Modulation of Synaptic Transmission by Adenosine in Layer 2/3 of the Rat Visual Cortex in Vitro

Nicholas M. Bannon
University of Connecticut - Storrs, nicholas.bannon@uconn.edu

Recommended Citation
https://opencommons.uconn.edu/gs_theses/391

This work is brought to you for free and open access by the University of Connecticut Graduate School at OpenCommons@UConn. It has been accepted for inclusion in Master's Theses by an authorized administrator of OpenCommons@UConn. For more information, please contact opencommons@uconn.edu.
Modulation of Synaptic Transmission by Adenosine in Layer 2/3 of the Rat Visual Cortex in Vitro

Nicholas Bannon
B.A., University of Connecticut, 2010

A Thesis
Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Arts
At the
University of Connecticut
2013
Masters of Arts Thesis

Modulation of Synaptic Transmission by Adenosine in Layer 2/3 of the Rat Visual Cortex in Vitro

Presented by

Nicholas Bannon, B.A.

Major Advisor ____________________________________________

Maxim Volgushev

Associate Advisor ___________________________________________

John D. Salamone

Associate Advisor ___________________________________________

Harvey A. Swadlow

University of Connecticut

2013
ACKNOWLEDGEMENTS

Thanks to my committee:

Maxim Volgushev Ph.D.
Harvey A. Swadlow Ph.D.
John D. Salamone Ph.D.

Thanks to my laboratory:

Maxim Volgushev Ph.D.
Marina Chistiakova Ph.D.
Vladimir Ilin M.D.
Roman Goz
Pei Zhang
Chris Lee

And for continued help in every aspect:

Patrick Randall
Eric Nunes
Adenosine (Ado) is an endogenous neuromodulator which is widespread in the central nervous system. It is a metabolite of ATP, hence its ubiquitous presence, yet this purine nucleoside has an importance that surpasses cellular energy maintenance. It is a known modulator of synaptic transmission, and its global presence places it at center stage for many physiological processes. Its ubiquitous presence is convoluted by different types of adenosine receptors which are coupled to diverse intracellular cascades and are expressed in a brain region-specific and cell-type specific fashion. This diversity allows adenosine to serve many behavioral roles.

Adenosine is well understood in the hippocampus, where it influences the induction of synaptic plasticity. Adenosine has been shown to decrease the magnitude of long-term potentiation (LTP), whereas blockade of specifically the A₁ receptor (A₁R) has been shown to facilitate the induction of LTP (reviewed by Costenla et al. 2010). As adenosine acts at metabotropic receptors which initiate vast and divergent intracellular cascades, there are many mechanisms by which it may facilitate or inhibit plasticity induction. The most obvious is the ability for A₁R activation to selectively inhibit NMDA currents, which are necessary for many types of LTP (de Mendonça et al. 1995, Klishin et al. 1995). A₁R activation also decreases presynaptic release probability by inhibiting presynaptic Ca²⁺ influx (Gundlfinger et al. 2007, Wu and Saggau 1994, 1997) and reduces cell excitability through increased K⁺ conductance and membrane hyperpolarization (Trussel and Jackson 1985, 1987, Thompson et al. 1992, Takigawa and Alzheimer 1999, 2002). This provides a straightforward hurdle in the induction of plasticity by limiting the efficacy of transmission. In addition, all adenosine receptors’ intracellular
cascades converge upon adenylate cyclase and subsequently cyclic-AMP (cAMP) production which have been implicated in the mechanisms by which cells express plastic changes (elevations in cAMP have been linked to the initiation of long term enhancements in synaptic transmission; Weisskopf et al. 1994, Tzounopoulos et al. 1998). Namely, activation of the A1R inhibits adenylate cyclase activity and decreases cAMP production whereas activation of A2AR receptors does the opposite (Dunwiddie and Masino 2001).

Adenosine’s capacity to modulate dopaminergic transmission is well documented within the striatum. The activation of adenosine A2AR receptors (A2ARs) on enkaphalin-positive medium spiny neurons directly inhibits the activity of co-localized dopamine (DA) D2 receptors through membrane receptor-receptor interactions (where it decreases the affinity of the D2 receptor for dopamine) and through convergence on the intracellular cascade (where A2AR activation increases adenylate cyclase activity and D2 activation decreases it; Ferré et al. 2007). On substance-P positive medium spiny neurons, antagonistic interactions occur between A1Rs and D1 receptors. A1R activation in these neurons changes the binding characteristics of the D1 receptor to a lower affinity state (Ciruela et al. 2011). The consequence behaviorally is that locomotor and motivational behavioral models which are regulated by dopaminergic transmission are also modulated by adenosine. A tonic activation of these adenosine receptors translates to a tonic suppression of dopamine transmission. Antagonism of these adenosine receptors thus results in enhanced dopaminergic transmission, resulting in therapeutically beneficial behavioral effects such as increased motivation, locomotion, and reduced tremor (For review see Salamone et al. 2008, Nunes et al. 2010, Collins-Praino et al. 2011, Salamone et al. 2012).
In the cortex, adenosine levels rise dramatically in response to traumatic events such as hypoxia and ischemia. Acute A₁R activation reduces neuronal damage following these types of trauma (for review see Cunha 2005, Gomes et al. 2011). Although the exact mechanism of neuroprotection by adenosine is not well elucidated, it appears to be a combination of adenosine’s capacity to powerfully suppress glutamatergic transmission as well as its position as a regulator of cellular metabolism. Adenosine accumulation, reflective of cellular energy use, initiates cascades inside the cytoplasm which limit further cell energy expenditure and transmission analogous to a negative feedback loop (Chen et al. 2013). Adenosine is also an endogenous anticonvulsant (Rho et al. 2013). Excessive adenosine clearance has been linked to the etiology of epilepsy (Boison 2013). Increasing adenosine levels and thus increasing the activation of A₁Rs has been shown to suppress seizures (Masino et al. 2011, Rho et al. 2013).

Adenosine signalling has also been implicated in the control of sleep and waking states in several brain areas. Thalamic neurons express two modes of firing. During awake behavioral states (and more depolarized membrane potentials) these neurons transmit action potentials reliably (tonic firing mode). Hyperpolarization of these neurons (corresponding to sleep states) causes them to engage in burst firing (bursting mode; Lu et al. 1992, Pape 1992, review by Bjorness and Greene 2009). By increasing K⁺ conductance and hyperpolarizing neurons, as well as through the inhibition of other modulatory systems such as acetylcholine, adenosine aids in the transition of these neurons’ firing patterns to a state associated with sleep (Pape 1992, for review see Bjorness and Greene 2009). There exist neurons within the basal forebrain, mesopontine tegmentum, and hypothalamus which are preferentially active during waking. Adenosine decreases release probability of neurotransmitters and decreases cell excitability in
these neurons many of which are part of the acetylcholinergic arousal system; Rainnie et al. 1994, reviewed by Strecker et al. 2000, Arrigoni et al. 2006, Liu and Gao 2007). Within the ventrolateral preoptic area and the preoptic/anterior hypothalamic area, there exist neurons which are active mainly during sleep. A selective suppression of GABAergic transmission by adenosine in these areas mediates a disinhibition of these neurons (see Strecker et al. 2000, Moriarty et al. 2004). Adenosine promotes sleep by inhibiting the acetylcholinergic arousal system and inhibiting wake-active neurons while simultaneously disinhibiting sleep-active neurons.

These examples illustrate a multitude of roles and effects of adenosine, while highlighting a significant diversity of action across the brain. Whereas the roles of adenosine in the hippocampus, striatum, and in subcortical structures are well defined, the role of adenosine in regulation of neocortical functions is much less understood. The neocortex is a critical brain area in the processing of sensory stimuli and higher order cognition. It is responsible for numerous functions many of which have been demonstrated to be influenced by adenosine signaling.

It is known that adenosine levels fluctuate in the neocortex as a function of behavioral state and sleep need. Generally, adenosine levels accumulate during waking and are highest after prolonged wake periods, and subsequently decrease during sleep, with lowest levels occurring at the end of sleep (discussed by Bjorness and Greene 2009). Adenosine administered exogenously is sleep promoting, while antagonism of adenosine receptors (such as by caffeine) is known to increase vigilance and waking (Bjorness and Greene 2009). It is also known that adenosine modulates sleep homeostasis and neocortical slow oscillations (Halassa et al. 2009).
Slow wave sleep (occurring during NREM sleep) is marked by slow cortical oscillations which are thought to be homeostatically regulated. If an organism has an increased need for sleep, such as after sleep deprivation, it will respond during subsequent sleep with increased sleep time and increased length of NREM bouts. Mice lacking adenosine tone do not demonstrate compensatory increases in these measures after sleep deprivation (Halassa et al. 2009).

Plasticity in the neocortex is thought to underlie permanent memory storage, an action necessary for long-term learning. A role for adenosine here is suggestive yet tentative due to the well documented influence of adenosine on plasticity in the different architecture of the hippocampus.

Adenosine receptor activation in the cortex in general has been implicated in a neuroprotective role, mitigating damage after traumatic events. As mentioned, epileptic seizures, which are cortical phenomena, have been demonstrated to be influenced in therapeutic ways by adenosine receptor activation (Boison 2013, Rho et al. 2013).

Adenosine’s ability to modulate glutamatergic and dopaminergic transmission within the frontal lobe has raised the possibility of therapeutic application in schizophrenia, where hypotheses propose glutamate hypofunction and dopamine hyperfunction play a role in etiology (Boison et al. 2012). It is presumed adenosine may modulate forebrain dopaminergic transmission in a way that is etiologically relevant to schizophrenia.

Heterogeneous expression of adenosine receptors in different brain areas provide unique environments at which adenosine is able to act and modulate other neurotransmitters. In each physiological role it serves, it is this profile of expression along with its specific neuroanatomical context that gives adenosine its relevance. The nature of
adenosine’s modulation of synaptic transmission then, is very diverse. This diversity demands explicit demonstrations in electrophysiology to provide a foundation on which adenosine’s actions in a given brain area can be understood. While thorough electrophysiological work has been done in other brain areas, a comparable demonstration in the neocortex is lacking. Understanding roles of adenosine in normal operation of the neocortex and during dysfunction requires demonstration of adenosine effects on specific types of cells and connections.

The goal of the present work, then, is to uncover the synaptic actions of adenosine in the neocortex. Here we sought to investigate adenosine’s effects on excitatory synaptic transmission in layer 2/3 of the rat visual cortex, and determine which receptors mediate these effects. This knowledge informs us of the synaptic capabilities of neocortical adenosine and provides groundwork for a mechanistic basis for known behavioral and physiological roles of this neuromodulator.

To frame the present work, it is best to first consider the general principles of adenosine as a neuromodulator. How it is synthesized and degraded, neuronal and glial sources, and what receptors for adenosine exist will all be considered. A survey of various physiological roles in several brain areas will then serve to highlight the diversity of adenosine’s synaptic, neuroanatomical, and behavioral actions. The focus will remain on the mechanistic bases of adenosine’s action and how they are utilized in different contexts. The importance of an explicit profile of neocortical action (and the inability to generalize from heterogenous brain areas) will then be clear, and the rationale for the present work justified.
Formation and Degradation

There are two major pathways which result in adenosine formation inside of the cell. Adenosine triphosphate (ATP) is hydrolyzed to adenosine diphosphate (ADP), which is broken down into adenosine monophosphate (AMP), and finally adenosine. Here endo-5’ nucleotidase catalyzes the final reaction, which is also the rate limiting step. (Dunwiddie et al. 1997, Park and Gupta 2013, Sebastião et al. 2013). In another pathway, S-adenosylhomocysteine hydrolase can catalyze a transmethylation reaction, converting S-adenosylhomocysteine into adenosine (and homocysteine). Intracellular ATP concentration is about 100,000 times greater than adenosine concentration, meaning that relatively modest use of ATP by a cell results in significant increases in adenosine levels (Cunha 2005). Extracellularly, membrane bound Ecto-5’ nucleotidase (ecto-5’ NT) can catalyze the formation of adenosine as endo-5’ nucleotidase does in the cytoplasm. This Ecto-5’ NT is predominantly on glial cells (namely astrocytes), an importance which will be emphasized when considering the regulation of extracellular adenosine which is capable of acting on receptors. As it is a metabolite of ATP, adenosine levels are linked to the energy use of a cell. During synaptic transmission, there is an activity-dependent ATP release which, after conversion to adenosine, results in a suppression of transmission. Therefore a modest energy use is coupled to a feedback mechanism which slightly conserves energy. More dramatic events that deplete intracellular ATP initiate increases in adenosine, which initiate reciprocal feedback through many mechanisms. Examples include increasing blood flow (through actions on endothelial A2A Rs), increasing glucose availability (by increasing glucose transport, glucose delivery, and glycogenolysis), and by boosting metabolic processes which result in increased ATP (Chen et al. 2013). In even more dramatic situations, in which traumatic events initiate
excitotoxicity and spur large increases in adenosine, adenosine receptor activation can help initiate astrogliosis and other reparative mechanisms. This coupling to the energetic use of a cell has earned it the title of a ‘retaliatory metabolite’.

The degradation of adenosine can be accomplished by its conversion to inosine by cytoplasmic adenosine deaminase and ecto-adenosine deaminase. Adenosine kinase (ADK) phosphorylates adenosine back into AMP, and is a major mechanism for the removal of adenosine. It is prominent in astrocytes, which will reveal themselves to be important players in the production and removal of adenosine.

**Where does adenosine come from/when is it released?**

The sources of adenosine are multiple, and the rules governing its release multifaceted. Passive bidirectional nucleoside transporters do exist in neuronal membranes, yet experimental evidence indicates these typically serve to take up adenosine into the cytoplasm rather than release it into the extracellular space (Wall and Dale 2008, Cunha 2008). Being passive, the net flow of adenosine through these transporters depends on the relative concentrations on either side of the membrane. Under specific circumstances, such as intracellular buildup due to excessive ATP use, cytoplasmic adenosine deaminase inhibition or deficiency, or excessive clearance in the extracellular space, these bi-directional transporters could serve to release adenosine directly. Recently, adenosine release through nucleoside transporters has been demonstrated in hippocampal neurons during physiologically relevant spiking activity (Lovatt et al. 2012).
Rather than being released as is, it appears much adenosine is formed within the extracellular space from ATP, which may be co-released with neurotransmitters or released from astrocytes. The ATP is then broken down into adenosine by the processes outlined above. The total time course for this conversion is about 200 milliseconds. Again, the final conversion of AMP to adenosine is the rate limiting step (Dunwiddie et al. 1997). While many enzymes catalyze the hydrolysis of ATP to ADP and ADP to AMP, only ecto 5 nucleotidase catalyzes the reaction that results in adenosine formation from AMP (Lovatt et al. 2012).

Indeed ATP can be released from nerve terminals in an activity dependent manner, particularly under high frequency firing, such as during stimulation protocols utilized for the induction of long-term potentiation (Wall and Dale 2008, Sperlagh and Vizi 1996, Cunha 2008). In this circumstance it would be broken down to adenosine by 5'-ectoNT and activate synaptically located receptors with high temporal and spatial specificity.

Recent research has confirmed the importance of astrocytes as a source of extracellular adenosine. Astrocytes, though electrically passive, can serve to release gliotransmitters like ATP in an activity-dependent manner (Halassa et al. 2007). Indeed astrocytes contain many neurotransmitter receptors and show responsiveness to glutamate. Activation of astrocytic receptors can cause Ca\(^{2+}\) influx which propagate (even to other gap-junction coupled astrocytes) via ‘Ca\(^{2+}\) waves.’ These Ca\(^{2+}\) waves can stimulate the release of vesicularly stored gliotransmitters (Halassa et al. 2007). Using transgenic mice in which SNARE-dependent vesicular release from astrocytes was impaired, it was demonstrated that vesicular ATP release from astrocytes (and its subsequent metabolism to adenosine) was responsible for a tonic inhibition of transmission at Schaffer collateral synapses in hippocampal slices (Pascual et
Antagonism of $A_1$Rs in wild-type mice was able to mimic the transgenic mice, and $A_1$ agonism was able to reverse the effects of impaired vesicle release in the transgenic mice, indicating it was astrocyte-derived adenosine, rather than other gliotransmitters contributing to these effects. Utilizing the same transgenic mice yet working in the neocortex, tonic inhibition of synaptic transmission by astrocytic-derived adenosine acting on the $A_1$ receptor was found (Halassa et al. 2009). Because the responsiveness of astrocytes is slower than neurons and their influence spatially broad, adenosine signaling sourced from these glial cells is postulated to be more broadband (that is low temporal and spatial resolution).

Whether from astrocytes or nerve terminals, the formation of extracellular adenosine is activity-dependent. Activity-dependent release of adenosine has been demonstrated in the caudate putamen, nucleus accumbens, hippocampus and neocortex (motor and prefrontal; Pajski and Venton 2012). The predominant source of adenosine release in the caudate-putamen and nucleus accumbens was glutamate receptor dependent. While glutamate receptor blockade by CNQX and AP5 decreased activity dependent adenosine release in these areas it did not in the hippocampus and cortex. In hippocampus and cortex, the major source of adenosine release was from the breakdown of nucleotides (presumably ATP, and quite possibly astrocytic in source). This suggests differential sources for adenosine in different brain areas. Notably, this group found the highest levels of activity-dependent adenosine release (and the most inconsistent detection by voltammetry) in the caudate-putamen and nucleus accumbens, areas associated with glutamate receptor dependent release. Lower levels of release (which were more reliably detected) were found in the hippocampus and cortex, areas associated with ATP-derived adenosine (Pajski and Venton 2012). In theory the source of adenosine in the
hippocampus and cortex is from a broadband and reliable (albeit more modest) breakdown of ATP. Astrocytes are good candidates for this source as they have been suggested to fulfill the role of providing temporally and spatially general rises in adenosine. In contrast, caudate-putamen and nucleus accumbens appear to have more extreme though less reliable rises in adenosine, possibly sourced synaptically and locally after ionotropic glutamate receptor activation. A less diffuse source of adenosine may underlie the less reliable detection.

**Receptors: their intracellular cascade and distribution**

There are four recognized metabotropic adenosine receptors coupled to different G-proteins: the $A_1$ ($G_{i/o}$ linked), $A_{2A}$ ($G_{i/o}$ linked), $A_{2B}$ ($G_s$ linked), and $A_3$ ($G_{i/q}$ linked). $A_1$ and $A_3$ receptors inhibit adenylate cyclase and decrease cyclic-AMP (cAMP) production, while $A_{2A}$ and $A_{2B}$ activate adenylate cyclase and increase cAMP production (Dunwiddie and Masino 2001). This action on cAMP subsequently results in the modification of Protein Kinase A (PKA) activity. The importance of adenosine’s actions on the intracellular cascade is further discussed as it becomes relevant to different physiological roles adenosine serves.

The $A_1$R is expressed in high concentrations all throughout the brain, notably in cerebral cortex. $A_{2A}$Rs are most dense in the striatum and olfactory bulb. $A_{2A}, A_{2B}$ and $A_3$ receptors were also found throughout the brain at lower levels (Dixon et al. 1996, Fredholm et al. 2001). $A_3$ receptors have been reported in the hippocampus and neocortex specifically (Lopes et al. 2003; Brand et al. 2001). The highest density of $A_3$ receptor protein mRNA has been reported in the hippocampus and cerebellum (Brand et al. 2001). Though some work revealing functional $A_3$ receptors has been published, limited work has covered $A_{2B}$ receptors
and their role in brain physiology. Being the most densely expressed and bearing the most explicit effects on brain physiology, the A₁Rs and A₂A Rs have been the focus of most adenosine receptor research, and are the focus of the present work as well.

It is important to note that while typically the receptors outlined couple to their respective G-proteins, an enormous diversity of exceptions exists. Depending on brain structure, cellular location, and even sub-cellular location, a given adenosine receptor can couple to a range of G-proteins and transduction mechanisms (Cunha 2005). One of the well-studied examples is the heteromerization of receptors, specifically within the striatum. As clarified by Ferré (Ferré et al. 2007): “A receptor heteromer can be defined as a complex molecule made up of different receptor molecules for the same or different neurotransmitters.” In essence, it is a protein complex comprised of two or more neurotransmitter receptors. Homomers (in which the complex is comprised of two or more of the same receptor) also exist. Whereas coupling to the Gₛ protein is typical for A₂A Rs, it was found that when CB1 receptors are present (and forming heteromers with A₂A Rs), the A₂A R couples to the Gᵢ protein instead (Orrú et al. Neuroscience 2012 abstract). Heteromerization has consequences for the binding and subsequent action of ligands as well. Research has shown distinct populations of A₂A Rs within the striatum. Specifically, presynaptic A₂A Rs coupled to A₁Rs and A₂A Rs which do not heteromerize formulate one population, while postsynaptic A₂A Rs heteromerizing with dopamine D₂ receptors (D2Rs) seem to comprise a different population. The two populations are pharmacologically separable through the use of A₂A R ligands, which demonstrate different effects in in vivo models and also different binding characteristics in in vitro preparations (Orrú et al. 2011). Extending these principles, there has been shown heterogenous binding
properties of supposedly selective A\textsubscript{2A}R ligands when comparing the striatum and the cortex (Lopes et al. 2004). Pharmacological and binding studies using the A\textsubscript{2A} agonist CGS 21680 show clear binding in striatum, but an unidentified atypical binding site in the cortex. The binding of this ligand in cortex appears to require the presence of the A\textsubscript{1}R, suggesting possible heteromeric complexes with unique binding properties. (Lopes et al. 2004) This heterogeneity of receptor distribution, cellular location, and transduction is a recurring theme in the diversity of adenosine’s actions, and as such will reappear often.

**Receptors: cellular/synaptic location and effects on other transmitters**

Adenosine’s noted electrophysiological actions come from action at pre, post, and extrasynaptic receptors. In general, presynaptic receptor activation modulates release probability through action on Ca\textsuperscript{2+} influx, and also by interfering downstream with release machinery. Receptor activation also has various effects on membrane properties and excitability of the neuron, including influencing Ca\textsuperscript{2+} and K\textsuperscript{+} conductance and altering the functioning of postsynaptic receptors for other ligands.

\textit{A\textsubscript{1}Rs}

Adenosine acting on presynaptic A\textsubscript{1}Rs reduces the release probability at a synapse by inhibiting calcium influx at the presynaptic terminal through N-type and P/Q-type Ca\textsuperscript{2+} channels (Gundlfinger et al. 2007, Wu and Saggau 1994, 1997). There is also evidence that adenosine is acting downstream of Ca\textsuperscript{2+} influx to reduce the efficacy of the exocytotic machinery (Gomes et al. 2011, Scanziani et al. 1992, Scholz and Miller 1992). A\textsubscript{1}Rs have been demonstrated to inhibit
the release of glutamate, aspartate, acetylcholine and serotonin (Lupica et al 1992, Silinsky 1984, Corradetti et al. 1984, Cunha 2005, Fontantez and Porter 2006). They have been discussed as preferentially inhibiting excitatory transmission. In some preparations within the neocortex however, GABAergic transmission is suppressed by adenosine (Kirmse et al 2008, Zhang et al. Neuroscience 2012 abstract).

Postsynaptic/nonsynaptic receptors also exist. Postsynaptic A1Rs have been linked to the activation of G-protein coupled inwardly-rectifying potassium channels (GIRKs), which increase potassium conductance (Trussel and Jackson 1985, 1987, Thompson et al. 1992, Takigawa and Alzheimer 1999, 2002). The major effect of this activation is the hyperpolarization of the membrane, effectively decreasing the excitability of the neuron, and consequentially the impact of excitatory transmission on the postsynaptic membrane. In hippocampal pyramidal cells, there is a tonic activation of GIRKs by adenosine acting on A1Rs (Takigawa and Alzheimer 2002). Hyperpolarization by GIRKs not only shunts excitatory post-synaptic potentials (EPSPs) in the direct manner, but also limits the conductance of voltage dependent channels (notably Ca^{2+} channels; Takigawa and Alzheimer 2002).

A2ARs

The A2AR functions in a manner nearly opposite to the A1R. Presynaptic A2AR activation facilitates glutamate, GABA, serotonin, noradrenalin and glycine release (Marchi et al. 2002, Cunha 2005). A2AR activation increases acetylcholine (ACh) release in cerebral cortex, hippocampus, striatum, and motor nerve terminals. Within the hippocampus, heterogeneity exists in the ability of adenosine to modulate ACh release. In CA3, A1Rs and A2ARs are present and modulate ACh release. In CA1, however, only A1Rs seem to modulate release of ACh
GABA release is enhanced by A2A R activation in hippocampus, yet in striatum, GABA release is inhibited by A2A R activation (summarized by Marchi et al. 2002). A2A Rs in striatum are present on corticothalamic to medium spiny neuron (MSN) contacts, as well as symmetric MSN contacts where they are mainly postsynaptic. Elsewhere in the brain (specifically cerebral cortex) they typically take a presynaptic locus and control transmitter release (Rebola et al. 20051,2, Chen et al. 2013) In addition to its direct actions, A2A Rs can affect transmission indirectly through receptor interactions with the A1 R. A2A R activation can shunt the effects of the A1 R causing an indirect excitation dependent on tonic A1 R activation (Phillis 2001, Lopes et al. 1999, Lopes et al. 2002). On presynaptic gluamatergic terminals in the striatum, A1 R-A2A R heteromers exist in which activation of the A2A R decreases the affinity of the A1 R for its ligand.

A2A R activation alters membrane properties of neurons as well, although the literature is less exhaustive for description of these actions. In hippocampal pyramidal neurons, A2A R agonism was shown to depolarize neurons and increase their input resistance, in direct contrast to A1 R activation (Li and Henry 1998). In the supraoptic nucleus, A2A R activation can modify membrane excitability and increase firing activity by depolarizing neurons (Ponzio et al. 2006). The mechanism by which this occurs appeared to be Na⁺ channel dependent (and possibly involving metabotropic glutamate receptors). At the mossy fiber synapse, A2A R activation selectively potentiates NMDAR-mediated EPSCs without effect on AMPAR-mediated EPSCs (Rebola et al. 2008). This supports a postsynaptic locus of action whereby A2A Rs facilitate transmission and increase excitability.
**A3Rs**

A3 receptor (A3R) mRNA has been found using techniques that dissect single pyramidal neurons of the hippocampus (Lopes et al. 2003). A3Rs are found to be lower affinity as well as much more sparsely expressed, leading researchers to presume that they are active only under special physiological circumstances, such as during traumatic events which result in the accumulation of extremely high adenosine concentrations (Lopes et al. 2003). Recordings from layer 5 pyramidal neurons within the associative frontal cortex (with stimulation in layer 1) demonstrated that the A3 agonist IB-MECA decreased the amplitude of postsynaptic potentials, probably through a reduction in glutamate release. IB-MECA had no influence on membrane properties such as resting potential or input resistance (Brand et al. 2001). In this study, neither A1R nor A3R agonism displayed measurable effects on membrane properties.

**Roles of Adenosine**

Adenosine has been implicated in many physiological roles. Despite the ubiquity of adenosine in the CNS, it is the source of this modulator and profile of receptor expression that truly determines the actions of adenosine. Adenosine’s actions in different brain areas demonstrate significant heterogeneity on the behavioral and physiological level owing to this diversity. The relevance of both adenosine source and receptor expression is expounded more appropriately in context, namely through discussion of the physiological roles served by adenosine. Several roles will be considered, with special attention to the synaptic and anatomical features that make these actions possible.
**Plasticity regulator: insight from the hippocampus**

The modulation of a synapse during the induction of plasticity can affect the induction's outcome, and thus exert influence on a long lasting timescale. In fact, adenosine receptor ligands have been demonstrated experimentally to affect synaptic plasticity (de Mendonça and Ribeiro 2001, Costenla et al. 2010 for review). Generally A₁R agonists inhibit long-term potentiation (LTP) while A₂A R agonists facilitate LTP. Adenosine receptors have several potential mechanisms by which they might influence the induction of synaptic plasticity. One mechanism of action for adenosine ligands on the induction of plasticity could be related to glutamatergic NMDA receptors (NMDARs). Ca²⁺ influx resulting from NMDAR activation is implicated as a critical step in the induction of many types of LTP. A₁R activation is able to inhibit NMDAR responses. The adenosine analog 2-Chloroadenosine, by acting on A₁Rs, was demonstrated to reversibly inhibit NMDA currents (de Mendonça et al. 1995). Conversely, in the hippocampus it was found that A₁R blockade by DPCPX preferentially increased the NMDA component of EPSCs (Klishin et al. 1995). A₁R regulation of NMDA receptors (NMDARs) has been demonstrated more recently in the cortex (Deng et al. 2011). Mice with impaired astrocytic vesicle release (dnSNARE mice), which subsequently show a lack of A₁R activation, demonstrated reduced NMDAR function while AMPA currents remained unaffected. This attenuation of NMDAR functioning was linked to a reduction in surface expression of NR2A and B NMDA receptor subunits due to reduced Src Family Kinase activity. Activation of A₁Rs in dnSNARE mice mimicked the wildtype phenotype, increasing NR2B subunit expression and NMDAR function. Conversely, A₁R antagonism in wildtype mice showed the same phenotype as the dnSNAREs. A₁Rs, activated by endogenous adenosine can regulate NMDARs in pyramidal
neurons (Deng et al. 2011). This selective inhibition of NMDAR function could make the induction of plasticity more difficult.

NMDARs are not the only possible locus for adenosine’s control of plasticity, however. An NMDA-independent LTP mediated by A2Rs at the mossy fiber synapse has been described (Kessey and Mogul 1997). This was presumably due to postsynaptic A2A receptor activation during induction and subsequent increase in AMPA receptor function or expression. The authors of this study also highlight the possible importance of cAMP levels after an induction in determining the outcome of a protocol. Adenosine receptor activation can manipulate cAMP levels through their actions on adenylate cyclase, offering another avenue by which potentiation may be influenced by adenosine. Weisskopf et al. 1994 suggest cAMP elevations contribute to long lasting potentiation of glutamatergic transmission at these synapses. According to Tzounopoulos et al. 1998, adenlyate cyclase activation and subsequent increase in cAMP levels and activation of the protein kinase A pathway would initiate a long-term potentiation. A2A receptor activation is known to increase cAMP, and thus may contribute to an intracellular cascade known to affect plasticity. In addition, Blockade of A1Rs may increase adenylate cyclase activity and increase cAMP through the removal of tonic A1R activation. Conversely, reduction in cAMP levels at the mossy fiber synapse (feasible through the blockade of A2A Rs and/or activation of A1Rs) may attenuate plasticity.

There exist additional avenues for adenosine receptor ligands to influence plasticity at a synapse. Postsynaptic A1R activation has also been linked to actions which hyperpolarize cells, decrease their input resistance, and reduce excitability of the cells. There is a well documented K+ conductance mediated by GIRKs which is activated by A1Rs. In addition, the inhibition of Ca^{2+}
currents by A$_1$R activation provides an obvious hurdle to plasticity induction. Adenosine has also been noted to preferentially inhibit excitatory transmission in hippocampal cell cultures (Yoon and Rothman 1991). A diminished impact of EPSPs with normal GABAergic transmission could bias a synapse towards quiescence, providing a more difficult environment in which to induce plastic changes. Lastly, while adenosine receptor ligands certainly operate to regulate plasticity in manners independent of changes to basal transmission, this does not remove adenosine’s ability to reduce glutamate release and inhibit EPSPs as an important factor. Of course, an inhibition of release will create a hurdle for the induction of long lasting synaptic changes.

A phenomenological assessment of the impact of different adenosine receptor ligands on the outcome of plasticity-inducing protocols within the hippocampus serves to link these known mechanisms of action to physiological relevance. The vast majority of work regarding synaptic plasticity and adenosine (and all referenced here unless otherwise stated) has been done in hippocampal slices (typically CA1). Ground-breaking work found an inhibition of LTP by 2-Chloroadenosine and related it to reduced Ca$^{2+}$ influx (Dolphin 1983). Both adenosine and 2-Chloroadenosine were shown to decrease the magnitude of high-frequency stimulation induced LTP as measured by population spike amplitude and field EPSP (fEPSP; de Mendonça and Ribeiro 1990, 1994). Using the adenosine uptake blocker NB-I, they also found this inhibitory effect on LTP, implicating endogenous adenosine in the mediation of plasticity. In contrast, blockade of A$_1$Rs by DPCPX increased LTP induced by this high frequency stimulation. A$_1$R blockade by DPCPX also increased the magnitude of LTP induced by theta burst stimulation, as measured by fEPSPs (Arai and Lynch 1993). It did not alter, however, the upper limit of
potentiating magnitude. This suggests $A_1$R blockade may increase the ability of weak or near-threshold protocols to induce LTP. This is corroborated by the observation that weak stimulation becomes more likely to induce LTP with DPCPX in the background (Forghani and Krnjević 1995). Previously subliminal trains can induce LTP on the background of DPCPX (reviewed by Costenla et al. 2010). Thus, LTP induction is facilitated by $A_1$R blockade, and under baseline conditions adenosine acting on $A_1$Rs is exerting a tonic inhibition of LTP induction.

The observation that LTP is attenuated by $A_1$R activation and facilitated by its blockade is compatible with the knowledge that $A_1$R activation inhibits NMDA currents and cAMP production, and that their blockade enhances both NMDA currents and cAMP production. As NMDAR activation and increased cAMP have been linked to sustained increases in synaptic efficacy, their manipulation by $A_1$Rs provide a reasonable mechanism by which adenosine modulates LTP.

Long-term depression (LTD) is also influenced by actions on adenosine receptors. DPCPX can increase the magnitude of homosynaptic LTD, indicating that endogenous adenosine acting on $A_1$Rs inhibits both LTP and LTD (de Mendonça et al. 1997). As with LTP, it appears $A_1$R blockade lowers the requirements for induction of LTD. At the Schaffer collateral-commissural pathway adenosine’s actions on homosynaptic LTD were tested. A low-frequency stimulation that could not produce lasting LTD on its own was able to induce LTD on the background of DPCPX or after the removal of tonic $A_1$R activation using adenosine deaminase (Kemp and Bashir 1997).

In contrast to homosynaptic LTD, heterosynaptic depression appears to be attenuated by a blockade/absence of $A_1$R activation. It was found that heterosynaptic depression induced
by theta-burst stimulation was absent in mice with impaired A₁R activation (Using the previously mentioned dnSNARE mice; Pascual et al. 2005). Similarly, DPCPX applied to a wildtype slice prevented the expression of heterosynaptic depression (Pascual et al. 2005). Corroborating this notion, more recent work found DPCPX prevented HFS-induced heterosynaptic depression (Lovatt et al. 2012). At least in the hippocampus, adenosine acting at A₁Rs is critical in inducing heterosynaptic depression.

A₂A receptors also mediate the induction of plasticity. Generally they operate inversely to the A₁R; A₂A R antagonists attenuate LTP whereas agonists facilitate it. Indeed, A₂A R antagonism by SCH 58261 decreased LTP in the hippocampus (Costenla et al. 2011) while A₂A R agonism via CGS 21680 facilitated potentiation (reviewed by Costenla et al. 2010). In addition, the non-selective antagonist caffeine appears to attenuate LTP as would an A₂A R antagonist (again reviewed by Costenla et al. 2010).

Concerns about effects of adenosine receptor ligands on basal transmission have been addressed by the above experiments in a few ways. Notably, LTP induced at one level of release probability occluded that induced at a different (lower or higher) level (Asztely et al. 1994). In most experiments, baseline responses were calibrated post ligand application to comparable levels with control slices. In addition, the use of antagonists mitigates the effects of changes in basal transmission as they typically have minimal effects on these measures (de Mendonça and Ribeiro 2001).

The heterogeneity of receptor expression again precludes the extrapolation of mechanism in one brain area to another. For example, adenosine’s ability to influence NMDARs is significant at Schaffer collateral synapses, but is irrelevant at the mossy fiber synapse. In
addition to the variety of induction requirements possible at differing synapses is the role that adenosine source and receptor profile may play at a slightly broader level of analysis. The localization of receptors provides the theater in which transmission is permitted to act. Rodrigo Cunha and colleagues (Cunha 2008, Costenla 2010) describe an elegantly coordinated series of events in which broadband, slow transmission due to widely expressed A₁Rs under tonic activation operates in conjunction with focal activation of A₂ARs sourcing adenosine which is released in an activity dependent manner during high frequency stimulation (such as would occur at a homosynaptic site during an LTP induction). In this situation basal or moderate levels of neural and astrocytic activity set an inhibitory tone mediated by a temporally and spatially broad inhibition of transmission through the A₁R. Conditions such as an induction protocol then cause a focal increase in adenosine at the active synapse, which reach levels sufficient to activate synaptic A₂ARs which overcome A₁R activation to facilitate transmission. Mechanistically, the activation of the A₂AR has been demonstrated to reduce the affinity of A₁Rs for their ligands in cortical synaptosomes (Lopes et al. 1999). This would result in a selective facilitation of transmission at the homosynaptic site and a more diffuse A₁R mediated inhibition at surrounding synapses. The consequences for plasticity induction could be facilitation of LTP (through A₂AR activation) at the active synapse and depression (through the A₁R) at heterosynaptic locations. In circumstances below the threshold this profile of adenosine may serve still to increase salience at an active synapse. This circumstance is only possible due to the unique combination of receptor expression (ubiquitous A₁Rs and synaptically restricted A₂ARs) and multiple sources of adenosine.
The striking difference between the relatively organized layout of pyramidal neurons in the hippocampus and the more dispersed presentation of pyramidal soma in the neocortex raises the question of generalizability. Whether this situation is relevant to the neocortex, or if other organizational principles enact similar outcomes remains undetermined. Exhaustive electrophysiological investigations in the hippocampus stand in contrast to the near-absence of investigation into adenosine in neocortical plasticity. If the spatial profile of receptor localization is critical to potentiation and depression, the role of adenosine in neocortical plasticity cannot be assumed from other areas. At the most basic level, knowledge of the pre and postsynaptic actions of adenosine precede any speculation of a role for adenosine within the neocortex, and would inform us of the parameters by which adenosine would be permitted to act.

**Adenosine and dopamine interact within the striatum:**

Within the striatum, adenosine receptor activation is able to influence dopaminergic transmission through receptor heteromerization, intracellular convergence, and interactions on the neuroanatomical/circuit level. Of specific concern here are the first two, as they highlight unique mechanistic actions of adenosine at the cellular level. As mentioned above, the expression of $A_{2A}$Rs in the brain is most dense in the striatum (Dixon et al. 1996, Fredholm et al. 2001). Here, $A_{2A}$ and D2 receptors are colocalized postsynaptically on enkephalin positive GABAergic medium spiny neurons where they can interact through receptor heteromerization and convergence on the intracellular cascade (Ferré et al. 2007, Salamone et al. 2008, Armentero et al. 2011). $A_1$ and D1 receptors also show a coexpression and heteromerization
within the striatum, yet on a distinctly separate population of substance-P positive neurons (Ferré et al. 2007, Ciruela et al. 2011). In addition, adenosine $A_1$R and $A_2A$Rs have been shown to colocalize and heteromerize on presynaptic glutamatergic terminals in the striatum (Reviewed by Ferré et al. 2007, Ciruela et al. 2011).

The consequence of heteromerization is the most direct interaction possible. In the case of postsynaptic $A_{2A}$-D2 heteromers, activation of the $A_{2A}$R decreases the affinity of ligands for the D2 receptor. Blockade of $A_{2A}$Rs removes a tonic inhibition by adenosine, increasing DA receptor affinity and consequentially facilitating dopaminergic transmission (Ferré et al. 2007, Ciruela et al. 2011). In $A_1$-D1 heteromers, adenosine receptor activation again decreases the affinity of the DA receptor for its ligand, and may also cause uncoupling of the D1 receptor from the $G_s$ protein (Ferré et al. 2007, Ciruela et al. 2011). In these scenarios the influence is unidirectional from the adenosine receptor to the dopamine receptor. Although it is not directly modulating dopamine transmission, it is suitable to mention a third adenosine receptor heteromer present in the striatum. Within presynaptic $A_1$-$A_{2A}$ heteromers, activation of the $A_{2A}$R ‘switches off’ $A_1$Rs by decreasing their affinity (Ciruela et al. 2006). Under normal circumstances and lower concentrations of extracellular adenosine, the inhibitory $A_1$R is tonically activated. As adenosine levels increase, the excitatory $A_{2A}$R becomes activated and $A_1$R mediated actions are shunted. At a glutamatergic synapse, the consequence is an inhibition of glutamate release at lower concentrations of adenosine, and a local facilitation of transmission as adenosine levels rise high enough to activate $A_{2A}$Rs. In addition to membrane interactions, adenosine and dopamine receptors both converge onto cAMP and PKA Pathways (discussed in Ferré et al. 2007). Whereas $A_{2A}$R activation stimulates adenlylate cyclase activity and increases
cAMP production, colocalized D2 receptors inhibit adenylate cyclase activity and decrease production of cAMP. Conversely, A3R activation inhibits cAMP production by decreasing adenylate cyclase activity while colocalized D1 receptors increase cAMP production by increasing adenylate cyclase activity. Here the integration of signaling from two different transmitter/modulatory systems is accomplished by converging upon adenylate cyclase in a mutually antagonistic way. Because of the implication of impaired dopamine transmission in Parkinson’s disease and parkinsonism, and the linkage of dopamine receptors to adenosine receptors, adenosine receptors have been investigated as a potential therapeutic target.

**Locomotion**

Animal models of parkinsonism typically utilize drugs which decrease extracellular DA levels or block DA receptors to induce motor deficits such as rigidity, catalepsy, tremor, and reduced locomotor activity. This is commonly achieved with DA antagonists such as haloperidol, DA-depleting agents such as reserpine and tetrabenazine, or by eliminating dopaminergic nerve terminals such as with the toxic 6-OHDA.

A2aR antagonists are effective in reversing the motor deficits present in these animal models. Rigidity, catalepsy, hypolocomotion and impaired locomotion induced by DA-depleting agents or DA antagonism were reversed by various A2aR antagonists (such as MSX-3, SCH 58261, KW-6002, and KF17837; reviewed by Salamone et al. 2008). Bilateral infusions of the reversing agent (MSX-3) directly into the nucleus accumbens shell, core, and the ventrolateral striatum (VLS) have highlighted the nucleus accumbens (specifically the core) as the locus for these locomotor manipulations (again reviewed in Salamone et al. 2008). Antagonism of the A2aR
alleviates a tonic suppression of D2 receptor functioning, effectively increasing the efficacy of the limited dopamine available.

_Tremor_

Parkinsonian tremor has also been modeled. The tremulous jaw movement (TJM) model typically employs striatal DA depletions or DA receptor antagonism to induce rapid deflections of a rat’s lower jaw. This movement is not directed at any stimulus and occurs in a range typical of resting tremor in parkinsonian patients (3-7 Hz). It is induced by the same manipulations that cause parkinsonism in humans (for example typical antipsychotics, 6-OHDA, reserpine, muscarinic receptor agonism, and anticholinesterases), and is relieved by interventions known to be antiparkinsonian (all reviewed by Collins-Praino et al. 2011).

Adenosine $A_{2A}$ receptor antagonism has been effective in reducing TJMs. TJMs induced by typical antipsychotics (DA antagonists) such as haloperidol and pimozide were reversed by $A_{2A}$ antagonism. MSX-3 injected directly into the VLS was effective in suppressing pimozide induced TJMs (again reviewed by Collins-Praino et al. 2011). For tremor, the locus of these pharmacological manipulations is the ventrolateral striatum.

_Motivation_

As noted in Salamone et al. 2012, animals in a natural environment are confronted with multiple avenues to obtain stimuli. Which behavior the animal engages in is influenced by the amount of effort, or cost, involved, and the ‘reinforcement value’ of the stimulus. The investigation of effort-related choice experimentally involves manipulating the cost/benefit ratio
of different options. This can be modeled with different food choices attached to different work requirements. In these paradigms, DA receptor antagonism or DA depletion within the nucleus accumbens shifts behavior away from the high cost/reward option and toward the low cost/low reward choice.

In a concurrent FR-5/chow procedure, a rat is given the choice between unrestricted access to laboratory chow, and pressing a lever to obtain a more palatable food pellet (a higher work requirement). A normal rat will mostly press the lever for a more preferred food. DA antagonists cause rats to press the lever less and reciprocally increase the free chow consumption. Effectively, a rat with diminished DA receptor activation would choose the easier task despite the food being less preferable. This is not demonstrably due to decreased appetite or motoric deficits (Nunes et al. 2010, Salamone et al. 2012). As with their ability to reverse parkinsonian locomotor deficits and TJMs, A_{2A} antagonists such as MSX-3 or KW-6002 administered after DA antagonism have been shown to reverse the shift in effort-related choice back towards a higher work output (Nunes et al. 2010).

Both D1 and D2 antagonism (via ecopipam and eticlopride, respectively), shifted behavior away from lever pressing and increased consumption of lab chow (Nunes et al. 2010). A_{2A} antagonist KW-6002 was able to shift lever pressing and chow consumption back toward baseline after either ecopipam or eticlopride, though the A_{2A} antagonist was much more effective at reversing D2 antagonism. Notably, neither DPCPX nor CPT (both A_{1}R antagonists) was able to reverse the effort related choice shift induced by ecopipam (D1 antagonist) or eticlopride (D2 antagonist).
The inability of A<sub>1</sub> antagonism to reverse D<sub>1</sub> antagonism, despite several known interactions between receptors (including heteromerization and intracellular cascade convergence) could be attributable to the overwhelming abundance of A<sub>1</sub>Rs throughout the striatum and rest of the brain. The majority of these receptors would not necessarily be directly linked with D<sub>1</sub>Rs and thus numerous other effects of A<sub>1</sub>R blockade may occlude or overwhelm potential effects on DA transmission (Nunes et al. 2010).

Adenosine’s role in the striatum is particularly insightful because it exemplifies the exclusivity of certain mechanisms within a brain area. Again, the expression of dopamine and adenosine receptors postsynaptically on medium-spiny neurons is a unique feature of the striatum by which adenosine signaling is integrated with dopaminergic transmission to tune behavior in a relevant manner. This ability of adenosine receptors to couple in an antagonistic way with dopamine receptors and integrate signaling on the membrane and in the intracellular cascade is an exception, not a ‘rule.’ As noted, A<sub>2A</sub>Rs elsewhere are often presynaptically located, and much sparser. In these presynaptic scenarios they serve restricted roles, activated only during specific circumstances (such as facilitating transmitter release locally at a synapse; Marchi et al. 2002, Ciruela et al. 2006, Cunha et al. 2008).

**Neuroprotection**

As adenosine levels tend to rise dramatically following hypoxic and ischemic insults, and given its known suppressant actions on excitatory transmission, it has been thought that it acts as an endogenous neuroprotector (reviewed in Gomes et al. 2011,). A wealth of culture, in vitro and in vivo experiments have followed and uncovered evidence that adenosine receptor
ligands do indeed incur neuroprotective benefits. The homeostatic role of adenosine found in eukaryotic cells in general will not be considered here, though significant actions of adenosine in peripheral tissues and within the brain in endothelial cells play a role in neuroprotective action, vasculature control, and permeability of the blood brain barrier (Reviewed by Gomes et al. 2011).

*In vitro*, hypoxic/ischemic conditions can be created through oxygen deprivation and hypoglycemia. Typically damage is assessed by infarcted area/neuronal cell loss. In both primary cortical and hippocampal cultures, ligands acting acutely on A₁Rs mitigated neuronal damage (reviewed by Cunha 2005). In direct contrast, antagonism of this receptor exacerbated morphologically assessed damage (reviewed by de Mendonça et al. 2000). In vivo, acute CPA (A₁R agonist) administration was able to decrease neural loss and mortality in gerbils following insult. A₁R agonists have also been found to prevent the damage associated with NMDA excitotoxicity (discussed by de Mendonça et al. 2000). The mechanisms by which A₁R activation acts to accomplish neuroprotection are not well elucidated, but likely center around its ability to inhibit Ca²⁺ influx, NMDAR function, and glutamate release.

While acute administration of A₁R agonists is neuroprotective, chronic administration exacerbates damage (discussed by de Mendonça et al. 2000). This inversion of protective benefit has been observed with antagonists as well. Whereas the nonselective adenosine antagonist caffeine (or the selective A₁R antagonist CPX) has been shown to exacerbate ischemic damage acutely, it confers protection at low chronic doses (Jacobsen et al. 1996). Chronic noxious conditions which cause sustained elevations of adenosine in the brain result in a down regulation of the A₁R (Rebola et al. 2005). The result is a decreased ability of A₁R
activation to be neuroprotective (Sweeney 1997, reviewed by Cunha 2005). The therapeutic window for adenosine acting on A₁Rs also appears to be acutely after (within 15 minutes) the traumatic event (Sweeney 1997). In this manner A₁R activation is not a sustainable therapy in the long term but only acutely. Considering traumatic brain events are difficult to predict and the acute administration of a drug during a seizure or stroke is not very realistic, the use of A₁R agonists therapeutically faces major challenges.

While the protective role of A₁R agonists is perhaps intuitive, A₂A antagonists have received attention for their neuroprotective actions as well. A₂A receptor blockade is in fact neuroprotective in response to ischaemic or hypoxic insult, particularly in cortex. (reviewed by Cunha 2005). In the rat, A₂A antagonist SCH 58261 reduced brain injury when administered 10 minutes after focal ischaemia (Monopoli et al. 1998). A₂A knockout mice also demonstrated attenuated damage due to ischemia (Chen et al 1999). The potential mechanisms conferring this protection are several, yet it is an area that remains poorly defined. It is possible that blockade of endogenous adenosine acting on A₂A receptors influences the intracellular cascades which control apoptosis. Blockade may also control inflammation (As supported by the observation that A₂A receptor blockade mitigates inflammation triggered by lipopolysaccharide in the hippocampus; Rebola et al. 2011), astrogliosis following trauma, and perhaps through known actions on glutamate release/clearance (reviewed by Cunha 2005, Gomes et al. 2011). A₂A receptors don’t desensitize as readily as A₁Rs, and clinically relevant doses have less peripheral side effects also making them a reasonable candidate for clinical application (Cunha 2005).
Epilepsy

The release of ATP and adenosine during seizure activity has been demonstrated, suggesting a role as a neuroprotective agent mitigating the damage of seizures, and mediating seizure termination (Dale and Frenguelli 2009). The A₁R specifically has been implicated in seizure suppression. The pre and post-synaptic actions of the A₁R, including a reduction in glutamate release and a hyperpolarization of the cell membrane, provide a hurdle which makes overexcitation more difficult (Gomes et al. 2011, Rosim et al. 2011). While adenosine deficiency has been linked to epilepsy, and it is etiologically more relevant than current sodium channel blockers, the activation of A₁Rs in a manner as coarse as the application of agonists incurs a wide range of side effects in the CNS and also peripheral tissues (including the heart). Fortunately, a more subtle influence of adenosine levels has been achieved through the manipulation of adenosine kinase (ADK) expression.

Astrocytes have more recently been recognized as significant contributors to transmission and network activity. They are a significant source of adenosine through the release of ATP, and a prominent source of ADK. The rapid clearance of intracellular adenosine intracellularly in astrocytes by ADK couples with their passive channels on the membrane to provide a gradient in which adenosine can be rapidly removed from the extracellular space. Excessive ADK activity can cause seizures by creating abnormally low levels of extracellular adenosine. Astrogliosis, an increase in astrocyte number owing to neuron loss from traumatic events, can provide the circumstances in which ADK becomes overexpressed and extracellular
adenosine is cleared too exhaustively. ADK overexpression in mice was sufficient to cause spontaneous seizures (Boison 2013). Supporting the role of ADK expression clinically is the observation that temporal lobe epilepsy patients have lower adenosine levels and higher ADK expression (Boison 2013). As might be expected, ADK inhibitors can indeed suppress seizures (Boison 2013).

Recently, a ketonergic diet (high-fat and low carbohydrate) has been found to suppress seizures by reducing adenosine kinase expression and increasing the activation of A1Rs in mice (Masino et al. 2011). The ketonergic diet switches cellular metabolism away from glycolosis (which is ATP-producing) and toward one that increases fatty acids, lowers glucose, and increases ketone bodies. A major consequence of this is a reduction in ADK expression. The result of decreased ADK expression is that more adenosine is available extracellularly to act upon A1Rs. The ketonergic diet was not effective in mice with a full knockout of the A1R. Increased activation of A1Rs caused by lower ADK expression is seizure-suppressing. The ketonergic diet’s subtle manipulation of A1R activation may avoid many of the negative side effects observed with pharmacological agents (Rho et al. 2013).

Adenosine is released in an activity-dependent manner, particularly during traumatic events where there is massive ATP expenditure and excessive excitation. Its release in response to these events, combined with its powerful suppressant effects on excitatory transmission place it at center stage as an endogenous ‘emergency response’ system within the cortex. Epileptic seizures are typically cortical phenomena, and in light of the complexities of mechanisms by which adenosine exerts a neuroprotective/antiepileptic effect, an explicit demonstration of adenosine’s synaptic actions within the neocortex may help to clarify the

synaptic and network basis for seizure suppression. Clearly astrocytic clearance of adenosine plays a role, but the mechanism by which restored A₁R activation actually suppresses seizures is suggestive. Decreased glutamate release and decreased cell excitability are both reasonable candidates, yet understanding of the pre and post synaptic actions of neocortical adenosine suffer from being mostly implied, rather than experimentally demonstrated.

Sleep

Due to the noted effects of the nonselective antagonist caffeine as a minor stimulant, and the ability of it and other adenosine receptor antagonists to promote wakefulness, adenosine has been implicated in sleep behavior.

Increasing adenosine levels in the brain promotes sleep while decreasing adenosine levels increases wakefulness (Bjorness and Greene 2009). Consistent with this is the converse observation that adenosine levels rise during waking and decrease during sleep. Specifically, rises during waking are seen in the basal forebrain and cortex whereas decreases during sleep are seen in the BF, cortex, brainstem, and hypothalamus (Bjorness and Greene 2009). Sleep deprivation in specific causes a rise in adenosine levels within the basal forebrain and the neocortex (Porkka-Heiskanen et al. 1997, Strecker et al. 2000, Stenberg 2007). Adenosine, by modulating transmission in the thalamus, hypothalamus, basal forebrain, and brainstem can influence the transition between behavioral states.

Thalamocortical (TC) and thalamic reticular nucleus (TRN) neurons display different modes of firing in response to inputs, depending on their resting potential. At depolarized states they transmit spikes reliably (tonic firing), while highly hyperpolarized TC/TRN neurons
display more time between bursts, resulting in a shift to oscillatory burst firing and an initiation of delta waves transmitted to the cortex (Bjorness and Greene 2009, Halassa 2011). In the lateral geniculate nucleus, adenosine has been demonstrated to act postsynaptically to increase K+ conductance and hyperpolarize cells, shifting their preference toward burst-firing (Pape 1992). Within the cortex, inputs from the thalamus are also modulated by adenosine. In a thalamocortical slice preparation of mouse, the responses of thalamocortical inputs onto layer 4 onto both interneurons and pyramidal neurons were measured. Adenosine acting on presynaptic receptors at thalamocortical terminals reduced the release probability of glutamate onto both inhibitory cells and excitatory cells (Fontanez and Porter 2006).

The basal forebrain (BF) contains both cholinergic and noncholinergic neurons which project to the cortex. These cholinergic inputs to the cortex are more active during waking and REM sleep than in NREM sleep. Adenosine causes an inhibition in wake-promoting cholinergic basal forebrain neurons (Strecker et al. 2000, Bjorness and Greene 2009). Local adenosine perfusion into the BF reduced wakefulness and cortical arousal. In contrast, A1R antagonism did the opposite (Strecker et al. 2000). Adenosine agonists can inhibit transmission in the mesopontine tegmentum as well. The result of inhibition here is again a reduction in the activity of the cholinergic system, which would facilitate sleep. Within the diagonal band of Broca (in the basal forebrain) and the mesopontine tegmentum, adenosine reduces excitability in part by acting postsynaptically through increased K+ conductance (Rainnie et al. 1994). The hypothalamus contains hypocretin/orexin neurons which again are active specifically during waking (Bjorness and Greene 2009). Adenosine here suppresses excitatory inputs to these neurons through presynaptic inhibition of glutamate release. The hypocretin/orexin neurons
themselves demonstrated reduced postsynaptic Ca\(^{2+}\) currents in response to adenosine, through no GIRK mediated current was observed (Liu and Gao 2007). Adenosine inhibiting glutamatergic and cholinergic transmission, and decreasing neuronal excitability in the above brain areas serves to inhibit arousal systems and excitatory inputs to cortex, promoting sleep.

In the ventrolateral preoptic area (VLPO) there exist ‘sleep-active’ neurons which are active, as indicated, primarily during sleep. Upon adenosine application in this area, spontaneous IPSP/Cs were decreased whereas EPSP/Cs were unaffected. The disinhibition of ‘sleep-active’ neurons in the VLPO then serves to promote the behavioral state of sleep (Strecker et al. 2000, Morairty et al. 2004). This preferential action of adenosine on inhibitory transmission is interesting because in other brain areas (such as hippocampus) adenosine preferentially inhibits excitatory transmission (Yoon and Rothman 1991). The intriguing consequence of this action is that the end result is still somnogenic, and therefore in agreement with the roles of adenosine in sleep in other areas. There also exist neurons in the preoptic/anterior hypothalamic area (POAH; ventral forebrain) which are primarily active during slow wave sleep. Adenosine again activates these neurons indirectly through an inhibition of GABAergic transmission (Strecker et al 2000). Adenosine then disinhibits ‘sleep-active’ neurons to promote sleep while simultaneously inhibiting arousal systems which when active promote waking.

Adenosine’s most generalizable effects, namely the inhibition of transmitter release and the reduction in postsynaptic excitability, have profound consequences due to the anatomical importance of the nuclei they act within. In the thalamus, intrinsic properties of these neurons which allow them to fire in two states are directly modulated by the ability of
adenosine to hyperpolarize neurons and reduce cell excitability. In the so-called arousal and somnogenic areas discussed, an inhibition or disinhibition through these general mechanisms contributes directly to the transition of behavioral state. While receptor profiles and multiple sources of adenosine certainly create a diversity of scenarios, even a simple and straightforward action of adenosine mediated inhibition can have profound consequences when applied to specific circuits. This highlights adenosine’s role as an important modulator, tuning the actions of other transmitter systems such as Acetylcholine, GABA, and glutamate.

**Sleep Homeostasis**

Adenosine also acts to modulate transmission within the state of sleep itself. Within sleep higher adenosine concentrations promote deeper sleep and higher slow wave activity (SWA; 0.5-4 Hz range of the EEG during NREM sleep). While not necessary for the production of SWA, $\text{A}_1$Rs play a role in its modulation (Bjorness and Greene 2009). A net potentiation of synapses cooccurring with an increase in sleep pressure is thought to occur during waking. A proposed role of sleep, and specifically slow-wave sleep, is that it restores metabolically sustainable synaptic distributions while maintaining the relative weights of synapses that presumably underlie memory. SWA is characteristic of the EEG during NREM and is thought to underlie this homeostatic ‘synaptic downscaling’ (Tononi and Cirelli 2006). Normal mice will respond to sleep deprivation (and increased sleep pressure) with increased SWA in subsequent sleep. $\text{A}_1$R knockout mice showed a largely absent SWA compensation after deprivation.

Following the knowledge that $\text{A}_1$Rs play an important role in sleep homeostasis, the importance of astrocytic sources of adenosine in these processes was demonstrated. Mice in
which vesicular release from astrocytes was selectively impaired were utilized (dnSNARE mice; Halassa et al. 2009). Adenosine, arising from vesicularally released ATP was found to be a major factor in A₁R activation and subsequent modulation of sleep homeostasis in the cortex. DnSNARE expression did not significantly change sleep time or architecture, however sleep pressure was reduced. This was demonstrated by a decrease in SWA, particularly in the lower frequencies. Low frequency slow waves (<1 Hz) have been shown to be a cortical phenomenon, present even in the absence of thalamic input (Contreras and Steriade 1995). Sleep deprivation, a manipulation which results in an increase in sleep pressure, is normally accompanied also by increased sleep time. In contrast to their wildtype counterparts, dnSNARE mice did not respond to sleep deprivation with an increased total sleep time. The A₁R antagonist CPT administered to wildtype mice mimicked the phenotype of dnSNARE mice, while CPT given to dnSNAREs had little to no effect. Sleep deprivation and increased sleep pressure also results in cognitive deficits, so behavioral assessments of the impact of impaired gliotransmission were assessed. In a novel object recognition task, wildtype mice were impaired following sleep deprivation, whereas dnSNARE mice (demonstrating decreased sleep pressure) were not affected. Again, manipulation of A₁R by ligands to mimic the dnSNARE condition (lack of A₁R activation) mimicked the phenotype of dnSNARE mice. The mechanisms of how neocortical adenosine modulates this SWA is poorly understood. Phasic release of D-Serine and ATP from astrocytes (Halassa 2011), alterations in the excitatory/inhibitory balance, and/or changes in neuronal excitability is likely to contribute, but explicit demonstration of many of these theories is lacking. These mechanisms would also rely on specific synaptic actions of adenosine, which are also poorly elucidated within the neocortex.
Within the Neocortex

Early electrophysiological research on purines in cerebral cortex documented the ability of adenosine and related purines to suppress excitatory transmission (Harms et al. 1978 on adenosine's ability to inhibit noradrenaline, Kostopoulos and Phillis 1977, Stone and Taylor 1980, Pedata et al. 1983 on the release of acetylcholine, Kostopoulos et al. 1989 and O'Shaughnessy et al. 1988 on suppression of epileptiform activity). The results were consistent with more detailed subsequent research, namely adenosine and other purines act to suppress synaptic transmission, and are able to inhibit the release of glutamate, noradrenalin, and acetylcholine. Thus adenosine came to be considered as a neuromodulator, with widespread and powerful effects on transmission.

In the neocortex, application of adenosine receptor agonists leads to a suppression of both excitatory transmission (specifically in layer 5; Brand et al. 2001, Murakoshi et al. 2001), and GABAergic transmission (Kruglikov and Rudy 2008, Kirmse et al. 2008 for the developmentally relevant inhibition of GABAergic transmission by adenosine during early postnatal development). In regards to inhibitory transmission, GABAergic inputs from fastspiking interneurons onto layer 5 pyramidal cells of the somatosensory cortex were inhibited by adenosine. The reduction in inhibitory postsynaptic currents (IPSCs) here was found to be due to presynaptic mechanisms (likely a reduction in release probability; Kruglikov and Rudy 2008). Regarding adenosine’s ability to inhibit excitatory transmission, EPSPs in layer 5 pyramidal neurons have been shown to be inhibited by adenosine (specifically through presynaptic A1Rs).

In intracellular recordings from pyramidal cells in layer 5 of the associative frontal cortex, post-
synaptic potentials (PSPs) evoked by stimulation in layer 1 were demonstrated to be suppressed by $A_1$R activation (Brand et al. 2001). In this study, the amplitude and slope of PSPs were decreased when the $A_1$R agonist CPA was bath applied. The NMDA and non-NMDA component of the PSP were not differentially affected which is explicable by invoking a strictly presynaptic site of action. Further supporting this, no changes in membrane potential or input resistance were observed after application of the $A_1$R agonist (Brand et al. 2001). Layer 5 pyramidal cells of the visual cortex have also been studied. Evoking excitatory postsynaptic currents (EPSCs) by stimulating neighboring layer 5 pyramidal cells, it was demonstrated that adenosine decreased the amplitude of elicited EPSCs and increased the paired-pulse ratio (PPR), indicating a presynaptic locus of action (Murakoshi et al. 2001). In addition, adenosine reduced the frequency of miniature EPSCs recorded under application of 1μM TTX. No effect of adenosine on the mean amplitude of miniature events was found, indicating no postsynaptic modulation of the EPSC. More recently transmission from layer 4 onto layer 2/3 pyramidal cells in the somatosensory cortex has been studied. Mice with impaired astrocytic vesicle release (dnSNARE mice), which subsequently show a lack of $A_1$R activation, demonstrated reduced NMDAR function while AMPA currents remained unaffected (Deng et al. 2011). In addition, mEPSC frequency was increased in dnSNARE mice, suggesting tonic activation of cortical $A_1$Rs by astrocytic-derived adenosine suppressed presynaptic release. The implication of A1Rs specifically is supported by the observation that $A_1$R antagonism mimics the dnSNARE condition (Deng et al. 2011). Again the locus of adenosine’s action appears to be presynaptic $A_1$Rs.

There is notable heterogeneity of effects within the neocortex itself. The magnitude of adenosine’s suppression and the locus of action (pre and post-synaptic) vary in different
preparations. Within the neocortex, it has been shown that different layers may be differentially affected by adenosine. Using optical imaging with voltage sensitive dye, these regional differences in the ability of adenosine to suppress transmission were investigated (Kovac et al. 2008). Stimulation in layer 1/2 or in the border between white/grey matter was used to evoke activity in the slice which traversed the lamina of the cortex. Adenosine significantly suppressed evoked activity in layers 4, and most profoundly in 2 and 3. Audioradiographic data in the visual cortex of the monkey has revealed distribution of A₁Rs is highest in layer 2/3 and 5, and lowest in layer 4 (Chaudhuri et al. 1998). The details of adenosine’s actions in any preparation, then, cannot be established through generalization, even across different synapses within the neocortex.

Our knowledge of the actions of adenosine in the neocortex is incomplete. How adenosine effects synaptic transmission to layer 2/3 pyramidal neurons of the visual cortex remains largely unknown. The primary source of feedforward inputs to layer 2/3 arises from neurons of layer 4 (which receive projections from the lateral geniculate nucleus; Tucker and Fitzpatrick 2004) Layer 2/3 neurons also have horizontal connections between each other, and mediate communication between cortical areas. This connectivity makes them a likely candidate to mediate network activity such as global oscillations.

**Rationale for the current work**

Different profiles of receptor expression set the stage for how adenosine is able to influence transmission. For example, interaction of the A₂AR with other pre, post, and extrasynaptic receptors results in different binding profiles and transduction mechanisms, and
very different consequences for transmission (Orrú et al. 2011). Within the striatum densely expressed postsynaptic A\textsubscript{2A}Rs integrate dopaminergic transmission on medium spiny neurons while incoming cortico-striatal glutamate terminals express A\textsubscript{2A}Rs and A\textsubscript{1}Rs which operate together to control glutamate release locally. Within the hippocampus it has been proposed that sparse, presynaptically located A\textsubscript{2A}Rs serve to facilitate transmission specifically during high frequency stimulation at homosynaptic sites. Knowledge of the expression of A\textsubscript{2A}R mRNA within the neocortex (Dixon et al. 1996, Fredholm et al. 2001), informs us little of the function of these receptors. The vast heterogeneity of A\textsubscript{1}R and A\textsubscript{2A}R expression, transduction, and interactions is the basis of a need for explicit demonstrations of synaptic capabilities of adenosine in any brain area of interest.

Adenosine’s modulation of plasticity outcomes is well documented in the hippocampus, but its role in neocortical plasticity is unknown. Because understanding of adenosine at the synaptic level in neocortex is understudied, educated theories about modulation of plasticity in the cortex are missing critical groundwork, yet one may not inappropriately generalize from the hippocampus. The well-documented, intricate, and unique interaction between dopamine and adenosine in the striatum further highlight the incredible complexity of receptor-receptor interactions and integration of modulatory systems. It is likely that adenosine’s modulation of transmitter systems involves complex receptor interactions in other brain areas as well. An explicit demonstration of what receptors for adenosine are functional and their pre- or postsynaptic location is a prerequisite for more detailed investigations into how these receptors interact with other transmitter systems. Again, this foundation is lacking within the neocortex. Adenosine modulation of neocortical slow waves is
demonstrated, yet again, the synaptic basis which makes this modulation possible is unexplored. The profound impact of adenosine on seizure activity is well known but typically assessed at a more macroscopic level than the synapse. Proposals for mechanism are suggestive, supported by knowledge of hippocampal and subcortical cellular actions. Known behavioral and physiological actions of adenosine in the neocortex all share a gap in understanding of the synaptic basis of their action. The work that has been done on the synaptic level within the neocortex has focused mostly on incoming thalamocortical inputs, or layer 5 pyramids. Knowing there is a heterogeneity of adenosine’s impact across the lamina, the synaptic actions of adenosine in the cortex are largely unexplored.

The rationale for explicit characterization of a given synapse’s ‘adenosine profile’ should then be clear. Important physiological events within the neocortex, such as sleep homeostasis, network oscillations, plasticity, and neuroprotection have been demonstrated explicitly to (or are likely to) be modulated by adenosine. The lack of electrophysiological characterization of adenosine’s actions in the neocortex then is a clear missing link. This link has been implicitly supplied by the assumption adenosine acts here as in other brain areas, but the experimental evidence exposes the dangers of such assumptions and questions the existence of a ‘typical’ role for adenosine.
**Introduction: the present work**

The primary aim of the present work is to gain explicit knowledge of the actions of adenosine in the neocortex on a cellular level. Here, we investigate the effects of adenosine on evoked excitatory postsynaptic potentials (eEPSPs), spontaneous miniature postsynaptic potentials (mEPSPs), and membrane properties in layer 2/3 pyramidal cells of the rat visual cortex *in vitro*. We show that adenosine suppresses excitatory synaptic transmission in layer 2/3 of the visual cortex. Adenosine acts presynaptically to reduce release probability and postsynaptically to decrease input resistance and hyperpolarize the cell. In agreement with the A₁R's role in the inhibitory actions of adenosine, we find selective blockade of this receptor by DPCPX to prevent the actions of adenosine in our preparations. Unlike other work in the cortex, we found a robust action of adenosine (through the A₁R) on membrane properties. There was hyperpolarization of the membrane and a reduction in input resistance consistent with the activation of K⁺ conductance. We also provide what is (to our knowledge) the first demonstration of A₂AR mediated actions on basal transmission in the neocortex. Using A₂AR antagonist SCH 58261, we found high concentrations of adenosine activate A₂ARs which exert a facilitatory effect on transmission.

**Methods**

*Slice Preparation*

All experimental procedures used in this study are in compliance with the US National Institutes of Health regulations and were approved by the Institutional Animal Care and Use
Committee of the University of Connecticut. Details of slice preparation and recording were similar to those used in previous studies (Volgushev et al. 2000; Lee et al 2012). Wistar rats (15-32 days old) were anaesthetized with isoflurane, decapitated, and the brain was quickly removed and placed into an ice-cold oxygenated artificial cerebrospinal fluid solution (ACSF), containing, in mM: 125 NaCl, 25 NaHCO₃, 25 glucose, 3 KCl, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgCl₂, bubbled with 95% O₂/5% CO₂, pH 7.4. Coronal slices (350 μm thickness) containing visual cortex were prepared from the right hemisphere. Slices were allowed to recover for at least an hour at room temperature. For recording, individual slices were transferred to a recording chamber mounted on an Olympus BX-50WI microscope equipped with infrared differential interference contrast (IR-DIC) optics. In the recording chamber slices were submerged in oxygenated ACSF at 28°-32°C.

*Intracellular recording and synaptic stimulation*

Layer 2/3 pyramidal cells from visual cortex were selected for recording in the whole cell configuration. Identification of pyramidal neurons using DIC microscopy was reliable as demonstrated in our previous work with biocytin labelling and morphological reconstruction of recorded neurons (Volgushev et al 2000). Intracellular pipette solution contained, in mM: 130 K-Gluconate, 20 KCl, 10 HEPES, 10 Na-Phosphocreatine, 4 Mg-ATP, 0.3 Na₂-GTP, (pH 7.4 with KOH).

Two pairs of stimulating electrodes (S1 and S2) were placed in Layer 4, below the Layer 2/3 recording site (Fig 1). Stimulation current intensities were adjusted to evoke monosynaptic excitatory postsynaptic potentials (EPSPs) in the recorded neuron. In some cases picrotoxin (100 µM Sigma) was used to block inhibitory transmission. We used paired-pulse stimulation
protocol with a 50 ms inter-pulse interval. Paired stimuli were applied to S1 and S2 in alternating sequence once per 7.5 seconds, so that each input was stimulated with paired pulses each 15 seconds.

For experiments measuring spontaneous miniature EPSPs (mEPSPs), tetrodotoxin (TTX; 0.1-0.5 µM, Tocris) was added to the extracellular solution, at least 25 minutes before recordings were started. All drugs were bath applied. All chemicals were obtained from Sigma-Aldrich unless stated otherwise. Adenosine was dissolved in ACSF to a 1 mM stock before being applied to the bath. DPCPX was dissolved in a 60% ethanol solution to a 0.5 mM stock. Final ethanol concentration in the bath ranged from $1.8 \times 10^{-4}$ to $1.8 \times 10^{-2}$ % for 1.5-150 nM DPCPX. SCH-58261 was dissolved in 100% ethanol to a 2.5mM stock. Final bath concentration of ethanol in SCH-58261 experiments was $12 \times 10^{-4}$ %. 
**Data analysis**

Data analysis was made using custom-written programs in MatLab (© The MathWorks, Natick, MA, USA). All inputs included in the analysis fulfilled the criteria of (1) stability of EPSP amplitudes during the control period, (2) stability of the membrane potential throughout the recording, and (3) stability of the onset latency and kinetics of the rising slope of the EPSP. EPSP amplitudes were measured as the difference between the mean membrane potential during two time windows. The first time window was placed before the EPSP onset and the second time window was placed on the rising slope of the EPSP, just before the peak. Amplitude of the
second EPSP in paired-pulse stimulation paradigm was measured using windows of the same duration, but shifted by the length of the inter-pulse interval (50 ms).

Spontaneous mEPSPs were detected as following. Two windows (a window 1, ‘baseline’, 2.5 ms in width and a window 2, ‘amplitude’ 1.5 ms in width) were spaced 2.5 ms and swept the recorded trace. Within each window the mean membrane potential was measured. When the difference between the mean membrane potential in the ‘amplitude’ and the ‘baseline’ window (window 2 – window 1) was at least 0.2 mV but no more than 2 mV, an event was detected. After this automatic detection, all detected events were visually checked for their shape, and erroneously detected traces were excluded from analysis. We measured frequency of mEPSCs, amplitude of each event and compiled their amplitude distributions. From the amplitude distributions, we calculated the skew of the distribution and determined median.

Significance tests were accomplished utilizing Student's t-tests or one-way ANOVAs with Post-hoc comparisons (Dunnett’s and Tukey’s HSD). Error bars represent the standard error of the mean (± SEM).

Results

Adenosine reduces the amplitude of evoked EPSPs and increases the paired pulse ratio

To study the effects of adenosine on synaptic transmission to layer 2/3 pyramidal neurons we recorded EPSPs evoked by paired-pulse electric stimuli in control and during bath application of adenosine (Sigma-Aldrich) at different concentrations. To determine an effective concentration, we used bath application of 5 µM to 50 µM adenosine (Fig. 2). Already with the lowest tested concentration of 5 µM, adenosine induced a clear decrease of the EPSP amplitude to 64.3 ± 4.3% of baseline (p <0.001). Application of increasing concentrations of adenosine led
to a progressive reduction of the EPSP amplitude (Fig 2A, B). 20 µM of adenosine had a robust effect, reducing the EPSP amplitude to 32 ± 4.6% of baseline (p < 0.001). This concentration was selected for further experiments.

The reduction in EPSP amplitude by adenosine was accompanied by an increase in the paired-pulse ratio (PPR; Figure 2B). The PPR is an index of release that is inversely related to the release probability (P_r) at a synapse (Stevens 1993; Voronin 1993). The increase in the PPR is suggestive of a decrease in release probability. Changes in the EPSP amplitude observed during adenosine application were negatively correlated with the changes in the PPR, indicating the adenosine acted on presynaptic receptors to reduce release probability (Fig 5, open circles; r = -0.620, n = 39, p < 0.001)
Fig 2. Adenosine reduces evoked EPSP amplitude and increases paired-pulse ratio (PPR) in a reversible and concentration dependent manner.

A. (Above) Traces of averaged EPSPs evoked in a layer 2/3 neuron from visual cortex by paired stimuli (50 ms interpulse interval) in control and through increasing concentrations of adenosine. (Below) The time course of amplitude changes of the responses to the first pulse in a pair (EPSP1, % of control). Data for same cell.

B. Changes of the amplitude of EPSP1, EPSP2 and paired-pulse ratio (PPR) induced by increasing concentration of adenosine. EPSP amplitudes were normalized by the amplitude of the EPSP1 in control for each cell, and then averaged for N=5 neurons (10 inputs). Adenosine reduces EPSP amplitude and increases PPR in a concentration dependent and reversible manner. Significance denoted as * p<0.05; **p<0.01; *** p<0.001. Significance for EPSP2 mirrored EPSP1 for all concentrations of adenosine tested, yet "****" is omitted for clarity.
Adenosine reduces the frequency of spontaneous miniature EPSPs

To corroborate the evidence that presynaptic mechanisms are involved in adenosine’s effects on synaptic transmission we recorded miniature EPSPs in the presence of 0.5 µM tetrodotoxin (TTX) in the bath. Figure 3A illustrates membrane potential traces and detected mEPSPs. Application of adenosine (20 µM) led to a decrease of the frequency of mEPSPs (Fig. 3A, C). On average, mEPSP frequency decreased from 9.0 ±0.9 events/sec in control to 6.5 ±0.7 events/sec (when normalized, 80.4 ±4.5% of baseline frequency) during adenosine application (Fig 3C; n = 20, p < 0.01). After washout of adenosine, mEPSP frequency recovered to 9.5 ±1.2 events/sec (when normalized, 104.3 ±9.8% of baseline frequency).

Adenosine application led to reduction of frequency of mEPSPs of all amplitudes: the amplitude distributions were scaled down, but their skew did not change (2.9 ±0.3 vs 3.1 ±0.4; n.s.). There was however a very small yet significant reduction in the median amplitude of events, from 0.29 ±0.02 mV to 0.28 ±0.02 mV, (or when normalized, to 95.0 ±1.2% of baseline; Fig 3D n = 20, p < 0.01). This reduction of the median mEPSP amplitude may be attributable to a ~20% decrease of the input resistance of neurons during adenosine application (see below).

A1R blockade prevents adenosine’s effects on evoked EPSPs

Which receptors mediate adenosine’s effects on synaptic transmission? In situ hybridization, reverse transcription-polymerase chain reaction, binding studies, and other
biochemical methods have demonstrated high expression of A$_1$Rs in the neocortex (Dixon et al. 1996, Fredholm et al. 2001). These receptors are classically responsible for the inhibitory actions of adenosine. Therefore we first studied how blockade of A$_1$Rs affect the inhibitory actions of adenosine on synaptic transmission. We applied 20 µM adenosine on the background of varying concentrations of the selective A$_1$R antagonist 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX). DPCPX blocked the adenosine-induced reduction in EPSP amplitude in a concentration dependent manner (Fig 4). The effect of DPCPX was clear even at the lowest concentration tested (1.5 nM), and in the presence of 30 nM DPCPX, application of 20 µM adenosine failed to induce significant reduction of EPSP amplitude. Figure 4B displays summary data on the EPSP amplitude and PPR changes during application of 20 µM adenosine as a percent of baseline. In control conditions (no DPCPX), 20 µM adenosine reduced the EPSP amplitude to 39.4 ± 4.6% of baseline (Fig. 4B, C; p<0.01, n= 13; significant reduction from baseline denoted in C). On the background of 1.5 nM DPCPX adenosine reduced the EPSP amplitude to 65.4 ± 6.7% of baseline. This reduction was significantly smaller than in the control group (Fig 4B, p < 0.05). On the background of 30nM DPCPX, adenosine reduced the EPSP amplitude to only 93.4 ± 6.6% of baseline, again significantly less than in the control condition (Fig 4B; p < 0.001). In fact, 30 nM DPCPX was sufficient to completely block the effect of adenosine on EPSP amplitude. No significant reduction in EPSP amplitude was found after application of adenosine on the background of 30nM DPCPX (Fig 4C, top).
Fig 3. Adenosine reduces miniature EPSP (mEPSP) frequency and decreases the median amplitude of events.

A. Example traces of spontaneous activity from one cell in control and in the presence of 20 µM adenosine. The slopes of detected mEPSPs are highlighted in pink and marked with arrows.

B. Examples of individual (top) and averaged (bottom; N=509 events for CTRL; N=355 events for Ado) miniature EPSPs and their amplitude distributions from 2.5 min recordings in control and during application of 20 µM adenosine.

C, D. Changes of the frequency of mEPSPs (C) and their median amplitude (D) during application of 20 µM adenosine and washout. Both the reduction in frequency of events and median amplitude of events recovered after washout of adenosine. Averaged data for N=18 cells.
The attenuation of the adenosine-induced reduction in EPSP amplitude in the presence of DPCPX was accompanied by an attenuation of the adenosine-induced increase in PPR. Figure 4B (bottom) shows a summary of the PPR changes induced by application of 20 µM adenosine on the background of different concentrations of DPCPX. In control conditions (no DPCPX), application of 20 µM adenosine increased the PPR to 144 ± 12.9% of baseline (Fig 4B, C; p < 0.01, significant increase denoted in C). In the presence of 1.5 nM DPCPX, the increase was smaller (122 ± 6.6%), and became significantly different from the control group at 7.5 nM (108 ± 6.1% of baseline; p < 0.05). With 30 nM DPCPX, adenosine did not elicit any significant increase in the PPR, just as it did not depress the EPSP amplitude (Fig 4B,C).

In experiments with adenosine applied alone or in the presence of DPCPX, the changes in EPSP amplitude were inversely proportional to the PPR changes (Fig 5). A strong negative correlation between EPSP amplitude changes and PPR changes was found in DPCPX experiments ($r = -0.693, n = 52, p < 0.001$), and experiments in which adenosine was applied alone at different concentrations ($r = -0.620, n = 39, p < 0.001$ for concentration dependence experiments and $r = -0.546, n = 13, p < 0.05$ for the control group for DPCPX experiments). The correlation remained strong when all groups were pooled together ($r = -0.644, n = 104, p < 0.001$) indicating that the relationship between EPSP/PPR change is maintained when manipulating the activation or blockade of A1Rs by various ligands. These results suggest that presynaptic A1Rs are mediating adenosine’s effects on EPSP amplitude by modifying release probability.

Adenosine-induced decrease of the frequency of mEPSPs was also found to be mediated by A1 receptors. On the background of 30 nM DPCPX, application of 20 µM adenosine
failed to induce any changes of the frequency of mEPSPs (9.8 ± 1.5 vs 9.6 ± 1.6 events/sec, n= 15, data not shown).

To uncover any tonic activation of A₁Rs, DPCPX was applied to the slice without the application of adenosine. The application of 7.5 nM DPCPX on the background of 0.1µM TTX and 100 µM picrotoxin led to a non-significant increase of excitatory mEPSP frequency from 7.9 ± 1.9 events/sec to 10.9 ± 3.1 events/sec (n = 6, n.s.; data not shown). An increase in mEPSP frequency would suggest that the blockade of A₁Rs by DPCPX relieves a tonic activation of these receptors and thus tonic inhibition of release by adenosine. The experiment described here trended toward this result but lacked statistical power. Although suspected, the present results cannot confirm physiologically relevant tonic levels of adenosine in the neocortex in vitro.

Taken together, the results of experiments with DPCPX demonstrate that the inhibitory effects of adenosine on synaptic transmission to pyramidal neurons in layer 2/3 are mediated via activation of presynaptic A₁ receptors which have a suppressive effect on release probability.
Fig 4. **A1 receptor antagonist DPCPX blocks adenosine’s effects on synaptic transmission.**

**A.** Example traces of evoked EPSPs before (black) and after application of 20 µM adenosine (red) on the background of DPCPX. Data for different concentrations of DPCPX are from different cells.

**B.** Concentration dependence of the blockade of adenosine’s effects on synaptic transmission by DPCPX. Changes of EPSP1 amplitude (top) and PPR (bottom) induced by bath application of 20 µM adenosine in control (no DPCPX) and on the background of different concentrations of DPCPX. Number of synaptic inputs studied with each DPCPX concentration is indicated in parentheses in the top plot. Solid curves show sigmoid fit to the data points. Significance was calculated for the difference between percent reduction of EPSP amplitude by 20 µM adenosine in control group vs. the reduction in the presence of DPCPX.

**C.** Effects of 20 µM adenosine on synaptic transmission are completely blocked by 30 nM DPCPX. Data from B, with washout of adenosine. Changes of EPSP1 amplitude (top) and PPR (bottom) during application and washout of 20 µM adenosine, in control (black bars) and in the presence of 30 nM DPCPX (grey bars).
Fig 5. Adenosine induced changes in EPSP amplitude are inversely related to changes in PPR. Changes in PPR plotted against changes in the EPSP1 amplitude. For experiments with the application of adenosine of different concentrations (5-50 µM, open circles), changes in PPR and EPSP1 amplitude were correlated ($r = -0.62$, $n = 39$, $p < 0.001$). These changes were again correlated in experiments utilizing application of 20 µM adenosine on the background of different concentrations of DPCPX (1.5 - 150 nM, closed circles; $r = -0.69$, $n = 52$, $p < 0.001$). The control group for these experiments, which utilized 20 µM adenosine but no DPCPX, also yielded a correlation between PPR change and EPSP1 amplitude change ($r = -0.55$, $n = 13$, $p < 0.05$). The relationship between changes in EPSP amplitude and PPR is linear, and manipulation of A$_1$R activation by different ligands alters these measures in a way that maintains this relationship.
Adenosine reversibly hyperpolarizes the membrane and decreases the input resistance of layer 2/3 pyramidal neurons

In addition to the presynaptically mediated effects on synaptic transmission, adenosine application led to a decrease of the input resistance and hyperpolarization of the cell membrane in layer 2/3 pyramidal neurons. Input resistance was calculated from membrane potential responses to small steps of positive and negative current applied through the recording electrode (Fig 6A). During application of 20 µM adenosine the input resistance of the neurons decreased from 396.5 ± 44.4 Mohm to 314.0 ± 36.3 MOhm (Fig 6B, right; n = 14 p < 0.001). The decrease of the input resistance could have been the reason for the minor decrease of the median amplitude of spontaneous mEPSPs during adenosine application described above. The resting membrane potential was hyperpolarized during adenosine application from -65.3 ± 1.5 mV to -67.7 ± 1.8 mV (Fig 6B, left; n = 14 p < 0.01). The adenosine elicited changes of the membrane potential and of the input resistance were absent when 20 µM adenosine was applied on the background of 30 nM DPCPX (n = 13; data not shown). This suggests these effects are also mediated by the A1R.
Fig 6. Adenosine decreases input resistance and causes hyperpolarization in pyramidal neurons from layer 2/3.

A. Membrane potential response of a pyramidal neuron from layer 2/3 to current steps in control, during application of 20 µM adenosine, and after washout. Decreased slope of the voltage-current relationship during adenosine application (red) indicates a decrease of the input resistance.

B. Changes of the membrane potential (left) and input resistance (right) during adenosine application and washout. Individual data for N=14 neurons (grey) and their average (black). 20 µM adenosine hyperpolarizes cells and decreases their input resistance. Washout of adenosine demonstrates the reversibility of these effects.
**Effects of high concentration of adenosine on synaptic transmission**

In order to discover a maximal effect of adenosine, we conducted a series of experiments which included high concentrations of adenosine (100 and 150 µM). On 18 inputs from 14 cells, we bath applied 20, 100 and 150 µM adenosine and recorded evoked activity and membrane properties (Fig 7). Increasing adenosine concentration from 20 µM to 100 µM led to a significant decrease of EPSP amplitude from 58.0 ± 5.3% to 33.9 ± 6.1 % of the control (Fig. 7A, p<0.001). In 2 out of 18 cases, responses were completely abolished during application of 100 µM adenosine, but recovered upon wash out. Further increase of adenosine concentration to 150 µM did not result in a further significant decrease of response (28.3 ± 5.4% of the baseline), and did not increase the proportion of inputs in which responses were completely abolished.

The input resistance of the membrane followed similar concentration dependence as the EPSP (Fig 7C): significantly decreasing from control to 20 µM adenosine, and again from 20 µM to 100 µM adenosine. There was no significant change between 100-150 µM adenosine however, suggesting a saturating or nearly saturating effect of adenosine on input resistance. The hyperpolarization of the cell caused by the application of adenosine appears to be the most easily saturated measure of adenosine’s actions. After an initial hyperpolarization upon application of 20 µM adenosine, membrane potential did not hyperpolarize any further upon increasing adenosine concentration to 100 and then 150 µM (Fig 7B). Because evoked activity was not blocked in these experiments, the membrane potential expressed stronger fluctuations (as indicated by larger standard error of these measurements) as compared to experiments conducted under TTX (see Fig. 6). The possibility of a small effect size being masked by large variability cannot be excluded.
Functional $A_{2A}$ receptors are present in layer 2/3 of neocortex

Results presented so far show that $A_1$R activation plays a major role in mediating suppressive effects of adenosine on excitatory synaptic transmission in our preparation. Evidence obtained using immunochemical methods and binding of $A_{2A}$R ligands in synaptosomes suggests presence of $A_{2A}$Rs in the cerebral cortex albeit at much lower levels than for example, in the striatum (Lopes et al. 1999, Marchi et al. 2002, Lopes et al. 2004). The activation of $A_{2A}$Rs is associated with a facilitation of excitatory transmission, which is in opposition to $A_1$R effects. Because affinity to adenosine of $A_1$Rs is about an order of magnitude higher than of $A_{2A}$Rs, and the expression of $A_1$Rs in the cortex is much higher than other subtypes (Ciruela et al. 2006, Ciruela et al. 2011, Fredholm et al. 2001), effects of low concentrations of adenosine might have been due to $A_1$R activation. High concentrations (100-
150 µM) of adenosine could activate both A₁ and A₂A receptors. We questioned if at such high concentrations adenosine has saturated the A₁Rs but also activated facilitatory A₂A Rs. In this scenario the maximal reduction in EPSP amplitude elicited by A₁R activation would be curbed by A₂A R activation. The effect of adenosine on EPSP amplitude described in Fig. 7 would then be a combination of inhibitory effect of A₁R activation with a smaller facilitatory component produced by A₂A R activation. If this is the case, then under high concentrations of adenosine, the blockade of A₂A Rs should further reduce the amplitude of the EPSP. To test this conjecture, we conducted a series of experiments in which application of 150 µM adenosine was followed by application of the selective A₂A R antagonist SCH58261 (30 nM). As described above, application of 150 µM adenosine profoundly reduced the EPSP amplitude (Fig 8; average of 36.8± 7.6% of baseline; p <0.001). Addition of SCH58261 elicited a small yet highly significant further reduction in the EPSP amplitude (to an average of 29.2 ± 6.6% of baseline; p <0.001). This suggests functional A₂A Rs are present in the neocortex, where they act in opposition to A₁Rs to modulate excitatory transmission. Subsequent addition of A₁R antagonist DPCPX, and thus blockade of both A₁ and A₂A receptors was expected to relieve EPSPs from suppression. The EPSP amplitude increased after addition of 30 nM of DPCPX. However, the recovery was not complete, (only to 59.1 ± 9.8% of baseline). We attribute the incomplete recovery to the inability of the competitive A₁R antagonist DPCPX to completely block the activation of A₁Rs by 150 µM adenosine. Whereas 30 nM DPCPX was sufficient to block the effects of 20 µM adenosine (Fig. 4), it was not enough to compete with 150 µM.
Fig 8. On the background of a saturating concentration of adenosine, blockade of facilitatory A$_{2A}$ receptors by SCH58261 leads to further reduction of EPSP amplitude. Average reduction in evoked EPSP amplitude as percent of baseline (n = 16 inputs from 13 cells). After application of 150 µM adenosine, 30nM SCH58261 reduced the EPSP amplitude further, suggesting that under a high concentration of adenosine, the suppressive effect of inhibitory A$_{1}$Rs is curbed by the activation of facilitatory A$_{2A}$ receptors.
Discussion

Results of the present study demonstrate that in rat visual cortex, adenosine suppresses synaptic transmission to layer 2/3 pyramidal neurons, decreases their input resistance and leads to membrane hyperpolarization. These pre- and postsynaptic effects of adenosine are mediated predominantly by $A_1$ receptors. Our results also present evidence for functional $A_{2A}$ receptors that are activated in cortical neurons at high concentrations of adenosine.

Presynaptic action of adenosine: a reduction in release probability.

The most pronounced effect of adenosine application in layer 2/3 of the rat visual cortex was the suppression of synaptic transmission. Adenosine reduced the evoked EPSP amplitude in a concentration dependent and reversible manner. Concurrent with the reduction in the EPSP amplitude was an increase in the paired-pulse ratio. Because the paired-pulse ratio is inversely related to release probability (Voronin 1993; Murthy et al. 1997, Dobrunz and Stevens 1997, Oleskevich et al. 2000), these results suggest that an inhibition of transmission concurrent with an observed increase in the PPR was due to a reduction in release probability. This conclusion is consistent with prior data showing that adenosine and $A_{1R}$ agonists can increase the paired-pulse facilitation in the hippocampus and in layer 1 and 5 of the visual cortex (Dunwiddie and Haas 1985, Murakoshi et al. 2001, Kirmse et al. 2008). In agreement with this view, we found that 20 µM adenosine reduces the frequency of spontaneous mEPSPs, which reflects presynaptic properties. Other groups as well have witnessed adenosine’s ability
to change the frequency of spontaneous mEPSPs (Scanziani et al. 1992, Murakoshi et al. 2001, Deng et al. 2011,).

These effects of adenosine were mediated by the A₁Rs, as they were abolished by application of the selective A₁R antagonist DPCPX (30 nM). In hippocampus, presynaptic A₁ receptors mediate inhibitory effects on synaptic transmission by reducing the influx of calcium through voltage-dependent calcium channels (VDCCs; Wu and Saggau 1994, Wu and Saggau 1997, Gundlfinger 2007), or by modulation of release machinery downstream from Ca²⁺ influx (Wu and Saggau 1997, Scanziani et al. 1992, Deng et al 2011). In both cases, the result of this presynaptic inhibition would be a reduction of release probability.

Our results on the presynaptic effects of adenosine most closely correspond with Murakoshi et al. 2001. Upon adenosine application in layer 5 neurons of rat visual cortex they found a reduction in evoked EPSC amplitude, an increase in the paired-pulse ratio, and a decrease in miniature EPSC frequency. However, they report only presynaptic changes, and did not see any changes in membrane conductance or an effect on the mean mEPSC amplitude. Layer/region-specific effects of adenosine have been demonstrated in neocortex and hippocampus (Kovac et al. 2008). In their experiments, adenosine induced the most significant suppression of transmission in layer 2/3 of the neocortex, consistent with the demonstration of highly concentrated adenosine expression in layers 2/3 in visual cortex (Chaudhuri et al. 1998). Taken together with the present work, these results suggest adenosine acts differentially in different layers of the neocortex.

In the hippocampus, a tonic inhibition of synaptic transmission by endogenous adenosine acting on the A₁R is documented both in vivo and in vitro (Dunwiddie and Diao 1994,
Work with mice in which astrocytic vesicle release is impaired has suggested that adenosine derived from astroctic ATP affects neurotransmission by providing an adenosine ‘tone’ to the neocortex (Halassa et al. 2009). Increase in the frequency of spontaneous mEPSPs upon application of DPCPX in the absence of exogenously applied adenosine lends support to this notion. Presence of the tonic inhibition of synaptic transmission by endogenous adenosine in the neocortex would substantiate the role of adenosine as a ubiquitous and persistent modulator of synaptic transmission.

**Postsynaptic actions of adenosine on layer 2/3 pyramidal neurons**

Adenosine hyperpolarized layer 2/3 pyramidal neurons and decreased their input resistance, suggesting functional postsynaptic receptors. None of these effects were observed during application of adenosine on the background of A<sub>1</sub>R blockade by DPCPX, suggesting that they were mediated by A<sub>1</sub> receptors. Indeed, A<sub>1</sub>Rs activate potassium currents through the activation of G-Protein coupled inwardly rectifying potassium channels (GIRKs; Trussel and Jackson 1985, Trussel and Jackson 1987, Greene and Haas 1991, Thompson et al. 1992, Takigawa and Alzheimer 1999). The resulting increase of K<sup>+</sup> conductance can explain the decrease in input resistance and hyperpolarization of the membrane potential. These changes of membrane properties may underlie a small decrease in median mEPSP amplitude observed in experiments with spontaneous events. Interestingly, no postsynaptic effects of adenosine were found in layer 5 neurons of rat visual or associative frontal cortex (Murakoshi et al., 2001, Brand et al. 2001), and have been found only in some neurons of layer 4 of the barrel cortex (spiny neurons but not interneurons; Feldmeyer et al. Neuroscience 2012 abstract).
demonstration of postsynaptic actions in layer 2/3 then is layer-specific and may underlie increased inhibition of transmission in this region.

* A role for $A_{2A}$ receptors in neocortex? Insight from other brain areas. 

Our results provide evidence for functional $A_{2A}$ receptors in the neocortex. Increasing adenosine concentration from 100 to 150 µM failed to induce further significant reduction of EPSP amplitude. However, application of a selective $A_{2A}$R antagonist SCH58261 on the background of 150 µM adenosine led to a further reduction of EPSP amplitude, suggesting high concentrations of adenosine increased EPSP amplitude via $A_{2A}$Rs.

The dense expression of $A_1$Rs in the neocortex far outweighs the presence of $A_{2A}$Rs. $A_{2A}$Rs then are likely to be strategically located and activated only under specific circumstances. Insight from known $A_1$R and $A_{2A}$R actions offer a possible scenario. At low concentrations, adenosine activates high-affinity $A_1$ receptors, which suppress synaptic transmission. High concentrations of adenosine (100-150 µM) activate also $A_{2A}$Rs that oppose suppressive effects of $A_1$Rs. Activation of $A_{2A}$Rs can enhance glutamate release (Marchi et al. 2002). Interaction between $A_1$Rs and $A_{2A}$Rs has been directly demonstrated in synaptosomes extracted from cortical and hippocampal regions from young adult rats. In this preparation, activation of $A_{2A}$Rs led to a protein kinase C dependent decrease of the binding affinity for ligands of $A_1$Rs (Lopes et al. 1999). In striatal neurons, $A_1$Rs and $A_{2A}$Rs can form heteromers in the membrane in which $A_{2A}$R activation decreases the affinity of the $A_1$R for its agonists through direct intramembrane receptor-receptor interactions (Ciruela et al. 2006, Ferré et al. 20071,2, Ciruela et al. 2011).
Application of SCH 58261 on the background of 150 µM adenosine blocks A$_{2A}$R-mediated effects, and thus unmask the full extent of suppression caused by A$_1$R activation.

In the neocortex, A$_{2A}$Rs are expressed at lower levels than A$_1$Rs. Moreover, A$_{2A}$Rs have lower affinity to adenosine (Fredholm et al. 2001, Ciruela et al. 2011), and their effect on synaptic transmission is less robust than that mediated via A$_1$ receptors. This brings into question the role of A$_{2A}$ receptors in normal physiology. Of what use are two receptors for the same ligand which act in opposition to each other? Evidence from other brain areas provides insight into a potential role for this receptor under specific circumstances.

Both neurons and astrocytes release adenosine and ATP into the extracellular space in activity-dependent manner (Pascual et al. 2005; Wall and Dale 2008; Halassa et al. 2009). This extracellular adenosine is suggested to set a global inhibitory tone. On the background of this inhibitory tone, activity of synaptic networks can be regulated both upwards and downwards. ATP can be co-released with transmitter from highly activated presynaptic terminals (as mentioned above). A high local concentration of adenosine that results from broken down ATP may activate A$_{2A}$Rs located at these synapses (Cunha 2008; Costenla et al. 2010). These A$_{2A}$Rs can locally suppress the function of A$_1$Rs by decreasing their affinity to adenosine, or by downregulating the receptor. The result is a facilitation of transmission (and possibly an LTP) at the active synapses, and a more diffuse (A$_1$R mediated) inhibition of transmission at surrounding synapses. This antagonistic interaction between A$_{2A}$ and A$_1$ receptors has been proposed to increase signal salience and mediate heterosynaptic depression (Cunha 2008, Pascual et al. 2005). The previously mentioned interaction between A$_1$ and A$_{2A}$ receptors at glutamatergic synapses in the striatum, where A$_1$Rs and A$_{2A}$Rs can form heteromers on the
membrane surface, also suggests a role for the A$_2A$R as a ‘concentration-dependent switch.’ At low concentrations, adenosine preferentially occupies A$_1$R$_s$, inhibiting transmission, while high concentrations can activate A$_2A$R$_s$ which then ‘switch off’ A$_1$ activation (by decreasing the receptor’s affinity) and facilitate transmission (Ciruela et al. 2006, Ciruela et al. 2011; Ferré et al. 2007). This indicates possible functional relevance of two adenosine receptors with opposing effects. These two examples provide some indication of how sparse A$_2A$R$_s$ could be relevant, but exactly how, and under what circumstances, these two receptors interact in the neocortex (as well as the implications of such an interaction) remain to be elucidated.

**Conclusion**

The data presented here are generally consistent with the known possibilities of adenosine’s actions. This work, however, stresses region and cell-type specificity of adenosine effects in the neocortex and beyond. The presence of both pre and postsynaptic effects, and a role for A$_2A$R$_s$ constitutes a characterization of layer 2/3 synapses which is different from other known neocortical synapses. A clearer picture of adenosine within the neocortex thus emerges, with recognition of heterogenous actions across layers. The accumulation of information about the mechanisms of adenosine action at neocortical synapses provides the foundation on which physiological events in the neocortex can be understood. It is known, for example, that adenosine mediates slow wave activity. These cortical oscillations are shaped and permitted by adenosine modulation. The postsynaptic effects discovered in layer 2/3 neurons (which mediate intracortical communication) can have powerful implications for the excitablility of these neurons and subsequently cortical network activity. In addition, the presence of functional
A$_{2A}$Rs coexisting with A$_{1}$Rs opens the possibility that adenosine can fulfill a role of increasing signal salience or mediating plasticity in the neocortex (through the circumstances outlined above). Whereas the significance of adenosine in the neocortex is well recognized, most work has been done on a broader physiological behavioral level. How adenosine’s actions on a synaptic level permit these broader actions has remained much less studied. The present work provides this characterization and elucidates the basis of action for adenosine in the neocortex.
References


de Mendonça A, Sebastião AM, Ribeiro JA (1995). Inhibition of NMDA receptor-mediated currents in isolated rat hippocampal neurons by adenosine A$_1$ receptor activation. NeuroReport 6, 1097-1100.


