Research Paper

ATP STIMULATES RAT HYPOTHALAMIC SYMPATHETIC NEURONS BY ENHANCING AMPA RECEPTOR-MEDIATED CURRENTS

Ferreira-Neto HC1,2; Antunes VR1*; Stern JE2*

1Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, Brazil. 2Department of Physiology, Georgia Regents University, Augusta, Georgia, USA

Running title: P2-AMPA receptor coupling in PVN neurons

*Corresponding authors:

Javier E. Stern, M.D. Ph.D.
Department of Physiology
Georgia Regents University
1120 15th Street, Augusta, GA 30912
United States
Email: jstern@gru.edu

or

Vagner R. Antunes, BS Pharm, PhD
Department of Physiology and Biophysics
Institute of Biomedical Sciences, University of Sao Paulo
1524 Professor Lineu Prestes Avenue, 05508-900, Sao Paulo, Brazil
Email: antunes@icb.usp.br

*V. R. Antunes and J. E. Stern are equal last authors.

Total numbers of words (excluding references and figures legend): 4723

Key words: ATP, AMPA, PVN, RVLM, hyperosmolarity

Figures: 5
AUTHORS CONTRIBUTIONS

Ferreira-Neto HC: Conception and design of the experiments, collection, analysis and interpretation of data, writing the article.

Antunes VR: Revising critically the article for important intellectual content, editing the manuscript

Stern JE: Conception and design of the experiments, interpretation of data, writing, and editing of the article
ABSTRACT

We have previously shown that ATP within the PVN induces an increase in sympathetic activity; an effect attenuated by the antagonism of P2 and/or glutamatergic receptors. Here, we evaluated precise cellular mechanisms underlying the ATP-glutamate interaction in the PVN, and assessed whether this receptor coupling contributed to osmotically-driven sympathetic PVN neuronal activity. Whole-cell patch clamp recordings obtained from PVN-RVLM neurons showed that ATP (100 µM, 1 min, bath applied) induced an increase in firing rate (89%) an effect blocked by kynurenic acid (1 mM) or PPADS (10 µM). While ATP did not affect glutamate synaptic function, AMPA receptors mediated-currents evoked by focal application of AMPA (50 µM, n=13) were increased in magnitude by ATP (AMPA amplitude: 33%, AMPA area: 52%). ATP potentiation of AMPA currents was blocked by PPADS (n=12) and by chelation of intracellular Ca²⁺ (BAPTA, n=10). Finally, a hyperosmotic stimulus (mannitol 1%, +55 mOsm, n=8) potentiated evoked AMPA currents (53%), an effect blocked by PPADS (n=6). Taken together, our data support a functional stimulatory coupling between P2 and AMPA receptors (likely of extrasynaptic location) in PVN sympathetic neurons, which is engaged in response to an acute hyperosmotic stimulus, which might contribute in turn to osmotically-driven sympathoexcitatory responses by the PVN.
ABBREVIATIONS

ATP, adenosine triphosphate; BAPTA, BAPTA tetrapotassium salt, cell impermeant; CVOs, circumventricular organs; IR-DIC, infrared differential interference contrast; $I_{AMP}$, AMPA current; IML, intermediolateral cell column; KYN, kynurenic acid; LSNA, lumbar sympathetic nerve activity; PIC, picrotoxin; PNA, phrenic nerve activity; PPADS, 4-[[4-Formyl-5-hydroxy-6-methyl-3-((phosphonoxy)methyl)-2-pyridinyl]azo]-1,3-benzenedisulfonic acid tetrasodium salt; PVN, paraventricular nucleus; RSNA, renal sympathetic nerve activity; RVLM, rostral ventrolateral medulla; SON, supraoptic nucleus.
INTRODUCTION

Body fluid homeostasis is tightly regulated by the integration of renal, cardiovascular and neuroendocrine systems (Share and Claybaugh 1972). An increase in plasma osmolality induces several responses including an increase in sympathetic activity, blood pressure elevation and the release of neurohormones, such as vasopressin and angiotensin II (Bealer 2000; Hatzinikolaou et al. 1980, 1981; Stocker and Toney 2005; Weiss et al. 1996). Part of these responses are mediated through activation of the hypothalamic paraventricular nucleus (PVN) driven by the circumventricular organs (CVOs) in which central osmoreceptors are located (Antunes–Rodrigues et al. 2004; Stocker et al. 2008). The PVN is composed of magnocellular and parvocellular neurons. Magnocellular neurons synthesize and release vasopressin and oxytocin systemically from the posterior pituitary, while parvocellular neurons project to pre-motor sympathoexcitatory neurons located either in the rostral ventrolateral medulla (RVLM) and/or intermediolateral cell column (IML) of the spinal cord.

Several neurotransmitters in the PVN are involved in the autonomic response evoked by a hyperosmotic stimulus. For example, the increase in renal sympathetic nerve activity (RSNA) after central sodium/osmotic activation is mediated, in part, by a mechanism involving angiotensin II-AT1-receptor activation within the PVN (Chen and Toney 2001). Furthermore, Badoer et al. (2003) showed that glutamatergic inputs into the PVN play an important role in the renal nerve response to elevations in osmolality. More recently, Son et al. (2013) have demonstrated that an acute osmotic challenge increased RSNA, which was attenuated by the antagonism of V1a receptors within the PVN, supporting the concept that local, dendritic release of vasopressin is another relevant signal regulating sympathoexcitatory outflow from the PVN.
In the last decades, adenosine triphosphate (ATP) has been recognized as an important neurotransmitter involved in many physiological and pathological mechanisms, besides its well-known role as an intracellular energy source. ATP actions as a neurotransmitter are mediated via P2 receptor activation, which are divided into two classes: P2Y and P2X. P2Y receptors are G protein-coupled receptors, while P2X receptors are ligand-gated ion channels, permeable to Na\(^+\), K\(^+\) and Ca\(^{2+}\). ATP acts as an excitatory neurotransmitter at synapses in the brain, spinal cord and peripheral nerve terminals (Burnstock 2007).

Immunohistochemical studies have identified the presence of P2 receptors in the hypothalamus (Guo et al. 2009; Song et al. 2011; Yao et al. 2003), including in RVLM-projecting PVN neurons (Cham et al. 2003), suggesting that ATP could act as a neurotransmitter within the PVN. This is in fact supported by our recent studies showing that P2 receptor activation within the PVN stimulated sympathetic nerve activity. Importantly, we found that ATP-induced sympathoexcitation within the PVN involved interactions between P2 receptors and non-NMDA glutamate receptors in the PVN (Ferreira–Neto et al. 2013), suggesting a significant role of the purino-glutamatergic neurotransmission within the PVN in the central regulation of sympathetic outflow. However, the precise cellular mechanisms underlying the ATP-glutamate interaction in the PVN, and whether this interaction contributed to osmotically-driven PVN sympathetic neuronal activity is still unknown.

Here, we tested the hypothesis that an osmotic challenge engages an ATP-glutamate crosstalk that contributes to increased firing activity in RVLM-projecting PVN neurons (PVN-RVLM). We found that exogenously applied ATP increased firing activity of PVN sympathetic neurons, an effect that was dependent on P2 and glutamatergic receptors. Furthermore, we report that bath application of ATP in PVN slices potentiated AMPA-receptor evoked current, an effect
that was blocked by PPADS and intracellular Ca$^{2+}$ chelation. Additionally, we demonstrate that
an acute hyperosmotic stimulus potentiated glutamatergic AMPA receptor-mediated currents in a
P2 receptor-dependent manner.

MATERIALS AND METHODS

Animals

All procedures were performed in agreement with guidelines of the Georgia Regents
University Institutional Animal Care and Use Committee and were approved by the committee.
Male Wistar rats (180–220 g) were used in this study for electrophysiological experiments and
purchased from Harlan Laboratories (Indianapolis, IN). Rats were housed in rooms with constant
temperature of 22–24 °C, relative humidity of 50–60% and under a controlled light/dark cycle
(12/12 hours) with normal rat chow and drinking water ad libitum.

Retrograde tracing

Hypothalamic, sympathetic rostral ventrolateral medulla (RVLM)-projecting
paraventricular nucleus (PVN) neurons (PVN-RVLM) were identified by injecting rhodamine
beads unilaterally into the brainstem region containing the RVLM as previously described
(Sonner et al. 2011). Rats were anesthetized (ketamine–xylazine mixture, 90 and 50 mg/kg,
respectively, i.p.) and a stereotaxic apparatus was used to pressure inject 500 nL of rhodamine-
labeled microspheres (Lumaflor) into the RVLM (starting from bregma, 12 mm caudal along the
lamina, 2 mm medial lateral, and 8 mm ventral). In general, RVLM injection sites were
contained within the caudal pole of the facial nucleus to ∼1 mm more caudal, and were ventrally
located with respect to the nucleus ambiguus. The location of the tracer was verified histologically (see Fig.1). Injections located either more rostral or lateral to the targeted area did not result in PVN labeling, and these animals were discarded from the study. Animals were used for electrophysiology experiments 3–5 days after surgery.

Hypothalamic slice preparation

Hypothalamic brain slices were prepared according to methods previously described (Potapenko et al. 2011; Stern 2001). Briefly, rats were deeply anesthetized with pentobarbital (80 mg/kg, i.p.). Then, rats were quickly decapitated by using a guillotine apparatus, brains dissected out and coronal slices cut (240 μm thick) using a vibroslicer (D.S.K. Microslicer, Ted Pella, Redding, CA). An oxygenated ice-cold artificial cerebrospinal fluid (aCSF) was used during slicing (containing in mM: 119 NaCl, 2.5 KCl, 1 MgSO\(_4\), 26 NaHCO\(_3\), 1.25 NaH\(_2\)PO\(_4\), 20 d-glucose, 0.4 ascorbic acid, 2 CaCl\(_2\), and 2 pyruvic acid; pH 7.4; 295 mOsm). Slices were placed in a holding chamber containing aCSF and kept at room temperature until used.

Patch-clamp electrophysiology

Slices were bathed with solutions (~2.0 mL/min) that were continuously bubbled with 95% O\(_2\)–5% CO\(_2\) and maintained at ~32 °C. Thin-walled (1.5 mm od, 1.17 mm id) borosilicate glass (G150TF-3, Warner Instruments, Sarasota, FL) was used to pull patch pipettes (3–7 MΩ) on a horizontal Flaming/Brown micropipette puller (P-97, Sutter Instruments, Novato, CA). The internal solution contained in mM: 140 potassium gluconate, 5 EGTA, 10 HEPES, 10 KCl, 0.9 MgCl\(_2\), 0.5 CaCl\(_2\), 4 MgATP, 0.3 NaGTP, and 20 phosphocreatine (Na\(^+\)); pH was adjusted to 7.2–7.3 with KOH. The osmolality of the intracellular solutions was 285 mOsm. Recordings
were obtained with an Axopatch 700A amplifier (Axon Instruments, Foster City, CA) from PVN-RVLM neurons using a combination of fluorescence illumination and infrared differential interference contrast (IR-DIC) videomicroscopy. The voltage output was digitized at 16-bit resolution, 10 kHz and was filtered at 2 kHz (Digidata 1440A, Axon Instruments). Data were discarded if the series resistance was not stable throughout the entire recording (>20% change) (Potapenko et al. 2011; Stern 2001). For current-clamp recordings, DC current injection was applied to bring the membrane potential near or at spike threshold (~-50 to -45 mV). The mean firing rate was analysed in segments of 1 min before, during and after ATP application, and the peak effect calculated and express as percent change from baseline levels (Li et al. 2003).

Spontaneous glutamate-mediated excitatory postsynaptic currents (sEPSCs) were recorded and analyzed as previously described (Potapenko et al. 2011). Briefly, sEPSCs were recorded as inward currents in aCSF containing the GABA_A receptor blocker picrotoxin (PIC, 100 µM), while holding the membrane at –70 mV. sEPSCs were detected using Mini Analysis software (Synaptosoft Inc, Leonia NJ), using a detection threshold of 12 pA. PSCs frequency and waveform parameters were analyzed using the same software. sEPSCs were analyzed in periods of one minute before (total of four minutes), during and after delivery of ATP.

Pharmacological activation of AMPA receptor-mediated current (I_{AMPA}) in PVN sympathetic neurons was assessed by measuring the peak and the integrated area of the evoked change in holding current (I_{holding}) following a focal puff of AMPA (50 µM, 50 ms) onto the recorded cell using a picospritzer device (Toohey, 5–10 psi) connected to a patch pipette positioned around ~10 µm from the recorded cell. Cell input resistance and cell capacitance were calculated in voltage clamp using a 5mV pulse while holding the cells at −70 mV. All data were analyzed using MiniAnalysis software. Adenosine 5'-triphosphate disodium salt (ATP), 4-[[4-
Formyl-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-2-pyridinylazo]-1,3-benzenedisulfonic acid tetratosodium salt (PPADS) were purchased from Abcam. Kynurenic acid sodium salt (KYN) was purchased from Ascent Scientific. (RS)-AMPA (AMPA) was purchased from Tocris Bioscience. BAPTA Tetrapotassium Salt, cell impermeant was purchased from Invitrogen. Picrotoxin and Mannitol were purchased from Sigma Aldrich.

Statistical Analysis

All values are expressed as mean± standard error mean (SEM), and passed a test for normality (D’agostino-Pearson test). One- or two-way analysis of variance (ANOVA) tests with Bonferroni post hoc tests were used as indicated. Pearson’s correlation test was used to determine if correlations existed between two parameters. Differences were considered significant at p<0.05 and n refers to the number of cells (for electrophysiology experiments. All statistical analyses were conducted using GraphPad Prism 5.00 (GraphPad Software).

RESULTS

Exogenous ATP evokes action potentials in PVN-RVLM neurons via activation of P2 and ionotropic glutamatergic receptors

To evaluate whether ATP modulates the firing activity of PVN sympathetic neurons, 100 µM of ATP was bath applied (1 min) in the recording chamber. In current-clamp, whole-cell configuration, ATP increased the frequency of action potentials in 70% of PVN sympathetic neurons (89±27% increase, Fig. 2A and D), an effect that was completely blocked by the P2 receptor antagonist PPADS (10 µM, Fig. 2B and D). In most cases (80%), the ATP-mediated change in firing activity did not completely wash out after removal of ATP, at least for the
duration of the recordings. To determine whether the ATP-evoked action potentials in PVN sympathetic neurons were dependent on the activation of ionotropic glutamatergic receptors, KYN (1 mM) was bath applied 5 min before ATP stimulation. The blockade of ionotropic glutamatergic receptors abolished the ATP-induced effect on the firing rate of PVN-RVLM neurons (Fig. 2C and D).

**ATP does not affect glutamate synaptic function in PVN-RVLM neurons**

To assess whether ATP altered glutamate-mediated excitatory postsynaptic currents (EPSCs) we monitored spontaneous synaptic activity in PVN sympathetic neurons. To isolate glutamate receptor-mediated EPSCs from GABAergic PSCs, picrotoxin (100 µM, GABA$_A$ receptor antagonist) was added 10 min before application of ATP. Picrotoxin decreased the frequency, amplitude, area and decay time of spontaneous PSCs in PVN sympathetic neurons (Fig. 3A), indicating an effective blockade of GABAergic currents. The properties of isolated glutamate EPSCs (frequency, amplitude, area and decay kinetics) were not significantly affected in the presence of ATP (100 µM, 1 min) or during washout periods evaluated (Fig. 3B–E) ($p>0.1$ for all parameters reported, one way ANOVA repeated measures). No changes in the holding current were observed as a consequence of ATP application (baseline: -12.5 ± 3.0 pA; ATP: -12.3 ± 2.7 p; washout: -11.3 ± 2.7 pA. $p>0.1$, one way ANOVA repeated measures). All synaptic currents were blocked following addition of KYN (*not shown*).

**ATP potentiates AMPA receptor-evoked currents in PVN-RVLM neurons**

Our previous study demonstrated that ATP induced-sympathoexcitation at the PVN level depends on non-NMDA receptors (Ferreira-Neto et al. 2013). Thus, we aimed to directly evaluate whether ATP affected AMPA receptor-mediated currents in PVN sympathetic neurons.
after focally applying AMPA onto those neurons, an approach that activates both synaptic and extrasynaptic receptors. As shown in Fig. 4A, a focal application of AMPA (50 µM, 50 ms) induced a large inward current ($I_{AMP A}$, amplitude: 304±65 pA, area: 5.5x10^5±0.7x10^5 pA*ms, decay time constant (τ): 2669±399 ms) in neurons. $I_{AMP A}$ was significantly increased in amplitude (Fig. 4B, ~9% increase, 332±73 pA, $p<0.05$, n=13) and area (Fig. 4D, ~22% increase, 6.7x10^5±1.0x10^5 pA*ms, $p<0.01$) without changing decay τ (Fig. 4C, 2968±392 ms, $p>0.05$) during bath application of ATP. Importantly, all $I_{AMP A}$ parameters continued to increase following ATP washout, reaching statistical significance after 5 min (~20, 36 and 15% for amplitude, area and τ, respectively). A plot of the percentage change of the amplitude and area of $I_{AMP A}$ 5 min after ATP application and the basal amplitude and area of $I_{AMP A}$ failed to reveal a significant correlation between these parameters (amplitude $R^2 = 0.085$, area $R^2 = 0.001$), indicating that the degree of ATP modulation was independent on the basal magnitude of AMPA currents. Due to the slowly developing and persistent effects of ATP, we were unable to address the recovery kinetics of the ATP effects during the period of our recordings.

**ATP-mediated potentiation of I$_{AMP A}$ in PVN-RVLM neurons depends on P2 receptor activation and changes in intracellular Ca$^{2+}$**

To determine whether the increased $I_{AMP A}$ induced by ATP stimulation was dependent of P2 receptor activation, the P2 receptor antagonist PPADS (10 µM) was bath-applied 10 minutes before the exogenous application of ATP. Furthermore, to evaluate whether the ATP-induced potentiation of $I_{AMP A}$ was dependent on a rise of [Ca$^{2+}$]$_i$, we performed additional experiments in which neurons were dialyzed with Ca$^{2+}$ chelator BAPTA (10 mM) through the recording patch pipette. Given that studies for the different groups (AMPA with ATP, AMPA with ATP+PPADS...
and AMPA with ATP+BAPTA) were obtained from different cells, data was expressed as percent change from control (first focal application of AMPA before ATP stimulus), to facilitate relative comparisons among groups. As shown in Fig. 5, PPADS completely blocked the ATP-mediated potentiation of $I_{AMPa}$ amplitude 10 min (Fig. 5A, AMPA washout 10 min: 29±6% vs AMPA+PPADS washout 10 min: $-2\pm7\%$, $p<0.001$) and 15 min (Fig. 5A, AMPA washout 15 min: 33±8% vs AMPA+PPADS washout 15 min: 1±7%, $p<0.001$) after the ATP stimulus. Moreover, P2 receptor antagonism reduced $I_{AMPa}$ decay $\tau$ 10 min after ATP bath application (Fig. 5B, AMPA washout 10 min: 19±4% vs AMPA+PPADS washout 10 min: 2±5%, $p<0.05$). In relation to $I_{AMPa}$ area (Fig. 5C), PPADS significantly abolished the increase in $I_{AMPa}$ area induced by ATP 5 min (AMPA washout 5 min: 36±5% vs AMPA+PPADS washout 5 min: 2±8%, $p<0.001$), 10 min (AMPA washout 10 min: 52±7% vs AMPA+PPADS washout 10 min: 2±7%, $p<0.001$) and 15 min after ATP application in PVN sympathetic neurons (AMPA washout 15 min: 51±9% vs AMPA+PPADS washout 15 min: 3±6%, $p<0.001$). Similar to PPADS, intracellular dialysis with BAPTA prevented ATP effects on $I_{AMPa}$ amplitude, decay $\tau$ and area (Fig. 5A–C). Taken together, these data are consistent with the notion that P2 receptor activation by ATP induces increase in [Ca$^{2+}$], which then leads to $I_{AMPa}$ enhancement in PVN sympathetic neurons.

**Hyperosmotic stimulation elicits a P2-AMPA receptor coupling in PVN sympathetic neurons**

Previous studies have shown that acute osmotic stimulation by intravenous hypertonic saline or mannitol solution induces a PVN-dependent sympathoexcitatory response (Antunes et al. 2006; Badoer et al. 2003; Chen and Toney 2001; Son et al. 2013; Stocker and Toney 2005). Thus, based on results outlined above, we addressed whether an osmotic challenge (aCSF...
containing mannitol 1%, 1 min) could engage a purinergic-AMPA receptor crosstalk in PVN sympathetic neurons. As shown in Figure 6A, we found that $I_{AMPA}$ magnitude in PVN neurons was progressively increased following hyperosmotic stimulation. Thus, the amplitude (Fig. 6B, washout 5 min: 39±14%, $p<0.01$) and area (Fig. 6D, washout 5 min: 53±25% and washout 15 min: 62±26%, $p<0.05$) of $I_{AMPA}$ was potentiated following the hyperosmotic stimulation, while $I_{AMPA}$ decay $\tau$ was not altered significantly (Fig. 6C, washout 5 min: 7±5%, $p>0.05$). Again, we have not found any correlation between the percentage change in $I_{AMPA}$ amplitude and area (5 min after mannitol stimulus) and the basal $I_{AMPA}$ amplitude and area of control (amplitude $R^2 = 0.22$, area $R^2 = 0.11$) indicating the degree of ATP modulation was independent on the basal magnitude of AMPA currents.

To elucidate whether the osmotically-evoked increase in $I_{AMPA}$ magnitude was dependent on P2 receptor activation, PPADS was bath-applied before the osmotic challenge. In the presence of PPADS, the hyperosmotically-mediated $I_{AMPA}$ potentiation was completely abolished (amplitude: Fig. 6B, washout 5 min: –8±6%; area: Fig. 6D, washout 5 min: –7±6% and washout 15 min: –2±5%). Taken together, these data suggest that an acute hyperosmotic stimulus can induce a purinergic signaling that enhance $I_{AMPA}$ and, consequently, contributes to increased excitability of PVN sympathetic neurons during an osmotic stimulation.

**DISCUSSION**

Results presented in this manuscript provide evidence for a signaling crosstalk between P2 and glutamatergic AMPA receptors in PVN-RVLM neurons, which influences neuronal excitability as well as neuronal responsiveness to an acute hyperosmotic stimulus. We showed that: (1) exogenous application of ATP increased the firing activity of PVN sympathetic neurons,
an effect dependent on P2 and ionotropic glutamatergic receptor activation; (2) ATP-induced
effect did not involve changes in the magnitude or frequency of spontaneous postsynaptic
glutamatergic currents; (3) ATP potentiated AMPA-evoked currents, an effect that was
prevented by chelation of intracellular Ca\(^{2+}\), or by P2 receptor blockade; and (4) an endogenous
purinergic-glutamatergic crosstalk was engaged in response to a hyperosmotic stimulus, which
resulted in a P2-mediated potentiation of AMPA-evoked currents. Taken together, our results
support a functionally relevant P2-AMPA receptor crosstalk in PVN-RVLM neurons, which
contributes to ATP-mediated excitatory effects on PVN sympathetic neurons. Moreover, our
studies indicate that the P2-AMPA receptor coupling is engaged during an osmotic challenge,
being thus a potential mechanism contributing to increased PVN neuronal activation and the
enhanced sympathoexcitation evoked during this physiological challenge (Antunes et al. 2006;
Stocker and Toney 2005).

**Methodological Considerations**

Regarding the microinjection of the retrograde tracer targeted the RVLM, it is important
to acknowledge that a proportion of PVN neurons that innervate the RVLM (~10-30%) also send
axon collaterals to spinal cord preganglionic neurons located in the intermediolateral cell column
(Badoer 2001; Shafton et al. 1998). Thus, it is likely that the sampled population of neurons in
this study includes both RVLM- and RVLM/spinal cord-projecting neurons.

Another caveat of our studies is that ATP was contained in the patch pipette internal
solution, and thus cells were briefly exposed to ATP as the cell was approached with positive
pressure to obtain a gigaOhm seal. Thus, it is possible that AMPA currents were already
enhanced before the actual ATP test was performed. The fact that we were still able to observe a
significant enhancement during bath-applied ATP indicate that even if that were the case, the
initial exposure to ATP from the pipette did not saturate or evoke a maximal modulation of AMPA currents. The fact that we did not find a significant correlation between the basal $I_{\text{AMPA}}$ magnitude and the magnitude of modulation evoked by bath-applied ATP also argues against a major precluding effect of ATP exposure during the membrane sealing process. We cannot rule out however, that the ATP effects reported herein were underestimated due to this potential technical limitation.

The magnitude and time course of the osmotic stimulation used in our study has been commonly used to study *in vitro* neuronal osmosensitive responses (Prager-Khoutorsky et al. 2014; Richard and Bourque 1995; Trudel and Bourque 2010; Zhang et al. 2007). We acknowledge however, that both the magnitude and time course of our stimulation is not representative of an osmotic stimulus that would occur *in vivo* under physiological conditions.

**P2 receptor activation increases PVN-RVLM neuronal excitability in an AMPA receptor-dependent manner**

In the last five decades ATP has emerged as an important neurotransmitter in several brain nuclei, including the hypothalamus (Burnstock 2007). Previous studies have shown that purinergic signaling in the PVN influences autonomic and neuroendocrine outflows (Cruz et al. 2010; Ferreira–Neto et al. 2013; Kapoor and Sladek 2000; Knott et al. 2008; Li et al. 2010; Mori et al. 1992; Song and Sladek 2006). Our results demonstrate that exogenous ATP increases the firing activity of PVN sympathetic neurons, an effect that was dependent on P2 receptor activation. These results are in line with our previous *in situ* study that showed that microinjection of ATP within the PVN evoked a rapid and robust P2 receptor-mediated sympathoexcitatory response (Ferreira–Neto et al. 2013).
The actions of ATP as a neurotransmitter are mediated by P2 purinoreceptors, which are divided into two main classes: P2Y and P2X (Burnstock 2007). The P2Y receptors are G protein-coupled receptors, while P2X receptors are ligand-gated ion channels, permeable to Na\(^+\), K\(^+\) and Ca\(^{2+}\). Several studies showed that PVN neurons express P2X receptors (Guo et al. 2009; Yao et al. 2003), including six subtypes of purinoceptors (P2X\(_1\)-P2X\(_6\)) in PVN neurons projecting to RVLM (Cham et al. 2006). While the expression of P2Y\(_4\) receptor in the PVN was also described (Song et al. 2011), our studies showing that ATP effects were blocked by 10 µM PPADS (a concentration that does not antagonize P2Y receptors (Ralevic and Burnstock 1998)), support that the ATP effects reported in this study were P2X-dependent. Still, the identity of the specific P2X receptor involved remains to be determined.

Glutamate is the most important excitatory neurotransmitter in the hypothalamus (van den Pol and Trombley 1993) and several studies support an important role for glutamate in regulating sympathetic PVN neuronal activity (Li et al. 2006) as well as PVN sympathoexcitatory outflows (Chen et al. 2003; Li et al. 2001), particularly in response to an osmotic challenge (Antunes et al. 2006). Importantly, ATP has been shown to act as a co-transmitter with glutamate (Braga et al. 2007; Pankratov et al. 1998, 2002; Passamani et al. 2011; Scislo and O’Leary 2000), and we recently showed that ATP-evoked sympathoexcitatory responses within the PVN were blunted in the presence of ionotropic glutamate receptor blockade (Ferreira–Neto et al. 2013). Similar to this previous study, we found here that the ionotropic glutamatergic receptor antagonist kynurenic acid abolished the ATP-evoked increase in firing activity in PVN sympathetic neurons. It is important to note however, that we observed differences in the kinetics of ATP actions between these two studies (slower onset and longer-lasting during in vitro recordings). These discrepancies could be due to at least in part, to a
variety of technical differences between the two preparations, including temperature, kinetic
differences in relation to drug application and washout out from the site of recordings, and the
effect cell dialysis, among others). Nonetheless, we believe that together, these two studies
support an interaction between ATP and glutamate in the regulation of neuronal activity and
sympathetic outflow at the PVN level.

**Purinergic receptor activation potentiates AMPA-evoked current in an intracellular Ca\(^{2+}\)-
dependent manner**

One possible scenario involved in the ATP-glutamate interaction in PVN-RVLM neurons
is an ATP-mediated modulation of glutamate synaptic transmission, acting either at pre- and/or
post-synaptic loci. To test whether this was the case, we measured isolated spontaneous
 glutamate excitatory postsynaptic currents (sEPSCs). Of note, we previously showed that the
frequencies of spontaneous and miniature PSCs were similar in PVN-RVLM neurons (Han et al.
2010), suggesting that in the slice preparation, most basal synaptic activity is action-potential
independent. We found that P2 receptor activation by ATP did not induce significant changes in
either the frequency, magnitude or time course of glutamate receptor-mediated sEPSCs,
suggesting that ATP did not affect the degree of glutamate release (i.e. no change in PSC
frequency) or the magnitude/kinetics of postsynaptic receptors (i.e., no change PSC
amplitude/kinetics) in PVN-RVLM neurons. This is different to previous reports in SON and
PVN magnocellular neurosecretory neurons showing that ATP increased glutamate EPSCs
frequency and amplitude (Gordon et al. 2005; Vavra et al. 2011), suggesting that ATP actions in
the hypothalamus may be cell-type dependent.

Both AMPA and NMDA receptors are major ionotropic glutamate receptors influencing
PVN neuronal activity and sympathoexcitatory responses from the PVN (Chen et al. 2003; Li et
al. 2001; Li et al. 2006). Moreover, NMDA receptors have been shown by other groups to contribute to PVN regulation of sympathetic outflow in response to a hyperosmotic stimulation (Bardgett et al. 2014, Holbein and Toney 2015; Jin et al. 2001). In a recent publication from our group however, we found that ATP-mediated effects on sympathoexcitatory PVN responses involved AMPA (but not NMDA) receptors (Ferreira-Neto et al. 2013). Based on this, and as a logical continuation from our previous studies, we tested in here whether ATP potentiated focally-evoked AMPA currents ($I_{\text{AMPA}}$) in PVN-RVLM neurons. Our results show that $I_{\text{AMPA}}$ magnitude was enhanced by ATP, an effect that was blocked by PPADS, as well as by chelation of intracellular Ca$^{2+}$. While we have not investigated the sources of intracellular Ca$^{2+}$ contributing to ATP effects, it is reasonable to speculate that influx of Ca$^{2+}$ through activated P2X receptors (which are Ca$^{2+}$ permeable) (Bhattacharya et al., 2013; Homma et al., 2008; Ohta et al., 2005; Song et al., 2007), contributed as a significant source to the ATP-mediated rise in intracellular Ca$^{2+}$.

The fact that ATP affected focally evoked AMPA currents (which activate both synaptic and non-synaptic receptors), but not EPSCs (primarily mediated by activation of synaptic AMPA receptors), may indicate that ATP selectively influences non-synaptic AMPA receptors. Expression of AMPA receptors at extrasynaptic sites is commonly observed throughout the brain, and they are increasingly recognized as being functionally important. They contribute, for example, to glutamatergic signaling during glutamate spillover (Allan and Rothwell 2001) and have been shown to play a critical role in activity-dependent long-term potentiation (LTP) (Granger et al. 2013; Makino and Malinow 2009) and during postnatal neurogenesis (Schmidt-Salzmann et al. 2014).
We recently showed that ambient extracellular glutamate levels surrounding SON and PVN neurons are sufficiently high to tonically activate extrasynaptic glutamate receptors. Under basal conditions however, we found the extrasynaptic glutamate current to be mediated mostly by NMDA (but not AMPA) receptors (Fleming et al. 2011; Naskar and Stern 2014; Potapenko et al. 2012, 2013). It is possible however, that activation of P2 receptors increases extrasynaptic AMPA receptor density and/or glutamate sensitivity, contributing under this condition to glutamate-mediated extrasynaptic actions. Arguing against this however, is the fact that we failed to observe a significant change in the holding current during ATP administration in voltage clamp experiments. This could be due however to limited resolution of our approach. In this sense, given the very high input resistance of PVN-RVLM neurons (~ 1 GΩ) (Sonner et al. 20011), a minimal change in a sustained current (i.e, 5 pA, which is within the noise level of our recordings) would be sufficient to result in a functionally significant membrane depolarization to evoke firing activity in these neurons.

Hyperosmotic stimulation reveals an endogenous P2-AMPA receptor crosstalk in PVN-RVLM neurons

Our results discussed thus far support the presence of a functional coupling between P2 and AMPA receptors in the regulation of PVN-RVLM neuronal excitability. However, these studies were based on exogenous application of ATP. In order to determine whether this coupling could also be engaged by endogenous ATP, we challenged the PVN with a hyperosmotic stimulus. A large bulk of evidence in the literature supports that a hyperosmotic challenge results in the activation of both magnocellular and parvocellular PVN neuronal populations (e.g., increased c-Fos expression) (Chu et al. 2005; Sharp et al. 1991). Moreover, a
hyperosmotic challenge was also shown to increase PVN neuronal excitability; evoke a PVN-mediated sympathoexcitatory response and consequently, an elevation in blood pressure (Antunes et al. 2006; Chen and Toney 2001; Chu et al. 2010; Son et al. 2013). However, whether an ATP-mediated potentiation of AMPA receptors in PVN-RVLM neurons occurs in response to an osmotic stimulation, contributing in turn to increased neuronal excitability and sympathetic outflow during this condition has not been explored thus far. Here, we found that a transient hyperosmotic stimulus increased the magnitude of $I_{\text{AMPA}}$, an effect that was blunted in the presence of PPADS. To the best of our knowledge, this is the first study to demonstrate a hyperosmotic-mediated potentiation of glutamate AMPA receptor function. Moreover, our studies suggest that ATP could be locally released within the PVN during an osmotic challenge, contributing in turn to the osmotic-mediated potentiation of AMPA receptors.

Since astrocytes constitute a major source of ATP within the SON and PVN, and this process can be evoked by physiologically relevant signals, including norepinephrine and dendritically-released vasopressin (Gordon et al. 2005; Haam et al. 2014), it will be important to assess in future studies whether astrocytes are key participants in osmotically-driven changes PVN neuronal excitability and sympathoexcitatory regulation.

Collectively, our findings support an ATP-mediated potentiation of AMPA-evoked currents in PVN-RVLM neurons. These effects involved activation of P2 receptors and changes in intracellular $\text{Ca}^{2+}$, and resulted in an ATP-mediated increase in PVN-RVLM firing activity. Furthermore, we demonstrate that the P2-AMPA signaling crosstalk is engaged in response to a local hyperosmotic stimulus, which we propose may contribute to osmotically-driven sympathoexcitatory responses by the PVN.


Han TH, Lee K, Park JB, Ahn D, Park JH, Kim DY, Stern JE, Lee SY, and Ryu PD. Reduction in synaptic GABA release contributes to target-selective elevation of PVN


**Li YF, Jackson KL, Stern JE, Rabeler B, and Patel KP.** Interaction between glutamate and GABA systems in the integration of sympathetic outflow by the paraventricular nucleus


COMPETING INTERESTS

The authors declare no competing financial interests.

FUNDING
This work was supported by a National Heart, Lung, and Blood Institute Grant NIH HL112225 (Stern JE) and by Sao Paulo Research Foundation, FAPESP, #07/04085-0; #10/17997-0 (Antunes VR), #10/05037-1, #12/12444-8 (Ferreira-Neto HC).
FIGURE LEGENDS

Figure 1. Representative example of a rhodamine beads microinjection onto the RVLM. Photomicrographs showing two superimposed images (fluorescence + dark field) through the brainstem at three different rostrocaudal levels (left Bregma: -11.30 mm; middle: Bregma: -12.30 mm; right: Bregma: -13.68 mm) showing the location of the microinjected beads (middle, arrow). 7: facial nucleus; AP: area postrema, CC: central canal; NA: nucleus ambiguus.

Figure 2. ATP increases the firing rate of PVN sympathetic neurons. A, Current-clamp whole-cell recording of membrane potential from PVN-RVLM neuron in slices before, during, and after the application (bar) of 100 µM ATP. ATP induced an increase in frequency of action potentials. The excitatory effect of ATP in PVN sympathetic neurons was blocked by the P2 receptor antagonist, PPADS 10 µM (B), and by the ionotropic glutamatergic receptors antagonist, kynurenic acid 1 mM (KYN, C). D, Summary data showing the percentage changes in action potentials frequency induced by ATP application (black bar, n= 11) and in the presence of PPADS (dark gray bar, n= 13) and KYN (light gray bar, n= 8). **p<0.01 vs Control, One-way repeated measures ANOVA with Bonferroni’s post hoc test.

Figure 3. Exogenous ATP did not significantly alter spontaneous glutamatergic postsynaptic currents (EPSCs). A, Representative trace of spontaneous postsynaptic currents in voltage-clamp whole-cell recording (holding potential: -70 mV) of a PVN sympathetic neuron in the presence of picrotoxin (100 µM) and before (white bars in bars
graphs), during ATP bath application (black bars in bars graphs) and during washout periods (gray bars in bars graphs). ATP did not change frequency (B), amplitude (C), decay time (D) and area (E) of spontaneous postsynaptic glutamatergic currents (n=7). p>0.1, One-way repeated measures ANOVA.

Figure 4. ATP potentiated $I_{AMPA}$ in sympathetic PVN-RVLM projecting neurons. A, Representative example of a voltage-clamp whole-cell recording (holding potential: -70 mV) of an inward current evoked by three puffs of AMPA (50 µM, 50 ms, 5 psi) with an interval of 40s between each before and during ATP application. Summary data showing (B) mean $I_{AMPA}$ amplitude, (C) mean $I_{AMPA}$ decay tau (τ) and (D) mean $I_{AMPA}$ area before and during ATP application (n=10). *p<0.05, **p<0.01 and ***p<0.001 vs basal, One-way repeated measures ANOVA with Bonferroni’s post hoc test.

Figure 5. ATP-induced potentiation of $I_{AMPA}$ in PVN-RVLM neurons are P2- and Ca$^{2+}$-dependent. Summary data showing (A) mean change of $I_{AMPA}$ amplitude, (B) decay tau (τ) and (C) area before, during and after ATP application in PVN RVLM neurons pre-treated the with PPADS (gray bars, n=12) and in cells dialyzed with BAPTA (black bars, n=10). *p<0.05 and ***p<0.001 comparing control vs PPADS in the same period evaluated. #p<0.05 and ###p<0.001 comparing control vs BAPTA in the same period evaluated, One-way repeated measures ANOVA with Bonferroni’s post hoc test.
Figure 6. Hyperosmotic stimulation potentiates AMPA currents in PVN-RVLM neurons via activation of ATP P2 receptors. A, Representative example of a voltage-clamp recording (holding potential: -70 mV) of inward currents evoked by puffs of AMPA (50 µM, 50 ms, 5 psi) before, during and after the hyperosmotic stimulus (Mannitol 1%, +55 mOsm). Summary data showing (B) mean percentage change of $I_{\text{AMPA}}$ amplitude, (C) decay tau ($\tau$) and (D) area before, during and after the hyperosmotic stimulus in control conditions (white bars, n=8) and in the presence of PPADS (black bars, n=6). *p<0.05 and **p<0.01 vs Control, Two-way repeated measures ANOVA with Bonferroni’s post hoc test.