Transcriptional Regulation of T-type Calcium Channel Ca\textsubscript{V}3.2

BI-DIRECTIONALITY BY EARLY GROWTH RESPONSE 1 (Egr1) AND REPRESSOR ELEMENT 1 (RE-1) PROTEIN -SILENCING TRANSCRIPTION FACTOR (REST)*

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Background: Expression of the T-type Ca\textsuperscript{2+} channel Ca\textsubscript{V}3.2 has to be tightly regulated for proper calcium homeostasis.

Results: Overexpression of the transcription factor Egr1 strongly activates the Ca\textsubscript{V}3.2 promoter and can be counteracted by the repressor REST.

Conclusion: Egr1 and REST “bi-directionally” regulate the Ca\textsubscript{V}3.2 promoter.

Significance: Our results have important implications for calcium homeostasis and dynamics in health and disease.

The pore-forming Ca\textsuperscript{2+} channel subunit Ca\textsubscript{V}3.2 mediates a low voltage-activated (T-type) Ca\textsuperscript{2+} current (\textit{I\textsubscript{CaT}}) that contributes pivotaly to neuronal and cardiac pacemaker activity. Despite the importance of tightly regulated Ca\textsubscript{V}3.2 levels, the mechanisms regulating its transcriptional dynamics are not well understood. Here, we have identified two key factors that up- and down-regulate the expression of the gene encoding Ca\textsubscript{V}3.2 (Cacna1h). First, we determined the promoter region and observed several stimulatory and inhibitory clusters. Furthermore, we found binding sites for the transcription factor early growth response 1 (Egr1) overexpression in \textit{vitro} and \textit{in vivo}. Subsequent chromatin immunoprecipitation assays in NG108–15 cells and mouse hippocampi confirmed specific Egr1 binding to the Ca\textsubscript{V}3.2 promoter. Congruently, whole-cell \textit{I\textsubscript{CaT}} values were significantly larger after Egr1 overexpression. Intriguingly, Egr1-induced activation of the Ca\textsubscript{V}3.2 promoter was effectively counteracted by the repressor element 1-silencing transcription factor (REST). Thus, Egr1 and REST can bi-directionally regulate Ca\textsubscript{V}3.2 promoter activity and mRNA expression and, hence, the size of \textit{I\textsubscript{CaT}}. This mechanism has critical implications for the regulation of neuronal and cardiac Ca\textsuperscript{2+} homeostasis under physiological conditions and in episodic disorders such as arrhythmias and epilepsy.

Low voltage-activated (T-type) Ca\textsuperscript{2+} channels are expressed in multiple organs, including the CNS and heart (1–4), where they play a key role in many cellular processes, such as shaping of neuronal discharge patterns, secretion of hormones and neurotransmitters, amplification of dendritic excitatory postsynaptic potentials, maintenance of circadian rhythms, and pacing of the heart (4–6). T-type Ca\textsuperscript{2+} channels comprise a subfamily of three Ca\textsubscript{V}3 pore-forming channel subunits (Ca\textsubscript{V}3.1, Ca\textsubscript{V}3.2, and Ca\textsubscript{V}3.3), encoded by members of the Cacna1 gene family (Cacna1g, Cacna1h, and Cacna1i).duction of these three Ca\textsubscript{V}3 channel subtypes are activation at subthreshold voltages, comparatively slow activation, and potentiation of Ca\textsubscript{V}3.2-dependent excitatory postsynaptic potentials (7). In addition to their common characteristics, the Ca\textsubscript{V}3 channels also exhibit diverging properties. Ca\textsubscript{V}3.3 channels display particularly slow inactivation kinetics, and Ca\textsubscript{V}3.2 channels are significantly more sensitive to nickel than Ca\textsubscript{V}3.1 and Ca\textsubscript{V}3.3 (6, 8–10).

The importance of T-type Ca\textsuperscript{2+} channels for normal cellular function is underscored by the pathophysiological alterations associated with genetic and acquired Ca\textsubscript{V}3.2 “channelopathies.” Gain-of-function mutations in the Ca\textsubscript{V}3.2 gene are associated with idiopathic generalized/absence epilepsy (11–13). Likewise, acquired increases in thalamic and hippocampal Ca\textsubscript{V}3.2 expression contribute to the development of chronic epilepsy (14, 15). In addition, overexpression of Ca\textsubscript{V}3.2 channels in myocytes may result in the development of several cardiac dysfunctions, including ventricular arrhythmias (16, 17). Epileptic seizures as well as cardiac arrhythmias share the episodic onset of symptoms. Because no overlapping mutations for both conditions have been identified in Ca\textsubscript{V}3.2, transcriptionally mediated changes in Ca\textsubscript{V}3.2 levels might constitute an attractive mechanism explaining the common episodic onset. However, despite the importance and potency of transcriptional regulation, only little is known about the key mechanisms controlling expression of the Ca\textsubscript{V}3.2 gene. Recently, an intriguing transcriptional...
CaV3.2 Gene Regulation

The CaV3.2 gene is regulated by various mechanisms, including transcriptional repression and transcription factor binding. This page discusses the experimental procedures and results of a study investigating the transcriptional regulation of CaV3.2.

EXPERIMENTAL PROCEDURES

Bioinformatic Analysis—The genomic sequence of the rat CaV3.2 gene was obtained from the UCSC genome browser. Potential transcription start sites were identified using the Epiphragm software (threshold value of 0.99) (23). Comparative analysis of the nucleotides of the CaV3.2 gene of different species was performed with PhyloP (PHAST package) and Vector NTI (9.0) using default parameters. Potential transcription factor (TF) binding sites were identified using the MathInspector RegionMiner software tool (Genomatix).

Cloning and Plasmids—The mammalian expression vectors pCMV-Egr1, pCMV-myc-REST and pCMV-FLAG-NLS-REST, were kindly provided by Prof. Gerald Thiel (University of Saarland Medical Center, Homburg, Germany). The rat full-length CaV3.2–1426 promoter region was amplified by PCR using rat genomic DNA as a template with the primer set forward (MluI) 5'-GGC ACG CGT AAG GGA GAA TGG GGT CAC TGT AAC CAC T-3' and reverse (XhoI) (5'-GCG GTC GAC GCT ACC CCA CAG GAA GGT-3') into the pGL3-CaV3.2–1426 vector digested with BamHI and Sall. Cloning was performed by ligating a BamHI-Sall-digested rat genomic PCR fragment (forward, 5'-GGC GGA TCC ACT CTG CTC TAA TGA GGG ACC CT-3'; reverse, 5'-GGC GTC GAC GCT ACC CCA CAG CAA GGT-3') into the pGL3-CaV3.2–1426 vector digested with BamHI and XhoI. For construction of the pAAV-Synapsin-Egr1-IRES-Venus plasmid, pAAV-Syn-IRES-Venus (kindly provided by Martin Schwarz, Heidelberg, Germany) was modified. The Egr1 sequence was amplified from pCMV-Egr1 using the primers: forward (Nhel) (5'-GGC GGT AGC ACC CCG CCA TGG CAG CG-3') and reverse (BamHI) (5'-GGG GGA TCC CCC TTT AGC AAA TTT CAA TTG TCC-3') and cloned in the Nhel-BamHI-digested pAAV-Syn-IRES-Venus vector. The correctness of the plasmids was confirmed by sequencing analyses.

Cell Culture, Transfections, and Luciferase Assays—NG108-15 cells were maintained at 37 °C and 5% CO2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin/streptomycin, 2 mM glutamine, and 1% (v/v) heat-inactivated fetal calf serum (HyClone), aminopterin, and thymidine (Invitrogen). Transfection was performed in 48-well tissue culture plates (80% confluence) using Lipofectamine (Invitrogen) following the manufacturer's protocol. Briefly, 0.05 μg of CaV3.2 luciferase reporter plasmid with firefly luciferase and 0.0125 μg of control pRL-TK vector with the Renilla luciferase gene (Promega) together with the amount of overexpression plasmids as indicated were mixed with 25 μl of Opti-MEM medium (Invitrogen). The mixture was incubated for 20 min at room temperature and then added to the appropriate wells. Cells were grown in serum-free culture medium at 37 °C and 5% CO2. After 12 h, the serum-free medium was replaced by serum containing medium. The cells were collected 48 h after transfection. The luciferase assay was performed using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's specifications. Renilla and firefly luciferase activities were determined using the Glomax Luminometer (Promega). The results are given as firefly/Renilla relative light units.

RT-PCRs—mRNA was isolated with a Dynabeads mRNA Direct Micro kit (Invitrogen) according to the manufacturer's protocol, and first-strand cDNA was prepared using RevertAid Reverse Transcriptase (Fermentas). The presence of CaV3.2, Egr1, REST, and the truncated REST4 variant was analyzed by RT-PCR. PCR samples contained 1X GoTaq buffer (Promega), 25 mM MgCl2, 0.1 mM each of dTTP, dATP, dCTP, and dGTP, 0.5 units of GoTaq (Promega), 10 pmol each oligonucleotide primer (Invitrogen), and 1 μl synthesized cDNA in a 25-μl volume. The following primers were used: CaV3.2 forward (5' - ATGC TCA TCA CCA TGT CCA TGG A-3'); CaV3.2 reverse (5' - ACAG TAG TGG CAC TAC TTA AGG GCC-3'); Egr1 (forward, 5' - GGA GCC GCC GGA AGG ACA ACC CT-3'); Egr1 reverse (5' - AAA GAG AAG AAG CCC GTA GT-3'); REST and REST4 forward (5' - AGG GAG TAC CAC TGG AGG AAA CA-3'); REST reverse (5' - ATT TAA GAG GGT GG GTG TAC CC-3'); REST4 reverse (5' - ATG GCC AGG ACA ACC CT-3'). PCR was performed with conditions as follows: 2 min at 94 °C, then 30 cycles of 45 s at 94 °C, 30 s at 60 °C, and 45 s at 72 °C followed by a final extension step at 72 °C for 10 min.

CaV3.2–1426-REST reporter plasmid was cloned by ligating a BamHI-Sall-digested rat genomic PCR fragment (forward, 5'-GGG GGA TCC ACT CTG CTC TAA TGA GGG ACC CT-3'; reverse, 5'-GGC GTC GAC GCT ACC CCA CAG CAA GGT-3') into the pGL3-CaV3.2–1426 vector digested with BamHI and Sall. For construction of the pAAV-Synapsin-Egr1-IRES-Venus plasmid, pAAV-Syn-IRES-Venus (kindly provided by Martin Schwarz, Heidelberg, Germany) was modified. The Egr1 sequence was amplified from pCMV-Egr1 using the primers: forward (Nhel) (5'-GGC GGT AGC ACC CCG CCA TGG CAG CG-3') and reverse (BamHI) (5'-GGG GGA TCC CCC TTT AGC AAA TTT CAA TTG TCC-3') and cloned in the Nhel-BamHI-digested pAAV-Syn-IRES-Venus vector. The correctness of the plasmids was confirmed by sequencing analyses.

The abbreviations used are: RE-1, repressor element-1; REST, RE-1-silencing transcription factor; Egr1, early growth response 1; TF, transcription factor; PI, protease inhibitor; rAAV, recombinant adeno-associated virus; ANOVA, analysis of variance; ERE, Egr1 responsive element; SE, status epilepticus.
products were analyzed on a 2% agarose gel. A control (no template) was included for each primer set.

**Quantitative Real Time RT-PCR**—Transcript quantification was performed by quantitative real time RT-PCR analysis according to the ∆∆Ct method. β-Actin was amplified from all samples to normalize expression. Quantitative RT-PCR was performed in a 6.25-μl reaction volume containing 3.125 μl of Maxima SYBR Green/Rox qPCR Master Mix (Fermentas), 1.5 μl of diethyl pyrocarbonate H2O, 1.25 μl of cDNA, and 0.1875 μl of each primer (10 pmol/ml; Ca3.2 and Egr1, same primers as described above; β-actin forward, 5'-CTG GAA AAG ATG ACC CAG ATC A-3'; β-actin reverse, 5'-GGA CAG CAC AGC CTG GAT G-3'). Reactions were performed in triplicate. After preincubation for 10 min at 94 °C, 40 PCR cycles (20 s at 94 °C, 30 s at 59 °C, and 40 s at 72 °C) were performed on an ABI Prism 9700HT system (PE Applied Biosystems, Foster City, CA).

**Chromatin Immunoprecipitation (ChIP) Assays-ChIP on Cultured Cells—**NG108-15 cells (6 wells; 80% confluency) were transiently transfected with pCMV-Egr1 or the empty pCMV vector (0.4 μg/well) using Lipofectamine as described above. 48 h after transfection the cells were cross-linked in DMEM with 1% formaldehyde for 10 min at 37 °C. Cells were washed twice in cold PBS containing protease inhibitors (Pis; Complete Protease Inhibitor Mixture Tablets; Roche Applied Science) and collected into conical tubes. Cells were spun down and lysed in 200 μl of SDS lysis buffer (1% SDS, 10 mm EDTA, 50 mm Tris, pH 8.1 with PIs) and incubated on ice for 10 min.

**ChIP on Brain Tissue—**Mice were decapitated under deep isoflurane anesthesia (Forene). Hippocampi were removed quickly, snap-frozen, and stored at −80 °C until further processing. 1% Formaldehyde was added to the tissue (200 μl/hippocampus), and the tissues were incubated for 10 min at 37 °C. Next, hippocampi were washed twice in cold PBS with PIs, sus- pended in 200 μl of SDS lysis buffer (1% SDS, 10 mm EDTA, 50 mm Tris, pH 8.1, with PIs), and incubated on ice for 10 min.

**ChIP Sample Processing—**After 10 min on ice, lysates (both NG108-15 cells and mice hippocampi) were sonicated using an Ultrasonic Processor UP50H (Hielers Ultrasound Technolog) with four sets of 10-s pulses at 50% of maximum power. This treatment yielded an average of 300–500-bp DNA fragments. Samples were centrifuged at 13,000 rpm for 10 min, and the cell supernatant was diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris, pH 8.1, 167 mM NaCl with PIs). To reduce nonspecific background, samples were precleared for 30 min with salmon sperm DNA/protein A agarose-50% slurry (Millipore). Next, samples were incubated overnight at 4 °C with 5 μg of anti-Egr1 SC110 antibody (Santa Cruz) or anti-NRFS (H-290) SC25398x antibody (Santa Cruz). Rabbit-IgG incubations were included as control for the immunoprecipitation. Salmon sperm DNA/protein A-agarose, 50% slurry was then added for 1 h at 4 °C. Non-specifically associated proteins and DNA were removed from the beads by sequentially washing with low salt washing buffer (20 mm Tris-HCl, pH 8.1, 150 mm NaCl, 2 mm EDTA, 0.1% SDS, 1% Triton X-100), high salt washing buffer (20 mm Tris-HCl, pH 8.1, 500 mm NaCl, 2 mm EDTA, 0.1% SDS, 1% Triton X-100), LiCl washing buffer (0.25 μl LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mm EDTA, 10 mm Tris-HCl, pH 8.1), and twice with TE buffer (10 mm Tris, pH 8.1, 1 mm EDTA). Next, immunoprecipitation complexes were eluted from the beads with 1% SDS and 0.1 M NaHCO3, and cross-links were reversed overnight at 65 °C by adding 20 μl of 5 M NaCl to 500 μl of eluates. Proteins were then digested by adding 20 μg of proteinase K (Sigma) for 1 h at 45 °C. DNA was recovered by phenol/chloroform extraction, ethanol-precipitated with glycogen as a carrier, and resuspended in 25 μl of water. The recovered DNA was analyzed by PCR with primers spanning the Ca3.2 promoter region: Egr-ChIP (ChIP on cultured cells and brain tissue): ChIP1 forward, 5'-CGT TTC CCG CAG CTC CGC TC-3'; ChIP1 reverse, 5'-GTG CCC TGC GTC ATG GTG GC-3'; ChIP2 forward, 5'-CCG GCC AGA AAA GGA GGG GG-3'; ChIP2 reverse, 5'-GCT CGC AGG GAT GCT CGG G-3'; ChIP3 forward, 5'-GAA GGG AGA ATC TTC ATG GAC AT-3'; ChIP3 reverse, 5'-CCA ATT GTA CTG GGC CCC TC-3'. REST-ChIP (ChIP on cultured cells) primers were: forward (5'-GAC CCT ACT CCG TTC TGG TTG GC-3') and reverse (5'-TAA AAA CCC CCT CAA TGC AG-3'); REST-ChIP (ChIP on brain tissue) forward (5'-AGG TGG GAC AAC TGC TTC AG-3') and reverse (5'-GGA TCA TCT TCA ATG CAC CA-3'). PCR amplification in a 25-μl reaction included 1 μl of immunoprecipitated DNA, 0.1 μm each of deoxyribonucleotide triphosphate (dNTP), 10 μl of each primer, 2.5 μm concentrations of a 10× Mg2+ reaction buffer and 0.5 unit of DNA Taq polymerase (Inverotien). Reactions were amplified for 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min followed by a final extension step at 72 °C for 10 min. PCR products were analyzed on 2% agarose gels and quantified using AIDA software.

**Electrophysiology Recordings and Analysis—**Patch clamp recordings were obtained from NG108-15 cells. Patch pipettes with a resistance of 3–4 megohms were fabricated from boro- silicate glass capillaries and filled with an intracellular solution containing 110 mM CsF, 20 mM tetraethylammonium, 2 mM MgCl2, 10 mM HEPES, 11 mM EGTA, 5 mM ATP, and 0.5 mM GTP, pH 7.2, adjusted with CsOH, 300 mosmol. Patch clamp recordings were performed in an artificial cerebrospinal fluid (ACSF) bath solution containing 125 mM sodium mesul- fonate, 3 mM KCl, 1 mM MgCl2, 5 mM CaCl2, 4 mM 4-aminopyri- dine, 20 mM tetraethylammonium, 10 mM HEPES, 10 mM glu- cose (pH 7.4, 315 mosmol). Tight-seal, whole-cell recordings were obtained at room temperature (21–24 °C) according to standard techniques. Membrane currents were recorded using a patch clamp amplifier (Axopatch 200B, Axon Instruments, Union City, CA). Series resistance compensation was employed to improve the voltage-clamp control (>80%) so that the maximal residual voltage error did not exceed 1.5 mV. Voltage recordings were corrected online for a liquid junction potential of 10 mV. Whole-cell Ca2+ currents were elicited with depolarizing voltage steps to −10 mV. The magnitude of ICa,t was quantified as the transient component of the resulting current traces. The recorded current can be attributed to Ca3.2 as they were largely blocked by application of 100 μM Ni2+.

**Viral Vector Production—**Recombinant AAV1/2 genomes were generated by large scale triple transfection of HEK293 cells. The adenovirus-associated virus (AAV)-Syn-Egr1-IRES-Ve- nus plasmid, helper plasmids encoding rep and cap genes.
CaV3.2 Gene Regulation

Bioinformatic Prediction of CaV3.2 Promoter Region—To determine key molecular mechanisms underlying CaV3.2 expression control, we first aimed to identify the CaV3.2 promoter region using bioinformatics. The sequence upstream of the translation start site of the rat CaV3.2 gene was analyzed for the presence of promoter region characteristics including transcription start sites and a high level of conservation between species. By using the Eponine software tool, four transcription start sites were found within the upstream region of the CaV3.2 gene, located 183, 467, 695, and 1064 bp upstream of the start ATG (Fig. 1). Furthermore, conservation analysis of the upstream CaV3.2 gene showed a high degree of homology throughout the first exon (80% identity between rat, mouse, and human) and within the first 700 bp upstream of the start ATG (>65% identity). A gradual decrease in sequence homology was observed for the more upstream sequences, with a sequence homology of less than 60% 1400 bp upstream of the start ATG (Fig. 1). Based on our bioinformatic analysis, we hypothesized that the 1400 nucleotides upstream of the start ATG contain the major regulatory promoter elements.

In Vitro Delineation of CaV3.2 Promoter Region—We next examined whether the bioinformatically predicted CaV3.2 promoter region is indeed sufficient for basal activity in neuronal cells. For this, we selected NG108-15 neural cells, which express CaV3.2 mRNA under naïve conditions (Fig. 2A). We cloned the predicted full-length rat CaV3.2 promoter region (CaV3.2–1426) into a firefly luciferase reporter plasmid and measured reporter activity in transiently transfected NG108-15 cells. Luciferase activity of the CaV3.2 promoter was ∼8-fold higher than the pGL3 control plasmid, which lacks a promoter (Fig. 2B), suggesting that this region of the CaV3.2 gene has significant promoter activity.

To pinpoint the exact region responsible for CaV3.2 promoter activity and to identify potential stimulatory and inhibitory regions, NG108-15 cells were transiently transfected with CaV3.2 deletion reporter constructs (CaV3.2–1188, –1020, –947, –312, –280, and –105). Each deletion fragment was tested multiple times with either three or four wells per construct and using two independent DNA isolations per construct. The basal activity of the first deletion fragment (CaV3.2–1188) was significantly lower (p ≤ 0.001) than the activity of the full-length CaV3.2–1426 construct (Fig. 2C), suggesting the presence of stimulatory elements in the 238 nucleotides upstream of the CaV3.2–1188 fragment. The activity of the second deletion fragment (CaV3.2–1020) was as high as that of full-length CaV3.2–1426, indicating the presence of an unknown repressor(s) in the 168 nucleotides upstream of the CaV3.2–1020 fragment. Moreover, the two subsequent deletion fragments (CaV3.2–947 and CaV3.2–312) again showed a reduced basal activity, comparable with the basal activity of the CaV3.2–1188 fragment, whereas the basal activity of the two smallest deletion fragments (CaV3.2–280 and CaV3.2–105) was again at the level of the basal activity of the full-length construct (CaV3.2–1426). These results indicate that several stimulatory and inhibitory regulatory elements are spread...
throughout the entire predicted CaV3.2 promoter region and that in vitro all seven CaV3.2 deletion fragments exhibit promoter activity (Fig. 2D). Furthermore, the strong promoter activity of the smallest CaV3.2/105 fragment suggests that this fragment functions as the CaV3.2 core promoter.

Identification of Putative Transcription Factor Binding Sites in CaV3.2 Promoter Region—To identify potential regulatory mechanisms underlying CaV3.2 expression, we first aligned the bioinformatically predicted rat CaV3.2 promoter region with genomic CaV3.2 sequences of mouse and human. The homologous sequences of rat (1464 nucleotides upstream from the start ATG), mouse (1468 nucleotides), and human (1597 nucleotides) were analyzed for the presence of enriched TF binding sites using the Genomatix RegionMiner software tool. TF binding sites were ranked based on their overrepresentation value calculated against either the whole genome (Z-score genome) and all annotated promoter regions of the genome (Z-score promoter). For the predicted rat CaV3.2 promoter region, 255 different TF matrices were found with the highest Z-score genome for Egr1 followed by zinc finger protein 161 (ZFP161 also known as ZF5) and Sp4 transcription factor (SP4) (Table 1). The highest Z-score genome was also found for Egr1 followed by SP4 and zinc finger protein 219 (ZNF219). For the mouse CaV3.2 promoter region, 239 different TF matrices were found with again the highest Z-score genome for Egr1 followed by ZF5 and brain and reproductive organ-expressed (BRE), whereas SP4, Egr1, and SP1 were found with the highest Z-scores calculated against all promoter regions. Finally, 203 TF matrices were found in the human CaV3.2 promoter region with nuclear respiratory factor 1 (NRF1), ZF5, and SP1 having the highest Z-score genome and SP1 (with two different matrices) and Egr1 with the highest Z-score promoter. Combining the overrepresentation values of rat, mouse, and human, we noticed that Egr1 was represented in the top Z-score lists of all three species (Table 1), pointing to a critical role of Egr1 for CaV3.2 promoter regulation.

Additional bioinformatic analysis of the CaV3.2 chromosomal region revealed that the overrepresentation of Egr1 was restricted to the evolutionary conserved promoter region (supplemental Fig. 1). Only one additional Egr1 binding site was found more than 1000 base pairs upstream of the identified CaV3.2 promoter region. Therefore, we decided to first examine if the cluster of Egr1 binding sites present in the conserved CaV3.2 promoter region mediates stimulation by Egr1.

Egr1 Strongly Activates CaV3.2 Promoter—To determine whether Egr1 actively regulates CaV3.2 promoter activity, an expression vector for Egr1 (24) was transfected into NG108-15 cells. Although NG108-15 cells express Egr1 constitutively (Fig. 3A), quantitative RT-PCR revealed significantly higher levels of CaV3.2 mRNA levels in Egr1-overexpressing NG108-15 cells
CaV3.2 Gene Regulation

TABLE 1

| TF matrix          | Number of matches | Z-Score genome | TF matrix          | Number of matches | Z-score promoter |
|--------------------|-------------------|----------------|--------------------|-------------------|------------------|
| Rat (1464 bp)      |                   |                | VSEGR1.02          | 19                | 17.75            |
| VSEGR1.02          | 19                | 54.57          | VSEGR1.02          | 19                |                  |
| VSSZ5.02           | 18                | 40.46          | VSSP4.01           | 19                | 16.86            |
| VSSP4.01           | 19                | 38.68          | VSZNFL219.01       | 22                | 15.72            |
| VSSPI.02           | 17                | 38.57          | VSSZ5.02           | 18                | 13.24            |
| VSSPI.03           | 17                | 37.88          | VSEGR1.03          | 17                | 12.81            |
| OSBRE.01           | 4                 | 28.86          | VSSPI.03           | 17                | 12.58            |
| VSCTCF.01          | 12                | 27.75          | VSSPI.02           | 17                | 12.46            |
| VSKL7F.01          | 10                | 27.15          | VSSZ9.01           | 17                | 12.12            |
| VSZF9.01           | 17                | 26.79          | VSSPI.01           | 18                | 11.27            |
| VSHDBP1_201        | 6                 | 25.68          | VSGC.01            | 18                | 11.08            |
| Mouse (1468 bp)    |                   |                | VSEGR1.02          | 16                | 14.81            |
| VSEGR1.02          | 16                | 42.69          | VSSP4.01           | 17                |                  |
| VSSZ5.02           | 16                | 40.17          | VSEGR1.02          | 16                | 14.39            |
| OSBRE.01           | 5                 | 35.16          | VSSP4.01           | 16                | 11.84            |
| VSSP4.01           | 16                | 35.13          | VSSP4.01           | 16                | 11.73            |
| VSSPI.02           | 15                | 34.11          | VSZNFL219.01       | 16                | 11.65            |
| VSSPI.03           | 15                | 32.91          | VSGC.01            | 19                | 11.05            |
| VSSPI.01           | 9                 | 29.14          | VSSPI.01           | 15                | 11.01            |
| VSZF9.01           | 20                | 23.29          | VSSZ9.01           | 16                | 10.90            |
| VSCTCF.01          | 16                | 23.18          | VSZNFL219.01       | 18                | 10.72            |
| VSHDBP1_201        | 11                | 22.15          | VSEF2.03           | 9                 | 9.59             |
| Human (1597 bp)    |                   |                | VSNRF1.01          | 31                | 21.29            |
| VSNRF1.01          | 31                | 76.50          | VSSP4.01           | 39                |                  |
| VSSZ5.02           | 27                | 75.03          | VSSP3.03           | 39                | 21.16            |
| VSSPI.03           | 35                | 67.26          | VSEGR1.02          | 35                |                  |
| VSEGR1.02          | 28                | 65.48          | VSSP4.01           | 28                | 19.48            |
| VSSPI.02           | 28                | 59.53          | VSSP4.01           | 26                | 18.77            |
| VSCTCF.01          | 29                | 56.18          | VSSPI.01           | 31                | 18.75            |
| VSSPI.01           | 39                | 56.10          | VSCTCF.01          | 29                | 18.43            |
| VSHDBP1_201        | 20                | 55.70          | VSNRF1.01          | 31                | 17.65            |
| VSZF9.01           | 26                | 51.35          | VSSZ5.02           | 27                | 17.37            |
| VSHDBP1_201        | 26                | 49.60          | VSZNFL219.01       | 27                | 16.71            |

(1.6-fold up-regulation; p = 0.0054; Fig. 3B). In addition, overexpression of Egr1 also induced a robust up-regulation of luciferase activity of the full-length CaV3.2–1426 reporter construct (p ≤ 0.001), whereas no up-regulation was observed for the promoterless pGL3 control plasmid (Fig. 3C). These results show that the cluster of Egr1 binding sites present in the 1426-bp fragment is sufficient to mediate stimulation of CaV3.2 transcription by Egr1.

Egr1 expression in cells is highly dynamic and can be altered by various stimuli. To examine the relationship between Egr1 levels in the cell and the transcriptional activity of the CaV3.2 promoter, we transfected NG108-15 cells with increasing amounts of Egr1 (2.5–75 ng/well) and determined the respective CaV3.2 luciferase activity. We observed that CaV3.2 activity was augmented as a function of gradually increasing Egr1 concentrations (Fig. 3D), with a plateau phase reached at ~20 ng/well. Higher amounts of Egr1 resulted in a decrease in CaV3.2 promoter activity, indicating a saturation of Egr1-induced CaV3.2 promoter activity at ~20 ng/well.

Egr1 Increases Functional Expression of T-type Ca2+ Channels—To examine whether increased neuronal Egr1 augments T-type Ca2+ channels on a functional level, NG108-15 cells were transfected with Egr1, and Ca2+ currents were recorded in whole cell condition. Ca2+ currents were isolated pharmacologically by blocking Na+ and K+ conductance (see “Experimental Procedures”). From a holding potential of −80 mV, Ca2+ currents were elicited by a voltage step to −10 mV (Fig. 3E). In Egr1-transfected cells, T-type currents were strongly increased (Fig. 3E, compare left and right current traces). On average, the magnitude of T-type currents was increased more than 2-fold in Egr1-transfected cells (Fig. 3F, 35.5 ± 4.5 pA, n = 13, versus 109.3 ± 14.2 pA, n = 13, in control and Egr1 transfected neurons respectively, p = 0.001), indicating a major functional role for Egr1 in regulating CaT. The recorded currents can be attributed to Ca3.2, as they were largely blocked by application of 100 μM Ni2+ (Fig. 3F, gray traces, n = 6 versus n = 5 in control and Egr1-transfected neurons).

Specific Binding of Egr1 to Ca3.2 Promoter—To determine the region of the Ca3.2 promoter involved in the strong Egr1-mediated up-regulation, Egr1 was cotransfected with the Ca3.2 reporter deletion constructs. Overexpression of Egr1 showed a gradual decrease in luciferase activity for the deletion fragments, with the largest luciferase activity for the full-length Ca3.2–1426 reporter fragment (10-fold up-regulation; p ≤ 0.001) and the lowest luciferase activity for the smallest Ca3.2–105 fragment (1.4-fold up-regulation; p = 0.0026) (Fig. 4A). These results imply that Egr1 effectively up-regulates promoter activity of Ca3.2 reporter constructs mainly via sequences located more upstream in the Ca3.2 promoter region.

To further support our hypothesis that the Ca3.2 promoter could be a target of Egr1 and to further pinpoint the region responsible for Egr1-induced Ca3.2 up-regulation, we performed ChIP experiments. 18 putative Egr1 responsive elements (EREs) are located within the Ca3.2 promoter region (supplemental Fig. 1 and 2), of which only the first two EREs are located within the full-length Ca3.2–1426 reporter construct. Because the up-regulation in luciferase activity after Egr1 stimulation was significantly different...
between full-length CaV3.2–1426 and CaV3.2–1188 (Fig. 4A), we hypothesized that the two most upstream EREs have the highest binding efficacy.

For the ChIP experiments, NG108-15 cells were transfected with Egr1 and compared with empty vector-transfected control cells. We examined binding of Egr1 to the CaV3.2 promoter by using an Egr1-specific antibody. A rabbit-IgG no-antibody reaction served as a negative control, and three different primer pairs were used to cover the CaV3.2 promoter region. Intriguingly, the most upstream ChIP fragment 3 of the CaV3.2 promoter (containing the two predicted upstream EREs; Fig. 4) was strongly enriched after immunoprecipitation with anti-Egr1 (Fig. 4, C and D). No significant difference was observed for the ChIP fragments 1 and 2. These observations indicate that Egr1 binds to the CaV3.2 promoter in neural cells and suggest that Egr1 overexpression leads to CaV3.2 promoter activation by the use of the upstream EREs.

**REST Binds CaV3.2 Gene in NG108-15 Cells**—Next, we examined whether Egr1-induced up-regulation of CaV3.2 can be counteracted by inhibitory elements located in the CaV3.2 gene. One repressor element known to be involved in CaV3.2 transcriptional regulation is REST. The CaV3.2 gene contains a highly conserved binding site for REST (RE-1) in its first intron (Fig. 5A), and this binding site has been reported capable of effectively binding REST (16, 18). To investigate whether Egr1-induced up-regulation of CaV3.2 could be counteracted by REST, we first analyzed NG108-15 cells for their endogenous REST mRNA expression. RT-PCR analysis revealed a clear band for the NG108-15 sample using primers against full-length REST and the truncated REST4 variant (Fig. 5B), indicating sufficient REST expression in the neuronal cell line.

Next, we cloned the CaV3.2 RE-1 sequence downstream of the full-length CaV3.2–1426 luciferase reporter construct (Fig. 5C) and compared basal activity of the CaV3.2–1426–REST reporter gene with the basal activity of CaV3.2–1426. We found no difference in basal activity for the two reporter constructs (Fig. 5D). In addition, overexpression of REST (25) or a dominant-negative variant of REST (RESTdN (26)) did not show an effect on the basal activity of any of the two reporter genes (Fig. 5E), suggesting no significant effect of REST on the CaV3.2 promoter fragment under study in naïve NG108-15 cells.

To investigate whether REST indeed binds the RE-1 site of the CaV3.2 gene, ChIP experiments on NG108-15 lysates were
performed using a REST antibody. In both basal and REST-overexpressing NG108-15 cells, PCR amplicons were obtained, indicating efficient binding of REST to the CaV3.2 gene (Fig. 5F). Nevertheless, no difference in binding efficiency was observed between immunoprecipitates generated from basal NG108-15 lysates and lysates from REST-overexpressing cells (Fig. 5G). Thus, under unstimulated conditions, up-regulation of REST has no effect on CaV3.2 promoter binding and CaV3.2 expression.

**REST Potently Counteracts Egr1-induced CaV3.2 Activation**—Next, we examined the effect of increasing REST concentrations on the CaV3.2 promoter activity of Egr1-stimulated NG108-15 cells. Cotransfection of Egr1 (25 ng/well) and REST resulted in a significant repression of the CaV3.2−1426-REST reporter gene when cells were treated with more than 100 ng/well REST (Fig. 6A). No down-regulation of Egr1-induced CaV3.2 promoter activity was observed in transfected cells harboring CaV3.2−1426 lacking a REST binding site (Fig. 6B). In addition, RESTdN did not have an effect on any of the two promoter constructs. Intriguingly, these results indicate that recruitment of REST to the RE-1 site of the CaV3.2 promoter effectively represses Egr1-induced CaV3.2 expression.

**Egr1 and REST Bind CaV3.2 Promoter in Vivo**—To analyze whether the above described effects in NG108-15 cells have any physiological relevance in vivo, ChIP analyses using anti-Egr1 and anti-REST antibodies were carried out on brain tissues. Because expression levels of the two target genes Egr1 and REST are relatively high in hippocampal tissue (Figs. 3A and 5B), we selected mouse hippocampi for our experiments. Anti-Egr1 and anti-REST hippocampal immunoprecipitates were analyzed for their binding to the CaV3.2 gene. Primers specific to the CaV3.2 promoter region (Fig. 4B) yielded PCR amplicons from the anti-Egr1 chromatin (Fig. 7A). In addition, PCR amplicons were also obtained from anti-REST immunoprecipitates when using primers specific for the RE-1 sequence of the CaV3.2 gene (Fig. 7A). Hence, consistent with the finding that
Egr1 and REST can bind the CaV3.2 gene in NG108-15 cells, ChIP analysis of mouse hippocampi revealed that Egr1 and REST also bind the CaV3.2 gene in vivo.

**Overexpression of Egr1 Increases CaV3.2 Expression in Vivo**—
Finally, we investigated whether the Egr1-induced up-regulation of CaV3.2 also occurs in vivo. Egr1 overexpression was accomplished by stereotaxical delivery of an AAV encoding the Egr1 protein in the hippocampus of adult mice. Two weeks after injection, hippocampal CaV3.2 and Egr1 mRNA expression levels were measured. We observed a significant up-regulation of Egr1 mRNA expression after rAAV-Egr1 transduction, indicating efficient rAAV infection in the hippocampus (Fig. 7B, left panel). Intriguingly, a significant up-regulation of CaV3.2 expression was also observed after infection with rAAV-Egr1 (Fig. 7B, right panel). Collectively, these data indicate that Egr1 can increase CaV3.2 expression not only in cultured cells but also in brain tissue.

**DISCUSSION**
Here, we have defined a regulatory element in the upstream CaV3.2 promoter that mediates activation of CaV3.2 transcription by Egr1. Stimulation of the CaV3.2 promoter by Egr1 thereby leads to an increase of I_{CaT}. Furthermore, we observed that Egr1-mediated promoter activation
CaV3.2 Gene Regulation

FIGURE 7. Egr1 and REST bind the CaV3.2 promoter in vivo. A, ChIP analysis of Egr1 and REST in mouse hippocampi is shown. Binding efficiency of Egr1 and REST to the CaV3.2 gene was tested using four primer sets spanning the CaV3.2 promoter region (Egr1-ChIP) and the RE-1 binding site in the first intron of the CaV3.2 gene (REST-ChIP). Notably, PCR amplicons were obtained for all four primer sets, indicating efficient Egr1 and REST binding to the CaV3.2 gene. A rabbit-IgG reaction served as negative control. B, shown is quantitative RT-PCR on RNA extracted from total hippocampi isolated from control (basal; n = 7) and rAAV-Egr1-injected (n = 4) mice. Egr1 and CaV3.2 mRNA expression levels were measured 14 days after injection, with β-actin as reference gene (t test: *p < 0.05; **, < 0.01).

can be effectively counteracted by binding of the transcriptional repressor REST to the CaV3.2 gene (Fig. 8). In contrast to short term modulatory effects on ICaT (for review, see Ref. 27), the mechanisms, we observed here, are well suited for prolonged dynamic regulation of neuronal and cardiac Ca2+-homeostasis and discharge behavior.

Our bioinformatic analyses revealed a striking accumulation of adjacent binding sites for Egr1 in the upstream CaV3.2 promoter region. Such “homotypic” TF binding clusters are a widespread genomic feature of higher eukaryotes (28) and may be utilized to control gene expression via sophisticated regulatory mechanisms such as high affinity cooperative binding of the corresponding TF (29). In general, cooperative TF binding can be translated into on/off transcriptional responses, regulating the functional state of the corresponding gene, namely, active or inactive. In contrast, non-cooperative TF binding does not switch between a digital (on/off) transcriptional response but results in a more gradual transcriptional activation (30). Our ChIP data reveal Egr1 binding to all Egr1 binding sites of the CaV3.2 promoter under basal conditions, indicating a cooperative mechanism of CaV3.2 transcriptional regulation. However, in the presence of increased Egr1 levels, augmented Egr1 binding occurred only at the two most upstream Egr1 binding sites, suggesting these binding sites to be of importance for strong stimulus-induced CaV3.2 up-regulation.

Egr1 is a zinc finger transcription factor and belongs to a larger family of early response genes that also include Egr2, Egr3, Egr4, and Wilms tumor 1 (Wt1). Egr1 is rapidly and transiently induced by a variety of stimuli, including serum, growth factors, mechanical injury, stress, and ischemia and has important roles in the regulation of cell growth, differentiation, development, and apoptosis (31, 32). Upon induction, Egr1 can bind ERE consensus sequences to regulate expression of downstream target genes, such as fibronectin (Fn1), fibroblast growth factor 2 (Fgf2), synapsin 1 (Syn1), transforming growth factor, β1 (Tgfβ1), phosphatase and tensin homolog (Pten), and p53 (for review, see Ref. 33). Furthermore, Egr1 has been reported to activate NGFI-A-binding protein 1 and 2 (Nab1 and Nab2). Interestingly, both proteins also appear to be important regulators of Egr1-mediated transactivation (34–36). By binding to Egr1, Nab1 and Nab2 can strongly inhibit Egr1 activity. Nab1 and Nab2 are also expressed in NG108-15 cells, but only Nab2 is significantly up-regulated after Egr1 stimulation.³ Congruently, Egr1 can regulate the induction of its own repressor Nab2, and can thus control its own transactivation efficiency in NG108-15 cells. This negative feedback loop provides a potential explanation for the saturation of Egr1-induced CaV3.2 up-regulation when increasing levels of Egr1 are transfected (Fig. 3D).

Previous studies have shown that Egr1 and Sp1, for which there are also several potential binding sites within the CaV3.2 promoter (Table 1), can generally compete for overlapping binding motifs (37, 38). Sp1 usually activates target promoter sequences but gives complex responses when in the presence of Egr-1 (39). Sp1 is also expressed in NG108-15 cells.³ Therefore, Sp1 and Egr1 could recognize and competitively bind overlapping Sp1/Egr1 consensus sequences located in the CaV3.2 promoter. Intrigually, of the predicted Egr1 binding sites in the CaV3.2 promoter (Fig. 4B), the two most upstream Egr1 binding sites, with the highest Egr1 binding efficiency in our ChIP experiments, do not overlap with Sp1 consensus sequences (supplemental Fig. 1). This bioinformatic finding together with our data suggest that the two upstream Egr1 binding sites that are critical for a dose-dependent CaV3.2 promoter control by Egr1 are exclusively controlled by Egr1 and not by Sp1.

We found that the transcriptional repressor REST counteracts the stimulatory effect of Egr1 on the CaV3.2 promoter. The CaV3.2 gene contains a highly conserved RE-1 that can interact with REST. Originally, REST was identified to be important for

³ K. M. J. van Loo, C. Schaub, K. Pernhorst, Y. Yaari, H. Beck, S. Schoch, and A. J. Becker, unpublished observations.
the silencing of neuronal-specific genes in non-neuronal cells (40). Nevertheless, REST also has a functional role within the nervous system by regulating expression of several target genes, including Syn1, synaptophysin (Syp), the type II sodium channel genes (Scn2A and Scn2B), and the genes encoding the potassium channel subunits Kv7.2 and Kv7.3 (Kcnq2 and Kcnq3) (41, 42). Recently a large scale chromatin immunoprecipitation assay (ChIPSeq) was performed to build a high resolution interactome map for REST (43). Here, CaV3.2 was identified as a REST-responsive gene as well as were other members of the voltage-dependent calcium channel subunit family (e.g. Cacna1a, Cacna1b, cacna1e and cacna2d2). In addition, many other ion channel genes were found positive for REST binding, including the sodium channels Scn3b and Scn10a, several potassium channels, and the hyperpolarization-activated cyclic nucleotide gated (Hcn) ion channel genes. In this context, REST may have a general role in coordinately regulating expression levels of ion channel proteins from different subfamilies, including the CaV3.2 gene.

REST did not repress the basal activity of the CaV3.2 promoter but strongly decreased Egr1-induced promoter activity. This observation suggests that because basal expression levels of CaV3.2 are low and the REST binding site in the CaV3.2 promoter is occupied during basal conditions (Figs. 5F and 7A), REST is involved in keeping basal CaV3.2 levels low. Therefore, an increase in REST levels cannot further down-regulate CaV3.2 expression. After Egr1 stimulation, REST binding to the RE-1 of the CaV3.2 gene might be relieved, resulting in augmented CaV3.2 expression levels. The Egr1-induced up-regulation can then only be repressed by higher REST availability. Therefore, REST may play an important role in keeping basal expression levels of CaV3.2 low before and after a transient activating stimulus. Transcriptional regulation by REST is even more complex due to the coexpression of REST4 (Fig. 5B). REST4, the truncated variant of REST that lacks the C-terminal zinc finger repressor domain can antagonize the action of full-length REST (44) and might thus interfere with REST-induced changes.

Transient transcriptional alterations of CaV3.2 relate to disorders with episodic onset of symptoms such as epileptic seizures and cardiac arrhythmias. The latter has been demonstrated to relate to the modulation of CaV3.2 transcription by REST (16, 18). Interestingly, highly dynamic changes of CaV3.2 expression have been observed in epileptogenesis after brain insults in thalamic as well as hippocampal principal neurons (14, 15). In hippocampal CA1 pyramidal cells, CaV3.2 mRNA is transiently up-regulated early in epileptogenesis that is triggered by an episode of status epilepticus (SE), leading to an increase in the propensity for intrinsic burst-firing (15, 45). Egr1 has been suggested as a critical factor for the establishment of long term neuronal plasticity in the hippocampal formation (46–48). Intriguingly, Egr1 expression is also strongly increased in hippocampal neurons after SE (49, 50). In addition, human twin studies showed a dysregulation of Egr1 mRNA expression in idiopathic absence epilepsies (51). In conjunction with our present data, these studies suggest that Egr1-
mediated transcriptional up-regulation of Ca\textsubscript{3.2} may be a mechanism generalizable to a number of CNS disorders.

Notably, Ca\textsubscript{3.2} mRNA peaks only transiently for 2 days after SE in CA1 pyramidal cells and afterward sharply returns to base-line levels (15). Neuronal REST expression is significantly up-regulated after global ischemia and epileptic insults (19–21, 52, 53). Extrapolating our present in vitro data to this condition, we suggest that augmented REST levels may be involved in Ca\textsubscript{3.2} repression 2 days after SE. A similar regulation by REST has been described for the hyperpolarization-activated cyclic nucleotide gated ion channel Hcn1 after kainic acid-induced SE. The SE-induced up-regulation of REST represses Hcn1 expression and the corresponding Hcn1-mediated currents (I\textsubscript{h}) (53). REST, therefore, may play the role of “a central switch” in SE-induced channelopathies. The fact that strong expression of REST and its binding to the RE-1 of the Ca\textsubscript{3.2} promoter raises the intriguing possibility that REST up-regulation may sharpen the temporal profile of Ca\textsubscript{3.2} up-regulation. This will depend to a great extent on the precise timing of Egr1 and subsequent REST induction. Further studies will be needed to analyze the effect of increased REST levels in vivo, e.g. after SE, on the Ca\textsubscript{3.2} transcriptional activity.

Interfering with a transcriptional complex that alters transcription of multiple disease-relevant genes represents a potential therapeutic approach. A detailed understanding of the responsible transcriptional regulatory mechanisms will allow for specific interference strategy. Therefore, our data showing that the Ca\textsubscript{3.2} promoter can be regulated by interplay of Egr1 and REST, whereby increases in cellular Egr1 activate the Ca\textsubscript{3.2} promoter while REST can counteract the Egr1-induced up-regulation, represents an important step in this direction.

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