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Downregulation of the $GABA_A$ receptor β2 subunit in a rat model of autism

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Conflict of interest

Authors of the present study have no conflict of interest to disclose.

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Author contributions

MLLM contributed to the study conceptualization and design, material preparation and provided study resources. The first draft of the manuscript was written by AAPL. FSV and AAPL performed the experiments and contributed to data acquisition and analysis. LDCC, CMV and LBP provided study resources and contributed to writing-review and editing. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethical Considerations

This study was carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and under the ARRIVE guidelines.

Decreased $GABA_A$ receptor β2 subunit immunoreactivity in a rat model of autism.

Abstract

Introduction: Gamma-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the brain, and activation of GABA type A ($GABA_A$) receptors mediates rapid inhibitory actions. Numerous studies have shown that individuals with autism spectrum disorder (ASD) exhibit abnormalities in the expression of $GABA_A$ receptors in several brain areas. In addition, animal models of ASD have suggested alterations in GABAergic neurotransmission and dysregulation of the balance between inhibitory and excitatory systems. **Objective:** We investigated the immunolabeling of GABAA receptor β2 subunit (GARB2) in the hippocampus, the amygdala, and the thalamus of infant rats prenatally exposed to valproic acid (VPA) as a model of ASD. **Methods:** Pregnant female rats were injected with VPA (600mg/Kg, i.p.) on embryonic day 12; control rats were injected with saline (SS group). On postnatal day 14, rats from both groups were anesthetized, transcardially perfused with 0.9% NaCl and 4% paraformaldehyde, and sequential coronal brain slices (40µm thickness) were obtained. Immunohistochemistry

was performed to detect GARB2, and the relative optical density (OD) of immunoreactivity was analyzed. **Results:** Our data showed a statistically significant decrease in GARB2 immunoreactivity in the lateral amygdaloid nucleus and the ventral and lateral thalamic nuclei of VPA group when compared to the SS group. No statistically significant differences were found in the hippocampus. Discussion: Our findings suggest that prenatal exposure to VPA reduces GARB2 immunoreactivity in limbic brain regions involved in social-emotional behavior, consistent with previous reports in individuals with ASD. Conclusion These findings support for the involvement of the GABAergic system in the pathogenesis of ASD.

Keywords: GABA, $GABA_A$, Autism, Valproic acid, GARB2.

Introduction

Autism spectrum disorder (ASD) is a complex neurodevelopment disorder characterized by difficulties in social communication (verbal and nonverbal), interaction and repetitive behaviors.[1] According to the Centers for Disease Control and Prevention in the United States, ASD affects 1 in 44 children, with a higher prevalence in boys than in girls (4.2 times more prevalent among boys).[2] However, the etiology of ASD remains unclear.

Individuals with ASD often exhibit abnormalities in glutamate[3-6] and gamma-aminobutyric acid (GABA) neurotransmission systems.[7-11] GABA receptors type A ($GABA_A$) are ligand-gated ion channel that mediate rapid inhibition in the brain.[12] This receptor is composed by five protein subunits with different isoforms: α1-6, β1-3, γ1-3, δ, ε,

θ, π.[13,14] The most common arrangement of $GABA_A$ receptors in the central nervous system (20 - 50% of all central synapses) is the α1β2γ2[15,16], with GABA binding at the junction between α and β subunits.[17] Autoradiography studies of brain tissue from individuals with ASD have revealed decrease density of $GABA_A$ and benzodiazepine receptors in the hippocampus and the anterior cingulate cortex.[18-20] In adition, reduced mRNA expression of $GABA_A$ receptor α6, β2, and γ2 subunits has been detected in the superior frontal cortex and the cerebellum of individuals with ASD.[10] Interestingly, the gene encoding the $GABA_A$ receptor β2 subunit has been associated with an increased risk of ASD.[21] Furthermore, 3-4% of individuals with ASD have chromosomal duplications in the proximal region of 15q11-q13, the most commonly observed chromosomal abnormality in these patients.[22] This chromosomal region contains the *GABRB3*, *GABRA5*, and *GABRG3* genes, which encode β3, α5, and γ3 subunits of the GABAA receptor, respectively.[23]

Preclinical studies using the valproic acid (VPA) rat model of ASD have demonstrated disruptions in the excitatory/inhibitory balance in the amygdala[24], the hippocampus[25,26] and cortex.[27] Impaired GABA-mediated

inhibition has been identified in the rat hippocampus in the VPA-induced model,[27] as well as reduced $GABA_A$ receptor α1, α2, α3 and β3 mRNA levels in the medial prefrontal cortex of adult rodents.[28] Thus, evidence supports the hypothesis of GABAergic dysfunction in ASD. The VPA autism model has been widely used as an environmental model of ASD in rodents; however, the effect of prenatal exposure to VPA on brain $GABA_A$ receptor expression has not been fully characterized. In particular, the hippocampus, amygdala and thalamus are brain areas involved in behavioral alterations or pathological changes observed in both individuals with ASD and animal models.[29] Therefore, we aimed to investigate the expression of the $GABA_A$ receptor β2 subunit (GARB2) in these brain areas in infant rats exposed to VPA *in utero*.

Methods

Animals

This study adhered to Mexican guidelines on the care and use of laboratory animals (NOM-062-ZOO-1999) and was approved by the Internal Committee for the Care and Use of Laboratory Animals of the Instituto de Investigaciones Cerebrales (CICUAL-CICE 2017-002-c). Wistar rats were

obtained from our local colony and housed in our vivarium. Throughout the study, rats were maintained in a 12:12 h light-dark cycle, with lights on at 08:00, under room temperature and humidity conditions, with free access to water and food (Rismart). Adult female rats with regulated fertility cycles were mated overnight with a sexually experienced male. The presence of spermatozoa in vaginal smears the following morning indicated the first day of pregnancy. On the twelfth and a half embryonic day, females received a single intraperitoneal injection of 600 mg/kg of VPA (sodium valproate Sigma-Aldrich, St. Louis, MO, dissolved in 0.9% NaCl for a concentration of 250 mg/mL) for the VPA group. Control rats were injected with 0.9% NaCl on the same embryonic day (SS group). Females were housed individually and allowed to rear their litters.[30] Experiments to assess GARB2 immunoreactivity were performed on postnatal day 14 (P14) rat pups. The SS group consisted of 9 rats (3 males and 6 females), while the VPA group consisted of 10 rats (8 males and 2 females).

Immunohistochemistry

The rats were deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and transcardially perfused with 0.9% NaCl

132 followed by 4% paraformaldehyde (prepared in 0.1 M
133 phosphate buffer [PB], pH=7.4) at a flow rate of 12 mL/min
134 flow. Brains were left *in situ* overnight at 4 °C. The next
135 day, they were removed and postfixed in the same fixative
136 for an additional 2 h. Subsequently, the brains were
137 cryoprotected with 30% sucrose (prepared in 0.1M PB) for 72
138 h at 4 °C. Brain coronal sections (40 µm thick) were
139 obtained at the level of the dorsal hippocampus using a
140 Leica cryostat.
141 For the immunohistochemical detection, the slices were
142 rinsed in 0.1 M PB containing 0.1% triton (0.1% PBT).
143 Endogenous peroxidases were quenched with 30% hydrogen
144 peroxide for 10 min. To block nonspecific binding, the
145 slices were treated with 5% horse serum in 0.3% PBT for 1 h
146 at room temperature. Subsequently, the slices were
147 incubated with the primary antibody against GARB2 (1:1000;
148 MAB341, Millipore) for 48 h at 4 °C. The slices were then
149 incubated with a biotinylated anti-mouse secondary antibody
150 (1:400; Vector Laboratories Inc.) for 90 min at room
151 temperature, followed by incubation with the avidin-biotin
152 complex (ABC kit PK-6100 Vector Laboratories Ellite-
153 Standard Inc.) for an additional 90 min at room
154 temperature. Immunodetection was visualized using 3,3′-

diaminobenzidine in the presence of nickel (SK-4100 Vector Laboratories Inc.). Brain slices were mounted on electrostatically charged glass slides (Superfrost, Fisher Scientific) and coverslipped using non-aqueous medium (Permount, Fisher). Immunolabeling was performed on some brain slides without the incubation with primary antibody to discard non-specific immunostaining (negative control); no unwanted immunoreactivity was found.

Densitometric analysis

Photomicrographs of three different brain sections per rat (from either left or right hemisphere) were taken using a Leica DM500 light microscope connected to a Leica ICC50 HD digital camera. The Leica Application System LAS EZ 4.8 software was used for this purpose. Photomicrographs were taken of the dorsal hippocampus (including the strata oriens, pyramidal, and radiatum in CA1, CA2, and CA3 fields, as well as the granule cell layer and hilus of the dentate gyrus), the lateral and basolateral nuclei of the amygdala, and the ventral and lateral nuclei of the thalamus. A standard brightness of 55% and a magnification of 40x were used.

The relative optical density (OD) of GARB2 immunoreactivity was analyzed using Fiji Image J software. The software was calibrated according to developer´s instructions, allowing the transformation of pixel values to a scale that correlates with optical density. This allowed the determination of the mean gray value of the region of interest (ROI).[31] The ROI was defined as 6,500 μm^2 for each stratum of the hippocampus and 70,000 μm^2 for both the amygdala and the thalamus. The presence of immunoreactivity to GARB2 appeared as gray to black, while its absence was indicated by a white color. The OD background was determined by averaging the optical density of the corpus callosum from the slices used. This brain region was chosen because it does not contain $GABA_A$ receptors.[32] The background was then subtracted from all images. The final GARB2 OD for each animal was obtained by averaging the OD from the three analyzed slices and expressed as arbitrary units (a.u.). A higher relative OD indicates increased expression of the protein of interest.

Statistical analysis

Data were initially assessed for normality of distribution using the Shapiro-Wilk test. Differences in GARB2 immunoreactivity between the VPA and SS groups in different brain regions were analyzed using either an unpaired two-tailed Student's t-test or a Mann-Whitney test, as appropriate. Analyses were performed using GraphPad Prism software (version 6), with a significance level of $\alpha = 0.05$.

Results

Statistical analysis showed that prenatal VPA administration significantly decreased GARB2 immunoreactivity in the basolateral nucleus of the amygdala ($t=2.814$, $df=17$; $p = 0.012$) compared to the SS group. A non-significant reduction was also observed in the lateral amygdaloid nucleus (MWU=21; p=0.0534). Similarly, VPA-treated rats exhibited significantly lower OD values, reflecting reduced GARB2 immunoreactivity in both the lateral ($t=2.804$, $df=17$; $p=0.0122$) and ventral ($t=3.281$, $df=17$; $p<0.004$) thalamic nuclei compared to SS group. No significant differences were found between VPA and SS groups in any hippocampal subregions or strata ($p>0.05$),

although a trend toward decreased GARB2 immunoreactivity was observed in the CA2 pyramidal layer (MWU=23.5; p=0.07; Figures 1 and 2).

Discussion

One hypothesis proposed to explain the etiology of ASD is the imbalance between neuronal excitation and inhibition, primarily mediated by glutamate and GABA, respectively.[33] In this study, we found that infant rats prenatally exposed to VPA exhibited reduced GARB2 immunoreactivity in specific regions of the amygdala and thalamus compared to control rats. These preclinical results support the relevance of GABA receptors in the pathophysiology of autism.

Several studies have reported a decrease in GABA levels in the frontal lobe and anterior cortex of patients with ASD[11,34], and decreased levels of glutamic acid decarboxylase (GAD) 65 and 67, the enzyme that catalyzes the conversion of glutamate to GABA, in the parietal cortex and cerebellum of post-mortem samples from adults with ASD,[8,35] and in the hippocampus and cerebellum of VPA-exposed rats.[36] With respect to GABA receptors, lower densities of $GABA_A$ receptors have been found in the

hippocampus, anterior and posterior cingulate cortex, and fusiform gyrus of post-mortem brain tissue from individuals with autism.[18-20] Additionally, there is a reduction in GARB2 protein levels in the superior frontal cortex and down-regulation of its mRNA in the cerebellum.[10] Interestingly, GARB2 polymorphisms have also been associated with ASD.[21] A (123) I-iomazenil (IMZ, a benzodiazepine ligand) SPECT study in children with ASD found decreased accumulation of (123) I-IMZ in the middle and superior frontal cortex.[37] However, a more recent study found no changes in $GABA_A$ receptor or $GABA_A$ α5 subunit availability in the hippocampal or amygdala regions of adults with ASD.[33]

Our results showed that infant rats prenatally exposed to VPA displayed decreased GARB2 immunoreactivity in the amygdala and thalamus compared to age-matched rats with standard gestation. These findings align with the excitation/inhibition imbalance hypothesis in individuals with ASD. Reduced expression of GARB2, which indirectly indicates reduced availability of $GABA_A$ receptors, may contribute to social deficits[25,38,39] and other neurological changes observed in the VPA rat model, such as increased seizure susceptibility.[25,30] The gene encoding GARB2 has been previously associated with a higher risk of ASD.[21] It

265 is noteworthy that both the amygdala and thalamus have been
266 implicated in behavioral alterations and pathological
267 changes observed in individuals with ASD and animal
268 models.[25,29]

269 Consisted with our findings, Yang et al.[28] also
270 described impaired inhibitory GABAergic neurotransmission
271 due to decreased GABA release and mRNA levels of $GABA_A$
272 receptor α1, α2, α3, and β3 subunits in the medial
273 prefrontal cortex of VPA-exposed mice. These authors also
274 demonstrated that acute administration of combined $GABA_A$
275 and $GABA_B$ receptor agonists reduced deficits in
276 sociability, anxiety, and repetitive behaviors in this ASD
277 model.[28] However, Bertelsen et al.[40] reported increased
278 binding of [11C] Ro15-4513 (an agonist with high affinity
279 for the $GABA_A$ receptor α-subunit) in the left amygdala of
280 VPA-treated rats as an ASD model, whereas no significant
281 differences were found in the thalamus compared to control
282 rats. That study differs from ours in the VPA
283 administration protocol and receptor detection methodology.
284 They administered 20 mg/kg of VPA daily during pregnancy,
285 while we injected a single dose of 600 mg/kg on embryonic
286 day 12. Therefore, it is essential to consider potential

differences in the assessed neurobiological changes depending on the methodological procedures used. Another study conducted in other ASD mouse models (*Cntnap2* or *Shank3* knockout mice and mice with the 16p11.2 deletion) did not identified differences in the levels of $GABA_A$ receptors or their α5 subunit in the frontal cortex, cingulate cortex, caudate/putamen, dorsal hippocampus, cerebellum, or amygdala between these three models or compared to control mice.[35] This discrepancy may be due to specific changes in $GABA_A$ receptor subunits or the different etiology of the ASD models (i.e., environmental versus genetic). Additional experimental protocols are needed to better understand the complex neurobiology of ASD.

Conclusions

Our study provides further evidence supporting the role of GABAergic dysfunction, specifically GARB2 expression, in the pathophysiology of ASD using a VPA-induced rat model. Our findings indicate that prenatal VPA exposure leads to reduced GARB2 immunoreactivity in the amygdala and thalamus, regions associated with social deficits and other

neurological alterations in ASD. These findings are consistent with the hypothesis of an excitation/inhibition imbalance in individuals with ASD. However, the complex neurobiology of ASD warrants further investigation to elucidate the contributions of specific $GABA_A$ receptor subunits and the varying etiologies of ASD models. A deeper understanding of the role of the GABAergic system in ASD, including the impact of hippocampal GABAergic receptors, could pave the way for novel therapeutic interventions and help improve the quality of life for individuals with ASD.

Figure legends

Figure 1. Effect of prenatal exposure to valproic acid (VPA) on GARB2 immunoreactivity in the amygdala, thalamus, and hippocampus of postnatal day 14 rat pups. Abbreviations: SS, saline solution; LaA, lateral nucleus of the amygdala; BLA, basolateral nucleus of the amygdala; LT, lateral nucleus of the thalamus; VT, ventral nucleus of the thalamus; Or, oriens; Py, pyramidale; Ra, radiatum; DG, Dentate gyrus; Gr, Granular layer, Hi, Hilus; CA1, CA2, and CA3 hippocampal regions. *p<0.05.

Figure 2. Photomicrographs show GARB2 immunoreactivity in the amygdala and thalamus of a postnatal day 14 rat pup exposed in utero to valproic acid (VPA) or saline solution (SS). The insets show greater GARB2 immunoreactivity in the rat from the SS group than in the rat from the VPA group (scale bars =100 µm). Arrowheads point to GARB2 immunoreactive cells. Abbreviations: LaA, lateral nucleus of the amygdala; BLA, basolateral nucleus of the amygdala; LT, lateral nucleus of the thalamus; VT, ventral nucleus of the thalamus.

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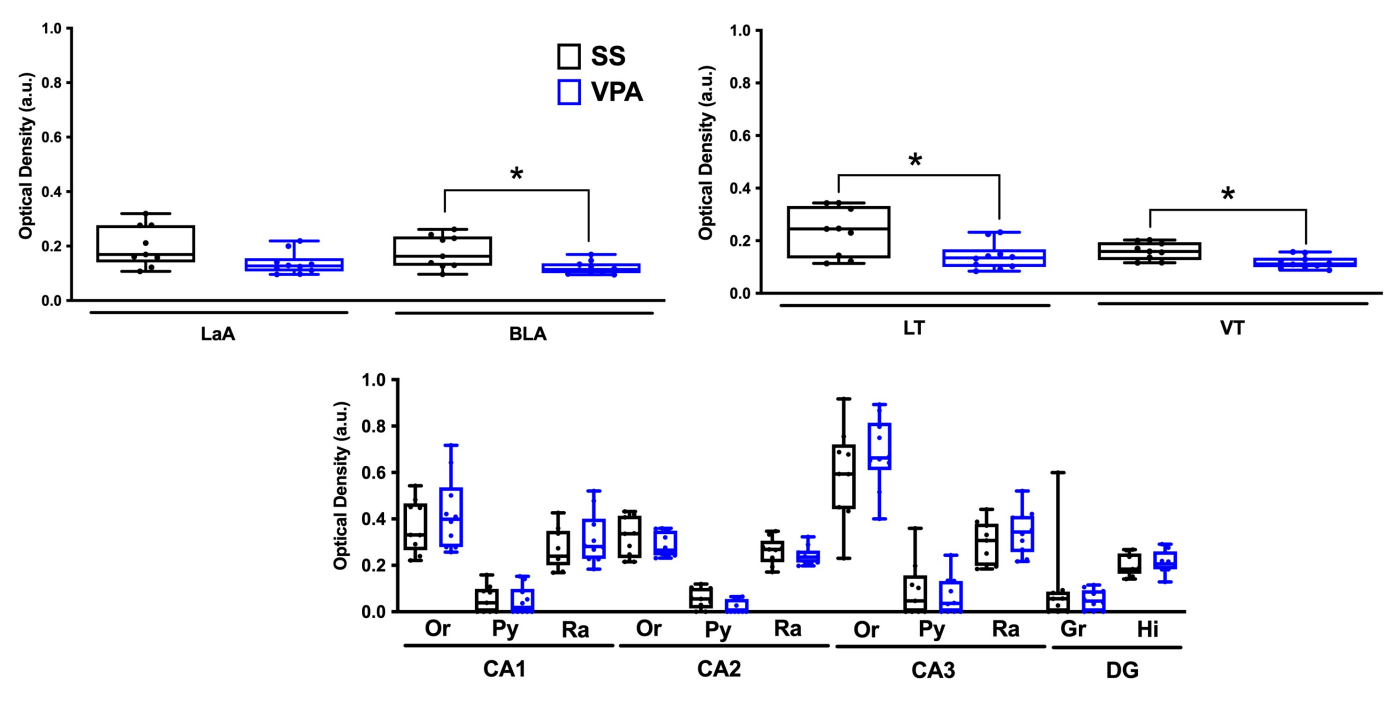
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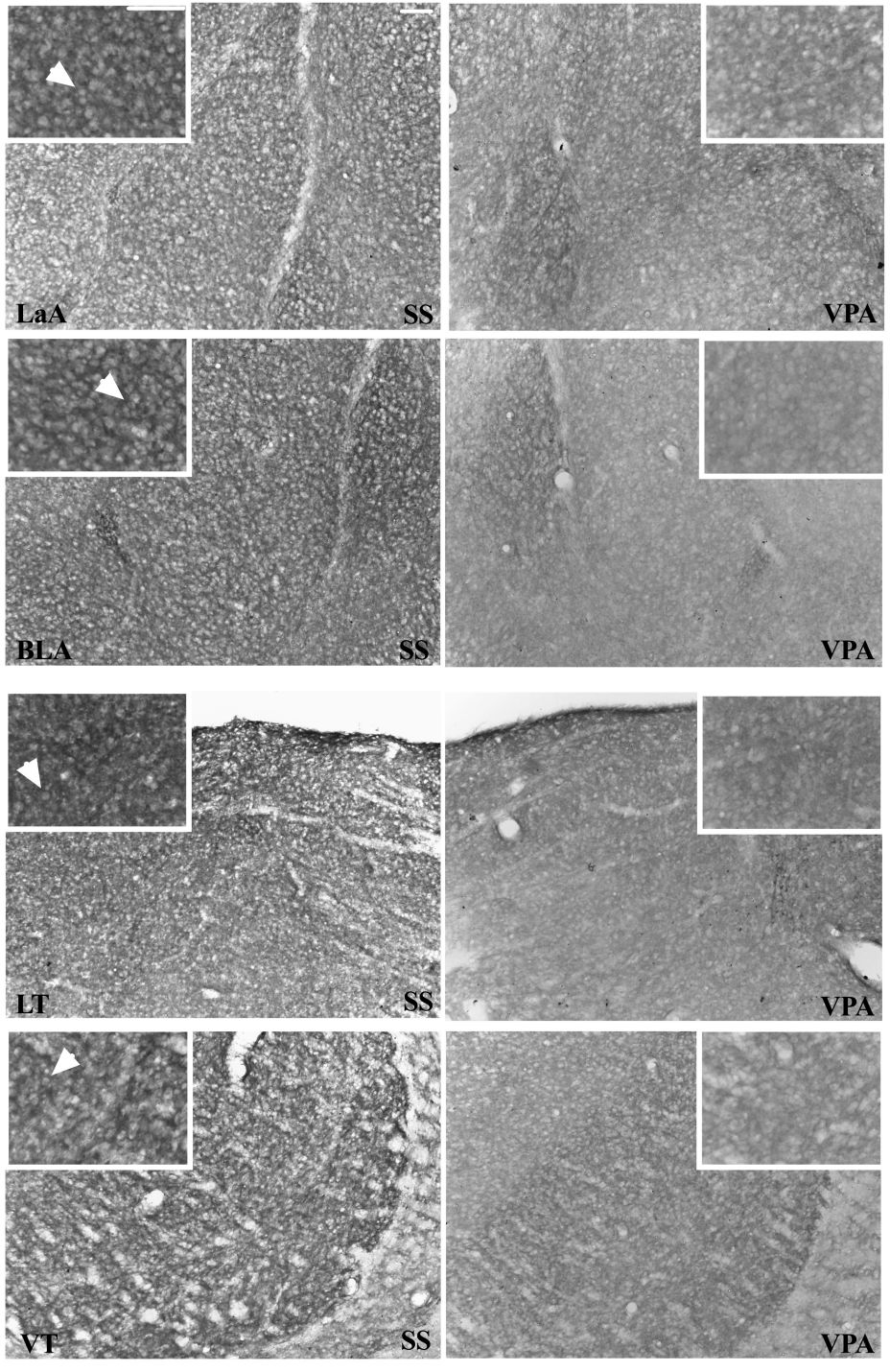
LaA
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