Introduction

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine kinase activity originally identified via the ability to phosphorylate glycogen synthase [1]. GSK-3 exists as two genetically distinct isoforms in vertebrates, GSK-3α and GSK-3β [2]. The activity of these kinases has been implicated in diabetes, cancer, and neurological diseases [3, 4]. Because of the involvement of GSK-3 in multiple disease settings, the development of specific GSK-3 inhibitors would prove beneficial. One well-characterized GSK-3 inhibitor is lithium [5, 6]. For example, it has been shown that lithium decreases β-amyloid plaque development in the brains of an Alzheimer’s mouse model [7]. One of the problems with current GSK-3 inhibitors is that they are not specific for GSK-3, possibly resulting in unintended side effects; thus, there is a need for more selective inhibitors.

GSK-3α and GSK-3β are largely functionally redundant [8], yet several studies also suggest the existence of distinct molecular roles for each isoform. A prime example of differential activity comes from the phenotype of GSK-3β knockout mice. GSK-3β knockout mice are embryonic lethal despite the presence of GSK-3α, demonstrating that GSK-3α is unable to compensate for the loss of GSK-3β [9]. Another example is a set of experiments in which each GSK-3 isoform was knocked down by RNA interference in cultured cells, and distinct effects were observed on the production of the Alzheimer’s disease-related β-amyloid peptides [7].

The reason for the observed differential roles for GSK-3 isoforms is not well understood, however, a possible explanation is isoform-specific protein-protein interactions that specifically regulate GSK-3α or GSK-3β activity. Identifying GSK-3 isoform-specific interacting proteins could provide a foundation for the development of therapeutics to selectively inhibit GSK-3 isoforms, a feature which is currently lacking in
existing small molecule inhibitors of GSK-3 activity.

The Receptor for Activated C-Kinase 1 (RACK1) has emerged as a binding partner to numerous other proteins involved in a broad range of functions. It was originally identified as a molecular scaffolding protein for activated Protein Kinase C (PKC) [10], but since then has been shown to interact with a host of binding partners, including Src family kinases [11, 12], β-integrin [13], IGF-1 receptor [14], and HIF-1α [15]. RACK1 contains 7 Trp-Asp (WD) repeats that are folded into a β-propeller structure closely resembling the G protein β-subunit. Each blade is thought to be a docking site for interacting proteins, allowing multiple proteins to bind to different propellers at once, facilitating the formation of protein complexes [16]. RACK1 has various functions from working as a ribosomal protein [17] to being involved in the hypoxic response [15] and TGF-β signaling [18] due to its diverse protein interactions. Therefore, RACK-1 is a versatile protein that has the potential to selectively bind proteins and regulate their function.

In this study, we identify RACK1 as a novel GSK-3 isoform-specific interacting protein. RACK1 binds to GSK-3α, but not GSK-3β, and this results in the inhibition of GSK-3α activity. We also show that the GSK-3α-RACK1 interaction is required for normal regulation of the circadian clock. Our data suggest that GSK-3 isoform-specific protein-protein interactions may provide a means by which to differentially inhibit either GSK-3α or GSK-3β activity in cultured mammalian cells.

Materials and methods

Plasmid constructs

RACK1 full length and deletion constructs were created by PCR amplification of human RACK1 cDNA (ATCC clone #7516839) using the primers listed in Table 1. GSK-3 full length and deletion constructs were created by PCR amplification of human GSK-3α (Origene, accession number NM_019884) or human GSK-3β cDNA (obtained from Peter Klein, University of Pennsylvania) using the primers listed in Table 2. The PCR products were then cloned into Gateway entry vector pCR8/GW/TOPO (Invitrogen). The sequence of each RACK1 and GSK-3 full length or deletion construct was confirmed by automated sequencing. Once confirmed, RACK1 constructs were directionally cloned into Gateway destination vector pDEST27 containing an N-terminal Glutathione S-Transferase (GST) tag (Invitrogen) using LR Clonase II (Invitrogen). GST-3 constructs were directionally cloned into Gateway destination vector pcDNA-DEST40 containing a C-terminal V5/6xHis tag (Invitrogen). Point mutations were generated via site-directed mutagenesis (Stratagene) of full length GSK-3α in pCR8/GW/TOPO, confirmed by automated sequencing, and subsequently cloned into pcDNA-DEST40 using LR Clonase II (Invitrogen). Mutagenesis primer sequences are shown in Table 3. EGFP was obtained from Stratagene. Renilla luciferase plasmid pRL-SV40 was obtained from Promega. pCS2+GW was made in our lab.

Antibodies

For detection of GST-RACK1, a rabbit polyclonal GST antibody (1:1000; Cell Signaling) was used. For secondary detection, an ECL rabbit IgG, Hrp-linked secondary antibody was used (1:5000; GE Healthcare) followed by detection with ECL reagent (GE Healthcare). For detection of GSK-3 constructs, anti V5-HRP monoclonal antibody (1:5000; Invitrogen) was used. Also a V5 monoclonal antibody (1:5000; Invitrogen) followed by secondary detection with an ECL mouse IgG, HRP-linked secondary antibody was used (1:5000; GE Healthcare) followed by detection with ECL reagent (GE Healthcare).

Cell culture

Neuro-2A cells (N2A; ATCC #CCL-131) were maintained in 50:50 Dulbecco’s Modification of Eagle’s Medium (Cellgro): Opti-MEM (Gibco) supplemented with 5% BGS (Hyclone) and 0.5% penicillin-streptomycin solution (Cellgro) at 37°C and 5% CO2. N2A cells were seeded in a 6-well plate at 8.0 x 10⁵ cells/well 24 hours prior to transfection. Wild-type and Gsk-3α/β mouse embryonic stem cells (generous gift from Brad Doble, McMaster University and Jim Woodgett, University of Toronto) were plated in a 6-well plate at 1.5 x 10⁶ cells/well 24 hours prior to transfection.

Transfection

24 hours after seeding, cells were transfected under normal serum conditions with 4 μl Lipoctefamine 2000 (Invitrogen) or under reduced serum conditions (2.5%) with 5 μl poly-
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<table>
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<tr>
<th>Construct</th>
<th>5' Primer</th>
<th>3' Primer</th>
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<td>RACK1 WD1-3</td>
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<td>RACK1 WD 1-4</td>
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<td>RACK1 WD 4-5</td>
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<tr>
<td>RACK1 WD 4-6</td>
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Table 1. Oligonucleotides used to create RACK1 deletions.

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<th>Antisense Primer</th>
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<td>GSK-3α Y279F</td>
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<td>GSK-3α S21A</td>
<td>CAGGGGCAGGACTAGCGGCTGCAGAGCCCCG</td>
<td>GCCTAGTCGCCGCCCTGCCCCAGGCAGAGCCCCAGG</td>
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<td>GSK-3α R159A</td>
<td>GTTCTCAGCGACAGGCCCTGAGGCTGGACTGACGACAC</td>
<td>CTCTCGGCTTCCTGAAAGCCCTGCTCTGAGAAGC</td>
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<tr>
<td>GSK-3α K148R</td>
<td>GAACATGTCCTCAGGGATGAGAGGTTACCTCCAGGAC</td>
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</tr>
</tbody>
</table>

Table 2. Oligonucleotides used for site-directed mutagenesis.

ethylamine (Polysciences, Inc.) dissolved in 50 mM HEPES, pH 7.05. For interaction experiments cells were transfected with 200 ng EGFP, 2 μg RACK1 construct and either 2 μg pDEST27, 2 μg pCS2+GW, or 2 μg GSK-3 construct. For luciferase assays, wild-type and Gsk-3α-/- embryonic stem cells were transfected with 1.5 μg Bmal1-Luciferase (provided by Mitch
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Figure 3. Oligonucleotides used to create GSK-3α deletions

<table>
<thead>
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<th>Construct</th>
<th>5' Primer</th>
<th>3' Primer</th>
</tr>
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<tbody>
<tr>
<td>GSK-3α FL</td>
<td>ATGAGCGGCGGCGGCTTCGGGA</td>
<td>GGAGGAGTTAGTGAGGTTAGGTG</td>
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<tr>
<td>GSK-3α ΔCT1</td>
<td>ATGAGCGGCGGCGGCGGCTTCGGGA</td>
<td>TGGGGACCTCAAGTGAGGAGGAT</td>
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<tr>
<td>GSK-3α ΔCT3</td>
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<td>GSK-3α ΔNT</td>
<td>ATGGTGACCACAGTCGTAGCC</td>
<td>GGAGGAGTTAGTGAGGTTAGGTG</td>
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</tbody>
</table>

Lazar, University of Pennsylvania), 200 ng pMax-GFP (Amaza), and 20 ng pRL-SV40 (Promega) using Liopfectamine2000 (Invitrogen). After 24 hours, transfection efficiency was determined and cells were re-plated in 24-well plates in triplicate. Cell lysates were then collected for luciferase assays.

GST pulldown

24 hours post-transfection, cells were lysed using 300 μl IP/lysis buffer (137 mM NaCl, 10 mM Tris pH 7.4, 1% NP-40) containing protease inhibitor cocktail (1:100; Sigma), incubated on ice for 30 minutes, vortexing every 10 minutes, and then centrifuged 14,000 rpm, 4°C for 15 minutes. Total lysate was set aside (30 μl), added to 30 μl 2x Tris/Tricine Sample Buffer (100 mM Tris, pH 6.8, 24% glycerol, 8% SDS, 31 mg/ml DTT, 0.2 mg/ml Coomassie Blue R250), and stored at -20°C. The remaining lysate was added to 20 μl Glutathione Sepharose 4B agarose beads (GE Healthcare) and rotated at 4°C for 2.5 hours. Samples were then centrifuged for 5 minutes at 500xg, 4°C and washed 4 times with 250 μl IP/lysis buffer containing protease inhibitor cocktail (1:100; Sigma). After the final wash, samples were eluted by adding 20 μl IP/lysis buffer and 20 μl 2x Tris/Tricine Sample Buffer and boiling the samples at 100°C for 5 minutes.

Western blot analysis

Samples were electrophoresed (20 μl/sample) through 10% Tris/Tricine gels and transferred onto nitrocellulose membrane (Whatman) at 100V for 1 hour. For detection of GSK-3 constructs, blots were blocked for 1 hour with 5% milk/TBST (150 mM NaCl, 50 mM Tris, pH 7.4, 0.1% Tween). Blots were then incubated in primary antibody diluted in 5% milk in TBST for 3 hours (anti-V5 Hrp 1:5000 or anti-V5 1:5000 diluted in 5% milk in TBST). For anti-V5 antibody, blots were then washed with TBST, and incubated in anti-mouse IgG Hrp secondary antibody (1:5000 in 5% milk in TBST) for 40 minutes. For RACK1 expression, blots were stripped with stripping buffer (100 mM b-Me, 2% SDS, 62.5 mM Tris pH 6.7 and incubated 55°C for 30 minutes), washed with TBST, and then blocked for 1 hour with 5% milk/TBST. Blots were then incubated in primary antibody diluted in 5% milk/TBST for 3 hours (anti-GST; 1:1000), washed with TBST, and incubated in anti-rabbit IgG Hrp secondary antibody (1:5000 in 5% milk in TBST) for 40 minutes. Proteins were visualized using ECL detection reagent (GE Healthcare).

Firefly and renilla luciferase assay

24 hours post-transfection, cells were trypsinized, collected in a 1.5 ml microcentrifuge tube, centrifuged 1500 rpm for 2 minutes and washed with 500 μl 1x PBS (Cellgro). Cells were then resuspended in 800 μl media and 100 μl of the cell suspension was added in triplicate to a 24-well plate containing media. The remaining cell suspension was centrifuged 1500 rpm for 2 minutes, washed with 500 μl 1x PBS, and lysed in 60 μl IP/Lysis buffer containing 1% NP-40 and protease inhibitor cocktail. 50 μl cleared lysate was transferred to a new 1.5 ml microcentrifuge tube containing 50 μl 2X Tris/Tricine sample buffer. The next day adherent cells were washed with 100 μl 1x PBS then lysed using 100 μl 1x Passive Lysis Buffer.
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(Biotium). 20 μl of lysate was used for the assay. Firefly and Renilla Luciferase Assays were performed according to manufacturer’s protocol (Biotium).

Results

A yeast-2-hybrid screen was performed in an effort to identify novel GSK-3 interacting partners. Using full-length human GSK-3α as bait, we screened a human fetal brain library. One of the interacting proteins we found in the screen was the Receptor for Activated C-Kinase1 (RACK1), a 318 amino acid protein that contains 7 WD40 domain repeats [10]. In order to confirm the interaction in mammalian cells and define the regions of interaction with GSK-3α, full length RACK1 and deletion constructs were generated and tagged with an N-terminal GST tag (Figure 1A). Similarly, the region of GSK-3α responsible for interaction with RACK1 was investigated using full length and deletion constructs tagged with a C-terminal V5/6His tag. In addition, GSK-3α point mutations were generated via site-directed mutagenesis and used to determine if GSK-3α activity was required for interaction with RACK1. The mutated constructs were also designed with a C-terminal V5/6His tag (Figure 1B).

To confirm the GSK-3α/RACK1 interaction in mammalian cells, GST-RACK1 full length and deletion constructs were transfected along with full length GSK-3α-V5/6His into Neuro2A (N2A) cells. Performing a GST pulldown 24 hours post transfection and immunoblotting for GSK-3α not only confirmed this interaction, but more specifically revealed that GSK-3α interacts with WD repeats.
repeats 4-7 at the C-terminus of RACK1 on Figure 2A).

In order to further define the region of RACK1 necessary for GSK-3α interaction, finer GST-RACK1 C-terminal WD repeat expression constructs were created (Figure 2B) and co-transfected along with full length GSK-3α-V5/6xHis into N2A cells. Pulldown of RACK1 and immunoblotting for GSK-3α showed that a smaller region of RACK1 (WD5-7) was able to maintain the interaction with GSK-3α while RACK1 deletions WD6-7 and WD7 were not able to maintain this interaction (Figure 2B). This proves WD4 is dispensable for interaction and suggests RACK1 WD repeat 5 must be required for the interaction while WD repeats 6 and 7 may or may not be necessary. To test this, GST-RACK1 WD5-6 and WD5 were cloned and co-transfected with full length GSK-3α-V5 into N2A cells. GST pulldowns were performed, and after immunoblotting for GSK-3, GSK-3α was found to pulldown with all of these finer deletions (RACK1 WD5-7, WD5-6 and WD5) (Figure 2C). Therefore, WD5, a 41 amino acid fragment of RACK1 (N-NCKLKTGHQGQALWDL-C), is the minimal RACK1 region necessary for interaction between GSK-3α and RACK1. Importantly, the interaction between GST-RACK1 and GSK-3α is specific as seen by lack of interaction between free GST and GSK-3α (Figure 2C). Taken together, these results show that the interaction between GSK-3α and GST-RACK1 is specific to RACK1 and the minimal region of RACK1 necessary for interaction with GSK-3α is WD repeat 5.

Interestingly, the screen revealed that RACK1 specifically interacted with GSK-3α, but not the closely related GSK-3β isoform (data not shown). Using GSK-3α as a positive control, interaction between RACK1 and GSK-3β was investigated. GST-RACK1 full length and WD4-7 plasmids were transfected into N2A cells with either full length GSK-3α or β containing a C-terminal V5 6xHis tag. GST pulldowns were performed 24 hours post transfection to selectively purify the GST-RACK1 protein and then immunoblotted with a V5 antibody to detect the presence of GSK-3α and β. Upon immunoblotting, neither GSK-3β nor β interacted with free GST, and while RACK1 strongly interacted with...
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GSK-3α there was no indication of an interaction with GSK-3β (Figure 3). This confirmed the isoform specific findings from the yeast-2-hybrid screen. Blots were stripped and re-probed with an anti-GST antibody to ensure the pulldown was successful. The presence of an interaction with GSK-3β cannot be entirely ruled out since expression levels of GSK-3β were significantly less than those of GSK-3α (Figure 3). It is also possible that RACK1 could be interacting with GSK-3β on WD repeats that may be physically inaccessible in either full length or RACK1 WD4-7, so full length GSK-3β was transfected with a panel of RACK1 WD deletion constructs. While an interaction between GSK-3β and RACK1 cannot be entirely dismissed, if such an interaction exists beyond methods used in this study, it must do so at a minimal level and functional importance of such an interaction is most likely irrelevant.

To determine the effect of various GSK-3α mutations on RACK1 interaction, GST-RACK1 WD 4-7 was transfected with GSK-3α-V5/6xHis functional point mutations. The K148R mutation renders the GSK-3α catalytic lysine inactive resulting in impaired function. The Y279F mutation is a phospho-mimic mutation where constitutive phosphorylation of this specific tyrosine residue is thought to boost activity of the kinase. Autophosphorylation of the serine 21 residue is an inhibitory event for which mutation of Ser21 to an alanine prohibits this site from being phosphorylated, thus prohibiting kinase inhibition in this manner. The R159A mutation prevents binding and phosphorylation of primed substrates. After purifying RACK1 via GST pull-down, interaction with the GSK-3α mutants was detected by immunoblotting for GSK-3α. Blots were stripped and reprobed with an anti-GST antibody to ensure the pulldown was successful (Figure 4). As shown in Figure 4, the various GSK-3α mutations have no effect on the interaction between RACK1 and GSK-3α. Therefore GSK-3α kinase activity is not required nor is a prerequisite for interaction with RACK1.

To classify regions required for interaction with RACK1, we utilized GSK-3α deletion constructs containing a V5/6xHis C-terminal tag. Constructs were deleted at either the N-terminus or sequentially at the C-terminus. Deletion constructs and GST-RACK1 WD4-7 were co-transfected into N2A cells. Upon GST pulldown and subsequent immunoblotting for the presence of GSK-3α, RACK1 was found to interact with GSK-3α FL, and δCT1, but not with δCT2 or δCT3. The interaction was also decreased between GSK-3α δNT and GST-RACK1 (Figure 5). Interestingly, GSK-3α C-terminal deletions δCT2 and δCT3 have been shown to be required for GSK-3α activity towards Tau phosphorylation and transcriptional activation of β-catenin target genes. It has also been implied that this region...
of GSK-3α is necessary for proper folding of the protein [19]. The results of this study suggest RACK1 is unable to interact with misfolded GSK-3α. The GSK-3α N-terminal deletion of amino acids 1-99 did interact with RACK1 WD4-7, however the interaction was much weaker than with full length GSK-3α (Figure 5). Deletion of GSK-3α’s N-terminus was shown to have no effect on GSK-3α’s activity toward Tau phosphorylation, interaction with Axin, or toward reporter activity of the β-catenin target gene, Tcf-Lef [19]. Therefore, the RACK1 interaction with GSK-3α may possibly enhance or inhibit kinase activity toward other proteins.

A recent report identified RACK1 as a critical regulator of the mammalian circadian clock [29]. Therefore, we investigated whether the RACK1-GSK-3α interaction was required for suppression of a reporter for Bmal1, a master regulator of the mammalian circadian clock. RACK-1 suppresses Bmal expression, so we reasoned that if the RACK1-GSK-3α interaction were relevant to the circadian clock, we should see increased Bmal expression in Gsk-3α-/- embryonic stem cells. Using a Bmal1-luciferase construct, we observe an increase in reporter activity in Gsk-3α-/- cells compared to wild-type cells (Figure 6). This result suggests that the interaction between RACK1 and GSK-3α is important in regulating Bmal1 expression, and thus the circadian clock.

**Discussion**

We find that the interaction between RACK1 and GSK-3α is isoform-specific, and have delineated the regions on each protein which are required for this interaction - WD5 on RACK1 and the N-terminus of GSK-3α. The interaction with RACK1 was abolished with partial deletion of the C-terminus, however these results are difficult to interpret because this deletion is thought to cause the protein to misfold [19].

**Figure 4.** Mutation of key GSK-3α regulatory residues does not affect interaction with RACK1. Point mutants of GST-tagged GSK-3α were co-expressed with GST-RACK 4-7 in Neuro2A cells. Total lysate (TL) and glutathione sepharose affinity purified protein (PD) were separated by Tricine-SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies that recognize GSK-3 (V5, top and middle panels) and RACK1 (GST, bottom panel).

**Figure 5.** Deletion of amino- or carboxyl-terminal portions of GSK-3α isoforms prevents interaction with RACK1. Deletions of V5-tagged GSK-3α were co-expressed with GST-RACK 4-7 in Neuro2A cells. Total lysate (TL) and glutathione sepharose affinity purified protein (PD) were separated by Tricine-SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies that recognize GSK-3 (V5, top and middle panels) and RACK1 (GST, bottom panel).

**Figure 6.** Increased expression of a Bmal1-luciferase reporter in Gsk-3α-/- embryonic stem cells. Wild-type and Gsk-3α-/- embryonic stem cells were transfected with a Bmal1-luciferase reporter. Cell lysates were collected 24 hours later. Error bars represent standard deviation. Experiment was performed in triplicate.
Deletion of the N-terminus, on the other hand, maintains activity toward Tau phosphorylation, β-catenin stability, and the ability to interact with Axin, and this deletion resulted in a decreased interaction with RACK1, suggesting this may be the preferred region of interaction.

RACK1 was able to bind to all functional GSK-3α mutations tested including S21A, K148R, R159A and Y279F. These data indicate that the interaction between GSK-3α and RACK1 occurs irrespective of RACK1 phosphorylation or GSK-3α phosphorylation, suggesting that this is not a transient interaction that occurs upon activation of upstream signaling pathways, but rather is a more stable association. In addition, the interaction was maintained with the GSK-3α K148R mutation, which renders the kinase catalytically dead, indicating that kinase activity is not essential for interaction.

RACK1 was originally identified as a PKC-interacting protein [10]. It has been reported that PKCβ selectively phosphorylates GSK-3β, resulting in kinase inactivation, while GSK-3α is not a PKC substrate [20]. It is unknown whether RACK1 is required for the PKCβ phosphorylation of GSK-3β. We did not find evidence of an interaction between GSK-3β and RACK1. However, our studies relied on the use of tagged overexpressed constructs, so we do not know whether there are certain cell types or conditions under which a RACK1/GSK-3β interaction occurs. Further experiments will be needed to dissect these details.

The misregulation of the circadian clock has been linked to numerous disease states [21-23], highlighting the importance of the coordinated regulation of the clock. GSK-3 isoforms have also been implicated in the regulation of the circadian clock in species ranging from Droso phila to human [24-28]. Recently, RACK1 was shown to be an important regulator of the mammalian circadian clock based on its physical interaction with Bmal1 [29]. Alterations in circadian rhythms are observed in almost all patients with bipolar disorder [30]. In fact, disruption of one of the master regulators of circadian rhythms, Clock, is sufficient to induce mania-like behavior, and lithium treatment is able to reverse this behavior [31]. In addition, polymorphisms in BMAL1 have been associated with bipolar disorder as well. Given the relationship between circadian rhythm genes, lithium, GSK-3 activity, RACK1 and bipolar disorder, these findings appear to be particularly relevant. Since GSK-3α/β mice are viable [32], it will be interesting to examine the circadian rhythms in this animal to see if there are alterations.

In conclusion, results of this study have identified RACK1 as a novel interacting protein for GSK-3α. Results also show that kinase activity is not required for this interaction. The absence of GSK-3α in cells disrupts RACK1 function in regulating the circadian clock. Findings from this study imply that isoform specific interacting proteins exist and differentially regulate the activity of GSK-3 isoforms.

Acknowledgment

We thank Drs. Jing Yang and Scott Harper for their helpful suggestions, discussions, and critical reading of the manuscript. We are also grateful to Dr. Peter Klein for the GSK-3β construct, Dr. Mitch Lazar for the Bmal1-luciferase reporter, and Dr. Brad Doble, McMaster University and Dr. Jim Woodgett, University of Toronto for the Gsk-3 knockout ES cells. This work was supported in part by National Institute on Aging Grant R01AG031883 (to C.J.P.).

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References


