Title:
Regulation and role of REST and REST4 variants in modulation of gene expression in vivo and in vitro in epilepsy models.

Running Title: REST and Epilepsy

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Abbreviations

SE, Status Epilepticus; SSSE, Self sustained status epilepticus; REST, Repressor element-1 Silencing Transcription Factor; Luc, luciferase; KA, Kainic acid; PP, Perforant path; rPPT-A, rat preprotachykinin-A; NRSF, Neuron Restrictive silencing Factor; AVP, Arginine Vaospressin; SCLC, small cell lung cancer

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Abstract

Repressor element-1 Silencing Transcription Factor (REST) is a candidate modulator of gene expression during status epilepticus in the rodent. In such models full length REST and the truncated REST4 variant are induced and can potentially direct differential gene expression patterns. We have addressed the regulation of these REST variants in rodent hippocampal seizure models and correlated this with expression of the proconvulsant, substance P encoding, PPT-A gene. REST and REST4 were differentially regulated following kainic acid stimulus both in *in vitro* and *in vivo* models. REST4 was more tightly regulated than REST in both models and its transient expression correlated with that of the differential regulation of PPT-A. Consistent with this, over-expression of a truncated REST protein (HZ4, lacking the C-terminal repression domain) increased expression of the endogenous PPT-A gene. Similarly the proximal PPT-A promoter reporter gene construct was differentially regulated by the distinct REST isoforms in hippocampal cells with HZ4 being the major inducer of increased reporter expression. Furthermore REST and REST4 proteins were differentially expressed and compartmentalized within rat hippocampal cells *in vitro* following noxious stimuli. This differential localization of the REST isoforms was confirmed in the CA1 region following perforant path and kainic acid induction of status epilepticus *in vivo*. We propose that the interplay between REST and REST4 alter the expression of proconvulsant genes, as exemplified by the PPT-A gene, and may therefore regulate the progression of epileptogenesis.

Introduction

Modulation of neurotransmitter expression after status epilepticus is observed with genes whose products can act either as pro or anticonvulsants. Neuropeptide transmitters represent such a target. *In vivo* studies have shown that there is a strong correlation between the propensity, severity and duration of seizures with augmented levels of substance P neuropeptide (proconvulsant) (Liu *et al.*, 1999a;Liu *et al.*, 1999b;Zachrisson *et al.*, 1998), reduced levels of galanin neuropeptide (anticonvulsant) (Fetissov *et al.*, 2003) and the integrity of the dentate gyrus in rodent models (Liu *et al.*, 1999a;Liu *et al.*, 1999b). Modulation of neuropeptide expression will result from changes in the active transcription factor complement within the cell in response to an epilepsy trigger. The repressor element-1 silencing transcription factor (REST), also termed neuron restrictive silencing factor (NRSF), is one such transcription factor that has been observed to be dynamically regulated during seizure (Palm *et al.*, 1998). This zinc finger protein binds to a conserved 23 bp consensus sequence named neuron restrictive silencer element (NRSE) (Schoenherr & Anderson, 1995;Chong *et al.*, 1995). Through binding to this consensus sequence in neuronal target genes and neural precursors it controls spatial and temporal plasticity of neuronal gene expression during neurogenesis (Schoenherr & Anderson, 1995;Chong *et al.*, 1995;Lunyak & Rosenfeld, 2005) and a similar role in altering gene expression profiles could be predicted during status epilepticus (SE).

Full length REST has been shown to possess at least two independent repression domains, one encompassed by the N-terminal 83 residues and the other by the C-terminal zinc finger domain (Tapia-Ramirez *et al.*, 1997). The N terminus of REST recruits the SIN3A/B histone deacetylase complex (HDAC) (Huang *et al.*, 1999;Roopra *et al.*, 2001;Grimes *et al.*, 2000) and the C-terminal repression has been shown to recruit a distinct HDAC complex via its interaction with CoREST (Andres *et al.*, 1999;Huang *et al.*, 1999;Ballas *et al.*, 2001). In combination with these co factors, REST is able to mediate gene silencing and cellular differentiation by decreasing histone acetylation, increasing DNA methylation (Lunyak *et al.*, 2002;Wood *et al.*, 2003;Toshiyuki & Ichiro, 2004) and thereby modulating chromatin structure (Roopra *et al.*, 2000;Naruse *et al.*, 1999;Murai *et al.*, 2004). Interestingly splicing variants have also been observed which may have distinct functions from the full length REST. In particular rodent REST4 and the analogous human sNRSF have been identified which contains only five zinc fingers of the eight in full length

REST and therefore lacks the C-terminal zinc finger repression domain. It is plausible that these variants bind different co factors and regulate gene expression in a different manner from the full length molecule.

A role for such chromatin remodelling properties in epilepsy, as predicted for REST, is suggested from the action of valproic acid, an 8-carbon, branched-chained fatty acid, a commonly used and effective antiepileptic drug (Loscher, 2002). It was recently reported that valproic acid inhibits histone deacetylases at concentrations well within the therapeutic range used to treat epilepsy. As such, valproic acid relieves repression by transcription factors that recruit histone deacetylases (Kramer *et al.*, 2003;Phiel *et al.*, 2001) and could therefore act upon REST and modulate downstream targets such as neuropeptide gene expression in epilepsy. Consistent with such a model REST is transiently expressed in neurons of the adult rat CNS and this expression of REST in hippocampal and cortical neurons is increased in an *in vivo* kainic acid (KA) rodent model of epilepsy (Palm *et al.*, 1998). Two REST isoforms are induced and differentially regulated in response to seizures in rodents (Palm *et al.*, 1998). REST can also regulate the rat preprotachykinin A gene (PPT-A), which encodes substance P, in *in vitro* models and we have previously postulated that REST is a key candidate to regulate PPT-A gene expression during epilepsy (Quinn *et al.*, 2002). PPT-A contains a motif, homologous to an NRSE, which has been shown to bind directly to a dominant repressor that silences proximal promoter activity in the vast majority of non neuronal cell lines tested, in part explaining the restriction of the proximal PPT-A promoter activity to neuronal cells (Mendelson *et al.*, 1995;Quinn *et al.*, 2002). We have demonstrated that the PPT-A NRSE motif is a target for REST binding and have shown that co-expression of REST can modulate reporter gene expression driven by the rPPT-A promoter in primary cultures of rat dorsal root ganglia (Quinn *et al.*, 2002).

We have previously demonstrated a key role for REST in the modulation of neuropeptide gene expression in small cell lung cancer (SCLC) (Coulson *et al.*, 1999a;Coulson *et al.*, 1999b). This tumour type has a neuroendocrine phenotype and aberrantly transcribes multiple neuropeptides including arginine vasopressin (AVP) and PPT-A. REST is both down-regulated and differentially spliced in SCLC (Coulson *et al.*, 2000;Gurrola-Diaz *et al.*, 2003) correlating with neuroendocrine properties and its function is best characterised for the AVP gene in this model. The novel splice variant expressed in these tumours was termed sNRSF (Coulson *et al.*, 2000) and

is closely related to the rodent REST4 (Palm *et al.*, 1998) and human neuroblastoma variants (Palm et al, 1999). We have proposed that the AVP promoter and hence AVP transcription can be affected by differential expression of REST isoforms in lung cancer (Coulson *et al.*, 2000). We have hypothesised that PPT-A is regulated by REST via a similar mechanism to that outlined for AVP given that the NRSE in both promoters is adjacent to the major transcriptional start site (Coulson *et al.*, 1999b;Quinn *et al.*, 2002). In human lung cancer models AVP transcription can be affected by differential expression of REST isoforms, where full length REST is a repressor of neuronal gene expression but sNRSF can activate at least some neuroendocrine genes (Coulson *et al.*, 1999b). However, the function of REST4 in other systems remains controversial (Magin *et al.*, 2002). One hypothesis for the action of REST4 is to antagonise the action of full length REST or indeed act as activator rather than repressor of genes containing an NRSE. This hypothesis is supported by the following evidence. REST4 was shown to de-repress choline acetyltransferase gene expression in a PC12 cell line by blocking wildtype REST activity (Shimojo *et al.*, 1999). Tabuchi *et al* confirmed the competitive interaction between REST and REST4 in primary rat cortical neurons, where REST4 was shown to reverse the silencing activity of REST (Tabuchi *et al.*, 2002). REST4 has been shown to increase expression of neuronal genes in PC12 cells (Shimojo *et al.*, 1999) and has potential to antagonize the full length REST resulting in anti-silencer function (Roopra *et al.*, 2001;Shimojo & Hersh, 2004). Therefore by analogy we hypothesised that alterations in expression of REST isoforms account, in part, for the changes in differential gene expression in the hippocampus following seizure (Palm *et al.*, 1998).

To further this hypothesis we have analysed the expression and regulation of REST isoforms and PPT-A in a series of *in vitro* and *in vivo* seizure models to determine whether REST isoforms could support similar differential functions in hippocampal epileptogenesis pathways and use the PPT-A gene as a marker of such variation. In this manner we hope to elucidate the regulation of proconvulsant neuropeptides following seizure induction.

Materials and Methods

Perforant Path Induction of Self Sustaining Status Epilepticus (SSSE)

This method has been described in detail previously (Walker *et al.*, 1999). In brief, male Sprague Dawley rats (270-330 gm) were anesthetized with 1-2% halothane in oxygen. An earth electrode was positioned subcutaneously, and a monopolar recording electrode was implanted stereotaxically into the right hippocampus (coordinates, 2.5 mm lateral and 4 mm caudal from bregma). A bipolar stimulating electrode was implanted in the right hemisphere and advanced into the angular bundle (coordinates, 4.4 mm lateral and 8.1 mm caudal from bregma) to stimulate the perforant path. The depths of the electrodes were adjusted to maximize the slope of the dentate granule cell field potential (Walker *et al.*, 1999). The electrodes were held in place with dental acrylic and skull screws. The animals were allowed to recover from anesthesia. Seven days later, the perforant path was electrically stimulated with 2-3 mA 50-150 µsec monopolar pulses at 20 Hz for 2 hours; this induced SSSE that was terminated after 3 hours with diazepam (10 mg/kg, intraperitoneally). Animals were killed 3 and 24 hours after SE with an overdose of pentobarbitone (500 mg/kg, intraperitoneal) and brains were fixed with 4% PFA by perfusion for histological and immunohistological examination.

Kainic Acid (KA) Induction of SSSE

Adult Male Sprague Dawley rats were intraperitoneally administered with 10mg/kg of KA in saline solution or saline alone as a control. Following 90 minutes rats underwent status epilepticus for 1 hour before 10mg/kg intraperitoneal diazepam was administered to arrest the seizures and 1ml saline subcutaeneously for rehydration. At 3 hour and 24 hour time points following SSSE rats were euthanised killed and the left hemisphere of the brain was fixed in 4% PFA for histological and immunohistological examination. The hippocampus was removed from the right hemisphere and frozen in liquid nitrogen for RNA extraction.

Organotypic cultures

Brains were removed from male Wistar rat neonates aged 1-10 days. Coronal sections (300 µm) containing the hippocampus, were sliced using a slice vibrotome (Campden Instruments). The hippocampus was dissected out, rinsed with growth media (50% MEM, 25%

horse serum, 25% HBSS, 20 mM HEPES, 1 mM glutamine, 5 mg/ml glucose supplemented with 10ng/ml GDNF) and placed on semiporous membrane inserts (0.4 μm, Millipore) housed in six-well plates. Hippocampal slices were incubated in a humid atmosphere of 5% CO_2 at 37°C for two days. Media was then replaced with that containing 5 μM KA (Sigma) or as a control, media alone for the duration of 18-20 hours. Following KA stimulation, media was then replaced with fresh growth media. Hippocampal slices were collected at time points 0, 2, 6 hours post stimulation, and snap frozen at -80°C for protein and RNA extraction.

Dissociated Hippocampal Cell Culture

Dissociated mixed neuronal cells from the rat hippocampus were produced for subsequent nucleofection using rat neuron nucleofector kit and machine (Amaxa GmbH). Briefly, the brain was removed from male Wistar rats (1-6 days old). Hippocampi were dissected out and collected in 10 ml dissecting solution (Hank's balanced salt solution with Calcium and Magnesium, 1M Hepes, 1M $MgCl_2$, 1% penicillin/streptomycin) at 4ºC. Tissue was centrifuged at 1000g for 4 minutes, supernatant was discarded and tissue was incubated in 0.25% trypsin for 30 minutes at 37ºC. 0.8 mg/ml Soybean trypsin inhibitor (Invitrogen) was added and incubated at room temperature for 5 minutes, tissue was centrifuged at 1000g for 5 minutes and supernatant was discarded. Tissue was washed in media [DMEM, 10% fetal calf serum (Invitrogen)] and centrifuged at 1000g for 5 minutes, three times. Cells were dissociated by passage through fire-polished Pasteur pipettes of decreasing diameter until homogenous. The resulting cell solution was passed through a 0.2 μm cell filter to remove undigested material. Suspended cells were then counted using a haemocytometer and immediately processed for nucleofection. Alternatively cells were plated in 6 well plates and media was then replaced following 24 hours with Neurobasal-A media (Invitrogen). Cultured cells were then stimulated overnight with 5 μM KA and 50 μM hydrogen peroxide. These cells were then fixed for immunoflourescence.

RNA extraction and Reverse Transcription PCR

Total RNA was extracted from cultured rat hippocampal slices (neonate) and rat whole hippocampi (adult) using the Gentra system protocol following manufacturer's instructions. RNA was treated with RQ1 DNAse (Promega) at 37°C for 30 minutes for DNA removal. 0.5 μg of oligo dT was bound to 0.6 μg of RNA in a 10 minute reaction at 70°C, 4°C for 5 minutes. A

reaction mixture of 4 µl of reverse transcriptase buffer, 2 µl of 10mM dNTPs, 0.5 µl ribonuclease inhibitor, 2.4 µl 25mM magnesium chloride, 5 µl dH_2O and 15 units reverse transcriptase (Promega) was added to the RNA mix to generate cDNA. The reverse transcription reaction was carried out at 25°C for 3 minutes, 42°C for 1 hour and 70°C for 15 minutes.

Quantitative PCR

Quantitative real-time PCR was performed in an Opticon qPCR machine (GRI) using the Dynamo SYBR Green qPCR Kit (Finnzymes). For each experiment a standard curve for each primer set was generated and used to derive the relative amounts in the unknown samples. Melting curve analysis was carried out to confirm the specificity of the products between 65-95 ^{o}C with 0.2 ^{o}C increments The content of unknown samples was calculated from the amount of the target gene, normalized to the amount of a housekeeping gene (GAPDH), with each derived from separate standard curves. The primer sequences were, GAPDH, forward primer (For) 5′-ACCACAGTCCATGCCATCAC-3′ and reverse primer (Rev) 5′-TCCACCACCCTGTTGCTGTA-3′; Galanin, For 5′-CGCGCCCGCCACTCTACG-3′ and Rev 5′-AGGCAGGGGCACAGCAACACTTC-3′; PPT-A, For 5′-TTCAGAGAATCGCCCGAAGAC-3′ and Rev 5′-CGCACTATCTATTCATCTCCATC-3′, REST For 5’-AGCGAATACCACTGGCGGAAACA-3’ and Rev 5’- AATTAAGAGGTTTAGGCCCGTTG- 3’, REST4 For 5’-AGCGAATACCACTGGCGGAAACA-3’ and Rev 5’-TCACCCAACTAGATCACACT-3’. BLASTN searches confirmed the mRNA gene specificity of the primer sequences chosen. Thermal cycling conditions were as follows: 95°C for 10 min, followed by 45 cycles of amplification of 94°C for 10 sec, 60°C for 20sec, 72°C for 20sec followed by data acquisition. Results were analyzed using software supplied with the Opticon machine (MJ Research). The default settings of the program were used to define both the threshold value and baseline for analysis of the raw data. The expression of each target gene was normalized with respect to 1000 copies of GAPDH and was calculated for all samples. Each experiment was performed in triplicate.

Nucleofection with PPT-A Reporter Gene Constructs and REST Expression Constructs

Transient delivery of promoter construct PPT-A -865+92 luciferase vector (Harrison *et al.*, 1999) and expression constructs RE-EX-1 (expressing full length human REST) and HZ4 (expressing a truncated form of human REST) (Schoenherr & Anderson, 1995;Chong *et al.*, 1995)

into rat hippocampal cells was achieved by nucleofection (Amaxa Ghbm). RE-EX-1 contains the full length cDNA of human REST cloned into pCMV vector and was a kind gift from Prof G.Mandel (Howard Hughes Medical Institute, Stony Brook, NY). HZ4 expression construct contains 2kb of human REST sequence under the control of a CMV promoter and comprises of the entire n terminal domain, DNA binding domain and lacking the C-terminal repression domain) and was a gift from Dr.D.J.Anderson (Howard Hughes Medical Institute, Pasadena, CA). As a control cells were transfected with commercial HSV-1 Thymidine kinase (Tk) promoter reporter gene construct supporting expression of luciferase in the presence of HZ4 or RE-EX1 to confirm specific regulation of -965 +92 PPT-A luciferase. Briefly, $3x10^6$ cells per plasmid sample were used for each nucleofection. Media was removed from cells and 100 µl of rat neuron nucleofector solution was added along with 1 µg of PPT-A or Tk reporter gene constructs plus 1 µg of RE-EX-1 or HZ4 expression construct. The resulting solution was nucleofected on programme 0-03. Following nucleofection cells were resuspended in media, and distributed between the wells of a poly-D-lysine/laminin coated six-well plate (one plate per plasmid). Media was changed 24 hours following plating to maintenance media containing neurobasal-A medium (Invitrogen) supplemented with 200 mM glutamine (Sigma) and 10% B27 supplement (Invitrogen). Stimuli were added with the media change following plating (at 24 hours). Cells were harvested and assayed for luciferase activity 48 hours following nucleofection.

Reporter Gene Assay

Media was removed from wells and cells were washed twice in PBS. Cells were lysed with reporter passive lysis buffer (Promega), lysate was spun at 10000rpm for 30 secs in a microcentrifuge and 20 µl of supernatant was loaded from each sample into a 96 well luminometer plate. Luciferase assay reagent (Promega) and Stop and Glow reagent were dispensed by a Lucy II luminometer (Anthos) at 200 µl per well to measure Firefly and Renillin luminescence respectively.

Immunofluorescence and Confocal Microscopy

Rat hippocampal cells on poly-D lysine (Sigma) coated glass coverslips were washed twice with PBS. Cells were fixed with 4 % paraformaldehyde (PFA) for 30 minutes and washed 3 times with TBS-T (0.15 M NaCl, 0.02 M Tris chloride, pH 7.4 with 0.1 % Triton X 100). Cells were

incubated in blocking buffer (1 x TBS-T, 0.1% Triton X 100, 2% BSA and 0.1 % sodium azide) for 10 minutes and then incubated in primary polyclonal REST4 and REST antibodies at 1 in 500 dilution in blocking buffer for 1 hour at room temperature. The REST4 antiserum was raised against the C-terminal epitope SGCDLAG (Belyaev *et al.*, 2004) and the REST antibody was raised to the alternative C terminal sequence CYFLEEAAEEQE. Both anti-sera were raised in rabbit. Following further washes in TBS-T anti-rabbit biotinylated antibody (Amersham Biosciences) was added and cells incubated at room temperature for 1 hour. After further washes, cells were incubated in streptavidin Texas red for 30 minutes. Cover slips were mounted in vectashield. Staining of the REST isoforms was viewed using two photon microscopy and excitation at wavelength 594 (Texas Red).

SDS-PAGE and Western Blotting

Organotypic hippocampal slices were homogenised in 1 x Laemmli buffer and 1 M DTT and heated to 95°C for 10 minutes. Protein samples were resolved on 12 % gel by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes according to standard protocols. 10 μg of protein extract from hippocampal slices and whole rat brain extract was loaded per lane of the gel. REST, REST4 and β actin (Sigma) primary antibodies were routinely used at a dilution of 1:500 with 3% milk in TBS. Anti-rabbit horseradish peroxidase linked secondary antibody (Amersham Biosciences) was used at a dilution of 1: 2000. Antibody visualization was performed using enhanced chemiluminescences according to standard protocols (Amersham Biosciences).

Histology and Immunohistology

Brains were extracted manually, fixed in 4% paraformaldehyde for 48-72 hours, cross sectioned into 2 mm thick slices with the help of a rat brain cutter and routinely embedded into paraffin wax. For histological evaluation and identification of brain areas, 3-5 μm thick sections were cut and stained with haematoxylin-eosin. For immunohistology, 3-5 μm thick sections were cut and placed on polysine-coated slides (BDH Laboratory Supplies, Lutterworth, UK).

Immunohistology for REST and REST4 was performed, using the rabbit anti-rat antibodies described above and the peroxidase anti-peroxidase (PAP) method. Briefly, after deparaffination and blocking of endogenous peroxidase, sections were pretreated with 10 mM citrate buffer (pH

6.0) for 30 minutes at 97°C. Non-specific binding was blocked by incubation with 50% swine serum in TBS for 30 minutes. Sections were then incubated at 4°C for 15-18 hours with the primary anti-REST and anti-REST4 antibodies (dilution 1 in 100 in TBS with 20% swine serum), washed with TBS and incubated with swine anti-rabbit IgG (DakoCytomation) and rabbit PAP complex (DakoCytomation), followed by visualisation with diaminobenzidintetrahydrochloride according to previously published protocols for rabbit polyclonal antibodies (Kipar *et al.*, 2001). Consecutive sections incubated with normal rabbit serum instead of the primary antibody served as negative controls. Both histological and immunohistological evaluations focused on the CA1 region of the hippocampus.

Results

Differential Expression of REST mRNA Splice Variants and PPT-A mRNA *in vitro* and *in vivo* in Response to Kainic Acid

Real time PCR analysis of PPT-A, REST4 and REST mRNA expression was carried out on rat hippocampi removed at 3 and 24 hours post SE induced by kainic acid and from saline injected controls (Fig. 1). PPT-A and REST variants were differentially regulated in this *in vivo* status epilepticus model. At 3 hours post SE there was a dramatic and transient upregulation of REST4. In this model REST4 was below detectable levels in both saline controls and at 24 hours post SE. The observed increase in REST4 expression correlates with a transient increase in expression of PPT-A mRNA. However REST expression is fairly constant at the time points analysed with a more modest but statistically significant increase at 3 hours post SE.

Organotypic hippocampal slices have been used previously as a model of epileptiform activity to observe mossy fibre sprouting and pathological changes induced by KA treatment (Routbort *et al.*, 1999;Holopainen *et al.*, 2004). We therefore used organotypic hippocampal slices to address the levels of REST mRNA isoforms and PPT-A expression in response to KA in an *in vitro* model which might mimic our observations *in vivo*. Two hours following KA stimulation an upregulation of PPT-A, REST and REST4 mRNA was observed (Fig. 2). REST4 mRNA expression increased the most dramatically with an 8 fold increase at two hours post stimulation, which correlates with the observed 3 fold upregulation of the proconvulsant PPT-A mRNA. This is comparable with *in vivo* modulation of these genes. Similar to the *in vivo* model, REST mRNA fluctuated less over this time course. These *in vivo* and *in vitro* findings together support our hypothesis that REST4 is a potential regulator of tachykinin gene expression due to the correlation of expression in these models.

REST4 has a Direct Regulatory Effect on the PPT-A Promoter and Endogenous PPT-A Expression in Primary Hippocampal Cultures

To investigate the role of REST in the regulation of PPT-A, primary rat hippocampal neurons were transfected with both the -865 +92 PPT-A promoter luciferase construct and REST expression plasmids. The HZ4 expression construct expresses a truncated form of human REST (Schoenherr & Anderson, 1995) and dramatically upregulated transcription from the rat PPT-A gene promoter (up to 7 fold, $p<0.01$**); no significant difference was observed with the Tk-

luciferase control promoter indicating a specific regulation of the PPT-A promoter. In contrast, the full length REST, expressed from RE-EX-1 (Chong *et al.*, 1995), resulted in only a 1.8 fold increase of the -865 +92 PPT promoter driven luciferase activity (Fig. 3). This provides evidence for a direct link between the regulation of REST molecules and altered PPT-A promoter activity.

Hippocampal cultures transfected with RE-EX-1, HZ4 or the equivalent CMV expression construct with no insert (as a control for modulation by transfection) were analysed by qPCR to determine the effect on endogenous PPT-A and galanin mRNA expression. Transfection of the cells with RE-EX-1 did not alter the endogenous PPT-A mRNA expression levels compared to controls whereas expression of HZ4 resulted in a significant 1.7 fold upregulation (P=<0.01**) of endogenous PPT-A expression (Fig. 4). Due the anticonvulsant properties of galanin, the effect on endogenous galanin mRNA was also measured as an internal control in the transfected cultures. Interestingly RE-EX-1 resulted in significant 2.5 fold upregulation of endogenous galanin expression (P=<0.01**) where as HZ4 had no effect (Fig. 4). Although a fuller data set of experiments must be performed on the galanin gene the data are suggestive that full length and truncated forms of REST may differentially modulate expression of anti or proconvulsant genes.

Differential Localization of REST, REST4 Proteins in Hippocampal Culture Following Noxious Stimuli

Regulation of transcription factor function can result from their de novo synthesis in the cell in response to a challenge; alternatively they can be activated from an inactive form, for example, sequestered in the cytoplasm. The action of NF-kB is a well characterised example of the latter mechanism (Yang *et al.*, 2000;Yang *et al.*, 2001;Sun *et al.*, 1996). We wanted to address REST/REST4 cellular localization as recent evidence has suggested that REST may also be sequestered in the cytoplasm. Huntingtin has been shown to activate the BDNF promoter II through its NRSE site and via the action of REST. Full length REST was shown to be sequestered in the cytoplasm of neurons by huntingtin protein thereby preventing REST from regulating NRSE nuclear binding sites (Zuccato *et al.*, 2003). Furthermore Shimojo and Hersh identified a nuclear localization signal within zinc fingers 2 to 5 which is responsible for translocation of both full length REST and REST4 into the nucleus (Shimojo & Hersh, 2003).

Antibodies specific to the C-terminal epitopes of either REST or REST4 recognised different sized proteins in rat tissue of predicted molecular weights by western blotting (Fig. 5a). In addition to transcriptional induction of the REST isoforms in response to challenge, a second layer of complexity was observed. Differential localization of these isoforms between the nucleus and cytoplasm of hippocampal primary culture cells correlated with response to stress induced by noxious stimuli. REST staining was observed predominantly in the cytoplasm of cells under control conditions. However, following 18 hours of KA or hydrogen peroxide treatment REST is translocated to the nucleus (Fig. 5b). Consistent with the fact that dissociated culture may cause severe stress to the cells, REST4 was found to be expressed strongly in the nucleus in control conditions. The nuclear localization in this case suggests it could be acting as a transcription factor. Post kainic acid and hydrogen peroxide stimuli REST4 moved into the cytoplasm and dendrites of hippocampal cells (Fig. 5b.).

Differential Expression and Localization of REST isoforms in the Perforant Path and Kainic acid model of Self Sustained Status Epilepticus (SSSE)

In CA1 hippocampal pyramidal cells, differential localization of REST and REST4 was observed in both the perforant path (PP) (Fig. 6) and the KA (Fig. 7) models of SSSE. Under control conditions (KA saline control, PP surgery control; Figs. 6a and 7a), pyramidal cells exhibited a weak to moderate cytoplasmic staining for REST (Figs. 6a, 7a). The staining for REST4 was also cytoplasmic, but generally much weaker (Figs. 6b, 7b). Following status epilepticus, cells, degeneration and necrosis of pyramidal cells was observed. From 3 hours post SSSE on, variable numbers of slightly contracted cells without any nuclear changes and cells with the typical morphology of dark neurons (contracted cells with shrunken nuclei) were observed, both representing degenerative changes (Csordas *et al.*, 2003; Figs. 6c, d and 7c, d). At 24 hours post SSLE, numerous necrotic cells were also viewed (Fujikawa *et al.*, 2000) (Figs. 6c, d and 7c, d). In the slightly contracted degenerating cells, moderate to strong staining for full length REST was seen both in cytoplasm and nucleus (Figs. 6c and 7c) and staining for REST4 was strong in the cytoplasm and weak in the nucleus (Figs. 6d, 7d). Dark neurons exhibited strong, solely cytoplasmic staining for both REST and REST4 (Figs. 6c, d and 7c, d). Necrotic pyramidal cells were negative for both antigens (Figs. 6c, d and 7c, d). Staining patterns for REST and REST 4 in

unaltered pyramidal cells were identical to those observed in control animals (Figs. 6c, d and 7c, d).

Consecutive negative control sections did not exhibit any staining (data not shown); proving that the weak cytoplasmic staining for REST4 observed in unaltered pyramidal cells was specific. Both of these seizure models show evidence of differential localization of REST isoforms in response to epileptic stimulation as seen *in vitro*. Although immunostaining is not quantitative the immunohistological results are consistent with the levels of mRNA observed at the same time points in real time PCR (Fig. 1) (Figs. 6 and 7).

Discussion

The differential expression of REST isoforms during epilepsy previously observed (Palm *et al*, 1998) and the proposed role of REST as a major regulator of neuronal gene expression and neurogenesis highlight the potential for this transcription factor to be a major modulator of gene expression during epilepsy. It is therefore important to compose a map of not only the temporal expression of REST isoforms but also their tissue specific regulation and cellular localization in response to seizure. The latter is important as more recently compartmentalized localization of REST has been observed (Zuccato *et al.*, 2003). Further, we and others, have previously proposed that different isoforms of REST can regulate distinct patterns of gene expression and, at least in certain cancer models, may function antagonistically (Coulson *et al.*, 2000). It is therefore important to define how differential regulation by these isoforms may relate to the changing patterns of gene expression associated with epilepsy. Indeed we could hypothesise that the duration and levels of expression of these isoforms could have important consequences for the progression and severity of seizure thereby leaving a molecular marker that might affect a predisposition to subsequent episodes.

In this communication we have addressed these mechanisms *in vitro* within rat hippocampus organotypic and dissociated primary cultures and validated our hypothesis *in vivo* in both a perforant path and kainic acid rodent model of status epilepticus. Our data support a hypothesis of differential expression and localization of REST and REST4 in response to the same insult. Further by addressing the proconvulsant PPT-A gene as a target for the REST modulation we can hypothesise that REST4 might orchestrate proconvulsant pathways.

Differential expression of REST and REST4 was examined by qPCR in organotypic cultures of rat hippocampus (Fig 2). Kainic acid should drive the cells to a state more reflective of the seizure phenotype. Both REST4 and PPT-A were again the genes that were markedly regulated with both demonstrating an early rise at 2 hours post induction (PI) which fell again by 6 hours PI. By the same criteria there was insignificant variation in levels of REST over the same time period. This data is similar to the expression pattern of these molecules observed *in vivo* in kainic acid induced SSSE (Fig 1). In this model there is a dramatic but transient, increase of REST4 which is correlated with a significant increase in PPT-A.

The similar differential expression patterns of both REST4 and PPT-A suggested that REST4 could directly modulate the level of PPT-A expression. Therefore we addressed whether REST isoforms could modulate both the endogenous PPT-A gene and the reporter gene construct containing the proximal 5' PPT-A promoter in rat hippocampal dissociated cultures. Dissociated cultures provide a useful model as it is difficult to transfect organotypic cultures with plasmid constructs. Consistent with our hypothesis, qPCR demonstrated that HZ4, which is a truncated REST molecule similar in structure to the REST4 variant, resulted in a two fold up-regulation of the endogenous PPT-A gene whilst REST itself had no affect on expression (Fig 4). Interestingly in a control for this experiment, endogenous galanin levels were significantly increased with RE-EX-1 but unaffected by HZ4. Although this observation requires further characterization, it might support our hypothesis that in general expression of REST4 would favour proconvulsant effects whilst REST would favour anticonvulsant effects. Supporting data on the function of REST4 modulation of PPT-A expression are demonstrated by analysis of the reporter gene expression supported by a well characterized promoter fragment of the PPT-A promoter spanning -865 to +92 which encompasses a repressor element at the start of the transcription previously identified as an REST regulated element/NRSE (Mendelson *et al.*, 1995; Quinn *et al.*, 2000; Quinn *et al.*, 2002). Expression of HZ4 but not the full length RE-EX-1 greatly increased the PPT-A promoter driven luciferase activity. RE-EX-1 had a lower but nevertheless statistically significant increase in reporter gene expression (Fig 3). It is possible in both co transfections that REST4/ REST are having an indirect effect on PPT-A promoter although the more likely explanation is via direct modulation promoter via the NRSE.

We have demonstrated that REST and REST4 are differentially expressed in hippocampal cells and that the REST4 expression appears to be more dynamic in both the absolute levels of expression that are induced and the duration of that expression in response to a stimulus. However, clearly the qPCR data does not tell us which cells are expressing these isoforms or whether both isoforms are co-expressed in the same cell. To begin to address the localization and co-expression of the isoforms within the cell we have used immunohistology and immunofluorescence. *In vitro* in rat hippocampal cells we were able to demonstrate that the location of REST isoforms was dependent on the stimulus applied to the cells. Two stimuli were applied separately to the cultures, kainic acid (which is used commonly to induce status epilepticus *in vivo*) and hydrogen peroxide (ischemic model *in vivo* and mimics oxidative stress seen during

seizures). In both cases these stimuli caused a relocation of both REST and REST4 in the cell. Under our normal growth conditions, REST4 was predominantly located in the nucleus whilst full length REST was in the cytoplasm. However at 24 hours post induction this was reversed, reflecting a stress mediated change in localization (Fig 5). We were not able to address whether this is trafficking or newly synthesized proteins going to different compartments within the cell. We are currently addressing this relocalization observation in more detail.

Clearly the localization of either REST or REST4 *in vitro* is stimulus regulated and we explored the consequence for this using *in vivo* rodent epilepsy models. Two commonly used rodent models of status epilepticus were tested, the kainic acid and the perforant path induced status epilepticus. Both models were used to determine temporal and spatial localization of the REST isoforms in control animals and in response to the insult in the CA1 region which exhibits considerable damage in the perforant path model. As can be seen REST and REST4 were both expressed in the cytoplasm of pyramidal neurons in the control animals, with REST being moderately expressed and REST4 generally only weakly expressed. However following SE, with the occurrence of degenerative changes in pyramidal cells, several observations were made: in degenerating cells which were identified on the basis of their morphology, either as slightly contracted neurons without nuclear changes (likely an early stage of degeneration) or as cells with the typical features of dark neurons (Csordas *et al.*, 2003), both REST and REST4 were expressed with higher intensity compared to intact neurons in the same section (Fig 6). In the slightly contracted pyramidal neurons, both REST isoforms were now also expressed in the nucleus, and REST with an intensity comparable to that present in the cytoplasm. The nuclear expression was again lost in the dark neurons. There is evidence that these stages of neuronal degeneration are still reversible (Csordas *et al.*, 2003). At 24 hours post SE, however, numerous necrotic pyramidal cells were seen, in addition to neurons with the above-mentioned degenerative changes, which lacked expression of both molecules. These data demonstrated that REST isoforms were both differentially expressed and localized during cell degeneration following the epileptic insult.

The differential expression and localization of REST isoforms during epilepsy could have profound consequences for the individual as they are known to alter expression of synaptic genes(Bruce *et al.*, 2004). We have also indicated that REST can alter expression of three neuropeptides genes PPT-A, AVP and galanin, albeit in different cell models (Quinn *et al.*, 2000;Coulson *et al.*, 2000).Taken together, these results imply that neuronal signalling will be

altered when REST function is modulated. The tissue specificity, duration and cellular expression of the different variants could all contribute to the final outcome and degree of cell damage from the seizure. Further, our findings indicate that REST4 could perhaps be a key player in the deregulation of proconvulsant neuropeptides in epileptogenesis.

Figure legends

Fig 1: Quantitative PCR of Endogenous PPT-A and REST expression in whole hippocampi post *in vivo* Kainic acid induction of self sustained status epilepticus(SSSE). A, PPT-A mRNA expression increases two fold 3 hours post status epilepticus induced with kainic acid compared to saline control and reduces to below the level of saline control at 24 hours.**B,** REST 4 mRNA expression is at undectable levels in saline control rats and 24 hours post SSSE but is significantly induced at 3 hours post status epilepticus.**C,** wild type REST mRNA expression is increased significantly 5 fold at 3 hours post SSSE compared to control. ($p < 0.05$ significant , $p < 0.01$ ** highly significant) In all cases adult rats were administered with 10mg/kg I.P injections of kainic acid in saline or saline as a control. Rats underwent status epilepticus for 1hr before being administered with 10mg/kg diazepam. The mean and S.E of copy number of gene of interest per 1000 copies of GAPDH (n=2 animals, done in triplicate) is shown.

Fig 2: Quantitative PCR of PPT-A and REST in the in vitro hippocampal slice model post kainic acid stimulation. A,B, PPT-A mRNA and REST 4 expression levels are significantly increased post kainic acid treatment at 2 hours post the end of stimulation compared to relative controls**. C,** REST mRNA expression is not significantly affected by kainic acid treatment. ($p < 0.05$ ** highly significant). Rat hippocampal slices were maintained in culture for 48 hours and incubated in 5microM Kainic acid for 18 hours. Post stimulation media was removed from both stimulated and control slices. RNA was extracted from tissue at indicated time points and analysed by Q-PCR. The mean and S.E of copy number per 1000 copies GAPDH (n=3) is shown.

Fig 3: Regulation of the -865 + 92 PPT promoter Luciferase by wt NRSF and truncated REST expression constructs in primary hippocampal cultures. Co- Transfection of primary rat hippocampal cultures with -865 +92 PPT luciferase and RE-EX-1 (full length) and HZ4 (truncated REST) expression constructs. HZ4 expression construct produced a highly significant increase in PPT luciferase activity. Tk driven luciferase was used as a negative control. Reex1 and HZ4 had no significant effect on the Tk luciferase activity. The mean and S.E of fold increase from n=5 experiments each done in triplicate is shown. ($p<0.05$ * significant, $p<0.01$** highly significant).

Figure 4: Regulation of endogenous PPT-A and Galanin mRNA expression in the *in vitro* rat hippocampal cells by REST isoforms. Endogenous PPT-A expression is not significantly affected by expression of REST from the RE-EX1 construct and is significantly upregulated 1.7 fold by HZ4. Galanin mRNA expression is significantly upregulated by RE-EX1 expression and is unaffected by transfection with HZ4 expression construct compared to control. Rat hippocampal cultures were transfected with RE-EX-1, HZ4 expression constructs and an empty CMV expression construct as a control. Cultures were maintained in culture for 48 hours and then RNA was extracted for quantitative PCR. The mean and S.E of copy number of gene of interest per 1000 copies of GAPDH housekeeping genes (n=3) is shown. ($p<0.05$ * significant ,$p<0.01$ ** highly significant).

Fig 5a: Western blot of rat and human tissues with REST and REST 4 antibodies. 1: whole rat brain extract, 2: Rat hippocampal slice, 3: Specific REST antibody binding is observed at 110kda in both rat tissue samples and REST 4 binds specifically at 40kDa.

Fig 5b: Immunoflourescence staining of REST and REST 4 in hippocampal cultures under control conditions (A,A'), post kainic acid treatment (B,B') and post hydrogen peroxide treatment (C,C'). Hippocampal cultures were stained REST and REST 4 (Texas red) post 48 hours in culture in control conditions and following 18 hour incubations with 50µM Kainic acid and 50µM hydrogen peroxide. REST 4 is present in the nucleus in control conditions (A) and trafficking to the cytoplasm and dendrites is viewed post kainic acid (B) and H2O2 stimulation (C).REST is predominantly in the cytoplasm in cells in control conditions (A') and shows strong nuclear localisation post both kainic acid (B') and H2O2treatment (C'). Images were visualised by two photon microscopy using x63 objective. (Scale bars are shown)

Fig 6: High power photomicrographs showing the localisation of REST isoforms in the CA1 pyramidal layer of the hippocampus in the perforant path in vivo model of status epilepticus. Changes in cell morphology and Trafficking of REST and REST 4 can be viewed in response to SSSE. In control conditions REST staining is observed

predominantly in the cytoplasm of viable cells. 24 hours post SSSE a moderate staining within the nucleus and cytoplasm of slightly contracted CA 1 layer cells is observed. Expression of REST is moderate in the cytoplasm of unaltered pyramidal cells and strong in cytoplasm of dark neurons**.** REST 4 showed a weaker staining pattern than REST and is present both in the nucleus and cytoplasm. Following seizure REST 4 staining has an increased intensity and is predominantly cytoplasmic and is nuclear is degenerating cells with shrunken nuclei. Necrotic pyramidal cells are negative for both forms of REST. In all cases adult rats were subjected to perforant path surgery. Experimental animals were underwent status epilepticus for 3hr before being administered with 10mg/kg diazepam. Photomicrographs are representative of n=2 animals. (Black arrow indicates pyramidal cells, blue arrow indicates slightly contracted pyramidal cells, red arrow indicates dark neurons and green arrow indicates necrotic neurons).

Fig 7: High power photomicrographs showing the localisation of REST isoforms in the CA1 pyramidal layer of the hippocampus in the kainic acid in vivo model of status epilepticus. In control conditions moderate REST staining can be viewed in the cytoplasm of viable pyramidal cells (A). 24 hours post SSSE strong nuclear staining is observed in slightly contracted neurons (blue) and strong cytoplasmic staining is seen in dark neurons (red). Necrotic neurons are negative for both isoforms. REST 4 staining was weak in the cytoplasm and the nucleus of pyramidal cells in control conditions (B). Following status epilepticus strong cytoplasmic staining can be viewed in degenerating and dark neurons (D). In all cases adult rats were administered with 10mg/kg I.P injections of kainic acid in saline or saline as a control. Rats underwent status epilepticus for 1hr before being administered with 10mg/kg diazepam. Photomicrographs are representative of n=2 animals. (Black arrow indicates pyramidal cells, blue arrow indicates slightly contracted pyramidal cells, red arrow indicates dark neurons and green arrow indicates necrotic neurons).

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Figure legends

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Fig 7: High power photomicrographs showing the localisation of REST isoforms in the CA1 pyramidal layer of the hippocampus in the kainic acid in vivo model of status epilepticus. In control conditions moderate REST staining can be viewed in the cytoplasm of viable pyramidal cells (A). 24 hours post SSSE strong nuclear staining is observed in slightly contracted neurons (blue) and strong cytoplasmic staining is seen in dark neurons (red). Necrotic neurons are negative for both isoforms. REST 4 staining was weak in the cytoplasm and the nucleus of pyramidal cells in control conditions (B). Following status epilepticus strong cytoplasmic staining can be viewed in degenerating and dark neurons (D). In all cases adult rats were administered with 10mg/kg I.P injections of kainic acid in saline or saline as a control. Rats underwent status epilepticus for 1hr before being administered with 10mg/kg diazepam. Photomicrographs are representative of n=2 animals. (Black arrow indicates pyramidal cells, blue arrow indicates slightly contracted pyramidal cells, red arrow indicates dark neurons and green arrow indicates necrotic neurons).

Figure 1: Regulation of REST variants in epilepsy

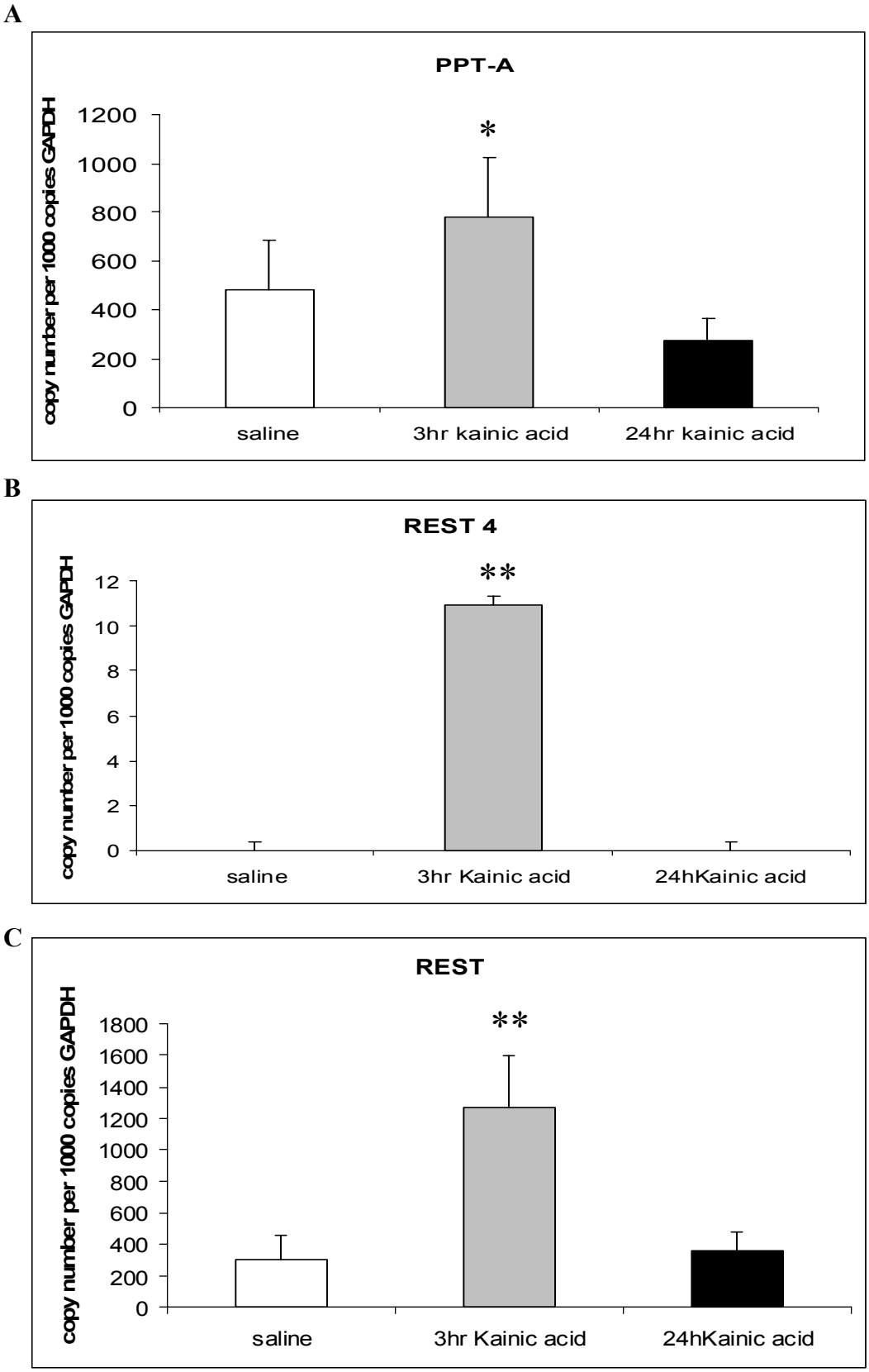


Figure 2: Regulation of REST variants in epilepsy

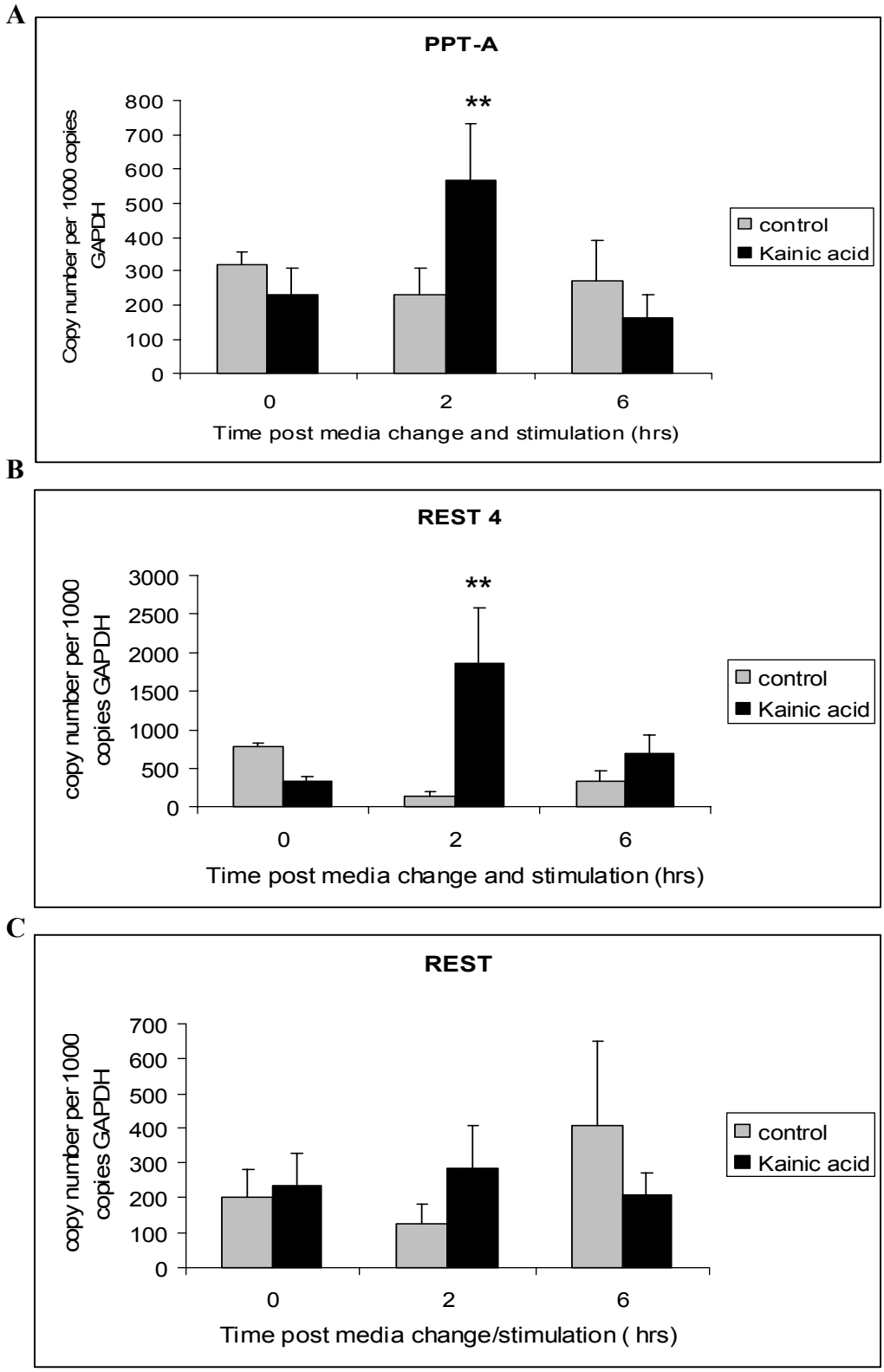


Figure 3: Regulation of REST variants in epilepsy

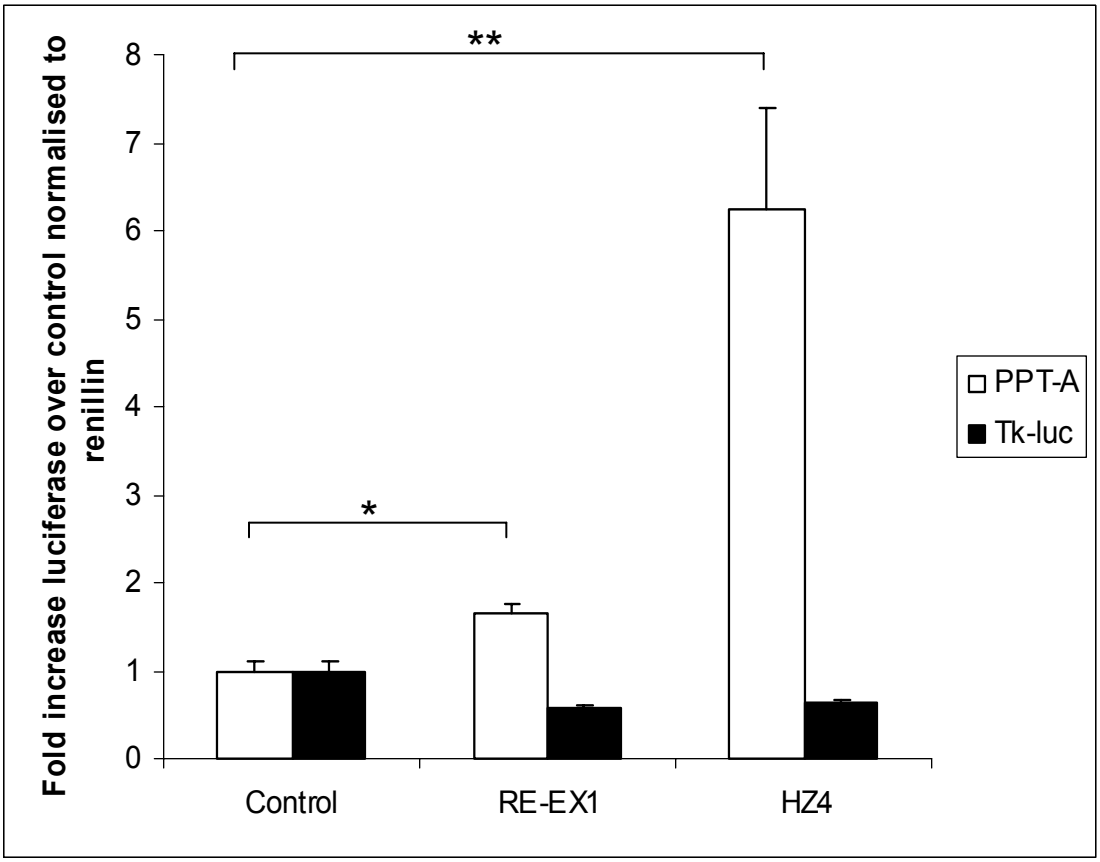


Figure 4: Regulation of REST variants in epilepsy

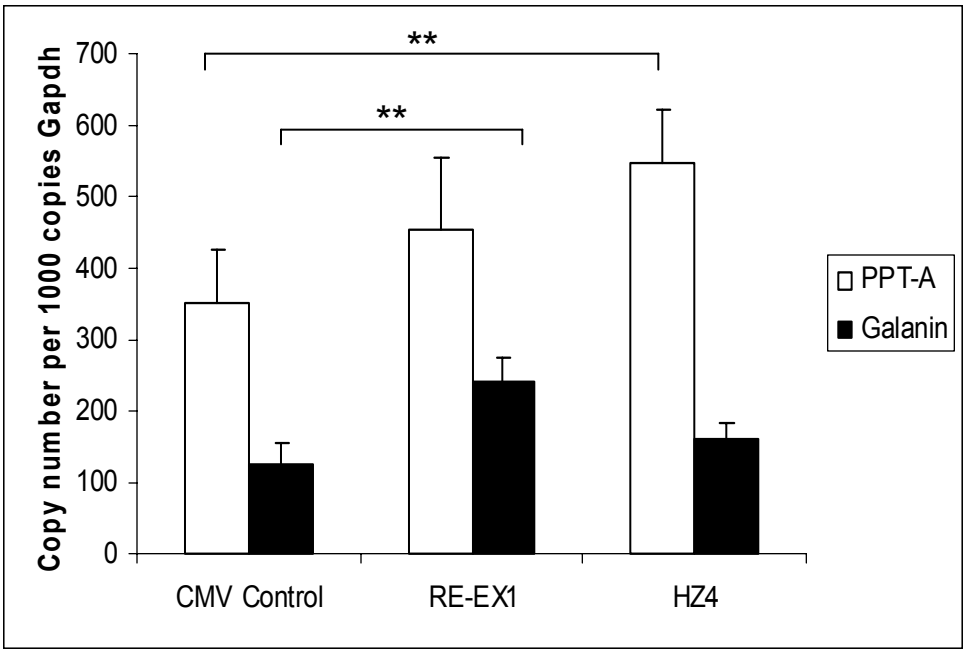


Figure 5: Regulation of REST variants in epilepsy

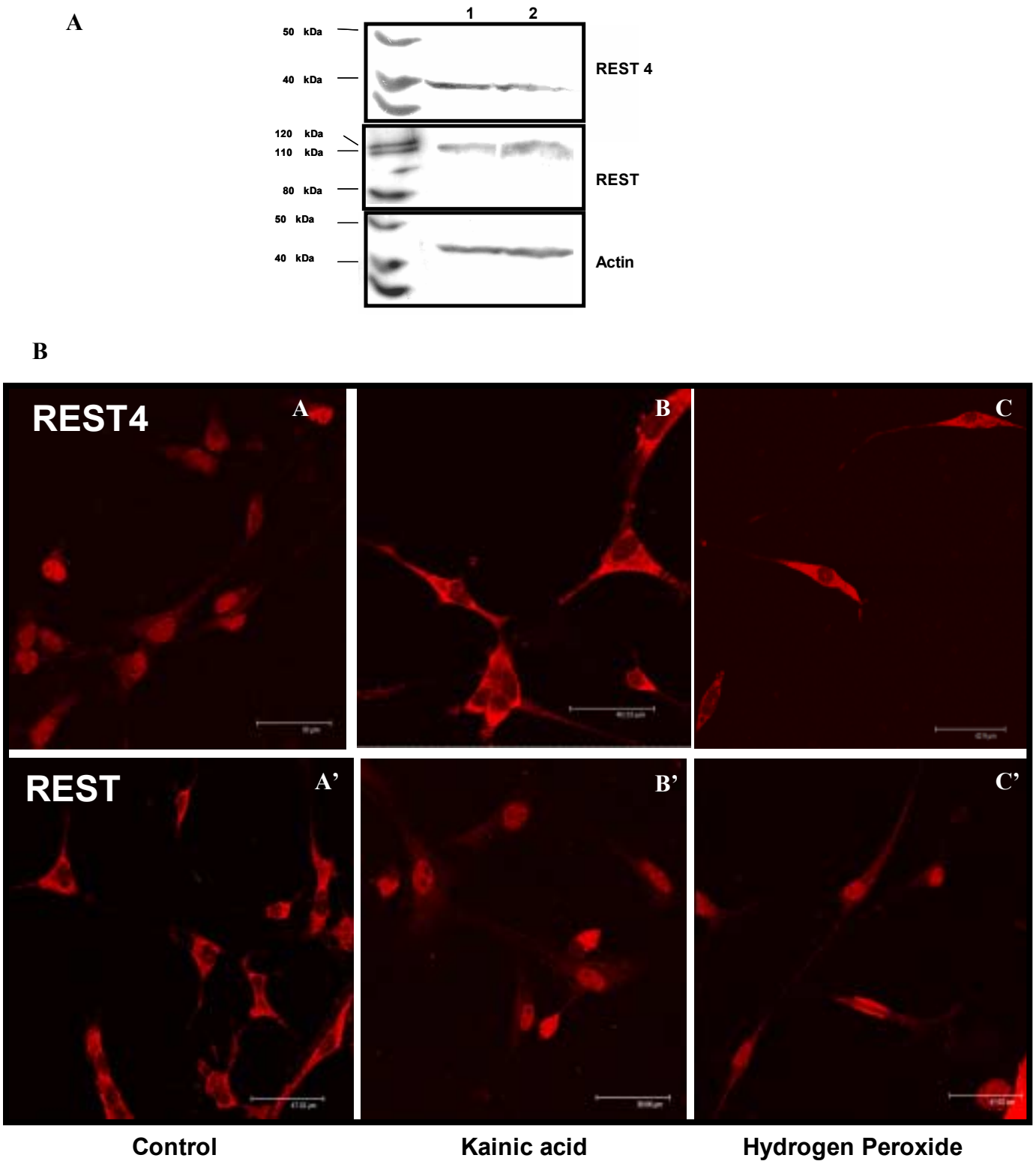


Figure 6: Regulation of REST variants in epilepsy

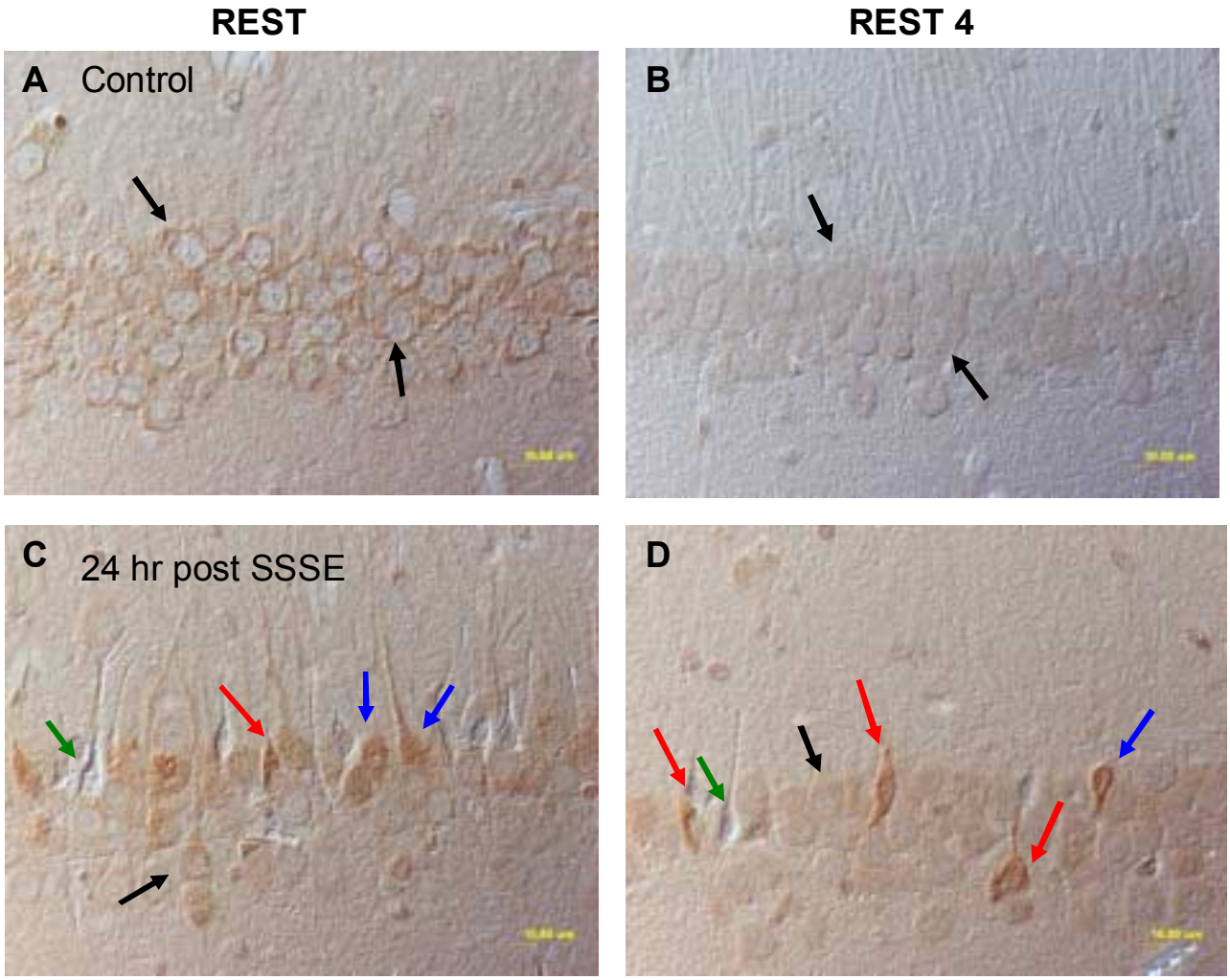
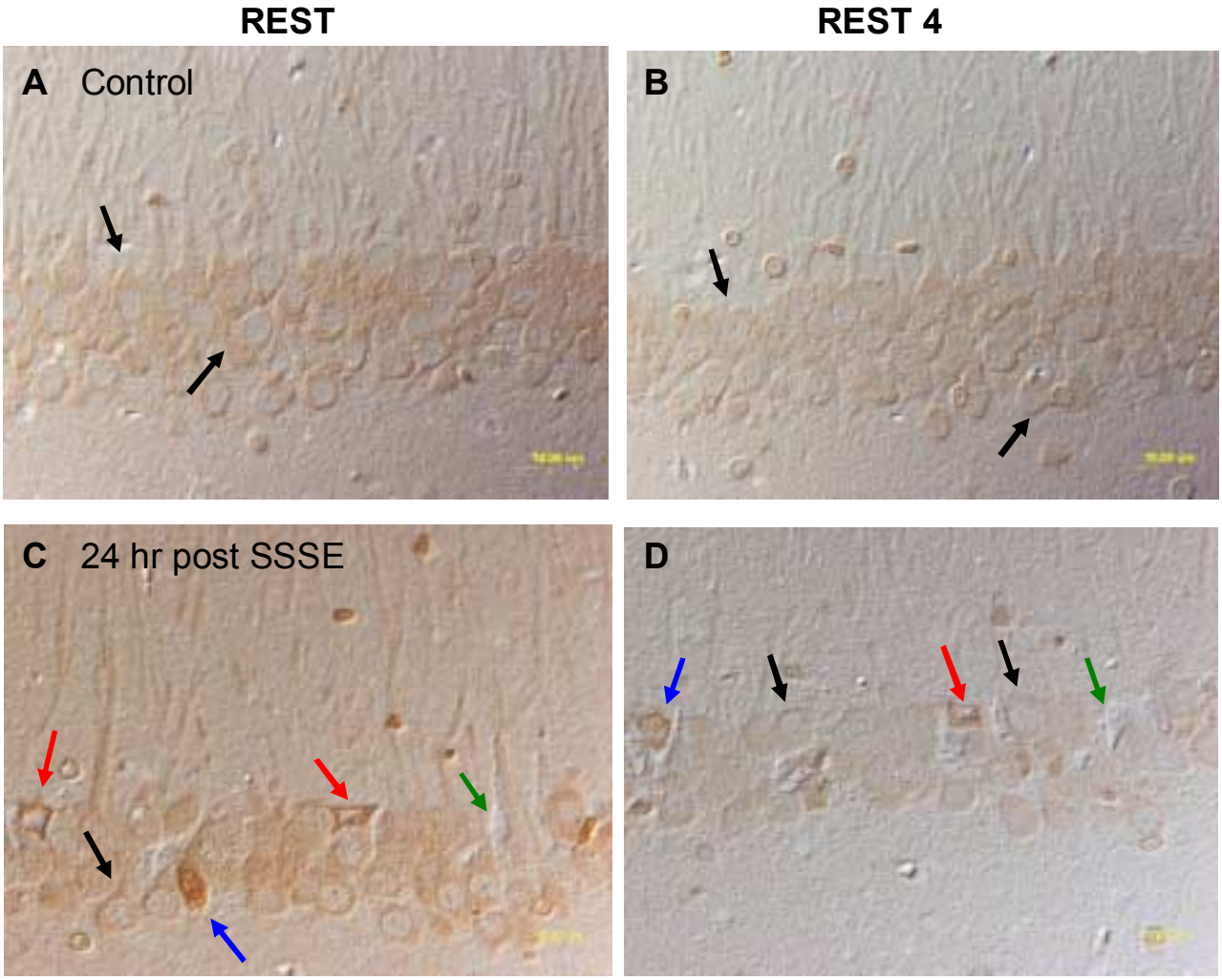


Figure 7: Regulation of REST variants in epilepsy



Supplementary Material;regulation of REST variants in epilepsy

Click here to download Supplementary Material: supplementary fig.ppt