European Neuropsychopharmacology (▮▮▮▮) ▮, ▮▮▮–▮▮▮

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SHORT COMMUNICATION

Epigenetic induction of melatonin MT_1 receptors by valproate: Neurotherapeutic implications

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Received 14 October 2016; received in revised form 30 March 2017; accepted 10 June 2017

KEYWORDS
Histone deacetylase inhibition;
Histone acetylation;
Chromatin remodeling;
Melatonin MT_1 receptor promoter;
Chromatin immunoprecipitation

Abstract
We have reported that the anticonvulsant/mood stabilizer and histone deacetylase (HDAC) inhibitor valproate (VPA) induces expression of melatonin receptors both in vitro and in vivo, but the mechanisms involved were not known. Here we show that pharmacological inhibition of CREB, PKC, PI3K, or GSK3β signaling pathways, which are known targets for VPA, do not prevent its upregulation of melatonin MT_1 receptors in rat C6 glioma cells. M344, an HDAC inhibitor unrelated to VPA, mimics the effects of VPA on MT_1 expression, whereas valpromide, a VPA derivative lacking HDAC inhibitory activity, does not. Furthermore, VPA, at a concentration which upregulates the MT_1 receptor, induces histone H3 hyperacetylation along the length of the MT_1 receptor promoter. These results show that an epigenetic mechanism involving histone acetylation underlies induction of MT_1 receptor expression by VPA. Given the neuropsychiatric effects of melatonin coupled with evidence that VPA upregulates melatonin receptors in the rat brain, these findings suggest that the melatonergic system contributes to the psychotropic effects of VPA.

1. Introduction

The indoleamine melatonin interacts with several second messenger pathways via its G protein-coupled receptors, MT_1 and MT_2, which are widely distributed in the mammalian brain (Lacoste et al., 2015). Melatonin modulates diverse physiological activities including circadian rhythmicity, neuroendocrine function and neuroprotection. Abnormalities in melatonin synthesis, phase shifts in its nocturnal peak or altered MT_1 receptor expression have

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http://dx.doi.org/10.1016/j.euroneuro.2017.06.002
0924-977X/

Please cite this article as: Bahna, S.G., Niles, L.P., Epigenetic induction of melatonin MT_1 receptors by valproate: Neurotherapeutic implications. European Neuropsychopharmacology (2017), http://dx.doi.org/10.1016/j.euroneuro.2017.06.002

been linked to depression (Wu et al., 2013). Valproate (VPA) an anticonvulsant and mood stabilizer, activates multiple protein kinase pathways (Monti et al., 2009). Importantly, VPA inhibits histone deacetylase (HDAC) activity, which enhances gene expression by inducing hyperacetylation of histones (Phiel et al., 2001). We have reported a significant VPA-induced increase in melatonin MT_1 and/or MT_2 receptor expression in rat C6 glioma cells (Castro et al., 2005; Kim et al., 2008), rat brain (Bahna et al., 2014; Niles et al., 2012) and human MCF7 breast cancer cells (Jawed et al., 2007), indicating the cross species and in vivo relevance of this positive regulatory effect. Notably, another HDAC inhibitor, trichostatin A, which is structurally distinct from VPA, also induces melatonin receptor expression (Kim et al., 2008), suggesting involvement of an epigenetic mechanism. This study further examined possible mechanisms underlying the upregulation of melatonin MT_1 receptors by VPA.

2. Experimental procedures

2.1. Cell culture and drugs

Rat C6 glioma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), as reported previously (Castro et al., 2005). The medium was changed to DMEM with 1% FBS 24 h prior to treatment of cells (passages 5–18) at a confluency of 55–70%. VPA and lithium chloride (LiCl; Sigma-Aldrich Canada, Oakville, ON) were prepared in DMEM. M344 (Tocris Bioscience, Ellisville, MO), valpromide, KG501 (CREB inhibitor – Sigma-Aldrich), AR-A014418 (GSK3β Inhibitor VIII), bisindolylmaleimide I (protein kinase C, PKC inhibitor), and LY294002 (phosphatidylinositol 3-kinase, PI3K inhibitor – Cayman Chemical, Ann Arbor, MI) were prepared in 100% dimethyl sulfoxide (DMSO). For drug treatments (with or without kinase blockade), 3 mM VPA for 24 h was used in order to maximize mRNA induction, as observed previously (Castro et al., 2005). For ChIP analysis, treatment with 1 mM VPA for 72 h was selected, based on preliminary concentration- and time-dependent studies of histone acetylation. For 72 h treatments, both drug and medium were replaced on the second day.

2.2. Reverse transcription-qPCR

Total RNA was isolated from C6 cells and cDNA was synthesized as reported previously (Niles et al., 2012). Quantitative PCR (qPCR) was conducted using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories Canada Ltd, Mississauga, ON), in a final volume of 25 μl containing SsoAdvanced™ Universal Inhibitor-Tolerant SYBR Green Supermix (Bio-Rad), and 1 μl cDNA. PCR conditions for MT_1 were as follows: 98 °C (2 min); 40 cycles at 98 °C (15 s); 63 °C (60 s). The internal control 18S ribosomal RNA, was amplified using 1 μl cDNA as follows: 40 cycles at 98 °C (15 s); 63 °C (30 s). Forward and reverse primers were: MT_1 – GAGGAAATAAGATCGCGGCC and CTGCGTTCCTGAGCTTCTTG (136 bp); 18S rRNA – CGTTCTTAGTTGGTGGAGCG and AACGCCACTTGTCCCTCTAA.

2.3. Chromatin immunoprecipitation-qPCR

ChIP assays were performed with the ChIP-IT Express Chromatin Immunoprecipitation Kit (Active Motif, Carlsbad, CA) as described by the supplier. Briefly, proteins were cross-linked to DNA with 1% (v/v) formaldehyde for 10 min at room temperature. Cells were washed, centrifuged and lysates were sonicated (10 s × 7 times) to yield 200–1000 bp fragments of DNA. Sheared DNA (25 μg) was immunoprecipitated using 5 μg of anti-acetyl-histone H3 (K9/18) polyclonal antibody (EMD Millipore Corporation, Billerica, MA), or 5 μg of normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) as a negative control. Protein/DNA complexes were captured with protein G magnetic beads, treated with proteinase K and reverse cross-linked overnight. After purification (QIAquick PCR Purification Kit; Qiagen Inc., Mississauga, ON), 2 μl of immunoprecipitated DNA was used to preamplify segments of the MT_1 promoter (~500 bp) by standard PCR (GeneAmp PCR System 2400 Thermal Cycler, Perkin Elmer), with HotStarTaq DNA Polymerase (Qiagen) as follows: 95 °C (5 min); 15 cycles at 94 °C (30 s); 55 °C (30 s); 72 °C (1 min), and a final incubation at 72 °C (10 min). Preamplified DNA was diluted 9-fold for qPCR amplification with primers for shorter segments (~200 bp or less) of the MT_1 promoter. qPCR was conducted in a final volume of 25 μl containing 12.5 μl SsoAdvanced™ Universal Inhibitor-Tolerant SYBR Green Supermix (Bio-Rad), 1.25 μl each of forward and reverse primers (10 μM), and 4 μl DNA as follows: 98 °C (3 min), followed by 40 cycles at 98 °C for 15 s and 60 °C for 60 s. Primers used for amplification of short (MT_1) promoter segments were: P1 – TGGCCTTGAACTTCTGATCC and CATGCTGACACCTTGACGAT (223 bp); P2 – CCCAAAGTGGCATTGATTCT and CATTTCTTCCAGAGTCCCTTTG (182 bp); P3 – TGGCTAATCCACTTCCCAGA and TAAAGGCTGTGCTGGATGCT (166 bp); P4 – TCATCCTCATTTTGCCGATA and GTCAAGTGCAGGGGAAACTT (122 bp).

2.4. Statistics

Analysis of qPCR data from pathway blockade studies was performed using the Relative Expression Software Tool (REST), which determines the crossing point (Cp) deviation between a sample and control group, normalizes data to a reference gene and performs a correction for amplification efficiency (Pfaffl et al. 2002). In addition, REST incorporates statistical analysis of normalized Cp data using a pairwise fixed reallocation randomization approach, with no assumptions about the distribution of data (Metzger et al., 2005; Pfaffl et al., 2002). Student's *t* test was used to analyze MT_1 promoter data.

3. Results

3.1. Effects of pathway inhibitors on MT_1 induction by VPA

Pharmacological blockade of CREB, PKC or PI3K signaling did not inhibit induction of MT_1 by VPA. Treatment with VPA upregulated MT_1 mRNA expression by mean factors of 38.06 ($p<0.05$; Figure 1A), 19.27 ($p<0.05$; Figure 1B) and 23.37 ($p<0.05$; Figure 1C), in the presence of KG501 (CREB inhibitor), BIM1 (PKC inhibitor) or LY294002 (PI3K inhibitor), respectively. In contrast, AR-AO14418 (GSK3β inhibitor) prevented the transcriptional induction of MT_1 by VPA ($p<0.05$; Figure 1D), suggesting that VPA acts via a GSK3β-sensitive mechanism. However, treatment with lithium, another antagonist of GSK3β, did not block MT_1 induction by VPA, which increased by a mean factor of 34.03 ($p<0.05$; Figure 1E). These results, which contradict the above findings with AR-A014418, do not support involvement of GSK3β in the induction of MT_1 by VPA.

3.2. M344 and VPA, but not valpromide, induce MT_1 mRNA expression

Valpromide, a VPA derivative without HDAC inhibition properties (Phiel et al., 2001), did not alter MT_1 expression at

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