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(Citation)
Journal of Psychiatric Research,135:237-242

(Issue Date)
2021-03

(Resource Type)
journal article

(Version)
Accepted Manuscript

(URL)
https://hdl.handle.net/20.500.14094/90007988

Clozapine increases macrophage migration inhibitory factor (MIF) expression via increasing histone acetylation of MIF promoter in astrocytes

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ABSTRACT

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine and promotes neurogenesis and neuroprotection in brains. In addition, MIF has been identified as a potential marker of schizophrenia (SCZ). Our recent study also showed that serum MIF level is higher in SCZ and positively correlated with antipsychotic doses, and that MIF promoter polymorphisms are associated with SCZ. Here, we investigated the effects of antipsychotics such as clozapine on MIF expression in primary cultured astrocytes derived from neonatal mouse forebrain. MIF mRNA expression was estimated with quantitative reverse-transcription polymerase chain reaction. MIF protein concentration was measured with enzyme-linked immunosorbent assay. The histone acetylation of MIF promoter was examined with chromatin immunoprecipitation assay. As a result, common antipsychotics, especially clozapine, increased MIF mRNA expression in a dose-dependent manner. Clozapine increased MIF mRNA expression and protein concentration in a time-dependent manner. Moreover, clozapine increased the acetylation of histone H3 at lysine 27 residues (H3K27) in MIF promoter. In conclusion, we provide novel evidence that antipsychotics such as clozapine increases MIF expression via the acetylation of H3K27 in astrocytes, and that MIF may have a potential role for astrocytes in the action mechanisms of antipsychotics.

Keywords

Antipsychotics, Clozapine, MIF, Astrocyte, Histone acetylation

INTRODUTCION

Schizophrenia (SCZ) is a chronic and disabling psychiatric syndrome (Marder and Cannon, 2019) and affects approximately 1% of the general population worldwide (Mueser and McGurk, 2004). Since the serendipitous discovery of chlorpromazine, a lot of antipsychotics have been developed and utilized for the treatment of SCZ for more than 50 years. However, the effects of existing antipsychotics on SCZ have been limited yet. Therefore, it is necessary for the improvement of the treatment of SCZ to develop new antipsychotics based on unknown action mechanisms.

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine, which works as a regulator in both innate and adaptive immunity. Not only in immune cells, MIF is also expressed in neurons (Bacher et al, 1998; Savaskan et al, 2012) and astrocytes (Conboy et al, 2011; Su et al, 2017), and plays an important role in neurogenesis and neuroprotection (Ohta et al, 2012), which are involved in the pathophysiology of SCZ (Kusumi et al, 2015). In addition, several global serum studies have suggested that MIF is a promising biomarker for SCZ (Chan et al, 2015; Schwarz et al, 2012). Furthermore, our previous study has shown that serum MIF levels are higher in patients with SCZ compared to controls and positively correlated with antipsychotic doses, and that higher-expression polymorphisms of MIF promoter are significantly minor for females with adolescent-onset SCZ (Okazaki et al, 2018). These results suggest that MIF may be involved in the pathophysiology of SCZ and the action mechanisms of antipsychotics.

Based on past studies described above, we hypothesized that MIF might be involved in unknown action mechanisms of antipsychotics. MIF is expressed predominantly in astrocytes among various cell types in brains (Conboy et al, 2011; Gellen et al, 2017). In addition, a lot of past studies have shown that the expression of

glial fibrillary acidic protein (GFAP), a common marker of astrocytes, is altered in postmortem brains of SCZ patients (Dietz et al, 2020; Kim et al, 2018), which suggests the involvement of astrocytes in the pathophysiology of SCZ. Therefore, here we focused on MIF and astrocytes, and examined the effects of antipsychotics on MIF expression in neonatal mice-derived primary cultured astrocytes (PCAs). Following it, we investigated the molecular mechanism underlying the effects of antipsychotics on MIF expression in PCAs.

MATERIALS AND METHODS

Ethical Statement

This study was approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimentation Regulations.

Drugs

Clozapine was obtained from Abcam (Cambridge, UK). Chlorpromazine, haloperidol, risperidone, olanzapine, and aripiprazole were obtained from FUJIFILM Wako Pure Chemical (Osaka, Japan). CBP-CREB interaction inhibitor was obtained from Calbiochem (La Jolla, CA, USA). BMS-345541, trichostatin A (TSA) and valproic acid (VPA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). SR11302 was obtained from Tocris Biosciences (Bristol, UK).

Cell culture

PCAs were prepared from neonatal C57BL/6J mice as previously described (Maruyama et al, 2020). Briefly, the isolated forebrain was minced and incubated with trypsin

(Thermo Fisher, Waltham, MA, USA) and DNase I (FUJIFILM Wako Pure Chemical) for dissociation. The dissociated tissues were suspended in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12; Thermo Fisher) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and 1% penicillin/streptomycin (PS; Thermo Fisher). The suspended cells were seeded in poly-L-lysine (PLL; ScienCell, Carlsbad, CA, USA)-coated 75mm2 flasks (10–15×10^6 cells/flask). The plated cells were cultured in a 5% CO2 incubator at 37 ℃. Every 8 to 12 days, the confluent cells were shaken for 10 min to separate them from microglial cells, released from the flasks with trypsin, and seeded into non-coated flasks. These cells were seeded onto non-coated 8 well-chamber slides (2.5×10^4 cells/well) and checked with immunocytochemistry for anti-GFAP antibody (Abcam).

Total RNA extraction and quantitative reverse transcription polymerase chain reaction (Q-RT-PCR) assay

PCAs were seeded onto non-coated 6 well-plates (2×10^5 cells/well) in DMEM/F12 supplemented with 10% FBS and PS. After 3 days, the medium was changed to FBS-free medium. After overnight, drug administration was performed and followed by incubation. Total RNA was extracted with RNeasy Mini Kit (Qiagen, Hilden, Germany). The extracted total RNA was converted to cDNA with Quantitect Reverse Transcription Kit (Qiagen). Quantitative PCR was performed with TB Green Advantage qPCR Premix (Takara Bio, Tokyo, Japan) and ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacture's protocol. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. The sequences of the forward and reverse primers were as follows:

ACCTGCCAAGTATGATGACATCA and GGTCCTCAGTGTAGCCCAAGAT for GAPDH; CCCAGAACCGCAACTACAG and GCAGCGTTCATGTCGTAATAGT for MIF; AAAATGCTCACACTCCAC and GAACAAATGCTGGTCTTT for BDNF, respectively. The PCR condition was as follows: 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 15 sec, and 60 ℃ for 30 sec.

Enzyme-Linked ImmunoSorbent Assay

PCAs were seeded onto non-coated 6 well-plates (2×10^5 cells/well) in DMEM/F12 supplemented with 10% FBS and PS. After 3 days, the culture medium was changed to FBS-free medium. After overnight, drug administration was performed and followed by incubation. Medium was collected and stored at −80 ℃. MIF protein concentration was measured with Mouse MIF Simplestep Enzyme-linked immunosorbent (ELISA) Kit (Abcam, Cambridge, UK) according to the manufacture's protocol. The absorbance was detected with Multiskan FC (Thermo Fisher) at 450 nm. All samples and standards were measured in duplicate.

Chromatin Immunoprecipitation Assay

PCAs were seeded onto non-coated 90 mm dish (2×10^6 cells/dish) in DMEM/F12 supplemented with 10% FBS and PS. After 3 days, the culture medium was changed to FBS-free medium. After overnight, reagent administration was performed and followed by incubation. Chromatin immunoprecipitation (ChIP) assay was performed with ChIP-IT express kit (Active Motif, La Hulpe, Belgium) according to the manufacture's protocol. Briefly, PCAs were fixed with 37% formaldehyde. Shearing chromatin of fixed PCAs Sonication was performed with Bioruptor II (BM Equipment, Tokyo, Japan) for 10 times

of homogenization cycles. Each homogenization cycle consists maximum setting for 30 sec and following interval for 30 sec. The sheared chromatin was utilized for immunoprecipitation with anti-histone H3K9ac antibody (61251, Active Motif) and anti-histone H3K27ac antibody (39685, Active Motif). Real-time PCR for the immunoprecipitated DNA was performed with TB Green Advantage qPCR Premix (Takara Bio) and QuantiStudio 3 Real-Time PCR System (Applied Biosystems). The primers were designed to cover 151 base pair of MIF promoter as follows: ATGTAATACTTCCTACAGCACCAGAAG and CTACGTGACCCAGCTCAGTACC, respectively (Supplementary Fig. S1). The PCR condition was as follows: 95 ℃ for 30 sec, followed by 40 cycles of 95 ℃ for 5 sec, 60 ℃ for 20 sec and 72 ℃ for 34 sec.

Histone Deacetylase Assay

PCAs were seeded onto non-coated 90 mm dish (2×10^6 cells/dish) in DMEM/F12 supplemented with 10% FBS and PS. After 3 days, the culture medium was changed to FBS-free medium. After overnight, the nuclear extracts were collected with Nuclear Extract Kit (Active Motif). Histone Deacetylase (HDAC) assay was performed with HDAC Assay Kit (colorimetric, Active Motif) according to the manufacture's protocol. The absorbance was detected with Multiskan FC (Thermo Fisher) at 405 nm. All samples and standards were measured in duplicate.

Statistics

Statistical analyses were performed with R version 3.5.2 (The R Foundation for Statistical Computing, Vienna, Austria) and EZR version 1.40 (Jichi Medical University, Saitama, Japan) (Kanda et al, 2013). The group differences were analyzed using one-way ANOVA

with Dunnett's multiple comparison test. Statistical significance was defined as two-tailed $p < 0.05$.

RESULTS

Effects of antipsychotics on MIF expression

The effects of common antipsychotics, such as chlorpromazine, haloperidol, clozapine, risperidone, olanzapine, and aripiprazole, on MIF mRNA expression in PCAs were examined with Q-RT-PCR. All of these antipsychotics increased MIF mRNA expression in a dose-dependent manner 48 hours after drug administration (Fig. 1). Especially, clozapine had the strongest effect on MIF mRNA expression increase. Therefore, we focused on clozapine in the subsequent examinations.

Next, the time-dependent effect of clozapine on MIF mRNA expression was examined. 50 μM of clozapine increased MIF mRNA expression, which reached significance 12 h after administration and maximum 48 h after administration (Fig. 2A). To examine whether clozapine also increases MIF protein production, the time-dependent effect of clozapine on MIF protein production was examined with ELISA. 50 μM of clozapine increased MIF protein production in a time-dependent manner along with mRNA expression increase (Fig. 2B).

Effects of transcription factor inhibitors on MIF mRNA expression

Human MIF promoter has putative binding sites of transcription factors, such as cAMP response element-binding protein (CREB), nuclear factor-kappa B (NFκB), and activator protein 1 (AP-1) (Baugh et al, 2002). All of CREB, NFκB and AP-1 regulate MIF expression in other types of cells than astrocyte (Baugh et al, 2006; Cho et al, 2009;

Lugrin et al, 2009; Roger et al, 2007; Shan et al, 2011). We confirmed that mouse MIF promoter also has putative binding sites of these transcription factors with LASAGNA-Search 2.0 (Lee and Huang, 2013) (Supplementary Fig. S1). Therefore, the involvement of these transcription factors in the effect of clozapine on MIF expression increase in PCAs was examined with CBP-CREB interaction inhibitor (CREB inhibitor), BMS-345541 (NFκB inhibitor), and SR11302 (AP-1 inhibitor). All of these inhibitors had no inhibitory effect on clozapine-increased MIF mRNA expression (Supplementary Fig. S2). These results suggest that clozapine may have no effect on the activities of CREB, NFκB, and AP-1.

Effects of clozapine on histone acetylation in MIF promoter

Instead of transcription factors-mediated mechanisms mentioned above, epigenetic mechanisms can be considered as the mechanisms underlying the effect of clozapine on MIF expression increase. Among various epigenetic mechanisms, here we focused on histone acetylation because histone acetylation regulates MIF expression in non-neural cell lines (Lugrin et al, 2009) and clozapine recovers phencyclidine-induced histone acetylation decrease in mice brains (Aoyama et al, 2014). Therefore, the involvement of histone acetylation in the effect of clozapine on MIF expression increase in PCAs was examined with HDAC inhibitors such as TSA and VPA. Both TSA and VPA increased MIF mRNA expression in a dose-dependent manner as clozapine did (Fig. 3).

Next, we examined the effects of clozapine on the acetylation of histone H3 at lysine 9 and 27 residues (H3K9 and H3K27, respectively), which are often acetylated in active promoters (Creyghton et al, 2010; Koch et al, 2007) with ChIP assay. We also tested TSA as a comparison. Both clozapine and TSA increased the acetylation of H3K27, but

not that of H3K9 (Fig. 4).

These results suggest that clozapine might work as a HDAC inhibitor in PCAs. Therefore, the effect of clozapine on HDAC activity was examined with nuclear extracts of PCAs. However, clozapine had no direct inhibitory effect on HDAC activity (Supplementary Fig. S3).

DISCUSSION

Here, we showed that commonly prescribed typical and atypical antipsychotics including clozapine increased MIF mRNA expression in PCAs in a dose-dependent manner. Clozapine increased MIF expression in a time-dependent manner. Next, we investigated how clozapine increased MIF expression in PCAs. The inhibitors of MIF promoter-binding transcription factors, such as CREB, NFκB, and AP-1, had no inhibitory effect on clozapine-induced MIF mRNA expression increase. On the other hand, HDAC inhibitors, such as TSA and VPA, increased MIF mRNA expression. Moreover, clozapine increased the acetylation of H3K27, but not that of H3K9. However, clozapine had no direct inhibitory effect on HDAC activity. These results suggest that antipsychotics may increase MIF expression in astrocytes via increasing the acetylation of H3K27 in MIF promoter. Our present study is the first study to show the involvement of MIF, astrocytes and histone acetylation in the action mechanism of antipsychotics.

MIF is involved in adult hippocampal neurogenesis and the effects of antidepressants (Conboy et al, 2011; Moon et al, 2012) and expressed predominantly in astrocytes in brains (Conboy et al, 2011; Gellen et al, 2017). Astrocytes play a role in adult hippocampal neurogenesis (Boku et al, 2018), which is involved in the pathophysiology of SCZ and the action mechanisms of antipsychotics (Kusumi et al,

2015). Therefore, these studies and our results suggest that antipsychotics-increased MIF in astrocytes may mediate the action mechanisms of antipsychotics via adult hippocampal neurogenesis. However, our present study is *in vitro* study. Therefore, it is necessary to investigate the involvement of MIF in antipsychotics-induced adult hippocampal neurogenesis with *in vivo* models.

Although we focused on MIF as an astrocyte-derived neurogenic factor, antipsychotics can increase the expressions of other neurogenic factors in astrocytes because astrocytes produce various neurogenic factors (Seth and Koul, 2008). In astrocyte-derived neurogenic factors, BDNF is extensively investigation showing that its serum level and gene polymorphism are associated with SCZ (Ikeda et al, 2008; Skibinska et al, 2004). In addition, systemic administration of MIF and antipsychotics including clozapine increases BDNF expression in mice brains (Bai et al, 2003; Moon et al, 2012). Therefore, we examined the time-dependent effect of clozapine on BDNF mRNA expression in PCAs. Although 50 μM of clozapine increased BDNF mRNA expression until 24h after administration of clozapine, the increasing effect of clozapine on BDNF mRNA expression was not continued after that unlike the case of MIF (Supplementary Fig. S4). This result suggests that antipsychotics may transiently increase BDNF mRNA expression in astrocytes and that continuously increased MIF by antipsychotics may not affect BDNF mRNA expression in astrocytes. Further investigation is required to understand the effects of antipsychotics on BDNF expression and the correlation between MIF and BDNF on the action mechanisms of antipsychotics.

It has been considered that the blockade ability of dopamine D2 receptor (D2R) is associated with the efficacy of antipsychotics on SCZ (Meltzer and Stahl, 1976) for a long time. However, clozapine, which has a low blockade ability of D2R, is more effective

than high potent antagonists of D2R such as serotonin-dopamine antagonist (SDA) and butyrophenone (Kusumi et al, 2015). Therefore, other biological phenomena than the blockade of D2R may play a role in the action mechanisms of antipsychotics. Here we focused on epigenetics as such biological phenomena because a lot of recent studies have shown the involvement of epigenetics including histone acetylation in the pathophysiology of SCZ (Cromby et al, 2019). In addition to our results, a past study also showed that clozapine increased histone acetylation in mice brains (Aoyama et al, 2014). These suggest that histone acetylation may be a potential biological phenomenon to explain the action mechanisms of antipsychotics. However, there are only a few studies to mention the involvement of histone acetylation in the action mechanisms of antipsychotics and, further studies are required.

DNA methylation is a common epigenetic mechanism as well as histone acetylation. Past studies have shown that DNA methylation may play a role in the pathophysiology of SCZ (Ovenden et al, 2018) and that clozapine decreases DNA methylation in reelin and GAD67 promoters of mice brains (Dong et al, 2008). These studies suggest the possibility that clozapine-induced DNA demethylation of MIF promoter might mediate the effect of clozapine on MIF expression increase. However, the effect of clozapine on MIF expression is short-term: it reaches a maximum 48 hours after clozapine administration, and it is well established that DNA methylation tends to mediate long-term epigenetic modification and that histone acetylation tends to mediate short-term epigenetic alternation (Handy et al, 2011). In addition, although olanzapine and haloperidol also increased MIF expression as well as clozapine, they have no effect on DNA methylation differently from clozapine (Dong et al, 2008). Therefore, in our current study we focused on histone acetylation, but not DNA methylation, as a target of clozapine. However, the

long-term effects of clozapine on DNA methylation may be involved in the long-term effects of antipsychotics and is worth to be investigated for the further understanding of the pathophysiology of SCZ and the action mechanisms of antipsychotics.

As a mechanism underlying the effect of clozapine on the histone acetylation increase of MIF promoter, the direct inhibition of HDAC by clozapine was presumed because clozapine and HDAC inhibitors had the similar effect on MIF mRNA expression. However, clozapine had no direct inhibitory effect on HDAC activity. It is established that HDAC activity is intricately regulated by cofactors, transcription, post-translational modification and subcellular translocation (Seto and Yoshida, 2014). Therefore, clozapine is considered to increase the histone acetylation of MIF promoter via such complex processes for the regulation of HDAC activity. Further elucidation may lead to identify new target molecules of antipsychotics.

We found that HDAC inhibitors, such as TSA and VPA, increased MIF mRNA expression. This result can be clinically very relevant, because VPA is one of mood stabilizers and sometimes combined to antipsychotics in the treatment for patients with refractory SCZ. Future studies are required to investigate the role of MIF on synergetic effects of antipsychotics and mood stabilizers. On the other hand, MIF has been reported to be involved in the effects of antidepressants (Conboy et al, 2011; Moon et al, 2012). Antidepressants and antipsychotics are sometimes combined in the treatment for depressive and psychotic symptoms of patients with SCZ or mood disorder. It is also required to elucidate the role of MIF on synergetic effects of antipsychotics and antidepressants.

In conclusion, we provide novel evidence that antipsychotics such as clozapine increases MIF expression via the acetylation of H3K27 in astrocytes, which suggests the

potential role of astrocyte-derived MIF in the molecular mechanism of antipsychotics.

ACKNOWLEDGEMENT

We thank M. Ohnishi, Y. Nagashima and H. Maeda for their expert technical assistance, as well as M. Takebayashi, K. Hisaoka-Nakashima, and N. Kajitani for their helpful advice about primary cultured astrocytes. This work was partly supported by grants from JSPS KAKENHI grant numbers 15K19727 and 18K15483 (SO), 15K09805 and 18K07556 (SB), as well as 17H04249 (AH).

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FIGURE LEGENDS

Fig. 1. The dose-dependent effects of antipsychotics on macrophage migration inhibitory factor (MIF) expression in primary cultured astrocytes (PCAs).

PCAs were incubated for 48 h in antipsychotics: 0, 10, 20, 50 μM of chlorpromazine (CHP), haloperidol (HPD), clozapine (CLO), risperidone (RIS) olanzapine (OLZ), or 0, 1, 2, 5 μM of aripiprazole (ARP), which showed cytotoxicity when 10 μM and over. MIF mRNA expression was analyzed by Q-RT-PCR. The values are shown as the ratio of MIF mRNA to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. The data are expressed as the mean ± SEM. *$p < 0.05$, **$p < 0.01$, or ***$p < 0.001$ vs. vehicle (Dunnett's test; n = 9–15).

Fig. 2. The time-dependent effects of clozapine on expression of macrophage migration inhibitory factor (MIF) in primary cultured astrocytes (PCAs). PCAs were treated with 50 μM of clozapine or DMSO for the indicated periods of time. (**A**) MIF mRNA expression was analyzed by Q-RT-PCR. The values are shown as the ratio of MIF mRNA to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. The lines connect the average values. *$p < 0.05$, **$p < 0.01$, or ***$p < 0.001$ vs. basal (time point = 0) (Dunnett's test; n = 3). (**B**) MIF protein concentration in culture medium was analyzed by ELISA. The lines connect the average values. *$p < 0.05$, **$p < 0.01$, or ***$p < 0.001$ vs. basal (time point = 0) (Dunnett's test; n = 3).

Fig. 3. The effects of histone deacetylate inhibitor on macrophage migration inhibitory factor (MIF) expression in primary cultured astrocytes (PCAs). PCAs were incubated for 48 h in histone deacetylate inhibitors: 0, 10, 25, 50 μM of trichostatin

A (TSA) or 0, 1, 2.5, 5 mM of valproic acid (VPA). MIF mRNA expression was analyzed by Q-RT-PCR. The values are shown as the ratio of MIF mRNA to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. The data are expressed as the mean ± SEM. *$p < 0.05$, **$p < 0.01$, or *** $p < 0.001$ vs. vehicle (Dunnett's test; n = 3-6).

Fig. 4. The effects of clozapine on histone acetylation of macrophage migration inhibitory factor (MIF) gene promoter in primary cultured astrocytes (PCAs). PCAs were incubated for 48 h in 50 μM of clozapine (CLO) or 10 μM of trichostatin A (TSA). The acetylation of histone H3 at lysine 9 and 27 residues (H3K9ac and H3K27ac, respectively) were analyzed by ChIP assay. Real-time PCR was performed on DNA purified from each of the ChIP reactions using a primer set specific for the MIF gene promoter. The % input values demonstrated the ratio of immunoprecipitated DNA fragments to the input DNA fragments. The data are expressed as the mean ± SEM. *$p < 0.05$, **$p < 0.01$, or ***$p < 0.001$ vs. vehicle (Dunnett's test; n = 4).

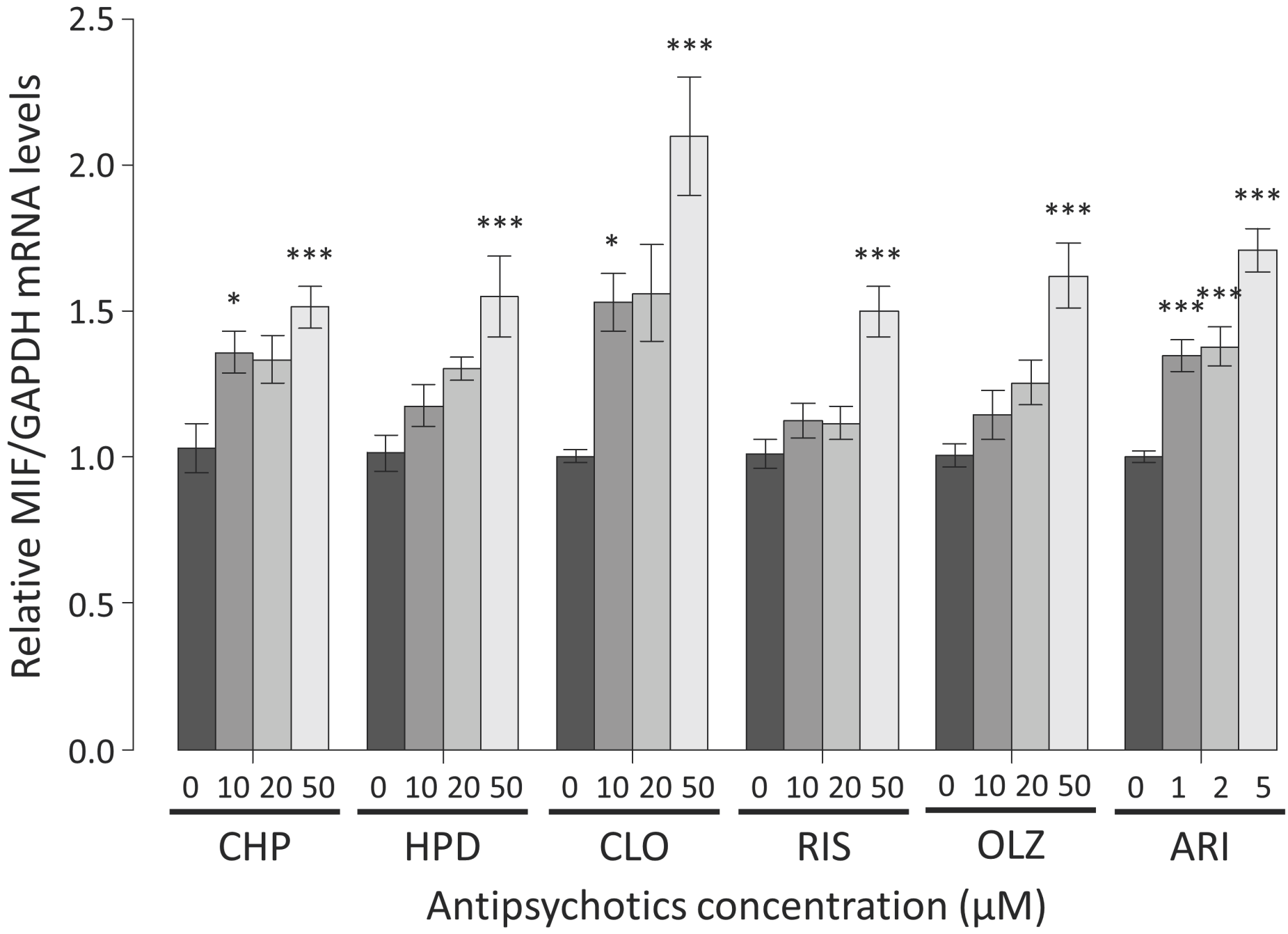
Supplementary Fig. S1 Mouse MIF promoter region. Putative binding sites of transcription factors, such as CREB, NFκB, and AP-1, as well as primers for ChIP assay.

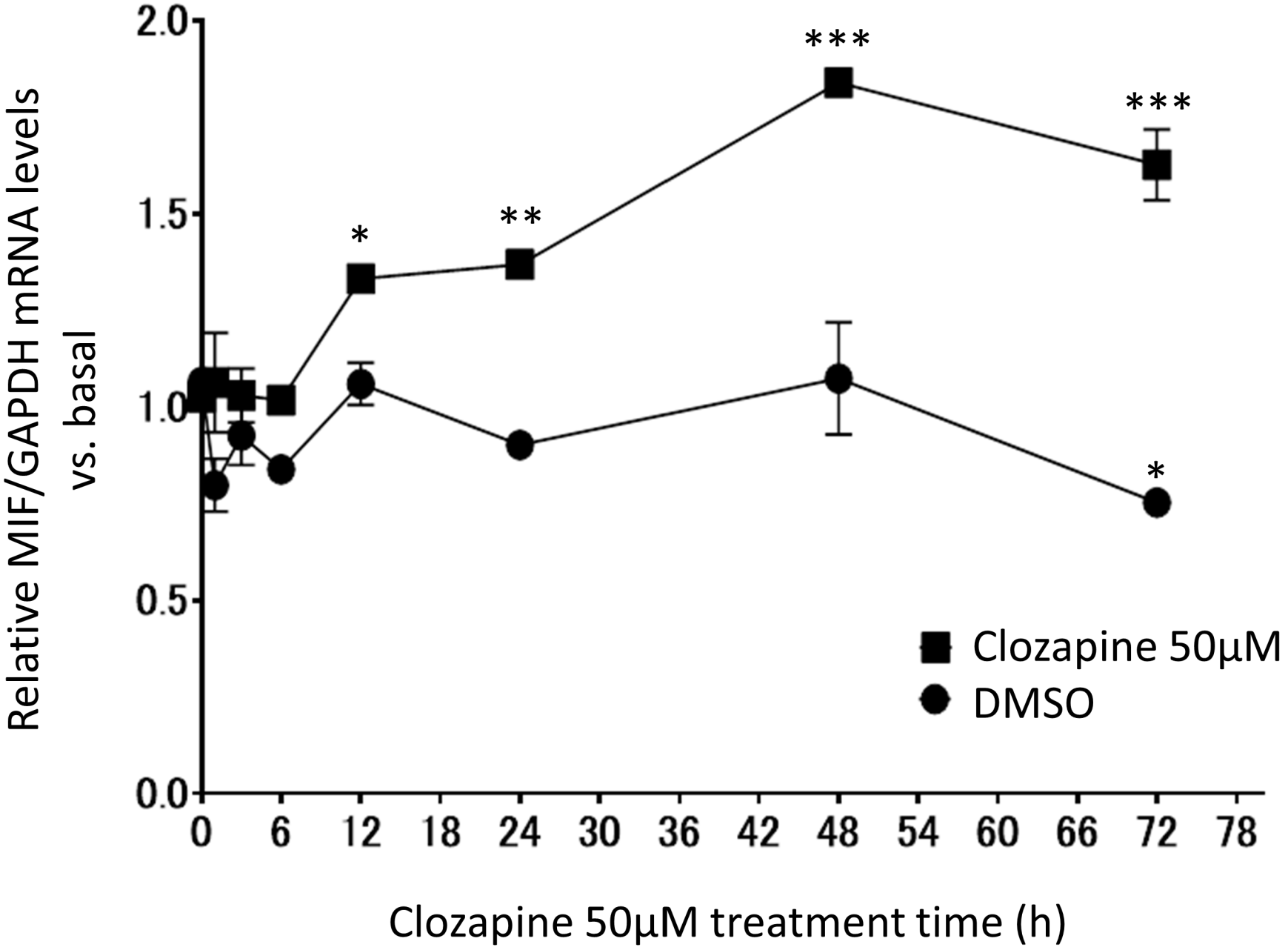
Supplementary Fig. S2. The effects of transcription factor inhibitors on macrophage migration inhibitory factor (MIF) expression in primary cultured astrocytes (PCAs). PCAs were incubated for 48 h in 50 μM of clozapine (CLO) and/or transcription factor inhibitors: 10 μM of CBP-CREB interaction inhibitor (CREB inhibitor), 5 μM of BMS-345541 (NFκB inhibitor), or 1 μM of SR-11302 (AP-1 inhibitor). MIF mRNA expression was analyzed by Q-RT-PCR. The values are shown as the ratio of MIF mRNA to

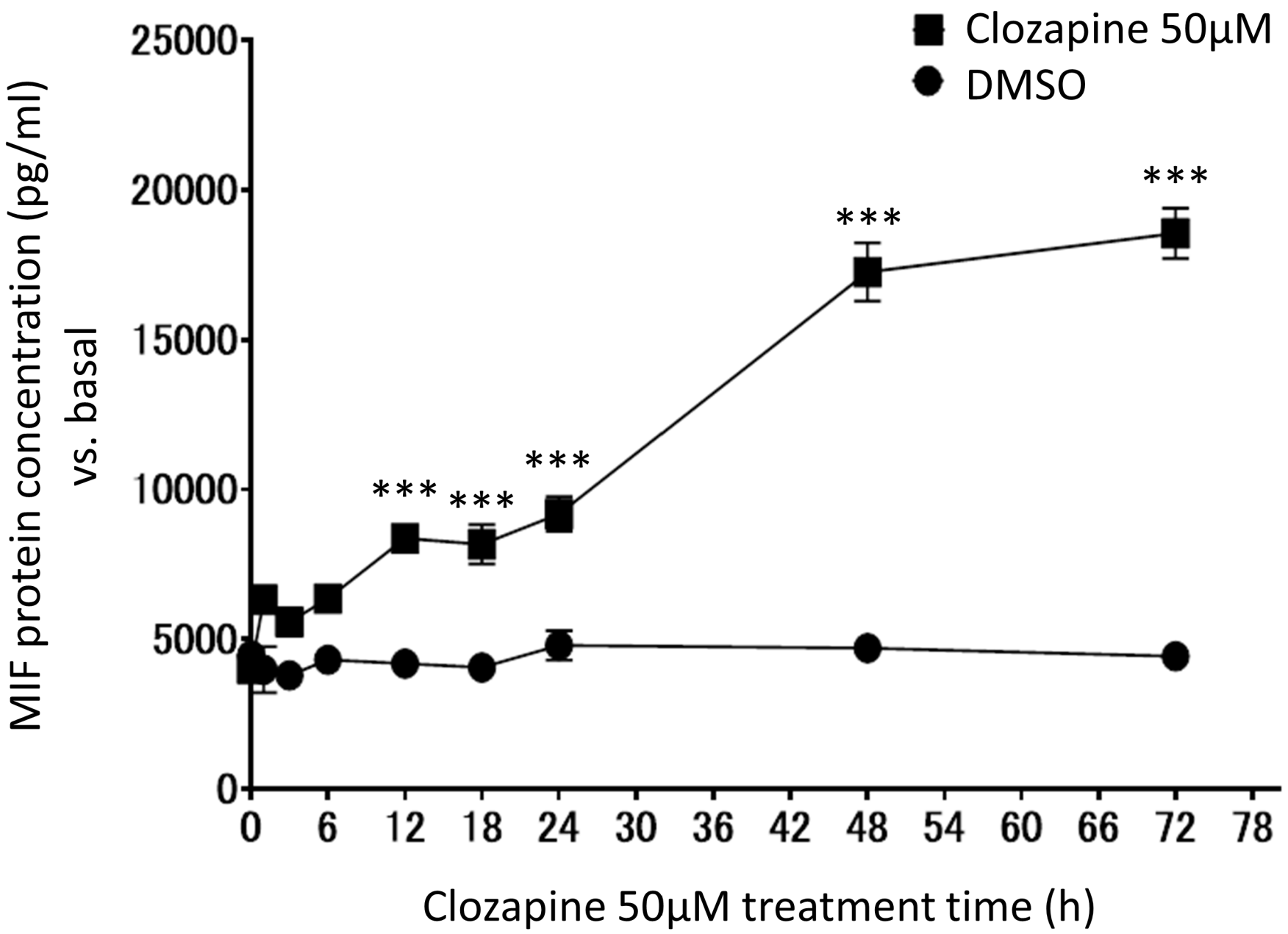
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. The data are expressed as the mean ± SEM. *$p < 0.05$, **$p < 0.01$, or ***$p < 0.001$ vs. vehicle (Dunnett's test; n = 3-6).

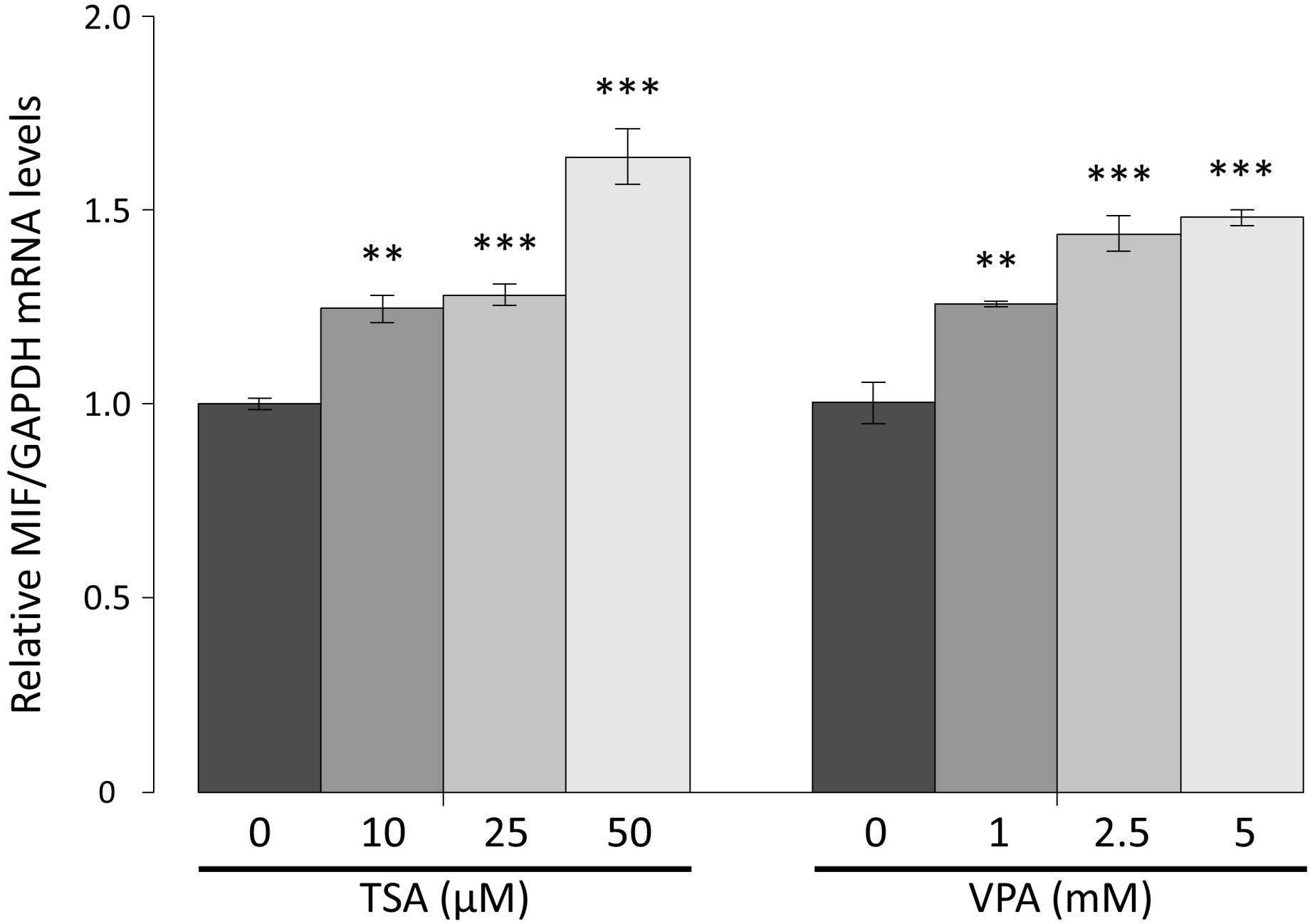
Supplementary Fig. S3. The effects of clozapine (CLO) on histone deacetylate (HDAC) activity in primary cultured astrocytes (PCAs). HDAC assay was performed using nuclear extracts from PCAs. The HDAC concentration (μM) was calculated from the standard curve. No inhibitor is negative control. Trichostatin A (TSA) is positive control and showed inhibition as expected. We compared DMSO with 50, 100, and 200 μM of CLO. The data are expressed as the mean ± SEM. No significance was observed vs. DMSO (Dunnett's test; n = 6).

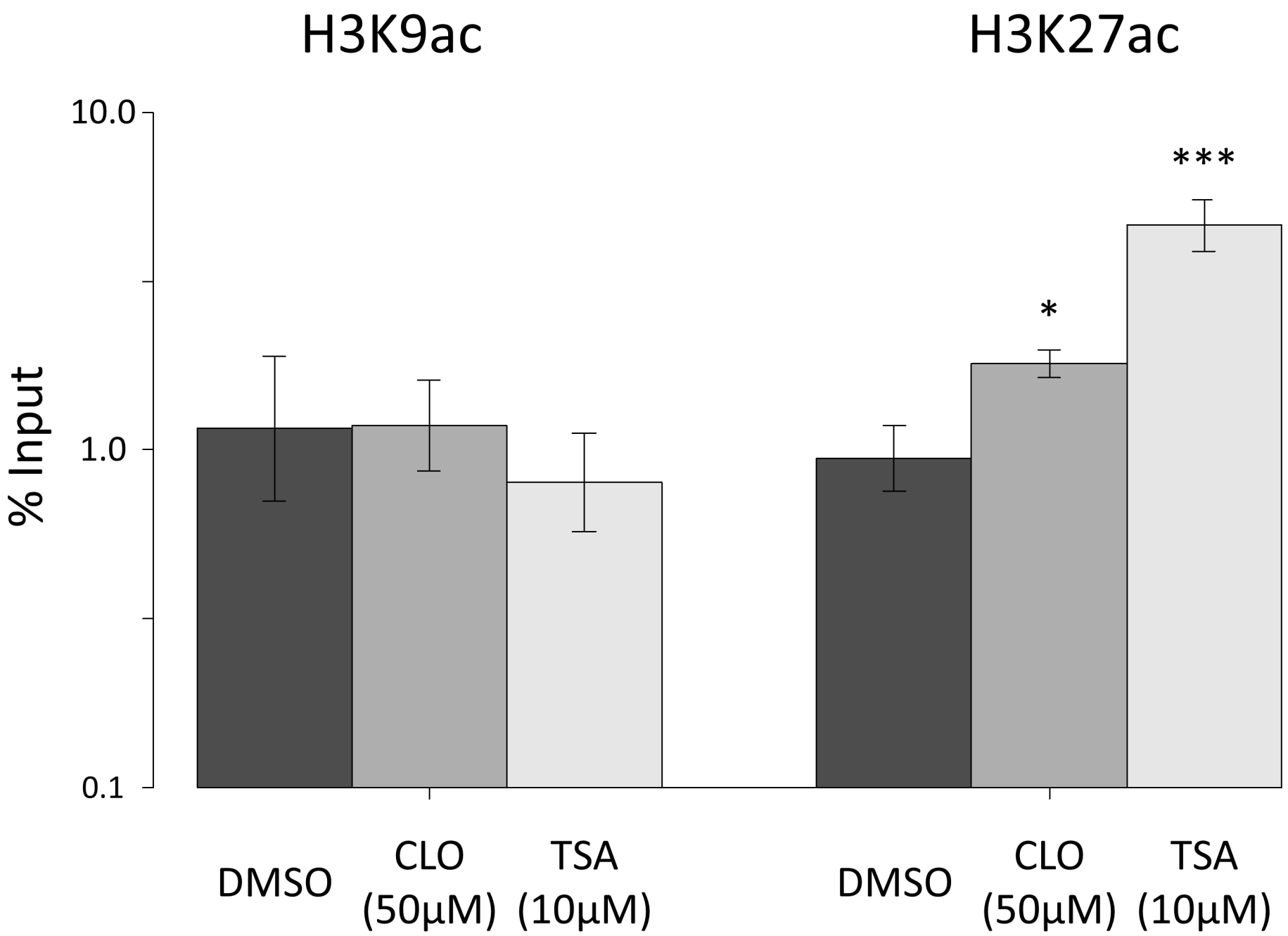
Supplementary Fig. S4. The time-dependent effects of clozapine on expression of brain-derived neurotrophic factor (BDNF) in primary cultured astrocytes (PCAs). BDNF mRNA expression was analyzed by Q-RT-PCR. The values are shown as the ratio of BDNF mRNA to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. The lines connect the average values (n = 3). *$p < 0.05$, **$p < 0.01$, or ***$p < 0.001$ vs. basal (time point = 0) (Dunnett's test). $^{\star}p < 0.05$, $^{\star\star}p < 0.01$, or $^{\star\star\star}p < 0.001$ vs. DMSO (Student's t-test).


Relative MIF/GAPDH mRNA levels
2.5
2.0
1.5
1.0
0.5
0.0
*
***
***
*
***
***
***
***
***
***
0 10 20 50
0 10 20 50
0 10 20 50
0 10 20 50
0 10 20 50
0 1 2 5
CHP
HPD
CLO
RIS
OLZ
ARI
Antipsychotics concentration (μM)

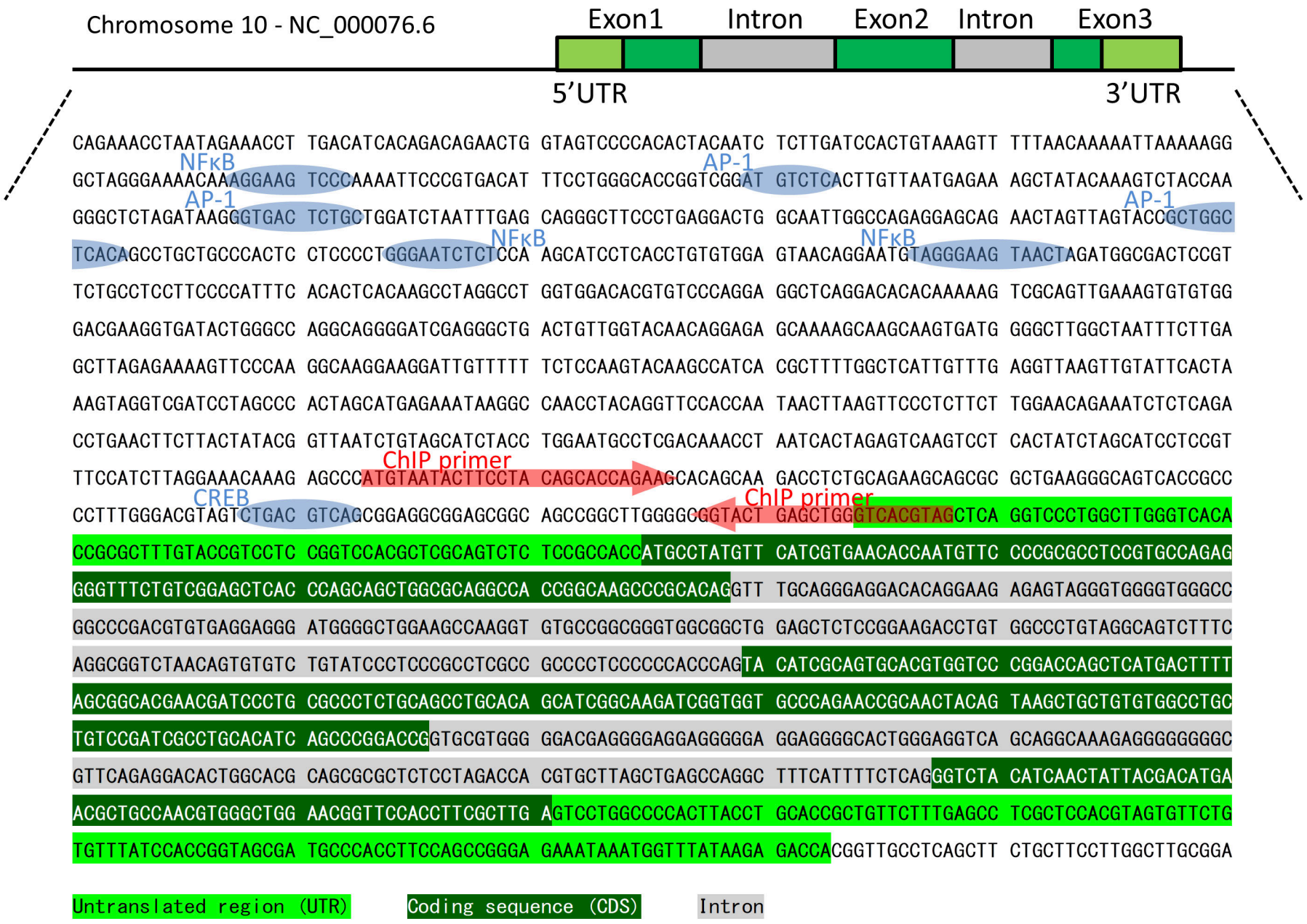

Relative MIF/GAPDH mRNA levels
vs. basal
2.0
1.5
1.0
0.5
0.0
0
6
12
18
24
30
36
42
48
54
60
66
72
78
*
**
***
***
*
Clozapine 50μM
DMSO
Clozapine 50μM treatment time (h)

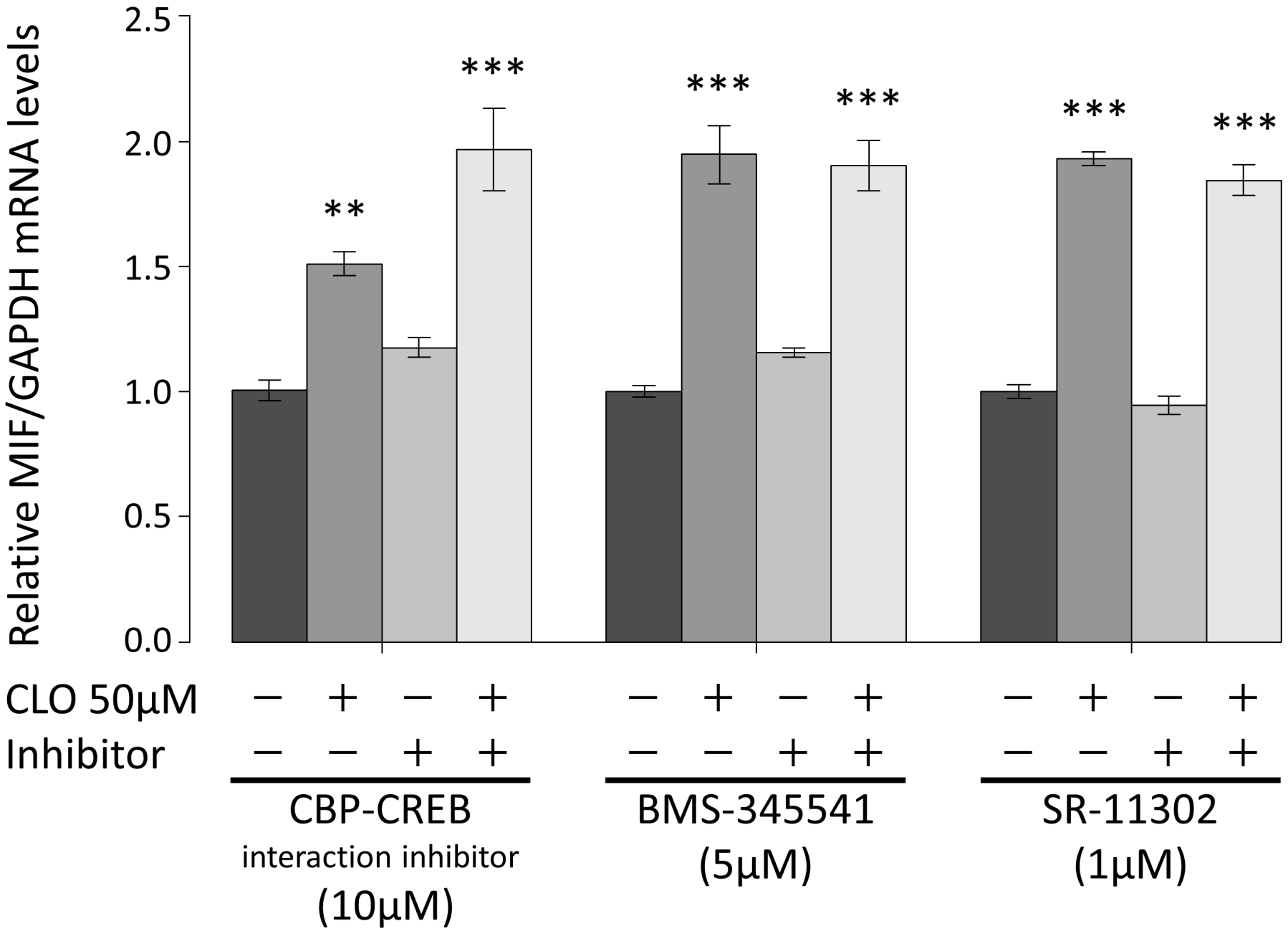

Clozapine 50μM
DMSO
MIF protein concentration (pg/ml)
vs. basal
25000
20000
15000
10000
5000
0
***
***
***
***
***
0
6
12
18
24
30
36
42
48
54
60
66
72
78
Clozapine 50μM treatment time (h)

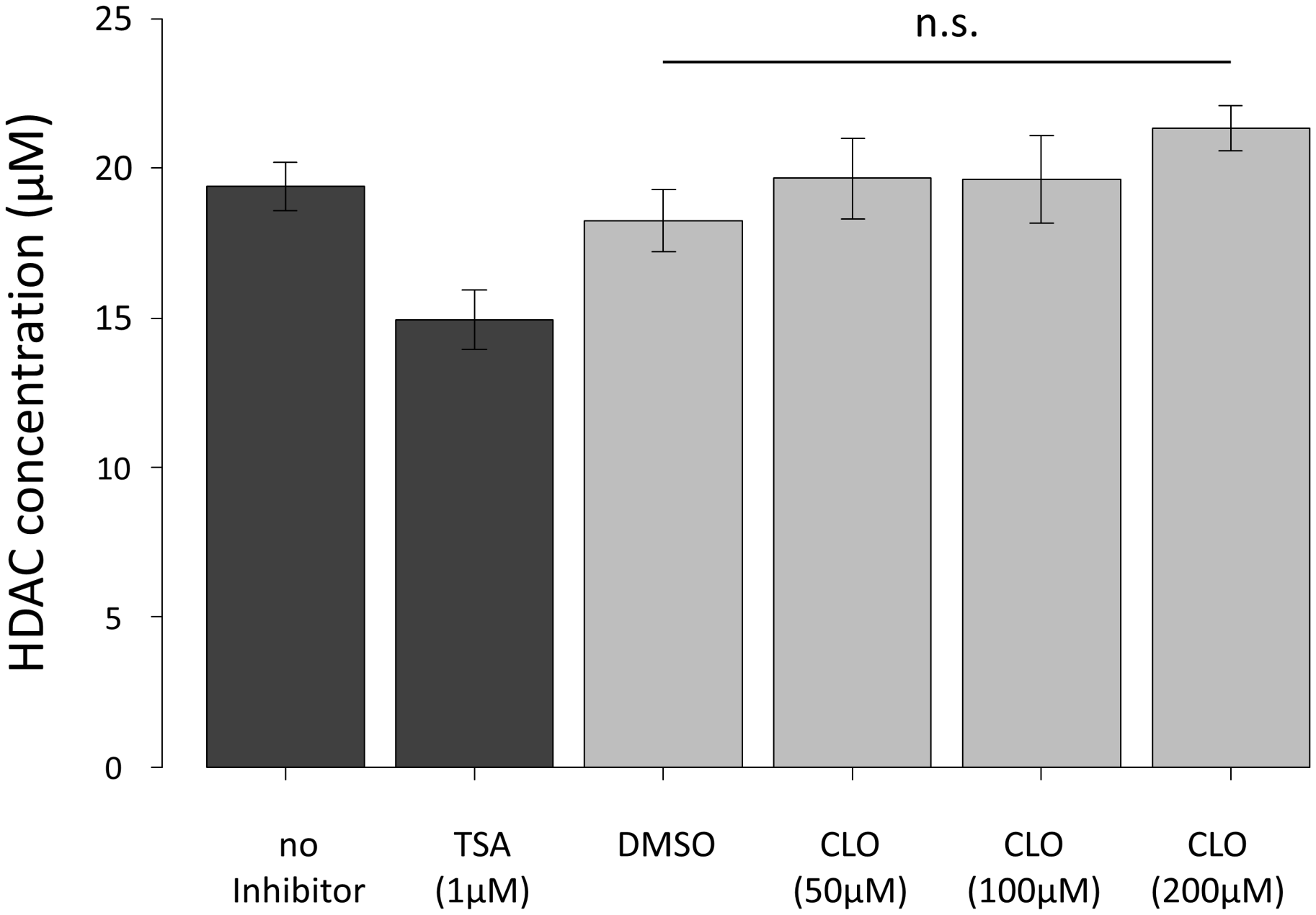

Relative MIF/GAPDH mRNA levels
2.0
1.5
1.0
0.5
0
**
***
***
**
***
***
0
10
25
50
0
1
2.5
5
TSA (μM)
VPA (mM)

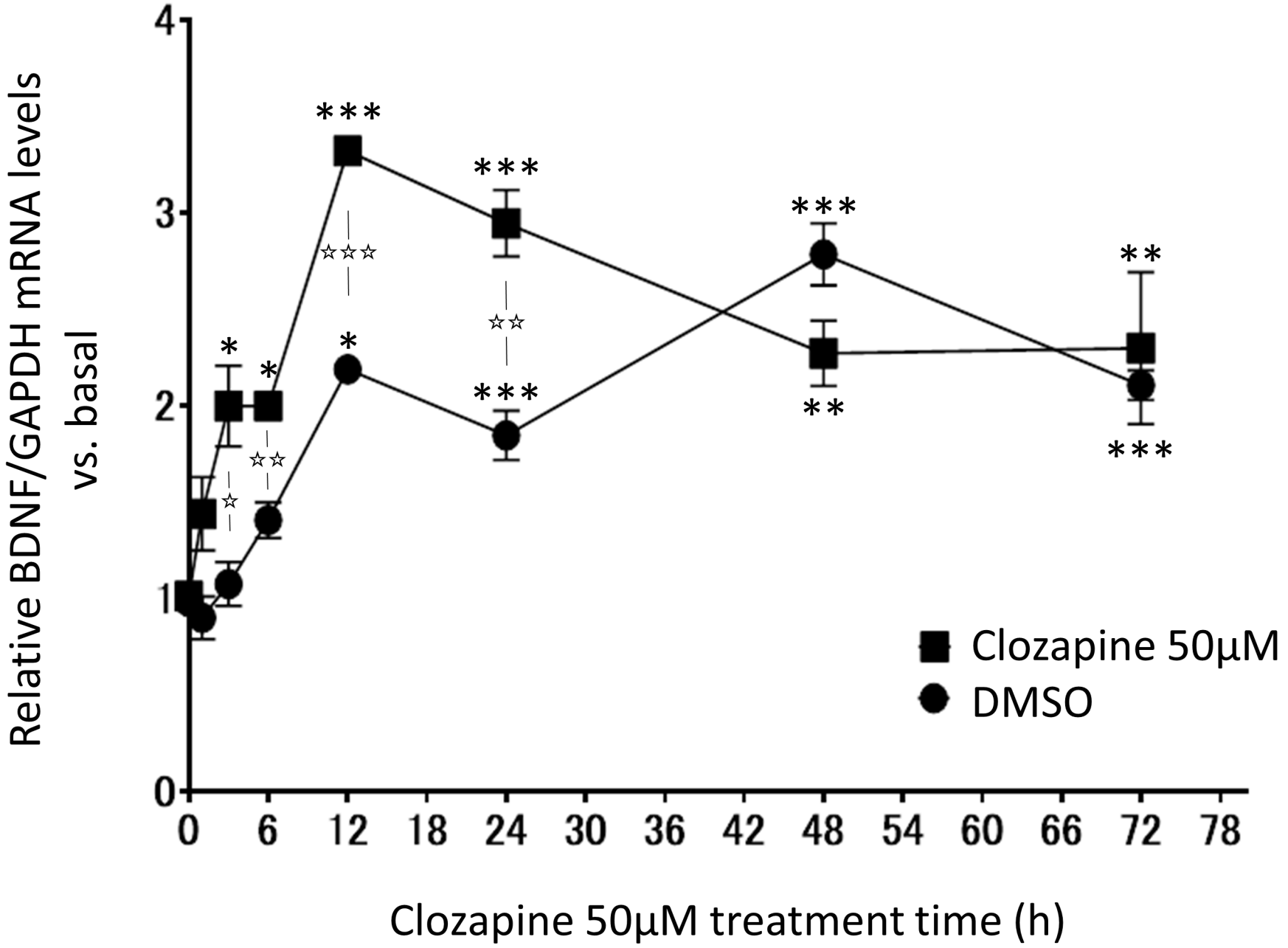

H3K9ac
H3K27ac
10.0
1.0
0.1
% Input
*
***
DMSO
CLO (50µM)
TSA (10µM)
DMSO
CLO (50µM)
TSA (10µM)

Mouse Macrophage migration inhibitory factor gene




Relative MIF/GAPDH mRNA levels
2.5
2.0
1.5
1.0
0.5
0.0
**
***
***
***
***
***
CLO 50μM
Inhibitor
CBP-CREB
interaction inhibitor
(10μM)
BMS-345541
(5μM)
SR-11302
(1μM)


n.s.
HDAC concentration (µM)
25
20
15
10
5
0
no Inhibitor
TSA (1µM)
DMSO
CLO (50µM)
CLO (100µM)
CLO (200µM)


Relative BDNF/GAPDH mRNA levels
vs. basal
4
3
2
1
0
0
6
12
18
24
30
36
42
48
54
60
66
72
78
Clozapine 50μM treatment time (h)
Clozapine 50μM
DMSO