Rhes, a striatal–enriched small G–protein, mediates mTOR signaling and L–DOPA–induced dyskinesia.

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Supplementary Text:

The striatum contains medium spiny neurons, which are the projection neurons, as well as interneurons. Two major populations of medium spiny neurons have been discriminated, those expressing dopamine D1 receptors and dynorphin and those expressing dopamine D2 receptors and preproenkephalin[1]. Santini et al[2] showed that mTOR signaling, monitored immunocytochemically with antisera to phospho-S6 and phospho-S6 kinase, is selectively enhanced by L-DOPA treatment in the D1 containing neurons. Consistent with these data, the stimulation of mTOR signaling by L-DOPA is abolished by D1 antagonists and unaffected by D2 antagonists. In situ hybridization analysis establishes that Rhes is expressed to a similar extent in D1 and D2 containing cells[3]. Thus, regulation of mTOR by Rhes is presumably restricted to the population of Rhes in D1-containing cells.

While the present study indicates that Rhes influences LID via mTOR rather than dopamine signaling, there are well established interfaces of Rhes with dopamine disposition. Thus, Rhes deleted mice have increased concentrations of Golf, which mediates links to adenylate cyclase, and manifest enhanced basal cAMP signaling; higher levels of PKA-dependent AMPA receptor (GluR1) phosphorylation at serine-845; and augmented motoric responses to D1 agonists[3]. Moreover, Santini et al[2] showed that the abnormal movements elicited by L-DOPA are mediated by mTOR signaling uniquely in D1 receptor-containing striatal receptors and prevented by a selective D1 antagonist drug.

Materials and Methods:

Reagents, Plasmids and Antibodies

Unless otherwise noted, reagents were obtained from Sigma. Antibodies for myc were from Roche. GST antibody (G7781) was from Sigma. mTOR, Raptor, Rictor, Gbl, Akt, S6K, S6, pAkt (pS473, pT308) pS6K (pT473), pS6 (pS-240/244), p4EBP1 (pS65), and tubulin antibodies were from Cell Signaling. Myc or myc-Rhes (pCMV-myc), GST, GST-Rhes (S33N or C263S, pCMV-GST) were cloned as reported[4,5]. GST, GST-Rhes or GST Rheb (in pGEX6P2) proteins were purified by recombinant techniques[5]. Striatal

cells, SThQ7/7 were a generous gift from Marcy MacDonald. HEK293 or SThQ7/7 were grown in DMEM media (10% serum, glutamine and antibiotics). Rhes antibodies were from Sigma (HPA005839).

Binding assay

GST, GST-Rhes-WT or GST-Rhes-C263S were transfected in striatal cells or HEK293 cells using polyfect (QIAGEN). After 48 h, cells were lysed in Chaps lysis buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 1% Chaps and 10% glycerol and complete protease inhibitors (Roche). Protein lysate (100 µg) was incubated with 50 µl glutathione beads in 500 µl of 0.5% Chaps lysis buffer overnight at 4°C. After washing three times with 0.5% Chaps buffer, beads and the inputs (10 µg) were dissolved in LDS lysis buffer and loaded onto 4-12% Tris gradient gel (Invitrogen). Endogenous mTOR and associated proteins were detected by western blotting. Similarly, 100 µg of striatal lysates were incubated with 5 µg of GST or GST-Rhes purified from bacteria in 500 µl of Tris buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 1% Triton-X100 and 10% glycerol and complete protease inhibitors), and mTOR components were detected by western blotting. Binding assay can be performed either in Triton or Chaps buffer, the latter being more efficient. For in vitro binding, recombinant GST, GST-mTOR catalytic domain (2148-2300)[6] and precession protease-cleaved Rhes[5] were produced in bacteria and incubated with equimolar concentrations of GST/Rhes or GST-mTOR-CD in Tris buffer (50 mM Tris, pH 7.6, 150 mM NaCl and 10% glycerol), for 4 h at room temperature along with glutathione beads. The amount of bound Rhes was detected by western blotting using antibodies against Rhes.

mTOR activity assay in cells

Rhes constructs (myc or GST-tagged) were transfected in striatal or HEK293 cells for 48 h. Where indicated, cells were washed and replaced with DMEM with or without 10% serum for two hours. Cells were lysed in Tris buffer, and protein (25µg) was loaded onto 4-12% gradient gels to detect phosphorylation by western blotting. Where indicated, 100 nM rapamycin was applied for 2 h before serum deprivation.

In vitro kinase assay for mTORC1 activity

In vitro mTORC1 kinase assay were performed as described previously[7]. HEK293T cells were incubated for serum- and leucine-deficient media for 1 h. Cells were rinsed once with ice-cold PBS and lysed in ice-cold lysis buffer (40 mM HEPES [pH 7.4], 2 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 0.3% CHAPS, 5 mg/mL aprotinin, 1 mg/mL leupeptin, 6 µg/mL chymostatin, 0.7 µg/mL pepstatin, and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were incubated at 4 °C for 10 min and the supernatant was collected by centrifuging lysates at 13,000 rpm for 10 min. Two micrograms of raptor antibody (Bethyl Laboratories) were added to the 1.5 milligrams of lysates and incubated with rotation for 1.5 hours at 4 °C. Fifteen microliters of rabbit TruBlot bead (eBioscience) were added and the incubation continued for an additional 1 h. Raptor immunoprecipitates were washed twice with the same lysis buffer and twice

with kinase wash buffer (25 mM HEPES [pH 7.4], 20 mM potassium chloride). Purified GST-Rhes or GST-Rheb (200 nM) was charged with GDP or GTP-γ-S as described previously[1] and incubated with mTORC1 isolated from leucine-stimulated HEK293T cells for 20 min at 30 °C. Kinase assays were performed for 20 min at 30 °C in a final volume of 15 microliters of mTORC1 kinase buffer (25 mM HEPES [pH 7.4], 50 mM KCl, 10 mM $MgCl_2$, 250 mM ATP) and 150 ng 4E-BP1 as a substrate.

6-OHDA lesioning

Surgical procedures were performed according to a previous protocol[2]. Mice were anesthetized with a mixture of fentanyl citrate (0.315 mg/ml), fluanisone (10 mg/ml) (VetaPharma, Leeds, UK), midazolam (5 mg/ml) (Hameln Pharmaceuticals, Gloucester, UK), and water (1:1:2 in a volume of 10 ml/kg) and mounted in a stereotaxic frame (David Kopf Instruments, Tujanga, CA) equipped with a mouse adaptor. 6-OHDA-HCl (Sigma, St Louis, MO) was dissolved in 0.02% ascorbic acid in saline at a concentration of 3 µg of free-base 6-OHDA per µl. Each mouse received two unilateral injections of 6-OHDA (2 µl per injection) into the right striatum, according to the following coordinates (mm) (40): anteroposterior (AP), +1; mediolateral (ML), −2.1; dorsoventral (DV), −3.2; and AP +0.3; ML, −2.3; DV, −3.2. Animals were allowed to recover for 3 weeks before behavioral evaluation and drug treatment were carried out. The efficacy of the lesion was assessed by the loss of tyrosine hydroxylase immunoreactivity in the striatum. Only animals displaying ≥ 75% decrease in striatal tyrosine hydroxylase immunoreactivity were used for western blotting and included in statistical analyses of the cylinder test and AIMs.

Cylinder test

The cylinder test, performed as previously described[2], was used to monitor the anti-akinetic effect of L-DOPA. Mice were individually placed in glass cylinders (diameter, 12 cm) and movements recorded for 5 min. Each 6-OHDA-lesioned mouse was tested before and 1 h after the first injection of 10 mg/kg L-DOPA. The number of contacts of either forelimb with the wall was counted by an observer blind to the mouse treatment. To discriminate between accidental touches and meaningful physiological movements, only wall contacts where the animal supported its body weight on the paw with extended digits were counted. The use of the impaired (left) forelimb was calculated as a percentage of the total number of supporting wall contacts. Statistical analysis was performed by two-way ANOVA (genotype x treatment).

AIMs rating

Abnormal involuntary movements (AIMs) were scored using the rating system described in Lundblad *et al*[8]. 6-OHDA-lesioned mice were treated for 9 consecutive days with one injection per day of 10 mg/kg L-DOPA plus benserazide (20 mg/kg). AIMs were assessed at day 3, 6 and 9 by an observer blind to the mouse treatment. Briefly, 20 min after L-DOPA administration, mice were placed in separate cages, and individual

dyskinetic behaviors were assessed for a 1 min monitoring period every 20 min for 180 min. Purposeless movements, distinguished from natural stereotyped behaviors (such as grooming, sniffing, rearing, and gnawing), were classified into four subtypes: locomotive AIMs (tight contralateral turns), axial AIMs (contralateral dystonic posture of the neck and upper body toward the side contralateral to the lesion), limb AIMs (jerky and fluttering movements of the limb contralateral to the side of the lesion) and orolingual AIMs (vacuous jaw movements and tongue protrusions). Each subtype was scored on a severity scale from 0 to 4: 0, absent; 1, occasional; 2, frequent; 3, continuous; 4, continuous and not interruptible by outer stimuli. Statistical significance over days was determined by two-way ANOVA (genotype x days of treatment) with repeated measures. Statistical significance over the 180-min test session of day 9 was determined by two-way ANOVA (genotype x observation periods) with repeated measures, while total AIMs were analyzed by *post-hoc* comparison.

Western blotting of striatal tissue

Mice were killed and their heads immediately immersed in liquid nitrogen for 6 s, as previously described[2]. The brains were then removed and the striata dissected out within 20 s on an ice-cold surface, sonicated in 1% SDS and boiled for 10 min. The effectiveness of this extraction procedure in preventing protein phosphorylation and dephosphorylation, hence ensuring that the level of phosphoproteins measured *ex vivo* reflects the *in vivo* situation, has been tested previously[2]. Aliquots of the homogenate were used for protein determination using Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA). Equal amounts of total proteins (70 μg) for each sample were loaded onto 10% polyacrylamide gels. Proteins were separated by SDS-PAGE and transferred overnight to membranes (PVDF) (Amersham Pharmacia Biotech, Uppsala, Sweden). The membranes were immunoblotted overnight using selective antibodies against pSer240/244-S6, pSer65-4EBP1, pThr202/Tyr204-ERK1/2 (each diluted 1:1000, Cell Signaling Technology, Beverly, MA) and pSer845-GluR1 (1:1000, PhosphoSolutions, Aurora, CO). Antibodies against S6, 4EBP1 (both diluted 1:1000, Cell Signaling Technology) and GluR1 (1:1000, Chemicon, Temecula, CA, USA) that are not phosphorylation state-specific were used to estimate the total amount of proteins. An antibody against tyrosine hydroxylase (1:1000, Chemicon) was used to assess the severity of 6-OHDA lesions. Both pThr202/Tyr204-ERK1/2 and tyrosine hydroxylase optical density values were normalized using an antibody against DARPP-32 (1:1000, Cell Signaling Technology). Blots were then incubated in horseradish peroxidase-conjugated secondary antibodies and target proteins visualized by ECL detection (Pierce, Rockford, IL), followed by quantification by Quantity One software (Biorad). Normalized values were averaged and used for statistical comparisons performed by ANOVA followed by *post-hoc* comparison.

Supplementary References:

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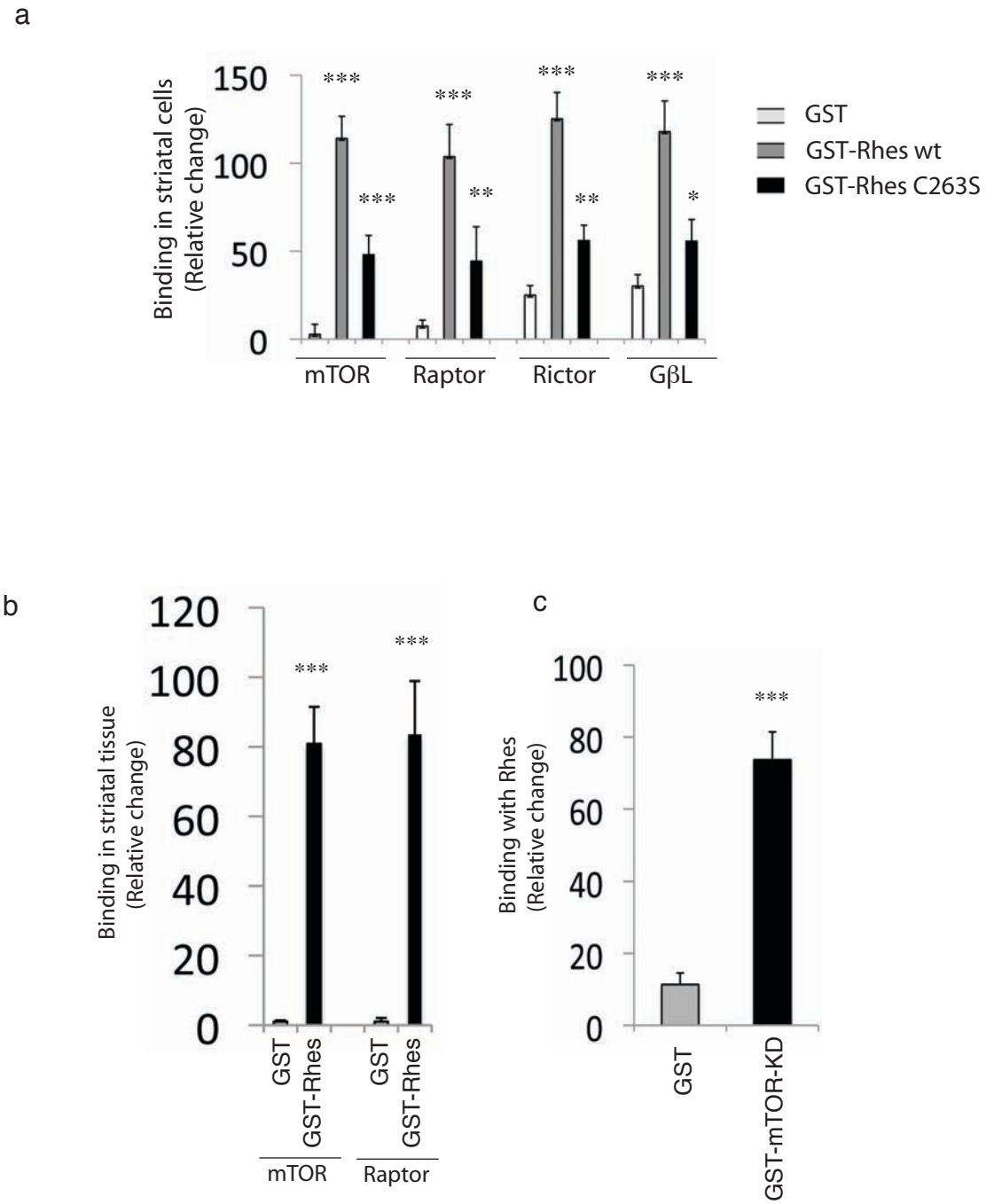
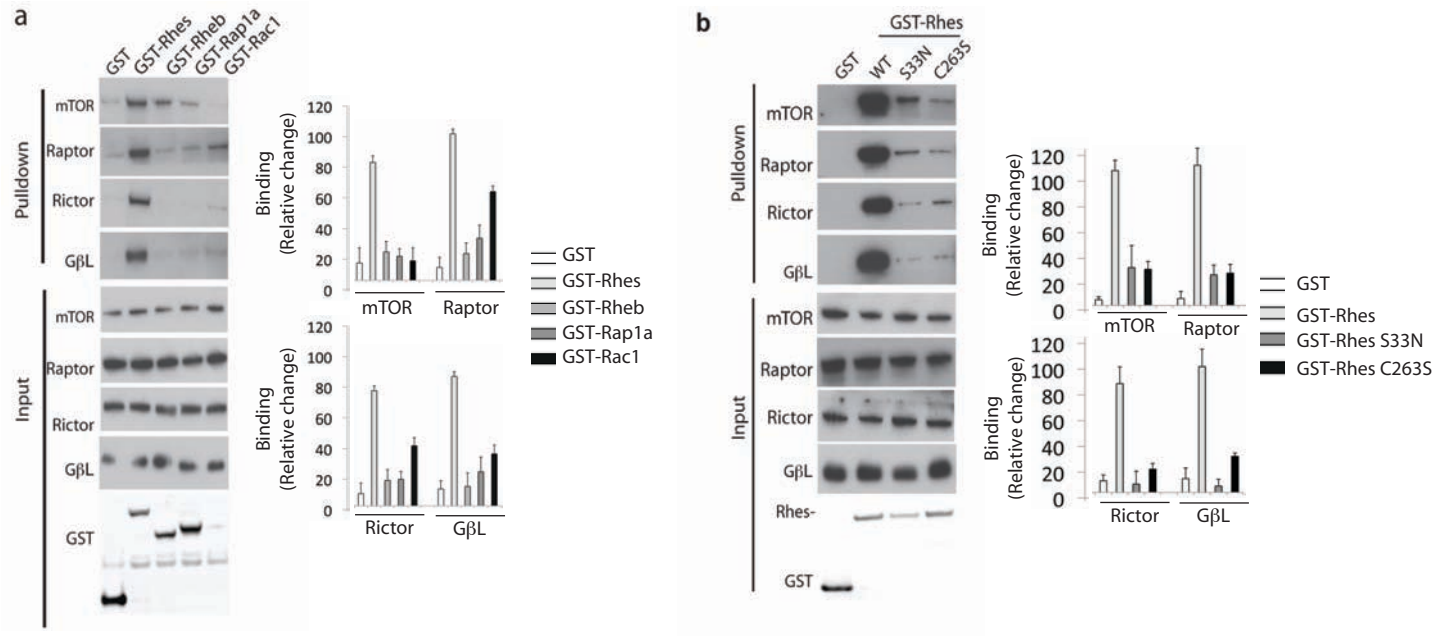


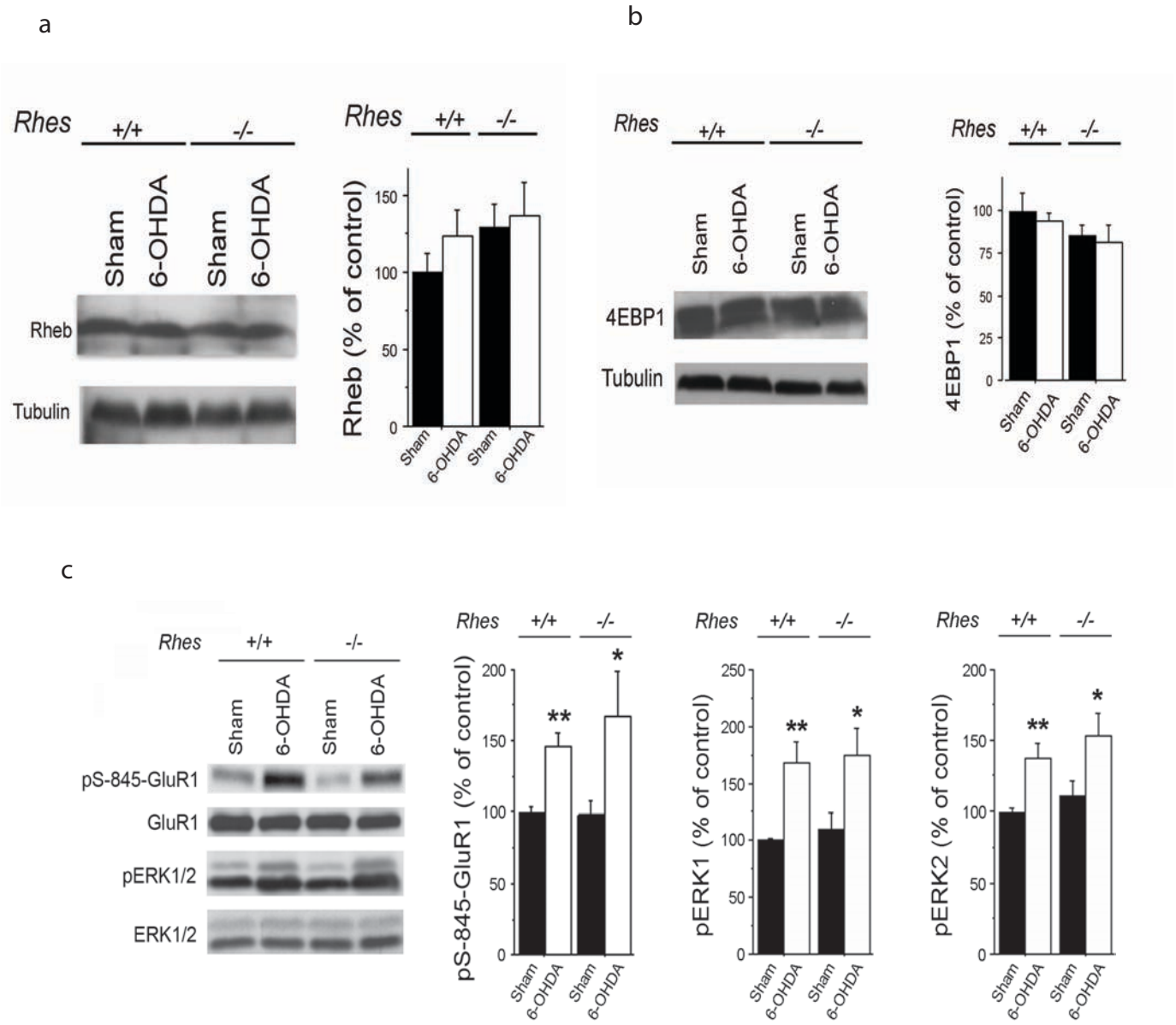
Supplementary Figure 1

Legend: Quantification of Rhes binding to mTOR. Quantification of Rhes binding to the endogenous mTOR-complex in striatal cells (a). Binding of mTOR and Raptor with recombinant Rhes, in striatal tissue (b). Binding of purified Rhes to recombinant GST tagged mTOR-kinase domain, in vitro (c). Data are shown as relative binding among different baits compared to GST alone. Data presented are means ± s.d. from at least three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to GST alone.



Supplementary Figure 2

Legend: Comparison of mTOR binding. Rhes binds more strongly to mTOR complex than to other small GTPases (a). Binding of endogenous mTOR, Raptor, Rictor and Gβl to overexpressed GST, GST-Rhes, GST-Rheb, GST-Rap1a and GST-Rac. Rhes mutants (S33N & C263S) bind less than wild-type to mTOR complex (b). Quantification of binding is plotted as described in Supplementary Figure 1. See Materials and Methods for more detail.

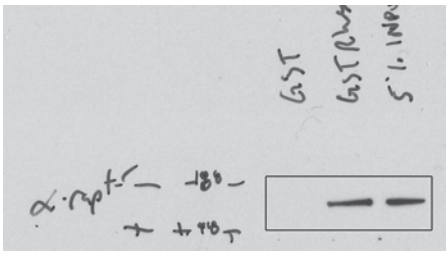


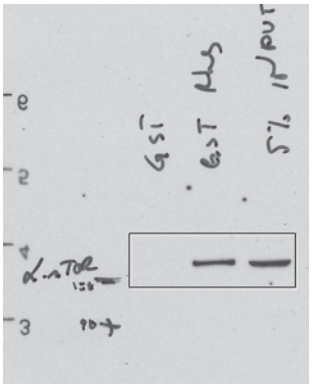
Supplementary Figure 3

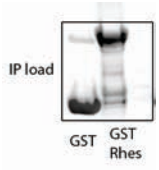
Legend: Determining the levels of Rheb, 4EBP1, GluR1 and ERK. Total levels of Rheb (a), 4EBP1 (b), and phosphorylation levels of GluR1 and ERK1/2, respectively at Ser845 and Thr202/Tyr204 residues (c), in the striatum of unilaterally 6-OHDA-lesioned $Rhes^{+/+}$ (n = 12) and $Rhes^{-/-}$ (n = 10) mice. Statistical analysis highlights that 6-OHDA lesion increases striatal pGluR1, pERK1 and pERK2 levels, irrespective of genotypes. All data are expressed as mean ± s.e.m. * $P < 0.05$, ** $P < 0.01$, as compared with unlesioned striata.

Raw blot for Figure 1a

Supplementary Figure 4

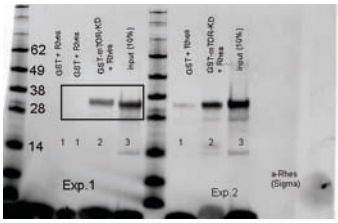


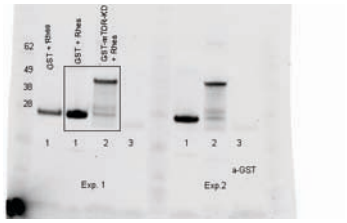




Raw blots for Figure-1b

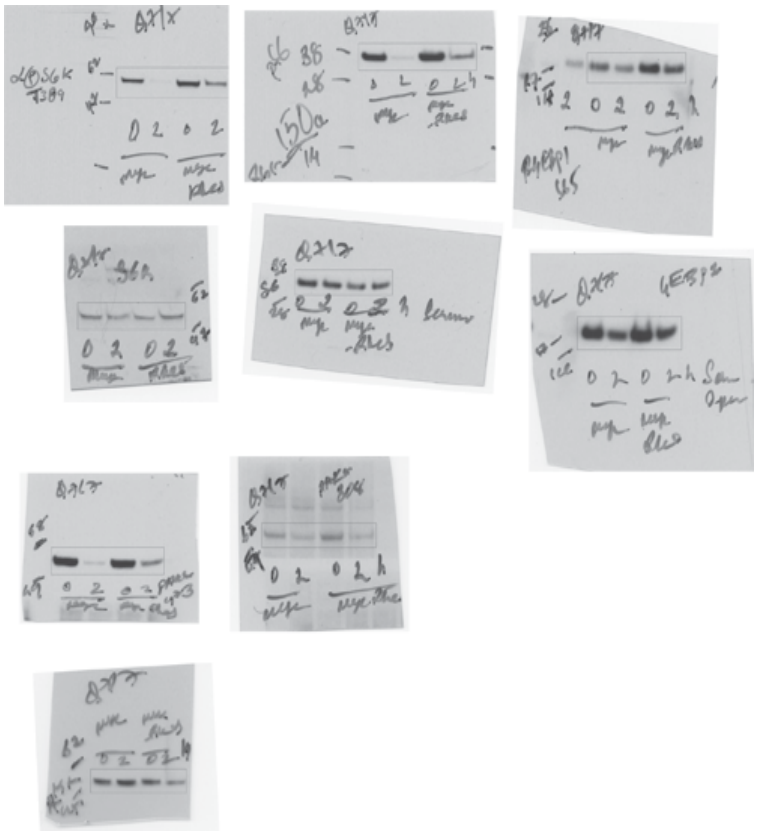
Supplementary Figure 5





Raw blots for Figure-1c

Supplementary Figure 6

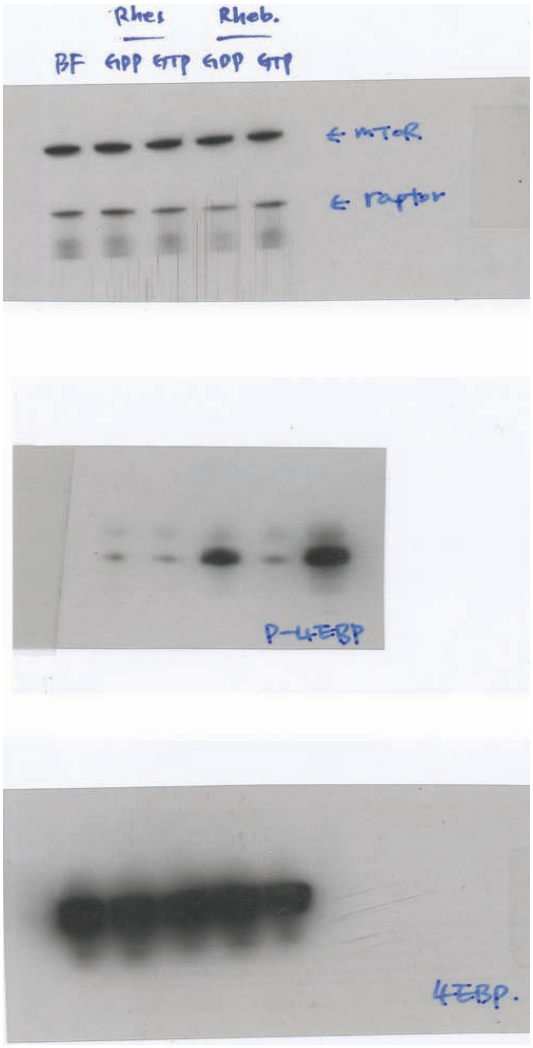


Raw blots Figure-1d

Supplementary Figure 7

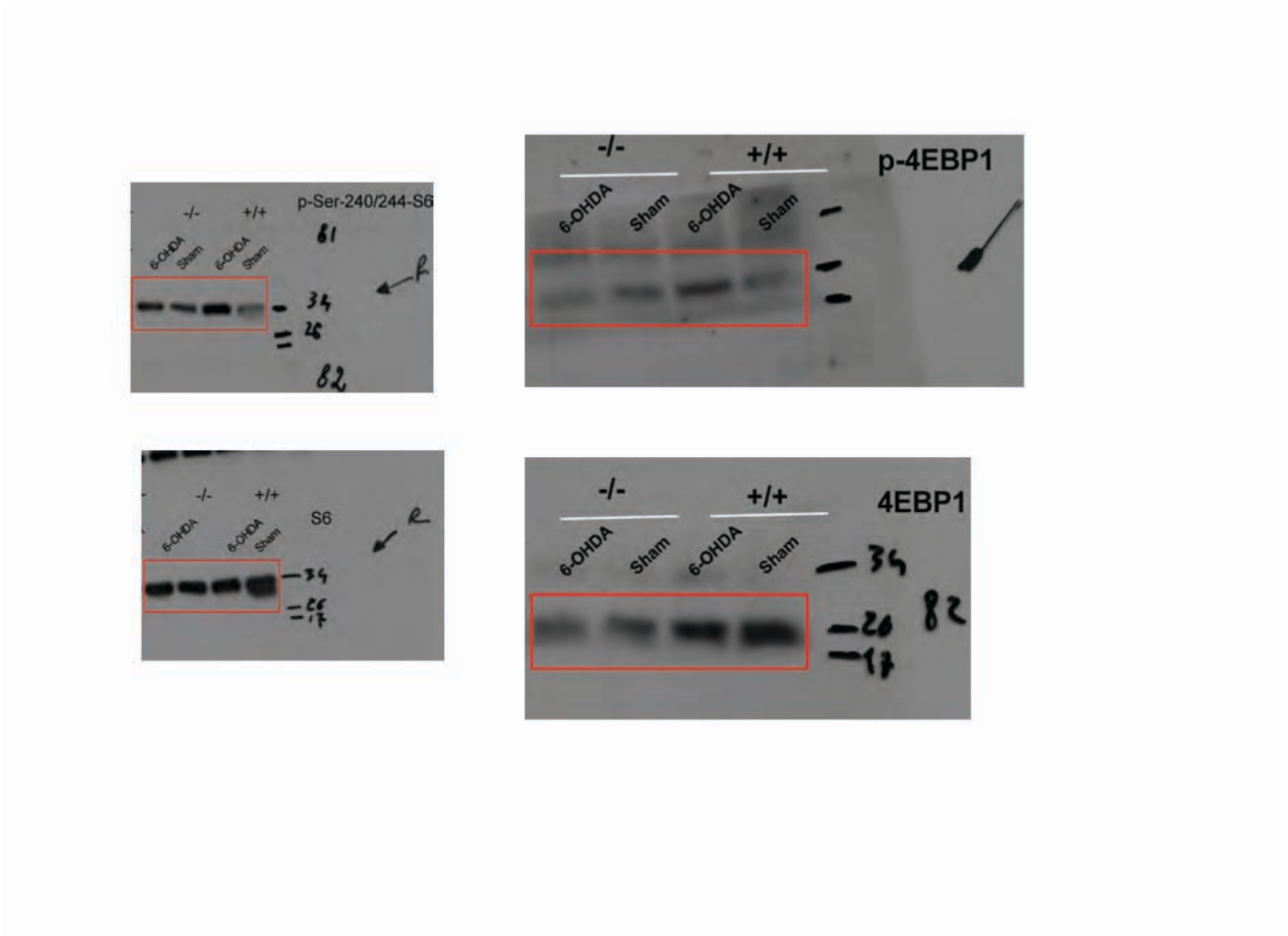
Raw blots for Figure-1e

Supplementary Figure 8



Raw blots Figure-1f

Supplementary Figure 9



Raw blots of Figure -2a

Supplementary Figure 10