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Ian Wood, University of Leeds

Mariusz Mucha, University of Leicester

Lezanne Ooi,

John Linley, University of Leeds

Pawel Mordaka, University of Leeds

Carine Dalle, Pfizer Global Research and Development

Brian Robertson, University of Leeds

Nikita Gamper, Uiversity of Leeds

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Transcriptional control of *KCNQ* channel genes and the regulation of neuronal excitability.

Abbreviated: *KCNQ* gene regulation

Mariusz Mucha[1, 2, 4], Lezanne Ooi[1,4], John E. Linley[1], Pawel Mordaka[1], Carine Dalle[1,3], Brian Robertson[1], Nikita Gamper[1] and Ian C. Wood[1].

[1]Institute of Membrane and Systems Biology, Faculty of Biological Sciences, University of Leeds, Leeds, LS2 9JT. [2]Present address: Department of Cell Physiology and Pharmacology, Faculty of Medicine and Biological Sciences, University of Leicester, Leicester LE1 9HN. [3]Present address: Pain Therapeutics, Pfizer Global Research and Development, Sandwich, CT13 9NJ.

Corresponding author: Dr Ian C. Wood, [1]Institute of Membrane and Systems Biology, Faculty of Biological Sciences, University of Leeds, Leeds, LS2 9JT. Email i.c.wood@leeds.ac.uk

[4]These authors contributed equally.

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Abstract.

Regulation of the resting membrane potential and the repolarisation of neurons are important in regulating neuronal excitability. The potassium channel subunits Kv7.2 and Kv7.3 play a key role in stabilising 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* while the transcriptional repressor, REST, represses expression of both of these genes. Further, 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 repolarisation of neurons are important in regulating neuronal excitability. One ionic current which plays a key role in stabilising neuronal activity is the M-current, 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 *KCNQ1-5,* each of which encodes an individual potassium channel subunit Kv7.1-7.5 (Jentsch, 2000; Robbins, 2001).

Of the five KCNQ gene family members, *KCNQ2* and *KCNQ3* are particularly important for regulating neuronal activity as these subunits are expressed widely throughout the central nervous system with expression patterns that almost entirely overlap (Tinel et al., 1998; Yang et

al., 1998). Kv7.2 and Kv7.3 form functional heteromultimers which are believed to represent a major M-channel isoform in central and peripheral nervous systems (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 (BFNC) 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 depolarisation 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) as 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 the Repressor Element 1-Silencing Transcription factor (REST) 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 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 ischaemia (Calderone et al., 2003) and in a model of neuropathc 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 Dulbecco's modified Eagle's medium/F12 supplemented with 10% foetal calf serum (PAA Laboratories), 6 g/l penicillin, 10 g/l streptomycin, and 2 mM L-glutamine at 37°C and 5% CO_2. Dorsal root ganglia (DRG) neurons were cultured as previously described (Crozier et al., 2007; Linley et al., 2008; Liu et al., 2010) ; briefly, ganglia were extracted from 7-10 day old rats from all spinal levels. Ganglia were enzymatically dissociated in Hanks balanced salt solution supplemented with collagenase type 1A (1.5 mg/ml) and dispase (15 mg/ml, Gibco, UK) at 37ºC for 15-20 minutes. Cells were then mechanically triturated, washed twice by centrifugation (800 rpm for 5 minutes), 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 days in a humidified incubator (37ºC, 5% CO2) in DMEM supplemented with GlutaMAX I (Gibco, UK), 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 hr: 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 was replaced with fresh media that did not contain inflammatory mediators for at least 2 hr prior to experiments, to remove any acute effects of inflammatory mediators on the M-current.

DNaseI hypersensitivity assay

SHSY-5Y cells were washed twice with ice-cold PBS, resuspended in DNaseI buffer (10 mM Tris (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 3 mM $CaCl_2$, 1 mM DTT, 0.1% Nonidet P40, 8% glycerol) and incubated on ice for ten minutes. Cells were counted and transferred to a Dounce homogeniser and lysed with 15 strokes of a type B pestle. Nuclei were divided into independent samples (~$1.6x10^5$ per reaction) and treated with 0, 1, 3, 5, and 10 U of DNaseI (Sigma) at 37°C for five minutes. Reactions were stopped by addition of equal volume of Stop/Lysis buffer (20 mM EDTA, 1% SDS, 0.1 mg/ml proteinase K) and incubated at 55 °C overnight. DNA was

extracted several times with phenol/chloroform, once with chloroform and ethanol-precipitated. DNA was resuspended in water and the concentration was measured by spectrophotometry. Ten micrograms of each sample was digested overnight with the appropriate restriction enzymes in a final volume of 30 µl and electrophoresed through 1xTAE, 0.6% (w/v) agarose gel at 0.5 V/cm. DNA was transferred to Hybond XL membranes (Amersham) using alkaline capillary transfer. DNA fragments were radiolabelled using Prime-It kit, (Stratagene) and [α-^{32}P]dATP. Hybridisation to Hybond XL membranes was performed as per the manufacturer's instructions.

Gel mobility shift assay

Nuclear extracts were prepared from SHSY-5Y and JTC-19 cells using the method described in (Andrews and Faller, 1991). DNA probes were radioactively labelled by Klenow (New England Biolabs) fill in of a restriction enzyme generated overhang using [α-^{32}P]dATP (Amersham) and incubated with protein extracts before electrophoresis through a 4% nondenaturing acrylamide gel. Specificity of DNA-protein binding was assessed by including 10 pmols of double-stranded oligonucleotides, or 1 µl anti-Sp1 (2 mg/ml, H-255 Santa Cruz Biotechnology) or 1 µl 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‘ Non Spec, 5‘-AATTCCCGCGAGGGGCGCCTAGTCC-CCATG-3‘ *CHRM4* RE1, 5’-GTACGGAGCTGTCCGAGGTGCTGAATCTGCCT-3’, Human *KCNQ2* RE1 5’-GATCCTGGTCAGGACCATGGCCAGCACCCC, Dog *KCNQ2* RE1 5’-GATC-TGCTGCTCAGGACCACGGCCAGCGCCTC, Mouse *KCNQ2* RE1 5’-GATCTTGAGTCCAGGA-CCATGGTCAGCACCAC, Rat *KCNQ2* RE1 5’-GATCTTTGCGTCCAGGACCATGGTCAGCGC-CAC, *KCNQ1* RE1 5’-GATCCGGGCCTGCACCCAGGACAGGGCC, *KCNQ3* RE1 5’-GATCA-GGCTCAGGACCTAGGACAGTTCC, *KCNQ4* 5’-GATCCTGTCCAGGACCTGAGCCAGGGCT, *KCNQ5* RE1 5’-GATCCTTGTCAGCACCTAGGACAGAGAT

Luciferase assay

Fragments of human *KCNQ2* gene corresponding to -64/+179 and -416 /+151 regions were amplified by PCR with primers: Sp1s 5'-AAAAGATCTCATGGTGCCTGGCGGGAGG and Sp1a 5'-AAGAGCTCAACGCGGGGC**GG**GGCGAG, and AscIs 5'-GGCGCGCCTCGGGCTCA-GGCTCAG 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 $Sp1s^{Mut}$ 5'- AAGAGCTCAACGCGGGGC**A-A**GGCGAG containing a substitution of GG with AA (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 pGL3 plasmid and 1 ng pRL CMV (*Renilla* luciferase; Promega) using 4 µl of Lipofectamine (Invitrogen). Each transfection was performed in triplicate for each experiment. Cells were harvested 48 hr post 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 RT-PCR

Total RNA was extracted from dorsal root ganglia cells using Tri-reagent (Sigma). DNase-treated (Ambion) RNA was reverse transcribed by using M-MLV 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 data sets. Significance was tested using Student's *t*-test. Primers used were: *U6*s 5'-CTCGCTTCGGCAGCACA, *U6*a 5'-AACGCTTCACGAATTTGCGT, *KCNQ2*s 5'-GTGGTCTACGCTCACAGCAA, *KCNQ2*a 5'-AGGGTAAACGTCGCTGCTAA, *KCNQ3*s 5'-GCCCACAGTCCTGCCCATCTTGAC, *KCNQ3*a 5'-CCGTTCCAGTTCCTCGTGGTTGACG,. RESTs 5'-CGAACTCACACAGGAGAACG. *REST*a 5'-GAGGCCACATAATTGCACTG, SCN2As 5'-GCTGCATGTCTCTCTTGCTG, *SCN2A*a 5'-GGACCGATTTGCTTCACTTC.

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 and transferred to Hybond C-Extra (Amersham) and screened with 1:1000 dilution of anti-Kv7.2 (Alomone) antibody and a 1:1000 dilution of horse radish peroxidase conjugated anti-rabbit antibody. Antibody staining was visualised using an ECL plus detection kit (Amersham). For immunocytochemistry, cells were fixed and permeabilised in acetone:methanol 1:1 for 5 min on ice, followed by 0.1M PBS Triton X 0.1% for 5 min at room temperature and blocked with 10% donkey serum in PBS for 2 hr at room temp. Primary antibody incubation was carried out in 10% donkey serum 0.4 M PBS overnight at 4°C at 1:100 dilution for rabbit anti-REST (H290, Santa Cruz), 1:1000 rabbit anti-Kv7.2 (kind gift from Dr Mark Shapiro) or 1:1000 guinea pig anti-TRPV1 (Neuromics) and 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 hr at room temperature. Coverslips were mounted in Vectashield with DAPI and imaged using a Zeiss LSM510 inverted confocal. Among comparisons between cell conditions, coverslips were mounted on the same slide and imaged with the same laser settings. Image analysis was carried out using ImageJ software

Plasmid and adenoviral delivery

The coding sequence for Sp1 was amplified using the primers 5'-GCGAATTCAATGAGCGAC-CAAGATCACTCA 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 days after transfection. The green fluorescent protein plasmid pmaxGFP (Amaxa) was co-transfected 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 hr prior to harvesting and RNA or electrophysiological analysis.

Electrophysiology

In patch clamp experiments the standard bath solution contained (in mM) NaCl (160), KCl (2.5), $CaCl_2$ (2), $MgCl_2$ (1), HEPES (10), pH adjusted to 7.4 with NaOH. For perforated patch experiments the patch pipette contained K-acetate (90), KCl (20), $CaCl_2$ (1), $MgCl_2$ (3), EGTA (3), HEPES (40), amphotericin B (400 µg/ml), pH adjusted to 7.4 with NaOH. Currents were amplified using an EPC-10 patch clamp amplifier (HEKA) and recorded using Patchmaster software (v2.2. 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 mV to -60 mV and was calculated as 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 minimise any contribution from other K^+ currents and capacitance artefacts. XE991 was obtained from Tocris. All analysis of patch clamp data was conducted using Fitmaster software (v2.11, HEKA).

Statistics

All data are given as mean ± sem. Differences between groups were assessed by Student's *t* test or 1-way ANOVA. The differences were considered significant at $P \leq 0.05$.

Results

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

In order 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 previously been shown 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 if 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 radiolabelled 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) which could be competed by excess of an unlabelled 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 this complex is the result of non-specific 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 radiolabelled probe was also competed by an excess of unlabelled 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 which 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 if 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/+151bp relative to the transcription start site into a luciferase reporter vector such that luciferase expression is driven by *KCNQ2* promoter sequences. This 568bp region encompasses the Sp1 site and 137bp 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/+151bp promoter fragment was 6 fold higher than the control plasmid (Fig 2A, compare -416 /+151bp 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/+151bp with -64/+179bp). Introduction of mutations that prevent Sp1 binding (see Methods, Fig 1D) however resulted in loss of over 50% luciferase activity (Fig 2A compare -64/+179 with -64/+179bp $Sp1^{mut}$) 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 though its score was less, suggestive of a lower affinity for Sp1 (Fig S1A). In gel mobility-shift experiments, this second site did indeed bind to Sp1, showing a lower affinity than our originally identified site (Fig S1B). 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 due to low levels of Sp1 recruitment. To determine if 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 1F). To determine if 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 which lacks the conserved Sp1 site. Luciferase activity from the -540/+336 promoter region drove high levels of luciferase expression in SHSY-5Y cells (Fig 2B) which was reduced by approximately 80% when the region containing the Sp1 sites was removed (Fig 2B). 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 wished 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 2C). To determine the role of Sp1 on endogenous gene expression we incubated neurons isolated from dorsal root ganglia (DRG) in the presence of 200 nM of mithramycin A for 48 hours and measured mRNA expression using quantitative PCR. In the presence of mithramycin A *KCNQ2* and *KCNQ3* levels were reduced by 90% (Fig 2D), 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 the effect on *KCNQ* expression was not the result of a global change in gene expression (Fig 2D). Incubation of SHSY-5Y cells with mithramycin A also resulted in a reduction of Kv7.2 protein (Fig 2E). As an alternative approach to determine if 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 3A). In whole cell voltage clamp experiments, M-current (I_M) was measured from a standard square voltage pulse protocol stepping to -60 mV from a holding potential of -30 mV (Fig. 3B Inset) as an amplitude of 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 to control neurons (Fig 3B and C, 1.54 ± 0.34 pA/pF n=8, compared to 0.91 ± 0.12 pA/pF $p<0.05$,). Consistently the Kv7.2 immunoreactivity was greater in Sp1 transfected than in control neurons (Fig 3A and D, relative pixel intensity of 9.53 ± 2.15 n=4, compared to 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 the Repressor Element 1-Silencing Transcription Factor (REST)**

Bioinformatic analysis of the second DNase I hypersensitive site (Fig 1A, HSS2) in the *KCNQ2* gene identified a putative repressor element 1 (RE1) binding site for the Repressor

Element 1-Silencing Transcription factor (REST). The sequence for this site is conserved between multiple mammalian species (Fig 1C), 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 if 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 4A, lane 1). One of these (indicated with an arrow on Fig 4A) was competed by an RE1 sequence from the *CHRM4* gene but not by an unrelated sequence (Fig 4A, lanes 2 and 3). Furthermore an anti-REST antibody produced an additional shift of this complex indicating the presence of REST protein (Fig 4A, 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 4A, lanes 5-12).

To determine if RE1 sites are present in other *KCNQ* genes we used our position specific scoring matrix (Johnson et al., 2006) to scan each of the 5 *KCNQ* genes for the best match to an RE1. High scoring RE1s were identified in *KCNQ2*, *KCNQ3* and *KCNQ5* while 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 4B). The RE1 sequences from *KCNQ2*, *KCNQ3* and *KCNQ5* were each able to compete for REST binding while the sequences in *KCNQ1* and *KCNQ4* could not (Fig 4B). To determine if REST could regulate the *KCNQ2* and *KCNQ3* promoters we cloned the RE1 sequences upstream of the luciferase reporter constructs (Fig 2A) 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 (unpublished observations). Inclusion of the RE1 site resulted in robust repression of luciferase activity for both *KCNQ2* and *KCNQ3* promoters in HEK293 (Fig 4C), 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 while the *KCNQ2* promoter did not show a significant reduction in activity (Fig 4D). REST is normally expressed in high levels in non-neuronal cells and only at low levels in neurons (Sun et al., 2005; Olguin et al., 2006). Our luciferase data suggests that the absence or the low levels of REST in neurons may be important for permitting *KCNQ2* and *KCNQ3* expression. However following epileptic seizures, global ischemia, or neuropathic injury – conditions characterized by periods of sustained neuronal hyperactivity - REST expression in neurons (Palm et al., 1998; Calderone et al., 2003; Uchida et al., 2010). To determine if 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 particles. Infection of DRG neurons with control adenovirus had no effect on *KCNQ2* mRNA whilst infection with adenovirus driving REST expression resulted in a significant reduction of *KCNQ2* and *KCNQ3* mRNA (Fig 4E).

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

KCNQ2 and *KCNQ3* encode two subunits Kv7.2 and Kv7.3 which together form a heterotetrameric potassium channel which is believed to be the most abundant M-channel isoform in the central and peripheral nervous systems (Delmas and Brown, 2005). The M-current (I_M), is a slowly activating and deactivating potassium current that provides a brake for repetitive action potential firing (Wang et al., 1998). To determine if 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, in order to restrict our study to a more homogeneous population of neurons, we applied TRPV1 agonist capsaicin (1 μM) at the end of each recording and only considered those neurons which were TRPV1-positive (nociceptive neurons). Whole cell currents were measured from small (whole cell capacitance of 23.7 ± 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 ± 4% inhibition of deactivation current, n = 8). In neurons infected with Ad REST, 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 to 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 non-specifically, 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 ± 0.26 pA/pF vs. 2.37 ± 0.48 pA/pF, n = 8, $p<0.05$) but was significantly larger than REST infected cells (0.31 ± 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 to uninfected neurons from the same dish (Fig 5A, Ad REST (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 neuronal firing of DRG neurons (Passmore et al., 2003; Linley et al., 2008). We therefore tested whether increasing REST expression affected the action potential (AP) firing properties and the resting membrane potential of DRG neurons. Using whole cell current clamp, the membrane potential was adjusted to -65 mV by injection of current. Injection of +400 pA produced 1 AP in the majority (7/9) of Ad infected cells and 2 action potentials in the remainder (2/9) of cells with a similar pattern of firing observed in non-infected AdREST cells (5/6 cells firing 1 AP). In contrast in REST over-expressing cells, 6/11 cells fired multiple action potentials (mean AP number = 6.5), which did not show accommodation (Fig 5B) indicating that the brake on neuronal firing had been removed. REST over-expressing neurons also were significantly depolarised 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 following REST over-expression is likely to reflect multiple changes in the expression of ion channels at the neuronal membrane. However experimental data (Gamper et al., 2006) and modelling (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 further support to this assumption, the selective Kv7 blocker, XE991 produced an increase in AP firing when acutely applied to a non-transfected DRG neuron (Fig. 5E), the increase in excitability was similar to that produced by REST over-expression. These data suggest that the regulation of *KCNQ* gene expression levels underlies the increased neuronal excitability resulting from expression of REST.

Though its levels are normally low neuronal REST expression was shown to increase following extended periods of neuronal over-activity seen in seizures, ischemia and neuropathic pain (Palm et al., 1998; Calderone et al., 2003; Uchida et al., 2010). 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 inflammatory mediators increase the excitability of nociceptive DRG neurons via inhibition of the M-current mediated through short term G-protein signalling 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 (reviewed in (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 ATO, 1 μM Substance P and 10 μM PAR-2 activating peptide) and analysed the expression of REST and Kv7.2 specifically in the nociceptive, TRPV1 positive, neurons (Fig 6A, B and C). In control conditions REST levels in nociceptive neurons were very low but they increased significantly in response to inflammatory mediators (Fig 6A, C). The levels of TRPV1 in nociceptive neurons did not change significantly (Fig 6C; 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 6B, C). We also analysed M-current density in capsaicin responsive neurons. For patch clamp, inflammatory mediators were

washed out for at least 2 hr prior to experiments to remove any acute effects on I_M. Inflammatory conditions dramatically reduced I_M compared to control (0.25 ± 0.04 compared to 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 sensitisation of inflamed sensory fibres.

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 while 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 which 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 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 upon 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).

Our data also highlight a second conserved mechanism for the regulation of *KCNQ2* and *KCNQ3:* repression by the repressor element 1-silencing transcription factor (REST). Both *KCNQ2* and *KCNQ3* contain evolutionarily conserved REST binding sites which can interact with REST *in vitro* and over expression 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 however 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 ischaemia 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; Zuccato et al., 2007).

Individuals who suffer an epileptic seizure become more susceptible to further 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, eg. 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), NF-κB, Ap-1 (Rong and Baudry, 1996), DREAM (Matsu-ura et al., 2002) and REST (Palm et al., 1998). One report has also shown that Sp1 levels increase after seizure (Feng et al., 1999), though 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 post-translational marks within chromatin (Ooi and Wood, 2007). Thus any changes induced upon 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 while regulation of the μ opiod 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 axotomised sensory fibres (Roza and Lopez-Garcia, 2008) and reduced behaviour 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 while 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 axotomisation 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, however two well known REST target genes, chromagranin A (*CGA*) and synaptosomal associated protein 25 (*SNAP25*) showed reduced expression in axotomised DRGs (Xiao et al., 2002). Recently expression of REST was shown to be upregulated following 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, which 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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Figure legends

Figure 1 DNaseI hypersensitive sites present in the *KCNQ2* gene. A) Top shows a schematic representation of the 5' of the *KCNQ2* gene. Filled boxes represent exons, 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 analysed for DNase I hypersensitive sites. DNA was digested with HindIII (upper panel) or AseI (bottom panel) and the Southern blot was hybridised 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 *KCNQ2* genes. 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. Labelled 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 and $Sp1^{mut}$ 1 μM lane 7, 10 μM lane 8, respectively). Position of complexes containing Sp1 (Sp1:DNA), α-Sp1 (Ab:Sp1:DNA) and resulting from non specific binding (Non spec) are shown on the left. E) A schematic representation of the 5' of the *KCNQ3* gene. Filled boxes represent exons, 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).

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 normalised luciferase values expressed relative to empty vector, pGL3 Basic (mean ± s.e.m. 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 normalised luciferase values expressed relative to empty vector (mean ± s.e.m. 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 which were subsequently treated with either with 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 normalised to *U6* gene (mean ± s.e.m. n=3 *p<0.05). E) Antibodies to Kv7.2 and β-actin were use to analyse respective protein levels in extracts from control or mithramycin A (Mith, 100 nM) treated SHSY-5Y cells.

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 plasmid (bottom). Transfected cells were identified by GFP expression (green). Kv7.2 levels were analysed using an anti-Kv7.2 antibody and Alexa Fluor 555 conjugated secondary antibody (red). Nucleii are counter stained with DAPI (blue). Scale bar 10 μM. B) 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 normalised to cell capacitance (I_M density, mean ± s.e.m. *p<0.05). C) 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 ± s.e.m. *p<0.05).

Figure 4. The *KCNQ2* and *KCNQ3* promoters are regulated by the transcriptional repressor REST.

A, B) Labelled 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 (A) oligonucleotides containing sequence from the human (lanes 5, 6), dog (lanes 7, 8), mouse (lanes 9, 10) and rat (lanes 11, 12) *KCNQ2* genes or (B) oligonucleotides containing potential RE1 sequences from the human, *KCNQ1-KCNQ5* genes (lanes 5 - 14). Concentration of competing oligonucleotides were 1 μM (lanes 5, 7, 9, 11 and 13) or 10 μM (lanes 2, 3, 6, 8, 10, 12 and 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 C) HEK293 cells or D) SHSY-5Y cells. 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 ± s.e.m. 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 ± s.e.m. n=3 *p<0.05).

Figure 5. REST expression inhibits M-current and increases excitability in small diameter DRG neurons.

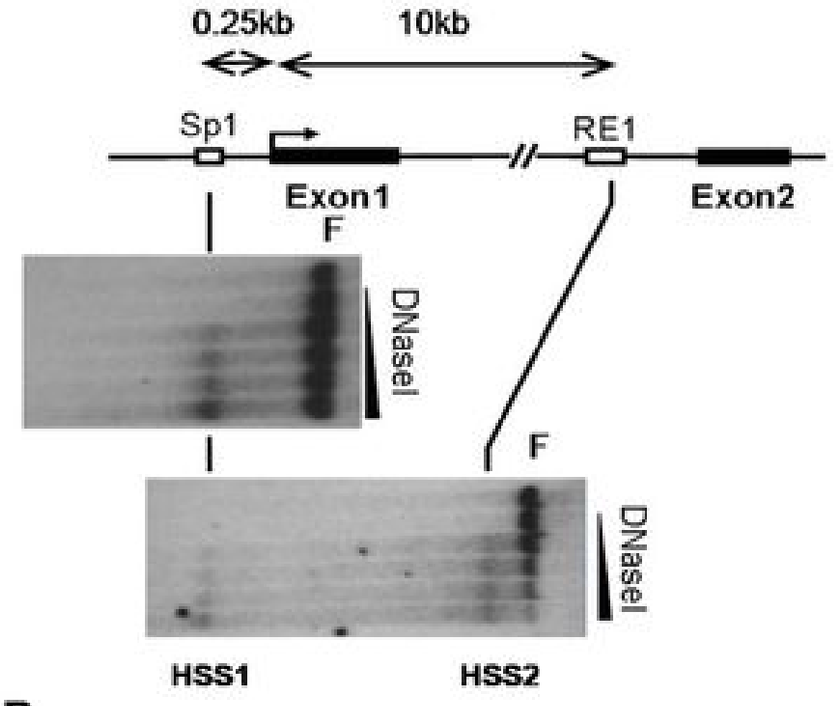
DRG neurons were infected with an adenoviral construct expressing GFP only (Ad) or REST and GFP (AdREST) and assessed functionally using whole cell patch clamp. In cultures infected with REST and GFP both green (AdREST) and non-green (AdREST non-infected) cells were tested. Only small diameter neurons (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 mV 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 normalised to cell capacitance (I_M density). Number of experiments is indicated within bars. D) Bars show mean resting membrane potentials of GFP, REST and non-infected neurons. Membrane potential was measured in current clamp mode (0 pA). E) Exemplary current-clamp experiment showing excitatory effect of 3 μM XE991. 400 pA current injections (inset) were applied to whole-cell current clamped DRG neuron 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 hr. REST (A, red), Kv7.2 (B, red) and TRPV1 (green) levels were analysed 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 represent 10 μm. C) Bars show the pixel intensity of TRPV1, REST and Kv7.2 staining in TRPV1 positive neurons in control conditions and after exposure to inflammatory mediators (mean ± s.e.m. n≥12 *p<0.05).

A

KCNQ2 promoter region



B

KCNQ2 Sp1 region

Consensus	GrGGCrGGGs
Human	GGGGTTAACGCGGGGCGGGGCGAGGCGGCG
Mouse	TAGGGTTAAGGCGGGCGGGGCGGGGCGGCG
Rat	TAGGGTTAAGGCGGGCGTGGGGCGGGGGCG

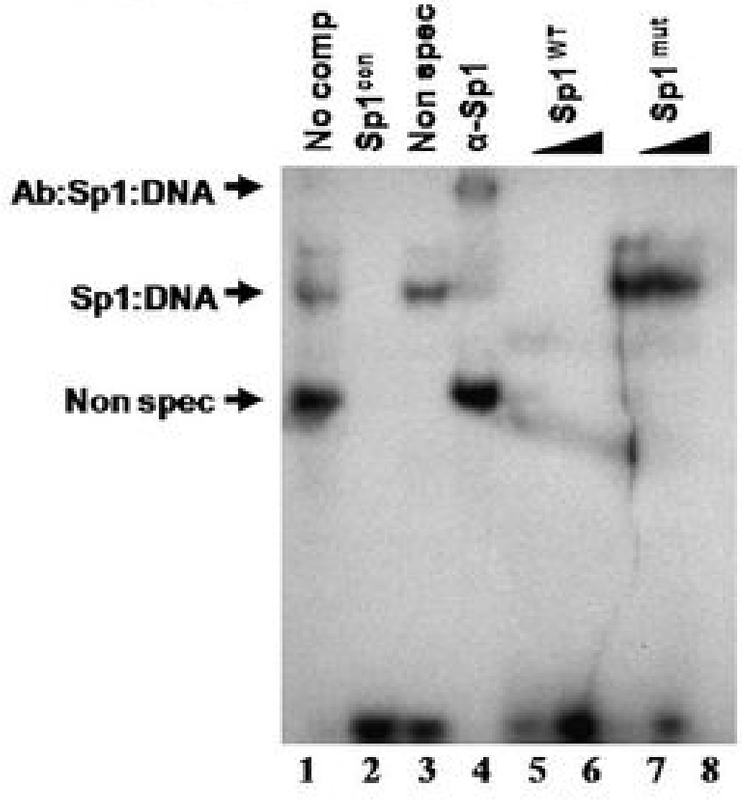
C

KCNQ2 RE1 region

Consensus	nTyAGmrCCnnrGmsAG
Human	GCTCCTGGTCAGGACCATGGCCAGCACCCC
Chimp	GCTCCTGGTCAGGACCATGGCCAGCACCCC
Rhesus	GCTCCTGGTCAGGACCACGGCCAGCA-CCC
Mouse	ACTTGAGTCCAGGACCATGGTCAGCACCAC
Rat	ATTTGCGTCCAGGACCATGGTCAGCGCCAC
Dog	CCTGCTGCTCAGGACCACGGCCAGCGCCTC
Cow	GTTCCTGATCAGGACCACGGCCAGCACCCC
Elephant	GCTCGTGTTCAGCACCAAGGCCAGCGCCAA

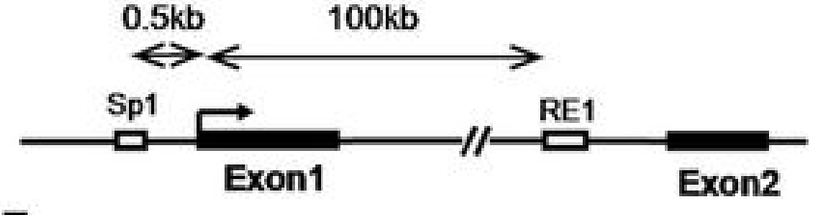
D

KCNQ2 Sp1 region



E

KCNQ3 promoter region



F

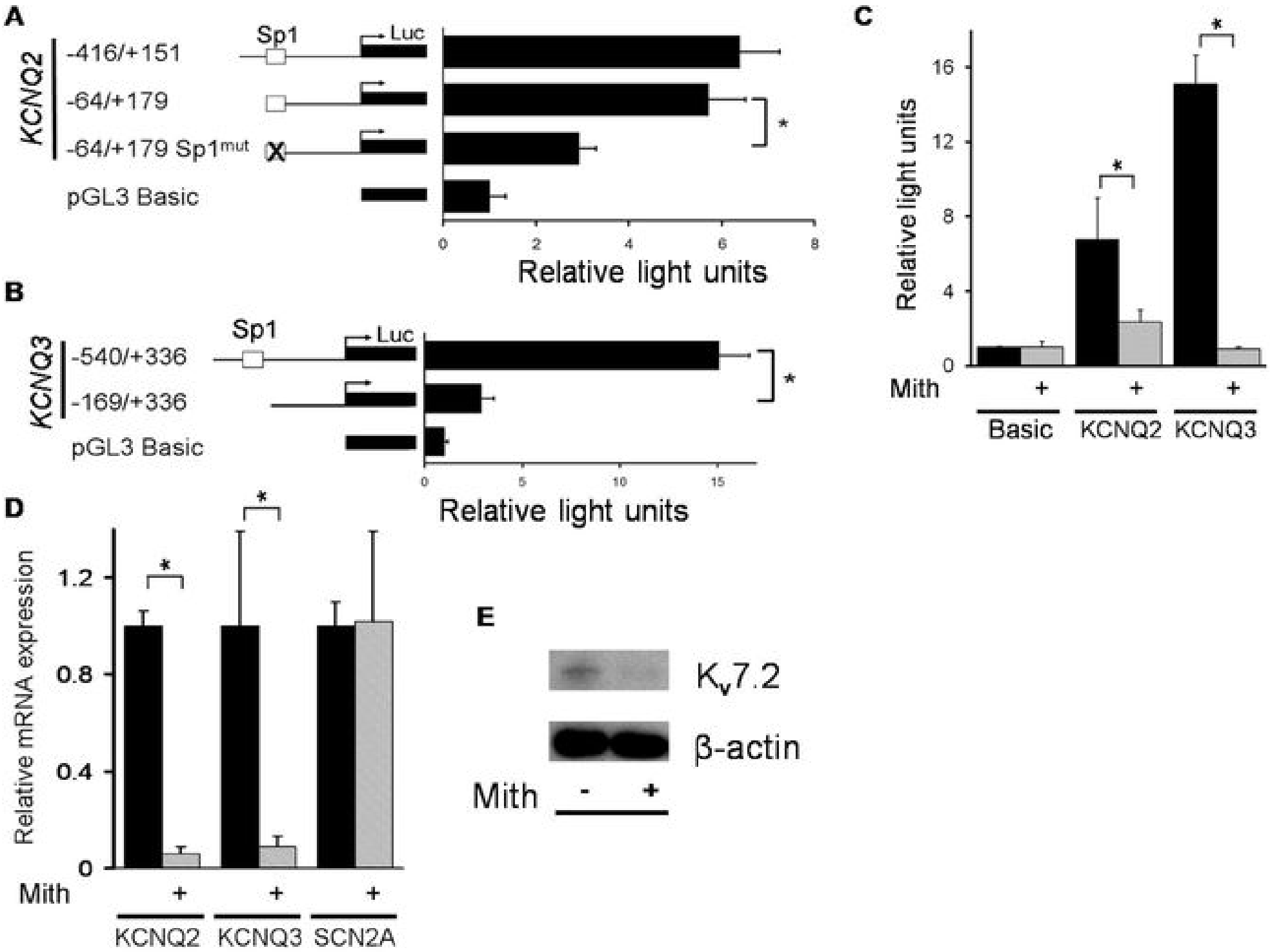
KCNQ3 Sp1 region

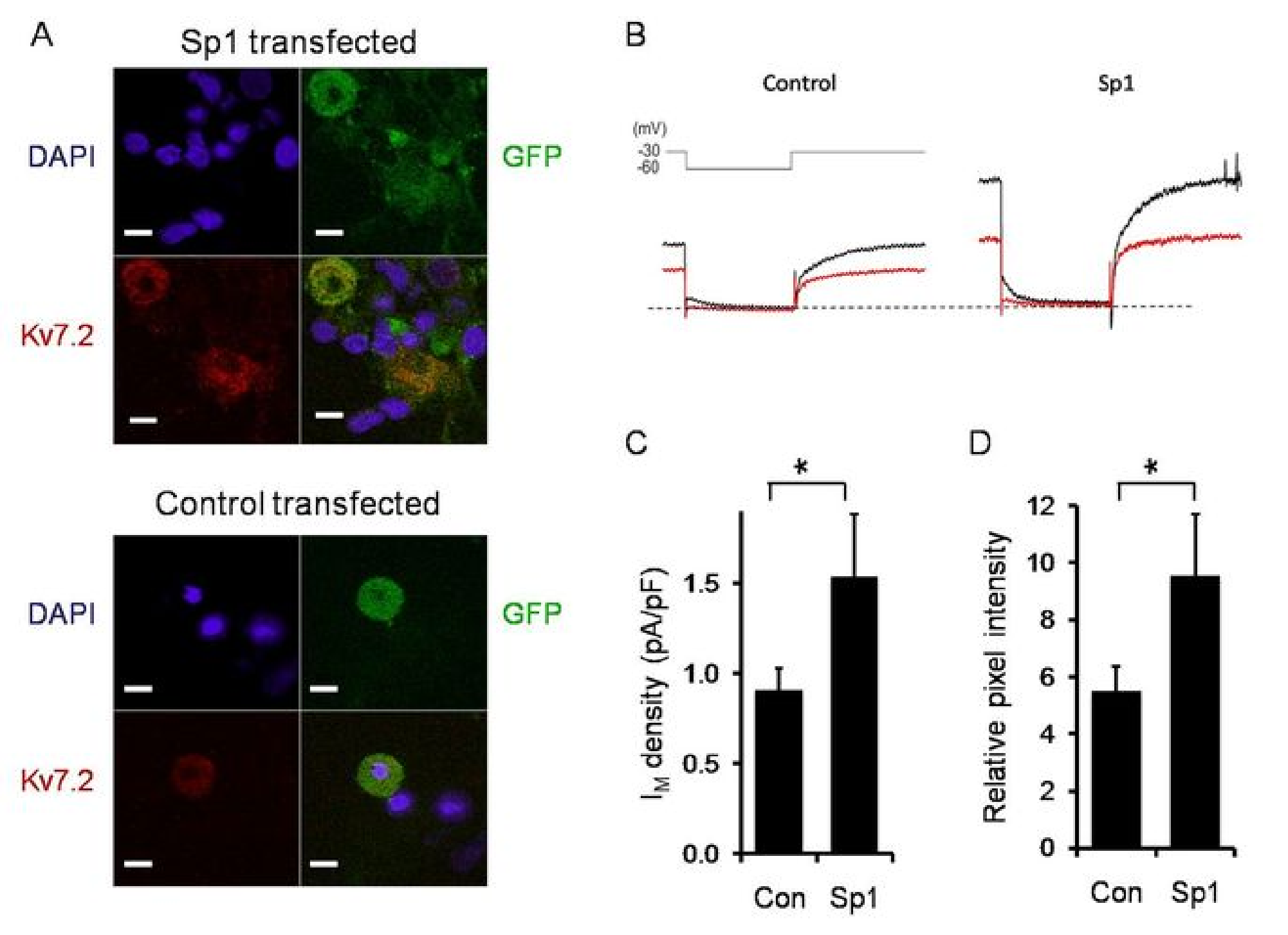
Consensus	GrGGCrGGGs
Human	GGTCGGCGGGAAGAGGCGGGGCGCCGCCGCC
Mouse	GGGCGGCGGGGAGAGGCGGGGCCCCGCCTGG
Rat	GGGCGGCGGGGAGAGGCGTGGCGCTGCCTGG

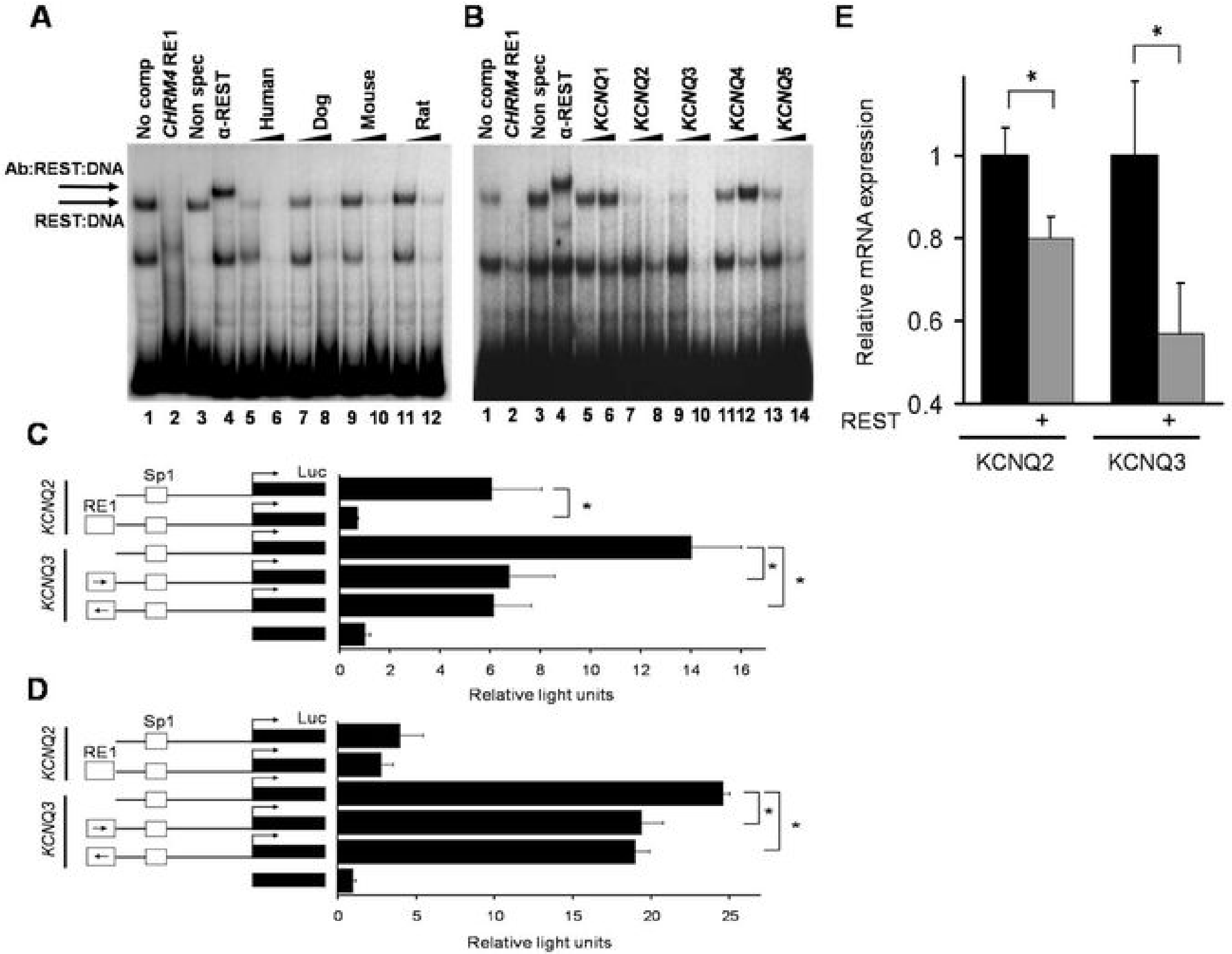
G

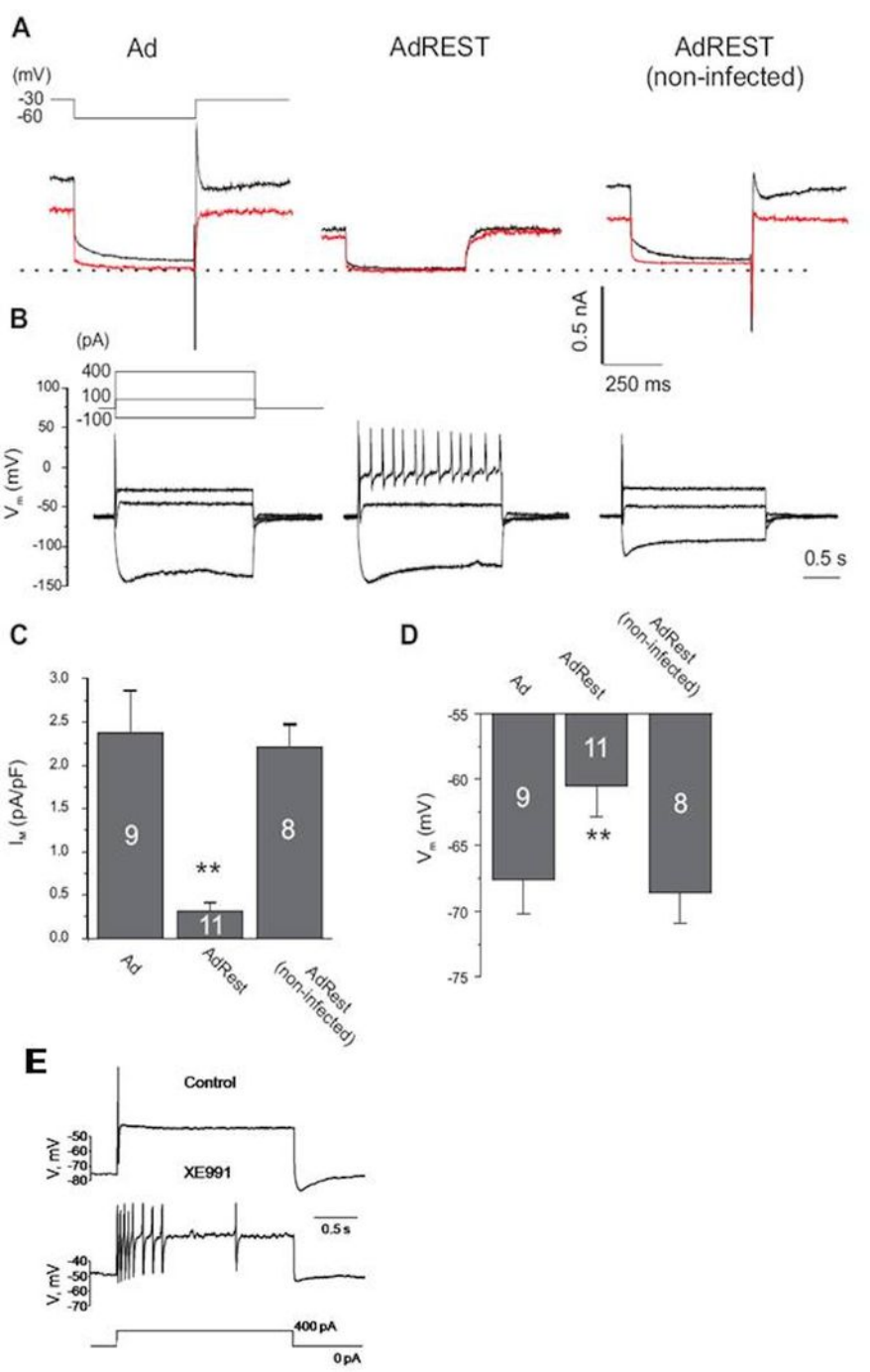
KCNQ3 RE1 region

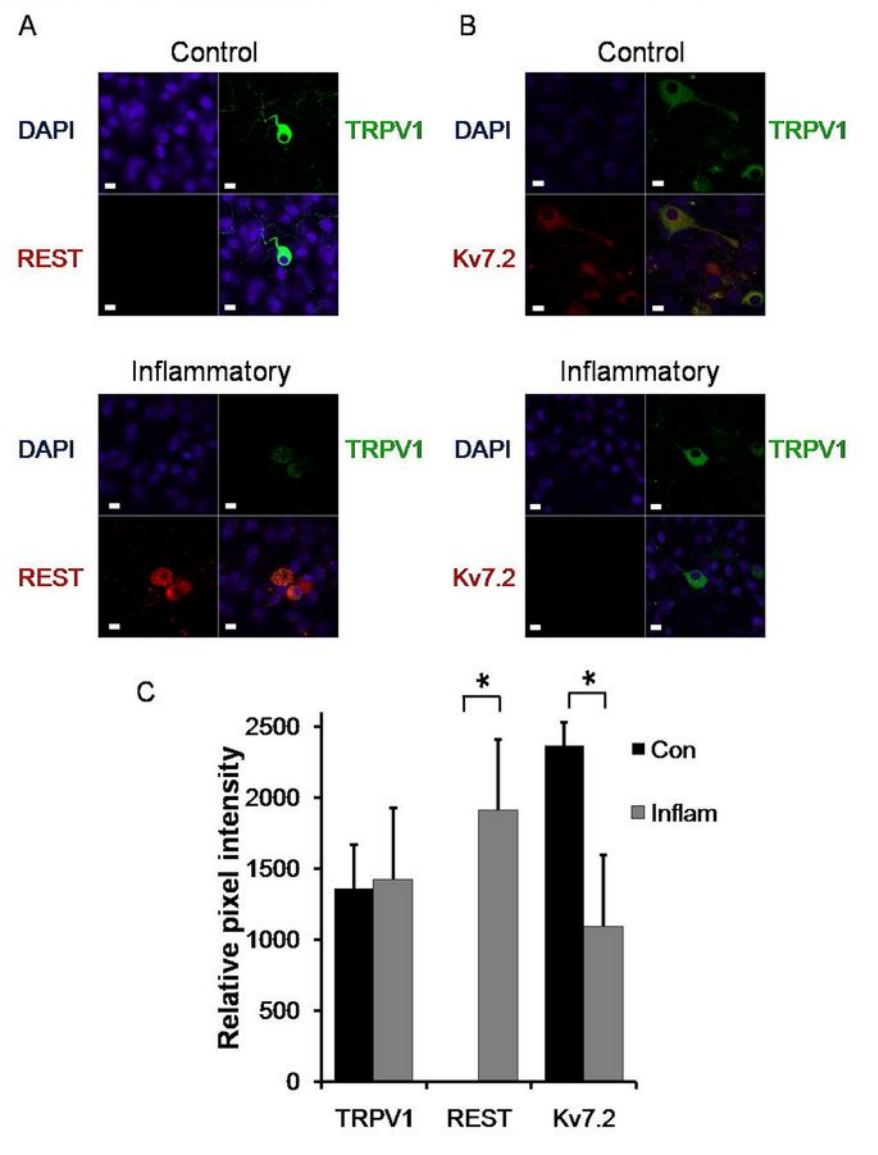
Consensus	nTyAGmrCCnnrGmsAG
Human	TTATAGGCTCAGGACCTAGGACAGTTCCTA
Chimp	TTATAGGCTCAGGACCTAGGACAGTTCCTA
Rhesus	TTACAGGCTCAGGACCTAGGACAGTTCCTA
Mouse	TTGTGTGATCTGGACCAAGGACAGTTCCTC
Rat	TGATGTGGTCAGGACCTGGGACAGTTCCTC
Dog	TTGTGT-GCAGAGCCCTGTATAGGCCTGGT
Cow	CTGTATGTCTAGGACCTAGCACAGGCCTGG
Elephant	ACTACATCTCAGGACCTAGCACAGTCCCTG


A
KCNQ2
-416/+151
-64/+179
-64/+179 Sp1mut
pGL3 Basic
Sp1
Luc
X
*
0
2
4
6
8
Relative light units
B
KCNQ3
-540/+336
-169/+336
pGL3 Basic
Sp1
Luc
*
0
5
10
15
Relative light units
C
Relative light units
16
12
8
4
0
*
*
Mith
+
+
+
Basic
KCNQ2
KCNQ3
D
Relative mRNA expression
1.2
0.8
0.4
0
*
*
Mith
+
+
+
KCNQ2
KCNQ3
SCN2A
E
Kv7.2
β-actin
Mith
-
+


A
Sp1 transfected
DAPI
GFP
Kv7.2
Control transfected
DAPI
GFP
Kv7.2
B
Control
Sp1
(mV)
-30
-60
C
*
I_M density (pA/pF)
1.5
1.0
0.5
0.0
Con
Sp1
D
*
Relative pixel intensity
12
10
8
6
4
2
0
Con
Sp1


A
No comp
CHRM4 RE1
Non spec
α-REST
Human
Dog
Mouse
Rat
Ab:REST:DNA
REST:DNA
1 2 3 4 5 6 7 8 9 10 11 12
B
No comp
CHRM4 RE1
Non spec
α-REST
KCNQ1
KCNQ2
KCNQ3
KCNQ4
KCNQ5
1 2 3 4 5 6 7 8 9 10 11 12 13 14
E
Relative mRNA expression
1
0.8
0.6
0.4
REST
+
+
KCNQ2
KCNQ3
C
KCNQ2
KCNQ3
Sp1
RE1
Luc
0 2 4 6 8 10 12 14 16
Relative light units
D
KCNQ2
KCNQ3
Sp1
RE1
Luc
0 5 10 15 20 25
Relative light units


A
Ad
AdREST
AdREST
(non-infected)
(mV)
-30
-60
0.5 nA
250 ms
B
(pA)
400
100
-100
100
50
0
-50
-100
-150
V_m (mV)
0.5 s
C
I_M (pA/pF)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
9
**
11
8
Ad
AdRest
AdRest
(non-infected)
D
AdRest
(non-infected)
AdRest
Ad
-55
-60
-65
-70
-75
V_m (mV)
11
9
**
8
E
Control
XE991
V, mV
-50
-60
-70
-80
0.5 s
-40
-50
-60
-70
400 pA
0 pA


A
Control
DAPI
TRPV1
REST
Inflammatory
DAPI
TRPV1
REST
B
Control
DAPI
TRPV1
Kv7.2
Inflammatory
DAPI
TRPV1
Kv7.2
C
Relative pixel intensity
2500
2000
1500
1000
500
0
*
*
Con
Inflam
TRPV1
REST
Kv7.2