Cardiovascular responses to microinjection of ATP into the
nucleus tractus solitarii of awake rats

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Running Title: ATP into the NTS of awake rats.

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ABSTRACT

Microinjection of increasing doses of ATP (0.31, 0.62, 1.25 and 2.5 nmol/50 nl) into the nucleus tractus solitarii (NTS) produced a dose-dependent pressor response. Prazosin abolished the pressor response and produced no change in the bradycardic response to ATP. Microinjection of PPADS (0.25 nmol/50 nl), a non-selective P2 receptor antagonist into the NTS, reduced the bradycardic response but had no effect on the pressor response to microinjection of ATP (1.25 nmol/50 nl) into the NTS. Microinjection of suramin (2 nmol/50 nl), another non-selective P2 receptor antagonist, had no effect on the pressor and bradycardic responses to microinjection of ATP (1.25 nmol/50 nl) into the NTS. Antagonism of A1 receptors of adenosine with DPCPX also produced no changes in the cardiovascular responses to microinjection of ATP into the NTS. The involvement of excitatory amino acid (EAA) receptors in the pressor and bradycardic responses to microinjection of ATP into the NTS was also evaluated. Microinjection of kynurenic acid, a non-selective EAA receptor antagonist (10 nmol/50 nl), into the NTS reduced the bradycardic response and had no effect on the pressor response to microinjection of ATP into the NTS. The data show that a) microinjection of ATP into the NTS of awake rats produced pressor and bradycardic responses by independent mechanisms, b) the activation of parasympathetic component may involve an interaction of P2 and EAA receptors in the NTS and c) the sympathoexcitatory response to microinjection of ATP into the NTS was not affected by the blockade of P2, A1 or EAA receptors.

Keywords: adenosine 5'-triphosphate (ATP), P2 purinergic receptors, kynurenic acid, suramin, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), 8-cyclo-pentin-1.3-dipropylxanthine (DPCPX).
INTRODUCTION

The concept that ATP may act as a non-adrenergic non-cholinergic neurotransmitter on peripheral nerves was established by Burnstock (4) and several recent studies indicate that ATP and adenosine can act as neurotransmitters or neuromodulators in the central nervous system (CNS), including areas involved in cardiovascular regulation such as the nucleus tractus solitarii (NTS) (1, 2, 3, 7, 8, 13, 19, 20, 24, 26, 27, 28, 33). Immunohistochemical studies documented the presence of different subtypes of P2 receptors in the NTS of rats (12, 15, 34, 35). ATP interacts with two distinct families of receptors: P2X and P2Y, which differ in structure and signal transduction mechanism (9, 21). There are evidences indicating that the available P2 receptors antagonists such as PPADS and suramin produced no blockade of P2X4 and P2X6 receptor subtypes (25).

Studies performed in anesthetized rats documented that microinjection of ATP into the NTS produced a fall in arterial pressure and bradycardia, which were altered in different manner by suramin (8). These cardiovascular responses to ATP in anesthetized rats exhibited a typical pattern of fast-transmitter similar to the responses to microinjection of L-glutamate into the NTS of anesthetized rats, i.e., a fall in arterial pressure and bradycardia (14, 32). Studies from our laboratory have shown that microinjection of L-glutamate into the NTS of conscious rats produced a dose-related pressor and bradycardic response, contrasting to the dose-related depressor response observed when the same rats were studied under urethane or chloralose anesthesia (16), indicating that anesthetics may affect the autonomic responses to microinjection of a putative neurotransmitter into the NTS. In order to avoid this potential problem, the present study was performed in awake rats and the following aspects related to the purinergic transmission in the NTS were...
evaluated: 1) the effect of microinjection of increasing doses of ATP into the NTS on mean arterial pressure (MAP) and heart rate (HR), 2) the autonomic components involved in the cardiovascular responses to microinjection of ATP, and 3) the effect of previous microinjection of P₂ receptors antagonists available (suramin or PPADS), A₁ receptors antagonist (DPCPX) or excitatory amino acid (EAA) receptors antagonist (kynurenic acid) into the NTS on the cardiovascular responses to microinjection of ATP into the NTS.

METHODS

All experimental approaches were performed in accordance with the Guide for the Care and Use of Laboratory Animals and the Ethical Principles for Animal Experimentation established by the Brazilian Committee for Animal Experimentation (COBEA) and approved by the Animal Care and Ethics Committee of the School of Medicine of Ribeirão Preto, University of São Paulo.

Four days before the experiments, male Wistar rats weighing 290-310 g were anesthetized with tribromoethanol (250 mg/kg, i.p., Aldrich Chemical, Milwaukee, WI, USA) and placed in a stereotaxic apparatus (David Kopf, Tujunga, CA, USA). The technique described by Michelini and Bonagamba (17) was adapted to implant bilateral guide cannulae in the direction of the lateral aspect of the commissural NTS (0.5 mm lateral to the midline and ~0.5 mm rostral to the calamus scriptorium). The guide cannulae were implanted according to the coordinates of the atlas of Paxinos and Watson (23). Additional anesthesia was provided when the rat reacted to frequent toe pinching during stereotaxic surgery. Bilateral guide cannulae [a 15-mm-long stainless steel guide cannula (22-gauge)] were introduced perpendicularly through a small window in the skull at the following
coordinates: 14.5 mm caudal to the bregma, 0.5 mm lateral to the midline, and 7.8 mm below the skull surface at the bregma. The tip of each guide cannula was positioned in the cerebellum ~1.0 mm above the dorsal surface of the brainstem.

The guide cannulae were fixed to the skull with methacrylate and watch screws and closed with an occluder until the day of the experiments. The needle (33-gauge) used for microinjection into the NTS was 1.5 mm longer than the guide cannula and was connected by PE-10 polyethylene tubing to a 1-µl syringe (Hamilton, Reno, NV, USA). After removal of the occluder, the needle for microinjection of drugs into the NTS was carefully inserted into the guide cannula. Manual injection was initiated 30 sec later and the volume microinjected in all experimental protocols was 50 nl. Guide cannulae were implanted bilaterally in order to increase the possibility that at least in one side, the cannula was correctly positioned in the direction of the NTS and only the first side responsive to microinjection was used for microinjections in all experimental protocols.

One day before the experiments, under tribromoethanol anesthesia, a catheter [PE-10 connected to PE-50 (Clay Adams, Parsippany, NJ, USA)] was inserted into the abdominal aorta through the femoral artery for measurement of pulsatile arterial pressure (PAP), MAP and HR. The catheter was tunneled subcutaneously and exteriorized through the back of the neck to be connected to the pressure transducer under conscious freely moving conditions on the subsequent day. PAP was measured with a pressure transducer (model CDX III, Cobe Laboratories, Lakewood, CO, USA) connected to a polygraph (Narcotrace 80, Narco Bio-Systems, Austin, TX, USA). MAP was derived from the PAP using a Universal Coupler (model 7189, Narco Bio-Systems) and HR was quantified from the PAP using a Biotachometer Coupler (model 7302, Narco Bio-Systems) and recorded in the same polygraph.
Five experimental protocols related to the microinjection of ATP into the NTS of awake rats were used in the present study: a) the dose-related effect of microinjection of increasing doses of ATP (0.31, 0.62, 1.25 and 2.5 nmol/50 nl) into the NTS on MAP and HR were studied in four distinct groups of rats and each rat received only one dose of ATP; b) the autonomic components involved in the cardiovascular responses to microinjection of ATP into the NTS were determined in two distinct groups of rats, in which prazosin (1mg/kg, i.v.) or methyl-atropine (2 mg/kg, i.v.) were used to block the efferent sympathetic or parasympathetic activity, respectively. In this protocol, ATP (1.25 nmol/50 nl, corresponding to the ED$_{50}$) was microinjected into the NTS before and after the selective autonomic blockade and the changes in MAP and HR were evaluated; c) ATP (1.25 nmol/50 nl) was microinjected into the NTS before and after microinjection of PPADS (0.25 nmol/50 nl) or suramin (2 nmol/50 nl) in different groups of rats; d) ATP was microinjected into the NTS before and after microinjection of kynurenic acid (10 nmol/50 nl) into the NTS; e) ATP was microinjected into the NTS before and after microinjection of an effective dose of DPCPX, a selective A$_1$ adenosine receptors antagonist (0.285 nmol/50 nl), in accordance with previous study from our laboratory (7). The drugs used in these experiments (Sigma Chemical, St. Louis, MO, USA) were adenosine-5’-triphosphate (ATP), prazosin, methyl-atropine, kynurenic acid, suramin, pyrinoxalphospathe-6-azophenil-2’,4’-disulphonic acid tetrasodium salt (PPADS) and 8-cyclo-pentil-1,3-dipropylxanthine (DPCPX). All drugs were freshly dissolved in saline (154 mM NaCl) and sodium bicarbonate was added to adjust the pH to 7.4.

At the end of each experiment, 50 nl of 2% Evans’ blue were microinjected into the same sites of the NTS for histological analysis. The animals were killed with an overdose of thiopental sodium (100 mg/kg, i.v.) and submitted to intracardiac perfusion with saline.
(154 mM) followed by 10% buffered formalin. The brains were removed and stored in buffered formalin for 2 days, and serial coronal sections (15 µm thickness) were cut and stained by the Nissl method. Only the rats with the center of the microinjection site located in the lateral aspect of the commissural NTS were considered for data analysis. All data are expressed as means ± SEM. The results were analyzed by one-way ANOVA, and the differences between individual means were determined by Student's t-test, with the level of significance set at p<0.05.

RESULTS

Dose-response curve to microinjection of ATP into the NTS

Figure 1 presents typical tracings of four rats representative of their respective groups showing the changes in HR, PAP and MAP in response to microinjection of increasing doses of ATP (0.31, 0.62, 1.25 and 2.5 nmol/50 nL) into the NTS. Unilateral microinjection of ATP into the commissural NTS produced a dose-dependent pressor response. In this range of doses the magnitude of the bradycardic response does not followed a dose-related pattern. Figure 1 also shows that ATP produced a consistent initial hypotensive response, which was secondary to the intense bradycardic response because intravenous injection of methyl-atropine abolished the bradycardia and the initial hypotensive response to microinjection of ATP into the NTS (data not shown). The data of pressor and bradycardic responses are summarized in Figure 2 and the upper panel shows that the pressor response to microinjection of ATP into the NTS is dose-dependent. The dose of 1.25 nmol/50 nl produced an increase in MAP corresponding to ~50% of the
maximal pressor response (ED$_{50}$) and it was used in the subsequent protocols against P$_2$, A$_1$ and EAA receptors antagonists. The bottom panel of Figure 2 summarizes the data related to HR and shows that the bradycardic response to microinjection of ATP into the NTS was at the maximal level with the lowest dose used (0.31 nmol/50 nl), indicating that this dose was already at the plateau of the dose-response curve.

Microinjection of the vehicle (50 nl, saline) into the NTS in a group of rats (n=8) used in this experimental protocol produced a negligible effect on the baseline MAP (-4±4 mmHg) and HR (-10±13 bpm).

_Peripheral autonomic blockade with prazosin or methyl-atropine_

Figure 3A shows that the pressor response produced by microinjection of ATP (1.25 nmol/50 nl) into the NTS was replaced by a hypotensive response 5 min after prazosin (+37±4 vs -25±12 mmHg), while the Figure 3C shows that the bradycardic response was not significantly altered after prazosin (-215±23 vs -291±22 bpm). Figure 3B shows that the pressor response to microinjection of ATP into the NTS 2 min after methyl-atropine was not statistically different in relation to the control pressor response (51±7 vs 68±5 mmHg). Figure 3D shows that microinjection of ATP into the NTS produced bradycardia, which was replaced by a tachycardic response 2 min after methyl-atropine (-238±21 vs +53±10 bpm).
**Blockade of P2 receptors with PPADS or suramin**

Figure 4 presents typical tracings of one rat, representative of the group, showing that 2 min after microinjection of PPADS (0.25 nmol/50 nl) into the commissural NTS the bradycardic response to microinjection of ATP into the same site of the commissural NTS was significantly reduced and that after 10 min it was back to the control level. Previous microinjection of PPADS produced no major change in the pressor response to microinjection of ATP into the commissural NTS. The data of this experimental protocol are summarized in the Figure 5 and the bottom panel shows that the bradycardic response was significantly reduced only on the 2\textsuperscript{nd} min after microinjection of PPADS (-224±10 vs -177±10 bpm) and the upper panel shows no significant change in the pressor response (25±3 vs 32±6 mmHg) to microinjection of ATP into the same site of the commissural NTS.

With respect to the suramin (2 nmol/50 nl) we verified that the pressor (19±7 vs 37±5 mmHg) and bradycardic responses (-161±23 vs -224±14 bpm) to microinjection of ATP into the NTS of a specific group of rats (n=7) were not significantly altered by previous microinjection of this P\textsubscript{2} receptor antagonist into the same site in the NTS.

**Blockade of A1 receptors of adenosine with DPCPX**

In another specific group of rats (n=4) the microinjection of ATP into the NTS was performed before and 2 and 10 min after local microinjection of DPCPX, an adenosine A\textsubscript{1} receptors antagonist. The data shows that pressor (42±8 vs 40±7 mmHg) and bradycardic responses (-225±25 vs -222±38 bpm) to microinjection of ATP on the 2\textsuperscript{nd} min after local
microinjection of DPCPX (0.285 nmol/50 nl) were not statistically different in relation to the control responses. At the 10th min after DPCPX the pressor (42±9 mmHg) and bradycardic responses (-237±12 bpm) to microinjection of ATP were also not different in relation to the control.

Blockade of EAA receptors with kynurenic acid

Figure 6 presents typical tracings of one rat, representative of the group, showing the effect of previous microinjection of kynurenic acid (10 nmol/50 nl) on the cardiovascular responses to ATP (1.25 nmol/50 nl) microinjected into the commissural NTS. Microinjection of kynurenic acid into the commissural NTS almost blocked the bradycardic response and produced no major changes in the pressor response to microinjection of ATP. The effect of kynurenic acid was reversible considering that 30 min later the bradycardic response was back to the control level. The data of this experimental protocol are summarized in Figure 7 and indicate that kynurenic acid microinjected into the commissural NTS produced no significant changes in the pressor responses (upper panel) and a significant reduction in the bradycardic response to microinjection of ATP into the commissural NTS on the 2nd (−64±23 bpm) and 10th min (−83±24 bpm) when compared to the control response (−234±15 bpm).

Histology

Figure 8A is a photomicrograph of a transverse section of the brainstem of one rat, representative of all groups studied, showing the site of unilateral microinjection into the
lateral aspect of the commissural NTS. Figure 8B is a line drawing of a transverse section of the brainstem (~13.7 mm caudal to the bregma), modified from Paxinos and Watson (20), showing the center of microinjections into the lateral aspect of the commissural NTS (dark circles) of 8 representative rats from a group of 54 animals presenting positive histology, which were used in the 5 experimental protocols of the present study.

DISCUSSION

The data of the present study shows that microinjection of ATP into the NTS of awake rats produced important cardiovascular responses. However, it is important to note that the endogenous release of ATP in the synapses of the NTS may not necessarily produce the same pattern of responses to microinjections, especially due to the relatively large volume of 50 nl used in this experimental approach, which may reach different subpopulations of neurons and interneurons related to a diverse neuronal network at the NTS level. In fact, there are several technical limitations for studying specific synaptic transmission in the central nervous system, especially in the NTS of whole animals in anesthetized or awake preparations.

The most important finding of the present study is related to the dose-dependent increase in MAP in the range from 10 to 40 mmHg in response to microinjection of ATP into the NTS of awake rats. In studies performed in anesthetized rats the microinjection of ATP or the noncatabolizing analogue $\alpha,\beta$-methylene ATP into the NTS usually produced a depressor response (8, 27, 28). It is conceivable that anesthetics play a critical role that may explain the difference in terms of the pattern of the cardiovascular response to microinjections of a putative neurotransmitter in the NTS. In previous study from our
laboratory we verified that microinjection of L-glutamate into the NTS of awake rats produced increase in arterial pressure, while the microinjection of the same dose into the NTS of the same rat, under anesthesia, produced depressor response. The observed increase in the MAP in response to microinjection of ATP into the NTS of awake rats strongly suggest that this nucleotide and P2 receptors may play an important role in the processing of the sympathoexcitatory responses at the NTS level.

The increase in the arterial pressure in response to microinjection of ATP into the NTS was really due to sympathoexcitation because the intravenous injection of prazosin, a α1 adrenoceptor antagonist, abolished the pressor response. In fact, microinjection of ATP after prazosin produced an important hypotensive response, which appear to be essentially dependent of the remaining intense bradycardic response. In the case of the blockade of the bradycardic response with methyl-atropine, we verified that microinjection of ATP produced an increase in heart rate (Figure 3, panel D) and a tendency of additional increase in the MAP (Figure 3, panel B). In spite of a predominant cardiac parasympathetic excitatory effect produced by ATP, it is remarkable that this nucleotide microinjected into the NTS also produced an increase in the sympathetic activity to the heart, observed only after the blockade of the parasympathetic component.

In the present study we verified that the pressor response to microinjection of ATP into the NTS was not blocked by PPADS or suramin. These data from the experimental protocols using different P2 receptor antagonists raise doubts about the selectivity and the efficacy of these compounds. Although we had used a relatively large concentration of these antagonists, compared with other studies performed in anesthetized rats (8) or in the working heart-brainstem preparation (22), we were not able to affect the pressor response to microinjection of ATP.
Considering that ATP activates P₂ receptors, we must consider other mechanisms than those explored in the present study in order to understand why the blockade of these receptors were not effective in reducing the pressor response to microinjection of ATP into the NTS. There is evidence indicating that PPADS and suramin, the available P₂ receptors antagonists used in the present study, are not effective in the blockade of P₂X₄ and P₂X₆ receptor subtypes (25). Therefore, further studies using different approaches such as: a) a more selective antagonists of P₂ receptors subtypes; b) antibodies to these specific receptors subtypes, and c) ATP antisense will be a requirement in subsequent studies for our understanding of the effective role of P₂ receptors in the sympathoexcitatory response to microinjection of ATP into the NTS.

Studies performed in anesthetized or in vitro preparations (6, 10, 18, 31) indicated that the responses to application of ATP into neural tissues were mediated by adenosine. In a study by Kato and Shigetomi (10) performed in brainstem slices, it was shown an inhibitory effect of ATP on the NTS neurons, which was not reproduced by α,β-methylene ATP, a non catabolizing ATP analogue, and also not affected by PPADS. In fact, Kato and Shigetomi (10) were able to block this inhibitory effect of ATP using adenosine A₁ receptor antagonists CPT or DPCPX, indicating that this inhibitory effect of ATP was mediated by adenosine A₁ receptors. For this reason, in the present study we also explored the possibility that the adenosine resulting from ATP catabolization may be acting on A₁ receptors and producing the observed pressor response. However, the data shows that A₁ receptors antagonism with DPCPX, in a dose that was effective in blocking the effect of microinjection of adenosine into the NTS (7), produced no effect on the cardiovascular responses to microinjection of ATP, ruling out the possible involvement of A₁ receptors in the pressor response to microinjection of ATP into the NTS of awake rats.
In a study performed in anesthetized rats, Ergene et al. (8) documented that microinjection of ATP into the NTS produced depressor response. In these experiments by Ergene et al. (8) performed in three different groups of rats, suramin produced a diverse pattern of changes in the depressor response to ATP: 1) transformed the depressor response in a transient increase in pressure, 2) produced an augmentation of the depressor response, or 3) completely blocked the depressor response to ATP. Therefore, suramin may affect the P₂ receptors in the NTS in a different manner, probably due to the fact that it may also affect P₂Y receptors and also because some of the P₂X receptors such as P₂X₄ and P₂X₆ are not blocked by suramin (25). Ergene et al. (8) also documented that suramin was effective in blocking the effect of α,β-methylene ATP, a noncatabolizing analogue of ATP. Therefore, it is plausible that the effects of different compounds resulting from the catabolization of ATP are not necessarily blocked by suramin.

In the present study, performed in awake rats, the data indicates that PPADS was effective because it produced a significant reduction in the bradycardic but not in the pressor response to microinjection of ATP. In order to explain the absence of the blockade of the pressor response we may also consider the possibility that the pressor effect of ATP after suramin or PPADS is related to the activation of a different neurotransmitter system, considering that ATP may act as a co-transmitter with different neurotransmitters (5). In this case, the excitatory amino acid L-glutamate is the natural candidate for this co-transmission and considering the possibility that ATP may also release L-glutamate (11), we performed the experimental protocol in which ATP was microinjected into the NTS before and after kynurenic acid, a non-selective antagonist of EAA receptors. We verified that the antagonism of the EAA receptors blocked the bradycardic response but does not
affect the pressor response to microinjection to ATP, suggesting that the sympatoexcitatory response induced by ATP in the NTS does not involve L-glutamate and EAA receptors. These data suggest that the cardiovascular responses to microinjection of ATP into the NTS are mediated by two different mechanisms: 1) a glutamatergic for the bradycardia (parasympathetic component) and 2) an unknown mechanism for the pressor response (sympathetic component). In a recent study by Scislo and O’Leary (29) it was documented that the blockade of ionotropic glutamatergic receptors abolished the fast but not the slow component of the response to activation of P2 receptors by α, β-methylene ATP. These findings indicate a complex interaction of P2 with glutamatergic ionotropic receptors at the NTS level, which requires further investigation.

The bradycardic response to microinjection of ATP into the NTS was not due to the activation of the baroreflex because the α1-adrenoceptor antagonism with prazosin blocked the pressor response to ATP but did not affected the bradycardic response, which was abolished only after muscarinic receptors blockade with methyl-atropine, indicating again that microinjection of ATP into the NTS trigger two independent autonomic pathways, i.e., a parasympathetic pathway related to the bradycardic response and a sympathetic pathway related to the pressor response. The pattern of the bradycardic response to microinjection of ATP into the NTS was not dose-dependent, suggesting that the magnitude of the bradycardic responses was already at the plateau of the dose response curve even with the lowest dose used. This possibility suggests a different sensitivity of the autonomic components activated by ATP in the NTS and also that the parasympathetic is more sensitive to ATP than the sympathetic component. We may also suggest an interaction of ATP and L-glutamate on EAA receptors, considering that PPADS, a P2 receptor antagonist,
or kynurenic acid, a non-selective EAA receptors antagonist, significantly reduced the bradycardic response to microinjection of ATP into the NTS. In this direction, there is evidence that ATP acting on P₂X receptors at presynaptic level in the dorsal root ganglion neurons results in the increase of glutamate release (11) and also that activation of presynaptic P₂X receptors with α,β-methylene ATP in the NTS triggered Ca²⁺-dependent glutamate release (30). In addition, recent studies by Paton et al. (22) using an anaesthetized decerebrate working heart-brainstem preparation, showed that bilateral microinjection of suramin or PPADS into the commissural NTS produced a significant reduction in the bradycardic responses to chemoreflex activation, suggesting that ATP and P₂ receptors are important in the processing of the parasympathetic component of the chemoreflex at the NTS level. Whether or not the bradycardic response to microinjection of ATP into the NTS is due to a presynaptic release of L-glutamate is an important matter for further investigation.

We conclude that microinjection of ATP into the NTS of awake rats produced two independent cardiovascular responses, i.e., pressor and bradycardic responses. The bradycardic response involves P₂ and EAA receptors, while the pressor response was not affected by the blockade of P₂ receptors of ATP or A₁ receptors of adenosine. The interaction of P₂ and EAA mechanisms in the processing of the parasympathetic component at the NTS level is still a matter for further investigation. A new series of functional experiments using more selective antagonists for each subtypes of P₂ receptors are required to elucidate the specific mechanisms involved in the pressor response to microinjection of ATP into the NTS of awake rats.
Perspectives

The pressor and bradycardic response to microinjection of ATP into the NTS open a very interesting possibility about the involvement of this purine in the processing of the cardiovascular reflexes at this level of the central nervous system. The most attractive possibility to be explored in further studies is related to the neurotransmission of the sympatoexcitatory component of the chemoreflex, considering that the pattern of the pressor response to microinjection of ATP into the NTS is similar to that observed in response to chemoreflex activation with potassium cyanide (KCN). However, the data of the present study clearly indicates that for a better pharmacological evaluation of the involvement of ATP and P2 receptors in the processing of the sympatoexcitatory component of the chemoreflex, at the NTS level, it will be necessary the use of selective antagonists for the different P2 receptors subtypes. The use of P2 receptors antibodies and/or ATP antisense will be also useful for a precise evaluation of the purinergic neurotransmission at the NTS level. Another important aspect that also requires further investigation is related to the interaction of glutamatergic and purinergic mechanisms in the neurotransmission of the cardiovascular reflexes at the NTS, especially on the bradycardic components of the baroreflex, chemoreflex, and cardiopulmonary reflex. The evaluation of the possible interaction of respiratory and sympathetic mechanisms related to the complex pattern of responses to microinjection of ATP into the NTS is another important subject to be investigated. The complete evaluation of the role of purinergic neurotransmission in the processing of the sympatoexcitatory component of the chemoreflex at the NTS level, for example, may bring important contribution to the understanding of the mechanisms
underlying the sympathetic overactivity observed in pathophysiological situations such as hypertension, obstructive sleep apnea and heart failure.
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Figure Legends

**Fig. 1.** Tracings of four rats, representative of their respective groups, showing the changes in heart rate (HR), pulsatile arterial pressure (PAP) and mean arterial pressure (MAP) in response to microinjection of increasing doses of ATP (0.31, 0.62, 1.25 and 2.5 nmol/50 nL) into the nucleus tractus solitarii (NTS) of awake rats.

**Fig. 2.** Changes in mean arterial pressure (MAP, upper panel) and heart rate (HR, bottom panel) in response to microinjection of increasing doses of ATP (0.31, 0.62, 1.25 and 2.5 nmol/50 nl) into the nucleus tractus solitarii (NTS) of awake rats. The number of rats in each group is given in parentheses.

**Fig. 3.** **Left panels** - Changes in mean arterial pressure (MAP, panel A) and heart rate (HR, panel C) in response to microinjection of ATP into the NTS before (control, closed bar) and 5 min after intravenous injection of prazosin [1 mg/kg, i.v., hatched bars, (n=7)]. **Right panels** - Changes in MAP (panel B) and HR (panel D) in response to microinjection of ATP into the NTS before (control, closed bar) and 2 min after intravenous injection of methyl-atropine [2 mg/kg, i.v., hatched bars, (n=6)]. *Significantly different from control (p<0.05)."
Fig. 4. Tracing of one rat, representative of the group, showing the changes in heart rate (HR), pulsatile arterial pressure (PAP) and mean arterial pressure (MAP) in response to unilateral microinjection of ATP (1.25 nmol/50 nl) before (control) and 2, 10, 30 and 60 min after microinjection of PPADS (0.25 nmol/50 nl) into the commissural NTS.

Fig. 5. Changes in mean arterial pressure (ΔMAP, upper panel) and heart rate (ΔHR, bottom panel) in response to unilateral microinjection of ATP (1.25 nmol/50 nl) into the NTS before (control) and 2, 10, 30 and 60 min after unilateral microinjection of PPADS (0.25 nmol/50 nL) into the commissural NTS (n=14). *Significantly different from ATP control (p<0.05).

Fig. 6. Tracing of one rat, representative of the group, showing the changes in heart rate (HR), pulsatile arterial pressure (PAP) and mean arterial pressure (MAP) in response to unilateral microinjection of ATP (1.25 nmol/50 nL) before (control) and 2, 10, 30 and 60 min after microinjection of kynurenic acid (10 nmol/50 nL) into the commissural NTS.

Fig. 7. Changes in mean arterial pressure (ΔMAP, upper panel) and heart rate (ΔHR, bottom panel) in response to unilateral microinjection of ATP (1.25 nmol/50 nL) into the NTS before (control) and 2, 10, 30 and 60 min after unilateral microinjection of kynurenic acid (10 nmol/50 nL) into the commissural NTS (n=11). *Significantly different from ATP control (p<0.05).
Fig. 8. Panel A - Photomicrograph of a transverse section of the brainstem showing a unilateral microinjection site in the lateral aspect of the commissural NTS. Arrow shows the center of microinjection at the level of the area postrema (30 x). AP, area postrema; CC, central canal. Panel B – Line drawing of a transverse section of the brainstem (~13.7 mm caudal to the bregma, adapted from Paxinos and Watson) showing the centers of microinjections in the commissural NTS (dark circles: 3 on the right and 5 on the left side) of 8 representative rats of the group of 16 animals used in the protocol of the dose-response curve to microinjection of ATP into the NTS. The site of the center of the microinjection into the NTS of the remaining 46 rats with positive histology used in the 5 different experimental protocols were located in the same sub-region of the NTS. AP, area postrema; 10, dorsal motor nucleus of the vagus; 12, hypoglossal nucleus; CC, central canal; NTS, nucleus tractus solitarii.
FIG. 1

HR (bpm)

PAP (mmHg)

MAP (mmHg)

0.31
0.62
1.25
2.5 nmol/50 nL

1 min.
FIG. 2
FIG. 3
FIG. 4
FIG. 5

$\Delta$ MAP (mmHg)

$\Delta$ HR (bpm)
FIG. 6