

Baiap3 regulates depressive behaviors in mice via attenuating dense core vesicle trafficking in subsets of prefrontal cortex neurons

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ABSTRACT

Selective serotonin reuptake inhibitors (SSRIs) are effective first line therapies for treating depression, but are plagued by undesirable side effects and are not effective in all patients. Because SSRIs effectively deplete the neuronal releasable serotonin (5-HT) pool, gaining a deeper understanding of intracellular mechanisms regulating 5-HT pools can help us understand the shortcomings of SSRIs and develop more effective therapies. In this study, we found that BAIAP3 (brain-specific angiogenesis inhibitor 1-associated protein 3) is significantly downregulated in two mouse models of depression (the IR- and CUMS-induced depressive mouse models). In BAIAP3 downregulated models (*in vitro* and *in vivo*), we discovered that trafficking of dense core vesicle (DCV), organelles that store, transport and release cargo via exocytosis, was reduced. Accordingly, 5-HT exocytosis and levels in the synapse were lowered, causing defective post-synaptic neurotransmission. In a screen of natural products, we identified eucalyptol, the active components of Eucalyptus, as uniquely capable of increasing neuronal Baiap3 expression and elevate synaptic 5-HT levels. Moreover, eucalyptol treatment relieved depressive behavioral symptoms and restored serotonin levels in mice. Mechanistically, eucalyptol restores Baiap3 expression by reducing inhibitory microRNAs (miR-329, miR-362). These findings illuminate how Baiap3 depletion propagates neurotransmission dysfunction and point to eucalyptol as a novel agent for restoring serotonin exocytosis, suggesting potential for developing eucalyptol as a therapy for treating depression.

1. Introduction

Depression, affecting over 260 million people of all ages world-wide (World Health Organization (January 2020)), is a several mental disorder that in its most severe form can lead to suicide. Decades of research have centered on identifying molecular and physiological changes in the limbic system, including the prefrontal cortex and hippocampus, that can explain the pathogenesis of depression (Pandya et al., 2012). Although several hypotheses based on these discoveries have been proposed, identification of specific molecular mechanisms that initiate and drive depression, and that can be harnessed to develop effective treatments, have remained out of reach.

Although various factors are associated with depression, serotonin is its most potent pathophysiological regulator. By blocking serotonin

reuptake and maintaining levels of synaptic serotonin, serotonin selective reuptake inhibitors (SSRIs) are effective first-line therapies for treating depression (Vaswani et al., 2003). Considering how effective SSRIs are, maintaining synaptic serotonin levels has become established as a central mechanism for treating depression (Cowen and Browning, 2015). However, whether the onset of depression and individual variation in response to SSRIs also involves a serotonin level-regulating mechanism in the synapse is less clear. Genome-wide association studies of patients with depressive disorders identified genetic variants associated with differing outcomes of SSRI therapy. More recent work goes further by pinpointing single-nucleotide polymorphisms (SNPs) in the promoter of the serotonin receptor 7 gene, and in the ERICH gene, an ORF associated with vesicular function in serotonergic and other neuronal cell types, suggest that studying regulation of receptor expression and vesicular transport can inform why patients with

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Abbreviations

Baiap3	Bai-1 associated protein 3
DCV	dense core vesicle
CUMS:	chronic unpredictable mild stress
IR	ionizing irradiation
PFC	prefrontal cortex
5-HT	5-Hydroxytryptamine
SSRIs	serotonin selective reuptake inhibitors
SNPs	single-nucleotide polymorphisms
TGN	trans-Golgi network
OFT	open field test
TST	tail suspension test
FST	forced swimming test
CSF	cerebrospinal fluid
CgA	Chromogranin A
Vamp4	Vesicle-associated membrane protein 4
BBB	blood-brain barrier
F.T	fragrance treatment
I.P	intraperitoneal
TFs	transcription factors

depression differ in their responses to therapy (Liu et al., 2020; Wei et al., 2020).

In both the nervous and endocrine systems, dense core vesicle (DCV) is essential for peptidergic and aminergic signaling (Persoon et al., 2018). DCVs mature in the cell body and dendrites and function in exocytosis of neuropeptides or neurotransmitters including serotonin. Distinct from synaptic vesicles, DCV release their contents relatively slowly (Kreutzberger et al., 2019) and undergo compensatory endocytosis, in which DCV membrane proteins are recycled through retrograde trafficking to the trans-Golgi network (TGN). If the recycling process is defective, retrograde trafficking and maturation of DCVs does not proceed normally and defective DCVs accumulate in the cell body. DCV abnormalities, including accumulation of defective DCV, can induce anxiety and other mental disorders (Wojcik et al., 2013). Considering the role of DCV in synaptic signaling homeostasis, DCV dysfunction has a high possibility of resulting in neuronal abnormalities and subsequent mental disorders, but the relationship between DCV and mental disorders is not well understood.

Bai1 associated protein 3 (Baiap3), initially identified as a protein-binding partner of p53-target gene Bai-1 (that encodes a brain-specific angiogenesis inhibitor) is reportedly involved in dense core vesicle (DCV) trafficking (Shiratsuchi et al., 1998). Baiap3 is a C2 domain-containing Munc13 protein, which play essential roles at multiple steps in anterograde DCV-mediated trafficking. Accordingly, Baiap3 knockdown results in accumulation of non-fusion DCV in neuroendocrine cells and lysosome degradation of insulin-containing DCV in INS-1 β cells (Zhang et al., 2017). Unlike other Munc13 proteins, BAIAP3 indirectly regulates DCV exocytosis by affecting DCV maturation through DCV protein recycling. DCVs play essential roles in the accumulation and transmission of monoamines such as adrenaline, noradrenaline, dopamine and serotonin prior to their release via SNARE-dependent exocytosis (Tao et al., 2018, 2019). As such, the function of Baiap3 and DCVs in neuroendocrine cells and neurons has been studied, but whether Baiap3-mediated regulation of DCV turnover and homeostasis might play a role in nervous system dysregulation of serotonergic signaling is not clear.

In this study, we identified Baiap3 as a significantly downregulated gene in a cross-comparison of RNA sequencing and microarray data from two mouse models of depression. We found that decreased Baiap3 expression leads to defective DCV trafficking and decreased serotonin exocytosis. Mechanistically, we show that Baiap3 is regulated by

microRNAs (miR-329, miR-362), and that the natural product eucalyptol restores Baiap3 levels and serotonin exocytosis and reduces depressive behavior in mouse models of depression.

2. Materials and methods

2.1. Animal protocol

Male C57BL/6 mice (6 weeks of age; Orient bio., Gyeonggi-do, Republic of Korea) were used for the *in vivo* experiments. The animal protocols were approved by the Institutional Animal Care and Use Committee of Pusan National University (Busan, Republic of Korea), and performed in accordance with the provisions of the NIH Guide for the Care and Use of Laboratory Animals. Mice were housed in groups of up to five in sterile cages. Animals were maintained in animal care facilities in a temperature-regulated room (23 ± 1 °C) under a 12 h light/dark cycle, allocated and randomized for the experiments by the technician of the facility, and quarantined for 1 week prior to the study. The animals were fed water and a standard mouse chow diet.

2.2. Open field test (OFT)

Locomotor activity and depressive-like behavior of mice were assessed in rectangular chambers (WxLxD = 50 cm \times 50 cm \times 38 cm). Mice were habituated for 3 min in the chamber (without recording) then placed for another 5min in center of the chamber (with recording). Locomotor activity and localization were recorded and analyzed by the time in center zone using Noldus EthoVision XT software (Noldus Information Technology, Leesburg, VA).

2.3. Elevated plus maze test (EPM)

Elevated plus maze is consist of two opposing open arms (WxL = 50 cm \times 10 cm) and two opposing closed arms (WxLxD = 50 cm \times 10 cm \times 40 cm) connected by a central platform (WxL = 10 cm \times 10 cm) and elevated 50 cm above the floor. Each mouse was put gently on the central platform, directed facing an open arm. Locomotor activity and localization were recorded for 5 min and analyzed using Noldus EthoVision XT software (Noldus Information Technology, Leesburg, VA). The maze was wiped with a 70% EtOH after each trial.

2.4. Injection of miR-329 and miR-362 mimics

Briefly, 0.75 mg/ml miRNA-329 and miRNA-362 mimics were prepared for miRNA injection. miRNA mimics in InvivoFectamine® solution was incubated for 30 min at 50 °C. C57BL/6J mice were anesthetized with mixture of Zoletil and Rompun solution (1 : 3 ratio, 1 ml/kg, i.p) and mounted in a stereotaxic apparatus (RWD Life Science Co., Ltd.). 2 μ l of miR-mimics in InvivoFectamine® solution was injected into each prefrontal cortex through a polyethylene stainless steel syringe intubation (RWD Life Science Co., Ltd.). The animals were anesthetized with isoflurane and fixed in a stereotaxic frame and a small craniotomy (0.5 mm) was made unilaterally over the mPFC, 1.7 mm anterior to bregma and 0.4 mm lateral to the midline. miR-mimics were delivered by a glass capillary attached to a stereotaxic injector (RWD Life Science Co., Ltd.). The entire infusion procedure took 5 min.

2.5. Primary cell culture

Pregnant C57BL/6 mice were obtained from a specific pathogen-free colony at Oriental Inc. (Seoul, Korea). Embryos were dissected from embryonic day 15.5 mice and prepared for culturing. The prefrontal cerebral cortices were dissected under sterile conditions, and the meninges were pulled off. The prefrontal cerebral cortices were then digested with 2.5% Trypsin-EDTA (Gibco BRL) in DMEM media for 15 min at 37 °C. After digestion, cells were dispersed through a pipette and

filtered through a 70 μm nylon net filter. The cells were then centrifuged 5 min at 500g; washed with Neurobasal medium containing B27 serum-free supplement (Thermo Scientific) and penicillin/streptomycin (Thermo Scientific). The cultured cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂. Mature cultured primary cortical neurons (DIV14) were confirmed by immunocytochemistry analysis using Syp, Map2 primary antibodies with DAPI.

2.6. Image analysis

Primary cultured neuron was attached by a poly-d lysine-coated confocal plate. Cells were fixed in 4% paraformaldehyde (5 min) and permeabilized with 0.1% Triton X-100 in PBS with 5% BSA (10 min). After blocking with 1% BSA in PBS (30 min), primary antibodies were incubated overnight at 4 °C and stained with secondary antibodies for 1 h. Coverslips were mounted with Fluoromount™ aqueous mounting medium. Samples were imaged with a confocal microscope (LSM 800 system). The microscopes were controlled by Zeiss black edition software. Secondary antibodies were labeled with Alexa Fluor 488, or 647. Nuclei were stained with DAPI mounting solution. GFP were used for live-cell studies.

2.7. Reagents

siRNA specific for Baiap3 (EMU192281) and control siRNA were purchased from Sigma (St. Louis, MO). Eucalyptol was purchased from Sigma (St. Louis, MO). Antibodies against MAP-2 (sc-20172), α -Tubulin (sc-23948) and β -actin (sc47778) were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specific for Baiap3 (256 003) was purchased from Synaptic Systems (Göttingen, Germany). Antibody for CgA (ab15160) was purchased from Abcam (Cambridge, UK). GFP-tagged CgA ORF clone (MG207386) was purchased from OriGene Technologies (Rockville, MD). Mmu-miR-329 and mmu-miR-362 mimics and inhibitors were synthesized by Macrogen (Seoul, Republic of Korea).

2.8. Tail suspension test (TST)

The TST was conducted as previously described (Kang et al., 2018). In brief, mice were suspended from a plastic rod mounted 50 cm above a surface by fastening the tail to the rod with adhesive tape. A 6 min trials were recorded and analyzed using Noldus EthoVision XT software (Noldus Information Technology, Leesburg, VA).

2.9. Forced swimming test (FST)

The FST was conducted as previously described (Kang et al., 2018). In brief, mice were placed in a glass cylinder (height: 30 cm, diameter: 16 cm) containing water at 24 °C and a depth of 14 cm, so that they could neither escape nor touch the bottom. Mice were forced to swim for 6 min. The animals were habituated for the first 1 min and behavior was monitored over the next 5 min. A 6 min trials were recorded and analyzed using Noldus EthoVision XT software (Noldus Information Technology, Leesburg, VA).

2.10. Sucrose preference test (SPT)

Anhedonic behavior was assessed by a sucrose preference test. Briefly, before testing, C57BL/6 mice were habituated with two bottles of pure water and 1% sucrose water. The positions of sucrose and water were alternated daily, and the amounts of sucrose solution and water consumed were determined by weighing each bottle when removed from the cage, and for sucrose solution. Sucrose preference was calculated as $100 \times [(\text{sucrose intake}) / (\text{sucrose intake} + \text{pure water intake})]$ by averaging results for 3 days.

2.11. Real-time qRT-PCR

The expression levels of mRNAs, miRNAs, and EMT-related genes were analyzed by real-time qRT-PCR, as previously described. Aliquots of the master mix containing all of the reaction components with the primers were dispensed into a real-time PCR plate (Applied Biosystems, Foster City, CA, USA). All PCR reagents were from a SYBR Green core reagent kit (Applied Biosystems). The expressions of all genes were measured in triplicate in the reaction plate. The qRT-PCR was performed using the StepOne Real-Time PCR System (Applied Biosystems). It was performed by subjecting the samples at 95 °C for 15 s and at 60 °C for 1 min for 40 cycles followed by thermal denaturation. The expression of each gene relative to Gapdh mRNA was determined using the $2^{-\Delta\Delta\text{CT}}$ method. To simplify the data, values for the relative expression were multiplied by 10^2 .

2.12. Western blot analysis

The assessment of protein levels was performed following previous study (Kang et al., 2018). In brief, Whole cell lysates (WCL) were prepared using radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 25 mM NaF, 1 mM dithiothreitol (DTT), 20 mM EGTA, 1 mM Na₃VO₄, 0.3 mM phenylmethanesulfonyl fluoride (PMSF), and 5 U/mL aprotinin) and the protein concentrations in the lysates were determined using a BioRad protein assay kit (BioRad Laboratories, Hercules, CA). Protein samples were subjected to SDS-PAGE, transferred to a nitrocellulose membrane and then blocked with 5% bovine serum albumin in TBST for 1 h at RT. Next, membranes were probed with specific primary antibodies and peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). The samples were subsequently analyzed using an ECL detection system (Roche Applied Science, Indianapolis, IN). Detection and densitometric analysis were conducted using the iBright Western blot imaging systems (ThermoFisher).

2.13. Statistical analysis

All numeric data are presented as the means \pm standard deviation (SD) from at least three independent experiments. Experimental results were analyzed by one-way ANOVA for ranked data followed by Tukey's honestly significant difference test. The Prism 9 software (GraphPad Software, San Diego, CA) was used to conduct all statistical analyses. A *p*-value < 0.05 was considered to be statistically significant.

3. Results

3.1. Baiap3 is down-regulated in two mouse models of depression

Animal models that mimic aspects of depressive behavior have provided insights into mechanisms associated with depression. Here, we performed a molecular cross comparison of two mouse models of depression; the chronic unpredictable mild stress (CUMS) model and the IR-induced depression model (Kang et al., 2018). To identify molecular targets associated with depression, we analyzed CUMS RNA-sequencing data, published in the NCBI database (Ma et al., 2016), and IR-induced depression microarray data, generated in a previous study (Kang et al., 2018) using the frontal cortex of the limbic system, brain regions that plays a key role in the control of depression. As neurotransmission dysfunction is considered the major driving force of depressive symptoms, 'neurotransmitter transport', 'regulation of neurotransmitter levels' and 'neurotransmitter metabolic process' GO process were filtered in both datasets. Through analysis of the intersection between the two filtered datasets with over 1.5 folds change, we identified the only gene, Baiap3 (Fig. 1A and B).

To confirm that Baiap3 is downregulated in mouse models of depression, we first set up and validated both IR-induced and CUMS

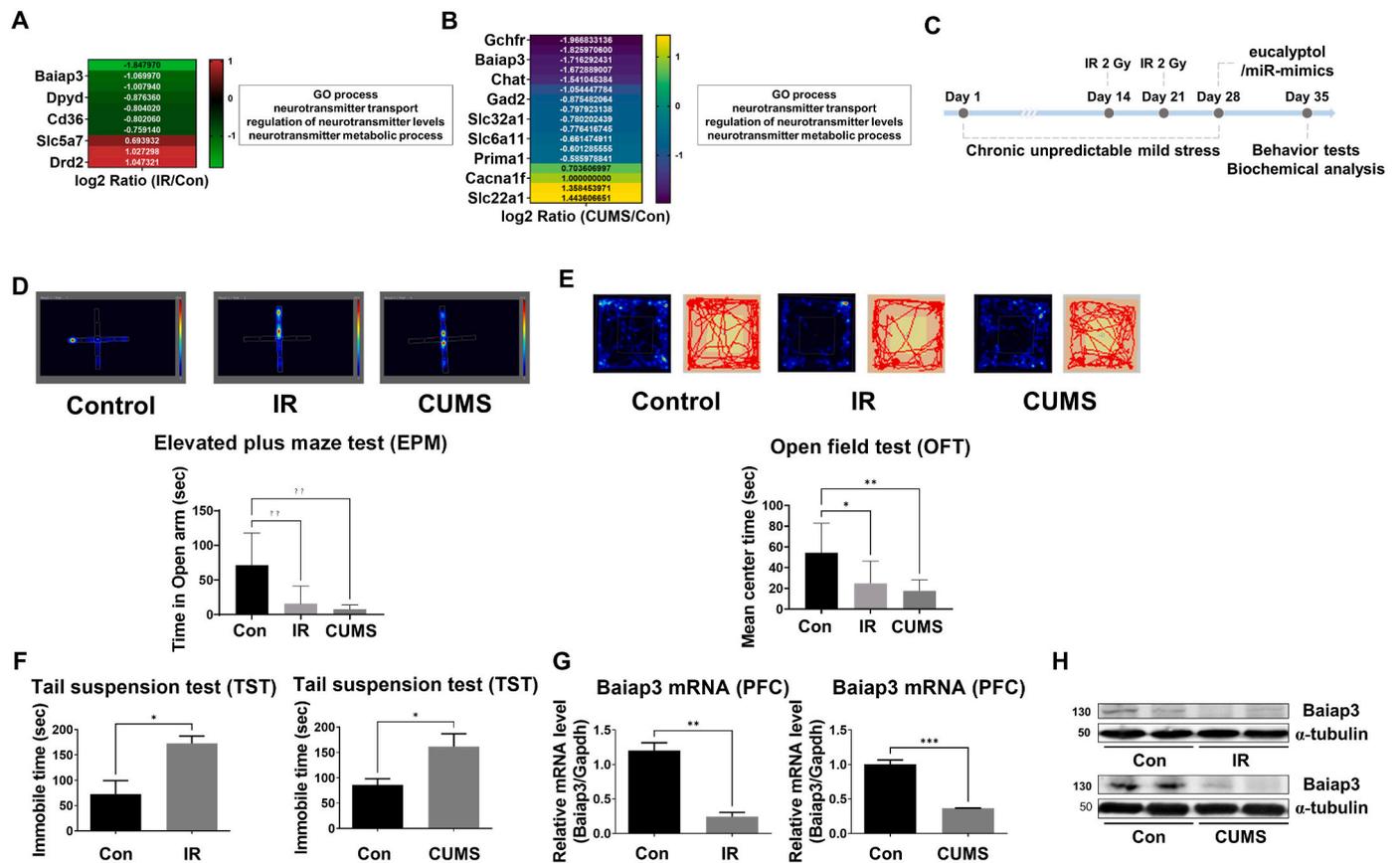


Fig. 1. Baiap3 is down-regulated in two mouse models of depression. **A.** Heat map representing gene expression changes (at least 1.5 fold) in mouse PFC which are related in 3 GO processes (neurotransmitter transport, regulation of neurotransmitter levels or neurotransmitter metabolic process) following cranial irradiation (IR-induced depression). Microarray experiments were performed using cDNA samples obtained from hippocampus tissues treated or non-treated with 2 Gy of radiation and microarray results were deposited in the Gene Expression Omnibus database (GSE94440). **B.** Heat map representing gene expression changes (at least 1.5 fold) in mouse PFC which are related in 3 GO processes (neurotransmitter transport, regulation of neurotransmitter levels or neurotransmitter metabolic process) following chronic unpredictable mild stress (CUMS-induced depression). Parental RNA-sequencing information are from published NCBI database (GSE81590). **C.** Time-course of the animal experiments. **D.** Qualitative, positional heatmaps show the average time spent in the closed arms (vertical axis) and open arms (horizontal axis). **E.** Open Field Test (OFT) of IR-induced and CUMS mice. Qualitative, positional heatmaps show the average time spent in the center. **F.** TST in IR-induced and CUMS mice to assess depressive behavior. C57BL/6 mice were treated with cranial radiation and CUMS and duration of immobility was checked after 1 week. **G.** Real-time qRT-PCR analysis of Baiap3 mRNA levels in IR-induced and CUMS mouse PFC tissue. **H.** The effects of irradiation and CUMS on Baiap3 protein expression in mouse PFC regions were confirmed by Western blot analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, using two tailed t -test or one-way ANOVA analysis.

mouse models through behavioral experiments including Elevated Plus-maze test, open field test (OFT), tail suspension test (TST) and forced swimming test (FST) (Fig. 1C). As expected, both mouse models showed increased depressive behaviors relative to control mice (Fig. 1D, E, F and Figs. S2A and B). When we analyzed PFC in both mouse models, we found that Baiap3 mRNA levels were reduced relative to control mice (Fig. 1G). In addition, Baiap3 protein levels were reduced in both mouse models (Fig. 1H). Collectively, these results show that Baiap3 expression is down regulated in both IR-induced and CUMS mouse models, consistent with deposited data.

3.2. Down-regulated Baiap3 expression disturbed the secretion of 5-HT

To investigate how reduced Baiap3 expression impacts on cellular function, we utilized primary neuronal cultures isolated from the prefrontal cortex of prenatal mice and cultured for 14 days *in vitro* (DIV14) (Fig. 2A). To elicit Baiap3 expression changes, we either irradiated the cultured neuron (4 Gy in two fractions) or transfected Baiap3 siRNA neuron, and found that both approaches reduced Baiap3 mRNA and protein levels (Fig. 2B and C). We confirmed that there was no change in Baiap3 expression after irradiation in hippocampal neurons, confirming that this was a prefrontal cortex neuron-specific change (Fig. S1). Given that Baiap3 is known to regulate DCV trafficking, we next assessed

serotonin secretion after irradiation and Baiap3 siRNA transfection. Both treatments reduced neuronal serotonin secretion, as detected by ELISA in cultured media (Fig. 2D). These observations were extended to IR-induced and CUMS depression mouse models, as we found in both cerebrospinal fluid (CSF) and serum that serotonin levels were down-regulated (Fig. 2E). Taken together, these data show that decreased Baiap3 expression is associated with lower serotonin secretion both *in vitro* and *in vivo*.

3.3. Accumulation of defective DCVs in neurons with low Baiap3 expression

To investigate whether DCV trafficking is impacted by Baiap3 expression, we next performed *in vitro* immunofluorescent experiments. Unfortunately, since the currently available primary antibodies to Baiap3 and CgA are only rabbit hosts, there is a limit to the immunocytochemical reaction by co-labeling, so DCV trafficking was confirmed except for simultaneous confirmation of Baiap3. In Baiap3-positive neurons, approximately 30% of irradiated and siRNA-treated neurons (Fig. S5), Baiap3 was decreased in Baiap3 positive neurons and colocalization of the DCV marker Chromogranin A (CgA) and the Golgi marker Vesicle-associated membrane protein 4 (Vamp4) increased compared to control neurons (Fig. 3A), in which based on previous

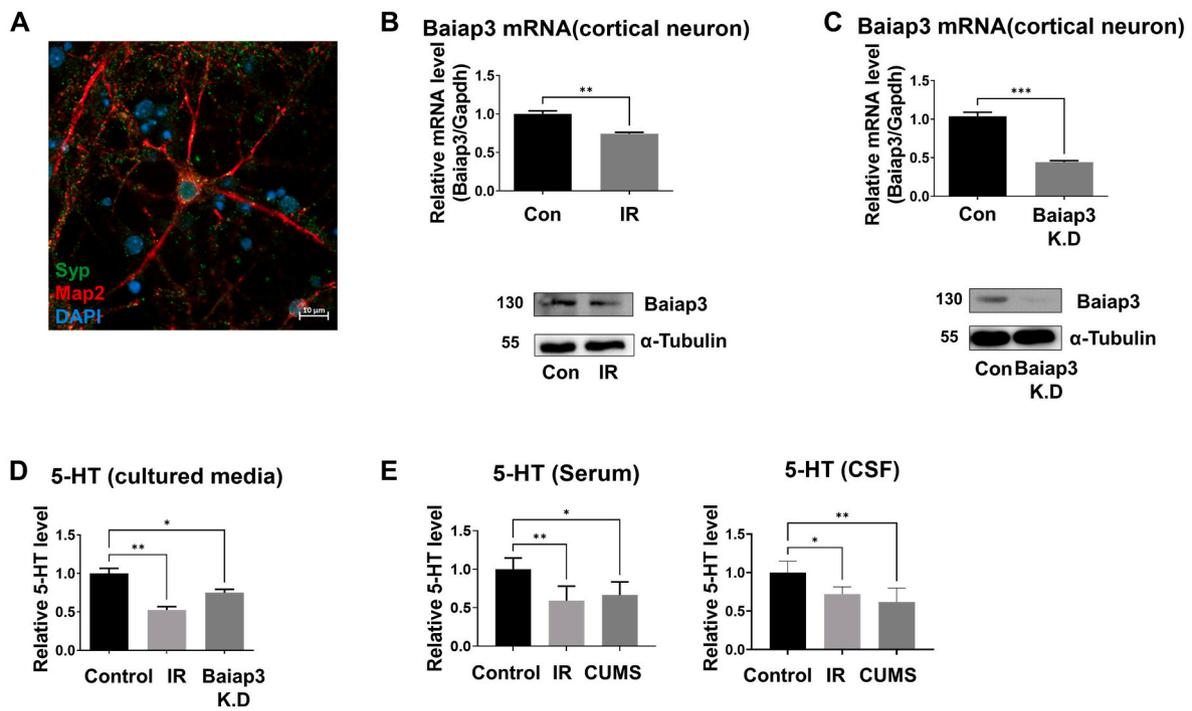


Fig. 2. Down-regulated Baiap3 expression disturbed the secretion of 5-HT. **A.** Primary cortical neurons cultured for 14 days (DIV14) were used for all experiments, using immunocytochemistry analysis for Map2 expression to confirm neuronal maturation. **B.** Baiap3 mRNA and protein levels after irradiation were analyzed and confirmed by qRT-PCR and Western blot. **C.** Baiap3 mRNA and protein levels after siBaiap3 transfection were analyzed and confirmed by qRT-PCR and Western blot analysis. **D.** 5-HT levels in cultured media after irradiation and Baiap3 knockdown was analyzed by ELISA. **E.** Serum and CSF 5-HT levels in IR-induced and CUMS models were analyzed by ELISA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, using two tailed *t*-test or one-way ANOVA analysis. Scale bar 10 μm .

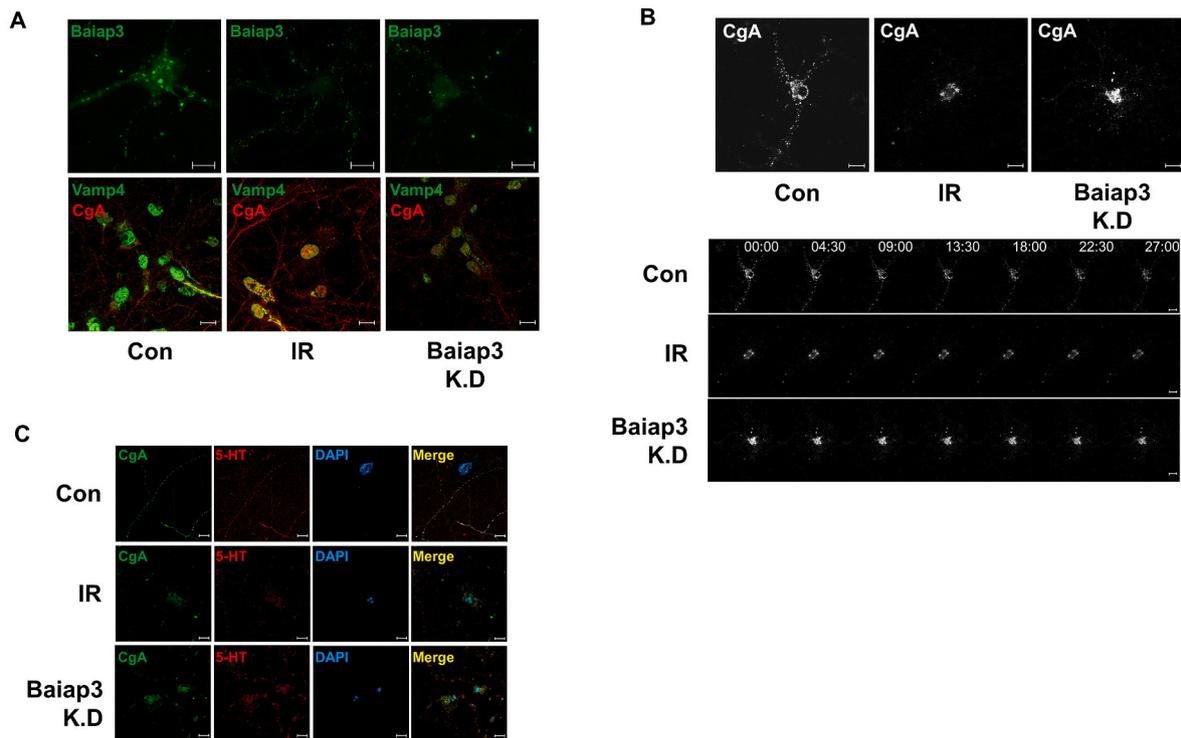


Fig. 3. Accumulation of defective DCVs in neurons with low Baiap3 expression. **A.** Co-immunofluorescent staining of the DCV marker CgA and the Golgi marker VAMP4 in irradiated and Baiap3 knockdown cortical neuron. **B.** Immunofluorescent staining of GFP-CgA in cortical neurons. The number in each panel refers to the time at which each image was captured (mm:ss). **C.** Co-immunofluorescent staining of the DCV marker CgA and 5-HT were performed in irradiated and Baiap3 knockdown cortical neuron. Scale bar 10 μm .

studies is an indication of a high proportion of defective DCVs (Zhang et al., 2017). In live cell imaging of GFP-CgA transfected cortical neurons, we found that DCV trafficking was delayed in both IR-irradiated and Baiap3 siRNA-treated neurons. Furthermore, we observed that DCVs primarily localized within the soma as opposed to being equally distributed in the soma and axon compartments (Fig. 3B), indicating that neurons with reduced Baiap3 have lower capacity to transport DCVs to the axonal region. This distribution was confirmed that the DCV carrying serotonin also appeared in the same way (Fig. 3C). These data suggest that decrease of Baiap3 levels lead to an increase in defective DCVs and dysfunctional DCV trafficking.

3.4. Eucalyptol treatment rescues Baiap3 expression and 5-HT secretion

Given that a decrease in Baiap3 results in defective neurotransmitter transport, we next sought to identify treatment strategies for upregulating neuronal Baiap3 expression. Because natural products are generally considered safe and have demonstrated efficiency in many studies, we selected a panel of candidates based two criteria; either a terpenoid, which could be used for fragrance therapy, or an agent with known capability to penetrate the blood-brain barrier (BBB), as the largest impediment to brain treatment through vascular injection of drugs is the BBB. According to the above criteria, we selected and screened 16 natural products in neurons for capacity to elevate Baiap3 mRNA expression. Of the set, only eucalyptol significantly increased both Baiap3 mRNA levels (Fig. 4A) and Baiap3 protein expression (Fig. 4B). In neurons in which Baiap3 levels had been reduced by IR or Baiap3 siRNA, eucalyptol treatment restored serotonin secretion (Fig. 4C).

In the mouse depression models, eucalyptol delivered via two routes of administrations; inhalation with incense (Fragrance treatment; F.T) or intraperitoneal (I.P) injections, restored Baiap3 protein levels in the frontal cortex (Fig. 4D). I.P injection was more efficient in restoring Baiap3 protein levels than the F.T, and we therefore used I.P injections in subsequent experiments. Eucalyptol treatment increased mobility relative to control in both IR-induced and CUMS mouse models as assessed in behavioral tests (Fig. 4E, Figs. S3A, B, C). Depression symptoms changes were also confirmed by the recovery of serotonin levels in CSF and serum (Fig. 4F). In both IR-treated and Baiap3 knockdown neurons, eucalyptol treatment decreased co localization of CgA and Vamp4, which indicate immature DCVs (Fig. 4G). In live cell imaging experiments, eucalyptol treatment restored CgA distribution in distal part of the cell body and increased the movement of DCV to distal part of the cell body in both IR-treated and Baiap3 knockdown neurons (Fig. 4H and Supplementary videos). Together, these results suggest that eucalyptol treatment restores Baiap3 expression in both IR-treated and Baiap3 knockdown neurons.

3.5. Eucalyptol rescued the Baiap3 expression and depressive behavior through inhibition of miRNA-329 and miRNA-362

Next, we sought to identify the molecular mechanisms by which eucalyptol regulates Baiap3 expression. Given that Baiap3 is a relatively new member of the neuronal retrograde trafficking machinery that returns secretory vesicle components to the TGN, little is known about its regulation. Here, we decided to focus on microRNA regulation and performed *in silico* screening to find microRNAs that specifically binding to Baiap3 mRNA. By using the TargetScan database, we identified two candidate miRNAs; mmu-miR-329 and mmu-miR-362 (Fig. 5A). We found that both mmu-miR-329 and mmu-miR-362 were upregulated in primary cortical neurons and in the PFC of IR-induced and CUMS depression mouse models, and that their levels were reduced upon eucalyptol treatment *in vitro* and *in vivo* (Fig. 5B, C, D). Next, to assess the specificity of the microRNA regulation, we performed *in vitro* experiments with miRNA inhibitors specific for miR-329 and miR-362. In irradiated cortical neurons, we found that both mRNA inhibitors

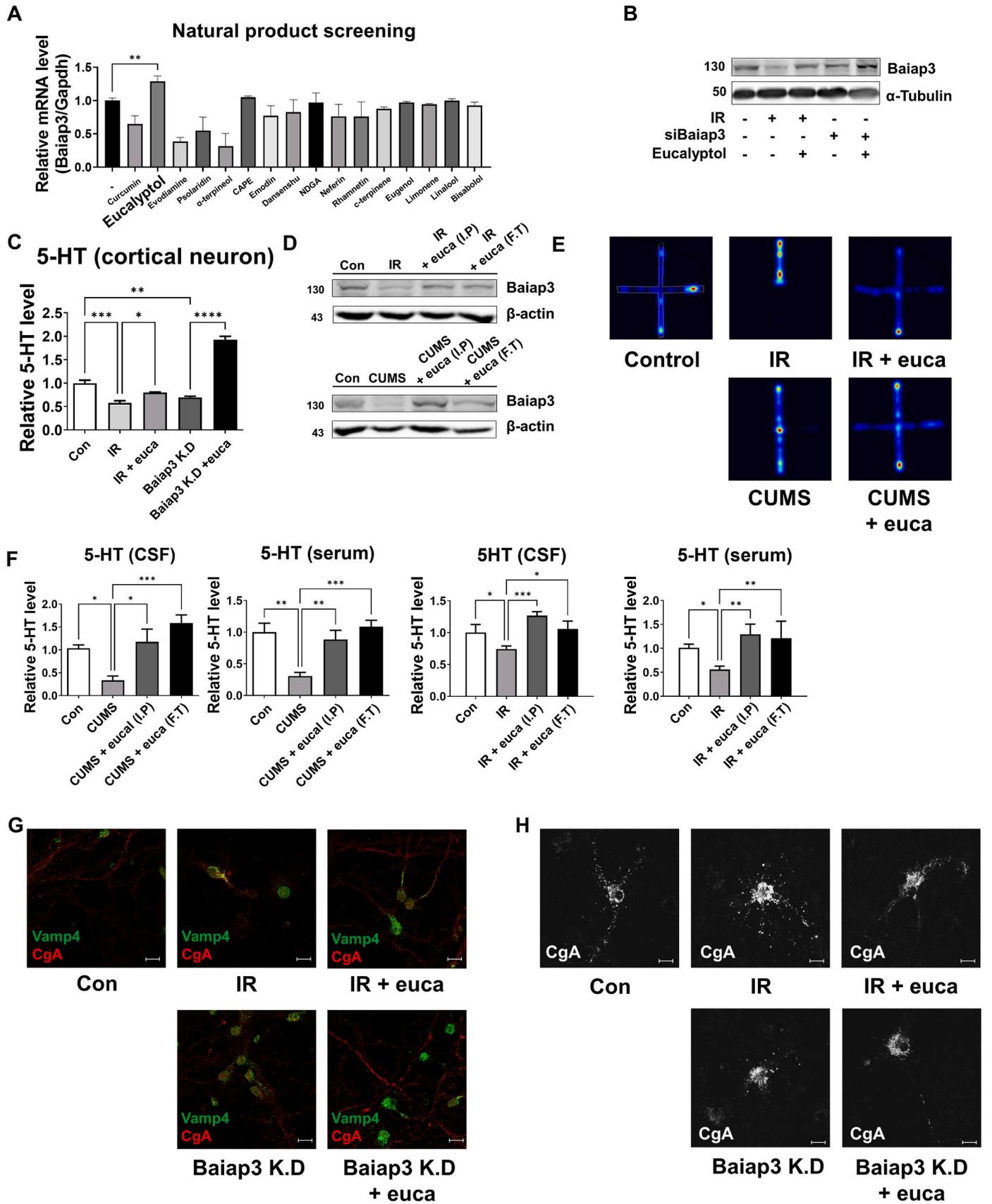
prevented upregulation of miR-329 and miR-362, respectively, concomitant with maintaining Baiap3 protein levels (Fig. 5E). The specific miR-329 and miR-362 inhibitors also reversed the accumulation of defective DCVs and impaired 5-HT containing DCV trafficking which indicates 5-HT transmission (Figs. 5F and S4A). Furthermore, miR-329 and miR-362 reversed the effects of eucalyptol treatment in neuron (Figs. 5G and S4B). Based on these data, miR-329 and miR-362 are key factors regulating Baiap3, and their inhibition can protect Baiap3 levels. Next, we sought to assess whether the microRNA-mediated mechanism was functional *in vivo*. To this end, IR-induced and CUMS mice were treated with eucalyptol only or eucalyptol with miR-329/miR-362 to cortical area. In behavioral assessments, we found that mice that had received eucalyptol and either miR-329 or miR-362 mimics, the behavioral improvement conferred by eucalyptol only was no longer present (Fig. 5H, Figs. S4C and S4D). As we had seen before *in vitro*, eucalyptol protected Baiap3 mRNA levels in the PFC of both depression models. However, in mice that had also received miR-329 or miR-362, Baiap3 mRNA levels were similar to untreated IR-induced and CUMS mice (Fig. 5I). In addition, serotonin secretion and miRNAs expression in PFC were also comparable between IR-induced and CUMS mice and mice treated with both eucalyptol and miR-329 or miR-362 (Fig. J and Fig. S4E). Taken together, these results suggest that Baiap3 mRNA levels are decreased by both miR-329 and miR-362 and miRNAs in a way that prevents eucalyptol from inducing antidepressant effects in IR-induced and CUMS mice.

4. Discussion

Neuronal serotonin transmission is believed to have a prominent role at the onset of depression. The regulation of emotions and behaviors by serotonin has been verified through various studies and clinical experiences (Kanen et al., 2021; Wolf et al., 2018), but both general and context-dependent upstream mechanisms that regulate serotonin are poorly understood. We here propose Baiap3 as a novel candidate emotion regulator based on our findings that Baiap3 regulates serotonin transmission and depressive behavior in two mouse models of depression. We found that Baiap3 was profoundly reduced in the PFC of IR-induced and CUMS mice, and accordingly, that reduction of Baiap3 in cultured cortical neurons was associated with DCV abnormalities and reduced serotonin synaptic release. Furthermore, we identified eucalyptol as a natural product capable of restoring beneficial Baiap3-dependent cellular phenotypes as well as reversing depressive behavior. Taken together, our findings places Baiap3 as a key regulator of synaptic serotonin and thereby potentially functioning as a gate-keeper for induction of depression.

DCV regulation according to Baiap3 expression in PFC was focused in this study. However, the expression level of Baiap3 in PFC is very low, and in fact, subset of neurons was Baiap3 positive neurons (about 30%) even in the primary neurons used in this study (Fig. S5). It is known that the expression level of Baiap3 varies by brain region, and the expression level in PFC is much lower than that in other regions (Lein et al., 2007). In the hippocampus, which plays an important function in regulating emotions, the expression level of Baiap3 was sufficient, but it was confirmed that the amount was not decreased by radiation and stress. In addition to the PFC and hippocampus, changes in other areas related to mood control (e.g. amygdala, hypothalamus, etc.) were not confirmed in this study. Whether Baiap3 affects mood regulation in other areas needs to be confirmed through follow-up studies. However, as revealed in this study, the phenotype differences were clearly shown according to the change in Baiap3 expression in PFC and the subsequent regulation of DCV trafficking. Therefore, despite the low basal level of Baiap3 in PFC, it can be seen that the decrease in Baiap3 expression may play an important role in mood regulation.

In this study, we measured the amount of serotonin available for exocytosis by DCVs through *in vitro* and *in vivo* experiments. Serotonin, released via exocytosis into the synaptic cleft, binds serotonin receptors



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Fig. 4. Eucalyptol treatment rescues Baiap3 expression and 5-HT secretion. **A.** A focused screen assaying 16 natural products for their capacity to affect Baiap3 mRNA level in cortical neurons, analyzed by real-time qRT-PCR. **B.** Western blot analysis of eucalyptol effects on Baiap3 expression after irradiation and knock-down of Baiap3 in cortical neurons. **C.** ELISA analysis of eucalyptol effects on 5-HT levels in cultured media after irradiation and knockdown of Baiap3. **D.** Western blot analysis of eucalyptol effects on Baiap3 protein expression in IR-induced and CUMS models. Eucalyptol was injected intraperitoneally or delivered as fragrance therapy. **E.** Qualitative, positional heatmaps show the average time spent in the closed arms (vertical axis) and open arms (horizontal axis). **F.** ELISA analysis of eucalyptol effects on 5-HT level in CSF and serum after irradiation and Baiap3 knockdown. $n = 5$ per group. **G.** Co-immunofluorescent staining of the DCV marker CgA and the Golgi marker VAMP4 in irradiated and Baiap3 knockdown cortical neuron after 2 days of eucalyptol treatment. **H.** Immunofluorescent staining of GFP-CgA in cortical neuron. Imaging analysis was performed in cortical neuron 2 days after eucalyptol treatment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, using two tailed t -test or one-way ANOVA analysis. Scale bar 10 μm .

on the postsynaptic neuron, causing neurotransmission, and is reabsorbed into the presynaptic neuron by serotonin reuptake channels (Belmer et al., 2017). In patients with depression, SSRIs suppresses the reuptake of serotonin and maintain the amount of serotonin between synapses to normalize neurotransmission (Sangkuhl et al., 2009). Although SSRIs increase the amount of serotonin available in the synaptic cleft, this therapeutic principle can be limited if insufficient serotonin is produced or released. Indeed, SSRIs are not effective in all patients; 10–30 percent are identified as SSRI-nonremitters. In recent paper investigating induced pluripotent stem cells (iPSCs) derived from patients with MDD, and known to be either SSRI-remitters or SSRI-nonremitters, 5-HT release and reuptake was unaltered, but postsynaptic hyperactivity and changes in neurite length and morphology was associated with SSRI-nonremitters (Vadodaria et al., 2019b). As a result of RNA-seq data provided by above study, it was confirmed that the expression level of BAIAP3 in the neurons of SSRI remitters was about 4.13 times higher than that of SSRI nonremitters (Vadodaria et al., 2019a). Thus, it can be inferred that BAIAP3 expression levels might determine the magnitude of serotonin secretion in a way that correlates with SSRI's antidepressant efficacy. From this point of view, the discovery of mechanisms regulating Baiap3 levels and serotonin exocytosis may be the basis of a more fundamental solution for SSRI non-responders.

Obtaining samples from patients with psychiatric disorders is often difficult, unlike in other diseases including cancer, and animal models of depression are therefore valuable. However, the use of a single animal model can be limiting, given the range of phenotypes associated with different types of depression (Wang et al., 2017). Here, we utilized the most common CUMS model and the IR-induced depression model. The latter simulates onset of depression, which can appear as a side effect after cranial irradiation. Cranial irradiation is a treatment method used in brain cancer, nasopharyngeal cancer, or prophylactic treatment to prevent brain metastasis and is known to induce emotional disorders as side effects (Le P  choux et al., 2011; Marusak et al., 2018). Based on clinically applied radiotherapy protocol, we utilized a dose of 2 Gy irradiated in two separated fractions, which we found to reproducibly produce robust depressive behavioral characteristics. On this basis, we conclude that the IR-induced depression model, which mimics onset of depression, complements the CUMS model, which represent general depression well, in a way that may enable identification of factors uniquely triggering onset of depression.

It is well known that the etiology of psychiatric disorder involves interactions between genetic vulnerabilities and environmental cues (Moran et al., 2016; Tsuang et al., 2004). Several studies have demonstrated a crucial role for epigenetics (e.g. DNA methylation, post-translational histone modifications and microRNA-mediated post-transcriptional regulation) to impact on adult behavioral outcomes, which can progress towards psychopathology (Forstner et al., 2015; Giridharan et al., 2016). Within this context, non-coding RNAs, especially microRNAs, came to light as pivotal regulators of many biological processes occurring in the CNS, including neurogenesis, emotion regulation and synapse development as well as learning and memory (Lang and Shi, 2012; Lopez et al., 2017; Moran et al., 2016; Qiao et al., 2020). On this basis, microRNA modulation and expression has been investigated in many brain disorders, including neurodegenerative disorders and mental illnesses, and more specifically, alterations in

miRNA-regulated systems in patients diagnosed with MDD or during major depressive episodes (Alural et al., 2017; Dwivedi, 2014; van den Berg et al., 2020). Overall, possibly given sample heterogeneity, the findings thus far have rarely been replicated between studies, with only a few miRNAs reported to be altered in more than one study, among which miR-132, miR-451a, and miR-34a-5p seem to be the most consistent (Cha et al., 2019; Hicks and Middleton, 2016; Samadian et al., 2021). Given that microRNAs have the advantage of being highly sensitive to drug treatment, many studies have identified specific microRNAs as potential therapeutic targets. Although the emergence of miRNA therapeutics has not yet translated into FDA-approved candidates for medical intervention, candidate miRNA drugs are in preclinical development or in phase 1 and 2 clinical trials (Hanna et al., 2019). In this study, we discovered miR-329 and miR-362 as novel regulators of Baiap3. Both miR-329 and miR-362 strongly inhibit Baiap3 expression, and we confirmed that Baiap3 expression was effectively restored by reducing miRNAs. In general, transcription factors (TFs) are challenging to target therapeutically because they regulate numerous genes and are often regulated by complex mechanisms. Considering the 1700–1900 human TF proteome, this amounts to an average ratio of 1.6 signaling genes per TF, indicating redundancy mechanisms for most TFs (Fontaine et al., 2015). Recently, in a lymphoblastoid cell line, the expression of 59 transcription factors and chromatin modifiers was independently knocked down (by at least 50%, using small interfering RNAs) and down-regulated genes were identified in three independent microarray experiments. The number of genes differentially expressed ranged from 40 to 4,000, depending on the knock down experiment (Cusanovich et al., 2014), illustrating the disadvantages that need to be overcome when targeting transcription factors therapeutically. However, with microRNAs, specific genes as therapeutic targets can be directly targeted. Here, we found that the decrease of Baiap3 in both depression models was caused by an increase in miR-329 or miR-362, and that microRNA inhibition restored Baiap3 expression. This finding can be said to be the discovery of an intuitive and potent treatment strategy.

Treatment of mental disorders including depression with natural products has been attempted, and although not used in clinical practice, there are examples of beneficial effects (Diers et al., 2008). However, given their undefined mechanism of action, benefits of treating depression using natural products is likely to differ from person to person. In this study, we propose eucalyptol as a natural product that can exert antidepressant effects by restoring Baiap3 expression. Eucalyptol is a monoterpene, with a unique scent as most monoterpenes, and is a highly safe compound used in foods and drugs (Hon  rio et al., 2015; Seol and Kim, 2016). Monoterpene-based natural products are widely used as essential oils and have been reported to relieve tension and stabilize mood, supporting the potential of eucalyptol as an antidepressant. Most antidepressant strategies using natural products have not achieved consistent efficacy, and underlying mechanisms are unknown (Zielińska-Błajet and Feder-Kubis, 2020). In this study, we found that the effect of eucalyptol was both consistent and potent in the mouse depression models. In addition, we show that eucalyptol can exhibit antidepressant effects by reducing the expression of microRNAs (miR-329, miR-362) that inhibit Baiap3 expression. This can be said to be a research achievement that supports the use of eucalyptol as a safe therapeutic strategy with a defined mechanism of action for treating depression.

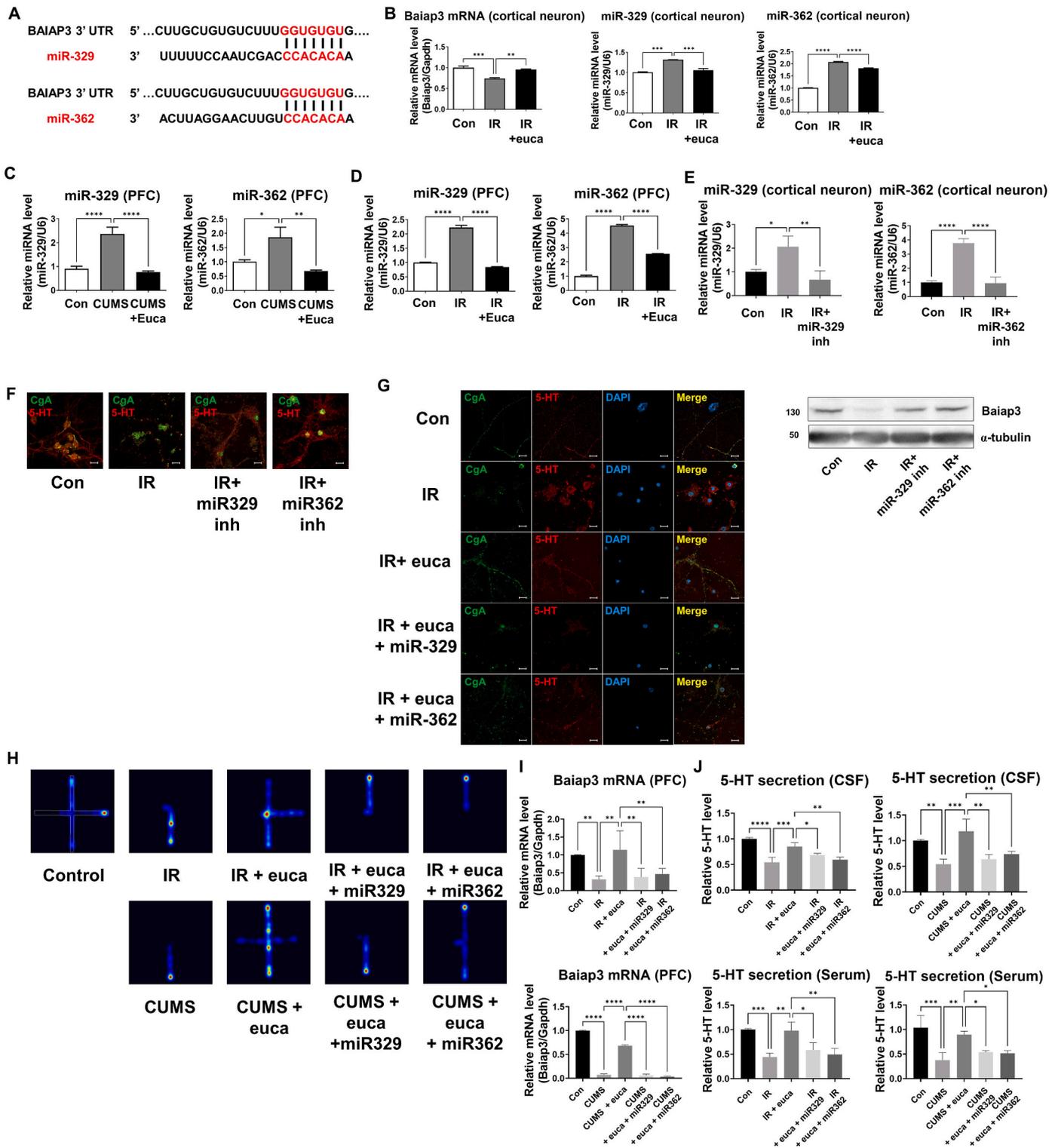


Fig. 5. Eucalyptol rescued the Baiap3 expression and depressive behavior through inhibition of miRNA-329 and miRNA-362. **A.** Schematic of the miR-329- and miR-362-binding sites in the Baiap3 3' UTR. **B.** qRT-PCR analysis of Baiap3 mRNA levels and levels of miR-329 and miR-362 level after irradiation and eucalyptol treatment in cortical neurons. **C, D.** miR-329 and miR-362 levels in PFC tissue after eucalyptol treatment in two depression mouse models analyzed by qRT-PCR. *n* = 6 per group. **E.** miR-329 and miR-362 levels in cortical neurons treated with miR inhibitors analyzed by qRT-PCR. Baiap3 protein levels in cortical neurons treated with miR inhibitors analyzed by Western blot analysis. **F.** Co-immunofluorescent staining of the DCV marker CgA and the Golgi marker VAMP4 in irradiated and Baiap3 knockdown cortical neuron 1 day after miR inhibition. **G.** Co-immunofluorescent staining of the DCV marker CgA and 5-HT were performed in irradiated and Baiap3 knockdown cortical neuron 1 day after eucalyptol treatment. **H.** Elevated plus maze test was assessed in IR-induced and CUMS depression models 1 day after eucalyptol treatment. Qualitative, positional heatmaps show the average time spent in the closed arms (vertical axis) and open arms (horizontal axis). **I.** Baiap3 mRNA levels in PFC tissues after injection of miR-329 and miR-362 mimics analyzed by real-time qRT-PCR. **J.** 5-HT levels in CSF and serum after injection of miR-329 and miR-362 mimics assessed by ELISA. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, using two tailed *t*-test or one-way ANOVA analysis. Scale bar 10 μm.

5. Conclusions

We demonstrated that novel target Baiap3 by regulating trafficking of serotonin-containing DCV has a crucial role in development of depression. Baiap3 levels are regulated by two microRNAs (miR-329, miR-362) in a mechanism that is regulated by eucalyptol, indicating potential for developing eucalyptol as a new antidepressant strategy. Given that neurotransmitters including serotonin is implicated in the etiology of several neuropsychiatric diseases (schizophrenia, autism and cognitive disorders), our findings contribute towards understanding pathophysiological causes of mental disease more broadly as well as development of therapeutic strategies targeting upstream mechanisms regulating synaptic serotonin to treat depression.

Ethics approval and consent to participate

The animal protocols were approved by the Institutional Animal Care and Use Committee of Pusan National University (Busan, Republic of Korea), and performed in accordance with the provisions of the NIH Guide for the Care and Use of Laboratory Animals. Mice were housed in groups of up to five in sterile cages. Animals were maintained in animal care facilities in a temperature-regulated room (23 ± 1 °C) under a 12 h light/dark cycle, allocated and randomized for the experiments by the technician of the facility, and quarantined for 1 week prior to the study. The animals were fed water and a standard mouse chow diet.

Consent for publication

All authors read and approved the final manuscript.

Availability of supporting data

The datasets and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Authors' contributions

H.K. and B.Y. designed research; H.K. and B.Y. wrote the paper; H.K., J.K., H.L., E.S. and H.K. performed experiments; J.J. performed irradiation experiments. All authors read and approved the final manuscript.

CRedit authorship contribution statement

Hyunwoo Kim: Conceptualization, Methodology, Investigation, Writing – original draft. **Jeongha Kim:** Investigation, Visualization. **Haksoo Lee:** Formal analysis. **Eunguk Shin:** Visualization. **Hyunkoo Kang:** Visualization. **Jaewan Jeon:** Resources. **BuHyun Youn:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare no competing interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jynstr.2021.100423>.

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