ATP stimulates rat hypothalamic sympathetic neurons by enhancing AMPA receptor-mediated currents

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Ferreira-Neto HC, Antunes VR, Stern JE. ATP stimulates rat hypothalamic sympathetic neurons by enhancing AMPA receptor-mediated currents. J Neurophysiol 114: 159–169, 2015. First published April 22, 2015; doi:10.1152/jn.01011.2014.—We have previously shown that ATP within the paraventricular nucleus (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-rostral ventrolateral medulla 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 4-[[4-Formyl-5-hydroxy-6-methyl-3-[(phosphonoxy)methyl]-2-pyridinyl]azo]-1,3-benzenedisulfonic acid tetrasodium salt (PPADS) (10 μM). Whereas ATP did not affect glutamate synaptic function, α-amino-3-hydroxy-5-methylisoxazole propionic acid (AMPA) receptor-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.

ATP; α-amino-3-hydroxy-5-methylisoxazole propionic acid; paraventricular nucleus; rostral ventrolateral medulla; hyperosmolarity

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 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, whereas parvocellular neurons project to premotor sympathoexcitatory neurons located either in the rostral ventrolateral medulla (RVLM) and/or intermediolateral cell column 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, whereas P2X receptors are ligand-gated ion channels, permeable to Na+, K+, and Ca2+. 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. 2006), 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-N-methyl-d-aspartate (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 are still unknown.

Here, we tested the hypothesis that an osmotic challenge engages an ATP-glutamate crosstalk that contributes to in-
creased 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 that was dependent on P2 and glutamatergic receptors. Furthermore, we report that bath application of ATP in PVN slices that was dependent on P2 and glutamatergic receptors. Furthermore, we report that bath application of ATP in PVN slices that was dependent on P2 and glutamatergic receptors. Furthermore, we report that bath application of ATP in PVN slices that was dependent on P2 and glutamatergic receptors. Furthermore, we report that bath application of ATP in PVN slices that was dependent on P2 and glutamatergic receptors. Furthermore, we report that bath application of ATP in PVN slices that was dependent on P2 and glutamatergic receptors. Furthermore, we report that bath application of ATP in PVN slices that was dependent on P2 and glutamatergic receptors. Furthermore, we report that bath application of ATP in PVN slices that was dependent on P2 and glutamatergic receptors. Furthermore, we report that bath application of ATP in PVN slices that was dependent on P2 and glutamatergic receptors. Furthermore, we report that bath application of ATP in PVN slices that was dependent on P2 and glutamatergic receptors. Furthermore, we report that bath application of ATP in PVN slices that was dependent on P2 and glutamatergic receptors. 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 h:12 h) with normal rat chow and drinking water ad libitum.

**Retrograde tracing.** Hypothalamic, sympathetic RVLM-projecting 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 ip, respectively), 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). Briefly, sEPSCs were recorded as inward currents in aCSF containing the GABAA receptor blocker picrotoxin (100 μM) while the membrane was held at −70 mV. sEPSCs were detected using Mini Analysis software (Synaptosoft, Leonia, NJ) using a detection threshold of 12 pA. PSC frequency and waveform parameters were analyzed using the same software. sEPSCs were analyzed in periods of 1 min before (total of 4 min), during, and after ATP application, and the peak effect was calculated and expressed as a percentage 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<sub>A</sub> receptor blocker picrotoxin (100 μM) while the membrane was held at −70 mV. sEPSCs were detected using Mini Analysis software (Synaptosoft, Leonia, NJ) using a detection threshold of 12 pA. PSC frequency and waveform parameters were analyzed using the same software. sEPSCs were analyzed in periods of 1 min before (total of 4 min), during, and after delivery of ATP.

Pharmacological activation of AMPA receptor-mediated current (I<sub>AMPA</sub>) in PVN sympathetic neurons was assessed by measuring the peak and the integrated area of the evoked change in holding current (I<sub>holding</sub>) following a focal puff of AMPA (50 μM, 50 ms) onto the recorded cell using a picospritzer device (Tooehy, 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 5-mV pulse while the cells were held.
at ~70 mV. All data were analyzed using MiniAnalysis software.
ATP and PPADS were purchased from Abcam (Cambridge, MA).
Kynurenic acid sodium salt (KYN) was purchased from Ascent
Scientific (Princeton, NJ). (RS)-AMPA was purchased from Tocris
Bioscience (Ellisville, MO). Cell-impermeable BAPTA tetrato-
sium salt was purchased from Invitrogen (Carlsbad, CA).
Picrotoxin and mannitol were purchased from Sigma-Aldrich (St.
Louis, MO).

Statistical analysis. All values are expressed as means ± SE and
passed a test for normality (D’agostino-Pearson test). One- or
two-way ANOVA tests with Bonferroni post hoc tests were used
as indicated. Pearson’s correlation test was used to determine
whether 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,
San Diego, CA).

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. 2, A and D), an effect that was completely
blocked by the P2 receptor antagonist PPADS (10 µM, Fig. 2,
B 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 glutama-
teric 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. 2, C and D).

ATP does not affect glutamate synaptic function in PVN-
RVLM neurons. To assess whether ATP altered glutamate-
mediated EPSCs, we monitored spontaneous synaptic activity
in PVN sympathetic neurons. To isolate glutamate receptor-
mediated EPSCs from GABAergic PSCs, picrotoxin (100 µM,
GABA<sub>A</sub> 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 GABAe-
gic 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. 3, B–E) (P > 0.1 for
all parameters reported, 1-way ANOVA, repeated measures).
No changes in the holding current were observed as a conse-
quence of ATP application (baseline: −12.5 ± 3.0 pA; ATP:
−12.3 ± 2.7 pA; washout: −11.3 ± 2.7 pA; P > 0.1, 1-way
ANOVA, repeated measures). All synaptic currents were
blocked following addition of KYN (data 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 re-
ceptor-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<sub>AMPA</sub>, amplitude: 304 ± 65
pA; area: 5.5 × 10<sup>5</sup> ± 0.7 × 10<sup>5</sup> pA/ms, decay time constant (τ):
2669 ± 399 ms) in neurons. I<sub>AMPA</sub> was significantly increased
in amplitude (Fig. 4B, ~9% increase, 332 ± 73 pA,
P < 0.05, n = 13) and area (Fig. 4D, ~22% increase, 6.7 ×
10<sup>5</sup> ± 1.0 × 10<sup>5</sup> 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<sub>AMPA</sub> parameters continued to increase
following ATP washout, reaching statistical significance after

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Fig. 2. ATP increases the firing rate of paraventricular nucleus (PVN) sympa-
thetic neurons. A: current-clamp whole-cell recording of membrane potential
from PVN-RVLM neuron in slices before, during, and after the application
of ATP 100 µM. ATP induced an increase in frequency of action
potentials (APs). The excitory effect of ATP in PVN sympathetic neurons
was blocked by the P2 receptor antagonist, 4-[[4-Formyl-5-hydroxy-6-methyl-
3-[(phosphonoxy)methyl]-2-pyridinyl][azo]-1,3-benzenedisulfonic acid tetra-
sodium salt (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 AP frequency induced by ATP application (solid bar,
n = 11) and in the presence of PPADS (dark shaded bar, n = 13) and KYN
(light shaded bar, n = 8). **P < 0.01 vs. control, 1-way repeated-measures
ANOVA with Bonferroni’s post hoc test.
5 min (~20, 36, and 15% for amplitude, area, and τ, respectively). A plot of the percentage change of the amplitude and area of $I_{\text{AMPA}}$ 5 min after ATP application and the basal amplitude and area of $I_{\text{AMPA}}$ 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 of the basal magnitude of AMPA currents. Because of 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.

Fig. 3. Exogenous ATP did not significantly alter spontaneous glutamatergic excitatory postsynaptic currents (sEPSCs). A: representative trace of sEPSCs in voltage-clamp whole-cell recording (holding potential: −70 mV) of a PVN sympathetic neuron in the presence of picrotoxin (100 µM) and before (open bars in bars graphs), during ATP bath application (solid bars in bars graphs), and during washout periods (shaded bars in bars graphs). ATP did not change frequency (B), amplitude (C), decay time (D), and area (E) of sEPSCs ($n = 7$). $P > 0.1$, 1-way repeated-measures ANOVA.
evaluate whether the ATP-induced potentiation of $I_{\text{AMPA}}$ was dependent on a rise of $[\text{Ca}^{2+}]_i$, we performed additional experiments in which neurons were dialyzed with $\text{Ca}^{2+}$-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 were expressed as percentage 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 $I_{\text{AMPA}}$ amplitude 10 min (Fig. 5A, AMPA washout 10 min: 29 ± 6% vs. AMPA + PPADS washout 10 min: −2 ± 7%, $P < 0.001$) 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 $I_{\text{AMPA}}$ 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 $I_{\text{AMPA}}$ area (Fig. 5C), PPADS significantly abolished the increase in $I_{\text{AMPA}}$ 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 $I_{\text{AMPA}}$ amplitude, decay $\tau$, and area (Fig. 5, A–C). Taken together, these data are consistent with the notion that P2 receptor activation by ATP induces increase in $[\text{Ca}^{2+}]_i$, which then leads to $I_{\text{AMPA}}$ 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, on the basis of results outlined above, we addressed whether an osmotic challenge (aCSF containing mannitol 1%, 1 min) could engage a purinergic-AMPA receptor cross talk in PVN sympathetic neurons. As shown in Fig. 6A, we found that $I_{\text{AMPA}}$ 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 $I_{\text{AMPA}}$ were potentiated following the hyperosmotic stimulation, whereas $I_{\text{AMPA}}$ 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 $I_{\text{AMPA}}$ amplitude and area (5 min after mannitol stimulus) and the basal $I_{\text{AMPA}}$ amplitude and area of control (amplitude $R^2 = 0.22$, area $R^2 = 0.11$), indicating that the degree of ATP modulation was independent of the basal magnitude of AMPA currents. To elucidate whether the osmotically evoked increase in $I_{\text{AMPA}}$ magnitude was dependent on P2 receptor activation, PPADS was bath applied before the osmotic challenge. In the presence of PPADS, the hyperosmotically mediated $I_{\text{AMPA}}$ potentiation was completely abolished (amplitude: Fig. 6B, washout 5 min: $-8 \pm 6\%$; area: Fig. 6D, washout 5 min: $-7 \pm 6\%$; and washout 15 min: $-2 \pm 5\%$). Taken together, these data suggest that an acute hyperosmotic stimulus can induce a purinergic signaling that enhances $I_{\text{AMPA}}$ and, consequently, contributes to increased excitability of PVN sympathetic neurons during an osmotic stimulation.

**DISCUSSION**

Results presented in this study 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 cross talk 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 cross talk 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 targeting the RVLM, it is important to acknowledge that a proportion of PVN neurons that innervate the RVLM ($\sim 10-30\%$) 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
**A**

AMPA 50 µM
50 ms

Mannitol 1% (+55 mosm) washout 5 min

Control

PPADS

**B**

Δ I_{AMPA} amplitude (%)

Control
Mannitol
washout 5 min
washout 10 min
washout 15 min

Control n=8
PPADS n=6

**C**

Δ I_{AMPA} τ (%)

Control
Mannitol
washout 5 min
washout 10 min
washout 15 min

**D**

Δ I_{AMPA} area (%)

Control
Mannitol
washout 5 min
washout 10 min
washout 15 min

* * *
exposed to ATP as the cell was approached with positive pressure to obtain a GΩ 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 indicates 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 because of 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 are 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 two purinoceptors, which are divided into two main classes: P2Y and P2X (Burnstock 2007). The P2Y receptors are G protein-coupled receptors, whereas 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<sub>1</sub>-P2X<sub>6</sub>) in PVN neurons projecting to RVLM (Cham et al. 2006). Although the expression of P2X<sub>1</sub> receptor in the PVN have also been 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 cotransmitter 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 KYNA 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 attributable to, at least in part, a variety of technical differences between the two preparations, including temperature, kinetic differences in relation to drug application, washout from the site of recordings, and the effect of 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 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 in kinetic properties) in PVN-RVLM neurons. This is different from previous reports in supraoptic nucleus (SON) and PVN magnocellular neurosecretory neurons showing that ATP increased glutamate EPSC 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, 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).

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**Fig. 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 ps) before, during, and after the hyperosmotic stimulus (mannitol 1%, +55 mosM). Summary data showing mean percentage change of $I_{\text{AMPA}}$ amplitude (B), decay (C), and area (D) before, during, and after the hyperosmotic stimulus in control conditions (open bars, $n = 8$) and in the presence of PPADS (solid bars, $n = 6$). *$P < 0.05$ and **$P < 0.01$ vs. control, 2-way repeated-measures ANOVA with Bonferroni’s post hoc test.

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al. 2013). On the basis of this, and as a logical continuation from our previous studies, we tested here whether ATP potentiated focally evoked AMPA currents (I_{AMPA}) in PVN-RVLM neurons. Our results show that I_{AMPA} magnitude was enhanced by ATP, an effect that was blocked by PPADS, as well as by chelation of intracellular Ca^{2+}. Although 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 nonsynaptic receptors), but not EPSCs (primarily mediated by activation of synaptic AMPA receptors), may indicate that ATP selectively influences nonsynaptic AMPA receptors. Expression of AMPA receptors at extrasynaptic sites is commonly observed throughout the brain, and they are increasingly recognized as 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 (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 attributable, however, to limited resolution of our approach. In this sense, given the very high input resistance of PVN-RVLM neurons (≈1 GΩ) (Sonnor et al. 2011), 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 cross talk 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. 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 the fact 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 and 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_{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.

Because 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 Ca^{2+} and resulted in an ATP-mediated increase in PVN-RVLM firing activity. Furthermore, we demonstrate that the P2-AMPA signaling cross talk is engaged in response to a local hyperosmotic stimulus, which we propose may contribute to osmotically driven sympathoexcitatory responses by the PVN.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

Author contributions: H.C.F.-N. and J.E.S. conception and design of research; H.C.F.-N. and J.E.S. interpretation of results; H.C.F.-N. performed experiments; H.C.F.-N. and J.E.S. analyzed data; H.C.F.-N. and J.E.S. wrote paper.

**REFERENCES**


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