What Knock-Out Animals Tell Us About the Effects of Caffeine

Jiang-Fan Chen\textsuperscript{a, *} , Liqun Yu\textsuperscript{a}, Hai-Ying Shen\textsuperscript{a}, Jin-Cai He\textsuperscript{b}, Xiaotong Wang\textsuperscript{b} and Rongyuan Zheng\textsuperscript{b}

\textsuperscript{a}Department of Neurology, Boston University School of Medicine, Boston, MA, USA
\textsuperscript{b}Department of Neurology, Wenzhou Medical College, Wenzhou, P.R. China

Abstract. Caffeine is well known for its complex pharmacological actions, in part reflecting the multiple molecular targets of caffeine. The adenosine receptors are the primary extracellular targets of caffeine. Since caffeine has similar affinity for several adenosine receptors, it has been difficult to determine which receptor subtypes mediate caffeine’s effects using pharmacological tools. The development of genetic mutant mice deficient in adenosine receptors and other signaling molecules has allowed targeted inquiry into the molecular targets by which caffeine elicits its biological effects on behavior and gene expression. This review summarizes recent work using genetic knockout models to elucidate the mechanisms of caffeine action in the brain. This review focuses on insights into caffeine action from genetic knockout models on: (1) the molecular basis for caffeine’s effects on psychomotor activity; (2) the involvement of adenosine receptors in caffeine-mediated arousal and cognitive effects; and (3) a novel approach using knockout animals coupled with microarray profiling to validate multiple molecular targets of caffeine in striatal gene expression.

Keywords: Adenosine A\textsubscript{2A} receptor, adenosine A\textsubscript{1} receptor, caffeine, arousal, DARPP-32, psychostimulation

INTRODUCTION

Caffeine, probably the most widely consumed psychostimulant substance, produces complex pharmacological actions. Habitual human consumption of caffeine-containing foods and beverages is estimated in a range of 70–350 mg/person/day or 5 to 8 mg/kg/day (equivalent to 3 cups of coffee). This human caffeine consumption is estimated to reach peak plasma concentration of 0.25 to 2 mg/L (or approximately 1 to 10 \( \mu \)M) and produces overall psychostimulant effects, reducing fatigue and enhancing performance, with relatively little risk of harmful effects [1,2]. However, at higher doses (above 400–500 mg/day), the effects of caffeine vary among individuals and may lead to undesired effects, including increased anxiety, increased blood pressure, headache, and confusion [1–4]. Furthermore, repeated exposure to caffeine results in rapid tolerance [1], and chronic caffeine exposure often produces effects opposite to that of acute caffeine, termed “effect inversion” [1,5]. These complex actions of caffeine are in part due to its multiple molecular effects, ranging from GABA\textsubscript{A} receptor (GABA\textsubscript{A}R) inhibition, phosphodiesterase (PDE) inhibition, to antagonism of adenosine receptors [1,3]. Differences in the affinity of caffeine for these multiple potential targets may contribute to the biphasic motor and cardiovascular, cognitive responses to increasing doses of caffeine seen in rodents [6,7] and to the anxiety, sleeplessness, and increases in blood pressure and heart rate associated with high doses of caffeine in human [1]. Furthermore, this complexity may also underlie the association of caffeine consumption with a variety of common disorders detected by large prospective epidemiological studies [2,4,8–10], including Parkinson’s disease [10,11] and possi-
by Alzheimer’s disease [12,13]. However, traditional pharmacological studies are limited in dissecting out the complex actions of caffeine, which probably reflect a summation of caffeine’s effects on a large array of molecular targets.

Genetic knockout (KO) mice deficient in adenosine A2A receptor (A2AR) and other associated signaling molecules have been used to gain the new insight into the complex actions of caffeine on various aspects of cognitive behaviors. Over the last 10 years, genetic knockout mice have been developed which are deficient in individual adenosine receptors, i.e. adenosine A1, A2A, A2B and A3 receptors (A1R, A2AR, A2BR and A3R) [14], or in other signaling molecules associated with caffeine action, such as dopamine D2 receptor (D2R) [15,16] and DARPP-32 (dopamine- and cyclic AMP-regulated phosphoprotein with molecular weight of 32 kDa) [17]. These knockout mice provide novel approaches to dissect out the complex actions of caffeine and yield new insights into the actions and mechanisms of caffeine in the brain. This review focuses on insights about caffeine actions from genetic knockout models on: (1) the molecular basis for caffeine’s psychomotor stimulant; (2) the involvement of adenosine receptors in caffeine-mediated arousal; and (3) a novel approach using knockout animals coupled with microarray profiling to validate multiple molecular targets of caffeine in striatal gene expression.

**MOLECULAR BASIS FOR PSYCHOSTIMULANT EFFECTS OF CAFFEINE**

**Adenosine receptors and caffeine effects**

At the concentration attainable by regular human consumption, caffeine’s effect on the brain is mediated primarily by blocking adenosine receptors. Although pharmacological characterization suggests that caffeine has similar affinities for the adenosine A1R and A2AR in the brain [1,18], recent studies using the genetic knockout models indicate that caffeine’s psychostimulant effect correlates better with its blockade of brain A2AR [1] [1–3,19,20]. Two genetic knockout (KO) mouse lines deficient in the A2AR but in different genetic backgrounds (CD1 versus C57BL/6) have been developed [21,22]. The psychomotor stimulant effect of caffeine in wild-type mice was completely abolished or converted into depressant effect A2AR KO lines [19,20,22]. Thus, the A2AR is required for the psychomotor stimulant of caffeine. Furthermore, using recently developed brain region and cell-type specific A2AR KO mice, we demonstrated that it is the A2ARs in forebrain neurons that are responsible for caffeine-induced psychomotor activity [23]. Thus, there is clear and strong evidence that the A2AR located on forebrain neurons is the molecular target by which caffeine exerts its psychomotor effect. In contrast, it appears that the A1R is not critical to caffeine’s psychomotor effect in the brain, despite the similar affinity of caffeine for the A1R and A2AR, since caffeine’s psychomotor effect is largely intact in mice lacking the A1R [24,25].

The generation of the A1R-A2AR double homozygous KO and their heterozygous mutants provided an opportunity to study the interaction between A1R and A2AR in mediating caffeine’s effect. Regardless of the A1R genotype, mice lacking the A2AR exhibited no psychomotor response to caffeine, confirming the critical requirement for the A2AR but not the A1R in caffeine’s motor stimulant effect [24,25]. However, A1Rs may still contribute to caffeine’s action through interactions with the A2AR since lack of the A1R seems to slightly enhance the caffeine stimulant effect via A2AR blockade [26]. Interestingly, it is noted that the inhibitory effect of high doses of caffeine (30–100 mg/kg) on motor activity was not altered by lack of either A1R or A2AR. Thus, it is likely that molecular targets other than A1R or A2AR play a role in this inhibitory effect of caffeine at high doses. Lastly, it is postulated that double heterozygous (not homozygous) A1−/A2AR mutant mice had approximately one-half the number of A1R and A2AR and may better mimic chronic effect of caffeine since, at the concentration attainable by regular human consumption, caffeine blocks both A1R and A2AR by ~30–50% [27]. Indeed, the ability of adenosine to activate receptors was shifted to the right in the dose-response study by caffeine or in double heterozygous A1−/A2AR mutant mice. Moreover, psychomotor activity by acute treatment with caffeine was reduced in double heterozygous A1−/A2AR mutant mice and in wild-type mice after chronic treatment with caffeine. Thus, the A1−/A2AR double heterozygous mutant mice capture some aspects of long-term caffeine use [27], and thus may be a useful model for studying effects of chronic caffeine treatment. It should be noted that chronic caffeine treatment is generally associated with upregulated A1R expression in the brain. Thus, additional mechanism may contribute to the reduced response of acute caffeine challenge in A1−/A2AR double heterozygous mutants and in mice chronically treated with caffeine.
Interestingly, a recent study of prenatal exposure to caffeine using A<sub>1</sub>R KO mice uncovered a possible role of epigenetic factors in prenatal caffeine’s effect on psychomotor activity through the A<sub>1</sub>R [28]. Bjorklund and colleagues found that perinatal (from gestational day 1 to postnatal day 21) exposure to caffeine (0.3 g/L in maternal drinking water) increased locomotor activity and enhanced the response to cocaine when mice were tested on postnatal day 60. Interestingly, the offspring of A<sub>1</sub>R heterozygous mutant mice exhibited increased psychomotor responses to caffeine and cocaine when tested as adults, which is very similar to the behavior of wild-type mice perinatally exposed to caffeine. This effect is unlikely attributed to the reduced A<sub>1</sub>Rs but rather adenosine receptor-mediated epigenetic modification as suggested in a recent study [29]. Remarkably, this behavioral change is manifested in the next generation. Moreover, it is the maternal, not offspring’s, genotype that determines this trans-generational behavioral alteration [28]. This finding suggests that perinatal exposure to caffeine, probably acting on the maternal A<sub>1</sub>R, produces a long-lasting, trans-generational behavioral change such as methylation of DNA and histone phosphorylation. The finding has public health implications since there are still some lingering concerns that caffeine consumption during pregnancy (particularly at high doses) may be associated with long-lasting behavioral changes in the offsprings.

Lastly, given the low affinity of caffeine for the A<sub>3</sub>R, it was surprising to find that caffeine-induced psychomotor activity is reduced in A<sub>3</sub>R KO mice [30]. Since A<sub>3</sub>R KO similarly attenuated both caffeine- and amphetamine-induced psychomotor activity, the possible developmental effect of A<sub>3</sub>R KO (such as compensatory changes in A<sub>1</sub>Rs and A<sub>2A</sub>Rs) needs to be taken into consideration [30].

**Dopamine and caffeine effect on psychomotor activity**

Based on the co-localization, heterodimerization and co-aggregation of the A<sub>2A</sub>R and D<sub>2</sub>R; and the profound antagonistic interaction between A<sub>2A</sub>R and D<sub>2</sub>R, it is widely believed that A<sub>2A</sub>R effects are mediated by the dopaminergic system, particularly dopamine signaling. As caffeine’s psychomotor effect is mediated mainly by the A<sub>2A</sub>R, caffeine’s psychomotor effect is also proposed to be at least partly mediated by D<sub>2</sub>Rs. Consistent with this view, psychomotor activity mediated by either A<sub>2A</sub>R antagonist or caffeine is markedly attenuated in D<sub>2</sub>R KO mice [20,31]. However, caffeine- and A<sub>2A</sub>R antagonist-induced psychomotor activity remains partially intact in D<sub>2</sub>R KO mice, albeit significantly attenuated [20,31]. Similarly, A<sub>2A</sub>R agonist-induced GABA release is still observed in the acute pharmacological blockade of the D<sub>2</sub>Rs [31] and A<sub>2A</sub>R activity modulates encephalin mRNA expression in the absence of the D<sub>2</sub>R [20]. These observations suggest that at least part of the A<sub>2A</sub>R-mediated effect, including caffeine’s psychomotor effect, is mediated by D<sub>2</sub>R-independent mechanisms. Thus, it is proposed that caffeine exerts its motor stimulant effect by both D<sub>2</sub>R-dependent (through its interaction with A<sub>2A</sub>R) and D<sub>2</sub>R-independent mechanisms [20,31–33].

A possible role for dopamine in mediating caffeine’s psychomotor effect is also suggested by some neurochemical studies showing that caffeine can induce dopamine release in the nuclear accumbens [34,35]. Consistent with this notion, caffeine’s motor stimulant effect and effect on feeding behavior are markedly reduced in mice deficient in dopamine, and these effects are restored by injection of dopamine [36]. Furthermore, the dopamine signaling molecule, DARPP-32 is
CAFFEINE, ADENOSINE A₁ AND A₂A RECEPTORS, SLEEP AND COGNITIVE BEHAVIORS

Caffeine, A₁, and A₂A receptors and sleep

Several lines of study support a key role for adenosine as an endogenous sleep factor [39,40]. Adenosine levels increase during prolonged wakefulness and decrease during sleep [41,42]. However, the source of the extracellular adenosine during the sleep-wake cycle is not clear. Pascual et al developed a transgenic mouse line that uses the tet-off system to allow conditional CNS knock-out of the A₁R [43]. Selective expression of dn-SNARE in astrocytes abolishes the sleep-suppression effect of the A₁R antagonist 8-cyclopentyltheophylline (CPT), but did not affect arousal effect induced by caffeine or the A₂A antagonist ZM241385 [44]. Thus, caffeine-induced wakefulness depends on A₂A, but probably not A₁. While additional studies are needed to clarify the modulatory role of the A₁R, this study raises an intriguing question: how does the A₂A R exert its effect on arousal state?

Global genetic inactivation of A₂AR abolishes caffeine’s effects on both psychomotor activity and arousal [48]. This, together with the predominant expression of the A₂AR in striatal neurons, raises the intriguing possibility that caffeine may affect arousal and motor activity via the same neuronal substrate. In support of this notion, our recent preliminary study found that caffeine (2–30 mg/kg) dose-dependently increased wakefulness, in parallel with increased motor activity, in wild-type mice and these caffeine-induced arousal and motor effects were abolished in global A₂AR KO mice as well as the mice with selective gene deletion of the A₂AR in striatal neurons [50]. Thus, both the arousal and motor-stimulation effects of caffeine are mediated by A₂ARs in striatal neurons, indicating that caffeine’s arousal effect may depend in part on its motor-stimulant activity.
Caffeine, adenosine receptors, and cognitive behaviors

Studies with genetic knockout mice indicate the $A_1R$ and $A_2AR$ are novel targets for modulating anxiety, depression, and memory. The $A_1R$ KO mice exhibited altered anxiety-related behaviors with an increased behavioral response in the classic light/dark box test and a reduced exploratory behavior in the open-field [25, 51]. In supporting the $A_{2A}R$ involvement of anxiety-like behavior, $A_2A$ KO mice displayed higher anxiety behavior scores than wild-type mice, and caffeine produced clear-cut anxiety-like effects in the plus-maze and light/dark box tests in wild-type mice but not in $A_2A$ KO mice. However, the anxiety-like effects of caffeine is not shared by the specific $A_2A$ antagonists ZM241385 and SCH58261 [52]. Similar to anxiety behavior, caffeine is also an effective anti-depressant strategy to confirm, at the level of striatal gene expression, the specificity of the effect of the $A_2A$ antagonist SCH58261 [60].

Using this strategy, we addressed the following questions about caffeine’s complex actions: (1) Is the $A_2A$ required to elicit and sufficient to account for changes in striatal gene expression induced by caffeine? (2) Does caffeine act at single or multiple molecular targets to elicit striatal gene expression? We found that caffeine (10 mg/kg) elicited a distinct profile of striatal gene expression in wild-type mice compared to the profile in untreated $A_2A$ KO mice or that of caffeine-treated $A_2A$ KO mice. Thus, $A_2A$ Rs are required but not sufficient to elicit the full striatal gene expression pattern induced by caffeine (10 mg/kg) [61]. This expression pattern of the large cohort of striatal genes by microarray profiling is consistent with the in situ hybridization finding that caffeine induces distinct striatal gene expression patterns in wild-type and $A_2A$ KO mice, resulting in a conversion of a biphasic dose response into a monophasic response [62]. Furthermore, 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 was elicited by caffeine in wild-type as well as $A_2A$ KO mice; and (3) the third subset was elicited by caffeine only in $A_2A$ KO mice. The identification of these distinct striatal gene populations and their corresponding molecular targets, including $A_2A$-dependent and independent, as well as their interactions following low and high doses of caf-

MULTIPLE MOLECULAR TARGETS OF CAFFEINE REVEALED BY A NOVEL DRUG TARGET VALIDATION WITH COMBINED MICROARRAY PROFILING AND GENETIC KNOCKOUT MODELS

It should be emphasized that while the adenosine receptor, particularly the $A_2A$R, is the main molecular target by which caffeine elicits its psychomotor stimulant effect, there is evidence that other targets could also play a role, particularly in the higher range of doses consumed by humans. To validate and identify multiple molecular targets of caffeine, particularly at the higher doses, we recently combined microarray profiling with genetic $A_2A$R KO models. This “drug target validation” approach was initially developed by Marton and coworkers studying effects of the immunosuppressants cyclosporine A or FK506 in yeast mutant strains deficient in calcineurin or immunophilins [59]. If the characteristic drug “signature” pattern of gene expression matches that in yeast cells with a particular mutation in a specific protein-encoding gene, a putative target is established. Furthermore, if the “signature” expression pattern of a drug in WT wild-type disappears when the drug is administered to a mutant strain, then it is concluded that the target missing in the mutant is required to generate the drug signature. Recently, we have successfully employed this drug target validation strategy to confirm, at the level of striatal gene expression, the specificity of the effect of the $A_2A$ antagonist SCH58261 [60].

It should be emphasized that while the adenosine receptor, particularly the $A_2A$R, is the main molecular target by which caffeine elicits its psychomotor stimulant effect, there is evidence that other targets could also play a role, particularly in the higher range of doses consumed by humans. To validate and identify multiple molecular targets of caffeine, particularly at the higher doses, we recently combined microarray profiling with genetic $A_2A$R KO models. This “drug target validation” approach was initially developed by Marton and coworkers studying effects of the immunosuppressants cyclosporine A or FK506 in yeast mutant strains deficient in calcineurin or immunophilins [59]. If the characteristic drug “signature” pattern of gene expression matches that in yeast cells with a particular mutation in a specific protein-encoding gene, a putative target is established. Furthermore, if the “signature” expression pattern of a drug in WT wild-type disappears when the drug is administered to a mutant strain, then it is concluded that the target missing in the mutant is required to generate the drug signature. Recently, we have successfully employed this drug target validation strategy to confirm, at the level of striatal gene expression, the specificity of the effect of the $A_2A$R antagonist SCH58261 [60].

Using this strategy, we addressed the following questions about caffeine’s complex actions: (1) Is the $A_2A$ required to elicit and sufficient to account for changes in striatal gene expression induced by caffeine? (2) Does caffeine act at single or multiple molecular targets to elicit striatal gene expression? We found that caffeine (10 mg/kg) elicited a distinct profile of striatal gene expression in wild-type mice compared to the profile in untreated $A_2A$ KO mice or that of caffeine-treated $A_2A$ KO mice. Thus, $A_2A$ Rs are required but not sufficient to elicit the full striatal gene expression pattern induced by caffeine (10 mg/kg) [61]. This expression pattern of the large cohort of striatal genes by microarray profiling is consistent with the in situ hybridization finding that caffeine induces distinct striatal gene expression patterns in wild-type and $A_2A$ KO mice, resulting in a conversion of a biphasic dose response into a monophasic response [62]. Furthermore, 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 was elicited by caffeine in wild-type as well as $A_2A$ KO mice; and (3) the third subset was elicited by caffeine only in $A_2A$ KO mice. The identification of these distinct striatal gene populations and their corresponding molecular targets, including $A_2A$-dependent and independent, as well as their interactions following low and high doses of caf-
in vivo as DARPP-32, that mediate caffeine’s effects also identified intracellular signaling molecules, such as cerebral cortex and hippocampus may provide molecular correlates of cognitive behaviors such as depression, anxiety and memory.

CONCLUSIONS

Recent studies using genetic mutant mice deficient in adenosine receptors and other signaling molecules have provided critical insight into the molecular targets by which caffeine elicits its biological effects. For example, studies with adenosine A1 and A2A receptor knockout mice have provided compelling evidence that, despite similar affinity of caffeine for these receptors, both the psychomotor stimulant and arousal effects of caffeine are mediated by the A2A receptor, while the A1 receptor plays a little, if any, role. Genetic mutant models also identified intracellular signaling molecules, such as DARPP-32, that mediate caffeine’s effects in vivo. Finally, coupling mutant mice with microarray profiling provides a novel strategy for validating drug targets. This approach has identified multiple molecular targets by which caffeine elicits striatal gene expression, implicating both A2A R-dependent and – independent mechanisms and their interaction, and opening new pathways of investigation. Future studies using genetic knockout mice with cell-type specific gene deletion will allow us to refine our understanding of caffeine’s action in the brain further by revealing the distinct cellular elements involved in psychomotor, arousal and cognitive effects of caffeine. Understanding how and where caffeine acts to stimulate psychomotor activity and arousal and associated cognitive functions may promote the development of novel pharmacological treatments for neuropsychiatric disorders including Alzheimer’s disease.

ACKNOWLEDGMENTS

The work from the authors was supported by NIH grants RO1NS48995, RO1NS41083, RO1DA19362, Department of Defense W81XWH-071-00012, and Bumpus Foundation, USA (J.F.C) and by the grants from Wenzhou Medical College Key Research Project (No. XNK05002), the Development Funding of Zhejiang Key Subject (Pharmacology and Biochemical Pharmaceutics), P.R. China.


REFERENCES


[38] Huang ZL, Wu WM, Eguchi N, Chen JF, Schwarzschild MA,


