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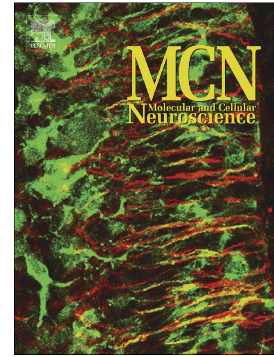

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SALT-LOADING PROMOTES EXTRACELLULAR ATP RELEASE MEDIATED BY GLIAL CELLS IN THE HYPOTHALAMIC PARAVENTRICULAR NUCLEUS OF RATS

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Running title: glial release of ATP in the PVN under osmotic challenge.

Key words: ATP, adenosine, astrocytes, hypothalamus, salt-loading.

HIGHLIGHTS

- Using the model of a salt-loaded laboratory rat we sought to determine the source(s) of ATP released PVN in response to high salt intake
- High salt intake evokes an increase in glial fibrillary acidic protein immunoreactivity in the PVN
- Salt loading stimulates the release of vesicular ATP from glial cells in the PVN with extracellular adenosine remaining unchanged
- Activity of ectonucleotidase responsible for the breakdown of ATP in the PVN was increased in SL group
- The evidence suggests that increased extracellular ATP seen in hyperosmotic conditions results from glial release and not reduced ectonucleotidase activity

ABSTRACT

Previously, we have shown that purinergic signalling is involved in the control of hyperosmotic-induced sympathoexcitation at the level of the PVN, *via* activation of P2X receptors. However, the source(s) of ATP that drives osmotically-induced increases in sympathetic outflow remained undetermined. Here, we tested the two competing hypotheses that either (1) higher extracellular ATP in PVN during salt loading (SL) is a result of a failure of ectonucleotidases to metabolize ATP; and/or (2) SL can stimulate PVN astrocytes to release ATP. Rats were salt loaded with a 2% NaCl solution replacing drinking water up to 4 days, an experimental model known to cause a gradual increase in blood pressure and plasma osmolarity. Immunohistochemical assessment of glial-fibrillary acidic protein (GFAP) revealed increased glial cell reactivity in the PVN of rats after 4 days of high salt exposure. ATP and adenosine release measurements *via* biosensors in hypothalamic slices showed that baseline ATP release was increased 17-fold in the PVN whilst adenosine remained unchanged. Disruption of Ca^{2+}-dependent vesicular release mechanisms in PVN astrocytes by virally-driven expression of a dominant-negative SNARE protein decreased the release of ATP. The activity of ectonucleotidases quantified *in vitro* by production of adenosine from ATP was increased in SL group. Our results showed that SL stimulates the release of ATP in the PVN, at least in part, from glial cells by a vesicle-mediated route and likely contributes to the neural control of circulation during osmotic challenges.

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INTRODUCTION

Excess dietary salt intake can increase plasma osmolarity and [Na^+] that triggers sympathoexcitation and the release of arginine vasopressin (AVP) into the circulation to produce pressor and antidiuretic effects (Antunes-Rodrigues *et al.*, 2004). The process of osmo-sensation occurs centrally within well-defined brain nuclei (Bourque, 2008), however, the brain cell types and transmitters involved in this process and their interaction with circuits controlling the autonomic nervous system are yet to be fully elucidated.

One such brain area is the paraventricular nucleus of the hypothalamus (PVN) which plays a major role in the regulation of AVP release and sympathetic nerve activity during hyperosmotic episodes as, positioned adjacent to the third ventricle, it receives excitatory input from primary osmo- and Na^+ sensors located in the circumventricular organs (CVOs) (Weindl & Joynt, 1973; Thrasher, 1985). Acting as an integrator with two output branches the PVN's magnocellular neurones control the release of AVP or oxytocin (OT) from the posterior pituitary while the parvocellular neurones connect extensively with pre-motor sympathoexcitatory neurones located either in the rostral ventrolateral medulla (RVLM) and/or intermediolateral cell column of the spinal cord (Geerling *et al.*, 2010). While this second group displays particularly diverse neurochemistry in its projections to target regions, within the nucleus has been largely confined to excitatory neurotransmitters such as glutamate, angiotensin and AVP -as seen in this the study of brain signalling mechanisms during osmotic stress by induced hyperosmolarity (Benarroch, 2005).

Recently, the importance of the role of adenosine triphosphate (ATP) has come to light from studies in our laboratory suggesting the possibility of co-transmission between ATP and glutamate acting to increase lumbar sympathetic nerve activity *via* the activation of P2 purinoceptors (P2R) and AMPA receptors (Ferreira-Neto *et al.*, 2013). Further evidence from whole-cell patch clamp recordings shows that ATP increases the activity of RVLM-PVN projecting neurones and potentiates AMPA-evoked currents (Ferreira-Neto *et al.*, 2015). Additionally, P2R-NMDAR coupling has been shown to be engaged in responses to acute hyperosmotic stimulation, contributing to osmotically driven activity in magnocellular neurones of the PVN (Ferreira-Neto *et al.*, 2021). Together, these data strongly suggest a role for ATP signalling in central osmo-sensation. However, all previous studies were conducted on an acute time-scale and to our knowledge the role of ATP signalling within the PVN has not been addressed in a chronic setting, as would be relevant for high salt intake. In the present

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study we challenge this apparent gap in knowledge and seek to localise the source of extracellular ATP in the PVN.

A multitude of previous studies have shown that glial cells, such as astrocytes are critical for central chemoreception and that they communicate *via* the extracellular release of ATP (see Marina et al, 2018). Indeed, mechanical deformation of astrocytic membranes in the brainstem induces release from internal Ca^{2+} stores inducing release of ATP and subsequently activating pre-sympathetic neurones (Marina *et al.*, 2020). Further, glutamate released by neuronal terminals also evoked Ca^{2+} waves in astrocytes, which in turn stimulated glial cells to release glutamate, as a positive feedback loop that potentiates the excitatory effect of this transmitter (Porter & McCarthy, 1996).

Another important element of the purinergic system is the role of ectonucleotidases that encompass several enzymes that metabolize ATP into other nucleotides and nucleosides. Different families of ectonucleotidases have been described: ectonucleoside triphosphate diphosphohydrolase (E-NTPD), ectonucleotide pyrophosphatase/phosphodiesterase (E-NPP), alkaline phosphatase (AP) and ecto-5'-nucleotidase (E-5'-NT) (Langer *et al.*, 2008). Some subtypes of E-NTPD family (subtypes 1, 2 and 3) are widely expressed in the CNS, including the in hypothalamus where they breakdown ATP and adenosine diphosphate (ADP) into adenosine monophosphate (AMP), increasing the extracellular concentration of AMP and inorganic phosphate (Zimmermann *et al.*, 2012). AP and E-5'-NT breakdown AMP into adenosine, a nucleoside that generally exerting an inhibitory effect on neuronal excitability by acting on P1-G_i coupled receptors (A1 and A3 subtypes) (Li *et al.*, 2010).

Collectively, two competing hypotheses have been raised in this study: I) PVN glial cells could directly and/or indirectly be activated by osmotic stimuli causing release of ATP into the extracellular space. Alternatively, II) ATP metabolization may be reduced as a result of a failure of ectonucleotidase activity, which

would increase its availability in the extracellular space. In order to address these questions, we used a rat model of salt loading in which animals received hypertonic saline (2% NaCl) in replacement of their normal drinking water (SL rats) for four days. Coronal hypothalamic slices containing PVN were prepared and amperometric enzyme-encapsulated biosensors were used to directly record ATP release in PVN. Next, we employed genetically-encoded tools to target astrocyte-specific vesicular ATP release by expression of dominant-negative SNARE protein (dnSNARE) under control of the glial fibrillary acidic protein (GFAP) promoter.

METHODS

Ethical approval

All experimental procedures were performed in accordance with the Ethical Principles in Animal Research mandated by the Brazilian College of Animal Experimentation and were approved by the Ethical Committee for Animal Research of the Institute of Biomedical Sciences, University of São Paulo (ICB/USP - protocol # 111-108/2011). The procedures were also in agreement with the European Commission Directive 2010/63/EU (European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes) and the UK Home Office (Scientific Procedures) Act (1986) with project approval from the UCL Institutional Animal Care and Use Committee.

Animals

Male Sprague Dawley rats 9-12 weeks old, weighing 250–350 g (n=36) were placed in individual cages and randomly assigned to one of two cohorts: 1) Euhydrated (EU) group, maintained on standard chow diet and given tap water; and 2) salt loading (SL) group, maintained on standard chow diet and given 2% NaCl solution in replacement of the tap water for 4 days. Rats were kept at a constant temperature of 22–24°C and a relative humidity of 50–60% under a controlled light-dark cycle (12:12 h; lights on 7:00 am) with normal rat chow *ad libitum.*

Drugs and reagents

The chloride anion and connexin channel blocker 5-nitro-2-(3-phenyl-propylamino) benzoic acid (NPPB), adenosine 5′-triphosphate (ATP) disodium salt hydrate, adenosine (ADO), sodium L-lactate and glycerol were all purchased from Merck Ltd. The P2X7 receptor antagonist, AZ 10606120 dihydrochloride, was purchased from Tocris Bioscience.

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In vitro acute slice preparations

After four days of salt load protocol (NaCl, 2%), rats were deeply anesthetised with isoflurane inhalation and immediately decapitated. The brains were removed and placed in ice cold artificial cerebrospinal fluid (aCSF) containing 120 mM NaCl, 3 mM KCl, 1 mM $NaH2PO_4$, 22 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 10 mM glucose at pH 7.4 when saturated with carbogen gas (95% O_2/5% CO_2). Coronal hypothalamic slices containing PVN (thickness 350 µm) were obtained using a vibratome (Campden Instruments 7000 smz; Loughborough, UK) and then transferred in to aCSF satured with carbogen gas and maintained at room temperature. After a recovery period of 15 min the slices were then transferred to custom made flow chamber and continuously perfused with aCSF at 37°C. Extracellular concentrations of purines (ATP and ADO) were recorded using biosensors (Sarissa Biomedical ltd., Coventry, UK)

Biosensor recordings

ATP and adenosine biosensors were used in a dual simultaneous amperometric recording set up (Whistonbrook Technologies ltd. Bedfordshire, UK) as previously described (Huckstepp *et al.*, 2016; Sheikhbahaei *et al.*, 2018). Coronal hypothalamic slices containing the PVN were immediately transferred to the recording chamber and submerged in aCSF with at a perfusion rate of 6 ml/min (32°C). Each biosensor tip was bent and positioned perpendicularly to the surface of the slice on each side of the PVN (Figure 3 A). In some experiments a null probe lacking enzymatic biolayer was used to confirm that no non-specific electro-active interferents were released during the experimental manipulations.

The ATP biosensor is a platinum microelectrode covered with an ultrathin biolayer containing two enzymes, glycerol kinase and glycerol-3-phosphate oxidase. The former enzyme converts extracellular ATP and glycerol into ADP and glycerol-3-phosphate, respectively. These products are subsequently converted to glycerone phosphate and H_2O_2 by the latter enzyme, and H_2O_2 is detected *via* oxidation of the electrode. The adenosine sensor is comprised of three enzymes: adenosine deaminase (that converts adenosine to inosine); nucleoside phosphorylase (converts inosine to hypoxanthine) and xanthine oxidase (converts hypoxanthine to urate and H_2O_2). Similarly, H_2O_2 is detected *via* oxidation at the electrode.

Biosensors were calibrated before placing the brain slices into the chamber with solutions containing a known concentration of ATP or adenosine. For this purpose, ATP (10

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µM) and adenosine (5 µM) were diluted in aCSF. Before calibration biosensors were cycled from −500 mV to +500 mV at a rate of 100 mV/s for 10 cycles to increase the sensitivity of signal. The sensors were polarized to +600 mV relative to an Ag/AgCl potentiostat reference electrode that was placed in the bath.

<u>(Glio-)transmitters release analysis</u>

Tonic release of ATP and ADO were quantified by measuring the peak response after each sensor contacted the PVN surface. The concentration of each substance in µM was derived from the initial calibration. The role of pannexin/connexin hemichannels in mediating tonic release of gliotransmitters was evaluated by quantifying the peak change of ATP and adenosine concentration immediately before and after application of NPPB and zero Ca^{2+} solution. This approach accounted for the natural decline of sensor sensitivity with repeated use.

<u>Molecular approaches to block astroglia signalling</u>

Three days before starting salt load protocol of 4 days, rats were anesthetised with a mixture of ketamine (60 mg kg^{-1}) and medetomidine (250 µg kg^{-1}, i.m.) and placed in a stereotaxic frame. The tooth bar was adjusted so that bregma and lambda were kept in the same dorsal-ventral level. The PVN was targeted unilaterally with an adenoviral vector AAV-sGFAP-dnSNARE-EGFP (titre: 7.7×10^9) to drive the expression of dominant-negative SNARE protein (dn-SNARE) under the control of an enhanced GFAP promoter. dnSNARE protein locks vesicles in the transient fusion stage, preventing the pore from widening to the full-fusion state (Guček *et al.*, 2016). In astrocytes, virally delivered transgenes that express the dnSNARE protein inhibit Ca^{2+}-dependent vesicular release mechanisms reducing astrocyte-derived paracrine signals (Sheikhbahaei *et al.*, 2018). The construct used in the present study was originally developed to target astrocytes in the hippocampus (Papouin *et al.*, 2017) and was made ready for our use by the Kasparov group (Univ. Bristol, UK).

Two microinjections (250 nL each) were delivered into the right side of the PVN using the following coordinates: (1º) anteroposterior -1.7 mm; lateral, +0.3 mm; ventral -7.0 mm relative to the bregma; and (2º) anteroposterior -1.8 mm; lateral, +0.3 mm; ventral -7.0 mm relative to the bregma. All injections were performed slowly, over a period of 5 min each, and once completed, another 5 min period was allowed before pipette removal to ensure full dispersal of the virus throughout the target region. After the microinjections the surgical wound was sutured and anaesthesia was reversed with atipamezole (1 mg kg^{-1}, i.m.). For

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postoperative analgesia, the animals received buprenorphine (0.05 mg^{-1} kg^{-1} per day, s.c.) for 2 days.

After a 3-day recovery period from the surgical procedure, animals received hypertonic saline (2% NaCl) during four days for subsequent analysis of ATP release. In this experiment we used two ATP sensors in parallel (instead of one ATP and another adenosine) that were vertically positioned on each side of PVN. ATP release was compared from the dnSNARE transfected side vs. non-transfected side.

Estimative of ectonucleotidases activity with biosensors

To estimate the enzymatic activity of ectonucleotidases we placed an adenosine biosensor on the surface of the PVN and superfused the slices with ATP solutions at two different concentrations, 10 and 50 µM, diluted in physiological buffer. If the stimulus of ATP superfusion is strong enough to cause a transient response in adenosine concentration in PVN we can use the gain to infer ectonucleotidases activity. Thus, we calculated the peak response of adenosine by taking the maximum value of adenosine concentration after ATP reached the brain slice and subtracted the baseline adenosine concentration taken when the slice was superfused with buffer alone.

Real-time polymerase chain reaction (RT-qPCR)

After four days of high salt exposure (2% NaCl), or normal drinking water only (EU), the rats were rapidly decapitated without anaesthesia by a trained researcher to quickly remove the brains and collect PVN samples from hypothalamic coronal sections (sliced with a brain matrix RVM-4000C, ASI Instruments®). Samples were homogenized and the total RNA were isolated using the General Electric IllustraRNAspin Mini Kit following the manufacturer's instructions. Samples were quantified by spectrophotometer ("Nanodrop 3300" - Thermo scientific, Bremen, Germany). The cDNA synthesis was performed with the SuperScript® III Reverse Transcriptase (Invitrogen ™) kit from 1 µg of total RNA.

The amplification reaction was performed on the Gene Q Rotor (Qiagen®) using the SYBR Green Jump Start Taq Ready Mix kit without $MgCl_2$ # S5193 400 RXN (Sigma-Aldrich Co.). The threshold values (ΔCt) and ΔΔCT were determined from the reference genes 36B4 and HPRT. Oligonucleotide sequences (primers) for *rattus norvegicus* were constructed from the GenBank (NLM / NCBI) database available at https://www.ncbi.nlm.nih.gov/genbank. The

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sequence-specific primers used in this study are delineated in supplementary table 1 (Exxtend; Campinas, São Paulo, Brazil).

Histology and immunohistochemistry

The GFAP expression in PVN, cortex and hippocampus were determined by immunofluorescence. For this purpose, after 4 days of 2% NaCl intake, rats were deeply anesthetised with pentobarbitone sodium overdose (200 mg kg^{-1}, i.p.) and immediately perfused transcardially with phosphate buffer (pH 7.4). Brains were removed and post-fixed in 4% paraformaldehyde for 24h and subsequently cryoprotected in 30% sucrose of 24h. Serial transverse sections (40 μm) of the hypothalamus were cut using a cryostat (Bright Instruments Ltd, UK). Free-floating sections were washed 3 times for 5 min in 0.1 M phosphate-buffered saline (PBS). Hypothalamic sections were then incubated in blocking buffer for 1h, to minimise non-specific binding of the antibody and for cell membrane permeabilisation (0.25% casein in 0.1% Triton in PBS. Tissue was incubated in primary antibodies diluted in blocking buffer for 48h at 4 °C [Rabbit anti-GFAP (1:500, Dako)]. Tissue was washed 3 times with PBS and subsequently incubated in the secondary antibody for 2h (Alexa Fluor 488 Donkey anti-rabbit, Dako). After 3 final washes in PBS, the tissue was mounted on glass slides and cover-slipped with Fluoroshield® (Sigma).

Image analysis

Digital images were captured using a Leica DM2000 fluorescence microscope (Leica Microsystems, Germany) coupled to a Retiga 3000C camera (Q Imaging, Canada). Two images were captured from 3 slices per animal with either 10× or 20× objectives. All images were captured by a blind investigator during the same session and using the same camera settings. Images were analysed by the mean grey value intensity using WCIF ImageJ software (Turlejski *et al.*, 2016).

Statistical Analysis

Relative gene expression (ΔΔCt) of ectonucleotidases and GFAP immunofluorescence (a.u.) intensity were compared between EU and SL by one-way ANOVA. ATP and adenosine

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(µM) release were compared between the EU and SL group by Student's t-test. Statistical analyses were performed using GraphPad Prism 6.01 software for Windows. Results were represented as mean ± standard deviation (SD). Differences between groups were considered statistically significant when $p<0.05$.

RESULTS

GFAP immunofluorescence is increased in the PVN of SL rats

To test the hypothesis that high salt exposure triggers a response of astrocytes in the PVN, we analysed GFAP immunofluorescence at the PVN level, cortex and hippocampus as comparative controls contained within the same slice from the same subject acquired under the same imaging conditions in rats having undergone 4 days of salt loading (Figure 1A). The PVN showed significantly increased GFAP immunofluorescence intensity when compared with the EU group [6.9±1.3 (EU) vs 27.2±6.2 a.u. (SL) $p=0.0184$]; Figure 1B and 1D. GFAP intensity in the cortex and hippocampus was unchanged between SL and EU groups (Figure 1C and 1D). Here it is worth noting that we found the GFAP immunoreactivity in PVN of EU animals was significantly lower when compared to cortex and hippocampus. Indeed, it has been previously reported that the profile of its expression can diverge amongst different brain areas (Middeldorp & Hol, 2011) and may not immunohistochemically detectable in many astrocytes throughout the healthy CNS (Sofroniew & Vinters, 2010). The difference could be explained by the low level of PVN glial cell activation in normal physiology in order to respond effectively to osmotic stress as observed in SL condition.

Basal release of ATP in the PVN is increased in SL rats

Once we had observed the increase in astroglial reactivity in PVN of SL rats we sought to understand whether this phenomenon would be accompanied by release of ATP and if it would affect the availability of adenosine. Using the experimental approach illustrated in Figure 2A, extracellular ATP was measured in the PVN of brain slices taken from SL animals. After calibration (Figure 2B), the sensors were placed on the slice surface in the recording chamber to determine the tonic ATP release from the tissue. The overlaid trace in Figure 2C showed that tonic ATP release was detected as soon as the biosensors contacted the PVN surface and revealed a dramatic difference between groups. The peak level of ATP was significantly higher in SL rats than in EU rats [1.1±0.8 µM (control) vs. 19.2±6.9 µM (SL) p =

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<0.0001]. However, no difference was observed in adenosine as shown in Figure 2E-F [2.1±0.9 µM (control) vs. 1.2±0.6 µM (SL)].

As ATP release from non-exocytotic pathways (Hamilton & Attwell, 2010) is well documented and ATP-permeable connexin hemichannels (Anselmi *et al.*, 2008) are highly expressed in brain astrocytes (Theis *et al.*, 2005) we sought to determine if connexins could be the source of the increased ATP release recorded in the PVN area of the brain slice. Connexins are sensitive to extracellular [Ca^{2+}] and open in conditions of zero Ca^{2+} (Lopez *et al.*, 2016), using this feature we increased connexin opening probability by superfusing the slice with Ca^{2+} -free aCSF for 10 min, while the two sensors (ATP and adenosine) were simultaneously recording the release of both purines directly from PVN. Then, after retuning back the slice to physiological buffer for an additional 10 min before superfusing the slices with the connexin blocker NPPB (50 µM), also for 10 min. As shown in Figure 2 G-H none of these approaches significantly altered the ATP and adenosine release between slices taken from the SL and EU subjects, suggesting that connexin-mediated release of ATP does not play a significant role in the increase of extracellular ATP seen in SL animals.

<u>Blockade of vesicular exocytosis reduces ATP release in the PVN astrocytes of SL rats</u>

Once it was established that ATP release observed was not interrupted by reducing connexin permeability with NPPB, we sought to investigate if the release occurs by exocytosis mechanisms. Accordingly, the vesicular release mechanisms in PVN astrocytes in SL rats were disrupted by virally driven expression of the dnSNARE protein under the GFAP promotor to block SNARE-dependent vesicular exocytosis in GFAP-positive cells (Fig 3A). The PVN of SL rats (n=4) was transduced unilaterally with an AAV encoding dnSNARE. A total of 500nL of virus suspension was delivered over 2 injection sites in order to completely transduce the right-hand side of the PVN without leakage to the contralateral side, see Methods for details. This allowed extracellular ATP concentration to be compared between the transduced PVN and the (control) contralateral non-transduced side. The data revealed that the tonic ATP release measured in the PVN of SL rats was significantly decreased in the PVN transduced to express dnSNARE (6.6±1.6 µM, p= 0.0117), compared to the contralateral control PVN (13.9±0.1 µM, Figure 3A). ATP release was not affected in either transduced or control PVN regions after application of the P2X7 antagonist AZ 10606 1 µM (Fig 3B-C).

<u>Ectonucleotidase activity and gene expression are not changed in the PVN of SL rats</u>

To test our second hypothesis that increased ATP availability in the PVN of SL rats might also occur due to a failure of ectonucleotidases to metabolize this nucleotide, we evaluated ectonucleotidase activity in PVN- containing slices. We quantified the maximal adenosine production in the PVN after superfusing the slices with 10 and 50 µM ATP (Figure 4A). 10 µM ATP was able to evoke a detectable response in adenosine concentration in PVN of SL rats [0.38 ± 0.2 µM (aCSF, SL) vs. 0.54 ± 0.2 µM (10 µM ATP, SL)] but not in EU [3.4 ± 0.1 µM (aCSF, EU) vs. 3.67 ± 1.1 µM (50 µM ATP, EU)], suggesting that ectonucleotidases activity is increased in these animals comparing to EU. When the slices were stimulated with 50 µM ATP, the adenosine response increased similarly in both groups [0.51±0.1 µM (aCSF, EU) vs. 1.33±0.3 µM (ATP 50 µM, EU)] and SL [0.77 ± 0.3 µM (aCSF, SL) vs. 1.82±0.2 µM (50 µM ATP, SL)] which could indicate that the maximal reaction rate (Vmax) of PVN ectonucleotidases was reached at this concentration.

The results in Figure 4B showed that the relative mRNA expression of the subtype E-NTPD2, E-NTPD3 and E-5'-NT in PVN was not different between EU and SL animals, suggesting that the increased degradation rate of ATP by ectonucleotidases in this nucleus cannot be explained by changes in their gene expression.

DISCUSSION

This study focused on the role of astrocytes as a putative source of ATP released at the PVN level, a key brain region involved in neuroendocrine and autonomic functions directly or indirectly related to the neural control of circulation during osmotic challenges. Here we show that rats receiving a hypertonic solution of 2% NaCl instead of regular drinking water exhibit a strongly increased immunoreactivity to GFAP in PVN. These findings correlate with a vesicular release of ATP in PVN that are potentially mediated by astrocytes, as the PVN expression of the dominant-negative SNARE protein under GFAP promoter was able to attenuate ATP release by approximately 50%. However, our results do not exclude the possibility of ATP being additionally released from other glial cells types or by secondary activation of purinergic neurones within the PVN. Indeed, there is evidence that PVN microglia are also known to be activated by salt loading (Gilman *et al.*, 2019) and microglia have been reported to release ATP by SNARE-dependent exocytosis (Imura *et al.*, 2013). Contrary to our initial hypothesis, the activity of the ectonucleotidases in PVN appeared to be increased by high salt intake.

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The primary contribution of our study is the increase GFAP expression specifically in PVN of SL rats which indicates glial cell activation. Reactive astrocytes exhibit a number of molecular and morphological features, with one of the hallmarks being the upregulation of GFAP. This protein belongs to the family of intermediate filaments that constitute the cytoskeleton of astrocytes, a structure that is fundamental for the transport and metabolism of amino acids, glucose and other molecules (Li *et al.*, 2008). Studies suggest that disorders in its expression can determine not only morphological changes, but also the function of astrocytes in the release of (glio-) transmitters that modulate neuronal excitability (Sheikhbahaei *et al.*, 2018).

The profile of GFAP expression can diverge amongst different brain areas and can be influenced by aging and circadian rhythm (Middeldorp & Hol, 2011). However, when comparing within brain area GFAP hyperexpression is one of the main characteristics defining the phenomenon of reactive astrogliosis, which is a glial reaction to cytokines such as transforming growth factor alpha (TGF-a), ciliary neurotrophic factor (CNTF), interleukin-6 (IL-6) and leukaemia inhibitory factor (LIF), whose production is usually increased during neurological disorders, such as neurotrauma, ischemic stroke and neurodegenerative disease (Hol & Pekny, 2015). Indeed, studies have shown that long term high salt intake can induce activation of astrocytes both *in vivo* and *in vitro* that is associated with the upregulation of tumour necrosis factor-α (TNF-α), IL-6, interleukin-1β (IL-1β) and vascular endothelial growth factor (VEGF) (Deng *et al.*, 2017).

For reasons not yet understood, the increased GFAP immunoreactivity as showed here differs from what is found in the literature during dehydration. For example, a reduction of GFAP and an increase of AVP immunoreactivities in PVN and in the supraoptic nucleus (SON) was observed in Meriones shawi (a rodent species adapted to desert life) when subjected to prolonged water deprivation (Gamrani *et al.*, 2011), which is, however, a reaction that can be fully normalized after a period of rehydration (Elgot *et al.*, 2012). Conflicting reports in primary culture of hypothalamic astrocytes also revealed that the exposure of these cells in hypertonic medium reduces GFAP immunoreactivity as well as the ability of astrocytes to promote glutamate reuptake (Souza *et al.*, 2020). Also, contradicting results were found in salt-sensitive rats fed with 4% NaCl diet for seven days, where GFAP expression in the PVN was unaffected (Moreira *et al.*, 2019). These divergent findings may suggest that the cyto-architecture of PVN astrocytes can behave in a complex manner according to the modality of osmotic challenge that is imposed. Additionally, it is important to note that there are significant ultrastructural changes and cellular re-organization in the hypothalamus, and that astrocytic processes

undergo a significant withdrawal in certain circumstances, such as, dehydration, salt loading and lactation (Tweedle & Hatton, 1977; Theodosis & Poulain, 1984; Montagnese et al, 1988). The present study cannot determine the state of the glial processes with the PVN as GFAP immunoreactivity does not fully recover the extent of the complex structure of glial cells including their processes (Wilhelmsson *et al.*, 2004). As a result, we cannot rule out the possibility of a glial retraction is a feature of the SL PVN that could affect the release of gliotransmitters to modulate synaptic efficacy.

GFAP hyperexpression in the brainstem has been observed in the spontaneously hypertensive rat and is associated with astrocytic release of ATP and others gliotransmitters (such as lactate) that have sympathoexcitatory properties (Marina *et al.*, 2015; Turlejski *et al.*, 2016). In our study, we also showed that the tonic release of ATP was profoundly increased in the PVN of SL rats, independently of the opening state of astrocytes connexins or hemichannels, once the manipulation of these structures with NPPB and aCSF with 0 Ca^{2+} had no effect in ATP release. Previous evidence also suggests that the pore-formed by activation of purinergic P2X7 receptors (expressed in astrocytes) are also a likely site for ATP release (Suadicani *et al.*, 2006), although in our study the P2X7 receptor antagonist AZ 10606 did not interrupt ATP release.

However, we showed that PVN unilateral transfection of the dominant negative SNARE protein under GFAP promoter was able to reduce ATP release by approximately 50%, comparing the transfected vs non-transfected side of the PVN. This result suggests that ATP is released by astrocytes *via* the exocytotic pathway (SNARE-dependent) similar to what has been observed in the brainstem in response to acute reductions in cerebral blood flow (Marina *et al.*, 2020) or during hypoxia and hypercapnia (Sheikhbahaei *et al.*, 2018).

Purinergic signalling has become an important aspect of neurotransmission in many brain nuclei involved in different pathophysiological conditions (Burnstock, 2007), influencing autonomic and neuroendocrine responses at the PVN level (Knott *et al.*, 2008; Cruz *et al.*, 2010; Ferreira-Neto *et al.*, 2013). Previous evidence has demonstrated the involvement of purinergic signalling in the hypothalamus in the regulation of body temperature (Gourine *et al.*, 2002) and in the secretion of AVP (Mori *et al.*, 1994). Besides, during almost a decade our laboratory has shown a series of studies showing that ATP acting on the PVN neurones elicit a dose-dependent increase of lumbar sympathetic nerve activity (Ferreira-Neto *et al.*, 2013). Moreover, the acute increasing of plasma osmolality activates $P2X_2$ receptor-expressing PVN neurones and elicits lumbar sympathoexcitation, an effect that is attenuated with a P2

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receptor antagonist, PPADS (Ferreira-Neto *et al.*, 2017). Based on these data, we suggest that astrocytic release of ATP in the PVN might be an important element involved in the neural control of circulation during a period of high salt intake.

Despite the increase of ATP release, the concentration of its metabolite adenosine was not changed in SL rats which is, however, not caused by a failure of ectonucleotidases to convert ATP into adenosine as our primary hypothesis predicted. Indeed, the opposite effect has been observed when we stimulated the slices by superfusion of ATP at 10 µM and only in PVN of SL rats it produced a detectable response in adenosine concentration, suggesting that ectonucleotidases seems to be more reactive to ATP at levels similar to what were detected to be tonically released. This may represent how these enzymes control the excess ATP in the PVN, as a negative feedback loop. Adenosine can be produced from the conversion of ATP or ADP into AMP by NTPDases (or CD39) and subsequently from the conversion of AMP into adenosine by E-5'-NT (or CD765) (Ballesteros-Yáñez *et al.*, 2018). Our results showed that the mRNA levels of E-NTPD2, E-NTPD2 either E-5'-NT NTPD in PVN are not suppressed in SL rats, suggesting that gene expression is not influenced by high salt intake. The fact that adenosine concentration remained unaffected even in the presence of copious amounts of extracellular ATP would be potentially explained by an increase of phosphorylation and deamination of adenosine by adenosine kinase or adenosine deaminase to produce AMP and inosine, respectively.

Final considerations

Figure 5 summarizes a schematic drawing that brings together the main findings that our group has been exploring over the last decade regarding purinergic neurotransmission between hypothalamic and brainstem pathways involved in neural control of circulation under osmotic challenges in experimental animal models with high salt intake.

The SFO, OVLT and MnPO have direct and indirect connections to the parvocellular subdivision of PVN, where the sympathetic pre-autonomic neurones that project to the brainstem, where the solitary tract nucleus and the RVLM are located (Toney & Stocker, 2010). In the PVN, the combination of PPADS and the non-NMDA glutamate receptor antagonist CNQX attenuates the sympathoexcitatory response evoked by ATP in a more marked way than PPADS individually, suggesting the hypothesis that ATP and glutamate are co-released in this environment (Ferreira-Neto *et al.*, 2013). Subsequent data support the hypothesis of a functional coupling between P2 and AMPA receptors (probably of extrasynaptic localization) in

PVN-RVLM neurones, which stimulates the activity of sympathetic pre-autonomic neurones of the PVN (Ferreira-Neto *et al.*, 2015).

Our results obtained here suggest that ATP is also released by PVN astrocytes *via* exocytotic pathway (SNARE-dependent) under an osmotic challenge seen in the condition of high salt intake. We suppose that excitatory inputs from CVOs to the PVN could promote functional changes in PVN astrocytes, which would lead to subsequent release of ATP. However, we cannot exclude the possibility that other gliotransmitters are released concurrently that stimulate ATP release from other sources. Previous functional data complement our findings, since microinjection of the P2 purinergic receptor antagonist into PVN attenuates the sympathetic-excitatory response mediated by an acute increase in plasma osmolarity (Ferreira-Neto *et al.*, 2017).

Taken together, our findings lead us to conclude that the high salt intake by the supply of hypertonic NaCl solution does not decrease ectonucleotidases activity, but it stimulates PVN astrocytes to release ATP *via* exocytotic pathway.

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Author contributions: RMS designed and performed experiments, acquired and analysed data. Conception and study design; VRA and NM. KMS, INC, SMT and BVC assisted with data acquisition and analysis. PSH analysed data and drafted the manuscript with VRA and RMS. All authors reviewed the manuscript critically and revised it for important intellectual content, approved the final version of the manuscript and agree to be accountable for all aspects of the

work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Disclosure: No conflicts of interest

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Zimmermann H, Zebisch M, Sträter N, Zimmermann H, Zebisch M & Sträter N. (2012). Cellular function and molecular structure of ecto-nucleotidases Madin-Darby canine kidney MS Mass spectrometry NDP Nucleoside diphosphate NLD Nuclease-like domain NMP Nucleoside monophosphate NMN Nicotinamide mononucleotide NMR Nuclear magnetic resonance NPP Nucleotide pyrophosphatase/ phosphodiesterase NR Nicotinamide riboside NTP Nucleoside triphosphate. *Purinergic Signalling* **8,** 437-502.

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Supplementary Table 1. Sequence-specific of forward (F) and reverse (R) primers.

Gene	***Primers* (5`→3`)**		
ENTPD1 (CD39)	AGTATGGGATTGTGCTGGATG (F)		TCTGAGCGTATTTTGAGATTCCG (R)
ENTPD2 (CD39/L1)	GATCCAGGCAACGAGGTTCA (F)		GAAGCGATGGATCTGGAGGG (R)
ENTPD3 (CCD39/L3)	AGATAGGGCCCCACTCAACT (F)		TCTCCCTCCGTATTCCTCCT (R)
E-5-NT (CD73)	CATTCGTGAAGATGCAGCCAT (F)		GCGGAGCCATTCAGGTAGAC (R)

FIGURES LEGENDS

Figure 1. (A) Schematic representation of a rat brain sliced in the regions 1, 2 and 3 referring to PVN, cortex and hippocampus, respectively, where GFAP immunoreactivity was detected. Epifluorescence micrographs of coronal section through the PVN (B), cortex and hippocampus (C) of one representative animal from the SL group subjected to 4 days of NaCl (2%) solution intake and from on rat from the EU group (normal drinking water only) immunolabeled with an antibody against GFAP. (D) The histogram represents the GFAP fluorescence intensity signal in arbitrary units in the brain of SL rats and EU group. The yellow square dotted line represents the PVN region analysed for fluorescence signal. 3V: third ventricle

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Figure 2. (A) Schematic drawings illustrating the experimental setup for real-time detection of ATP and adenosine release in the PVN with amperometric biosensors. (B) calibration of biosensors with exogenous application of ATP (10 uM) and adenosine (5 uM). ATP (C and D) and adenosine (E and F) release detected from the moment that biosensors tips contacted PVN surface. The release of both purines has not changed after manipulation of astrocytes permeability with aCSF 0 Ca^{2+} and NPPB (50 µM). Summary data (D and F) are shown as means ± SD

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Figure 3. (A) Schematic of AAV-sGFAP-dnSNARE-EGFP vector layout and the illustration of PVN unilateral transfection with dnSNARE. (B) Recording of the real time signals of ATP release on transfected PVN side with dnSNARE (red line) compared to the non-transfected side (greenline)in SL rats. (C) Concentration of ATP (µM) release in transfected side (dnSNARE) compared to non-transfected side of the PVN (control). (D) Plot showing the concentration of ATP (µM) release before (in aCSF) and after application of the P2X7 antagonist AZ 10606 1 µM in transfected side (dnSNARE) compared to non-transfected side of the PVN (control).

Figure 4. (A) Ectonucleotidases activity tested with biosensors in slices by quantifying the maximal adenosine production in PVN after the exogenous application of ATP 10 and 50 µM. (B) Quantification of mRNA expression in the PVN for tri(di)phosphohydrolases (E-NTPDs) 1 and 2 and the ecto 5'-Nucleotidase (E-5'-NT) in SL and EU rats.

Figure 5. Schematic representation summarizing the hypothalamic and brainstem neuronal integration under osmotic challenges that elicits a phenotype of neurogenic hypertension induced by salt-loading with the involvement of the purinergic and glutamatergic neurotransmission at the paraventricular nucleus of the hypothalamus (PVN). Glu (glutamate); ANG-II (angiotensin II) subfornical organ (SFO); median preoptic nucleus (MnPO); organum vasculosum of the lamina terminalis (OVLT); rostral ventrolateral medulla (RVLM); intermediolateral nucleus (IML) in the spinal cord; circumventricular organs (CVOs), 3°V: third ventricle.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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HIGHLIGHTS

- Using the model of a salt-loaded laboratory rat we sought to determine the source(s) of ATP released PVN in response to high salt intake
- High salt intake evokes an increase in glial fibrillary acidic protein immunoreactivity in the PVN
- Salt loading stimulates the release of vesicular ATP from glial cells in the PVN with extracellular adenosine remaining unchanged
- Activity of ectonucleotidase responsible for the breakdown of ATP in the PVN was increased in SL group
- The evidence suggests that increased extracellular ATP seen in hyperosmotic conditions results from glial release and not reduced ectonucleotidase activity

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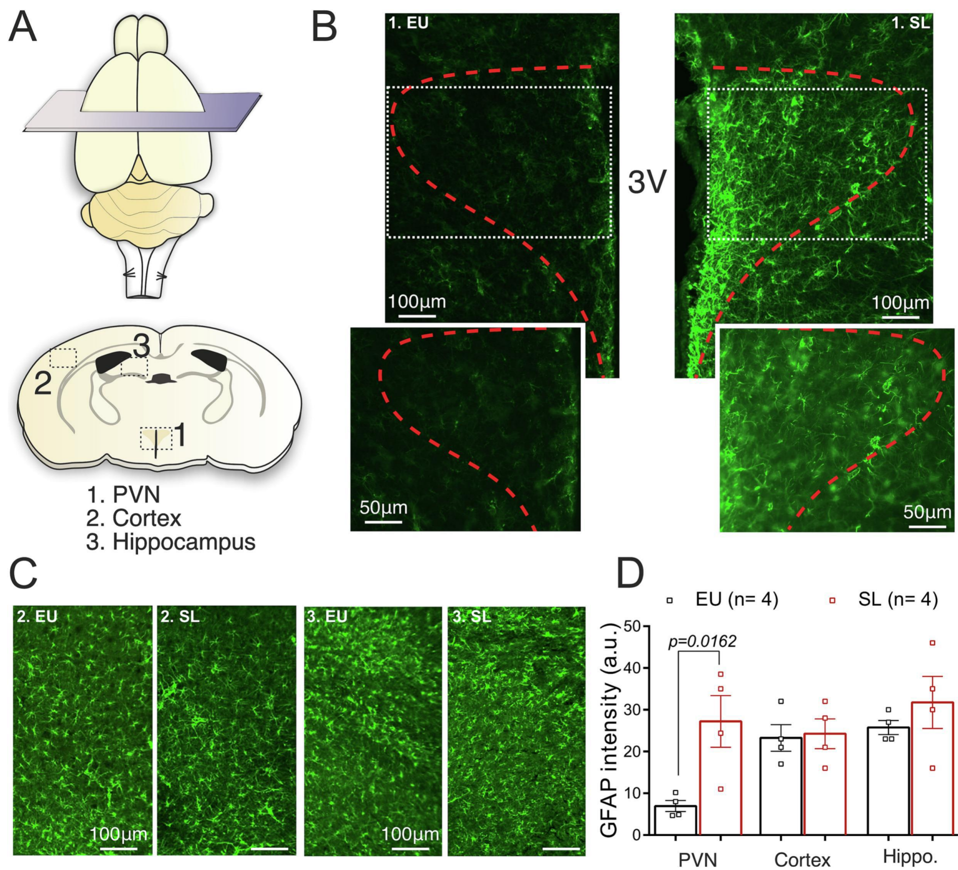


Figure 1

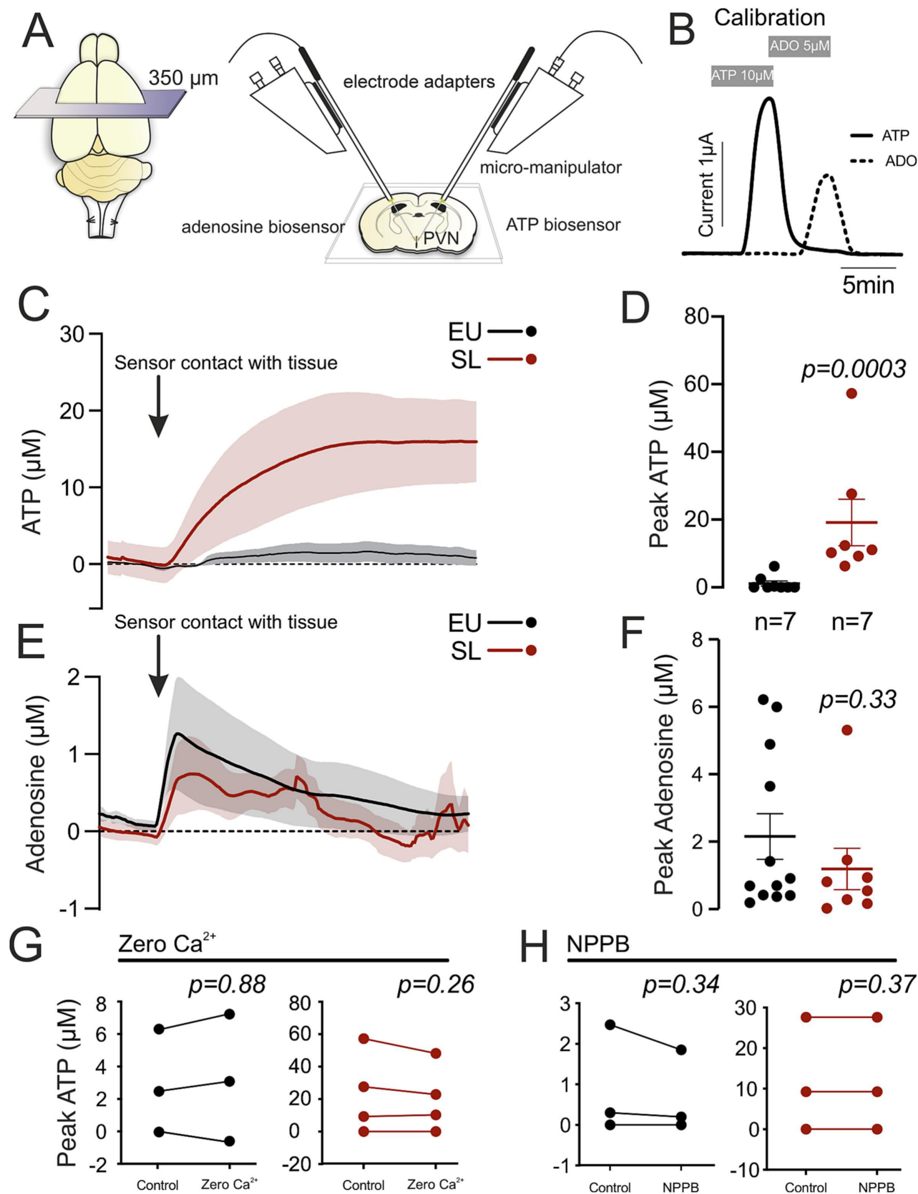
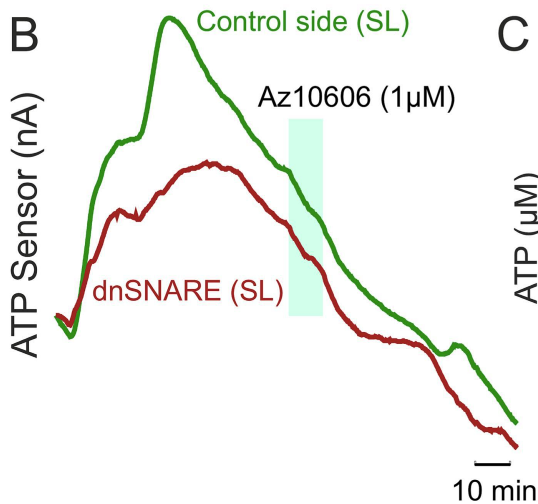
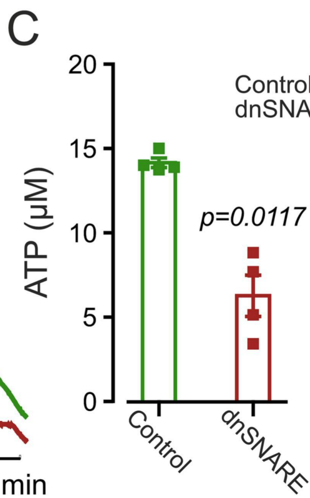


Figure 2

A

GAL4p65
mCMV
5xGAL4 BS
GfaABC$_1$D
dnSNARE-EGFP
SL Rat
PVN
PVN
Transfected side
Non-transfected





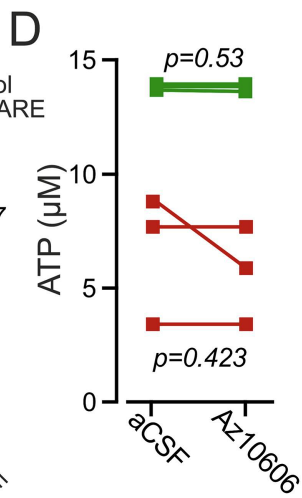


Figure 3

A

EU

p=0.12

SL

p = 0.03

Adenosine Production (μM)

15

10

5

0

aCSF

ATP 10μM

1.5

1.0

0.5

0

aCSF

ATP 10μM

p=0.03

p = 0.01

Adenosine Production (μM)

3

2

1

0

aCSF

ATP 50μM

aCSF

ATP 50μM

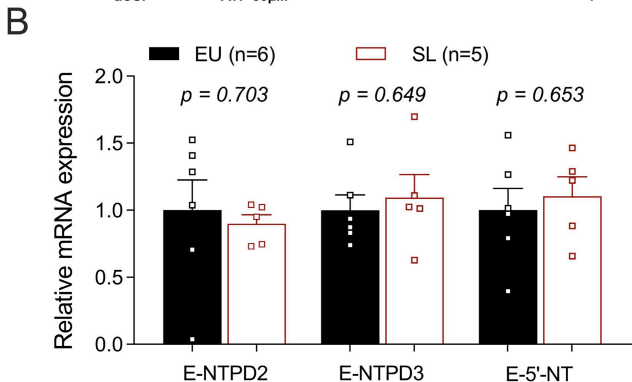


Figure 4

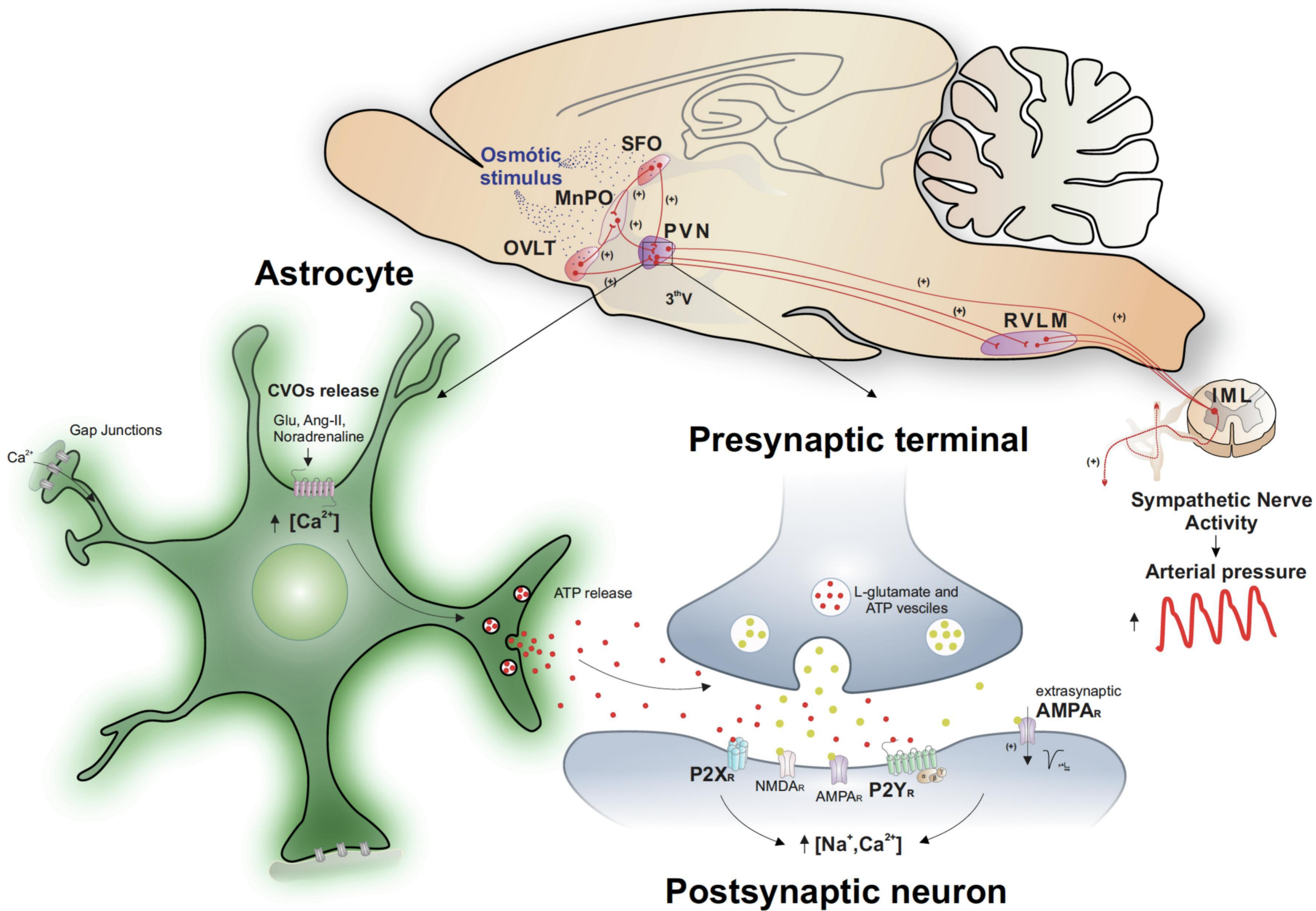


Figure 5