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Research paper

Antidepressant treatment is associated with epigenetic alterations of *Homer1* promoter in a mouse model of chronic depression

Lu Sun[a,b], Rikst-Nynke Verkaik-Schakel[c], Knut Biber[a,b], Torsten Plösch[c], Tsvetan Serchov[d,e,*]

[a] Department for Psychiatry and Psychotherapy, Medical Center - University of Freiburg, Faculty of Medicine, University of Freiburg, Hauptstr. 5, 79104, Freiburg, Germany
[b] Department Biomedical Sciences of Cells & Systems, Section Molecular Neurology, University of Groningen, University Medical Center Groningen, 9713 AV, Groningen, The Netherlands
[c] Department of Obstetrics and Gynecology, University of Groningen, University Medical Center Groningen, 9713 RB, Groningen, The Netherlands
[d] Department of Stereotactic and Functional Neurosurgery, Medical Center - University Freiburg, Faculty of Medicine, University of Freiburg, Breisacher Str. 64, 79106, Freiburg, Germany
[e] Centre National de la Recherche Scientifique, Université de Strasbourg, Institut des Neurosciences Cellulaires et Intégratives, 67000, Strasbourg, France

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ABSTRACT

Background: Understanding the neurobiology of depression and the mechanism of action of therapeutic measures is currently a research priority. We have shown that the expression of the synaptic protein Homer1a correlates with depression-like behavior and its induction is a common mechanism of action of different antidepressant treatments. However, the mechanism of Homer1a regulation is still unknown.
Methods: We combined the chronic despair mouse model (CDM) of chronic depression with different antidepressant treatments. Depression-like behavior was characterized by forced swim and tail suspension tests, and via automatic measurement of sucrose preference in IntelliCage. The Homer1 mRNA expression and promoter DNA methylation were analyzed in cortex and peripheral blood by qRT-PCR and pyrosequencing.
Results: CDM mice show decreased Homer1a and Homer1b/c mRNA expression in cortex and blood samples, while chronic treatment with imipramine and fluoxetine or acute ketamine application increases their level only in the cortex. The quantitative analyses of the methylation of 7 CpG sites, located on the *Homer1* promoter region containing several CRE binding sites, show a significant increase in DNA methylation in the cortex of CDM mice. In contrast, antidepressant treatments reduce the methylation level.
Limitations: Homer1 expression and promotor methylation were not analyzed in different blood cell types. Other CpG sites of *Homer1* promoter should be investigated in future studies. Our experimental approach does not distinguish between methylation and hydroxymethylation.
Conclusions: We demonstrate that stress-induced depression-like behavior and antidepressant treatments are associated with epigenetic alterations of *Homer1* promoter, providing new insights into the mechanism of antidepressant treatment.

1. Introduction

Major depression disorder (MDD) is a mental disorder, which affects millions of people worldwide and causes an enormous burden for the society (Mrazek et al., 2014). The specific pathophysiology of MDD is still under debate. In spite of the numerous antidepressant drugs available, such as selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants (TCA), which are widely prescribed, effective pharmacotherapies are limited and their mechanism of action is still rudimentary. Understanding the pathophysiology of depression and the mechanism of action of therapeutic measures have been an area of intense investigation in modern psychiatry.

One of the hypotheses on the neurobiology of depression involves an inability of neuronal systems, especially under stress conditions, to show adaptive change, a mechanism known as neuronal plasticity (Pittenger and Duman, 2008; van Calker et al., 2018). We have recently shown that the brain expression of the synaptic plasticity protein Homer1a correlates with the depression-like behavior and its induction is a common mechanism of action of several antidepressant treatments (Serchov et al., 2015; Serchov et al., 2016). Homer1 belongs to a family of scaffolding proteins that facilitate clustering of specific synaptic proteins and modulate their activities at neuronal synapses. The *Homer1* gene consists of full-length constitutive isoforms Homer1b/c and short lacking C-terminal

* Corresponding author.
E-mail address: serchov@inci-cnrs.unistra.fr (T. Serchov).

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domain splicing variant Homer1a (Bottai et al., 2002; Brakeman et al., 1997). General deletion of *Homer1* enhances anxiety- and depression-like behaviour in mice (Szumlinski et al., 2006), while the expression of the short Homer1a isoform is upregulated by various antidepressant treatments (Kato, 2009; Serchov et al., 2015; Sun et al., 2011). Moreover, several other Homer proteins have been implicated in the pathophysiology of different psychiatric disorders (Szumlinski et al., 2006).

The interaction between specific genes and environmental factors (GxE), such as stress exposure, has been strongly linked to MDD vulnerability. Epigenetic processes, like DNA methylation and histone methylation/acetylation, regulate gene transcription and have been shown to dynamically respond to environmental stressors, thus mediating the GxE interaction in the complex etiology of MDD (Dalton et al., 2014; Lockwood et al., 2015; Roth, 2013). Particularly, DNA methylation has been considered as a potential link between GxE and depression (Bakusic et al., 2017; Chen et al., 2017; Li et al., 2019). Remarkably, epigenetic modulation of *Homer1a* transcription has been reported in amygdala and hippocampus in an animal model of post-traumatic stress disorder (Mahan et al., 2012). Indeed, *Homer1* promoter contains several cAMP-response element (CRE) binding sites that are preferential target for epigenetic alterations (Mahan et al., 2012; Robison and Nestler, 2011; Saavedra et al., 2016). In humans, there are significant influence of single nucleotide polymorphisms (SNPs) located in the regulatory region of *Homer1* gene on prefrontal activity during cognitive and motivational processes (Rietschel et al., 2010) and are associated with risk for MDD and suicide attempts (Rao et al., 2017; Strauss et al., 2012). However, potential epigenetic regulation of *Homer1* in depression and antidepressant treatment has not been investigated.

In this study, we utilized the chronic despair model (CDM) as a mouse model of chronic depression induced by repeated swimming, in combination with different antidepressant treatments and analyze the methylation of *Homer1* promoter and gene expression of Homer1a and Homer1b/c isoforms. In summary, we identified that the effects of both the repeated swim stress in CDM model and the antidepressant treatment on depression-like behavior are associated with epigenetic alterations of *Homer1* promoter, thus expanding our understanding of the mechanism of Homer1 regulation in depression and antidepressant response.

2. Materials and methods

2.1. Mice

Wild type C57BL/6 mice (8 weeks old) were obtained from Charles River Laboratories (CEMT-Freiburg, Germany). All procedures were performed in accordance with the German animal protection law (TierSchG), Dutch animal care and use laws, FELASA (Federation of European Laboratory Animal Science Associations), the national animal welfare body GV-SOLAS guide for the care and use of laboratory animals and were approved by the animal welfare committee of the University of Freiburg and the University of Groningen. Animals were housed in a temperature and humidity-controlled vivarium with a 12 h light-dark cycle, and food and water available ad libitum.

2.2. Behavioral experiments

Activity and behavior of mice were observed using an automatic video tracking system for recording and analysis (VideoMot2 system V6.01, TSE) and an IntelliCage system (TSE system) unless otherwise specified. All the animal behavior experiments contain male and female mice. In order to avoid aggressive behaviours only female mice were used for the IntelliCage system experiments.

2.3. Chronic behavioral despair model (CDM)

To induce chronic depression-like behavior in mice, we utilized the chronic behavioral despair model (CDM), where the forced swimming paradigm was used (Holz et al., 2019; Normann et al., 2018; Serchov et al., 2015; Sun et al., 2011) The mice were subjected to repeated swimming for 10 min daily for 5 days in a transparent cylinder (15 cm diameter), which contained 20 cm of water (22-25°C) (induction phase). From day 6 on, the mice were kept undisturbed in the home cage for 4 weeks, then the test phase (last 10 min swim session and tail suspension test) was performed on day 32. The immobility time was recorded and analyzed.

2.4. Antidepressant drug treatment

The mice were chronically treated for 4 weeks with: imipramine (15 mg/kg, imipramine hydrochloride; Sigma) or fluoxetine (15 mg/kg, fluoxetine hydrochloride; Sigma) both given in the drinking water. Ketamine (3mg/kg ketamine hydrochloride; Pfyzer) was administered acutely via single intraperitoneal injection, as a positive control substance having rapid antidepressant effect 1h prior to the mouse behavioral assessment or 7 days before the sacrifice.

2.5. Tail suspension test (TST)

The TST was used to assess antidepressant efficacy of drugs and use the previously described protocol (Holz et al., 2019; Serchov et al., 2015). Mice were suspended with its tail on a hook to a horizontal bar located inside a white box (30 × 50 × 20 cm). There was about 20 cm from the mouse head to the floor and the mouse is taped 1-1.5 cm from the tip of the tail. The test was carried out for 6 min (360s), the behavior was recorded by the video and the immobility time was scored by two independent persons blinded to the experimental condition of the mice. The mice which climbed their tails (>10% of total time) were removed from further analyses.

2.6. Analysis of sucrose preference and motivation-oriented behavior in IntelliCage

The IntelliCage system (TSE Systems, Bad Homburg, Germany) was utilized for simultaneous and automatic analysis of sucrose preference and motivation oriented behavior (Holz et al., 2019; Serchov et al., 2019). It allows the recording of up to 16 group-housed mice implanted with radio-frequency identification (RFID) transponders. The unit contains 4 operant corners and one open common space with 4 red shelters in the center. Food can be accessed for mice in the middle of the IntelliCage, while water is available behind remote-controlled guillotine doors. The drinking bottles in each corner can be visited by only one mouse at the same time. The durations and the number of visits to any of the four corners, the licks on the bottles and the nosepokes towards the doors were recorded by PC-based tracking software (IntelliCage Plus, TSE Systems). At the outset, the mice adapt to the IntelliCage with water ad libitum in all corners for at least 7 days. The animals were habituated to the sucrose taste: one bottle was filled with 1% sucrose solution and other one was filled with water. All the bottles are freely accessible with both doors open in the corner. During the nosepoke adaptation protocol all doors were closed: in order to open the door, the mice had to apply a nosepoke. After 5s of drinking the door closes automatically. Every 24h the positions of the bottles were exchanged in the tasks involving sucrose filled bottles. The nosepoke SPT protocol was applied to measure anhedonia and motivation-oriented behavior by scoring the sucrose preference with gradually increasing effort (number of nosepokes). During this paradigm, all doors open based on a nosepoke and close after 5s licking. After every 24 h the number of nosepokes needed to open a door to a side with a sucrose containing bottle increases (1, 2, 3, 4, 5, 6, 7). The number of licks was recorded and the averaged preference for sucrose was calculated as a percentage of the total number of licks for each bottle.

2.7. RNA isolation

Mice were killed by cervical dislocation, then the brains were rapidly removed, the cortices were dissected and quickly frozen on dry ice

and stored at -80°C. The brains were always dissected by the same investigator. The RNA extraction was performed as previously described (Chomczynski and Sacchi, 2006). Briefly, the guanidine thiocyanate/2-mercaptoethanol buffer used to homogenize the tissues and total RNA was extracted with the sodium acetate/phenol/chloroform/isoamylacohol step. Samples were isopropanol precipitate and washed twice with 70% ethanol. The pellets were dissolved in the RNase free Tris-HCl buffer (pH 7.0), then RNA concentrations were measured by spectrophotometer (BioPhotometer; Eppendorf) and stored at −80°C until further processing.

2.8. Quantitative real-time PCR (qRT-PCR)

The isolated RNA was converted to cDNA using M-MLV reverse transcriptase (Promega) with 1 mg of total RNA. The quantitative real-time PCR was done by the C1000TM Thermal Cycler (CFX96 real-time PCR system, Bio-Rad) with iQ SYBR Green Supermix (BioRad) system. The target genes mRNA levels were normalized by the levels of actin, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), and s12 RNA automatically by CFX Manager software (Version 3.0.1224.1051, 2012, Bio-Rad) using the "Gene study" option. The primer sequences used were as follows: Homer1a: 5′-CAAACACTGTTTATGGACTG-3′, 5′-TGCTGAATTGAATGTGTACC-3′; Homer1b: 5`-AGAGCTGAACCAGACAGTGC-3`, 5`-GGTTACTGCGGAAAGCCTCT-3`; actin: 5′-CTAAGGCCAACCGTGAAAAG-3′, 5′-ACCAGAGGCATACAGGGACA-3′; GAPDH: 5′-TGTCCGTCGTGGATCTGAC-3′, 5′-CCTGCTTCACCACCTTCTTG-3′; s12: 5′-GCCCTCATCCACGATGGCCT-3′, 5′-ACAGATGGGCTTGGCGCT TGT-3′.

2.9. DNA isolation and bisulfite conversion

DNA was isolated from venous EDTA-blood and brain tissues using the FlexiGene DNA Kit (Qiagen) according to manufacturer's instructions and stored at −80°C until further processing. DNA concentration and quality were assessed with a ND-1000 spectrophotometer (NanoDrop). Then the DNA was bisulfite-converted and purified by the EpiTect 96 Bisulfite Kit (Qiagen) according to manufacturer's instructions.

2.10. DNA methylation analysis

Bisulfite specific primers were designed with the PyroMark Assay Design software 2.0 (Qiagen) for the *Homer1* promoter. The seven examined CpG positions were located between position -791 and -749 in relation to the transcription start site of *Homer1* (Fig. 4A). The primers used to amplify a 190 bp region: forward: 5′-Biotin-AGAGAATAGGGAGTAGGTGG-3′ and reverse: 5′-CATCCTCCCCCCAAAAAAACCTT-3′. Amplification was performed using HotStarTaq Master Mix (Qiagen) using the following steps: DNA polymerase activation (95°C, 15 minutes), 3-step cycle of denaturation (94°C, 30 seconds), annealing (56°C, 30 seconds) and extension (72°C, 30 seconds) repeated for 45 cycles in a row. The final extension was performed at 72°C for 7 minutes. The methylation status was examined by pyrosequencing using the PyroMark Q24 system with 15 µl of PCR product, performed as described in the PyroMark Q24 Vacuum Workstation Guide (Qiagen). The sequencing primer used for *Homer1*: 5′-CAAAAAAACCTTTAAATAACAAC-3'. The sequence to analyze was RTCAACRCRCTCCTCTCCRCACATACRTCACCCTCCRCTCACRTCAACTTATAC. Finally, the PyroMark Q24 2.0.6 software (Qiagen) was used to determine the methylation percentage of individual CpG positions.

2.11. Statistical analyses

All values are expressed as means ± SEM. Statistical analyses were done by GraphPad Prism 8.1.1. (GraphPad Software Inc.) using one- or two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test to compare the means of two or more groups or unpaired two-tailed Student's t-test to compare the means of two groups. P value ≤ 0.05 was considered as significant (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001). Prior to statistical analyses data assumptions (for example normality and homoscedasticity of the distributions) were verified using Anderson-Darling, D'Agostino-Pearson, Shapiro-Wilk and Kolmogorov-Smirnov tests (GraphPad Prism 8.1.0 software). Mice were randomly assigned to groups for all studies and investigators were blinded to the treatment group until the data has been collected. Sample sizes were determined on the basis of extensive laboratory experience and were verified via power analysis.

3. Results

3.1. Chronic despair paradigm (CDM) – mouse model for chronic depression and antidepressant response

In order to investigate the mechanism of Homer1 regulation in stress-induced depression and antidepressant treatment, we used the chronic despair model (CDM) of chronic depression-like behavior in combination with different antidepressant treatments. In this paradigm (Holz et al., 2019; Serchov et al., 2015; Serchov et al., 2019), mice are subjected to repetitive swimming of 10 min for 5 consecutive days, which leads to significant increase of the immobility time in days 2 to 5 ($F_{4,120} = 35.60$; $P < 0.0001$; Fig. 1A). The increased immobility time during the test phase demonstrates that the stress-induced behavioral despair is chronically maintained for 4 weeks. The chronic treatment with the TCA imipramine and the SSRI fluoxetine strongly reduce the immobility time at day 32. Moreover, we used acute ketamine application as a positive control, which as expected had a robust antidepressant effect ($F_{9,140} = 18.86$; $P < 0.0001$; Fig. 1B). Similar results were obtained with tail suspension test (TST) that was performed during the test phase – repeated swimming increased the immobility time of CDM mice, while the antidepressant treatment decreased it ($F_{5,44} = 23.30$; $P < 0.0001$; Fig 1C).

In order to further analyze the depression-like behavior, we performed a sucrose preference test, as a measure of anhedonia and reward-oriented behavior. To avoid possible stress by the isolated housing, we utilized the IntelliCage system, in which locomotor, explorative and drinking behavior of socially grouped mice can be automatically monitored in undisturbed condition (Endo et al., 2011). We subjected the mice to an experimental paradigm for automated measurement of sucrose preference and motivation-oriented behavior with gradually increasing effort to reach the sucrose solution (Fig 2A and 2C) (Alboni et al., 2017; Serchov et al., 2019). Initially, we evaluated this paradigm by comparing the sucrose preference of the control and the CDM mice (Fig. 2A and 2B). The naive control mice showed a high sucrose preference with a small decrease of the sucrose preference correlating with the increased effort to get access to the sucrose, while the CDM mice exhibited a pronounced anhedonia phenotype with a marked decrease of the sucrose preference associated with the increasing number of nosepokes (nosepokes $F_{6,70} = 6.484$; $P < 0.0001$; treatment $F_{1,70} = 107.4$; $P < 0.0001$; interaction $F_{6,70} = 1.015$; $P = 0.4228$; Fig 2B). The chronic treatment with imipramine or fluoxetine for 4 weeks, as well as the acute injection of ketamine (Fig. 2C), which has a rapid and sustained antidepressant effect up to seven days (Autry et al., 2011), inhibited the anhedonic phenotype of CDM mice, increasing significantly their drinking preference towards the sucrose (nosepokes $F_{6,105} = 5.147$; $P = 0.0001$; treatment $F_{3,105} = 51.31$; $P < 0.0001$; interaction $F_{18,105} = 0.75455$; $P = 0.7471$; Fig. 2D).

3.2. Homer1a and Homer1b/c mRNA expression in the cortex and blood

To investigate Homer1 as a potential marker for depression and antidepressant response, we analyzed the mRNA expression of its two isoforms - Homer1a and Homer1b/c in the cortex and the peripheral blood of control, CDM and antidepressant treated CDM mice (Fig 3A). The chronically despaired mice showed a reduced mRNA expression of both Homer1a and Homer1b/c in the cortex and blood (Fig 3). While chronic treatment with imipramine, fluoxetine and acute ketamine

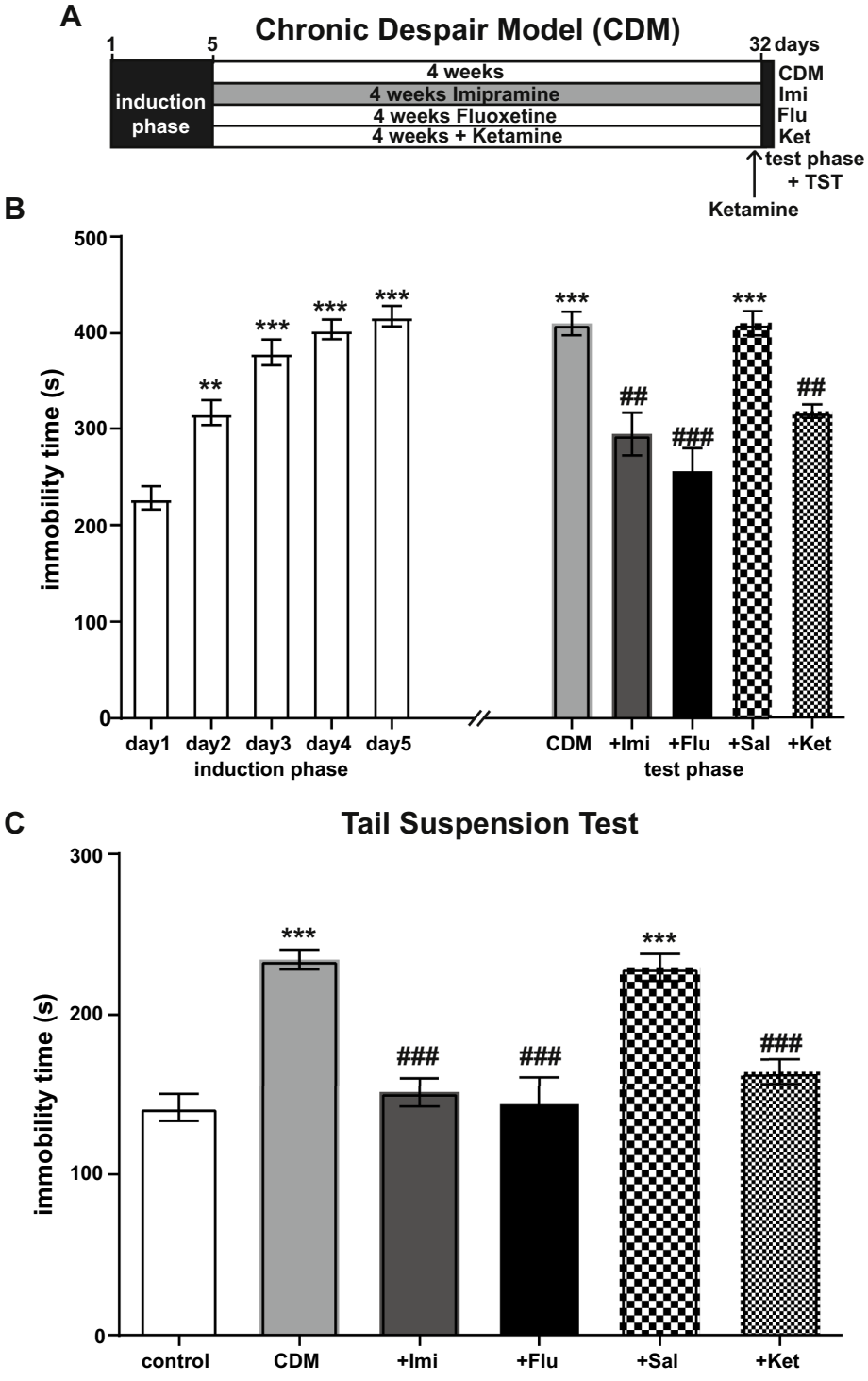

Fig. 1. Chronic despair model for stress-induced chronic depression. (A) Schematic illustration of the experimental protocol: The mice were despaired by 10 min swim sessions for 5 consecutive days: days1–5 (induction phase). For the following 4 weeks the mice were kept undisturbed in their home cages and divided into 4 groups: without any antidepressant treatment (CDM); treated with 15mg/kg imipramine for 4 weeks (Imi), treated with 15mg/kg fluoxetine for 4 weeks (Flu), injected with 3mg/kg ketamine (Ket) or saline (Sal) on day 32 1h prior to testing. On day 32 the last 10 min swim session and tail suspension test (test phase) were performed. (B) Immobility time spent during the induction phase (day1 to day5) and the test phase (day32) of CDM (n = 15), imipramine treated (n = 5), fluoxetine treated (n = 5), ketamine and saline (n = 5 per group). (C) Immobility time in tail suspension test (TST) of control (n = 5), CDM (n = 15), imipramine treated (n = 5), fluoxetine treated (n = 5), ketamine and saline injected (n = 5 per group). One-way ANOVA with Bonferroni post hoc test: *p < 0.05, **p < 0.01, ***p < 0.001 in comparison to day1 and control; ##p < 0.01, ###p < 0.001 in comparison to CDM or saline. Data are expressed as means ± SEM in all graphs.

administration increased Homer1a and Homer1b/c mRNA expression in the cortex ($F_{4,40} = 13.82$; $P < 0.0001$; Fig. 3B and $F_{4,40} = 8.832$; $P < 0.0001$; Fig. 3D), they were not able to significantly change Homer1 gene expression in blood ($F_{4,40} = 6.903$; $P = 0.0003$; Fig. 3C and $F_{4,40} = 8.865$; $P < 0.0001$; Fig. 3E). Thus, our data demonstrate that stress-induced depression-like behavior correlates with the decreased *Homer1* gene expression in both cortex and blood. However, antidepressant drugs increased only the cortical expression of Homer1, whereas its levels in the blood remained unchanged, suggesting for a differential regulation of *Homer1* gene by antidepressant treatments in the brain and peripheral blood.

3.3. Stress-induced depression-like behavior correlates with increased CpG DNA methylation of the Homer 1 promoter

To explore whether the chronic stress was associated with epigenetic regulation of *Homer1* gene, we investigated the DNA CpG methylation of a part of its promoter region. Our initial analyses using UCSC Genome Browser on Mouse Dec. 2013 assembly (GRCm38/mm10) identified a 1111 bp long CpG island (position: chr13: 93,303,527-93,304,637) containing 136 CpG sites covering parts of the promoter and exon 1 of the *Homer1* gene. For our quantitative methylation analysis, we particularly focused on 7 CpG sites located on the

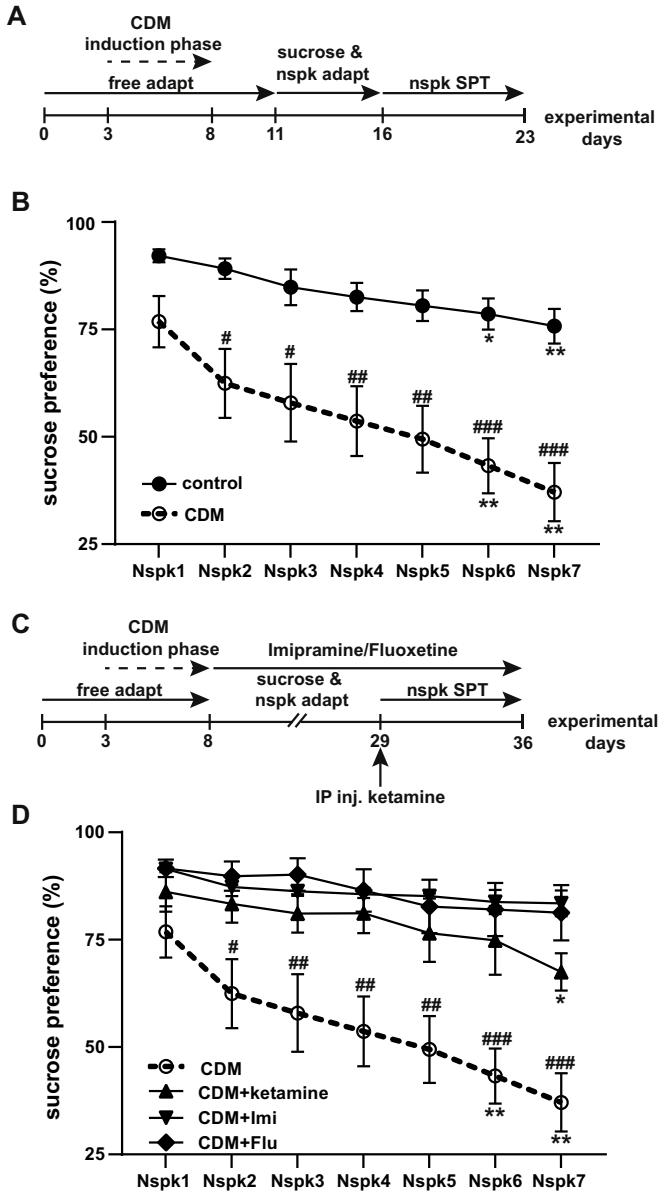

Fig. 2. Automated analyses of sucrose preference. (A) Schematic illustration of the experimental protocol: During the free adaptation phase, the mice were acclimatized to the IntelliCage - water was available in all corners and doors to water were open. Induction phase – the mice were subjected to repeated swimming for 10 min daily for 5 consecutive days to induce chronic despair. Then divided the mice into 2 groups: control and chronically despaired (CDM) groups; sucrose & nspk adapt – the bottle in every corner, one was filled with 1% sucrose and another with water. The nosepoke opened the respective door for 5s during the nosepoke adaptation; Nspk SPT – the door opens with nosepoke and closes in response to 5s licking session. After every 24 h, the number of nosepokes that needed to open a door to the sucrose-containing bottle side increases (1, 2, 3, 4, 5, 6, 7). (B) Sucrose preference in nspk SPT. (n = 8 per group, two independent experiments) Two-way ANOVA followed by Bonferroni post hoc test: *p < 0.05, **p < 0.01 in comparison to Nspk1; ### < 0.001 in comparison to control; (C) Schematic illustration of the experimental protocol. (D) Sucrose preference in nspk SPT1 in antidepressant treated CDM mice. (n = 5 per group) Two-way ANOVA followed by Bonferroni post hoc test: *p < 0.05, in comparison to Nspk1; #p < 0.05, ##p < 0.01, ###p < 0.01 in comparison to antidepressant treated mice; Data are expressed as means ± SEM in all graphs.

CRE binding sites between position -791 and -749 in relation to the transcription start site of *Homer1* promoter region (Fig 4A). The cortex samples from the chronic despair group mice showed a robust increase in the DNA methylation (5mC) at all 7 CpG sites compared to the control group (CpG $F_{6,196} = 10.71$; $P < 0.0001$; treatment $F_{1,196} = 99.95$; $P < 0.0001$; interaction $F_{6,196} = 0.6925$; $P = 0.46561$; Fig. 4B). However, we did not observe any significant changes in the 5mC levels in the blood sample of CDM group (CpG $F_{6,196} = 7.995$; $P < 0.0001$; treatment $F_{1,196} = 0.01269$; $P = 0.9104$; interaction $F_{6,196} = 0.1052$; $P = 0.9958$; Fig 4D). These data suggest that the stress induced increase of the DNA methylation of *Homer1* promoter might be a potential mechanism mediating its reduced expression in the CDM mice.

3.4. Monoaminergic antidepressants downregulate DNA methylation at the Homer1 promoter

To investigate whether antidepressant drug treatment affects DNA methylation at the *Homer1* promoter, we used two different monoaminergic-based antidepressant drugs - fluoxetine and imipramine to chronically treat the CDM mice. The 5mC level was significantly decreased in CpG site 1 and 7 in cortex after both chronic antidepressant drug treatments (CpG $F_{6,154} = 10.67$; $P < 0.0001$; treatment $F_{2,154} = 7.752$; $P = 0.0006$; interaction $F_{12,154} = 0.8251$; $P = 0.6244$; Fig. 4C). The blood sample show similar changes in the DNA methylation only at CpG site 7 after both treatments (CpG $F_{6,154} = 3.73$; $P = 0.0017$; treatment $F_{2,154} = 3.186$; $P = 0.0441$; interaction $F_{12,154} = 1.021$; $P = 0.4321$; Fig 4E).

4. Discussion

In our previous studies, we have shown that Homer1a expression correlates with the depression-like behavior and that the antidepressant effects of different antidepressant treatments are mediated via induction of Homer1a (Serchov et al., 2015). In order to investigate the regulation of Homer1a in stress-induced depression and antidepressant response, we subjected the mice to a chronic model of depression (CDM) followed by different antidepressant treatments and then analyzed the mRNA expression of both Homer1 isoforms in the brain cortex and peripheral blood and *Homer1* gene promoter DNA methylation. Our data show that the mRNA expression of Homer1a and Homer1b/c is downregulated in both cortex and blood of the CDM mice. In contrast, chronic treatment with different monoaminergic-based antidepressants, like imipramine and fluoxetine, as well as the fast-acting ketamine, increases their expression only in the cortex. The quantitative analysis of the methylation of 7 CpG sites located in the *Homer1* promoter in cortex sample from the CDM mice show a significant increase in DNA methylation at all analyzed CpG sites. In contrast, monoaminergic antidepressant treatments reduce the methylation level of CpG site 1 and 7. Thus, our study shows that stress-induced depression-like behavior and antidepressant treatment is associated with epigenetic alterations of *Homer1* promoter. It provides new insights into the mechanisms of the antidepressant drug treatment in major depression.

Chronic depression-like behavior can be induced via a variety of approaches. Here, we used the repetitive forced swimming as a model of inducing chronic state of behavioral despair, anhedonia and reduced motivation- and reward-oriented behavior. The advantage of our CDM model is the simplicity that chronic depression-like behavioral is induced and maintained without any other stressors compared to other paradigms. After the induction phase, the CDM mice were treated with antidepressant drugs for 4 weeks and the antidepressant effect was validated by TST and automatic measurement of sucrose preference and motivation-oriented behavior. The depression-like state lasts for more than 4 weeks and thus the therapeutic attempts can be validated during this period. Similarly, human depression also has a latent period of weeks for responding to antidepressants and thus the present CDM paradigm has a high face validity in a reference to the human depression in combination with a high predictive validity showing a comparable time course of antidepressant treatments to humans in terms of ameliorating the depression-like behavior (Hellwig et al., 2016; Holz et al., 2019; Serchov et al., 2019).

Considerable evidence suggests a crucial role for *Homer1* in the pathogenesis of MDD (Banerjee et al., 2016; Leber et al., 2017; Rietschel et al., 2010; Serchov et al., 2015). Moreover, we have

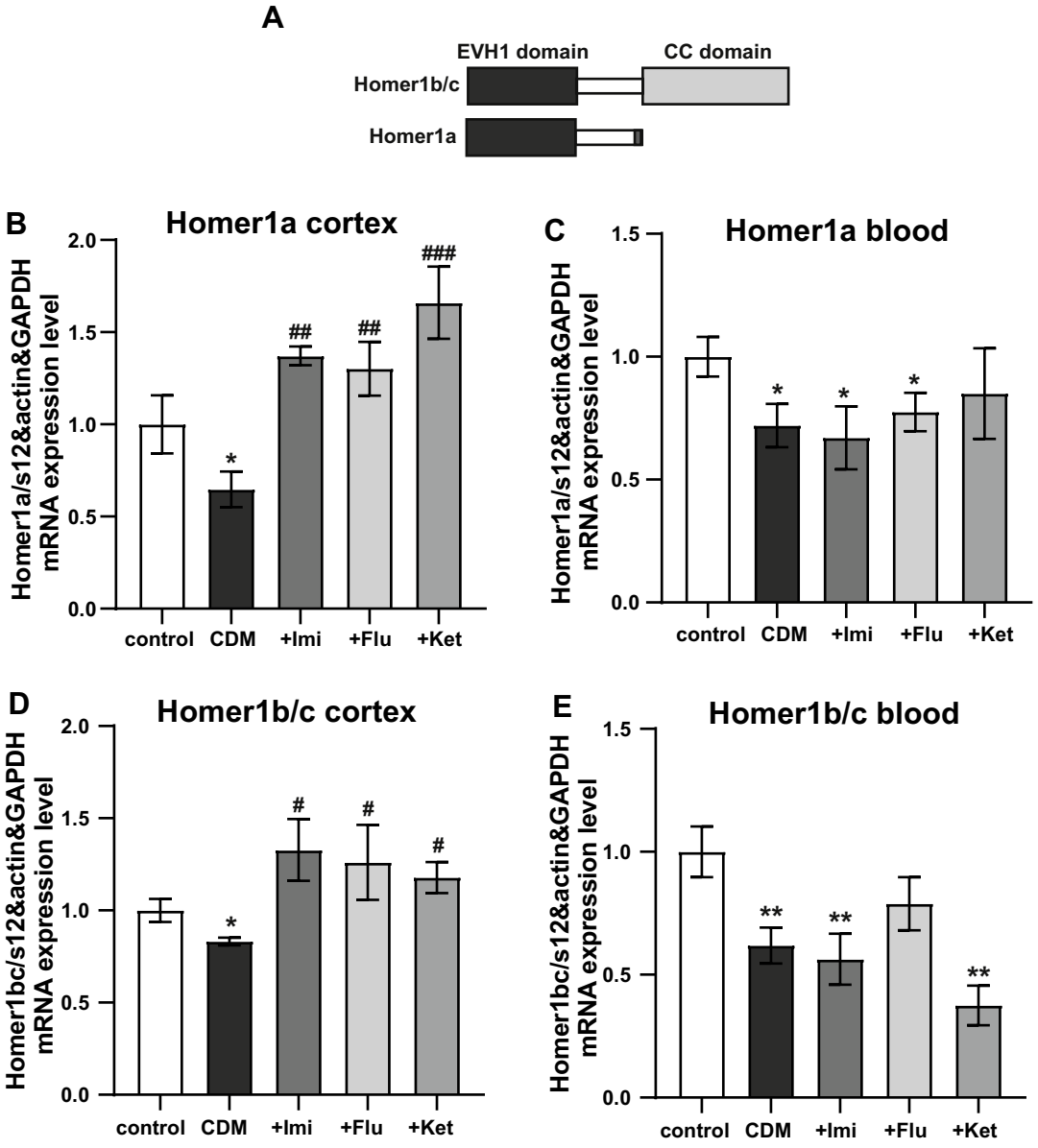

Fig. 3. Homer1a and Homer1b/c mRNA expression in the cortex and blood. (A) Homer1 isoforms and domain structure. Relative mRNA expression of Homer1a (B, C) and Homer1b/c (D, E) in cortex (B, D) and blood (C, E) of control (n = 15), mice subjected to the chronic despair (CDM) paradigm (n = 15) and chronically treated with 15mg/kg imipramine (n = 5) and 15mg/kg fluoxetine (n = 5) for 4 weeks or acutely injected with ketamine (n = 5) 1h prior killing. One-way ANOVA followed by Bonferroni post hoc test: *p < 0.05, **p < 0.01 in comparison to control; #p < 0.05, ##p < 0.01, ###p < 0.001 in comparison to CDM. Data are expressed as means ± SEM in all graphs.

previously shown that the induction of the short Homer1a isoform is a common mechanism of action of several different antidepressant treatments (Holz et al., 2019; Serchov et al., 2015; Serchov et al., 2016). In humans, SNPs located in the regulatory region of *Homer1* gene have been associated with a risk for MDD and suicide attempts (Rao et al., 2017; Rietschel et al., 2010; Strauss et al., 2012). In animal models, general deletion of Homer1 enhances depression-like behaviour (Szumlinski et al., 2006), while the expression of the short Homer1a isoform is upregulated by various antidepressant treatments (Conti et al., 2007; Kato, 2009; Serchov et al., 2015; Sun et al., 2011). Similarly, we show here that Homer1a expression is low in the CDM mice and selectively upregulated only in the cortex by the chronic treatment with monoaminergic based antidepressants. However, the expression of the long splice variant Homer1b/c, has not been previously reported to be affected in the chronic stress model nor the antidepressant drugs treatment (Serchov et al., 2015). The inconsistency across these reports might be explained by the higher number of mice used in the current study.

DNA methylation, as one of the most prominent forms of epigenetic variability, is involved in regulating the transcription and function of selected genes in the adult mammalian nervous system (Moore et al., 2013; Nelson et al., 2008) and also been associated with human depression (Dalton et al., 2014). The gene expression was typically suppressed by 5mC DNA methylation at the promoter thus preventing transcription (Bustamante et al., 2016; Gutierrez et al., 2019; Hervouet et al., 2009). Moreover, the methylation of sites in certain genes has been shown to be a predictive marker of antidepressant response (Okada et al., 2014). Indeed, our data demonstrates that the methylation level of 7 CpG sites located inside and around the two binding sites for cAMP-response element binding protein (CREB) correlates with the depression-like behavior. Several reports have shown that Homer1a expression is regulated by CREB (Mahan et al., 2012; Sala et al., 2003; Sato et al., 2020). Moreover, numerous studies have reported that the under-functioning of CREB is associated with major depressive disorder (Belmaker and Agam, 2008; Saavedra et al., 2016) and CREB protein is one of the most studied transcription factors implicated in

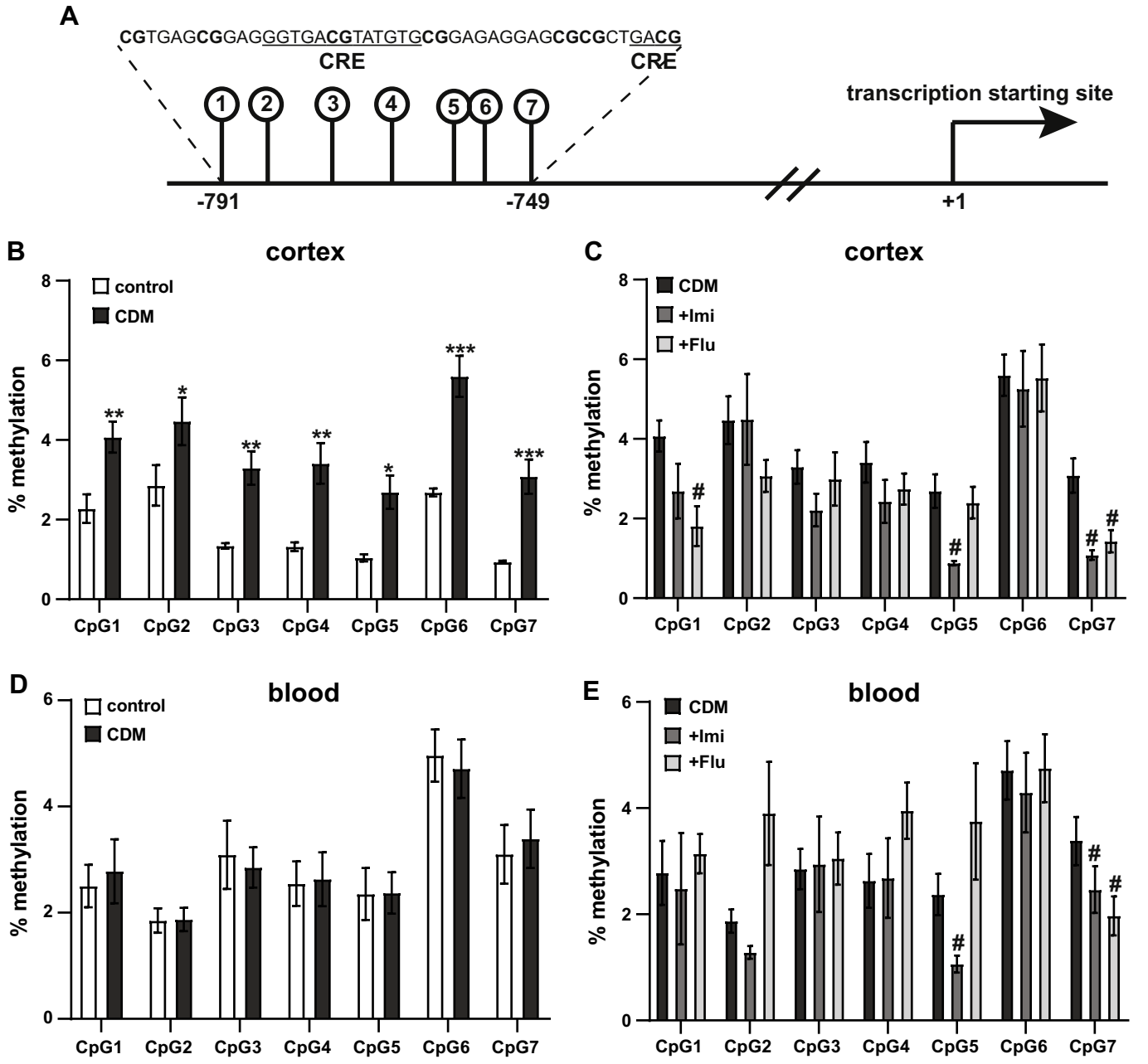

Fig. 4. DNA methylation analyses of Homer1a promoter. (A) The analyzed sequence containing 7 CpG sites covering the mouse Homer1 promoter region: CRE – CRE binding sites; (B,C) Quantification of the DNA methylation levels of Homer1 promoter in the cortex among different groups; (D,E) Quantification of the DNA methylation levels of Homer1 promoter in the peripheral blood among different groups; control, n = 15; CDM, n = 15; + Imi, n = 5; + Flu, n = 5; Two-way ANOVA followed by Bonferroni post hoc test analyses: *p < 0.05, **p < 0.01 in comparison to control, #p < 0.05 in comparison to CDM. Data are presented as mean ± SEM in all graphs.

depression (Jiang et al., 2017). Here, this is the first study that indicates that *Homer1* promoter methylation is associated with the depression-like behavior. Particularly we show that fluoxetine and imipramine treatments decrease DNA methylation of two CpG sites at *Homer1* promoter localized on or closely to the CRE binding sites, suggesting that the antidepressant treatment might be regulated by epigenetic alterations of *Homer1* promoter.

5. Limitations

Many studies have examined epigenetic biomarkers for MDD, however convenient biological diagnostic tests for facilitating MDD diagnosis are lacking (Cui et al., 2016; Nemoda et al., 2015; Numata et al., 2015). In this study, we try to investigate the diagnostic potential of *Homer1* as a peripheral biomarker for depression and a predictor of an antidepressant treatment response. Here, we have analyzed an important, but rather short, regulatory region of the *Homer1* promoter and we were unable to identify changes in the DNA methylation after chronic stress in blood. DNA methylation specifically varies depending on tissue types or cell types (Reik, 2007). However, we show that the antidepressant drugs treatment decreased *Homer1* promoter methylation in the blood in a similar manner like in the brain cortex. In blood leukocytes DNA methylation of specific genes is associated with individual variations in therapeutic response to the SSRI paroxetine (Takeuchi et al., 2017). Thus further studies examining the potential role of the DNA methylation in distinct blood cell types or other regions of the *Homer1* promoter will extend our understanding of the mechanism of antidepressant drugs therapy.

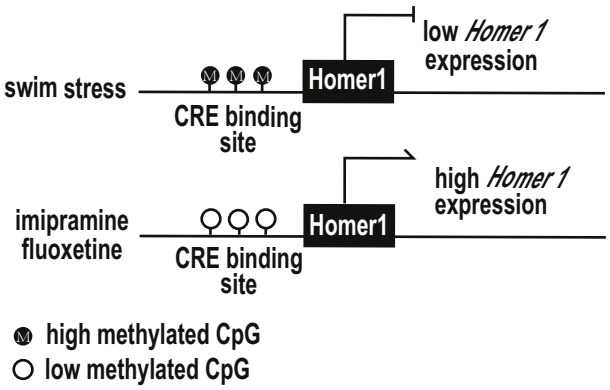

Fig. 5. Model. The repeated swim-stress in CDM paradigm increases the DNA methylation of the CpG site located on and around CRE sites of *Homer1* promoter, which in turn inhibits the *Homer1* gene expression and correlates with increased depression-like behavior. In contrast, antidepressant drugs (imipramine and fluoxetine) treatment, decrease the methylation of the *Homer1* promoter, which leads to increased *Homer1* gene expression and antidepressant response.

DNA hydroxymethylation (5hmC) is another covalent modification that is highly prevalent in the mammalian brain (10-20% of total 5mC levels) and has distinctive interactions with transcription factors (Croft et al., 2010). Previous studies have shown that 5hmC is associated with DNA demethylation and regulates gene expression (Li et al., 2017). Indeed, hippocampal overexpression of the neuronal-activity regulated enzyme Tet methylcytosine dioxygenase 1 leads to conversion of 5mC into 5hmC, and modulates the expression of several memory-associated synaptic genes, including Homer1a (Kaas et al., 2013). However, our experimental approach using bisulfite sequencing can not discriminate between methylated and hydroxymethilated cytosine (Huang et al., 2010).

6. Conclusions

In conclusion, we have studied the epigenetic regulation of *Homer1* as a potential mechanism and marker in the antidepressant treatments. Several reports suggested that Homer1a has a general importance for antidepressant therapy (Holz et al., 2019; Serchov et al., 2015; Serchov et al., 2019). We show here decreased levels of Homer1 mRNA expression, associated with a higher DNA methylation in the promoter region, in the cortex of the swim stress-induced chronic depression mouse model. Moreover, we demonstrated that monoaminergic antidepressant drugs can downregulate the DNA methylation on the *Homer1* promoter site containing CRE transcriptional binding sites, which may relate to the increased levels of Homer1 mRNA expression (Fig 5). Thus, our data point out that the antidepressant effect of fluoxetine and imipramine in chronically despaired mice is associated with the epigenetic modification in *Homer1* promoter. Understanding the mechanism of action of the antidepressant treatments could lead to an improved, personalized and target-specific therapy. However, further research is needed to determine which enzymes are involved in the epigenetic regulation, as well as more CpG sites should be analyzed to extend our knowledge of the DNA methylation/hydroxymethylation-mediated mechanism in antidepressant response.

Author contribution statement

Conceptualization, T.S., K.B.; Methodology, T.S., T.P.; Investigation, L.S., R.N.V.-S., T.S.; Formal Analysis, L.S., T.P., T.S.; Visualization, L.S., T.P., T.S.; Writing-Original draft, L.S., T.S.; Writing-Review and Editing, L.S., T.P., T.S.; Funding Acquisition, T.S.; Supervision, T.S., T.P., K.B.; All authors contributed to and approved the final manuscript.

Declaration of Competing Interest

The authors declare no competing financial interests.

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