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Review Article

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

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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_{2A} receptor, adenosine A_1 receptor, caffeine, arousal, DARPP-32, psychostimulation

INTRODUCTION

Caffeine, probably the most widely consumed psychoactive 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 μM) 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 $GABA_A$ receptor ($GABA_AR$) 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-

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bly 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 A_{2A} receptor ($A_{2A}R$) 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 A_1, A_{2A}, A_{2B} and A_3 receptors (A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R) [14], or in other signaling molecules associated with caffeine action, such as dopamine D_2 receptor (D_2R) [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 A_1R and $A_{2A}R$ 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 $A_{2A}R$ [1] [1–3,19,20]. Two genetic knockout (KO) mouse lines deficient in the $A_{2A}R$ 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 $A_{2A}R$ KO lines [19,20,22]. Thus, the $A_{2A}R$ is required for the psychomotor stimulant of caffeine. Furthermore, using recently developed brain region and cell-type specific $A_{2A}R$ KO mice, we demonstrated that it is the $A_{2A}Rs$ in forebrain neurons that are responsible for caffeine-induced psychomotor activity [23]. Thus, there is clear and strong evidence that the $A_{2A}R$ located on forebrain neurons is the molecular target by which caffeine exerts its psychomotor effect. In contrast, it appears that the A_1R is not critical to caffeine's psychomotor effect in the brain, despite the similar affinity of caffeine for the A_1R and $A_{2A}R$, since caffeine's psychomotor effect is largely intact in mice lacking the A_1R [24,25].

The generation of the A_1R-$A_{2A}R$ double homozygous KO and their heterozygous mutants provided an opportunity to study the interaction between A_1R and $A_{2A}R$ in mediating caffeine's effect. Regardless of the A_1R genotype, mice lacking the $A_{2A}R$ exhibited no psychomotor response to caffeine, confirming the critical requirement for the $A_{2A}R$ but not the A_1R in caffeine's motor stimulant effect [24,25]. However, A_1Rs may still contribute to caffeine's action through interactions with the $A_{2A}R$ since lack of the A_1R seems to slightly enhance the caffeine stimulant effect via $A_{2A}R$ 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 A_1R or $A_{2A}R$. Thus, it is likely that molecular targets other than A_1R or $A_{2A}R$ play a role in this inhibitory effect of caffeine at high doses. Lastly, it is postulated that double heterozygous (not homozygous) A_1-$A_{2A}R$ mutant mice had approximately one-half the number of A_1R and $A_{2A}R$ and may better mimic chronic effect of caffeine since, at the concentration attainable by regular human consumption, caffeine blocks both A_1R and $A_{2A}R$ by $\sim$30–50% [27]. Indeed, the ability of adenosine to activate receptors was shifted to the right in the dose-response study by caffeine or in double heterozygous A_1-$A_{2A}R$ mutant mice. Moreover, psychomotor activity by acute treatment with caffeine was reduced in double heterozygous A_1-$A_{2A}R$ mutant mice and in wild-type mice after chronic treatment with caffeine. Thus, the A_1-$A_{2A}R$ 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 A_1R expression in the brain. Thus, additional mechanism may contribute to the reduced response of acute caffeine challenge in A_1-$A_{2A}R$ double heterozygous mutants and in mice chronically treated with caffeine.

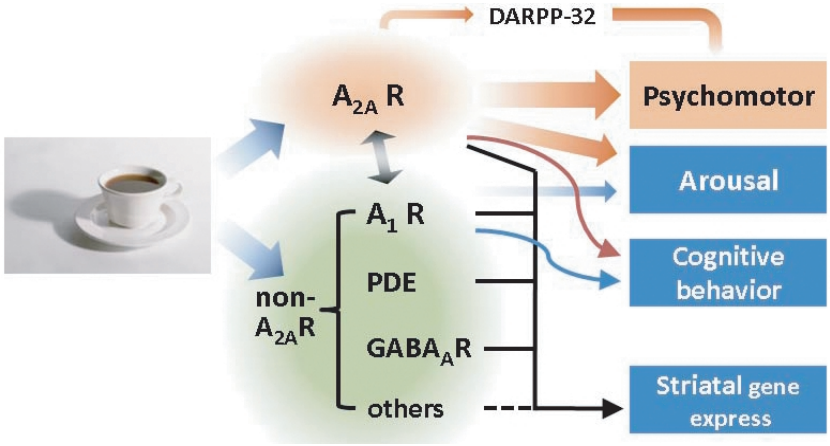

Fig. 1.

Interestingly, a recent study of prenatal exposure to caffeine using A_1R KO mice uncovered a possible role of epigenetic factors in prenatal caffeine's effect on psychomotor activity through the A_1R [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_1R 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_1Rs 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_1R, produces a long-lasting, transgenerational 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_3R, it was surprising to find that caffeine-induced psychomotor activity is reduced in A_3R KO mice [30]. Since A_3R KO similarly attenuated both caffeine- and amphetamine-induced psychomotor activity, the possible developmental effect of A_3R KO (such as compensatory changes in A_1Rs and $A_{2A}Rs$) 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 $A_{2A}R$ and D_2R, and the profound antagonistic interaction between $A_{2A}R$ and D_2R, it is widely believed that $A_{2A}R$ effects are mediated by the dopaminergic system, particularly dopamine signaling. As caffeine's psychomotor effect is mediated mainly by the $A_{2A}R$, caffeine's psychomotor effect is also proposed to be at least partly mediated by D_2Rs. Consistent with this view, psychomotor activity mediated by either $A_{2A}R$ antagonist or caffeine is markedly attenuated in D_2R KO mice [20,31]. However, caffeine- and $A_{2A}R$ antagonist-induced psychomotor activity remains partially intact in D_2R KO mice, albeit significantly attenuated [20,31]. Similarly, $A_{2A}R$ agonist-induced GABA release is still observed in the acute pharmacological blockade of the D_2Rs [31] and $A_{2A}R$ activity modulates enkephalin mRNA expression in the absence of the D_2R [20]. These observations suggest that at least part of the $A_{2A}R$-mediated effect, including caffeine's psychomotor effect, is mediated by D_2R-independent mechanisms. Thus, it is proposed that caffeine exerts its motor stimulant effect by both D_2R-dependent (through its interaction with $A_{2A}R$) and D_2R-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

demonstrated as an intracellular mediator of caffeine's psychomotor action. In DARPP-32 KO mice, the stimulatory effect of caffeine on motor activity is greatly reduced. Caffeine increased phosphorylation of DARPP-32 at Thr 75 in mouse striata through inhibition of protein phosphatase-2A (PP-2A) activity, further supporting a role for DARPP-32 in mediating the psychomotor action of caffeine [37].

A recent genetic knockout study also implicates a role of the transcription factor nuclear factor kappa B (NF-κB) in caffeine's effect on psychomotor activity. After caffeine ingestion, mice lacking the p50 subunit of NF-κB (p50 KO mice) display greater locomotor activity without change in caffeine metabolism, indicating that NF-κB may also be involved in modulation of caffeine's psychomotor stimulation [38].

CAFFEINE, ADENOSINE A_1 AND A_{2A} RECEPTORS, SLEEP AND COGNITIVE BEHAVIORS

Caffeine, A_1, and A_{2A} 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 expression of the cytosolic portion of the soluble N-ethylmaleimide sensitive fusion attachment protein receptor (SNARE)-domain of synaptobrevin 2 [dominant-negative SNARE (dn-SNARE)] selectively in astrocytes [43]. Selective expression of dn-SNARE in astrocytes was achieved by using an astrocyte-specific glial fibrillary acid protein (GFAP) promoter. In this transgenic line, inhibiting gliotransmission and reducing extracellular adenosine levels attenuated the accumulation of sleep pressure, and prevented cognitive deficits associated with sleep loss [44]. The development of these transgenic mice with selective inhibition of gliotransmission provides direct evidence that adenosine released from astrocytes acts at the A_1R to modulate the accumulation of sleep pressure and its cognitive consequences.

Pharmacological studies have suggested that both the A_1R and A_{2A}R contribute to adenosine-mediated modulation of the sleep-wake cycle [45,46]. A_1R KO and A_{2A}R KO mice have been used to validate the critical involvement of these receptors in mediating caffeine-induced arousal effects. In a recent study, mice with conditional CNS knock-out of the A_1R displayed selective attenuation of rebound of slow wave activity (SWA), a widespread, synchronized neuronal activity that varies directly with previous waking duration [47]. Moreover, this attenuation has functional consequence, interfering with normal working memory function [44]. These results suggest that extracellular adenosine acting at A_1Rs is required for normal rebound SWA and consequent working memory function. These findings are complementary to a large body of pharmacological work implicating endogenous adenosine acting at the A_1R in basal forebrain in the sleep-wake cycle [39].

On the other hand, using mice deficient in the A_{2A}R, caffeine-induced arousal effects were largely attributed to blockade of the A_{2A}R in the brain [48]. Huang et al found that caffeine (at 5, 10 and 15 mg/kg) dose-dependently increased wakefulness in wild-type mice, but this effect was completely abolished in A_{2A}R KO mice. The contribution of the A_{2A}R to caffeine's arousal effect is further supported by a recent study showing that a genetic variant of the A_{2A}R is associated with individual sensitivity to caffeine's effect on sleep in humans [49]. Surprisingly, caffeine-induced arousal remains intact in A_1R KO mice. Consistent with this notion, inhibition of gliotransmission by transgenic expression of dn-SNARE in astrocytes abolished the sleep-suppression effect of the A_1R antagonist 8-cyclopentyltheophylline (CPT), but did not affect arousal effect induced by caffeine or the A_{2A}R antagonist ZM241385 [44]. Thus, caffeine-induced wakefulness depends on A_{2A}R, but probably not A_1R. While additional studies are needed to clarify the modulatory role of the A_1R, this study raises an intriguing question: how does the A_{2A}R exert its effect on arousal state?

Global genetic inactivation of A_{2A}Rs abolishes caffeine's effects on both psychomotor activity and arousal [48]. This, together with the predominant expression of the A_{2A}R 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 A_{2A}R KO mice as well as the mice with selective gene deletion of the A_{2A}R in striatal neurons [50]. Thus, both the arousal and motor-stimulation effects of caffeine are mediated by A_{2A}Rs 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 $A_{2A}R$ 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, $A_{2A}R$ 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 $A_{2A}R$ KO mice. However, the anxiety-like effects of caffeine is not shared by the specific $A_{2A}R$ antagonists ZM241385 and SCH58261 [52]. Similar to anxiety behavior, caffeine is also an effective anti-depressant in forced swimming and tail suspension tests, an effect that is mimicked by $A_{2A}R$ KO mice but not by specific $A_{2A}R$ antagonists [53]. Thus, the exact role of the $A_{2A}R$ in mediating caffeine's effect on anxiety and anti-depression remain to be clarified.

Recent studies using genetic $A_{2A}R$ KO mice also support a role of the $A_{2A}R$ in modulation of learning and memory. Consistent with pharmacological studies [54], transgenic over-expression of the $A_{2A}R$ in forebrain impair working memory in Morris water maze test, without affecting reference memory [55]. In keeping with this notion, we recently showed that $A_{2A}R$ KO mice displayed selective enhancement in working memory in 8-arm radial maze and Morris water maze tests [56]. On the other hand, in contrast to the early pharmacological studies, A_1R KO mice showed an intact spatial memory function [51,57]. There is also sparse evidence for the A_1R and $A_{2A}R$ in spatial reference memory and social recognition memory [58]. Given the known cognitive enhancement of caffeine, it would be interesting to determine whether the learning and memory property of caffeine is mediated by the A_1R or $A_{2A}R$ using the genetic knockout models.

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 $A_{2A}R$ antagonist SCH58261 [60].

Using this strategy, we addressed the following questions about caffeine's complex actions: (1) Is the $A_{2A}R$ *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 $A_{2A}R$ KO mice or that of caffeine-treated $A_{2A}R$ 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 $A_{2A}R$ 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 $A_{2A}R$ KO mice; and (3) the third subset was elicited by caffeine only in $A_{2A}R$ KO mice. The identification of these distinct striatal gene populations and their corresponding molecular targets, including $A_{2A}R$-dependent and independent, as well as their interactions following low and high doses of caf-

feine, provide molecular insights into the acute pharmacological effects of caffeine in the brain. Analysis of caffeine-induced gene expression in other brain regions (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 A_1 and A_{2A} 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 A_{2A} receptor, while the A_1 receptor plays 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 A_{2A}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.

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