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Rem2 signaling affects neuronal structure and function in part by regulation of gene expression

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Abstract

The central nervous system has the remarkable ability to convert changes in the environment in the form of sensory experience into long-term alterations in synaptic connections and dendritic arborization, in part through changes in gene expression. Surprisingly, the molecular mechanisms that translate neuronal activity into changes in neuronal connectivity and morphology remain elusive. Rem2, a member of the Rad/Rem/Rem2/Gem/Kir (RGK) subfamily of small Ras-like GTPases, is a positive regulator of synapse formation and negative regulator of dendritic arborization. Here we identify that one output of Rem2 signaling is the regulation of gene expression. Specifically, we demonstrate that Rem2 signaling modulates the expression of genes required for a variety of cellular processes from neurite extension to synapse formation and synaptic function. Our results highlight Rem2 as a unique molecule that transduces changes in neuronal activity detected at the cell membrane to morphologically relevant changes in gene expression in the nucleus.

Keywords

Rem2; Excitatory synapse; activity-regulated; Lrrtm4; Glypican-5 (Gpc5)

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

KK, MTM and SP designed the experiments. KK, XC, ARM and LR performed the experiments. KK, XC, LR and MTM analyzed the data. ARM created the *Rem2^{flx/flx}* mouse line used in this study. KK and SP wrote the manuscript. KK, SP, MTM, LR, ARM and XC edited the manuscript.

Introduction

During central nervous system (CNS) development, processes such as synapse formation and dendritic and axonal outgrowth establish connectivity between neurons in order to construct functional neuronal circuits. This developmental program utilizes “hard-wired” genetic programming, but is further refined by sensory input [1–3]. Experience-driven structural changes require, in part, the precise regulation of activity-dependent signaling pathways [4–6]. These pathways activate downstream transcription factors that ultimately result in changes in gene expression [4–7]. Thus, not surprisingly, a large proportion of the studies elucidating the molecular mechanisms of synapse formation and dendritic arbor elaboration have focused on either transcription factors or cell surface receptors [8–13]. Therefore, much remains to be determined regarding the identity and function of cytosolic signaling molecules that transduce changes in neuronal activity into changes in gene expression and ultimately, neuronal structure and function.

Rem2 is a positive regulator of excitatory synapse formation in cultured rodent hippocampal neurons and an activity-dependent, negative regulator of dendritic complexity in both cultured rodent neurons and in *Xenopus laevis* optic tectum [14–18]. Rem2 is a member of the Rad/Rem/Gem/Kir (RGK) family of non-canonical Ras-like GTPases and is primarily expressed in the nervous system [19]. Unlike canonical Ras-like GTPases, Rem2 has amino acid substitutions at conserved residues in its GTP binding domain, which results in a low rate of GTP hydrolysis [20, 21]. The nucleoside diphosphate kinase, Nm23, has been identified as a GTPase activating protein (GAP) for Rad [22, 23]. However, neither GAPs nor Guanine exchange factors (GEFs) have been identified for Rem2 or the other RGK proteins thus far. These data and other lines of evidence [14] suggest that Rem2 does not transduce signals like a classical GTPase, regulated by its nucleotide binding state, but is instead activated by other means, for example by post-translational modification [20]. In addition, in both cultured rat neurons and in *Xenopus* optic tectum, *Rem2* mRNA expression is upregulated in response to neuronal depolarization [15]. The increase in *Rem2* mRNA expression is dependent on calcium entry specifically through voltage-gated calcium channels [15]. Therefore, Rem2 is well poised to be a cytosolic signal transducer that translates changes in neuronal activity into changes in dendritic branching and neuronal connectivity.

To identify the mechanism(s) by which Rem2 regulates synapse formation and neuronal morphology, we took an unbiased approach to investigate Rem2-dependent changes in gene expression. We employed RNA-sequencing (RNA-seq) of mRNA isolated from cultured mouse neurons in which *Rem2* had been knocked out. Using this approach, we identified 24 genes whose expression was altered by *Rem2* knockout. Importantly, we observed that *Rem2* knockout caused a decrease in expression of at least two genes that regulate synapse formation: Leucine Rich Repeat Transmembrane Neuronal 4 (*Lrrtm4*) and Glypican-5 (*Gpc5*). *Gpc5* is a GPI-linked heparan sulfate proteoglycan that can act as a ligand for *Lrrtm4* to promote excitatory synapse formation [24].

In a separate set of experiments, we identified *Rem2* and activity-dependent changes in gene expression by depolarizing *Rem2* knockout or control neurons with high extracellular

potassium followed by RNA-Seq. Using this approach, we identified 217 genes whose expression was altered by *Rem2* knockout in an activity-dependent manner. We found that in the context of neuronal depolarization, the expression of 94 genes is normally promoted by *Rem2* signaling while expression of 123 genes is normally repressed by *Rem2* signaling. Our results suggest that *Rem2* is an important component of a signaling network that translates changes in neuronal activity to changes in gene expression and ultimately, synaptic connectivity.

Results and Discussion

Rem2 knockout results in altered gene expression

We sought to identify genes whose expression is modulated by *Rem2* using next generation RNA-sequencing of samples isolated from cortical neurons in the presence or absence of *Rem2*. To perform these studies, we took advantage of a Cre-dependent conditional allele of *Rem2*, *Rem2^{flx/flx}*, that was generated in our laboratory [25]. In brief, exons 2 and 3 of the *Rem2* allele, which encode the GTP-binding domain, were flanked by LoxP sites. Therefore, when Cre recombinase is expressed, exons 2 and 3 are excised, resulting in a *Rem2* null allele [25].

Dissociated cortex from E16 *Rem2^{flx/flx}* mice was cultured at high density and infected at 1 DIV with either a control virus (AAV-GFP) or a virus expressing Cre-recombinase (AAV-Cre-GFP) to knockout *Rem2*. By DIV 5, GFP expression was observed in approximately 85% of the neurons in each condition. All neurons were treated with the voltage-gated sodium channel blocker, tetrodotoxin (TTX), overnight to suppress activity-dependent transcriptional programs in the culture. On DIV 6, total RNA from cells cultured in each condition (Control and *Rem2* KO) was harvested and reverse transcribed. For each biological replicate, quantitative PCR (q-PCR) was performed to verify that *Rem2* exons 2 and 3 were deleted (Fig. 1A). Next, we enriched mature mRNA from total RNA pool by performing a polyA selection and prepared RNA-seq libraries using standard protocols for high throughput sequencing. We confined our analysis to differential expression of protein coding genes only.

To begin, we determined the effect of knocking out *Rem2* on gene expression. To that end, we compared the gene expression profile in the control neurons to the expression profile of *Rem2* KO neurons. When analyzing the RNA-sequencing data, a gene was considered to be a “hit” if the FPKM (fragments per kilobase of transcript per million mapped reads) was greater than 0.5, its expression changed 2-fold in the *Rem2* knockout condition compared to the control condition, and the expression changes were observed in both biological replicates. We found that knockout of *Rem2* led to increased expression (using our 2-fold cutoff) of 11 protein-coding genes, suggesting that *Rem2* signaling normally functions to inhibit the expression of these genes (Fig. 1B). We also found decreased expression of 13 protein-coding genes, suggesting that *Rem2* normally functions to promote expression of these genes (Fig. 1B). From these data, we conclude that *Rem2* signaling both positively and negatively regulates gene expression.

To confirm that the results from our RNA-sequencing analysis were representative of the changes in gene expression observed between conditions in our biological replicates, we performed qPCR using probe sets designed to quantify expression of 11 genes identified by our RNA-seq experiment. We chose 6 genes whose expression decreased and 5 genes whose expression increased with *Rem2* KO. For the qPCR validation, we probed RNA from each RNA-seq biological replicate and RNA from a third, independent biological replicate that was not subjected to deep sequencing. We found that 10 of the 11 genes identified as having significant expression changes by RNA-seq were validated by qPCR in all three biological replicates tested (Fig. 1C). Expression of the 11th gene, *Klhl4*, shows a small but not significant decrease in the *Rem2* KO compared to control by qPCR from the first biological replicate, so its expression in the subsequent biological replicates was not determined (Fig. S1). Taken together, these data suggest that our RNA-seq experiment has a false discovery rate of <10%.

Knockdown of *Rem2* target genes *Lrrtm4* and *Gpc5* decrease excitatory synapse density

Rem2 is a positive regulator of excitatory synapse formation [16, 18]. Therefore, we reasoned that at least one function of a subset of genes affected by *Rem2* deletion could be to regulate synapse formation. Further support of this hypothesis comes from the fact that it has been previously reported that *Lrrtm4*, whose expression is decreased upon *Rem2* deletion (Fig. 1B–C), promotes excitatory synapse formation [24, 26]. In addition, *Gpc5* acts as a ligand for *Lrrtm4* to mediate synapse formation [24]. However, a loss of function analysis of *Gpc5* in neurons has never been performed.

We obtained siRNA Smartpools (Dharmacon) targeting *Gpc5* and *Lrrtm4* and assayed the effect of RNAi-mediated knockdown on excitatory synapse density in cultured neurons. To perform these experiments, we utilized our previously established assay to quantify excitatory synapse formation [14–18]. Hippocampal neurons were sparsely transfected with GFP and the siRNA Smartpools using the calcium phosphate method such that the vast majority of synaptic connections made onto the transfected neurons are from non-transfected, “wildtype” neurons. Thus, the sparseness of our transfection is a key element of the experimental design as it allows us to test a role for the targeted gene in the postsynaptic neuron only. This approach differs from the high percentage of transduced *Rem2*^{flx/flx} cultured neurons achieved with Cre-expressing AAV infection which was necessary for our RNA-seq experiments. As a result, we also included a well-validated RNAi reagent that specifically targets *Rem2* [14–18] in our sparse transfection paradigm as a positive control for decreased excitatory synapse density. We have previously shown that the decreased synapse density observed with transfection of this *Rem2* shRNA can be rescued by co-transfection of a vector encoding an RNAi-resistant *Rem2* cDNA [14–17].

Neurons were fixed at 14 DIV and stained for the excitatory synaptic markers PSD-95 and Synapsin. The transfected neurons were then imaged and analyzed for changes in excitatory synapse density by quantifying the co-localization of PSD-95 and Synapsin on a transfected neuron. We found, as previously reported [24, 26], that knockdown of *Lrrtm4* expression led to a decrease in excitatory synapse density, as did knockdown of *Rem2* (Fig. S2A, B). This

result serves to verify the biological significance of our RNA-seq findings and further, places Rem2 signaling upstream of and positively regulating *Lrrtm4* expression.

Interestingly, we also observed that knockdown of *Gpc5* expression was sufficient to decrease excitatory synapse density (Fig. S2A, B), consistent with the hypothesis that *Rem2* promotes the expression of genes that regulate synapse formation. In addition, these data support the model that *Gpc5* is a ligand for *Lrrtm4* in regulating synapse formation, as knockdown of *Lrrtm4* and *Gpc5* yields similar phenotypes. Lastly, these data add new insight into a possibly novel signaling mechanism of *Gpc5* and *Lrrtm4* as knockdown of either gene in the postsynaptic neuron causes a decrease in synapse density, suggesting that *Gpc5* could interact with *Lrrtm4* in *cis* to promote synapse formation.

We sought to confirm that the decreased synapse density phenotype we observed with the knockdown of *Lrrtm4* and *Gpc5* was due to the knockdown of the targeted gene and not an off-target effect of the siRNA pools. To accomplish this, we designed an shRNA targeting *Gpc5* and utilized a previously published shRNA targeting *Lrrtm4* [26]. We also engineered *Lrrtm4* and *Gpc5* cDNA constructs containing silent mutations in the nucleotide sequence targeted by the respective shRNAs, rendering the cDNAs RNAi resistant. We confirmed that the shRNAs knocked down *Lrrtm4* or *Gpc5* protein expression and that the RNAi resistant cDNAs were indeed resistant to shRNA targeting in HEK 293T cells by Western blotting of cellular lysates (Fig. S2C).

Next, we transfected cultured hippocampal neurons with shRNAs targeting *Lrrtm4* or *Gpc5* alone or with the appropriate RNAi resistant cDNA; the cultures were subsequently processed for immunostaining for synaptic markers at 14 DIV. We found that decreased expression of either *Lrrtm4* or *Gpc5* resulted in a decrease in excitatory synapse density which could be rescued to control synapse density upon co-transfection of the corresponding RNAi resistant cDNA (Fig. 2). These results demonstrate that the loss of synapse density was due to knockdown of either *Lrrtm4* or *Gpc5* specifically, and not to an off-target effect of the RNAi. Taken together, our data indicate that the function of both *Lrrtm4* and *Gpc5* is required in postsynaptic neurons to regulate the formation of excitatory synapses. Glypican proteins can be either membrane associated or cleaved from the cell surface by phospholipases [27], suggesting multiple possible signaling configurations for *Gpc5* and *Lrrtm4*. Taken together with previous studies demonstrating that *Gpc5* can signal through *Lrrtm4* to mediate synapse formation [24], our data suggests that *Gpc5* may signal in an autocrine manner to regulate this process.

Overexpression of *Lrrtm4* or *Gpc5* restores the Rem2 RNAi-dependent decrease in synapse density

In order to determine if *Lrrtm4* and *Gpc5* function downstream of Rem2 in a signaling pathway that promotes excitatory synapse formation, we performed a series of epistasis experiments in cultured hippocampal neurons. We asked if overexpression of either *Lrrtm4* or *Gpc5* could restore decreased synapse density that occurs upon Rem2 knockdown by RNAi. Cultured hippocampal neurons were transfected at 4DIV with either a shRNA targeting *Rem2* or cDNAs that express either *Lrrtm4* or *Gpc5*. We also co-transfected Rem2 shRNA and *Lrrtm4* cDNA, or Rem2 shRNA and *Gpc5* cDNA. At 14 DIV we fixed and

stained the neurons as described above to assay excitatory synapse density. We found that overexpression of either *Lrrtm4* or *Gpc5* alone significantly increased excitatory synapse density, suggesting that both *Lrrtm4* and *Gpc5* are sufficient to promote excitatory synapse density [24] (Fig. 3). This result again suggests that *Gpc5* acts in an autocrine manner to promote synapse formation, as overexpression of *Gpc5* increases synapse density in that same neuron.

Importantly, overexpression of either *Lrrtm4* or *Gpc5* was able to compensate for the decreased synapse density observed upon *Rem2* knockdown and restore synapse density to control levels (Fig. 3). One interpretation of this experiment is that *Lrrtm4* and *Gpc5* function downstream of *Rem2* in a linear signaling pathway: *Rem2* promotes the expression of *Gpc5* and *Lrrtm4* who signal together, in the same cell, to promote synapse formation. Thus, in the absence of *Rem2*, increased expression of either the receptor *Lrrtm4* or its ligand *Gpc5* can promote excitatory synapse formation. Alternatively, it could be that *Rem2*, *Lrrtm4*, and *Gpc5* function in parallel pathways to regulate synapse formation. Given our RNA-seq data indicating that *Rem2* signaling promotes the expression of *Lrrtm4* and *Gpc5* (Fig. 1), we believe that the linear pathway model is the most parsimonious explanation for our results.

***Rem2* regulates activity-dependent gene expression**

Treatment of cultured rodent neurons with high extracellular potassium has been shown to mimic the cellular response to neuronal activity. Increased activity stimulates the transcription of immediate early genes (IEGs) such as *Fos*, *Jun* and *Egr1* which encode transcription factors [28, 29]. This process occurs in a transcription-independent manner [30]. Once active, IEGs promote the transcription of late response genes in an activity-dependent and cell-type specific manner [4, 6, 28, 31–33]. Importantly, these transcriptional responses mimic gene induction events observed in intact rodent brain in response to physiological stimuli such as visual experience, seizures and cocaine exposure [34–37].

Therefore, in parallel to the above experiments, we performed RNA-seq using RNA isolated from control and *Rem2* KO neurons that were depolarized with high extracellular potassium (55mM KCl) for 4 hours to determine the effect of *Rem2* deletion on the subset of genes that are specifically upregulated in response to neuronal depolarization. Our previous work demonstrated that a 4 hour exposure to high extracellular potassium produced a robust increase in *Rem2* mRNA expression while still displaying a significant induction of the IEG *Fos* [15]. For each biological replicate, we performed qPCR to confirm that the expression of the activity-regulated genes *Rem2* and *Fos* were upregulated following a 4 hour treatment of high extracellular potassium (KCl, Fig. 4A).

Using this experimental paradigm, we identified 316 genes whose expression increased greater than 2-fold following KCl stimulation in the control condition (i.e. infection with AAV-GFP virus; Fig. 4B, yellow circle). Included among this gene set are known activity-regulated genes *Fos*, *Jun* and *Npas4* (Table. S2). Additionally, we found increased expression of 345 genes upon KCl stimulation in the absence of *Rem2* (Fig. 4B; blue circle). Comparison of these two data sets revealed that 222 of the genes were upregulated both in the presence and absence of *Rem2*, indicating that *Rem2* signaling has no effect on their

expression in the context of KCl stimulation (Fig. 4B; green, Table. S2). Thus, expression of 94/316 genes were upregulated upon KCl stimulation in a *Rem2*-dependent manner, indicating that Rem2 signaling is required to increase expression of these genes in the context of neuronal depolarization (Fig. 4B; yellow, Table. S3). Conversely, expression of 123/345 genes were up-regulated in response to KCl stimulation only in neurons in which *Rem2* was deleted, suggesting that Rem2 signaling normally functions to inhibit expression of these genes in response to neuronal depolarization (Fig. 4B; blue, Table. S4).

We validated the expression profiles for 12 differentially regulated genes, 6 from the 94 genes promoted by Rem2 signaling and 6 from the 123 genes repressed by Rem2 signaling. For the qPCR validation, we probed RNA from each RNA-seq biological replicate and RNA from a third, independent biological replicate that was not subjected to deep sequencing. We found that 5/6 genes whose expression is promoted by Rem2 signaling and 4/6 genes whose expression is repressed by Rem2 signaling matched the expression profile identified in our RNA-seq experiments (Fig. 4C). The qPCR expression profiles for the genes that did not recapitulate results from the RNA-seq are shown in Figure S3. Surprisingly we observed that, with the exception of the gene *Slc7a1*, the set of genes regulated by Rem2 signaling at basal levels of activity versus those regulated with KCl stimulation were non-overlapping. Specifically, our data demonstrate that *Slc7a1* mRNA expression decreases with *Rem2* knockout (Fig. 1), but is upregulated upon *Rem2* deletion with KCl stimulation (Fig. 4C). Thus, without stimulation, Rem2 signaling promotes *Slc7a1* expression while KCl stimulation causes a switch, perhaps in signaling pathways upstream of Rem2, such that Rem2 signaling represses *Slc7a1* expression. Taken together, these data indicate that depletion of *Rem2* modifies the neuronal transcriptome in a complex manner in response to changes in neuronal activity.

Rem2 signaling promotes the expression of genes that encode membrane targeted proteins

We next utilized the DAVID functional annotation classification tool [38, 39] to further analyze the subset of genes whose expression is promoted by Rem2 signaling (Fig. 4B, yellow circle). The DAVID algorithm groups genes from large datasets together by their known biological process, cell component, or molecular function [38, 39], by identifying enriched gene ontology (GO) terms in a given dataset. We began our gene ontology analysis by determining the cell component classification for genes whose expression is promoted by Rem2 signaling (Fig. 5; purple bars, Table. S5). Only GO terms that were significantly enriched ($P < 0.05$) using Fisher's exact test were considered in the analysis. We found that enriched GO terms for genes promoted by *Rem2* signaling encode a multitude of membrane protein classifications including: postsynaptic membrane, integral component of plasma membrane, membrane, presynaptic membrane and plasma membrane (Fig. 5; purple bars, Table. S5). Further, we observed that a large proportion of these genes encode ion channels and receptors including *Kcna1*, *Grm2* and *Gabrb3* (Table. S5). We validated the expression profiles of *Grm2* and *Gabrb3*, two genes identified by the GO analysis as membrane targeted, confirming that Rem2 signaling does promote the expression of genes that code for membrane localized proteins (Fig. 4C).

One well-established role for Rem2 is to promote the formation of excitatory and inhibitory synapses [16–18]. Therefore, finding that Rem2 signaling promotes the expression of genes which encode membrane localized protein. A number of well-described synaptogenic factors are membrane associated proteins [11], suggesting that these genes might represent additional molecules through which Rem2 mediates synapse formation. Interestingly, the DAVID GO analysis also suggests a role for Rem2 signaling in synaptic transmission, a previously unexplored output of Rem2 signaling. We found that Rem2 signaling promotes the expression of *Snap25* and *Gabrb3*, which encode the presynaptically localized protein SNAP25 and postsynaptically localized $\beta 3$ subunit of the GABA_A receptor. SNAP25 is part of the SNARE complex which is critical for vesicle fusion and neurotransmitter release at both excitatory and inhibitory synapses [40–42]. In addition, the GABA_A receptor is a ligand-gated channel that is a crucial receptor responsible for mediating inhibitory neurotransmission in the brain [43, 44]. Mutations in *Gabrb3* have been linked to childhood absence epilepsy [45]. Further, *Kcna1*, identified here as a gene whose expression is promoted by Rem2 signaling, encodes the potassium channel Kv1.1, which localizes to axons and dendrites and is specifically enriched in synaptosomes [46]. Taken together, these data suggest that in response to neuronal depolarization, Rem2 signaling promotes the expression of membrane targeted genes required for multiple aspects of synaptic function, a previously unsuspected role for this protein.

Rem2 signaling represses the expression of genes that encode proteins that regulate the cytoskeleton

As above, we used the DAVID functional annotation classification tool [38, 39] to further analyze the subset of genes whose expression is repressed by Rem2 signaling (Fig. 4B; blue circle). Using cell component analysis, we identified uniquely enriched GO terms in our list of genes whose expression is repressed by Rem2 signaling: actin filament, early endosome, synapse, intracellular and cell-cell adherens junction classifications (Fig. 5; blue bars, Table. S6). We found that many of the genes repressed by Rem2 signaling encoded proteins that localized to the cytoplasm or specifically associate with actin filaments including *Cpeb2*, *Rhoq* and *Dlc1*. Using q-PCR, we validated the expression profile of *Cpeb2* using RNA from all three biological replicates to confirm our result that Rem2 signaling represses its expression in an activity-dependent manner (Fig. 4C).

Another known function of Rem2 is as an activity-dependent negative regulator of dendritic branching [14–16]. Interestingly, we find that *Rem2* signaling represses the induction of *Cpeb2*; the closely related family member *Cpeb1* promotes dendritic elaboration [47]. Both *Cpeb2* and *Cpeb1* are RNA-binding molecules and while little is known about the function of *Cpeb2*, *Cpeb1* was identified in the *Xenopus laevis* visual system to be required for proper dendritic outgrowth *in vivo* [47]. Thus, *Rem2* repression of *Cpeb2* could represent one molecular mechanism by which Rem2 inhibits activity-dependent dendritic branching.

Additionally, Rem2 signaling negatively regulates the expression of the actin cytoskeleton regulating genes *Rhoq* and *Dlc1*. *Dlc1* can act as a GTPase activating protein (GAP) for several Rho GTPases including CDC42 and Rho A–C which have been implicated in cytoskeleton regulation [48]. Furthermore, the Rho GTPase *Rhoq* promotes neurite

outgrowth through a Rab11 and Exo70 dependent mechanism [49]. Therefore, repression of *Rhoq* and *Dlc1* expression by Rem2 signaling could represent another mechanism by which Rem2 restricts dendritic complexity.

Taken together, our genetic analysis suggests a formerly unappreciated mechanism where Rem2 signaling promotes the expression of genes that encode membrane proteins in response to increased neuronal depolarization, while inhibiting the expression of genes that encode cytosolic signaling proteins in this same context (Fig. 5). We postulate that these results represent a functional “division of labor” of Rem2 signaling that matches previously identified Rem2 cell biological outputs: promoting the expression of genes that build synapses, while repressing the expression of genes that promote dendrite growth and branching.

Rem2 regulates the activation of activity-dependent transcription factors

One known output of CaM kinase signaling is the activation of the activity-regulated transcription factor CREB [50, 51]. Given our RNA-seq data, coupled with our previously published results demonstrating that Rem2 functions in a signaling network with CaM kinases [14], we sought to determine if Rem2 knockdown affected the activity-dependent activation of CREB. To address this issue, we quantified CREB transcriptional activity using CRE (CREB response element) reporter dual luciferase assays. We transiently transfected cultured neurons with a reporter construct containing 4× CRE sites upstream of a minimal promoter driving firefly luciferase [52]. As a control, we transfected an SP1 reporter construct containing GC box promoter elements upstream of a minimal promoter driving firefly luciferase [53]; SP1 activation should not be dependent on neuronal depolarization. Rat cortical neurons were chosen for this experiment as they are particularly robust to both the culture and transfection paradigm used in this experiment. Neurons were transfected at 4 DIV with the CREB or SP1 luciferase reporter plus a plasmid expressing renilla-luciferase under control of a thymidine kinase promoter, and either a control shRNA (Control) and our verified shRNA targeting Rem2 (Rem2 RNAi) [16–18]. At 6 DIV, all neurons were treated with TTX overnight, thereby lowering the baseline activation of activity-dependent transcriptional programs in our cultures. At 7 DIV, a subset of neurons from each condition were left untreated, or treated with 55mM KCl to depolarize the neurons and activate CREB [52]. After 6 hours, cell lysates were harvested and a dual luciferase assay performed to quantify firefly and renilla luminescence in each condition [54].

As expected, we found that KCl stimulation was sufficient to induce CREB activation (Fig. 6A). Importantly, we found that RNAi-mediated knockdown of Rem2 led to a significant decrease in the KCl-induced activation of CREB (Fig. 6A, filled bars). This result suggests that Rem2 normally functions to promote the activation of CREB in response to neuronal depolarization. In addition, close examination of the untreated condition revealed that RNAi-mediated knockdown of Rem2 increases CREB activity, suggesting that in the absence of neuronal activity, Rem2 functions to repress transcriptional activation of CREB (Fig. 6A, hollow bars). Taken together, these data imply that Rem2 has differential effects on CREB transcriptional pathways, depending on the activity state of the cell. As predicted, neither

Rem2 knockdown nor KCl stimulation had a significant effect on the activation of SP1 (Fig. 6B).

Given the effect of Rem2 RNAi on CREB activation, we asked whether depletion of Rem2 altered the activation of other activity-regulated transcriptional pathways by examining the activation of the transcription factors MEF2, NFAT, RARE and NF- κ B using dual luciferase assays (see methods for details about these plasmids). Interestingly, in untreated conditions, Rem2 negatively regulates MEF2, NFAT and NF- κ B pathways and positively regulates the RARE pathway (Fig. 6C–F). One interpretation of these data is that Rem2 acts upstream in a common signaling pathway that regulates multiple activity-dependent transcriptional programs. Alternatively, it could be that Rem2 plays a modulatory role in a number of independent signaling pathways that regulate activity-dependent transcriptional programs. Interestingly, a recent publication identified another RGK family member, Rad, as a modulator of the NF- κ B pathway [55]. This study demonstrated that Rad binds to the RelA/P65 subunit of NF- κ B causing decreased promoter binding and subsequently decreased expression of NF- κ B target genes [55]. Thus, we hypothesize that regulation of gene expression is an unappreciated role for RGK family members.

CREB-dependent genes are not particularly enriched in the activity-dependent, Rem2-dependent gene set

Since our data demonstrates that Rem2 affects CREB transcriptional activity, and our previous work implicated Rem2 in a CaMK signaling pathway [14], we sought to determine if Rem2 preferentially modulated the expression of genes regulated by CREB. To accomplish this, we compared our dataset of genes regulated by *Rem2* with two previously published datasets examining CREB or CBP (CREB binding protein) occupancy on the promoters of genes using ChIP-seq [33, 56]. Interestingly, we found that approximately 80% of genes promoted or repressed by *Rem2* signaling had been identified in the ChIP-seq datasets to have either CREB or CBP occupancy at their promoters. These genes include the previously characterized CREB-regulated genes *Grm2*, *Npas2* and *Per1* [57–59]. Somewhat surprisingly, we failed to observe a change in *Fos* expression with Rem2 knockout using our RNA-seq approach (Fig. 4A), despite the fact that we observe a change in CREB transcriptional activation using a luciferase reporter assay (Fig. 6A). We postulate that this discrepancy is due to the fact that the endogenous *Fos* promoter contains numerous binding sites for other transcription factors which contribute to *Fos* activation. Conversely, the CRE-luciferase reporter construct (Fig. 6A) only interrogates CREB activation without the influence of additional factors. Consequently, changes in Rem2 signaling that may modulate CREB activity are detectable in our 4 $\times$ CRE-luciferase reporter assay, but are not dramatic enough to produce a change in *Fos* expression at its endogenous locus.

Next, we compared the *Rem2*-independent genes (green overlap, Fig. 4B) to the genes identified in the CREB or CBP ChIP-seq and found that 86% and 88% of these genes had CREB or CBP occupancy respectively. For comparison, we also determined the CREB occupancy of a subset of genes that were neither regulated by neuronal depolarization nor *Rem2* signaling. To accomplish this, we randomly picked 100 protein-coding genes whose expression was unchanged with *Rem2* KO or neuronal depolarization and cross referenced

that list to the genes from the CREB or CBP ChIP-seq analyses. We found that 74% and 63% of the activity and *Rem2*-independent genes were identified as having CREB or CBP binding sites in their promoters, respectively. Taken together, these analyses suggest that neuronal depolarization upregulates CREB-dependent genes both in the presence and absence of *Rem2*; however, CREB-dependent genes are not particularly enriched in the subset of genes whose expression is both activity- and *Rem2* dependent. These findings favor our hypothesis that *Rem2* plays a modulatory role in a number of independent signaling pathways that regulate activity-dependent transcriptional programs.

Conclusions

The proper development, maintenance, and function of brain circuits depends critically on both genes and activity. The molecular programs that alter neural circuits in response to activity are still poorly understood. Our lab discovered that the activity-regulated GTPase *Rem2* is a key molecule linking sensory experience to changes in synapse and dendrite development. Specifically, *Rem2* promotes synapse formation, yet inhibits activity-dependent increases in dendritic complexity [14–16, 18]. However, the signaling mechanisms by which *Rem2* and other RGK proteins mediate these processes are not well-understood. Our exploration of changes in gene expression in response to *Rem2* deletion represents a critical first step towards uncovering these signaling networks.

Our unbiased genetic analysis of *Rem2*-dependent changes in gene expression led to the finding that expression of known synaptogenic factors *Lrrtm4* and *Gpc5* are regulated by *Rem2* signaling (Fig. 1, 2). Thus, we have uncovered a novel signaling pathway that further elucidates the genetic pathway by which neurons regulate synapse formation (Fig. 3). Further, we found that *Rem2* signaling both promotes and represses the expression of non-overlapping sets of genes in the context of neuronal depolarization (Fig. 4), indicating that the role of *Rem2* signaling in the regulation of the transcriptional response to neuronal depolarization is complex. Additionally, we were surprised to discover that *Rem2* modulates the activity of several activity-dependent transcription factors (Fig. 6), suggesting a general role for *Rem2* as a modulator of activity-dependent transcriptional programs.

Interestingly, inhibition of high-voltage activated Ca^{2+} channels (VGCCs; Ca_v1 and Ca_v2 families) by overexpression of *Rem2* and other RGK family members has been reported in numerous cell types [17, 60, 61], suggesting that *Rem2*-dependent effects on gene expression could be due to increased calcium flux through VGCCs that occurs upon *Rem2* deletion. However, a number of lines of evidence undermine this model. First, a previously published electrophysiological study from our lab [17, 62] and others [17, 62] using verified RNAi reagents to knock down *Rem2* in cultured neurons, failed to uncover a role for *Rem2* loss of function in modulation of calcium currents. In addition, we found no difference in the resting Ca^{2+} levels of cortical neurons obtained from *Rem2*^{-/-} mice compared to WT controls using ratiometric Ca^{2+} imaging [25]. The Ikeda lab also reports a similar result using an electrophysiological approach: no change in calcium currents in dissociated cultures of striatal medium spiny neurons in which *Rem2* was deleted [63]. Taken together, existing data do not support the hypothesis that loss of *Rem2* affects calcium flux into the cell.

In summary, we propose that Rem2 functions at the nexus of signaling pathways that sense and respond to changes in neuronal activity to modulate both structural and functional plasticity, in part by regulating changes in gene expression. Our gene ontology analysis suggests that *Rem2* signaling primarily promotes the expression of genes that code for membrane localized proteins that regulate synaptic transmission and represses the expression of genes that encode cytoplasmic regulator proteins that affect the cytoskeleton, consistent with Rem2 function in promoting synapse formation and repressing dendritic branching (Fig. 5). Therefore, we favor a model where Rem2 functions as a cytosolic signaling molecule to mediate changes in gene expression in the nucleus, perhaps by interacting with the CaMK signaling pathways [14, 64].

Materials and Methods

Ethics Statement

All experimental procedures involving the use of animals were approved by the Institutional Animal Care and Use Committee at Brandeis University.

Neuronal cell culture and transfection

Hippocampal neurons from E18 Long Evans rats of either sex were cultured in Neurobasal media with B27 Supplement (Invitrogen) at a low density (8×10^4) cells/well in a 24 well dish on a confluent astrocyte feeder layer, as previously described [16]. AraC (Sigma) was added 24 hours after neuron plating to a final concentration of 5 μ M. For synapse assays, neurons were transfected at 4 DIV using the calcium phosphate method [65]. All conditions were transfected with a CMV-GFP plasmid at 500ng/well. For control conditions, neurons were also transfected with an empty pSuper plasmid at either 33 or 100ng/well. For Rem2 RNAi, neurons were also transfected with a pSuper plasmid containing an shRNA targeting Rem2 [18] at 33ng/well. For siRNA knockdown of *Lrrtm4* and *Gpc5*, neurons were also transfected with pools of 4 siRNAs targeting the indicated gene at 20nM. For *Lrrtm4* or *Gpc5* RNAi, neurons were also transfected with a pSuper plasmid containing an shRNA targeting *Gpc5* or *Lrrtm4* [26] expressed at 33ng or 100ng/well respectively. It has been previously reported that co-transfection efficiency of up to three plasmids is 93% [14, 16]. On DIV 8 and 11, half of the neuronal media is removed and replaced with fresh Neurobasal with B27 supplement and 5 μ M AraC.

Synapse assays

At 14 DIV, the neuronal media was replaced with 1 $\times$ PBS and fixed using 4% paraformaldehyde/4% sucrose for 8 minutes at room temperature. The coverslips were then washed three times with 1 $\times$ PBS and moved to a humidified chamber. Neurons were co-stained with mouse anti PSD-95 (1:500; Neuromab) and rabbit anti Synapsin I (1:500; Fisher) diluted in 1 $\times$ GDB (0.1% gelatin, 0.3% Triton X-100, 4.2% of 0.4M phosphate buffer and 9% 5M NaCl). The coverslips were incubated with primary antibodies overnight at 4°C and then washed three times with 1 $\times$ PBS. Cy3- or Cy5-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were diluted 1:500 in 1 $\times$ GDB and incubated on coverslips for two hours at room temperature. Coverslips were then washed

three times with 1× PBS and mounted on glass slides using Aqua-mount mounting media (Lerner Laboratories).

12-bit images of GFP transfected neurons were taken on an Olympus Fluoview300 confocal microscope using a 60× oil objective. Identical settings within biological replicates were used for laser power, amplifier offset and detector gain. Z-stacks of 5–10 optical sections with a step size of .5µm were taken for each neuron. Images of GFP-expressing neurons from three separate coverslips were taken for each condition for each biological replicate. Maximum intensity projections were made from the z-stacks of each neuron using ImageJ (NIH). Synapse density was quantified using Metamorph image analysis software and was defined as the overlap of GFP, anti-PSD-95 and anti-Synapsin staining. For each biological replicate, a threshold was set for Synapsin and PSD-95 staining using a control transfected neuron. The set threshold was then applied to every neuron within that particular experiment. The average synapse density for each condition was then calculated. At least two biological replicates were analyzed for each condition.

HEK 293T cell culture and western blotting

HEK 293T cells were grown in a 12 well plate and transfected using the calcium phosphate method [65] with 200 ng GFP (GFP), or 200 ng GFP and either 100 ng myc-Lrrtm4 (Lrrtm4 OE), 100 ng myc-Lrrtm4 and 1 µg shRNA targeting Lrrtm4 (Lrrtm4 RNAi), 1 µg shRNA targeting Lrrtm4 and 100 ng RNAi resistant Lrrtm4 cDNA (Lrrtm4 Rescue), 100 ng myc-Gpc5 (Gpc5 OE), 100 ng myc-Gpc5 and 1 µg shRNA targeting Gpc5 (Gpc5 RNAi), or 1 µg shRNA targeting Gpc5 and 100 ng RNAi resistant Gpc5 cDNA (Gpc5 Rescue). For the Western blot, the HEK 293T cells were lysed in 3× Sample Buffer (SDS, bromphenol blue, 1M Tris pH 6.8, glycerol) and proteins were separated by electrophoresis on a 10% SDS/PAGE gel. Proteins were then transferred to a nitrocellulose membrane and blocked for one hour in a solution containing 5% milk in TBST. The membrane was then incubated overnight at 4°C with anti-myc (Santa Cruz Biotechnologies; 1:500) and anti-β-actin (Abcam; 1:5000) primary antibodies. The membrane was washed three times for 15 minutes in TBST and incubated in appropriate secondary antibodies (Licor; 1:5000) for two hours at room temperature. The membrane was washed three times for 15-minutes with TBST and developed using the Odyssey Western Blotting system (Licor).

Luciferase assays

Cortical neurons from E18 Long Evans rats of either sex were dissociated and plated at a high density (5×10^5 /well) in a 24 well dish. At 4 DIV, the neurons were transfected using the calcium phosphate method as described above. One well was transfected with a GFP plasmid at 500ng/well which was used as a transfection control. The firefly and renilla readings from the GFP well were subtracted from the firefly and renilla readings to remove background luminescence. All remaining wells were transfected with a thymidine kinase renilla plasmid at 200ng/well which was used as an internal control for transfection variability between wells in an experiment. All of the firefly reporters (CRE-Luc [52], MRE-Luc [66], SP1-Luc [53], NFAT-Luc [67], RARE-Luc [68] or NF-κB-Luc [69]) were transfected into neurons at 500ng/well. In conditions where Rem2 was knocked down (Rem2 RNAi), a pSuper plasmid containing a shRNA targeting Rem2 was also transfected

into neurons at 33ng/well. To make sure the effect we were seeing was from knocking down *Rem2* and not from activating the RNAi machinery, a control shRNA was transfected into control neurons at 33ng/well. At 6DIV, neurons were quieted overnight with 1 μ M TTX. On DIV 7, a subset of neurons was treated with 55mM KCl for 6 hours, lysed with passive lysis buffer (Promega) and assayed using a dual luciferase assay. Firefly luciferase was measured by adding 100 μ l of 75mM HEPES pH 8.0, 5mM MgSO₄, 20mM DTT, 100mM EDTA and 530 μ M ATP, .5mM D-luciferin and .5mM coenzyme A. Renilla luciferase was measured using 100 μ l of 25mM Na₄PPi, 10mM NaOAc, 15mM EDTA, .5M Na₂SO₄, 1M NaCl, and .1mM Coelenterazine, pH 5.0. All conditions were assayed at least three times in triplicate and normalized to renilla luciferase then to the control untreated condition for each firefly reporter.

Cell culture, infection, stimulation and harvesting of RNA for expression analysis by qPCR

Cortical neurons from E16 *Rem2^{flx/flx}* mice of either sex with either black or agouti coat color were harvested and dissociated as previously described. They were plated at a high density (1.5×10^7)/10cm dish in glia conditioned Neurobasal media with B27 supplement (Invitrogen). At 1DIV, neurons were infected with either AAV1.hSyn.eGFP.WPRE.bGH or AAV1.hSyn.HI.eGFP-Cre.WPRE.SV40 (UPenn virus core). On DIV 5, the neurons were quieted overnight with 1 μ M TTX and then on DIV6 a subset of neurons were treated with 55mM KCl for 4 hours. RNA was extracted using Trizol Reagent (Invitrogen) following the manufacturer's protocol and DNase free RNA was then reverse transcribed using Random Primer Mix (New England Biologicals). Quantitative PCR was run using IQ-SYBR Green Supermix (Quanta Biologicals, VWR) on a Rotor Gene thermal cycler (Roche). Bands from genes of interest were normalized to β -actin (*Actb*) and shown as fold change over baseline using the delta-delta T_T method [70]. Primers for *Rem2* and *Fos* were previously validated [15] and the additional primers listed in Table 1 were validated by analyzing melting curves for reproducibility.

RNA-sequencing and Analysis

500ng of total RNA from each condition (Control, *Rem2* KO, Control + KCl, *Rem2* KO + KCl) was used to make mRNA libraries. mRNA was isolated, fragmented into regions about 300bp in length and ligated to condition specific adaptors using the TruSeq RNA library prep kit (Illumina). The quality of the libraries from each biological replicate were assayed using a 2100 BioAnalyzer (Agilent technologies). The libraries were sequenced on an Illumina HiSeq 2000 deep sequencer following the standard protocol for 75bp single-end non-stranded reads. The libraries were then analyzed using Galaxy (usegalaxy.org) [71]. The reads were mapped to the mm10 mouse genome using TopHat (Galaxy version 2.1.0) and FPKM for known protein-coding genes was determined using Cufflinks (Galaxy version 2.2.1.0). Fold changes in gene expression between conditions in each biological replicate were calculated manually using Microsoft Excel. The RNA-sequencing data from this publication has been deposited to GEO. GEO accession number: GSE97891.

Statistics

Within each biological replicate, the average synapse density value was obtained for control and each experimental condition. The normalized synapse density for each experiment

represents the experimental average synapse density divided by the control synapse density. The standard error of this ratio is then determined using the following equation:

$$\text{std error of the ratio} = \text{ratio} * \sqrt{[(\text{std error experimental} / \text{mean experimental})^2 + (\text{std error control} / \text{mean control})^2]}$$

The normalized synapse density values from each biological replicate were averaged for each condition and the error was calculated as follows:

$$\text{std error of the average ratio} = \sqrt{[(\text{std error of the ratio})^2 + (\text{std error of the ratio})^2 + \dots + (\text{std error of the ratio})^2] / n}$$

Statistical analysis was determined comparing each experimental condition to control using combined raw data from all biological replicates using SPSS Software (version 24) to run a two-way between- effect ANOVA followed by Tukey's post-hoc test for significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Rem2 signaling regulates gene expression.
- Rem2 signaling promotes the expression of known synaptogenic factors *Lrrtm4* and *Gpc5*.
- The Gpc5 ligand functions in the postsynaptic neuron to regulate excitatory synapse formation.
- Rem2 both promotes and represses gene expression in the context of neuronal depolarization.
- Rem2 knockdown modulates activity-dependent transcriptional pathways.

Figure 1. Knockout of *Rem2* results in changes in gene expression

A) Fold change in *Rem2* mRNA by qPCR after 5 days of Cre-mediated *Rem2* exon 2 and 3 deletion. mRNA levels were first normalized to *Actb* and then to the control condition within each replicate then averaged across biological replicates. Data is presented as mean $\pm$ SEM. *Rem2* KO condition is significantly decreased from control by student's t-test ($P < 0.05$) **B)** RNA-seq results: Genes up or downregulated greater than 2-fold with *Rem2* knockout. Orange bars indicate genes whose expression change with *Rem2* knockout was validated by qPCR while the white bar shows the gene whose expression profile that did not recapitulate the RNA-seq results. The gray bars show genes that were not chosen for qPCR validation. Data is presented as the mean fold change across biological replicates in FPKM (Fragments per kilobase of transcript per million mapped reads) from control $\pm$ SEM. **C)** Validation of gene expression changes (of the genes shown in orange in **B**) using qPCR.

Fold change in mRNA expression for each indicated gene was averaged across 3 biological replicates. First, mRNA levels were normalized to *Actb* and then to the control condition. Data is presented as mean $\pm$ SEM. All *Rem2* KO conditions are significantly increased (top row) or decreased (bottom row) from controls by student's t-test ($P < 0.05$).

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Figure 2. Lrrtm4 and Gpc5 are required for proper excitatory synapse formation

A) Immunostaining for PSD-95 (red) and Synapsin I (blue) in the dendrites of transfected neurons. A synapse is defined as the overlap of red and blue puncta (shown in white) on a GFP positive dendrite. Scale bar = 5 μ m. **B)** Quantification of PSD-95/Synapsin puncta for neurons transfected with GFP and either: a pSuper empty vector (Control), shRNA targeting Rem2 (Rem2 RNAi), shRNA targeting Gpc5 (Gpc5 RNAi), shRNA targeting Gpc5 and an RNAi resistant Gpc5 cDNA (Gpc5 Rescue), shRNA targeting Lrrtm4 (Lrrtm4 RNAi) or a shRNA targeting Lrrtm4 and an RNAi resistant Lrrtm4 cDNA (Lrrtm4 Rescue). Data is presented as mean of normalized PSD-95/Synapsin puncta density $\pm$ SEM. Asterisks indicate $p < 0.05$ compared to control by two-way ANOVA with Tukey post-hoc test. Normalized values are the average of two biological replicates and $n = 30$ neurons for each condition.

Figure 3. Overexpression of Gpc5 or Lrrtm4 restores the Rem2 RNAi-dependent decrease in synapse density

A) Immunostaining for PSD-95 (red) and Synapsin I (blue) in the dendrites of transfected neurons. A synapse is defined as the overlap of red and blue puncta (shown in white) on a GFP positive dendrite. Scale bar = 5 μ m. **B)** Quantification of PSD-95/Synapsin puncta for neurons transfected with GFP and either: (Top) a pSuper empty vector (Control), shRNA targeting Rem2 (Rem2 RNAi), Gpc5 cDNA (Gpc5 OE), or shRNA targeting Rem2 and Gpc5 cDNA (Rem2 RNAi + Gpc5 OE) and (Bottom) a pSuper empty vector (Control), shRNA targeting Rem2 (Rem2 RNAi), Lrrtm4 cDNA (Lrrtm4 OE), or shRNA targeting Rem2 and Lrrtm4 cDNA (Rem2 RNAi + Lrrtm4 OE). Data is presented as mean of normalized PSD-95/Synapsin puncta density $\pm$ SEM. * indicates $p < 0.005$ and # indicates $P < 0.085$ between conditions indicated by bars using two-way ANOVA with Tukey post-hoc test. In both top and bottom graphs, control versus Rem2 RNAi was different at $P < 0.005$ but this was not indicated on the graph for clarity. Normalized values are the average of 2 biological replicates and $n = 32$ neurons for each condition.

Figure 4. *Rem2* knockout causes changes in activity-dependent gene expression

A) Fold change in *Rem2* or *Fos* mRNA expression by qPCR averaged across biological replicates. mRNA levels were first normalized to *Actb* and then to the control untreated condition within each biological replicate. Data is presented as mean $\pm$ SEM. **B)** Venn diagram showing the number of genes upregulated 2-fold following KCl stimulation. The 316 genes represented by the yellow circle were upregulated 2-fold by KCl treatment in control neurons. Of the 316 genes upregulated 2-fold in control neurons, 94 of those genes were only upregulated 2-fold in the control condition. The blue circle represents the 345 genes upregulated 2-fold by KCl stimulation in the *Rem2* KO condition. 123 of the 345 genes were upregulated 2-fold in the *Rem2* KO condition only. **C)** Validation of expression profiles of genes identified in panel **B**. Fold change in mRNA expression by qPCR for each indicated gene was averaged across biological replicates. Hollow bars represent untreated conditions while yellow bars represent KCl treated conditions for genes promoted by *Rem2* signaling and blue bars represent KCl treated conditions for genes repressed by *Rem2* signaling. mRNA levels were first normalized to *Actb* and then to the control untreated (UT) condition within each biological replicate. Data is presented as mean $\pm$ SEM. Data shown

for the untreated control and *Rem2* KO conditions for *Rem2* and *Slc7a1* are the same data as shown in Figure 1A and 1C respectively. The data is included here again for clarity.

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Figure 5. *Rem2* signaling promotes the expression of genes encoding membrane targeted proteins and represses the expression of genes encoding cytoplasmic proteins that regulate the cytoskeleton

DAVID Gene Ontology (GO) Cell Component analysis for genes whose expression is promoted by *Rem2* signaling (Left, purple) or repressed by *Rem2* signaling (Right, blue). Only the GO terms with a p value <0.05 by Fisher's Exact test were analyzed. The number of genes identified for each GO term are shown by each bar. The P value for each GO term is shown next to the corresponding bar.

Figure 6. Rem2 specifically regulates the activation of activity-dependent transcription factors
Quantification of firefly/renilla luciferase luminescence acquired from cortical neuron lysate from (A) CREB (B) SP1, (C) NFAT, (D) MEF2, (E) NF-κB or (F) RARE reporter transfected cells. Filled bars represent conditions treated with 55mM KCl for 6 hours before harvesting cell lysate while hollow bars represent untreated (UT) conditions. For each reporter, all conditions were normalized to the control UT condition within each biological replicate and averaged between replicates. Data is presented as mean of normalized luciferase activity $\pm$ SEM. * indicates $p < 0.05$ by Student's t-test between KCl treated conditions. # indicates $p < 0.05$ by Student's t-test between UT conditions. Each bar represents at least 3 biological replicates assayed in triplicate.

Table 1

Primers used for qPCR

Gene targeted		Sequence
<i>Actb</i>	Forward	ACGGTCAGGTCATCACTATCG
	Reverse	AGCCACCAATCCACACAGA
<i>Fos</i>	Forward	ATGGGCTCTCCTGTCAACACAC
	Reverse	ATGGCTGTACCGTGGGGATAAAG
<i>Rem2</i>	Forward	TGAGAGACGGATCATGGTGGACAA
	Reverse	AGCCGAAGAAGGGTTTCTGGAAGT
<i>Lrrtm4</i>	Forward	AGAAGAAAGCTGAGAGAGGGG
	Reverse	CATCTGCAGTTCTTTGGGCA
<i>Slc7a1</i>	Forward	CTCCTGGCTTACTCTTTGGTGG
	Reverse	GATCTAGCTCCTCGGTGGTTCT
<i>Npnt</i>	Forward	TGGGTCCTACAAGTGCCAAT
	Reverse	ATATGGTGTCTTCAGGGGCC
<i>Dcn</i>	Forward	CTGGCCAATGTTCTCATCT
	Reverse	AAGTCATTTTGCCCAACTGC
<i>Gpc5</i>	Forward	TGCTGGAATGGGGAGGAAAT
	Reverse	CTTGTTGGGCTTGGGTGATC
<i>Exo5</i>	Forward	GCTTCCTGGTTCGTTGACACCT
	Reverse	CCCAAGCATCTTCTTTGTGGCG
<i>Nif3</i>	Forward	CTACTACGGCAACAGAGACGCT
	Reverse	GGTGAGGTCTATTGGCTACCAC
<i>Renbp</i>	Forward	CGTGTTGAGCTTCTGGATGCAG
	Reverse	AAATGGTCCGCTGCACCTTAC
<i>Rasl12</i>	Forward	CCAAGGAGACACAACGTGGCTA
	Reverse	TCAAAGTCCAGGCAGGCAGAGA
<i>Glis3</i>	Forward	CCATACTTGTCAGCATCC
	Reverse	TCTCGGGAAGAATGTGCCTT
<i>Klhl4</i>	Forward	GTGGGAGGAAGAGATGGCTT
	Reverse	TAGCTCCATCCATCGTGACC
<i>Gabrb3</i>	Forward	CCTCACGCTTGACAATCGAG
	Reverse	CGTAGTGATCCTGAGCCCAT
<i>Grm2</i>	Forward	TATCATCTGGCTGGCTTTCC
	Reverse	GGCTCACCACGTTCTTCTGT
<i>Lin54</i>	Forward	GATCCAGCCCAACCTCACTA
	Reverse	TGTGTCTGGATGCCAGATGT
<i>Klhl14</i>	Forward	CCCATGCAAGAAAGAAGAGC
	Reverse	GCTGAGGCAGAGATGACACA
<i>Pard6a</i>	Forward	CTGCTTGGCTATACGGATGC

Gene targeted		Sequence
	Reverse	CCCTTTCTTGCGCCTTTGTA
<i>Pacsin3</i>	Forward	GAGCATTGAGGCTGCCAGTGAT
	Reverse	GCTCTCTGTGTGTCTAACGACC
<i>Kctd6</i>	Forward	CCATCACCACAAAGGTCCACTC
	Reverse	GGTGGTAATCGCACGGTCCAAA
<i>Cpeb2</i>	Forward	AGCTCAGTCCAGGCACTCAT
	Reverse	TTCCAAGGACGGATTGAAC