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Effects of the Ras Homolog Rhes on Akt/Protein Kinase B and Glycogen Synthase Kinase 3 Phosphorylation in Striatum

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Abstract

G protein-coupled receptors signal not only through heterotrimeric G proteins, but also through alternate pathways. Thus, dopamine D2 receptors in striatum signal through G_{α}i/o and also by promoting formation of a multi-protein complex containing β-arrestin2, protein phosphatase 2A, and Akt in order to dephosphorylate Akt. Lithium, on the other hand, disrupts this complex to increase Akt phosphorylation. Rhes is a striatally-enriched GTP binding protein that has been shown to inhibit dopamine receptor-mediated behavior and signaling through heterotrimeric G proteins. Therefore, our objective was to test whether Rhes similarly affects signaling through the Akt/GSK3 pathway in striatum. $Rhes^{-/-}$ mice showed basally increased Akt and GSK3β phosphorylation relative to $rhes^{+/+}$ mice that was not further enhanced by lithium treatment. Furthermore, they responded to the D1/D2 agonist apomorphine with increased Akt and GSK3 phosphorylation. Co-immunoprecipitation experiments revealed that apomorphine treatment recruits protein phosphatase 2A-C to Akt in both $rhes^{+/+}$ and $rhes^{-/-}$ mice. Lithium did not disrupt their interaction in $rhes^{-/-}$ mice as there was little basal interaction. Rhes co-immunoprecipitated with β-arrestins, suggesting that it is integral to the multi-protein complex. Thus, Rhes is necessary for Akt dephosphorylation by the striatal multi-protein complex, and in its absence, a lithium-treated phenotype results.

Keywords

dopamine; lithium; G protein; striatum; rasd2; β-arrestins

Rhes (Ras Homolog Enriched in Striatum) is an intermediate size GTP-binding protein with preferential expression in striatum (Usui et al.,1994; Falk et al., 1999; Harrison and LaHoste, 2006; Harrison et al., 2008). It has multiple effects in striatal cells and has recently

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L.M.H. designed experiments, performed experiments, analyzed data, and wrote the manuscript.
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been shown to act as an E3 ligase for sumoylation (Subramaniam et al., 2009, 2010). In addition, Rhes has been shown to inhibit signaling by G protein-coupled receptors (GPCRs). For example, reporter gene activation by agonist-occupied β-adrenergic and thyroid stimulating hormone receptors is inhibited by Rhes (Vargiu et al., 2004), as well as cAMP accumulation by dopamine receptor agonists (Harrison and He, 2011; Harrison, 2012). In vivo, rhes$^{-/-}$ mice show up-regulated protein kinase A (PKA) signaling as evidenced by increased phosphorylation of GluR1 at the PKA site (Ser845) (Errico et al., 2008). These findings point to a role for Rhes in signaling through traditional pathways of GPCRs—heterotrimeric G proteins, cAMP, and adenylyl cyclase (AC). However, as alternate pathways for GPCR signaling have now been defined, the question arises as to whether Rhes affects this signaling as well.

Although the family of GPCRs was so named because of members' ability to activate heterotrimeric G proteins in order to transmit signals into the cell (Hepler and Gilman, 1992; Oldham and Hamm, 2008), alternate signaling pathways are being increasingly identified. For example, β-arrestins, originally described as contributing to the termination of GPCR-mediated signals, are now known to scaffold alternate signaling pathways upon termination of heterotrimeric G protein signaling. Phosphorylation of receptors by G protein-coupled receptor kinases, followed by binding of β-arrestins, "uncouples" the receptor from the G protein, a process that allows for internalization of the receptor for recycling or degradation (Krupnick and Benovic, 1998; Perry and Lefkowitz, 2002). However, this uncoupling from the G protein pathway can actually allow the receptor to signal by alternate pathways. The role of β-arrestins has been expanded to include scaffolding of multi-protein complexes. For example, they can scaffold ERK kinases to allow signaling through this pathway independent of the G protein pathway (Wei et al., 2003; Shenoy et al., 2006). Thus, the family of G protein-coupled receptors may more accurately be termed "7 transmembrane receptors" (TMRs) to denote that post-receptor signaling can occur through more than one major pathway (Shenoy and Lefkowitz, 2011; Shukla et al., 2011).

A recently-defined β-arrestin scaffolded pathway of 7 TMRs involves signaling by Akt (protein kinase B). Upon activation of dopamine receptors in striatum, a multi-protein complex forms, consisting of at least β-arrestin2, Akt, and protein phosphatase 2A (PP2A). This complex allows PP2A to dephosphorylate Akt at the threonine 308 residue, thus decreasing activation of the kinase. This decreased Akt activation, in turn, results in less phosphorylation of the downstream target glycogen synthase kinase-3 (GSK3) (Beaulieu et al., 2005). As GSK3 is constitutively active and is inhibited by phosphorylation at an N-terminal serine (Ser21 in GSK3α and Ser9 in GSK3β), this decreased phosphorylation activates the kinase (Sutherland et al., 1993; Cross et al., 1995). Although it is not yet known whether other striatal receptors can promote formation of this complex, or whether it is formed in other areas of brain, it has been demonstrated that among dopamine receptors, D2 receptors, but not D1, promote its formation. D3 receptors contribute to complex formation, but are not necessary for it (Beaulieu et al., 2007).

GSK3, originally described for its ability as one of the kinases that phosphorylate glycogen synthase (Embi et al., 1980; Woodgett, 1990), is now appreciated to play major roles in neuronal function, including neurogenesis, synapse formation, and neurite outgrowth (Cole, 2012). Also, several neuropsychiatric disorders are postulated to involve perturbations in Akt/GSK3 signaling. For example, schizophrenia is associated with decreased Akt activity, likely from increased activity of dopamine D2 receptors, which would be postulated to increase GSK3 activity (Emamian et al., 2004; Blasi et al., 2011). The anti-manic drug lithium also affects Akt/GSK3 signaling. It has been known for many years that lithium can directly inhibit GSK3 (Klein and Melton, 1996; Stambolic et al., 1996). Recently, it has been shown that lithium can also inhibit GKS3 indirectly by disrupting the striatal Akt-

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containing multi-protein complex that favors GSK3 activation (Beaulieu et al., 2004, 2008). Studies with GSK3β$^{+/-}$ mice have shown that this kinase is an important locus for the behavioral effects of lithium as these mice show a lithium-treated phenotype in forced swim and locomotor tests (Beaulieu et al., 2004; O'Brien et al., 2004). Due to the importance of Akt/GSK3 signaling in neuropsychiatric disorders and the presence of a protein complex regulating this signaling in striatum, we tested whether Rhes is involved in the regulation of striatal Akt and GSK3.

Experimental Procedures

Materials

Lithium chloride (LiCl) and R-(−)-apomorphine hydrochloride hemihydrate were purchased from Sigma (St. Louis, MO). The following antibodies were purchased from Cell Signaling Technology (Danvers, MA): panAkt, phospho-Akt Thr308, phospho-Akt Ser473, GSK3α/β, phospho-GSK3α/β, PP2A-C, beta-arrestin 1/2, GAPDH. An additional anti-β-arrestin1/2 antibody from Santa Cruz (Santa Cruz, CA) was also used. The anti-Rhes antibody was from FabGennix (Frisco, Texas).

Animals

Rhes$^{+/+}$ and rhes$^{-/-}$ mice were generated as described previously (Spano et al., 2004) and backcrossed for ten generations onto the C57BL/6 background. Male and female mice, aged 2–4 months, from heterozygous and homozygous matings were used for all experiments. Genotypes were verified by using PCR of tail biopsies. Mice were group-housed in a climate controlled vivarium placed on a 12-h light/dark cycle (lights on at 0600) with food and water provided ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee of the Louisiana State University Health Sciences Center and were in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The ARRIVE guidelines were used in the design and execution of the animal experiments, and every effort was made to minimize the suffering of the animals and to reduce the number of animals used. For LiCl experiments, mice were injected intraperitoneally (IP) with 200 mg/kg LiCl or vehicle (sterile saline) and sacrificed by rapid decapitation 30 minutes later. Apomorphine (3 mg/kg) or its vehicle (0.1% ascorbate) was injected subcutaneously (SC), and mice were sacrificed 1 hour later by rapid decapitation. All injections were in a volume of 10 ml/kg.

Western blotting for phosphoproteins

For analysis of phosphoproteins, striata were rapidly dissected and homogenized in a buffer containing 50 mM Tris (pH = 8), 150 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, Sigma protease inhibitor cocktail, 0.3 μM okadaic acid, and 2 mM sodium orthovanadate. Protein concentration was determined with a Bio-Rad (Hercules, CA) Detergent Compatible Protein Assay kit. Proteins (20 μg cell lysate) were separated by SDS-PAGE using any kD polyacrylamide TGX gels (Bio-Rad) and transferred to PVDF membranes. After blocking with 5% Carnation® milk in TBS-T (20 mM Tris, 150 mM NaCl, 0.1% Tween-20), membranes were incubated at 4°C overnight in TBS-T/5% BSA with primary antibody [pAkt308, pAkt473, Akt, pGSK3α/β, GSK3α/β (all 1:1000), or GAPDH (1:5000)]. Membranes were washed and incubated in HRP-conjugated secondary antibody (1:20,000; Pierce/Thermo Scientific; Rockford, Ill), followed by additional washing. Proteins were visualized by enhanced chemiluminescence (Pierce/Thermo) and imaged with a GE LAS4010 (GE Healthcare; Pittsburgh, PA). Blots were initially probed for phosphoproteins, then stripped (One Minute stripping buffer, GM Biosciences, Inc; Frederick, MD) and re-probed for respective total protein. GAPDH was used as a loading control.

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Immunoprecipitations

Mice were injected as described above with LiCl, apomorphine, or their respective vehicles and sacrificed either 30 minutes (LiCl) or 1 hour (apomorphine) later. Brains were rapidly dissected out, immediately frozen in isopentane at −50°C, and stored at −80°C until processing. Striata were rapidly dissected on ice by cutting a section containing anterior and middle striatum, and taking a 2 mm punch from each hemisphere. Striata were homogenized in a buffer containing 20 mM Tris, pH = 8, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM sodium orthovanadate, 2 μM okadaic acid, and Sigma protease inhibitor cocktail. Protein was determined as above. Immunoprecipitations were performed with the Catch and Release system (Millipore; Billerica, MA). Lysates (500 μg) were incubated with anti-Akt or anti-β-arrestin1/2 antibody (1:100) for 30 minutes at room temperature. In some apomorphine experiments, lysates were also incubated for 30 minutes at room temperature with 10 μM apomorphine or vehicle prior to immunoprecipitation, in addition to the in vivo treatment of mice with 3 mg/kg apomorphine or vehicle 1 hour prior to sacrifice. Samples were washed and eluted according to the manufacturer's instructions. Western blotting was performed as described above with 50 μl of elutions. For Western blotting following immunoprecipitations, the anti-PP2A-C and anti-Rhes antibodies were used at a dilution of 1:500, and the anti-Akt and anti-β-arrestin1/2 antibodies were used at 1:1000. Clean Blot (Thermo Scientific) secondary antibody was used to avoid visualization of the light and heavy chains of immunoprecipitating antibodies in the elutions.

Data Analysis

ImageQuant TL software (GE) was used to obtain relative values for band intensity. For analysis of phosphoproteins, values were expressed as a ratio of phospho- to total protein. Values for drug-treated mice of each genotype were expressed as a percent of vehicle-treated mice for that genotype. For immunoprecipitations, values for band intensity of PP2A-C were expressed as a ratio of values for Akt in order to account for any differences in the level of immunoprecipitated Akt. Data were analyzed by Two-factor ANOVA, followed by Bonferroni post-tests where appropriate, or by Student's t-test. Significance was set a p value <0.05.

Results

Lithium disrupts a β-arrestin/Akt-containing multi-protein complex in striatum and, by thus removing the influence of PP2A on Akt, causes increased phosphorylation of Akt at the threonine 308 site (Beaulieu et al., 2008). We tested whether Rhes affects such signaling by lithium. Wild type and rhes mutant mice were administered LiCl or vehicle and tested for Akt and GSK3 phosphorylation 30 minutes later. For phosphorylation of Akt at the 308 site, Two-factor ANOVA showed a significant main effect of genotype ($F = 14.2$, $p<0.05$) and a significant treatment x genotype interaction ($F= 14.2$, $p<0.05$). Bonferroni post-tests showed that as expected, LiCl caused a significant increase in pAkt308 in rhes$^{+/+}$ mice ($p<0.05$). However, the drug was without effect in rhes$^{-/-}$ mice (Figure 1A and B). We examined basal pAkt308 in the two genotypes and found it to be significantly increased in rhes$^{-/-}$ mice relative to rhes$^{+/+}$ mice ($t = 2.64$, $p<0.05$; Figure 1C). LiCl had no effect on Akt phosphorylation at the 473 site in either genotype, and this phosphorylation was not altered basally in rhes$^{-/-}$ mice (Figure 1D, E, and F). The levels of total Akt were not different in rhes$^{+/+}$ versus rhes$^{-/-}$ mice, and they were not affected by LiCl treatment (Figure 1G).

Akt phosphorylates and thus inhibits GSK3, and GSK3 inhibition is likely to be a salient feature of lithium action (Beaulieu et al, 2004; O'Brien et al., 2004). Therefore, we examined GSK3 phosphorylation in the rhes mutant mice. As shown in Figure 2A and B, LiCl was without effect on phosphorylation of GSK3α in either genotype. Also, the two

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genotypes showed similar basal levels of GSK3α phosphorylation (Figure 2C). However, LiCl was differentially effective in phosphorylating GSK3β in the two genotypes (Figure 2A and D). Two-factor ANOVA indicated a significant effect of treatment ($F = 10.7$, $p<0.05$) and statistical trends for effects of genotype ($F = 8.03$, $p = 0.06$) and genotype x treatment interaction ($F = 8.03$, $p = 0.06$). Post-hoc Bonferroni tests showed a significant effect of LiCl in rhes$^{+/+}$ mice ($p<0.05$), but not in rhes$^{-/-}$ mice. As with phosphorylation of Akt at the 308 site, basal GSK3β phosphorylation was significantly increased in rhes$^{-/-}$ mice relative to rhes$^{+/+}$ mice ($t = 2.7$, $p<0.05$; Figure 2E). The levels of total GSK3α and β were not different in rhes$^{+/+}$ versus rhes$^{-/-}$ mice, and they were not affected by drug treatment (Figure 2F and G).

In contrast to lithium, which disrupts the multi-protein complex and thus increases phosphorylation of Akt and GSK3, dopamine receptor activation promotes complex formation and thus decreases their phosphorylation (Beaulieu et al., 2005, 2008). As shown in Figure 3A and B, apomorphine not only failed to decrease Akt phosphorylation at the 308 site in rhes$^{-/-}$ mice, but there was a significant increase in phosphorylation in these mice in response to this drug. Two-factor ANOVA revealed a significant effect of genotype ($F = 17.96$, $p<0.01$) and a significant genotype x treatment interaction ($F = 20.37$, $p<0.01$). Bonferroni post-tests indicated a significant effect of the drug in rhes$^{-/-}$ mice ($p<0.01$). There were no genotype or treatment effects on phosphorylation at the 473 site (Figure 3C and D). Apomorphine treatment did not affect levels of total Akt in either genotype (Figure 3E). GSK3β was affected by drug and genotype in a similar pattern to Akt (Figure 4A and C). Two-factor ANOVA indicated significant effects of treatment ($F = 13.95$, $p<0.05$) and genotype ($F = 11.23$, $p<0.05$), and a significant treatment x genotype interaction ($F = 11.23$, $p<0.05$). Bonferroni post-tests showed a significant effect of drug in rhes$^{-/-}$ mice ($p<0.01$). The decrease in pAkt308 in rhes$^{+/+}$ mice (Figure 3A and B) was not reflected in a decrease in pGSK3β at the single time point measured. GSK3α was not significantly affected by drug or genotype (Figure 4A and B). Although there was a slight increase (20%) in pGSK3α in rhes$^{-/-}$ mice, the effect was not significant ($p = 0.288$). Apopmorphine treatment did not affect GSK3α or GSK3β total protein levels in either genotype (Figure 4D and E).

Both lithium and apomorphine have been shown to act through the β-arrestin2-containing multi-protein complex that regulates Akt phosphorylation in striatum (Beaulieu et al., 2005, 2008). Thus, we tested whether this complex is altered in rhes$^{-/-}$ mice. The ability of the multi-protein complex to form upon agonist treatment was tested by administering apomorphine (3 mg/kg) or vehicle to mice and sacrificing 1 hour later. Striatal lysates were immunoprecipitated with an anti-Akt antibody, and Western blots were probed with antibodies to Akt and PP2A–C. In order to protect complexes formed by the in vivo treatment, lysates were incubated with 10 μM apomorphine for 30 minutes before addition of immunoprecipitating antibody (anti-Akt). Densitometric values are presented as a ratio of PP2A-C/Akt in order to account for any variability in the amount of Akt in the initial immunoprecipitation reaction. Although there was no difference between genotypes or with drug treatment in the amount of total Akt in striatum (see Figures 1G and 3E), Akt was differentially immunoprecipitated by the anti-Akt antibody according to genotype and apomorphine treatment. In each genotype, apomorphine caused a decrease in the amount of Akt immunoprecipitated by the anti-Akt antibody ($F = 10.55$, $p<0.0001$, Figure 5A). This effect was more pronounced in rhes$^{-/-}$ than rhes$^{+/+}$ when expressed as a percent of own genotype control ($t = 4.209$, $p = 0.003$, Figure 5B). This finding suggests that the immunoprecipitating antibody (anti-Akt) may have differential access to Akt in the two genotypes and upon drug treatment. We therefore normalized the co-immunoprecipitating protein (PP2A-C) as a ratio to immunoprecipitated Akt for all quantification.

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As shown in Figure 6A and B, apomorphine promoted Akt and PP2A-C interaction in both rhes$^{+/+}$ and rhes$^{-/-}$ mice. Two-factor ANOVA indicated significant main effects of treatment ($F = 85.18$, $p<0.0001$) and genotype ($F = 6.10$, $p<0.05$), but no treatment x genotype interaction. Bonferroni post-tests indicated a significant effect for drug in rhes$^{+/+}$ and rhes$^{-/-}$ mice, showing that Akt and PP2A-C physically interact upon agonist stimulation in rhes mutant mice. However, as this same apomorphine treatment results in Akt dephosphorylation in rhes$^{+/+}$, but not rhes$^{-/-}$ mice, it is possible that this interaction between Akt and PP2A-C in rhes$^{-/-}$ mice is not functional. We therefore tested whether Rhes itself is present in the complex, enabling the complex to function. Deletion of the rhes gene results in loss of the expected ~30 kDa protein, but also loss of a ~48kDa protein that is highly expressed and a ~39 kDa protein that is expressed at a low level. This pattern has been detected previously upon rhes gene deletion. One of the larger proteins has been proposed to be that encoded by a transcript containing rhes and an N-terminal extension (accession number BC036988; Spano et al., 2004). It is not known at this time if post-translational modification or additional transcripts account for the multiple bands. As shown in Figure 6C, the anti-Rhes antibody recognizes all three proteins, which are absent in rhes$^{-/-}$ mice. In order to determine if Rhes is part of the β-arrestin-containing multi-protein complex, we immunoprecipitated with an anti-β-arrestin1/2 antibody. Rhes was detected by Western blotting as a co-immunoprecipitating protein, as was Akt (Figure 6D). Rhes$^{-/-}$ striata were used as a negative control. The 48 kDa form of Rhes was detected in the elutions. Thus, Rhes interacts with β-arrestin, and this interaction may be critical for the multi-protein complex to allow PP2A-C to dephosphorylate Akt.

The ability of the multi-protein complex to be influenced by lithium was also tested in rhes$^{+/+}$ and rhes$^{-/-}$ mice through co-immunoprecipitation experiments. Mice were injected with either LiCl (200 mg/kg) or vehicle and sacrificed 30 minutes later. Basal interaction between Akt and PP2A-C in rhes$^{-/-}$ mice was low and not further decreased by lithium. There was a significant genotype effect by Two-factor ANOVA ($F = 51.23$, $p<0.05$), but no significant effect of treatment or a treatment x genotype interaction (Figure 6E and F). Levels of PP2A-C and β-arrestins did not differ between rhes$^{+/+}$ and rhes$^{-/-}$ mice (Figure 6G–J).

Discussion

The striatally-enriched protein Rhes has previously been shown to have roles in heterotrimeric G protein (Vargiu et al., 2004; Thapliyal et al., 2008; Harrison and He, 2011) and mTOR signaling (Subramaniam et al., 2011b), and to act as an E3 ligase for sumoylation in striatum (Subramaniam et al., 2009, 2010, 2011a). Here we show that in addition to these roles, Rhes participates in signaling through Akt and GSK3 pathways. The major findings of this study are: (1) Rhes$^{-/-}$ mice display basally increased phosphorylation of Akt and GSK3β. (2) Although administration of the anti-manic drug LiCl increases Akt and GSK3β phosphorylation in rhes$^{+/+}$ mice, it is without effect on these measures in rhes$^{-/-}$ mice. (3) Rhes$^{-/-}$ mice respond to dopamine agonist administration with an increase, rather than a decrease, in Akt and GSK3β phosphorylation. (4) Rhes interacts with β-arrestins, and a striatal multi-protein complex that regulates Akt phosphorylation is altered in rhes$^{-/-}$ mice. Thus, Rhes appears necessary for proper signaling through this complex to regulate Akt and GSK3β phosphorylation. Drugs that promote both disruption and formation of the protein complex show differential effects in rhes$^{+/+}$ versus rhes$^{-/-}$ mice. Such a role would be complementary to the participation by Rhes in signaling through heterotrimeric G proteins, as β-arrestins are a link between these two pathways.

The effects of LiCl and apomorphine on Akt and GSK3 signaling are likely mediated by the striatal multi-protein complex described by Beaulieu, Caron, and colleagues (Beaulieu et al.,

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2005, 2008). Although LiCl can act directly on GSK3, the finding of a similar pattern with Akt phosphorylation found here indicates an indirect effect through the multi-protein complex. In β-arrestin2 knockout mice, co-immunoprecipitation of PP2A-C with Akt is not detected, indicating that this protein is critical to formation of the complex (Beaulieu et al. 2008). Our findings suggest that Rhes, like β-arrestin2, may also participate in the multi-protein complex. Indeed, we found that Rhes and Akt co-immunoprecipitated with β-arrestins. However, there are some key differences. First, we were able to detect some basal interaction of Akt and PP2A-C in the absence of Rhes. This basal interaction was increased when the lysates were pre-incubated in vehicle or drug for the complex formation experiments (Figure 6A and B). Furthermore, in both rhes$^{+/+}$ and rhes$^{-/-}$ mice, apomorphine caused increased interaction between Akt and PP2A-C. However, as this treatment also caused dephosphorylation of Akt in rhes$^{+/+}$ and not rhes$^{-/-}$ mice, it is possible that in rhes$^{-/-}$ mice, the proteins interact in a non-functional manner. That is, Rhes is necessary for proper alignment of the component proteins, and in its absence a non-functioning complex forms. As an indication of altered complex formation in rhes$^{-/-}$ mice, we found a distinct and very consistent pattern in the amount of Akt immunoprecipitated by the anti-Akt antibody: within a given genotype, apomorphine treatment always caused a decrease in Akt immunoprecipitated, and this effect was more pronounced in rhes$^{-/-}$ mice (Figure 5). This effect did not occur with lithium pretreatment to disrupt the complex. Alternatively, as only one time point was tested, we cannot rule out the possibility that a shift in the time course of Akt/GSK3 phosphorylation events accounts for the differential effects in rhes$^{-/-}$ mice.

It is also possible that the phosphorylation differences in rhes$^{-/-}$ mice are not due to effects at the β-arrestin-containing complex. Alternatively, there could be differences in initial phosphorylation of Akt, rather than complex-mediated dephosphorylation. Indeed, Rhes has been shown to bind to PI3 kinase in pull-down assays (Vargiu et al., 2004). More recently, it has been shown that the C-terminal tail of Rhes binds to the p85 regulatory subunit of PI3 kinase and that Rhes interacts with Akt. However, in these studies, Rhes enhanced growth factor-mediated Akt phosphorylation, as well as recruitment of Akt to the plasma membrane. A model was proposed in which growth factor stimulation promotes Rhes interaction with PI3 kinase and Akt in order to position Akt with membrane components that lead to its phosphorylation (Bang et al., 2012). A possible reason for the discrepancy between these studies and our current results, in which Rhes promoted Akt dephosphorylation, is the locus of the effect at PI3 kinase and PIP3 versus the β-arrestin-containing complex. Our results involved 7TM receptor-mediated effects on Akt and likely involved the β-arrestin complex, as Rhes interacts with this protein.

The biochemical phenotype of increased Akt and GSK3 phosphorylation in rhes$^{-/-}$ mice is surprising in light of the documented correlations between these proteins and behavior. Increased phosphorylation/inhibition of GSK3 has been associated with decreased locomotor activity, whereas locomotor-producing drugs cause decreased phosphorylation/activation of GSK3. For example, lithium (which increases pGSK3β) is used clinically for the treatment of bipolar disorder, where it has particular efficacy in the manic phase (Cade, 1949; Bowden, 2000). In addition, lithium is effective in decreasing amphetamine-induced hyperlocomotor activity, an established rodent model of mania (Cox et al., 1971). Genetic and pharmacological studies have shown that the inhibition of GSK3 is a salient feature of lithium's effects on behavior. Thus, GSK3β$^{+/-}$ mice show a lithium-treated phenotype in locomotor and forced swim behavioral tests. In addition, several selective GSK3 inhibitors decrease locomotor activity (Beaulieu et al., 2004; O'Brien et al., 2004). Beta-arrestin2 knockout mice, which show increased basal phosphorylation of Akt and GSK3, also show decreased novelty-induced locomotor activity, in agreement with the established role of these phosphoproteins in locomotion (Beaulieu et al., 2008). Rhes$^{-/-}$ mice, on the other hand, show increased phosphorylation of Akt and GSK3 but a behavioral phenotype of

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increased locomotor activity in several different assays: (1) novelty-induced and open field locomotor activity (Errico et al., 2008), (2) locomotor response to the D1 agonist SKF81297 (Errico et al., 2008), (3) profound hyperlocomotor response to the D1 agonist SKF83822 (unpublished observations), and (4) stereotypy in response to apomorphine (Quintero et al., 2008). Apomorphine treatment results in decreased Akt and GSK3 phosphorylation in wild type mice, but increases in rhes$^{-/-}$ mice, again emphasizing the disconnect between the biochemistry and the behavior. One possible explanation for this discrepancy is the involvement of Rhes in both D1 and D2 receptor-mediated signaling. As a mixed D1/D2 agonist, apomorphine would affect both D1/PKA signaling, which is known to be up-regulated in rhes$^{-/-}$ mice (Errico et al., 2008), and D2/Akt signaling. The exact contribution of each signaling pathway to locomotor behavior is unknown, and it is possible that the up-regulated D1/PKA pathway predominates in rhes$^{-/-}$ mice. Furthermore, the degree of GSK3 inhibition in rhes$^{-/-}$ mice may not be great enough to influence behavior.

Signaling through Akt and GSK3 is clinically relevant not only for lithium and bipolar disorder but also for schizophrenia. Schizophrenics show decreased Akt protein levels and decreased GSK3β phosphorylation, which would be in agreement with enhanced signaling by D2 receptors through this pathway. In addition, an Akt1 haplotype resulting in lower protein levels has been associated with schizophrenia (Emamian et al., 2004). Antipsychotic drugs such as haloperidol increase phosphorylation of Akt (Emamian et al., 2004), and all classes of antipsychotics (typical, atypical, and D2 partial agonists) disrupt the interaction of the D2 receptor with β-arrestin 2 (Masri et al., 2008). The human gene for rhes, rasd2, is localized to chromosome 22q13.1, and an SNP has been highly significantly associated with a subset of schizophrenics without deficits in sustained attention (Liu et al., 2008). Furthermore, in a mouse study, the rhes gene was found to be authentically cis-modulated and to be likely to regulate the expression of downstream genes in disease etiology (Ciobanu et al., 2010). Thus, by participating in striatal signaling through pathways critical to neuropsychiatric disorders, Rhes may be involved in both their etiology and treatment. Furthermore, the interaction between Rhes and β-arrestins positions Rhes to influence both heterotrimeric G protein and alternate signaling pathways.

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Highlights

- The GTPase Rhes is required for lithium to acutely phosphorylate Akt and GSK3
- D1/D2 agonist apomorphine increases Akt and GSK3 phosphorylation in rhes$^{-/-}$ mice
- Rhes interacts with beta-arrestins
- A multi-protein complex in striatum in altered in the absence of Rhes protein

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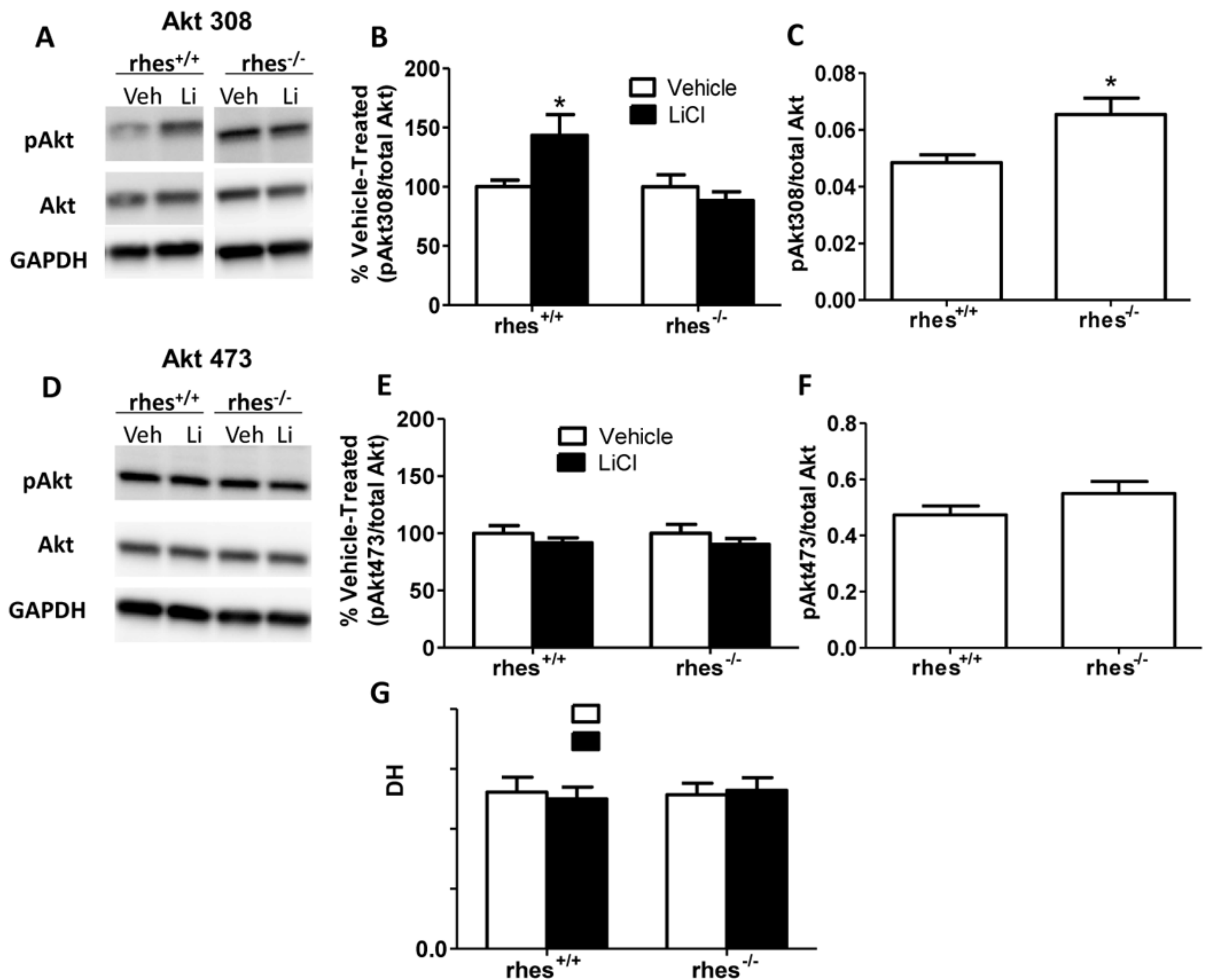

Figure 1.

Phosphorylation of Akt at the 308 and 473 sites in response to lithium treatment in rhes$^{+/+}$ and rhes$^{-/-}$ mice. Mice were injected with 200 mg/kg LiCl (IP) and sacrificed 30 minutes later. Shown are representative Western blots from rhes$^{+/+}$ and rhes$^{-/-}$ mice showing pAkt308 (A) and pAkt473 (D), with total Akt, as well as GAPDH as a loading control. Densitometric values for the ratio of pAkt308 (B) or pAkt473 (E) to total Akt in lithium-treated animals are presented as percent of own genotype's vehicle control. (C) and (F): Basal phosphorylation of Akt308 (C) or Akt473 (F), expressed as ratio to total Akt, in vehicle-treated mice of each genotype. (G), Amounts of total Akt do not differ between rhes$^{+/+}$ and rhes$^{-/-}$ mice or with drug treatment. Densitometric values are presented as ratio to GAPDH, which was used as a loading control. Values are mean ± SEM. n = 8–10. *$p<0.05$

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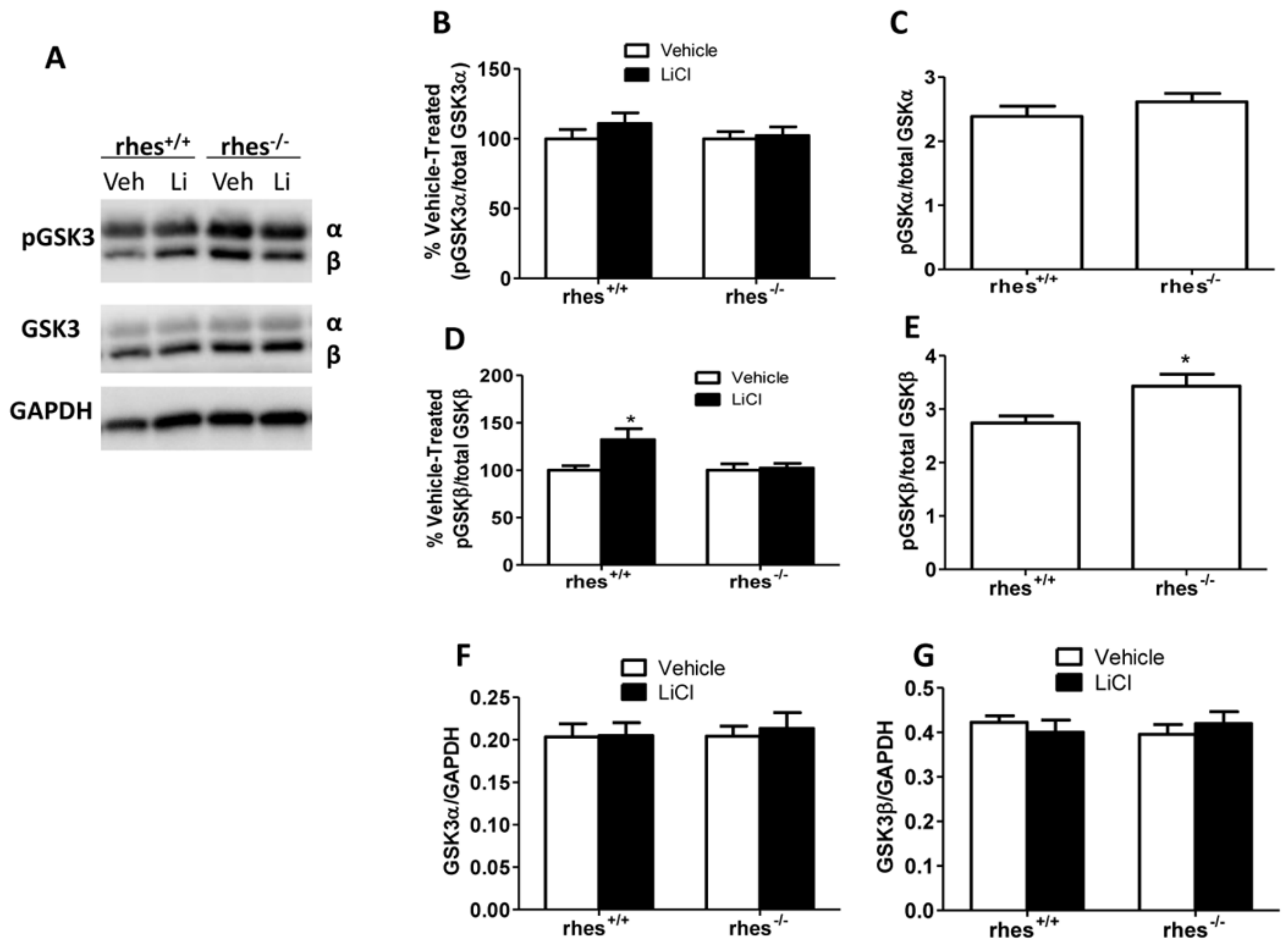

Figure 2.

Phosphorylation of GSK3α and GSK3β in response to lithium treatment in rhes$^{+/+}$ and rhes$^{-/-}$ mice. Mice were injected with 200 mg/kg LiCl (IP) and sacrificed 30 minutes later. (A) Representative Western blots of pGSK3α and β, total GSK3α and β, and GAPDH. Densitometric values for the ratio of phospho- to total GSK3 in lithium- and vehicle-treated mice are presented as percent own genotype's control for GSK3α (B) and GSK3β (D). Basal phosphorylation is shown as the ratio of densitometric value for phospho- to total protein in vehicle-treated controls for GSK3α (C) and GSK3 β (E). Levels of total GSK3α (F) and GSK3β (G) did not differ between rhes$^{+/+}$ and rhes$^{-/-}$ mice or with LiCl treatment. Densitometric values are shown as a ratio to GAPDH. Values are mean ± SEM. n = 8–10. *$p<0.05$

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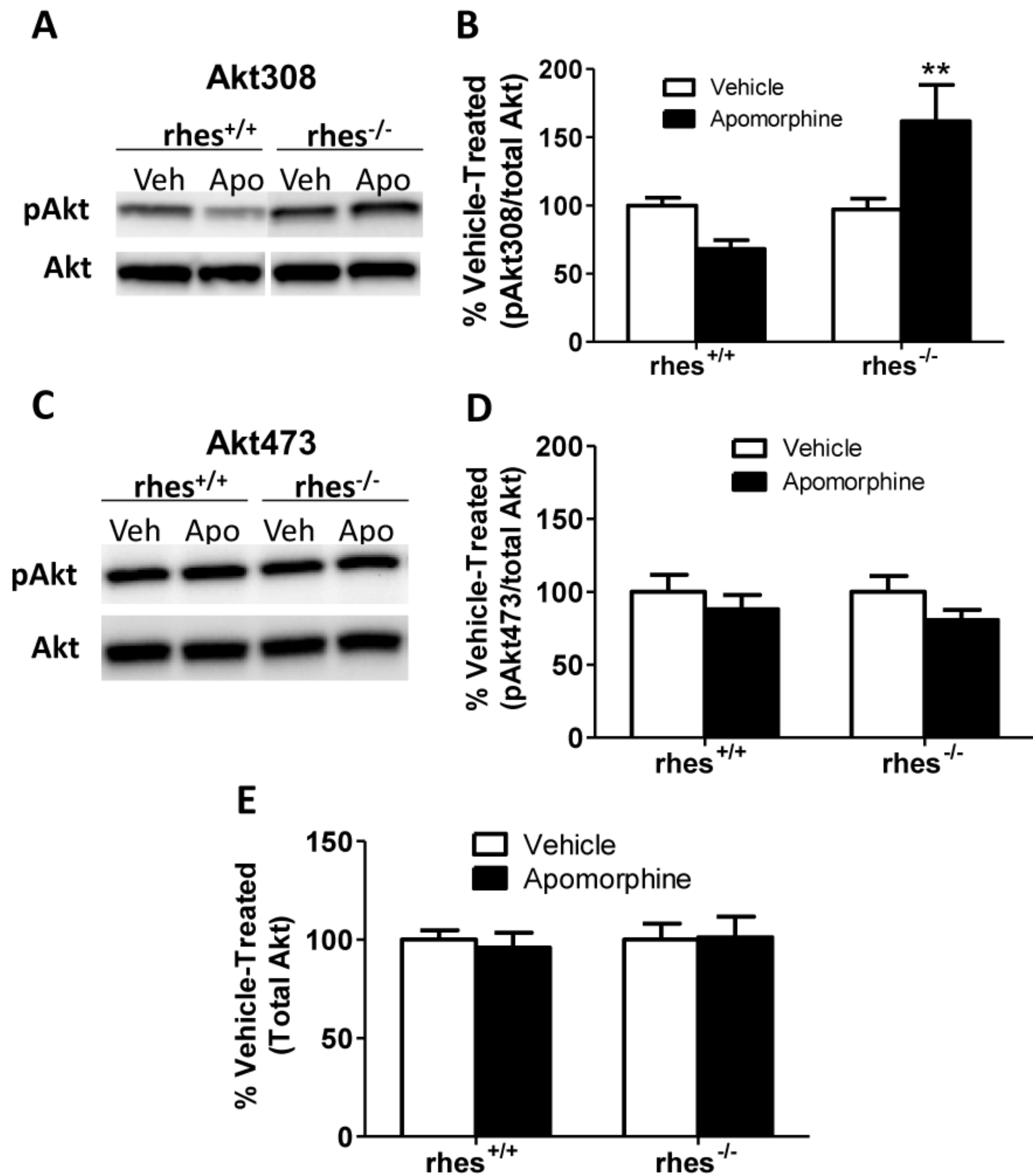

Figure 3.

Phosphorylation of Akt at the 308 and 473 sites in response to apomorphine treatment in rhes$^{+/+}$ and rhes$^{-/-}$ mice. Mice were administered 3 mg/kg apomorphine or vehicle and sacrificed 1 hour later. (A) and (C), representative Western blots of pAkt308 and pAkt473, and total Akt. (B) and (D), densitometric values for apomorphine-treated mice, expressed as ratio of phospho- to total Akt, are presented as percent vehicle control of own genotype. (E) Total Akt, presented as percent vehicle control of own genotype, was not affected by apomorphine treatment. Values are mean ± SEM. n = 9–10. **p<0.01

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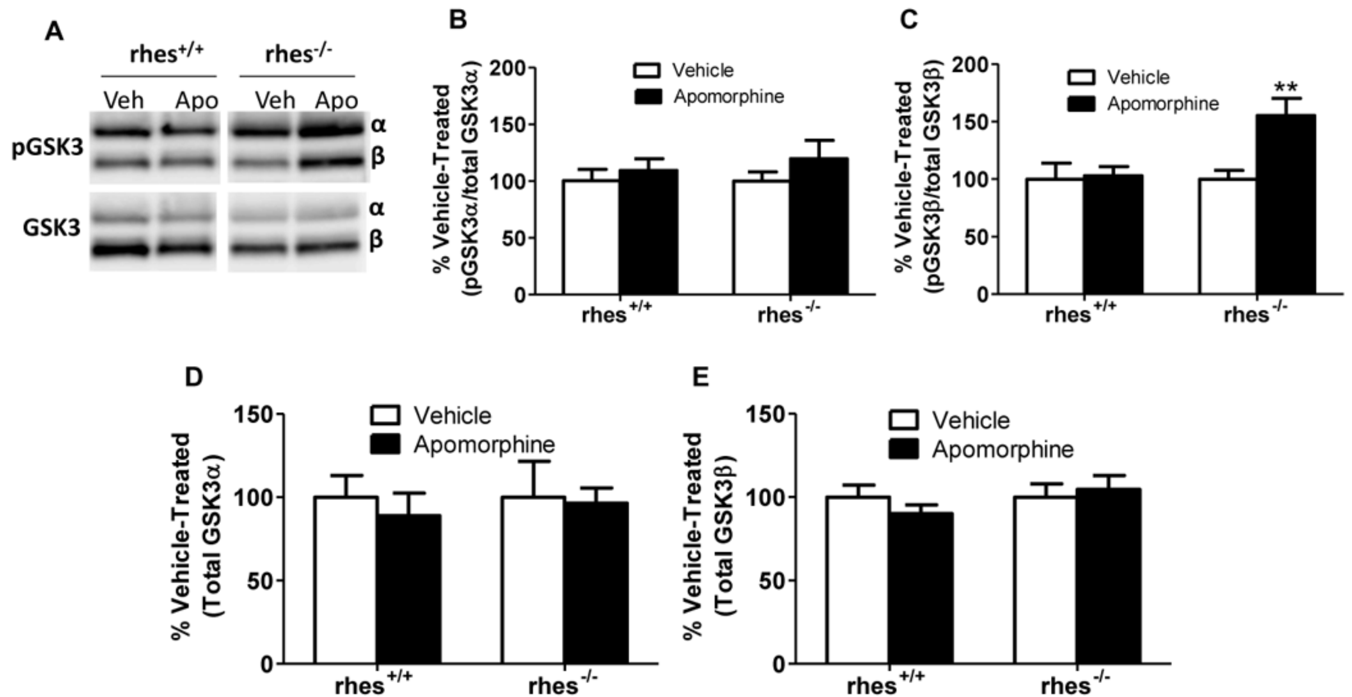

Figure 4.

Phosphorylation of GSK3α and β in response to apomorphine treatment in $rhes^{+/+}$ and $rhes^{-/-}$ mice. Mice were administered 3 mg/kg apomorphine or vehicle and sacrificed 1 hour later. (A) Representative Western blots of phospho- and total GSK3α and β. Densitometric values for phosphorylation of GSK3α (B) and GSK3β (C), expressed as ratio of phospho- to total GSK3, are presented as percent of own genotype's control. Densitometric values for total GSK3α (D) and GSK3β (E), expressed as percent own genotype control, were not affected by apomorphine treatment. Values are mean ± SEM. n = 9–10. **$p<0.01$

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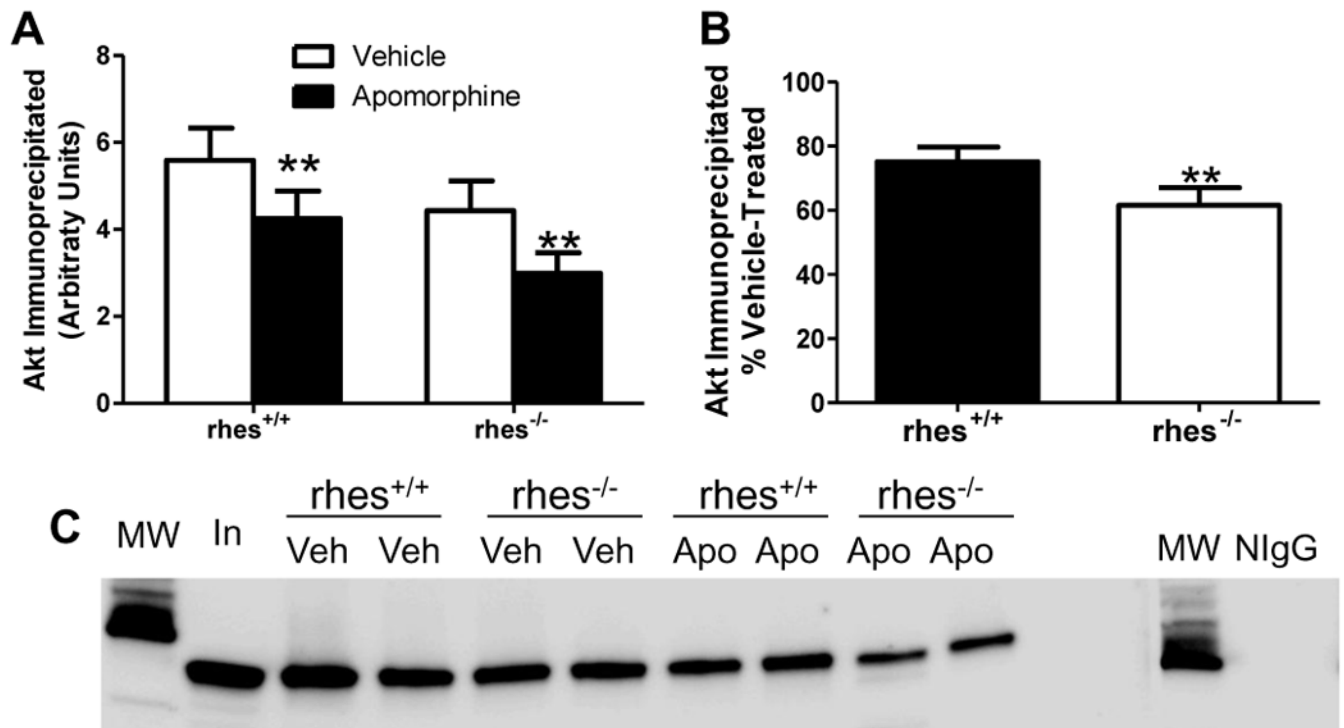

Figure 5.

Immunoprecipitation of Akt in rhes$^{+/+}$ and rhes$^{-/-}$ mice. Mice were treated with vehicle or 3 mg/kg apomorphine and sacrificed 1 hour later. Striatal lysates were immunoprecipitated with an anti-Akt antibody, and Western blots were probed with the same antibody. (A) Apomorphine treatment caused a significant decrease in the amount of Akt immunoprecipitated. (B) Data from drug-treated mice are presented as percent vehicle-treated mice of own genotype. ** $p<0.01$, $n = 10$. (C) Representative Western blots. MW = molecular weight standards, In = input from rhes$^{+/+}$ vehicle-treated mouse, NIgG = normal rabbit IgG.

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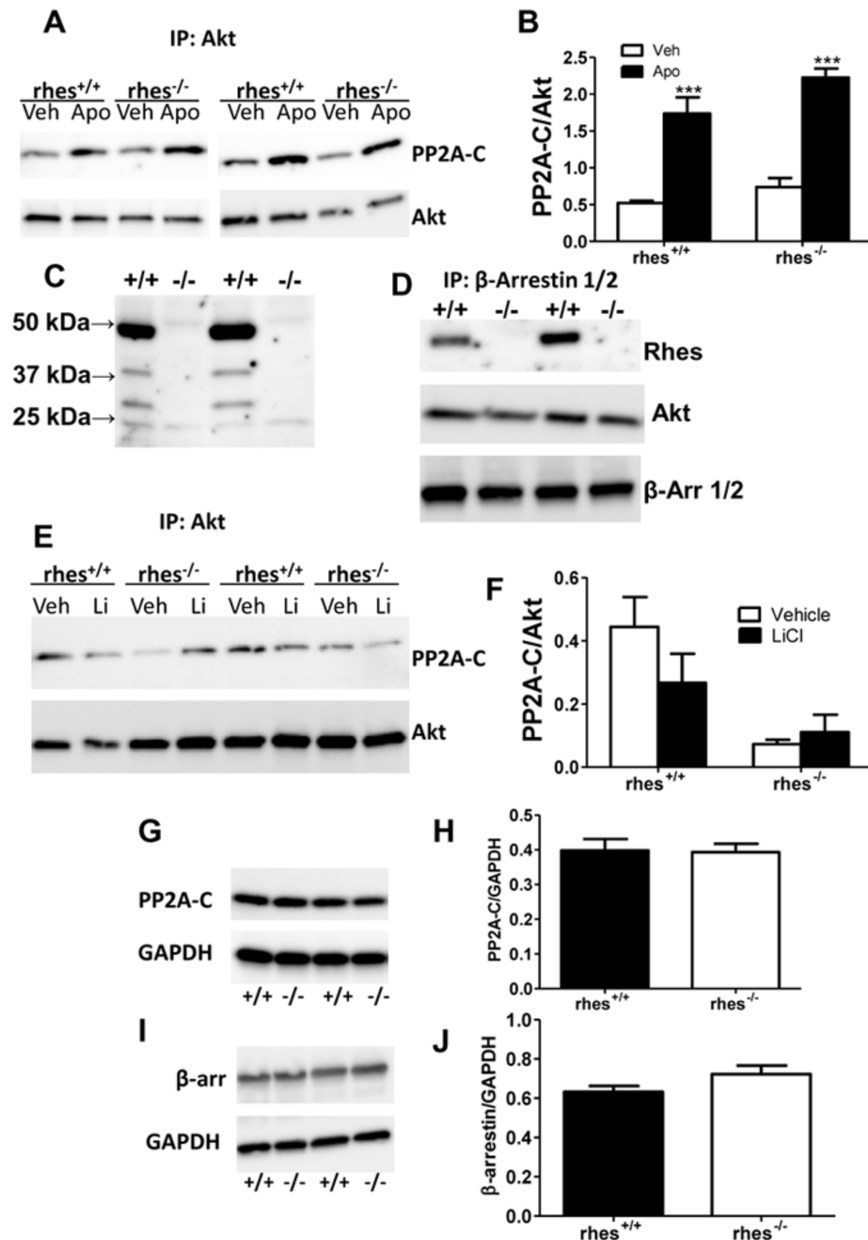

Figure 6.

Striatal multi-protein complex formation in rhes$^{+/+}$ and rhes$^{-/-}$ mice. (A) and (B), PP2A-C co-immunoprecipitated with Akt in both rhes$^{+/+}$ and rhes$^{-/-}$ mice. Mice were pre-treated with 3 mg/kg apomorphine and sacrificed 1 hour later. Striatal lysates were also incubated in vitro for 30 minutes with either vehicle or 10 μM apomorphine. Panel A shows representative Western blots. Panel B shows densitometric values for the ratio of PP2A-C/Akt. Values are mean ± SEM. n = 2–3. ***p<0.001. (C) An anti-Rhes antibody recognizes 48 kDa, 39 kDa, and 30 kDa proteins in wild type mice that are not present in the Rhes mutant mice. (D) Striatal lysates were immunoprecipitated with an anti-β-arrestin1/2 antibody and analyzed by Western blotting. In wild type mice (+/+), Rhes co-immunoprecipitated with β-arrestin1/2. Similar results were found with two different anti-β-arrestin1/2 antibodies. Akt also immunoprecipitated with β-arrestin. (E) and (F): PP2A-C

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and Akt co-immunoprecipitation after lithium treatment in rhes$^{+/+}$ and rhes$^{-/-}$ mice. Mice were treated with 200 mg/kg lithium, and striatal lysates were subjected to immunoprecipitation. n = 2–3. The amounts of PP2A-C and β-arrestin did not differ between rhes$^{+/+}$ and rhes$^{-/-}$ mice, as shown by representative blots in (G) and (I) and densitometric values as a ratio to GAPDH in (H) and (J). n = 9–10. Values are mean ± SEM.