Published in final edited form as:

Brain Res. 2007 January 19; 1129(1): 1–14.

Evaluation of neuronal phosphoproteins as effectors of caffeine and mediators of striatal adenosine A_{2A} receptor signaling

Bogachan Sahin¹, Stacey Galdi², Joseph Hendrick², Robert W. Greene^{1,3}, Gretchen L. Snyder², and James A. Bibb^{1,*}

¹Department of Psychiatry, The University of Texas Southwestern Medical Center, Dallas, TX 75390

²Intra-Cellular Therapies, Inc., New York, NY 10032

³Veterans Administration Medical Center, Dallas, TX 75216

Abstract

Adenosine A_{2A} receptors are predominantly expressed in the dendrites of enkephalin-positive γ -aminobutyric acidergic medium spiny neurons in the striatum. Evidence indicates that these receptors modulate striatal dopaminergic neurotransmission and regulate motor control, vigilance, alertness, and arousal. Although the physiological and behavioral correlates of adenosine A_{2A} receptor signaling have been extensively studied using a combination of pharmacological and genetic tools, relatively little is known about the signal transduction pathways that mediate the diverse biological functions attributed to this adenosine receptor subtype. Using a candidate approach based on the coupling of these receptors to adenylate cyclase-activating G proteins, a number of membranous, cytosolic, and nuclear phosphoproteins regulated by PKA were evaluated as potential mediators of adenosine A_{2A} receptor signaling in the striatum. Specifically, the adenosine A_{2A} receptor agonist, CGS 21680, was used to determine whether the phosphorylation state of each of the following PKA targets is responsive to adenosine A_{2A} receptor stimulation in this tissue: Ser40 of tyrosine hydroxylase, Ser9 of synapsin, Ser897 of the NR1 subunit of the N-methyl-D-aspartate-type glutamate receptor, Ser845 of the GluR1 subunit of the α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid-type glutamate receptor, Ser94 of spinophilin, Thr34 of the dopamine- and cAMP-regulated phosphoprotein, M_r32,000, Ser133 of the cAMP-response element-binding protein, Thr286 of Ca²⁺/calmodulin-dependent protein kinase II, and Thr202/Tyr204 and Thr183/Tyr185 of the p44 and p42 isoforms, respectively, of mitogen-activated protein kinase. Although the substrates studied differed considerably in their responsiveness to selective adenosine A_{2A} receptor activation, the phosphorylation state of all postsynaptic PKA targets was up-regulated in a time- and dose-dependent manner by treatment with CGS 21680, whereas presynaptic PKA substrates were unresponsive to this agent, consistent with the postsynaptic localization of adenosine A_{2A} receptors. Finally, the phosphorylation state of these proteins was further assessed *in vivo* by systemic administration of caffeine.

Section: Cellular and Molecular Biology of Nervous Systems

Keywords

Caffeine; Striatum; Adenosine A_{2A} receptor PKA; MAPK; Signal transduction

*Corresponding author. Fax: 214 648-1293. E-mail: james.bibb@utsouthwestern.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

In the central nervous system, the actions of adenosine are mediated by four subtypes of G protein-coupled receptors, namely A₁, A_{2A}, A_{2B}, and A₃ (Fredholm, et al., 1994). Of these subtypes, adenosine A₁ and A_{2A} receptors may be activated in response to the low levels of extracellular adenosine found in the mammalian brain under physiological conditions (Svenningsson, et al., 1999). Therefore, it is believed that these receptors represent the most relevant sites of action for adenosine and adenosine-like compounds, including the psychomotor stimulant caffeine and its major metabolites, which act as adenosine receptor antagonists (Snyder, et al., 1981). Adenosine A₁ receptors are coupled to Gα_i or Gα_o proteins that inhibit adenylate cyclase and reduce cAMP formation (Akbar, et al., 1994; Freund, et al., 1994; Jockers, et al., 1994). In contrast, A_{2A} receptors associate with Gα_s or Gα_{olf} proteins, which stimulate adenylate cyclase activity and increase cAMP generation (Corvol, et al., 2001; Herve, et al., 2001; Kull, et al., 2000; Olah, 1997). These receptors also exhibit different regional distributions in the brain. A₁-type receptors are abundantly expressed throughout the brain, with high levels found in the cerebral cortex, septum, hippocampus, and the basal ganglia, including the striatum (Ochiishi, et al., 1999). In the striatum, many A₁ receptors are located presynaptically on dopaminergic, glutamatergic, and cholinergic inputs to medium spiny neurons (Brown, et al., 1990; Flagmeyer, et al., 1997; Okada, et al., 1996), where they inhibit neurotransmitter release (Fredholm, et al., 1988). Some striatal A₁ receptors may also be present in postsynaptic locations (Alexander, et al., 1989; Ferre, et al., 1997). In contrast, A_{2A} receptors are almost exclusively found in the striatum (Schiffmann, et al., 1991), where they colocalize with dopamine D₂ receptors on the enkephalin-positive, GABAergic medium-sized spiny projection neurons of the striatopallidal pathway (Fink, et al., 1992; Schiffmann, et al., 1991; Svenningsson, et al., 1998). The majority of striatal A_{2A} receptors are postsynaptic and occur predominantly at excitatory synapses on the dendrites and dendritic spines of GABAergic medium spiny neurons, although some may be expressed presynaptically at glutamatergic nerve terminals (Hettinger, et al., 2001; Rosin, et al., 2003). Thus, they are well positioned to modulate cortical glutamatergic excitatory input to the striatum. Recently it has been shown that presynaptic A₁ receptor activation in the striatum attenuates tonic D₂ receptor activity, thereby facilitating postsynaptic A_{2A} signaling in medium spiny neurons (Yabauucki, et al., 2006).

Striatal A_{2A} receptors have been shown to modulate dopaminergic neurotransmission (Ferre, et al., 1997; Ferre, et al., 1991; Fuxe, et al., 2005; Fuxe, et al., 1998) and mediate a number of neuronal phenomena, including motor control (Barraco, et al., 1993; El Yacoubi, et al., 2000; Kanda, et al., 1998), level of arousal (Huang, et al., 2005), and vigilance (Lieberman, et al., 2002). Furthermore, selective A_{2A} receptor antagonists hold promise as therapeutic treatments for Parkinson's disease (Fredholm, et al., 2003). Although pharmacological (Bruns, et al., 1986; Fredholm, 1995) and genetic tools (Johansson, et al., 2001; Ledent, et al., 1997; Salmi, et al., 2005) developed in the past twenty years have revealed a wealth of information about the physiological and behavioral consequences of A_{2A} receptor signaling, relatively little is known about the intracellular signaling cascades that mediate these diverse biological functions.

In the following studies, a candidate approach was adopted to identify phosphoprotein targets likely to be regulated by A_{2A} receptors in the striatum. In keeping with the ability of these receptors to activate adenylate cyclase, the proteins included in this analysis comprised a heterogeneous group of pre- and postsynaptic cAMP-dependent protein kinase (PKA) substrates as well as phosphoproteins indirectly regulated by PKA-mediated signaling. The experimental design involved an initial screen for candidate phosphoproteins, followed by the evaluation and characterization of each target in terms of responsiveness to A_{2A} receptor

stimulation. Specifically, the phosphorylation state of the following PKA substrates was assessed in acute striatal slices treated with a PKA activator or an adenosine A_{2A} receptor agonist: in the presynaptic compartment, the rate-limiting enzyme in catecholamine biosynthesis, tyrosine hydroxylase (Nagatsu, et al., 1964), and the synaptic vesicle-associated neuronal protein, synapsin (De Camilli, et al., 1983; Ueda, et al., 1977); in the postsynaptic compartment, the ionotropic glutamate receptor subunits, N-methyl-D-aspartate (NMDA)-type glutamate receptor 1 (NR1, an obligate NMDA receptor subunit) (Monyer, et al., 1992; Moriyoshi, et al., 1991) and glutamate receptor 1 (GluR1, a subunit of the α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type glutamate receptor) (Boulter, et al., 1990; Hollmann, et al., 1989), the protein phosphatase 1 (PP-1)- and actin-binding dendritic protein, spinophilin (Allen, et al., 1997), the striatal PP-1 inhibitor, DARPP-32 (Walaas, et al., 1983), and the transcriptional regulator, cAMP-response element-binding protein (CREB) (Montminy, et al., 1987). Three additional phosphoproteins indirectly regulated by PKA were also evaluated: the protein serine/threonine kinases, Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) and the p44 and p42 isoforms of mitogen-activated protein kinase (p44 and p42 MAPK, or extracellular signal-regulated kinase (ERK) 1 and 2). Finally, the phosphorylation state of these proteins was assessed *in vivo* by systemic administration of caffeine.

PKA activity has been reported to exert profound effects on the functions of each of these phosphoproteins. The PKA-dependent phosphorylation of tyrosine hydroxylase at Ser40 (Campbell, et al., 1986) stimulates enzymatic activity (Wu, et al., 1992). Synapsin Ser9 phosphorylation by PKA (Czernik, et al., 1987) causes synapsin to disassociate from synaptic vesicles (Hosaka, et al., 1999). The PKA-dependent phosphorylation of NR1 at Ser897 (Tingley, et al., 1997) promotes NR1 trafficking from the endoplasmic reticulum to the cell surface (Scott, et al., 2003). There is substantial evidence that PKA-dependent GluR1 phosphorylation at Ser845 potentiates AMPA-type glutamate receptor-mediated currents (Roche, et al., 1996). Spinophilin no longer binds actin when phosphorylated at Ser94 by PKA (Hsieh-Wilson, et al., 2003). Finally, the striatal PP-1 inhibitor, DARPP-32, and the transcription factor, CREB, are activated by PKA-dependent phosphorylation at Thr34 (Hemmings, et al., 1984) and Ser133 (Gonzalez, et al., 1989), respectively.

As for the non-PKA substrates included in this analysis, there is substantial evidence that both the CaMKII and ERK pathways serve as secondary effectors under the indirect regulation of PKA-mediated signaling in a number of systems. The autophosphorylation of CaMKII at Thr286 results in the Ca²⁺-independent activation of this protein kinase (Thiel, et al., 1988). In the hippocampus, this autophosphorylation event also mediates the translocation of active CaMKII from the cytosol to the postsynaptic density, where phospho-Thr286 CaMKII is dephosphorylated and inactivated by PP-1 (Strack, et al., 1997). In turn, PP-1 activity toward CaMKII in this tissue is negatively regulated by PKA-mediated signaling through protein phosphatase inhibitor-1 (Blitzer, et al., 1998). A similar PKA-dependent pathway may exist in the striatum. With regard to the ERK pathway, ERK1 and ERK2 are activated by MAPK/ERK kinase (MEK)-dependent dual phosphorylation of Thr202/Tyr204 and Thr183/Tyr185, respectively (Anderson, et al., 1990; Charest, et al., 1993; Crews, et al., 1992; Gomez, et al., 1991; Payne, et al., 1991). There is evidence that some forms of synaptic plasticity involve the activation of the ERK pathway through PKA-mediated signaling upstream from MEK (Kanterewicz, et al., 2000; Roberson, et al., 1999; Yuan, et al., 2002).

2. Results

2.1 Effect of PKA activation on the phosphorylation state of striatal phosphoproteins

In proof-of-principle experiments designed to optimize the use of phosphorylation state-specific antibodies to selected phosphoproteins directly or indirectly regulated by PKA, striatal slices were incubated in the presence or absence of the adenylate cyclase activator, forskolin,

and the levels of phosphorylation were measured (Fig. 1, Table 1). Forskolin treatment resulted in the increased phosphorylation of PKA-dependent sites in the presynaptic proteins, tyrosine hydroxylase and synapsin. Forskolin also induced the up-regulation of PKA-dependent sites in the postsynaptic proteins, NR1, GluR1, spinophilin, DARPP-32, and CREB. For three additional targets, phosphorylation sites not directly phosphorylated by PKA were also tested. Forskolin caused an increase in the level of MEK-dependent phosphorylation of ERK1 and ERK2 at Thr202/Tyr204 and Thr183/Tyr185, respectively. On the other hand, no significant change in the autophosphorylation of CaMKII at Thr286 was detected in response to forskolin. Thus, with the exception of phospho-Thr286 CaMKII, adenylate cyclase activation reproducibly increased the levels of all pre- and postsynaptic phosphoproteins evaluated. PKA activation by forskolin did not alter the total amount any of the proteins with the exception of NR1 levels, which decreased 2-fold (1 ± 0.09 versus 0.5 ± 0.11 , $p = 0.025$, Student's unpaired *t* test) in response to constitutive PKA activation. It is conceivable that phosphorylation at Ser897 protects NR1 from degradation, possibly as a result of phosphorylation-dependent NR1 trafficking to the cell membrane, while on the other hand a global increase in PKA activity accelerates NR1 turnover and thus sets in motion a process whereby dephospho-NR1 is selectively eliminated, limiting the further translocation of NR1 subunits to membrane sites.

2.2 Effect of A_{2A} receptor activation on the phosphorylation state of striatal phosphoproteins

Phosphoprotein levels were next assessed in striatal slices treated with the selective A_{2A} receptor agonist, CGS 21680. In rat striatal membranes, this compound has been shown to be approximately 160-fold more selective for adenosine A_{2A} receptors than for the A₁ receptor subtype (Kim, et al., 1994). To characterize the relative responsiveness of each putative target to A_{2A} receptor activation by CGS 21680, a series of time course and dose-response experiments was conducted. Striatal slices were incubated in the absence or presence of varying concentrations (1-5 mM) of CGS 21680 for 0-30 min. Levels of phospho-Ser40 tyrosine hydroxylase, phospho-Ser9 synapsin, phospho-Ser897 NR1, phospho-Ser845 GluR1, phospho-Ser94 spinophilin, phospho-Thr34 DARPP-32, phospho-Ser133 CREB, diphospho-Thr202/Tyr204 ERK1, and diphospho-Thr183/Tyr185 ERK2 were measured in untreated and drug-treated slices (Fig. 2). With the exception of diphospho-Thr202/Tyr204 ERK1, CGS 21680 caused smaller increases in phosphoprotein levels relative to forskolin. In order of decreasing responsiveness to CGS 21680, the levels of phospho-Ser897 NR1, diphospho-Thr202/Tyr204 ERK1, diphospho-Thr183/Tyr185 ERK2, phospho-Thr34 DARPP-32, phospho-Ser94 spinophilin, phospho-Ser845 GluR1, and phospho-Ser133 CREB were optimally elevated relative to baseline by the treatments indicated in Table 2. The kinetics of the response to CGS 21680 varied considerably from target to target. Phospho-Ser897 NR1, phospho-Ser845 GluR1, diphospho-Thr202/Tyr204 ERK1, and diphospho-Thr183/Tyr185 ERK2 exhibited relatively rapid initial responses to CGS 21680 while phospho-Thr34 DARPP-32, phospho-Ser133 CREB, and phospho-Ser94 spinophilin showed responses that were delayed to varying degrees. All responses achieved maximal levels by 15 min. Moreover, with the exception of ERK1/2, the levels of all phosphoproteins evaluated were substantially diminished by 30 min of exposure to the A_{2A} receptor agonist. Consistent with the postsynaptic localization of adenosine A_{2A} receptors, CGS 21680 had no effect on the phosphorylation of tyrosine hydroxylase at Ser40, a presynaptic PKA substrate. Similarly, CGS 21680 did not alter phospho-Ser9 synapsin levels (data not shown). Thus, the selective activation of A_{2A} receptors in striatal tissue mediated increases in the phosphorylation of all postsynaptic targets that were also responsive to direct PKA activation.

2.3 Effect of systemic administration of caffeine upon the phosphorylation state of striatal phosphoproteins in vivo

The effect of an acute dose of caffeine (15 mg/kg, IP) on the phosphorylation state of these proteins over a 1-h time period was next assessed (Fig. 3). Consistent with the ability of caffeine

to antagonize tonically active A_{2A} receptors, caffeine injection caused a time-dependent reduction in the basal phosphorylation state of several PKA substrates, including phospho-Ser897 NR1, phospho-Ser845 GluR1, phospho-Ser94 spinophilin, and phospho-Thr34 DARPP-32. Interestingly, caffeine did not induce a decrease in the phosphorylation level of CREB at Ser133, perhaps because this site also serves as a substrate for other protein kinases, including CaMKII and ERK1/2, which might compensate for caffeine-induced changes in the phosphorylation of this transcriptional factor (Pozzi, et al., 2003). Caffeine did not significantly reduce the basal activity of ERK1/2 as indicated by lack of a caffeine effect on phospho-Thr202 ERK1 and phospho-Thr183 ERK2 levels, despite the ability of adenylate cyclase and A_{2A} receptor activation to induce increases in these sites.

The ability of DARPP-32 to be converted into a PP-1 inhibitor by PKA-dependent phosphorylation of Thr34 is regulated through phosphorylation at other sites (Svenningsson, et al., 2004). For example, phosphorylation of Ser130 by casein kinase I prevents dephosphorylation at Thr34 (Desdouits, et al., 1995). Furthermore, phosphorylation of Thr75 by cyclin-dependent kinase 5 (Cdk5) prevents DARPP-32 from being phosphorylated by PKA and converts it into a PKA inhibitor (Bibb, et al., 1999). Caffeine had no significant effect upon basal phospho-Ser130 DARPP-32 levels. However, as has been previously reported (Lindskog, et al., 2002), it induced a significant increase in the levels of phospho-Thr75 DARPP-32, which may partly explain the decrease in phospho-Thr34 DARPP-32 levels due to caffeine. Consistent with the inability of A_{2A} receptor activation to alter the PKA-dependent phosphorylation of presynaptic targets, caffeine had no effect on phospho-Ser40 tyrosine hydroxylase levels.

3. Discussion

These results support a role for striatal A_{2A} receptors in the regulation of a diverse array of postsynaptic signaling events in the striatum (Fig. 4). Glutamatergic innervation from the cerebral cortex and thalamus and dopaminergic innervation from the substantia nigra constitute the primary afferent inputs to this part of the basal ganglia (Lovinger, et al., 1996), where D_2 and A_{2A} receptors differentially regulate PKA activity through their opposing actions on adenylate cyclase in enkephalin-positive medium spiny neurons. All of the primary and secondary downstream effectors of PKA shown here to be responsive to A_{2A} receptor stimulation and antagonism are involved in the regulation of synaptic strength and plasticity. Furthermore, A_{2A} receptors have been shown to contribute to synaptic plasticity in the striatum (d'Alcantara, et al., 2001; Svenningsson, et al., 2003). Thus, these findings implicate adenosine as a regulator of key aspects of neurotransmission in this brain region.

The phosphoproteins examined differed considerably in the extent to which their phosphorylation state was regulated in response to a given dose of CGS 21680 over time. This observation may have biological significance with respect to the strength and duration of the adenosine stimulus required by each target for optimal phosphorylation under physiological conditions. Based on these results, one could conjecture, for instance, that a process with potentially long-range consequences, such as the up-regulation of CREB-dependent gene expression, does not readily lend itself to modulation by levels of adenosine that normally suffice to regulate a more acute phenomenon like excitatory neurotransmission. By the same token, A_{2A} receptor activation in the striatum may differently regulate glutamatergic transmission mediated by NMDA-*versus* AMPA-type glutamate receptors. Interestingly, doses of CGS 21680 that increased NR1, but not GluR1, phosphorylation in these experiments have been reported to receptors in the striatum (Gerevich, et al., 2002; Wirkner, et al., 2004).

Studies by others have already shown that the PKA-dependent phosphorylation of DARPP-32 and spinophilin is directly regulated by A_{2A} receptor activation in the striatum (Svenningsson,

et al., 1998; Uematsu, et al., 2005). However, there is little or no direct evidence linking A_{2A} receptors to the other phosphoproteins evaluated in this study. Although a growing body of evidence indicates that striatal A_{2A} receptors modulate NMDA receptor-mediated glutamatergic neurotransmission in this tissue (Gerevich, et al., 2002; Norenberg, et al., 1998; Norenberg, et al., 1997; Popoli, et al., 2003; Popoli, et al., 2002; Tebano, et al., 2004; Wirkner, et al., 2004), there is no definitive indication that these effects are dependent on NR1 phosphorylation at Ser897. A_{2A} receptors acting in a PKA-dependent fashion have been reported to potentiate the effects of metabotropic glutamate receptor 5 activation on NMDA-evoked responses in the striatum (Domenici, et al., 2004), and more recently, the activation of group I metabotropic glutamate receptors has been shown to increase striatal levels of phospho-Ser897 NR1 (Choe, et al., 2006). Thus, the findings described here constitute the first direct evidence that NR1 phosphorylation at Ser897 is regulated by A_{2A} receptor signaling in the striatum.

It has been reported recently that D₂ receptors negatively regulate phospho-Ser845 GluR1 levels in the striatum in a PKA/DARPP-32-dependent manner (Hakansson, et al., 2006). The same study also showed that tonic activation of A_{2A} receptors by endogenous adenosine is required for the D₂ receptor antagonist-evoked increase in GluR1 phosphorylation at this PKA/PP-1 site. However, the effect of A_{2A} receptor signaling on Ser845 phosphorylation has so far not been directly evaluated. Interestingly, the core promoter of the A_{2A} receptor gene may be regulated by phospho-Ser133 CREB through an A_{2A} receptor- and PKA-dependent mechanism in the striatum (Chiang, et al., 2005). In light of the results described here, A_{2A} receptors may therefore directly regulate their own expression by stimulating the phosphorylation and activation of CREB.

A_{2A} receptors regulate ERK activity in peripheral tissues and transformed cell lines (Schulte, et al., 2000; Seidel, et al., 1999; Sexl, et al., 1997). Moreover, in primary striatal cultures, ERK1 and ERK2 may be activated by A_{2A} receptors through a MEK- and PKA-dependent pathway (Canals, et al., 2005). However, a direct relationship between A_{2A} receptors and ERK activation has not been shown in the striatum, or the central nervous system at large. These results are the first demonstration that ERK1 and ERK2 are phosphorylated, and presumably activated, in the striatum in response to A_{2A} receptor activation. Notably, diphospho-ERK levels were among the most sensitive to stimulation with CGS 21680. Consistent with the findings here, A_{2A} receptor-dependent regulation has not been reported for phospho-Ser9 synapsin, phospho-Ser40 tyrosine hydroxylase, or phospho-Thr286 CaMKII. The lack of PKA-mediated responses in the presynaptic proteins, synapsin and tyrosine hydroxylase suggests that there may be too few striatal presynaptic A_{2A} receptors present to mediate detectable PKA activation, or presynaptic A_{2A} receptors are not coupled to the requisite signaling machinery to mediate PKA-dependent signaling.

Caffeine caused a decrease in the basal phosphorylation levels of most of the postsynaptic PKA targets evaluated in this study, indicating that they may function as downstream effectors of this widely self-administered stimulant. In keeping with its reputation as a non-addictive or mildly addictive agent, caffeine did not induce changes in the phosphorylation state of the transcription factor, CREB, which has been suggested to mediate neuroadaptive changes associated with stronger psychomotor stimulants such as cocaine through its ability to alter gene expression (Carlezon Jr., et al., 2005). Caffeine caused an elevation in the Cdk5-dependent phosphorylation of DARPP-32. The mechanism for this effect may involve not the activation of Cdk5, but rather the inactivation of PP-2A, which dephosphorylates this site and has been shown to mediate up-regulation of this site in response to activation of PKA by D₁ receptor agonists (Nishi, et al., 2000). Thus, the ability of caffeine to increase arousal and vigilance, as well as its effects as a motor stimulant that elevates stress may be mediated, at least in part, through these downstream effectors.

In addition to these central observations, the present study also illustrates the feasibility of a more extensive screen for the identification of additional phosphoprotein effectors involved in striatal A_{2A} receptor signaling pathways. However, the candidate approach is inherently limited by the availability and quality of phosphorylation state-specific antibodies for selected phosphoproteins. A viable alternative is provided by proteomics. Proteomic analysis has been used successfully in our hands to identify novel Cdk5 substrates in the central nervous system, such as the microtubule-binding protein, stathmin (Hayashi, et al., 2006). Thus, proteomic analysis holds the potential to reveal the involvement of unknown or poorly characterized phosphoproteins in A_{2A} receptor-mediated effects. By circumventing the assumption that all biochemical signaling pathways relevant to A_{2A} receptor signaling require the direct or indirect actions of PKA, it also offers the possibility of bringing to light previously unsuspected targets. Indeed, some of the salient signaling events regulated by A_{2A} receptors in the striatum may be PKA-independent (Svenningsson, et al., 1999). On the other hand, it is important to bear in mind that the proteomic approach requires high-throughput mass spectrometry technology in order to process the large sample sizes generated by time course and dose-response analyses. Therefore, it may be of limited use for defining the pharmacological parameters required by each target protein to respond optimally to A_{2A} receptor activation. This is in contrast to the immunodetection method used in the present study. Our findings point to the potential utility of the immunodetection approach for studying the relative responsiveness of different targets to differential A_{2A} receptor stimulation.

In conclusion, this analysis confirms or extends what is currently known about the relationship between the above phosphoproteins and striatal A_{2A} receptor signaling. Future investigations could address the biochemical, physiological, and behavioral consequences of these effects and assess the influence of A₁ and D₂ receptors on these pathways using a combination of immunodetection and proteomic analyses.

4. Experimental procedures

4.1 Chemicals

Forskolin was from LC Laboratories. All other chemicals were from Sigma.

4.2 Preparation and incubation of acute striatal slices

Male C57BL/6 mice (8-10 weeks old) were killed by decapitation. The brains were rapidly removed and placed in cold, oxygenated Krebs buffer (124 mM NaCl, 4 mM KCl, 26 mM NaHCO₃, 1.5 mM CaCl₂, 1.25 mM KH₂PO₄, 1.5 mM MgSO₄, and 10 mM D-glucose, pH 7.4). 350- μ m coronal sections were prepared using a vibrating blade microtome. Striatal slices were dissected from these sections in cold, oxygenated Krebs buffer using a dissecting light microscope. Each slice was transferred to a polypropylene incubation tube containing 2 ml of Krebs buffer and allowed to recover at 30°C under constant oxygenation with 95% O₂/5% CO₂ for 60 min, with a change of buffer following the first 30 min of recovery. Slices were subsequently treated with drugs as specified for each experiment, transferred to microfuge tubes, snap-frozen on dry ice, and stored at -80°C until further analysis.

4.3 In vivo studies

Normal adult male C57BL/6 mice (n = 6/treatment group/experiment) were injected systemically (IP) with caffeine (15mg/kg; prepared fresh in 0.9% NaCl solution). Mice were sacrificed by focused microwave irradiation (4.5-5 kW for 1.4 sec) using a small-animal microwave (Muromachi Kikai Co, Tokyo, Japan) at 15, 30 or 60 min after drug treatment. Striata were dissected from the fixed brains on a cold plate and stored at -80°C until assayed for phosphoprotein levels. Control samples were taken from mice 1 hr following injection with vehicle alone.

4.4 Immunoblot analysis

Frozen striatal tissue samples were sonicated in boiling 1% sodium dodecyl sulfate (SDS) containing 50 mM NaF and boiled for 5 min. Protein concentrations were determined by the bicinchoninic acid protein assay (Pierce) using bovine serum albumin as standard. 100 µg of total protein from each sample were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (12.5 or 15% polyacrylamide) followed by electrophoretic transfer to nitrocellulose membranes (0.2 µm) (Whatman). The membranes were immunoblotted using primary antibodies for phospho-Ser40 tyrosine hydroxylase (1:1000) (Chemicon), total tyrosine hydroxylase (1:2000) (Chemicon), phospho-Ser9 synapsin (1:500) (Calbiochem), total synapsin (1:1000) (Cell Signaling), phospho-Ser897 NR1 (1:1000) (Calbiochem), total NR1 (1:2000) (BD Biosciences), phospho-Ser845 GluR1 (1:500) (Upstate), total GluR1 (1:500) (Upstate), phospho-Ser94 spinophilin (1:1000) (Hsieh-Wilson, et al., 2003), total spinophilin (1:800) (Allen, et al., 1997), phospho-Thr34 DARPP-32 (1:750) (Snyder, et al., 1992), total DARPP-32 (1:8000) (Ouimet, et al., 1984), phospho-Ser133 CREB (1:1000) (Upstate), total CREB (1:250) (Zymed), phospho-Thr286 CaMKII (1:200) (Santa Cruz), total CaMKII (1:1000) (Santa Cruz), diphospho-Thr202/Tyr204 and Thr183/Tyr185 ERK1 and ERK2 (1:1000) (Cell Signaling), or total ERK1 and ERK2 (1:1000) (Cell Signaling). The antibodies to phospho-Thr34 and total DARPP-32, and phospho-Ser94 and total spinophilin were kindly provided by Paul Greengard (The Rockefeller University). For striatal slice homogenates, incubation with primary antibody was followed by incubation with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (1:8000) (Chemicon) and binding was detected by autoradiography using the enhanced chemiluminescence immunoblotting detection system (Amersham Biosciences) and quantified by densitometry using NIH Image software. For the *in vivo* studies, immunoblotting was carried out using phosphorylation state-specific antibodies (and respective phosphorylation state-insensitive antibodies for measuring the total protein levels) raised against phospho-Thr34, phospho-Thr75, and phospho-Ser130 DARPP-32 and phospho-Ser94 spinophilin (proprietary to ITI); phospho-Thr202/183 of ERK1/2 (Promega); phospho-Ser897 NR1 (Upstate). All other antibodies were from sources listed above. Whenever possible the phospho-form and total levels of a particular protein of interest were detected using dual fluorescent detection in the same sample by revealing individual antibody binding using Alexa 680 labeled goat anti-mouse IgG (Molecular Probes, Eugene OR) or IRdye 800CW labeled goat anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA). Antibody binding was detected and quantitated using a LiCor Odyssey infrared fluorescent detection system (LiCor, Lincoln, NE). In all experiments, apparent phosphoprotein levels were adjusted to reflect relative levels of total protein loaded per lane.

4.5 Statistical analysis

For each phosphorylation site, phosphoprotein levels were normalized to total protein levels in striatal homogenates, and expressed as percent of baseline values derived from untreated or vehicle dosed controls. Levels of phosphorylation in caffeine-treated animals were expressed as a percentage of phosphorylation levels in samples from vehicle-injected mice. The difference in phosphorylation for each site compared to control was depicted as mean ± SEM. Data are analyzed by one-way ANOVA using the Newman-Keuls post-hoc test or Student's *t* test of unpaired data. $p < 0.05$ was considered statistically significant.

Acknowledgments

This work was supported by funding from the United States Army Medical Research and Materiel Command NETRP (W81XWH-04-2-0009, G.L.S. and J.A.B.), the National Institute of Mental Health (MH67777, R.W.G. and J.A.B.), the Department of Veterans Affairs (R.W.G.), the National Institute on Drug Abuse (DA16672, J.A.B.), and the Ella McFadden Charitable Trust Fund at the Southwestern Medical Foundation (J.A.B.). We thank Paul Greengard (The Rockefeller University) for the antibodies to phospho-Thr34 and total DARPP-32, and phospho-Ser94 and total spinophilin. The excellent technical assistance of Minal Rana and Jennifer Pick is gratefully acknowledged. We would also like to express our gratitude to the Medical Scientist Training Program at UT Southwestern Medical Center.

Abbreviations:

AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
 CaMKII, Ca^{2+} /calmodulin-dependent protein kinase II
 CREB, cAMP-response element-binding protein
 D32 or DARPP-32, dopamine- and cAMP-regulated phosphoprotein, M_r 32,000
 ERK, extracellular signal-regulated kinase
 FSK, forskolin GABA, γ -aminobutyric acid
 GluR1, glutamate receptor 1
 MAPK, mitogen-activated protein kinase
 MEK, MAPK/ERK kinase
 Cdk5, cyclin-dependent kinase 5
 NMDA, N-methyl-D-aspartate
 NR1, NMDA-type glutamate receptor 1
 PAGE, polyacrylamide gel electrophoresis
 PKA, cAMP-dependent protein kinase
 PP, protein phosphatase
 SDS, sodium dodecyl sulfate
 TH, tyrosine hydroxylase.

REFERENCES

- Akbar M, Okajima F, Tomura H, Shimegi S, Kondo Y. A single species of A1 adenosine receptor expressed in Chinese hamster ovary cells not only inhibits cAMP accumulation but also stimulates phospholipase C and arachidonate release. *Mol. Pharmacol* 1994;45:1036–1042. [PubMed: 8190094]
- Alexander SP, Reddington M. The cellular localization of adenosine receptors in rat neostriatum. *Neuroscience* 1989;28:645–651. [PubMed: 2710337]
- Allen PB, Ouimet CC, Greengard P. Spinophilin, a novel protein phosphatase 1 binding protein localized to dendritic spines. *Proc. Natl. Acad. Sci. U. S. A* 1997;94:9956–9961. [PubMed: 9275233]
- Anderson NG, Maller JL, Tonks NK, Sturgill TW. Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature* 1990;343:651–653. [PubMed: 2154696]
- Barraco RA, Martens KA, Parizon M, Normile HJ. Adenosine A2a receptors in the nucleus accumbens mediate locomotor depression. *Brain Res. Bull* 1993;31:397–404. [PubMed: 8490738]
- Bibb JA, Snyder GL, Nishi A, Yan Z, Meijer L, Fienberg AA, Tsai LH, Kwon YT, Girault JA, Czernik AJ, Haganir RL, Hemmings HC Jr, Nairn AC, Greengard P. Phosphorylation of DARPP-32 by Cdk5 modulates dopamine signalling in neurons. *Nature* 1999;402:669–671. [PubMed: 10604473]
- Blitzer RD, Connor JH, Brown GP, Wong T, Shenolikar S, Iyengar R, Landau EM. Gating of CaMKII by cAMP-regulated protein phosphatase activity during LTP. *Science* 1998;280:1940–1942. [PubMed: 9632393]
- Boulter J, Hollmann M, O'Shea-Greenfield A, Hartley M, Deneris E, Maron C, Heinemann S. Molecular cloning and functional expression of glutamate receptor subunit genes. *Science* 1990;249:1033–1037. [PubMed: 2168579]
- Brown SJ, James S, Reddington M, Richardson PJ. Both A1 and A2a purine receptors regulate striatal acetylcholine release. *J. Neurochem* 1990;55:31–38. [PubMed: 2355224]
- Bruns RF, Lu GH, Pugsley TA. Characterization of the A2 adenosine receptor labeled by [^3H]NECA in rat striatal membranes. *Mol. Pharmacol* 1986;29:331–346. [PubMed: 3010074]
- Campbell DG, Hardie DG, Vulliet PR. Identification of four phosphorylation sites in the N-terminal region of tyrosine hydroxylase. *J. Biol. Chem* 1986;261:10489–10492. [PubMed: 2874140]
- Canals M, Angulo E, Casado V, Canela EI, Mallol J, Vinals F, Staines W, Tinner B, Hillion J, Agnati L, Fuxe K, Ferre S, Lluís C, Franco R. Molecular mechanisms involved in the adenosine A1 and A2a receptor-induced neuronal differentiation in neuroblastoma cells and striatal primary cultures. *J. Neurochem* 2005;92:337–348. [PubMed: 15663481]

- Carlezon WA Jr, Duman RS, Nestler EJ. The many faces of CREB. *Trends. Neurosci* 2005;28:436–445. [PubMed: 15982754]
- Charest DL, Mordret G, Harder KW, Jirik F, Pelech SL. Molecular cloning, expression, and characterization of the human mitogen-activated protein kinase p44erk1. *Mol. Cell. Biol* 1993;13:4679–4690. [PubMed: 7687743]
- Chiang MC, Lee YC, Huang CL, Chern Y. cAMP-response element-binding protein contributes to suppression of the A2A adenosine receptor promoter by mutant Huntingtin with expanded polyglutamine residues. *J Biol Chem* 2005;280:14331–14340. [PubMed: 15689617]
- Choe ES, Shin EH, Wang JQ. Regulation of phosphorylation of NMDA receptor NR1 subunits in the rat neostriatum by group I metabotropic glutamate receptors in vivo. *Neurosci. Lett* 2006;394:246–251. [PubMed: 16300895]
- Corvol JC, Studler JM, Schonn JS, Girault JA, Herve D. Galpha(olf) is necessary for coupling D1 and A2a receptors to adenylyl cyclase in the striatum. *J. Neurochem* 2001;76:1585–1588. [PubMed: 11238742]
- Crews CM, Alessandrini A, Erikson RL. The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. *Science* 1992;258:478–480. [PubMed: 1411546]
- Czernik AJ, Pang DT, Greengard P. Amino acid sequences surrounding the cAMP-dependent and calcium/calmodulin-dependent phosphorylation sites in rat and bovine synapsin I. *Proc. Natl. Acad. Sci. U. S. A* 1987;84:7518–7522. [PubMed: 3118371]
- d'Alcantara P, Ledent C, Swillens S, Schiffmann SN. Inactivation of adenosine A2A receptor impairs long term potentiation in the accumbens nucleus without altering basal synaptic transmission. *Neuroscience* 2001;107:455–464. [PubMed: 11719000]
- De Camilli P, Cameron R, Greengard P. Synapsin I (protein I), a nerve terminal-specific phosphoprotein. I. Its general distribution in synapses of the central and peripheral nervous system demonstrated by immunofluorescence in frozen and plastic sections. *J. Cell. Biol* 1983;96:1337–1354. [PubMed: 6404910]
- Desdouits F, Sciliano JC, Greengard P, Girault J-A. Dopamine- and cAMP-regulated phosphoprotein DARPP-32: Phosphorylation of Ser-137 by casein kinase I inhibits dephosphorylation of Thr-34 by calcineurin. *Proc. Natl. Acad. Sci. U. S. A* 1995;92:2682–2685. [PubMed: 7708705]
- Domenici MR, Peponi R, Martire A, Tebano MT, Potenza RL, Popoli P. Permissive role of adenosine A2A receptors on metabotropic glutamate receptor 5 (mGluR5)-mediated effects in the striatum. *J. Neurochem* 2004;90:1276–1279. [PubMed: 15312183]
- El Yacoubi M, Ledent C, Menard JF, Parmentier M, Costentin J, Vaugeois JM. The stimulant effects of caffeine on locomotor behaviour in mice are mediated through its blockade of adenosine A(2A) receptors. *Br. J. Pharmacol* 2000;129:1465–1473. [PubMed: 10742303]
- Ferre S, Fredholm BB, Morelli M, Popoli P, Fuxe K. Adenosine-dopamine receptor-receptor interactions as an integrative mechanism in the basal ganglia. *Trends Neurosci* 1997;20:482–487. [PubMed: 9347617]
- Ferre S, Fredholm BB, Morelli M, Popoli P, Fuxe K. Adenosine-dopamine receptor-receptor interactions as an integrative mechanism in the basal ganglia. *Trends Neurosci* 1997;20:482–487. [PubMed: 9347617]
- Ferre S, von Euler G, Johansson B, Fredholm BB, Fuxe K. Stimulation of high-affinity adenosine A2 receptors decreases the affinity of dopamine D2 receptors in rat striatal membranes. *Proc. Natl. Acad. Sci. U. S. A* 1991;88:7238–7241. [PubMed: 1678519]
- Fink JS, Weaver DR, Rivkees SA, Peterfreund RA, Pollack AE, Adler EM, Reppert SM. Molecular cloning of the rat A2 adenosine receptor: selective co-expression with D2 dopamine receptors in rat striatum. *Brain Res. Mol. Brain Res* 1992;14:186–195. [PubMed: 1279342]
- Flagmeyer I, Haas HL, Stevens DR. Adenosine A1 receptor-mediated depression of corticostriatal and thalamostriatal glutamatergic synaptic potentials in vitro. *Brain Res* 1997;778:178–185. [PubMed: 9462890]
- Fredholm BB. Astra Award Lecture. Adenosine, adenosine receptors and the actions of caffeine. *Pharmacol. Toxicol* 1995;76:93–101. [PubMed: 7746802]

- Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA, Leff P, Williams M. Nomenclature and classification of purinoceptors. *Pharmacol. Rev* 1994;46:143–156. [PubMed: 7938164]
- Fredholm BB, Dunwiddie TV. How does adenosine inhibit transmitter release? *Trends Pharmacol. Sci* 1988;9:130–134. [PubMed: 2907698]
- Fredholm BB, Svenningsson P. Adenosine-dopamine interactions: development of a concept and some comments on therapeutic possibilities. *Neurology* 2003;61:S5–9. [PubMed: 14663001]
- Freund S, Ungerer M, Lohse MJ. A1 adenosine receptors expressed in CHO-cells couple to adenylyl cyclase and to phospholipase C. *Naunyn Schmiedebergs Arch. Pharmacol* 1994;350:49–56. [PubMed: 7935854]
- Fuxe K, Ferre S, Canals M, Torvinen M, Terasmaa A, Marcellino D, Goldberg SR, Staines W, Jacobsen KX, Lluís C, Woods AS, Agnati LF, Franco R. Adenosine A2A and dopamine D2 heteromeric receptor complexes and their function. *J. Mol. Neurosci* 2005;26:209–220. [PubMed: 16012194]
- Fuxe K, Ferre S, Zoli M, Agnati LF. Integrated events in central dopamine transmission as analyzed at multiple levels. Evidence for intramembrane adenosine A2A/dopamine D2 and adenosine A1/dopamine D1 receptor interactions in the basal ganglia. *Brain Res. Brain Res. Rev* 1998;26:258–273. [PubMed: 9651540]
- Gerevich Z, Wirkner K, Illes P. Adenosine A2A receptors inhibit the N-methyl-D-aspartate component of excitatory synaptic currents in rat striatal neurons. *Eur. J. Pharmacol* 2002;451:161–164. [PubMed: 12231386]
- Gomez N, Cohen P. Dissection of the protein kinase cascade by which nerve growth factor activates MAP kinases. *Nature* 1991;353:170–173. [PubMed: 1716348]
- Gonzalez GA, Montminy MR. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* 1989;59:675–680. [PubMed: 2573431]
- Hakansson K, Galdi S, Hendrick J, Snyder G, Greengard P, Fisone G. Regulation of phosphorylation of the GluR1 AMPA receptor by dopamine D2 receptors. *J. Neurochem* 2006;96:482–488. [PubMed: 16336634]
- Hayashi K, Pan Y, Shu H, Kansy JW, White CL, Tamminga CA, Sobel A, Curmi PA, Bibb JA. Phosphorylation of the tubulin binding protein stathmin by Cdk5 and MAP kinases in the brain. *J. Neurochem.* 2006in press.
- Hemmings HC Jr. Greengard P, Tung HY, Cohen P. DARPP-32, a dopamine-regulated neuronal phosphoprotein, is a potent inhibitor of protein phosphatase-1. *Nature* 1984;310:503–505. [PubMed: 6087160]
- Herve D, Le Moine C, Corvol JC, Belluscio L, Ledent C, Fienberg AA, Jaber M, Studler JM, Girault JA. Galpha(olf) levels are regulated by receptor usage and control dopamine and adenosine action in the striatum. *J. Neurosci* 2001;21:4390–4399. [PubMed: 11404425]
- Hettinger BD, Lee A, Linden J, Rosin DL. Ultrastructural localization of adenosine A2A receptors suggests multiple cellular sites for modulation of GABAergic neurons in rat striatum. *J. Comp. Neurol* 2001;431:331–346. [PubMed: 11170009]
- Hollmann M, O'Shea-Greenfield A, Rogers SW, Heinemann S. Cloning by functional expression of a member of the glutamate receptor family. *Nature* 1989;342:643–648. [PubMed: 2480522]
- Hosaka M, Hammer RE, Sudhof TC. A phospho-switch controls the dynamic association of synapsins with synaptic vesicles. *Neuron* 1999;24:377–387. [PubMed: 10571231]
- Hsieh-Wilson LC, Benfenati F, Snyder GL, Allen PB, Nairn AC, Greengard P. Phosphorylation of spinophilin modulates its interaction with actin filaments. *J. Biol. Chem* 2003;278:1186–1194. [PubMed: 12417592]
- Huang ZL, Qu WM, Eguchi N, Chen JF, Schwarzschild MA, Fredholm BB, Urade Y, Hayaishi O. Adenosine A2A, but not A1, receptors mediate the arousal effect of caffeine. *Nat. Neurosci* 2005;8:858–859. [PubMed: 15965471]
- Jockers R, Linder ME, Hohenegger M, Nanoff C, Bertin B, Strosberg AD, Marullo S, Freissmuth M. Species difference in the G protein selectivity of the human and bovine A1-adenosine receptor. *J. Biol. Chem* 1994;269:32077–32084. [PubMed: 7798201]
- Johansson B, Halldner L, Dunwiddie TV, Masino SA, Poelchen W, Gimenez-Llort L, Escorihuela RM, Fernandez-Teruel A, Wiesenfeld-Hallin Z, Xu XJ, Hardemark A, Betsholtz C, Herlenius E, Fredholm

- BB. Hyperalgesia, anxiety, and decreased hypoxic neuroprotection in mice lacking the adenosine A1 receptor. *Proc. Natl. Acad. Sci. U. S. A* 2001;98:9407–9412. [PubMed: 11470917]
- Kanda T, Jackson MJ, Smith LA, Pearce RK, Nakamura J, Kase H, Kuwana Y, Jenner P. Adenosine A2A antagonist: a novel antiparkinsonian agent that does not provoke dyskinesia in parkinsonian monkeys. *Ann. Neurol* 1998;43:507–513. [PubMed: 9546333]
- Kanterewicz BI, Urban NN, McMahon DB, Norman ED, Giffen LJ, Favata MF, Scherle PA, Trzaskos JM, Barrionuevo G, Klann E. The extracellular signal-regulated kinase cascade is required for NMDA receptor-independent LTP in area CA1 but not area CA3 of the hippocampus. *J. Neurosci* 2000;20:3057–3066. [PubMed: 10777769]
- Kim HO, Ji X-D, Siddiqi SM, Olah ME, Stiles GL, Jacobson KA. 2-Substitution of N6-benzyladenosine-5'-uronamides enhances selectivity for A3 adenosine receptors. *J Med Chem* 1994;37:3614–3621. [PubMed: 7932588]
- Kull B, Svenningsson P, Fredholm BB. Adenosine A(2A) receptors are colocalized with and activate g (olf) in rat striatum. *Mol. Pharmacol* 2000;58:771–777. [PubMed: 10999947]
- Ledent C, Vaugeois JM, Schiffmann SN, Pedrazzini T, El Yacoubi M, Vanderhaeghen JJ, Constantin J, Heath JK, Vassart G, Parmentier M. Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor. *Nature* 1997;388:674–678. [PubMed: 9262401]
- Lieberman HR, Tharion WJ, Shukitt-Hale B, Speckman KL, Tulley R. Effects of caffeine, sleep loss, and stress on cognitive performance and mood during U.S. Navy SEAL training. *Sea-Air-Land. Psychopharmacology (Berl)* 2002;164:250–261.
- Lindskog M, Pozzi L, Svenningsson P, Fienberg A, Bibb JA, Fredholm BB, Greengard P, Fisone G. The stimulant action of caffeine is mediated by an increase in the state of phosphorylation at the Cdk5 site of DARPP-32. *Nature*. 2002(in press).
- Lovinger DM, Tyler E. Synaptic transmission and modulation in the neostriatum. *Int. Rev. Neurobiol* 1996;39:77–111. [PubMed: 8894845]
- Montminy MR, Bilezikjian LM. Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. *Nature* 1987;328:175–178. [PubMed: 2885756]
- Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B, Seeburg PH. Heteromeric NMDA receptors: molecular and functional distinction of subtypes. *Science* 1992;256:1217–1221. [PubMed: 1350383]
- Moriyoshi K, Masu M, Ishii T, Shigemoto R, Mizuno N, Nakanishi S. Molecular cloning and characterization of the rat NMDA receptor. *Nature* 1991;354:31–37. [PubMed: 1834949]
- Nagatsu T, Levitt M, Udenfriend S. Tyrosine Hydroxylase. The Initial Step In Norepinephrine Biosynthesis. *J. Biol. Chem* 1964;239:2910–2917. [PubMed: 14216443]
- Nishi A, Bibb JA, Snyder GL, Higashi H, Nairn AC, Greengard P. Amplification of dopaminergic signalling by a novel positive feedback loop. *Proc. Natl. Acad. Sci. U. S. A* 2000;81:832–841.
- Norenberg W, Wirkner K, Assmann H, Richter M, Illes P. Adenosine A2A receptors inhibit the conductance of NMDA receptor channels in rat neostriatal neurons. *Amino Acids* 1998;14:33–39. [PubMed: 9871438]
- Norenberg W, Wirkner K, Illes P. Effect of adenosine and some of its structural analogues on the conductance of NMDA receptor channels in a subset of rat neostriatal neurones. *Br. J. Pharmacol* 1997;122:71–80. [PubMed: 9298530]
- Ochiishi T, Chen L, Yukawa A, Saitoh Y, Sekino Y, Arai T, Nakata H, Miyamoto H. Cellular localization of adenosine A1 receptors in rat forebrain: immunohistochemical analysis using adenosine A1 receptor-specific monoclonal antibody. *J. Comp. Neurol* 1999;411:301–316. [PubMed: 10404255]
- Okada M, Mizuno K, Kaneko S. Adenosine A1 and A2 receptors modulate extracellular dopamine levels in rat striatum. *Neurosci. Lett* 1996;212:53–56. [PubMed: 8823761]
- Olah ME. Identification of A2a adenosine receptor domains involved in selective coupling to Gs. Analysis of chimeric A1/A2a adenosine receptors. *J. Biol. Chem* 1997;272:337–344. [PubMed: 8995267]
- Quimet CC, Miller PE, Hemmings HC Jr, Walaas SI, Greengard P. DARPP-32, a dopamine- and adenosine 3':5'-monophosphate-regulated phosphoprotein enriched in dopamine-innervated brain regions. III. Immunocytochemical localization. *J Neurosci* 1984;4:111–124. [PubMed: 6319625]

- Payne DM, Rossomando AJ, Martino P, Erickson AK, Her JH, Shabanowitz J, Hunt DF, Weber MJ, Sturgill TW. Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). *EMBO J* 1991;10:885–892. [PubMed: 1849075]
- Popoli P, Frank C, Tebano MT, Potenza RL, Pintor A, Domenici MR, Nazzicone V, Pezzola A, Reggio R. Modulation of glutamate release and excitotoxicity by adenosine A2A receptors. *Neurology* 2003;61:S69–71. [PubMed: 14663014]
- Popoli P, Pintor A, Domenici MR, Frank C, Tebano MT, Pezzola A, Scarchilli L, Quarta D, Reggio R, Malchiodi-Albedi F, Falchi M, Massotti M. Blockade of striatal adenosine A2A receptor reduces, through a presynaptic mechanism, quinolinic acid-induced excitotoxicity: possible relevance to neuroprotective interventions in neurodegenerative diseases of the striatum. *J. Neurosci* 2002;22:1967–1975. [PubMed: 11880527]
- Pozzi L, Hakansson K, Usiello A, Borgkvist A, Lindskog M, Greengard P, Fisone G. Opposite regulation by typical and atypical anti-psychotics of ERK1/2, CREB and Elk-1 phosphorylation in mouse dorsal striatum. *J. Neurochem* 2003;86:451–459. [PubMed: 12871586]
- Roberson ED, English JD, Adams JP, Selcher JC, Kondratieck C, Sweatt JD. The mitogen-activated protein kinase cascade couples PKA and PKC to cAMP response element binding protein phosphorylation in area CA1 of hippocampus. *J. Neurosci* 1999;19:4337–4348. [PubMed: 10341237]
- Roche KW, O'Brien RJ, Mammen AL, Bernhardt J, Huganir RL. Characterization of multiple phosphorylation sites on the AMPA receptor GluR1 subunit. *Neuron* 1996;16:1179–1188. [PubMed: 8663994]
- Rosin DL, Hettinger BD, Lee A, Linden J. Anatomy of adenosine A2A receptors in brain: morphological substrates for integration of striatal function. *Neurology* 2003;61:S12–18. [PubMed: 14663003]
- Salmi P, Chergui K, Fredholm BB. Adenosine-dopamine interactions revealed in knockout mice. *J. Mol. Neurosci* 2005;26:239–244. [PubMed: 16012197]
- Schiffmann SN, Jacobs O, Vanderhaeghen JJ. Striatal restricted adenosine A2 receptor (RDC8) is expressed by enkephalin but not by substance P neurons: an in situ hybridization histochemistry study. *J. Neurochem* 1991;57:1062–1067. [PubMed: 1713612]
- Schiffmann SN, Libert F, Vassart G, Vanderhaeghen JJ. Distribution of adenosine A2 receptor mRNA in the human brain. *Neurosci. Lett* 1991;130:177–181. [PubMed: 1795877]
- Schulte G, Fredholm BB. Human adenosine A(1), A(2A), A(2B), and A(3) receptors expressed in Chinese hamster ovary cells all mediate the phosphorylation of extracellular-regulated kinase 1/2. *Mol. Pharmacol* 2000;58:477–482. [PubMed: 10953039]
- Scott DB, Blanpied TA, Ehlers MD. Coordinated PKA and PKC phosphorylation suppresses RXR-mediated ER retention and regulates the surface delivery of NMDA receptors. *Neuropharmacology* 2003;45:755–767. [PubMed: 14529714]
- Seidel MG, Klinger M, Freissmuth M, Holler C. Activation of mitogen-activated protein kinase by the A(2A)-adenosine receptor via a rap1-dependent and via a p21(ras)-dependent pathway. *J. Biol. Chem* 1999;274:25833–25841. [PubMed: 10464324]
- Sextl V, Mancusi G, Holler C, Gloria-Maercker E, Schutz W, Freissmuth M. Stimulation of the mitogen-activated protein kinase via the A2A-adenosine receptor in primary human endothelial cells. *J. Biol. Chem* 1997;272:5792–5799. [PubMed: 9038193]
- Snyder GL, Girault JA, Chen JY, Czernik AJ, Kebedian JW, Nathanson JA, Greengard P. Phosphorylation of DARPP-32 and protein phosphatase inhibitor-1 in rat choroid plexus: regulation by factors other than dopamine. *J. Neurosci* 1992;12:3071–3083. [PubMed: 1494946]
- Snyder SH, Katims JJ, Annau Z, Bruns RF, Daly JW. Adenosine receptors and behavioral actions of methylxanthines. *Proc. Natl. Acad. Sci. U. S. A* 1981;78:3260–3264. [PubMed: 6265942]
- Strack S, Barban MA, Wadzinski BE, Colbran RJ. Differential inactivation of postsynaptic density-associated and soluble Ca²⁺/calmodulin-dependent protein kinase II by protein phosphatases 1 and 2A. *J. Neurochem* 1997;68:2119–2128. [PubMed: 9109540]
- Svenningsson P, Fredholm BB. Exciting news about A2A receptors. *Neurology* 2003;61:S10–11. [PubMed: 14663002]
- Svenningsson P, Le Moine C, Aubert I, Burbard P, Fredholm BB, Bloch B. Cellular distribution of adenosine A2A receptor mRNA in the primate striatum. *J. Comp. Neurol* 1998;399:229–240. [PubMed: 9721905]

- Svenningsson P, Le Moine C, Fisone G, Fredholm BB. Distribution, biochemistry and function of striatal adenosine A2A receptors. *Prog. Neurobiol* 1999;59:355–396. [PubMed: 10501634]
- Svenningsson P, Lindskog M, Rognoni F, Fredholm BB, Greengard P, Fisone G. Activation of adenosine A2A and dopamine D1 receptors stimulates cyclic AMP-dependent phosphorylation of DARPP-32 in distinct populations of striatal projection neurons. *Neuroscience* 1998;84:223–228. [PubMed: 9522376]
- Svenningsson P, Nishi A, Fisone G, Girault JA, Nairn AC, Greengard P. DARPP-32: an integrator of neurotransmission. *Annu. Rev. Pharmacol. Toxicol* 2004;44:269–296. [PubMed: 14744247]
- Tebano MT, Pintor A, Frank C, Domenici MR, Martire A, Peponi R, Potenza RL, Grieco R, Popoli P. Adenosine A2A receptor blockade differentially influences excitotoxic mechanisms at pre- and postsynaptic sites in the rat striatum. *J. Neurosci. Res* 2004;77:100–107. [PubMed: 15197743]
- Thiel G, Czernik AJ, Gorelick F, Nairn AC, Greengard P. Ca^{2+} /calmodulin-dependent protein kinase II: identification of threonine-286 as the autophosphorylation site in the alpha subunit associated with the generation of Ca^{2+} -independent activity. *Proc. Natl. Acad. Sci. U. S. A* 1988;85:6337–6341. [PubMed: 2842767]
- Tingley WG, Ehlers MD, Kameyama K, Doherty C, Ptak JB, Riley CT, Huganir RL. Characterization of protein kinase A and protein kinase C phosphorylation of the N-methyl-D-aspartate receptor NR1 subunit using phosphorylation site-specific antibodies. *J. Biol. Chem* 1997;272:5157–5166. [PubMed: 9030583]
- Ueda T, Greengard P. Adenosine 3':5'-monophosphate-regulated phosphoprotein system of neuronal membranes. I. Solubilization, purification, and some properties of an endogenous phosphoprotein. *J. Biol. Chem* 1977;252:5155–5163. [PubMed: 194903]
- Uematsu K, Futter M, Hsieh-Wilson LC, Higashi H, Maeda H, Nairn AC, Greengard P, Nishi A. Regulation of spinophilin Ser94 phosphorylation in neostriatal neurons involves both DARPP-32-dependent and independent pathways. *J. Neurochem* 2005;95:1642–1652. [PubMed: 16300646]
- Walaas SI, Aswad DW, Greengard P. A dopamine- and cyclic AMP-regulated phosphoprotein enriched in dopamine-innervated brain regions. *Nature* 1983;301:69–71. [PubMed: 6296685]
- Wirkner K, Gerevich Z, Krause T, Gunther A, Koles L, Schneider D, Norenberg W, Illes P. Adenosine A2A receptor-induced inhibition of NMDA and GABAA receptor-mediated synaptic currents in a subpopulation of rat striatal neurons. *Neuropharmacology* 2004;46:994–1007. [PubMed: 15081796]
- Wu J, Filer D, Friedhoff AJ, Goldstein M. Site-directed mutagenesis of tyrosine hydroxylase. Role of serine 40 in catalysis. *J. Biol. Chem* 1992;267:25754–25758. [PubMed: 1361189]
- Yabauucki K, Kuroiwa M, Shuto T, Sotogaku N, Snyder GL, Higashi H, Tanaka M, Greengard P, Nishi A. Role of adenosine A1 receptors in the modulation of dopamine D1 and adenosine A2A receptor signaling in the neostriatum. *Neuroscience* 2006:19–25.
- Yuan LL, Adams JP, Swank M, Sweatt JD, Johnston D. Protein kinase modulation of dendritic K^{+} channels in hippocampus involves a mitogen-activated protein kinase pathway. *J. Neurosci* 2002;22:4860–4868. [PubMed: 12077183]

Fig. 1.

Regulation of selected phosphoproteins by the adenylate cyclase activator, forskolin, in striatal slices. Quantitative immunoblot analysis of phospho-Ser40 tyrosine hydroxylase (*TH*), phospho-Ser9 synapsin, phospho-Ser897 NR1, phospho-Ser845 GluR1, phospho-Ser94 spinophilin, phospho-Thr34 DARPP-32 (*D32*), phospho-Ser133 CREB, phospho-Thr286 CaMKII, diphospho-Thr202/Tyr204 ERK1, and diphospho-Thr183/Tyr185 ERK2 levels in striatal slices treated with or without forskolin (*FSK*, 10 μ M, 10 min). Immunoblots of total protein levels are also included for all phosphoproteins except synapsin and tyrosine hydroxylase. *, $p < 0.01$ compared to baseline, Student's unpaired t test, $n = 4-5$.

Fig. 2. Regulation of selected phosphoproteins by the adenosine A_{2A} receptor agonist, CGS 21680, in striatal slices. Quantitative immunoblot analysis of phospho-Ser40 tyrosine hydroxylase (TH), phospho-Ser897 NR1, phospho-Ser845 GluR1, phospho-Ser94 spinophilin, phospho-Thr34 DARPP-32 (D32), phospho-Ser133 CREB, diphospho-Thr202/Tyr204 ERK1, and diphospho-Thr183/Tyr185 ERK2 levels in striatal slices treated with CGS 21680 (0-5 μ M, 0-30 min). Immunoblots for total protein are also included for all phosphoproteins except spinophilin. In some experiments, control slices were treated with forskolin (FSK, 10 μ M, 10 min).

Fig. 3.

Regulation of selected phosphoproteins by the A_1/A_{2A} receptor antagonist, caffeine, after systemic administration to mice *in vivo*. Quantitative immunoblot analysis of phospho-Ser40 tyrosine hydroxylase (TH), phospho-Thr34, phospho-Thr75, and phospho-Ser130 DARPP-32 (D32), phospho-Thr202 ERK1, phospho-Thr183 ERK2, phospho-Ser133 CREB, phospho-Ser94 spinophilin, phospho-Ser845 GluR1, and phospho-Ser897 NR1. Phosphoprotein levels were normalized to the total amount of the protein in the sample as detected by dual channel fluorescence detection (see Methods). Data are expressed as mean percent change in phosphorylation ($\pm$ SEM) detected 60 min after caffeine injection. Data are analyzed by

ANOVA with Newman-Keuls multiple comparison (post-hoc) test ($^*p < 0.05$; $^{**}p < 0.001$ versus vehicle-injected control; n = 6 mice/group).

Fig. 4. Schematic representation of several PKA-mediated postsynaptic signaling pathways activated by striatal A_{2A} receptors. The dendrite of a medium spiny neuron is depicted as receiving glutamatergic and dopaminergic inputs via ionotropic glutamate receptors and D₂ receptors, respectively. These inputs are, in turn, modulated by the tonic actions of adenosine at A_{2A} receptors. All pre- and post-synaptic phosphoproteins evaluated as effectors of A_{2A}/cAMP/PKA signaling are depicted graphically. Inhibitory signaling steps are denoted by boxed-end arrows.

Table 1

Quantification of the percent increase in the relative levels of phospho-Ser40 tyrosine hydroxylase (*TH*), phospho-Ser9 synapsin, phospho-Ser897 NR1, phospho-Ser845 GluR1, phospho-Ser94 spinophilin, phospho-Thr34 DARPP-32 (*D32*), phospho-Ser133 CREB, phospho-Thr286 CaMKII, diphospho-Thr202/Tyr204 ERK1, and diphospho-Thr183/Tyr185 ERK2 in striatal slices treated with forskolin (10 μ M, 10 min).

Phosphoprotein	Fold increase (Forskolin)
P-S40 TH	4.2 $\pm$ 0.4
P-S9 synapsin	8.8 $\pm$ 0.5
P-S897 NR1	6.4 $\pm$ 0.9
P-S845 GluR1	9.6 $\pm$ 0.3
P-S94 spinophilin	8.3 $\pm$ 0.6
P-T34 D32	9.5 $\pm$ 0.7
P-S133 CREB	2.8 $\pm$ 0.4
P-T286 CaMKII	-
P-T202/Y204 ERK1	1.6 $\pm$ 0.2
P-T183/Y185 ERK2	2.7 $\pm$ 0.4

Table 2

Quantification of the percent increase in the relative levels of phospho-Ser897 NR1, diphospho-Thr202/Tyr204 ERK1, diphospho-Thr183/Tyr185 ERK2, phospho-Thr34 DARPP-32, phospho-Ser94 spinophilin, phospho-Ser845 GluR1, phospho-Ser133 CREB, and phospho-Ser40 tyrosine hydroxylase (*TH*) in striatal slices subjected to the optimal treatment with CGS 21680 defined for each phosphoprotein in Figure 2.

Phosphoprotein	CGS 21680 Treatment	Fold increase
P-S897 NR1	1 μ M; 5 min	3.9
P-T202/Y204 ERK1	1 μ M; 10 min	2.9
P-T183/Y185 ERK2	1 μ M; 10 min	1.8
P-T34 D32	1 μ M; 15 min	6.7
P-S94 spinophilin	2 μ M; 15 min	4.2
P-S845 GluR1	5 μ M; 10 min	2.9
P-S133 CREB	5 μ M; 15 min	2.0
P-S40 TH	-	-