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Proportional Suppression of NMDA and AMPA Mediated Synaptic Responses in Layer 2/3 Pyramids from Rat Visual Cortex by Adenosine

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Introduction

Adenosine (ADO) is an abundant endogenous neuromodulator, with a generally suppressive effect on excitatory synaptic transmission. It is a metabolite of adenosine triphosphate (ATP), hence its ubiquitous presence in the central nervous system. Due in part to its role as a cellular energy molecule, adenosine finds its way into regulating key functions in the brain during both non-pathological and pathological states. In particular, the A_1 adenosine receptor subtype has the unique role of inhibiting synaptic transmission both pre and postsynaptically. Much attention has been focused on the role of adenosine in modulating specific excitatory glutamatergic channels, alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA). The heterogeneity of adenosine receptors and their sometimes opposing affects on excitatory transmission has made the exact role of adenosine complex. Part of the excitement around adenosine is created by a desire to understand how NMDA receptors are modulated by it. Previous research has shown that in different brain structures adenosine may have heterogeneous effects on the balance of NMDA receptor and non-NMDA receptor-mediated currents. In pyramidal neurons of the hippocampus, adenosine proportionally reduced the NMDA and non-NMDA mediated excitatory currents (Garaschuk, Kovalchuk, & Krishtal, 1992), whereas in dopaminergic neurons of rat midbrain, adenosine preferentially reduced NMDA currents (Y.-N. Wu, Shen, & Johnson, 1999), thus leading to a decreased ratio of NMDA/non-NMDA currents. It is known that in layer 2/3 pyramidal neurons of rat visual cortex, adenosine suppresses excitatory synaptic transmission (N. Bannon, Zhang, Ilin, Chistiakova, &

Volgushev, 2014). However, it remains unknown whether adenosine has the same effect on NMDA and AMPA-mediated currents. Here we asked whether NMDA-mediated currents are suppressed by adenosine differentially from AMPA-mediated currents, or whether the NMDA/AMPA balance is maintained in synaptic responses of layer 2/3 pyramidal neurons during application of adenosine.

To test this, we conducted *in vitro* whole-cell recordings from layer 2/3 pyramidal neurons in slices from rat visual cortex and, studied synaptic currents evoked with stimulating electrodes located in layer 4. Mixed excitatory postsynaptic currents composed of AMPA and NMDA-mediated components were recorded on the background of blockade of inhibition by 2-100μM picrotoxin (PTX). The identity of AMPA and NMDA currents was confirmed pharmacologically using selective antagonists DNQX and APV. Application of adenosine (20μM) led to a proportional decrease of NMDA and AMPA mediated currents, so that their balance in compound responses was maintained. These results were confirmed with recording of isolated NMDA currents and isolated AMPA currents, which showed a similar reduction in amplitude. Thus, in synaptic inputs to layer 2/3 pyramidal neurons of rat visual cortex, adenosine reduces the amplitude of NMDA and AMPA-mediated currents proportionally.

Adenosine at the synapse

In order to appreciate the larger consequences of adenosine modulation it is important to consider how adenosine is introduced to the synapse. Vesicular release of neurotransmitters such as acetylcholine, norepinephrine, and 5-HT is often accompanied by adenine nucleotides (Dunwiddie, Diao, & Proctor, 1997; Silinsky, 1975), and activation of adenylyl cyclase leads to diffusion of cAMP into the

extracellular space (Rosenberg & Li, 1995). Both sources of adenine nucleotides are metabolized from their precursors to adenosine monophosphate (AMP) and subsequently to the purine adenosine by 5'-nucleotidase in a rate-limiting step (Dunwiddie et al., 1997). An additional source of adenosine at the synapse may also be generated during times of high energy demand when the cell metabolizes ATP. Since the intracellular concentrations of ATP are in the millimolar range (approx. 3 mM) and AMP in the nanomolar, a relatively small amount of ATP must be metabolized to create a significant increase in AMP concentration (R.A. Cunha, 2001; Rodrigo A. Cunha, 2008; Dunwiddie & Masino, 2001). Intracellular AMP is then converted into adenosine via 5'-nucleotidase—an opposing reaction with adenosine kinase (Decking, Schlieper, Kroll, & Schrader, 1997; Kroll, Decking, Dreikorn, & Schrader, 1993). In an alternative pathway adenosine is generated by a transmethylation reaction where S-adenosylhomocysteine hydrolase converts S-adenosylhomocysteine into adenosine and homocysteine. Once sufficient concentrations of adenosine accumulate within the cell it may flow via facilitated diffusion to the extracellular space through nucleoside transporters (Dunwiddie & Masino, 2001).

In the hippocampus, it has been reported that astrocytes may also contribute to an increase in extracellular adenosine (Pascual et al., 2005). Gliotransmission begins with activation of astrocytic receptors by neurotransmitters, which cause an increase of intracellular Ca^{2+} leading to release of ATP into the extracellular space (Halassa, Fellin, & Haydon, 2007). Once in the extracellular space, ATP is quickly metabolized to adenosine by ecto-nucleotidases.

Despite the variety of ways adenosine is introduced to the synapse there exists a common theme, that is—adenosine release is activity-dependent. In a study conducted by Pajski and Venton (2013), they demonstrated an activity-dependent release of adenosine following extracellular stimulation in caudate-putamen, nucleus accumbens, hippocampus, and cortex. This was corroborated by introduction of a Ca^{2+}chelator or tetrodotoxin that nearly abolished adenosine release (Pajski & Venton, 2013). The local concentrations of adenosine also varied depending on brain region with the caudate-putamen having the highest concentrations (0.34 μM) and the secondary motor cortex the lowest (0.06μM). The extracellular breakdown of ATP following vesicular release was the primary mechanism of adenosine formation in nucleus accumbens, hippocampus, and prefrontal cortex whereas adenosine in the caudate-putamen was glutamate-receptor dependent. The differing sources and concentrations of adenosine by brain regions highlights that adenosine may have differential effects on synaptic transmission in differing brain regions.

The clearance of adenosine from the extraceullar space can be accomplished by two prevailing mechanisms. Adenosine can be degraded at the synapse into its inert form inosine by adenosine deaminase. However, under non-energetically demanding circumstances intracellular concentrations of adenosine favor diffusion into the cell where adenosine kinase captures it by phosphorylation. The latter mechanism is thought to play a larger role in clearing adenosine from the synapse as adenosine deaminase antagonism has little effect on extracellular adenosine concentration (Dunwiddie & Masino, 2001; Lloyd & Fredholm, 1995; Pak, Haas, Decking, & Schrader,

1994). This result makes intuitive sense due to the concentration gradient favoring adenosine flowing into the cell from the extracellular space.

Adenosine Receptors (A_1, A_{2A}, A_{2B}, A_3)

Currently, there are four adenosine receptors (A_1R, $A_{2A}R$, $A_{2B}R$, A_3R) that have been isolated and cloned in variety of species including human (Olah & Stiles, 1995). Each adenosine receptor is part of the G-protein coupled receptor family (Dunwiddie & Masino, 2001; Olah & Stiles, 1995). The functions of each receptor depend on both their location at the synapse (pre- or postsynaptic) and their effects on intracellular targets.

The A_1R is the most widely expressed adenosine receptor in all regions of the brain (Dixon, Gubitz, Sirinathsinghji, Richardson, & Freeman, 1996). The most common function of the A1 receptor is the reduction in vesicular release probability by reducing calcium influx into the presynaptic terminal through N-type Ca^{2+} channels (Gomes, Kaster, Tomé, Agostinho, & Cunha, 2011; Gundlfinger et al., 2007; L.-G. Wu & Saggau, 1994, 1997). There may also be an effect of presynaptic A_1R agonism downstream of Ca^{2+} influx that reduce release probability but the mechanisms are unclear (Yawo & Chuhma, 1993). However, the net effect of presynaptic A_1R activation appears to be the reduction in release of glutamate, aspartate, acetylcholine, and serotonin (Corradetti, Lo Conte, Moroni, Beatrice Passani, & Pepeu, 1984; Fontanez & Porter, 2006; Lupica, Proctor, & Dunwiddie, 1992; Silinsky, 1984). It has also been demonstrated that A_1R activation can reduce GABAergic inhibitory transmission in the neocortex (Kirmse, Dvorzhak, Grantyn, & Kirischuk, 2008; Zhang, Bannon, Ilin, Volgushev, & Chistiakova, 2015). Postsynaptic A_1 receptors affect synaptic transmission in both cortical and

subcortical brain regions (Kim & Johnston, 2015; Takigawa & Alzheimer, 1999; L. O. Trussell & Jackson, 1985). Post-synaptic A_1 receptors act on G-protein coupled inwardly rectifying potassium channels (GIRKs) causing a hyperpolarization of the membrane—making the neuron less excitable (Thompson, Haas, & Gähwiler, 1992; L. Trussell & Jackson, 1987; L. O. Trussell & Jackson, 1985). Thus, the main action of the A_1R is to suppress excitatory synaptic transmission regardless of its location pre- or postsynaptic.

The A_{2A} receptor opposes the suppressive effects of the A_1 receptor which may allow for a dynamic tuning of synapses. However, compared to the A_1 receptor the A_{2A} receptor is not widely distributed but is found in high concentrations in the basal ganglia and low concentrations in other brain areas like cortex (Rodrigo A. Cunha, 2005). In addition, the A_{2A} receptors in the basal ganglia are largely postsynaptic where they may depolarize neurons (Rodrigo A. Cunha, 2005). In the hippocampus, postsynaptic A_{2A} receptors activated by the selective agonist CGS 21680 slowly depolarized the membrane and increased the amplitude of evoked excitatory post synaptic potentials (H. Li & Henry, 1998). However, presynaptic A_{2A} receptors facilitate the release of neurotransmitters such as glutamate, GABA, glycine, acetylcholine, noradrenaline, and serotonin (Rodrigo A. Cunha, 2005). The co-localization of A_1 and A_{2A} receptors in the hippocampus and striatum suggests there may be a tuning of glutamatergic synapses between the inhibitory A_1Rs and facilitating A_{2A}Rs (Rodrigo A. Cunha, 2005; Rebola, Rodrigues, et al., 2005). This effect may be achieved by presynaptic A_1R-A_{2A}R heteromers where A_{2A} agonism can decrease the affinity of the A_1R for adenosine (Luísa V. Lopes, Cunha, & Ribeiro, 1999; L.V. Lopes, Cunha, Kull, Fredholm, & Ribeiro,

2002). This effect on excitatory synaptic transmission highlights the potential for a dynamic relationship between the A_1 and A_{2A} receptors pre and postsynaptically.

The A_{2B} receptor has been found in all tissues of the central nervous system by in situ hybridization (Dixon et al., 1996; Feoktistov & Biaggioni, 1997). However, the function of the A_{2B} receptor is unclear, it has been characterized as a low affinity receptor (Feoktistov & Biaggioni, 1997) which makes studying the physiological implications of A_{2B}R agonism difficult. Similar to the A_{2A} receptor, A_{2B}R activation converge on stimulation of adenyl cyclase but only A_{2B} agonism leads to the activation of phospholipase C. The consequence of this difference is still under debate but A_{2B} receptors are thought to function similar to A_{2A} in that they facilitate excitatory synaptic transmission by an increase of presynaptic Ca^{2+} influx at terminals (Feoktistov & Biaggioni, 1997). In all likelihood, the A_{2B} receptor is not a key player under normal physiological conditions but rather when adenosine tone increases dramatically during pathological states such as ischemia or trauma.

The A_3 receptor has been identified in many brain tissues of the central nervous system but had lower concentrations in the cortex as compared to subcortical structures (striatum, nucleus accumbens, olfactory bulb, hippocampus, cerebellum, hypothalamus, and thalamus; Dixon et al., 1996). Similar to the A_{2B}R, the A_3 receptor is a low affinity adenosine receptor (Luísa V. Lopes et al., 2003). The A_3 receptor is the most poorly understood adenosine receptor and likely only serves a function during pathophysiological states (Gessi et al., 2008). Most of the effects of adenosine as a neuromodulator can be attributed to the actions of the A_1R in most brain areas and A_{2A}R in the basal ganaglia.

Adenosine in normal physiology

While adenosine may act first at the molecular and synaptic level it is important to consider the broader implications adenosine has on normal physiological phenomena. Adenosine has long been studied in the context of arousal or neuronal excitability, the sleep/wake cycle, and its role in neuroprotection. In addition, it should be mentioned that while adenosine is ubiquitous in the central nervous system it modulates the activity of brain regions differently due to both their intrinsic differences in structure and adenosine receptor distributions. In the section that follows the heterogeneity of adenosine's actions in specific brain regions will be highlighted.

Sleep

Perhaps the most familiar way to modulate the normal role of adenosine is through caffeine, a mild stimulant frequently consumed to promote arousal and wakefulness. When consumed, caffeine acts as a non-selective adenosine antagonist which likely has an affect on A_1 and A_{2A} receptors leading to an increase in neuron excitation (Bjorness & Greene, 2009; Ferre et al., 2008). As one may expect, the opposing affect of adenosine agonists is to promote sleep behavior (Bjorness & Greene, 2009). Thus, it makes intuitive sense that adenosine levels rise during the day to promote sleep behavior and decrease during sleep. However, this simplified model of

sleep falls short of its true complexity due to the functional differences between brain areas.

In the mesopontine tegmentum, adenosine inhibits neurons in the cholinergic arousal system by reducing evoked EPSCs and GABAergic IPSCs. This reduction in glutamatergic, cholinergic, and GABAergic synaptic transmission promotes sleep behavior and influences thalamocortical neural activity characteristic of slow wave sleep (Bjorness & Greene, 2009; Rainnie, Grunze, McCarley, & Greene, 1994). The A_1 receptor is thought to mediate slow wave activation via the interaction between the brain stem and thalamocortical neurons. Under an increased adenosine tone, thalamic and cortical neurons have increased GIRK channel conductance and relatively less activity due to A_1R presynaptic inhibition (Bjorness & Greene, 2009). The end result of this modulation is a switch from a tonic firing to burst firing pattern of thalamocortical neurons; this firing pattern corresponds to delta waves seen on an EEG (Bjorness & Greene, 2009; Halassa, 2011).

Another key player in sleep behavior is the basal forebrain. Much like the mesopontine tegmentum, the basal forebrain contains cholinergic neurons responsible for arousal which are more active during waking states or REM sleep. Adenosine agonists perfused to the basal forebrain promote sleep behavior whereas antagonists achieved the opposite effect (Bjorness & Greene, 2009; Strecker et al., 2000). In addition, experiments involving sleep deprivation show increased adenosine tone in cortex as well as basal forebrain. However, after prolonged deprivation adenosine tone is only stable or increasing in basal forebrain (Bjorness & Greene, 2009). The implications of this finding suggest that rising adenosine levels during waking periods

reduce the activity of cholinergic and non-cholinergic neurons in the basal forebrain which results in sleep behavior.

The preoptic/anterior hypothalamus (POAH) and ventrolateral preoptic area (VLPO) have ‘sleep active’ neurons that preferentially fire during SWS. Interestingly, these neurons are indirectly activated by adenosine via the presynaptic reduction of $GABA_A$ release from inhibitory neurons onto the ‘sleep active’ neurons. Again, the net effect of this disinhibition of GABAergic transmission is a transition from wake behavior to SWS (Strecker et al., 2000).

Generally, adenosine’s role in the sleep/wake cycle is to reduce synaptic activity through the activation of A_1 receptors. However, this activation has different consequences in different brain regions. Thalamocortical neurons experience a shift between tonic and burst firing due to increased GIRK conductance and a reduction in presynaptic release probability. Whereas hypothalamic ‘sleep active’ neurons are disinhibited by presynaptic A_1R activation. Also, the cholinergic and non-cholinergic neurons responsible for arousal in mesopontine tegmentum and basal forebrain are inhibited by rising adenosine tone. The ability of adenosine to modulate these vastly different networks in such a concerted manner truly highlights its potential to influence other phenomena in the central nervous system.

Neuroprotection

The function of adenosine as an endogenous neuroprotector stems from its role in reducing excitatory synaptic transmission. As was stated before, during times of high energetic demand a neuron will expend large amounts of ATP which lead to an increase

of adenosine tone. This mechanism seems to be important in mitigating the damage caused during epileptic seizures (During & Spencer, 1992) and other excitotoxic events. Evidence of increased adenosine concentrations following ischemic or hypoxic events have also been reported in both *in vitro* and *in vivo* studies. The following section will highlight the function of adenosine as an endogenous neuroprotector during times when a neural network may be stressed.

When considering the neuroprotecive role of adenosine, the A_1 receptor becomes the center of focus due to its role in dampening excitatory glutamatergic transmission. Selective A_1R agonists have been shown to reduce neuronal death in experimentally induced hypoxia, ischemia, and epilepsy (Rodrigo A. Cunha, 2005; de Mendonça, Sebastião, & Ribeiro, 2000). Conversely, selective A_1R antagonists exacerbate the damage caused by these pathologies. The exact mechanisms have yet to be determined but likely reduce NMDA receptor activation by inhibiting glutamate release at the synapse. However, adenosine receptors located in tissue outside the CNS such as heart make clinical application of adenosine agonists dangerous; A_1R activation in heart causes bradycardia (Dunwiddie & Masino, 2001).

Although considerably less abundant than the A_1R in healthy tissue, the A_{2A} receptor may provide a clinical target for treatments. The logic behind this argument rests in a long-term downregulation of A_1Rs and upregulation of A_{2A}Rs in tissue that is chronically challenged with noxious stimuli such as epileptic seizures or ischemia (Rodrigo A. Cunha, 2005; Rebola et al., 2003). The consequence of this ‘flipped’ adenosine receptor density is a shift from a tonic inhibitory effect of adenosine on excitatory transmission to a facilitating. Interestingly, this phenomena was observed in

the cerebral cortex where A_{2A}Rs are typically less concentrated (Rebola, Porciúncula, et al., 2005). Multiple experiments have confirmed that A_{2A} antagonists provide robust neuroprotection when neurons are challenged with noxious stimuli (Rodrigo A. Cunha, 2005).

Astrocytes have become increasingly important in understanding the role of adenosine during epileptic seizures. Evidence that astrocytes contribute to endogenous adenosine tone comes from experiments with dominant negative mutation of soluble N-ethylmaleimde-sensitive factor mice (dsSNARE-mice). In these mutants, astrocytes are unable to release ATP normally because they lack the docking proteins required for release; this caused a loss in tonic adenosine suppression (Pascual et al., 2005). Under normal ATP release from astrocytes, ATP is rapidly metabolized to adenosine via several ectonucleotidases. As was discussed above, the extracellular adenosine is taken up by astrocytes and captured by adenosine kinase (ADK) thus changes in ADK will dramatically effect adenosine tone (Boison, 2012). In the hippocampus, an *in vitro* experiment demonstrated pharmacological blockade of ADK caused an increase in synaptic adenosine which decreased glutamatergic excitatory synaptic transmission in an A_1R dependent fashion (Boison, 2012; Etherington et al., 2009). Seizure activity induced by high frequency stimulation was significantly reduced under ADK inhibition. However, the blockade of ADK did not effect activity-dependent release of adenosine therefore the increase in adenosine is likely attributable ADK housed in astrocytes (Boison, 2012; Etherington et al., 2009). Further evidence suggesting astrocytes role in epilepsy comes from an *in vivo* study that induced mesial temporal lobe epilepsy (MTLE) seizures by causing astrogliosis (an increase in astrocytes secondary to neuron

death) and overexpression of ADK in CA3 region of the hippocampus and amygdala (Boison, 2012).

Adenosine clearly has a role as a natural break on seizure activity. When the neurons fire during a seizure there is an activity-dependent release of adenosine which acts on A_1Rs to reduce further excitation. In addition, astrocytes may release ATP to increase local adenosine concentrations. However, it should be mentioned that much of the research in epilepsy has been conducted *in vitro* and in subcortical structures; more research is needed in order to elucidate adenosine's role in seizures in the neocortex.

NMDA and AMPA Receptors

Excitatory synaptic transmission is mediated by four types of ionotropic glutamate receptors, delta-, kainate, alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid (AMPA), and N-methyl-D-aspartate (NMDA) receptors. The latter two receptors, AMPA and NMDA, have been researched extensively for their roles in synaptic transmission. The following section discusses the structure and function of AMPA and NMDA receptors with an emphasis on the NMDA receptor due to its key role as a detector of pre/post-synaptic activity and synaptic plasticity.

In general, ionotropic glutamate receptors are integral membrane proteins composed of four subunits that form a channel in the membrane that allows the passage of cations. The AMPA receptor is composed of four subunits (GluA1-GluA4) which can form both homo- or heteromers. The NMDA receptor is composed of combinations of homomers (two GluN1 with two GluN2 subunits or two GluN1 with one GluN1 and GluN3). In addition to binding glutamate, NMDA receptors must bind glycine

to open; the GluN1/GluN3 subunits bind glycine and GluN2 glutamate (Traynelis et al., 2010). Functionally, the binding of glutamate is the true determinant of NMDAR activation as tonic levels of glycine nearly saturate the co-activation site (Vyklicky et al., 2014). Another functional caveat of the NMDAR is the variety of GluN2 subunits (GluN2A, GluN2B, GluN2C, GluN2D) which have been the focus of recent research but will not be mentioned further.

The selective ion permeability of AMPA and NMDA receptors is determined by a three alpha helical transmembrane domain which creates a pore in the membrane. The key difference between AMPA and NMDA receptors in this region is the residues in a critical region of the pore known as the QRN site. In AMPA receptors, the QRN site has a glutamine or arginine residue whereas the NMDAR has an asparagine residue (Traynelis et al., 2010). One consequence of these residues is that AMPA and NMDA receptors selectively pass cations such as Na^+ and Ca^{2+}. However, NMDA receptors are three or four times more permeable to Ca^{2+} than calcium permeable AMPA receptors (Traynelis et al., 2010). Another consequence of the residues in the NMDA pore is a reduction of NMDAR activity at hyperpolarized potentials by a Mg^{2+} ion ‘stuck’ in the channel (Mayer, Westbrook, & Guthrie, 1984; Nowak, Bregestovski, Ascher, Herbet, & Prochiantz, 1984; Traynelis et al., 2010). This allows the NMDAR to act as a coincidence detector of pre/postsynaptic activity; the NMDAR is only active during postsynaptic depolarization and presynaptic release of glutamate (Mayer et al., 1984; Nowak et al., 1984; Tabone & Ramaswami, 2012).

NMDAR-dependent Long Term Potentiation (LTP)/ Long Term Depression (LTD)

It has been well established that the NMDA receptor is important in inducing long term changes in synaptic transmission; a phenomenon known as plasticity. The classic way to induce long-term potentiation (LTP) is by short (1-2 seconds), high frequency (>20Hz) stimulation of fibers which in turn causes the postsynaptic cell to fire. When postsynaptic NMDARs are activated during stimulation protocol, local Ca^{2+} concentration increases briefly (<2 seconds) which activates calcium/calmodulin dependent-protein kinase II (CAMKII) (Bliss & Collingridge, 1993; Malenka & Bear, 2004). The net result of Ca^{2+} influx appears to be two-fold: the recruitment of additional AMPARs and/or phosphorylation of AMPARs (Malenka & Bear, 2004). The effect of this modification to the synapse will be a greater response to glutamate released at the synapse over the course of hours, days or weeks.

In direct opposition of strengthening a synapse via LTP, a neuron must also have the ability to weaken a synapse. Interestingly, the NMDAR plays a critical role in inducing LTD as well as LTP. The induction of LTD is often accomplished experimentally by low frequency stimulation (0.5-5 Hz) and a slight postsynaptic depolarization to alleviate the Mg^{2+} ion blocking the NMDAR (Malenka & Bear, 2004). When NMDA antagonists are used LTD does not occur (Dudek & Bear, 1992) but experiments using NMDA itself as an agonist cause LTD (R. Li et al., 2004). The mechanism also appears to require Ca^{2+} influx through the NMDA receptor as experiments show uncaging of Ca^{2+} in dendritic spines causes LTD (Malenka & Bear, 2004). In contrast to LTP, the induction of LTD via increased Ca^{2+} in dendritic spines occurs only when NMDARs are activated with low frequency stimulation. The specific intracellular cascades have not been entirely worked out. However, there is evidence

that suggests the dephosphorylation of protein kinase A (PKA) allows LTD to occur. Furthermore, there is evidence that protein phosphatase 1 (PP1) acts on PKA to induce LTD (Malenka & Bear, 2004). As one might expect, the consequence of LTD is a mirror of LTP: AMPA receptor density declines in a clathrin/dynamin-dendent manner and are dephosphorylated to reduce the probability of opening (Malenka & Bear, 2004).

The mechanisms of LTP and LTD discussed above are by no means exhaustive. There exists other means by which a neuron may change the strength of its synapses independent of the NMDA receptor. For instance, Shaffer collateral-CA1 synapses can undergo LTD independent of NMDA receptor via metabotropic glutamate (mGlut) receptors. Also, endogenous endocannabinoids released postsynaptically can activate presynaptic CB1 receptors to depress excitatory synaptic transmission in cerebellum and hippocampus (Malenka & Bear, 2004). However, it is clear from the abundant research in NMDA-dependent LTP/LTD that it is a robust mechanism of plasticity.

Methods

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. Slice preparation details were similar to those used in previous studies (N. M. Bannon et al., 2016; N. Bannon et al., 2014; Zhang et al., 2015). Male Wistar rats of postnatal age (P) 21-38 days were decapitated under deep isoflurane anesthesia. The brain was quickly extracted, and 350 µm-thick coronal slices containing the visual cortex were prepared using a Leica VT100S vibratome. The brain was constantly bathed in ice-cold oxygenated artificial-cerebral spinal fluid (ACSF in mM: 125 NaCl, 25 $NaHCO_3$, 25 glucose, 3 KCl, 1.25

NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$ bubbled with 95% O_2/5% CO_2, pH 7.4) during slice preparation. Coronal slices were allowed to recover for at least 1h at room temperature. Recordings were conducted by transferring slices to a recording chamber mounted on an Olympus BX-50WI microscope equipped with infrared differential interference contract (IR-DIC) optics. During recording, slices were submerged in oxygenated ACSF at 30-32 °C.

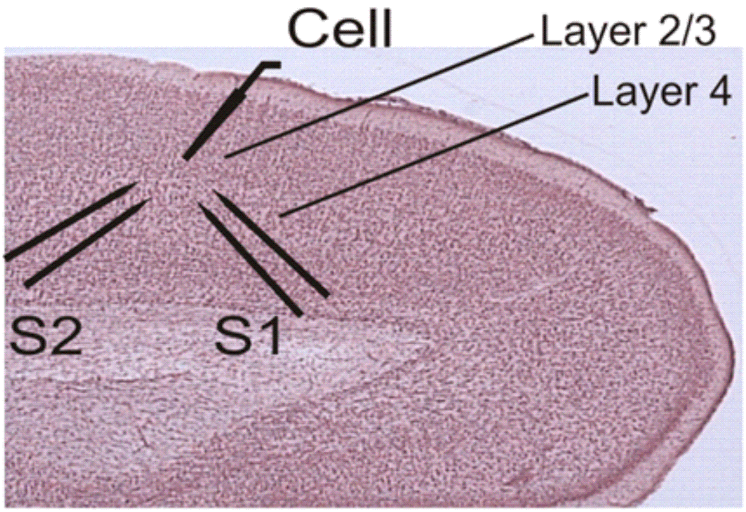

Fig. 1 Slice of visual cortex showing location of stimulation and recording electrodes
Pyramidal cells in layer 2/3 of rat visual cortex were visually identified and whole-cell patch recordings were conducted. Stimulation electrodes were placed in Layer 4 while pyramids recorded in Layer 2/3.

We made whole-cell recordings from visually and physiologically identified pyramidal cells in layer 2/3 of the visual cortex using DIC microscopy. Identification of pyramidal neurons was reliable as demonstrated in previous work with biocytin labeling and morphological reconstruction of recorded neruons (M Volgushev, Vidyasagar, Chistiakova, & Eysel, 2000). Intracellular pipette solution contained in mM: 130 K-Gluconate, 20 KCl, 10 HEPES, 10 Na-Phosphocreatine, 4 Mg-ATP, 0.3 Na_2-GTP (pH 7.4 with KOH). All excitatory postsynaptic currents (EPSCs) were recorded in Layer 2/3

and induced by a pair of stimulation electrodes (S1 and S2) in Layer 4 (Fig. 1). Stimulation current intensities were tuned to evoke monosynaptic EPSCs in the recorded neuron. Paired stimuli (50 ms interstimulus interval) 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.

Control protocol for isolated and mixed EPSCs is as follows. Recordings were conducted in voltage-clamp mode with holding potential at -50mV. Pharmacologically isolated AMPA EPSCs were recorded under 5µM picrotoxin (PTX; Sigma, St. Louis MO, USA) and D-(--)-2-amino-5-phosphonopentoic acid (APV 20µM; Tocris, Bristol, UK). Picrotoxin was dissolved in the ACSF directly. APV (Tocris, Bristol, UK) was dissolved in water to a 50mM stock before being added to ACSF. Pharmacologically isolated NMDA EPSCs were recorded under 5µM PTX (Sigma, St. Louis MO, USA) and 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX 10µM; Sigma, St. Louis MO, USA). DNQX (Sigma, St. Louis MO, USA) was dissolved in water to a 5mM stock before being added to ACSF. Mixed currents were recorded with 2-100µM PTX (Sigma, St. Louis MO, USA), 1mM glycine, and 1nM tetrodotoxin (TTX; Tocris, Bristol UK). Following control protocol, 20µM Adenosine (Sigma, St. Louis MO, USA) was applied to the bath and evoked EPSCs were recorded. Adenosine (Sigma, St. Louis MO, USA) was dissolved in ACSF to a 1mM stock before being applied to the bath. Control solution was then used to wash ADO from the bath and evoked EPSCs were recorded. The identity of all evoked EPSCs were confirmed using selective antagonists APV and DNQX. After recordings were conducted at -50mV, the holding potential was varied to determine current-voltage relationship at -30, -40, -60, -70, and -80mV. In addition, depolarized

holding potentials (-30 and -40mV) allowed us to determine if EPSCs were contaminated with inhibitory currents. Only currents that were still depolarizing at these holding potentials were considered excitatory and included in the analysis.

Data analysis

Using custom-written programs in MatLab (© The MathWorks, Natick MA, USA), EPSC amplitudes were measured as the difference between the mean membrane potential during two or three measurement windows. The amplitude of EPSCs was determined by placing a baseline window before the onset of an event and a measurement window at the peak of the EPSC. For mixed current experiments the baseline window was placed in the same manner as stated before. The AMPA window was set at the fast onset peak and the NMDA window was set when an event returned to baseline under APV (a selective NMDA antagonist; Fig. 2B). All inputs included in the analysis fulfilled the criteria of (1) stability of EPSC amplitude during the control period, (2) stability of the onset latency and kinetics of the slope of the EPSC, (3) absence of inhibitory currents when the holding potential was depolarized to -30 and -40mV during APV and DNQX conditions (Fig. 2B). Measurement windows for paired pulse stimulation paradigm were the same duration but displaced by the inter-pulse interval (50 ms).

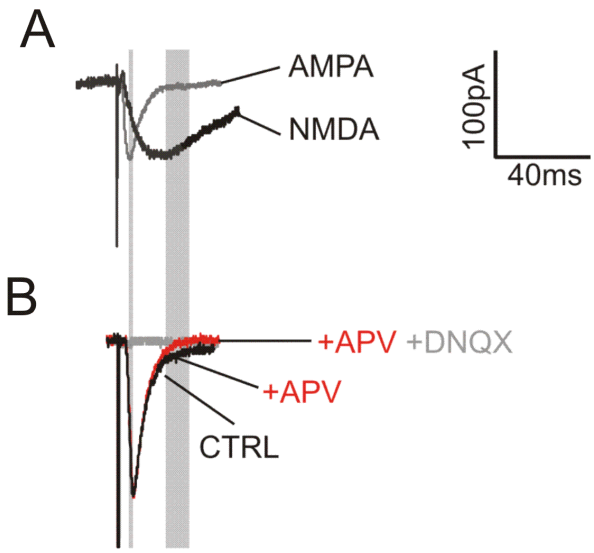

Fig. 2 Example traces illustrate NMDA window placement
(**A**) Pharmacologically isolated AMPA and NMDA currents overlayed demonstrate differences in the timing of peak onset. (**B**) Compound EPSC composed of AMPA and NMDA-mediated components (black). Wash-in of APV abolishes NMDA-mediated component (red). Wash-in of DNQX inhibits all excitatory transmission (grey). First grey window measures AMPA-mediated component of compound EPSCs. Second grey window measures NMDA-mediated component of compound EPSCs.

Statistical tests were conducted using a Student's t-test, repeated measures ANOVA, or univariate ANOVA with post-hoc comparisons (Bonferroni). Error bars represent the standard error of the mean.

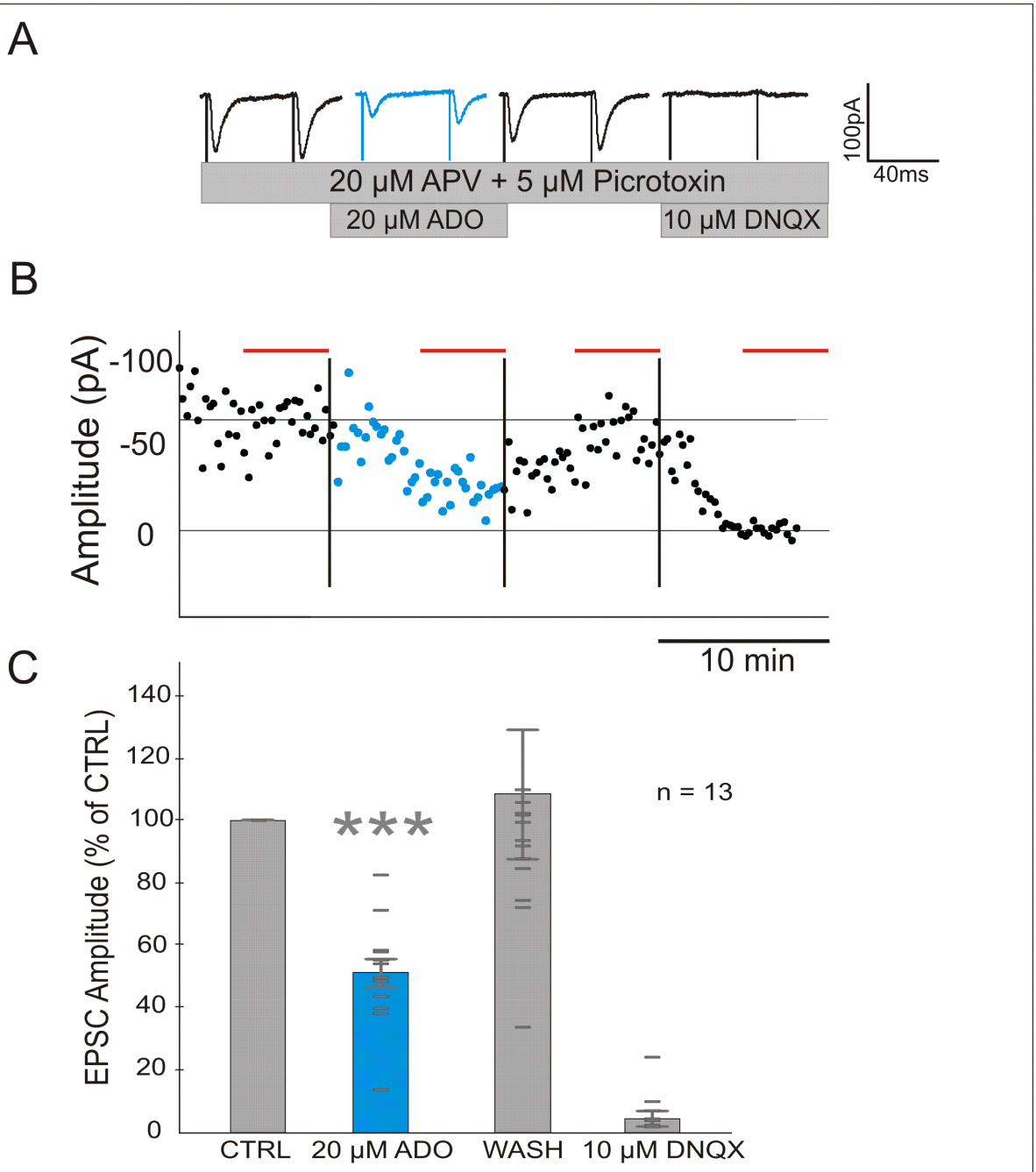

Fig. 3 Adenosine reduces the amplitudes of pharmacologically isolated AMPA currents
(**A**) Pharmacologically isolated AMPA currents recorded in layer 2/3 pyramidal neurons with paired-pulse stimuli under the background of inhibition 5µM PTX and 20µM APV, an NMDA antagonist. Measurement windows were placed before the peak of the event; window placement was consistent across each condition. Wash in of 20µM adenosine (blue) reduces the amplitude of AMPA-mediated EPSCs; wash out of adenosine restores AMPA EPSCs to baseline. Blockade of AMPA-mediated currents with 10µM DNQX confirms the identity of EPSCs.(**B**) Time course of evoked EPSCs throughout recording intervals. Black line indicates baseline EPSC in control. Wash in of adenosine (blue) reduces the amplitude of EPSCs. Red bars indicate sweeps incorporated into average. (**C**) Normalized AMPA-mediated EPSCs demonstrate a potent reduction (51% of baseline) in EPSC amplitude under the influence of 20µM ADO (n = 13; Repeated measures ANOVA,$F(2,24)=19.399$, $p<0.001$, Bonferroni post-hoc testing). Pharmacological blockade by 10µM DNQX confirms AMPA-mediated EPSCs. Individual normalized evoked EPSCs are shown as grey dashes.

Results

The experiments that follow aim to elucidate whether NMDA-mediated currents are suppressed differentially from AMPA-mediated currents in layer 2/3 of rat visual cortex. In order to accomplish this, we conducted experiments that systematically isolated different components of excitatory postsynaptic currents. We investigated the effect of adenosine on pharmacologically isolated AMPA and NMDA-mediated currents. In these experiments, we demonstrate a similar reduction in EPSC amplitude concurrent with an increase in the paired pulse ratio (suggesting a presynaptic action). In addition, the voltage-dependence of the evoked AMPA and NMDA-mediated currents further corroborates their identity. In our final preparation, we measured the effect of adenosine on co-occurring AMPA- and NMDA-mediated components in which the NMDA/AMPA ratio was maintained.

Adenosine reduces the amplitude of pharmacologically isolated AMPA currents

To study the effects of adenosine in layer 2/3 pyramidal neurons on AMPA-mediated currents we recorded EPSCs evoked by a paired-pulse stimulus under the background of a selective NMDA-antagonist (APV) and $GABA_A$ antagonist (PTX; Fig. 3A and Fig. 3B). After recording 80-100 sweeps to establish a stable baseline, 20μM adenosine (ADO) was washed into the bath (Fig. 3A and Fig. 3B). It was assumed that ADO concentrations in the bath reached 20μM after approximately 5 minutes of wash in (Fig. 3B). The concentration of adenosine that robustly reduced the amplitude of excitatory potentials (20μM) was established by previous experiments conducted by our laboratory (N. Bannon et al., 2014). Adenosine was subsequently washed out of the bath using control solution for approximately 5 minutes (Fig. 3B). After washout, DNQX

was washed into the bath to both confirm the identity of the EPSC as an AMPA-mediated event and to determine a potential inhibitory current contamination. All inputs with inhibitory events were not included in our data set.

Under the application of adenosine, AMPA-mediated EPSCs were potently reduced to 51.1 ± 4.59% of baseline (Fig. 3C; $p<0.001$). During washout of adenosine,

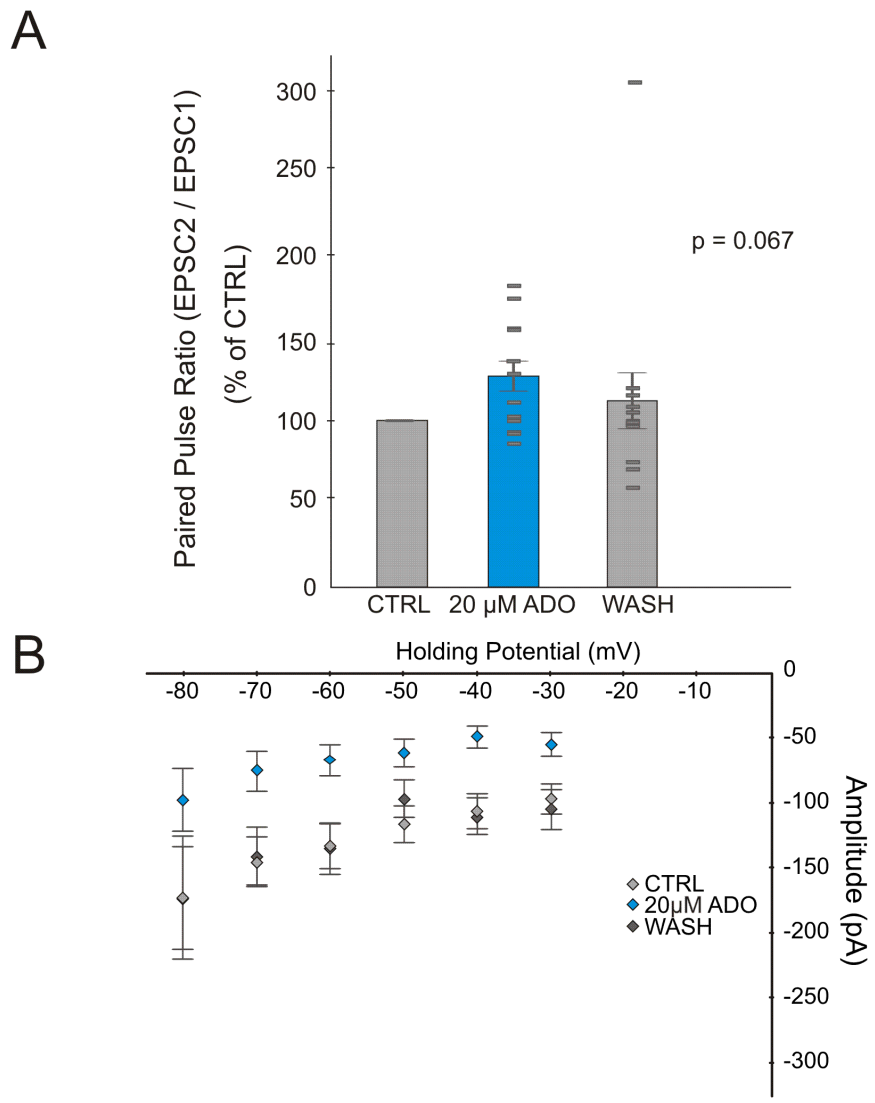

Fig. 4 Adenosine reduces the amplitudes of pharmacologically isolated AMPA currents
(**A**) Normalized paired pulse ratio, measured as EPSC2/EPSC1, increased under 20µM ADO (Repeated measures ANOVA, $F(2,24)=1.495$, $p=0.244$). This suggests a decrease in release probability which is characteristic of A1R activation. (**B**) Voltage-dependence on AMPA-mediated EPSCs between -30mV and -80mV demonstrates less ion conductance at depolarized potentials as compared to hyperpolarized potentials.

the AMPA-mediated EPSC returned to 108 ± 20.7% of baseline (Fig. 3C; n.s). The bath application of DNQX, a selective AMPA-antagonist, reduced the AMPA-mediated EPSC to 4.31 ± 2.43% of baseline (Fig. 3C; $p<0.001$). Concurrently, we observed a modest increase in the paired-pulse ratio (PPR) under bath application of adenosine (Fig. 4A; $p=0.067$). An increase in PPR is commonly interpreted as a decrease in release probability (Stevens, 1993) thus suggesting a presynaptic mechanism of ADO

modulation. At differing holding potentials (-80, -70, -60, -50, -40, and -30 mV) a clear

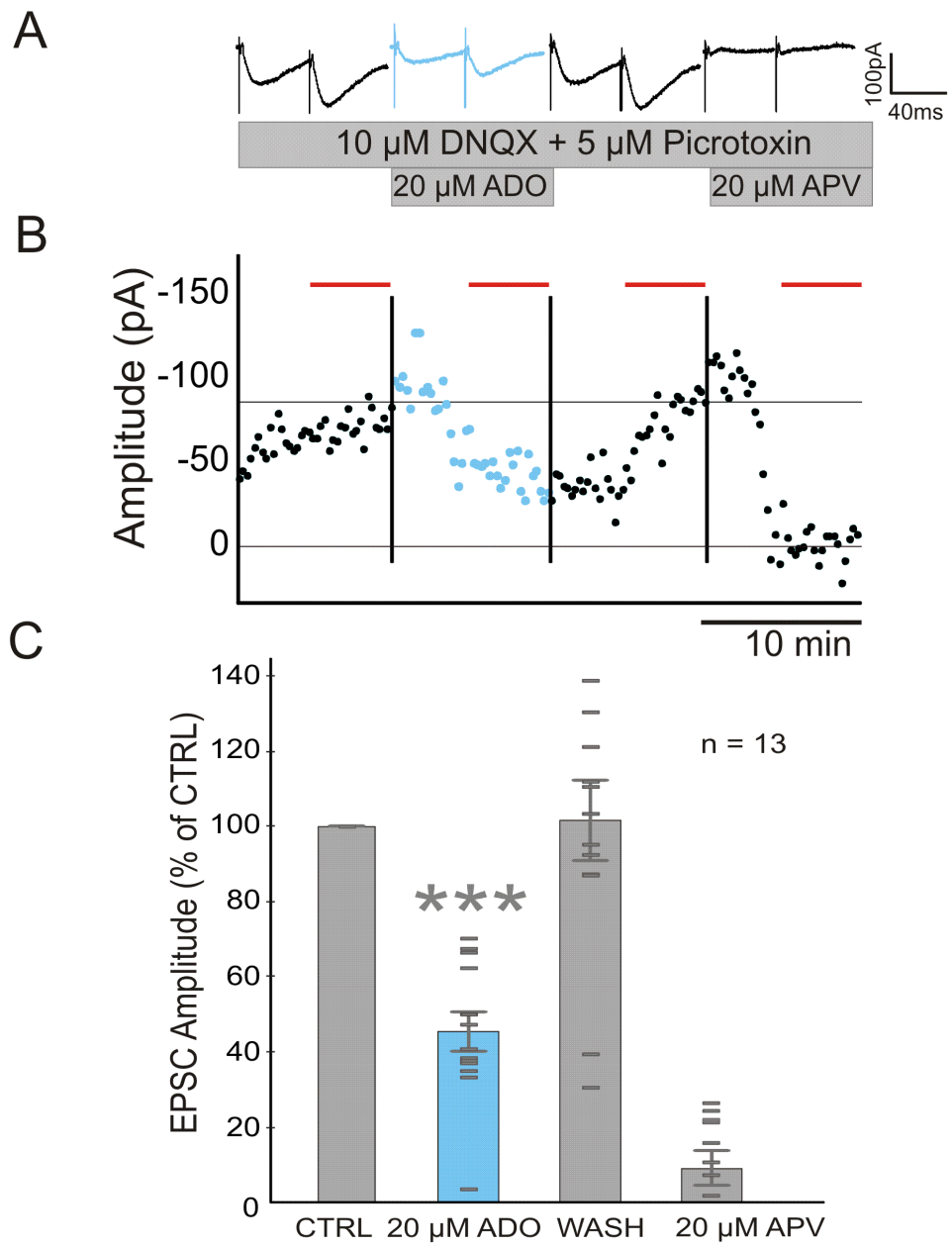

Fig. 5 Adenosine reduces the amplitudes of pharmacologically isolated NMDA currents
(**A**) Pharmacologically isolated NMDA currents recorded in layer 2/3 pyramidal neurons with paired-pulse stimuli under the background of inhibition 5μM PTX and 10μM DNQX, an AMPA antagonist. Measurement windows were placed at the peak of the event; window placement was consistent across each condition. Wash in of 20μM adenosine (light blue) reduces the amplitude of NMDA-mediated EPSCs; washout of adenosine restores NMDA EPSCs to baseline. Blockade of NMDA-mediated currents with 20μM APV confirms the identity of EPSCs.(**B**) Time course of evoked EPSCs throughout recording intervals. Black line signifies baseline EPSC in control. Wash in of adenosine (light blue) reduces the amplitude of EPSCs. Red bars indicate sweeps incorporated into average. (**C**) Normalized NMDA-mediated EPSCs demonstrate a potent reduction (56% from baseline) in EPSC amplitude under the influence of 20μM ADO (n = 13; Repeated measures ANOVA,$F(2,24)=21.684$, $p<0.001$, Bonferroni post-hoc testing). Pharmacological blockade by 10μM DNQX confirms NMDA-mediated EPSCs. Individual normalized evoked EPSCs are shown as grey dashes.

voltage-dependent relationship was seen during all conditions (Fig. 4B).

Adenosine reduces the amplitude of pharmacologically isolated NMDA currents

The effects of adenosine in layer 2/3 pyramidal neurons on NMDA-mediated currents were studied by pharmacologically isolating NMDA currents with DNQX and

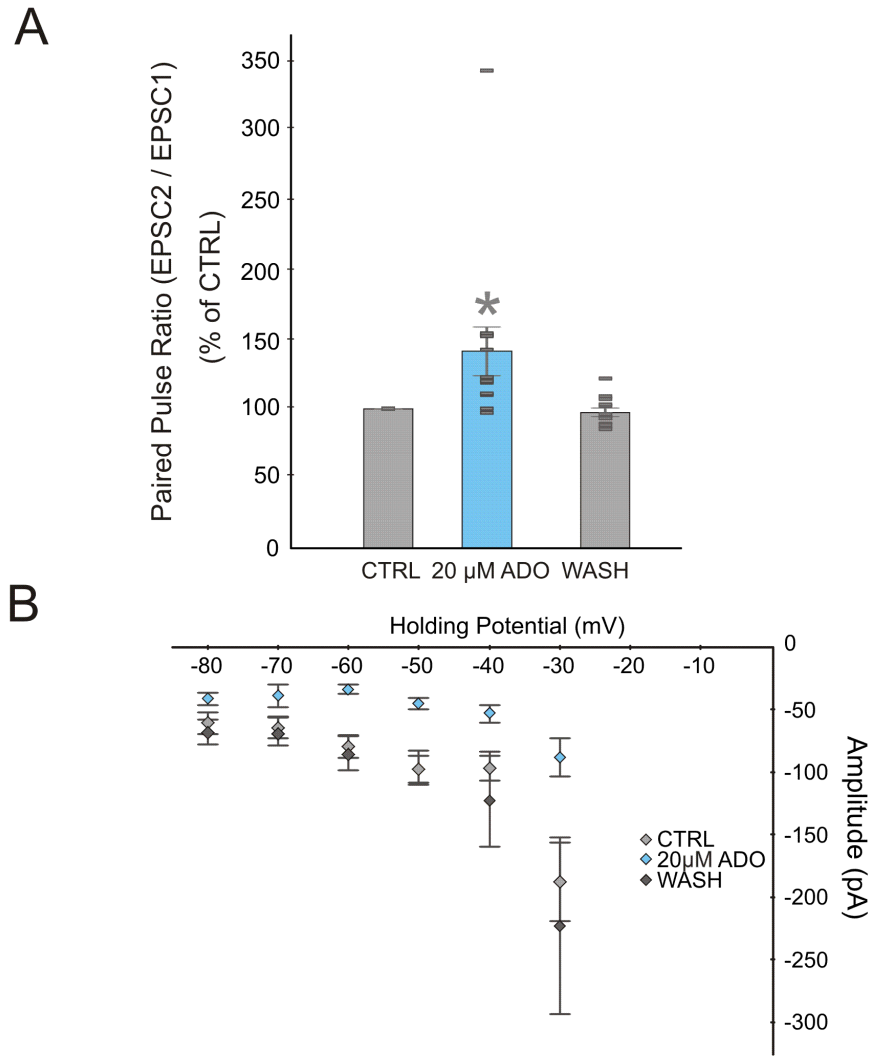

Fig. 6 Adenosine reduces the amplitudes of pharmacologically isolated NMDA currents

(**A**) Normalized paired pulse ratio, measured as EPSC2/EPSC1, increased under 20µM ADO (Repeated measures ANOVA, $F(2,24)=9.266$, $p=0.001$). This demonstrates a decrease in release probability which is characteristic of A1R activation. (**B**) Voltage-dependence on NMDA-mediated EPSCs between -30mV and -80mV demonstrates a greater conductance at depolarized potentials as compared to hyperpolarized potentials due to Mg ion occluding the channel. This voltage-dependent relationship confirms the identity of NMDA-mediated EPSCs.

PTX in control solution and subsequent bath application of adenosine. Similar to AMPA-mediated currents, adenosine robustly reduced the amplitude of NMDA-mediated EPSCs to 44.5 ± 4.94% of baseline (Fig 5C; $p<0.001$). During the washout of adenosine, NMDA-mediated EPSCs returned to 101 ± 10.5% of baseline (Fig. 5C; n.s). Wash-in of the selective NMDA-antagonist (APV) reduced NMDA-mediated EPSCs to 8.83 ± 4.55% of baseline (Fig. 5C; $p<0.001$). We observed a significant increase in the paired-pulse ratio to 142 ± 17.3% of baseline (Fig. 6A; $p=0.001$) suggesting a reduction in release probability and therefore a presynaptic action of adenosine. In addition, we observed a characteristic voltage-dependent relationship of the NMDA receptor at holding potentials between -30 and -80mV, where holding potentials less than -50mV passed little current due to a Mg^{2+} ion obstructing the channel (Fig. 6B; Nowak et al., 1984). It is important to note that our results show a clear difference in the voltage dependence of AMPA-mediated (Fig. 4B) and NMDA-mediated currents (Fig. 6B). These results indicate that we are truly recording isolated AMPA- and NMDA-mediated currents. Our results show similar voltage dependent relationships that have been previously identified for NMDA and non-NMDA-mediated currents (Klishin et al., 1995).

Adenosine reduces the amplitudes of mixed NMDA and AMPA currents proportionally

To study the effect of adenosine in layer 2/3 pyramidal neurons on mixed, AMPA and NMDA-mediated, currents we recorded EPSCs evoked by a single pulse under the background of PTX, TTX, and NMDAR co-activator glycine. A single pulse paradigm was used in conjunction with a low concentration of PTX (2µM) and TTX (1nM) to reduce slice seizures. Once a stable baseline was recorded (80-100 sweeps or approximately 10mins), 20µM ADO was washed into the bath (Fig. 7B and Fig.7C).

ADO was allowed to fully wash in (5mins) and the last 20 sweeps were averaged for analysis (Fig. 7B and Fig. 7C). ADO was subsequently washed out of the bath (10mins; Fig. 7B and Fig. 7C). After washout, 20µM APV was washed into the bath to isolate AMPA-mediated currents (Figure 7C). This allowed us to determine appropriate placement of NMDA-mediated measurement windows (see *Methods*). DNQX, a selective AMPA antagonist, was washed into the bath at the end of the experiment to confirm the absence of inhibitory currents at all holding potentials (-30 through -80mV; not shown).

During bath application of adenosine, we observed a reduction in the amplitude of both AMPA-mediated (Fig. 8A, 37.8 ± 5.92% of baseline; $p<0.001$) and NMDA-mediated (Fig. 8A, 35.7 ± 8.91% of baseline; $p<0.001$) components of EPSCs. The effects of adenosine could be reversed during washout where the AMPA and NMDA components returned to 80.1 ± 5.79% ($p=0.079$) and 86.3 ± 7.19% ($p=0.778$) of baseline, respectively (Fig. 8A). The application of the NMDA-antagonist, APV, reduced NMDA-mediated currents to 14.8 ± 5.05% of baseline (Fig. 8A; $p<0.001$). When compared, the mean amplitudes of AMPA or NMDA-mediated EPSCs under adenosine were not statistically different from each other (Fig. 8A, T-test; $p=0.157$). When a ratio was made of NMDA to AMPA-mediated EPSCs there was no significant difference

between control and the wash-in of adenosine (Fig. 8B, T-test; p=0.644). Furthermore, when the amplitudes of isolated AMPA and NMDA-currents under adenosine were

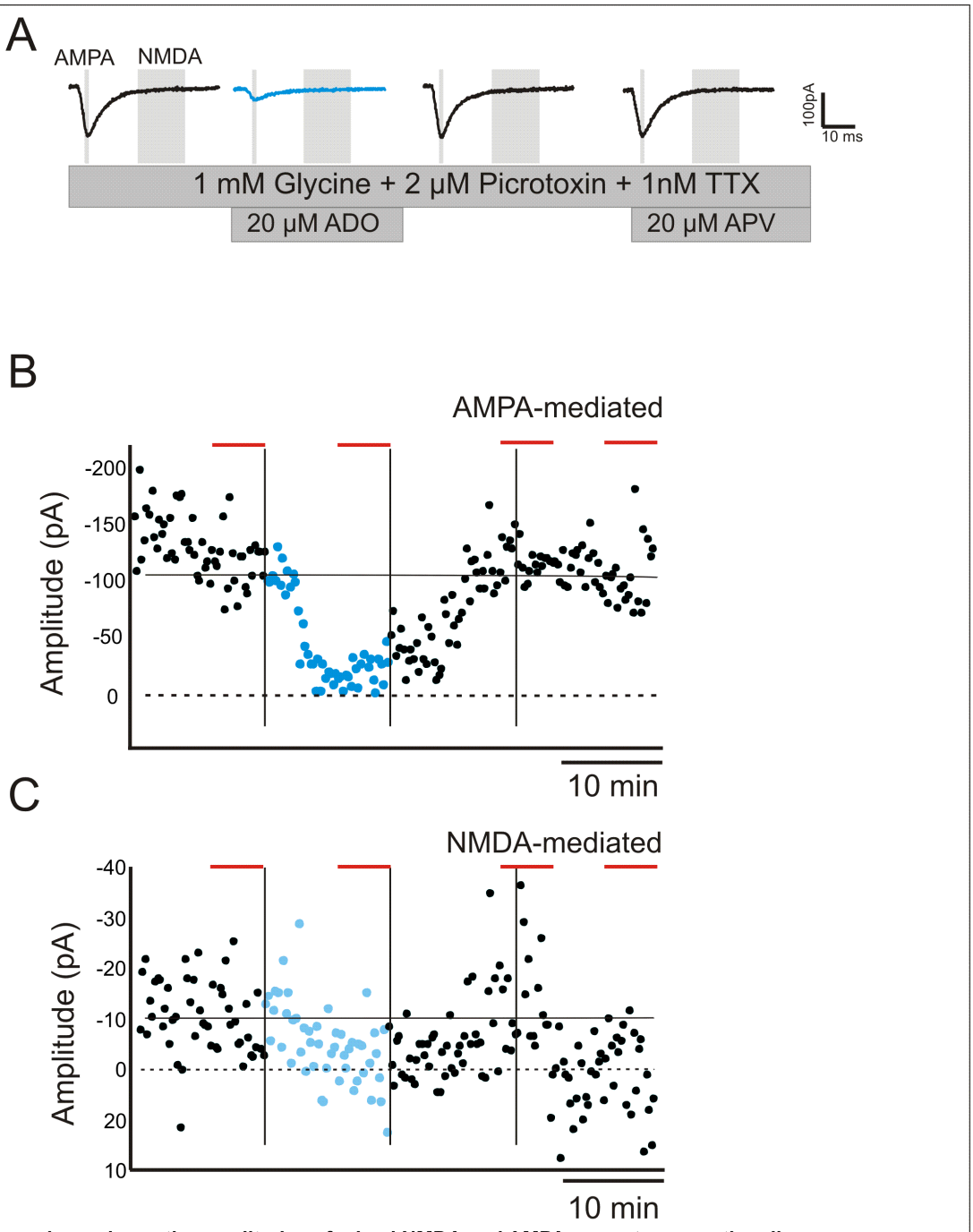

Fig. 7 Adenosine reduces the amplitudes of mixed NMDA and AMPA currents proportionally
(**A**) Mixed NMDA and AMPA currents recorded in layer 2/3 pyramidal neurons with single pulse stimulus under the background of inhibition 2µM PTX, 1nM TTX, and 1mM glycine. Representative traces show compound AMPA and NMDA-mediated EPSCs. The AMPA-component was measured by placing windows just before the peak of the EPSC. The NMDA-component windows were deduced by pharmacological isolation of AMPA-mediated EPSCs with 20µM APV after washout. (**B, C**) Time courses of evoked EPSCs throughout recording intervals .Grey line signifies baseline EPSC in control. Wash in of adenosine (blue-light blue) shows reduction of both AMPA and NMDA-mediated components, respectively. Red bars indicate sweeps incorporated into average.

compared there was no statistical difference (not shown, T-test; p=0.338).

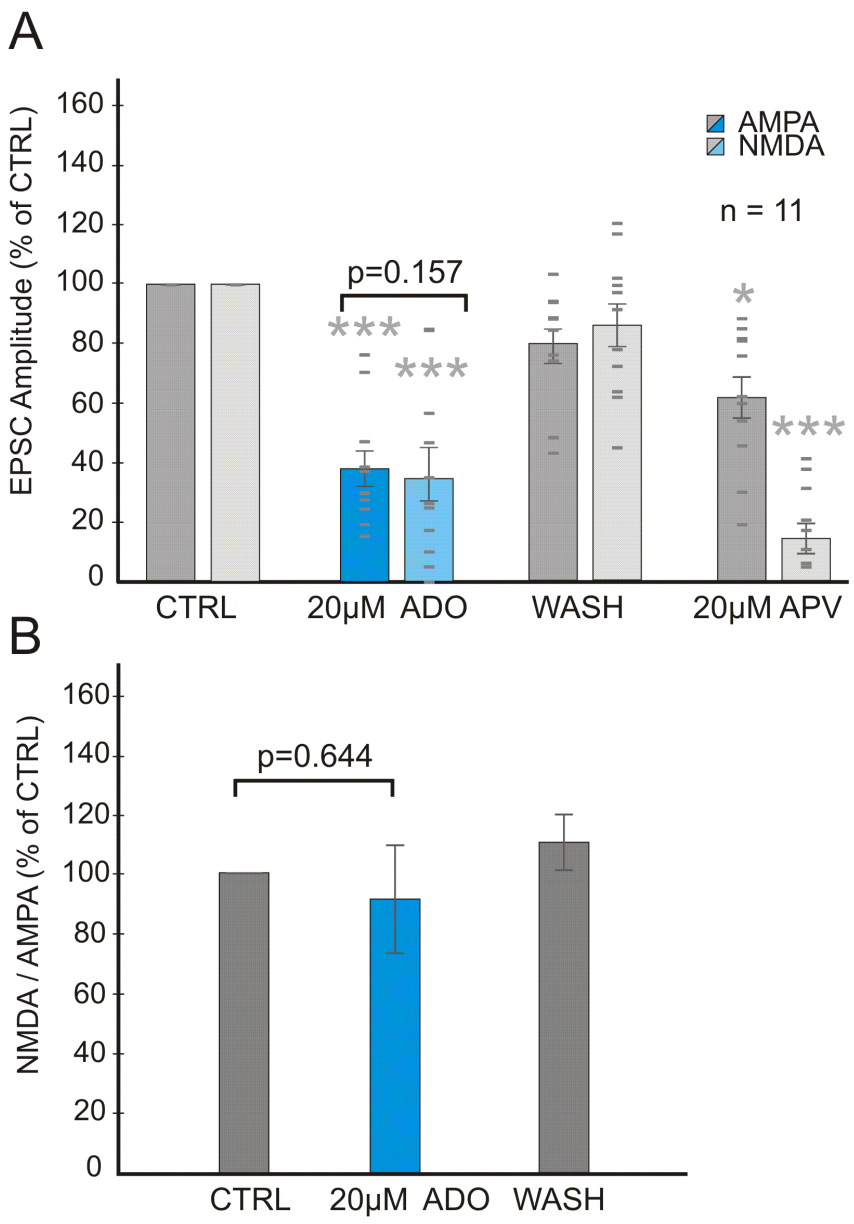

Fig. 8 Adenosine reduces the amplitudes of mixed NMDA and AMPA currents proportionally

(**A**) Normalized AMPA and NMDA-mediated EPSCs are potently reduced to 37.8% and 35.7% respectively under adenosine (n=11; AMPA-component; Univariate ANOVA, $F(3, 40)=23.885$, $p<0.001$, NMDA-component; Univariate ANOVA $F(3,40)=41.846$, $p<0.001$). However, AMPA and NMDA-mediated components were not significantly different (T-test; p=0.157). (**B**) Normalized NMDA/AMPA ratio demonstrates that under the influence of adenosine, NMDA- and AMPA-mediated components were reduced proportionally. Note that control is not significantly different to application of adenosine (T-test, p=0.644).

Discussion

In accord with previous work from our lab, adenosine robustly reduced the amplitude of EPSCs in L2/3 of rat visual cortex (N. Bannon et al., 2014). However, it was not previously known whether adenosine worked preferentially on NMDA-mediated or AMPA-mediated currents in the cortex. In dopamine neurons of the rat midbrain, adenosine via activation of presynaptic A_1Rs reduced NMDA-mediated currents 20 times more potently than AMPA-mediated currents (Y.-N. Wu et al., 1999). Their proposed mechanism suggests that the majority of NMDAR activation is due to glutamate spillover therefore a dilute concentration of glutamate would preferentially reduce NMDA-mediated events (Y.-N. Wu et al., 1999). In contrast, work conducted in the hippocampus has shown that AMPA and NMDA-mediated events are reduced equally by adenosine (Garaschuk et al., 1992; Perkel & Nicoll, 1993) and increases in release probability change NMDA/AMPA-mediated events proportionally (Tong & Jahr, 1994).

In our experiments we demonstrate that NMDA- and AMPA-mediated currents are reduced proportionally in mixed current recordings of L2/3 rat visual cortex (Fig. 8a). Thus, the NMDA/AMPA ratio was maintained during wash-in of adenosine (Fig. 8b). In our pharmacologically isolated preparations, we observe an increase in the paired-pulse ratio (EPSC2/EPSC1) which is inversely related to release probability (Stevens, 1993; Fig. 4a and Fig. 6a). This result suggests that adenosine may be acting on A_1Rs thus reducing the amount of glutamate released. Therefore, adenosine did not confer with a selective reduction in NMDA-mediated currents (Fig. 8a).

The implications of our findings reach beyond a fundamental understanding of adenosine's role in synaptic transmission in the visual cortex. A_1R activation during the induction of plasticity has been demonstrated to have long term effects on synaptic transmission. In CA1 region of the hippocampus, long-term potentiation (LTP) was nearly blocked by an adenosine analog, 2-chloroadenosine (CADO). Further studies have shown that adenosine itself may block LTP induction and antagonizing the A_1 receptor using 8-cylcopentyl-1,3-dipropylxanthine (DPCPX) prevents adenosine-mediated inhibition of LTP (De Mendonça & Ribeiro, 2001). A plausible mechanism for the effect of adenosine on preventing LTP could be a reduction in the amount of activated NMDARs. LTP requires an influx of Ca^{2+} through the NMDA receptor. NMDA currents were inhibited by CADO in pyramids of dissociated hippocampus (de Mendonça, Sebastião, & Ribeiro, 1995). In CA1-CA3 regions of the hippocampus, A_1 antagonist, CPT, was shown to increase NMDA-mediated but not AMPA-mediated currents (Klishin et al., 1995). This potential preference for modulation of NMDA-mediated currents could be explained by phosphorylation of NMDA receptor subunits downstream of protein kinase A activation via adenosine receptors (Chen & Roche, 2007).

Previous work from our lab has demonstrated that adenosine may serve a homeostatic role in regulation of synaptic plasticity. The Hebbian model of learning generally states that synapses activated before postsynaptic firing will be potentiated (Magee & Johnston, 1997). Hence the common interpretation, 'synapses that fire together, wire together'. However, Hebbain-type long-term plasticity creates a positive feedback loop that cannot explain the variability of synaptic weights (N. M. Bannon et

al., 2016). In layer 2/3 of rat visual cortex, synapses are modulated at homosynaptic (active) as well as heterosynaptic (non-active) inputs (Maxim Volgushev et al., 2016). Furthermore, heterosynaptic inputs were modulated by the same activity as homosynaptic inputs. The direction of change was weight-dependent so that overall synaptic input was preserved (Maxim Volgushev et al., 2016). Recent experiments have demonstrated that in L2/3 of rat visual cortex, adenosine strengthened weight-dependent plasticity whereas adenosine antagonists prevent it (N. M. Bannon et al., 2016).

The present study adds to the narrative of adenosine's role in modulation of plasticity. Here, we demonstrate that NMDA-mediated currents are not preferentially reduced by adenosine in L2/3 pyramids of visual cortex. Thus, activation of adenosine receptors and postsynaptic modulation of NMDA-mediated currents are likely not mediating the effects of adenosine on weight-dependent plasticity.

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