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Biochemistry

Melatonin Induces Redistribution of mGluR5 and NHERF1 Heterocomplex in Jurkat Lymphoid Cells

Nana Narmania[*,], Maia Sepashvili[*,**], David Mikeladze[*,**,§]**

[*] *School of Natural Sciences and Medicine, Ilia State University, Tbilisi, Georgia*
[**] *Laboratory of Biochemistry, Iv. Beritashvili Center of Experimental Biomedicine, Tbilisi, Georgia*
[§] *Academy Member, Georgian National Academy of Sciences, Tbilisi, Georgia*

The mGluR5 is constitutively expressed in different immune cells, such as macrophages and T-lymphocytes, which influence cytokine release against bacteria and viruses; however, in some types of lymphoid cells, mGluR5 promotes tumor growth and drives neoplastic transformation. Conversely, melatonin, a frequently measured circadian marker, has been shown to attenuate neoplastic transformation and suppress tumor development through the metabolic reprogramming of cancer cells. However, the extent of melatonin's involvement in regulating mGluR5 activity in immune cells remains unclear. Therefore, we investigated the effect of melatonin on mGluR5 expression in Jurkat lymphoid cells. Through subcellular fractionation of mGluR5, we observed a significant receptor presence in plasma membranes, with a smaller fraction localized in endoplasmic and nuclear membranes. Furthermore, our findings demonstrated that melatonin treatment increased the expression of mGluR5 in the microsomal and nuclear fractions. Additionally, by utilizing co-immunoprecipitation techniques, we discovered that mGluR5 co-localized with the NHERF1 scaffold protein in endomembranes, suggesting a potential role for this protein in the trafficking and regulation of mGluR5. Notably, in the nuclear fraction of melatonin-treated cells, we observed an increase in mGluR5-dependent phosphorylation of ERK, while the phosphorylation of Akt remained unchanged. Our findings suggest a novel regulatory mechanism by which melatonin modulates mGluR5 expression and activity, particularly in immune cells. This could have implications for understanding the complex interactions between circadian rhythms, immune responses, and cancer.

jurkat cell, melatonin, metabotropic glutamate receptor

The metabotropic glutamate receptor 5 (mGluR5) is an essential regulator of excitatory and inhibitory transmission in the brain. Its improper activity is associated with several neurological and psychiatric disorders [1]. In nonneuronal cells, overexpression of mGluR5 is correlated with cancer progression [2]. The main part of the receptor is localized in the plasma membrane, and a lesser amount of the receptor is found in the nuclear and endoplasmic membranes. In the nuclear membranes, mGluR5 appears tethered in place via its C-terminal domain interactions with the DNA/chromatin. By stably anchoring on the chromatin, nuclear mGluR5 regulates transcription and chromosomal activities [3]. However, the mechanisms responsible for controlling the internalization of mGluR5

have not been fully characterized, despite their clear importance for receptor function.

Group I/5 mGluR receptors are regulated via dynamic protein-protein interactions. In addition to the guanine nucleotide-binding proteins (G proteins), mGluR5 interacts with many other proteins. Several protein kinases, scaffolding, and adaptor proteins were found to bind directly to the mGluR1/5 receptors [4]. Constitutive or activity-dependent interactions between mGluR1/5 and their interacting partners modulate trafficking, anchoring, and expression of the receptors. Among others, mGluR5, via the PDZ domain, binds to CAL/Gopc/PIST and NHERF/EBP50 proteins [5]. These proteins can selectively modulate the trafficking of mGluR5, which may contribute to specific intracellular signaling of mGluR5. In lymphocytes, both proteins bind to the mGluR1/5 that modulates the trafficking of other PDZ-domain-containing proteins, like CFTR (cystic fibrosis transmembrane conductance regulator) [6].

It has been suggested that the mGluR5 may regulate various physiological processes, including the sleep/wakefulness cycle. [7] mGluR5 availability increases by 10% during the sleep phase in rats [8] and a circadian pattern of mGluR5 availability has been demonstrated in healthy individuals [9]. mGluR5 activity can alter various metabolic processes, including the tryptophan-melatonin pathway. Glutamate, through activation of mGluR5, can increase the N-acetylserotonin/melatonin ratio in cells, which changes the survival of cells through activation of the TrkB receptor [10]. The activation of TrkB by N-acetylserotonin fluctuates in a circadian rhythm [11], which, for its part, is regulated by various endocrine compounds, including melatonin. Melatonin is one of the most frequently measured circadian markers. In health, sleep propensity increases approximately two hours after melatonin secretion rises, and these levels remain high in nighttime darkness [12]. However, the involvement of the intracellular mGluR5 in the mitochondrial melatonergic pathway is unknown. It should be noted that sleep deprivation, the light-induced suppression of melatonin, and lifestyle changes are important mechanisms that could explain the possible link between shift work and cancer risk [13]. Thus, when circadian homeostasis is disrupted, deregulation of the immune system produces immune suppression and the accelerated development of tumors. An important connection between the oncostatic effects of melatonin and circadian machinery is its ability to reduce the levels of c-Myc, which leads to antitumor and antiproliferative effects [14]. However, the extent of melatonin's involvement in regulating mGluR5 activity in immune cells remains unclear. Therefore, we investigated the effect of melatonin on mGluR5 expression and activity in Jurkat lymphoid cells.

Material and Methods

Cell line. Jurkat cells (clone E6-1, a human acute T-cell leukemia cell line) were purchased from ATCC (Manassas, VA, USA) and cultured in suspension in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (Invitrogen, USA), 1% (v/v) sodium pyruvate, 1% l-glutamine, and 100 Units/ml penicillin–streptomycin (Invitrogen, USA). The cultured cells were kept under humidity and at 37°C in a 5% CO_2 incubator. The Jurkat cells were seeded onto a 96-well plate at a density of 1×10^5 cells/well.

Cell lysate preparation and subcellular fractionation. Cells (1×10^5 cells) were incubated with 10^{-6} M melatonin for a 24 h. The viability of cells was measured using a trypan blue colorimetric assay. Incubated cells were lysed using a hypotonic buffer (20 mM Tris-HCl, pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, 0.5 mM PMSF, 0.1% IGEPAL and passed through a 25 Ga needle 10 times using a 1 mL syringe. Subcellular fractions of Jurkat cells were obtained after the lysis of cells by sequential centrifugation, as described previously [15]. The nuclear pellet was obtained from a 7-min 1000 g spin, the plasma membrane

fraction from a 20-min 20000 g spin, the microsomal fraction from a 1-h 100000 g spin, and the cytoplasm from the remaining supernatant. Proteins obtained from subcellular fractionation were resuspended by buffer A (20 mM HEPES, 150 mM NaCl, 1 mM DTT, 10 mM EDTA, 2 mM EGTA, 0.1% SDS, 1% Triton x-100, 1% CHAPS, 0.5% IGEPAL, 0.1% BSA, pH 7.5, and protease inhibitors) incubated overnight at 4°C temperature and re-centrifuged for 40 min at 14.000 × g. Solubilized supernatants were used for Western blotting.

Immunoprecipitation and Western blotting. Aliquots of protein fractions with similar protein amounts were incubated overnight at 4°C with mGluR5 antibody-bound protein A/G-Agarose beads. After washing, the protein A/G-Agarose pellets were resuspended in 100 mM glycine at a pH of 3.0 for 10 min, and then to eluates a pretitrated volume of 1.0 M Tris (pH 9.5) was added to adjust the pH to 7.4. Protein complexes in the supernatants after centrifugation (2,500 × g, 10 min) were analyzed by Western blotting.

For immunoblotting experiments, 50-70 μg of protein was separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose sheets. Labeled bands were visualized using enhanced chemiluminescence (Amersham, California, USA) and analyzed by densitometric scanning. Protein concentrations were determined using a BCA protein assay kit (Pierce). Differences in loading were corrected using the quantification of the total protein amount, and immunoblot signals were normalized to the controls.

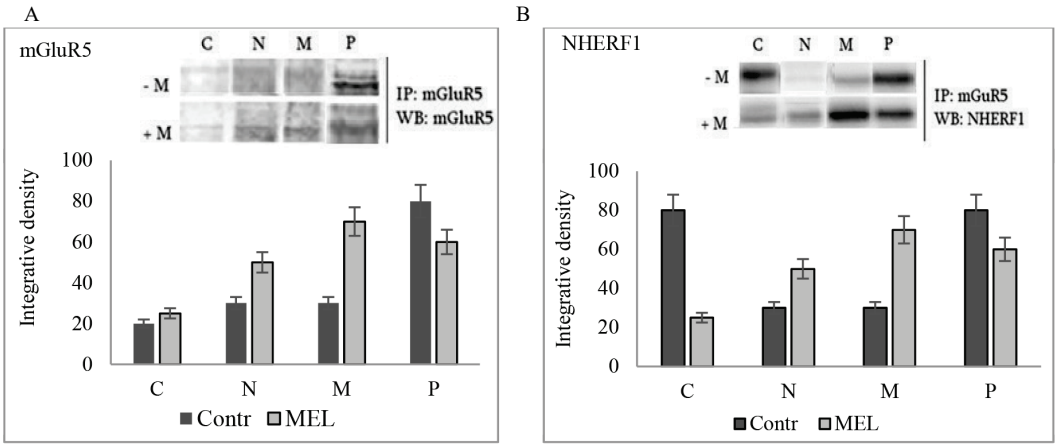
ERK and Akt phosphorylation assay. Nuclei (50-80 μg) were incubated in the medium containing 10 mM glutamate, 2 mM ATP, 25 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, 0.1 M sucrose, pH 7.5. In corresponding experiments, 10 μM MPEP (2-methyl-6-(phenylethynyl) pyridine) (mGluR5 antagonist) were added. The samples were incubated 1 h, and ERK, pERK, Akt, and pAkt were analyzed by Western blotting.

Statistical analysis. All data are presented as mean ± SEM. Statistical analysis was performed by one-way ANOVA, followed by Scheffe's post hoc comparison test. $P<0.05$ was considered statistically significant.

Results

To determine the specific subcellular membranes where mGluR5 is localized, we employed centrifugation-based fractionation of cell structures followed by Western blot analysis. Considering the low constitutive expression of mGluR5 in Jurkat cells [16], we concentrated the receptor using anti-mGluR5 loaded protein A/G-agarose. Our results demonstrate a significant enrichment of mGluR5 in 20,000 x g precipitates and the microsomal fraction, which mainly consists of plasma and endoplasmic membranes, respectively. Conversely, mGluR5 expression in nuclear membranes was relatively minimal. Nevertheless, the treatment of cells with melatonin increased the content of mGluR5 in nuclear membranes (Fig. 1A).

Immunohistochemical investigations reveal a similar expression pattern between the NHERF1 scaffold protein and mGluR5, suggesting a possible relationship between these proteins [17]. Notably, NHERF1 has been shown to modulate the activity of mGluR5 selectively. In light of this, we conducted co-immunoprecipitation experiments to examine the association between NHERF1 and mGluR5. To assess the potential co-localization of mGluR5 with NHERF1, aliquots eluted from anti-mGluR5-protein A/G-agarose beads were blotted against anti-NHERF1 and anti-mGluR5. Our findings confirm that mGluR5 co-immunoprecipitates with NHERF1, particularly in the plasma and microsomal membranes (Fig. 1B). These data support the hypothesis that the NHERF1 scaffold protein may be involved in the trafficking and regulation of mGluR5 [18]. Importantly, that melatonin increa-



Figs. 1A and **1B**. Immunoblot and densitometry analysis of co-immunoprecipitated mGluR5(A) and NHERF(B) in subcellular fractions after treatment of Jurkat cells with melatonin. C-cytosol; N-nuclear; M-microsomal and P-plasma membranes fractions.

ses the content of mGluR5-bound NHERF1 in microsomal and nuclear fractions while decreasing it in plasma membranes and cytosol fractions. This suggests that mGluR5 may undergo translocation between the cytosol and ER membranes facilitated by the involvement of the NHERF1 scaffold protein.

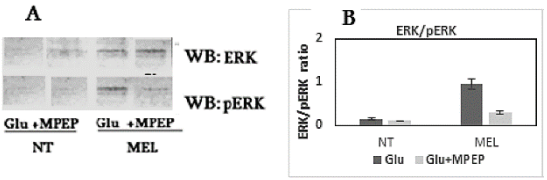


Fig. 2. mGluR5-dependent phosphorylation of ERK in the nuclei of Jurkat cells after treatment with melatonin. A- Western blot; B-Quantification of Western blots.

Besides canonical Gq-dependent signaling, mGluR5 mediates noncanonical b-arrestin-dependent ERK activation [19], To investigate the potential involvement of the nuclear mGluR5 receptor in activating ERK, we conducted an experiment using the nuclear fraction incubated with glutamate, with and without the mGluR5 antagonist MPEP. Subsequently, we measured the levels of phosphorylated ERK. Our results showed that in the control cells, both the glutamate-dependent phosphorylation of ERK and the overall content of ERK were lower compared to cells treated with melatonin (Fig. 2). These findings strongly indicate that melatonin enhances the content and activity of mGluR5, specifically within the nuclear fractions of Jurkat cells. Thus, the activation of nuclear mGluR5 leads to an enhancement in the activity of the ERK protein kinase.

Next, we examined the impact of mGluR5 on the activity of another regulatory protein kinase, Akt. We assessed the phosphorylation status of Akt and found that it remained unchanged following cell treatment with melatonin, as well as in the presence of both mGluR5 agonist and antagonist (data not shown). These observations suggest that mGluR5 signaling does not directly influence the phosphorylation of Akt in the specific experimental context of our study.

Discussion

Our study has shown that melatonin treatment resulted in increased mGluR5-dependent activation of ERK in the nuclear fractions of the Jurkat cells. This indicates that melatonin may play a crucial role in enhancing mGluR5 activity within lymphoid cells, consequently leading to increased ERK protein kinase activity. Furthermore, our study revealed that melatonin treatment led to the redistribution of NHERF1, a scaffold protein, from the cytosol and plasma membranes to the endoplasmic and nuclear membranes. NHERF1 interacts with and regulates various proteins, including G-protein coupled receptors, and serves as a linker

between integral membrane and cytoskeletal proteins. These interactions can protect signaling molecules from inactivation and contribute to the subcellular localization of proteins involved in the signaling pathway, thereby positioning NHERF1 as a coordinator of multiple signaling pathways [20].

In conclusion, our study provides evidence for the involvement of intracellular in the activation of ERK. The results indicate that melatonin induces the redistribution of NHERF1 and enhances the activity of mGluR5 in Jurkat cells, resulting in increased ERK protein kinase activity. These findings contribute to the existing data on mGluR5, melatonin, and their roles in cellular signaling, circadian regulation, and potential implications for sleep and disease processes. Further research is warranted to explore the precise mechanisms and downstream effects of nuclear mGluR5 activation on cellular functions.

ბიოქიმია

მელატონინი Jurkat-ის ლიმფოიდურ უჯრედებში იწვევს mGluR5 და NHERF1 ჰეტეროკომპლექსის გადანაწილებას

ნ. ნარმანია[*,**], მ. სეფაშვილი[*,**], დ. მიქელაძე[*,**,§]

[*] *ილიას სახელმწიფო უნივერსიტეტი, საბუნებისმეტყველო მეცნიერებებისა და მედიცინის ფაკულტეტი, თბილისი, საქართველო*
[**] *ივ. ბერიტაშვილის ექსპერიმენტული ბიომედიცინის ცენტრი, ბიოქიმიის ლაბორატორია, თბილისი, საქართველო*
[§] *აკადემიის წევრი, საქართველოს მეცნიერებათა ეროვნული აკადემია, თბილისი, საქართველო*

mGluR5 ტვინში არეგულირებს სხვადასხვა ფიზიოლოგიურ პროცესებს, მათ შორის, ძილ-ღვიძილის ციკლს. ცენტრალურ ნერვულ სისტემაში ლოკალიზაციის გარდა, mGluR5 ასევე ექსპრესირდება იმუნურ უჯრედებშიც. ხშირ შემთხვევაში, mGluR5-ის აქტივაცია პერიფერიულ უჯრედებში ხელს უწყობს სიმსივნის ზრდას და იწვევს ნეოპლასტიკურ ტრანსფორმაციას. მელატონინი, ნაერთი რომელიც არეგულირებს ცირკადულ რითმს, პირიქით, ასუსტებს ნეოპლასტიკურ ტრანსფორმაციას და თრგუნავს სიმსივნის განვითარებას, ჩვენ მიერ გამოკვლეული იყო მელატონინის ეფექტი mGluR5-ის ექსპრესიაზე Jurkat-ის ლიმფოიდურ უჯრედებში. ცდების შედეგად გაირკვა, რომ mGluR5 ლოკალიზებულია პლაზმურ მემბრანასა და მიკროსომებში, ხოლო შედარებით მცირე რაოდენობით იგი გვხვდება ბირთვებსა და ციტოზოლში. გარდა ამისა აღმოჩნდა, რომ მელატონინის თანაობისას იზრდება mGluR5-ის ექსპრესია უჯრედების ბირთვებში. დადგინდა, რომ mGluR5 ლოკალიზებულია NHERF1 ცილასთან ერთად, რაც მიუთითებს ამ ცილის პოტენციურ როლზე mGluR5-ის ტრეფიკინგსა და რეგულაციაში. აღსანიშნავია, რომ მელატონინით დამუშავებული უჯრედების ბირთვულ ფრაქციაში დაფიქ-

სირდა ERK- პროტეინკინაზას mGluR5-დამოკიდებული ფოსფორილირების ზრდა. გამოთქმულია მოსაზრება ახალ მექანიზმზე, რომლითაც მელატონინი არეგულირებს mGluR5-ის ექსპრესიას და აქტივობას იმუნურ უჯრედებში.

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