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Research Report

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Multi-level regulation of ENT1 in synaptic terminals and lipid rafts

1. INTRODUCTION

1.1. Retina: a biological model of neurochemical interactions of the Central Nervous System (CNS)

The retina originates from the neural ectoderm, the same embryonic origin of other structures of the Central Nervous System (CNS). Its function consists in the reception and conversion of luminous stimuli to electrical signals, as well as its conduction to higher centers such as the visual cortex, which is responsible for information processing.

The retina is composed of the following cell types: ganglion, horizontal cells, amacrine, cone and rod photoreceptors, bipolar cells and Müller glial cells. These cellular types are arranged in five distinct layers, such as a nuclear outer layer, where the photoreceptor cell bodies can be found, an outer plexiform layer, rich in synaptic processes between horizontal cells and bipolar cells and photoreceptors (dos Santos-Rodrigues et al., 2015). Below this region, composed of the nucleus of the bipolar cells, amacrine cells and Muller glial cells, there is an internal nuclear layer, followed by inner plexiform layer and a layer of ganglion cells.

One of the advantages of using the embryonic retina of chicken (*Gallus gallus*) is that it is structurally similar a human retina, and in addition, it presents almost all the neurotransmitters/neuromodulators that are found in other areas of the CNS such as GABA, glutamate, dopamine, acetylcholine and adenosine (Paes-de-Carvalho, 2002). Another positive point is that retina is easily isolated from other tissues during most of the embryonic development and is also an economical and easily accessible model for a dissection.

1.2. Adenosine

Adenosine is a nucleoside composed of ribose and a purine base, adenine. It is released by both neurons and glial cells of the retina and the CNS as a whole. It is a neuromodulator found in several areas of the CNS and interferes with the activity of neurotransmitters, especially in the excitatory synapses, promoting neuroprotection in the retina by the modulation of glutamatergic synapses (Jonsson & Eysteinsson, 2017).

Adenosine receptors are classified as metabotropic because they are associated with a G protein and are subdivided into 4 subtypes, A1, A2A, A2B and A3. The main functional difference between them is the type of G protein to which they are coupled. A1 and A3 receptors are classically bound to the Gi protein, which upon activation inhibits the adenyl cyclase enzyme, thus preventing the formation of cAMP, whereas the receptors A2A and A2B are associated with a Gs protein, which results in increased cAMP levels (dos Santos-Rodrigues et al., 2015).

The accumulation of cAMP in the embryonic retina of chicken induced by activation of A2A adenosine receptors, as well as the presence of nucleoside transporters, are examples of the presence of purinergic markers in the retinal tissue. These transporters, in addition to metabolic enzymes located both intracellularly and intracellularly, are responsible for the regulation of intracellular and extracellular levels of nucleosides (dos Santos-Rodrigues et al., 2015).

1.3. Nucleoside Transporters

Nucleoside transporters (NTs) are integral membrane proteins and may be either concentrative (CNTs), which are sodium-dependent or equilibrative (ENTs), which carry nucleosides independently of sodium and always in favor of the concentration gradient (Paes-de-Carvalho, 2002).

Four subtypes of ENTs are found in the CNS, but ENT1 and ENT2 are the predominant ones. Oliveros and colleagues (2016) demonstrated in rat that the deletion of ENT1 promotes the decrease of the extracellular levels of adenosine and its A1 receptor at the presynaptic terminal, producing a hyperglutamatergic state. This contributes to the recognition of the importance of these carriers in the regulation of adenosine levels.

The contribution of ENTs to purinergic signaling in the brain is fundamental, but our understanding of the regulatory mechanisms of ENT1 is still limited. NTs are regulated post-transcriptionally by PKC (Coe et al., 2002), JNK (Leisewitz et al., 2011), ERKs (dos Santos Santos et al., 2011) and CAMK II (Paes Carvalho et al., 2005).

In terms of transcriptional regulation, HIF-1 (Eltzschig et al., 2005) and c-Jun (Leisewitz et al., 2011) have already been shown to be regulators of ENT1. However, very little is known about other mechanisms of transcriptional regulation, such as regulation by microRNAs.

1.4. MicroRNAs

MicroRNAS are a group of non-coding RNAs, which has shown important regulatory role. More than half of the human transcription is subject to regulation by miRNA. They are formed from 21 to 24 nucleotides that when are bound to argonaute protein, it will form the miRNA-induced silencer complex (miRISC), regulating the expression of mRNAs that present complementary sequences. The binding with argonaute protein is fundamental, because only with this interaction, miRNAs will be able to interact with its target. This

interaction between microRNA and mRNA occurs in the untranslated region 3'-UTR, and generally represses gene expression, promoting blockade or degradation of mRNA (Pasquinelli, 2012).

The miRNAs present great regulatory potential during the development of the organism, in death and cell proliferation, in immunity and hematopoiesis, and therefore many diseases are related to the malfunction of this molecule, such as cancer, neurological and metabolic disorders (Ferrari, 2016). The miR-124 is one of the most found miRNAs in the CNS and we think it may be a possible regulator of ENT1, based on *in silico* analysis.

1.5 Lipid Rafts

Lipid rafts are specialized membrane microdomains enriched in cholesterol, glycosphingolipids and specific proteins. These microdomains act as platforms for the assembly of signaling molecules, and in regulation of different cellular processes such as intracellular trafficking and cellular signaling pathways. Lipid rafts are important for synaptic transmission and plasticity (Sebastiao et al., 2013) and therefore play critical roles in the CNS.

Neurotransmitter/neuromodulator receptors and/or transporters are found in in lipid rafts in the CNS and these microdomains are important for the activity of excitatory amino acid transporters (Butchbach et al., 2004), dopamine transporters (Adkins et al., 2007; Foster et al., 2008; Cremona et al., 2011) and serotonin transporters (Magnani et al., 2004). NTs have been shown to be involved in lipid rafts but not in the CNS (Errasti-Murugarren et al., 2010) and there appears to be a correlation with lipid raft localization and the affinity of the NT with nucleoside-derived drug (Errasti-Murugarren et al., 2011) suggesting that the presence of the NT in the lipid raft affects function.

2. Objectives:

In this project, we were especially interested in two main areas of regulation of ENT1:

1) At the membrane, specifically in the membrane microdomains known as lipid rafts.

2) At the transcriptional level, via microRNAs applied exogenously.

3. Results

3.1. The uptake of 3 [H]-Adenosine is differentially regulated according to the incubation time and the concentration of miR-124

Firstly, the activity of ENT1 in 8-day mixed chicken embryo retinal mixed cultures (E8) was analyzed by the uptake of 3-[H] Adenosine when exposed to different concentrations of miR-124 (25 nM, 50 nM and 100 nM nM) (FIG. 1). These conditions were done within 24 hours (FIG 1: A) and at 48 hours (FIG 1: B).

Exposure for 24 hours did not induce any significant decrease in uptake of 3-[H] Adenosine when compared to the control condition (FIG 1: A). We only detected a slight decrease in activity under miR124 (25 nM) and (50 nM) conditions, but it did not appear to be relevant.

However, in the 48-hour chronic treatment with different concentrations of miR-124, a reduction in adenosine uptake activity of approximately 40% was observed, especially under conditions of miR-124 (50 nM) and (100 nM) (FIG1: B). These results suggest that miR-124 may be modulating the activity of nucleoside transporters in these retinal cultures.

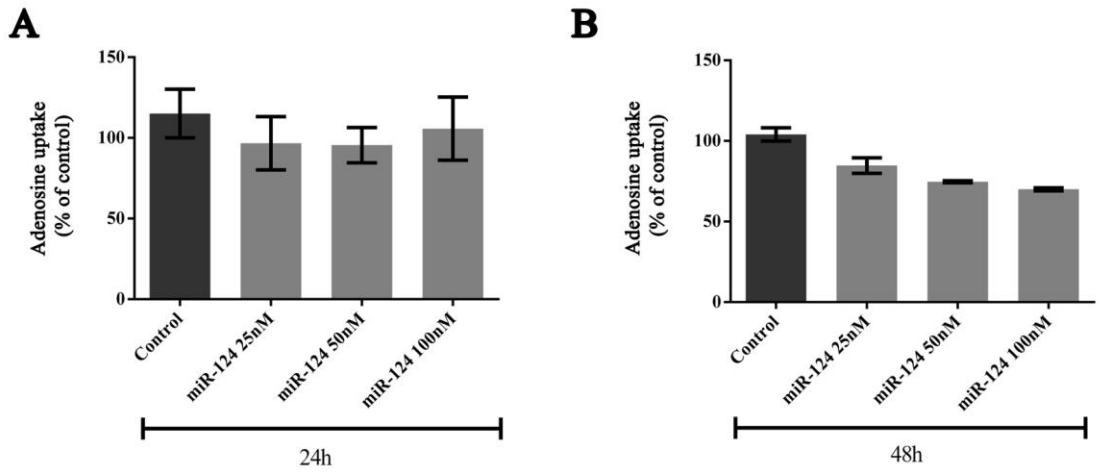

Figure 1. A and B: 3-[H] Adenosine uptake assays, with control conditions and miR-124 at different concentrations (25nM; 50nM; 100nM). A: 24 hours incubation. B: 48 hours incubation.

3.2. Long-term treatment with miR-124 induces changes in ENT1 levels in mixed chicken retina cultures

In parallel with the analysis of the quantification of the activity of nucleoside transporters in these cultures, we also analyzed the protein levels of ENT1, the main transporter expressed in these mixed chicken retina cultures, under the same conditions used in the adenosine uptake assays (control and miR-124 at concentrations of 25 nM, 50 nM and 100 nM (FIG 2) lasting 24 hours (FIG 2: A and B) and 48 hours (FIG 2: C and D).

The data indicate that there was a trend towards increased levels of ENT1 at concentrations of 50nM and 100nM in the treatment for 24 hours (FIG 2: A and B). However, in cultures that were kept for 48 h after transfection with the different treatments, we observed a decrease of approximately 50% of ENT1 levels in the miR-124 (100 nM) condition relative to the control (FIG 2: C and D). These data reinforce the hypothesis that miR-124 may have as one of its regulatory targets the mRNA of ENT1, and consequently a reduction in the protein density of ENT1 levels.

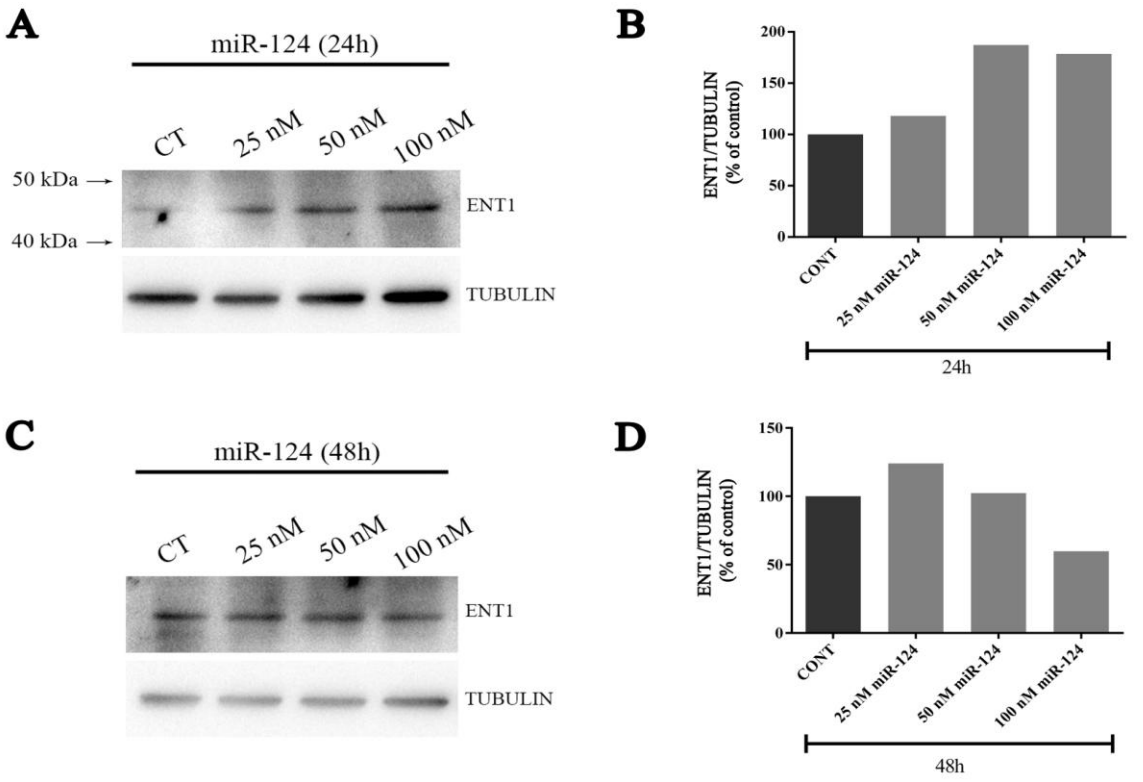

Figure 2. A-D: Quantification of ENT1 protein levels by Western Blot in control conditions and miR-124 at different concentrations (25nM; 50nM; 100nM). A and B: 24 hours. C and D: 48 hours.

3.3. Lipid rafts disruption modulates adenosine uptake activity

As mentioned previously, lipid rafts are specialized membrane microdomains enriched in cholesterol, glycosphingolipids and specific proteins. These microdomains act as platforms for the assembly of signaling molecules, and in regulation of different cellular processes such as intracellular trafficking and cellular signaling pathways. Our aim in this part of the project was to evaluate if ENTs function is regulated by a lipid rafts disruptor, methyl-beta-cyclodextrin (MβC), in mixed cultures of chick retinal cells. We measured ENTs activity through [^{3}H]-adenosine uptake assays. Treatment of mixed cultures with MβCD for 45 minutes significantly decreased adenosine uptake in a concentration-dependent fashion (68.8 ± 6.7% of control levels using MβC 5mM, $p<0.01$, $n=3$) (FIG 3). We did not detect any changes in terms of cellular viability with concentrations of this drug ranging from 0.1 mM to 5 mM.

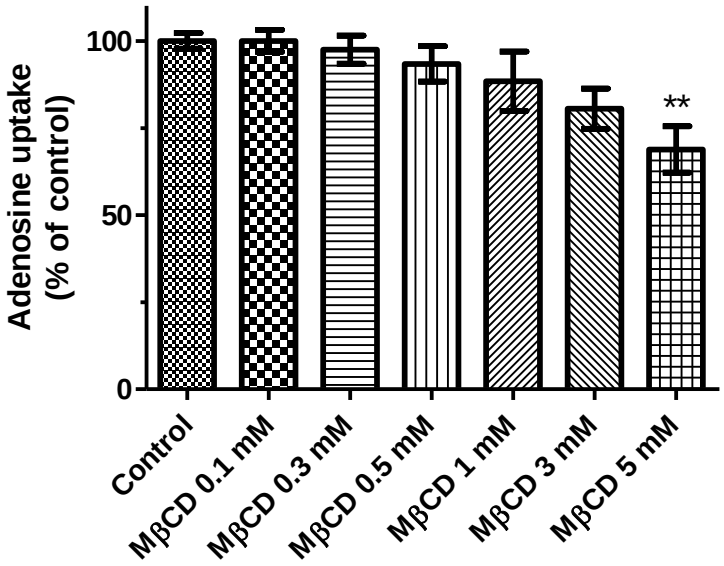

Figure 3. 3-[H] Adenosine uptake assays, with control conditions and MβCD for 45 minutes at different concentrations.

4. Conclusions

Our preliminary results show the possibility that miR-124 could be considered, in the future, as another mechanism for the regulation of ENT1 and, therefore, interfere in intra and extracellular nucleoside levels. However, more experiments need to be performed to actually confirm this hypothesis.

In terms of regulation of nucleoside transporters at lipid rafts, our initial findings reveal a role for these microdomains in ENTs activity, which suggests that changes in plasma membrane cholesterol could modulate purinergic transmission in the brain.

5. Acknowledgements

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The fund from ISN was spent on the acquiring antibodies against ENT1, chemicals, kit of micropipettes, a roller equipment for western blotting membranes and laboratory consumables.

6. Conferences/ Presentations

Part of these results were presented at:

VI Meeting of the Brazilian Purine Club, João Pessoa, Paraíba, Brazil - May 12-14, 2016

"New mechanisms of regulation of ENT1 in the chicken retina" (Invited Speaker)

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