

Valproic Acid Influences MTNR1A Intracellular Trafficking and Signaling in a β -Arrestin 2-Dependent Manner

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Abstract Valproate exposure is associated with increased risks of autism spectrum disorder. To date, the mechanistic details of disturbance of melatonin receptor subtype 1 (MTNR1A) internalization upon valproate exposure remain elusive. By expressing epitope-tagged receptors (MTNR1A-EGFP) in HEK-293 and Neuro-2a cells, we recorded the dynamic changes of MTNR1A intracellular trafficking after melatonin treatment. Using time-lapse confocal microscopy, we showed in living cells that valproic acid interfered with the

internalization kinetics of MTNR1A in the presence of melatonin. This attenuating effect was associated with a decrease in the phosphorylation of PKA (Thr197) and ERK (Thr202/Tyr204). VPA treatment did not alter the whole-cell currents of cells with or without melatonin. Furthermore, fluorescence resonance energy transfer imaging data demonstrated that valproic acid reduced the melatonin-initiated association between YFP-labeled β -arrestin 2 and CFP-labeled MTNR1A. Together, we suggest that valproic acid influences MTNR1A intracellular trafficking and signaling in a β -arrestin 2-dependent manner.

Ling-juan Hong and Quan Jiang contributed equally to this work.

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Abbreviations

VPA	2-Propylpentanoic acid
MTNR1A	Melatonin receptor subtype 1
PKA	Protein kinase A
PKC	Protein kinase C
FRET	Fluorescence resonance energy transfer

Introduction

The hypofunction of pineal endocrine signals is implicated in autism patients [1–3]. Pathway-biased and deleterious melatonin receptor mutants have also been reported in an autism spectrum disorder population [4]. Both prospective and retrospective studies have suggested that children who are exposed to valproic acid (2-propylpentanoic acid, VPA) in utero have an increased risk of neuropsychological developmental delays and mood disorder symptoms [5–7]; however, the cellular basis for the action of VPA remains elusive.

Recently, disruptions in hippocampal CaMKII/PKA/PKC signaling were observed in VPA-treated animals [8]. Interestingly, melatonin was shown to increase the phosphorylation of presynaptic and postsynaptic substrates of CaMKII and the induction of long-term potentiation (LTP), which correlated with the amelioration of social behavior dysfunction in VPA-treated rats [8]. Consistently, clinical data have indicated that melatonin may be useful in improving the behavior of children with autism spectrum disorder [9–14]. However, the role of melatonin receptors in autism spectrum disorder with or without melatonin treatment is another unaddressed issue.

Desensitization, endocytosis, and recycling are major mechanisms of G protein-coupled receptor (GPCR) regulation [15–17]. Reduced melatonin levels are associated with pathological process of central nervous system diseases [14, 18–20]. The melatonin-induced internalization of human melatonin receptor subtype 1 (MTNR1A) results in the activation of downstream mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling, which has been shown to be dependent on G protein activation and β -arrestin 2 trafficking in CHO cells [21]. It has been reported that clinically relevant concentrations of VPA are sufficient to alter MTNR1A, HDAC, and MeCP2 mRNA expression [22]. Given the opposing roles of the pineal endocrine systems and VPA, a precise understanding of the mechanistic details of VPA's effect on melatonin-mediated MTNR1A internalization may translate into pharmacologic therapies for autism.

In this study, we sought to investigate the effect of VPA on MTNR1A internalization and the underlying mechanism. We applied biochemical, fluorescence resonance energy transfer (FRET), and electrophysiological techniques to characterize the effect of VPA on MTNR1A internalization in its agonist-activated state. Our findings indicate that the disassociation between MTNR1A and β -arrestin 2 underlies the VPA-mediated inhibition of intracellular MTNR1A trafficking and signaling.

Materials and Methods

Reagents

The mouse neural crest-derived cell line, Neuro-2a, and Human Embryonic Kidney 293 cells, HEK-293, were used as described previously [23]. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA). Lipofectamine 2000 was obtained from Invitrogen. Unless otherwise stated, all reagents and chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Plasmid Construction and Transfection

MTNR1A (in pFLAG-CMV-3) and β -arrestin 2 (in pEGFP-N1) was a kind gift of Zhou Naiming (Zhejiang University, China) [23]. MTNR1A was modified by PCR and subcloned into EGFP, ECFP, or mCherry. β -Arrestin 2 was subcloned into pEYFP-C1 (Clontech). mRFP-Rab5, GFP-Rab7, and GFP-Rab11 were purchased from Addgene. HEK-293 cells were transiently co-transfected with MTNR1A-EGFP/mRFP-Rab5, MTNR1A-mCherry/GFP-Rab7, MTNR1A-mCherry/GFP-Rab11, or MTNR1A-CFP/YFP- β -arrestin 2 using Lipofectamine 2000 according to the manufacturer's instructions.

Internalization Measurements by Confocal Microscopy

For confocal time-lapse imaging, glass-bottomed dishes in an experimental chamber were placed on an Olympus IX-81 confocal microscope equipped with a $\times 60$ oil-immersion lens, a polychrome IV light source (Till Photonics), a 505 DCXR beam splitter, and a CCD camera (ANDOR iXon3). MTNR1A-EGFP and other cDNA plasmid constructs were transfected into HEK-293 and Neuro-2a cells using Lipofectamine 2000 according to the manufacturer's instructions. Twenty-four hours after VPA (0.5 mM) treatment, the dynamic changes of MTNR1A internalization were recorded with or without melatonin (100 nM) stimulation.

Measurement of Cell Surface Receptor by ELISA

The cell surface expression of MTNR1A was quantitatively assessed by ELISA as described previously [24]. Briefly, after transfection with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol, HEK-293 cells were grown in DMEM at 37 °C in a humidified atmosphere containing 95 % air and 5 % CO₂ overnight and then split into 24-well plates that were coated with poly-L-lysine. On the following day, cells were stimulated with melatonin at the indicated concentrations. Medium was aspirated and the cells were washed once with Tris-buffered saline. After fixing for 5 min at room temperature with 3.7 % formaldehyde in Tris-buffered saline, cells were washed three times with Tris-buffered saline and then blocked for 45 min with 1 % bovine serum albumin/Tris-buffered saline. Cells were then incubated for 1 h with an alkaline phosphatase-conjugated monoclonal antibody directed against the FLAG epitope at 1:3000 dilution. Cells were washed three times, and antibody binding was visualized by adding 0.25 ml of alkaline phosphatase substrate (Bio-Rad). Development was stopped by removing 0.1 ml of the substrate to a 96-well microtiter plate containing 0.1 ml of 0.4 M NaOH. Plates were read at 405 nm in a multimode detector (BECKMAN COULTER DTX 880) using Multimode Detection software.

Assessment of cAMP Level

The cAMP accumulation was measured through an assay that used a pCRE-Luc vector (Clontech) consisting of the firefly luciferase coding region under control of a promoter containing cAMP response elements (CREs) [25]. HEK-293 cells stably transfected with pCRE-Luc and MTNR1A were grown to 90–95 % confluence in a 48-well plate, followed by stimulation with 10 μ M forskolin alone or different concentrations of melatonin in DMEM incubated for 4 h at 37 °C. When required, cells were pretreated with VPA (0.05, 0.5, 5 mM) in serum-free DMEM for 24 h before the start of the experiment. Luciferase activity was detected by use of a firefly luciferase assay kit (Promega, Madison, WI).

Cellular Electrophysiology

Current clamp recordings for Neuro-2a cells were obtained as previously described [26]. Briefly, the cells were visualized with an infrared-sensitive CCD camera with a $\times 40$ water-immersion lens (Olympus) and the current readings were recorded using whole-cell techniques (MultiClamp 700B Amplifier, Digidata 1440A analog-to-digital converter) and pClamp 10.2 software (Axon Instruments/Molecular Devices). Patch pipettes (3–4 M Ω) were filled with a solution containing 140 mM KCl, 10 mM NaCl, 1 mM MgCl₂, 10 mM HEPES, and 10 mM EGTA (pH adjusted to 7.3–7.4 with KOH). The bath solution contained 4 mM KCl, 140 mM NaCl, 1 mM MgCl₂, 2 mM CaCl₂, 5 mM glucose, and 10 mM HEPES (pH adjusted to 7.3–7.4 with NaOH). Membrane capacitance and access resistance were calculated with pClamp 10.2 software. Access resistance ($R_a < 20$ M Ω) of Neuro-2a cells was not compensated and continually monitored throughout each experiment. When access resistance increased by >30 %, recordings were discarded. Specifically, cells with leak currents below 100 pA were used, and current recordings were therefore not leak-subtracted.

The current recordings were performed at room temperature (21–23 °C). Whole-cell currents were recorded for 400 ms using a series of voltage pulses from -70 to $+100$ mV at 10-mV increments. Peak current was defined as the maximal amplitude of response during melatonin or VPA/melatonin application. Responses were plotted as current density (pA/pF).

Cell Fractionation and Immunoblotting Analysis

The immunoblotting was carried out in total cell lysates extracts after determination of protein concentrations using Bradford's solution. Total cell lysates extracts from Neuro-2a containing equivalent amounts of protein were applied to 10 % acrylamide denaturing gels (SDS-PAGE) [27]. Proteins were then transferred to an immobilon polyvinylidene

difluoride membrane for 1 h at 50 V. Membranes were blocked in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1 % Tween 20 (TBS-T) containing 5 % fat-free milk powder for 1 h and immunodetected with antibodies to anti-phospho-protein kinase (PKC, Ser657) and anti-phospho-protein kinase A (PKA, Thr197) (1:1000; Millipore, Billerica, MA, USA); anti-phospho-ERK1/2 (Thr202/Tyr204), anti-ERK1/2, anti-PKC, and anti-PKA (Cell Signaling Technology, Beverly, MA); and β -actin monoclonal antibody (Sigma, St Louis, MO). After incubation, membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody. Immunoreactivity was visualized by enhanced chemiluminescence (Biological Industries, Kibbutz Beit Haemek, ISRAEL).

Fluorescence Resonance Energy Transfer

Fluorescence resonance energy transfer (FRET) was detected using the acceptor photobleaching method [28]. CFP-labeled MTNR1A (MTNR1A-CFP) and YFP-tagged β -arrestin 2 (YFP- β -arrestin 2) plasmid constructs were transfected into HEK-293 cells for FRET analysis. Acceptor photobleaching experiments were performed on an Olympus BX-61 confocal microscope (FV1000-fluoview) with a 40 mW argon laser. YFP (acceptor) was excited with the 515 nm line of the argon laser and CFP (donor) with the 405 nm line. Cells were examined with a $\times 60$ oil-immersion objective. We bleached cells in the YFP channel by scanning a region of interest (ROI) using the 515 nm argon laser line at 100 % intensity (95 mW laser power). The time of bleaching ranged from 3 to 10 s, depending on the locations of bleached ROIs. Before and after YFP photobleaching, CFP images were taken to assess changes in donor fluorescence. Any increase in CFP fluorescence that was caused by bleaching of the YFP acceptor could be masked by unintended bleaching of CFP during the imaging process. To minimize the excessive photobleaching, images were collected at 0.15 % of the laser intensity, which was 500 times less than the bleaching intensity. In addition, we monitored the level of bleaching in each experiment by collecting two CFP/YFP image pairs before the bleach and six after the bleach. Percent fluorescence was calculated as average fluorescence intensity (AI) of post-bleach/AI of pre-bleach $\times 100$.

Statistical Analysis

The significance of differences between different groups was first determined by using one-way ANOVA. Differences were considered significant at $P < 0.05$. All data are expressed as the mean \pm SEM.

Results

Spatio-temporal Changes of MTNR1A Internalization After Melatonin Stimulation

Receptor internalization following agonist exposure is a well-documented response for a wide variety of GPCRs [15–17]. We stimulated cells that transiently express MTNR1A-EGFP with or without melatonin for 60 min. Fluorescence analysis showed that, in the absence of melatonin, MTNR1A-EGFP was localized to the HEK-293 cell membrane (Fig. 1a, b). By contrast, melatonin induced MTNR1A internalization dose-dependently (Fig. 1c).

Once internalized from the cell surface, GPCRs can be sorted along multiple pathways. They may be either recycled back to the plasma membrane or targeted for lysosomal degradation [29, 30]. The Rab family GTPases are typical markers for identifying intracellular vesicular traffic routes of membrane proteins. For example, Rab5, Rab7, and Rab11 are widely used for tracing early, late, and recycling endosomal pathway, respectively [31, 32]. HEK-293 cells were stimulated for various periods of time (0–60 min) with melatonin (100 nM), followed by fluorescence analysis of colocalization between MTNR1A and various Rab isoforms (Fig. 2). We found that mRFP-Rab5 appeared to be contained in vesicles that were associated with the tubulovesicular structure, which was positive for MTNR1A-EGFP (Fig. 2a, b and Supplementary Movie 1). Moreover, both GFP-Rab7- (Fig. 2c, d and Supplementary Movie 2) and GFP-Rab11-positive (Fig. 2e, f and Supplementary Movie 3) endosomal compartments partially colocalized with MTNR1A-mCherry-positive structures. In addition, internalized MTNR1A was recycled back

to the cell surface after removal of the ligand (Supplementary Fig. 1a, b and Supplementary Movie 4).

Effect of VPA on Melatonin-Mediated MTNR1A Internalization in HEK-293 Cells

To assess the functional significance of VPA on melatonin-mediated MTNR1A internalization, we examined changes in the internalization kinetics. The proportion of internalized MTNR1A was significantly decreased in HEK-293 cells that were pretreated with VPA (0.5 mM) before melatonin stimulation in comparison with cells that received melatonin treatment alone (Fig. 3a, Supplementary Movie 5 and Supplementary Movie 6). The internalization kinetics of Flag-tagged MTNR1A was quantitatively assessed by ELISA, which compared the decrease in fluorescence intensity at the membrane with the increase in fluorescence intensity in the cytoplasm. The ELISA data validated that melatonin induced MTNR1A internalization in a dose-dependent manner, whereas it was significantly blocked in the presence of 0.5 mM VPA (Fig. 3b).

Effect of VPA on Melatonin-Mediated MTNR1A Internalization in Neura-2a Cells

The temporal changes of melatonin-mediated MTNR1A internalization were further confirmed in Neura-2a cells, a well-characterized neuroblastoma cell line [33]. MTNR1A-EGFP signals were equally distributed throughout the cell membrane prior to melatonin stimulation (Fig. 3c, Supplementary Movie 7 and Supplementary Movie 8). Upon exposure to melatonin, a large number of strong MTNR1A-EGFP puncta (green) were redistributed

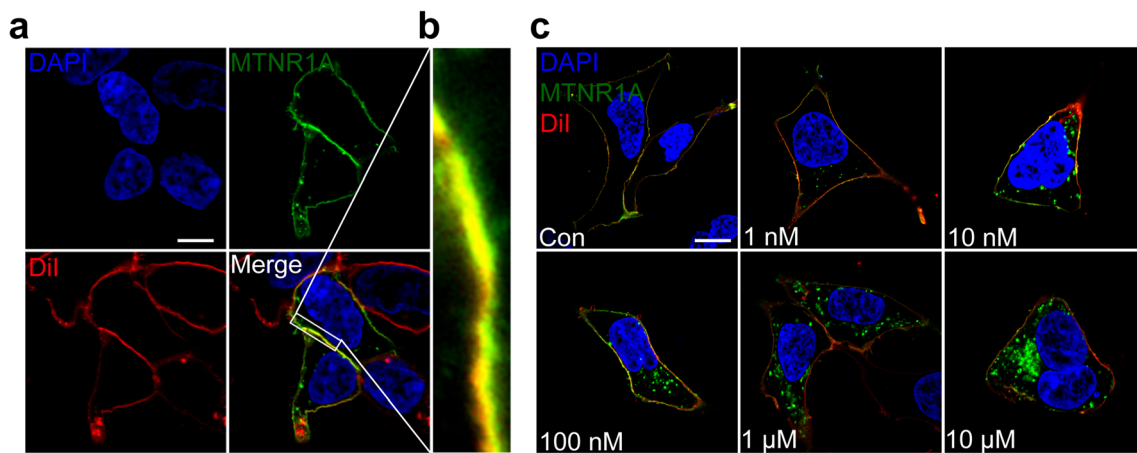
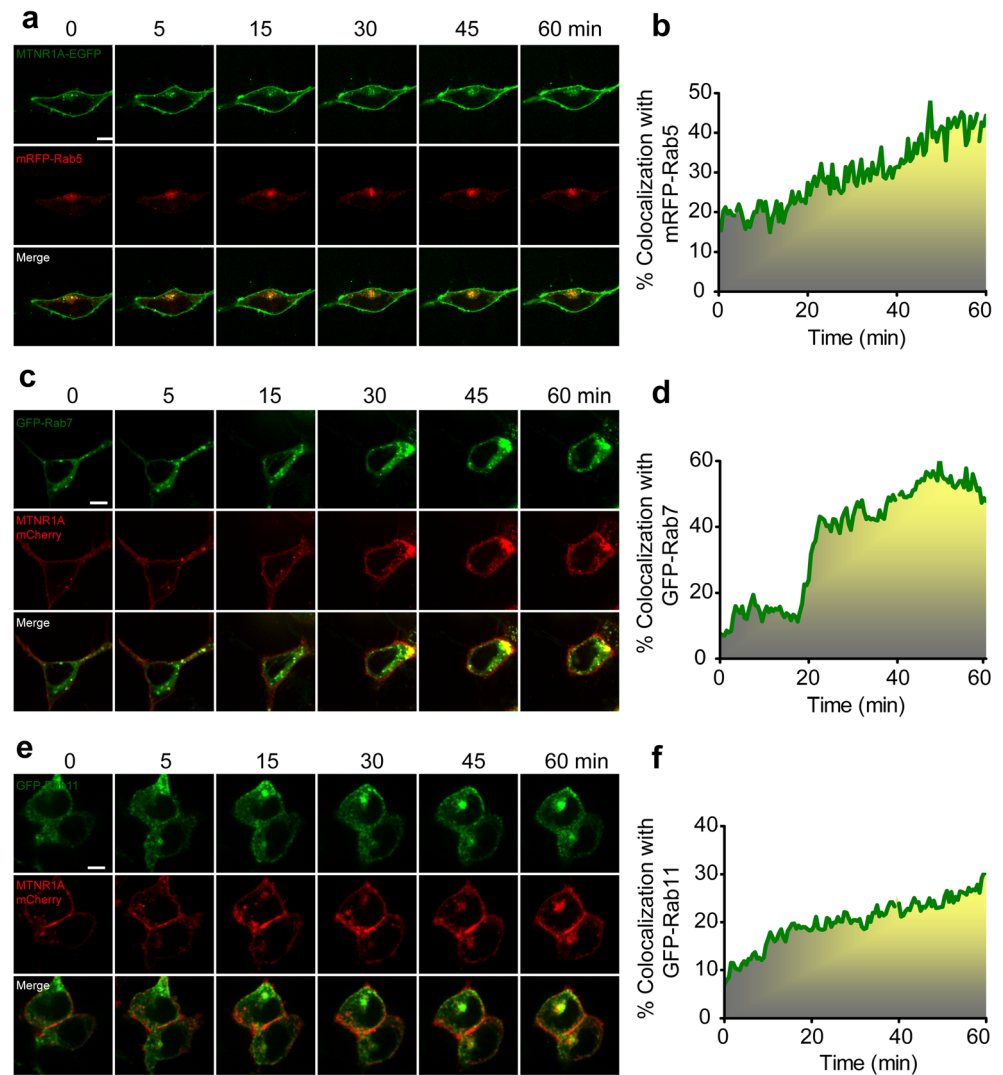


Fig. 1 Melatonin induced MTNR1A receptor internalization. Characterization of transiently expressed MTNR1A-EGFP in HEK-293 cells. **a** HEK-293 cells transiently expressing MTNR1A-EGFP were stained with a membrane plasma probe (DiI) and a nucleus probe (DAPI). The cells were seeded on glass-bottom six-well plates overnight, incubated with DiI (5 μM) and DAPI, and examined by confocal microscopy. MTNR1A-EGFP was found to be localized to the

plasma membrane. Higher-magnification image of membrane MTNR1A staining from the insets shown in (b). **c** HEK-293 cells expressing MTNR1A-EGFP were stimulated with different concentrations of melatonin for 60 min. The representative confocal images show that MTNR1A internalization was induced by melatonin dose-dependently. DiI shows the plasma membrane staining (red). DAPI counterstaining indicates nuclear localization (blue). Scale bar = 10 μm

Fig. 2 Rab GTPases were involved in MTNR1A trafficking. **a** Fluorescence analysis of colocalization between MTNR1A and Rab5. HEK-293 cells were stimulated with melatonin for 60 min and then analyzed for the colocalization between mRFP-Rab5 and MTNR1A-EGFP. **b** Quantitative analysis of MTNR1A-EGFP colocalization with mRFP-Rab5 after melatonin (100 nM) stimulation. **c** MTNR1A was localized to Rab7-positive vesicles after melatonin (100 nM) stimulation in HEK-293 cells that were transiently co-transfected with GFP-Rab7 and MTNR1A-mCherry. **d** Quantitative analysis of MTNR1A-mCherry colocalization with GFP-Rab7. **e** MTNR1A was transported through the Rab11-positive compartment. **f** Quantitative analysis of MTNR1A-mCherry colocalization with GFP-Rab11. Scale bar=10 μ m



to form aggregates (Fig. 3c, Supplementary Movie 7 and Supplementary Movie 8). By contrast, we found that VPA (0.5 mM) pretreatment partially abolished the melatonin-mediated MTNR1A internalization (Fig. 3c, Supplementary Movie 7 and Supplementary Movie 8).

Several functional responses to melatonin are mediated by the regulation of ion channels [34]. To establish a direct role for VPA on whole-cell currents, therefore, we turned to current clamp studies in Neura-2a cells (Fig. 3d). The membrane capacitance (C_m) of patch-clamped cells ranged from 11.87 to 39.54 pF (21.19 ± 0.79 pF, $n=43$). Our data demonstrated that VPA pretreatment did not significantly alter whole-cell currents in the presence of melatonin (Fig. 3d).

Effect of VPA on Melatonin-Induced Phosphorylation of PKA and ERK

Both PKA and MAP kinase pathways are among the numerous primary and secondary signaling cascades that are

activated by the binding of melatonin to MTNR1A at the cell surface [21, 35]. To rule out the involvement of receptor tyrosine kinases on these processes, PKA, PKC, and ERK signaling were investigated in Neura-2a cells. Western blot analysis showed that the level of phospho-PKC (Ser657) remained constant while phospho-PKA (Thr197) and phospho-ERK (Thr202/Tyr204) were both elevated in the presence of melatonin. However, VPA treatment partially reduced the melatonin-induced phosphorylation of PKA (Thr197) and ERK (Thr202/Tyr204) (Fig. 4a, b). Based on our results, we conclude that VPA negatively affects melatonin-mediated MTNR1A internalization, thereby disrupting melatonin/MTNR1A-related intracellular cell signaling. No significant changes were observed in total PKC, PKA, or ERK proteins in the same context (Fig. 4a). To further confirm whether VPA treatment directly affects PKA activity, we detected the cAMP accumulation in reporter pCRE-Luc expressing HEK-293 cells. The pCRE reporter genes are

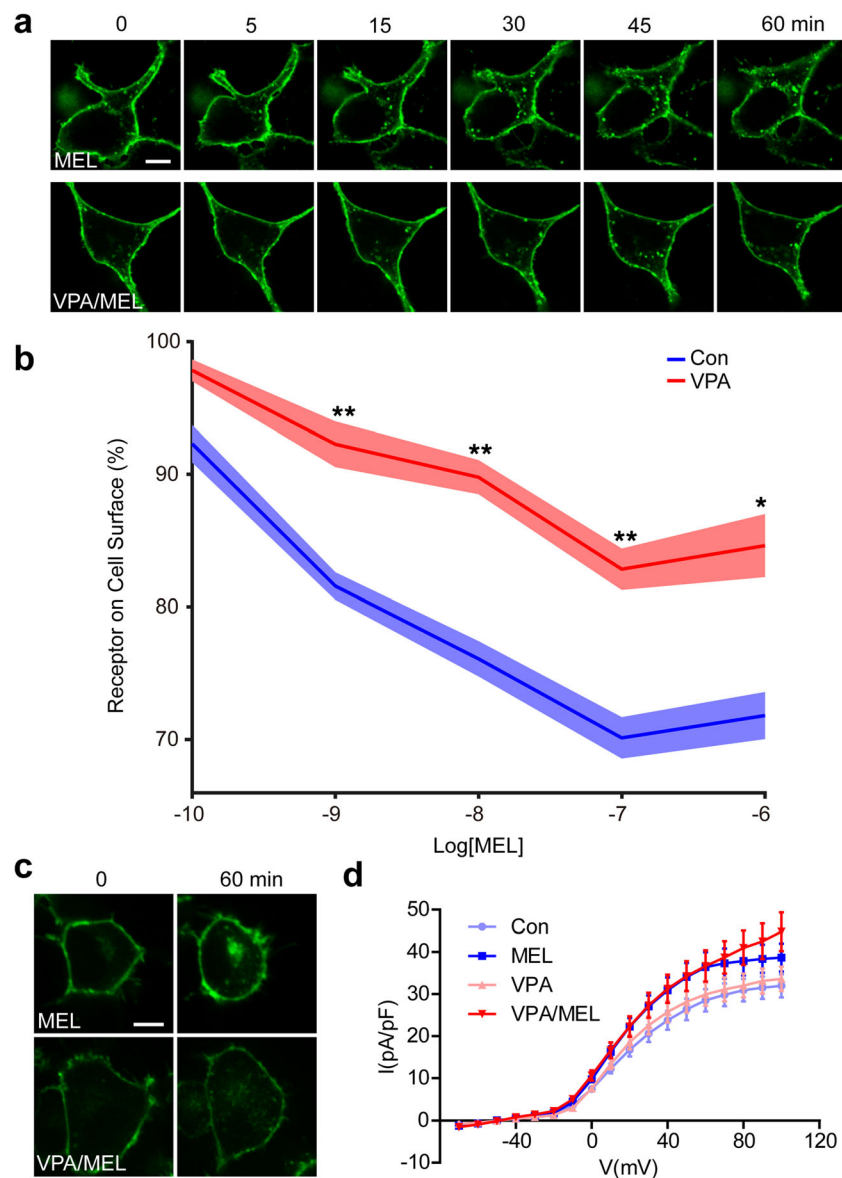


Fig. 3 VPA inhibits melatonin-induced MTNR1A internalization. **a** Melatonin-induced MTNR1A internalization was time-dependent. A time-lapse series of single confocal plane images taken from living HEK-293 cells expressing MTNR1A-EGFP were used to evaluate the internalization kinetics after stimulation by melatonin (100 nM) with or without VPA (0.5 mM) pretreatment. **b** ELISA measurement of MTNR1A expression on the cell surface after treatment of HEK-293 cells with the indicated concentrations of melatonin for 60 min in the presence or absence of VPA (0.5 mM, 24 h). **c** Representative time-lapse recording of melatonin-induced MTNR1A internalization with or

without VPA (0.5 mM) pretreatment in Neura-2a cells. Confocal studies in living cells were performed on MTNR1A-EGFP-transfected Neura-2a cells in glass-bottom dishes. **d** VPA treatment did not affect the whole-cell current. Whole-cell patch clamp recordings from Neura-2a cells at the holding potential of -50 mV. Application of melatonin (100 nM) in the presence or absence of VPA (0.5 mM) is indicated. *Error bars*, SE for four replicates. Data were analyzed using Student's *t* test ($*P < 0.05$; $**P < 0.01$). All images and data are representative of at least three independent experiments. *MEL* melatonin, *VPA* valproic acid. *Scale bar* = $10 \mu\text{m}$

highly responsive to PKA activation by forskolin as well as melatonin (Fig. 4c). Consistently, Chen et al. recently reported that melatonin/MTNR1A also activate ERK1/2 through a Gs/cAMP/PKA pathway [36]. Pretreatment with VPA for 24 h had no significant effect on the accumulation of cAMP with or without melatonin treatment (Fig. 4c).

Effect of VPA on Melatonin-Induced Membrane Trafficking of β -Arrestin 2

β -Arrestins function as intermediary endocytic adaptor proteins that recruit β -adaptins to target membrane receptors to clathrin-coated pits for internalization [37, 38]. To address whether β -arrestin 2 is involved in the removal of ligand-

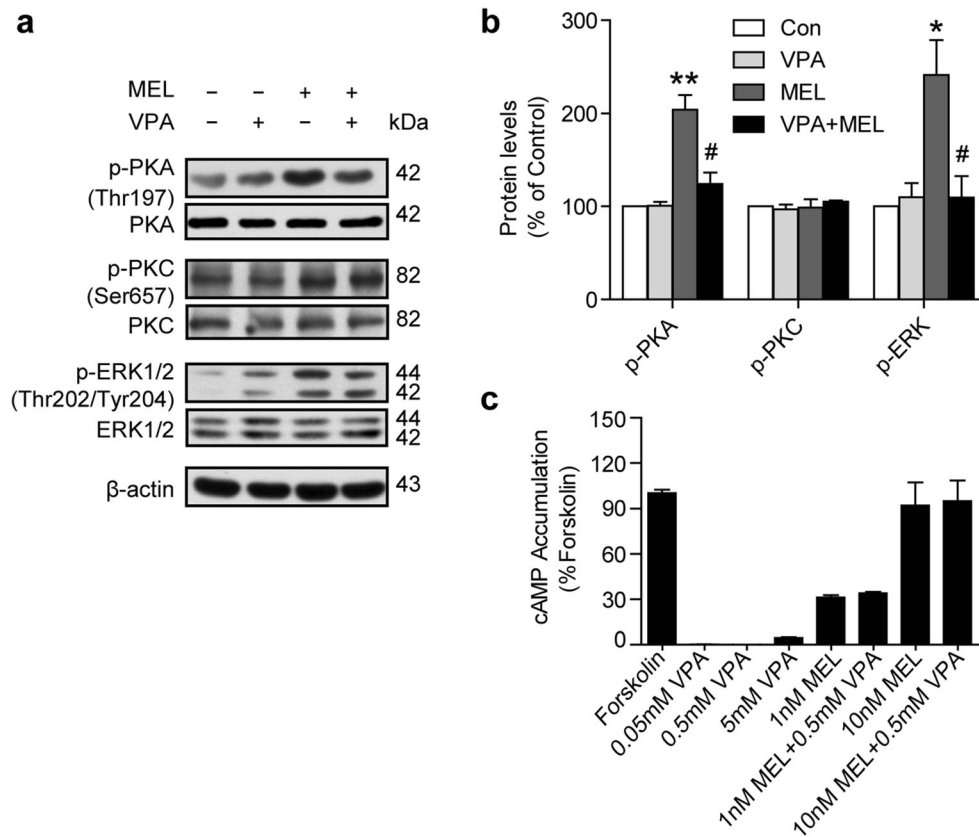


Fig. 4 Decreasing of melatonin-induced phosphorylation of PKA and ERK upon VPA treatment. **a** Effect of VPA on melatonin-induced downstream signaling in HEK-293 cell. Representative immunoblots from melatonin-treated cell lysates in the absence or presence of VPA (0.5 mM) when probed with anti-phospho-PKA(Thr197), anti-phospho-PKC(Ser657), or anti-phospho-ERK(Thr202/Tyr204) antibodies. **b** The quantitative analysis of relative phospho-proteins in the indicated groups. The data are expressed as percentages of the values of the control (mean±

SEM, $n=6$). * $P<0.05$; ** $P<0.01$ versus control; # $P<0.05$, ## $P<0.01$ versus melatonin-treated cells. β-Actin was used as a loading control. MEL melatonin, VPA valproic acid. **c** cAMP accumulation in HEK-293 cells stably expressing pCRE-Luc was determined in response to melatonin and VPA. The luciferase activity of forskolin was normalized to 100 %. The data shown are representative of at least three independent experiments

activated GPCRs from the plasma membrane, we coexpressed Flag-tagged MTNR1A and β-arrestin 2-EGFP in HEK-293 cells. Consistent with the enhancement of MTNR1A internalization by melatonin, time-lapse microscopy showed that membrane localization of β-arrestin 2 was gradually increased after melatonin stimulation (Supplementary Fig. 2a). However, VPA treatment did not significantly affect the plasma membrane localization of β-arrestin 2 in the presence of melatonin (100 nM) (Supplementary Fig. 2b).

FRET Analysis of β-Arrestin 2 Binding to MTNR1A in the Presence of VPA and Melatonin

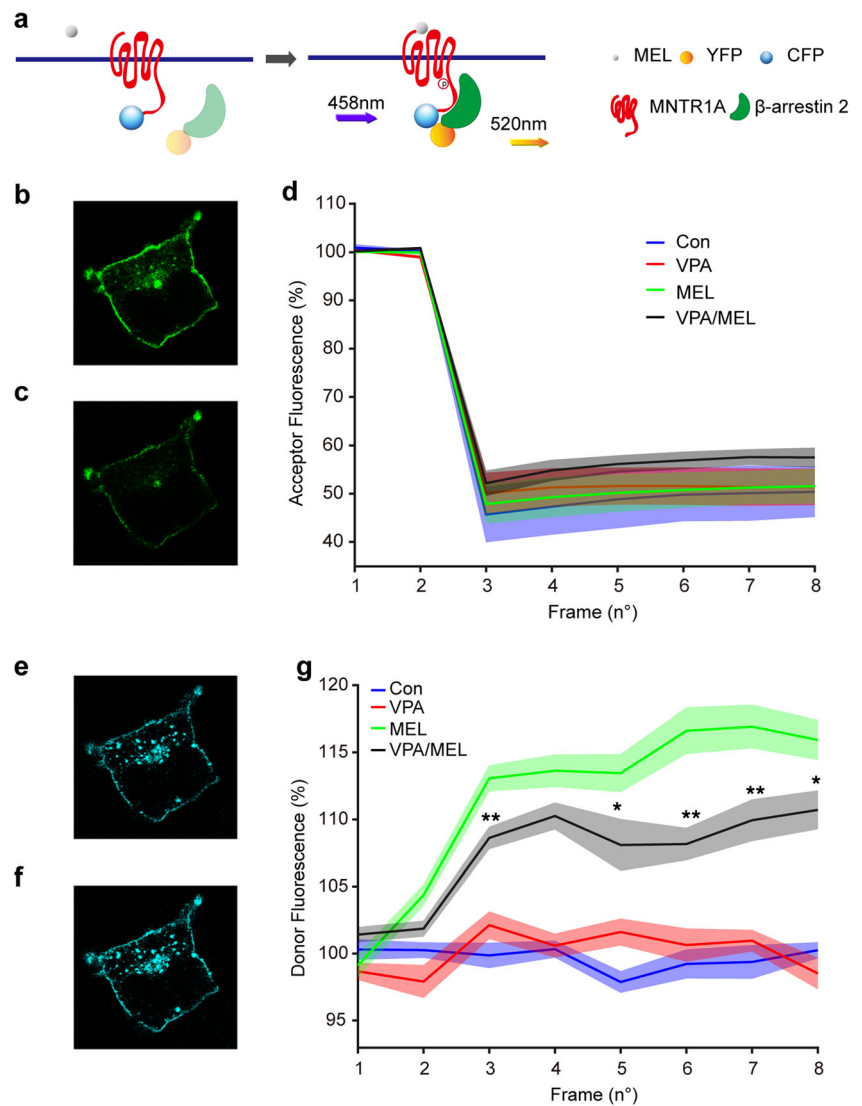
To monitor in real time the kinetics of β-arrestin 2 binding to MTNR1A, we developed a FRET-based assay using YFP-labeled β-arrestin 2 (YFP-β-arrestin 2) and CFP-labeled MTNR1A (Fig. 5a). Before (Fig. 5b) and after (Fig. 5c) YFP photobleaching, CFP images were collected to assess changes in acceptor fluorescence. Our data demonstrated that there were no significant differences among the groups with respect

to the acceptor fluorescence (Fig. 5d). Furthermore, before (Fig. 5e) and after (Fig. 5f) YFP photobleaching, CFP images were collected to assess changes in donor fluorescence under various stimulation. In the presence of melatonin (100 nM), we observed an increase in CFP fluorescence (donor) that was caused by bleaching of YFP (acceptor) (Fig. 5g). By contrast, pretreatment of HEK-293 cells coexpressing YFP-β-arrestin 2 and MTNR1A-CFP with VPA (0.5 mM) for 24 h resulted in a significant decrease in CFP emission (Fig. 5g).

Discussion

There is accumulating evidence for a relationship between in utero exposure to VPA and autism spectrum symptoms [39–41]. The effect and mechanism by which VPA impacts the intracellular trafficking and function of MTNR1A are not clear. By expressing epitope-tagged receptors (MTNR1A-EGFP) in cells, we here demonstrated the dynamic changes of MTNR1A internalization after melatonin treatment.

Fig. 5 The inhibitory effect of VPA on β -arrestin 2 binding to MTNR1A. **a** CFP-labeled MTNR1A (MTNR1A-CFP) and YFP-tagged β -arrestin 2 (YFP- β -arrestin 2) constructs were used for FRET analysis. Acceptor fluorescence was recorded before (**b**) and after (**c**) photobleaching of whole cell. **d** There were no significant differences among the groups with respect to the acceptor fluorescence. Percent donor fluorescence was recorded frame by frame before (**e**) and after (**f**) laser-induced acceptor photobleaching. **g** VPA reduced the association between β -arrestin 2 and MTNR1A. HEK-293 cells were transfected with MTNR1A-CFP/YFP- β -arrestin 2 and stimulated with melatonin (100 nM) for 15 min while the emission signals of excited MTNR1A-CFP (FRET donor) were recorded. Data were analyzed using Student's *t* test (* $P < 0.05$; ** $P < 0.01$)



Furthermore, the present study characterized the effect of VPA on the trafficking properties of MTNR1A in in vitro expression system. We found the detrimental role of VPA in altering the internalization kinetics of MTNR1A, which resulted in the disruption of β -arrestin 2-dependent trafficking of MTNR1A and related signaling.

Previous data have suggested that pineal endocrine hypofunction is associated with autism spectrum and that melatonin is useful in improving the behavior of children and youth with such disorders [10, 12]. In VPA-treated animals, melatonin-induced phosphorylation of CaMKII/PKC/PKA contributes to the amelioration of social behavior dysfunction [8]. However, the effect of VPA on the intracellular trafficking of MTNR1A remained elusive until now. Therefore, improved knowledge of the cellular trafficking properties of MTNR1A in autism spectrum disorders might provide insights into clinical treatment options.

MTNR1A-mediated signal transduction is the result of a coordinated balance between processes governing the desensitization and re-sensitization of this receptor [42, 43]. Different Rab isoforms regulate different aspects of receptor intracellular trafficking, such as internalization (Rab5), recycling (Rab11), and degradation (Rab7) [44, 45]. Following internalization, we found that Rab5-positive early endosomes and Rab11-positive recycling endosomes were largely involved in MTNR1A intracellular trafficking. In addition, Rab7-positive late endosomes also colocalized with MTNR1A-mCherry-positive structures in melatonin-treated HEK-293 cells. Therefore, Rab GTPases play an important role in the internalization, endocytic trafficking, and vacuolar sorting of MTNR1A. Our observations suggest that internalized MTNR1A may be either sequestered in early endosomes in a Rab5-dependent manner or transported back to the plasma membrane via Rab11-dependent recycling.

Melatonin signaling activates a complex cascade of events including cAMP-dependent PKA [8, 36, 46]. Using time-lapse confocal microscopy, we observed that VPA interfered with both MTNR1A internalization kinetics and MTNR1A signaling in vitro. Melatonin modulates glycine currents through the PLC/PKC signaling pathway, which is partially dependent on Ca^{2+} [47]. Our data suggest that VPA may partially abolish melatonin-mediated phosphorylation of PKA (Thr197) and ERK (Thr202/Tyr204), which normally occurs downstream of MTNR1A internalization. Parallel with the observation that melatonin did not elicit significant changes in whole-cell currents, the level of phospho-PKC also remained constant under our experimental conditions.

β -Arrestin 2 has a large impact on the regulation of MTNR1A trafficking, localization, and pharmacological properties [21]. We found that VPA treatment alone had no direct effect on whole-cell currents, G-protein-dependent signaling, or β -arrestin 2 recruitment. However, as demonstrated by the FRET data, when the cells were pretreated with VPA before melatonin stimulation, the association between β -arrestin 2 and MTNR1A was effectively blunted. No significant changes were observed in cAMP accumulation upon VPA alone treatment. Together, these observations indicate that the effect of VPA on the endocytic trafficking of MTNR1A might be due to the disassociation between MTNR1A and β -arrestin 2. Nevertheless, the molecular architecture and stoichiometry of the MTNR1A protein complex are still poorly understood [48, 49]. In addition, GPCR heterodimers may undergo alternative ligand-dependent trafficking compared with their monomeric or homodimeric counterparts [50]. Therefore, further investigation is required to evaluate whether other components of the MTRNR1A protein complex also play a role in the disruptive effect of VPA on melatonin-mediated MTNR1A internalization.

By demonstrating the attenuation of melatonin-mediated MTNR1A internalization and signaling by VPA, this study provides the first cellular and molecular mechanisms for the detrimental effect of VPA on neuropsychological development. Therefore, novel therapies that relieve the effect of VPA on MTNR1A intracellular trafficking may help lower the risk of autism spectrum disorders.

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Conflict of interests The authors declare no conflicts of interest.

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