Neuroscience Letters 590 (2015) 161–165

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Galanin up-regulates the expression of M1 muscarinic acetylcholine receptor via the ERK signaling pathway in primary cultured prefrontal cortical neurons

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HIGHLIGHTS

- Galanin up-regulates the expression of M1 receptor in the neurons.
- The ERK signaling pathway mediates the galanin induced up-regulation of M1 receptor.
- Our study supports the hypothesis that galanin is neurotrophic in the progress of AD.

ARTICLE INFO

Article history:
Received 21 November 2014
Received in revised form 3 February 2015
Accepted 6 February 2015
Available online 9 February 2015

Key words:
Galanin
M1 muscarinic acetylcholine receptor
Alzheimer's disease
ERK

ABSTRACT

The expression of galanin and galanin receptors are up-regulated in the brains from patients with Alzheimer's disease (AD). However, the role of galanin in the progress of AD is still controversial. Here we demonstrated that galanin increased the protein expression of M1 muscarinic acetylcholine receptor (M1) in the primary cultured prefrontal cortical neurons by ELISA and Western Blot. Moreover, we showed that the mRNA expression of M1 was also up-regulated by galanin treatment. We further explored the mechanism of the galanin induced up-regulation of M1. We found that galanin activated the ERK signaling pathway in the primary cultured prefrontal cortical neurons. In addition, our results showed that the up-regulation of M1 mRNA was blocked by an ERK inhibitor, U0126. Taken together, our results demonstrated that the ERK signaling pathway mediated the galanin induced up-regulation of M1 in the primary cultured prefrontal cortical neurons, supporting the hypothesis that galanin plays a beneficial role in the development of AD.

Galanin is a 29 amino acids neuropeptide with wide expression in the central and peripheral nervous systems [18]. Galanin has been reported to be involved in many biological functions such as nociception, feeding, waking and sleeping regulation, learning and memory [16,18]. Moreover, galanin is linked to a number of neurological disorders, including epilepsy, depression and Alzheimer's disease (AD) [18]. Galanin had been found to be up-regulated in the post-mortem brains of patients with AD more than twenty years ago [1]. However, the role of galanin during the onset of AD is still poorly understood.

AD is a progressive neurodegenerative disease with amyloid beta and tau protein as two major hallmarks [14]. Another major hallmark for AD is the hypofunction of cholingergic system in the brain. It has been reported that there are reduction of acetylcholine synthesis and acetylcholine receptor level as well as significant loss of cholinergic neurons in the brains from patients with AD [15]. The neurotransmitter acetylcholine activates either ionotropic nicotinic acetycholine receptors or metabotrophic muscarinic acetylcholine receptors (mAChRs) to have biological functions [15]. Five mAChR (M1–M5) subtypes have been identified to date and they are involved in various physiological functions in the nervous system. Of the five mAChRs, M1 plays a crucial role in neurological diseases including AD [17]. It has been shown that activation of M1 attenuated amyloid beta induced neurotoxicity through the WNT signaling pathway [13]. Moreover, deficiency in M1 was found to further exacerbate AD-like pathology in a mouse model of AD [22]. Interestingly, a study found that a selective M1 agonist reduced amyloid beta and tau pathology in a transgenic mice model of AD [2]. Thus M1 has been postulated as an important therapeutic target for treating AD.

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http://dx.doi.org/10.1016/j.neulet.2015.02.011
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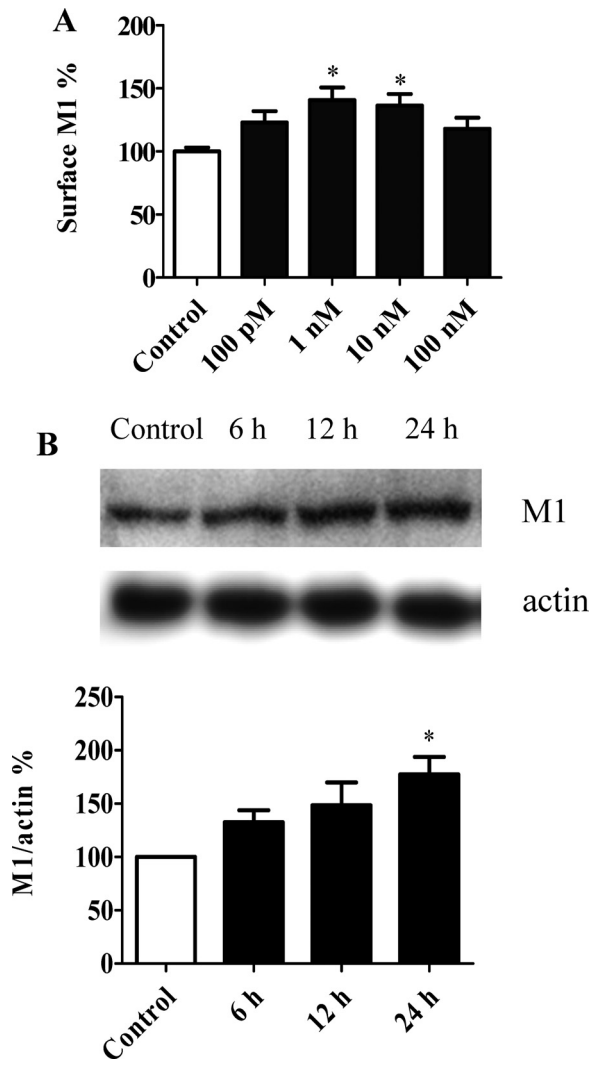

Fig. 1. Galanin up-regulated the M1 protein expression in the primary cultured prefrontal cortical neurons.
(A) Galanin increased the surface M1 expression in the primary cultured prefrontal cortical neurons as tested by ELISA assay. Note galanin had the maximum effect on the expression of surface M1 at the concentration of 1 nM. Values are mean ± SEM, one-way ANOVA followed Tukey post-hoc test, $^*p < 0.05$, compared with control. (B) Top panel: Representative Western Blot analysis of total M1 protein expression after galanin treatment at different time points in the neurons. Actin served as internal control. Bottom panel: Bar graphs showing the quantification of the total M1 protein signals normalized to actin. Note galanin increased the total M1 protein expression at a time depend manner. Values are mean ± SEM, one-way ANOVA followed Tukey post-hoc test, $^*p < 0.05$, compared with control. Four duplicates were run for the Western Blot.

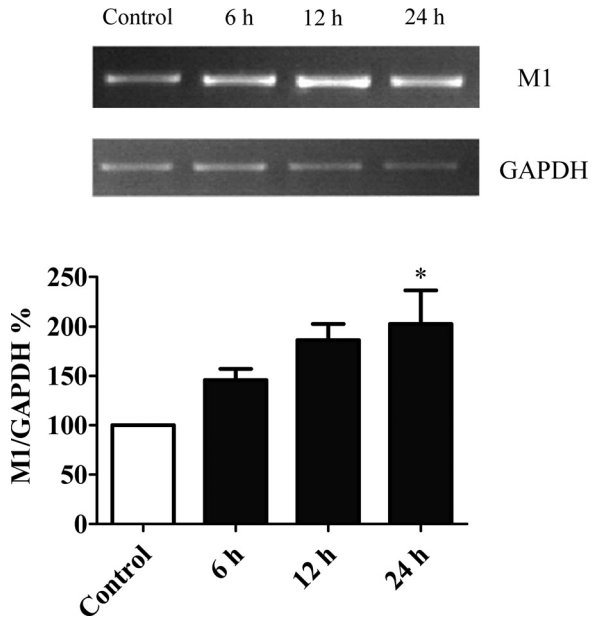

Fig. 2. Galanin increased the expression of M1 mRNA in the neurons.
Top panel: Representative RT-PCR analysis of M1 mRNA expression after galanin treatment at different time points in the neurons. GAPDH served as internal control. Bottom panel: Bar graphs showing the quantification of the M1 mRNA signals normalized to GAPDH. Note galanin increased the M1 mRNA expression at a time depend manner. Values are mean ± SEM, one-way ANOVA followed Tukey post-hoc test, $^*p < 0.05$, compared with control.

Although the regulatory role of galanin on the release of acetylcholine had been reported by many groups [9,21,25], there is no study showing whether galanin regulates the acetycholine receptors in the brains. Here we demonstrated that galanin up-regulated the expression of M1 in the primary cultured prefrontal cortical neurons, both in protein and mRNA levels. Furthermore, we found that the ERK signaling pathway mediated the galanin induced up-regulation of M1.

The primary cultured prefrontal cortical neurons of rats were prepared as described previously [27]. Briefly, the dissociated neurons from postnatal day zero to one pups (mixed gender) were plated at 10^5 cells/ml in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2 g/l HEPES, 2 g/l $NaHCO_3$, 100 U/mlstreptomycin and penicillin. To inhibit glial cell growth, cytosine arabinoside (10 μM; Sigma, St. Louis, Missouri) was supplemented after plating for 3 days. The neurons were used for experimentation after 7–8 days culture in vitro (DIV7-8). Galanin (Tocris, Bristol, UK) and 1,4-diamino-2,3-dicyano-1,4-*bis*[2-aminophenylthio]butadiene (U0126, Sigma, St. Louis, Missouri) were added freshly into the culture medium during treatments. U0126 is a highly selective inhibitor for extracellular-signal-regulated kinase 1/2 (ERK1/2).

RT-PCRs were performed as described previously [20]. Briefly, primary cultured prefrontal cortical neurons were harvested and total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, California). Total RNA (2 ug) was reverse-transcribed using M-MLV Reverse Transcriptase (Invitrogen) and oligo-d(T) 15 random primers (Takara, Shiga, Japan). PCRs were done using Pre-mix Taq (Takara, Shiga, Japan). Sequences of the primers for the experiments were: rat M1: sense 5′-CAGTTCCTCTCCCAACCCAT-3′ and antisense 5′-TGGGCATCTTGATCACCACT-3′; rat GAPDH: sense 5′-CGTATCGGACGCCTGGTT-3′ and antisense 5′-CCCTTCCACGATGCCAAAA-3′. Amplification was done at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min with a final extension cycle for 10 min at 72 °C in an Eppendorf Master (Eppendorf, Hamburg, Germany). PCR products were electrophoresed on agarose gels, stained with ethidium bromide (Sigma, St. Louis, Missouri) and visualized under UV light. The results were assessed with a BioRad Chemi Doc XRS imaging system (BioRad Hercules, CA).

Cell ELISA was used to detect M1 level on cell surface under non-permeabilized condition. The primary prefrontal cortical neurons of rats were treated and then gently washed with 0.01 M PBS before being fixed with 4% paraformaldehyde in PBS. Cells were then washed with PBS and blocked using PBS containing 10% donkey serum. After blocking, the cells were incubated with M1 primary antibody (1:200, Santa Cruz, CA, USA) diluted in 3% serum-PBS at 4 °C overnight. Then cells were incubated with biotin-conjugated donkey anti-rabbit second antibody (1:1000) Jackson ImmunoResearch Laboratories, Inc., PA, USA) after washing with PBS, following by 0.3% H_2O_2 incubation for 30 min to eliminate endogenous