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Review

Normal and abnormal functions of adenosine receptors in the central nervous system revealed by genetic knockout studies☆

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ABSTRACT

Endogenous adenosine is a widely distributed upstream regulator of a broad spectrum of neurotransmitters, receptors, and signaling pathways that converge to contribute to the expression of an array of important brain functions. Over the past decade, the generation and characterization of genetic knockout models for all four G-protein coupled adenosine receptors, the A1 and A2A receptors in particular, has confirmed and extended the neuromodulatory and integrated role of adenosine receptors in the control of a broad spectrum of normal and abnormal brain functions. After a brief introduction of the available adenosine receptor knockout models, this review focuses on findings from the genetic knockout approach, placing particular emphasis on the most recent findings. This review is organized into two sections to separately address (i) the role of adenosine receptors in normal brain processes including neuroplasticity, sleep–wake cycle, motor function, cognition, and emotion-related behaviors; and (ii) their role in the response to various pathologic insults to brain such as ischemic stroke, neurodegeneration, or brain dysfunction/disorders. We largely limit our overview to the prominent adenosine receptor subtypes in brain–the A1 and A2A receptors–for which numerous genetic knockout studies on brain function are available. A1 and A2A receptor knockouts have provided significant new insights into adenosine's control of complex physiologic (e.g., cognition) and pathologic (e.g., neuroinflammation) phenomena. These findings extend and strengthen the support for A1 and A2A receptors in brain as therapeutic targets in several neurologic and psychiatric diseases. However, they also emphasize the importance of considering the disease context-dependent effect when developing adenosine receptor-based therapeutic strategies. This article is part of a Special Issue entitled: "Adenosine Receptors".

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1. Extracellular adenosine level and adenosine receptors

1.1. Source and regulation of extracellular adenosine level

Adenosine is found in all cells where it is formed as a by-product of purine nucleotide metabolism and other key metabolic cell processes [1]. Beyond its role in energy-homeostasis, in the central nervous system (CNS), adenosine also serves a neuromodulatory role where it is capable of affecting neuronal excitability, release of various neurotransmitters including glutamate, γ-aminobutyric acid (GABA), acetylcholine, and dopamine, and synaptic plasticity [2]. Unlike neurotransmitters, however, adenosine is not stored and released from vesicles but rather is generated by the highly regulated intracellular metabolism of AMP (and transported out of the cell through bi-directional facilitated diffusion transporters). Over the last decade, it is increasingly recognized that the rapid (1 ms) extracellular conversion of locally released adenine nucleotides like ATP and also cAMP through a series of ectonucleotidases such as CD73 and CD39 represents another important source of extracellular adenosine in tissue [3–5]. Several processes operate in concert to maintain this equilibrium in which extracellular adenosine levels are estimated to fall within the 25–250 nanomolar range under basal conditions [4,6]. Antagonist studies have demonstrated that adenosine exerts a tonic inhibitory effect on synaptic transmission, implying that under basal conditions, adenosine levels are sufficient to tonically activate relevant adenosine receptor subtypes [1]. Under pathologic circumstances when cell function is compromised, however, extracellular adenosine levels can rise as much as 100 fold, perhaps reaching a concentration that activates the lower-affinity adenosine receptor subtypes, which then alters cellular function [7,8].

1.2. Adenosine receptors (ARs)

1.2.1. Classification of adenosine receptors

Adenosine receptors (ARs) are cell-surface receptors belonging to the G-protein-coupled receptor family [9]. These receptors were initially classified based on their pharmacologic response profiles to agonism by adenosine analogues and antagonism by methylxanthines as determined by their ability to inhibit (i.e., A1 and A3 subtypes) or stimulate (i.e., A2 subtype) adenylate cyclase (AC) [10,11]. The A2 receptor subtype was subsequently further categorized according to the presence of high-affinity (A2A) or low-affinity (A2B) binding sites for adenosine in brain [12]. However, similar affinities for A2A or A2B receptor stimulation of MAP kinase activity have also been reported in cell culture studies [13]. To date, four AR subtypes have been identified, purified, cloned, and expressed from mouse, rat, human, and other mammalian as well as non-mammalian species [9,14]. Successful molecular cloning and expression of these receptors have not only definitively verified the presence of four different subtypes but have also segued to genetic knockout and transgenic overexpression studies to further elucidate the functions of each AR subtype.

1.2.2. Expression, distribution, and signaling of adenosine receptors

1.2.2.1. A1 receptor (A1R). The A1R is the most highly conserved AR subtype between species [9], and it probably exhibits the greatest affinity for adenosine compared to the other subtypes [15]. It is expressed throughout the body with the highest levels observed in brain, notably in neurons of cortex, hippocampus, and cerebellum, as well as dorsal horn of spinal cord, eye, adrenal gland, and atria [9,16,17]. The A1R is also expressed at intermediate levels elsewhere in brain and in other peripheral tissues such as liver, kidney, white adipose tissue, testis, and colon; it is most lowly expressed in lung tissue [9]. In brain, A1Rs are found at both pre-synaptic and post-synaptic sites [18]. A1R stimulation suppresses neuronal activity through pre-synaptic and post-synaptic mechanisms by coupling with Gi to inhibit the AC-cAMP-protein kinase A (PKA) signaling pathway [11]. In striatum, A1Rs have also been shown to interact with dopamine D1 receptors (D1Rs) on striatonigral medium spiny neurons (MSNs) [19–21], providing yet another means by which A1Rs can influence neuronal activity.

1.2.2.2. A2A receptor (A2AR). The A2AR also exhibits a high potency for adenosine [15] and is widely expressed in different tissues, albeit at varying levels. A2AR expression is highest in brain, spleen, thymus, leukocytes, and blood platelets and intermediate in heart, lung, and blood vessels [22,23]. Within brain, A2AR expression levels are highly concentrated in dorsal and ventral striatum (on striatopallidal MSNs) as well as in olfactory tubercle [24–29]. It is also recognized that A2ARs are expressed at substantially lower levels outside of striatum in brain regions including hippocampus and cortex [16,28]. In brain, A2ARs are found predominantly at post-synaptic neurons in striatum, but they are also detected at significantly lower levels at pre-synaptic sites in cortico-striatal terminals and in hippocampus [30]. Although A2AR activation of the AC-cAMP-PKA pathway was originally assumed to result from A2AR coupling to Gs [9], anatomic and biochemical evidence later indicated that, at least in striatum, this activation mainly occurs through coupling to Golf [31–33], and in some cases, G-protein coupling may also require the $\beta\gamma_7$ subunit [34,35]. In addition to signaling via a PKA-dependent pathway, A2AR signaling through a protein kinase C-dependent pathway in

hippocampal synaptosomes has also been demonstrated [36,37]. Furthermore, $A_{2A}R$ activation can trigger alternative signaling pathways via interaction with other receptors and signaling molecules. For instance, A2AR heterodimer and/or functional interactions with A1Rs [38–40], dopamine D2 receptors (D2Rs) [41,42], group I metabotropic glutamate 5 receptors (mGlu5Rs) [43,44], *N*-methyl-D-aspartate receptors (NMDARs) [40,45], and cannabinoid CB1 receptors (CB1Rs) [46,47] have been reported. A_{2A}Rs have also been noted by *in vitro* assays and/or electrophysiology to affect signaling through brain-derived neurotrophic factor (BDNF) in hippocampus [48,49] and fibroblast growth factor (FGF) as well as glial cell line-derived neurotrophic factor in striatum [50] to modulate synaptic transmission.

1.2.2.3. A2B receptor (A2BR). The A2BR possesses lower affinity for adenosine than does the A1R or A2AR subtype [51]. Regionally defining A2BR expression has largely been restricted to mRNA evidence where A2BRs are thought to be broadly expressed but at low levels [9]. Specifically, RT-PCR, Northern blot, and immunochemical analyses have revealed higher expression in colon, cecum, and bladder; intermediate expression in blood vessels, eye, and lung; and lower expression in brain, adipose tissue, kidney, liver, ovary, adrenal gland, and pituitary gland of rat [9,16,52]. No significant level of A2BR expression was detected in brain. Like A2ARs, A2BR signaling is coupled to the Gs protein-AC-cAMP-PKA pathway. In addition, A2BRs can couple with Gq/11 to activate phospholipase C [53,54], and some evidence also suggests that they can interact with the arachidonic acid pathway [55].

1.2.2.4. A3 receptor (A3R). The A3R was the last subtype to be identified when it was first isolated as an orphan receptor in rat testis [56] and later cloned and expressed in other cell types [57]. Among the AR subtypes, the A3R exhibits the greatest species variation in expression pattern [9] and is notably insensitive to antagonism by methylxanthine. Nonetheless, A3Rs are generally widely expressed in the body in most species, and A3R transcript has been found in most peripheral organs including testis, lung, liver, spleen, thyroid, kidney, and heart [9,16,57] as well as in bone marrow-derived mast cells [58]. In brain, A3R expression levels are, at best, moderate in hippocampus and cerebellum, and low elsewhere [9]. Within hippocampus, A3Rs have been detected in neurons (i.e., CA1 pyramidal cells via single-cell PCR analysis of mRNA) and their terminals (via western blot analysis) [59]. A3Rs have also been detected in pial and intercerebral arteries [60]. Like A1Rs, A3Rs can signal through coupling to Gi proteins to inhibit AC activity and reduce intracellular cAMP concentrations [57,61]. However, Gq protein activation of phospholipase C leading to regulation of calcium status comprises the primary signaling pathway utilized by these receptors [62–64].

1.2.3. Brain regional and cell type A1R and A2AR expression patterns

As noted in the previous section, A1Rs and A2ARs show a complementary expression pattern in brain; that is, A1Rs are most prominently expressed in hippocampus and cortex and only moderately expressed in striatum while A2ARs are most abundantly expressed in striatum and minimally expressed in hippocampus and cortex. Moreover, within a specific tissue, ARs may show varying sub-regional and cellular expression patterns. For example, in striatum, A1Rs are localized to post-synaptic striatonigral MSNs of the direct pathway [19] whereas A2ARs are localized to post-synaptic striatopallidal MSNs of the indirect pathway [26]. At the sub-cellular level, A1Rs are highly expressed at pre-synaptic terminals [65] while A2ARs are predominantly expressed at post-synaptic sites in striatum [30,66]. This anatomic segregation is functionally significant and is relevant for understanding the role of these receptors in motor control, Parkinson's disease (PD), and L-dopa-induced dyskinesia as well as the therapeutic potential of A2AR antagonists for the motor symptoms of PD [41,66,67]. Interestingly, A2ARs appear to localize mainly to asymmetric, excitatory striatal synapses (expressing VGLUT) [68] where pre-synaptic A2ARs were shown to co-localize with A1Rs to fine-tune glutamate release [38]. These synapses were recently identified to largely comprise glutamatergic cortical afferents onto direct pathway striatonigral MSNs [69]. The functional significance of A2ARs' fine-tuning of glutamatergic neurotransmission in striatum from pre-synaptic or post-synaptic locations are likely highly relevant for A2AR-dependent effects on physiologic and pathologic states. These implications have been thoroughly discussed in a recent review by Schiffmann and colleagues [70].

In summary, the presence of various AR subtypes in distinct brain regions, sub-regions, cell types, and synaptic sites provides an array of paths by which adenosine can differentially modulate brain functions. Such diversity also points to an important strategy for targeted therapeutic interventions. On the other hand, this complexity makes characterization of adenosine and AR functions in brain more difficult. The development of genetic engineering strategies has facilitated the dissection of these functions and highlighted potential pathways in the CNS.

2. Genetic adenosine receptor animal models relevant to understanding adenosine and adenosine receptor modulation of brain function

Transgenic and knockout animal models represent highly valuable tools to assess protein functions *in vivo* [71,72]. Earlier pharmacologic strategies are intrinsically limited by their partial specificity or selectivity. Moreover, AR ligands often exhibit poor solubility, and systemic injection of these ligands showed variable CNS penetration and had the potential to produce non-specific effects. Thus, the use of a pharmacologic strategy alone to elucidate AR function in brain was less than ideal. Genetic strategies have helped circumvent many of these limitations. Through genetic engineering, AR gene knockout by the targeted deletion of a critical AR exon, or transgenic over-expression of the AR gene, has been achieved in rodents (mostly mouse). Furthermore, the conditional knockout of some AR genes has been attained using the Cre-*loxP* system. This strategy places the expression of Cre recombinase (and thus AR gene deletion) under the control of a region/tissue- or cell type-specific promoter. Such a strategy allows for the localization of AR functions to particular brain regions or cell types. Moreover, depending on the promoter, a degree of temporal specificity can be attained, with most gene deletions restricted to the early postnatal period. These advantages therefore render conditional knockout a particularly useful strategy to address AR functions in brain. Today, knockout mouse models have been generated for each of the four AR subtypes, and several brain region-specific as well as cell type-specific AR knockout mice are available (see overview below in section 2.1). These genetic strategies have substantially advanced the adenosine and AR field, extending findings from pharmacologic studies and revealing novel and important physiologic functions of the different receptor subtypes.

2.1. A1 receptor transgenic models

2.1.1. Global A1R knockout mice

Two constitutive, global A1R knockout mouse lines (gb-A1R KO) from similar mixed genetic backgrounds, 129sv/C57BL/6J [73] or 129/OlaHsd/C57BL [74], have been generated. Gb-A1R KO mice in both lines were viable, without gross anatomic abnormalities, developmentally normal, and fertile. Body weight measured up to six months of age, heart rate, and blood pressure were all unaffected by gb-A1R KO in both lines, and normal body temperature was also reported in one line [74]. Subsequent studies in these knockout lines, however, reported that male gb-A1R KO mice produced fewer pups and exhibited sub-optimal spermatozoa capacitation [75]. These mice

also showed reduced survival rates around 15 months of age that may be attributable to accelerated aging processes and the accumulation of cardiovascular, hepatic, and renal dysfunction over time [76].

2.1.2. Brain-specific conditional A1R knockout mice

Brain-specific conditional A1R knockout mice (mixed 129SvJ-C57BL/6 genetic background) were recently generated [77] using the Cre/*loxP* strategy in which *cre* transgene expression was placed under the control of a *CaMKII-α* promoter to provide both regional and temporal specificity of Cre expression and thus A1R gene knockout [78]. Successful A1R deletion was reported throughout most of the brain but most notably in cortex and hindbrain and to a lesser extent in thalamus by X-gal staining, mRNA autoradiography, and mRNA quantification.

2.1.3. Adeno-associated virus (AAV)-mediated brain region-specific A1R knockout

Focal deletion of A1Rs in hippocampal CA1 or CA3 neurons has been attained by local injection of AAV vectors containing the *cre* transgene construct into the brains of mice with a critical exon flanked by *loxP* sites [79]. This strategy allowed for a temporal and regional specificity that has helped to distinguish the functional roles of pre-synaptic *versus* post-synaptic A1Rs.

2.2. A2A receptor transgenic models

2.2.1. Global A2AR knockout mice

Four constitutive, global A2AR knockout mouse lines (gb-A2AR KO) from different genetic backgrounds, CD1 [80], mixed Sv-129-C57BL/6, Sv-129 [81] or C57BL/6 [82,83], have also been generated. Gb-A2AR KO mice from all four lines were viable, without gross anatomic abnormalities, and fertile. However, increased body weight, heart rate, blood pressure, platelet aggregation, as well as striatal D1R and D2R expression were only reported in gb-A2AR KO mice from a CD1 background [80,84], thus emphasizing the importance of considering the influence of genetic background on phenotypic analyses of knockout mouse lines. Given the constitutive deletion of A2ARs in gb-A2AR KO mice and A2AR interactions with dopaminergic pathways, it is important to note that, at least in two different gb-A2AR KO lines, striatal dopamine receptor levels as well as tyrosine hydroxylase (TH) immunoreactivity and dopamine transporter (DAT) function were indistinguishable between gb-A2AR KO and wildtype control mice from one group [85].

2.2.2. Forebrain- or striatum-specific conditional A2AR knockout mice

Brain-regional deletion of A2ARs has now been achieved in the forebrain (i.e., striatum, cortex, hippocampus) [86] or striatum [87] only. A2AR KO mice derived from both conditional knockout mouse lines were viable, without gross anatomic abnormalities, and fertile. Compensatory changes in A1R, D1R, or D2R expression levels in striatum and cortex as well as tyrosine hydroxlase immunoreactivity were also absent in both knockout mouse lines (Wei et al., unpublished data). Forebrain-specific A2AR KO mice (fb-A2AR KO; now near congenic C57 BL/6 genetic background) were generated using the Cre/*loxP* strategy in which *cre* transgene expression was placed under the control of the forebrain neuron-specific *CaMKII-α* promoter [86]. The postnatal deletion of A2ARs circumvents potential developmental effects of constitutive A2AR gene deletion. Striatum-specific A2AR KO mice (st-A2AR KO; mixed 129-Steel-C57BL/6-FVB genetic background) were later generated using the same Cre/*loxP* strategy but with *cre* transgene expression driven instead by the embryonic striatal neuron-specific *Dlx5/6* promoter elements [87]. Neuron-specific deletion in both conditional A2AR KO mouse lines was confirmed by PCR analysis of brain cells sorted by flow cytometry [87].

2.2.3. Transgenic over-expression of A2AR in rat brain

Transgenic rats over-expressing human A2ARs under the control of the neuron-specific enolase promoter have also been generated [88]. The transgene was mainly expressed in neurons of the cortex, hippocampus, cerebellum, and striatum. Transgenic mice were reported to express lower levels of D2Rs and mGlu5Rs only in striatum; A1R and D1R expression levels were unchanged in striatum and cortex.

2.3. A2B receptor transgenic models

Constitutive, global A2BR knockout mice (A2BR KO) of an 80% C57BL/6J genetic background have recently been developed [89]. These mice were viable, without gross anatomic features, and normotensive. Mice lacking the A2BR, however, exhibited higher basal levels of pro-inflammatory cytokines (e.g., TNF-alpha, Il-6) and an up-regulation of several vascular adhesion molecules (e.g., E-selectin, P-selectin, ICAM-1), differences that were augmented upon challenge. These findings suggest an important anti-inflammatory role for A2BRs, and the presence of baseline phenotypes highlights that, at least in some tissues, basal adenosine levels are sufficient to activate A2BRs to produce an effect, despite adenosine's low affinity for the receptor. Also of note, the use of a receptor knockout/reporter gene knock-in strategy permitted the visualization of A2BR expression throughout the body and revealed regional- and cell type-specific A2BR expression, which would be unattainable by other less sensitive detection methods. To our knowledge, A2BR KO mouse studies on brain function have not yet been reported.

2.4. A3 receptor transgenic models

Constitutive, global A3R knockout mice (A3R KO) of a mixed B6D2 and C57BL/6 genetic background have been generated [58]. These mice are reported to be viable, fertile, developmentally normal, lacking gross anatomic defects, and free from compensatory changes in the expression of the other AR subtypes [58]. In addition, body weight, heart rate, and blood pressure were indistinguishable between these A3R KO mice and their wildtype controls. Interestingly, A3R KO reduced intraocular pressure, thus highlighting the potential for targeting A3Rs to treat glaucoma [90]. Although global over-expression of A3Rs led to embryonic lethality [90], successful A3R over-expression was achieved in heart [91]. Several pharmacologic studies have examined A3R function and/or its role in hypoxia-ischemia models [e.g., 92, 93, 94], but some evidence suggests that the A3R agonist CI-IBMECA used in these studies also activates A1Rs [59,94,95]. Thus, the partial selectivity of this and other A3R ligands limits one from concluding that any effect is strictly an A3R-mediated effect. With the development of targeted A3R KO mouse models, its various CNS functions are now beginning to be validated and/or newly revealed [96,97].

2.5. Double receptor knockout mice and genetic models targeting adenosine metabolism or transport

Additional genetic models are available to study adenosine and AR function but will not be discussed in this review. Several double knockout mice to study A2AR interactions with other receptors including A1Rs [98], D2Rs [99], and CB1Rs [100] have been generated. In addition, several models have targeted important regulators of adenosine metabolism (e.g., adenosine kinase (ADK), adenosine deaminase (ADA), CD73, and CD39) and transport (e.g., equilibrative nucleoside transporter type 1 (ENT1)) [101–106]. Transgenic mice with reduced brain adenosine tone due to over-expression of ADK (under the control of the human ubiquitin promoter), ENT1 knockout mice [103,107–109], CD73 knockout mice [110], and CD39 knockout mice [111] have been described and provide an important snapshot of

adenosine's role in multiple neurologic functions and/or related pathologic conditions [112,113]. While ADA knockout mice surviving to adulthood are now available and show increased adenosine levels in most tissue types including brain [114], we are unaware of any reported phenotypes in these mice that confirm ADA's impact on brain processes, as might be expected given the neurologic abnormalities observed in ADA-deficient patients [115,116]. This knowledge gap is presumably because their assessment would largely be hindered/confounded by the marked immunologic phenotypes resulting from ADA deficiency.

3. Knockout models to study adenosine receptor function in physiologic and pathologic conditions in the central nervous system

Since the 1970s, when research on the neuromodulatory functions of adenosine first took flight, adenosine has been noted to influence numerous critical brain functions under both physiologic and pathologic conditions. The following two sections will focus on findings from the above mentioned genetic AR knockout models, with particular emphasis on the most recent findings, to separately address (i) the role of ARs on normal brain processes and (ii) their role in response to various pathologic insults to brain such as ischemic stroke, neurodegeneration, or brain dysfunction/disorders. We largely limit our overview to the prominent AR subtypes in brain–the A1R and A2AR–for which numerous genetic knockout studies on brain function are available. The lower expressed A2BR and A3R subtypes will only be briefly touched upon in this review due to limited genetic knockout studies on brain functions. A review of adenosine's peripheral actions can be found elsewhere [82,117–120]. Earlier reviews have also provided a nice overview of pharmacologic studies addressing the role of these receptors on brain function [2,4,14,41,65,121–125].

3.1. Brain adenosine receptor functions revealed by knockout mouse models

3.1.1. Sleep–wake physiology

An important role for adenosine as an endogenous sleep factor is supported by several lines of experimental evidence [126,127]. Extracellular adenosine levels in the basal forebrain increase during prolonged wakefulness and decrease during sleep [128]; however, the source of extracellular adenosine during the sleep–wake cycle is not clear. Using a transgenic mouse line expressing dominant-negative SNARE to suppress gliotransmission [129], Hallass et al. demonstrated that the reduction in glial cell-derived extracellular adenosine was associated with an accumulation of sleep pressure and cognitive impairment from sleep deprivation. This provides direct evidence that adenosine released from astrocytes modulates the accumulation of sleep pressure and its cognitive sequelae [130].

Adenosine exerts its effect on the sleep–wake cycle by acting at ARs. Both A1Rs and A2ARs contribute to adenosine-mediated modulation of the sleep–wake cycle [131,132]. A large body of pharmacologic studies suggests that endogenous adenosine acts at A1Rs in basal forebrain to modulate the sleep–wake cycle [126]. Consistent with this notion, brain-specific conditional A1R KO mice showed selective attenuation of slow wave activity (SWA) rebound and a widespread, synchronized neuronal activity that varied directly with previous waking duration [77]. Consequently, these mice also exhibited impaired working memory [130], which suggests that extracellular adenosine acting at A1Rs is required for normal rebound SWA and downstream working memory functions.

On the other hand, evidence for A2AR involvement in sleep–wake physiology largely stems from studies examining the arousal effects of the non-selective AR antagonist caffeine. Recent pharmacologic as well as genetic knockout studies suggest an important role for A2ARs in mediating this effect. For example, caffeine-induced arousal was largely intact in gb-A1R KO mice but essentially abolished in gb-A2AR KO mice [133]. The contribution of A2ARs to caffeine's arousal effect is consistent with recent studies showing that a genetic variant of the A2AR gene (*ADORA2A*) in humans is associated with individual sensitivity to caffeine's effect on sleep [134]. Furthermore, striatum-specific deletion of A_{2A}Rs, like global deletion, was found to blunt this caffeine-mediated arousal effect (Shen et al., unpublished data). This raises an intriguing possibility that caffeine's arousal effects are mediated by A_{2A}Rs in striatal neurons, a brain region traditionally associated with motor and motivational behavior.

3.1.2. Motor function

AR knockout mouse models have permitted confirmation that A2ARs are the main effectors of adenosine-based modulation of motor activity, isolation of the motor-enhancing effects of A2AR antagonists to A2ARs on striatal post-synaptic neurons, attribution of caffeine's motor stimulant effects largely to A2AR blockade (at least at lower doses), and identification of D2R-independent functionally significant motor outcomes of A2ARs.

3.1.2.1. Spontaneous activity. Since A2ARs are highly expressed on indirect pathway MSNs throughout striatum, a brain region critically involved in motor control, these striatal neuronal A2ARs were largely presumed to function as the main effectors of adenosine-based modulation of motor activity and thus the molecular targets of A2AR antagonist-induced motor stimulation. The recent demonstration of absent A2AR antagonist-induced motor stimulation in st-A2AR KO mice provided the first definitive evidence that post-synaptic striatal neuronal A2ARs are required for the motor stimulating effects of A2AR antagonists such as KW-6002 [87]. This effect was previously shown to depend only partially on D2Rs, thus revealing that A2ARs can facilitate striatal neuronal activity to produce outcomes that are independent of their antagonistic interaction with D2Rs [99].

Interestingly, while A2AR antagonists are well-known to induce motor stimulation, genetic A2AR KO has failed to produce a similar effect on basal motor activity. Instead, adult gb-A2AR KO mice (from different genetic backgrounds) consistently exhibited reduced spontaneous activity compared to their wildtype controls [80,81,85,135,136], an effect that was more pronounced during the dark phase [85] and not related to accelerated habituation [80,85], general motor impairment assessed by the accelerating rotarod [85,137], or abnormal circadian rhythm [85]. At least in one line, this phenotype might be explained by heightened anxiety-like behavior [80] and reduced dopaminergic tone [84]. On the other hand, differences in basal activity levels were not reported for fb-A2AR KO or st-A2AR KO mice harboring brain region- and neuron-specific deletions of the A2AR [86,87,138]. This difference between gb-A2AR KO and conditional A2AR KOs suggests that the phenotype observed in gb-A2AR KO mice might also stem from non-specific or adaptive effects of constitutive global gene deletion, or alternatively, may also reflect the activity of A2ARs at non-neuronal (i.e., astrocytic or microglial) sites. Confirmation of the latter awaits the development of A2AR KO restricted to these non-neuronal cell types. Nonetheless, the clear discrepancy between genetic (i.e., reduced or no effect on activity) and pharmacologic (i.e., enhanced activity) studies may relate to partial blockade and/or acute/short-term blockade of A2ARs by selective antagonists compared to complete absence and/or long-term depletion of the receptor in these knockout models.

Unlike gb-A2AR KO mice, gb-A1R KO produced minimal impact on spontaneous motor activity [74,76,98,136]. Moreover, gb-A1R KO failed to affect motor coordination but was accompanied by a reduction in muscle strength [76]. Recently, it was shown that A1R-A2AR double KO mice exhibited reduced peak spontaneous activity relative to A1R KO, A2AR KO, and wildtype controls [136]. This further suggests that while A2ARs appear to be most important AR subtype in effecting adenosine's modulation of spontaneous motor activity, A1Rs

still exert a small effect that is not revealed by A1R knockout alone (in which A2ARs are still present) but only uncovered when A2ARs are also eliminated. Lastly, A3R KO mice were also reported to exhibit reduced spontaneous activity that was restricted to the dark phase of the light-dark cycle [96,139] and more pronounced among the female mice [139]. This effect of A3R KO was postulated to relate to a potential role for A3Rs in the control of arousal since responses to caffeine and amphetamine were also impaired in these mice [96]. No evidence was found to indicate that the A2BR is involved in modulating motor activity [136].

3.1.2.2. Psychomotor activity by caffeine. Caffeine is a widely consumed psychoactive substance that is also a non-selective AR antagonist. The use of AR KO mice has also helped to elucidate the molecular mechanisms of caffeine's motor stimulant effects. This topic has been covered in detail in a recent review [140]. Briefly, studies in gb-A2AR KO mice confirmed the A2AR dependence of this effect [80,81,98,136,141], which was further attributed to A2ARs on forebrain neurons [138]. However, these studies also suggest that caffeine has other targets because gb-A2AR KO does not result in complete abolishment of caffeine's stimulatory effect and is without effect at higher doses of caffeine [135,141]. It appears, however, that caffeine can have A2AR- and A1R-independent effects on motor activity since gb-A1R KO mice also did not reliably differ from their wildtype controls when administered caffeine at a high dose [135,136]. It is possible still that these A2AR- and A1R-dependent effects at higher doses of caffeine may result from effects on A3R function since A3R KO mice showed attenuated responses to caffeine [96] at a dose (15 mg/kg, i.p.) that failed to produce, in other studies, a difference in gb-A2AR KO or gb-A1R KO mice relative to their wildtype controls.

3.1.2.3. Psychomotor activity by dopamine agonists and NMDAR blockade. Dopaminergic and glutamatergic function in striatum are posited to underlie psychomotor effects [142]. Adenosine and its receptor targets are known to modulate dopaminergic and glutamatergic signaling in striatum, and ARs, namely A2ARs, have been shown by knockout studies to modulate psychomotor effects produced by various drugs such as cocaine, amphetamine, and phencyclidine. Evidence from A2AR knockout studies paints a general picture of reduced psychomotor stimulation by the dopaminergic compounds cocaine and amphetamine and by the NMDAR antagonist phencyclidine. Gb-A2AR KO mice (from different genetic backgrounds) exhibited a selective attenuation in the motor response to cocaine or amphetamine without any effect on D1R or D2R direct agonist-induced motor stimulation or suppression, respectively [85]. In keeping with these results, fb-A2AR KO mice also displayed a reduction in their motor responses to cocaine [87] or amphetamine [86]. These KO mice also showed an attenuated hyperlocomotor response to phencyclidine [87]. Thus, stimulation of A2ARs on forebrain neurons appears to be important for the full expression of hyperlocomotor responses to cocaine, amphetamine, and phencyclidine.

In contrast, A2AR deletion restricted to post-synaptic striatal neurons in st-A2AR KO mice enhanced rather than attenuated the hyperlocomotor response to a single injection of cocaine or phencyclidine [87]. These results indicate that striatopallidal A2ARs predominantly inhibit psychomotor activity and are consistent with the A2AR-D2R antagonistic interaction at striatopallidal MSNs [41]. Thus, the enhanced psychomotor response in st-A2AR KO mice is likely attributable to increased striatopallidal D2R activity. Most importantly, comparative analysis of the psychomotor response profile to cocaine or phencyclidine in fb-A2AR KO and st-A2AR KO mice revealed for the first time, a critical role for the previously under-recognized extra-striatal A2ARs in modulating psychomotor activity [87]. The opposite behavioral phenotypes observed in fb-A2AR KO mice (i.e., attenuation) and st-A2AR KO mice (i.e., enhancement) following cocaine or phencyclidine treatment suggested that the excitatory effect of extra-striatal A2ARs predominates and counters the inhibitory effect of striatopallidal A2ARs on psychomotor activity. This idea was further substantiated by combining pharmacologic and genetic knockout strategies. Specifically, the impact of extra-striatal A2AR blockade on cocaine-induced psychomotor activity was assessed by administering KW-6002 to st-A2AR KO mice. These mice, like fb-A2AR KO mice, showed attenuated cocaine-induced psychomotor activity. This effect of extra-striatal A2ARs was speculated to result from pre-synaptic A2AR modulation of glutamate release at cortico-striatal nerve terminals.

Lastly, the A3R has recently been demonstrated to affect amphetamine-induced hyperactivity [96]. Notably, global loss of A3Rs attenuated the response to amphetamine.

3.1.3. Neuronal synaptic plasticity

Synaptic plasticity is often inferred by examining long-term potentiation (LTP) and long-term depression (LTD), phenomena which have been documented in brain regions such as hippocampus and striatum and which are generally thought to form the molecular and cellular basis of associated learning and memory processes [143–145]. Several of these processes involve neurotransmitters like glutamate and dopamine and their receptor targets (e.g., NMDARs, D1Rs). Adenosine acting at ARs is well known to modulate the release of multiple neurotransmitters, including glutamate and dopamine, in brain regions relevant for cognition including hippocampus and striatum [40]. Several lines of evidence suggest that ARs participate in modulating different forms of plasticity.

3.1.3.1. A1 receptor modulation of synaptic plasticity. In hippocampus, purines such as adenosine, ATP, and cAMP were shown to negatively modulate glutamatergic transmission via an A1R-dependent mechanism since both selective A1R antagonism and A1R KO eliminated this inhibitory effect of adenosine [74,146]. In the Schaffer-collateral CA3-CA1 pathway, NMDAR-dependent LTP elicited by either tetanic stimulation or theta-burst as well as CA1 LTD were unaffected by A1R KO [147], thus suggesting that A1Rs do not crucially participate in CA3–CA1 hippocampal post-synaptic plasticity. These A1R KO mice did, however, exhibit impaired (nearly absent) paired pulse facilitation, which is consistent with a pre-synaptic locus of short-term plasticity control by A1Rs at this synapse. This set of outcomes in A1R KO mice is in stark contrast to abundant pharmacologic evidence using A1R agonists and antagonists that supports the hypothesis that endogenous adenosine can stimulate A1Rs to modify excitatory synaptic transmission in terms of pre-synaptic glutamate release or post-synaptic NMDAR responses [1,4,148–150] and attenuate several measures of synaptic plasticity including LTP, LTD, and depotentiation in the CA1 region of hippocampus *in vitro* [151–155]. At hippocampal mossy fiber synapses, basal pre-synaptic A1R activation by endogenous adenosine was suggested to underlie activity-dependent synaptic plasticity through tonic suppression of basal release probability [156]. Removal of the adenosine tonus by pre-treatment with the A1R antagonist DPCPX, genetic A1R knockout, or enzymatic degradation by ADA selectively augmented mossy fiber basal transmission [156]. Importantly, these manipulations individually attenuated *in vitro* measures of both short-term plasticity (e.g., frequency facilitation and paired pulse facilitation) and LTP (a NMDAR-independent process) at this synapse, effects that could be bypassed by bath application of a GABA agonist [156]. That A1R inactivation depresses frequency facilitation at this synapse was recently replicated *in vivo* in freely moving rats [157]. It has been suggested that some of these depressant effects of adenosine on mossy fiber synaptic transmission may involve pre-synaptic A1R stimulation and the direct inhibition of pre-synaptic calcium channels [158]. The generally opposite effects of A1R inactivation on mossy

fiber synapses compared to CA3-CA1 synapses are suggested to arise from differences in the nature of plasticity at these synapses; i.e., pre-synaptic NMDAR-independent *versus* post-synaptic NMDAR-dependent, respectively [156]. In contrast to the above findings, however, the magnitude of frequency facilitation, paired-pulse facilitation, or post-tetanic potentiation at mossy fiber synapses was unaffected by A1R knockout or A1R antagonism in another *in vitro* study [159]. This led the authors to conclude that the basal release probability likely does not require local adenosine or the critical control of A1R activation at mossy fiber synapses. These discrepancies were, in part, attributed to some differences in experimental conditions, and additional studies are required to clarify this issue.

In striatum, evidence in cortico-striatal brain slice preparations from gb-A1R KO mice suggests that A1Rs may predominantly mediate adenosine's modulation of NMDAR-triggered LTD: A1R gene deletion nullified fEPSP/PS depression elicited by application of either NMDA or adenosine [160]. This result in gb-A1R KO mice is inconsistent with an earlier pharmacologic study which reported that induction of short-term depression but not LTD at these synapses was blocked by A1R antagonism and triggered by A1R agonism [161]. Thus, the nature of A1R modulation on LTD or other forms of striatal plasticity remains unclear.

The discrepancy between pharmacologic and genetic A1R manipulation in hippocampus and striatum may be partly due to compensatory developmental effects in gb-A1R KO mice and partly due to the complex action of A1Rs in different cellular (e.g., neuronal *versus* non-neuronal) or subcellular (e.g., pre-synaptic *versus* post-synaptic) elements and brain regions, all with potentially different effects. Therefore, A1R's modulation of synaptic plasticity in hippocampus and striatum is likely complex and may depend on the local milieu and which subset of A1Rs is activated to guide its outcome; i.e., enhancement or attenuation of synaptic plasticity.

3.1.3.2. A2A receptor modulation of synaptic plasticity. A critical role for A2ARs in a unique form of post-synaptic NMDAR-dependent LTP but not pre-synaptic plasticity at hippocampal mossy fiber synapses has been recently demonstrated at the pharmacologic level [162]. Intriguingly, a recent pharmacologic study recording activity in live, behaving mice demonstrated the inhibitory impact of A2AR blockade on the fEPSP slope at the CA3-CA1 synapse and the conditioned response behaviors in a trace eyeblink conditioning paradigm [163]. These *in vivo* measures were confirmed in the same study in which experimentally evoked LTP was also reported to be abolished by the selective A2AR antagonist SCH58261. Some additional pharmacologic evidence suggests that A2AR stimulation, at least in the Schaffer-collateral pathway, may augment BDNF-induced LTP in hippocampus [48]. Indeed, the only known study examining hippocampal LTP in A2AR KO mice confirmed this effect by demonstrating a loss of BDNF-induced LTP and reduced BDNF levels in hippocampus of gb-A2AR KO mice and that of wildtype mice treated with the selective A2AR antagonist ZM241385 [49]. This study suggested that tonic activation of A2ARs is required for maintaining normal levels of BDNF tone in hippocampus and for facilitating BDNF-induced LTP at this synapse. Interestingly, recent evidence using A2AR ligands suggests that A2AR activation in hippocampus may also potentiate BDNF-induced responses including CA3-CA1 LTP through recruitment of TrkB receptors to lipid rafts [164].

In addition to affecting hippocampal long-term plasticity, several studies using slices obtained from gb-A2AR KO mice suggest that A2ARs are capable of modulating cortico-striatal plasticity. Specifically, the induction of LTP but neither the basal synaptic transmission nor the paired-pulse facilitation index of pre-synaptic function at cortico-accumbal synapses was attenuated in gb-A2AR KO mice compared to wildtype control mice [165]. This phenotypic profile was recapitulated by treatment with either an A2AR antagonist or a specific PKA inhibitor, thereby suggesting that the A2AR-dependent LTP process occurs through a PKA-dependent pathway. This finding agrees with a recent report that LTP at glutamatergic synapses onto striatopallidal MSNs requires A2AR activation since pharmacologic A2AR blockade abolished spike timing-dependent LTP in this neuronal population [166]. Furthermore, dominant-negative A2AR mutant constructs as well as pharmacologic manipulations in BAC transgenic mice to tag D1R-expressing direct and D2R-expressing indirect pathway MSNs led to the proposal that A2ARs and FGF receptors synergize to facilitate a NMDAR-dependent cortico-striatal LTP in striatopallidal MSNs through activation of MEK1/2 and subsequent ERK1/2 phosphorylation [50]. To date, we are unaware of any genetic A2AR knockout studies specifically examining LTD in striatum, although A2AR agonism was reported to produce at best, inconsistent effects on cortico-striatal short-term depression and LTD [161]. Interestingly, A2ARs interact with both D2Rs and CB1Rs, which are regarded as important mediators of striatal LTD [47,167–169]. It would be interesting to examine cortico-striatal LTP and LTD in the recently developed st-A2AR KO mice and the concomitant impact of D2R or CB1R pathway manipulations.

3.1.3.3. A3 receptor modulation of synaptic plasticity. Some pharmacologic work suggests that A3Rs may modulate synaptic plasticity in the CNS. A selective A3R agonist was shown to only exert effects on synaptic transmission through antagonizing A1R-mediated inhibition of excitatory neurotransmission at hippocampal Schaffer-collateral CA3-CA1 synapses [170], an effect that may result from the partial specificity of the drug which evidence suggests can bind to native A1Rs [59,94,95]; no direct effects of A3R stimulation on CA1 EPSPs, paired pulse facilitation, or LTP were reported [94,170]. On the other hand, pharmacologic A3R stimulation alone selectively increased theta-burst LTP [94] and blocked pre-synaptic $mGlu_5R$ inhibitory effects on fEPSPs [171] at hippocampal CA3-CA1 synapses as well as blocked EPSPs in pyramidal cells of rat frontal cortex [92]. These effects were blocked by treatment with a selective A3R antagonist. That A3R antagonism alone had no effect suggests that at least under normal physiologic conditions, endogenous adenosine is not acting at A3Rs to significantly affect synaptic plasticity in these brain regions. In contrast, another study showed that A3R agonism could not modify hippocampal synaptic transmission either alone or through effects on A1R-mediated functions [59]. The functional significance of A3Rs in the CNS, particularly on neuronal plasticity, however, has been largely unexplored using A3R KO mice. Recently, the induction of hippocampal Schaffer-collateral CA3-CA1 LTP [97] or EPSC depression [172] by the chemokine CX3CL1 was shown to be prevented in A3R KO mice. Therefore, the function of A3Rs in modulating synaptic plasticity remains muddy and may or may not involve A1Rs in hippocampus.

3.1.4. Cognition

The notion that adenosine potentially modulates cognition probably arises from the general belief that consumption of caffeine, the most widely used psychoactive compound, improves cognitive performance in humans. Evidence for a strictly pro-cognitive effect of caffeine, however, is inconsistent and may reflect caffeine's impact on other processes such as arousal, attention, and mood (e.g., during withdrawal), which can in turn influence performance on cognitive tasks [173]. Recent work in transgenic mice possessing low adenosine tone in the brain due to over-expression of adenosine kinase (ADK) demonstrated profound deficits in spatial reference and working memory and in cued fear conditioning [113], thus providing strong genetic evidence that adenosine regulates cognition under physiologic conditions. These behavioral phenotypes were also accompanied by perturbations in their response to dopaminergic and glutamatergic compounds, suggesting that adenosine-mediated modulation of these respective pathways contributes to its cognitive profile.

As discussed in the previous section, adenosine acts mainly at A1Rs and A2ARs to modulate neurotransmitter systems, neuronal

excitability, and synaptic plasticity (e.g., LTP and LTD) in brain regions relevant for learning and memory. The precise contribution of A1Rs and A2ARs to adenosine's regulation of cognitive functions, however, remains to be established. Traditionally, A1Rs were largely thought to execute adenosine's potential modulatory effects on cognition because of its relative abundance in regions classically studied in learning and memory like hippocampus. Studies in AR knockout mice in the realm of cognition are only beginning to reveal the complexities and vastness of adenosine's functions in brain. The importance of A2ARs for some forms of learning and memory is now gradually reaching the forefront. The following sections provide an overview of the impact of genetic manipulation of ARs on learning and memory performance, emotional regulation, and sensorimotor processing. Only by considering all these cognitive phenotypes together can one gain a balanced and more accurate insight into ARs' modulation of cognition.

3.1.4.1. Learning and memory performance. Earlier work, largely using antagonists and agonists, has suggested a role for adenosine and its receptor targets in learning and memory, but these findings have been inconsistent. It is possible that this diverse array of findings reflects the different contributions of the different AR subtypes in distinct brain regions, all of which are more difficult to precisely target using only pharmacologic tools or even a global AR KO strategy. The current set of brain region-specific AR KO mice provide the first set of tools to begin dissecting the contribution of the different AR subtypes in different brain regions on several types of learning and memory processes. In addition to probable differences in brain regional contributions by different AR subtypes, these apparent inconsistencies may also likely reflect differences in the timing of the pharmacologic manipulation across studies, a factor that the currently available brain region-specific AR KO models cannot adequately address. Alternative strategies such as inducible/reversible, conditional AR knockout or expression models or well-timed and locally delivered selective pharmacologic and/or genetic agents would be required to provide greater temporal (i.e., within a timescale more suitable for learning and memory testing) and regional specificity of AR inactivation. Lastly, differences in the behavioral tasks and cognitive domains examined, both of which are generally restricted in number and scope, may also contribute to the apparent discrepancies. Therefore, a comprehensive and systematic assessment of the impact of each AR subtype in each relevant brain region (e.g., hippocampus, cortex, striatum) on the multiple phases of learning and memory (e.g., encoding, storage, consolidation, retrieval) using different behavioral tasks to evaluate distinct cognitive domains is necessary to adequately dissect the nature of AR-modulation of learning and memory. This is a formidable endeavor that now seems to be underway and gaining momentum.

3.1.4.1.1. A1 receptors in learning and memory. Evidence in A1R KO mice suggests that A1Rs may not play as critical a role as once believed in mediating some of the mnemonic effects of adenosine. Of note, global deletion of the A1R failed to produce any performance effect in the water maze in three separate experiments from two different knockout mouse lines [76,147,174]. In these studies, gb-A1R KO mice showed normal acquisition and retention of a spatial reference memory, normal spatial working memory, and normal ability to learn the new position of a fixed platform during reversal learning. These findings in gb-A1R KO mice suggest that the A1R receptor is not critical for the expression of normal spatial reference memory or working memory under physiologic conditions. However, old gb-A1R KO mice (19 months old) were reported to show spatial working memory deficits in the 6-arm radial tunnel maze [147], a finding that was consistent with that from an earlier pharmacologic study showing that hippocampal A1Rs influence working memory [175]. This earlier finding, however, was attributed to reduced test environment habituation rather than to a mnemonic process [147]. Interestingly, in disease models, A1R stimulation has been shown to prevent scopolamine-induced working memory deficits [176] while A1R blockade has been shown to prevent morphine-induced impairment in the retrieval of a spatial reference memory [177]. These findings suggest that A1Rs may gain relevance under certain pathologic conditions. It should be noted that these studies in gb-A1R KO mice were all on the background of potentially confounding alterations of emotional processing/behaviors; these changes will be reviewed in the next section (see section 3.1.4.2).

3.1.4.1.2. A2A receptors in learning and memory. A growing body of evidence now suggests that A2ARs may play an important role in adenosine's modulation of learning and memory. Transgenic and knockout studies have recently provided some of the direct evidence that A2ARs are major players in adenosine's control of working memory. For example, gb-A2AR KO mice showed improved spatial recognition memory in an elevated Y-maze, but this effect might instead be explained by maze hypoactivity [178]. We recently also showed that similar deletion of A2ARs (on a different genetic background) selectively enhanced working memory performance in both the spatial water maze and radial arm maze [179]. Importantly, these effects were unaccompanied by changes in spatial reference memory performance or activity assessed by swim speed in the water maze and distance traveled in the radial arm maze. A2AR-dependent modulation of working memory is also consistent with the finding that transgenic rats over-expressing A2ARs in brain exhibited impaired working memory in several behavioral paradigms including the water maze, 6-arm radial tunnel maze, and novel object recognition tasks [88]. These phenotypes were also selective, with spatial reference memory, motor function, and anxiety-like behavior left intact. Since working memory is typically thought to require intact cortical as well as striatal function, these findings in rodents with global changes in A2AR gene expression illuminate the possibility that A2ARs in cortex, despite their relative low expression in brain, can produce functionally significant outcomes on learning and memory. This notion is supported by our recent conditional knockout mice study showing that striatal and extra-striatal (i.e., cortical and/or hippocampal) A2ARs exert opposite effects on motor stimulation induced by cocaine or phencyclidine [87], which suggests that extra-striatal and not striatal A2ARs direct the principal outcome of A2AR stimulation. To obtain a broader understanding of A2AR modulation of learning and memory, the role of A2ARs should be explored in brain region-specific A2AR knockout mice.

A2ARs in striatum, namely those on post-synaptic striatal neurons, have also been recently demonstrated to critically modulate learning and memory and notably habit formation [180]. In this study, st-A2AR KO mice were trained in an instrumental learning task to learn to lever press at a stable level that in wildtype mice had become habitual. While st-A2AR KO mice successfully learned to lever press, their behavior remained sensitive (i.e., more goal-directed/flexible) to both devaluation and reversal/omission procedures, as evidenced by the st-A2AR KO mice readily reducing their lever presses. These results suggest that st-A2AR KO mice exhibited weaker habit formation compared to wildtype mice, a result that is consistent with impaired cortico-striatal LTP in gb-A2AR KO mice [165]. Thus, this targeted knockout approach allowed for the identification of a novel role for A2ARs in habit formation and further localized their effect to striatopallidal pathway neurons. The mechanism behind this phenotype might relate to A2AR interactions in striatum with the dopaminergic or endocannabinoid systems, which are both linked to habit learning [181,182] and capable of affecting striatal plasticity [145]. In light of this phenotype in st-A2AR KO mice and the observation that working memory is enhanced in gb-A2AR KO mice, weaker habit learning in the face of deficient striatal A2AR activity may in fact partially explain a greater flexibility in response patterns, or *vice versa*. Additional studies will be necessary to elucidate these possibilities.

Thus, findings from genetic A2AR studies generally support the notion that suppression of A2AR activity is pro-cognitive and raise the possibility that the A2AR may represent a target for improving cognitive function under normal and pathologic conditions, as first suggested by Cunha and Agostinho [183]. That A2AR blockade reverses memory impairment caused by various brain insults from aging [184], beta-amyloid deposition [185,186], or spontaneous hypertension [187] is encouraging and suggests that the A2AR antagonists may present a therapeutic strategy for ameliorating memory dysfunction under these pathologic conditions.

3.1.4.1.3. A3 receptors in learning and memory. Studies of A3R modulation of learning and memory in knockout mice have not yet been reported. However, the A3R agonist IB-MECA was reported to reduce scopolamine-induced deficits in spontaneous alternation in the Y-maze and in passive avoidance via an A1R-independent mechanism [188]. Given the impact of A3R KO on cytokine-elicited electrophysiologic changes in hippocampus, it will be interesting to see whether hippocampal-dependent task performance is altered in A3R KO mice, particularly in disease models.

3.1.4.1.4. Summary. Studies using AR knockouts have provided some clarification of the potential impact of A1Rs and suggest an important role for A2AR signaling on some types of learning and memory. Nonetheless, additional studies are needed to examine other forms of learning and memory and to provide brain regional specification as well as temporal dissection of each AR's functions.

3.1.4.2. Emotional and sensorimotor regulation. Adenosine is thought to modulate anxiety, exploration, aggressive behaviors, depressive responses, and sensorimotor gating. The following sections provide an overview of the impact of AR knockout on these behaviors. Adenosine's impact on anxiety has recently been thoroughly reviewed and thus will only briefly be discussed here [189].

3.1.4.2.1. Anxiety. The anxiogenic effects of caffeine are largely attributed to A1R antagonism [190]. Indeed, studies in A1R KO mice have generally revealed consistent phenotypes that are suggestive of augmented emotional reactivity. For example, gb-A1R KO mice have been shown to exhibit more anxiety-like behaviors in a variety of tasks including the elevated plus maze, light-dark test, novel open field, emergence test, and o-maze [74,76,174]. These mice were also reported to exhibit emotional instability as reflected by increased wall hugging behavior in response to reversal in a water maze task [174]. Yet, it remains unclear whether these mild anxiogenic phenotypes reflect changes in arousal since gb-A1R KO mice also exhibited reduced habituation to more familiar environments [98,147].

The possibility that the A2AR, another target of caffeine, might modulate anxiety and related behaviors is supported by a positive correlation between the acute anxiogenic effects of caffeine and specific A2AR gene polymorphisms in humans [191] and the observation that the A2AR gene may be linked to panic disorder [192]. However, unlike A1Rs, the role of A2ARs in modulating anxiety-like states in transgenic animal studies is less consistent. While mice on a CD1 background with global A2AR deletion were initially reported to respond more to anxiogenic stimuli [80], basal anxiety-like behavior did not differ between A2AR KO and wild-type mice in another study [193], although the A2AR KO mice in the latter study were reported to exhibit greater sensitivity to the anxiolytic effect of ethanol. This lack of consistency might be explained by potential compensation in mice globally lacking the A2AR. Indeed, it has been shown in gb-A2AR KO mice on a CD1 background that purinergic utilization, as determined by the density of nucleoside transporters and A1Rs, is altered in brain regions like hippocampus or hypothalamus that are relevant for anxiety and arousal [194].

These changes in adenosine levels in this gb-A2AR KO mouse line might also affect anxiety through actions at A3Rs. Nevertheless, although A3R KO mice showed some evidence of an anxiety phenotype on the elevated plus maze and light-dark box, these findings were largely attributed to the hyperactive phenotype which was noted in the novel open field, elevated plus maze, and light-dark box [195].

3.1.4.2.2. Aggression. Adenosine may also impact aggressive behaviors. This has been observed in both global A1R and A2AR KO mouse lines, with knockout mice showing more aggression than their wildtype controls in a resident-intruder test [74,80]. It is also possible that these aggressive phenotypes may simply reflect anxiety status.

3.1.4.2.3. Depression. Adenosine's potential role in depressive behaviors, as in anxiety-like phenomena, stems largely from clinical studies in which patients with major depression were found to have reduced serum ADA activity (thus increasing adenosine tone), which was inversely correlated with disease severity [196]. Both selective A2AR antagonists as well as global A2AR deletion have been shown to reverse signs of behavioral despair in the tail suspension and forced swim tests independently of its hyperlocomotor effect [197]. However, A2AR and A1R antagonists were also shown to reverse adenosine's anti-depressant effects while A2AR and A1R agonists mimicked adenosine's actions [198]. On the other hand, A3R KO mice exhibited heightened signs of behavioral despair, spending more time immobilized in both the tail suspension and forced swim tests [195].

3.1.4.2.4. Sensorimotor processing. A2ARs are most abundantly expressed in striatum, including nucleus accumbens, which is a critical locus for prepulse inhibition (PPI), a measure of sensorimotor gating function [199]. A2ARs might therefore be expected to contribute to PPI expression. Indeed, pharmacologic evidence has shown that A2ARs in nucleus accumbens are important modulators of PPI [200], and A2AR agonists can reverse the apomorphine-induced PPI deficit [201]. In mice where A2ARs were globally deleted, startle amplitude, startle habituation, and prepulse inhibition of the acoustic startle response were reduced [202]. It should be noted, however, that interpretation of the PPI deficit here may be confounded by a baseline difference in startle response among gb-A2AR KO and wildtype mice [203]. To our knowledge, PPI has not yet been examined in A1R or A3R KO mice.

3.1.4.2.5. Summary. In summary, the contributions of the AR subtypes to different types of learning and memory are gradually being unveiled. Studies in A2AR KO mice, in particular, have largely focused on typical striatum-dependent learning processes, where A2ARs are most highly concentrated. These studies have revealed novel roles for A2ARs, likely in striatum, in the control of associative learning (instrumental learning in particular), working memory, and habit learning. The influence of A1Rs on these cognitive domains is less clear. It appears that A1Rs may play a greater role in the regulation of emotional expression and processing. Collectively, the findings from these studies emphasize the importance of broadly studying the phenotypes in AR KO mice when characterizing KO mice. These studies raise the exciting possibility that A2ARs may represent a target for improving cognitive function under physiologic or pathologic states.

3.2. Adenosine receptor function in disorders of the central nervous system

3.2.1. Neuroprotection

Adenosine acting primarily at A1Rs is generally believed to protect brain tissue against multiple types of brain insults including ischemia, hypoxia, excitotoxicity, trauma, and neurodegenerative disease. On the other hand, recent evidence also suggests that adenosine acting primarily at A2ARs may contribute to neurotoxicity, neuronal damage, and cell death. The following sections describe recent findings on the neuroprotective effects of ARs in different brain injury models in an attempt to reveal the therapeutic potential of AR agents in these pathologic conditions.

3.2.1.1. Ischemia and hypoxia

3.2.1.1.1. A1 receptor role in ischemia and hypoxia. Adenosine stimulation of A1Rs is well known to exert an inhibitory effect on

synaptic transmission, largely through pre-synaptic A1R control of glutamate release. A1Rs have therefore been hypothesized to protect against neuronal damage in the face of ischemia or hypoxia or similar pathologic conditions characterized by supra-physiologic elevations of adenosine levels in brain. Although multiple studies using pharmacologic strategies have largely supported this neuroprotective benefit of A1R stimulation, studies in gb-A1R KO mice have confirmed this conclusion only in certain experimental paradigms.

The first confirmation of this neuroprotective hypothesis in gb-A1R KO mice examined responses to hypoxia induced by carbon monoxide. Compared to hippocampal slices from wildtype mice, slices from gb-A1R KO mice showed a markedly reduced and delayed protective response to hypoxia, as determined by measuring the expected dampening of fEPSPs and thus glutamatergic neurotransmission [74]. Moreover, whereas slices from wildtype mice fully recovered functionality (i.e., restoration of fEPSP responses) upon re-oxygenation of the medium, slices from gb-A1R KO mice displayed no such improvement, thus suggesting that the lack of A1Rs during hypoxia contributed to significant and lasting hippocampal neuronal impairment. The attenuation but incomplete abolishment of the response to hypoxia in gb-A1R KO mice also suggests that while A1Rs are important and critical in this pathologic process, other mechanisms regulating hypoxia's effects on neuronal damage are likely also involved. Nonetheless, additional work examining hypoxic damage in primary astrocytes prepared from gb-A1R KO and wildtype mice supports the original study with evidence of greater cytotoxicity observed in astrocytes from gb-A1R KO mice [204]. This finding is interesting because it suggests that A1Rs on non-neuronal cells might contribute to the potential neuroprotective effects of A1R stimulation.

On the other hand, A1Rs have also been demonstrated in genetic knockout models to have no effect on the level of ischemia/hypoxia-induced damage. For example, the level of brain damage induced by global ischemia was indistinguishable between gb-A1R KO and wildtype mice [205]; a similar result was also obtained from gb-A1R KO and wildtype hippocampal slices placed under *in vitro* ischemic conditions [205]. However, consistent with a neuroprotective role for A1R stimulation, wildtype mice pretreated with a selective A1R antagonist prior to the ischemic insult showed marked exacerbation of neuronal damage [205]. These data highlight the potential differences in outcomes generated by pharmacologic *versus* genetic models, and they suggest that some of A1Rs effects, at least in regard to hypoxia-induced neuroprotection, may be compensated for.

In immature brain, pharmacologic A1R stimulation has been shown to exacerbate rather than attenuate ischemic injury [206]. Consistent with this surprising result, genetic deletion of A1Rs revealed neuroprotective effects in immature gb-A1R KO mice raised under hypoxic conditions; these mice were indistinguishable from wildtype mice raised under normoxic (i.e., room air) conditions when using assessments of ventriculomegaly and white matter loss as endpoints [207]. Interestingly, such hypoxia-induced changes were also reduced in A1R+/− mice. This strongly suggests that even a partial reduction in A1R activity provided some protection against hypoxia-induced injury and raises the possibility that A1R antagonism, even if incomplete, may still represent a useful strategy for treating hypoxia-based injuries during development. These “surprising” results of exacerbation of damage by pharmacologic A1R stimulation and neuroprotection by genetic A1R knockout in immature brain may also reflect the phenomenon of “effect inversion", which has been reported for the A1R in ischemia/hypoxia animal models [208]. That is, the impact of pharmacologic A1R manipulation will largely depend on the timing of the manipulation relative to the insult, where chronic *versus* acute drug administration can produce opposite effects. While acute A1R agonism or chronic A1R antagonism can be neuroprotective, chronic A1R agonism or acute A1R antagonism can exacerbate ischemic injury [208]. In contrast to inactivation by A1R ligands, constitutive gb-A1R KO produces a persistent inactivation of the receptor. Therefore, the neuroprotective effect of gb-A1R KO in this particular study, which was not observed in the pharmacologic studies showing a different outcome, may relate to the chronicity of the A1R deletion and the timing of the insult.

From this brief overview, compared to the generally believed protective role of A1Rs, which is supported by studies using pharmacologic tools, evidence from genetic A1R deletion studies using knockout mouse models only partially confirm the potential for A1R activation as a neuroprotective strategy—a neuroprotective effect of A1Rs is only observed in certain experimental paradigms, and in other paradigms, a relatively complex and contradictory role for A1Rs in neuroprotection is revealed. Specifically, studies in A1R KO mice have demonstrated protection, no protection, or exacerbation of damage in ischemia/hypoxia models of brain injury. This may partly reflect the different role of A1Rs in various stages of development. Depending on the particular brain injury model used, as well as the age of the animal at the time of insult, it appears that A1Rs may produce a variable effect when it comes to neuroprotection following brain ischemia and/or hypoxia. Possible compensatory mechanisms in knockout animals may also mask the consequences of A1R deficiency or loss. Additional research will need to conscientiously control for various factors and dissect out the contributions of each before A1Rs can be brought to the clinic as a pharmacotherapeutic strategy.

3.2.1.1.2. A2A receptor role in ischemia and hypoxia. A2ARs, like A1Rs, have also been shown by multiple studies to assume an important role in animal models of focal and global ischemia. In fact, due to the relatively reduced potential for peripheral side effects, A2ARs have been considered a more attractive target for therapeutic exploration. Several pharmacologic studies using first generation A2AR antagonists have demonstrated that A2AR blockade confers neuroprotection in animal models of brain ischemia [209–211]. Evidence from gb-A2AR KO mice has overcome the concern of potential non-specific effects inherent to pharmacologic approaches, and it strongly supports that A2AR blockade might confer neuroprotection against brain damage induced by transient focal ischemia [81]. Specifically, gb-A2AR KO significantly reduced the brain damage and functional neurologic sequelae produced by transient middle cerebral artery occlusion (MCAO). However, similar to observations from A1R studies, the effect of A2AR blockade on neuroprotection against ischemia/hypoxia when examined in immature animals differed from that observed in adult animals. Using a cerebral hypoxia ischemia model in seven-day-old mice, for instance, gb-A2AR KO was found to exacerbate brain injury and produce impairments in several behavioral indices of motor function [137]. This “surprising” result suggested that A2AR stimulation, not blockade, might also play a protective role under the circumstance of neonatal ischemic/hypoxic brain injury. Therefore, when studying and interpreting the potential neuroprotective effects of ARs, one should consider the developmental stage of the animal (e.g., brain slices and primary cell cultures derived from early developmental stages).

Since A2ARs are not only expressed at high levels in neurons, but are also highly expressed in inflammatory cells and glial cells, the possibility that A2ARs on inflammatory cells contribute to adenosine's action in brain injury was subsequently explored. The nature of A2AR's involvement in neuroprotection after ischemia/hypoxia was examined using a chimeric mouse model in which A2ARs on bone marrow-derived cells (BMDCs) were selectively inactivated or reconstituted by bone marrow transplantation [212]. This procedure permitted the isolation of A2AR's effects to this particular cell type. Selective deletion of A2ARs in BMDCs captured the neuroprotective effect observed in gb-A2AR KO mice while selective reconstitution of A2ARs in BMDCs reinstated the brain damage. Thus, A2ARs on BMDCs also contribute to neuroprotection against ischemic/hypoxic insult, and targeting A2AR blockade to these cell types might offer an effective therapeutic strategy against ischemic brain injury.

3.2.1.1.3. A3 receptor role in ischemia and hypoxia. Despite the low expression of A3Rs in the CNS, several studies have attempted to gain

insight into the effects exerted by A3Rs [93]. The neuroprotective function of A3Rs was first demonstrated using mice with genetic deletion of the A3R [195]. Although all mice exhibited histologic and morphologic changes in hippocampus following repeated bouts of hypoxia (induced by carbon monoxide), A3R KO mice displayed a more pronounced loss of CA2-3 hippocampal pyramidal neurons. This effect of A3R KO was mimicked by the repeated administration of an A3R-selective antagonist to wildtype mice and was also accompanied by deficient hippocampal-dependent contextual fear conditioning. These data suggest that A3R agonists might afford protection against hypoxia-induced neuronal damage. Consistent with this, A3R KO mice were shown in another study to also exacerbate cerebral infarction induced by MCAO, and wildtype but not A3R KO mice benefited from pre-treatment with an A3R selective agonist [213]. The mechanism of A3R agonist-induced neuroprotective effects in hypoxia is unclear but may involve the suppression of apoptosis [213] or may involve non-neuronal targets. The latter possibility is supported by recent evidence that glial cells engineered to lack the A3R were more sensitive to hypoxia than were wildtype cells [204]. These data also pointed to a cytoprotective role of adenosine that is mediated by both A1Rs and A3Rs in primary mouse astrocytes. Collectively, these data highlight the neuroprotective potential of A3R agonists.

3.2.1.2. Parkinson's disease (PD). The high abundance of A2ARs in striatopallidal neurons, coupled with its ability to interact antagonistically with D_2Rs and its motor improving effects, has led to the proposal that A2ARs represent a non-dopaminergic target for PD treatment [214–217]. In recent years, epidemiologic and complimentary animal studies have provided evidence that raised the exciting possibility that A2AR blockade might present the additional benefit as a neuroprotective strategy to slow or halt dopaminergic neuronal degeneration [216–219]. As result, the A2AR is emerging as a leading, non-dopaminergic drug for the treatment of PD, and several A2AR antagonists have entered and completed clinical phase II and III trials for advanced PD patients, thus confirming their motor benefits [220–224]. These developments have been expertly reviewed recently [216,217,219,222,225]. Therefore, we only provide a brief overview of the genetic A2AR knockout studies in animal models of PD.

Studies using genetic A2AR knockout models have contributed to this exciting development by demonstrating the A2AR antagonists exert dual benefits through several distinct mechanisms—A2AR antagonists act at striatopallidal neurons to enhance motor activity and probably act through other mechanisms to exert their neuroprotective effect against dopaminergic neurodegeneration [138]. By selectively deleting A2ARs in forebrain neurons [138], or more selectively, in striatal neurons [87], our studies provide definitive evidence that A2AR antagonists act at striatal neurons to enhance motor activity. These findings not only confirm the early pharmacologic [226,227] and genetic studies, but also localize this therapeutic effect of A2AR antagonists to striatal neurons. Furthermore, A2AR knockout work has provided important evidence supporting the neuroprotective property of A2AR antagonists in animal models of PD and has also provided a neurobiological basis for the inverse relationship between human caffeine consumption and reduced PD risk [228,229]. Using gb-A2AR KO mice, we showed that A2AR loss conferred protection in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD [218]. The neuroprotective effect of A2AR inactivation was also observed in PD models induced by the toxin 6-hydroxydopamine (6-OHDA) and more recently by transgenic expression of alpha-synuclein (Schwarzschild et al., SFN abs. 2009).

Despite the consistent demonstration of neuroprotection by A2AR inactivation, the mechanism by which A2AR inactivation protects dopaminergic neurons is still largely unknown. Until recently, perhaps one of the main obstacles has been the uncertainty and scarcity of direct evidence regarding whether A2ARs are expressed at appreciable levels in dopaminergic neurons (early studies failed to detect A2AR expression in these neurons), despite several functional studies showing that A2AR agonists and antagonists could modify dopamine release in striatum [230,231]. Recently, A2AR-immunoreactivity in TH- or DAT-immunopositive dopaminergic terminals in striatum was demonstrated in purified nerve terminal preparations [232], thereby providing a potential direct mechanism by which A2ARs can impact the survival of dopaminergic neurons in PD models.

In recent years, the contribution of A2ARs in different brain regions and cell types has been specifically examined. Recently, we showed that selectively inactivating forebrain neuronal A2ARs was not sufficient to confer neuroprotection against acute MPTP exposure, despite being able to eliminate the motor stimulant effects of the selective A2AR antagonist KW-6002 [138]. Only when A2AR antagonists were delivered via intracerebroventricular injection to fb-A2AR KO mice could a neuroprotective effect be observed in these mice [138]. This observation strongly suggested that A2AR antagonists act at non-forebrain neurons to exert a neuroprotective effect. However, a recent study using the same fb-A2AR KO mouse line showed complete prevention of dopamine neuron degeneration and gliosis in substantia nigra pars compacta and partial prevention of gliosis in striatum following sub-chronic MPTP administration [233]. The divergent observations derived from the same fb-A2AR KO mouse line in these two studies are likely the result of differences in the experimental paradigm such as the duration of MPTP toxin exposure (i.e., acute *versus* subchronic). The impact of different MPTP paradigms, however, still needs further assessment.

In addition to their potential neuroprotective benefits, A2AR antagonists may possess anti-tremor and anti-dyskinesic properties. Indeed, A2AR antagonists have recently shown promise as an alternative non-dopaminergic PD therapeutic drug [214,234]. We limit our discussion here to the potential anti-dyskinesic effects since, to our knowledge, A2AR's impact on animal models of PD tremor has only been examined using pharmacologic tools [235–238]. Although pharmacologic dopamine replacement with L-dopa remains the mainstay drug treatment for PD [239,240], up to 75% of patients will develop disruptive dyskinesias, coined L-dopa-induced dyskinesia (LID), within ten years of starting L-dopa therapy [241]. While several studies suggest a role for A2ARs in the development and maintenance of LID, the ability of A2AR antagonists to modify LID remains unclear: co-administration of KW-6002 with L-dopa failed to alter LID in rats [242] and non-human primates [243,244], and more importantly, clinical data now suggest that KW-6002 may slightly increase dyskinesia [220,221,223,224,245]. While some discrepancies may relate to the dose of L-dopa used (since KW-6002 may permit the use of a lower and therefore less dyskinesogenic dose of L-dopa [220]), they might also be partially explained by different effects of A2ARs in distinct relevant brain regions or cellular elements.

Recent AR knockout studies have now begun the process of dissecting out their contribution to LID. In the 6-OHDA PD model, gb-A2AR KO or fb-A2AR KO mice exhibited attenuated contralateral rotational sensitization to repeated L-dopa treatment [246–248], an effect that was paralleled by reduced abnormal involuntary movements (AIMS measure of LID) and striatal preproenkaphalin mRNA [247,248]. A similar phenotypic profile was observed in A1R KO mice but not in A1R-A2AR double knockout mice [248]. These findings in multiple genetic knockout mouse lines collectively suggest that blockade of either A2ARs (likely in forebrain neurons) or A1Rs may prevent or reduce LID. The mechanism behind this effect nonetheless remains elusive: while blockade of A2ARs on post-synaptic striatopallidal neurons accounts for the motor stimulant effect of A2AR antagonists, this cannot account for the observed anti-dyskinesic effect in gb-A2AR KO or fb-A2AR KO mice. Additional studies, which further break down the contribution of A2ARs in different brain regions and cellular elements, will be necessary. Moreover, whether

the anti-dyskinesic effect of A2AR gene deletion can be consistently demonstrated by A2AR antagonists still requires further investigation.

3.2.1.3. Huntington's disease (HD). The enrichment of A2ARs to striatum and the cellular localization of these receptors to striatopallidal neurons (the most susceptible neurons undergoing neurodegeneration in HD) indicated that A2ARs might also contribute to the pathologic processes underlying HD [225,249]. This possibility is supported by the observation that A2AR expression is reduced in HD patients [250]. Several studies have now examined the consequences of pharmacologic and/or genetic A2AR inactivation on striatal damage produced by the mitochondrial complex II inhibitor 3-nitropropionic acid (3-NP), a toxin model for HD, and in R6/2 mice, a genetic model of HD. Evidence from pharmacologic studies showed that both A2AR agonists and antagonists were capable of producing neuroprotection in HD models. For example, five-week repeated treatment with the A2AR agonist CGS21680 at postnatal week seven was shown to prevent striatal atrophy, ventricular enlargement, and HTT aggregations in R6/2 mice [251]. In agreement, A2AR activation in cultured cells protected against HTT-induced neuronal cell death through a cAMP-PKA pathway [252]. On the other hand, the A2AR antagonist SCH58261 has also been shown to reduce behavioral abnormalities and neurochemical changes in R6/2 mice [253,254]. In a recent study, A2AR inactivation by chronic administration of SCH58261 significantly increased the number of nNOS-immunoreactive striatal neurons in R6/2 mice, thus also suggesting a protective effect of A2AR antagonism in the HD model [255]. In agreement with this notion, 3-NP-induced striatal damage was attenuated by treatment with A2AR antagonists [256]. This dichotomous profile might reflect the opposite effects of A2ARs located at pre-synaptic (exacerbating role due to stimulatory effects on glutamate release) *versus* post-synaptic (protective role) striatal sites as well as the striatal sub-region affected [257].

Similar to pharmacologic studies, A2AR KO mouse models have also yielded a complex picture of A2AR modulation of striatal neuronal death in HD. Knockout mice lacking A2ARs everywhere were reported to either attenuate or exacerbate striatal damage depending on the dose of 3-NP insult [257]. Another study, however, reported exacerbation of 3-NP striatal damage as well as neurologic deficits in gb-A2AR KO mice but not in fb-A2AR KO mice [83]. This exacerbatory phenotype was attributed to A2ARs on BMDCs since transplantation of bone marrow cells derived from gb-A2AR KO mice to wildtype mice recapitulated the level of striatal damage observed in gb-A2AR KO alone following 3-NP insult. Together, these findings in genetic and pharmacologic models indicate that the effect of A2ARs in HD is also complex, both at the synaptic and cellular levels. Further investigation is needed before A2AR-targeted strategies can be used to treat HD patients.

3.2.1.4. Traumatic brain injury (TBI). Extracellular adenosine increases rapidly and dramatically during traumatic brain injury (TBI) and may modulate the cascade of secondary damaging effects including excitotoxicity and inflammation triggered by TBI. Recent studies have evaluated the effects of A2ARs on TBI using an A2AR KO model. Global deletion of A2ARs was found to protect against acute cortical impact injury as assessed by brain water content, histology, cell death, and neurological deficit scores [258]. These effects were associated with a suppression of glutamate and inflammatory cytokine levels. A later study extended these findings by using selective inactivation of A2ARs on BMDCs to demonstrate that A2AR loss on either BMDCs or non-BMDCs was sufficient to confer protection against TBI [259]. These studies suggest that control of TBI by A2ARs can occur at multiple cellular targets and via multiple cellular mechanisms. Interestingly, using this same paradigm, A2ARs were found capable of producing dichotomous effects on TBI responses and outcomes in a manner that depended on local glutamate concentrations [260]. Notably, when glutamate levels were low, A2AR agonists attenuated the morphologic, behavioral, cellular, and cytokine changes induced by TBI. In contrast, when glutamate levels were high (as is typically the case following TBI), A2AR antagonists (not agonists) were found to produce a similar protective outcome. These findings collectively raise the prospect that A2AR antagonism may represent a potential therapeutic strategy for TBI. More importantly, they bring attention to the need to consider local glutamate levels when determining the appropriate administration of select A2AR-based pharmacologic compounds following TBI.

3.2.1.5. Summary. Evidence from ischemia/hypoxia, PD, HD, and TBI all point to a potential role for adenosine, A1Rs, and A2ARs in their pathologic processes in terms of neuroprotection. Overall, genetic knockout models have confirmed the neuroprotective effect afforded by A1R activation and A2AR inactivation against various brain insults. However, the results from these lines of work also reveal that the effect of A1Rs and A2ARs on brain injury is highly context-dependent —the outcome of adenosine modulation of brain injury may depend on the mode of brain insult (e.g., acute *versus* sub-chronic), distinct cellular elements, chronologic age, extracellular glutamate concentration, and/or the different time points in which the outcomes are evaluated.

3.2.2. Neuroinflammation

Neuroinflammatory reactions participate in a wide range of acute and chronic neurodegenerative disorders. Adenosine is thought to critically contribute to inflammation and tissue damage [261], and recently, evidence has accumulated to suggest that ARs partake in brain inflammatory processes and might serve important therapeutic targets for treating diseases of the brain in which inflammation plays a key role in its etiology or progression [262]. However, as observed in studies examining AR function in neuroprotection, the influence of ARs on neuroinflammatory processes is also perplexing: pharmacologic studies have reported an anti-inflammatory outcome following either stimulation or blockade of ARs [225]. Although all four AR subtypes have been implicated in neuroinflammation to date [225], the neuroinflammatory impact of ARs has up to now only been examined in A1R and A2AR genetic knockout models, with the majority of these studies focusing largely on the role of the A2AR subtype. Therefore, we provide here an overview of the role of ARs in neuroinflammation as revealed by studies using A1R or A2AR KO mice.

3.2.2.1. A1 receptor role in neuroinflammation. Studies of neuroinflammation in gb-A1R KO mice suggest that A1R blockade exacerbates damage induced by experimental allergic encephalomyelitis (EAE) model and TBI. Firstly, gb-A1R KO mice were reported to develop a severe progressive-relapsing form of EAE [263]. This was characterized by more pronounced demyelination and axonal injury as well as greater microglial/macrophage activation in these knockout mice compared to wildtype controls. Further analysis in macrophages obtained from gb-A1R KO mice revealed increased pro-inflammatory gene expression. Moreover, significant oligodendrocyte cytotoxicity was observed upon exposure to soluble factors derived from these perturbed macrophages. These data strongly suggest that A1Rs on macrophages contribute to neuroinflammation and related cell death. Interestingly, A1R expression was down-regulated in the microglia of wildtype mice showing neuroinflammation in the EAE model, and caffeine reduced EAE severity, an effect that was accompanied by up-regulation of microglial A1Rs. Since EAE is a model for multiple sclerosis, it has been suggested that A1Rs could be targeted to limit the demyelination characteristic of this disease [263]. Secondly, a neuroinflammatory role for A1Rs has also recently been demonstrated in a cortical TBI model. In this study, the microglial response, notably Iba-1+ microglial cells, was markedly enhanced in several brain

regions including cortex, CA1 and CA3 regions of hippocampus, and thalamus of gb-A1R KO mice compared to wildtype mice [264]. Along with the demonstration that A1R stimulation inhibits microglial proliferation, these results support the notion that activation of A1Rs reduces TBI-induced neuroinflammation, likely by suppressing the microglial proliferative response. Collectively, these two gb-A1R KO studies using two different methods of damage and inflammation induction strongly indicate that A1Rs serve an anti-inflammatory function in brain and suggest that modulation of microglial activity can be therapeutic.

3.2.2.2. A2A receptor role in neuroinflammation. Evidence for AR modulation of neuroinflammation has largely focused on the A2AR subtype. Several studies using A2AR KO mice generally seem to suggest that the brain inflammatory response and/or resultant damage from different injury models (e.g., ischemia/hypoxia, TBI, MPTP) is less severe following A2AR deletion (but see discussion below), and thus point to the potential for A2AR antagonists as a therapeutic strategy for CNS disorders characterized by high levels of brain inflammation.

Using the MCAO brain ischemia model, selective deletion of A2ARs on BMDCs were clearly shown to attenuate transient MCAO-induced brain infarct volume 22 hours post-reperfusion, an effect that was associated with a reduction in the expression of several pro-inflammatory cytokines [212]. A similar anti-inflammatory profile in gb-A2AR KO mice was observed following TBI [258]. Interestingly, the duration of elevated inflammatory cytokines (TNF-α and IL-1β) was apparently shorter (peak at 12 h) in the KO mice than that in the wildtype group (peak at 24 h) following TBI, thereby revealing the complex and dynamic nature of A2AR-regulation of TBI-induced pro-inflammatory cytokines in brain. Lastly, an anti-inflammatory effect of A2AR blockade has also been demonstrated in the MPTP-PD model [138]. This effect was attributed to A2ARs on microglial cells since fb-A2AR KO failed to alter acute MPTP neurotoxicity or inflammatory status, but intracerebroventricular injection of KW-6002 in fb-A2AR KO mice produced neuroprotection and attenuated MPTP-induced striatal microglial and astroglial activation. The impact of A2AR blockade on microglial activation was confirmed by flow cytometric analysis demonstrating that A2AR antagonism largely attenuated the progression of microglial cells to a fully activated state. It is interesting to note that the broad spectrum of anti-inflammatory effects from A2AR inactivation in brain is apparently at odds with the prominent anti-inflammatory effect of A2AR activation in peripheral organs [119,265,266]. To reconcile this difference, Dai et al. recently demonstrated in microglial primary cell cultures following treatment with lipopolysaccharide and in intact animals following TBI that extra-synaptic glutamate levels can direct the switch of A2AR-mediated responses from an anti-inflammatory and neuroprotective role to a pro-inflammatory and cytotoxic role [260]. Thus, the level of glutamate released from neurons and glial cells in response to brain insults may dictate A2ARs' modulation of neuroinflammation in brain, which is distinct from their modulation in peripheral tissues. Together, these results suggest an important role for A2ARs in regulating neuroinflammation and consequent brain injury. The finding that glutamate critically controls the direction of A2ARs' modulation of neuroinflammation reveals a novel mechanism for neuronal control of inflammation through regulation of extra-synaptic glutamate to modify A2ARs' functional effects in brain.

3.2.3. Pain

Nociception involves both peripheral and central structures and mechanisms and is another important adenosine-regulated neural function. Evidence suggests a role for A1Rs, A2ARs, and A3Rs in pain regulation.

A1Rs are abundant in mouse spinal cord, with the highest levels in the outer lamina of the dorsal horns [74]. Therefore, most attention has been devoted to pain pathways involving A1Rs, which have been well documented in a wide range of animal models including both acute nociceptive tests and models of neuropathic and inflammatory pain [267]. Previous work in which A1R agonists were administered intrathecally pointed to A1R-mediation of analgesia [121]. Genetic A1R knockout models later confirmed the anti-nociceptive effects of central A1Rs and suggested that A1Rs might represent new targets for anti-nociceptive drug development since gb-A1R KO mice exhibited a faster reaction to thermal pain than did wildtype mice [74].

Unlike A1Rs which are concentrated in anatomic regions relevant for pain, A2ARs are found at low levels in pain-relevant areas such as somatosensory cortex as well as dorsal spinal cord and dorsal root ganglia. Nonetheless, it has been proposed that A2ARs in the CNS, perhaps located on pre-synaptic inhibitory terminals of descending fibers in spinal cord, may modulate pain [268]. Previous pharmacologic studies have shown that both A2AR agonists [269,270] and A2AR antagonists [271] produce anti-nociception in different pain tests. Tests in gb-A2AR KO mice have helped clarify these mixed results, showing a hypoanalgesic effect in thermal tests of pain [80]. This initial observation was later confirmed and extended to the tail immersion test, hot-plate test, and intraplantar formalin injection paradigm [100,272,273]. Thus, studies using gb-A2AR KO mice confirm that A2AR blockade may possess some therapeutic potential in alleviating certain pain states.

A3R KO mice also showed decreased sensitivity only to some painful stimuli as assessed by the increase in latency in the hot plate test [195], but no difference was detected between A3R KO and wildtype mice in the nociceptive response to painful stimuli in the tail-flick test [195] or in response to mechanical or radiant heat stimuli [274]. Hence, the exact role of A3R in modulation of various painful stimuli remains to be determined.

3.2.4. Epilepsy

Extracellular adenosine is a critical determinant of the brain's susceptibility to seizure activity [275]. This notion is recently supported by transgenic studies where over-expression (140% of normal) of ADK to reduce extracellular adenosine levels increased susceptibility to status epilepsy while reduced expression (60% of normal) offered resistance to status epilepsy [276]. Since astrogliosis is a pathologic hallmark of the epileptic brain and contributes to seizure generation through a variety of mechanisms [277–279], and since ADK is induced in astrocytes following status epilepsy, this finding provides a molecular link between astrogliosis and neuronal dysfunction in epilepsy [276].

Adenosine's effect on epilepsy is largely mediated by A1Rs since their activation reduces susceptibility to epilepsy and seizure-induced excitotoxicity while their blockade produces the opposite effect [280]. A1Rs' critical role in the development of epilepsy is validated by the demonstration of increased susceptibility to epilepsy after kainate treatment [281] or mild cortical control impact [264,282] in gb-A1R KO mice compared to wildtype mice. Studies with transgenic models of AR/kinase have thus confirmed the critical gating effect of adenosine, largely acting at A1Rs, in the development of epilepsy. Currently, adenosine delivery into epileptic brain regions is postulated as a potential treatment strategy [283].

While A1Rs play a critical role in controlling epilepsy development, the possible role of A2ARs in seizure development is less clear. Pharmacologic studies suggest that A2AR activation can either suppress [284,285] or promote seizures [286,287]. Genetic knockout studies indicate that gb-A2AR KO mice are somewhat resistant to seizure induced by ethanol withdrawal [288] and kindled seizures induced by PTZ [289]. Additional experiments are needed to clarify the exact nature of A2AR's involvement in controlling seizure development.

3.2.5. Drugs of abuse: addiction and withdrawal

The ventral striatum or nucleus accumbens is widely regarded as the brain's reward center and the link between the brain's limbic and

motor systems [290]. The addictive potential of most, if not all, drugs of abuse including cocaine, amphetamine, opiates, and ethanol has been linked to this brain region [291]. The A2AR is the most prominent AR subtype in ventral striatum where it is known to interact closely with dopaminergic and glutamatergic pathways, two neurotransmitter systems that are now generally recognized to contribute to drug addiction and reward [291–293]. Consequently, the majority of literature, particularly in studies with knockout mice, has largely described work pertaining only to A2AR effects. The following sections therefore provide an overview of the contributions of A2ARs to cocaine, amphetamine, opiate, and ethanol addiction as understood from A2AR KO studies.

3.2.5.1. Cocaine and amphetamine. Genetic A2AR KO models have linked A2ARs to some of the maladaptive responses to the indirect dopamine agonist cocaine and the dopamine releaser amphetamine, thus leading to the suggestion that A2AR antagonists might be employed to treat drug addiction. The rate of self-administration of cocaine, motivation for cocaine (i.e., reduced breakpoint), and efficacy of its reinforcing effects (i.e., vertical shift in dose-response curve) were all reduced in gb-A2AR KO mice compared to wildtype mice [294]. Locomotor sensitization and conditioned place preference, in contrast, remained undisturbed. In addition to cocaine, A2AR KO mice have also consistently shown attenuated responses to amphetamine. Both gb-A2AR KO and fb-A2AR KO mice failed to develop sensitized locomotor responses to daily amphetamine [86,295], and in gb-A2AR KO mice, this effect was associated with a lack of amphetamine-induced striatal dynorphin mRNA expression [295]. Together these findings point to an important role for A2ARs in the addictive properties of cocaine and amphetamine.

3.2.5.2. Opiates (e.g., morphine). Adenosine may act at receptor targets in different brain regions to modulate several actions of opiods including opiod dependence. Several lines of pharmacologic evidence support this notion, but are limited by the partial specificity of drugs. Multiple studies examining the reward, motivational, and withdrawal responses to morphine have been performed in gb-A2AR KO mice and suggest that A2ARs are important modulators of the rewarding and motivational properties of morphine. Gb-A2AR KO completely abolished the acute rewarding effects of morphine [296,297] and the aversive effects of morphine withdrawal [297]. These phenotypes were accompanied by a reduction in morphine self-administration and breakpoint compared to wildtype mice, suggesting that gb-A2AR KO mice were less motivated, possibly the result of morphine's reduced rewarding effects in the KO mice [296]. Gb-A2AR KO mice also failed to develop tolerance to chronic morphine in this study despite responding normally to acute morphine administration and despite demonstrating comparable locomotor sensitization to morphine relative to wildtype mice [296]. It should be noted, however, that several studies have observed enhanced withdrawal signs in gb-A2AR KO mice [100,298]. This enhancement was associated with the functional activation status of μ-receptor-stimulated [^{35}S]GTPγS binding in nucleus accumbens [298]. Together, these data highlight that A2AR antagonism might represent a therapeutic strategy for treating or preventing drug addiction.

3.2.5.3. Ethanol. Adenosine may also modulate responses to ethanol. Genetic A2AR KO studies provide some evidence that A2ARs are important for mediating some behavioral effects of ethanol, although these phenotypes were only observed in gb-A2AR KO mice generated on a CD1 background and not on a C57BL/6 background. Gb-A2AR KO mice from a CD1 background consumed more ethanol and were less sensitive to the intoxicating effects (e.g., sedation, hypothermia) of acute ethanol administration [299] as well as to its withdrawal [288]. Greater ethanol consumption among gb-A2AR KO mice was associated with weakened rewarding properties of ethanol (i.e., reduced ethanol-induced conditioned place preference) and heightened anxiolytic and acute locomotor responses; no effects on ethanol-induced conditioned taste aversion or locomotor sensitization were reported [193].

3.2.5.4. Summary. Evidence from genetic AR knockouts provides compelling evidence for A2AR modulation of phenomena underlying drug addiction. The broad inhibitory effect of A2AR knockout on the development of locomotor sensitization to amphetamine suggests that A2ARs may play a critical role in modulating dopaminergic signaling, particularly when administered intermittently. A2AR interaction with mGlu5Rs or CB1Rs might play a greater role. Interestingly, it was reported that a synergy between D2Rs, CB1Rs,

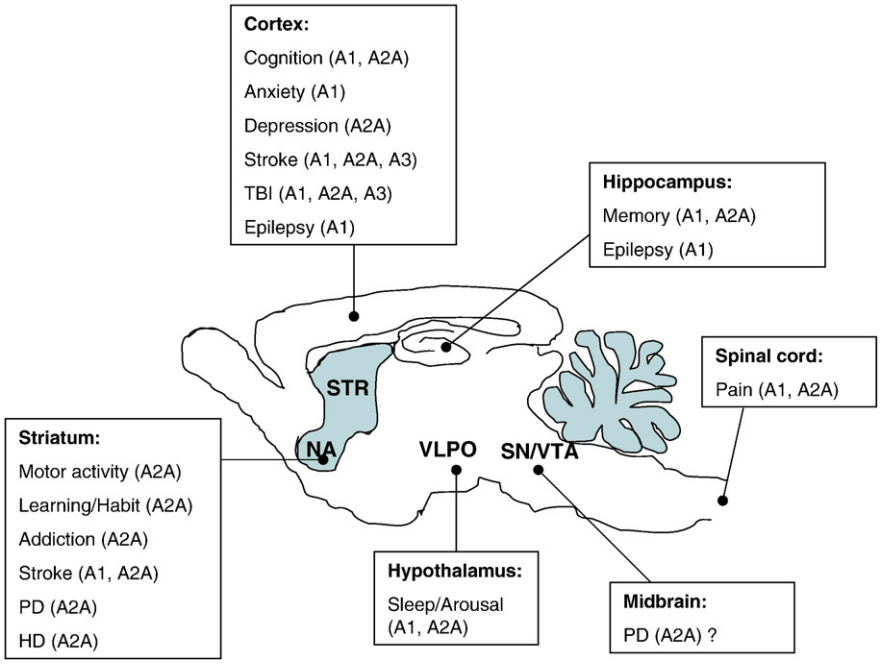

Fig. 1. Controversial role of adenosine receptors in different physiologic and pathologic conditions and brain regions suggested by knockout studies.

and A2ARs that requires beta-gamma dimers can occur in striatum and enhances sensitivity to D2R signaling [300]. Deletion of A2ARs might prevent this synergy and thus prevent the maladaptive cascade that might lead to addiction and/or other disorders that might arise from aberrant plasticity such as LID. Future studies might explore any of these avenues in AR knockout mice.

4. Concluding remarks

Endogenous adenosine is a widely distributed upstream regulator of a broad spectrum of neurotransmitters, receptors, and signaling pathways that converge to contribute to the expression of an array of important and complex behaviors. Consequently, genetic knockout analysis of adenosine and ARs reveal a broad spectrum of modulatory effects on various normal and abnormal functions of the brain. These functions range from neuronal plasticity, motor, motivation, sleep–wake cycle, cognition, and emotion in the normal brain to neuroprotection, neuroinflammation, and maladaptive behavioral and neuropsychiatric disorders in the pathologic brain (Fig. 1 and Table 1). Collectively, the findings from these studies emphasize the importance of broadly studying the phenotypes in AR knockout mice when characterizing these knockout mouse lines. This ability of adenosine to interact and integrate these critical brain processes is, for instance, most evident in its modulation of cognitive functions, which usually

Table 1
Controversial role of adenosine receptors suggested by knockout in different situations.

		AR	Effect revealed by AR KO	Brain region	References
Physiologic	Sleep/arousal	A1	Promote sleep/inhibit arousal	Basal forebrain	Bjorness et al., 2009 [77]
		A2A	Promote sleep/inhibit arousal	Hypothalamus Striatum	Huang et al., 2005 [133]
	Cognition	A1	Anxiogenic Increase aggression	Cortex	Johansson et al., 2001 [74] Lang et al., 2003 [174] Gimenez-Llort et al., 2002 [76]
		A2A	Improve spatial recognition memory Improve working memory	Cortex	Wang et al., 2006 [178] Zhou et al., 2009 [179]
			Impair habit learning	Striatum	Yu et al., 2009 [180]
			Impair startle habituation and prepulse inhibition Increase aggression		Wang et al., 2003 [202] Ledent et al., 1997 [80]
	Spontaneous locomotion	A2A	Reduce locomotor activity (gb-A2AR KO) No effect (fb-A2AR KO and st-A2AR KO)	Striatum	Chen et al., 1999 [81] Chen et al., 2000 [85] Bastia et al., 2005 [86] Shen et al., 2008 [87]
Pathologic	Ischemia/hypoxia	A1	Reduce or exacerbate brain damage	Cortex	Johansson et al., 2001 [74] Björklund et al., 2008 [204] Turner et al., 2003 [207] Olsson et al., 2004 [205]
		A2A	Reduce or exacerbate brain damage	Cortex	Chen et al., 1999 [81] Yu et al., 2004 [212] Aden et al., 2003 [137]
		A3	Reduce or exacerbate brain damage	Cortex	Fedorova et al., 2003 [195] Chen et al., 2006 [108]
	Parkinson's disease (PD)	A2A	Neuroprotection Increase locomotor activity	Striatum	Chen et al., 2001 [218] Yu et al., 2008 [138] Carta et al., 2009 [233]
	Huntington's disease (HD)	A2A	Reduce or exacerbate striatal damage	Striatum	Blum et al., 2003 [257] Fink et al., 2004 [256] Huang et al., 2006 [83]
	Traumatic brain injury (TBI)	A2A	Reduce brain damage	Cortex	Li et al., 2009 [258] Dai et al., 2010 [259]
	Epilepsy/Seizure	A1	Lower threshold and promote epilepsy	Cortex Hippocampus	Fedele et al.2006 [281] Haselkorn et al., 2010 [264] Kochanek et al., 2006 [282]
		A2A	Confer some resistance to seizures	Cortex	El Yacoubi et al., 2001 [288] El Yacoubi et al., 2008 [289]
	Neuroinflammation	A1	Enhance microgial response	Cortex	Tsutsui et al., 2004 [263] Haselkom et al., 2010 [264]
		A2A	Inhibit or promote neuroinflammation	Cortex	Yu et al., 2004 [212] Yu et al., 2008 [138]
				Striatum	Li et al., 2009 [258] Dai et al., 2010 [259]
	Pain	A1	Antinociceptive	Spinal cord	Johansson et al., 2001 [74]
		A2A	Antinociceptive	Striatum Somatosensory cortex Spinal cord	Ledent et al., 1997 [80] Bailey et al., 2002 [272] Berrendero et al., 2003 [100] Hussey et al., 2007 [273]
		A3	Antinociceptive or no effect	Maybe in peripheral tissues	Fedorova et al., 2003 [195] Wu et al., 2002 [274]
	Drugs of abuse: addiction and withdrawal	A2A	*Cocaine and morphine:* reduce reward, motivation, rate of self-administration *Amphetamine:* reduce locomotor sensitization	Striatum (nucleus accumbens)	Soria et al., [294] Chen et al., 2003 [295] Bastia et al., 2005 [86] Castane et al., 2008 [297] Brown et al., 2009 [296]
			Ethanol: reduce reward, sensitivity to intoxicating effects, withdrawal		Naassila et al., [299] Houchi et al., [193] El Yacoubi et al., 2001 [288]

involves highly inter-connected brain circuits and multiple neurotransmitters. The development of genetic knockout mice with targeted deletion of the A1R or A2AR subtypes has especially been instrumental in providing several new insights into the nature of cognitive modulation by adenosine and its receptors. Notably, A2AR knockout models highlight the pro-cognitive potential of A2AR blockade, particularly under circumstances in which the cognitive load is greater, as in the case of working memory. Moreover, region-specific manipulation of the A2AR in striatum has revealed a novel role for this receptor in the formation of habitual behavior and the control of psychomotor activity. Together, these main recent findings extend the functions of the A2AR beyond motor control to modifying cognitive performance under physiologic and possibly pathologic conditions as well. These studies raise the exciting possibility that A2ARs may represent a target for improving cognitive function under physiologic and/or pathologic states.

AR knockouts have also provided in recent years new insights regarding the role of the ubiquitous ligand and its receptor targets in brain pathology. They confirm the fundamental role of the adenosine system as an endogenous defense mechanism through its "retaliatory" metabolite and neuromodulatory effects in response to a variety of brain insults. The elevated extracellular adenosine in injured brain, acting through multiple ARs, is commonly recognized and now largely validated by genetic knockout models to produce an overall neuroprotective effect. However, studies in knockout mice lacking the A1R and/or A2AR gene have unveiled an increasingly complex and injury context-dependent effect in brain pathology. For example, while activation of A1Rs generally exerts a broad neuroprotective profile, activation of A2ARs may in fact contribute to brain damage. Moreover, while a general neuroprotective effect of A2AR antagonism has emerged against a broad spectrum of brain insults ranging from ischemia to PD to TBI, both A2AR agonists and antagonists have been shown to protect against striatal damage in animal models of HD. Such a disease context-dependent effect of ARs may thus yield different net outcomes depending on the brain region, disease, and/or timing of the manipulation (e.g., drug or gene knockout) and/or insult. These complex and even opposing phenotypes may reflect the nature of adenosine's multi-level interactions with a vast array of neurotransmitter and inflammatory/immune systems. A prominent example of such a context-dependency of adenosine's effects is the demonstration of the close interaction between local glutamate levels and adenosine acting at A2ARs to control neuroinflammation. Increased local levels of glutamate in injured brain regions can switch the anti-inflammatory effect of A2AR activation to that of a pro-inflammatory effect and consequently promote brain damage. This finding unexpectedly revealed a novel mechanism by which extra-synaptic glutamate, in addition to its well-documented neuronal excitotoxic effects, can control neuroinflammation via regulation of the A2AR during brain injury.

In summary, AR knockouts, particularly for the A1R and A2AR, have provided significant insight into adenosine's control of complex physiologic and pathologic phenomena. These findings extend and strengthen support of A2ARs and A1R in brain as targets for several neurologic and psychiatric diseases. However, they also emphasize the importance of considering the disease context-dependent effect when developing AR-based therapeutic strategies and support the notion that new developments of such drugs will likely need to be specifically tailored to a given disease.

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