Inhibition of histone deacetylases enhances the function of serotoninergic neurons in organotypic raphe slice cultures

Nozomi Asaoka[1], Kazuki Nagayasu[1, 2, 3], Naoya Nishitani[1], Mayumi Yamashiro[1], Hisashi Shirakawa[1], Takayuki Nakagawa[1, 4, *] and Shuji Kaneko[1]

[1]*Department of Molecular Pharmacology, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan.*

[2]*Drug Innovation Center, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan*

[3]*Laboratory of Molecular Neuropharmacology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan*

[4]*Department of Clinical Pharmacology and Therapeutics, Kyoto University Hospital, Kyoto, Japan*

**Corresponding author*

Takayuki Nakagawa

Department of Clinical Pharmacology and Therapeutics, Kyoto University Hospital

54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan

Tel/Fax: +81-75-751-4560

E-mail: tknakaga@kuhp.kyoto-u.ac.jp

ABSTRACT

Inhibition of histone deacetylases (HDACs) is a promising approach for the treatment of mood disorders. However, the effects of HDAC inhibition on the serotonin (5-HT) system, a common target for psychiatric disorders, are poorly understood. Here, we show that a broad-spectrum HDAC inhibitor, trichostatin A (TSA), enhances the function of 5-HT neurons in organotypic raphe slice cultures. Sustained treatment with TSA (1 μM) for 2 or 4 days significantly increased the 5-HT tissue content and tryptophan hydroxylase 2 (TPH2) expression, which were accompanied by hyper-acetylation of histone H3 in the promoter region of the TPH2 gene. TSA treatment for 4 days increased the extracellular 5-HT level, which was significantly suppressed in the presence of the selective AMPA receptor (AMPAR) antagonist NBQX. Moreover, the expression of both the AMPAR subunit GluA2 and Ca^{2+}/calmodulin-dependent kinase II α (CaMKIIα) mRNAs were significantly increased by TSA treatment. Co-treatment with the CaMKII inhibitors KN-62 and KN-93 prevented the TSA-induced increase in 5-HT release, but had no effect on the increases in 5-HT tissue content. These results suggest that inhibition of HDACs increases 5-HT synthesis and release by epigenetic mechanisms, and that 5-HT release is mediated by the enhancement of AMPAR-mediated excitatory inputs and CaMKII signaling.

Keywords

Histone deacetylase; Serotonin; Raphe slice cultures; Trichostatin A; AMPA receptor; Ca^{2+}/calmodulin-dependent kinase II

1. Introduction

Chromatin modification is one mechanism of epigenetic gene regulation that changes gene expression and cellular function without changing the DNA sequence [1]. Histone acetylation enhances mRNA transcription, and is regulated by the balance between histone acetyltransferase and histone deacetylase (HDAC). HDAC inhibitors increase the acetylation of histones at lysine residues and enhance gene expression [2]. Growing evidence suggests that epigenetic mechanisms affect an individual's vulnerability to psychiatric disorders [3]. Environmental factors, such as early-life stress and chronic stress, can change epigenetic modifications to alter the response to further stress [4]. While HDAC inhibitors are clinically used as anticancer agents, recent evidence suggests a therapeutic potential for HDAC inhibitors in the treatment of psychiatric disorders such as depression [5-7]. However, the mechanisms by which HDAC inhibitors exhibit an antidepressant effect are not fully understood.

The serotonin (5-HT) system plays a key role in pathogenic mechanisms and treatment of mood disorders. In animal models of chronic stress, the activity of 5-HT neurons in the dorsal raphe nuclei (DRN) is decreased [8]. On the other hand, conventional antidepressants such as selective serotonin reuptake inhibitors (SSRIs) potentiate 5-HT signaling by increasing extracellular 5-HT levels, while it takes several weeks to show therapeutic effects [9]. Several studies suggest that not only acute effects, i.e., serotonin transporter inhibition, but also chronic changes, including epigenetic modification, play important roles in the therapeutic effects induced by SSRIs [10, 11]. However, whether epigenetic modification of the 5-HT system is involved in the

antidepressant effect of HDAC inhibitors is currently unclear.

We previously reported that organotypic raphe slice cultures that possess functional 5-HT neurons and local neuronal circuits provide a useful tool for investigation of the effects of antidepressants on the 5-HT system [12, 13]. In this study, we examined whether a pan-HDAC inhibitor, trichostatin A (TSA), affects 5-HT neuronal function using organotypic raphe slice cultures.

2. Materials and Methods

Details are presented in the Supplementary Materials and Methods.

2.1. Preparation of rat organotypic raphe slice cultures

All animal care and experimental procedures were conducted in accordance with the ethical guidelines of the Kyoto University Animal Research Committee. Rat raphe slice cultures were prepared as previously described [12, 13]. Briefly, coronal sections (350 μm thick) containing the dorsal and median raphe nuclei were prepared from Wistar/ST rat pups, and maintained in culture for 14–16 days.

2.2. Measurement of tissue contents and extracellular levels of 5-HT

Tissue contents and extracellular levels of 5-HT were measured as previously described [13]. Briefly, for measurement of tissue contents of 5-HT, the brain slices were homogenized and centrifuged, and the supernatants were analyzed by high performance liquid chromatography with an electrochemical detector (HPLC-ECD)

(Eicom, Kyoto, Japan). For measurement of extracellular 5-HT levels, slices were incubated in 0.7 mL Krebs-Ringer-Henseleit (KRH) buffer in the presence of citalopram (1 μM) for 30 min. The 5-HT concentration in the conditioned KRH buffer was measured by HPLC-ECD.

2.3. Real-time RT-PCR

Raphe slice cultures were cut and 1/3 of both lateral sides and 1/4 of the dorsal side was removed to isolate the raphe. Total mRNA was isolated from the slices, and reverse transcribed. Real-time quantitative PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), tryptophan hydroxylase 2 (TPH2), GluA1, GluA2 and Ca^{2+}/calmodulin-dependent protein kinase II α (CaMKIIα) were performed.

2.4. Western blot

Western blot analysis for TPH was performed as previously described [12, 13]. Briefly, proteins extracted from slices were subjected to SDS-PAGE. Resolved proteins were probed with an anti-TPH antibody, and visualized by enhanced chemiluminescence.

2.5. Immunohistochemistry

Immunohistochemistry for TPH was performed as previously described [12]. Fixed, permeabilized and blocked slices were incubated with an anti-TPH antibody, followed by a fluorescent secondary antibody.

2.6. Quantitative chromatin immunoprecipitation (ChIP) assay

Chromatin samples prepared from slices were incubated with an anti-acetyl-histone H3 antibody. Antibody conjugated chromatin was precipitated using Protein G-coupled Dynabeads. Acetylated histone H3 levels in the upstream regions of the TPH2, GluA2 or CaMKIIα genes were analyzed by quantitative PCR (qPCR).

2.7. Statistical analysis

All data are presented as the mean ± S.E.M. Differences between two groups were compared by Student's *t*-test. Data with more than two groups were compared using a one-way or two-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test for multiple comparisons. Differences with a value of $p < 0.05$ were considered significant.

3. Results

3.1. TSA treatment increased 5-HT synthesis by increasing TPH2 gene transcription

To examine whether TSA increases 5-HT synthesis, slices were treated with vehicle or TSA (1 μM) for 1, 2 or 4 days. Sustained treatment with vehicle had no effect on the tissue content of 5-HT, or of 5-hydroxyindolacetic acid (control: 0.74 ± 0.07 nmol/mg protein; 2 days: 0.59 ± 0.02 nmol/mg protein; 4 days: 0.67 ± 0.13 nmol/mg protein; n = 3), while the TSA treatment significantly increased the tissue content of 5-HT, compared with vehicle-treated slices (TSA effect: $F_{1,24} = 21.91$, $P < 0.001$; time effect:

$F_{2,24}$ = 7.73, P < 0.01; TSA × time interaction: $F_{2,24}$ = 1.65, P = 0.2137) (Fig. 1A). The level of TPH2 mRNA was significantly increased by 2 or 4 days of treatment ($F_{3,14}$ = 10.58, P < 0.01) (Fig. 1B). TSA treatment for 4 days significantly increased TPH protein levels (Fig. 1C). Immunostaining for TPH showed that TSA treatment increased the fluorescence intensity of the TPH-positive cells in the raphe slices, while it had no effect on the morphology (Fig. 1D). Furthermore, pan-acetylation levels of histone H3 in the upstream region of the TPH2 gene were significantly increased ($F_{3,14}$ = 3.792, P < 0.05) after 2 days of TSA treatment, but not after 1 or 4 days (Fig. 1E).

3.2. TSA treatment increased 5-HT release from organotypic raphe slice cultures

We examined whether TSA treatment affected the 5-HT release from raphe 5-HT neurons. Acute treatment with TSA (0.1–10 μM) for 30 min in KRH buffer did not change the extracellular 5-HT level (Fig. 2A), whereas sustained treatment for 4 days significantly increased the extracellular 5-HT levels in a concentration-dependent manner ($F_{3,10}$ = 15.06, P < 0.001) (Fig. 2B). Sustained treatment with TSA (1 μM) for 1–4 days significantly increased the extracellular 5-HT levels (TSA effect: $F_{1,12}$ = 24.17, P < 0.001; time effect: $F_{2,12}$ = 5.96, P < 0.05; TSA × time interaction: $F_{2,12}$ = 4.81, P < 0.05). A significant increase was observed after 4 days of treatment (Fig. 2C), which was delayed compared to the increase in 5-HT tissue content. Similarly, sustained treatment with another HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA; 1 and 10 μM), for 4 days significantly increased the extracellular 5-HT levels ($F_{2,10}$ = 21.79, P < 0.001) (Fig. 2D). We previously reported that sustained treatment with SSRIs

dramatically enhanced exocytotic 5-HT release in the raphe slice cultures [13]. Here, we examined whether TSA treatment further augments the enhanced 5-HT release in response to sustained treatment with an SSRI, citalopram. Consistent with our previous report [13], sustained treatment with citalopram (1 μM) for 4 days increased the extracellular 5-HT level ($F_{1,23}$ = 13.72, P < 0.01). Sustained co-treatment with TSA (1 μM) significantly augmented the elevated 5-HT level by sustained citalopram treatment ($F_{1,23}$ = 77.25, P < 0.001), although a two-way ANOVA revealed no interaction between citalopram and TSA ($F_{1,23}$ = 2.56, P = 0.123) (Fig. 2E).

3.3. TSA treatment increased the expression levels of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and CaMKII

Since HDAC inhibitors potentiate excitatory synaptic function by increasing gene transcription [14, 15], we examined whether AMPAR-mediated excitatory inputs were involved in the TSA-induced increase in 5-HT release. The increase in 5-HT release in response to 4 days of TSA treatment (1 μM) was significantly suppressed in the presence of the selective AMPAR antagonist NBQX (30 μM) (Fig. 3A). We next examined the gene expression of AMPAR subunits and CaMKIIα, which plays an important role in AMPAR trafficking [16] (Fig. 3B-D). TSA treatment (1 μM) for 1–4 days significantly increased the mRNA levels of GluA2 ($F_{3,11}$ = 10.98, P < 0.01) and CaMKIIα ($F_{3,11}$ = 8.707, P < 0.01). The level of GluA1 mRNA also tended to be increased by TSA treatment, but the effect was not significant ($F_{3,11}$ = 2.006, P = 0.1715). Furthermore, TSA treatment significantly increased pan-acetylation levels of

histone H3 in the upstream regions of the GluA2 and CaMKIIα genes ($F_{3,14}$ = 12.59, P < 0.001, and $F_{3,12}$ = 3.017, P < 0.05, respectively). The increases peaked after 2 days of treatment, and tended to return to basal levels by 4 days of treatment (Fig. 3E, F).

3.4. CaMKII signaling is required for the TSA-induced increase in 5-HT release, but not 5-HT synthesis

We examined whether CaMKII-mediated signaling is involved in the effects of TSA. Co-treatment with the CaMKII inhibitors, KN-62 (10 μM) or KN-93 (10 μM), and TSA (1 μM) for 4 days significantly blocked the TSA-induced increase in 5-HT release (KN-62 × TSA interaction: $F_{1,12}$ = 6.22, P < 0.05 and KN-93 × TSA interaction: $F_{1,8}$ = 6.61, P < 0.05, respectively), while they failed to affect the TSA-induced increase in 5-HT tissue content (KN-62 × TSA interaction: $F_{1,12}$ = 0.07, P = 0.79 and KN-93 × TSA interaction: $F_{1,8}$ = 0.77, P = 0.4057, respectively) (Fig. 4).

4. Discussion

In this study, we showed that prolonged treatment with the broad-spectrum HDAC inhibitor TSA increased 5-HT synthesis and release in organotypic raphe slice cultures. This effect was mediated primarily through the enhancement of gene expression related to 5-HT synthesis and excitatory inputs. These data suggest that inhibition of HDACs potentiates the function of 5-HT neurons by an epigenetic mechanism.

TPH2 is a rate-limiting enzyme for 5-HT synthesis in the central nervous system [17]. While the mechanisms that control TPH2 expression are not fully understood, several

reports suggest that the neuron-restrictive silencer factor, which recruits corepressor complexes containing HDAC1/2 [18], negatively regulates TPH2 expression [19]. Consistent with these reports, the increase in TPH2 expression was accompanied by hyper-acetylation of histone H3 at the promoter region of the TPH2 gene in the raphe slice cultures. By contrast, HDAC6 that exists mainly in the cytosol indirectly regulates TPH2 expression by modulating nuclear transport of glucocorticoid receptors [20]. Since TSA also inhibits HDAC6, inhibition of HDAC6 may also be involved in TSA-induced increases in 5-HT synthesis in the raphe slice cultures, although possible involvement of other off-target effects of TSA and SAHA cannot be excluded.

AMPAR-mediated excitatory inputs regulate the DRN 5-HT neuronal activity and 5-HT release [13, 21]. Several lines of evidence suggest that HDAC inhibitors enhance excitatory synaptic function and plasticity in a transcription-dependent manner [14, 15]. Consistent with the present results, class I HDACs that are specifically localized in the nucleus control the expression of the AMPAR subunit GluA2 [22] and of CaMKIIα [23]. CaMKII promotes AMPAR trafficking [24] and gating [25]. Taken together, the results of this study suggest that inhibition of HDACs enhances CaMKII signaling, and consequently, contributes to the increase in 5-HT release through the enhancement of AMPAR-mediated excitatory inputs, although the enhanced CaMKII signaling is not involved in increased 5-HT synthesis. However, considering the concentration we used in this study, we cannot rule out the possible involvement of other kinases which KN-62 and KN-92 may weakly inhibit. Furthermore, additional research will be needed to clarify the contribution of non-5-HT neurons and glial cells in the raphe slice cultures to

the effects of the HDAC inhibitors.

Recent evidence suggests that chronic stress exposure decreases TPH2 expression in the DRN [26]. By contrast, chronic treatment with the SSRI fluoxetine increases TPH2 expression in the midbrain, which is correlated with the antidepressant-like effect in the forced swim test [27]. In this context, our present results suggest that the increased TPH2 expression in response to the HDAC inhibitor may indicate that such inhibitors can be used in the treatment of psychiatric disorders. We previously demonstrated that the sustained SSRI-induced elevation of extracellular 5-HT levels is caused by the facilitation of AMPAR-mediated exocytotic 5-HT release, but not by increases in 5-HT synthesis [13]. Therefore, it is conceivable that a TSA-induced increase in 5-HT synthesis and AMPAR-mediated 5-HT release by epigenetic mechanisms can further enhance the effects of SSRIs.

In the present study, we used broad-spectrum HDAC inhibitors, TSA and SAHA, which potently inhibit both class I HDACs and class II HDACs, such as HDAC5 and HDAC6 [28]. Class II HDACs exist both in the nucleus and cytosol, and catalyze lysine acetylation of various proteins other than histones. In addition to class II HDACs, class I HDACs may also be responsible for the antidepressant effect [6, 10, 20]. Although we showed that TSA increased gene expression, potentially by histone H3 hyper-acetylation, we cannot exclude the possibility that cytoplasmic HDACs are involved as well. Furthermore, it is possible that anti-inflammatory effect of HDAC inhibitors on glial cells [29] may affect excitatory inputs to 5-HT neurons.

In conclusion, we have shown that sustained treatment with the HDAC inhibitor TSA

increased AMPAR-mediated 5-HT release from the raphe slice cultures, through the enhancement of 5-HT synthesis and CaMKII signaling by epigenetic mechanisms. Although it has not been determined whether TSA itself exhibits antidepressant effects, our results suggest that the inhibition of HDACs potentiates the function of the 5-HT system, which may underlie the mechanism for the antidepressant effect of HDAC inhibitors.

Acknowledgements

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Figure Legends

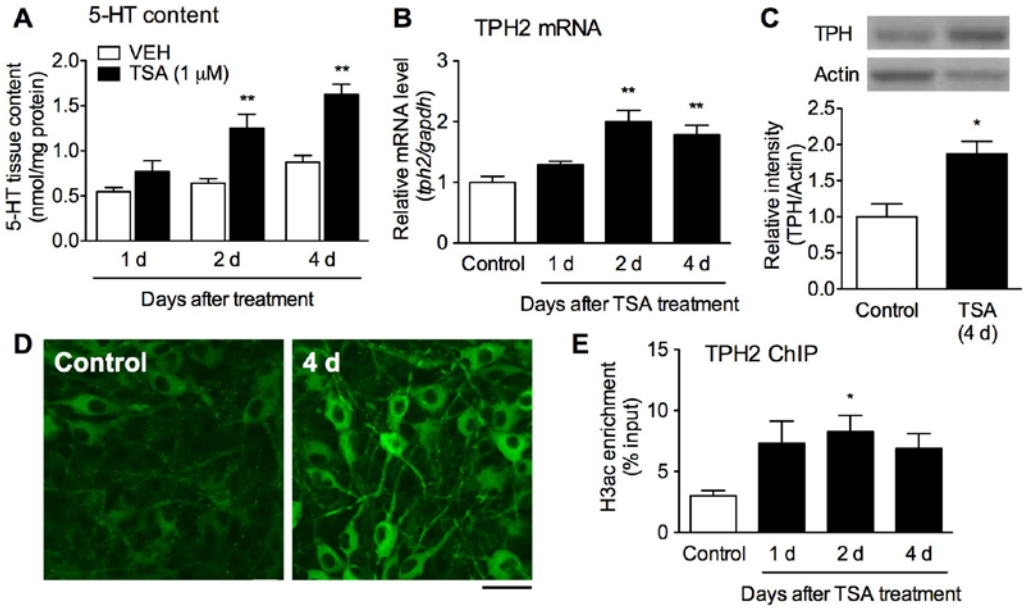

Fig. 1. Sustained treatment with TSA enhanced 5-HT synthesis by increasing TPH2 expression. Slices were treated with vehicle or TSA (1 μM) for 1, 2 or 4 days. (A) 5-HT tissue content. $^{**}P < 0.01$ vs vehicle. n = 3–8. (B) qPCR for TPH2. n = 3–4. (C) Western blot analysis of TPH. n = 3. (D) Representative images of TPH immunoreactivity in the slices. Scale bar = 40 μm. (E) ChIP assays were performed using an anti-acetyl-histone H3 antibody. qPCR for the upstream region of the TPH2 gene were conducted. n = 4–5. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control.

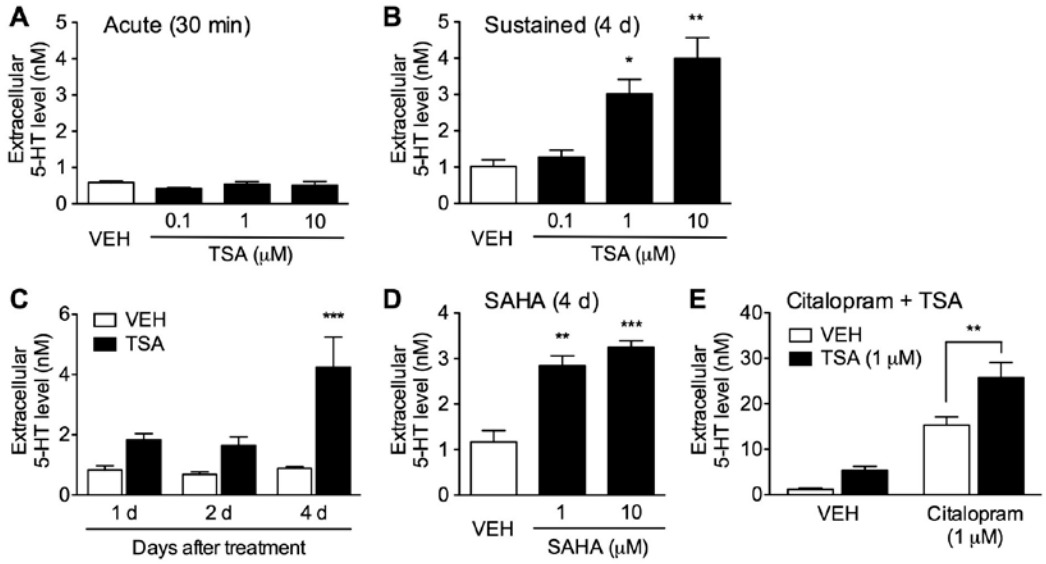

Fig. 2. Sustained treatment with TSA or SAHA increased extracellular 5-HT levels in the raphe slice cultures. (A-D) Slices were treated with TSA (0.1–10 μM) for 30 min (A; n = 3–6) or 4 days (B; n = 3–4), with TSA (1 μM) for 1, 2 or 4 days (C; n = 3), or with SAHA (1 or 10 μM) for 4 days (D; n = 3–6), and extracellular 5-HT levels were measured. VEH: vehicle. *P < 0.05, **P < 0.01, ***P < 0.001 vs vehicle or control. (E) Effect of TSA on the enhanced 5-HT release in response to sustained SSRI treatment. Slices were co-treated with TSA (1 μM) and citalopram (1 μM) for 4 days, and the extracellular 5-HT levels were measured. **P < 0.01. n = 6–9.

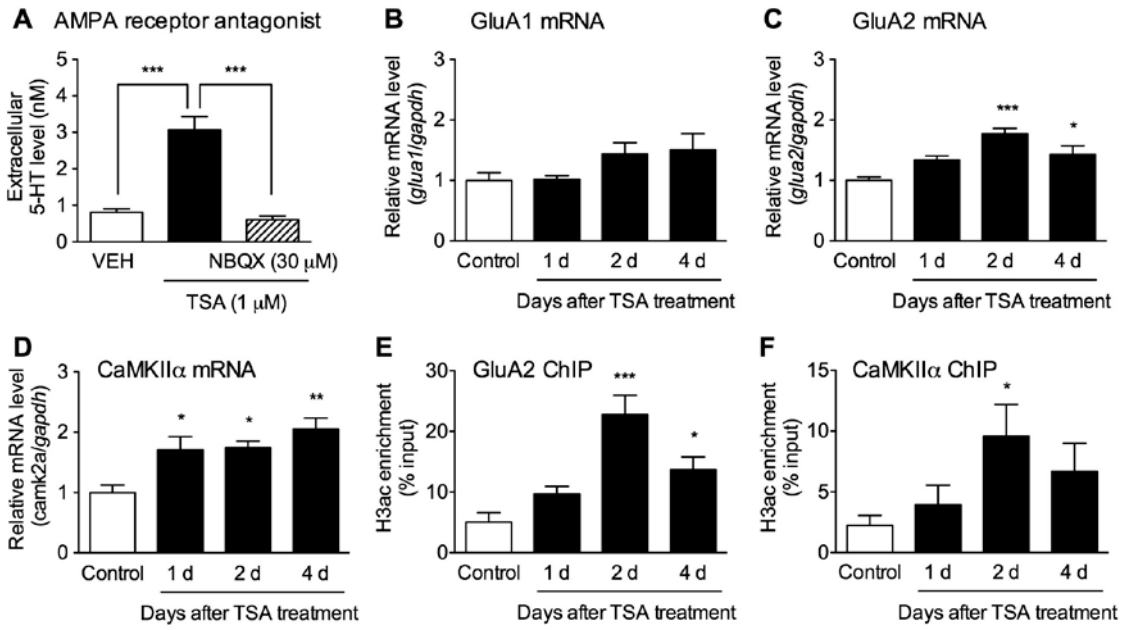

Fig. 3. TSA treatment enhanced AMPAR-mediated input by increasing the mRNA levels of AMPAR and CaMKIIα. (A) Slices were treated with vehicle or TSA (1 μM) for 4 days, and extracellular 5-HT levels were measured in the presence or absence of the AMPAR antagonist NBQX (30 μM). VEH: vehicle. n = 3–4. (B-F) Slices were treated with TSA (1 μM) for 1, 2 or 4 days, and qPCR for GluA1 (B), GluA2 (C), or CaMKIIα (D) was performed. n = 3–4. (E, F) ChIP assays were performed using an anti-acetyl-histone H3 antibody. qPCR for the upstream regions of the GluA2 (E) and CaMKIIα (F) genes was conducted. n = 3–5. *P < 0.05, **P < 0.01, and ***P < 0.001 vs control.

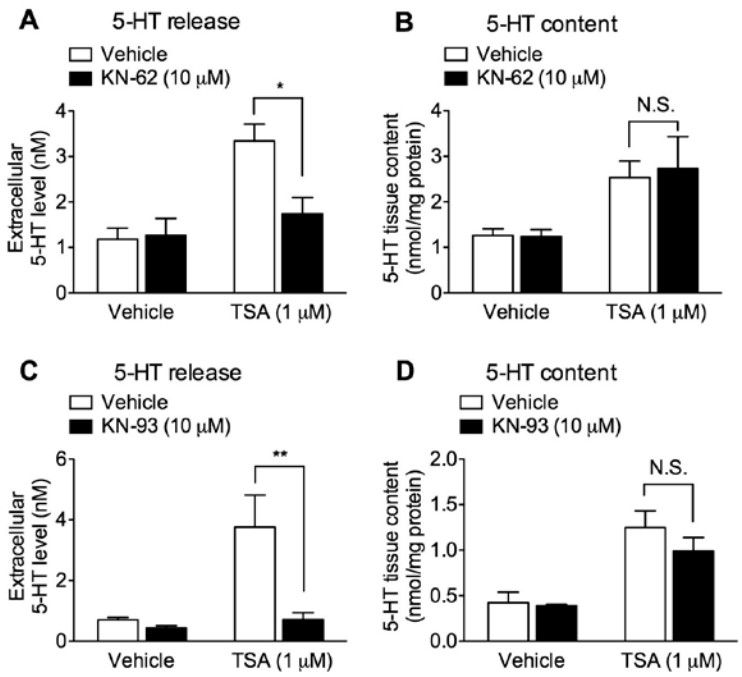

Fig. 4. CaMKII signaling is necessary for TSA-induced increase in 5-HT release, but not in 5-HT tissue content. Slices were co-treated with TSA (1 μM) and the CaMKII inhibitors KN-62 (10 μM) or KN-93 (10 μM) for 4 days. Extracellular 5-HT level and 5-HT tissue content were measured. n = 3–4. N.S.: not significant, *P < 0.05, **P < 0.01.