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ACTIONS OF ADENOSINE AT ITS RECEPTORS IN THE CNS: Insights from Knockouts and Drugs

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■ **Abstract** Adenosine and its receptors have been the topic of many recent reviews (1–26). These reviews provide a good summary of much of the relevant literature—including the older literature. We have, therefore, chosen to focus the present review on the insights gained from recent studies on genetically modified mice, particularly with respect to the function of adenosine receptors and their potential as therapeutic targets. The information gained from studies of drug effects is discussed in this context, and discrepancies between genetic and pharmacological results are highlighted.

GENETICALLY MODIFIED MICE

Adenosine Receptor Knockouts

Mouse strains lacking the genes for three of the four adenosine receptors have been generated (Table 1). Two groups have generated adenosine A_1 receptor knockouts (A_1R KO) (27, 28). A_1R KO mice develop normally. They are fertile, but appear to have a smaller number of offspring per litter, perhaps because sperm capacitation is compromised in these animals (29). Their body temperature is normal, but, as expected, the hypothermia elicited by A_1R agonists is absent in A_1R KO mice. Interestingly, A_1R KO mice had reduced survival rates as compared to A_1R wild-type (WT) mice (30), although the maximal life span was unaffected. The increased mortality in midlife may be linked to disturbances in cardiovascular, hepatic, and renal systems, where A_1Rs are likely to play an important role in the normal physiology.

TABLE 1 Target disruption of adenosine receptors and adenosine kinase and deaminase in mice

Target	Disrupted portion of the gene	Parent strains	Congenic	Lethality	References
A_1 receptor	Major portion of coding exon 2 + ~5 kb adjacent 3′ genomic seq	129/OlaHsd × C57BL/6	No	No	(27)
	3′ portion of coding exon 1 + intron 5′ portion of coding exon 2	129/SvJ × C57BL/6	No	No	(28)
A_{2A} receptor	Entire coding exon 2	129/Sv × CD1	CD1, N12	No	(31)
	3′ portion of coding exon 2 ~1.0 kb immediate intron seq	129/Sv × C57BL/6	129/Sv, N = 1 C57BL/6 N = 6	No No	(32, 104)
A_3 receptor	Entire coding exon 1 + 7.5 kb immediate intron seq	129 × C57BL/6 129 × B6D2	No No	No No	(34)
Adenosine kinase	In frame insertion at exon amino acid Gly169-Thr225	129/JEms × C57BL/6	No	Die at P4	(39)
Adenosine deaminase	In frame insertion at exon 5	129/Sv × C57BL/6	No	Perinatal death, die at 3 weeks after trophoblast rescue	(42)

Two groups of scientists have generated adenosine A_{2A} receptor knockout (A_{2A}R KO) mice. In the line generated by Ledent et al. (31), the mice were on a CD1 outbred background, whereas in the two lines generated by Chen et al. (32) the mice were bred onto C57BL/6 and Sv-129 backgrounds. All three lines of A_{2A}R KO mice were viable and bred normally. Blood pressure and heart rate were increased, as well as platelet aggregation, in mice on a CD1 background (31), but blood pressure and heart rate were not affected in mice with a C57BL/6 or Sv-129 background (32, 33).

Adenosine A_3 receptors have been implicated in a variety of peripheral organ system functions, including the regulation of cellular components of the immune system (34) and cardiovascular function (35). The A_3R KO mouse also had significantly lower intraocular pressure, suggesting that these receptors might be a target for the development of drugs against glaucoma (36). However, an understanding of the functions of A_3 receptors in the central nervous system (CNS) has been impeded both by a lack of specific ligands and the low density of these receptors (37).

All the KO mice mentioned so far lack one adenosine receptor subtype from a very early developmental stage. Recently, a mouse with LoxP elements flanking the second coding exon of the A_1 gene was reported, as well as the combination with an adenovirus expressing Cre recombinase. This opens the interesting possibility of time- and tissue-specific inactivation of the adenosine A_1R (38).

Metabolic Pathways

In addition to mice lacking specific adenosine receptor subtypes, there are mouse strains in which the metabolic pathways controlling the levels of adenosine have been genetically modified (Table 1). It is well known that adenosine levels are regulated by adenosine kinase (phosphorylates adenosine to AMP) and adenosine deaminase (converts adenosine to inosine).

A mouse strain with a targeted disruption of the adenosine kinase gene was recently reported (39). These mice developed normally until birth, but they died soon after birth. Low levels of adenine nucleotides and high levels of S-adenosyl homocysteine are signature features of this genetic manipulation (39). It has been shown in studies on yeast KOs that adenosine kinase plays an important role in methyl transfer reactions (40). The fatal outcome in adenosine kinase KO mice may be due, in particular, to the high levels of S-adenosyl homocysteine and the consequent depression of several transmethylation reactions. For this reason, we have to await the generation of region- and time-dependent KOs for adenosine kinase before we can get clear information about its roles in the CNS. It is also worth noting that adenosine may play a role in the association between cardiovascular morbidity and hyperhomocysteinemia (41).

An adenosine deaminase KO mouse has been generated and provides a model for increased adenosine levels (23, 42). Lack of adenosine deaminase is classically associated with immune deficiency, but this is probably due to an accumulation of 2-deoxy-adenosine and subsequent accumulation of dATP, and not to adenosine accumulation (43). Indeed, blockade of adenosine kinase in adenosine deaminase

KO mice, which is expected to massively increase adenosine accumulation, decreased thymocyte death in parallel with decreased dATP accumulation (44).

Deamination of adenosine to inosine largely, but not completely eliminates the actions of adenosine on adenosine receptors: The A_1 and particularly the A_3R can also respond to inosine, although inosine is not a full agonist (45–47). Indeed, studies on KO mice have shown that the A_3 receptor can mediate some of the effects of inosine in the immune system, but for other effects of administered inosine only mice lacking both the A_{2A} and the A_3 receptor were unresponsive (48), suggesting that A_{2A} receptors are also involved in mediating the effects of inosine. This does not necessarily mean that inosine acts on A_{2A} receptors, however. It could mean that inosine increases levels of adenosine, which in turn acts on A_{2A} receptors. In addition, inosine may influence energy levels and polyADP-ribosylation (49).

Use of Heterozygotes

Attention is most often paid to the phenotype of the homozygous KO. However, detailed examination of heterozygotes (HZ) can also be very revealing.

a) How well adjusted is the receptor level? If receptor number is directly proportional to gene dosage—as is the case in A_1R and A_{2A}R HZ—this argues against strong autoregulation of transcription. Therefore, it seems likely that neither A_1 nor A_{2A}R levels are regulated to a major extent by the ongoing signaling via these receptors.

b) Heterozygotes often provide a better model for the effects likely to be seen with antagonists because it is only rarely the case that antagonists can be given at a dose that will inhibit all the receptors all the time. An especially relevant aspect is that caffeine in doses commonly consumed by humans gives plasma concentrations very close to the K_D for caffeine at human A_1 and A_{2A}Rs (3). Because responses to adenosine are shifted to the right, and because there are only half the normal number of receptors in heterozygous mice, it seems possible that heterozygous mice can be used as a genetic model for caffeine use.

c) Heterozygous mice have also been used to circumvent the problems associated with the developmental effects that can potentially confound studies on homozygous KO mice (50). In this approach, pharmacological agents are given to heterozygous mice at doses that are subthreshold in WT mice. There is no biological effect in WT mice treated with a subthreshold dose of the drug or in HZ mice treated with vehicle, but a subthreshold dose elicits a biological effect when combined with heterozygous genetic inactivation of the target molecule (51). This approach may be particularly useful in examining some adenosine receptor functions where discrepancies between the pharmacological and genetic approaches have been reported (such as the psychostimulant effect of A_{2A}R KO and A_{2A}R antagonists; see below under the section on striatum and dopamine receptors).

d) Heterozygotes can also be used to examine aspects of coupling, e.g., the so-called receptor reserve in different tissues (52).

Limitations and Alternatives to Genetic Knockout Approaches

As powerful as it may be, the genetic KO approach also has its intrinsic limitations that may confound the correct interpretation of the phenotypic analysis of KO mice. The two major limitations are the confounding developmental effect and the lack of tissue specificity. The genes for adenosine receptors or enzymes affecting adenosine level are depleted from early development throughout life in KO mice, resulting in a phenotype that represents a developmental effect, rather than an immediate consequence of receptor inactivation. Reproduction of KO phenotypes by adenosine receptor antagonists—given acutely or long-term—would rule out developmental confounding effects. If developmental confounding effects are strongly suspected, the development of inducible knockouts (53–55) may be worth the effort. However, differences between acute effects of a purportedly selective drug and phenotype in a KO could have many other causes than developmental effects. One potentially useful method to test specificity of drugs, and the consequences of incomplete blockade, is to administer subthreshold doses of pharmacological antagonists to heterozygotes (as described above). To address the issue of lack of tissue specificity, a LoxP strategy has been used to create a brain-specific depletion of A_1Rs (38), and a similar approach can be applied to other adenosine receptor knockouts. Finally, many studies have demonstrated the effects of genetic backgrounds on differential phenotypic expression (56–58). An example could be the differences in blood pressure and heart rate of A_{2A}R KO mice in CD1 versus C57BL/6 or Sv-129 backgrounds, but methodological differences might also explain the different results. Another interesting example is the finding that the A_1R KO mouse possesses the Ren-2 renin gene derived from the 129 strain, whereas WTs do not (59). This is related to the fact that the Ren-2 gene is positioned relatively close to the A_1R gene (some 850 kb apart) on chromosome 1. Thus it is imperative to employ appropriate breeding strategies to control for potentially confounding genetic backgrounds and flanking genes (60).

PRE- AND POSTSYNAPTIC EFFECTS

As a neuromodulator, adenosine affects synaptic transmission in a number of brain regions (see Reference 6). Thus far, studies manipulating adenosine receptors have focused primarily on characterizing responses in brain regions where that receptor subtype is known to be important; subtle alterations or compensations in these or other brain regions may yet be revealed.

The A_1R subtype tonically inhibits synaptic transmission both pre- and postsynaptically in brain regions with a high concentration of A_1Rs, such as the hippocampus (see Reference 6). Heterozygote mouse brain contains half of the number of receptors, and the EC_{50} for adenosine in the hippocampus is exactly

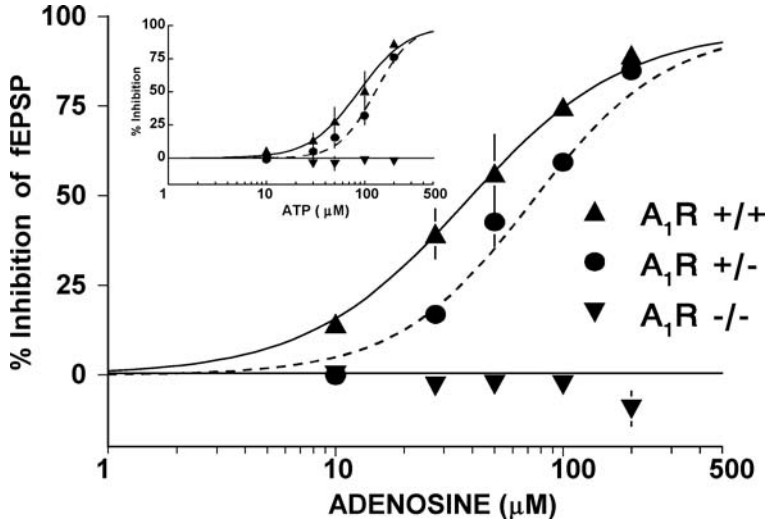

Figure 1 Critical role of A_1R in mediating inhibition of excitatory neurotransmission by adenosine and ATP (insert) in hippocampus. Note that neither adenosine nor ATP had any clear effect in A_1R KO mice, and that the dose-response curve is shifted to the left in the heterozygous mice, with no change in the maximal effect. Redrawn from data in (27, 62).

twice that of the WT, whereas E_{MAX} is unaffected (see Figure 1). Despite the loss of tonic inhibition, no compensatory responses were found in other receptor subtypes mediating similar G protein–coupled presynaptic inhibition of synaptic transmission (27), but a wide range of potential compensatory mechanisms remain to be explored.

Slices from homozygous A_1R KO show no evidence of any remaining endogenous inhibitory influence of adenosine in the Schaffer collateral pathway in the CA1 region of the hippocampus or at the mossy fiber synapses in the CA3 region (61). Furthermore, there is no inhibition of synaptic transmission when large concentrations of adenosine (100 μM) are applied exogenously (27). Using the *Cre-loxP* system and an adeno-associated viral vector, a targeted deletion of the A_1R was induced separately in the CA1 and in the CA3 region of the hippocampus (38). This approach holds promise for dissecting out specific pre- and postsynaptic actions of adenosine and its synaptic interactions with other molecules and neurotransmitters, but so far no major results have been reported. Similar to the constitutive KO model, there was no response to adenosine in the targeted inducible knockout. Application of adenine nucleotides such as ATP was also ineffective in hippocampal slices from KO mice (62) (see Figure 1). Together, these data suggest that the inhibitory effects of both adenosine and adenine nucleotides in the hippocampus are mediated ultimately through the adenosine A_1 receptor (27, 38),

or alternatively, that effects of the other receptors require the presence of A_1Rs, as suggested for A_{2A}Rs (63). It will be important to determine the potency of adenosine in regulating neurotransmission via A_1R in mice lacking the other receptors to determine if there is such an interaction.

Dunwiddie and coworkers, using pharmacological tools (64), reported some role of A_3Rs in modulating the responses to A_1 stimulation. This question was re-examined recently and no significant interactions between A_1 and A_3Rs were discovered using a host of different methods, including binding studies and electrophysiological studies (65). Thus, if A_3Rs do play a role it is likely to be small and indirect.

ISCHEMIA

It is generally believed that adenosine can protect tissues against the negative consequences of hypoxia or ischemia (1, 66), and that A_1Rs play a particularly important role. Hence, survival after a hypoxic challenge may be reduced if A_1Rs are absent or blocked (27). One consequence is that use of caffeine or other methylxanthines in doses that would completely block A_1Rs may be hazardous in hypoxic human newborns. In keeping with the proposed role for adenosine acting at A_1Rs, hippocampal slices taken acutely from adult mice do show greater functional recovery from both hypoxic and ischemic insults when A_1Rs are intact (27, 67). Moreover, acute administration of an A_1R antagonist did enhance ischemic damage in vivo, giving further evidence that compensatory mechanisms may be providing protection in the knockout (68).

However, the severity of ischemic damage either in vivo or in organotypic hippocampal slice cultures is not increased in the A_1R KO model (68). The lack of any obvious difference between the WT and the KO after pathophysiological insult where A_1Rs are considered neuroprotective is surprising. In immature brain, blockade of A_1Rs in fact attenuated ischemic injury. For example, the loss of white matter that is a typical consequence of hypoxia in the newborn actually appears to be mediated by adenosine acting on A_1Rs (69). Thus, blockade of adenosine receptors—even incomplete blockade like that achieved by caffeine—reduces such white matter loss (69). In addition, the consequences of prenatal hypoxic ischemia in rats are reduced if the dams have been given caffeine (70).

Brain damage after focal ischemia has been reported to be attenuated in adult A_{2A}R KO mice compared with WT mice (32). On the other hand, aggravated brain damage is observed after hypoxic ischemia in immature seven-day-old A_{2A}R KO mice (71). These results suggest that, in contrast to the situation in adult animals, A_{2A}Rs play an important protective role against hypoxic ischemic brain injury in neonates. Interestingly, a recent study using a novel approach where A_{2A}R KO is combined with bone marrow transplantation demonstrated that selective reconstitution of the A_{2A}R in bone marrow–derived cells of A_{2A}R KO mice abolished the neuroprotection against ischemic brain injury afforded by global depletion of A_{2A}R

(72). Conversely, selective $A_{2A}R$ inactivation by transplantation of bone marrow cells from $A_{2A}R$ KO mice into WT mice reduced the volume of MCAO-induced infarct in brain. This neuroprotection did not relate to the number of infiltrating neutrophils and macrophages, but was associated with reduced MCAO-induced expression of IL-6, IL-1, and IL-12 in the ischemic brain after gene inactivation. These findings reveal a critical role for $A_{2A}Rs$ on bone marrow–derived cells following transient focal ischemia and suggest that targeting peripheral $A_{2A}Rs$ in bone marrow–derived cells may be therapeutic against ischemic brain injury.

The role of A_3Rs is enigmatic. Part of the reason for this is that the drugs used to test their importance are not very selective, especially on rodent receptors (7). Indeed, some of the purportedly selective antagonists have effects in A_3R KO mice (36). A_3 receptors are, however, clearly implicated in ischemia in the heart, where the knockout shows significantly improved tolerance (21, 35, 73; J. Yang, H. Sommerschild, G. Valen & B.B. Fredholm, unpublished data). In particular, recovery after myocardial ischemia was improved (74). By contrast, in a model of carbon monoxide–induced hypoxia, the hippocampal neuronal damage was increased in A_3R KO mice (75). The histological changes, along with possible cognitive consequences, were also observed after administration of the A_3R antagonist MRS 1523 [5-propyl-2-ethyl-4-propyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate 1 mg/kg i.p.]. These results, and the observation that deletion of the A_3R had a detrimental effect in a model of mild hypoxia, suggest the possible use of A_3R agonists in the treatment of ischemic, degenerative conditions of the CNS (75).

The effects observed in these models and in in vivo models for other diseases are summarized in Tables 2 and 3.

CAFFEINE

One reason why studies of adenosine and its receptors attract interest is that adenosine receptors (A_1, A_{2A}, and A_{2B}) are the targets for the most widely used of all psychoactive drugs, caffeine. Studies on KO animals have provided compelling evidence that the psychostimulant effects of caffeine require blockade of $A_{2A}Rs$. Caffeine has a mild stimulant effect in $A_{2A}R$ WT mice, but becomes a depressant of locomotor activity in $A_{2A}R$ KO mice (31). Thus, $A_{2A}Rs$ appear to be required for the stimulant effect of caffeine (see Figure 2). In fact, caffeine dependently decreases locomotion in $A_{2A}R$ KO mice over a wide range of doses (76). This effect probably results from the other biological effects of caffeine, the blockade of A_1Rs being a candidate. Examining immediate early gene expression in WT and $A_{2A}R$ KO mice, the Schiffmann group also concluded that A_1R blockade was important for some of the high-dose effects of caffeine (77). However, the role of A_1R in the effects of caffeine on motor activity is less clear. Recently, Halldner et al. (78) showed that the A_1R is not crucial for the stimulatory effect of caffeine, although the effect is facilitated in the A_1R KO mice. The results also suggest that the inhibitory effects

TABLE 2 Adenosine receptor knockouts show a variety of behavioral changes that differ according to receptor subtype. The different phenotypes may help to define brain functions of adenosine and to discover novel targets for drugs against neurologic and psychiatric disorders

Function	Receptor knockout	Modifications
Aggressiveness	A_1	Increased
	A_{2A}	Increased
	A_3	Not determined
Anxiety	A_1	Increased/no change
	A_{2A}	Increased
	A_3	No change
Despair-like	A_1	Not determined
	A_{2A}	Decreased
	A_3	Increased
Memory	A_1	No change
	A_{2A}	Not determined
	A_3	Not determined
Motor activity	A_1	No change/decreased
	A_{2A}	Decreased/slightly increased
	A_3	Slightly increased
Neuroprotection	A_1	No effect in adults, beneficial in newborns
	A_{2A}	Beneficial in adults, detrimental in newborns
	A_3	Detrimental effect
Sensorimotor gating	A_1	Not determined
	A_{2A}	Reduced startle inhibition and prepulse inhibition
	A_3	Not determined
Thermal nociception	A_1	Hyperalgesia
	A_{2A}	Hypoalgesia
	A_3	Hyperalgesia/no change

of higher doses of caffeine are not due to blockade of the A_1R. Rather, this effect is likely to be independent of adenosine receptors. Clearly, many more studies of the actions of caffeine in single and double KOs are necessary to delineate which effects are entirely due to adenosine receptor blockade and which are not.

SLEEP

One of the best-known effects of caffeine is its effect on sleep (3). There is also considerable evidence that adenosine is an endogenous promoter of sleep (for references see 79). Adenosine levels, particularly in the basal forebrain, increase

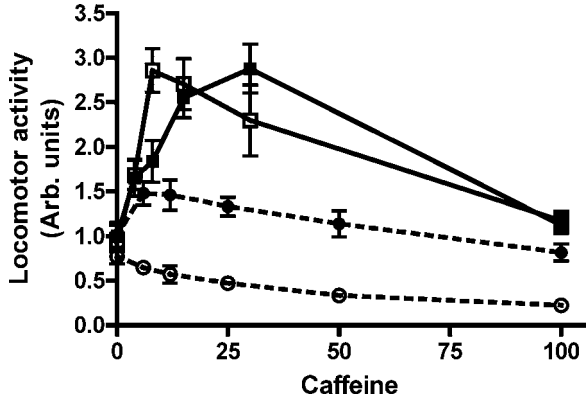

Figure 2 Biphasic effects of caffeine on locomotor behavior in mice. Data redrawn from (78) and (76). The studies using A_1R mice (*squares*) used mice on a mixed C57BL/6 × 129OlaHsd background; those on A_{2A} mice (*circles*) used CD1 mice. Furthermore, the exact experimental setup differs. Hence, the two sets of data are not strictly comparable. Filled symbol, WT mice; open symbols, KO mice.

during wakefulness and become particularly high during prolonged wakefulness (80). Part of the reason for these changes in adenosine levels could be changes in adenosine kinase and 5′-nucleotidase activities in the basal forebrain (81), but it remains unclear if the basal forebrain differs from other brain regions and if there are any changes upon sleep deprivation (82).

Most of the pharmacological data implicate A_1R in the regulation of sleep (79). Thus, A_1R agonists induce sleep and sleep-like EEG (83, 84), whereas antagonists reduce sleep (85). There are several mechanisms by which A_1R stimulation may induce sleep. First, there is evidence that A_1Rs are present on the cell bodies of long cholinergic neurons and reduce their firing rate tonically (86), presumably by increasing potassium conductances. In hypothalamic slices, adenosine disinhibits the GABAergic input to ventrolateral preoptic neurons (87). One possible additional substrate are the orexin-containing neurons, which express A_1R (88).

Further support for an important role of A_1R was given by studies showing that an A_1R antisense construct infused into the basal forebrain could decrease the amount of REM sleep and increase wakefulness (89). However, the A_1R KO mouse did not show any difference from controls in the amount of sleep or in rebound after sleep deprivation, even though an A_1R antagonist produced the expected effect in the control animals (90). Thus, there is evidence that the A_1R is important in sleep regulation in normal animals, but also that it is not absolutely necessary for sleep regulation. Thus, when A_1Rs are eliminated (and presumably when they are profoundly antagonized for long periods of time) other regulatory mechanisms take over. The nature of these adaptive changes is not known, and it remains to be

shown if some level of adaptation also occurs after long-term, high-dose exposure to caffeine.

Despite the fact that most attention has been focused on the role of A_1 receptors, there is increasing evidence that A_{2A} receptors also play a role. For example, in fetal sheep there is evidence for a tonic role of A_{2A}Rs in regulating REM sleep state (91), and administration of A_{2A}R agonists into the subarachnoid space close to the preoptic area increases sleep (92). The possibility exists that the abundant A_{2A}Rs present in the nucleus accumbens (92) or tuberculum olfactorium (93) play a role. Thus, there are changes in A_{2A}Rs and the corresponding mRNA in tuberculum olfactorium following sleep deprivation (93). Furthermore, the sleep-inducing effect of the A_{2A}R agonist CGS 21680 was eliminated in A_{2A}R KO mice (94). It will be of considerable interest to examine sleep in mice that lack both A_1 and A_{2A}Rs and to determine whether caffeine has any effect in such mice.

STRIATUM AND INTERACTIONS WITH DOPAMINE

Although A_{2A}Rs are commonly believed to couple to G_s proteins, it is now established that in striatum, A_{2A}Rs (as well as dopamine D_1 receptors) signal via G_{olf} proteins instead (95, 96). Indeed, full activity of A_{2A} and D_1 ligands requires the presence of the normal number of G_{olf} molecules, as shown by the reduced response in mice heterozygous for G_{olf} deletion (96). In agreement with this, the same authors also found that the disruption of A_{2A} or D_1 receptors led to an altered expression of G_{olf}.

There is excellent evidence that A_{2A}Rs and dopamine D_2 receptors are coexpressed on striatopallidal neurons and that they are functionally antagonistic (97, 98). Thus, blockade of D_2 receptors would be expected to increase activity mediated by A_{2A}Rs, and vice versa. One might therefore expect adaptive changes in KOs. Indeed, in dopamine D_2 receptor KO mice there is a functional decrease in A_{2A}R signaling (99), and A_{2A}R KO mice are somewhat hypodopaminergic (100). This might explain why A_{2A}R deficiency selectively attenuates amphetamine-induced and cocaine-induced locomotor responses (101), even though A_{2A} receptor agonists can also attenuate psychostimulant responses (101, 102).

Even though A_{2A} and D_2 receptors are colocalized, interact at the membrane level, and may form functional heterodimers, studies using A_{2A} and D_2 KO mice show that endogenous adenosine can act independently of D_2 receptors on A_{2A}Rs and exerts a tonic influence. This tonic A_{2A} stimulation is opposed by dopamine acting at D_2 receptors (98, 103–105).

Locomotion

Basal locomotion is marginally affected in A_1R KO mice (30, 78). Thus, no differences in overall spontaneous motor activity were detected over a 23-h monitoring period, but activity was reduced in some parts of the light-dark cycle.

A_{2A}Rs are highly expressed in the dorsal and ventral striatum, where they could be involved in the physiological control of motor activity, and a major role of A_{2A}R stimulation is to modulate locomotor activity. In most studies performed so far (31, 32, 101, 106), the exploratory behavior of A_{2A}R KO mice was reduced as compared to A_{2A}R WT mice. As expected, treatment with the A_{2A} agonist CGS 21680 strongly reduced locomotor activity in A_{2A}R WT mice and had no significant effect on A_{2A}R KO mice (31, 32). However, this reduction of locomotor activity is not an invariable characteristic of A_{2A}R KO mice because as Ådén et al. (71) observed there is a small increase in basal locomotor activity at four weeks of age in A_{2A}R KO mice compared with A_{2A}R WT mice. Locomotor behavior was reported to be increased in A_3R KO mice (75).

Parkinson's Disease

Among new therapeutic approaches for Parkinson's disease, one possibility being investigated is modulation of dopamine-mediated striatal functions through the blockade of A_{2A}Rs. The past ten years have witnessed significant progress in the development and characterization of a new generation of A_{2A}R antagonists for use in Parkinson's disease. The motor enhancement afforded by A_{2A} antagonists was well documented in early pharmacological studies (19), and activity at A_{2A}Rs reduces motor responses in both normal and dopamine-depleted animals. The multiple benefits of A_{2A}R inactivation (seen either after genetic deletion, as in A_{2A}R KO mice, or after treatment with A_{2A} antagonists) advance the prospects of A_{2A}R antagonists as a novel treatment strategy for Parkinson's disease (18, 107) (see Table 3).

Proof of principle comes from large epidemiological studies that firmly establish an inverse relationship between caffeine consumption and the risk of developing Parkinson's disease (108–110). Studies carried out in mice support these epidemiological findings, providing evidence for neuroprotective effects of caffeine and specific A_{2A}R antagonists, as well as genetic deletion of the A_{2A}R (111). Several other pharmacological studies employing various A_{2A}R antagonists also support this neuroprotective effect (112, 113).

A_{2A}R antagonism also provides symptomatic relief of surgical lesion or drug-induced motor dysfunction. Catalepsy, a state where the animals remain immobile for long periods, even if they are placed in awkward postures, can be induced by dopamine D_1 or D_2 receptor antagonists or a muscarinic acetylcholine receptor agonist. In A_{2A}R KO mice, such catalepsy was reduced as compared with A_{2A}R WT mice (104, 114). These results suggest that A_{2A}Rs influence not only dopamine D_1 and D_2 receptor–mediated neurotransmission but also that mediated via muscarinic acetylcholine receptors. Interestingly, caffeine and muscarinic antagonists act in synergy to inhibit haloperidol-induced catalepsy (115). The results on catalepsy show that deletion of the A_{2A} receptor alleviates dysfunction of basal ganglia motor circuitry caused by drugs acting at dopamine and acetylcholine receptors. These preclinical studies led to the clinical trial of the A_{2A}R antagonist KW6002 in patients with Parkinson's disease, and the initial results were encouraging (116, 117).

TABLE 3 Therapeutic implications for neurological disorders as suggested by genetic knockout and pharmacological analysis

Disorder	Animal models	Effect	References
Parkinson's disease	MPTP	Reduced neurotoxicity in $A_{2A}R$ KO mice	(111)
	MPTP	Reduced neurotoxicity by $A_{2A}R$ antagonists	(111, 153)
	6-OHDA	Reduced SN neuron loss by $A_{2A}R$ antagonists	(112)
	Haloperidol-catalepsy	Enhanced locomotor activity by $A_{2A}R$ KO	(111, 114)
	D_2R KO-induced hypolocomotion		
	MPTP-bradykinesia in monkey	Reversed by $A_{2A}R$ antagonists	(154, 155)
Huntington's disease	3-NP	Reduced striatal damage in $A_{2A}R$ KO	(111)
	3-NP	Reduced striatal damage by $A_{2A}R$ antagonists	(111)
	3-NP	Enhanced/reduced striatal damage in $A_{2A}R$ KO	(120)
	3-NP	Reduced striatal damage by A_1 agonists	(156)
Multiple sclerosis	Experimental autoimmune encephalomyelitis (EAE)	Increased demyelination and axonal degeneration in A_1R KO	(132)
Stroke (ischemic brain injury)	Hypoxia	No change in organotypic hippocampus slices in A_1R KO mice	(27)
	Hypoxic-ischemia	Reduced white matter in neonate A_1R KO	(69)
	Prenatal hypoxic ischemia	Aggravated damage in neonate $A_{2A}R$ KO	(71)
	MCAO	Reduced infarct volume and neurological deficit score in $A_{2A}R$ KO mice	(32)
	MCAO	No effect on damage in A_1R KO	(68)
	MCAO	Reduced infarct volume in chimeric mice with selective depletion of bone marrow-derived cells	(72)
	Carbon monoxide	Increased hippocampus neuronal damage in A_3R KO mice	(75)
Alzheimer's disease	Beta-amyloid aggregation	Reduced neurotoxicity in cerebellum neurons	(157)

Finally, recent studies with $A_{2A}R$ KO mice and pharmacological agents suggest the possibility of another potentially beneficial effect of $A_{2A}R$ antagonists, namely prevention of the development of dyskinesia after repeated treatment with L-DOPA (118, 119). Debilitating motor complications such as dyskinesia are the major limiting factors of management in the later stages of Parkinson's disease. Thus the finding that repeated administration of L-DOPA did not lead to behavioral sensitization in $A_{2A}R$ KO mice indicates that the $A_{2A}R$ may be required for the development of maladaptive changes after long-term treatment with L-DOPA (113). This notion is further supported by recent studies in MPTP-treated nonhuman primates, showing that coadministration of KW6002 with the dopamine agonist apomorphine completely abolished apomorphine-induced dyskinesia (119).

Huntington's Disease

The cellular localization of the $A_{2A}R$ in striatopallidal neurons suggests that the $A_{2A}R$ may contribute to selective vulnerability to neurotoxins in Huntington's disease. Indeed, there is also evidence that $A_{2A}Rs$ may play a role in Huntington's disease (16, 120) (Table 3). In a neurochemical model of Huntington's disease, pharmacological and genetic inactivation of the $A_{2A}R$ have been shown to attenuate striatal damage induced by the mitochondrial toxin 3-nitropropionic acid (121) or the excitotoxin quinolinic acid (122). However, the role of the receptors is complex, and it is difficult at present to envision A_{2A} antagonism as a therapy in this disorder. The complex actions were interpreted as resulting from a balance between negative effects owing to blockade of presynaptic A_{2A} receptors regulating glutamate release and positive effects owing to blockade of postsynaptic receptors (120). However, glutamate levels are also regulated by glutamate transporters on glial cells, which express $A_{2A}R$. Therefore, the interpretation of glutamate changes is not yet clear.

Schizophrenia

Another pathological condition involving both adenosine and dopamine in the striatum—and where A_{2A} agonists might be beneficial—is schizophrenia. Patients with schizophrenia show impaired sensorimotor gating. Normally, this gating prevents excessive irrelevant sensory stimuli from disturbing integrative mental processes in the brain. In schizophrenic patients, the impairment in sensorimotor gating results in reduced prepulse inhibition (PPI) and reduced startle habituation. In experimental animals, both parameters are modulated by dopaminergic and adenosine receptor agonists and antagonists. Wang et al. (123) recently found that startle amplitude, startle habituation, and PPI were significantly reduced in $A_{2A}R$ KO mice, which provides evidence that this receptor may be involved in the regulation of these phenomena. In addition, responses to an NMDA antagonist and amphetamine were altered (123). These data suggest substances with A_{2A} receptor agonist properties may be of interest in the development of antipsychotic drugs (124).

ACTIONS IN OTHER PARTS OF THE NERVOUS SYSTEM

Addictive Drugs

A popular belief is that coffee can antagonize the intoxicating effects of alcohol. However, the molecular mechanisms that might underlie this offsetting action of coffee remain poorly identified. To investigate the possible involvement of the $A_{2A}R$ in the behavioral sensitivity to high doses of ethanol, the hypnotic effect of ethanol on $A_{2A}R$ KO mice and $A_{2A}R$ WT mice has been assessed (125). The righting reflex was lost following acute ethanol administration, but the effect lasted longer in $A_{2A}R$ WT mice than in $A_{2A}R$ KO mice. The fall in body temperature was not different between the two phenotypes. Dipyridamole, an inhibitor of adenosine uptake, increased the sleep time observed following administration of ethanol in

A_{2A}R WT mice but not in A_{2A}R KO mice. The selective A_{2A}R antagonist SCH 58261, but not the selective A_1 receptor antagonist DPCPX, shortened the duration of the loss of righting reflex induced by ethanol, thus mimicking the lack of the receptor in A_{2A}R-deficient mice. Caffeine (25 mg/kg) also reduced ethanol-induced hypnotic effects. These results indicate that the activation of A_{2A} receptors plays a role in the hypnotic effect of ethanol.

The cessation of chronic ethanol intake or "ethanol withdrawal" is an experimental procedure recognized to produce seizures in mice. This convulsant activity is associated with an increase in excitatory neurotransmission in the brain. Whereas A_{2A}R KO mice and controls ingested similar amounts of ethanol during forced ethanol consumption, the severity of handling-induced convulsions during withdrawal was significantly lower in the A_{2A}R KO mice than in A_{2A}R WT mice. Because the selective A_{2A}R antagonist ZM 241385 also attenuated the intensity of withdrawal-induced seizures, it was suggested that selective A_{2A}R antagonists may be useful in the treatment of alcohol withdrawal (126). The role of A_{2A}Rs in ethanol consumption and neurobiological responses to this drug of abuse was further characterized by Naassila et al. (127). Male and female A_{2A}R KO mice consumed more ethanol than WT mice. This slightly higher ethanol consumption was also related to ethanol preference. Relative to A_{2A}R WT mice, A_{2A}R KO mice were found to be less sensitive to the sedative and hypothermic effects of ethanol. No major difference in the development of tolerance to ethanol-induced hypothermia was found between the two phenotypes, although female A_{2A}R KO mice showed a lower tolerance-acquisition rate. These results suggest that activating the A_{2A}Rs may play a role in suppressing alcohol-drinking behavior and be associated with sensitivity to the intoxicating effects of acute ethanol administration.

There is also evidence that morphine dependence is modified by A_{2A}Rs. Opiate withdrawal was enhanced in mice lacking A_{2A} receptors, and this enhancement was abolished when both the cannabinoid CB1 receptor and A_{2A}R were eliminated (106).

Because there is considerable evidence for interactions between adenosine receptors and central stimulants (see above), for a role of adenosine in some actions of morphine (17), for various interactions between adenosine and ethanol (128), and because adenosine receptors are very important in regulating dopaminergic transmission in the reward pathways in nucleus accumbens, it is important to further examine the effects of addictive drugs in AR KO mice.

Seizures

It has long been known that adenosine can suppress repetitive neuronal firing, and a role of adenosine as an endogenous modifier of seizures has been suspected. This notion recently received support (129) when it was found that seizure-inducing lesions can increase the level of adenosine kinase in astrocytes, and that this, by reducing adenosine levels, contributes to increased seizure susceptibility. This raises the possibility that modifying the extracellular adenosine level in brain may be of therapeutic value against seizures. Indeed, cells that generate adenosine have

been transplanted into rat brain and this has led to decreased seizure susceptibility (130, 131). In particular, activation of A_1Rs appears to be an interesting target for therapy in drug-resistant epilepsy (131). Unless there are major compensatory mechanisms in effect, seizure thresholds would be expected to be lower in A_1R KO animals, but this awaits further investigation.

Multiple Sclerosis

There is some evidence that adenosine may play a role in multiple sclerosis—at least there are effects in an experimental model (132). Thus, in A_1R KO mice the demyelination and axonal degeneration was much more pronounced than in WT littermates. There was also a stronger activation of microglia/macrophages. Furthermore, macrophages from A_1R KO animals exhibited increased expression of the proinflammatory genes IL-1β and matrix metalloproteinase-12 on immune activation compared to control cells from A_1R WT animals (132). This would imply first that A_1Rs are very important in regulating macrophages/microglial cells. However, this is not immediately obvious from other data where these cells have been examined (e.g., 133). Furthermore, the role of A_1Rs in regulating oligodendrocyte function and survival appears to differ between the adult spinal cord (132) and the immature brain (69). This again emphasizes that the roles of adenosine receptors may be complex, and that they could differ with age, location, and pathology.

Memory

Despite some hints from experiments with drugs that affect adenosine receptors, the evidence from KO animals does not reveal any clear effect of the A_1R KO genotype on memory (30, 134). Minor effects in the water maze were suggested (134) to be due to the altered emotional stability reported for these mice (27, 30). Long-term potentiation (LTP), an in vivo model of memory formation, has generally been observed to be inhibited by A_1R activation (135) and enhanced by A_{2A}R activation (136, 137). Deletion of adenosine A_{2A}Rs did not alter ongoing synaptic transmission in either striatum (138) or nucleus accumbens (137), but accumbens neurons showed significantly reduced LTP when the effects of the A_{2A}R were removed (137). LTP was reduced greatly in the mossy fiber pathway in hippocampal slices from A_1R KO mice as well as rat hippocampal slices pretreated with an A_1R antagonist (61), providing strong evidence that adenosine acting at the A_1R augments LTP in this pathway.

Anxiety

The neurobiology of anxiety, including the role of adenosine, was recently comprehensively reviewed (139). Interestingly, anxiety-related behavior in the classical light/dark box test was increased in the A_1R KO mice, as shown by a reduction in the number of entries into as well as the total time spent in the lit compartment compared with A_1R WT mice (27, 30). The A_1R KO mice also showed a decrease in exploratory behavior in the open-field and in the hole-board, results that could reflect an anxiogenic state in A_1R KO mice. However, another strain of A_1R KO

mice with a similar genetic background displayed a normal overall level of motor activity, with very modest behavioral changes in the direction of increased anxiety (134). It is likely that different environmental conditions have contributed substantially to the behavioral discrepancies between the two lines. This might prompt us to ask whether the increased sensitivity to caffeine reported in patients with panic disorders (140) is indeed linked to a disorder of adenosine neuromodulation at A_1Rs in the brain.

A_{2A}R KO mice showed higher rates of spontaneous anxiety-like responses in two different anxiety-like behavioral tests, the elevated plus-maze and the light/dark box (31, 106, 141). Thus, A_{2A}R KO mice and at least one strain of A_1R KO mice exhibit increased anxiety, consistent with the well-known, pronounced, anxiogenic effects of high doses of caffeine. High doses of caffeine will presumably block most of these adenosine receptor subtypes, but low doses will not. Despite several studies using pharmacological tools and performed in rodent models (141–144) there is no clear consensus concerning the role of A_1 and A_{2A}Rs in anxiety. However, on the basis of screening tests, it has been proposed that A_1R agonists exert anxiolytic effects, whereas A_1R antagonists in some cases, but not consistently, exert anxiogenic effects. On the other hand, it is still unclear whether the A_{2A}R also plays a major role in anxiety states. Selective A_{2A}R antagonists seem to be devoid of effects in tests on rodents (141). However, recent data from humans shed fresh light on the potential role of A_{2A}Rs in the anxiogenic effects of caffeine. In a study conducted by Alsene et al. (145), the association between variations in anxiogenic responses to caffeine and polymorphisms in the adenosine A_1 and A_{2A}R genes has been examined. They found a significant association between self-reported anxiety after oral administration of 150 mg of caffeine and two linked polymorphisms on the A_{2A}R gene. Individuals with the 1976T/T and the 2592Tins/Tins genotypes reported greater increases in anxiety after caffeine administration than the other genotypic groups. Moreover, in patients with panic disorder, a psychiatric condition characterized by recurrent panic attacks and anticipatory anxiety, a single-nucleotide polymorphism haplotype in the A_{2A}R gene was found to be associated with the disease (146). Alpha-melanocyte-stimulating hormone (alpha-MSH) influences anxiety, aggressiveness, and motor activity, all of which are also influenced by A_{2A}R gene disruption. In A_{2A}R KO mice, significantly increased alpha-MSH content was observed in the amygdala and cerebral cortex. Plasma corticosterone concentration was significantly higher in A_{2A}R KO mice, revealing hyperactivity of their pituitary-adrenocortical axis. Results suggest that A_{2A}Rs are involved in the control of POMC gene expression and biosynthesis of POMC-derived peptides in pituitary melanotrophs and corticotrophs (147).

Aggression

Several studies have suggested that adenosine receptors are involved in the modulation of aggressive behavior. In agreement with the decrease of offensive behavior induced by a selective stimulation of A_1Rs (148), A_1R KO mice isolated for the resident-intruder aggression test showed enhanced aggressive behavior (27). A

similar enhancement was also observed in isolated male $A_{2A}R$ KO mice in the resident-intruder test (31). The increased aggressiveness observed in both A_1R KO mice and $A_{2A}R$ KO mice is in agreement with the increase of offensive behavior induced by selective blockade of either A_1 or $A_{2A}Rs$ (M. El Yacoubi & J.M.Vaugeois, unpublished observations). These results suggest that both adenosine receptor subtypes are involved in the effect of adenosine on aggressiveness. The link between these effects and the increase in nervousness and irritability reported in humans (3) after chronic administration of high doses of caffeine remains a matter of speculation.

Depression

In behavioral procedures used to screen potential antidepressants, such as tail suspension and forced swim tests, $A_{2A}R$ KO mice were found to be less sensitive to "depressant" challenges than their WT littermates, which were less immobile than $A_{2A}R$ WT mice in both tests (149). Consistently, $A_{2A}R$ blockers reduced the immobility times in tail suspension and forced swim tests. Taken together, the results support the hypothesis that blockade of the $A_{2A}R$ might be an interesting target for the development of effective antidepressant agents. Although their mode of action in potentially alleviating mood disorders is unknown, modulation of dopamine transmission might play a role (149). Future clinical trials with selective $A_{2A}R$ antagonists as potential therapeutic agents for major depressive episodes will help to delineate the role of adenosine in the pathophysiology of mood disorders. Whereas $A_{2A}R$ antagonists have been proposed as antidepressants (149), A_3R KO mice showed an increase in the amount of time spent immobile in the two tests of behavioral depression, the forced swim test and the tail suspension test (75).

Pain

The role of adenosine as an endogenous analgesic substance has also been evaluated (27). A_1Rs are abundant in mouse spinal cord, with the highest levels in the outer lamina of the dorsal horns, where the density of receptors was close to that observed in the hippocampus. A_1Rs are responsible for the analgesic effects of intrathecally administered A_1 agonists. A_1R KO mice react faster to thermal pain than A_1R WT mice. However, this increase is not matched by an increased sensitivity to mechanical stimulation. The authors suggested that endogenous adenosine acting at A_1Rs decreases nociception, mediated via C fibers. These results also suggest that the A_1R may be a target for the development of antinociceptive drugs.

The response of $A_{2A}R$ KO mice to acute pain stimuli is slower in the hot plate and tail-flick tests compared to $A_{2A}R$ WT mice (31). Similar reduced pain responses were also found when a tail-immersion test was used (106). This higher nociceptive threshold suggests that the peripheral lack of $A_{2A}Rs$ predominates over the spinal defect. Thus, depending on the site of action and the receptor activated (A_1 or A_{2A}), adenosine may exert very different effects on pain. This variety of effects may explain why caffeine has analgesic effects against some, but not all, types of pain (3).

A_3R KO mice show decreased sensitivity to some painful stimuli, as evidenced by the increase in latency in the hot plate but not tail-flick test (75). Another study (150) found no evidence for a role of A_3R in nociception or in the antinociceptive effect of the adenosine analog R-phenylisopropyl adenosine (R-PIA). Thus, no difference was seen between A_3R KO and A_3R WT mice in nociceptive response to mechanical or radiant heat stimuli. The antinociceptive response to intrathecal R-PIA was also unchanged in the A_3R KO mice. In contrast, heat hyperalgesia, plasma extravasation, and edema following carrageenan-induced inflammation in the hind paw were significantly reduced in A_3R KO mice compared to the A_3R WT controls. Thus, mice lacking A_3R had deficits in generating the localized inflammatory response to carrageenan, supporting a proinflammatory role of A_3Rs in peripheral tissues.

CONCLUSIONS

Whereas deletion of genes for enzymes critically involved in adenosine metabolism leads to lethal phenotypes, deletion of A_1, A_{2A}, and A_3 receptors has rather subtle effects and the mice are remarkably normal. This agrees well with the conclusion drawn before, i.e., that adenosine receptors are involved in modulating physiological responses and that they are particularly important under pathophysiological conditions. Thus, to determine the roles of the adenosine receptors, the genetically modified mice must be subjected to various types of challenges.

The results obtained so far have both confirmed previous data and yielded some surprises. The important role of the A_1R in modulating excitatory transmission and its role in pain transmission was expected, as was the critically important role of A_{2A}Rs in striatal function. Among the major surprises were the noncritical role of A_1Rs in brain ischemia and in sleep and the finding that A_{2A}Rs mediate aggravated brain damage mainly via peripheral receptors. Our examination of the literature has also indicated studies that can and should be performed to further define the roles of adenosine receptors in the nervous system. Because the goal of these studies is to examine the possibility for novel drug therapies, the use of KO mice to determine that the drugs are indeed selective is very important. Indeed, data obtained already suggest that some of the drugs used to delineate adenosine receptor effects are not as selective as previously hoped (36, 151, 152).

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