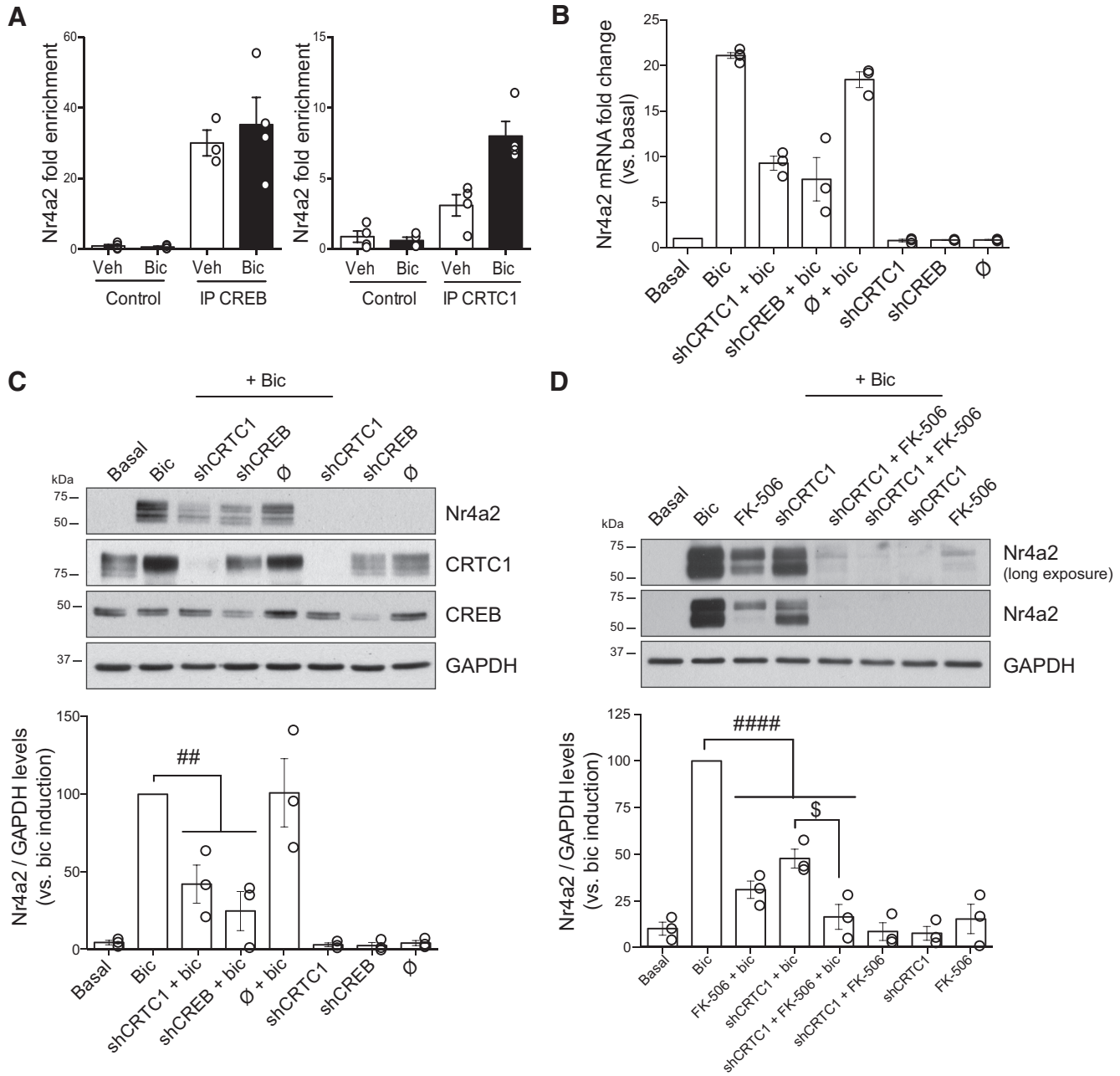

Figure 2. Activity-dependent increase of Nr4a2 levels in hippocampal neurons requires CRTC1 and CREB. ***A***, *Nr4a2* promoter occupancy by CREB and CRTC1 proteins measured by ChIP analysis after bicuculline (bic; 50 μM) treatment in DIV18-DIV21 primary cultures. ***B***, *Nr4a2* mRNA and (***C***) protein levels in CREB and CRTC1 shRNA-transduced neurons. ***D***, Nr4a2 protein levels in CRTC1 shRNA-transduced neurons treated with calcineurin inhibitor FK-506 (10 μM) or control conditions. $n = 4$ independent cortical cultures (***A***). $n \geq 3$ independent hippocampal cultures (***B***-***D***). ∅ represents empty vector. Data are mean ± SEM. Values were normalized versus basal (***A***,***B***) or bicuculline (***C***,***D***). Statistical analysis was determined by one-way ANOVA followed by Bonferroni (or Tukey in ***D***) *post hoc* test. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$ (* vs basal, # vs bic, $ vs shCRTC1 + FK-506 + bic).

reduced activity-dependent increases in Nr4a2 protein levels (Fig. 2*D*, ~70% decrease vs bicuculline treatment; $p < 0.0001$). Together, our results strongly suggest that neuronal activity-dependent upregulation of Nr4a2 protein levels in the hippocampus is CREB/CRTC1-dependent.

Nr4a2 mediates activity-dependent BDNF increase in hippocampal neurons

BDNF is known to impact several neurobiological processes in the mature brain. The complex multipromoter organization of the *Bdnf* gene indicates that *Bdnf* expression levels could be regulated by several or selective stimuli, targeting *Bdnf* promoter sequences (West et al., 2014; Yao et al., 2021). Hence, the identity of the factors that regulate *Bdnf* following neuronal stimulation remains incomplete (Lyons and West, 2011; Bloodgood et al., 2013). We previously described that iGluR-mediated activation of Nr4a2 was involved in the regulation of *Bdnf* expression in cerebellar granule cells (Barneda-Zahonero et al., 2012), but it has been reported that Nr4a2 does not mediate BDNF regulation in cortical neurons (Abdollahi and Fahnestock, 2022). Thus, the role of Nr4a2 as a regulator of *bdnf* expression seems to be neuronal type-specific. We therefore tested whether the CREB/

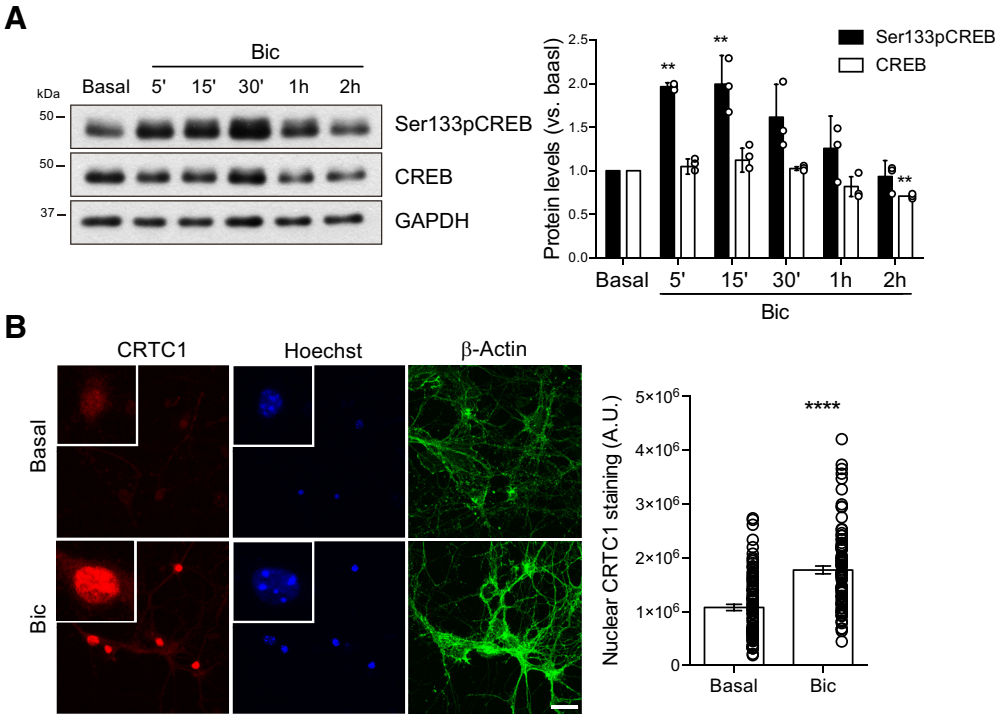

Figure 3. Bicuculline activates the CRTC1-CREB signaling pathway. ***A***, Time course of total and Ser133-phosphorylated CREB levels in hippocampal-cultured neurons after bicuculline (bic; 50 μM) treatment. ***B***, CRTC1 nuclear staining after 15 min of bic treatment. Original magnification ×40. Scale bar, 25 μm. $n = 3$ independent cultures (***A***,***B***). $n \geq 25$ neurons/culture ($n \geq 100$ neurons/condition) (***B***). Data are mean ± SEM. Statistical analysis was determined by one-way ANOVA followed by Bonferroni *post hoc* test (***A***) or unpaired *t* test (***B***). $^{**}p < 0.01$. $^{****}p < 0.001$.

CRTC1/Nr4a2 pathway could modulate BDNF levels in an activity-dependent manner in hippocampal neurons. Indeed, bicuculline treatment produced a significant elevation in *Bdnf* mRNA in hippocampal cultured neurons after 2 h (Fig. 4*A*; 4.5 ± 0.23-fold change vs basal; $p = 0.002$) that peaked at 4–6 h (Fig. 4*A*; *bdnf* mRNA after 4 h of bicuculline: 6.67 ± 0.27; $p = 0.0002$ and 6 h of bicuculline: 8.4 ± 0.53-fold change vs basal; $p < 0.0001$). Meanwhile, BDNF protein levels rose after 4 h and peaked at ~8–12 h (Fig. 3*B*). In addition to elevating BDNF levels, bicuculline treatment also enhanced BDNF secretion (Fig. 4*C*; BDNF secretion after 4 h of bicuculline: 2.02 ± 0.32; $p = 0.0138$ and 8 h of bicuculline: 3.12 ± 0.26 vs basal; $p = 0.0006$).

Activity-mediated modulation of *Bdnf* expression mirrored *Nr4a2* upregulation in mature hippocampal cultures, and BDNF levels seemed to accumulate after *Nr4a2* induction (Fig. 4*A*,*B*), raising the possibility Nr4a2 acts upstream of BDNF. To determine the impact of Nr4a2 on BDNF levels, we knocked down *Nr4a2* expression using shRNA-containing lentivirus. A dramatic reduction in both *Bdnf* mRNA (Fig. 4*D*) and protein levels was detected in shNr4a2 conditions (Fig. 4*E*; BDNF protein levels [fold change vs basal] after bicuculline: 1.56 ± 0.26, $p = 0.0354$ vs basal; shNr4a2 + bicuculline: 0.88 ± 0.28, $p = 0.0086$ vs bicuculline; shNr4a2: 0.52 ± 0.09; $p = 0.0086$ vs basal). A strong reduction in *bdnf* secretion was also observed in the shNr4a2 conditions following bicuculline treatment (Fig. 4*F*; BDNF secretion after bicuculline: 2.09 ± 0.34, $p = 0.0432$ vs basal; shNr4a2 + bicuculline: 1.09 ± 0.18, $p = 0.99$ vs basal). Further evidence supporting Nr4a2 modulation of BDNF levels was obtained by pharmacological activation of Nr4a2. Incubation with the Nr4a2 activator chloroquine (CQ) produced an increase in BDNF protein levels (Fig. 4*G*; fold change vs basal; BDNF protein levels after CQ: 1.72 ± 0.08, $p = 0.0002$ vs basal; shNr4a2 + CQ: 0.81 ± 0.21, $p = 0.0002$ vs CQ and $p > 0.99$ vs basal) and BDNF secretion (Fig. 4*H*; BDNF secretion after CQ: 2.06 ± 0.56, $p = 0.1464$ vs basal; shNr4a2 + CQ: 0.84 ± 0.24, $p = 0.0486$ vs CQ and $p = 0.96$ vs basal).

Nr4a2 modulates both iGluR levels and hippocampal LTD

It is well known that BDNF is an important factor involved in synaptic plasticity (Peng et al., 2009; Zagrebelsky and Korte, 2014) and impacts expression of the AMPAR subunit GluA1 (Caldeira et al., 2007). Given our findings that Nr4a2 enhances BDNF levels, we explored the potential modulatory role of Nr4a2 on iGluR expression. First, we transfected cultured hippocampal neurons with shNr4a2 and detected reduced GluA1 and NMDAR subunit GluN1 protein levels (Fig. 5*A*; AMPAR-GluA1 protein levels in shNr4a2: 0.691 ± 0.03 vs basal, $p < 0.0001$; NMDAR-GluN1 protein levels in shNr4a2: 0.686 ± 0.08 vs basal, $p = 0.0029$). No changes in protein levels were observed for GluA2, GluN2A, and GluN2B (Fig. 5*A*). Consistent with these observations, pharmacological activation of Nr4a2 with CQ for 8 h increased GluA1 and GluN1 protein levels in a Nr4a2-dependent manner. This CQ-dependent increase (fold change vs basal) was abolished in shNr4a2 transfected neurons (Fig. 5*B*; AMPAR-GluA1 protein levels after CQ: 1.22 ± 0.05, $p < 0.0001$ vs basal; shNr4a2 + CQ: 0.67 ± 0.08, $p < 0.0001$ vs CQ); NMDAR-GluN1 protein levels after CQ: 1.24 ± 0.07, $p = 0.0226$ vs basal; shNr4a2 + CQ: 0.8 ± 0.12, $p = 0.7314$ vs basal). To determine the impact of BDNF signaling on iGluRs subunit regulation, we blocked tropomyosin receptor kinase B (TrKB; BDNF receptor) activation using the antagonist ANA-12. In the presence of ANA-12 (50 μM), CQ did not enhance GluA1 expression (Fig. 5*C*; AMPAR-GluA1 protein levels [fold change vs basal] after CQ: 1.22 ± 0.05, $p = 0.004$ vs basal;

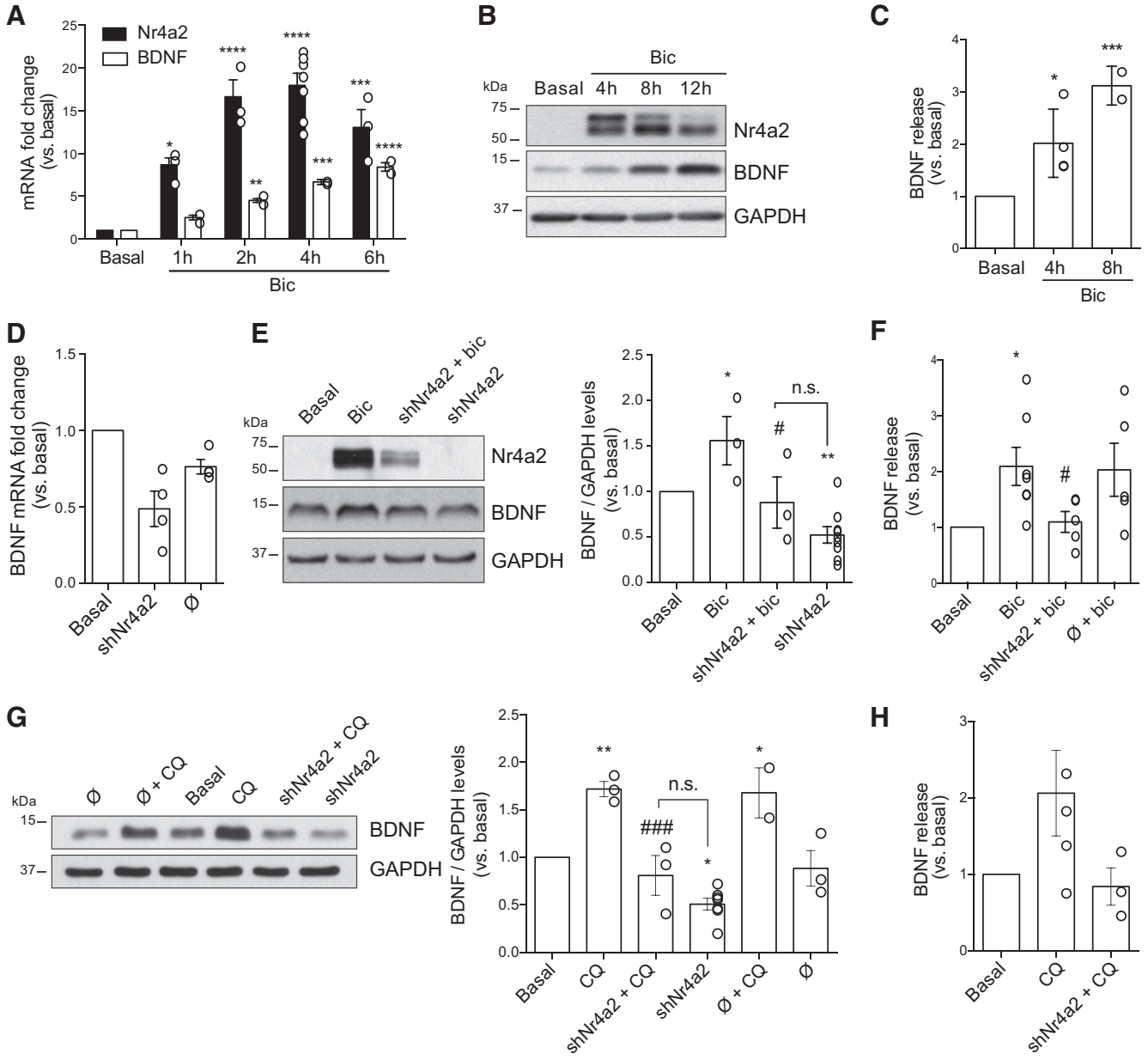

Figure 4. Nr4a2 is necessary for basal and activity-mediated BDNF production in hippocampal neurons. ***A***, Time course of *Nr4a2* and *bdnf* mRNA and (***B***) protein levels after bicuculline (bic; 50 μM) treatment in DIV18-DIV21 primary cultures. ***C***, Extracellular BDNF secreted measured by Luminex bead assay after bic application. ***D***, *bdnf* mRNA levels in Nr4a2 shRNA-transduced neurons. ***E***, BDNF protein levels in Nr4a2 shRNA-transduced neurons in the presence or absence of bic. ***F***, BDNF secreted in Nr4a2 shRNA-transduced neurons in the presence or absence of bic. ***G***, BDNF protein levels in the presence of the Nr4a2 agonist CQ (10 μM) in Nr4a2 shRNA-transduced neurons. ***H***, BDNF secreted after chloroquine (CQ) treatment. $n \geq 3$ independent hippocampal cultures. ∅ represents empty vector. Data are mean ± SEM. Values were normalized versus basal. Statistical analysis was determined by one-way ANOVA followed by Bonferroni (or Tukey in ***E***,***G***) *post hoc* test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ (* vs basal, # vs bic in ***E***,***F*** or vs CQ in ***G***,***H***).

ANA-12 + CQ: 0.88 ± 0.04, p = 0.7428 vs CQ). In contrast, ANA-12 did not inhibit the CQ-mediated increase in GluN1 levels. Thus, although Nr4a2 can impact both GluA1 and GluN1 protein levels, only GluA1 regulation appears to be BDNF-dependent.

Next, we wanted to evaluate whether Nr4a2 regulated synaptic AMPAR levels in a more intact system that preserves neuronal circuitry by using acute hippocampal slices. LTD is mainly associated with a reduction of AMPAR function in the postsynaptic compartment (Beattie et al., 2000; Lee et al., 2000). Given that Nr4a2 agonists upregulate AMPARs in hippocampal neurons, we reasoned that Nr4a2 activation could impair/affect LTD. Incubation for 4 h (3 h out to 1 h in the recording chamber) with the Nr4a2 agonists CQ or AQ produced a blockade of LTD elicited by LFS (1 Hz, 900 pulses) of the Schaffer collateral (Fig. 6*A*). In neuronal cultures, chemical LTD (cLTD) elicited by a 5 min application of 50 μM NMDA produces dephosphorylation at Ser845 GluA1 (Oh et al., 2006; Cheng et al., 2020). However, cLTD-induced GluA1 Ser845 dephosphorylation was markedly reduced in the presence of Nr4a2 agonists (Fig. 6*B*). In addition, Nr4a2 activation increased GluA1 Ser845 phosphorylation in control conditions (Fig. 6*B*). Together, our results demonstrate that pharmacological activation of Nr4a2 prevented LTD phenomena both in acute slices and cultured hippocampal neurons. Moreover, Nr4a2 regulation of synaptic AMPAR was further confirmed by measuring the

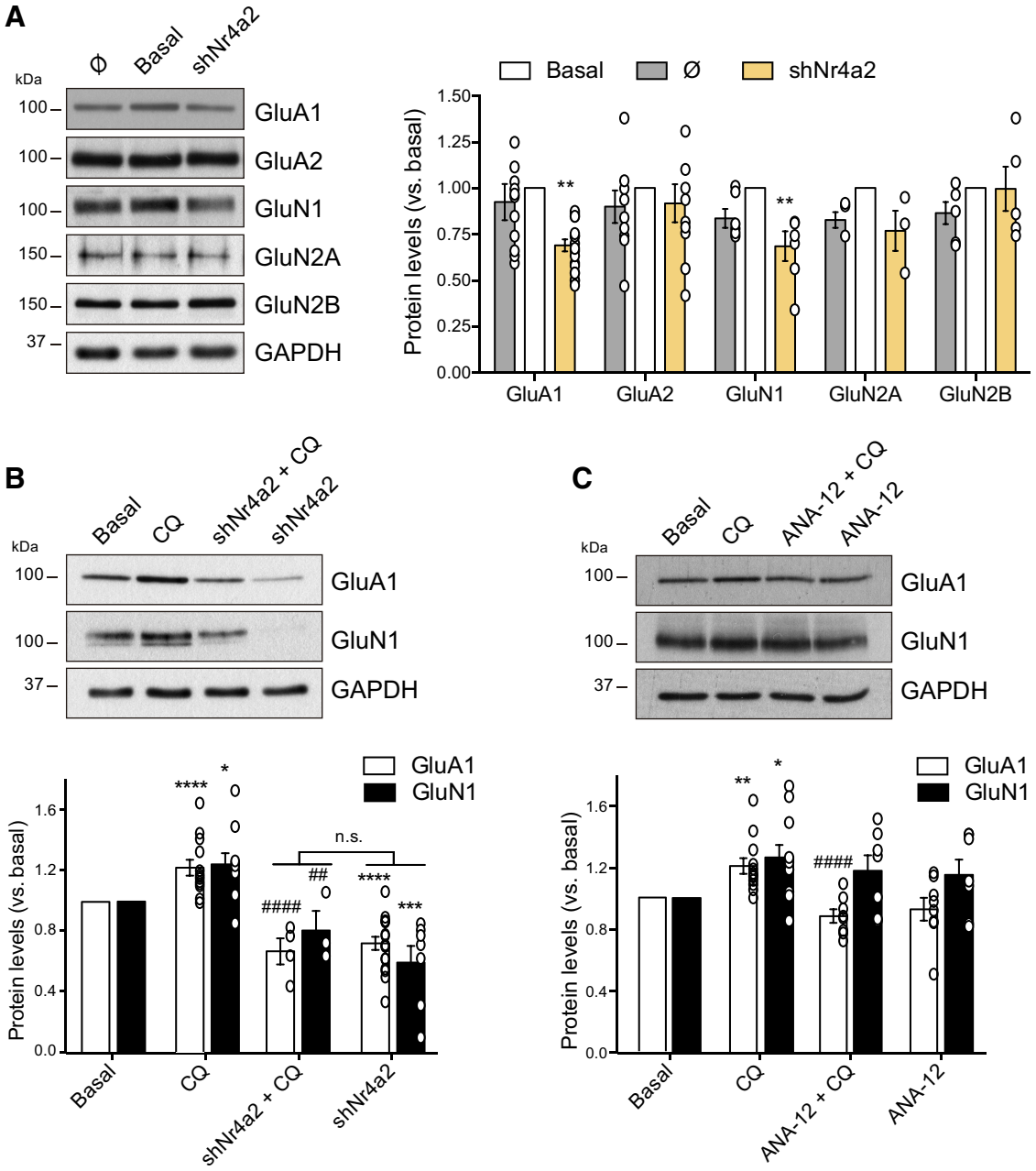

Figure 5. Nr4a2 modulates GluA1-AMPAR and GluN1-NMDAR expression in hippocampal neurons. ***A***, Protein levels of different subunits of AMPARs and NMDARs in Nr4a2 shRNA-transduced neurons. ∅ represents empty vector. Primary cultures DIV18-DIV21. ***B***, GluA1 and GluN1 protein levels in the presence of the Nr4a2 agonist chloroquine (CQ) (10 μM; 8 h) in Nr4a2 shRNA-transduced neurons. ***C***, GluA1 and GluN1 protein levels in the presence of the Nr4a2 agonist CQ in neurons treated with the TrKB inhibitor ANA-12 (50 μM). $n \geq 3$ independent hippocampal cultures. Data are mean ± SEM. Values were normalized versus basal. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; one-way ANOVA followed by Tukey *post hoc* test (* vs basal, # vs CQ).

AMPA/NMDA ratio at the Schaffer-CA1 synapses in acute hippocampal slices. Incubation for 2-3 h followed by bath application of the Nr4a2 agonist amodiaquine (AQ) revealed an increase in AMPA/NMDA ratio (Fig. 6*C*), suggesting AMPAR upregulation. As a complementary approach, action potential-independent spontaneous release in the form of mEPSCs was recorded in the presence of TTX (0.5 μM) and picrotoxin (100 μM). Enhancement of mEPSC frequency and amplitude was detected in Nr4a2 agonist conditions compared with control (Fig. 6*D*). Presynaptic release assessed by PPR analysis was not affected by Nr4a2 agonists (Fig. 6*E*,*F*). Hence, increased mEPSC frequency and amplitude likely resulted from modulation of postsynaptic AMPARs. To determine the role of Nr4a2 more directly, we took a genetic approach and delivered AAV-Nr4a2 shRNAs targeting CA1 by performing stereotaxic injections in P17-P19 mice (Fig. 7*A*). Since our results showed that pharmacological activation of Nr4a2 prevented LTD, we hypothesized that Nr4a2 knockdown would enhance LFS-LTD. Four weeks after surgery, a significant increase in LTD was observed in shNr4a2 animals compared with control (Fig. 7*B*). Together, our findings strongly support a role for Nr4a2 in modulating AMPAR function and synaptic plasticity.

Discussion

Activity-dependent changes in synaptic strength are involved in several brain functions, including learning and memory (Magee and Grienberger, 2020). Long-term synaptic plasticity requires

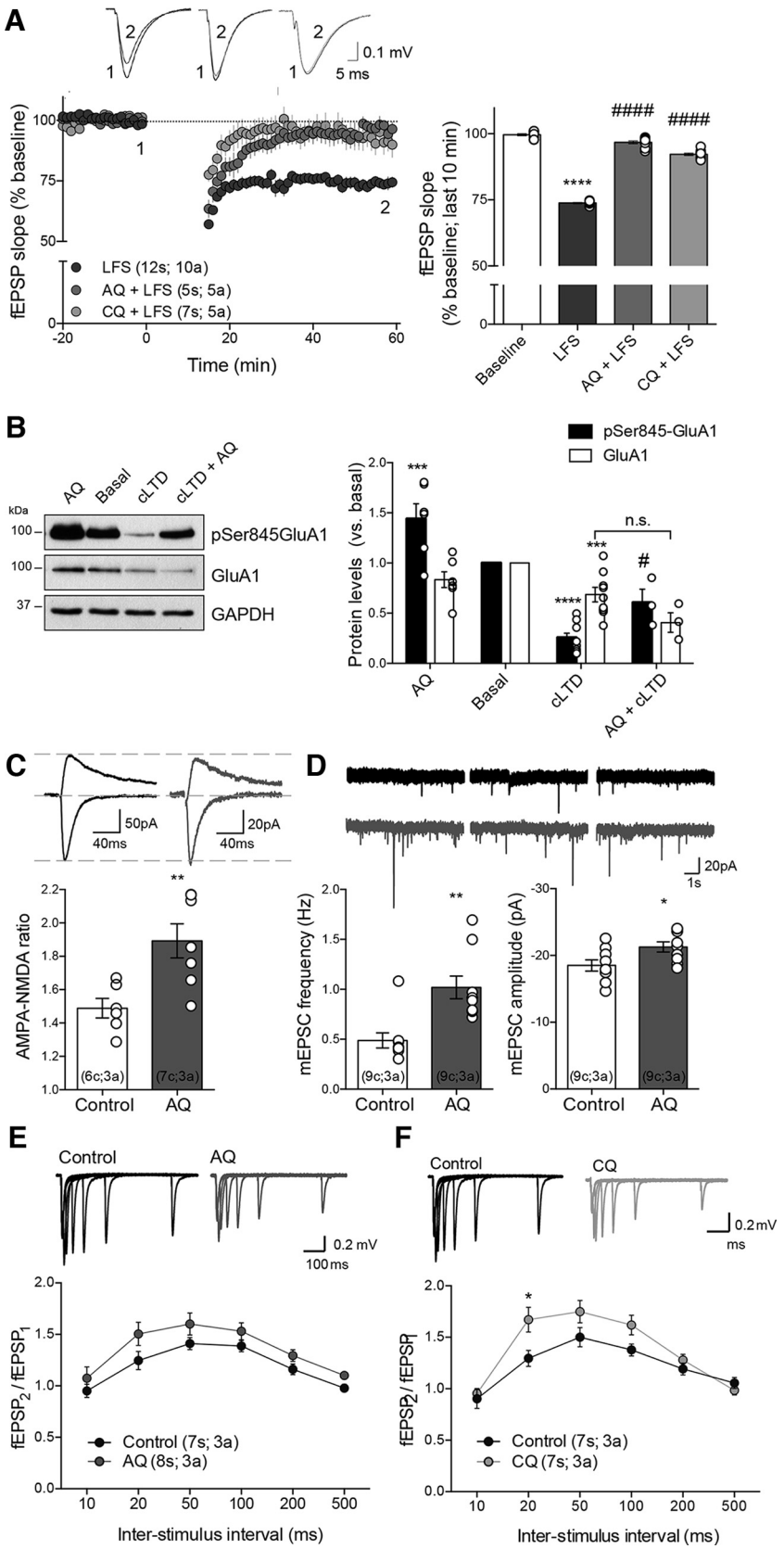

Figure 6. Nr4a2 activation enhances postsynaptic AMPAR function and blocks LTD. ***A***, ***C***, Electrophysiological recordings were performed in rat acute hippocampal slices P17-P27. ***A***, fEPSP responses of Schaffer collateral inputs before and after LTD induction (LFS: 900 pulses at 1 Hz) in the presence or absence of amodiaquine (AQ; 30μM) or chloroquine (CQ; 60μM). Slices were preincubated for 3 h with CQ or AQ and afterward transferred to the recording chamber where LTD was triggered after an additional 1 h incubation with the Nr4a2 agonists. ***B***, Total and Ser845-phosporylated GluA1-AMPAR protein levels after cLTD protocol (NMDA 50 μM, 5 min) in the presence or absence of AQ (10 μM, 4 h). Values were normalized versus basal. ***C***, Representative averaged EPSCs and summary data of AMPA/NMDA ratio from whole-cell CA1 pyramidal neurons in AQ (30 μM) and naive conditions. ***D***, Frequency and amplitude of mEPSCs measured in CA1 pyramidal neurons in the presence or absence of AQ (30 μM). ***E***, PPR of Schaffer collateral-CA1 synapses after incubation with the Nr4a2 agonists CQ or (***F***) AQ measured at 10, 20, 50, 100, 200, and 500 ms interstimulus intervals. Illustrated traces represent averages of 30 responses. Numbers in parenthesis indicate the number of animals (a), cells (c), or slices (s). Data are mean ± SEM. Statistical analysis was determined by one-way ANOVA followed by Bonferroni *post hoc* test (***A***,***B***) or unpaired *t* test (***C***,***D***). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ (* vs basal or control conditions, # vs LFS or cLTD).

transcriptional regulation and understanding, which factors are involved is necessary to advance our knowledge of these processes (Hegde and Smith, 2019). Several studies have linked the Nr4a family of orphan nuclear receptors to memory and synaptic plasticity in the hippocampus (Bridi and Abel, 2013; Bridi et al., 2017). More specifically, Nr4a2 activation was reported to rescue deficits in spatial object recognition and contextual fear conditioning in aged rodents and to enhance LTP duration, thereby highlighting the functional consequences of Nr4a2 activity (Hawk et al., 2012; Aldavert-Vera et al., 2013; Kwapis et al., 2019; Chatterjee et al., 2020). In this study, we have identified an activity-dependent molecular pathway that recruits Nr4a2 and impacts synaptic plasticity in the hippocampus. Future endeavors toward determining the intricacies of this mechanism could help design strategies to ameliorate synaptic plasticity deficits related to cognitive impairment observed in several brain pathologies (Benarroch, 2018; O'Reilly et al., 2019).

We have previously demonstrated that elevation of extracellular K^+ or intracellular cAMP upregulates *Nr4a2* mRNA in mature cortical cultures (España et al., 2010; Parra-Damas et al., 2014). In this study, we show activity-dependent upregulation of *Nr4a2* mRNA and protein levels in mature hippocampal neurons in an activity-dependent manner. Neuronal activity-dependent gene transcription associated with synaptic plasticity typically requires calcium-mediated signaling (West et al., 2002). iGluRs and/or L-type VDCCs commonly calcium influx into the postsynaptic compartment and could shape the mode(s) of activity-dependent transcriptional regulation (West et al., 2001; Hagenston and Bading, 2011). In cultured hippocampal neurons, calcium chelation using BAPTA-AM and iGluR antagonism attenuated activity-dependent activation of *Nr4a2*. These findings strongly suggest that calcium influx through iGluRs supports activity-dependent *Nr4a2* induction. Whereas a previous study reported that L-type VDCCs mediated KCl + bicuculline Nr4a2 regulation in hippocampal neurons (Tokuoka et al., 2014), we found that L-type VDCC

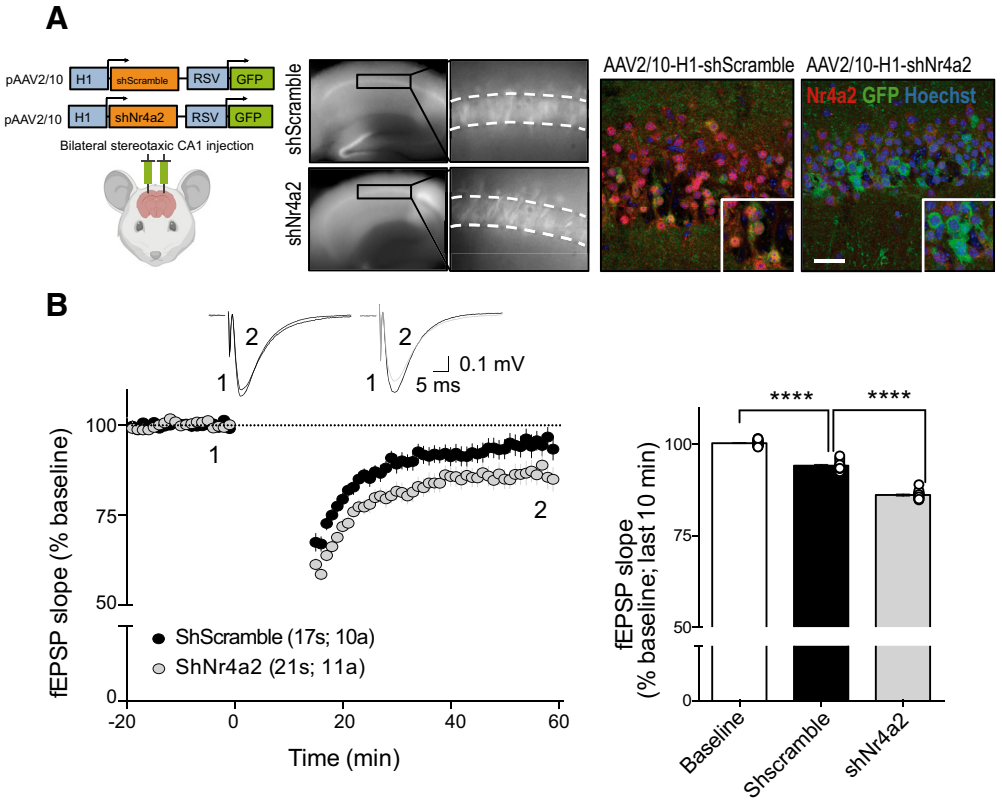

Figure 7. Nr4a2 downregulation enhances hippocampal LTD. ***A***, Schematic representation of the AAV used to silence Nr4a2 (left). Representative images of bright field (middle) and fluorescent CA1 pyramidal cells (right) after 3-4 weeks of injection. Scale bar, 200 μm. ***B***, fEPSP responses of Schaffer collateral inputs in acute hippocampal slices before and after LTD induction (900 pulses at 1 Hz) in scramble or shNr4a2-injected mice P35-P42. Traces represent averages of 30 responses. Numbers in parenthesis indicate the number of animals (a) and slices (s). Data are mean ± SEM. ****$p < 0.0001$ (one-way ANOVA followed by Bonferroni *post hoc* test).

blockade had no impact on Nr4a2 modulation when only bicuculline was used. A possible explanation for this apparent discrepancy is the different stimulation protocols. While Tokuoka et al. (2014) applied KCl to enhance neuronal activity by boosting depolarization that is widespread and cell type-unspecific, $GABA_A$ receptor inhibition with bicuculline likely elevates neuronal activity by promoting excitatory synaptic drive. These two stimulation protocols could engage distinct signaling mechanisms that are dependent or independent of the L-type VDCCs to activate Nr4a2.

It is well known that activity-dependent transcriptional responses could be modulated by recruitment of regulatory signaling cascades and transcription factors, such as CREB, FOXO, or NFAT (Flavell and Greenberg, 2008). Among them, CREB has been widely implicated in synaptic plasticity and long-term memory formation (Kaldun and Sprecher, 2019), and the Nr4a family of orphan nuclear receptors are reported to be targeted by CREB (Fass et al., 2003; Lam et al., 2010). Previously, we described that NMDAR-mediated induction of *Nr4a2* in cerebellar granule cells was CREB-dependent (Barneda-Zahonero et al., 2012). Thus, it was not surprising to find that CREB depletion, using shRNA in cultured hippocampal neurons, produced a strong reduction in *Nr4a2* levels. However, CREB may not be the determining factor involved in activity-dependent *Nr4a2* induction since we found that CREB was constitutively bound to the *Nr4a2* promoter, as previously reported by our laboratory and others (Zhang et al., 2005; T. K. Kim et al., 2010; Parra-Damas et al., 2017). CREB transcriptional activity is modulated by cofactors whose recruitment is important for the formation of gene regulatory complexes supporting distinct CREB-regulated transcriptional programs (Saura and Cardinaux, 2017). Among these cofactors, CRTCs have been suggested to be putative synapto-nuclear messengers. CRTC1 is mainly found in the brain (Watts et al., 2011), and several lines of evidence suggest that CRTC1 is involved in the transcription-dependent phase of neuronal plasticity (Ch'ng et al., 2012; Pan et al., 2021). More importantly, the dysregulation of CREB/CRTC1 is associated with learning and memory impairments (Saura and Cardinaux, 2017). In the present study, we observed that CRTC1 recruitment to the *Nr4a2* promoter was modulated in an activity-dependent manner, and that CRTC1 downregulation reduced Nr4a2 activation.

Interestingly, when we reduce CRTC1 activation by blocking calcineurin activity with FK-506, we observe a complete reduction of one Nr4a2 isoform in the presence of bicuculine and a partial reduction in the other. However, when CRTC1 was downregulated, bicuculine-mediated increase in both Nr4a2 variants was completely blocked. At present, it is not known whether these hippocampal Nr4a2 variants have differential functions, and it is not possible to envision if the result observed with FK-506 could be physiologically relevant. Together, our data are consistent with a model wherein an iGluR-Ca^{2+}-CREB/CRTC1 pathway mediates activity-dependent *Nr4a2* upregulation in hippocampal neurons.

The *Bdnf* gene has been widely implicated in hippocampal plasticity and memory formation (Korte et al., 1995; Hall et al., 2000). Among Nr4a family members, Nr4a2 was reported to modulate BDNF levels in dopaminergic neurons (Volpicelli et al., 2007) and cerebellar glutamatergic neurons (Barneda-Zahonero et al., 2012). Using pharmacology and genetic manipulation of

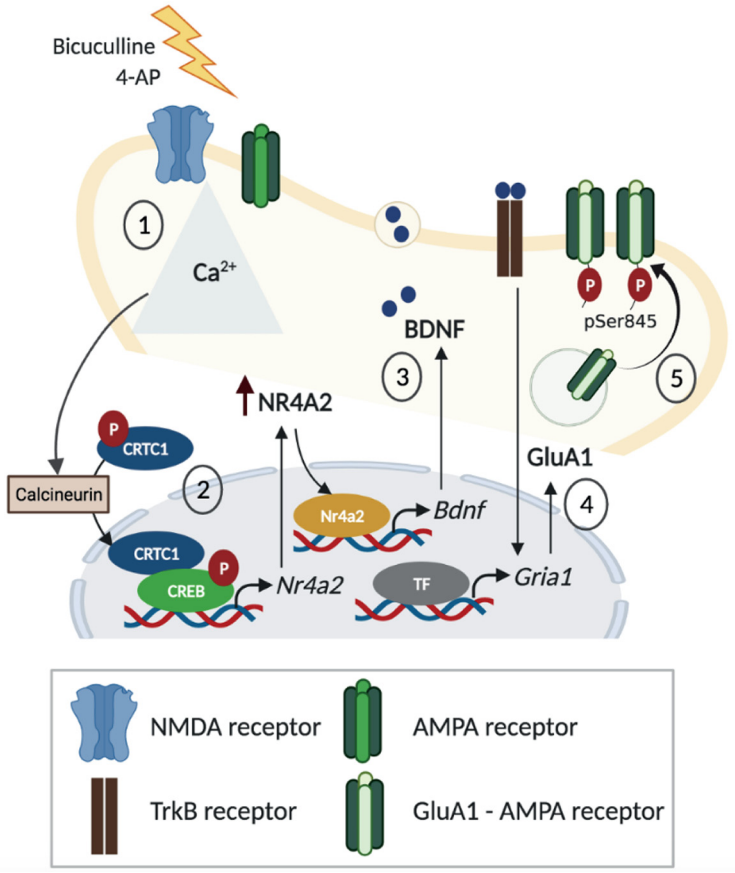

Figure 8. Proposed model for Nr4a2 role in glutamatergic synaptic plasticity. Activity-dependent induction of Nr4a2 in hippocampal neurons is dependent on Ca^{2+} influx through iGluRs and requires the CREB-CRTC1 signaling pathway. Nr4a2 is necessary for basal and activity-mediated BDNF production in hippocampal neurons, leading to an increase in postsynaptic GluA1-AMPAR and consequently decreasing LTD. Numbers in circles indicate the figure supporting the depicted component of the proposed model.

Nr4a2 in cultured hippocampal neurons, we confirm the requirement of Nr4a2 for activity-mediated production and release of BDNF. Although several factors are involved in *Bdnf* regulation, our results revealed a dramatic reduction of BDNF levels in shNr4a2 conditions. Our finding suggests that Nr4a2 plays a key role in BDNF modulation that could have an important impact on BDNF-dependent mechanisms. Moreover, while we show that activity-dependent *Bdnf* expression is mediated by a CREB/CRTC1-Nr4a2 pathway, a recent study demonstrated that TrkB recruitment of the CREB-CBP/p300 complex can bind the *Bdnf* promoter (Esvald et al., 2020). Thus, our results also highlight that different mechanisms are involved in activity-dependent and TrkB-dependent regulation of *Bdnf* gene expression. Further efforts to improve our understanding of the complex molecular mechanisms involved in BDNF modulation in the hippocampus are required.

The cellular and molecular mechanisms underlying hippocampal synaptic plasticity and memory enhancement supported by the Nr4a family of orphan nuclear receptors are still largely unknown. Our findings revealing Nr4a2 modulation of BDNF drove our attention to BDNF-dependent mechanisms involved in synaptic plasticity. It is known that BDNF regulates the expression of the AMPAR subunit GluA1 (Carvalho et al., 2008). Moreover, BDNF impacts the presence of GluA1 in hippocampal synapses by receptor trafficking and local synthesis (Nakata and Nakamura, 2007; Fortin et al., 2012), raising the possibility that Nr4a2 could be involved in BDNF-mediated iGluR regulation. *Nr4a2* knockdown caused a decrease in GluA1 and GluN1 levels without affecting other AMPAR or NMDAR subunits. The role of Nr4a2 in GluA1 and GluN1 regulation was further supported by Nr4a2 pharmacological activators CQ and AQ (J. Kim et al., 2016), which increased GluA1 and GluN1 levels. The pharmacological impact of CQ pplication was confirmed to be Nr4a2-dependent by shNr4a2 knockdown. These results suggest that Nr4a2 may play an important role in modulating AMPAR and NMDAR function. However, we observed that the Nr4a2-mediated increase in GluA1 levels was only observed when cells were treated for >6 h with the agonists. Intriguingly, TrkB receptor antagonism showed that BDNF specifically supports Nr4a2-mediated regulation of GluA1 but not GluN1. Although evidence indicates that CREB modulates GluA1 transcription, controversy exists whether CREB acts on the *gria1* promoter (Borges and Dingledine, 2001) or participates in BDNF signaling (Caldeira et al., 2007) to modulate GluA1 levels. Our data support a role for BDNF in CREB/CRTC1-mediated GluA1 regulation. Moreover, we identified Nr4a2 as a key factor downstream CREB/CRTC1 and upstream BDNF that may control GluA1 levels in an activity-dependent manner. Other transcription factors, such as NRF-1, Sp1 and MEF2C, and CREB, are implicated in regulating *grin1* by binding to its promoter (Krainc et al., 1998; Lau et al., 2004; Dhar and Wong-Riley, 2009; J. H. Kim et al., 2012). We observed that CREB is bound to the *Nr4a2* promoter in basal conditions and has no impact on GluN1 levels. However, activity-dependent recruitment of CRTC1 to the *Nr4a2* promoter led to elevations of GluN1. Our study highlights the impact of Nr4a2 on modulating iGluR subunit levels through BDNF-dependent (GluA1) and BDNF-independent (GluN1) signaling pathways. Moreover, the observation that downregulation of *Nr4a2* produced a decrease of GluA1 in control conditions, whereas inhibition of TrkB only blocked Nr4a2 agonist-mediated increase in GluA1 levels, suggests that Nr4a2 could also activate BDNF-independent downstream mechanisms involved in GluA1 regulation.

Activity-dependent changes in the number of AMPARs at hippocampal excitatory synapses represent a fundamental molecular mechanism underlying synaptic plasticity processes, such as LTP and LTD. Given that BDNF promotes synthesis of GluA1-AMPARs to be incorporated into synapses (Caldeira et al., 2007), we predicted pharmacological activation of Nr4a2 could induce functional changes of AMPARs in acute hippocampal slices. Whole-cell recordings in CA1 pyramidal neurons treated with the Nr4a2 activator AQ increased AMPA/NMDA ratios and mEPSC amplitude indicating enhanced AMPAR function. An increase in mEPSC frequency was also observed but could not be explained by an increase in the probability of glutamate release since we did not observe alterations in the PPR. The Nr4a2-mediated increase in AMPAR-mediated transmission could explain how LFS-LTD was prevented in the presence of AQ and CQ. Similar results were obtained in mature hippocampal cultured neurons since incubation for 4 h with Nr4a2 agonists blocked the

described cLTD-mediated dephosphorylation of GluA1Ser845 (Ehlers, 2000), supporting a role for Nr4a2 in the mechanisms that control perisynaptic and synaptic localization of AMPARs (Nakata and Nakamura, 2007). Moreover, Nr4a2 depletion using AAV-shRNA increased LFS-LTD magnitude, thereby confirming the modulatory role of Nr4a2 on AMPARs. The impact of Nr4a2 on AMPAR function following synaptic plasticity stimulation paradigms in acute slices could also explain the enhancement of LTP by Nr4a2 pharmacological activation (Bridi et al., 2017).

Our data demonstrate that Nr4a2 is a key player in activity-dependent modulation of GluA1 levels through a BDNF signaling pathway that enhances synaptic AMPAR-mediated transmission and reduces LTD of CA1 synapses (Fig. 8). Several compounds have been reported to activate Nr4a2 (for review, see Català-Solsona et al., 2021). Although some of the existing Nr4a2 agonists present important side-effects or lack sufficient brain permeability, they could be used as chemical scaffold to develop novel chemical molecules without undesirable characteristics. Thus, a therapeutic approach could be envisioned for brain disorders where Nr4a family function seems to be impaired and cursed with synaptic and cognitive dysfunction associated with dysregulated GluA1-AMPAR function.

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