Electrical Stimulation Normalizes c-Fos Expression in the Deep Cerebellar Nuclei of Depressive-like Rats

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ORIGINAL PAPER

Electrical Stimulation Normalizes c-Fos Expression in the Deep Cerebellar Nuclei of Depressive-like Rats: Implication of Antidepressant Activity

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Abstract The electrical stimulation of specific brain targets has been shown to induce striking antidepressant effects. Despite that recent data have indicated that cerebellum is involved in emotional regulation, the mechanisms by which stimulation improved mood-related behaviors in the cerebellum remained largely obscure. Here, we investigated the stimulation effects of the ventromedial prefrontal cortex (vmPFC), nucleus accumbens (NAc), and lateral habenular nucleus on the c-Fos neuronal activity in various deep cerebellar and vestibular nuclei using the unpredictable chronic mild stress (CMS) animal model of depression. Our results showed that stressed animals had increased number of c-Fos cells in the cerebellar dentate and fastigial nuclei, as well as in the spinal vestibular nucleus. To examine the stimulation effects, we found that vmPFC stimulation significantly decreased the c-Fos activity within the cerebellar fastigial nucleus as compared to the CMS sham. Similarly, there was also a reduction of c-Fos expression in the magnocellular part of the medial vestibular nucleus in vmPFC- and NAc core-stimulated animals when compared to the CMS sham. Correlational analyses showed that the anxiety measure of home-cage emergence escape latency was positively correlated with the c-Fos neuronal activity of the cerebellar fastigial and magnocellular and parvicellular parts of the interposed nuclei in CMS vmPFC-stimulated animals. Interestingly, there was a strong correlation among activation in these cerebellar nuclei, indicating that the antidepressant-like behaviors were possibly mediated by the vmPFC stimulation-induced remodeling within the forebrain-cerebellar neurocircuitry.

Keywords High-frequency stimulation · Ventromedial prefrontal cortex · Deep cerebellar nuclei · Vestibular nuclei · Antidepressant-like behaviors

Highlights • CMS increased c-Fos expression in the Dent Fast and SpVe.
• vmPFC HFS decreased c-Fos expression in the Fast and MVeMC.
• In CMS, HCET anxiety is correlated with the Fast, IntMC, and IntPC after vmPFC HFS.
• In CMS, the Fast is correlated with the IntMC and IntPC after vmPFC HFS.
• vmPFC HFS induced antidepressant via forebrain-cerebellar neurocircuitry remodeling.

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Abbreviations

CMS	Chronic mild stress
Dent	Dentate nucleus of the cerebellum
Fast	Fastigial cerebellar nucleus
HCET	Home-cage emergence test
HFS	High-frequency stimulation

IntMC	Interposed cerebellar nucleus, magnocellular part
IntPC	Interposed cerebellar nucleus, parvicellular part
LHb	Lateral habenular nucleus
MVePC	Medial vestibular nucleus, parvicellular part
MVeMC	Medial vestibular nucleus, magnocellular part
NAc core	Nucleus accumbens core
NAc shell	Nucleus accumbens shell
SpVe	Spinal vestibular nucleus
vmPFC	Ventromedial prefrontal cortex

Introduction

Currently, electrical deep brain stimulation (DBS) is an emerging clinical treatment option for debilitating neurological and psychiatric diseases. In mood-related disorder, it has become an alternative therapeutic approach especially for treatment-resistant depression [1–3]. Converging evidence from experimental and clinical findings have shown that depression is a multisystem disorder affecting several integrated pathways including the cortical, subcortical, and limbic regions [4, 5]. For example, DBS has been shown to influence this network system by modulating other brain regions which causes various biochemical changes in the normalization of its pathological mood state [2, 5, 6].

In clinical and experimental animal models, the application of DBS in different brain regions has been reported to alleviate mood and anxiety-related symptoms [5, 7–9]. At present, studies in patients with treatment-resistant depression have shown promising results when stimulating electrode were implanted within the subcallosal cingulate gyrus or the ventromedial prefrontal cortex (vmPFC) [4, 10, 11], the inferior thalamic peduncle [12], the nucleus accumbens (NAc) [13–15], the anterior limb of the internal capsule [16, 17], the lateral habenula (LHb) [18], and the medial forebrain bundle [19]. Recently, we investigated the effects of electrical stimulation in different brain regions using the unpredictable chronic mild stress (CMS) paradigm as an animal model of depression [5]. The latter study has shown that high-frequency stimulation (HFS) of the NAc core, LHb, and of particular interest, the vmPFC produced significant antidepressant effects [5]. In addition, a wide range of brain regions has been reported to be specifically modulated by the HFS of the vmPFC, including the cortical, limbic structures, and the deep cerebellar nuclei [20].

The cerebellum is a brain region that functions mainly outside of the sphere of consciousness. Besides motor coordination, there is an accumulating body of evidence that supports the involvement of cerebellum in learning and emotional regulation [21, 22]. In addition, experimental animal and patient studies with cerebellar damage or lesioning have confirmed the roles of cerebellum in a number of cognitive tasks, such as language, working memory, and executive tasks and affective-related processes, for instance, the regulation of sadness, panic, stress, pain, empathy, and impulsive behavior [23–25]. Besides, Schmahman and Sherman also found that personality changes of either flattening of emotion or disinhibited and inappropriate behavior were commonly diagnosed in patients with lesions involving the cerebellum especially the anterior and posterior lobes of the cerebellum, as well as the vermal cerebellar pathology [26]. Indeed, converging lines of evidence from many studies have also demonstrated that the cerebellum is involved in the pathology of depression. Of particular interest, the reduction of cerebellar gray matter was seemingly observed in major depressive disorder [27] and functional MRI also detected abnormalities on the cerebellar activity of bipolar disorder, major depression, or geriatric depression patients [28–30]. In line with these studies, the depression scores were significantly increased in patients with cerebellar stroke [31] and cerebellar mass lesions [32]. Interestingly, some antidepressant medications have been shown to regulate the cerebellar function during improvement of affective symptoms and cognitive function [33] and normalization of the white matter volume reduction in patients with depression [34]. Furthermore, it is important to note that the patterns of cerebellum regulation can be considered also for the treatment comparison of antidepressant outcome effects [34].

In this study, we investigated the effects of electrical stimulation in different brain targets on the neuronal activity of the deep cerebellar and vestibular nuclei using immunohistochemical detection of c-Fos expression, a transcription factor expressed in response to both the intracellular and extracellular stimuli [35–37]. This neuroanatomical mapping method is widely used to identify the functional connectivity after an induction of electrical stimulation or behavioral responses [38–40]. Since pathological alterations in the cerebellum have been described in patients with mood-related problems, we hypothesized that effects of electrical stimulation on antidepressant response would induce a network remodeling of the dysfunctional neural circuitry within the deep cerebellar and vestibular nuclei.

Materials and Methods

Design of the Study

We aimed to investigate the effects of different stimulation brain targets on the neurocircuitry activity within the deep cerebellar and vestibular nuclei. The structure samples of the cerebelli and vestibular nuclei were obtained from previous experimental works [5]. In this study, using c-Fos immunohistochemistry, we compared the neuronal activation pattern of the deep cerebellar and vestibular nuclei in animals with

exposure to either stressed or non-stressed condition. In each condition, animals received electrode implantation within the vmPFC, NAc core, NAc shell, and LHb. The sham animals were also implanted with electrodes but without the deliverance of electrical stimulus (Supplementary Table 1a).

Subjects

Sprague Dawley male rats ($n = 62$; Charles River, Sulzfeld, Germany) were kept in standard cages with control of room temperature (20–22 °C), humidity (60–70 %), and 12-h reversed dark or light cycle. The animals were having continuous ad libitum access to food and water. All experimental procedures were conducted in accordance to the guidelines of the institutional animal care provided by the Animal Experiments and Ethics Committee of Maastricht University.

Electrode Implantation and Stimulation Procedures

The details of stereotactic surgery for electrode implantation procedure have been previously described [41, 42]. In brief, stimulating electrodes were implanted in the vmPFC (AP +2.70 mm; L ±0.60 mm; V −4.60 mm), NAc core (AP + 2.20 mm; L: ±1.50 mm; V −6.80 mm), NAc shell (AP +1.70 mm; L ±0.60 mm; V −7.20 mm), and LHb (AP −3.80 mm; L ±0.60 mm; V −5.00 mm) based on the rat brain atlas of Paxinos and Watson [43]. After surgery, animals were given a recovery period of 2 weeks.

For electrical stimulation, a digital stimulator (DS8000, World Precision Instruments or WPI, Berlin, Germany) and stimulus isolators (DLS100, WPI) were used to deliver the stimuli. The stimulation parameters of 100 μA (amplitude), 100 hz (frequency), and 100 μs (pulse width) were utilized based on previous experiments [5, 7].

The Unpredictable Chronic Mild Stress Model

The stressed animals were initially exposed to 3 weeks of chronic unpredictable stress paradigm, while the non-stressed controls were gently handled once daily. The CMS and the control non-CMS animals were separately placed in different rooms. The stress procedures were continued throughout the experimental period and details of the paradigm were described in a previous study [5]. In brief, the CMS protocol comprised sessions of intermittent illumination (on/off every 2 h), placement in mouse cage, regular flashes of light (stroboscopic lamp; frequency at 2.5 Hz), wet bedding with 300 ml of cold water, paired-housing in uncleaned cages, food and water deprivation, and without stress condition. The duration of each stressor ranged from 10 to 14 h. The order of the CMS stressors was randomized and it usually took place in the morning and followed by another in the evening.

Histological Processing

After the final experiment, animals were stimulated for 1 h and returned to their home-cage for 1 h before sacrifice. All rats were anesthetized with Nembutal (75 mg/kg), and then perfused intracardially with saline and fixative solution containing 4 % paraformaldehyde. Subsequently, all brains were removed, cryoprotected in sucrose solution, and stored in a −80 °C freezer prior to cryosectioning. Sections in coronal plane (10 μm) were obtained in a cryostat at −28 °C and mounted onto the SuperFrost/Plus slides (Menzek-Gläser, Braunschweig, Germany) and then stored again in the −80 °C freezer until further immunohistochemical staining. Histological study of the electrode tip localization was performed using the standardized hematoxylin–eosin (Merck, Darmstadt, Germany) method.

Immunohistochemistry

c-Fos immunohistochemical staining was performed on the microscopic slides using previously established methods with minor modifications [25, 44]. The coronal sections of cerebelli were obtained from Bregma −11.00 to −11.80 mm based on the rat brain atlas of Paxinos and Watson [43]. The cerebellar sections were incubated in the primary polyclonal rabbit anti-c-Fos antibody (sc-52; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at the concentration of 1:300 in room temperature for 48 h. Then, the sections were incubated in the secondary biotinylated donkey anti-rabbit antibody (Jackson Immunoresearch Laboratories Inc., Westgrove, PA, USA) at the concentration of 1:400 in room temperature for 24 h, followed by 3 h of incubation of avidin–biotin–peroxidase complex (Elite ABC-kit, Vectastatin; Vector, Burlingame, CA, USA). Finally, the immune complex reaction of horseradish peroxidase was visualized by 10 min of incubation of 3, 3′-diaminobenzidine tetrahydrochloride/nickel chloride solution. After staining, all sections were subsequently dehydrated and cover-slipped using Vectamount™ mounting medium (Vector Laboratories Inc., Burlingame, CA, USA). In the negative-control sections, no staining was detected when the primary antibody was omitted.

Image Acquisition and Analysis

The images were obtained using a BX-41 Olympus microscope that was connected to a DP-70 Olympus digital camera with objective of ×10 magnification power. The quantification of c-Fos immunopositive nuclei was performed using the ImageJ software (http://rsb.info.nih.gov/ij/) as previously described [23, 25]. The regions of interest from the deep

cerebellar and vestibular nuclei, which included the dentate nucleus of the cerebellum (Dent); the interposed cerebellar nucleus, magnocellular part (IntMC); the interposed cerebellar nucleus, parvicellular part (IntPC); the fastigial cerebellar nucleus (Fast); the medial vestibular nucleus, parvicellular part (MVePC); the medial vestibular nucleus, magnocellular part (MVeMC); and the spinal vestibular nucleus or inferior vestibular nucleus (SpVe), were delineated (Fig. 1). The artifacts and control of background staining were taken into consideration in order to avoid false-positive findings. The optimal threshold for Gray value and particle size were determined for each area and remained the same for all sections. Quantification was conducted in six different sections per animal according to the rat brain atlas of Paxinos and Watson [43].

Statistical Analyses

The results were analyzed using the IBM SPSS Statistics 23 and the data were presented in boxplots with interquartile ranges. A multivariate analysis with general linear model and ANOVA with Bonferroni post hoc test were performed for detailed multiple comparisons. Independent sample t test was used to compare differences between the variables of the CMS and non-CMS groups. All p values <0.05 were considered significant. In addition, Pearson correlation coefficients were performed to study the association of antidepressant-related behaviors (forced swim test, home-cage emergence test, food intake test, sucrose intake test, and open-field test) and c-Fos activities within the cerebellar and vestibular nuclei. Bonferroni correction was calculated and the p value was adjusted for multiple variable comparisons.

Results

Electrode Localization and Behavior

The localization of electrode tips and the behavioral effects of antidepressant after electrical stimulation in different brain regions (vmPFC, NAc core, NAc shell, and LHb) have been previously reported [5]. The animals with misplacement of electrodes were discarded from the data analysis. A total of 62 rats were used and the data were derived from the average cell count of c-Fos positive nuclei per mm^2 with respect to the deep cerebellar and vestibular nuclei (see Table 1; and Supplementary Table 1a).

Stimulation-Induced Changes of c-Fos Expression in Cerebellar–Vestibular Nuclei

Electrical stimulation in specific brain structure has been previously shown to modulate the expression of c-Fos immediate-early gene within the deep cerebellar nuclei [23, 25]. Here, we further investigated the stimulation effects of the vmPFC, NAc core, NAc shell, and LHb on the c-Fos induction in various deep cerebellar and vestibular nuclei of non-CMS and CMS animal models of depression. Since no significant differences were observed in the c-Fos cell count between the ipsilateral and contralateral regions of the cerebellum (all p-values >0.05), the results were pooled from the right and left hemispheres to create an overall estimation. The multivariate general linear model analysis revealed significant main effects for animals with either non-CMS or CMS condition ($F = 4.609$, $p = 0.002$), stimulation target group ($F = 1.498$, $p = 0.070$; marginal difference), and the interaction ($F = 1.570$, $p = 0.050$). In the CMS group, one-way ANOVA revealed significant effects in the Fast ($F_{(4,\ 24)} = 3.668$,

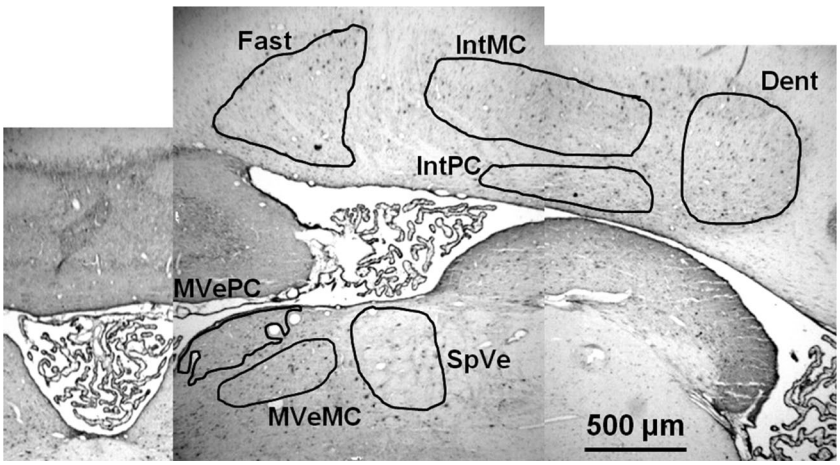

Fig. 1 Representative microphotograph showing the digitally superimposed images of the deep cerebellar and vestibular nuclei at Bregma level −11.6 mm. The delineation of the regions of interest was conducted from Bregma −11.0 to −11.8 mm. Abbreviations: *Dent* dentate nucleus of the cerebellum, *IntMC* interposed cerebellar nucleus, magnocellular part, *IntPC* interposed cerebellar nucleus, parvicellular part, *Fast* fastigial cerebellar nucleus, *MVeMC* medial vestibular nucleus, magnocellular part, *MVePC* medial vestibular nucleus, parvicellular part, *SpVe* spinal vestibular nucleus. Scale bar: 500 μm

Table 1 Comparison between the non-CMS and CMS animals with respect to the average cell count of c-Fos-positive nuclei per mm^2 within the cerebellar (Dent, IntMC, IntPC, and Fast) and vestibular (MVeMC, MVePC, and SpVe) nuclei of animals receiving either vmPFC HFS, NAc core HFS, NAc shell HFS, LHb HFS, or sham implantation

		Sham Mean ± S.E.M.	vmPFC HFS Mean ± S.E.M.	NAc core HFS Mean ± S.E.M.	NAc shell HFS Mean ± S.E.M.	LHb HFS Mean ± S.E.M.
Non-CMS	Dent	73.03 ± 9.38	102.23 ± 29.24	67.17 ± 3.74	61.20 ± 13.89	89.38 ± 6.55
	IntMC	88.31 ± 3.40	66.99 ± 13.36	80.09 ± 8.86	78.73 ± 13.24	92.38 ± 16.27
	IntPC	112.25 ± 10.73	110.56 ± 24.32	108.23 ± 11.87	102.83 ± 10.79	109.01 ± 19.00
	Fast	25.57 ± 4.56	35.26 ± 9.01	35.89 ± 0.85	29.06 ± 7.70	43.98 ± 5.06
	MVeMC	22.63 ± 4.63	17.97 ± 4.73	17.00 ± 3.09	21.22 ± 2.52	27.88 ± 3.26
	MVePC	21.88 ± 4.85	15.12 ± 3.73	13.88 ± 2.41	18.38 ± 1.41	23.63 ± 6.18
	SpVe	47.06 ± 5.61	25.82 ± 6.05	34.31 ± 7.69	67.40 ± 9.71	58.97 ± 9.95
CMS	Dent	107.68 ± 3.76	92.03 ± 13.79	91.43 ± 10.03	95.44 ± 11.91	80.69 ± 4.68
	IntMC	118.03 ± 12.95	81.56 ± 6.05	95.04 ± 6.72	110.36 ± 3.46	91.16 ± 6.43
	IntPC	114.09 ± 7.08	87.15 ± 12.32	117.67 ± 15.48	97.41 ± 3.44	110.95 ± 10.04
	Fast	59.85 ± 6.66	33.27 ± 5.11	42.50 ± 6.60	36.90 ± 3.10	49.24 ± 4.18
	MVeMC	34.76 ± 2.05	21.53 ± 2.70	20.75 ± 2.09	30.02 ± 4.36	23.03 ± 2.69
	MVePC	26.00 ± 4.65	17.78 ± 2.09	18.83 ± 2.62	19.70 ± 3.64	19.27 ± 0.89
	SpVe	68.80 ± 4.00	60.85 ± 7.95	65.38 ± 0.93	76.04 ± 8.62	60.97 ± 2.02
		Significant effects ($p < 0.05$)				
Comparison between animals of non-CMS and CMS	Dent	$t_{14} = 3.092$, $p = 0.008$.	$t_{10} = -0.315$, p = n.s.	$t_{10} = 2.267$, $p = 0.047$.	$t_9 = 1.827$, p = n.s.	$t_7 = -1.110$, p = n.s.
	IntMC	$t_{11} = 2.061$, $p = 0.064$.	$t_9 = 0.926$, p = n.s.	$t_{10} = 1.345$, p = n.s.	$t_8 = 1.885$, p = n.s.	$t_9 = -0.075$, p = n.s.
	IntPC	$t_{13} = 0.127$, p = n.s.	$t_9 = -0.804$, p = n.s.	$t_{10} = 0.484$, p = n.s.	$t_8 = -0.394$, p = n.s.	$t_9 = 0.095$, p = n.s.
	Fast	$t_{13} = 4.340$, $p = 0.001$.	$t_9 = -0.180$, p = n.s.	$t_9 = 0.901$, p = n.s.	$t_9 = 0.875$, p = n.s.	$t_9 = 0.810$, p = n.s.
	MVeMC	$t_{12} = 2.139$, $p = 0.054$.	$t_9 = 0.618$, p = n.s.	$t_9 = 0.962$, p = n.s.	$t_8 = 1.748$, p = n.s.	$t_8 = -1.145$, p = n.s.
	MVePC	$t_{13} = 0.609$, p = n.s.	$t_9 = 0.587$, p = n.s.	$t_{10} = 1.392$, p = n.s.	$t_8 = 0.339$, p = n.s.	$t_6 = -0.699$, p = n.s.
	SpVe	$t_{14} = 2.979$, $p = 0.010$.	$t_9 = 3.386$, $p = 0.008$.	$t_9 = 3.638$, $p = 0.005$.	$t_9 = 0.652$, p = n.s.	$t_7 = 0.221$, p = n.s.

The structure samples of the cerebelli and vestibular nuclei were obtained from previous experimental works [5]. The data were analyzed by independent sample *t* test and are presented in mean ± standard error of mean (S.E.M). All *p* values <0.05 were considered statistically significant. Source: Lim LW, Prickaerts J, Huguet G, Kadar E, Hartung H, Sharp T and Temel Y. Electrical stimulation alleviates depressive-like behaviors of rats: investigation of brain targets and potential mechanisms. Transl Psychiatry 2015: 5:e535. doi 10.1038/tp.2015.24

$p = 0.018$) and MVeMC ($F_{(4, 22)} = 4.780$, $p = 0.006$) and marginally different in the IntMC ($F_{(4, 23)} = 2.746$, $p = 0.053$). However, no remarkable differences were found in the Dent, IntPC, MVePC, and SpVe (all $F_{(4, 22–24)} < 1.167$, p = n.s.) (Figs. 2 and 3; Supplementary Table 1b). Interestingly, the Bonferroni post hoc tests demonstrated that the HFS of the vmPFC decreased c-Fos expression in the Fast ($p = 0.027$) and MVeMc ($p = 0.032$) and a marginal reduction in the IntMC ($p = 0.078$) as compared to the sham (Fig. 4). Likewise, the HFS of the NAc core also decreased the c-Fos expression level within the MVeMC ($p = 0.020$), but not the other cerebellar and vestibular nuclei.

In the non-CMS group, we detected no significant effects in the Dent, Fast, IntMC, IntPC, MVeMC, and MVePC (all $F_{(4, 24–27)} < 1.280$, p = n.s.), except for the SpVe ($F_{(4, 25)} = 4.465$, $p = 0.007$) (Supplementary Table 1b). The Bonferroni post hoc test analysis showed no statistical differences when compared to the sham.

For comparison between the sham non-CMS and CMS animals, Student's *t* tests showed that CMS sham animals significantly increased the number of c-Fos-positive cells in the Dent ($t_{14} = 3.092$, $p = 0.008$), Fast ($t_{13} = 4.340$, $p = 0.001$), and SpVe ($t_{13} = 2.979$, $p = 0.010$); a tendency of increase was also found in the IntMC ($t_{11} = 2.061$, $p = 0.064$) and MVeMC ($t_{12} = 2.139$, $p = 0.054$). No significant differences were found in the IntPC ($t_{13} = 0.127$, p = n.s.) and MVePC ($t_{13} = 0.609$, p = n.s.) (Table 1). For additional comparison of results between individual stimulated-groups and the sham animals, see Supplementary Table 1c.

Correlation Analyses of Behavior and c-Fos Expression

The correlations of the stimulation-induced antidepressant effects and the c-Fos expression within the cerebellar and vestibular nuclei are presented in Fig. 5 and Supplementary Table 2a–e. For correlational analyses, there was a significant positive correlation of the Fast with the IntMC ($R^2 = 0.985$, $p = 0.0001$) and IntPC ($R^2 = 0.962$, $p = 0.001$) after HFS of the vmPFC in the CMS group (Fig. 5; Supplementary Table 2b). Interestingly, CMS animals with vmPFC HFS showed a remarkable association between the IntMC and the IntPC ($R^2 = 0.958$, $p = 0.0001$), as well as association between the MVeMC and the MVeMC ($R^2 = 0.988$, $p = 0.0001$). To further investigate the stimulation-induced c-Fos expression on antidepressant-related behaviors, we found a significant positive correlation of the escape latency in the home-cage emergence test with c-Fos activity in the Fast ($R^2 = 0.928$, $p = 0.002$), IntMC ($R^2 = 0.967$, $p = 0.0001$), and IntPC ($R^2 = 0.919$, $p = 0.003$) (Fig. 5c–e; Supplementary Table 2b).

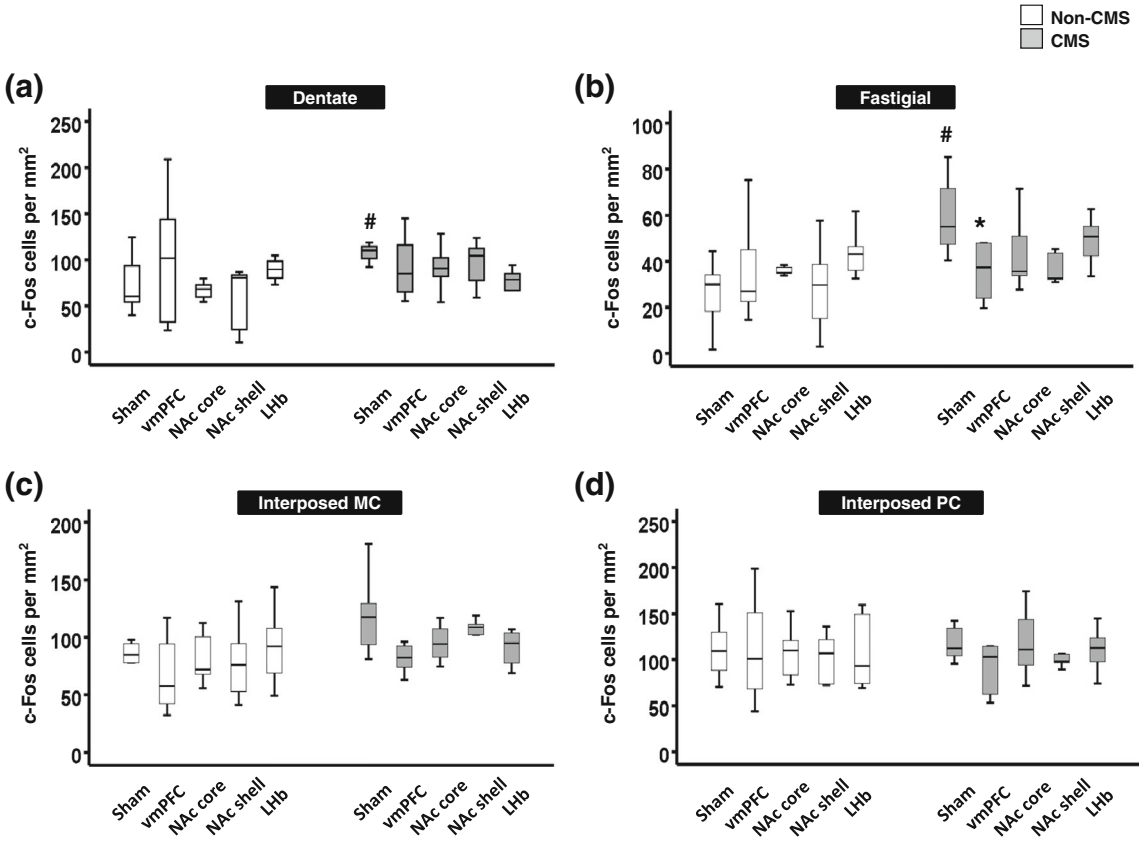

Fig. 2 The *boxplots* show the high-frequency stimulation effects of the vmPFC, NAc core, NAc shell, and LHb on c-Fos activity within the deep cerebellar nuclei of the non-CMS and CMS animal models of depression. Note: In the CMS group, sham animals had remarkable increase of c-Fos-positive cells in the dentate (**a**) and fastigial (**b**) nucleus of the cerebellum. No significant result was found in the interposed cerebellar nuclei (**c** & **d**). Interestingly, vmPFC stimulation reduced significantly the c-Fos expression in the fastigial nucleus as compared to the sham. Indication: #significant difference from the non-CMS sham; *significant difference from the CMS sham

In the non-CMS animals, after HFS of the LHb, there was a positive correlation between the IntMC and the open-field center zone ($R^2 = 0.965$, $p = 0.003$), as well as significant association between the IntPC and the escape latency in the home-cage emergence test ($R^2 = 0.960$, $p = 0.003$). In the non-CMS vmPFC HFS and CMS LHb HFS groups, no significant correlation was detected between the c-Fos activity of the cerebellar–vestibular nuclei and the antidepressant-related behaviors (Supplementary Table 2). Overall, no correlational association was found between the c-Fos expression in the cerebellar–vestibular nuclei and the antidepressant-related behaviors in the sham, NAc core HFS, and NAc shell HFS of both the CMS and non-CMS groups.

Discussion

The present findings show that the CMS paradigm induced a remarkable increase in the number of c-Fos-positive cells within the cerebellar Dent and Fast, as well as within the SpVe. Further, we have demonstrated that the HFS of the vmPFC reduced significantly the c-Fos neuronal activation within the Fast as compared to the CMS sham. Likewise, HFS of the vmPFC and NAc core also decreased the c-Fos expression levels within the MVeMC in comparison with the CMS sham. Interestingly, the activation of Fast, IntMC, and IntPC nuclei are highly correlated among each other and with the anxiety level of escape latency in the home-cage emergence test after HFS of the vmPFC in CMS animals.

In stress animal models, several studies have demonstrated the hemispheric lateralization that resulted from gender differences [45–47]. Despite the hemispherical asymmetries in the cerebellar or vestibular nuclei that have been described in patients with major and bipolar depression [28, 29, 48], the current CMS model of depression in male rats showed no major differences in c-Fos expression in the cerebellar hemispheres. This suggested a possible lack of cerebellar lateralization in mood-related behaviors of this animal model, and this finding was in line with our previous observations [23, 25, 44]. Currently, no studies of hemispheric lateralization have been reported to associate the mood-related behaviors in the cerebellum of rats. However, it would be an interesting subject to investigate the possibility of gender differences and other

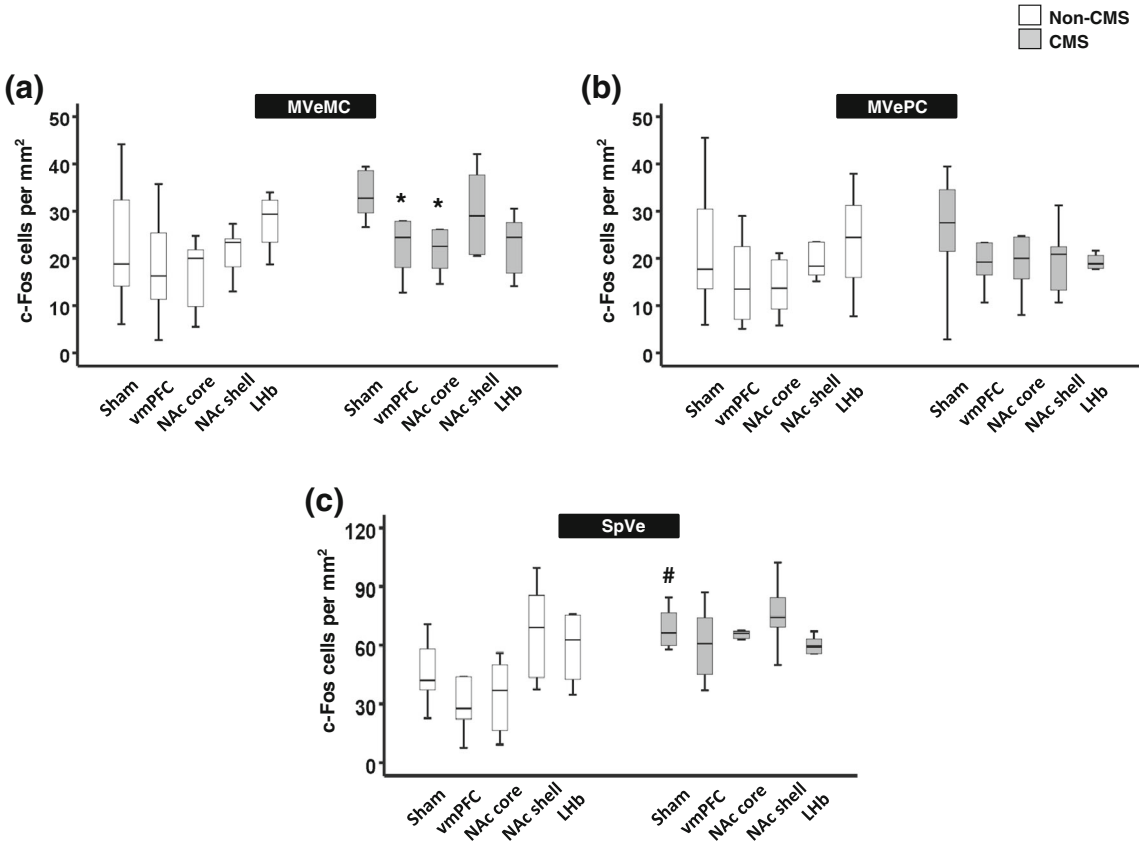

Fig. 3 The *boxplots* show the high-frequency stimulation effects of the vmPFC, NAc core, NAc shell, and LHb on c-Fos activity within the vestibular nuclei of the non-CMS and CMS animal models of depression. Note: CMS sham animals significantly increased the c-Fos-positive cells in the spinal vestibular nucleus (**c**), and a marginal increase was also found in the magnocellular part of the medial vestibular nucleus (**a**). Interestingly, vmPFC and NAc stimulation showed a remarkable reduction of c-Fos activity within the magnocellular part of the medial vestibular nucleus (**a**). No significant result was found in the parvicellular part of the medial vestibular nucleus (**b**). Indication: #significant difference from the non-CMS sham; *significant difference from the CMS sham

indicators in various brain regions in the animal models of depression for potential cerebellar lateralization.

In human imaging studies, there have been several reports demonstrating that the changes of cerebellar activities are associated with the resting frontal function in patients with social anxiety disorders [49, 50], posttraumatic stress disorder [51, 52], and generalized anxiety disorder [53]. The reciprocal connections between the deep cerebellar nuclei and the forebrain regions, namely, the limbic system and the frontal cortical areas, support the involvement of cerebellum in modulation of mood and anxiety responses. In particular, using functional connectivity magnetic resonance imaging (MRI), Allen et al. demonstrated the coherence of MR signal in the Dent to be correlated with signal in the cerebellar, thalamic, limbic, striatal, and cortical regions [54]. This study further provided a functional coherence and connectivity between the cerebellum and forebrain regions. It also supported the non-human primate findings of anatomical projections from the Dent to the prefrontal and posterior parietal cortical regions via the thalamus and the corticopontine–cerebellar connections. The corticopontine projections comprise the motor and associative cortical connections. From the cortical regions, the motor connection projects to the interpeduncular and peripeduncular nuclei and terminate in the anterior cerebellar lobe, while the associative cortical connection projects to the rostromedial pons (from prefrontal cortex); the rostrolateral pons (from parietal cortex); and the dorsolateral, lateral, and dorsal pons (from temporal and visual cortex), and their projections are terminated in the posterior cerebellar lobe which is involved in intellectual and emotional regulation [55, 56].

The Dent plays an important role in motor functions, and to a greater extent, it is also involved in processing cognitive and emotional responses [21]. In lower mammals, the Dent is connected to several associative areas in the forebrain, for instance, the vmPFC [57], and lesions within the Dent have been shown to produce cognitive deficits in the maze-learning tasks of rodents [58]. These findings were in accordance with the clinical data obtained from human studies, where Dent has expanded massively and interconnected with the cortical areas in the regulation of executive, language, mnemonic, and other cognitive functions [21, 22]. It has been demonstrated that the Dent projects to the prefrontal cortex in both the motor and non-motor cortical areas [59, 60]. Thus, stimulation of the Dent in patients produced subjective

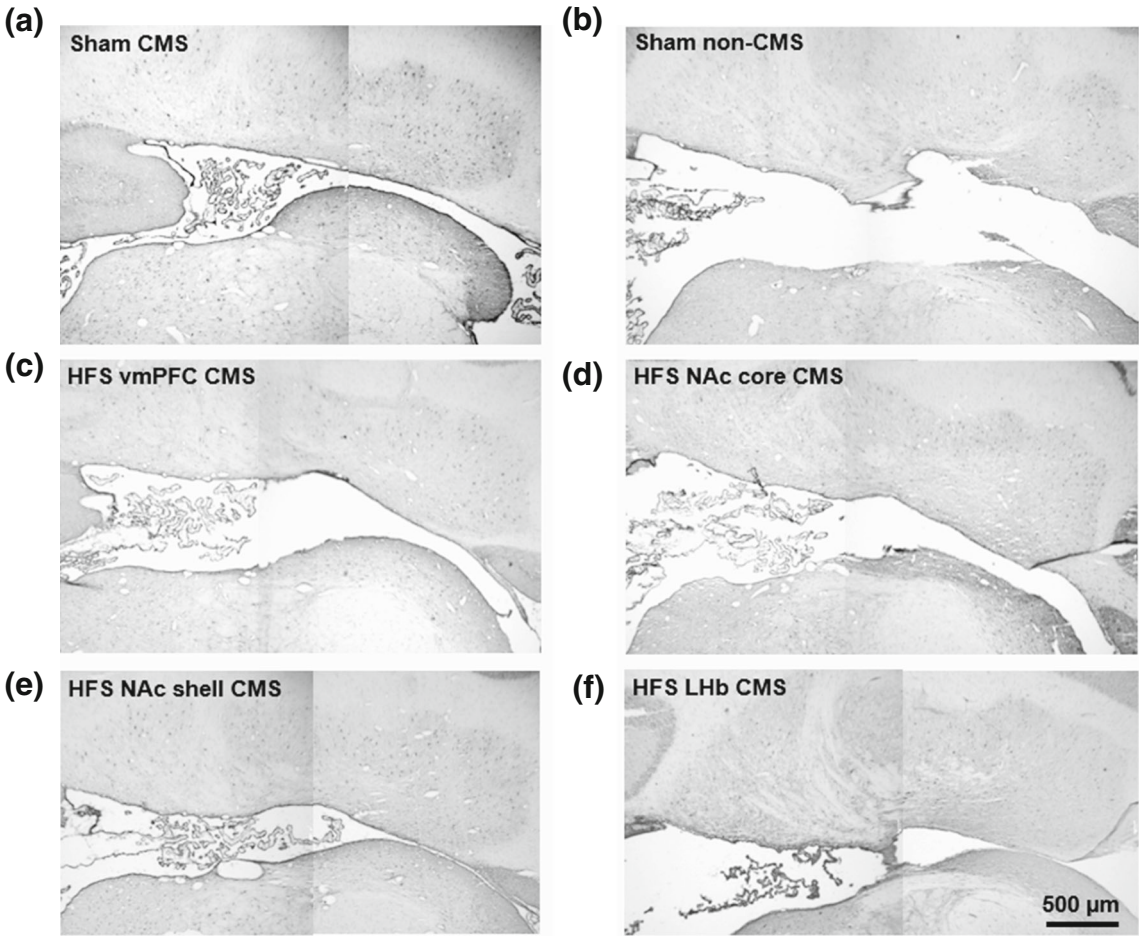

Fig. 4 Representative photomicrographs of superimposed images showing the effects of high-frequency stimulation of the vmPFC (**c**), NAc core (**d**), NAc shell (**e**), and LHb (**f**) on c-Fos neuronal activity within the deep cerebellar and vestibular nuclei (Bregma level from −11.30 to −11.60 mm) as compared to the sham CMS (**a**) and sham non-CMS (**b**) groups. Note: CMS sham animals significantly increased the number of c-Fos cells (**a**). Scale bar: 500 μm

feelings of unpleasant sensation and fear, indicating the emotional component of this cerebellar nucleus [61]. Besides, the experience of fear sensation has also been reported when the Dent and superior cerebellar peduncle of patients were stimulated [62]. In rats, lesions of the Dent have caused incredible reduction of hedonic and purposive motivational interest [63]. Interestingly, our current data show activation of the Dent in the CMS animals, indicating a possible role of this nucleus in the pathophysiology of emotional responses.

The Fast receives strong afferent projection from Purkinje cells in the vermis, forming the "limbic cerebellum" [64, 65]. This neural circuit has robust anatomical connections with the reticular formation, hypothalamus, and other forebrain limbic structures, which plays a substantial role in the arousal system, autonomic functions, and emotional behaviors [64, 65]. It has been shown that lesions occurring within the Fast and the vermis caused mood dysregulation, and it is generally known as the cerebellar cognitive affective syndrome or the "Schmahmann syndrome" [26]. Likewise, the increase of activity within these areas was correlated with worsening of mood in humans, especially during the premenstrual dysphoric disorder [66].

In the present study of stressed animals, there was a significant increase of c-Fos neuronal activation within the Fast, and HFS of the vmPFC induced a remarkable reduction of its level of expression. In line with this observation of neuronal activity reduction, there was a study also demonstrating a decrease of ΔFosB in the Dent and Fast during the interictal phase of fully kindled rats for epileptic behavior [67]. Although both studies found a similar result of Fos activity reduction with respect to different experimental conditions of either antidepressant effect or epileptic interictal phase, it is important to highlight that the decrease of ΔFosB during the interictal phase can be possibly due to the neuronal network reorganization of the motor function after an episode of seizure attack. In support of this notion, previous studies have indicated that decreased neuronal activity promotes the synaptic strength of neuronal circuits [68, 69].

Nevertheless, the present finding is well-supported by the established anatomical connection of the cerebellum, and importantly, it provides strong evidence on the role of Fast, as

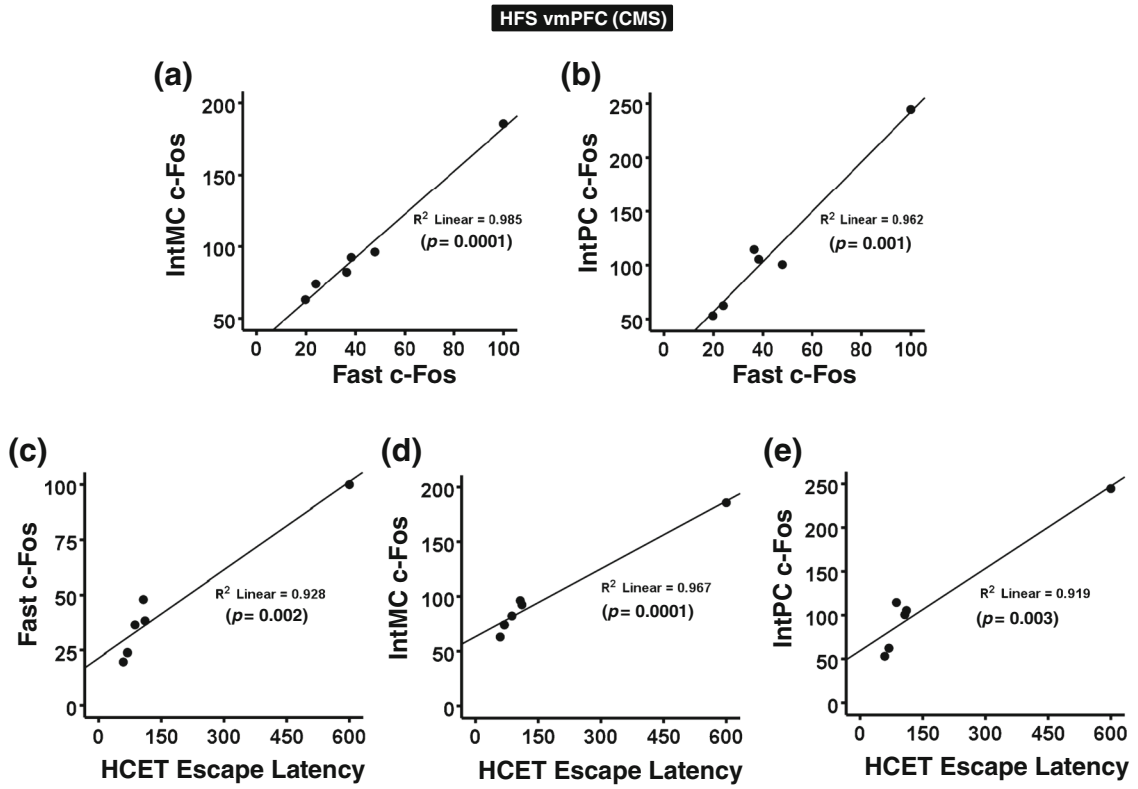

Fig. 5 *Scatter plots* show the correlational association between the deep cerebellar nuclei c-Fos expression and the antidepressant-like behavior. Note: In CMS animals, the fastigial nucleus is highly correlated with the magnocellular (**a**) and parvicellular (**b**) parts of the interposed cerebellar nucleus after high-frequency stimulation of the vmPFC. Interestingly, there is a positive correlation in the escape latency of the home-cage emergence test with the fastigial nucleus (**c**), as well as the magnocellular (**d**) and parvicellular (**e**) parts of the interposed cerebellar nucleus. Bonferroni correction was calculated and the p value (significant at $p < 0.0036$) was adjusted for multiple comparisons

part of the "limbic cerebellum" for emotional processing. Interestingly, after vmPFC HFS, we found a positive correlation between the anxiety measure of escape latency in the home-cage emergence test and the deep cerebellar nuclei, in particular of the Fast, IntMC, and IntPC in CMS animals. To further examine the intrinsic relationship of these nuclei, our data revealed that a strong correlation was found among the Fast, IntMC, and IntPC in CMS animals after vmPFC HFS. In anatomical and functional connection, the forebrain–cerebellum relationship was previously established using the anatomical retrograde transneuronal transport of rabies virus [70] and classical eye blink conditioning experiments [71, 72]. Taken together, these observations further support the interactions between the prefrontal cortex and the roles of cerebellum in emotion and cognition.

Since vestibular nuclei have extensive afferent and efferent projections with the deep cerebellar nuclei, we investigated the stimulation effects of vmPFC, NAc, and LHb on these nuclei in both the CMS and non-CMS conditions. The vestibular nuclei received robust cerebellar inputs from the fastigial nuclei, and they were also involved in the control of emotional behaviors and vestibulocerebellum functions [73]. In clinical investigation, there were reports demonstrating that patients with vestibular impairment have high incidence of mood and anxiety-related disorders [74–76], as well as cognitive dysfunction [77]. In the present CMS model, there was a statistical increase of c-Fos expression found in the SpVe, while the MVeMC showed a marginal increase, when compared to the control non-CMS group. Interestingly, after HFS of the vmPFC and NAc core, we showed a reversal of the increased c-Fos neuronal expression in the MVeMC, but not the SpVe. Although we found no direct correlation between the c-Fos expression of the vestibular nuclei and the antidepressant-like behaviors, there was a possibility that the behaviors were modulated by the other brain regions. One of the possible explanations could be due to the non-cerebellar inputs of serotonergic and non-serotonergic pathways from the dorsal raphe nuclei [78, 79], as well as the polysynaptic pathways of hippocampal formation and amygdaloidal connection [80–82] to the vestibular nuclei. In support of this explanation, we have previously demonstrated that HFS of the vmPFC, which enhanced mood-related behaviors, modulated the serotonergic system within the dorsal raphe nucleus, as well as activation of certain brain areas associated with the vestibular nuclei in the CMS animal model of depression [5].

In addition, it is also postulated that the decrease of Fast and MVeMC c-Fos activity after vmPFC stimulation was allegedly caused by the influence of Purkinje cells. Purkinje

cells play an inhibitory function on both the Fast and vestibular nuclei that originate from the anterior and posterior cerebellar vermis. It has been demonstrated that the neuronal activity of individual ensemble of Purkinje cells promotes the induction of plasticity and consolidation in the cerebellar and vestibular nuclei by neural interaction with either the mossy fiber or climbing fiber collaterals or among each other [83]. Besides the inhibitory role of Purkinje cells on motor function [84], our present results have suggested a possible antidepressant function of Fast and MVeMC within the deep cerebellar nuclei that is regulated by the inhibitory projections of Purkinje cells. Importantly, recent evidence from theta oscillation studies have revealed the synchronization of neuronal activities between the vmPFC and the cerebellum, particularly in experimental models of associative learning using trace eye blink conditioning [71, 72, 85]. Although the present study has implicated a potential antidepressant role of vmPFC stimulation on the cerebellum, its neural mechanisms by which the interaction of Purkinje cells with the vmPFC and deep cerebellar nuclei remain largely obscure, and comprehensive studies are required to unravel their neural–behavioral interactions.

In conclusion, our present CMS animal model induced specific phenotypic changes of c-Fos neuronal activation within the cerebellar Dent and Fast, as well as the SpVe. However, HFS targeted in the vmPFC significantly reduced the neuronal activity within the Fast as compared to the CMS sham. Our correlation analyses show that the antidepressant-like effects were highly associated with the activation of Fast, IntMC, and IntPC nuclei, and these nuclei were strongly correlated among each other. Taken together, the vmPFC stimulation-induced antidepressant-like behaviors were possibly mediated by a dynamic neurocircuitry remodeling within the forebrain–cerebellum interactions for emotional regulation.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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