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# Protein kinase A mediates adenosine $A_{2a}$ receptor modulation of neurotransmitter release *via* synapsin I phosphorylation in cultured cells from medulla oblongata

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ABSTRACT

Synaptic transmission is an essential process for neuron physiology. Such process is enabled in part due to modulation of neurotransmitter release. Adenosine is a synaptic modulator of neurotransmitter release in the Central Nervous System, including neurons of medulla oblongata, where several nuclei are involved with neurovegetative reflexes. Adenosine modulates different neurotransmitter systems in medulla oblongata, specially glutamate and noradrenaline in the nucleus tractussolitarii, which are involved in hypotensive responses. However, the intracellular mechanisms involved in this modulation remain unknown. The adenosine A2a receptor modulates neurotransmitter release by activating two cAMP protein effectors, the protein kinase A and the exchange protein activated by cAMP. Therefore, an *in vitro* approach (cultured cells) was carried out to evaluate modulation of neurotransmission by adenosine A2a receptor and the signaling intracellular pathway involved. Results show that the adenosine A2a receptor agonist, CGS 21680, increases neurotransmitter release, in particular, glutamate and noradrenaline and such response is mediated by protein kinase A activation, which in turn increased synapsin I phosphorylation. This suggests a mechanism of A2aR modulation of neurotransmitter release in cultured cells from medulla oblongata of Wistar rats and suggest that protein kinase A mediates this modulation of neurotransmitter release via synapsin I phosphorylation.



## 1. Introduction

Neurotransmitter (NT) release occurs through the very complex process of exocytosis, which is subject to modulation by several factors, such as the presence of NT or neuromodulators in the synapse. This process, at the presynaptic level, is tuned regulated by auto- and hereto-receptors which, by way of a negative or positive feedback mechanism, can adjust the exact amount of NT released for the information to pass to postsynaptic elements, which is important because an imbalance in this process may trigger different brain disorders (Langer, 2008).

Many presynaptic receptors are able to modulate NT release (Ribeiro et al., 1996; Marchi and Raiter, 1996; Kato and Shigetomi, 2001), by direct physical interactions, such as receptor–receptor interaction, as the case of G protein-coupled receptor heterodimerization (Fuxe et al., 2008), or it may involve intracellular signaling pathways, such as kinase protein activation (Fresco et al., 2004). Moreover, the coexistence of more than one NT, which are simultaneously released from the same nerve endings, in particular purines, strongly suggests a modulatory role for adenosine and ATP as neuromodulators in the NT release (Dunwiddie, 1985; Ribeiro and Sebastiao, 1987).

Adenosine is released by most cells and acts as a homeostatic molecule in the nervous system where it influences NT release (Ribeiro et al., 2002). Adenosine $A_{2a}$ receptor ($A_{2a}$R), a $G_s$ protein-coupled receptor, is widely found in the brain, including brainstem regions involved in autonomic functions (Thomas et al., 2000; Zaidi et al., 2006; Matsumoto et al., 2010). Previous results suggest that $A_{2a}$R activation facilitates the release of different neurotransmitters

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such as glutamate (GLU) and noradrenaline (NA), γ-aminobutyric acid, acetylcholine and serotonin in several areas of the central nervous system (CNS) (Castillo-Melendez et al., 1994; Barraco et al., 1995; Cunha and Ribeiro, 2000; Cunha et al., 1995; Rebola et al., 2002; Okada et al., 2001).

The highest density of adenosine uptake sites in the CNS has been observed in the nucleus tractus solitarii (NTS) (Bisserbe et al., 1985), which suggests a special role of adenosine in this nucleus in medulla oblongata. The NTS plays a major role in the regulation of many physiological functions that include cardiovascular, gustatory and respiratory function in several mammalian species (Lawrence and Jarrot, 1996). It has been demonstrated that adenosine, *via* $A_{2a}R$ activation, induce hypotensive responses when administered in the NTS contributing to modulation of autonomic responses mediated by this nucleus (Castillo-Melendez et al., 1994; Barraco et al., 1995; Scislo and O'Leary, 1998; Thomas et al., 2000). In distinct works, Castillo-Melendez et al. (1994) and Barraco et al. (1995) showed, respectively, that $A_{2a}R$ activation increases glutamate and noradrenaline release in the NTS. Nevertheless, the intracellular mechanisms involved in these processes are still not known. Thus, using cultured cells from medulla oblongata the present work analyze the mechanism involved in adenosine $A_{2a}$ receptor activation-induced neurotransmitter release.

$G_s$ protein-coupled receptor activation increases cAMP levels. Second messenger activation is implicated in mediating different aspects of short- and long-term changes in synaptic transmission (Leenders and Sheng, 2005). Furthermore, activation of protein kinases in presynaptic terminals, in particular a classical cAMP target, protein kinase A (PKA), is correlating with increased transmitter release (Capogna et al., 1995; Trudeau et al., 1996, 1998). Nevertheless, a new cAMP receptor was identified, the exchange protein activated by cAMP (EPAC) (de Rooij et al., 1998; Kawasaki et al., 1998). Since then, EPAC-mediated modulation of secretory mechanisms was reported in melanotrophs (Sedej et al., 2005), the crayfish neuromuscular junction (Zhong and Zucker, 2005), as well as in cortical neurons (Huang and Hsu, 2006).

Synaptic vesicle (SV) release depends on an accurate event sequences, including NT transporting to SV, traffic to action zone, docking, fusion and endocytosis. In addition, neurotransmitter release also depends on several specialized proteins, which control different steps leading to exocytosis. Synapsin (syn) is a major SV-specific phosphoprotein, which plays multiple roles in several stages of synaptic transmission, including vesicles clustering, maintenance of the reserve pool, vesicles delivery to active zones, and synchronization of neurotransmitter release events (Bykhovskaia, 2011). These processes are modulated *via* a dynamic phosphorylation/desphophorylation cycle, which involves several kinases, such as PKA (Jovanovic et al., 2001).

The knowledge of these interactions and the detailed comparative analysis of their mechanisms may therefore be relevant for a better understanding not only of the receptor-mediated modulation of secretory mechanisms, but also of the neurotransmitter release physiology and its disorders, which might represent suitable targets for pharmacological intervention by exogenous compounds.

Here, we were interested in the evaluation of intracellular signaling pathway to the $A_{2a}R$-mediated facilitation of neurotransmitter release in cell culture from medulla oblongata of Wistar rats. We show that application of the $A_{2a}R$ agonist CGS 21680 increases synapto-pHLuorin (SpH) and NADH fluorescence as well glutamate and noradrenaline levels in working solution. Only PKA pathway inhibition was able to counteract CGS 21680-mediated GLU and NA increasing levels. Moreover, CGS 21680 induced an increase in synapsin I ($serine_9$) (syn I $ser_9$) phosphorylation and H89 was capable to abolish this response, whereas no change was observed in the CGS 21680-induced GLU and NA release when the cells were treated with EPAC inhibitor. Therefore, our results suggests that $A_{2a}R$ modulation of GLU and NA release in cultured cells from medulla oblongata of Wistar rats is mediated by PKA through synapsin I phosphorylation.

## 2. Methods

### 2.1. Drugs and solutions

Brefeldin A, CGS 21680, H89, GDH and $NAD^+$ (Sigma, USA); ZM 241385 (TOCRIS, USA) and $N^6$ benzoyladenosine-3′,5′-cyclic monophosphate (6Bnz) and chlorophenylthio-2′-O-methyladenosine-3′,5′-cyclic monophosphate (8pCPT) (BioLog, Germany). Working solution (WS) is composed of (mM) 140 NaCl, 5 KCl, 5 $ClCa^2$, 1 $MgCl^2$, 10 HEPES/NaOH and 20 Glucose, pH 7.3 was used for all experiments unless otherwise indicated.

### 2.2. Dissociated dorsomedial medulla oblongata cell culture

All the procedures and protocols were performed in accordance with The Institutional Guidelines for Animal Experimentation (Protocol 114/2010). All efforts were carried out to minimize animal suffering and to reduce the number of experimental animals. Cell cultures were performed using a pool of rats. The rats were euthanized by decapitation and their medulla oblongata were rapidly removed from the skull. Dorsomedial medulla oblongata portion was dissected from one-day old Wistar rats and prepared as previously described by Kivell et al. (2001). Briefly, cells were dissected out and dissociated in cold isotonic salt solution, pH 7.4. Cells were suspended in Neurobasal A media (Invitrogen, USA) supplemented with L-glutamine (250 μmol/L, Sigma), GlutaMax (250 μmol/L, Gibco, USA), B27 (2%, Gibco) and gentamicin (40 mg/L, Gibco). Viable cells were counted using dye exclusion method with Trypan Blue (Gibco, USA) and plated on poly-D-lysine-coated culture 12-well plates (mean of fifteen rats *per* plate) (TTP, USA) for neurotransmitter dosage assays, 96-well plates (mean of six rats *per* plate) for in-cell western assay and confocal dish (mean of six rats *per* plate) (Corning, USA) for image experiments at the concentration of 1800 cells/$mm^2$. Cultures were kept in a humidified incubator with 5%$CO_2$ and 95% air, at 37 °C, for 7 days. On the seventh day the medium was replaced by working solution and the cell culture submitted to pharmacological treatments.

### 2.3. Superecliptic Synapto-pHLuorin transfection

To monitor SV release we used SpH, a reporter in which the pH-sensitive super-ecliptic pHluorin is fused to the intravesicular vesicle protein synaptobrevin2 (Miesenböck et al., 1998). Cells were transfected after 7 days *in vitro* using Lipofectamine LTX in OPTMEM according to the manufacturer's instructions (Invitrogen, Paisley, UK).

One day after transfection, cells in working solution were analyzed by means of time series image showing the SpH response during stimulation. Cell image was analyzed using a Zeiss LSM 510 Axiovert microscope (Zeiss, Germany). A 488 nm line of argon laser and a 364 nm UV light were used to excite SpH. Laser light reflected from a dichroic mirror (HFT/UV/488, Zeiss, Germany) passed through a 40x/1.30 oil lens. Fluorescence transmission passed through an LP485 (Zeiss, Germany) filter for collected 485 nm fluorescence and a LP385 (Zeiss, Germany) filter for collected 385 nm fluorescence, according to Miesenböck et al. (1998) with some minor modification. To calculate fluorescence kinetics, regions of interest (ROIs) were identified, and fluorescence time course of the treatments was recorded. The ROIs were analyzed for 90 frames, which frame took approximately 8 s. Cells were treated on the frames 10 (CGS21680), 30 (ZM241385), 50 (CGS21680) and