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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.

1. 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 programing, but is further refined by sensory input (Lendvai et al., 2000; Hubel and Wiesel, 1970; Wiesel and Hubel, 1963). Experience-driven structural changes require, in part, the precise regulation of activity-dependent signaling pathways (West and Greenberg, 2011; West et al., 2002; Flavell et al., 2008). These pathways activate downstream transcription factors that ultimately result in changes in gene expression (West and Greenberg, 2011; West et al., 2002; Flavell et al., 2008; Loebrich and Nedivi, 2009). 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 (Guan et al., 2005; Biederer et al., 2002; Biederer and Stagi, 2008; Chia et al., 2013; Sudhof, 2008; Scheiffele, 2003). 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 (Ghiretti et al., 2013; Ghiretti et al., 2014; Ghiretti and Paradis, 2011; Moore et al., 2013; Paradis et al., 2007). 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 (Finlin et al., 2000). 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 (Sasson et al., 2011; Reymond et al., 2012). The nucleoside diphosphate kinase, Nm23, has been identified as a GTPase activating protein (GAP) for Rad (Zhu et al., 1999; Yang and Colecraft, 1828). 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 (Ghiretti et al., 2013) 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

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modification (Sasson et al., 2011). In addition, in both cultured rat neurons and in *Xenopus* optic tectum, *Rem2* mRNA expression is upregulated in response to neuronal depolarization (Ghiretti et al., 2014). The increase in *Rem2* mRNA expression is dependent on calcium entry specifically through voltage-gated calcium channels (Ghiretti et al., 2014). 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 (Siddiqui et al., 2013).

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.

2. Results and discussion

2.1. 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 (Moore et al., 2017). 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 (Moore et al., 2017).

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 > 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 $< 10\%$.

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

Rem2 is a positive regulator of excitatory synapse formation (Ghiretti and Paradis, 2011; Paradis et al., 2007). 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 (Siddiqui et al., 2013; de Wit et al., 2013). In addition, *Gpc5* acts as a ligand for *Lrrtm4* to mediate synapse formation (Siddiqui et al., 2013). 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 (Ghiretti et al., 2013; Ghiretti et al., 2014; Ghiretti and Paradis, 2011; Moore et al., 2013; Paradis et al., 2007). 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* (Ghiretti et al., 2013; Ghiretti et al., 2014; Ghiretti and Paradis, 2011; Moore et al., 2013; Paradis et al., 2007) 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 (Ghiretti et al., 2013; Ghiretti et al., 2014; Ghiretti and Paradis, 2011; Moore et al., 2013).

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