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Adenosine A1 receptors modulate the Na^+-Hypertonicity induced glutamate release in hypothalamic glial cells

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ABSTRACT

Glutamate release in response to a hypertonic stimulus is a well described phenomenon in the hypothalamus. Evidence suggests that hypothalamic glial cells release glutamate into the extracellular environment in hypertonic conditions. In the current study, we described autocrine regulation of adenosine on glutamate release induced by Na^+ hypertonicity in hypothalamic glial cell cultures. We showed that glial cells cultured from the cerebral cortex did not release glutamate or adenosine under hypertonic conditions. The findings suggest that the hypothalamus has specialized glial cells, which are responsive to osmotic variations. Stimulation or inhibition of adenosine A1 receptors modulates extracellular glutamate levels in hypothalamic glial cell cultures under hypertonic stimulation. Our results extend previous observations regarding the role of glial cells in the control of hypothalamic physiology. They further demonstrate for the first time that hypothalamic glial cells regulate Na^+-hypertonicity-induced glutamate release by activation of adenosine A1 receptors via adenosine release.

1. Introduction

The hypothalamus is a brain region involved in distinct physiological functions including hydroelectrolytic regulation of body fluids (Antunes-Rodrigues et al., 2004). The adjustment of salt-intake behavior and neurohypophysial hormone release in response to variations in blood osmolarity are controlled by distinct hypothalamic nuclei (Antunes-Rodrigues et al., 2004; Johnson and Thunhorst, 1997; Mckinley et al., 2004). The hypothalamus is considered to be a specialized sensory apparatus of the brain through which minor variations of plasma osmolarity can be detected. In fact, the hypothalamus works as an "osmosensor" in the brain exerting a central control on the blood plasma osmolarity (see Verbalis, 2010). It is well documented that minor variations in hypothalamic osmolarity trigger neuronal activation and hormonal release in distinct hypothalamic nuclei. However, it is not fully clear how glial cells housed in the hypothalamus respond to hyperosmolarity.

Glial cells have emerged as important protagonists of the central nervous system (CNS) physiology (Chen and Swanson, 2003; Kettenmann and Verkhratsky, 2008; Parpura and Verkhratsky, 2012). As widely described in the literature, astrocytes regulate glutamatergic synapses by clearance of glutamate in the synaptic cleft (Currie and Kelly, 1981; Danbolt, 2001; Schousboe and Waagepetersen, 2005). Glutamate uptake and release are mediated by Na^+-dependent and Na^+-independent glutamate transporters present in neuronal and glial cells. The importance of these protein transporters in the maintenance of CNS homeostasis is evident in some neurodegenerative diseases which are associated with dysfunctions in glutamate transport (see King et al., 2011).

Glial participation in the hypothalamic response to hypertonic stimuli has already been reported in the literature (Gamrani et al., 2011; Wang et al., 2006; Yuan et al., 2010). Previous studies have described that hypothalamic astrocytes show a neurochemical response to a hypertonic stimulus (Jiang et al., 2011). Cao et al. (2008) demonstrated that hypothalamic glial cells synthesize and release glutamate under hypertonic conditions. Several studies described that glutamate

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transport is modulated by different neurotransmitters, such as GABA (Héja et al., 2009), dopamine (Amara, 2014; Gainetdinov et al., 2001) and acetylcholine (Araque et al., 2002). Gliotransmitters such as ATP and adenosine can also regulate glutamate levels in the extracellular environment (Fellin et al., 2006; Fields and Burnstock, 2006; Jeremic et al., 2001; Paes-de Carvalho et al., 2005). Although previous studies have described that adenosine inhibits the glutamate release in brain preparations (Harvey and Lacey, 1997), it is not still clarified if the control of glutamate release induced by Na^+-hypertonicity is controlled by purinergic system. The current study aimed to evaluate the mechanism by which glial cells control glutamate release when submitted to a hypertonic stimulus as well as the role of the adenosinergic system in this regulation.

2. Material and methods

2.1. Glial cell cultures

Glial cells were cultured from the hypothalamus or the cerebral cortex of 1-2-day-old Wistar rat pups. Briefly, animals were deeply anesthetized with a ketamine/xylazine solution (1:1) and quickly decapitated before the subsequent craniotomy procedures. The hypothalamus was dissected and isolated from brains of two rat pups at 1 day old and then transferred to assay tubes containing trypsin solution (0.25%). This procedure was followed by mechanical tissue dissociation using cold Dulbecco's Modified Eagle's Medium (DMEM) media supplemented with 10% of fetal bovine serum (FBS). Similar steps were performed with the cerebral cortex samples. Cell homogenates were centrifuged for 10 min at 5000 rpm and supernatant was discarded. The pellet containing the cells was resuspended in DMEM +10% FBS and 6 × 105 cells were seeded in 12-well culture plates and cultured in a humidified incubator at 37 °C and 5% CO2. Hypothalamic and cortical glial cells had their culture medium replaced each 3 days until the 15th day in vitro. The purity of glial cell cultures was determinated by GFAP immunofluorescence and flow citometry as described in supporting information 1. All experiments were conducted in accordance with the ethical protocol for animal use of the Federal University of Pará (CEPAE-UFPA 140–13).

2.2. Hyperosmotic stimulus and pharmacological treatments

Glial cell cultures from the hypothalamus or the cerebral cortex were incubated with isosmotic (300 mOsm/L) pH 7.4 Hank's solution (128 mM NaCl, 4 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 12 mM glucose and 20 mM HEPES) or hyperosmotic pH 7.4 Hank's solution (340 mOsm/L) (148 mM NaCl, 4 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 12 mM glucose and 20 mM HEPES) for 0; 5; 10; or 15 min in a CO2 stove at 37 °C. In order to verify the role of sodium on the glutamate release experiments were conducted in Hank's solution without NaCl supplemented with 40 mM mannitol (345 mOsm/L) (40 mM mannitol, 4 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 12 mM glucose and 20 mM HEPES). To evaluate the mechanism involved in the adenosine release from glial cells, cell cultures were incubated for 15 min with 10 μM glutamate. Adenosinergic regulation of glutamate release was evaluated in glial cell cultures maintained in medium containing 0.1 μM of adenosine, 1 μM RR-PIA ([R(−)N6-(2phenylisopropyl) adenosine), 10 μM 8-CPT (8-cyclopentyl-1,3-dimethylxanthine) or 10 μM dipyridamole for 5 min. All drugs were diluted in isotonic or hypertonic Hank's solution. After all experimental procedure, the extracellular medium was collected from separate cell cultures under isotonic or hypertonic conditions and then prepared for HPLC analysis.

2.3. Quantification of glutamate and adenosine levels

Extracellular levels of glutamate and adenosine were measured using high performance liquid chromatography (HPLC). Stock solutions of adenosine, glutamate, and internal standard were dissolved in ultrapure water at 100 μg/mL. For determination of the extracellular glutamate levels a standard curve with different glutamate concentrations was used (0.1 μg/mL, 0.5 μg/mL, 1.0 μg/mL, 2.5 μg/mL, 5.0 μg/mL, 10.0 μg/mL and 20.0 μg/mL). The quantification of adenosine levels in the supernatant was also based on a standard curve with different adenosine concentrations (0.5 μM, 1 μM, 5 μM, 10 μM, 25 μM, 50 μM, 100 μM and 200 μM). Retention time, linearity, calibration, selectivity, limit of detection and quantification, accuracy, precision, recovery and stability of glutamate and adenosine were determined in accordance with the guideline for validation methods described by the US Food and Drug administration (US-FDA, 2001). The HPLC system used in the current study was manufactured by Shimadzu (LC-10 AD, Tokyo, Japan) with a 20 μl injection loop and a fluorescent detector (RF-10AXL) coupled to an LC-20AT pump. The system was equipped with a Shimadzu C18 analytical column (Shim-pack VP-ODS 4.6*250LC, internal diameter 4.6 mm) and a pre-packed column holder. The column was heated to 29 °C with a thermostat system (CTO-20A). An integrator was also used to analyze the chromatographic data. The mobile phase was composed of 50 mM sodium acetate, methanol 5% and 2-propanol (pH 5.67) as phase A and methanol 70% as phase B. These phases were eluted in a lowpressure gradient as follows: Initially, 100% phase A, after 20 min, 50%, and finally 100% at 25 min elution time. Mobile phases were filtered using Millipore 0.22-μm Durapore membrane filters before use. Glutamate was quantified by fluorescence detection utilizing homoserine as an internal standard. The fluorescent detector was set at 340 nm (excitation wavelength) and 460 nm (emission wavelength) and glutamate was derivatized using a mix of glutamate with 13 mg o-phthalaldehyde (OPA) in borate buffer (pH 9.5). Adenosine was quantified using ultraviolet detection at 257 nm without chemical derivatization.

3. Results

Both hypothalamic and glial cell cultures presented high purity for astrocytes as observed in supplementary material (Supplementary Figs. 1A–G). Our data also have shown that hypertonic stimulation evoked evident decrease in the cell volume of hypothalamic astrocytes in culture. This phenomenon was not observed in astrocytes cultured from brain cortex (Supplementary Figs. 1A–D).

3.1. The glutamate release from glial cells during exposure to hypertonic medium

Our results showed that hypothalamic glial cells increased the extracellular glutamate concentration threefold when being maintained for 5 min under hypertonic stimulus conditions. After this period, we observed that glutamate levels decreased significantly within the first 10 min and returned to basal levels 15 min after the hypertonic stimulus (Fig. 1A). When glial cells from the cerebral cortex were submitted to the same stimulation protocol, biphasic response of extracellular glutamate levels was not observed (Fig. 1B). Our data showed that glial cells from hypothalamus and cerebral cortex have different responsiveness to a hypertonic stimulus. In the current study, we also verified the involvement of Na + -dependent glutamate transporters as mediators of glutamate release induced by hypertonic stimulation of hypothalamic glial cells. It has been often described that glial cells transport glutamate using Na^+-dependent and Na^+-independent transporters. Our data show that sodium withdraws prevents the Na^+-hypertonicity-induced glutamate released in hypothalamic glial cultures. This result suggests participation of Na^+-dependent glutamate transporters in the glutamate release induced by hypertonic stimulus (Fig. 1C).

Extracellular changes in glutamate levels in response to a hypertonic medium lead to the question about which cellular mechanism is involved in the downregulation of the glutamate transport dynamics in

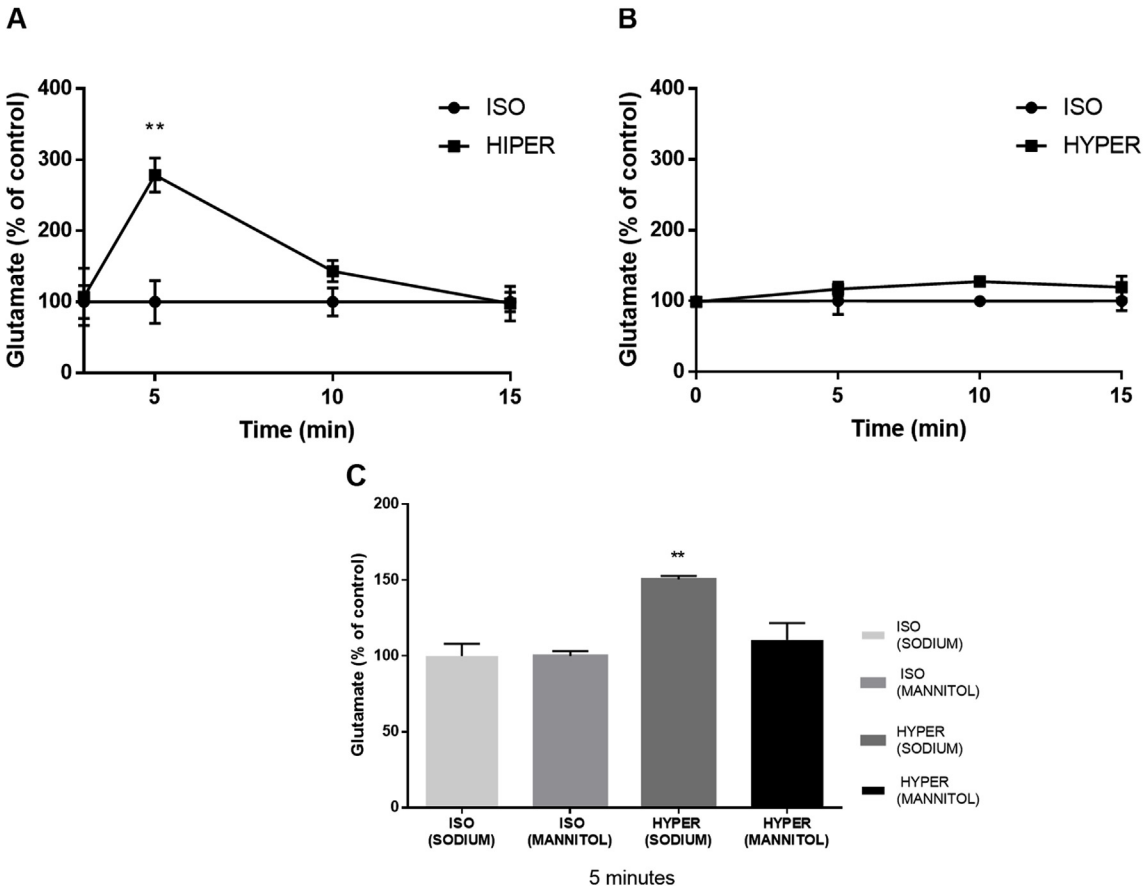

Fig. 1. Extracellular glutamate levels (A) in hypothalamic glial cell cultures and (B) brain cortical cell cultures incubated with hypertonic medium. (C) Effect of sodium withdrawal on the glutamate release induced by hypertonic medium in hypothalamic glial cell cultures. Data were expressed as media ± SD (n = 6, **p ≤ 0.01 and * p ≤ 0.05 ANOVA – One way – post test Tukey).

hypothalamic glial cells over the time analyzed.

3.2. The adenosine release from glial cells during exposure to hypertonic medium

Our results showed that hypothalamic glial cells increase the extracellular adenosine level only 10 and 15 min after the hypertonic stimulus (Fig. 2A). As observed for glutamate, no accumulation of adenosine was evident in the extracellular medium of cortical glial cell cultures submitted to hypertonic stimulation (Fig. 2B).

We also verified whether adenosine treatment can modulate glutamate release induced by hypertonic stimuli. As observed in Figs. 2C and 0.1 μM of adenosine blocked glutamate release evoked by hypertonic stimulation of hypothalamic glial cells. Previous studies have already described that hypothalamic glial cells show a high expression of adenosine A1 receptors. Based on this, we have tested if activation of the A1 receptor using R-PIA ([R(−)N6-(2-phenylisopropyl) adenosine]) can control glutamate release during a hypertonic stimulus. Fig. 2D shows that 1 μM R-PIA inhibited glial glutamate release induced by Na + -hypertonicity.

After showing that adenosine controlled the Na^+-hypertonicity-induced glutamate release, we tested if the blockage of adenosine release or the inhibition of adenosine A1 receptors could maintain the elevation of extracellular glutamate levels, which was observed in the late periods of hypertonic stimulation. Our results showed that 10 μM dipyridamole treatment elevated the extracellular glutamate levels in hypothalamic glial cell cultures incubated with hypertonic medium (Fig. 3A). This phenomenon was also observed when hypothalamic glial cells were treated with antagonist of A1 adenosine receptor 8-CPT (8-cyclopentyl-1,3-dimethylxanthine) at 10 μM (Fig. 3B).

4. Discussion

4.1. Hypothalamic glial cells as sensors for hypertonic environment

It is well characterized that glutamate released from hypothalamic neurons is essential for the control of body homeostasis (Pal et al., 1993). However, there are few data about the physiologic response of hypothalamic glial cells to Na^+-hypertonicity. The current study has shown that glutamate levels change in the extracellular environment when hypothalamic glial cells are stimulated with hypertonic medium. The fast increase followed by progressive decrease in extracellular glutamate levels suggests that hypothalamic glial cells under hypertonic stimulation can control their own glutamate release. This autoregulatory mechanism was not observed in glial cells cultured from the cortex. Taken together these findings support the hypothesis that hypothalamic glial cells are specialized to respond to osmotic variations. The hypothesis that glial cells present phenotypic differences in distinct areas of the CNS has already been suggested previously (Matyash and Kettenmann, 2010; Schitine et al., 2015). However, the present study demonstrated for the first time that hypothalamic glial cells themselves have a mechanism for control of extracellular glutamate level in hypertonic conditions. These findings ratiffy previous studies showing glial cells as important protagonists of hypothalamic physiology (Garcia-segura et al., 2004, 2008; Simard and Nedergaard, 2004).

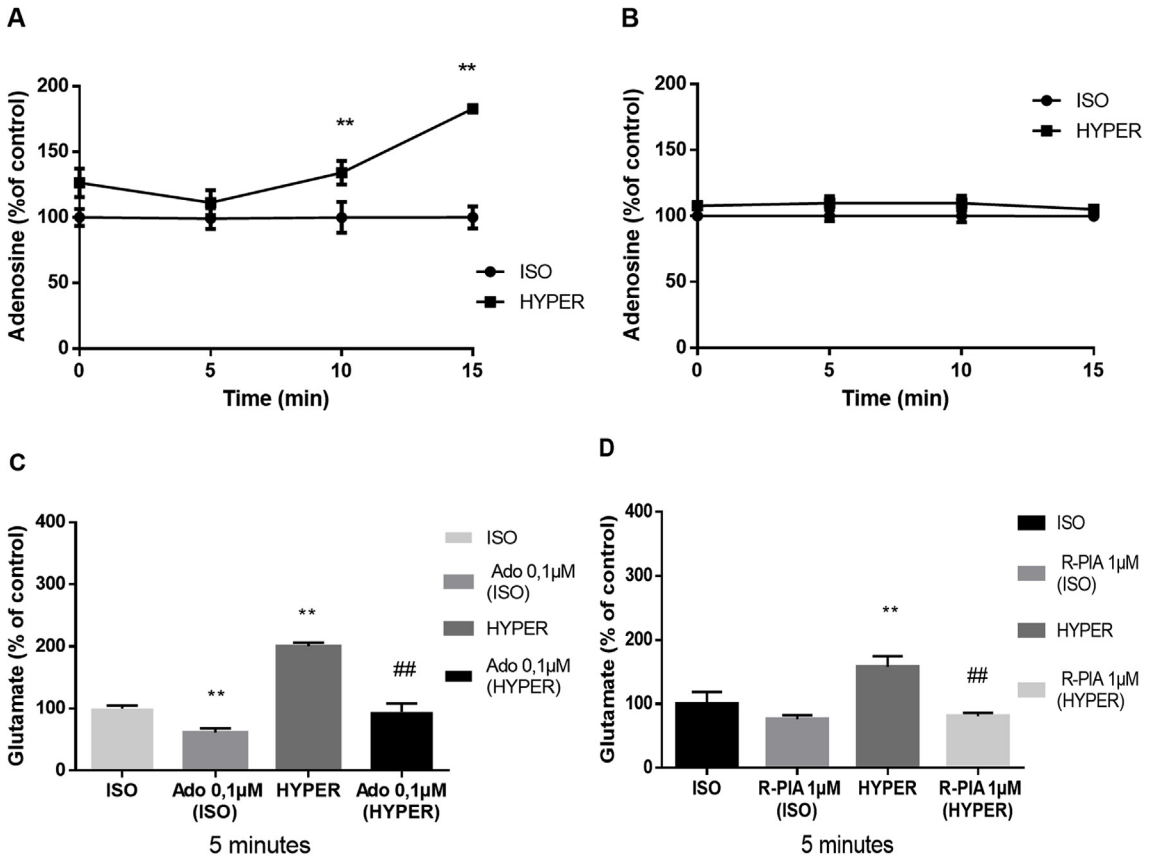

Fig. 2. Adenosine levels in (A) hypothalamic glial cell cultures and (B) brain cortical cell cultures submitted to hypertonic stimulation. (C) Effect of adenosine 0.1 μM and (D) A1 adenosine receptor agonist R-PIA at 1 μM on the glutamate release in hypothalamic glial cell cultures. Data were expressed as media ± SD (n = 6, **p ≤ 0.01 and * p ≤ 0.05 ANOVA – One way – post test Tukey).

4.2. The properties of glutamate release from hypothalamic glial cells under hypertonic stimulation

Although the cellular mechanism involved in glutamate release from astrocytes in hypertonic conditions remains unclear, Jiang et al. (2011) already have described that glial cells under hyperosmotic stimulation release glutamate through Na^+-independent Cx43 hemichannels. It is well established that glutamate transport by astrocytes is controlled by distint Na^+-dependent and Na^+-independent glutamate transporters (Vandenberg and Ryan, 2013). In the current study, it was observed that Na^+ withdrawal inhibits glutamate release from hypothalamic glial cells in a hypertonic medium. These finds support that beyond Na^+-independent Cx43 hemichannels, Na^+-dependent glutamate transporters also mediate glutamate transport induced by Na^+-hypertonicity. In fact, other important cellular mechanisms could be involved in this pheneomenon such as transient inhibition of glutamate uptake and posterior activation of glutamate transport at 10 and 15 min post hypertonic stimulus as well as a hypertonic-induced glutamate release by exocytosis from glial cells. Posterior studies should be performed in order to clarify the precise mechanism controlling glutamate release from hypothalamic glial cells under hypoertonic conditions.

4.3. The purinergic autoregulation of Na^+-hypertonic-induced glutamate release from hypothalamic glial cells

The increase of adenosine levels observed in late periods of hypertonic stimulation strongly suggest an involvement of the purinergic system in the regulation of glutamate release induced by Na + -hypertonicity. Our data supports that isotonic conditions favor adenosine uptake while hypertonic conditions favor adenosine release through

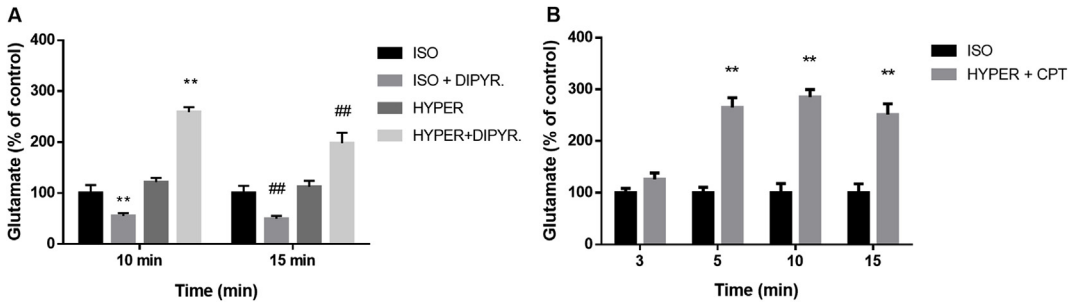

Fig. 3. Effect of (A) adenosine release inhibitor dipirydamole 10 μM and (B) adenosine A1 receptor antagonist CPT at 10 μM on the glutamate release in hypothalamic glial cell cultures under hypertonic stimulus. Data were expressed as media ± SD (n = 6, **p ≤ 0.01 and * p ≤ 0.05 ANOVA – One way – post test Tukey).

adenosine transporters. In this way, dipyridamole treatment inhibits adenosine transporter and accumulates adenosine in the extracellular environment of glial cells exposed to isotonic medium. This phenomenon is followed by intense stimulation of A1 adenosine receptors and consequent decrease in the extracellular glutamate levels. On the other side, dipyridamole incubation in hypertonic medium inhibits adenosine release from glial cells which promote decreased stimulation of A1 adenosine receptors. This phenomenon could explain why we have observed elevated extracellular levels in hypothalamic glial cell cultures exposed to dipyridamole in hypertonic medium. Hypothalamic glial cell cultures treated with a blocker of adenosine release provided further important evidence that elevation of adenosine levels in the extracellular hypertonic environment controls glutamate release. Inhibition of adenosine release lead to maintenance of high extracellular glutamate levels in hypothalamic glial cells exposed to a hypertonic medium. In other words, the absence of adenosine in the extracellular medium does not allow that hypothalamic glial cells remove glutamate from the hypertonic extracellular environment. The use of an adenosine A1 receptor antagonist also led to elevated levels of glutamate in hypothalamic glial cell cultures under a hypertonic stimulus. These results reinforce the participation of the adenosine A1 receptor as a mediator of glutamate release.

5. Conclusions

Taken together these results conclude that (1) hypothalamic glial cells respond to a hypertonic stimulus by the release of glutamate into the extracellular medium and (2) this phenomenon is followed by an increase of extracellular adenosine which (3) activates adenosine A1 receptors (4) blocking the initial glutamate release. The current study demonstrates for the first time that hypothalamic glial cells present a specialized neurochemistry that responds physiologically to hypertonic changes. They utilize the purinergic system to regulate Na^+-dependent glutamate release induced by a hypertonic stimulus.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.02.013.

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