Activation of Adenosine A$_{2A}$ Receptors Alter Postsynaptic Currents and Depolarizes Neurons of the Supraoptic Nucleus

Todd A. Ponzio$^{1,2}$, Yu-Feng Wang$^1$ & Glenn I. Hatton$^1$

$^1$ Department of Cell Biology & Neuroscience, University of California, Riverside, CA 92521
$^2$ Present address: Laboratory of Neurochemistry, NIH, Bldg 49/5C68, Bethesda, MD 20892

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Correspondence: ponziot@mail.nih.gov

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ABSTRACT

Supraoptic nucleus (SON) neurons secrete oxytocin or vasopressin in response to various physiological stimuli (e.g., lactation/suckling, dehydration). Released near fenestrated capillaries of the neurohypophysis, these peptides enter the blood and travel to peripheral target organs. The pervasive neuromodulator adenosine, acting at A₁ receptors, is an important inhibitory regulator of magnocellular neuroendocrine cell activity. Another high-affinity adenosine receptor exists in this system, however. We examined the physiological effects of adenosine A₂A receptor activation and determined its localization amongst various cell types within the SON.

In whole-cell patch clamp recordings from rat brain slices, application of the selective adenosine A₂A receptor agonist, CGS 21680, caused membrane depolarizations in SON neurons, often leading to increased firing activity. Membrane potential changes were persistent (>10 min) and could be blocked by the selective A₂A receptor antagonist ZM 241385, or GDP-β-S, the latter suggesting postsynaptic sites of action. However, MCPG or tetrodotoxin also blocked CGS 21680 effects, indicating secondary actions on postsynaptic neurons. In voltage clamp mode, application of CGS 21680 caused a slight increase (~8%) in high-frequency clusters of excitatory postsynaptic currents. Using specific antibodies, adenosine A₂A receptors were immunocytochemically localized to both the magnocellular neurons and astrocytes of the SON. Ecto-5’ nucleotidase, an enzyme involved in the metabolism of ATP to adenosine, was also localized to astrocytes of the SON. These results demonstrate that adenosine acting at A₂A receptors can enhance the excitability of SON neurons and modulate transmitter release from glutamatergic afferents projecting to the nucleus. We suggest that adenosine A₂A receptors may function in neuroendocrine regulation through both direct neuronal mechanisms and via actions involving glia.
INTRODUCTION

Magnocellular neurons of the supraoptic nucleus (SON) and paraventricular nucleus synthesize either oxytocin (OT) or vasopressin (VP), peptide hormones that participate in a variety of physiological phenomena including lactation, parturition, and water balance. OT and VP released within the neural lobe enter the general circulation and ultimately activate receptors on their target organs. These hormones however, can be released from anywhere along the SON neuronal plasma membrane (38) and peptide release profiles from magnocellular dendrites do not necessarily parallel those observed from the neural lobe (22). Therefore, knowledge concerning endogenous regulators of magnocellular excitability contributes to the understanding of neuronal, and in this case, hormonal regulation.

Adenosine is a ubiquitous neuromodulator that tends to exert primarily inhibitory actions on synaptic transmission and transmitter release. With four known receptors, it is through adenosine’s activation of A1 receptors that its suppressive effects are mostly executed. In addition to A1 receptors, adenosine A2A receptors have also been shown to be present in the SON (19, 39). The staining pattern reported within the SON in those studies, however, left unclear its exact location on various cellular elements, e.g., axon terminals, glia, postsynaptic neurons and/or blood vessels. The A2A receptor’s function in vasodilation and its presence on endothelial cells are well documented. In this regard, it is noteworthy that these magnocellular nuclei have the highest capillary densities found in the brain (31).

That A2A receptor activation can cause glutamate release from astrocytes has been reported (21). Evidence for astrocyte-neuron signaling in the SON has long suggested that the glial cells in this nucleus behave proactively in the regulation of neuronal excitability (2, 15, 34). Astrocytes of the SON are uniquely organized, with most having their somata in the ventral glial lamina, often sending long processes coursing dorsally to permeate the entire nucleus. Glutamate release from astrocytes has been shown to be capable of modulating neuronal activity (33) and the possible presence of A2A receptors on its astrocytes would suggest that this could be the case in the SON.

As previous studies have shown, adenosine A1 receptor activation hyperpolarizes the membrane and causes inhibitory effects on synaptic transmission (30, 35). Here we investigate the physiological effects and anatomical location of adenosine A2A receptors in the SON. We report that A2A receptor activation causes a small membrane depolarization that can lead to
neuronal firing changes. We also report an A$_{2A}$ receptor-mediated increase in the frequency of spontaneous postsynaptic currents, and the immunolocalization of adenosine A$_{2A}$ receptors on astrocytes and neurons of the SON.

METHODS

Chemicals

All salts used in the perifusion media and whole-cell recording solution were purchased from Fisher Scientific (Fair Lawn, NJ). Adenosine, the A$_{2A}$ agonist 2-p-(2-carboxyethyl)phenethyl-amino-5'-N-ethylcarboxamidoadenosine (CGS 21680), tetrodotoxin (TTX), tetraethyl ammonium chloride (TEA), ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), N-(2-Hydroethyl) piperazine-N’-(2-ethanesulfonic acid) (HEPES), (±)-α-Methyl-(4-carboxyphenyl)glycine (MCPG), and 3(N-morpholino) propanesulfonic acid (MOPS) were purchased from RBI/Sigma (St. Louis, MO).

Slice preparation and procedures

Slices were prepared similarly to those previously described (35) and procedures were in accordance with the University of California, Riverside animal handling and use guidelines. Briefly, adult male Sprague-Dawley rats (45-70 days old) were decapitated and the brains were rapidly removed and placed in ice-cold oxygenated artificial cerebral spinal fluid (aCSF) consisting of (in mM) NaCl, 126; KCl, 3; NaH$_2$PO$_4$, 1.25; MgSO$_4$, 1.3; D-Glucose, 10; NaHCO$_3$, 26; CaCl$_2$, 2.4; MOPS, 5, pH 7.4. The combination of this organic buffer and NaHCO$_3$ has been found in earlier studies to better stabilize the pH over prolonged recording sessions than does the use of the bicarbonate buffer alone. Brains were then placed ventral side up, blocked for slicing and glued to a specimen holder of a Vibratome. Gassed (95% O$_2$/5% CO$_2$), ice-cold aCSF bathed the brain block and coronal hypothalamic slices containing the SON (300-400 µm thick) were cut and placed in a bath of gassed aCSF for slice bisection and cropping. One hemi-slice was then placed in a recording chamber held at 34°C, the others in a holding chamber at room temperature.

Whole-cell recording

Methods were similar to those described previously (35). Briefly, patch electrodes (borosilicate, tip diameter: 1-2 µm) were pulled using a multi-stage pipette puller (P-97, Sutter Instruments) and filled with a recording solution consisting of (in mM): K$^+$-gluconate, 130; KCl
8; MgCl₂, 2; HEPES, 10; EGTA 0.4, K₂-ATP, 2; Na₂-GTP, 1 and Alexa Fluor 488 (Molecular Probes Eugene, OR). The final pH and osmolality of the recording solution was 7.3 (adjusted with KOH) and 298 ± 3 mOsm/kg (adjusted with H₂O). The final DC resistance of the pipettes was 3-4 MΩ.

Patch pipettes were guided to cells visualized under near infrared differential interference contrast video microscopy (Leica DMLFSA equipped with a Dage IR-1000 camera) and GΩ seals were obtained. Brief suction resulted in the establishment of whole-cell configuration and the bridge circuitry of the amplifier (Axoclamp 2-B, Axon Instruments) was immediately engaged and optimized.

Drugs were applied by bath perifusion at a flow rate of 1.5 ml/min. At the close of each experiment, images of the Alexa Fluor 488 -filled neuron were collected with a charged-coupled device (CCD) camera using the microscope under epifluorescence illumination for morphological verification that the recorded cell was magnocellular. Images were stored on a PC and only SON neurons having an observed diameter greater than 12 µM, were used in the study.

**Immunocytochemistry**

Adult male rats were deeply anesthetized with sodium pentobarbital and transcardially perfused with 4% paraformaldehyde. Coronal sections (thickness = 50 µm) were cut using a Vibratome and stored in 24-well culture plates. All immunocytochemical reactions were done in the wells. Sections were washed in 0.1 M phosphate buffered saline (PBS) and treated with 0.3% Triton X-100 for 30 min. Following a PBS wash, sections were incubated in a 0.3% gelatin blocking buffer for 30 min. Primary antibodies used were against the adenosine A₂A receptor (Alpha Diagnostics, San Antonio, TX), the OT-neuron identifying protein neurophysin I (NP I, Santa Cruz Biotechnology, Santa Cruz, CA), ecto-5´nucleotidase (Santa Cruz Biotechnology) and glial fibrillary acidic protein (GFAP, Sigma, St. Louis, MO and Santa Cruz Biotechnology). Incubation in primary antibody solutions was done at room temperature for 3 h with gentle agitation on an orbital shaker. All antibodies were diluted in blocking buffer and were combined as outlined in Table 1. Following four PBS washes, the sections were again incubated in blocking buffer for 30 min, followed by secondary antibody solutions (Table 1) for 2 h at room temperature. All secondary antibodies were from Molecular Probes (Eugene, OR) and made in donkey. Control experiments with the absence of primary antibody were done in parallel with those including primary antibodies. Images of the sections were collected on a confocal
microscope (Leica SP2) under sequential scan mode. The pinhole was set to 1 airy unit corresponding to an optical slice thickness of < 300 nm and images are presented as z-series projections.

**Data analysis**

All electrophysiological data were digitized at 10 kHz, filtered at 5 kHz and collected for off-line analysis using pClamp 8 software (Axon Instruments). Statistical analysis using SigmaStat software consisted of the paired or unpaired Student’s *t* tests or their non-parametric equivalents. Minimal statistical significance was taken as *p* < 0.05.

**RESULTS**

*A2A receptor activation depolarizes SON neurons*

As shown in figure 1, application of the selective adenosine A2A receptor agonist CGS 21680 caused a slight, but lasting depolarization of the membrane. This occurred over all concentrations tested (20 nM-1 µM) and often led to increased firing activity (Fig. 1A and B). The depolarization could be blocked by delivery of 1 mM GDP-β-S into the postsynaptic cell through the patch electrode (n=4) or by extracellular application of the selective A2A receptor antagonist ZM 241385 (n=5, 1 µM). To obtain membrane potential values, recordings were low-pass filtered at 1 Hz using an 8-pole Bessel filter algorithm. This resulted in the removal of action potentials and fast synaptic potentials, leaving only the slower membrane processes intact. The average membrane potential occurring 3 min into CGS 21680 application (measured over 1 min) was compared to control values taken just prior to the application. A summary of changes in membrane potential observed under several conditions is presented in figure 1 C. As shown in our previous work and in contrast to activating only A2A receptors, application of adenosine 50-100 µM caused a strong hyperpolarization (-3.64 mV, *p* < .01, n=8). This adenosine-induced hyperpolarization prevailed even in the presence of selective A2A receptor activation (Fig. 1D.) Additionally, unlike the inhibitory effects of adenosine which were repeatable in a given cell, only the first application of CGS 21680 induced the depolarization, which is consistent with the A2A receptor’s rapid desensitizing characteristics (1, 24, 32). Therefore, each cell was exposed to only a single application of CGS 21680.
$A_2A$ receptor activation slightly increases EPSC clustering

To investigate the effects of adenosine $A_2A$ receptor activation on afferent activity, voltage clamp recordings were obtained from SON neurons. Clamping the membrane at either -35 or -60 mV reveals bicuculline-sensitive IPSCs or CNQX-sensitive EPSCs, respectively. With the addition of 20 mM KCl, the number of events observed increased dramatically (from ~5 to ~20 Hz). The further addition of CGS 21680 (500 nM) did not cause a change in EPSC amplitude (-18.7±1.4 vs. -17.0±3.5 pA, p<0.42, n = 6), nor was there a significant further increase in EPSC frequency (17.9±7.7 vs. 20.7±7.5 Hz, n = 6). However, activation of $A_2A$ receptors slightly, but significantly increased the number of EPSC clusters (by +7.7 ± 2% over control period, p< 0.02, n = 6), where a ‘cluster’ was defined as a minimum of 5 consecutive events occurring with a maximum inter-event interval of 50 ms (Fig. 2). The same analysis applied to 5 cells voltage clamped at -35 mV to reveal IPSCs did not reveal an effect of $A_2A$ receptor activation on frequency, amplitude, or clustering of IPSCs.

Adenosine $A_2A$ receptor activation has been associated with increases in intracellular calcium. To test for an $A_2A$ receptor-mediated enhancement of voltage-gated calcium currents, cells were treated with 0.6 µM TTX + 5 mM TEA and given current pulses to evoke Ca$^{2+}$ spikes. CGS 21680 did not change the amplitude or duration of evoked calcium spikes, nor did it change individual components of the action potential (Fig. 3). Several components were measured including the spike peak, width, AHP amplitude and descending slope. The effect of CGS 21680 on these action potential components for 6 cells is presented in figure 3B.

Action Potentials and Metabotropic Glutamate Receptors Participate in the $A_2A$-mediated Depolarization

Recent studies have suggested that metabotropic glutamate receptors are functionally linked to adenosine $A_2A$ receptors (6, 9). To investigate involvement of metabotropic glutamate receptors and TTX-sensitive channels in the adenosine $A_2A$ receptor mediated depolarization, we bath-applied either the nonselective metabotropic glutamate receptor antagonist MCPG (200 µM, n=5) or TTX (0.6 µM, n=6) prior to CGS 21680 (500 nM). Consistent with metabotropic glutamate receptors playing a permissive role for $A_2A$ receptor activation (26), MCPG prevented CGS 21680 from depolarizing the SON neurons (Fig. 4). An earlier study in this system using TTX found no membrane depolarization in response to application of the natural ligand, adenosine (17). Consistent with those results, no $A_2A$-mediated depolarization was observed in...
the presence of TTX, suggesting an involvement of action potentials/Na⁺ channels in the observed depolarization.

**Localization of Adenosine A₂ₐ receptors and ecto-5’nucleotidase in the SON**

Immunocytochemical experiments using antibodies to the adenosine A₂ₐ receptor, GFAP, and NP I were performed. The A₂ₐ receptor antibody used was a rabbit polyclonal antibody made against the canine A₂ₐ receptor. The reactivity of this antibody to rat adenosine A₂ₐ receptors has previously been described (25) and it recognizes an intracellular portion of the protein’s C-terminal. In agreement with a previous report (39), A₂ₐ receptor immunoreactivity is apparent in the SON (Fig. 5 A). Large puncta were seen along large diameter blood vessels (diameter >12 µm) entering the brain. Within the SON parenchyma, the heaviest labeling occurred in the ventral glial lamina, followed by the somatic zone, with lighter A₂ₐ receptor immunoreactivity in the dendritic zone. Furthermore, ventral to the astrocytes of the ventral glial lamina, meningeal cells also expressed A₂ₐ receptor immunoreactivity. Dual immunolabeling of the astrocyte intermediate filament protein GFAP together with the adenosine A₂ₐ receptor revealed colocalization in both astrocytic somata and along the GFAP-positive processes that extend toward the interior of the nucleus (Fig. 5 B). Acquisition of a confocal z-series on the immunoprocessed sections revealed the presence of A₂ₐ receptor immunoreactivity completely surrounded by GFAP labeling (Fig. 5 D). Light A₂ₐ receptor immunoreactivity was also seen in magnocellular somata both positive and negative for neurophysin I immunoreactivity (Fig. 5 C), consistent with the physiological data that the receptor is expressed by both OT and VP neurons.

There are two main sources of endogenous extracellular adenosine, one from bidirectional nucleoside transporters and the other from metabolism of ATP. In the SON, the nucleoside transporter inhibitor, dilazep, produced effects similar to adenosine A₁ receptor activation, suggesting there exists a constant supply of extracellular adenosine and that nucleoside transporters are important in maintaining extracellular adenosine concentrations (35). Dendritic release of OT and VP from large dense-core vesicles, however, is a well-described phenomenon in this system, and also ATP is highly concentrated in secretory vesicles. Because we were interested in knowing if breakdown of ATP might be contributing to extracellular adenosine levels, we investigated the presence of ecto-5’nucleotidase, an enzyme involved in the metabolism of ATP to adenosine. Strong immunoreactivity to the enzyme was observed in the SON (Fig. 6). Interestingly, some of the strongest immunoreactivity for ecto-5’nucleotidase...
colocalized with long GFAP-positive processes, suggesting an astrocytic source of this enzyme and ascribing another function to this dynamic cell type in this region (Fig. 6 C).

DISCUSSION

Adenosine is a ubiquitous neuromodulator that exerts strong effects on several cell types of the magnocellular hypothalamic neuroendocrine system. Within this system, adenosine acting on A<sub>1</sub> receptors induces astrocyte stellation (40), reduces Ca<sup>2+</sup> currents (29), reduces PSC frequency (30), and hyperpolarizes SON neurons resulting in reduced spike firing (35). Although the presence of adenosine A<sub>2A</sub> receptors in this system has been known for several years prior to this report, direct effects of activating A<sub>2A</sub> receptors on SON neuronal physiology and their specific locations had not been investigated. The present evidence suggests that activation of SON adenosine A<sub>2A</sub> receptors results in a depolarization of SON neuronal membrane potential, and slightly increases high-frequency excitatory postsynaptic currents. We also present evidence suggesting the presence of A<sub>2A</sub> receptors both on SON neurons and astrocytes, and for astrocytic expression of ecto-5′nucleotidase.

A<sub>2A</sub> receptor modulation of PSCs

Evidence for adenosine A<sub>2A</sub> receptor influence on transmitter release has been reviewed (8, 41). Studies have suggested that A<sub>2A</sub> receptor activation can result in enhanced levels of several neurotransmitters including glutamate and GABA. It has been suggested that these effects may not be due to activation of terminally-located A<sub>2A</sub> receptors, but result from disinhibition of axon terminals (8).

Conversely, a study monitoring A<sub>2A</sub> receptor-mediated modulation of GABA release from synaptosomes concluded that activation of this receptor enhances GABA release (3). It has been shown that A<sub>2A</sub> receptor activation leads to an increase in Ca<sup>2+</sup> levels within terminals (10), likely through an enhancement of N- and P-type Ca<sup>2+</sup> currents (11). In this study we did not address the specific location of A<sub>2A</sub> receptors on the glutamatergic afferents. It is interesting that in the SON, activation of adenosine A<sub>2A</sub> receptors did not significantly alter the overall frequency of PSCs, however, it did increase the occurrence of high-frequency EPSC clusters. High-frequency EPSCs are more apt to summate and, in turn, may bring the membrane potential above threshold. An enhancement of high frequency EPSCs, but not IPSCs, by adenosine A<sub>2A</sub> receptor activation is consistent with 1) an adenosine A<sub>2A</sub> receptor-induced disinhibition of
glutamatergic afferents and 2) a direct enhancement of N- and P/Q-type channels on glutamatergic neurons. Either process could produce a net A2A-mediated excitatory effect ultimately leading to increased firing activity.

**A2A receptor functions in the SON**

Spontaneous firing is a characteristic of SON neurons both *in vivo* and *in vitro*. OT cells tend to fire intermittently or continuously until they become activated, at which point they can then undergo periodic high-frequency bursts (37). VP cells, on the other hand, fire phasically upon their activation by dehydration. These cells undergo periods of spike firing that are followed by quiescent periods, and this phasic cycle repeats. It has been suggested that both cell types regulate their own firing activity through autocrine or retrograde transmission via dendritic release of OT or VP. Dendritically released OT tends to reduce inhibitory synaptic transmission to OT neurons. Interestingly, this effect can be mimicked by exogenous application of either OT or adenosine and is blocked by antagonists for both OT and adenosine A1 receptors (4). These results suggest that retrogradely transmitted adenosine (or its precursor) is released along with OT from the dendrites of OT neurons.

ATP is known to be colocalized with a number of neurotransmitters and peptides (16, 20) in neurons that send projections to the SON (13). Along with its capacity to activate P2 receptors, ATP is also readily metabolized to adenosine in the extracellular environment (7, 42). Here we present evidence that the SON itself is endowed with sources of ecto-5´nucleotidase, one of the enzymes involved in the metabolism of ATP in the extracellular space. Glial processes were seen to be particularly immunoreactive for the enzyme. As the glia of the SON are especially influential on SON neuronal activity, these results suggest that they may also function to regulate extracellular levels of ATP and adenosine. SON astrocytes retract from between adjacent neurons and dendrites during activated states (14). This would tend to reduce their effectiveness in metabolizing ATP to adenosine under these conditions, and promote prolongation of ATP action.

Finding an adenosinergic influence on SON cells was not surprising. Adenosine is fast becoming known as a potent inhibitory neurotransmitter. However, that adenosine tends to so tightly regulate SON neuronal firing activity and synaptic input in this system is of considerable interest. Also, that both excitatory A2A receptors and inhibitory A1 receptors are capable of altering the physiological activity of SON neurons in an opposing manner suggests that
adenosine may play an integral role in neuroendocrine function. Whereas activation of A₁ receptors leads to a sustained hyperpolarization (35), activation of A₂A receptors leads to a depolarization. Here, however, we show that under conditions in which both receptors are activated, the inhibitory influence of the A₁ receptors can override the excitatory effect of A₂A receptor activation.

**A₂A receptors and SON neuronal membrane depolarization**

A₂A immunoreactivity on SON neurons combined with the ability of GDP-β-S to block the effects of the A₂A agonist, CGS 21680 on membrane potential may suggest the observed excitatory effects on neurons were due to activation of postsynaptic A₂A receptors. However, this effect could be blocked by both TTX and MCPG, suggesting an involvement of action potentials and metabotropic glutamate receptors. Basal activation of metabotropic glutamate receptors has been shown to be necessary for observing effects of A₂A receptor activation (26). Blockade of the A₂A receptor-mediated depolarization is consistent with this occurring in the SON. TTX blocks the propagation of action potentials and thereby reduces the likelihood and amount of glutamate released. MCPG blocks the direct action of glutamate on metabotropic receptors.

There are several potential sources of glutamate release in the mHNS, possibly including the SON neurons themselves (36). Additionally, there may be a glial-induced effect via A₂A receptor activation on astrocytes. It has been shown that astrocytes can release glutamate nonsynaptically in response to A₂A receptor activation (27, 28), and that astrocytes can contain vesicular glutamate transporters (VGLUTs) (23). Furthermore, the astrocytes of the ventral glial lamina play an integral role in SON neuronal excitability (12, 14) and are immunopositive for VGLUT type 3 (36) and taurine (5, 18). It is established that the astrocytes of the SON can release taurine in response to hypo-osmotic changes. They may similarly release glutamate in response to activating stimuli. If A₂A receptors are involved in this release, then the immunoreactivity seen along glial processes coursing through the SON, suggests a non-synaptic mechanism for intranuclear glutamate release, leading to excitation of those neurons near the release sites.

In this study, we report physiological effects of adenosine A₂A receptor activation on SON neurons and their specific localization amongst SON astrocytes and neurons. Adenosine A₂A receptor activation causes a depolarization of the neuronal membrane potential that can lead to increased firing activity and an increase in high-frequency EPSC clusters. These effects
contrast with those seen following activation of adenosine A1 receptors, suggesting this widespread neuromodulator can play both an activating and an inhibiting role in the SON.

**Conclusions**

Our data suggest that activation of adenosine A2A receptors results in the depolarization of magnocellular neurons of the SON and in a slight enhancement of high frequency EPSC clustering. Metabotropic glutamate receptor activation plays a permissive role in this depolarization. Also, the previous anatomical findings of Rosin et al [34] were extended by immunolocalization of the adenosine A2A receptor to both neurons and astrocytes of the SON. We further explored the source of endogenous adenosine, showing that the presence of an enzyme participating in its production, ecto-5’nucleotidase, was found throughout the SON and was particularly enriched within GFAP-labeled astrocytes of the ventral glial lamina. Together these results suggest ATP-derived adenosine may activate postsynaptic A2A receptors leading to membrane depolarization and subsequent selectively enhanced excitability.

**FIGURE LEGENDS:**

Figure 1. Adenosine A2A receptor activation causes a depolarization of SON neurons. A. An example of CGS 21680 application inducing a depolarization that leads to increased firing activity. B. Two examples of selective A2A receptor-evoked firing activity. C. Summary graph showing average changes in membrane potential under various conditions. The CGS 21680-induced depolarization was blocked by intracellular infusion of GDP-β-S and by the A2A receptor antagonist ZM 241385. D. 1 and 2, Low-pass filtered voltage recordings showing that the slow depolarization produced by CGS 21680 (500 nM) does not preclude an adenosine-mediated hyperpolarization. Arrows in D indicate resting membrane potential just prior to adenosine application. * Indicates significant difference from 0 mV (p < 0.05).

Figure 2. Adenosine A2A receptor activation increases EPSC clustering. In the presence of bicuculline (10 µM) and additional KCl (20 mM), afferent glutamate activity increases and numerous EPSCs can be recorded. Incoming EPSCs often cluster, such that several (minimum of
5) EPSCs occur sequentially with short (less than 50 ms) inter-event intervals. In each case $V_h$ was -60 mV. A.1. A voltage-clamp recording under control conditions (bicuculline + KCl), wherein a cluster (circled) has been recorded. A.2. An expansion of the circled cluster shown in A.1. The numbers above the trace refer to the inter-event intervals in milliseconds. An arrow spans the duration of the cluster in which the interval between each successive EPSC is less than 50 ms. B.1. The addition of CGS 21680 slightly increased the number of EPSC clusters. Two clusters have been circled. B.2. An expansion of the top cluster (circled and asterisked) is shown. The numbers above the trace refer to the time, in milliseconds, between successive EPSCs.

Figure 3. Adenosine $A_{2A}$ receptor activation does not alter SON neuron action potentials or Ca$^{2+}$ spikes. A. Although $A_{2A}$ receptor activation induces membrane depolarization, there were no changes in several components of recorded action potentials. B. Summary graph addressing various aspects of the action potential. There were no significant differences from control, $p>0.05$, paired t-test. C. In the presence of 0.6 µM TTX and 5 mM TEA, brief (50 or 100 ms) current pulses evoke Ca$^{2+}$ spikes. Traces are shown for which application of CGS 21680 ($N = 2$) did not alter the Ca$^{2+}$ spike amplitude or duration, or the ensuing calcium-generated afterhyperpolarization (AHP).

Figure 4. The $A_{2A}$ receptor mediated depolarization is blocked by MCPG or TTX. A,B. Raw traces from four cells. Bath application of MCPG (200 µM) blocked the CGS 21680-induced depolarization. C. Raw traces from two cells showing that the CGS 21680-induced depolarization is blocked in the presence of TTX (0.6 µM). D. Summary graph comparing changes in membrane voltage under three different conditions, CGS 21680 alone, CGS 21680 + MCPG, and CGS 21680 + TTX. No significant changes in membrane voltage were observed in the presence of MCPG or TTX.

Figure 5. Localization of adenosine $A_{2A}$ receptors in the SON. A. Immunoreactivity for adenosine $A_{2A}$ receptors (green) in the SON. Yellow arrowheads indicate large $A_{2A}$ puncta along a blood vessel (bv). Red arrowheads in A and B indicate $A_{2A}$ puncta along astrocytic processes (blue). B. Colocalization of GFAP and $A_{2A}$ immunoreactivity. White arrows indicate $A_{2A}$ puncta in astrocytic somata. C. Scattered $A_{2A}$ immunoreactivity is
colocalized in Neurophysin I- positive and negative cells (white arrows). D. A representation of a z-series at the point of the crosshairs shows A2A immunoreactivity is completely surrounded by GFAP. Z distance is 8 µm. SZ, somatic zone; DZ, dendritic zone; VGL, ventral glial lamina.

Figure 6. Presence of ecto-5’nucleotidase in astrocytes of the SON. A. Immunoreactivity for ecto-5’nucleotidase (green), an enzyme that participates in the formation of adenosine from ATP. B. Immunoreactivity for GFAP (red), showing astrocytes within the ventral glial lamina (left). Many long glial processes extend dorsally through the nucleus. C. Overlay of A and B showing several GFAP-positive processes containing immunoreactivity for ecto-5’nucleotidase (yellow). D. Inclusion of the nuclear stain, Hoechst. Neither GFAP nor ecto-5’nucleotidase colocalized with Hoechst (insets).

REFERENCES:


Table 1. Probe sources and dilutions.

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Abbreviations: GFAP, glial fibrillary acidic protein (a marker for astrocytes); NP I, neurophysin I (a marker for oxytocin neurons); m, mouse; r, rabbit; g, goat; d, donkey; A, Alexa Fluor. * The nuclear stain Hoechst was made at a stock concentration of 1 mg/ml in water.