



Effect of the β -amyloid peptide on microglia activation: ATP release

Title in Spanish: *Efecto del péptido β -amiloide en la activación de la microglía: liberación de ATP*

Aida Menéndez Méndez¹, Juan Ignacio Díaz Hernández¹, Felipe Ortega¹, Rosa Gómez Villafuertes¹, Javier Gualix^{1,*}

¹Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria. Instituto Universitario de Investigación en Neuroquímica (IUN). Instituto de Investigación Sanitaria del Hospital Clínico San Carlos (IdISSC). Universidad Complutense de Madrid, 28040 Madrid, España.

ABSTRACT: Previous studies have shown a key role of microglial cells in the neuroinflammatory processes associated with some neurodegenerative diseases, such as Alzheimer's disease (AD). Microglia sense several types of diffusible molecules that regulate the multiple repertoire of microglial functions. Among them, extracellular nucleotides, acting on microglial P2 receptors, have central roles. In this sense, the ionotropic P2X7 receptor has gained recognition as a key regulator of microglial-mediated inflammatory responses.

It is known that microglia releases ATP and other nucleotides to the extracellular medium. Although several mechanisms, such as release through connexins or panexins, has been proposed, a vesicular origin for this released nucleotides, relying on the activity of the vesicular nucleotide transporter (VNUT), cannot be ruled out.

In this work we evaluated whether the expression of VNUT and the P2X7 receptor, as well as the ATP release, could be modified in the reactive microglia. To achieve microglia activation we stimulated the cells with the lipopolysaccharide (LPS). Moreover, we analyzed the effect of the β -amyloid peptide β_{1-42} , which is also able to activate the microglial cells, on the expression of VNUT and the ATP release in the microglia.

RESUMEN: Estudios previos han mostrado un papel clave de las células microgliales en los procesos neuroinflamatorios asociados con algunas enfermedades neurodegenerativas, como la enfermedad de Alzheimer (EA). La microglía detecta varios tipos de moléculas difusibles que regulan el múltiple repertorio de funciones microgliales. Entre ellos, los nucleótidos extracelulares, actuando sobre los receptores P2 microgliales, llevan a cabo un papel central. En este sentido, el receptor P2X7 ionotrópico ha sido reconocido como un regulador clave de las respuestas inflamatorias mediadas por la microglia.

Se sabe que la microglía libera ATP y otros nucleótidos al medio extracelular. Aunque se han propuesto varios mecanismos, tales como la liberación a través de conexinas o panexinas, no se puede descartar un origen vesicular para estos nucleótidos liberados, basándose en la actividad del transportador vesicular de nucleótidos (VNUT).

En este trabajo hemos analizado si la expresión de VNUT y el receptor P2X7, así como la liberación de ATP, podrían modificarse en la microglía reactiva. Para lograr la activación de la microglía estimulamos las células con el lipopolisacárido (LPS). Además, analizamos el efecto del péptido β 1-amiloide, β_{1-42} , que puede activar también las células microgliales, sobre la expresión de VNUT y la liberación de ATP en la microglía.

*Corresponding Author: jgualix@ucm.es

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1. INTRODUCTION

Microglial cells constitute the resident immune cell population of the mammalian central nervous system (CNS). These cells monitor environmental changes and serve as damage sensors within the brain. Any type of injury or pathological process leads to activation of the microglia from their surveillant/resting state. In response to injury, microglial cells change their morphology taking on an amoeboid appearance and then start to migrate to the

site of injury, proliferate, produce/release several substances that affect pathological processes, such as pro-inflammatory cytokines, tumor necrosis factor (TNF)- α and interleukin (IL)-6 or IL-12, or even phagocytose damaged cells or debris to maintain the brain homeostasis (1).

However, excessive activation of the microglia could contribute to the progression of chronic neurodegenerative

diseases, though the underlying mechanisms are still unclear. Regarding Alzheimer's disease (AD), this disorder is characterized by complex molecular and cellular alterations, including the development of extracellular amyloid-beta ($A\beta$) deposits, intracellular aggregated phosphorylated tau, dystrophic neurites, loss of synapses and neurons, and a prominent gliosis. The reactive gliosis involves alterations in morphology and function of microglia and astrocytes. It has long been recognized that $A\beta$ neuritic plaques are surrounded by activated microglial cells that could contribute to $A\beta$ phagocytosis and/or compaction (2, 3). In this sense, microglial activation could improve the AD pathology reducing the $A\beta$ levels (4). However, the release of pro-inflammatory mediators, such as cytokines or chemokines, from reactive microglia is thought to contribute to the chronic inflammatory response associated to AD, which has a prominent role in the progression and severity of the disease (5, 6).

Nucleotides act as widespread extracellular signaling molecules. The physiological effects of these compounds are mediated through an extended family of purinoceptors that are classified into ionotropic P2X or metabotropic P2Y receptors (7, 8). Microglia express multiple P2 receptors that differentially sense extracellular nucleotides and, therefore, P2 receptor-mediated microglial responses have received much attention (9-12). Among these purinoceptors, the P2X7 receptor has gained prominent recognition as a regulator of inflammatory responses (13). Microglial cells express P2X7 receptors (14, 15) which drive microglial activation and proliferation (16). P2X7 receptors are overexpressed in microglial cells in the neuroinflammatory foci of numerous neurodegenerative conditions (17) and the activation of these P2X7 receptors is coupled to the maturation and secretion of the key pro-inflammatory cytokine IL-1 β (18), thereby triggering or potentiating neuroinflammation. These results lead to propose the use of P2X7 receptor antagonists for the treatment of CNS inflammation (19, 20). Interestingly, the P2X7 receptor seems to be also involved in the regulation of the processing of the amyloid precursor protein (APP) (21) and the *in vivo* inhibition of this receptor conducted to a reduction of the amyloid plaques in a mouse model of AD (22).

Microglia sense ATP or other nucleotides by multiple P2 receptors after which they change in different phenotypes. However, it is necessary to investigate what is the origin of extracellular ATP in the brain under both physiological and pathological conditions. Microglia have been shown to release ATP when the cells are exposed to different stimuli, such as ATP or glutamate (23, 24). The signaling pathways that mediate the ATP release from microglia remain unclear, although Connexin 43 (Cx43) hemichannels have been shown to be involved (25). However, a recent work showed that microglial cells express the nucleotide vesicular transporter (VNUT), which transports cytosolic nucleotides into vesicles using a proton-mediated membrane potential as a driving force,

and these cells are able to release ATP by VNUT-dependent exocytotic mechanisms (26).

Due to the close relationship that exists among ATP, the P2X7 receptor and microglial cells in inflammatory and neurodegenerative processes, where the extracellular nucleotide can have a vesicular origin, we evaluated whether the expression of VNUT and the P2X7 receptor, as well as the ATP release, could be modified in the reactive microglia. To achieve microglia activation we use the lipopolysaccharide (LPS), one of the main components of gram-negative bacterial cell wall, that induces microglial cells activation through interaction with the Toll-like receptor 4 (TLR4) (27). Additionally, we analyzed the effect of the β -amyloid peptide β_{1-42} , which is able to activate and stimulate the phagocytic activity of the microglial cells (28), on the expression of VNUT and the ATP release in the microglia.

2. MATERIALS AND METHODS

2.1. Cell Culture

Primary cultures of microglial cells were performed according to the procedure described by (29), with some modifications. Briefly, the whole brain was removed aseptically from postnatal day 5 C57BL/6 mice and submitted to digestion with papain 100 U/ml (Worthington, Lake Wood, NJ) (previously activated in Earle's Balanced Salt Solution (EBSS) buffer containing 5 mM L-Cys and 2 mM EDTA), in the presence of 100 U/ml of DNase (Worthington, Lake Wood, NJ), 1 mM CaCl₂ and 1 mM MgCl₂. Then, cells were resuspended in Dulbecco's Modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum, 25 mM glucose, 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 2.5 μ g/ml amphotericin, and they were plated in culture flasks at a density of 70,000 cells/cm². Cells were maintained in culture until they reached confluence (approximately 10–12 days), replacing the medium every 3–4 days. This procedure allows obtaining an astroglial culture, as neurons do not survive under such conditions. To separate astrocytes and microglia, flasks were shaken at 250 rpm for 2 h at 37°C in an orbital shaker, and after that, the supernatant contains microglia whereas astrocytes remains adhered to the culture surface. Culture medium was then centrifuged at 200 x g for 10 min and microglial cells were resuspended and seeded onto culture plates.

Treatment with the lipopolysaccharide (LPS) was initiated 48h after seeding and the compound was added at 1 μ g/ml concentration for 24h (26). In the case of the treatment with β_{1-42} , the β -amyloid peptide was prepared according to (30, 31) and added at a final concentration of 5 μ M for 18h. Control non-amyloidogenic β_{42-1} peptide was prepared and added under the same conditions.

2.2. Reverse Transcription PCR and Quantitative Real-Time PCR

Total RNA was obtained from cultured microglia using a SpeedTools Total RNA Extraction kit (Biotools), following the manufacturer's instructions. After

purification, the total RNA was quantified and 1 μ g was reversed transcribed using M-MLV reverse transcriptase, 6 μ g of random primers and 350 μ M deoxynucleotide triphosphates (dNTPs) (all from Thermo Fisher Scientific). Quantitative real-time PCR (qPCR) reactions were performed in final volume of 25 μ l using the LuminoCt qPCR Ready Mix (Sigma–Aldrich), 5 μ l of the cDNA synthesized previously and 1.25 μ l of specific commercial TaqMan[®] gene expression assays for mouse: VNUT (Mm00805914_m1), P2X7 (Mm00440582_m1), P2Y₂ (Mm02619978_s1) and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Mm99999915_g1), all from Applied Biosystems. The qPCR assays were carried out using a StepOnePlus Real-Time PCR System (Applied Biosystems) as follows: denaturation at 95°C for 20 s, followed by 40 cycles of 95°C for 1 s and 60°C for 20 s. The expression of each gene was normalized to that of the endogenous housekeeping gene GAPDH amplified in parallel.

2.3. Western Blotting

Microglial cells were lysed and homogenized for 1 h at 4°C in lysis buffer (pH 7.4) containing 50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P40 and Complete[™] Protease Inhibitor Cocktail Tablets (Roche Diagnostics GmbH). The cell lysate was then centrifuged at 16,000 \times g for 9 min and the supernatants collected. Protein extracts were resolved on 10% SDS-PAGE gels and transferred to nitrocellulose membranes, which were then blocked for 1 h at room temperature (~25°C) with 3% bovine serum albumin (BSA) in Tris buffered saline (TBS) (10 mM Tris, 137 mM NaCl, 2.7 mM KCl, 5 mM Na₂HPO₄, 1.4 mM KH₂PO₄ [pH 7.5]) with 0.1% Tween 20. The membranes were probed with primary antibodies raised against VNUT (1:500, MBL) or P2X7 (1:1000, Alomone Labs). Antibody binding was detected by ECL chemiluminescence (Amersham GE Healthcare), and images were captured with ImageQuant LAS 500 (GE Healthcare Life Sciences) and analyzed using ImageQuant TL.

2.4. Immunofluorescence

Microglial cells cultured on coverslips in 35 mm dishes were fixed for 15 min with 4% paraformaldehyde and then rinsed with phosphate buffered saline (PBS) twice for 10 min. Afterward, coverslips were blocked and permeabilized for 1 h at room temperature with blocking solution (0.1% Triton X-100, 5% fetal bovine serum and 10% bovine serum albumin in PBS). The coverslips were then incubated overnight at 4°C with the corresponding primary antibodies: rabbit anti-VNUT (1:100, MBL), mouse anti-Iba1 (1:300, Wako) or rat anti-CD11b (1:500, ThermoFisher Scientific). The following day, the cells were washed three times with PBS and incubated with the corresponding fluorescent-tagged secondary antibodies

(Thermo Fisher Scientific) for 1 h at room temperature. The nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific) and the coverslips were finally mounted on glass slides using FluoroSave[™] Reagent (Calbiochem). Images were acquired on a Leica TCS SPE confocal microscope.

2.5. ATP release

Adenosine triphosphate release was measured using the ENLITEN[®] rLuciferase/Luciferin reagent (Promega). Microglial cells were maintained for 30 min at 37°C in Mg²⁺-free Locke's buffer (140 mM NaCl, 4.5 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 5.5 mM glucose and 10 mM HEPES [pH 7.4]) containing 100 μ M ARL67156, a competitive inhibitor of ecto-ATPases (32), and subsequently, 50 μ l of extracellular medium was collected to measure the ATP levels. The samples were centrifuged at 600 \times g for 5 min at 4°C and 10 μ l of supernatant were transferred onto 96-well plate placed on ice. The plate was then situated in a FLUOstar OPTIMA Microplate Luminometer (BMG LABTECH GmbH) and 100 μ l of rLuciferase/Luciferin reagent was automatically injected into each well at room temperature.

2.6. Statistical Analysis

The data shown are mean values \pm standard error of the mean (s.e.m) and all independent experiments shown were reproduced 3–6 times. The figures were generated and the statistical analyses performed using GraphPad Prism 6 (GraphPad Software). The results were analyzed using an unpaired Student's *t*-tests. A *p*-value \leq 0.05 was considered statistically significant.

3. RESULTS

3.1. Effect of LPS treatment in the expression of purinergic receptors and VNUT in the microglia

Microglial cells were stimulated with 1 μ g/ml LPS for 24 h and mRNA levels of the P2X7 receptor and VNUT were analyzed. In order to confirm the effectiveness of the treatment, mRNA levels of the purinergic P2Y₂ receptor, which expression increases after stimulation with the lipopolysaccharide (33), were also measured.

As can be observed in Figure 1, LPS treatment induced a significant increase in metabotropic P2Y₂ receptor expression, whereas P2X7 mRNA levels were not affected. On the other hand, VNUT mRNA levels were significantly reduced after LPS stimulation (Figure 1A).

The protein levels of the nucleotide vesicular transporter and the P2X7 receptor were also measured by Western blot analyses and a marked reduction in the amount of both proteins was observed after LPS treatment (Figures 1D-F).

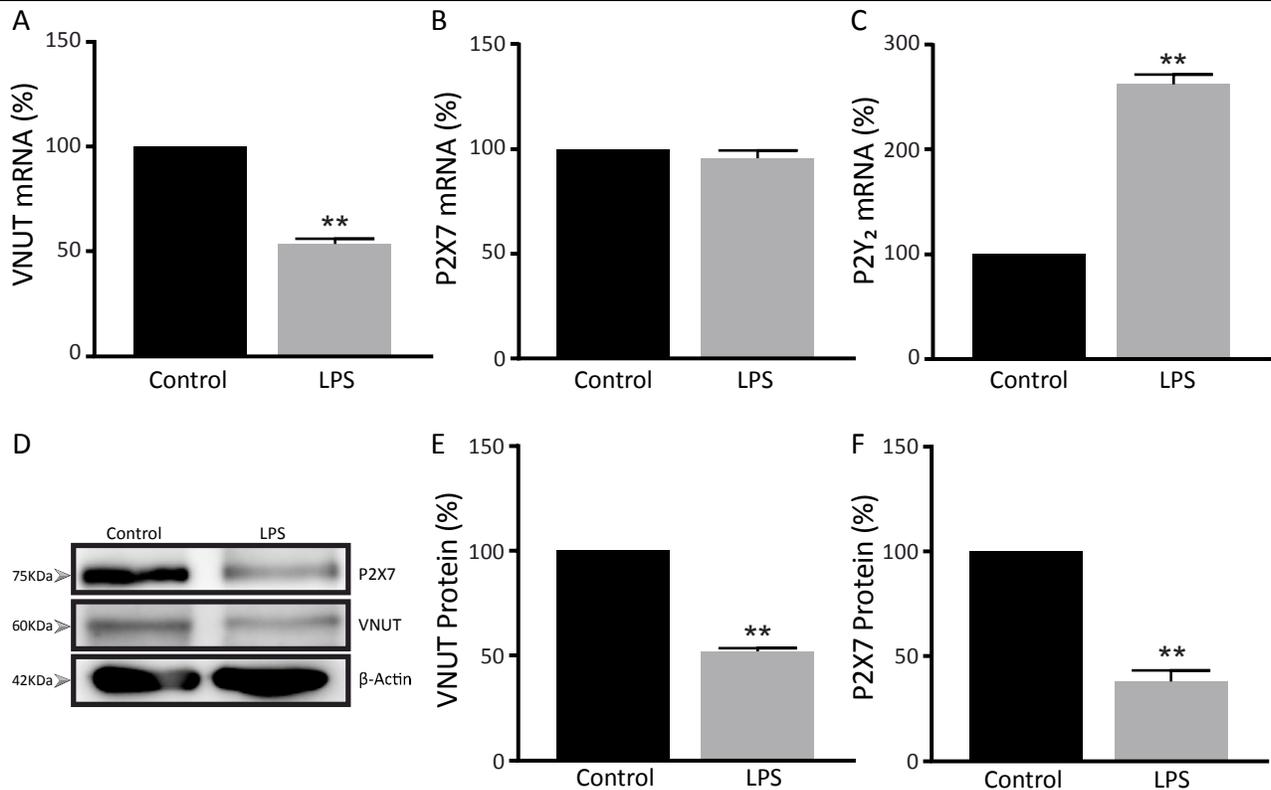


Figure 1. Effect of LPS treatment in the expression of purinergic receptors and VNUT in microglia cultures. Q-PCR analysis of mRNA levels of VNUT (A), P2X7 receptor (B) and P2Y₂ receptor (C) after stimulation with LPS (1µg/ml) for 24h. Values are represented as a percentage of those obtained in untreated (control) cells, which were set as a 100%. (D) Western blot detection of VNUT and P2X7 proteins in cellular lysates of both LPS treated and untreated microglial cells. VNUT (E) and P2X7 receptor (F) protein levels were normalized with respect to β-actin. Values are represented as a percentage of those obtained in untreated cells, which were set as a 100%. Data are the mean ± SEM of three independent experiments. **p < 0.01 (Student's *t*-test).

3.2. Effect of LPS treatment in the ATP release by the microglia

The effect of LPS in the microglial culture was visualized by immunofluorescence techniques, using a specific marker of microglial cells, such as the Iba1 (ionized calcium-binding adapter molecule 1) protein (34). As can be observed in figure 2A, microglial cells acquire an amoeboid morphology that is characteristic of the reactive microglia after 24h of treatment with the lipopolysaccharide, which confirmed that the LPS treatment was effective. Regarding the distribution of the nucleotide vesicular transporter, this was mainly localized

in the proximal zone to the cell nucleus (Figure 2A).

The release of ATP after the treatment with LPS was measured by luminometric techniques. As can be observed in Figure 2B, the treatment with the lipopolysaccharide for 5 min induced a significant increase in the luminescence values, which means a higher liberation of the nucleotide to the extracellular medium. These results are in agreement with those obtained by other research groups (35). However, the stimulation with LPS for 24h did not significantly modify the recorded luminescence values (Figure 2C).

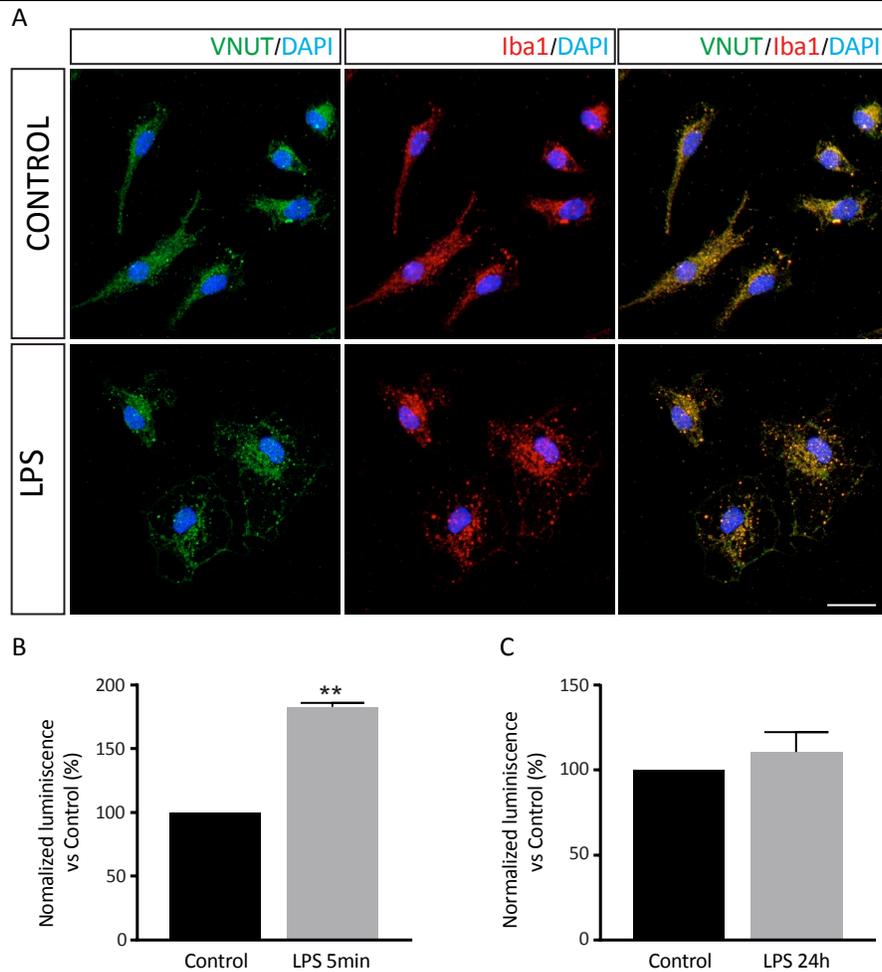


Figure 2. Effect of LPS treatment in the ATP release by the microglia. (A) Representative photomicrographs of quiescent (upper panels) and LPS treated (lower panels) microglial cells showing VNUT (green) and Iba1 (red) immunodetection. Nuclei are counterstained with DAPI (blue). A merge image is shown. Scale bar = 15 μ m. (B, C) Luminometric measure of the extracellular ATP in microglial cells treated with LPS for 5 min (B) or 24 h (C). Luminiscence values were normalized with respect to those obtained in untreated (control) cells and are represented as a percentage. Data are the mean \pm SEM of three independent experiments. ** $p < 0.01$ (Student's t -test).

3.3. Effect of the amyloid peptide β_{1-42} in VNUT expression in microglial cells. Release of ATP

Stimulation of the microglia with the amyloid peptide β_{1-42} at a 5 μ M concentration for 18h resulted in a significant increase in the ATP release to the extracellular medium when compared with the effect of the non-amyloidogenic (control) β_{42-1} peptide (Figure 3A). However, VNUT expression was unaffected by the

treatment with the amyloidogenic peptide as both the mRNA and protein levels of the transporter remained constant in β_{1-42} or β_{42-1} treated cells (Figura 3B).

Moreover, the treatment with the amyloidogenic peptide induced a morphological change in the microglial cells which acquired the amoeboid morphology that is characteristic of the reactive microglia (Figure 3C).

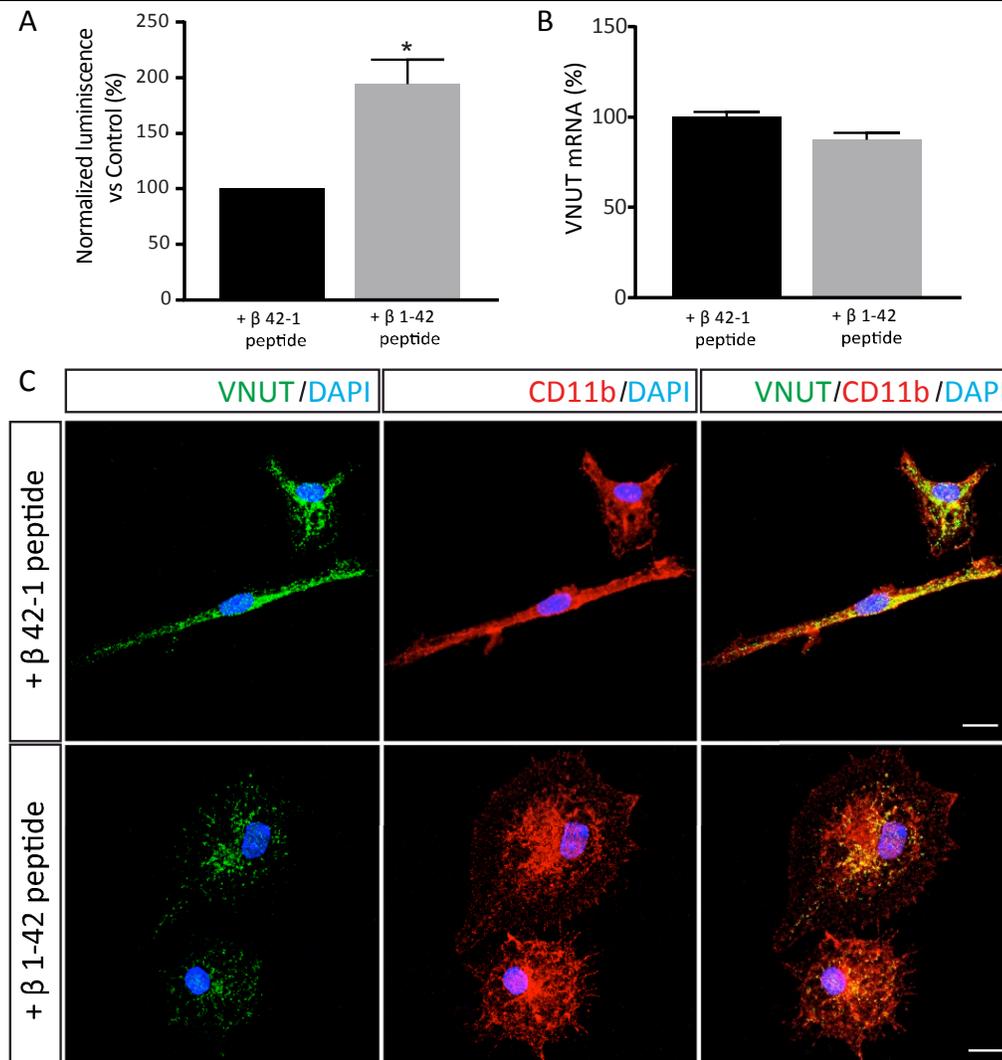


Figure 3. Effect of the amyloid peptide β_{1-42} in VNUT expression in microglial cells. Release of ATP. Luminometric measure of extracellular ATP (A) and Q-PCR analysis of the VNUT mRNA levels (B) in microglial cells treated with the control peptide β_{42-1} or the amyloidogenic peptide β_{1-42} for 18h. Values were normalized with respect to those obtained in cells treated with the control peptide and are represented as a percentage. Data are the mean \pm SEM of three independent experiments. * $p < 0.05$ (Student's *t*-test). (C) Representative photomicrographs of microglial cells treated with the control peptide β_{42-1} (upper panels) or the amyloidogenic peptide β_{1-42} (lower panels) showing immunodetection for VNUT (green) and the microglial cell marker CD11b (red). Nuclei are counterstained with DAPI (blue). Scale bar = 10 μ m.

4. DISCUSSION

A common characteristic of the CNS diseases that courses with inflammation is that the activation of microglial cells occurs at the initial stages (36, 37). Then, cells migrate to the damaged area where they carry out phagocytic processes and mediate the inflammatory responses (1). Purinergic system plays an important role in the regulation of such processes, ATP being a key molecule in the development of microglial functions. Due to the relevance of the presence of extracellular ATP in the physiopathological context of the microglia, we asked whether such presence could be mediated by a VNUT-dependent mechanism.

Our first methodological approach was the activation of the microglia through the use of the lipopolysaccharide

LPS. Stimulation with this bacterial endotoxin has been used as a study model for neuroinflammatory processes, which are present in neurodegenerative diseases (38, 39). The conditions of the treatment of the microglia with LPS were based on previous studies carried out in a microglial cell line (26). The effectiveness of this stimulation was confirmed by the morphological change observed, microglial cells acquiring an amoeboid morphology, as well as by the increase in the expression of the P2Y₂ receptor (33). LPS treatment did not modify the mRNA levels of the P2X7 receptor, although a significant reduction in the amount of the protein was observed. These results could reflect a change in the turnover or degradation of the P2X7 protein induced by the lipopolysaccharide. In the case of VNUT, the 24-hour treatment with LPS produced a significant decrease in its

expression both at the mRNA and protein levels. These data does not agree to those described in a previous study in which the vesicular transporter was identified in the microglia (26). In this case, although the conditions tested were the same, the authors of the work used a cell line instead of a primary microglial culture. Although it is a widely used study model, it is important to consider the origin of this microglial cell line, obtained from the brain of a transgenic mouse deficient in the p53 gene (40). The absence of this gene, known as the guardian of the genome, enhances the proliferative potential of the cells (41), so that the pattern of gene expression can differ considerably from that of the primary microglia from wild type animals. Likewise, in other microglial cell lines, discrepancies have been observed in the responses to the same treatment, not only with respect to the primary microglial culture, but also in the same cell line used (42). Therefore, this could explain the differences observed regarding the expression of VNUT.

On the other hand, LPS caused a significant increase in the amount of ATP released to the extracellular medium in a five-minute interval, which is coincident with previous data obtained by other research groups (35). These results confirm that LPS induces the release of ATP in the microglia, with the initial response being very pronounced. However, the luminescence values recorded in LPS treated cells for 24 hours did not significantly differ from the control values. We cannot define the mechanism involved in LPS-induced ATP release from microglial cells. Despite this, the robust expression of VNUT in both LPS and untreated cells make possible that such release could be mediated at least in part by VNUT-dependent exocytotic mechanism. However, the decrease in transporter expression by treatment of the microglial cells with LPS suggests that another mechanism other than exocytosis, such as release through connexins or panexins (25, 43), could mediate the ATP release from these cells.

Our second methodological approach was based in the use of the amyloid peptide β_{1-42} . Again, the microglial cells acquired an amoeboid morphology, similar to observed after LPS treatment and typical of reactive microglia, following stimulation with the β_{1-42} peptide. In this case, treatment with the amyloid peptide β_{1-42} for 18 hours did not alter the expression of VNUT, but produced a significant increase in the extracellular levels of ATP. β_{1-42} -induced ATP release from microglial cells has been described in a previous work (44). However, in this case acute (less than one hour) stimulation periods were used. Our results, however, showed long-term potentiation of ATP release by β_{1-42} peptide treatment in microglial cells. Although it cannot be confirmed that the release of ATP is mediated by a VNUT-dependent exocytotic mechanism, this idea cannot be ruled out, taking into account the expression of the transporter in the microglial cells that, unlike the treatment with LPS, remains unchanged in β_{1-42} stimulated cells.

It has been previously shown that microglial cells express the amyloid precursor protein (APP) and hence

these cells are able to produce and release the β -amyloid peptide (45-47). The ATP released by the microglia in response to β_{1-42} peptide stimulation could activate microglial P2 receptors that in turn regulate β -amyloid peptide formation. It has been described that P2X7 and P2Y₂ receptors modulate the α -secretase-dependent processing of the APP protein, these receptors having opposite effects on β -amyloid peptide generation (21). Activation of the P2X7 receptor leads to a reduction of α -secretase activity which enhances the amyloidogenic processing of the APP and the formation of β -amyloid peptide. However, activation of the P2Y₂ receptor increases α -secretase activity, thus precluding the generation of the β -amyloid peptide (21). As activation of the microglial cells lead to reduction in the expression of the amyloidogenic P2X7 receptor and to an increase in the expression of the non-amyloidogenic P2Y₂ receptor, this could represent a negative feedback mechanism by which the β -amyloid peptide limits or reduces its own formation and secretion by the microglia. Additionally, it has been previously described that the nucleotides released by the microglia in response to β_{1-42} peptide stimulation enhance cell migration and promote β -amyloid peptide phagocytosis through activation of the P2Y₂ receptor (44). However, to have a complete image of this process it is necessary to take also into account the effect of the microglial ATP release on purinergic receptors expressed in cells other than microglia, such as surrounding neurons and astrocytes.

5. CONCLUSIONS

ATP and other nucleotides are important signal molecules that regulate a wide diversity of microglial functions. One of the possible sources of extracellular nucleotides in the CNS is their release from microglial cells. It has been demonstrated that microglia are able to release ATP and other nucleotides in response to a variety of stimuli.

Our results demonstrate that activation of microglial cells by LPS or the β -amyloid peptide β_{1-42} induces an increase in the ATP release, measured by luminometric techniques. This release could be mediated, at least in part, by a VNUT-dependent exocytotic mechanism, as VNUT appear to be robustly expressed by both resting and reactive microglia. However, in the case of the activation by LPS, the reduction in the expression of the transporter in activated cells, suggests that a different mechanisms, other than exocytosis, could be also involved in the ATP release from these cells.

Moreover, activation of microglial cells induces a reduction in the expression of the purinergic P2X7 receptor and an increase in P2Y₂ receptor expression. In the case of the activation by β_{1-42} peptide, it can be hypothesized that this could represent a negative feedback mechanism by which the β -amyloid peptide limits their own formation and release by microglial cells.

Taken together, our results highlight the relevance of a new element, the nucleotide vesicular transporter (VNUT),

and its role in the ATP release, in the complex interplay among extracellular ATP, purinergic receptors and microglial cells in the context of neuroinflammatory responses in neurodegenerative diseases.

6. ACKNOWLEDGEMENTS

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