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RGS4 over-expression in the rat dorsal striatum modulates mGluR5- and amphetamine-mediated behavior and signaling

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Abstract

Rationale—Regulator of G-protein signaling 4 (RGS4) is a brain-enriched negative modulator of G-protein-coupled receptor signaling. Decreased availability of RGS4 in the frontal cortex and striatum has been described in animal models of schizophrenia and drug addiction. However, cellular and behavioral consequences of dysregulated RGS4-dependent receptor signaling in the brain remain poorly understood.

Objective—To investigate whether RGS4, through inhibiting function of mGluR5 receptors in the dorsal striatum (dSTR), regulates cellular and behavioral responses to acute amphetamine.

Methods—After HSV-RGS4 was infused into dSTR, RGS4 overexpression as well as binding of recombinant RGS4 to mGluR5 were assessed. The effect of RGS4 overexpression on behavioral activity induced by the intra-striatal mGluR5 agonist, DHPG, or amphetamine was recorded. Activation of extracellular signal-regulated kinase (ERK) and Akt (protein kinase B) was measured in the dSTR tissue at the end of each behavioral experiment.

Results—RGS4 overexpressed in the dSTR co-immunoprecipitated with mGluR5 receptors and suppressed both behavioral activity, as well as phospho-ERK levels induced by DHPG. RGS4 over-expression or the mGluR5 antagonist, MTEP attenuated amphetamine-induced phospho-ERK (but not phospho-Akt) levels. RGS4 suppressed amphetamine-induced vertical activity and augmented horizontal activity over 90 min. Similarly, MTEP augmented amphetamine-induced horizontal activity but did not affect vertical activity.

Conclusions—The present data demonstrate that RGS4 in the dSTR attenuates amphetamine-induced ERK signaling and decreases the behavioral efficacy of acute amphetamine likely by limiting mGluR5 function.

Keywords

Akt; amphetamine; dorsal striatum; ERK; locomotor activity; mGluR5; overexpression; RGS4

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Introduction

Regulator of G-protein signaling 4 (RGS4) is a member of the large family of RGS proteins that function as negative modulators of G-protein-coupled receptor (GPCR)-mediated signaling pathways (Siderovski and Willard 2005). All RGS proteins bind directly to the GTP-bound G α i or G α q subunit of activated heterotrimeric G-proteins and increase the rate of GTP hydrolysis. This accelerated “turn-off” of activated G-proteins provides a cellular mechanism for limiting temporal and spatial resolution of GPCR-signaling and GPCR-mediated synaptic plasticity by RGS proteins (Abramow-Newerly et al. 2006; Kimple et al. 2011). A growing body of evidence indicates that disruption of this regulatory mechanism is a critical component of pathophysiologies underlying various human diseases (Emilsson et al. 2006; Nishiguchi et al. 2004; Talkowski et al. 2006; Tekumalla et al. 2001).

RGS4 is a small RGS protein enriched in the brain, with highest levels of RGS4 protein present in the frontal cortex, dorsal striatum (dSTR), amygdala and thalamus (Gold et al. 1997). As such, dysregulation of RGS4 has been linked to several neuropsychiatric disorders including schizophrenia (Ding and Hegde 2009; Mirnics et al. 2001), Parkinson's (Ding et al. 2006; Zhang et al. 2005), Alzheimer disease (Emilsson et al. 2006) and drug addiction (Hooks et al. 2008). With regard to addiction, our laboratory as well as others have documented that acute exposure to psychostimulants or opiates results in rapid downregulation of RGS4 mRNA and protein levels, predominately in the striatum (Chase et al. 2010; Gonzalez-Nicolini and McGinty 2002; Schwendt et al. 2006; Yuferov et al. 2003). Furthermore, chronic exposure to both experimenter- and self-administered cocaine resulted in a lasting RGS4 decrease in the prefrontal cortex and striatum, followed by a rapid up-regulation of RGS4 levels as a result of cue-induced drug-seeking (Schwendt et al. 2007). This suggests that RGS4 can play a role in cellular adaptations underlying acute dopaminergic activity (hyperlocomotion) as well as enduring drug-induced behaviors (such as relapse to drug-seeking). However, the identity of GPCRs and signaling pathways affected by fluctuation of RGS4 levels remains obscure.

Although a number of receptors regulated by RGS4 have been identified in heterologous cell-lines (as reviewed by (Bansal et al. 2007)), few studies have addressed receptor-specificity and the regulatory function of RGS4 in native neuronal cultures or directly in the brain. In one of those studies (Saugstad et al. 1998), it was demonstrated that RGS4 is a potent inhibitor of mGluR1/5-induced signaling in hippocampal neurons. In the striatum, which contains high concentrations of both RGS4 and mGluR5 (but not mGluR1) receptors, this functional interaction is likely to occur via direct physical association of RGS4 with the mGluR5 receptor signaling complex (Schwendt and McGinty 2007). mGluR5 receptors in the brain are coupled to downstream signaling pathways either through a conventional G α q-to-phospholipase C, subtype β 1 (PLC β 1) pathway or through a pathway utilizing Homer scaffolding proteins (Conn and Pin 1997; Ribeiro et al. 2010). While the conventional pathway leads to a release of calcium from intracellular stores and activation of mitogen-activated protein kinase (MAPK) signaling pathways, the Homer-dependent pathway is calcium-independent and leads to an activation of both MAPK and PI3K-Akt-mTOR pathways (Mao et al. 2005; Ronesi and Huber 2008). In agreement, local administration of the mGluR1/5 agonist, DHPG, into the dSTR dose-dependently increased phosphorylation

of extracellular signal-regulated kinase (ERK) 1/2 MAPK, that was blocked by pretreatment with an mGluR1/5 antagonist (Choe and Wang 2001). Activation of mGluR5 receptors in the striatum is also necessary for induction of ERK1/2 signaling by Amph (Choe et al. 2002) and it is likely that this signaling mechanism is also involved in regulating mGluR5-dependent synaptic plasticity after exposure to psychostimulants (Fourgeaud et al. 2004; Grueter et al. 2006). On the other hand, regulation of PI3K-Akt-mTOR signaling in the STR by mGluR5 receptors has not been investigated. In addition to cellular signaling, striatal mGluR5s can regulate behavior in experimental animals, as demonstrated by the finding that intra-striatal administration of DHPG induced complex locomotor and stereotypical behaviors (Wang and Mao 2000). However, despite a number of studies investigating the cellular and behavioral significance of striatal mGluR5 receptors, there are considerable gaps in our understanding of their role in psychostimulant addiction.

Therefore, in the present study, we hypothesized that RGS4 limits mGluR1/5 signaling in the dSTR and further, that the mGluR5-RGS4 functional relationship is manifested by altered behavioral responses to an mGluR5 agonist and/or to Amph. Since psychostimulants decrease the levels of RGS4 in the dSTR, we employed a technique of viral-mediated gene transfer to over-express RGS4 within the dSTR in order to study the effects of elevated RGS4 protein levels on cellular signaling and behavior.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (275-300 g) (Charles River Laboratories, Wilmington, MA, USA) were single-housed in clear plastic cages and maintained on a 12 h light/dark cycle with food and water available *ad libitum*. All animals were acclimated to their home cage environment for a minimum of 3 days prior to surgery and handled for an additional 5 days prior to drug administration to minimize effects of stress on behavioral and neurochemical parameters. Every animal procedure in this study was approved by the Institutional Animal Care and Use Committee and was performed in strict accordance with the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, National Academy Press 1996).

Drugs

D-amphetamine sulfate (Amph) was purchased from Sigma-Aldrich (St. Louis, MO) or acquired from the NIDA Controlled Substances Program (Research Triangle Institute, NC). The mGluR1/5 agonist, (S)-3,5-DHPG, was purchased from Tocris Bioscience (Ellisville, Missouri) and the selective mGluR5 antagonist, MTEP hydrochloride, was purchased from Ascent Scientific (Bristol, UK). All drugs were freshly prepared on the day of the experiment. Amph was dissolved in physiological saline (Sal) and injected intraperitoneally (i.p.) in a volume of 1 ml/kg. For intrastriatal infusions, DHPG was first dissolved in 25% dimethylsulfoxide (DMSO) and then diluted in artificial cerebrospinal fluid (aCSF) (in mM: NaCl 123, CaCl₂ 0.86, KCl 3.0, MgCl₂ 0.89, NaH₂PO₄ 0.50 and Na₂HPO₄ 0.25, pH 7.4) to a final concentration of 125 or 250 nmol/μl. MTEP was dissolved in 1% Tween 80 in aCSF to a final concentration of 5μg/μl. DMSO in combination with aCSF or Tween80 in

combination with aCSF was therefore used as vehicle control for the respective agents. Solutions of all drugs were neutralized to pH 7.2–7.4 with 1N NaOH, if necessary. The concentrations of the drugs used were determined based on the results of previously published studies (Choe and Wang 2001; Gass and Olive 2009; Gonzalez-Nicolini and McGinty 2002; Molina-Hernandez et al. 2006), as well as our preliminary data.

Stereotaxic surgery

On the day of surgery, rats were anesthetized with ketamine/xylazine (66 mg/kg and 1.33 mg/kg i.p.), followed by equithesin (0.5 ml/kg i.p.) and ketorolac (2.0 mg/kg, i.p.). Rats were mounted onto a stereotaxic device (Stoelting, Wood Dale, IL) and bilateral stainless steel guide cannulae pre-cut (24-gauge, Plastics One, Roanoke, VA, USA) were implanted 2 mm above the dSTR infusion target (+1.2 mm antero-posterior, ± 3.4 mm medio-lateral and -3.4 mm dorso-ventral relative to bregma according to Paxinos and Watson, 2007). Animals were allowed to recover for 5 days prior to the beginning of behavioral experiments.

HSV-Mediated Gene Transfer

Recombinant herpes simplex virus (HSV)-LacZ, and HSV-RGS4 constructs were gifts from Drs. Stephen Gold and David Self (UT Southwestern, Dallas, TX). They were generated in the laboratory of Dr. Rachel Neve (McGovern Institute for Brain Research, MIT, Cambridge, MA) and were previously described (Neve et al. 1997; Rahman et al. 2003). The average titer of the purified virus stocks was $>10^8$ infectious units/ μ l. Two microliters of HSV vectors were bilaterally infused into dSTR at the rate of 0.2 μ l/min using a 33-gauge injector cannula inserted to a depth of 2 mm below the tip of the guide cannula. The injector was connected via polyethylene tubing to a 10 μ l gas-tight Hamilton syringe mounted in an infusion pump (PHD 2000, Harvard Apparatus, Holliston, MA). The injector remained in place for 5 min after the infusion to prevent backflow along the cannula track. HSV-mediated transgene expression was confirmed by β -galactosidase stain for HSV-LacZ or by immunohistochemistry and immunoblot for the HSV-RGS4. All behavioral experiments were performed on day 3 after HSV injection, at the peak of HSV-driven transgene expression (Rahman et al. 2003).

Drug administration and locomotor activity

DHPG (125, 250 nmols), MTEP (5 μ g), or the appropriate vehicle were bilaterally infused into dSTR (1 μ l/side) through a 33-gauge injector over a period of 4 min using an infusion pump (as described in detail for HSV microinfusions). After the infusions, the injectors were left in place for an additional 1 min. Amph (2.5–3 mg/kg, i.p.) or Sal was administered to rats 15 min later. This dose of Amph has been shown to reliably stimulate horizontal and vertical activity in rats (Wang and McGinty 1995). To measure baseline and drug-induced behavioral activity, animals were placed in automated photocell beam activity chambers (Accuscan Instruments, Columbus, OH, USA) and horizontal (total distance traveled) as well as vertical activity (rearing) was recorded for 15 min – 3h as described previously (Schwendt et al. 2006). At the end of each behavioral experiment, whole brains or selected brain tissues were harvested for protein analysis as described below.

Immunohistochemistry

For the immunohistochemical analysis of HSV-driven LacZ or RGS4 expression, rats were anesthetized and perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS) (in mM: NaCl 137, KCl 2.7, Na₂HPO₄ 4.3, KH₂PO₄ 1.47, pH 7.4). Brains were removed and post-fixed for 4 h and saturated in 30% sucrose/PBS solution. After embedding and freezing, 30 µm sections were cut on a freezing microtome and used for histology (cannulae placements) or for confirmation of transgene expression. Detection of β-galactosidase expression (LacZ-staining) was performed as described previously (Neve et al. 1997). Viral over-expression of RGS4 was detected via immunohistochemistry using a specific antibody against *FLAG*-tagged RGS4, anti-*FLAG* M2 HRP-conjugated antibody (1:1000, Sigma, Aldrich). The staining was visualized using VIP substrate (Vector Labs, Burlingame, CA). The sections were viewed and photographed with an Olympus microscope equipped with a digital camera.

Co-immunoprecipitation

For immunoprecipitation of *FLAG*-tagged RGS4, dSTR tissue was homogenized by a brief sonication followed by solubilization of striatal membrane proteins in a mild Triton-based lysis buffer (50 mM Tris HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing the following inhibitors: Complete Mini protease inhibitor (Roche Diagnostics, Indianapolis, IN, USA), Halt phosphatase inhibitor (Pierce Chemical, Rockford, IL, USA) and 5 mM MG-132 proteasome inhibitor (Tocris). Insoluble proteins were sedimented at 15,000g for 10 min and supernatants (500 µg of protein at 1 µg/µl concentration) were used for immunoprecipitation of *FLAG*-tagged RGS4 with anti-*FLAG* M2 agarose resin according to the manufacturer's instructions (Sigma-Aldrich). After a series of washes, immunocomplexes were dissociated from the beads under native conditions by a competition with a 3×*FLAG* peptide. Proteins were then resolved by SDS-PAGE and analyzed by immunoblotting as described below. Co-immunoprecipitation experiments included a positive control (immunoprecipitation of *FLAG*-BAP fusion protein; Sigma-Aldrich) as well as negative controls (omission of lysate, *FLAG* M2 beads or no over-expression of *FLAG*-tagged RGS4).

Immunoblotting

For immunoblotting analysis, 5 mm-thick forebrain coronal slices containing dSTR were collected using a Precision brain slicer (Braintree Scientific, Braintree, MA) and rapidly frozen in isopentane on dry ice. Frozen coronal slices were then trimmed in the cryostat to a 2 mm thickness corresponding to ~ 2.7-0.7 mm anterior to bregma (Paxinos and Watson 2007) and the dSTR was bilaterally dissected using a 2 mm tissue micropuncher and stored at -80°C until processed. This approach allowed for monitoring of cannulae placement and potential tissue damage. Total tissue protein was extracted from dSTR by sonication in 1% SDS/PBS buffer with inhibitors (as described above), denatured for 10 min at 85°C and centrifuged for 10 min at 12,000g to pellet insoluble proteins. Protein concentrations were measured with the Micro-Bicinchoninic Acid assay kit (Pierce Chemical, Rockford, IL, USA). Equal protein amounts (15 µg) were separated by SDS-PAGE (4–15% polyacrylamide) and transferred onto polyvinylidene difluoride membranes. Membranes

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were blocked for 1 h in 5% milk/Tris-buffered saline and probed overnight at 4°C with a primary antibody diluted in 3 or 5% milk/Tris-buffered saline with 0.1% Tween 20. The following primary antisera were used: rabbit phospho-ERK (1:2500), rabbit tERK (1:15000), rabbit phospho-Akt-Thr308 (1:1000), rabbit phospho-Akt-Ser473 (1:2000), rabbit tAkt (1:7500); all from Cell Signalling Technology (Danvers, MA), rabbit RGS4 (1:5000), rabbit mGluR5 (1:5000; Millipore, Billerica, MA), mouse *FLAG* M2 (1:1000, Sigma-Aldrich), mouse β -galactosidase (1:2500, Promega, Madison, WI), mouse D1 receptor (1:250; Millipore), rabbit D2 receptor (1:1000; Abcam, Cambridge, MA) and calnexin (1:10000; Enzo Life Sciences, Farmingdale, NY). After the incubation with an appropriate HRP-conjugated secondary antiserum (Jackson Immuno Research, West Grove, PA), immunoreactive bands on the membranes were detected by ECL+ chemiluminescence reagents on an X-ray film (GE Healthcare, Piscataway, NJ). Equal loading and transfer of proteins were confirmed by stripping and re-probing of the same membranes either for proteins independent of their phosphorylation state (tERK, tAkt) or for calnexin, an intracellular protein that was not altered by any experimental treatment. The integrated band density of each protein sample was measured using Image J software (U. S. National Institutes of Health, Bethesda, Maryland).

Statistical Analysis

Results are expressed as Mean $\pm$ Standard Error of the Mean (S.E.M.). Behavioral data were analyzed by calculating the area under the curve (AUC) for the total distance traveled and vertical activity plotted against time starting at time zero. For the immunoblotting data, the ratios of phospho-to-total protein levels (ERK and Akt), or ratios of protein-to-calnexin levels are presented. Because of the less abundant levels of phospho-ERK1 in the dSTR and because p-ERK2 has been identified as the isoform that mediates the neurochemical and behavioral effects of psychostimulants (Girault et al. 2007), only the phospho-ERK2/tERK2 ratio was analyzed. Behavioral and immunoblotting data were evaluated using a one-way ANOVA followed by Student–Newman–Keuls (SNK) multiple comparison tests. Sigma Stat (Systat Software, Chicago, IL, USA) software was used for all statistical analyses.

Results

Infusion of HSV-RGS4 into the dSTR resulted in a robust over-expression of recombinant RGS4 protein and binding to mGluR5 receptors

In order to study the role of RGS4 in the dSTR in mGluR5- and psychostimulant-induced behaviors and signaling, we over-expressed the protein specifically in the dSTR using HSV vectors previously demonstrated to be effective for in vivo gene transfer (Carlezon et al. 2000; Han et al. 2010; Rahman et al. 2003). A single bilateral microinfusion of HSV-RGS4 or HSV-LacZ directed into dSTR (as depicted on Fig 1A, top panel) resulted in expression of *FLAG*-tagged RGS4 or β -galactosidase restricted to an area of $\sim$ 1-1.5 mm in diameter as detected 3 days post-infection by immunohistochemical staining (Fig 1A, middle panels). Microinfusion of HSV vectors was associated with minimal tissue damage, comparable to the damage seen after HSV vehicle (10% sucrose) as demonstrated by a Nissl stain of dSTR sections collected at the site of infusion (Fig 1A, lower panel). Over-expression of RGS4 was also confirmed by immunoblotting, again at 3 days post-infection (Fig.1B).

Microinfusion of HSV-RGS4 (but not HSV-LacZ) resulted in a robust expression of *FLAG*-tagged RGS4 as detected by antibody against the *FLAG* tag as well as antibody against the endogenous RGS4 protein (Fig 1B). Anti-RGS4 antibody recognized both endogenous RGS4 and over-expressed *FLAG*-tagged RGS4 which has a higher molecular weight due to the presence of the *FLAG* tag.

We have previously shown that endogenous RGS4 protein co-immunoprecipitates with mGluR5 receptors in rat dSTR (Schwendt and McGinty 2007). In order to assess the behavioral and neurochemical significance of this interaction, we first had to examine whether *FLAG*-tagged RGS4 over-expressed in the dSTR also interacts with mGluR5 receptors. To that effect, a specific anti-*FLAG* antibody was used to immunoprecipitate the RGS4-*FLAG* protein fraction from dSTR lysates obtained 3 days after HSV-RGS4-*FLAG* microinfusion. As illustrated in Fig. 1C (top panel), probing the immunoprecipitated sample (+++) and total (T) striatal lysates with anti-mGluR5 antibody revealed 130- and 250-kDa bands corresponding to monomer and dimer isoforms of mGluR5 receptors. In contrast, no bands were detected in negative control samples in which lysate or Flag-IP beads were omitted, as well as in immunoprecipitates from the dSTR with no RGS4-*FLAG* over-expression. To confirm the efficiency and specificity of immunoprecipitation, samples were also probed with antibody against the *FLAG* tag itself. As demonstrated in a middle panel (Fig.1C), *FLAG* signal was only detected in total dSTR lysate (T) and complete *FLAG*-tag immunoprecipitates (+++), but not in a series of negative controls. In addition to mGluR5, other G α i- and G α q-coupled GPCRs have been shown to interact with RGS4 protein when co-expressed in various cell lines (Jaen and Doupnik 2006; Ruiz de Azua et al. 2010; Wang et al. 2009). In this study, we tested the ability of *FLAG*-tagged RGS4 to interact with D1 and D2 dopamine receptors, which are both abundant in the STR and critical for psychostimulant-induced behaviors (Berke and Hyman 2000). However, co-immunoprecipitation experiments revealed no interaction of RGS4-*FLAG* with D1 or D2 receptors in dSTR lysates (Fig. 1D). These results suggest a specific interaction of over-expressed RGS4 with native mGluR5 (but not dopamine) receptors in the rat dSTR under these experimental conditions.

Over-expression of RGS4 in the dSTR attenuated mGluR5-dependent behavior and phospho-ERK signaling

In the next series of experiments, we sought to determine whether increasing levels of RGS4 in the dSTR interferes with mGluR5 function, assessed as a suppression of mGluR5-dependent behavioral activation and phosphoprotein signaling.

In agreement with previous observations (Choe and Wang 2001), intrastratial infusion of DHPG (125 and 250 nmol) dose-dependently increased phospho-ERK2 levels 30 minutes post infusion (Fig. 2A left panel). A one-way ANOVA ($F_{(2, 13)} = 14.92$, $P < 0.001$) followed by SNK multiple comparisons revealed not only significantly higher phospho-ERK2 levels in both DHPG-treated groups vs. the vehicle-treated group ($p < 0.01$), but also a significant dose-dependent effect (DHPG250 vs. DHPG125, $p < 0.05$). Similarly, there was a dose-dependent effect of DHPG on phospho-Akt-Thr308 levels in the dSTR (Fig.2A right panel). However in the latter case, DHPG induced a dose-dependent de-phosphorylation of Akt-

Thr308 as demonstrated by one-way ANOVA ($F_{(2, 13)} = 12.85$, $P = 0.001$) followed by SNK multiple comparison test (DHPG250 vs. DHPG125, $p < 0.05$). Because phosphorylation of both Thr308 and Ser473 is thought to be required for full kinase activity (Alessi et al. 1996; Manning and Cantley 2007), the level of Akt phosphorylation at Ser 473 was also examined throughout this study. However, no changes in phospho-Akt-Ser473 were found among any groups in all the experiments (data not shown). It should be noted, that the two doses of DHPG used in this study did not alter total levels of ERK2 or Akt in the dSTR. In addition, microinfusion of 125 or 250 nmols DHPG had no effect on protein levels of endogenous RGS4 in the dSTR as measured 2 hrs post-infusion (Fig. 2B). This was not due to the inability of DHPG to induce mGluR5-dependent protein expression because 250 nmol significantly induced Arc protein levels in the dSTR (data not shown).

In a subsequent study, the effect of RGS4 over-expression on DHPG-induced behavior and signaling was investigated. Rats received bilateral microinfusions of HSV-RGS4 into dSTR in order to over-express RGS4 or they were infused with HSV-LacZ. Three days later, 250 nmol DHPG (or vehicle) was delivered into dSTR and behavior was analyzed for the first 30 min after the infusion. As shown in Fig. 3A, DHPG-infused rats demonstrated a robust increase in locomotor activity (total distance traveled) compared to vehicle-infused rats 20-30 min post-infusion. Quantitative analysis of total distance traveled over the last 10 min of the test (20-30 min post-infusion) revealed a significant difference in AUC between treatment groups as detected by ANOVA ($F_{(3,14)} = 19.60$, $P < 0.01$). Pairwise comparisons showed that in control animals over-expressing β -galactosidase in the dSTR, DHPG microinfusion induced a significant increase of total distance traveled (LacZ-DHPG vs. LacZ-Veh, $p < 0.01$). Interestingly, behavioral activation by DHPG was blunted in animals over-expressing RGS4 in the dSTR (RGS4-DHPG vs. LacZ-DHPG, $p < 0.01$). The effects of DHPG on vertical activity (rearing) were not significantly different in any treatment group (data not shown).

At the end of the experiment (30 min post Veh/DHPG microinfusion), the dSTR was harvested for phosphoprotein analysis (Fig. 3B and C). In accordance with the previous experiment, DHPG induced ERK phosphorylation, but to a different degree across treatment groups (Fig 3B, $F_{(3,14)} = 7.84$, $P < 0.01$). SNK pairwise comparison tests revealed a significantly greater increase in phospho-ERK2 levels in the dSTR of LacZ-DHPG animals than in LacZ-Veh rats ($p < 0.01$). In addition, RGS4 over-expression suppressed DHPG-induced phospho-ERK2 levels (RGS4-DHPG vs. LacZ-DHPG, $p < 0.01$). On the other hand, DHPG-induced dephosphorylation of Akt-Thr308 was not prevented by RGS4 over-expression (Fig 3C). One-way ANOVA ($F_{(3,14)} = 10.29$, $P < 0.01$) followed by SNK pairwise comparisons revealed that phospho-Akt-Thr308 levels were downregulated by DHPG regardless of β -galactosidase or RGS4 over-expression (LacZ-DHPG vs. LacZ-Veh, $p < 0.01$ and RGS4-DHPG vs. RGS4-LacZ, $p < 0.05$).

The effects of RGS4 over-expression in the dSTR on early-onset Amph-induced locomotion and phosphoprotein signaling: comparison to intra-dSTR mGluR5 receptor blockade

The preceding experiments suggested that RGS4 regulates mGluR5-mediated locomotor behavior and ERK phosphorylation in the dSTR. Follow-up experiments sought to determine whether RGS4-mGluR5 functional interactions play a role in psychostimulant-induced behaviors and protein phosphorylation in the dSTR. Therefore, the effects of RGS4 over-expression were compared to a site-specific blockade of mGluR5s in the dSTR after acute Amph exposure.

Animals received microinfusion of HSV-LacZ or HSV-RGS4 into dSTR and 3 days post infection, locomotor and phosphorylation responses to Amph were measured (Fig. 4). A single injection of Amph (2.5 mg/kg i.p.) induced significant locomotor hyperactivity ($F_{(3,19)}=4.57$, $P<0.05$) in both LacZ-Amph as well as RGS4-Amph groups when compared to their saline counterparts (Fig.4A; LacZ-Amph vs. LacZ-Sal, $p<0.05$ and RGS4-AMPH vs RGS4-Sal, $p<0.05$). Phosphoprotein analysis revealed that Amph increased ERK2 phosphorylation as previously reported (Shi and McGinty 2007), although to a different degree across treatment groups (Fig.4B; $F_{(3,20)}=6.64$, $P<0.05$). There was a significant increase in phospho-ERK2 levels in the LacZ-Amph group ($p<0.01$, vs. LacZ-Sal), which was attenuated in animals over-expressing RGS4 (RGS4-Amph vs, LacZ-Amph, $p<0.05$; Fig 4B). Amph treatment also induced phospho-Akt-Thr308 levels ($F_{(3,21)}=4.24$, $P<0.05$) as previously reported (Shi and McGinty 2007). SNK tests revealed that phospho-Akt-Thr308 levels were significantly greater in the LacZ-Amph group than in the LacZ-Sal group ($p<0.05$). RGS4 over-expression did not attenuate Amph-induced phosphorylation of Akt-Thr308 when compared to LacZ-Sal animals ($p=0.4$); nevertheless, it prevented the Amph-induced rise in phospho-Akt308 levels (RGS4-Amph vs. RGS4-Sal, $p=0.21$; Fig.4C).

In the next experiment, the selective mGluR5 antagonist, MTEP, was bilaterally infused into dSTR 15 mins before Amph (2.5 mg/kg i.p.) or Sal injection. Amph induced significant locomotor hyperactivity (total distance traveled) when compared to Sal rats ($t_{(21)}=2.77$, $P<0.05$) during the 15 min period following i.p. injections (Fig. 4D). However, no group-specific effects of Veh or MTEP pretreatment on Amph-induced locomotor activity emerged during this short time interval. As expected, acute Amph induced an increase in phospho-ERK2 levels (Fig 4E; $F_{(3,19)}=10.96$, $P<0.01$). SNK multiple comparison tests showed that elevated phospho-ERK2 levels were present in the Veh-Amph group when compared to the Veh-Sal group ($p<0.01$) whereas phospho-ERK2 was significantly suppressed in the Amph group pretreated with MTEP (MTEP-Amph vs. Veh-Amph; $p<0.01$). Amph also induced phospho-Akt-Thr308 levels ($F_{(3,19)}=3.44$, $P<0.05$), specifically in Veh-pretreated animals (SNK test: $p<0.05$ Veh-Sal vs. Veh-Amph). MTEP microinfusion showed a trend toward partial inhibition of Amph-induced phosphorylation of Akt-Thr308 ($p=0.06$, Fig.4F).

The effects of RGS4 over-expression in the dSTR or intra-dSTR mGluR5 receptor blockade on extended behavioral activity after acute Amph

The previous experiments suggested that RGS4 and mGluR5 may share a common mechanism in regulating Amph-induced phosphoprotein signaling in the dSTR. However,

due to the short time period analyzed, it remained unclear whether local manipulation of RGS4 (and/or mGluR5s) in the dSTR alters the overall pattern of post-Amph behavioral activation. Therefore, in the following experiment, the effects of RGS4 over-expression or mGluR5 inhibition in the dSTR on Amph-induced behaviors were measured over a 2 hr period.

First, HSV-LacZ or HSV-RGS4 was microinfused into dSTR as illustrated by the distribution of cannulae placement sites on Fig. 5A. Three days later, rats were injected i.p. with Sal or Amph (2.5 mg/kg) and behavioral activity was recorded for the following 2 hours. Quantitative analysis of total distance traveled (over the period of peak activity, 15-90 min post injection) revealed significant differences between AUC values among the treatment groups as detected by ANOVA ($F_{(3,13)} = 14.06$, $P < 0.01$; Fig. 5B). Pairwise SNK comparisons showed that in LacZ animals, Amph induced a significant increase in total distance traveled (LacZ-Amph vs. LacZ-Sal, $p < 0.01$). Interestingly, Amph-induced distance traveled was augmented in animals over-expressing RGS4 in the dSTR (RGS4-Amph vs. LacZ-Amph, $p < 0.05$). Similarly, Amph significantly increased the peak vertical activity of animals ($F_{(3,13)} = 17.93$, $P < 0.01$; Fig. 5C). Further, pairwise comparisons revealed that Amph induced a significant increase in vertical activity of LacZ animals (LacZ-Amph vs. LacZ-Sal, $p < 0.01$). In contrast to horizontal activity, Amph-induced vertical activity was blunted in animals over-expressing RGS4 in the dSTR (RGS4-Amph vs. LacZ-Amph, $p < 0.05$; Fig. 5C).

In the next experiment, MTEP was microinfused into dSTR 15 mins before an i.p. Sal or Amph (2.5 mg/kg) injection and behavior was again monitored for 2 hours. Distribution of individual cannulae placement sites is shown on Fig. 5D. Quantitative analysis of the total distance traveled (over the period of the peak Amph-induced activity, 15-90 min post injection) revealed significant differences between AUC values among the treatment groups as detected by ANOVA ($F_{(3,13)} = 18.48$, $P < 0.01$; Fig. 5E). Pairwise SNK comparisons showed that in Veh-treated rats, Amph induced a significant increase in total distance traveled (Veh-Amph vs. Veh-Sal, $p < 0.01$, Fig. 5E). In a striking similarity to the effect of RGS4 over-expression, Amph-induced distance traveled was augmented in animals that received intrastriatal MTEP infusion (MTEP-Amph vs. Veh-Amph, $p < 0.05$). As expected, Amph significantly increased the peak vertical activity of rats ($F_{(3,13)} = 11.55$, $P < 0.01$; Fig. 5F). Pairwise comparisons revealed that Amph induced a significant increase in vertical activity of Veh-treated rats (Veh-Amph vs. Veh-Sal, $p < 0.01$). In contrast to RGS4 over-expression, Amph-induced vertical activity was not blunted by intra-striatal administration of MTEP (MTEP-Amph vs. Veh-Amph, $p = 0.15$).

Discussion

The present study investigated the role of striatal RGS4 protein in regulating mGluR5- and Amph-dependent cellular signaling and behavioral output. First, infusion of HSV-RGS4 into the dSTR resulted in a robust over-expression of RGS4 protein, which co-immunoprecipitated with mGluR5 receptors. Second, over-expression of RGS4 in the dSTR suppressed both early locomotor activation as well as increased phospho-ERK2 levels induced by local administration of the mGluR5 agonist, DHPG. However, RGS4 over-

expression did not reverse DHPG-induced dephosphorylation of Akt-Thr308 in the dSTR. Third, HSV-driven RGS4 over-expression attenuated Amph-induced phospho-ERK2 levels in a manner similar to the mGluR5 antagonist, MTEP. In contrast, RGS4 (and MTEP) only partially inhibited phospho-Akt-Thr308 upregulation by Amph. Fourth, although neither RGS4 nor MTEP showed an effect on early onset Amph-induced behaviors, analysis of the extended 3h behavioral profile of acute Amph revealed that RGS4 over-expression caused a bi-directional regulation of behavior, augmenting peak Amph-induced horizontal activity while suppressing peak vertical activity. Similarly, microinfusion of MTEP into dSTR also augmented peak Amph-induced horizontal locomotion.

A growing body of evidence suggests that group I mGluRs play an important role in the behavioral and neurochemical actions of Amph as well as other abused drugs (for review see: Bird and Lawrence 2009). As such, systemic administration of mGluR5 antagonists inhibited Amph-induced hyperactivity (Gormley and Rompre 2011; McGeehan et al. 2004; Pietraszek et al. 2004) and expression of Amph-induced conditioned place preference (Herzig et al. 2005). However, some studies provided contradictory evidence regarding the role of mGluR5 in regulating rewarding and locomotor-stimulating effects of psychostimulants (Gormley and Rompre 2011; McGeehan and Olive 2003; Veeneman et al. 2011). Discrepancies could stem from the fact that whereas different drugs exert region-specific effects on the brain, a population of mGluR1/5 receptors in the whole brain was typically blocked in these studies. Furthermore, there is only limited information regarding brain region-specific effects of mGluR1/5 antagonists on psychostimulant-induced behavior and cellular signaling. In one study, intra-dSTR blockade of group I mGluRs suppressed Amph-induced ERK and CREB phosphorylation within this brain region (Choe et al. 2002). This finding was not surprising considering that: (1) STR receives major excitatory inputs from all regions of the cortex and from the thalamus (McGeorge and Faull 1989; Mengual et al. 1999; Smith et al. 2004), (2) striatal medium spiny neurons are densely populated with glutamate receptors, including group I mGluRs, and (3), Amph-induced glutamate release plays an important role in controlling Amph-induced behaviors and activation of immediate early genes (Ferguson and Robinson 2004; Gray et al. 1999; Mao and Wang 1999). The present study was the first one to investigate the effects of intra-dSTR delivery of an mGluR5 antagonist on both cellular signaling and behavior after acute Amph. The suppressive effect of MTEP on ERK phosphorylation is an agreement with previous findings (Choe and Wang 2001) and is likely mediated by a suppression of both conventional Gq/PLC β 1- and Homer-dependent pathways (Mao et al. 2005). The precise mechanism of mGluR5-mediated regulation of Akt activity is not clear, but it seems to be of a transient character. In the hippocampus, administration of DHPG resulted in a rapid increase in phospho-Akt308 at 5 minutes, returning back to baseline levels within 20 minutes post-administration (Hou and Klann 2004). Further, the current study suggests that DHPG treatment actually produces delayed dephosphorylation of Akt-Thr308 in the dSTR 30 minutes after the treatment. Surprisingly, manipulating mGluR5 function (by RGS4 or MTEP) in combination with DHPG or Amph treatment revealed none or very limited control of this receptor over the phosphorylation/dephosphorylation equilibrium of Akt-Thr308 in the dSTR. In agreement, delayed dephosphorylation of phospho-Akt308 in the STR after Amph treatment has been found to depend on activation of D2 receptors

(Beaulieu et al. 2007; Shi and McGinty 2011), a GPCR likely not regulated by RGS4 (Ghavami et al. 2004; Ding et al. 2006).

A unique observation of the current study was the striking resemblance between the effects of an mGluR5 antagonist and those of RGS4 over-expression, supporting the hypothesis that RGS4 negatively regulates mGluR5 signaling in the dSTR. Indeed, over-expression of RGS4 in non-neuronal cell lines inhibited Gαq-coupled GPCRs and attenuated MAPK activation (Yan et al. 1997). It is also significant that both the long-form Homer protein and RGS4 are necessary to fine-tune calcium oscillations downstream from Gαq-coupled receptors (Shin et al. 2003). Taken together, these findings suggest that aberrant regulation of RGS4 levels has direct consequences for the cellular signaling downstream of mGluR5 receptors. Since abused drugs (for the most part) downregulate RGS4 levels in the striatum, it could be hypothesized that this leads to hypersensitivity of mGluR5 receptors manifested as augmented ERK phosphorylation during re-exposure to the drug or drug-paired cues (Valjent et al. 2006; Schwendt et al. 2010).

Besides cellular signaling, over-expression of RGS4 and mGluR5 inhibition altered Amph-induced behavioral profiles in a similar, but complex, manner. It is possible that both the mGluR5 antagonist and RGS4 decrease the behavioral efficacy of Amph, since increased time spent in horizontal activity at the expense of vertical activity corresponds to a behavioral repertoire characteristic of lower doses of Amph (Antoniou and Kafetzopoulos 1991; Kuczenski and Segal 1989; Wang and McGinty 1995). It is likely that cellular mechanisms of this complex behavioral shift are not limited to a single signaling pathway or exclusive to inhibition of mGluR5 receptors in the dSTR. For example, direct inhibition of ERK activity results in suppression of both horizontal and vertical post-Amph hyperactivity (Shi and McGinty 2006; Sutton et al. 2000), whereas selective inhibition of PI3K/Akt activity in the dSTR results in a behavioral profile similar to one observed in animals with RGS4 overexpression (Shi et al. 2009). However, we observed that both MTEP administration and RGS4 overexpression result in greater inhibition of ERK than Akt activity in the STR. It is possible that in our study, manipulating mGluR5 function has different outcomes depending on the cell-type-specific localization of the receptor. Although mGluR5s are abundantly expressed in both D1- and D2- positive populations of striatal medium spiny neurons (Tallaksen-Greene et al. 1998), evidence suggests that mGluR5 receptors are co-localized and closely interact with D1 receptors to regulate striatal neurotransmission (Paolillo et al. 1998; Schotanus and Chergui 2008; Voulalas et al. 2005) and many of the long-term effects of addictive drugs (Novak et al. 2010). On the other hand, mGluR5s can dimerize with D2 receptors resulting in suppressed function and cellular signaling of D2 receptors present in this heterodimer (Fuxe et al. 2009). Thus inhibition of mGluR5 function (by MTEP or RGS4) could also alter Amph-induced activation of D2 receptors and behaviors mediated by the indirect (striato-pallidal) pathway. Due to a complex character of signaling and behavioral consequences of mGluR5 (and RGS4) manipulations in the dSTR, future studies will have to examine the functional mGluR5/RGS4 relationship in a cell-type specific manner.

In conclusion, the data presented in this study show novel evidence for the significance of mGluR5 receptors and mGluR5-RGS4 interactions in the dSTR in modulating

psychostimulant-induced behaviors and signaling. Genetic disruption of either mGluR5 (Chiamulera et al. 2001) or mGluR5-interacting proteins (Atkinson et al. 2006; Chuang et al. 2001; Kammermeier et al. 2000; Swanson et al. 2001) produces profound alterations in responses to psychomotor stimulants, similar to direct pharmacological inhibition of group I mGluRs (Herzig and Schmidt 2004; McGeehan and Olive 2003). Since, our data suggest that RGS4 tightly regulates mGluR5 signaling, dynamic regulation of striatal RGS4 levels by psychostimulants could have a dramatic effect on the behavioral and neural functions of mGluR5 receptors. Therefore, modulating RGS4-mGluR5 interactions may represent a novel mechanism suitable for therapeutic interventions for psychostimulant addictions as well as for other psychiatric disorders (Sjogren et al. 2010).

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Fig 1. Infusion of HSV-RGS4 into the dSTR results in over-expression of RGS4 protein as well as binding of recombinant RGS4 to mGluR5 receptors

Animals received bilateral intra-dSTR infusion of HSV-RGS4-FLAG or a control vector HSV-LacZ (2μl/side) and brains were analyzed 3 days later. **A**, *Upper panel*: Outline of the rat brain coronal section depicting a representative placement of HSV infusions; adapted from Paxinos and Watson (2005). *Lower panels*: Immunohistochemical detection of RGS4 and β-galactosidase over-expression in the dSTR near the infusion site (black box represents a tip of the injector). Representative image of Nissl-stained striatal section showing no necrosis or abnormal cytoarchitecture in the proximity of the infusion site. **B**, Protein levels of β-galactosidase, RGS4, RGS4-FLAG and calnexin as measured by immunoblotting in tissue lysates prepared from the dSTR. **C**, FLAG-tagged RGS4 co-immunoprecipitates with mGluR5, but not with, dopamine D1 or D2 receptors in the dSTR (**D**). Immunoprecipitates (IP) were analyzed by immunoblotting (IB) using antibodies against FLAG-tag, mGluR5 or D1 and D2 receptors. T - total lysate used as an immunoprecipitation input. co-IP analysis included negative controls in which lysate, FLAG IP beads or RGS4-FLAG over-expression was omitted.

Fig.2. Intrastriatal infusion of mGluR5 agonist DHPG dose-dependently regulates phospho-ERK, and phospho-Akt-Thr308, but not RGS4 protein levels in the dSTR

Animals received bilateral infusion of DHPG (125 or 250 nmol) and brain were analyzed 30 min or 2hr post-infusion. **A**, Levels of phospho-ERK and phospho-Akt-Thr308 in the dSTR as analyzed by immunoblotting 30 min after intrastriatal infusion of DHPG or vehicle. **B**, RGS4 protein levels in the dSTR as analyzed by immunoblotting 2h after intrastriatal infusion of DHPG or vehicle. Data are expressed as mean $\pm$ S.E.M. of phospho-ERK2/tERK2, phospho Akt/tAkt and RGS4/calnexin integrated density ratio (n=4-6 samples/group). *p<0.05 vs. Vehicle, **p<0.01 vs. Veh, ^p<0.05 vs. DHPG 125 nmol dose.

Fig. 3. Over-expression of RGS4 in the dSTR attenuates DHPG-induced behavior and ERK phosphorylation

Animals received bilateral infusion of HSV-RGS4-*FLAG* or a control vector HSV-LacZ (2 μ l/side) followed by the bilateral infusion of either DHPG (250 nmol) or vehicle into dorsolateral STR 3 days later. **A**, Locomotor activity (total distance traveled) as recorded for 60 min pre- and 30 min post-infusion of DHPG. Mean total distance traveled (per 5 mins) $\pm$ S.E.M. plotted over time and analyzed as area under the curve (AUC). * p <0.05 vs. LacZ-Vehicle, ^ p <0.05 RGS4-DHPG vs. LacZ-DHPG. **B** and **C**, Immunoblotting analysis of phospho-ERK (**B**) and phospho-Akt-Thr308 (**C**) levels in the dSTR 30 min after the infusion of DHPG or Veh. Mean integrated density ratio $\pm$ S.E.M. of phospho-ERK2/tERK2 and

phospho-Akt-Thr308/tAkt (n=4-5 samples/group). *p<0.05 vs. LacZ-Vehicle, **p<0.01 vs. LacZ-Vehicle, ^p<0.05 vs. LacZ-DHPG, #p<0.05 vs. RGS4-Vehicle.

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Fig. 4. The effects of RGS4 (or blockade of mGluR5 receptors) on early onset Amph-induced behavior and phosphoprotein levels in the dSTR

Left column (A-C): Animals received bilateral infusion of HSV-RGS4-FLAG or a control vector HSV-LacZ (2µl/site). Three days later animals received Amph (2.5 mgs/kg, i.p) or saline injection and behavioral activity and phosphoprotein levels in the dSTR were analyzed. **A**, Total distance traveled as recorded for 60 min pre- and 15 min post- Sal or Amph administration. Mean total distance traveled (per 5 mins) $\pm$ S.E.M. plotted over time and analyzed as area under the curve (AUC). * $p < 0.05$ vs. LacZ-Sal, $^{\wedge}p < 0.05$ RGS4-Amph

vs. LacZ-Amph. **B and C**, *Upper panels*: Representative immunoblot images for each treatment group. *Lower panels*: Immunoblotting analysis of pERK2 and tERK2 (**B**), phospho-Akt-Thr308 and tAkt (**C**) levels in the dlSTR 20min after the injection Sal or Amph. Mean integrated density ratio $\pm$ S.E.M. of phospho-ERK2/tERK2 and phospho-Akt-Thr308/tAkt (n=5-7 samples/group). *p<0.05, **p<0.01 vs. LacZ-Sal, ^p<0.01 vs. LacZ-Amph. *Right column (D-F)*: Animals received bilateral infusion of Veh or MTEP (5 μ g/side). Fifteen minutes later animals received Amph (2.5 mgs/kg, i.p) or saline injection and behavioral activity and phosphoprotein levels in the dSTR were analyzed. **D**, Total distance traveled as recorded for 60 min pre- and 15 min post- Sal or Amph administration. Mean total distance traveled (per 5 mins) $\pm$ S.E.M. plotted over time and analyzed as area under the curve (AUC). **E and F**, *Upper panels*: Representative immunoblot images for each treatment group. *Lower panels*: Immunoblotting analysis of phospho-ERK2 and tERK2 (**E**), pAkt-Thr308 and tAkt (**F**) levels in the dlSTR 20min after the injection Sal or Amph. Mean integrated density ratio $\pm$ S.E.M. of pERK2/tERK2 and pAkt-Thr308/tAkt (n=5-7 samples/group). *p<0.05, *p<0.01 vs. Veh-Sal, ^p<0.01 vs. Veh-Amph.

Fig. 5. The effects of RGS4 overexpression (or a blockade of mGluR5 receptors) in the dSTR on extended Amph-induced behavioral activity

Left column (A-C): Animals received bilateral infusion of HSV-RGS4-FLAG or a control vector HSV-LacZ (2 μ l/side) into dSTR. Three days later animals received Amph (2.5 mgs/kg, i.p) or saline injection and behavioral activity was recorded for 2hrs. **A**, Outline of the rat brain coronal section depicting distribution of cannulae placements adapted from Paxinos and Watson (2005). **B**, Total distance traveled as recorded for 60 min pre- and 120 min post- drug administration. Mean total distance traveled (per 5 mins) $\pm$ S.E.M. plotted over time and analyzed as area under the curve (AUC). ** $p < 0.01$ vs. LacZ-Sal, ^ $p < 0.05$

RGS4-Amph vs. LacZ-Amph. **C**, Vertical activity (rearing) is shown as mean number of beam breaks (per 5 mins) $\pm$ S.E.M. plotted over time and analyzed as area under the curve. ** $p < 0.01$ vs. LacZ-Sal, $\hat{p} < 0.05$ RGS4-Amph vs. LacZ-Amph. *Right column (D-F)*: Animals received bilateral infusion of Veh or MTEP (5 μ g/side). Fifteen minutes later animals received Amph (2.5 mgs/kg, i.p) or saline injection and behavioral activity was recorded for 2hrs. **D**, Outline of the rat brain coronal section depicting distribution of cannulae placements adapted from Paxinos and Watson (2005). **E**, Total distance traveled as recorded for 60 min pre- and 120 min post- Sal or Amph administration. Mean total distance traveled (per 5 mins) $\pm$ S.E.M. plotted over time and analyzed as area under the curve (AUC). * $p < 0.05$ vs. LacZ-Sal, $\hat{p} < 0.05$ RGS4-Amph vs. LacZ-Amph. **F**, Vertical activity (rearing) is shown as mean number of beam breaks (per 5 mins) $\pm$ S.E.M. plotted over time and analyzed as area under the curve. ** $p < 0.01$ vs. LacZ-Sal.