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Uncovering multiple molecular targets for caffeine using a drug target validation strategy combining A_{2A} receptor knockout mice with microarray profiling

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Yu L, Coelho JE, Zhang X, Fu Y, Tillman A, Karaoz U, Fredholm BB, Weng Z, Chen J. Uncovering multiple molecular targets for caffeine using a drug target validation strategy combining A_{2A} receptor knockout mice with microarray profiling. *Physiol Genomics* 37: 199–210, 2009. First published March 3, 2009; doi:10.1152/physiolgenomics.90353.2008.—Caffeine is the most widely consumed psychoactive substance and has complex pharmacological actions in brain. In this study, we employed a novel drug target validation strategy to uncover the multiple molecular targets of caffeine using combined A_{2A} receptor (A_{2A}R) knockouts (KO) and microarray profiling. Caffeine (10 mg/kg) elicited a distinct profile of striatal gene expression in WT mice compared with that by A_{2A}R gene deletion or by administering caffeine into A_{2A}R KO mice. Thus, A_{2A}Rs are required but not sufficient to elicit the striatal gene expression by caffeine (10 mg/kg). Caffeine (50 mg/kg) induced complex expression patterns with three distinct sets of striatal genes: *1*) one subset overlapped with those elicited by genetic deletion of A_{2A}Rs; *2*) the second subset elicited by caffeine in WT as well as A_{2A}R KO mice; and *3*) the third subset elicited by caffeine only in A_{2A}R KO mice. Furthermore, striatal gene sets elicited by the phosphodiesterase (PDE) inhibitor rolipram and the $GABA_A$ receptor antagonist bicucullin, overlapped with the distinct subsets of striatal genes elicited by caffeine (50 mg/kg) administered to A_{2A}R KO mice. Finally, Gene Set Enrichment Analysis reveals that adipocyte differentiation/insulin signaling is highly enriched in the striatal gene sets elicited by both low and high doses of caffeine. The identification of these distinct striatal gene populations and their corresponding multiple molecular targets, including A_{2A}R, non-A_{2A}R (possibly A_1Rs and pathways associated with PDE and $GABA_A$R) and their interactions, and the cellular pathways affected by low and high doses of caffeine, provides molecular insights into the acute pharmacological effects of caffeine in the brain.

adenosine A_{2A} receptor; striatum; phosphodiesterase

CAFFEINE IS THE MOST WIDELY consumed psychoactive substance. It is estimated that >50% of the world's adult population consumes caffeine in behaviorally significant doses on a daily basis (25). Habitual human consumption of caffeine-containing foods and beverages produces overall psychostimulant effects (reducing fatigue and enhancing performance) with relatively little risk of harmful effects (25, 42). However, at higher doses (>400–500 mg/day), the effects of caffeine vary among individuals and may lead to various undesired effects, including increased anxiety, increased blood pressure, headache, and confusion (17, 25, 32, 42). Some epidemiological studies have linked relatively moderate and high (>300 mg/day) caffeine intake with an increased risk of spontaneous abortion (13, 22, 34, 54) and low birth weight (5, 53). Furthermore, large prospective epidemiological studies have implicated human caffeine consumption in a variety of human disease including diabetes (32, 51, 52), hypertension (32), liver cancer (32, 45), and Parkinson's disease (3, 44). However, the molecular basis for the association between caffeine consumption and these disorders are not clear. Thus, many key questions remain concerning the issues of efficacy and safety regarding low and high dosages of caffeine consumption.

Pharmacologically, caffeine produces complex actions through multiple molecular targets (17, 25, 42). Three different molecular targets for caffeine have been suggested to account for its molecular mechanism of action, namely $GABA_A$ receptor ($GABA_A$R) inhibition, phosphodiesterase (PDE) inhibition, and antagonism of adenosine receptors (17, 25). Caffeine was initially found to inhibit the binding of benzodiazepines to the sites on $GABA_A$Rs, which might explain the anxiogenic and convulsant effects induced by a high dose of caffeine (39). Furthermore, caffeine and other xanthine-based compounds inhibit PDE, the enzyme that degrades cAMP, thus elevating intracellular cAMP level (10). However, the $GABA_A$R inhibition (K_i = 280 μM) and PDE inhibition requires concentrations 10–100 times higher than those achieved with typical dietary doses in humans (17, 25). By contrast, adenosine receptor blockade takes place at concentrations many times lower than those required for PDE inhibition or inhibition of $GABA_A$R binding, occurring at an affinity (2–50 μM) compatible with the caffeine plasma concentrations attainable by normal human caffeine consumption (23). Despite similar affinities for the adenosine A_1 and A_{2A} receptors in brain, caffeine's psychostimulant effect is better correlated with its blockade of brain A_{2A} receptors (A_{2A}R) (25). There is both pharmacological (24, 25) and genetic knockout (KO) (8, 17, 21, 25, 42) evidence to support this. However, it should be emphasized that there is evidence that other targets could also

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play a role, particularly in the higher range of doses taken by humans.

These multiple potential targets of caffeine may contribute to the biphasic effects of increasing doses of caffeine on motor and cardiovascular responses in rodents (47, 48) and to anxiety, sleeplessness, and high blood pressure and heart rate associated with high dose of caffeine. This complexity may also underlie the association of caffeine consumption with a variety of common disorders detected by epidemiological studies (32, 42, 44, 45, 50). Traditional pharmacological studies focus on a single molecular target and are thus are unlikely to capture the complex actions of caffeine, which may reflect a summation of activities from a large array of molecular targets. Thus, an effective way to decipher such complex actions of caffeine (particularly with regarding to the biphasic dose response curve) is needed to explore the multiple molecular targets, multiple signaling pathways, and their functional connections at the whole genome level. DNA microarray may be suitable for addressing complex actions of drugs like caffeine because it can simultaneously monitor thousands of gene expression patterns at the whole genome level in brain regions under normal and pathophysiological conditions (2, 28, 38, 41).

In this study, we combined the microarray profiling with genetic KO models and pharmacological approaches to uncover the molecular basis for caffeine's complex actions. This "drug target validation" approach was initially developed by Marton and coworkers (40) studying effects of the immunosuppressants cyclosporine A or FK506 in yeast mutant strains deficient in calcineurin or immunophilins. If the characteristic drug "signature" pattern of altered gene expression matches to yeast cells with a particular mutation in the gene encoding a specific gene product, a putative target is established. Furthermore, if the signature expression pattern by a drug in wild type (WT) disappears when the drug is administered to a particular mutant, then it is concluded that the target missing in the mutant is required to generate the drug signature. We have recently successfully employed this approach to confirm that the $A_{2A}R$ is the only molecular target for the selective $A_{2A}R$ antagonist SCH58261 in eliciting striatal gene expression (56). In this study, using this drug target validation strategy, we dissected out the multiple molecular targets for the low and high doses of caffeine by comparing striatal gene expression patterns resulting from administering caffeine at low and high doses into $A_{2A}R$ KO and WT littermates and from inhibition of PDE and $GABA_AR$s. Specifically, at the level of striatal gene expression, we addressed the following five questions about caffeine's complex actions: *1*) Is the $A_{2A}R$ required to elicit striatal gene expression by caffeine? *2*) Is the $A_{2A}R$ sufficient to account for the striatal gene expression pattern by caffeine? *3*) Does caffeine act at single or multiple molecular targets to elicit striatal gene expression? *4*) Is the molecular pathway associated with PDE inhibition or $GABA_AR$ antagonism resembles the gene expression of caffeine acting at non-$A_{2A}R$ molecular targets? (5) What molecular pathways are particularly enriched in the striatal gene sets elicited by caffeine?

METHODS

Generation of $A_{2A}R$ KO mice and drug treatment. $A_{2A}R$ KO mice in a near congenic (N6) C57BL/6 genetic background were generated as previously described (7, 8, 56). Heterozygous cross-breeding was used to generate $A_{2A}R$ KO and WT littermate mice. All the mice in this study were handled in accordance with protocols approved by IACUC at Boston University. For each experiment, $A_{2A}R$ KO mice and WT littermates were matched for sex and age. $A_{2A}R$ KO and WT mice (male, ~6–8 wk of age) were habituated for 2 h and then treated with either saline or caffeine (10 or 50 mg/kg ip), rolipram (3 mg/kg ip) or bicuculline (2 mg/kg ip). Their locomotion was recorded for 120 min at which point the mice were killed and striata were dissected. Mouse striatum was dissected out and immediately frozen with dry ice for RNA isolation.

RNA isolation and hybridization to Affymetrix GeneChip. Total RNA was isolated using the TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). RNA concentration and integrity were assessed by spectrophotometry and gel electrophoresis. The labeling of RNA samples, Mouse GeneChip 430 2.0 (Affymetrix) hybridization, and array scanning were performed according to the Affymetrix GeneChip Expression Analysis Manual (Affymetrix, 2001) at the Harvard Genome Research Center. An average yield of 40 μg of biotin-labeled target cRNA was obtained from 5–7 μg of total RNA from each sample, and 20 μg of cRNA was applied to each chip. The chips were hybridized overnight in a rotating oven at 45°C and then washed and stained on a fluidics station and scanned at a resolution of 3 μm in a confocal scanner (Agilent Affymetrix GeneArray Scanner). The RNA from each mouse was processed individually to allow independent DNA chip analysis. A total of 24 GeneChips were used in this study.

Microarray data processing and statistical analysis of microarray data. The Affymetrix Command Console was used to calculate the overall noise of each image (Qraw) to ensure that background noise was similar across arrays in all groups. The robust multiarray analysis algorithm was used to scale and normalize the array data with "affy" (27) package (version 1.03). *P* values for the significance of changes in mRNA abundance between the control and experimental conditions were calculated with a permutation test. This test computes the significance of a simplified *t*-test statistic proposed by Golub and colleagues (29), using a null distribution of the statistic consisting of all possible permutations of the experimental condition labels. This test requires fewer statistical assumptions than a standard *t*-test but provides a narrow range of *P* values with the small samples sizes available for our study. Genes showing an absolute fold-change of $\geq$1.5 and permutation *P* value $\leq$0.05 were considered as significantly altered in expression. These sets of genes with significant expression changes were used for Gene Set Enrichment Analysis (GSEA). Affymetrix annotation database (www.affymetrix.com) and National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov) were used for the gene information associated with each probe set.

GSEA. We used GSEA (46) to determine if the gene sets from rolipram or bicuculline and publicly available gene sets were unevenly distributed in the ranked genes from caffeine-affected microarray data sets. GSEA software was obtained from the GSEA website (http://www.broad.mit.edu/gsea/). A total of 245 publicly available curated gene sets from online pathway databases, publications in PubMed, and Knowledge of domain experts were tested for the enrichment. These gene sets are in the Molecular Signature Database maintained by the Broad Institute at Massachusetts Institute of Technology (http://www.broad.mit.edu/gsea/msigdb/index.jsp, version 2). GSEA was performed on the 45,101 probe sets, and the data were scaled and normalized as described above. The genes corresponding to the probe sets were ranked with a signal-to-noise metric according to the differential expression observed between the control and treatment group (i.e., WT mice treated with saline or caffeine at 50 or 10 mg/kg). The significance (*P* value) of the distribution of gene sets within the ranked list was determined by gene set permutation (PMID: 16199517) and corrected for multiple hypothesis testing (*q*-value).

Quantitative PCR analysis. In a separate experiment, C57BL/6 male mice were treated either with saline or with 10 or 50 mg/kg caffeine ($n = 3$ for each group). The mice were killed and striata were

isolated 120 min after the treatment, and total RNA was extracted as described above. We then reverse-transcribed cDNA from total RNA using an Omniscript RT Kit (Qiagen, Valencia, CA) and an oligo(dT) primer (Invitrogen). We carried out quantitative PCR (qPCR) for 19 genes that were most consistently affected by multiple treatments (i.e., jointly affected by low and high doses of caffeine and/or $A_{2A}R$ KO) using a SYBR Green kit (Applied Biosystems, Warrington, UK). PCR reactions were performed in an ABI PRISM 7900HT Sequence Detection System (PE Applied Biosystems). Reaction conditions were 50°C for 2 min, 95°C for 10 min followed by 45 cycles of the amplification step (95°C for 15 s, 60°C for 30 s, and 72°C for 45 s). An endogenous control mouse cDNA, *gapdh*, was included in each amplification using a TaqMan PCR Core Reagent kit (Perkin-Elmer, Roch, NJ). The specificity of qPCR products was verified by melting curve analysis and by visualizing PCR products in 1% agarose gel at the expected molecular sizes. The relative abundance of target genes was obtained by the $\Delta\Delta^{-CT}$ method. The qPCR primer sequences for 19 genes tested were provided as the Supplemental Table S1.[1]

RESULTS

Low and high doses of caffeine produce distinct striatal gene expression profiles. After normalization, we performed nonsupervised, hierarchical clustering analysis for all probe sets (i.e., entire 45,000 probe sets) to assess the quality of our microarray data and whether three biological replicates per group were sufficient to distinguish different treatment groups. Hierarchical clustering analysis showed that most of 18 samples were clustered with their corresponding groups, indicating the high quality of the microarray data (see Fig. 1). We used a statistical cut-off (P value ≤ 0.05, permutation test and fold-change ≥ 1.5) to select a cohort of caffeine-regulated genes. These cut-off criteria generated 103 genes for the WT-caf10 (i.e., the WT mice treated with caffeine at 10 mg/kg) vs. WT-veh (i.e., the WT mice treated with vehicle) comparison and 276 genes for the WT-caf50 (i.e., the WT treated with caffeine at 50 mg/kg) vs. WT-veh comparison.

To validate the microarray results, we used qPCR to measure the expression levels of 19 genes that were most consistently affected by multiple treatments (i.e., jointly affected by low and high doses of caffeine and/or $A_{2A}R$ KO). Independent brain samples obtained after caffeine or saline treatment were used for this validation by qPCR analysis. Data from microarray and qPCR experiments are presented as fold-change in expression of genes in the experimental group relative to the control group. In total, qPCR analysis confirmed microarray results in 14 out of 19 or 74% assays (Table 1). The consistency between the microarray analysis and qPCR validation is slightly lower than our previous study (56), 87%, and other recent microarray studies (4, 11, 18).

Caffeine induced significantly more genes at the high dose (276 genes) than the low dose (103 genes). However, there were 16 genes that were similarly affected by the low and high doses of caffeine. Among these (16) shared genes, 15 genes were regulated in the same direction by both low and high doses of caffeine. Except for this small set of shared striatal genes, caffeine at low and high doses elicited largely distinct striatal gene expression.

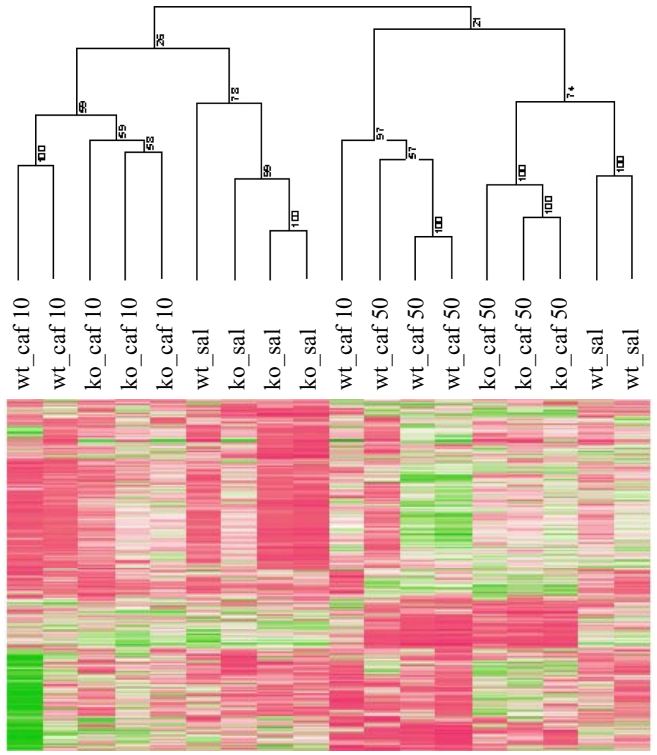

Fig. 1. Unsupervised hierarchical clustering analysis of striatal gene expression by low and high doses of caffeine in wild-type (wt) and A_{2A} receptor (R) knockout (ko) mice. Using whole normalized datasets without any gene filtering (i.e., entire 45,000 probe sets), we performed unsupervised hierarchical clustering analysis for striatal gene expression profiles in all mice after treated with low (10 mg/kg, caf10) and high (50 mg/kg, caf50) doses of caffeine in WT and $A_{2A}R$ KO mice (3 mice/group). Large majority of microarray profiles are clustered with their corresponding groups perfectly, indicating the high quality of the microarray data. sal, Saline.

$A_{2A}R$ is required but not sufficient to elicit striatal gene expression by the low dose of caffeine. We first tested the hypothesis that the $A_{2A}R$ is the required molecular target for caffeine-induced gene expression at the low dose (10 mg/kg). We reasoned that if the signature striatal gene expression pattern elicited by the low dose of caffeine in WT disappears when caffeine is administered to the $A_{2A}R$ KO mice, then the $A_{2A}R$ is required to generate caffeine's signature striatal expression pattern. Almost all (98 out of 103 total) striatal genes affected by the low dose of caffeine in WT mice (see Fig. 2*B*) disappeared in $A_{2A}R$ KO background, and instead caffeine elicited a new set of striatal genes (black dots, distinct gene set in Fig. 2*D*), indicating that the $A_{2A}R$ is required for the expression of the striatal gene elicited by the low dose of caffeine in WT mice.

Next, we tested whether the inactivation of $A_{2A}Rs$ was sufficient to recapture the striatal gene expression pattern elicited by the low dose of caffeine. We determined the overlap between the striatal genes affected by genetic deletion of the $A_{2A}R$ or that of the low dose of caffeine in WT mice. If the characteristic caffeine signature profile of striatal gene expression matches the expression profile elicited by $A_{2A}R$ gene deletion in $A_{2A}R$ KO mice, the $A_{2A}R$ inactivation is sufficient to produce striatal gene expression similar to caffeine. We detected 152 genes that were significantly affected by $A_{2A}R$ KO (compared with their WT littermates, Fig. 2*A*) and 103

[1] The online version of this article contains supplemental material.

Table 1. *The striatal gene expression elicited by caffeine (50 mg/kg) as assessed by microarray and qPCR*

Gene	Microarray	qPCR
A2M	2.4	1.3
ADA	−2.4	−3.3
ARC	1	1.8
CART	−1.5	−1.5
CD38	1.3	−1.5
DDX3Y	2	−6.7
FOS	−1.8	−1.3
FOSB	−1.9	−1.1
GALNTL2	−1.5	−2.7
HSPA1A	−1.5	1.1
LBP	2.2	2.1
NFKBIA	−2.1	−3.1
PTGDS	1.5	−3.7
PTGS2	−1.6	−1.2
SLC6A1	−2	−1.1
UTY	1.9	−1.2
ARPP21	1	−1.3
PDK4	1.9	2.9

Note: Boldfaced genes indicate the discrepancy between microarray and qPCR analyses. The full names for gene symbols are: A2M: α2 macroglobulin; ADA: adenosine deaminase; ARC: activity regulated cytoseletal associated protein; CART: cocaine and amphetamine regulated transcript; CD38: CD38 antigen; DDX3Y: DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked; FOS: FBJ osteosarcoma oncogene; FOSB: FBJ osteosarcoma oncogene B; GALNTL2: UDP-*N*-acetyl-α-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase-like 2; HSPA1A: heat shock 70 kDa protein 1A; LBP: lipopolysaccharide binding protein; NFKBIA: nuclear factor of κ-light polypeptide gene enhancer in B-cell inhibitor-α; PTGDS: prostaglandin D2 synthase (brain); PTGS2: prostaglandin-endoperoxide synthase 2; SLC6A1: solute carrier family 6 (neurotransmitter transporter, GABA), member 1; UTY: ubiquitously transcribed tetratricopeptide repeat gene, Y chromosome; ARPP21: cyclic AMP-regulated phosphoprotein, 21; PDK4: pyruvate dehydrogenase kinase, isoenzyme 4.

striatal genes that were affected by the low dose of caffeine in WT mice (compared with the saline-treated WT mice, Fig. 2*B*), respectively. However, there were only five striatal genes that were jointly affected by the low dose of caffeine and by $A_{2A}R$ KO (green dots in Fig. 2, *A* and *B*). This particularly low overlap indicates that inactivation of the $A_{2A}R$ gene alone, although required (as discussed above), is probably not sufficient to produce the striatal gene expression profile elicited by the low dose of caffeine. Thus, the $A_{2A}R$ coupled with additional molecular targets (i.e., multiple targets) are required to elicit the striatal gene expression profile elicited by the low dose of acute caffeine.

To further confirm the presence of multiple molecular targets by the low dose of caffeine, we reasoned that if the $A_{2A}R$ is indeed the only (single) molecular target for caffeine, caffeine is not expected to elicit a new set of striatal gene in addition to the striatal gene expression in the $A_{2A}R$ KO mice. On the other hand, if caffeine acts on multiple molecular targets, we would then expect that in the absence of the $A_{2A}R$ caffeine would act only at the non-$A_{2A}R$ molecular targets to elicit a new set of striatal genes in $A_{2A}R$ KO mice. Consistent with the prediction of the multiple targets for caffeine, administering the low dose of caffeine into $A_{2A}R$ KO induced a distinct striatal gene set (black dots in Fig. 2*D*). This new set of striatal genes remained even after accounting for the effect of $A_{2A}R$ KO alone or the effect of the low dose of caffeine in WT. Thus, this unique set of striatal genes primarily reflects the activity of caffeine acting at non-$A_{2A}R$ targets.

Caffeine at the high dose elicits complex striatal gene expression profiles with three distinct components, each involving the $A_{2A}R$, non-$A_{2A}R$ targets, and the interactions between the $A_{2A}R$ gene deletion and non-$A_{2A}R$ targets. In contrast to the single set of striatal gene expression being elicited by a low dose of caffeine for which the $A_{2A}R$ is required but not sufficient, a high dose of caffeine produces three distinct subsets of striatal genes, each apparently involving distinct molecular targets. First, a subset of striatal genes affected by high dose of caffeine in WT mice (66 out 276, green dots in Fig. 3*B*) was jointly affected by $A_{2A}R$ gene deletion in $A_{2A}R$ KO mice (green dots in Fig. 3*A*). This suggests that the $A_{2A}R$ is required and sufficient for the expression of this subset of the striatal genes elicited by high dose of caffeine. Of note, this subset of striatal genes that were jointly affected by $A_{2A}R$ KO and high dose of caffeine was largely upregulated striatal genes.

Second, the majority of the striatal genes (114 of 276 caffeine-affected genes, 41.3%) elicited by a higher dose of caffeine in WT mice (blue dots in Fig. 3*B*) persisted after we administered caffeine into $A_{2A}R$ KO mice (Fig. 3*D*), indicating that their expression is not $A_{2A}R$ dependent. Since these genes were elicited by a high dose of caffeine in $A_{2A}R$ KO mice (after accounting for the $A_{2A}R$ KO effect), this subset of striatal genes is due to caffeine acting at non-$A_{2A}R$ targets in either WT or $A_{2A}R$ KO background.

Third, a high dose of caffeine in $A_{2A}R$ KO mice elicited a distinct striatal gene expression profile that was not seen in WT background (113 genes, red dots in Fig. 3, *C* and *D*). This subset of striatal gene expression in response to caffeine (50 mg/kg) in $A_{2A}R$ KO was revealed after accounting for the effect of $A_{2A}R$ gene deletion (Fig. 3*D*) or the effect of high dose of caffeine in WT (Fig. 3*C*). Thus, this subset of striatal genes identified likely reflects the gene expression due to the interaction between $A_{2A}R$ gene deletion and non-$A_{2A}R$ targets by high doses of caffeine.

Notably, comparative analysis of the effect of low and high doses of caffeine revealed that after the low dose of caffeine was administered into $A_{2A}R$ KO (Fig. 2*D*), a significant portion of the striatal gene expression overlapped with the striatal genes affected by caffeine at 50 mg/kg in WT (63 out 276 genes, 22.8%; Fig. 3*B*) or in $A_{2A}R$ KO background (127 out of 469, 27%; Fig. 3*D*), supporting a common non-$A_{2A}R$ mediated mechanism responsible for the striatal gene expression profiles in these three comparisons.

PDE inhibitor rolipram and $GABA_AR$ antagonist bicucullin partially recapture the expression profiles of distinct subsets of striatal genes by the low and high doses of caffeine. To further identify the molecular identity and pathways of the multiple, non-$A_{2A}R$ targets for the high and low doses of caffeine, we compared the striatal gene sets affected by caffeine at the low and high doses with those affected by inhibition of PDE or $GABA_ARs$. Using the same cut-off (see above), we identified two cohorts of the striatal genes elicited by the treatment with the PDE inhibitor rolipram (3.0 mg/kg, total 328 genes) and by the $GABA_AR$ antagonist bicucullin (2.0 mg/kg, total 935 genes). For the striatal gene expression elicited by the low dose of caffeine (103 genes), 28 and 34 genes overlap with the rolipram and bicucullin, respectively. Among the overlapping genes, a common subset of the genes (20 genes) jointly affected by rolipram and bicucullin. For the low dose of

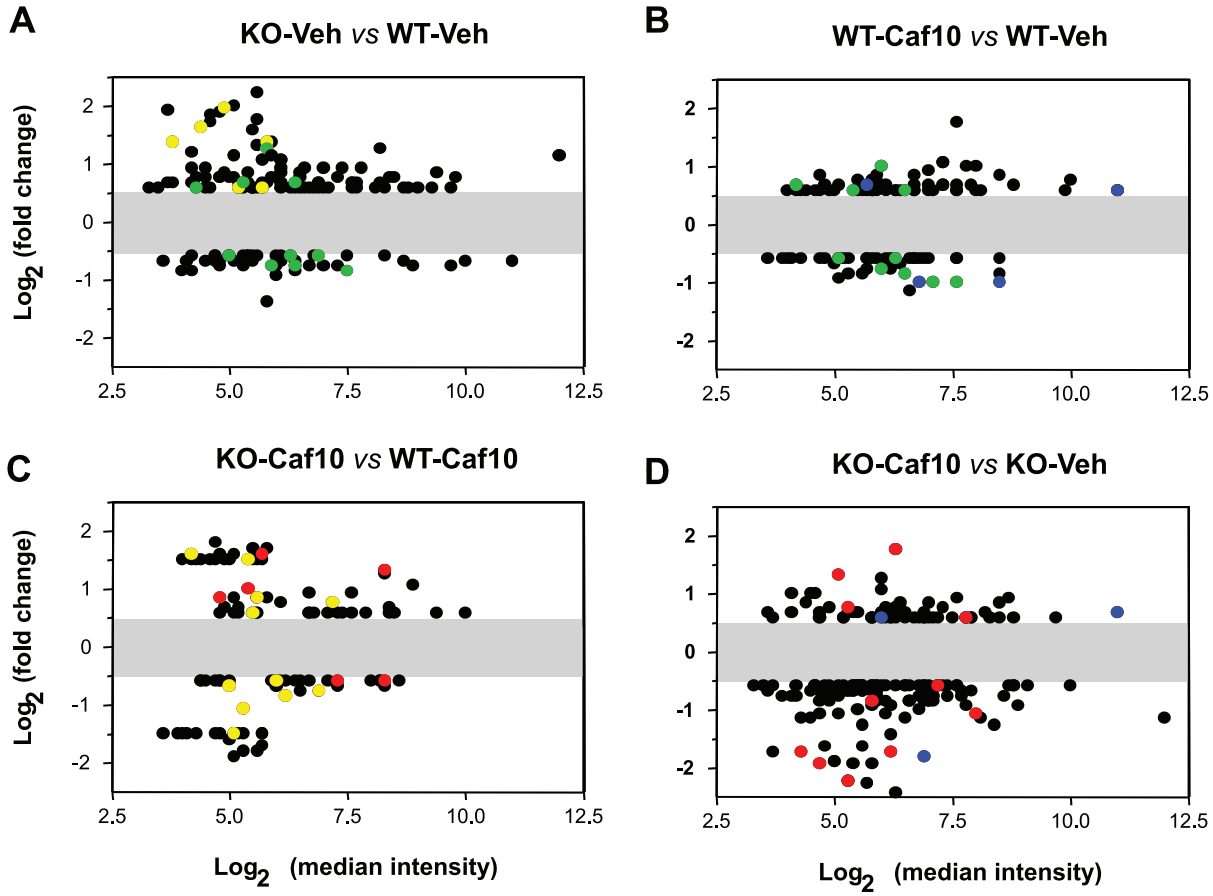

Fig. 2. Distinct patterns of striatal gene expression by caffeine (10 mg/kg) in WT and $A_{2A}R$ KO mice. Total RNA was isolated, quantified using Affymetrix Mu11kSubB oligonucleotide chips, and analyzed by permutation test as described in METHODS. The genes that passed the threshold for significant expression change (P value ≤ 0.05, fold-change ≥ 1.5) for each comparison are shown as points on the chart. The y-axis represents $\log_2$ fold-change, and the x-axis represents expression (Affy fluorescent) intensity. Red dots represent those genes common to the $A_{2A}R$ KO-Veh. vs. WT-Veh and the WT-Caf10 vs. WT-Veh comparisons. Green dots represent those genes common to the $A_{2A}R$ KO-Veh vs. WT-Veh and $A_{2A}R$ KO-Caf10 vs. WT-Caf10. Blue dots represent those genes common to the $A_{2A}R$ KO-Caf10 vs. WT-Caf10 and $A_{2A}R$ KO-Caf10 vs. $A_{2A}R$ KO-Caf10. Black dots indicate the striatal genes that were distinct from each other in the 2 comparisons. Veh, vehicle.

caffeine given to the $A_{2A}R$ KO mice, there were, however, significantly higher numbers of striatal genes that overlapped with the genes affected by rolipram (56 genes) than bicuculline (31 genes). For the striatal genes elicited by the high dose of caffeine in WT, 114 striatal genes were jointly affected by rolipram and by the high dose of caffeine. Despite significantly larger numbers of striatal genes affected by bicuculline than rolipram (935 vs. 328 genes), there were only 62 striatal genes that were affected both by bicuculline and the high dose of caffeine. This significantly higher overlap between caffeine (the low dose in $A_{2A}R$ KO mice or the high dose in WT) and rolipram rather than bicuculline suggests that the non-$A_{2A}R$-mediated mechanisms preferentially involve the molecular pathways and striatal gene expression associated PDE activation, rather than that associated with $GABA_AR$ inhibition.

Furthermore, we performed a detailed analysis to determine the relative contribution of PDE and $GABA_AR$ to three distinct gene expression profiles elicited by the high dose of caffeine in $A_{2A}R$ KO mice. Among the three distinct subsets of striatal genes elicited by the high dose of caffeine, the subset of striatal gene expression by the high dose of caffeine that overlapped with that elicited by $A_{2A}R$ KO (i.e., green dots in Fig. 3, *A* and *B*; 66 genes) shows 61% (40 out of 66 genes) overlap with rolipram-affected genes and only 15% (10 out of 66 genes) overlap with bicuculline-affected genes. Similarly, the subset of striatal genes elicited by a high dose of caffeine in WT as well as in $A_{2A}R$ KO mice (i.e., blue dots in Fig. 3, *B* and *D*) also shows 65% (74 out of 114 genes) overlap with the rolipram-affected genes and only 11% (13 out of 114 genes) overlap with bicuculline-affected genes. The relatively higher percentages of overlap between the genes elicited by caffeine (50 mg/kg) and rolipram than bicuculline are even more evident given that the threefold difference in the number of genes affected by bicuculline (935 genes) and rolipram (328 genes). On the other hand, there were only 10 out of 66 and 13 out of 74 genes that overlapped with the bicuculline-affected striatal genes. These results indicate that the molecular pathways associated with PDE are likely responsible for these two subsets of striatal genes induced by the high dose of caffeine in WT and $A_{2A}R$ KO mice. On the other hand, the subset of striatal genes elicited by a high dose of caffeine only in $A_{2A}R$ KO (i.e., interaction between high dose of caffeine and $A_{2A}R$ KO, red dots, $n = 113$) showed significant overlap with bicuculline (70/113, 62% genes) and a relatively lower overlap (34/113, 30% genes) with rolipram (Table 2). This indicates

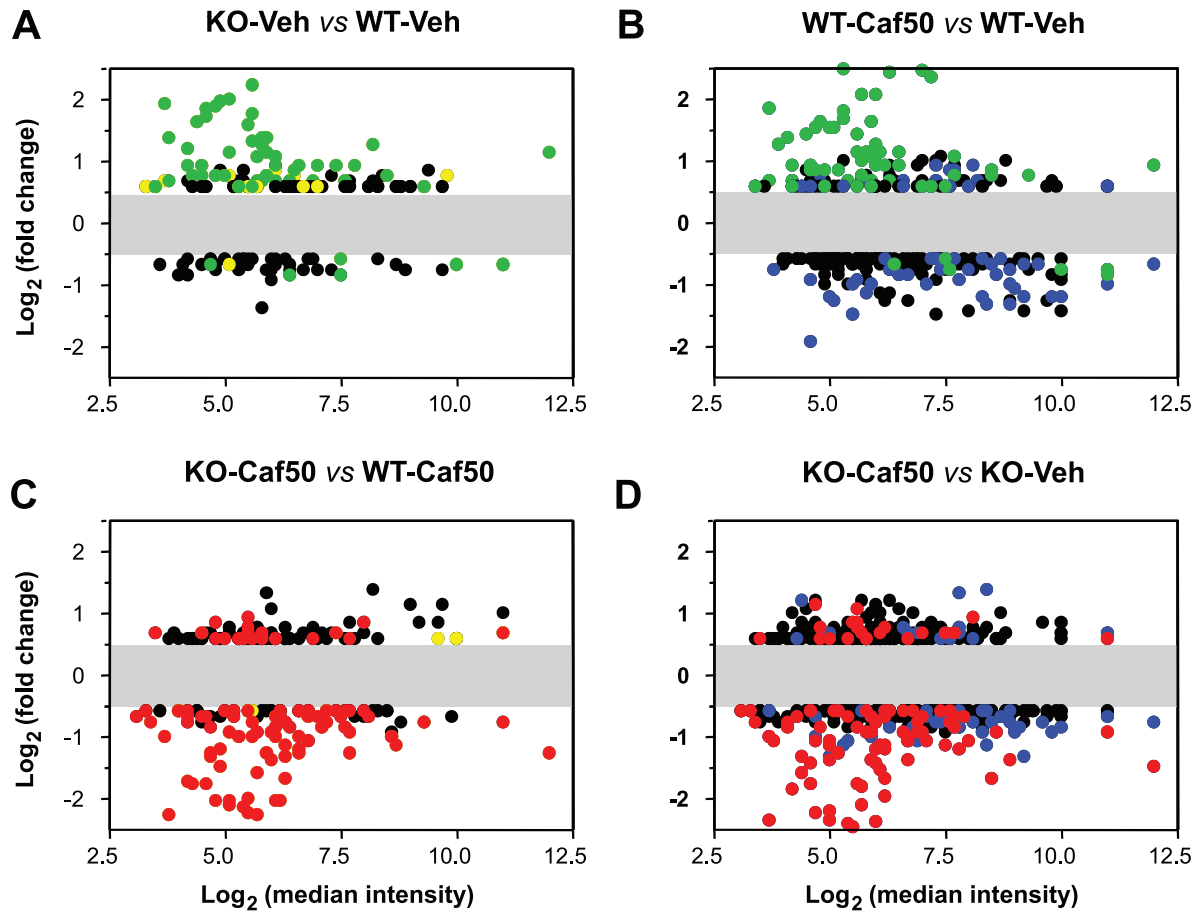

Fig. 3. Distinct patterns of striatal gene expression by caffeine (50 mg/kg) in WT and $A_{2A}R$ KO mice. The microarray gene expression was processed as described in Fig. 2. The *y*-axis represents $\log_2$ fold-change, and the *x*-axis represents expression (Affy fluorescent) intensity. Red dots represent those genes common to the $A_{2A}R$ KO-Veh vs. WT-Veh and the WT-Caf50 vs. WT-Veh comparisons. Green dots represent those genes common to the $A_{2A}R$ KO-Veh vs. WT-Veh and $A_{2A}R$ KO-Caf50 vs. WT-Caf50. Blue dots represent those genes common to the $A_{2A}R$ KO-Caf50 vs. WT-Caf50 and $A_{2A}R$ KO-Caf50 vs. $A_{2A}R$ KO-Caf50. Black dots indicate the striatal genes that were distinct from each other in the 2 comparisons.

that $GABA_AR$ antagonism can better mimic the expression of this particular subset of striatal genes than PDE.

To further elucidate the relative contribution of the molecular pathways associated with PDE inhibition and $GABA_AR$ antagonism to caffeine-induced striatal gene expression, we employed GSEA using our microarray data sets to address which of the gene lists, from rolipram-affected or bicuculline-affected, are enriched in any particular subset (e.g., upregulated and downregulated) of caffeine-affected genes. Using the default cut-off [false discovery rate (FDR) <0.25], we first examined enrichment of gene lists using the caffeine 50-WT (i.e., WT mice treated with caffeine at 50 mg/kg vs. saline) as the expression sets and the rolipram- or bicuculline-affected gene lists generated by the cut-off [i.e., $P < 0.05$ by permutation test, fold-change (over saline) ≥ 1.5]. We found that the rolipram-affected gene set is enriched in the gene set downregulated by caffeine (FDR q-value = 0.006), while the bicuculline-affected gene sets are enriched in the gene sets upregulated by caffeine (FDR q-value = 0). These results suggest that PDE inhibition and $GABA_AR$ antagonism partially capture the downregulated and upregulated gene expression by a high dose of caffeine, respectively.

Table 2. *Overlapping between the distinct subsets of striatal genes elicited by low and high doses of caffeine in WT and A_{2A} receptor KO mice with the genes by rolipram and bicuculline*

	Rolipram (329)	Bicuculline (935)
Caffeine 10-WT (103)	28	34
Caffeine 10-KO (191)	56	31
Caffeine 50-WT (276)	114	62
Caffeine 50-WT-G (66)	40	10
Caffeine 50-KO-B (114)	74	13
Caffeine 50-KO-R (113)	34	70

Overlap between distinct subsets of striatal genes elicited by caffeine in wild-type/knockout (WT/KO) mice and by rolipram and bicuculline.

GSEA revealed the novel "molecular signatures" that were enriched in the striatal genes elicited by low and high doses of caffeine. Using GSEA (46) (www.broad.mit.edu/gsea/), we further examined the molecular signatures/pathways that were enriched in caffeine-induced gene expression data. Table 3 lists the distinct gene sets (molecular signature) that overlap significantly with the caffeine induced genes by a low and a high dose of caffeine at the default cut-off FDR q <0.25. GSEA revealed that among 245 curated gene sets, 11 and 20 molecular signatures are distinctly enriched in the upregulated striatal

Table 3. *List of molecular signatures that are enriched in the striatal gene expression by low and high doses of caffeine*

	A. Molecular signatures (pathways) enriched in the striatal gene sets elicited by the low dose of caffeine (10 mg/kg)
	Upregulation
1.	Upregulated by nickel(II) in sensitive A/J mouse lung tissue
2.	Genes identified as time indicators in mouse SCN
3.	Upregulated at 2 h during differentiation of 3T3 fibroblasts into adipocytes (cluster 2)
4.	Downregulated by insulin in murine adipocytes, but response is blunted by TNF-α treatment
5.	Upregulated in the gastrocnemius muscle of aged (30 mo) vs. young (5 mo) adult mice
6.	Upregulated following conditional knockout of DNMT1 in MEFs
7.	Downregulated at 8-96 h during differentiation of 3T3 fibroblasts into adipocytes with IDX (cluster 3)
8.	Downregulated in hepatoma tissue in Acox1 KO mice
9.	Downregulated over 24 h during differentiation of 3T3-L1 fibroblasts into adipocytes (cluster 1)
10.	Co-regulated and transregulated putative targets of the *cis*-regulated HSC transcript, Runx1
11.	Downregulated in glomeruli isolated from Pod1 KO mice vs. WT controls
	Downregulation
1.	Downregulated by γ-irradiation and PARP inhibitors
2.	Downregulated at 2 h during differentiation of 3T3 fibroblasts into adipocytes with IDX (cluster 6)
3.	Upregulated in the nucleus accumbens of mice after 8 wk of induction of transgenic ΔFosB
4.	Upregulated in the hippocampus of 16-mo-old aged vs. 3-mo-old young mice
5.	Upregulated by treatment with insulin of NIH3T3 cells overexpressing insulin receptor
6.	Upregulated genes in *day 6* Hex/embryoid bodies
7.	Downregulated in mouse embryonic fibroblasts following targeted deletion of BRCA1 (exon 11)
8.	Downregulated by LIF treatment in AtT20 cells
9.	Upregulated in the hippocampus of 16-mo-old aged mice vs. 3-mo-old young controls
10.	Downregulated at 2-96 h during differentiation of 3T3-L1 fibroblasts into adipocytes with IDX (cluster 5)
11.	Upregulated in glomeruli isolated from Pod1 KO mice vs. WT controls
12.	Downregulated in the nucleus accumbens of mice after 8 wk of induction of transgenic CREB
13.	Upregulated in 3T3 fibroblasts following transduction with MyoD and incubation in differentiation medium
	B. Molecular signatures (pathways) enriched in the striatal gene sets elicited by the high dose of caffeine (50 mg/kg)
	Upregulation
1.	Upregulated at 2 h during differentiation of 3T3-L1 fibroblasts into adipocytes (cluster 2)
2.	Downregulated in the nucleus accumbens of mice after 8 wk of induction of transgenic CREB
3.	Genes identified as time indicators in mouse SCN
4.	Downregulated by insulin in murine adipocytes and continue to respond TNF-α treatment
5.	Upregulated in liver tissue from mice deficient in the lamin-protease Zmpste24/Face1
6.	Upregulated by nickel(II) in sensitive A/J mouse lung tissue
7.	Neurologically relevant downstream targets of a regulatory locus near the D6Mit150
8.	Upregulated in pro-B cells (FL5.12) following treatment with methotrexate
9.	Upregulated in pro-B cells (FL5.12) following treatment with cisplatin
10.	Upregulated in pro-B cells (FL5.12)by at least 2 of 4 cancer drugs (cisplatin, camptothecin, methotrextate, and/or paclitaxel)
11.	50 neurologically relevant transcripts with highest abundance fold range among strains
12.	Downregulated at 8-96 h during differentiation of 3T3 fibroblasts into adipocytes with IDX (cluster 3)
13.	Upregulated in mature, differentiated adipocytes following treatment with troglitazone
14.	Upregulated in pubertal mammary glands from CITED1 KO mice
15.	Upregulated in pro-B cells (FL5.12) following treatment with camptothecin
16.	Upregulated in pubertal mammary glands from CITED1 KO mice (vs. heterozygotes)
17	Upregulated at 2 h during differentiation of 3T3 fibroblasts into adipocytes with IDX (cluster 1)
18.	Downregulated in mouse C2AS12 myoblasts by treatment with IGF1 (vs. PDGF)
19.	Upregulated following 6 h differentiation of murine 3T3-L1 fibroblasts into preadipocytes
20.	Upregulated at 8 h during differentiation of 3T3 fibroblasts into adipocytes with IDX (cluster 2)
	Downregulation
1.	Upregulated by treatment with insulin of NIH3T3 cells overexpressing insulin receptor
2.	Genes downregulated by LIF treatment in AtT20 cells
3.	Genes expressed specifically in mouse kidney tissue
4.	Upregulated at 24 h during differentiation of 3T3 fibroblasts into adipocytes (cluster 5)
5.	Downregulated in hepatoma induced by diethylnitrosamine
6.	Upregulated following transduction of MyoD in NIH3T3 cells
7.	Co-regulated and transregulated putative targets of the *cis*-regulated HSC transcript, Runx1
8.	Upregulated in the nucleus accumbens of mice after 2 wk of induction of transgenic ΔFosB
9.	Upregulated in the nucleus accumbens of mice after 8 wk of induction of transgenic CREB
10.	Upregulated in glomeruli isolated from Pod1 KO mice

IDX, insulin, dexamethasone, and isobutylxanthine.

genes by the low and high doses of caffeine, respectively, while 13 and 10 molecular signatures are enriched in the downregulated genes by the low and high doses of caffeine, respectively.

Interestingly, adipocyte differentiation and insulin signaling pathways are highly enriched in caffeine-affected genes. Specifically, among the 10–20 enriched molecular signatures, nearly half of these gene sets (clusters, highlighted in boldface in Table 3) that were strongly downregulated during differentiation of 3T3-L1 fibroblasts into adipocytes after treatment with IDX (insulin, dexamethasone, and isobutylxanthine) (6) were similarly enriched in the striatal gene sets by both low and high doses of caffeine. Furthermore, another gene set that was downregulated by insulin treatment in murine adipocytes (6) was enriched in the upregulated gene sets by both low and high doses of caffeine. In addition, a set of the genes that was upregulated by treatment with insulin of NIH3T3 cells overexpressing insulin receptor (20) was enriched in the downregulated gene sets by both low and high doses of caffeine.

Furthermore, the nucleus accumben's genes that were downregulated after 8 wk of induction of transgenic expression of CREB were also enriched in the downregulated or upregulated genes by the low and high doses of caffeine, respectively (Table 3). On the other hand, the nucleus accumben's genes that were upregulated by 2 and 8 wk of induction by transgenic expression ΔFosB were enriched in the downregulated genes by both the low and high doses of caffeine (Table 3). Thus, these transcription factors are possibly involved in caffeine-induced striatal gene expression.

Lastly, GSEA revealed that genetic deletion of POD1 produced the opposite effect of caffeine (at low and high doses) on gene expression. Specifically, the gene set that was most strongly upregulated in glomeruli isolated from Pod1 (also called Tcf21/capsulin/epicardin, Table 3) KO mice (14) was enriched in the downregulated genes by both low and high doses of caffeine. Conversely, the gene set that was most strongly downregulated in glomeruli of Pod1 KO mice (14) was enriched in the upregulated gene sets by again both low and high dose of caffeine.

Thus, GSEA reveals that in spite of largely distinct striatal gene expression [with only 15 genes shared by low (103) and high (328) doses of caffeine], there are remarkably similar molecular signatures elicited by the low and high doses of caffeine, including adipocyte differentiation and insulin signaling, as well as gene expression associated with transcription factors CREB, ΔFosB, and POD.

DISCUSSION

A_{2A}R is required but probably not sufficient for eliciting striatal gene expression profiles by the low dose of caffeine. Using a novel drug target validation strategy of the combined targeted gene deletion and microarray profiling, we demonstrated that striatal gene expression elicited by the low dose of caffeine (10 mg/kg) is largely A_{2A}R-dependent since the caffeine-induced striatal gene expression is essentially absent when the low dose of caffeine was administered to A_{2A}R KO mice. This requirement of the A_{2A}R on the caffeine-induced striatal gene expression is consistent with the previous finding that the caffeine-induced behavioral responses including locomotor activity (9, 21), arousal effect (33), and basal metabolism (Yang JN and Fredholm BB, unpublished data) were abolished in A_{2A}R KO mice. Thus, at the level of gene expression this study validates that the A_{2A}R is a physiological molecular target for caffeine's action in the brain.

However, the drug target validation strategy reveals additional molecular targets for caffeine (10 mg/kg) in eliciting striatal gene expression. In the absence of A_{2A}R, caffeine (10 mg/kg) acts on targets other than the A_{2A}R to elicit a completely different set of striatal genes. This is in contrast to the selective A_{2A}R antagonist SCH58261, which elicited a set of striatal genes in WT mice but did not elicit any gene expression in A_{2A}R KO mice (after accounting for A_{2A}R KO effect). Therefore, the A_{2A}R is the only target for SCH58261, while caffeine (10 mg/kg) can act on other targets to elicit a distinct set of striatal genes in A_{2A}R KO mice.

While these additional targets are best manifested in the A_{2A}R KO mice, these targets (such as the A_1R, see discussion below) may also be partially responsible for striatal gene expression in WT mice. This view is consistent with the finding that A_{2A}R gene deletion by itself is not sufficient to mimic the striatal gene expression by caffeine (10 mg/kg). Thus, the A_{2A}R is required, but probably not sufficient to account for the striatal gene expression by caffeine in WT mice. Caffeine acts at the A_{2A}R as well as non-A_{2A}R targets to elicit the gene expression profile. However, the comparisons between pharmacological drugs and genetic KO should be interpreted with extreme cautions for two reasons: *1*) caffeine at 10 mg/kg would likely produce only partial blockade of the A_{2A}R and thus, A_{2A}R KO, which produce complete deletion of the A_{2A}R gene, would likely produce different striatal gene expression. On the other hand, heterozygous A_{2A}R KO mice (which delete the A_{2A}R gene and reduce A_{2A}R binding by 50%) probably better model the effect of caffeine (10 mg/kg) on striatal gene expression (55). *2*) Caffeine was administered to adult animals, while gene deletion (in most cases) takes place from very early development and persists throughout life. Thus, A_{2A}R gene deletion may produce developmental effects that cannot be recaptured by drug treatment such as caffeine to adult mice. Indeed, our early study shows that SCH58261 captures only one aspect of the A_{2A}R KO effect, namely upregulation of gene expression with ~30% shared genes (56). Therefore, the lack of overlap between the striatal gene sets by caffeine (10 mg/kg) and by A_{2A}R KO may not necessarily be indicative of insufficiency of the A_{2A}R as the primary molecular target for eliciting striatal gene expression. Instead, this may largely reflect the fundamental difference between these two manipulations and may be attributed to various other factors such as neuroadaptation in A_{2A}R KO mice and functional interdependence among A_1R and A_{2A}R. Nonetheless, the demonstration of the existence of non-A_{2A}R targets in A_{2A}R KO mice for caffeine-induced gene expression confirms the multiple molecular targets responsible for neurochemical and behavioral changes by caffeine.

While the existence of additional molecular targets is demonstrated by the drug target validation strategy, the nature of additional, non-A_{2A}R targets and their involvement in mediating caffeine's actions are yet to be determined. We speculate that A_1Rs may be involved in mediating caffeine-elicited striatal gene expression. This is in agreement with early pharmacological finding that caffeine has similar affinities for the A_1R and A_{2A}R (K_d = 12–20 and 2–8 nM for A_1Rs and A_{2A}Rs

in human and rat, respectively) (25) and that the effect of caffeine in the brain may be better correlated with simultaneous blockade of both A_1Rs and A_{2A}Rs (36). This interdependence of A_{2A}R-A_1R activation for the effects of caffeine is also consistent with the recent experimental evidence for the close proximity of these receptors in a proposed A_1R-A_{2A}R heterodimer in the brain (37) (12). However, the interdependence of A_1R-A_{2A}R function in mediating caffeine's effect seems to be specific at the molecular and neurochemical levels, since the caffeine-induced psychomotor (30) and arousal (33) effects were abolished in A_{2A}R KO mice but remained intact in A_1R KO mice, which is difficult to reconcile with an obligatory role of receptor heterodimers. Additional experiments with the same drug target validation strategy using caffeine combined with the A_1R KO and A_1R-A_{2A}R double KO should be performed to clarify the role(s) played by A_1Rs. In addition to adenosine receptors, the molecular pathways associated with PDE or less likely the $GABA_A$R may represent important components for caffeine at 10 mg/kg, particularly in the absence of the A_{2A}R. This notion is supported by the finding that PDE-affected genes are particularly enriched the subset of striatal genes elicited after administration of caffeine (10 mg/kg) in A_{2A}R KO mice (Table 2).

High doses of caffeine elicit complex striatal gene expression profiles with three distinct components associated with the A_{2A}R, non-A_{2A}R targets, and the interaction between A_{2A}R gene deletion and non-A_{2A}R targets. It has been suggested by pharmacological studies that a high dose of caffeine likely acts at the A_{2A}R as well as non-A_{2A}R targets to elicit biological responses (15, 16, 25). The nature of the molecular targets for high dose effects of caffeine is largely unknown, although A_1R antagonism, PDE inhibition, and $GABA_A$R antagonism have been proposed. It has, for example, been noted that whereas caffeine has a biphasic effect on motor activity (stimulation at low and depression at high doses) in WT mice, only a depressant effect was noted in A_{2A}R KO mice (21, 30). It was postulated that the depressant effect was due to the A_1R blockade (21), but this could not be confirmed in A_1R KO mice (30). As noted above, we fully expect that blockade of A_1Rs will also contribute to some aspects of the caffeine-mediated gene expression that are not attributable to A_{2A}R blockade, which can be further examined with the same strategy also using A_1R KO and A_1R-A_{2A}R double-KO mice. Our analyses, instead, focus on providing evidence for the contribution of distinct, multiple molecular targets, including the molecular pathways associated with PDE and $GABA_A$R, to the expression of distinct populations of striatal genes elicited by a high dose of caffeine.

Behavioral analysis demonstrates that even at a dose of 50 mg/kg, the caffeine-induced motor stimulant effect in WT mice was largely abolished, indicating the critical involvement of the A_{2A}R. Consistent with this notion, a subpopulation of striatal genes induced by the high dose of caffeine given to WT mice (66/328, 20%) was found to overlap with the striatal gene expression induced by deletion of the A_{2A}R. This subpopulation disappeared when the high dose was administered to A_{2A}R KO mice (after accounting for the A_{2A}R KO effect). These findings support the idea that the A_{2A}R is the molecular target responsible for the expression of this subpopulation of striatal genes induced by the high dose of caffeine. A distinct feature of this A_{2A}R-mediated subpopulation of striatal genes is that they are largely upregulated by high doses of caffeine or by A_{2A}R KO. This is consistent with our previous finding that the striatal gene expression induced by the A_{2A}R antagonist SCH58261 overlaps with the A_{2A}R KO expression pattern, with an also largely upregulated striatal gene expression (56). This suggests that adenosine acting at A_{2A}Rs probably exerts an inhibitory effect on striatal gene expression. This subpopulation could represent potentially important downstream target genes of A_{2A}Rs.

Furthermore, the drug target validation strategy also revealed two distinct subsets of striatal genes that are elicited by caffeine acting on non-A_{2A}R targets. First, the subpopulation of striatal genes that were jointly affected by the high dose of caffeine given to WT or A_{2A}R KO mice (blue dots in Fig. 3, *B* and *D*) represents the second component of the multiple molecular target of caffeine at 50 mg/kg. This subset of striatal genes is likely mediated by non-A_{2A}R targets since it can also be elicited in A_{2A}R KO mice and significantly overlaps with striatal genes elicited by the low dose of caffeine given to the A_{2A}R KO mice. The nature of this non-A_{2A}R target for this subset of striatal genes is not clear, but the striatal gene expression pattern can be partially mimicked by PDE (but not $GABA_A$R) inhibition, indicating that PDE or PDE-associated signaling/molecular pathways (such as cAMP level) may underlie the expression of this particular subset of striatal genes. Second, there is also a subpopulation of striatal genes induced by high dose of caffeine when administered to the A_{2A}R KO mice only. Of note, the majority of this particular set of the striatal genes are downregulated by caffeine. We identified this unique set of striatal genes after filtering the effect of A_{2A}R KO or the effect of high dose caffeine in WT. Thus, this subset of striatal genes (i.e., red dots in Fig. 3, *C* and *D*) may result from caffeine's action at yet another new molecular target that was only manifested in the absence of the A_{2A}R. Consistent with this unique, non-A_{2A}R-mediated gene expression by caffeine, we have also noted that even modest doses of caffeine (30 mg/kg) can produce some effects only seen in A_{2A}R KO mice, including behavioral depression and a decrease in body temperature (Yang JN, Chen JF, and Fredholm BB, unpublished observations). Since these behavioral effects are also clearly seen in mice that lack both A_1Rs and A_{2A}Rs, adenosine receptor-independent effects at doses close to normal human usage can be demonstrated. Indeed, the molecular pathway associated with $GABA_A$R are potential molecular targets for this novel subset of striatal genes since inhibition of $GABA_A$R (but not PDE) activity can partially mimic the expression profiles of this subset of striatal genes. The notion that the molecular pathways associated with PDE inhibition and $GABA_A$R antagonism play a prominent role in distinct components of non-A_{2A}R-mediated striatal gene expression is further supported by the finding that inhibition of PDE and $GABA_A$R recapture the downregulation and upregulation of striatal gene expression, respectively, when the entire gene set is considered. It should be noted that the significant overlap between PDE- or $GABA_A$R-affected and caffeine-affected gene expression in the striatum may be due to either an indirect effect on the molecular signaling pathways that activated by PDE and $GABA_A$R or less likely a direct effect of caffeine on the PDE and $GABA_A$R. The latter is relevant since caffeine's action on PDE and $GABA_A$R can be achieved at doses several-

fold higher than those required to block A_1Rs and A_{2A}Rs (17, 24–26).

Dissection of multiple molecular targets for striatal gene expression by low and high doses of caffeine provides molecular insights into complex pharmacological actions of caffeine. Caffeine is known for its complex pharmacological actions, such as biphasic dose-response effect after acute treatment, rapid development of tolerance, and "effect inversion" after chronic treatment, but the molecular targets by which caffeine produces complex pharmacological actions are largely unknown. The dissection of multiple molecular targets for caffeine at the low and high doses provides molecular insights into the molecular changes associated with complex actions of caffeine. For example, our study with the drug target validation strategy reveals that the majority (85%) of striatal gene expression profiling by caffeine at low and high doses are distinct, with only a small set (16 out of 103 genes) of the striatal genes being jointly affected by low and high doses of caffeine. Furthermore, caffeine at 10 mg/kg elicits a single set of striatal genes (mediated by the A_{2A}R and probably also non-A_{2A}R targets), while caffeine at 50 mg/kg produced three distinct striatal gene populations, each involving distinct and largely separate molecular targets (as discussed above). The largely distinct striatal gene expression patterns by low and high doses of caffeine suggest that biphasic responses by low and high doses of caffeine (including psychomotor, cardiovascular, and cognitive effect) are associated with the distinct striatal gene expression, rather than the same set of striatal gene with opposite directions by low and high doses of caffeine. Similarly these findings also provide partial explanations for the distinct side effects of a high dose that are not associated with low doses of caffeine. Furthermore, the lack of overlap between the striatal gene expression by caffeine (particularly at low doses) and by A_{2A}R KO (as well as by the A_{2A}R antagonist SCH58261) may provide a molecular explanation for how tolerance to caffeine develops rapidly after treatment (25), while A_{2A}R antagonists do not (31, 43). Indeed, there is some evidence that A_1Rs may be particularly important in the development of tolerance to caffeine (35, 48).

Importantly, given the lingering question about the possible side effects of high dose of caffeine, identification of multiple striatal gene expression patterns and possible associated multiple molecular targets and pathways by high doses of caffeine allow for the study of caffeine-mediated striatal gene expression at the network level. Lastly, these findings reinforce the notion that it is unlikely to capture the caffeine action by manipulating individual molecular targets (including the A_{2A}R). The complex yet unique pharmacological features of caffeine need to be investigated by the integrate analysis of multiple molecular targets of caffeine.

Insulin signaling and POD-1 transcription may be associated with caffeine-induced striatal gene expression and possibly long-term effects. GSEA shows that the molecular signature associated with adipocyte differentiation/insulin signaling and POD1 transcription are highly enriched in striatal gene expression by low and high doses of caffeine. It is remarkable that four gene clusters (the gene cluster 1, 2, and 3 at 2, 6, and 86 h, respectively, during adipocyte differentiation following treatment of insulin) were highly enriched in the upregulated striatal gene sets by both low and high doses of caffeine. Conversely, two sets of gene clusters (the gene clusters 5 and 6 during adipocyte differentiation following treatment with insulin or overexpressing insulin receptor) are highly enriched in the downregulated striatal genes by both low and high doses of caffeine. This remarkable enrichment in the gene sets associated with insulin signaling and adipocyte differentiation indicates that these molecular pathways may be involved in metabolic responses in brain and consequently long term effects of caffeine in the brain. These results may provide a molecular correlate with the epidemiologically demonstrated association between caffeine consumption and type II diabetes (1, 49). It is also noted that genetic deletion of POD1 transcription factor produces an opposite effect of caffeine on striatal gene expression. If confirmed, these findings would indicate that POD1 as a transcription factor may participate, in the opposite modulation direction, with caffeine-mediated transcriptional regulation of striatal gene expression.

Novel drug target validation strategy may have broad implications for drug development targeting multiple molecular pathways. We are fully aware that the gene expression studied here is likely the summed responses of primary and secondary targets of caffeine. Indeed, the gene expression studied here may not be the most important acute effect of caffeine, and caffeine could have major effects outside the striatum. We are also aware that there may be differences between acute administration of caffeine and its long-term effects. However, none of these caveats detract from the fact that the strategy we have chosen permits the dissection of distinct molecular elements (the A_{2A}R and different components and pathways of non-A_{2A}R targets) by which low and high doses of caffeine exert complex pharmacological actions in the brain. The development of a drug target validation strategy for dissecting complex drugs like caffeine to uncover the required as well as sufficient components and to determine single vs. multiple targets by drugs will facilitate the development of the treatment strategy targeting multiple pathways. Increasing evidence suggest that many neuropathological processes such as neurodegeneration involve multiple molecular signaling pathways in the brain. Thus, targeting a single pathway may be not sufficient in achieving the therapeutic goal of reversing pathological processes. Consequently, a treatment strategy targeting multiple molecular pathways (either by combinatory drugs or by a drug targeting multiple pathways) becomes an increasingly attractive option. The drug validation strategy we demonstrated here is applicable to other types of drugs with possible complex actions. This strategy may help to delineate the multiple molecular pathways affected by complex drug action and facilitate the development of drug treatment strategy targeting for multiple molecular targets.

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