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Transcriptional Control of *KCNQ* Channel Genes and the Regulation of Neuronal Excitability

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Regulation of the resting membrane potential and the repolarization of neurons are important in regulating neuronal excitability. The potassium channel subunits Kv7.2 and Kv7.3 play a key role in stabilizing neuronal activity. Mutations in *KCNQ2* and *KCNQ3*, the genes encoding Kv7.2 and Kv7.3, cause a neonatal form of epilepsy, and activators of these channels have been identified as novel antiepileptics and analgesics. Despite the observations that regulation of these subunits has profound effects on neuronal function, almost nothing is known about the mechanisms responsible for controlling appropriate expression levels. Here we identify two mechanisms responsible for regulating *KCNQ2* and *KCNQ3* mRNA levels. We show that the transcription factor Sp1 activates expression of both *KCNQ2* and *KCNQ3*, whereas the transcriptional repressor REST (repressor element 1-silencing transcription factor) represses expression of both of these genes. Furthermore, we show that transcriptional regulation of *KCNQ* genes is mirrored by the correlated changes in M-current density and excitability of native sensory neurons. We propose that these mechanisms are important in the control of excitability of neurons and may have implications in seizure activity and pain.

Introduction

Regulation of the resting membrane potential and the repolarization of neurons are important in regulating neuronal excitability. One ionic current that plays a key role in stabilizing neuronal activity is the M-current (I_M), a slowly deactivating, non-inactivating potassium current first identified nearly 30 years ago as the one underlying the excitatory effect of acetylcholine (Brown and Adams, 1980). The M-current is produced by the action of Kv7 channels encoded by members of the *KCNQ* gene family *KCNQ1–KCNQ5*, each of which encodes an individual potassium channel subunit Kv7.1–Kv7.5 (Jentsch, 2000; Robbins, 2001).

Of the five KCNQ gene family members, *KCNQ2* and *KCNQ3* are particularly important for regulating neuronal activity because these subunits are expressed widely throughout the CNS with expression patterns that almost entirely overlap (Tinel et al., 1998; Yang et al., 1998). Kv7.2 and Kv7.3 form functional hetero-multimers that are believed to represent a major M-channel isoform in the CNS and peripheral nervous system (Delmas and Brown, 2005). The importance of the appropriate functioning of

Kv7.2 and Kv7.3 in the nervous system is highlighted by the fact that mutations in *KCNQ2* and *KCNQ3* are associated with benign familial neonatal convulsions, an autosomal dominant neonatal epilepsy (Charlier et al., 1998; Singh et al., 1998; Yang et al., 1998; Biervert and Steinlein, 1999). The M-current is also one of the key players in nociceptive transmission; inhibition of M-current leads to membrane depolarization and hyperexcitability of nociceptive neurons (Passmore et al., 2003; Crozier et al., 2007; Linley et al., 2008; Liu et al., 2010), an important determinant of many pain conditions. Pharmacological openers of Kv7.2/Kv7.3 channels therefore now represent important analgesic targets (Surti and Jan, 2005; Gribkoff, 2008; Wickenden and McNaughton-Smith, 2009) because current therapies are not efficacious for the majority of inflammatory and chronic pain conditions.

Despite their importance, very little is known about the mechanisms responsible for regulating *KCNQ2* and *KCNQ3* expression. Here we use a functional assay to identify important regulatory regions within the *KCNQ2* and *KCNQ3* genes. We show that both *KCNQ2* and *KCNQ3* contain GC box motifs and provide evidence that their transcription is enhanced by the Sp1 transcription factor. We also show that expression of both *KCNQ2* and *KCNQ3* is repressed by REST (repressor element 1-silencing transcription factor) and that expression of REST in neurons is sufficient to repress *KCNQ2* and *KCNQ3* expression, inhibiting functional expression of the M-current and resulting in hyperexcitable neurons. We show that REST levels are increased in dorsal root ganglia (DRG) neurons in response to inflammatory mediators and that Kv7.2 levels and M-current density are reduced, suggesting a potential role in regulating inflammatory pain responses. Neuronal expression of REST is increased in response to sustained neuronal hyperactivity, i.e., in

epileptic insults (Palm et al., 1998), cerebral ischemia (Calderone et al., 2003), and a model of neuropathic pain (Uchida et al.); we therefore suggest that, by repressing *KCNQ2* and *KCNQ3* expression, REST may contribute to chronic overexcitability of neuronal circuits seen in epilepsy and chronic pain.

Materials and Methods

Cell culture. SHSY-5Y and HEK293 cells were grown in DMEM/F-12 supplemented with 10% fetal calf serum (PAA Laboratories), 6 g/liter penicillin, 10 g/liter streptomycin, and 2 mM L-glutamine at 37°C and 5% CO_2. DRG neurons were cultured as described previously (Crozier et al., 2007; Linley et al., 2008; Liu et al., 2010); briefly, ganglia were extracted from 7- to 10-d-old rats from all spinal levels. Ganglia were enzymatically dissociated in HBSS supplemented with collagenase type 1A (1.5 mg/ml) and dispase (15 mg/ml; Invitrogen) at 37°C for 15–20 min. Cells were then mechanically triturated, washed twice by centrifugation (800 rpm for 5 min), resuspended in 800 μl of growth medium, and plated onto glass coverslips coated with poly-D-lysine and laminin. DRG neurons were cultured for 2–5 d in a humidified incubator (37°C, 5% CO_2) in DMEM supplemented with GlutaMAX I (Invitrogen), 10% fetal bovine serum, penicillin (50 U/ml), and streptomycin (50 μg/ml). For the inflammatory conditions, cells were incubated with the following inflammatory mediators for 48 h: 1 μM bradykinin, 1 μM histamine, 1 μM ATP, 1 μM substance P, and 10 μM PAR-2 activating peptide. For patch-clamp experiments, cells were washed twice and media were replaced with fresh media that did not contain inflammatory mediators for at least 2 h before experiments, to remove any acute effects of inflammatory mediators on the M-current.

DNase I hypersensitivity assay. SHSY-5Y cells were washed twice with ice-cold PBS, resuspended in DNase I buffer (10 mM Tris, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 3 mM $CaCl_2$, 1 mM DTT, 0.1% Nonidet P-40, and 8% glycerol) and incubated on ice for 10 min. Cells were counted and transferred to a Dounce homogenizer and lysed with 15 strokes of a type B pestle. Nuclei were divided into independent samples ($\sim 1.6 \times 10^5$ per reaction) and treated with 0, 1, 3, 5, and 10 U of DNase I (Sigma) at 37°C for 5 min. Reactions were stopped by addition of equal volume of stop/lysis buffer (20 mM EDTA, 1% SDS, and 0.1 mg/ml proteinase K) and incubated at 55°C overnight. DNA was extracted several times with phenol/chloroform and once with chloroform and then ethanol precipitated. DNA was resuspended in water, and the concentration was measured by spectrophotometry. Ten micrograms of each sample were digested overnight with the appropriate restriction enzymes in a final volume of 30 μl and electrophoresed through 40 mM Tris-acetate, 1 mM EDTA, pH 8, and 0.6% (w/v) agarose gel at 0.5 V/cm. DNA was transferred to Hybond XL membranes (GE Healthcare) using alkaline capillary transfer. DNA fragments were radiolabeled using Prime-It kit (Stratagene) and [α-^{32}P]dATP. Hybridization to Hybond XL membranes was performed as per the instructions of the manufacturer.

Gel mobility shift assay. Nuclear extracts were prepared from SHSY-5Y and JTC-19 cells using the method described previously (Andrews and Faller, 1991). DNA probes were radioactively labeled by Klenow (New England Biolabs) fill in of a restriction-enzyme-generated overhang using [α-^{32}P]dATP (GE Healthcare) and incubated with protein extracts before electrophoresis through a 4% nondenaturing acrylamide gel. Specificity of DNA–protein binding was assessed by including 10 pmol of double-stranded oligonucleotides, or 1 μl of anti-Sp1 (2 mg/ml, H-255; Santa Cruz Biotechnology) or 1 μl of anti-REST (2 mg/ml, P-18; Santa Cruz Biotechnology) antibodies. Complimentary oligonucleotides with the following sequences were annealed and used for competition experiments: consensus Sp1, 5′-GATCCCGATCGGGGCGGGGCG-3′; nonspecific, 5′-AATTC-CCGCGAGGGGCGCCTAGTCCCCATG-3′; *CHRM4* repressor element 1 (RE1), 5′-GTACGGAGCTGTCCGAGGTGCTGAATCTGCCT-3′; human *KCNQ2* RE1, 5′-GATCCTGGTCAGGACCATGGCCAGCACCCC; dog *KCNQ2* RE1, 5′-GATCTGCTGCTCAGGACCACGGCCAGCGCCTC; mouse *KCNQ2* RE1, 5′-GATCTTGAGTCCAGGACCATGGTCAG-CACCAC; rat *KCNQ2* RE1, 5′-GATCTTTGCGTCCAGGACCATGGT-CAGCGCCAC; *KCNQ1* RE1, 5′-GATCCGGGGCCTGCACCCAGG-ACAGGGCC; *KCNQ3* RE1, 5′-GATCAGGCTCAGGACCTAGGACAGT-

TCC; *KCNQ4*, 5′-GATCCTGTCCAGGACCTGAGCCAGGGCT; and *KCNQ5* RE1, 5′-GATCCTTGTCAGCACCTAGGACAGAGAT.

Luciferase assay. Fragments of human *KCNQ2* gene corresponding to −64/+179 and −416/+151 bp regions were amplified by PCR with the following primers: Sp1 sense (s), 5′-AAAAGATCTCATGGTGCCTG-GCGGGAGG; Sp1 antisense (a), 5′-AAGAGCTCAACGCGGGGC-**GG**GGCGAG; AscIs, 5′-GGCGCGCCTCGGGCTCAGGCTCAG; and AscIas, 5′-GGCGCGCCCTTGGTCCCTTCTGCC. Underlined nucleotides correspond to newly formed restriction sites. Additionally, we introduced mutations into the Sp1 binding site to prevent Sp1 binding using the primer for Sp1s^{Mut}, 5′- AAGAGCTCAACGCGGGGC**AA**GGCGAG, containing a substitution of GG with AA (in bold). Amplified fragments were digested with appropriate restriction enzymes and cloned into pGL3 Basic (Promega). Cells were transfected in 24-well plates with 250 ng of pGL3 plasmid and 1 ng of pRL cytomegalovirus (*Renilla* luciferase; Promega) using 4 μl of Lipofectamine (Invitrogen). Each transfection was performed in triplicate for each experiment. Cells were harvested 48 h after transfection into passive lysis buffer, and luciferase and *Renilla* luciferase expression were quantified using a Dual Luciferase Assay kit (Promega) on a Mediators PHL luminometer.

Quantitative reverse transcriptase-PCR. Total RNA was extracted from dorsal root ganglia cells using Tri-reagent (Sigma). DNase-treated (Ambion) RNA was reverse transcribed by using Moloney murine leukemia virus reverse transcriptase (Promega) and purified using Qiaquick columns (Qiagen). Quantitative PCR was performed using SYBR Green incorporation (Bio-Rad) for duplicate samples in each experiment. The specificity of PCR was verified by melt curve analysis of products obtained from cDNA as well as controls in which the reverse transcriptase was omitted. Levels of signal were normalized to levels of *U6* small nuclear RNA, which were not different between any of the datasets. Significance was tested using Student's *t* test. Primers used were as follows: *U6*s, 5′-CTCGCTTCGGCAGCACA; *U6*a, 5′-AACGCTTCACGAATTTGCGT; *KCNQ2*s, 5′-GTGGTCTA-CGCTCACAGCAA; *KCNQ2*a, 5′-AGGGTAAACGTCGCTGCTAA; *KCNQ3*s, 5′-GCCCACAGTCCTGCCCATCTTGAC; *KCNQ3*a, 5′-CCGT-TCCAGTTCCTCGTGGTTGACG; *REST*s, 5′-CGAACTCACACAG-GAGAACG; *REST*a, 5′-GAGGCCACATAATTGCACTG; *SCN2A*s, 5′-GCTGCATGTCTCTCTTGCTG; and *SCN2A*a, 5′-GGACCGATTTGCT-TCACTTC.

Western blot and immunocytochemistry. For Western blots, 10 μg of protein extracts from control and mithramycin A-treated SHSY-5Y cells were electrophoresed through an SDS/10% (w/v) polyacrylamide gel using standard procedures, transferred to Hybond C-Extra (GE Healthcare), and screened with 1:1000 dilution of anti-Kv7.2 (Alomone Labs) antibody and a 1:1000 dilution of horse radish peroxidase-conjugated anti-rabbit antibody. Antibody staining was visualized using an ECL plus detection kit (GE Healthcare). For immunocytochemistry, cells were fixed and permeabilized in acetone/methanol 1:1 for 5 min on ice, followed by 0.1 M PBS/0.1% Triton X-100 for 5 min at room temperature and blocked with 10% donkey serum in PBS for 2 h at room temperature. Primary antibody incubation was performed in 10% donkey serum/0.4 M PBS overnight at 4°C at 1:100 dilution for rabbit anti-REST (H290; Santa Cruz Biotechnology), 1:1000 rabbit anti-Kv7.2 (kind gift from Dr. Mark Shapiro, University of Texas Health Science Center at San Antonio, San Antonio, TX) or 1:1000 guinea pig anti-TRPV1 (Neuromics), followed by 1:1000 Alexa Fluor-555 donkey anti-rabbit or Alexa Fluor-488 donkey anti-guinea pig secondary antibody (Invitrogen) in 0.1 M PBS for 2 h at room temperature. Coverslips were mounted in Vectashield with 4′,6′-diamidino-2-phenylindole (DAPI) and imaged using a Carl Zeiss LSM510 inverted confocal microscope. Among comparisons between cell conditions, coverslips were mounted on the same slide and imaged with the same laser settings. Image analysis was performed using NIH ImageJ software.

Plasmid and adenoviral delivery. The coding sequence for Sp1 was amplified using the primers 5′-GCGAATTCAATGAGCGACCAAGATCACTCA and 5′-CGGAATTCTCAGAAACCATTGCCACTGAT and cloned into the expression plasmid pTARGET (Promega). Freshly isolated DRG neurons were transfected with pTARGET Sp1 or control plasmid using a Nucleofector device (Amaxa) and used 3–5 d after transfection. The green fluorescent protein (GFP) plasmid pmaxGFP (Amaxa) was cotransfected as a marker of the efficiency of transfection. The adenoviral constructs have been described

previously (Wood et al., 2003). Green fluorescent protein was a marker of the efficiency of viral infection. Cells were cultured and infected with adenoviral particles for 48 h before harvesting and RNA or electrophysiological analysis.

Electrophysiology. In patch-clamp experiments, the standard bath solution contained the following (in mM): 160 NaCl, 2.5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, and 10 HEPES, pH adjusted to 7.4 with NaOH. For perforated-patch experiments, the patch pipette contained 90 mM K-acetate, 20 mM KCl, 1 mM CaCl$_2$, 3 mM MgCl$_2$, 3 mM EGTA, 40 mM HEPES, and 400 μg/ml amphotericin B, pH adjusted to 7.4 with NaOH. Currents were amplified using an EPC-10 patch-clamp amplifier (HEKA) and recorded using Patchmaster software (version 2.2; HEKA). The current signal was sampled at 1 kHz and filtered online at 500 Hz using a software-based Bessel filter. Patch pipettes were fabricated from borosilicate glass (Harvard Apparatus) using a horizontal puller (DMZ-universal puller; Zeitz-Instrumente GmbH) and heat polished to a resistance of 2–4 MΩ. Cells were mounted on an inverted microscope (TE-2000; Nikon) in a low-profile perfusion chamber fed by a gravity perfusion system flowing at ~2 ml/min, resulting in a bath exchange time of ~15 s. Series resistance was corrected online by up to 70% using the Patchmaster software, and liquid junction potentials were corrected. The magnitude of the neuronal M-current was measured from the deactivation current when stepping the membrane voltage from -30 to -60 mV and was calculated as the difference between current amplitude at 10 ms into the voltage pulse and that at the end of the pulse. This analysis method was designed to minimize any contribution from other K$^+$ currents and capacitance artifacts. XE991 [10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone dihydrochloride] was obtained from Tocris Bioscience. All analysis of patch-clamp data was conducted using Fitmaster software (version 2.11; HEKA).

Statistics. All data are given as mean $\pm$ SEM. Differences between groups were assessed by Student's t test or one-way ANOVA. The differences were considered significant at $p \leq 0.05$.

Results

Identification of DNase I hypersensitive sites in the human *KCNQ2* gene

To identify regions of the *KCNQ2* gene that are important for regulation of expression *in vivo*, we used a DNase I hypersensitivity assay. DNase I hypersensitive sites (HSS) correlate well with important transcriptional regulatory regions, such as promoters, enhancer, and repressor elements (Fritton et al., 1987; Steiner et al., 1987; Elgin, 1988; Wong et al., 1997), and map to regions that recruit transcription factors (Wong et al., 1995; Boyes and Felsenfeld, 1996). A DNase I HSS assay was performed in SHSY-5Y cells, which are known to express *KCNQ2* and *KCNQ3* (Wickenden et al., 2008) and identified two HSS within the *KCNQ2* gene (Fig. 1A). We performed a bioinformatic analysis of the regions identified to look for transcription factor binding sites. An analysis of HSS1 identified a putative Sp1 transcription factor binding site (Fig. 1B) that is conserved within the promoters of the human, rat, and mouse *KCNQ2* genes. It has been shown previously that regions of DNA that recruit Sp1 display hypersensitivity to DNase I (Philipsen et al., 1990; Pruzina et al., 1991; Jiang et al., 1997; Sinha and Fuchs, 2001). An analysis of the region surrounding HSS2 identified a putative binding site for the transcriptional repressor REST that is conserved within multiple mammalian species (Fig. 1C). REST binding to DNA is known to be sufficient to produce a DNase I HSS (Wood et al., 2003).

KCNQ2 and *KCNQ3* genes contain similar regulatory elements

To determine whether the potential Sp1 factor binding sites are important for *KCNQ2* gene regulation, we used an *in vitro* DNA–protein interaction experiment to examine the recruitment of Sp1. A radiolabeled fragment of the *KCNQ2* promoter containing the Sp1 site was incubated with nuclear protein extracts from SHSY-5Y cells. Incubation of this region of DNA with nuclear

protein produced two slowly migrating bands (Fig. 1D, lane 1, Sp1:DNA, Non spec) that could be competed by excess of an unlabeled Sp1 consensus sequence (Fig. 1D, lane 2). The faster migrating DNA–protein complex could also be competed by an unrelated sequence (Fig. 1D, lane 3), indicating that this complex is the result of nonspecific protein–DNA interactions. The slower migrating protein–DNA complex could only be competed with a consensus Sp1 sequence and was further retarded by an anti-Sp1 antibody (Fig. 1D, lane 4, Sp1:Ab:DNA), indicating the presence of Sp1 protein within this complex. Binding of the radiolabeled probe was also competed by an excess of unlabeled wild-type Sp1 sequence from the *KCNQ2* promoter (Sp1^{WT}) (Fig. 1D, lanes 5, 6) but not by a *KCNQ2* sequence containing mutations predicted to destroy the Sp1 site (Sp1^{mut}) (Fig. 1D, lanes 7, 8). These data indicate that the Sp1 site within the *KCNQ2* promoter can recruit Sp1 protein present in SHSY-5Y cells. Given the overlap of *KCNQ2* and *KCNQ3* expression and their shared evolutionary origin by duplication from a common ancestor gene (Hill et al., 2008), it is quite likely that these two genes would share at least some regulatory mechanisms. We therefore searched the *KCNQ3* gene for potential Sp1 and RE1 sites. We identified a conserved Sp1 site in the *KCNQ3* gene just upstream of the first exon, at an equivalent location to the Sp1 site in the *KCNQ2* gene (Fig. 1E,F). We also identified an RE1 site in the *KCNQ3* gene that is conserved across multiple mammalian species (Fig. 1E,G). Thus, the two regulatory elements that we identified within the *KCNQ2* gene are also present in the *KCNQ3* gene.

Sp1 regulates KCNQ2 and KCNQ3 expression

To determine whether the Sp1 site is functionally important in regulating *KCNQ2* and *KCNQ3* transcription, we used a reporter gene assay with the *KCNQ* promoter regions. We cloned a region of the *KCNQ2* promoter encompassing nucleotides $-416/+151$ bp relative to the transcription start site into a luciferase reporter vector such that luciferase expression is driven by *KCNQ2* promoter sequences. This 568 bp region encompasses the Sp1 site and 137 bp of the first exon. We also produced a truncated region that lacks some of the sequence upstream of the Sp1 site ($-64/+179$), and we introduced mutations into the Sp1 site that prevent Sp1 binding ($-64/+179$ Sp1^{mut}) (Fig. 1D, lanes 7, 8). We transfected these DNA constructs into SHSY-5Y cells and measured the resulting luciferase activity. Luciferase activity from the $-416/+151$ bp promoter fragment was sixfold higher than the control plasmid (Fig. 2A, compare $-416/+151$ bp with pGL3 Basic), suggesting that this region of the *KCNQ2* gene and the Sp1 site within it elevate expression of *KCNQ2*. Removal of the upstream region did not result in a significant loss of expression, suggesting that this region is not important in regulating transcription in SHSY-5Y cells (Fig. 2A, compare $-416/+151$ with $-64/+179$ bp). Introduction of mutations that prevent Sp1 binding (see Materials and Methods) (Fig. 1D), however, resulted in loss of >50% luciferase activity (Fig. 2A, compare $-64/+179$ with $-64/+179$ bp Sp1^{mut}), consistent with the hypothesis that *KCNQ2* expression is regulated by Sp1. In our bioinformatic analysis, we noticed that the *KCNQ2* gene contained a second Sp1 site that overlapped with the first although its score was less, suggestive of a lower affinity for Sp1 (supplemental Fig S1A, available at www.jneurosci.org as supplemental material). In gel mobility shift experiments, this second site did indeed bind to Sp1, showing a lower affinity than our originally identified site (supplemental Fig S1B, available at www.jneurosci.org as supplemental material). This second Sp1 site is unlikely to be bound by Sp1 in the wild-type promoter because the presence of Sp1 at the

high-affinity site, which overlaps with the low-affinity site, would presumably occlude it. Mutation of the high-affinity site, however, would uncover this second site, and the low level of promoter activity seen for the construct $-64/+179$ Sp1^{mut} is likely to be attributable to low levels of Sp1 recruitment. To determine whether the *KCNQ3* gene could also be regulated by Sp1, we conducted a bioinformatic search of the human *KCNQ3* promoter region. We identified a sequence matching a consensus Sp1 site that was highly conserved between the human, mouse, and rat genomes (Fig. 1*F*). To determine whether this potential Sp1 site is functional, we cloned an 876 bp region of the human *KCNQ3* gene encompassing nucleotides -540 to $+336$ relative to the transcription start site and including the Sp1 site into the same luciferase reporter vector as for the *KCNQ2* promoter. We also cloned a region of the promoter encompassing nucleotides -169 to $+336$ that lacks the conserved Sp1 site. Luciferase activity from the $-540/+336$ promoter region drove high levels of luciferase expression in SHSY-5Y cells (Fig. 2*B*), which was reduced by $\sim$80% when the region containing the Sp1 sites was removed (Fig. 2*B*). Together, these data suggest that Sp1 can regulate both *KCNQ2* and *KCNQ3* promoter activity.

Sp1 regulates the endogenous *KCNQ2* and *KCNQ3* genes

Having shown that Sp1 can be recruited to the *KCNQ2* promoter *in vitro* and the Sp1 site within the promoter is important for driving reporter gene activity, we wanted to test the relevance of Sp1 transcription factor activity to endogenous *KCNQ2* and *KCNQ3* expression. To do this, we took advantage of mithramycin A, a compound that binds to Sp1 binding sites (GC box) and inhibits the function of Sp1 (Ray et al., 1989; Liu et al., 2002; Kim et al., 2006). Transfection of *KCNQ2* and *KCNQ3* luciferase reporter constructs into SHSY-5Y cells in the presence of mithramycin A resulted in a 66 and 90% reduction in luciferase activity, respectively (Fig. 2*C*). To determine the role of Sp1 on endogenous gene expression, we incubated neurons isolated from DRG in the presence of 200 nM of mithramycin A for 48 h and measured mRNA expression using quantitative PCR. In the presence of mithramycin A, *KCNQ2* and *KCNQ3* levels were reduced by 90% (Fig. 2*D*), suggesting that Sp1 is critical for driving endogenous *KCNQ2* and *KCNQ3* expression. Expression of the *SCN2A* gene, which encodes the type II sodium channel, was unaffected by mithramycin A, suggesting that the effect on *KCNQ* expression was not the result of a global change in gene expression (Fig. 2*D*). Incubation of SHSY-5Y cells with mithra-
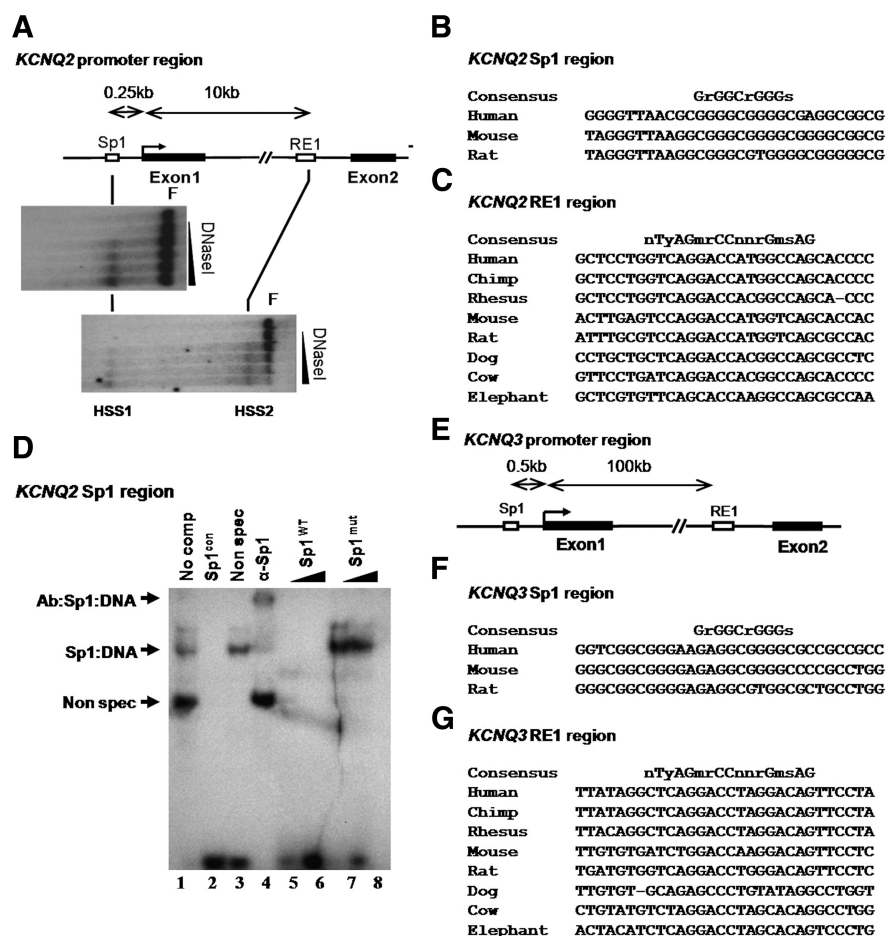

Figure 1. DNase I hypersensitive sites present in the *KCNQ2* gene. *A*, Top shows a schematic representation of the 5′ of the *KCNQ2* gene. Filled boxes represent exons, and the transcription start site is marked by an arrow. The location of identified regulatory elements (Sp1 and RE1) are shown as open boxes. Below shows two DNase I hypersensitivity assays. Nuclei were isolated from SHSY-5Y cells and analyzed for DNase I hypersensitive sites. DNA was digested with HindIII (top) or AseI (bottom), and the Southern blot was hybridized with a *KCNQ2*-specific probe. The positions of the hypersensitive sites (HSS1 and HSS2) are indicated on the diagram as is the position of the full-length DNA fragment (F). *B*, DNA sequence alignment of the Sp1 sequence identified in the HSS1 region of the human *KCNQ2* gene with the equivalent regions of the mouse and rat genomes. The consensus Sp1 sequence is shown above the alignment (r, A or G; s, C or G). *C*, DNA sequence alignment of the RE1 sequence identified in the HSS2 region of the human *KCNQ2* gene with the equivalent regions of the chimp, rhesus, mouse, rat, dog, cow and elephant *KCNQ2* genes. The consensus RE1 sequence is shown above the alignment (n, A, C, G, or T; y, C or T; m, A or C). *D*, Gel mobility shift assay of the Sp1 region of the *KCNQ2* promoter. Labeled DNA was incubated with protein isolated from SHSY-5Y cells in the presence of no competitor (No comp, lane 1), oligonucleotides containing a consensus Sp1 sequence (Sp1^{con}, 1 μM, lane 2), an unrelated sequence (Non spec, 1 μM, lane 3), an anti-Sp1 antibody (α-Sp1, 1 μg, lane 4), or oligonucleotides containing the wild-type or mutated Sp1 sequence from the *KCNQ2* promoter (Sp1^{wt}: 1 μM, lane 5; 10 μM, lane 6; Sp1^{mut}: 1 μM, lane 7; 10 μM, lane 8). Position of complexes containing Sp1 (Sp1:DNA), α-Sp1 (Ab:Sp1:DNA), and resulting from nonspecific binding (Non spec) are shown on the left. *E*, A schematic representation of the 5′ of the *KCNQ3* gene. Filled boxes represent exons, and the transcription start site is marked by an arrow. The location of identified regulatory elements (Sp1 and RE1) are shown as open boxes. *F*, DNA sequence alignment of the Sp1 sequence identified in the human *KCNQ3* gene with the equivalent regions of the mouse and rat *KCNQ3* genes. The consensus Sp1 sequence is shown above the alignment (r, A or G; s, C or G). *G*, DNA sequence alignment of the RE1 sequence identified in the human *KCNQ3* gene with the equivalent regions of the chimp, rhesus, mouse, rat, dog, cow, and elephant *KCNQ2* genes. The consensus RE1 sequence is shown above the alignment (n, A, C, G, or T; y, C or T; m, A or C).

mycin A also resulted in a reduction of Kv7.2 protein (Fig. 2*E*). As an alternative approach to determine whether Sp1 was important in regulating expression of *KCNQ2* and *KCNQ3*, we transfected DRG neurons with an Sp1 expression plasmid and measured Kv7.2 expression and M-current densities. We transfected a plasmid expressing GFP alongside either the Sp1-expressing or the control plasmid to identify transfected neurons (Fig. 3*A*). In whole-cell voltage-clamp experiments, I_M was measured from a standard square voltage pulse protocol stepping to -60 mV from a holding potential of -30 mV (Fig. 3*B*, inset) as an amplitude of
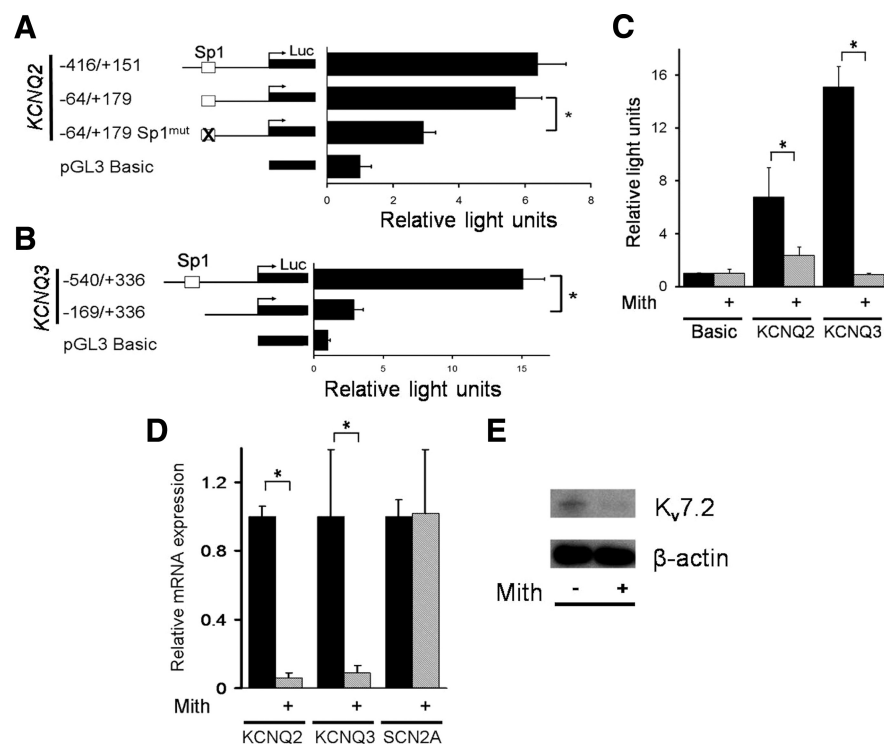
Figure 2. The *KCNQ2* and *KCNQ3* promoters contain functional Sp1 sequences. *A*, Regions of the *KCNQ2* promoter were cloned upstream of luciferase and transfected into SHSY-5Y cells. Shown are normalized luciferase values expressed relative to empty vector, pGL3 Basic (mean ± SEM; $n = 3$; *$p < 0.05$). Base pair numbers are expressed relative to transcription start sites. *B*, Regions of the *KCNQ3* promoter were cloned upstream of luciferase and transfected into SHSY-5Y cells. Shown are normalized luciferase values expressed relative to empty vector (mean ± SEM; $n = 3$; *$p < 0.05$). *C*, Luciferase reporter vector containing the *KCNQ2* or *KCNQ3* promoter regions or empty vector, pGL3 Basic (Basic), were transfected into SHSY-5Y cells that were subsequently treated with either 100 nM mithramycin A or water control. *D*, Reverse transcriptase-PCR analysis of *KCNQ2*, *KCNQ3*, and *SCN2A* mRNA levels in control and mithramycin A-treated cultures of rat dorsal root ganglia cells. Levels are normalized to *U6* gene (mean ± SEM; $n = 3$; *$p < 0.05$). *E*, Antibodies to Kv7.2 and β-actin were used to analyze respective protein levels in extracts from control or mithramycin A (Mith, 100 nM)-treated SHSY-5Y cells.

deactivating tail current at −60 mV sensitive to specific M channel blocker XE991 (3 μM) applied at the end of each recording. M-current density was significantly larger in Sp1-transfected compared with control neurons (Fig. 3 *B,C*) (1.54 ± 0.34 pA/pF, $n = 8$ compared with 0.91 ± 0.12 pA/pF, $p < 0.05$). Consistently, the Kv7.2 immunoreactivity was greater in Sp1-transfected than in control neurons (Fig. 3 *A,D*) (relative pixel intensity of 9.53 ± 2.15, $n = 4$ compared with 5.49 ± 0.85, $n = 3$, $p < 0.05$). Together, these data implicate an important role for Sp1 in promoting both *KCNQ2* and *KCNQ3* gene expression and enhancing levels of the M-current in neurons.

KCNQ genes are regulated by REST
Bioinformatic analysis of the second DNase I hypersensitive site (Fig. 1*A*, HSS2) in the *KCNQ2* gene identified a putative RE1 binding site for REST. The sequence for this site is conserved between multiple mammalian species (Fig. 1*C*), suggesting that it is functionally important. REST was first identified as a repressor of neuronal gene expression (Chong et al., 1995; Schoenherr and Anderson, 1995) but also has roles in heart, blood vessels, and epithelial cells (Ooi and Wood, 2007). We used a gel mobility shift assay to determine whether the RE1 site identified in the *KCNQ2* gene can recruit REST protein. Incubation of a radioactive, RE1-containing, *SCN2A* promoter region with nuclear extracts resulted in DNA–protein complexes (Fig. 4*A*, lane 1). One of these (indicated with an arrow on Fig. 4*A*) was competed by an RE1 sequence from the *CHRM4* gene but not by an unrelated

sequence (Fig. 4*A*, lanes 2, 3). Furthermore, an anti-REST antibody produced an additional shift of this complex, indicating the presence of REST protein (Fig. 4*A*, lane 4). Oligonucleotides containing RE1 sequences from human, dog, mouse, and rat were all able to compete for REST binding, indicating that the *KCNQ2* gene contains an evolutionary conserved, functional RE1 site (Fig. 4*A*, lanes 5–12).

To determine whether RE1 sites are present in other *KCNQ* genes, we used our position-specific scoring matrix (Johnson et al., 2006) to scan each of the five *KCNQ* genes for the best match to an RE1. High-scoring RE1s were identified in *KCNQ2*, *KCNQ3*, and *KCNQ5*, whereas the closest sequences in *KCNQ1* and *KCNQ4* to an RE1 site had low scores with our matrix, suggesting they would not bind REST. We tested each of these potential RE1 sequences in a gel mobility shift assay to determine which would bind REST with high affinity (Fig. 4*B*). The RE1 sequences from *KCNQ2*, *KCNQ3*, and *KCNQ5* were each able to compete for REST binding, whereas the sequences in *KCNQ1* and *KCNQ4* could not (Fig. 4*B*). To determine whether REST could regulate the *KCNQ2* and *KCNQ3* promoters, we cloned the RE1 sequences upstream of the luciferase reporter constructs (Fig. 2*A*) and transfected them into two cell lines, HEK293, which express robust levels of full-length REST natively, and SHSY-5Y, which express low levels of REST as well as a truncated version of REST resulting from an alternative splice variant (our unpublished observations). Inclusion of the RE1 site resulted in robust repression of luciferase activity for both *KCNQ2* and *KCNQ3* promoters in HEK293 (Fig. 4*C*), indicating that recruitment of REST to the RE1 site represses *KCNQ2* and *KCNQ3* expression. Consistent with the observation that SHSY-5Y cells express less REST protein, inclusion of the RE1 site resulted in only modest repression of luciferase activity for the *KCNQ3* promoter in SHSY-5Y, whereas the *KCNQ2* promoter did not show a significant reduction in activity (Fig. 4*D*). REST is normally expressed in high levels in non-neuronal cells and only at low levels in neurons (Sun et al., 2005; Olguín et al., 2006). Our luciferase data suggest that the absence or the low levels of REST in neurons may be important for permitting *KCNQ2* and *KCNQ3* expression. However, after epileptic seizures, global ischemia, or neuropathic injury, conditions characterized by periods of sustained neuronal hyperactivity, REST expression was seen in neurons (Palm et al., 1998; Calderone et al., 2003; Uchida et al., 2010). To determine whether such increased REST expression in neurons may affect *KCNQ2* and *KCNQ3* expression, we infected cultured DRG neurons with either GFP alone (control) or REST and GFP-expressing adenovirus (Ad) particles. Infection of DRG neurons with control adenovirus had no effect on *KCNQ2* mRNA, whereas infection with adenovirus driving REST expression resulted in a significant reduction of *KCNQ2* and *KCNQ3* mRNA (Fig. 4*E*).

Overexpression of REST reduces M-current density and changes firing properties of DRG neurons

KCNQ2 and *KCNQ3* encode the two subunits Kv7.2 and Kv7.3, which together form a heterotetrameric potassium channel that is believed to be the most abundant M-channel isoform in the CNS and peripheral nervous system (Delmas and Brown, 2005). I_M is a slowly activating and deactivating potassium current that provides a brake for repetitive action potential (AP) firing (Wang et al., 1998). To determine whether increased neuronal REST expression could downregulate the endogenous M-current, we infected cultured DRG neurons with REST-expressing adenovirus and examined the electrophysiological properties of these neurons. DRG neurons significantly differ in their function and channel expression profile; thus, to restrict our study to a more homogeneous population of neurons, we applied the TRPV1 agonist capsaicin (1 μM) at the end of each recording and only considered those neurons that were TRPV1 positive (nociceptive neurons). Whole-cell currents were measured from small (whole-cell capacitance of 23.7 $\pm$ 2.8 pF, $n = 28$) DRG neurons using a standard voltage protocol for M-current (Fig. 5A, inset). In control cells infected with adenoviral construct coding for GFP alone, the hyperpolarizing test pulse resulted in a slowly deactivating whole-cell current, characteristic of M-current, which was sensitive to the specific M-channel blocker XE991 (3 μM; 62 $\pm$ 4% inhibition of deactivation current, $n = 8$). In neurons infected with AdREST, I_M was dramatically reduced to 14% of the value of control Ad neurons ($p < 0.01$, $n = 9$ for GFP and $n = 11$ for GFP + REST-expressing neurons) (Fig. 5C). We also compared responses of virally infected neurons with 1 μM of the TRPV1 agonist capsaicin (measured as the inward current at -60 mV). Capsaicin responses were unaltered by REST overexpression (control, -21.8 ± 8.9 pA/pF, $n = 9$; REST, -26.6 ± 7.5 pA/pF, $n = 11$; ANOVA, $p = NS$) (data not shown), suggesting that the effect of REST was specific to its target genes. To further confirm that viral infection did not affect I_M nonspecifically, whole-cell currents were measured from non-infected neurons from AdREST-exposed neuronal cultures. In these cells, I_M was not significantly different from Ad controls (2.21 $\pm$ 0.26 vs 2.37 $\pm$ 0.48 pA/pF, $n = 8$; $p < 0.05$) but was significantly larger than REST-infected cells (0.31 $\pm$ 0.10 pA/pF, $n = 8$; ANOVA, $p < 0.01$) (Fig. 5C). Thus, increasing REST expression (Fig. 5A, Ad-REST) resulted in almost a complete absence of the M-current when compared with uninfected neurons from the same dish [Fig. 5A, AdREST (non-infected)] or neurons infected with a control adenovirus (Fig. 5A, Ad).

M-current is known to contribute to the resting membrane potential and acts as a brake on AP firing of DRG neurons (Passmore et al., 2003; Linley et al., 2008). We therefore tested whether increasing REST expression affected the AP firing properties and the resting membrane potential of DRG neurons. Using whole-cell current clamp, the membrane potential was adjusted to -65
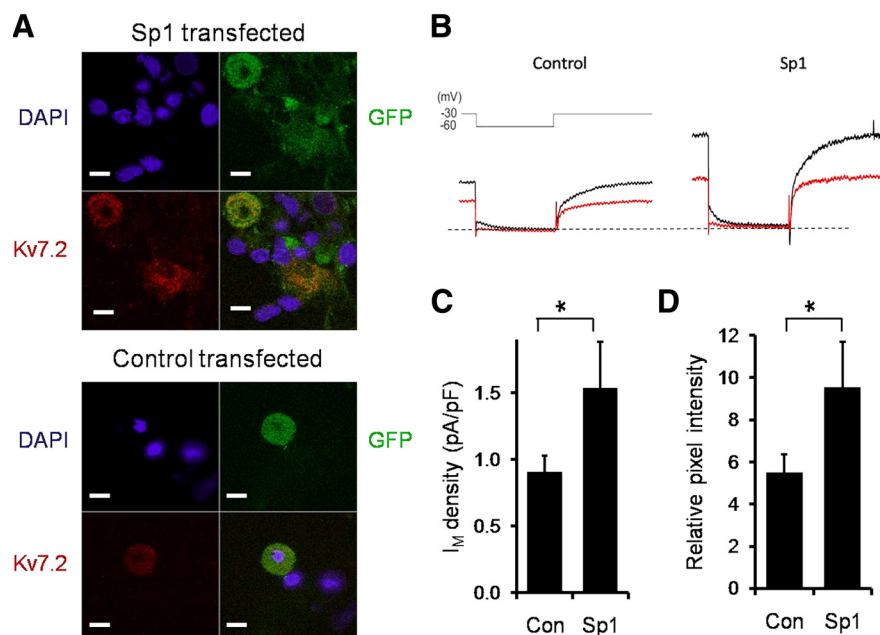
Figure 3. Sp1 enhances Kv7.2 expression and M-current density. *A*, Fluorescent images of DRG neurons transfected with a GFP-expressing plasmid and an Sp1-expressing (top) or control (bottom) plasmid. Transfected cells were identified by GFP expression (green). Kv7.2 levels were analyzed using an anti-Kv7.2 antibody and Alexa Fluor-555-conjugated secondary antibody (red). Nuclei are counterstained with DAPI (blue). Scale bars, 10 μm. *B*, Whole cell voltage-clamp traces obtained by 500 ms voltage pulses from -30 to -60 mV as indicated by the voltage pulse protocol (inset). Black trace represents steady state basal current; red trace represents current in the presence of the specific M-channel blocker XE991 (3μM). Dotted line indicates zero current. *C*, Bars show pooled current density data determined from the DRG neurons transfected with an Sp1-expressing (Sp1; $n = 8$) or control (Con; $n = 7$) plasmid. Magnitude of M-current was calculated as the XE991-sensitive deactivation current when stepping from -30 to -60 mV and normalized to cell capacitance (I_M density, mean $\pm$ SEM; *$p < 0.05$). *D*, Bars show pixel intensity of Kv7.2 staining in DRG neurons transfected with Sp1-expressing (Sp1; $n = 4$) or control (Con; $n = 3$) plasmid (mean $\pm$ SEM; *$p < 0.05$).

mV by injection of current. Injection of $+400$ pA produced one AP in the majority (seven of nine) of Ad-infected cells and two action potentials in the remainder (two of nine) of cells with a similar pattern of firing observed in non-infected AdREST cells (five of six cells firing one AP). In contrast in REST overexpressing cells, 6 of 11 cells fired multiple action potentials (mean AP number of 6.5), which did not show accommodation (Fig. 5B), indicating that the brake on neuronal firing had been removed. REST-overexpressing neurons also were significantly depolarized compared with controls (Fig. 5D: AdREST, -60.5 ± 2.4 mV; Ad, -67.6 ± 2.6 mV; Non-infected, -68.6 ± 2.3 mV; $n = 8$; ANOVA, $p < 0.01$), consistent with a reduction in M-current in these cells. REST has many gene targets (Bruce et al., 2004; Johnson et al., 2009) in addition to the *KCNQ* genes, and the change in the excitability profile of a DRG neuron after REST overexpression is likely to reflect multiple changes in the expression of ion channels at the neuronal membrane. However, experimental data (Gamper et al., 2006) and modeling (Zaika et al., 2006) suggest that inhibition of M-current alone is sufficient to explain the increase in DRG neuron firing rate observed here. In additional support to this assumption, the selective Kv7 blocker XE991 produced an increase in AP firing when acutely applied to a nontransfected DRG neuron (Fig. 5E), the increase in excitability was similar to that produced by REST overexpression. These data suggest that the regulation of *KCNQ* gene expression levels underlies the increased neuronal excitability resulting from expression of REST.

Although its levels are normally low, neuronal REST expression was shown to increase after extended periods of neuronal overactivity seen in seizures, ischemia, and neuropathic pain (Palm et al., 1998; Calderone et al., 2003; Uchida et al., 2010).
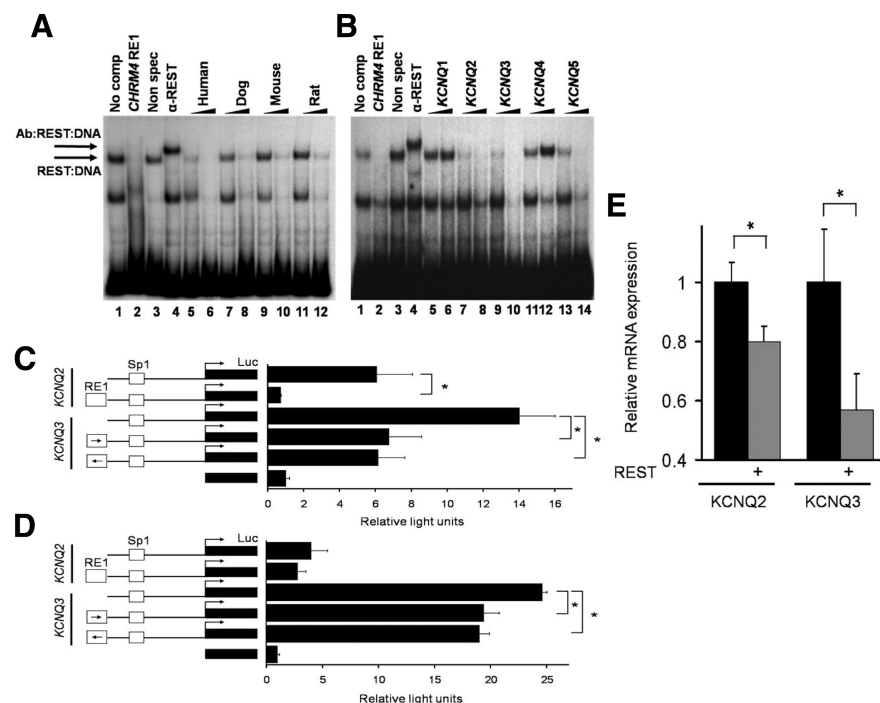
Figure 4. The *KCNQ2* and *KCNQ3* promoters are regulated by the transcriptional repressor REST. ***A***, ***B***, Labeled DNA containing the *SCN2A* RE1 site was incubated with nuclear protein in the presence of no competitor (No comp, lane 1), oligonucleotides containing an RE1 site from the *CHRM4* promoter (*CHRM4*, lane 2), an unrelated sequence (Non spec, lane 3), an anti-REST antibody (α-REST, lane 4), and oligonucleotides containing sequence from the human (lanes 5, 6), dog (lanes 7, 8), mouse (lanes 9, 10), and rat (lanes 11, 12) *KCNQ2* genes (***A***) or oligonucleotides containing potential RE1 sequences from the human *KCNQ1–KCNQ5* genes (lanes 5–14) (***B***). Concentration of competing oligonucleotides were 1 μM (lanes 5, 7, 9, 11, 13) or 10 μM (lanes 2, 3, 6, 8, 10, 12, 14). Positions of REST–DNA and REST–DNA–antibody complexes are identified by arrows on the left. ***C***, ***D***, Luciferase activity driven from *KCNQ2* and *KCNQ3* promoters with or without the RE1 site, transfected into HEK293 cells (***C***) or SHSY-5Y cells (***D***). The arrows on the *KCNQ3* RE1 site represent the direction of the sequence. Expression levels were normalized for transfection efficiency with *Renilla* luciferase and are expressed relative to empty plasmid, pGL3 basic (mean $\pm$ SEM; $n = 3$; *$p < 0.05$). ***E***, Reverse transcriptase-PCR analysis of *KCNQ2* and *KCNQ3* mRNA levels in cultured rat dorsal root ganglia cells infected with control adenovirus or adenovirus expressing full-length REST (+). Data are normalized to *U6* gene and expressed relative to cells infected with control adenovirus (mean $\pm$ SEM; $n = 3$; *$p < 0.05$).

before experiments to remove any acute effects on I_M. Inflammatory conditions dramatically reduced I_M compared with control (0.25 ± 0.04 compared with 0.95 ± 0.16 pA/pF; $p \leq 0.01$; $n = 9$ for control and $n = 5$ for inflammatory), consistent with a downregulation of *KCNQ2* and *KCNQ3*. These data are consistent with a mechanism whereby REST levels are increased in nociceptive neurons in response to inflammatory signals, resulting in an increase in excitability brought about by the reduced expression of *KCNQ2* and *KCNQ3*. Such a mechanism could contribute to a long-term peripheral sensitization of inflamed sensory fibers.

Discussion

In this work, we have identified two common mechanisms that regulate expression of *KCNQ2* and *KCNQ3*. We have identified consensus sequences for the Sp1 transcription factor in each of the promoter regions. These sequences are evolutionarily conserved, and we show that Sp1 directly interacts with the *KCNQ2* promoter *in vitro* and that removal or mutation of these sequences results in reduced activity of the promoters. Furthermore, chemical inhibition of Sp1–DNA interactions result in reduced *KCNQ2* and *KCNQ3* mRNA expression, whereas ectopic Sp1 results in increased M-current and Kv7.2 expression in cultured neurons. Our data implicating Sp1 as an important positive-acting transcription factor that regulates *KCNQ2* expression are consistent with a previous report on the human *KCNQ2* promoter (Xiao et al., 2001). Xiao et al. used a bioinformatic analysis to identify GC boxes in the proximal promoter of *KCNQ2*. Our new data show that these GC box regions are evolutionarily conserved and are also present in the *KCNQ3* promoter. Furthermore, Sp1 does in fact interact with these GC boxes, and this interaction is important for expression of the endogenous *KCNQ2* and *KCNQ3* mRNA in neurons. The physiological relevance of Sp1 regulation of *KCNQ2* and *KCNQ3* remains to be determined, and, although Sp1 is often thought of as a constitutive transcriptional activator, recent evidence suggests that Sp1 activity is important for mediating changes in neuronal gene expression in response to developmental or disease stimuli. For example, induced expression of the reelin gene during neuronal differentiation is dependent on Sp1, and mutation in the binding site for Sp1 within the reelin promoter is sufficient to prevent increased reelin expression (Chen et al., 2007). Sp1 is also important for the increased expression of the damage-induced neuronal endopeptidase (DINE) gene in response to nerve injury (Kiryu-Seo et al., 2008). In this context, Sp1 acts as a platform to which other transcription factors are recruited in response to nerve injury and activate expression of DINE (Kiryu-Seo et al., 2008).

Having shown that REST can regulate the expression of *KCNQ2* and *KCNQ3* in DRG neurons, we sought to determine whether REST levels in DRG neurons may be increased physiologically. We recently reported that inflammatory mediators increase the excitability of nociceptive DRG neurons via inhibition of the M-current mediated through short-term G-protein signaling events (Linley et al., 2008; Liu et al., 2010). Long-term changes in nociceptive neurons have been proposed to be important in the development of chronic pain (for review, see Basbaum et al., 2009; Linley et al., 2010) and are likely to be the result of changes in gene expression. We therefore cultured DRG neurons in the presence of a mix of inflammatory mediators to mimic inflammation (1 μM bradykinin, 1 μM histamine, 1 μM ATP, 1 μM substance P, and 10 μM PAR-2 activating peptide) and analyzed the expression of REST and Kv7.2 specifically in the nociceptive, TRPV1-positive, neurons (Fig. 6*A–C*). In control conditions, REST levels in nociceptive neurons were very low, but they increased significantly in response to inflammatory mediators (Fig. 6*A*,*C*). The levels of TRPV1 in nociceptive neurons did not change significantly (Fig. 6*C*) (TRPV1 is not a predicted target gene of REST). TRPV1-positive neurons cultured in inflammatory conditions showed lower Kv7.2 immunoreactivity than those in control conditions (Fig. 6*B*,*C*). We also analyzed M-current density in capsaicin-responsive neurons. For patch clamp, inflammatory mediators were washed out for at least 2 h

Our data also highlight a second conserved mechanism for the regulation of *KCNQ2* and *KCNQ3*: repression by REST. Both

KCNQ2 and *KCNQ3* contain evolutionarily conserved REST binding sites that can interact with REST *in vitro*, and overexpression of REST in neurons results in reduced *KCNQ2* and *KCNQ3* mRNA and loss of M-current. Interestingly, we also identified a functional binding site for REST in the *KCNQ5* gene, suggesting that *KCNQ5* may also be repressed by REST. Like *KCNQ2* and *KCNQ3*, *KCNQ5* is widely expressed throughout the nervous system, and it has been suggested that Kv7.5 subunits may contribute to the M-current *in vivo* either on their own or in complex with Kv7.3 (Lerche et al., 2000; Schroeder et al., 2000). In a whole genome chromatin immunoprecipitation analysis to look at REST binding in human Jurkat cells, REST is enriched at the RE1 sites for *KCNQ2* and *KCNQ3* (Johnson et al., 2007). In that study, REST was not found to be present at the *KCNQ5* RE1 site we identified, but this may be a cell-specific effect because enrichment of REST at the *KCNQ5* RE1 site has been identified in several other human cell lines (Bruce et al., 2009). Although REST was originally proposed to be important in silencing neuronal-specific genes in non-neuronal cells (Chong et al., 1995; Schoenherr and Anderson, 1995), it clearly has a functional role within the nervous system. REST is expressed at low levels in neurons and regulates some target genes (Wood et al., 2003). Neuronal REST expression is upregulated in response to epileptic insults and cerebral ischemia resulting in reduced expression of brain derived neurotrophic factor (BDNF) and the glutamate receptor subunit gene *GRIA2* and increased expression of the substance P gene *PPT-A* (Palm et al., 1998; Calderone et al., 2003; Garriga-Canut et al., 2006; Spencer et al., 2006). In addition, nuclear levels of REST protein are elevated in neurons in Huntington's disease, leading to reduced BDNF expression (Zuccato et al., 2003, 2007).

Individuals who suffer an epileptic seizure become more susceptible to additional seizure activity (Hauser and Lee, 2002). The molecular mechanisms underlying this long-term change are not known, although, like other features of the brain that show long-lasting changes, e.g., long-term potentiation, they most likely involve changes in gene expression. The expression of several transcription factors has been shown to be increased in response to seizure activity in rodents. These include c-Fos (Morgan et al., 1987; Hiscock et al., 2001), nuclear factor-κB, Ap-1 (Rong and Baudry, 1996), DREAM
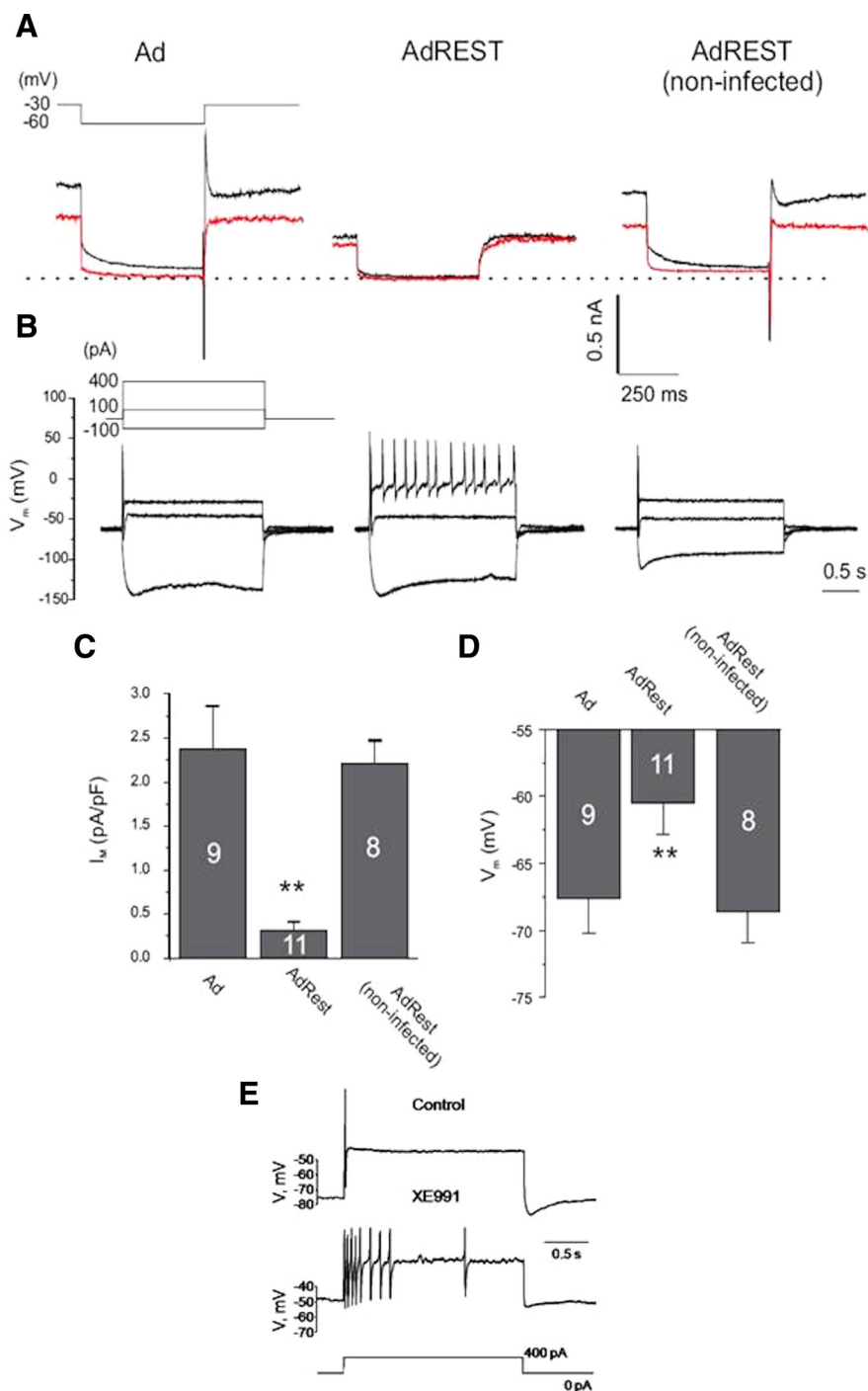

Figure 5. REST expression inhibits M-current and increases excitability in small-diameter DRG neurons. DRG neurons were infected with an adenoviral construct expressing GFP only (Ad) or REST and GFP (AdREST) and assessed functionally using whole-cell patch clamp. In cultures infected with REST and GFP, both green (AdREST) and non-green (AdREST non-infected) cells were tested. Only small-diameter neurons (18 –35 pF) responsive to the TRPV1 agonist capsaicin (1 μM) were investigated. ***A***, Whole-cell voltage-clamp traces obtained by 500 ms voltage pulses from −30 to −60 mV as indicated by the voltage pulse protocol (inset). Black trace represents steady-state basal current; red trace represents current in the presence of the specific M-channel blocker XE991 (3 μM). Dotted line indicates zero current. ***B***, Whole-cell current-clamp traces in which voltage was adjusted to −65 mV by current injection and 4 s square current pulses to different test currents were applied (inset). ***C***, Bars show pooled current density data determined from the experiments shown in ***A***. Magnitude of M-current was calculated as the XE991-sensitive deactivation current when stepping from −30 to −60 mV and normalized to cell capacitance (I_M density). Number of experiments is indicated within bars [**$p < 0.01$ compared to Ad and AdRes+ (noninfected)]. ***D***, Bars show mean resting membrane potentials of GFP, REST, and non-infected neurons. Membrane potential was measured in current-clamp mode (0 pA) [**$p < 0.01$ compared to Ad and AdRest (noninfected)]. ***E***, Exemplary current-clamp experiment showing excitatory effect of 3 μM XE991. Current injections at 400 pA (inset) were applied to whole-cell current-clamped DRG neurons before and during XE991 application. No voltage adjustment was performed.
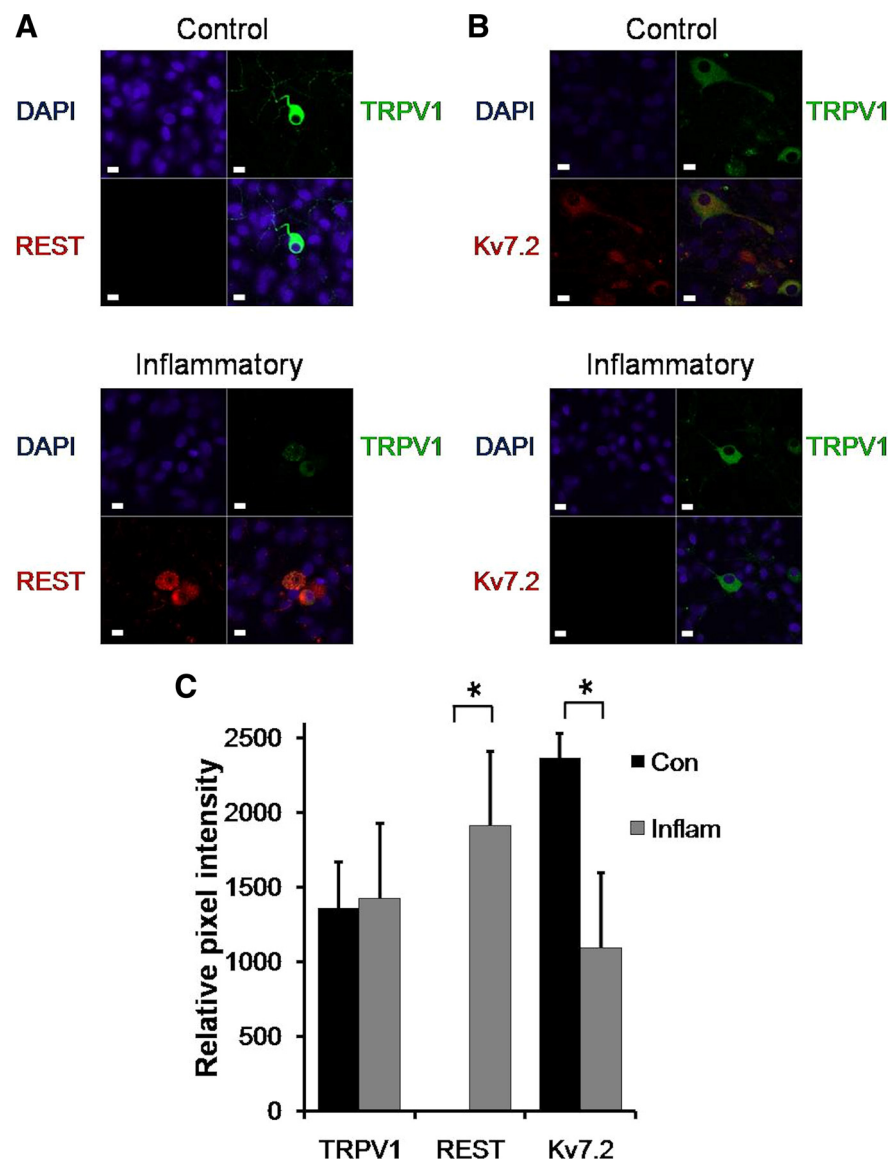
Figure 6. Inflammatory stimuli results in an increase in neuronal REST levels. *A*, *B*, Fluorescent images of DRG neurons cultured in control conditions (top) or exposed to a mixture of inflammatory mediators (bottom) for 48 h. REST (*A*, red), Kv7.2 (*B*, red), and TRPV1 (green) levels were analyzed by anti-REST, anti-Kv7.2, and anti-TRPV1 antibodies and Alexa Fluor-555 (REST and Kv7.2) or Alexa Fluor-488 (TRPV1) conjugated secondary antibodies. Scale bars, 10 μm. *C*, Bars show the pixel intensity of TRPV1, REST, and Kv7.2 staining in TRPV1-positive neurons in control conditions (Con) and after exposure to inflammatory mediators (Inflam) (mean $\pm$ SEM; $n \geq 12$; *$p < 0.05$).

(downstream regulatory element antagonist modulator) (Matsuura et al., 2002), and REST (Palm et al., 1998). One report has also shown that Sp1 levels increase after seizure (Feng et al., 1999), although another found no changes in levels of Sp1 (Rong and Baudry, 1996). Given our findings, an increase in expression of the transcriptional REST would result in a decrease in *KCNQ2* and *KCNQ3* expression, resulting in an increase in the excitability of the affected neurons. REST represses gene expression by recruiting protein complexes that modify the posttranslational marks within chromatin (Ooi and Wood, 2007). Thus, any changes induced on REST recruitment have the potential to be stably maintained even if REST expression is subsequently reduced, providing a prospective mechanism for long-term changes in gene expression levels. In addition to repressing transcription by modifying chromatin, REST has also been shown to interact with Sp1 and inhibit the ability of Sp1 to enhance transcription (Plaisance et al., 2005). Such a model is consistent with our data in which the ability of REST to repress *KCNQ2* and *KCNQ3* appears to be dominant over the ability of Sp1 to activate these genes and suggests that interaction between REST and Sp1 may be important for regulating the expression of many neuronal-specific genes. Consistent with such a hypothesis is the observation that, in addition to *KCNQ2* and *KCNQ3*, many REST-regulated genes are also known to be regulated by Sp1. For example, *Chrm4* (Wood et al., 1996), *Nmdar1* (Bai et al., 1998), *Gria2* (Myers et al., 1998), synatophysin (Lietz et al., 2003), and *Bsx* (Park et al., 2007) genes are all regulated by both REST and Sp1, whereas regulation of the μ-opioid receptor gene involves an interaction with REST and the Sp1 family member Sp3 (Kim et al., 2006).

Recently, much interest has been placed on Kv7 channels as a potential target for analgesics in pain (Passmore et al., 2003; Linley et al., 2008; Brown and Passmore, 2009; Wickenden and McNaughton-Smith, 2009). Activators of Kv7 channels had an analgesic effect in a hindpaw model of chronic pain (Passmore et al., 2003), inhibited ectopic firing of axotomized sensory fibers (Roza and Lopez-Garcia, 2008), and reduced behavior associated with visceral pain (Hirano et al., 2007). Furthermore, pharmacological inhibition of M-channels at peripheral nerve terminals by acute injection of M-channel blocker XE991 produced spontaneous pain (Linley et al., 2008; Liu et al., 2010). Our data suggest that REST expression increases whereas Kv7.2 expression and M-current density decreases in DRG neurons under chronic inflammatory treatment. All these findings suggest that transcriptional downregulation of M-channel expression in nociceptive pathways may result in painful phenotype and contribute to chronic pain conditions. Moreover, peripheral axotomization in a rat model for neuropathic pain resulted in changes in the gene expression profile of DRGs (Xiao et al., 2002). Levels of neither *KCNQ* nor *REST* genes were reported in this study, but two well known REST target genes, chromagranin A and *SNAP25* (synaptosomal-associated protein 25), showed reduced expression in axotomized DRGs (Xiao et al., 2002). Recently, expression of REST was shown to be upregulated after neuropathic injury (Uchida et al., 2010), thus it is tempting to speculate that the *KCNQ*–REST pathway discovered here may contribute to neuropathic pain pathophysiology.

Here we identify two transcription factors, Sp1 and REST, that are important for regulating expression of the potassium channel genes *KCNQ2* and *KCNQ3*. Regulation of *KCNQ2* and *KCNQ3* by these transcription factors may be important for long-term changes in expression levels in response to epileptic seizure activity and during chronic pain syndromes.

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