Early postnatal stress affects 5-HT_{1A} receptor function in the medial prefrontal cortex in adult rats

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Abstract

Traumatic events in early life are associated with an increased risk of psychiatric diseases in adulthood. 5-hydroxytryptamine (5-HT)$_{1A}$ receptors are known to play a pivotal role in the 5-HTergic mechanisms associated with the etiology of stress-related disorders. The goal of the present study was to investigate whether early postnatal stress influences 5-HT$_{1A}$ receptor function in the medial prefrontal cortex in adult rats. Rats were subjected to aversive foot shock (FS) during the third week of the postnatal period (3wFS group). During the postadolescent period (10-14 weeks postnatal), immunohistochemical experiments were carried out to investigate c-Fos expression following the administration of R-(+)-8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), a 5-HT$_{1A}$ receptor agonist. In the 3wFS group, the 8-OH-DPAT-induced c-Fos expression in the medial prefrontal cortex was significantly attenuated compared to that in the non-FS control group. A dual immunofluorescence study revealed that a small proportion of c-Fos positive cells co-express parvalbumin, and a relatively large proportion of c-Fos positive cells co-express glutaminase, suggesting that most c-Fos positive cells are glutamatergic neurons. We found that local perfusion of 8-OH-DPAT via a dialysis probe decreased extracellular 5-HT levels in the medial prefrontal cortex of the non-FS group, but not in the 3wFS group. However, the

levels of 8-OH-DPAT-induced 5-HT syndrome were not significantly different between the non-FS and 3wFS groups. Therefore, aversive stress in the third week of the postnatal period attenuates 5-HT_{1A} receptor function in the medial prefrontal cortex in adulthood and produces feedback inhibition of the raphe nuclei via postsynaptic 5-HT_{1A} receptors.

Keywords: 5-HT1A receptor, early postnatal stress, medial prefrontal cortex

1. Introduction

Several lines of evidence have shown that traumatic events (e.g., instability of family relationships, parental loss, sexual or physical abuse) in early life are associated with an increased risk of psychiatric diseases in adulthood (Brewin et al., 2000; Heim et al., 2001). In animal studies, prolonged maternal separation increased hypothalamic-pituitary-adrenal (HPA) responsivity to stress (De Kloet et al., 2005) and enhanced anxiety-like behavior (Kalinichev et al., 2002, Ladd et al., 2004), while neonatal brief handling has been shown to reduce HPA responsiveness to stress and anxiety-like behavior (Kalinichev et al., 2002, Ladd et al., 2004).

The development of the 5-hydroxytryptamine (5-HT) neural systems is incomplete at birth, and marked developmental changes in their structure and function continue during the pre-pubertal period (Lidov & Molliver, 1982; Ugrumov et al., 1986). 5-HT also affects neurogenesis (Lauder et al., 1981) and the development of the cerebral cortex (Cases et al., 1996). Moreover, the expression of 5-HT receptors in the prefrontal cortex changes with growth (Zhang, 2003; Beique et al., 2004).

Also, there is much evidence to correlate dysfunctions in central monoamine neurotransmission, particularly that related to 5-HT, with a variety of psychiatric disorders, including major depression (Mann et al., 1995; Drevets et al., 2000). In

animal studies, it is well known that 5-HT in the limbic system is involved in the regulation of emotional stress (Yoshioka et al., 1995; Roche et al., 2003).

Among the multiple 5-HT receptors, 5-HT_{1A} receptors are known to play a pivotal role in the 5-HTergic mechanism associated with the etiology of stress related disorders. In positron emission tomography studies, decreases in 5-HT_{1A} receptor expression were observed in psychiatric disorders such as depression (Meltzer et al., 2004). Indeed, 5-HT_{1A} receptor agonists have been used for the treatment of generalized anxiety disorder (Feighner et al., 1989). 5-HT_{1A} receptor knockout mice show increased anxiety-like behavior (Heisler et al., 1998), while 5-HT_{1A} receptor-overexpressing mice display reduced levels of anxiety-like behavior (Kusserow et al., 2004). Maternal separation reduces the expression of the 5-HT_{1A} receptor in the rat brain (Stamatakis et al., 2006; Vicentic et al., 2006). These findings have led us to assume the involvement of 5-HT_{1A} receptors in the 5-HTergic regulation of emotional stress during postnatal development of the brain.

We have recently reported that foot shock (FS) during the third week of the postnatal period (3wFS group) alters behavioral responses to emotional stimuli in the postadolescent period of rats. In that study, the 3wFS group showed an increased percentage of time spent in open arms in an elevated plus-maze test, and the number of

5-HT-like immunoreactive cells in the median raphe nuclei was reduced compared to the number in the non-FS control group (Konno et al., 2007). Moreover, extinction of contextual fear conditioning was significantly attenuated in the 3wFS group compared to that in the non-FS controls, and this attenuation was inhibited by administration of the 5-HT_{1A} receptor partial agonist tandospirone (Matsumoto et al., 2008).

In this study, we investigated whether early postnatal stress influences 5-HT_{1A} receptor function in adult rats. For this purpose, rats were subjected to footshock stress during the third week of the postnatal period, and during the postadolescent period immunohistochemical experiments were carried out to estimate the function of 5-HT_{1A} receptors in the brain, using the 5-HT_{1A} receptor agonist (R-(+)-8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT)) to induce c-Fos expression. Then, we used the double immunostaining technique to identify the profile of the c-Fos-positive cells that are induced by 8-OH-DPAT, using NeuN, glutaminase, and parvalbumin as neuronal cell markers. In *in vivo* microdialysis experiments, several studies have reported a long-loop feedback inhibition mechanism, in which postsynaptic 5-HT_{1A} receptors in the medial prefrontal cortex exert distal feedback control of 5-HTergic activity through the modulation of descending excitatory afferent neurons that extend into the raphe nucleus (Casanovas et al., 1999; Celada et al., 2001;

Martín-Ruiz et al., 2001). In this study, we investigated the feedback mechanism in the medial prefrontal cortex of the rats of a 3wFS group by local application of 8-OH-DPAT. In addition, we observed the 5-HT related behavior mediated by 5-HT_{1A} receptors.

2. Material and methods

2.1. Animals

Wistar rats were bred in our laboratory, with the exception of the first-breeder adult rats, which were supplied by Sankyo Labo Service, Ltd. (Shizuoka, Japan). The day of birth was denoted postnatal day 0. Sex was determined on postnatal day 10, and weaning occurred on postnatal day 21. Male pups were used. Female pups were used for other experiments. The rats were housed in a room with a 12 h light-dark cycle (light on at 19:00 h) and a temperature-controlled environment (22 ± 1 °C) with food and water ad libitum. All animals were treated in accordance with the guidelines for the Care and Use of Laboratory Animals of the Animal Research Committee of the Hokkaido University Graduate School of Medicine.

2.2 Drugs

For the in vivo microdialysis study, R-(+)-8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) (Sigma, USA) and (±)-1-(3-dimethylaminopropyl)-1-(4-fluoro-phenyl)-1,3-dihydroisobenzofuran-5-carbon nitrile hydrobromide (citalopram, a generous gift from Lundbeck, Ltd (Copenhagen, Denmark)) were dissolved and diluted in artificial cerebrospinal fluid (aCSF) (2.7 mM KCl, 140 mM NaCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.3 mM NaH_2PO_4, and 1.7 mM

Na_2HPO_4, pH 7.2). For the behavioral study, 8-OH-DPAT was dissolved in 0.9% NaCl solution (saline). The drug was administered by intraperitoneal injection (i.p.) at a dose of 1 ml.

2.3. Early postnatal stress

The rats were subjected to early postnatal stress as previously described (Matsumoto et al., 2005). All male pups were numbered serially and divided into the control and postnatal stress groups. The rats with the numbers 4n+1 (n=0, 1, 2…) and 4n+4 were assigned to the control group, while the rats with the numbers 4n+2 and 4n+3 were assigned to the postnatal stress group. The rats of the postnatal stress group were placed into the footshock box for 5 min and subjected to five footshocks (shock intensity: 0.5 mA; intershock interval: 30 s; shock duration: 2 s). They remained in the box for 5 min after the last footshock on postnatal days 21-25 (3wFS group). The rats of the control group were placed in the footshock box for 12.5 min without footshocking (non-FS group). In the postadolescent period (10-14 weeks old), behavioral, histological, and neurochemical experiments were performed.

2.4. Immunohistochemistry

All rats of the control group were numbered serially and divided into the saline and 8-OH-DPAT groups. The rats with the numbers 8n+1 (n=0, 1…), 8n+3, 8n+6, and 8n+8

were assigned to the saline group, while the rats with the numbers 8n+2, 8n+4, 8n+5, and 8n+7 were assigned to the 8-OH-DPAT group. All rats of the 3wFS group were divided into the saline and 8-OH-DPAT groups similarly. The rats were anesthetized with pentobarbital (50 mg/kg i.p.) and perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 2 h after the administration of 8-OH-DPAT (1 mg/kg i.p.) or saline. Their brains were sectioned into 30 μm slices. The sections were incubated in 1% hydrogen peroxidase in 50% methanol for 15 min. Then, the sections were incubated in 10% normal goat serum (NGS) (Jackson Immuno Research, USA) for 1 h followed by further incubation in rabbit polyclonal anti-c-Fos antibody (1:20,000, Calbiochem, Darmstadt, Germany) with 3% NGS for 24 h, before being incubated in biotinylated goat anti-rabbit IgG (Histofine MAX-PO®, Nichirei Co. Ltd. Tokyo, Japan) for 1 h. The reaction product was visualized by transferring the sections to a 50 mM Tris-HCl buffer (pH 7.6) containing 0.05% diaminobenzidine (DAB), 0.6% nickel ammonium sulfate, and 0.01% H_2O_2.

For dual immunofluorescence staining, the sections were blocked with 10% NGS for 1 h and then incubated in a mixture of rabbit anti-c-Fos antibody together with mouse anti-NeuN antibody (1:2000, Chemicon, USA & Canada), mouse anti-parvalbumin antibody (1:2,000, Sigma), or guinea pig anti-glutaminase antibody

(1:300, as previously reported in Hoshino et al. (2005)) for 24 h, followed by incubation in a mixture of the appropriate secondary antibodies such as goat anti-rabbit Cy3 antibody (1:400, Jackson ImmunoResearch, USA), goat anti-mouse FITC antibody (1:200, Jackson ImmunoResearch, USA), and donkey anti-guinea pig Alexa Fluor 488 antibody (1:200, Molecular Probes, Eugene, USA) for 1 h. The sections were analyzed with a confocal laser-scanning microscope (Axiophot®, Carl Zeiss, Germany) attached to a scanning confocal system (MRC-1024®, Bio-Rad, UK).

2.5. Cell quantification

Cell quantification was carried out according to the method described previously (Izumi et al., 2008). Briefly, according to the atlas of Paxinos and Watson (1997), sections including the medial prefrontal cortex, motor cortex, somatosensory cortex, and central amygdala (Fig. 1.) were selected for cell counting of c-Fos immunoreactivity with a densitometric video image analysis system (MCID system®, Imaging Research, CA, USA). Each unit area (200 × 200 μm) was digitally recorded with a CCD camera (CCD-IRIS®, Sony, Japan) connected to a photomicroscope (B×50, Olympus, Japan). The number of c-Fos positive cells was assessed by automated selection of the cells within the unit areas that satisfied the following criteria: (1) the gray value of the cell nucleus was higher than the threshold value (threshold gray value = higher than 2 fold

of the background gray value), (2) a nuclei diameter of 4–12 μm (to exclude cell debris and artifacts). The background gray value was determined in a part of each unit area that contained no nuclei. The measurement of the background gray value and the c-Fos positive cell counting were repeated 3 times, and the values were averaged. Cell quantification was performed by an investigator who was blinded to the treatment.

2.6. In vivo microdialysis

The rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and a guide cannula was stereotaxically implanted into the right medial prefrontal cortex (3.3 mm anterior and 0.6 mm lateral from the bregma, 1 mm ventral from the dura) according to the atlas of Paxinos and Watson (1997). The dialysis probe (A-I-4-02, Eicom Co. Ltd., Kyoto, Japan) was the same length as the length of the dialysis membrane that was exposed to the brain tissue; i.e., 2 mm. The probe was perfused with aCSF containing 1 μM citalopram at a flow rate of 1 μl/min, to maintain sufficient basal 5-HT levels. After the 5-HT levels were stabilized, the local application of 300 μm of 8-OH-DPAT dissolved in aCSF was performed by reverse dialysis through the probe.

The extracellular levels of 5-HT were determined using high-performance liquid chromatography with a reverse-phase column (Eicompak PP-ODS® 4.6 mm i.d. × 30 mm: Eicom) and an electrochemical detector (ECD-300®, Eicom). The mobile phase,

which consisted of 2.1 mM sodium 1-decansulfonate, 0.1 mM EDTA-2Na/0.1 M phosphate buffer (pH 6.0), and 1% (v/v) methanol, was pumped at a rate of 1 ml/min.

2.7. 5-HT syndrome

5-HT syndrome was observed in individual clear Plexiglas cages. Signs of 5-HT syndrome were studied for up 26 min after 8-OH-DPAT (1 or 3 mg/kg i.p.) administration. Specifically, signs of 5-HT syndrome were recorded in 5 different 1-min periods separated by 5-min intervals, starting 5 min after 8-OH-DPAT injection. Scoring was performed according to the method of Izumi et al. (2006). Intermittent behavior (forepaw treading, head weaving, and backward movement) was scored on a 0-4 scale: 0: absent, 1: present once, 2: present several times, 3: present frequently, 4: present continuously. Continuous behavior (hind limb abduction, straub tail, and flat body posture) was scored on a 0-4 scale of relative intensity: 0: absent, 1: perceptible, 2: weak, 3: medium, 4: maximal. Behavior was scored by an investigator who was blind to the treatment.

2.8. Data analysis

Multiple group comparisons were performed using 1-way and 2-way analysis of variance (ANOVA). When there was no interaction between factors, Bonferroni-Dunn's post hoc test was performed. When there was an interaction between factors, multiple

comparisons with Bonferroni's correction were also conducted. The alpha level was set at 5%; i.e., the alpha level was set at 0.83% (5% / 6) for multiple comparisons because the number of comparisons was six. The values obtained by microdialysis techniques were expressed as a percentage of the baseline before treatment. The area under the curve (AUC) (% min/10^3) was calculated 120 min after drug perfusion to evaluate the overall effect of 5-HT levels.

3. Results

3.1. The effects of early postnatal stress on 8-OH-DPAT-induced c-Fos expression in the medial prefrontal cortex

c-Fos-like immunoreactivity was sparsely distributed in almost all the cortical and subcortical areas of the brains of the rats in the non-FS and 3wFS groups. Two-way ANOVA (FS×8-OH-DPAT) indicated significant main effects of FS ($F(1, 12)=12.9$, $P<0.01$) and 8-OH-DPAT ($F(1, 12)=17.7$ $P<0.01$) in the medial prefrontal cortex. Two-way ANOVA also indicated a significant interaction between FS and 8-OH-DPAT in the medial prefrontal cortex ($F(1, 25)=15.8$, $P<0.01$). Multiple comparisons indicated a significant difference between the non-FS/saline group and the non-FS/8-OH-DPAT group ($P<0.0083$) and between the non-FS/8-OH-DPAT group and the 3wFS/8-OH-DPAT group ($P<0.0083$) in the medial prefrontal cortex, but the difference

between the non-FS/saline group and the 3wFS/saline group was not statistically significant (Fig. 2A, 2B). In other regions (the motor cortex, somatosensory cortex, and central amygdala), there were no significant differences between the non-FS and 3wFS groups (Table 1).

3.2. Characterization of the 8-OH-DPAT-induced c-Fos positive cells in the medial prefrontal cortex

The staining patterns of NeuN, parvalbumin, and glutaminase were consistent with the findings of previous studies: the nucleus except for the nucleolus was intensely stained for NeuN with relatively light staining in the cytoplasm (Fig. 3B) (Mullen et al., 1992), the majority of parvalbumin-labeled cells had smooth-surface cell bodies that radiated dendrites (Fig. 3C) (Gabbott et al., 1997), and mitochondrial thread-like staining of glutaminase was intense in the perikarya and proximal dendrites of large, pyramidal cells (Fig. 3D) (Donoghue et al., 1985). NeuN-positive cells were distributed widely in layers Ⅱ-Ⅴ of the medial prefrontal cortex, and 98% of c-Fos positive cells were positive for NeuN (Fig. 3E, Table 2). Parvalbumin-positive cells were scattered in layers Ⅱ-Ⅴ of the medial prefrontal cortex (Fig. 3A), and 15% of c-Fos positive cells were positive for parvalbumin (Fig. 3F, Table 2). Glutaminase-positive cells were mainly located in layers Ⅴ-Ⅵ of the medial prefrontal cortex (Fig. 3A), and 65% of

c-Fos positive cells were positive for glutaminase (Fig. 3G, Table 2).

3.3. The effects of early postnatal stress on decreases in the extracellular 5-HT levels induced by the local application of 8-OH-DPAT to the medial prefrontal cortex

There was no significant difference between the basal 5-HT levels (in pg/fraction) of the non-FS group and those of the 3wFS group in the medial prefrontal cortex (data not shown). In the non-FS group, the application of 300 μM 8-OH-DPAT to the medial prefrontal cortex significantly reduced the extracellular 5-HT levels to approximately 50% of the baseline, whereas no reduction was observed in the 3wFS group (Fig. 4A). Thus, the 8-OH-DPAT-induced 5-HT decrease in the 3wFS group was significantly attenuated compared to that in the non-FS group ($P<0.05$) (Fig. 4B).

3.4. The effects of early postnatal stress on the 5-HT syndrome induced by 8-OH-DPAT

Doses of 1 and 3 mg/kg of 8-OH-DPAT elicited forepaw treading, straub tail, flat body posture, and hind limb abduction, but no head weaving or backward movement was observed. Two-way ANOVA (FS×8-OH-DPAT) indicated no significant interaction with or effect of FS on any of the observed behaviors. However, 2-way ANOVA indicated significant main effects of 8-OH-DPAT in forepaw treading, straub tail, and flat body posture ($F(1, 21)=9.2$, $P<0.01$; $F(1, 21)=21.4$, $P<0.01$; $F(1, 21)=10.9$, $P<0.01$, respectively). Post hoc comparisons indicated that there were significant differences

between the 1 mg/kg 8-OH-DPAT and 3 mg/kg 8-OH-DPAT treatments in forepaw treading ($P<0.01$), straub tail ($P<0.01$), and flat body posture ($P<0.01$) (Table 3).

4. Discussion

In c-Fos immunohistochemistry, 8-OH-DPAT-induced c-Fos expression in the non-FS group was increased in the medial prefrontal cortex, motor cortex, somatosensory cortex, and central amygdala. The 8-OH-DPAT-induced c-Fos expression of the 3wFS group was only attenuated compared to that of the non-FS group in the medial prefrontal cortex.

The possibility exists that the differences in the number of cells in a certain area among the treatment groups were derived from differences in the sizes of the cells that were counted. To eliminate this possibility, unbiased stereological methods (West., 1993; Howard., 1997; Mouton., 2002) that take into account section thickness, mean profile cell diameter, and cell shape and number, which are the most accurate methods for counting the absolute numbers of cells, were used. However, the purpose of the present experiment is to assess the relative difference in c-Fos-positive cell numbers between the control and postnatal stress groups. So, we adopted the method described in the Materials and Methods section, and determine the size of the cell nuclei. All sections were treated similarly, and cell counting was performed by a blind investigator using

pre-defined standards and an automatic image analyzing system. For these reasons, we concluded that the results of the c-Fos-positive cell counts in this study are relative but sufficiently objective. While the c-Fos-labeled cell nuclei areas of the non-FS/8-OH-DPAT and 3wFS/8-OH-DPAT groups were 39.9 ± 0.76 μm^2 (n=105) and 41.0 ± 0.88 μm^2 (n=99), respectively, there were no significant differences between the cell nuclei areas of the non-FS/8-OH-DPAT and 3wFS/8-OH-DPAT groups. Taken together, the possibility that the reduction in the number of 8-OH-DPAT-induced c-Fos positive cells in the 3wFS group compared to that of the non-FS group was derived from a change in the cell nuclei area was ruled out. Therefore, it was thought that the 5-HT_{1A} receptor function of the 3wFS group was reduced in a site-specific manner in the medial prefrontal cortex.

There are many 5-HT_{1A} receptors in the hippocampus, septum, and dorsal raphe nucleus (Verge et al., 1986), but c-Fos was not expressed in these regions in response to 8-OH-DPAT administration in this experiment and previous studies (Hajós et al., 1999). Then, we could not assess the 5-HT_{1A} receptor function in these regions.

Whereas 5-HT_{1A} receptor activation usually inhibits neural activity via $G_{i/o}$ proteins, the present study and other studies have reported that 5-HT_{1A} receptor agonists induce c-Fos expression. Although the cause of this phenomenon is unknown, several

mechanisms have been suggested. Some neurons in the medial prefrontal cortex are excited by systemic administration of 8-OH-DPAT (Hajós et al., 1999). Cadogan et al. (1994) reported that systemic administration of 8-OH-DPAT increases extracellular cAMP efflux in the hippocampus. Albert and Tiberi (2001) indicated that 5-HT_1 receptors mediate a stimulatory pathway involving $G_{\beta\gamma}$-mediated stimulation of phospholipase C_β and mitogen-activated protein kinase. Santana et al. (2004) reported that glutamatergic neurons of the medial prefrontal cortex are disinhibited by stimulation of 5-HT_{1A} receptors on GABA interneurons.

The dual immunofluorescence study revealed that almost all of the 8-OH-DPAT-induced c-Fos positive cells in the medial prefrontal cortex co-expressed NeuN, a marker of mature neurons; a small proportion of c-Fos positive cells co-expressed parvalbumin, a marker of GABA neurons; and a relatively high proportion of c-Fos positive cells co-expressed glutaminase. These results suggest that a large number of the 8-OH-DPAT-induced c-Fos positive cells were glutamate neurons. Santana et al. (2004) reported that >50% of glutamatergic cells and approximately 20% of GABAergic cells co-express 5-HT_{1A} receptors in the medial prefrontal cortex. Therefore, our present results are consistent with those of previous studies.

In the *in vivo* microdialysis study, local perfusion of 8-OH-DPAT via a dialysis

probe decreased the extracellular levels of 5-HT in the non-FS group, but not in the 3wFS group. The postsynaptic 5-HT_{1A} receptor mediates the long-loop feedback inhibition of 5-HT release. For example, local infusion of 5-HT_{1A} receptor agonists in the medial prefrontal cortex reduces the cell firing rate in the raphe (Hajós et al., 1999) and 5-HT release in the raphe (Celada et al., 2001) and medial prefrontal cortex (Casanovas et al., 1999; Celada et al., 2001; Martín-Ruiz et al., 2001). In this study, the regulatory mechanism that acts via the postsynaptic 5-HT_{1A} receptors in the medial prefrontal cortex was attenuated in the 3wFS group. Since the glutamate neurons of the medial prefrontal cortex project into a variety of brain regions (Gabbott et al., 2005), the possibility exists that this attenuation is related to changes in behavioral response to emotional stress caused by early postnatal stress.

In the 5-HT syndrome observation, there were no significant differences between the 8-OH-DPAT-induced 5-HT syndrome of the non-FS group and that of the 3wFS group. The symptoms of 5-HT syndrome, head weaving, forepaw treading, hind limb abduction, straub tail, tremors, and flat body posture, are mediated via overstimulation of the 5-HT_{1A} receptor (Lucki et al., 1984). Irwin et al. (1984) reported that the 5-HT receptors of the spinal cord and lower brain stem are involved in 5-HT syndrome. Therefore, these data suggest that early postnatal stress does not affect the 5-HT_{1A}

receptors of the spinal cord or brain stem.

In this study, we used 8-OH-DPAT as a 5-HT_{1A} receptor agonist. However, 8-OH-DPAT also has 5-HT_7 receptor agonistic properties (Ruat et al., 1993). Hajós et al. (1999) reported that the 8-OH-DPAT-induced c-Fos expression in the medial prefrontal cortex was greatly attenuated by pretreatment with the selective 5-HT_{1A} receptor antagonist WAY-100,635. Moreover, our preliminary data (data not shown) and a previous report (Casanovas et al., 1999) indicated that local application of 8-OH-DPAT decreased the levels of 5-HT in the medial prefrontal cortex, and this effect was antagonized by concurrent application of WAY-100,635. These suggest that the 5-HT_{1A} receptor agonistic properties of 8-OH-DPAT are mainly responsible for the effects seen in the present study.

When an animal is exposed to stress, several mechanisms (e.g., HPA axis) are activated to restore homeostasis. Many studies have indicated that corticosterone regulates expression of the 5-HT_{1A} receptor via mineralcorticoid and glucocorticoid receptors (reviewed by Lanfumey et al., 2008). Fairchild et al. (2003) reported that chronic, but not acute, administration of corticosterone changes the electrophysiological function of 5-HT_{1A} receptors in the dorsal raphe. It is suggested that changing 5-HT_{1A} receptor function is necessary for the prolonged elevation of corticosterone levels.

However, maternal separation does not affect the basal corticosterone level in adult rats (Macrì et al., 2008). Therefore, it is questionable whether an increase in corticosterone levels due to postnatal stress would alter 5-HT_{1A} receptor function. There may be other mechanisms that induce 5-HT_{1A} receptor functional changes after postnatal stress.

Moreover, Béïque et al. (2004) investigated developmental change in 5-HT receptor function in the medial prefrontal cortex using whole-cell patch-clamp recordings in brain slices. 5-HT perfusion elicited depolarization during the second postnatal week, and a gradual shift from depolarization to hyperpolarization was observed in the third postnatal week. From these studies, we concluded that since the 5-HT neural system in the medial prefrontal cortex change dramatically during the third postnatal week, the neurons there might be more easily affected by stress. As a result, it is anticipated that long-lasting changes in the number and/or function of 5-HT_{1A} receptors in the medial prefrontal cortex are induced by early postnatal stress.

In summary, the present study revealed that a 3wFS group showed reduced 5-HT_{1A} receptor function in the medial prefrontal cortex. Thus, aversive stress present in the third postnatal week can cause changes in 5-HT_{1A} receptor function in the medial prefrontal cortex during the postadolescent period. Previous studies have shown that early life aversive treatment such as maternal separation changes 5-HT_{1A} function in the

amygdala (Vicentic et al., 2006) and hippocampus (Stamatakis et al., 2006). This is the first study to indicate that 5-HT_{1A} receptor function in the medial prefrontal cortex is changed by early postnatal stress. We hope to clarify the relationship between early life aversive events and psychiatric diseases in adulthood by pursuing the molecular mechanism of this phenomenon.

Acknowledgements

This study was supported by Japanese Ministry of Education grant No. 0677074 (T. I.).

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Figure Legends

Fig. 1. Areas in which the cell nuclei were counted. 1: medial prefrontal cortex, 2: motor cortex, 3: somatosensory cortex, 4: central amygdala

Fig. 2. (A) Photomicrographs of the medial prefrontal cortex showing the expression of 8-OH-DPAT-induced c-Fos positive cells. Scale bar=200μm. (B) The effects of early postnatal stress on the expression of 8-OH-DPAT-induced c-Fos positive cells in layer Ⅴ of the medial prefrontal cortex. The results are means plus S.E.M. of the data and are expressed as c-Fos positive cell nuclei per square millimeter. The number of rats per group was as follows: non-FS/saline group: n=6, 3wFS/saline group: n=6, non-FS/8-OH-DPAT group: n=7, 3wFS/8-OH-DPAT group: n=7. non-FS: rats exposed to the footshock box without footshocking at the third postnatal week, 3wFS: rats exposed to footshocks at the third postnatal week. *P<0.0083

Fig. 3. (A) Photomicrographs of the medial prefrontal cortex showing the distribution of glutaminase and parvalbumin positive cells in the medial prefrontal cortex. Parvalbumin-positive cells were scattered in layers Ⅱ-Ⅴ, and glutaminase-positive cells were mainly located in layers Ⅴ-Ⅵ. The arrows indicate examples of

parvalbumin positive cells, whereas the arrowheads indicate examples of glutaminase-positive cells. (B-D) Typical photomicrographs of NeuN, parvalbumin, and glutaminase in the medial prefrontal cortex, respectively. (E-G) 8-OH-DPAT-induced c-Fos colocalizes with NeuN, parvalbumin, and glutaminase in the medial prefrontal cortex. The arrows indicate examples of c-Fos and marker co-expression, whereas the arrowheads indicate examples of single-labeled c-Fos nuclei. Scale bars: (A) 200 μm, (B-G): 50 μm.

Fig. 4. The effects of early postnatal stress on extracellular 5-HT levels after the local application of 8-OH-DPAT in the medial prefrontal cortex. (A) The time course of extracellular 5-HT level changes, (B) the area under the curve (AUC) of extracellular 5-HT levels changes after local perfusion of 8-OH-DPAT into the medial prefrontal cortex. Data are given as a percentage of the baseline ± S.E.M. *P<0.05, **P<0.01 aCSF: artificial cerebrospinal fluid, non-FS group: rats exposed to the footshock box without footshocking at the third postnatal week, 3wFS group: rats exposed to five footshocks at the third postnatal week.

Table legends

Table 1

Positive c-Fos cell counts (/mm2) in each area

Values are the mean ± S.E.M. of total cell counts.

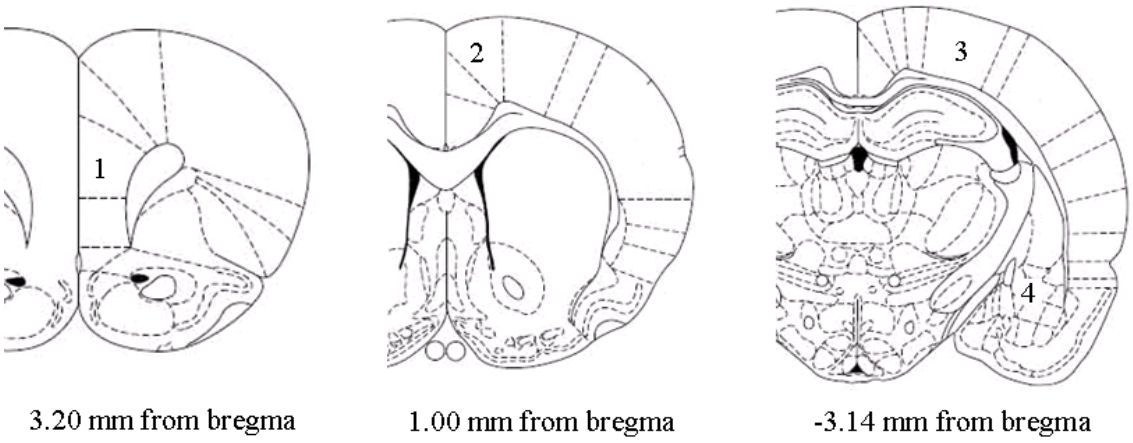
Table 2

Counted of c-Fos and its co-localization with NeuN, parvalbumin, and glutaminase

Table 3

The effects of early postnatal stress on 5-HT syndrome induced by 8-OH-DPAT

Each value represents the mean of summed behavioral score ± S.E.M.



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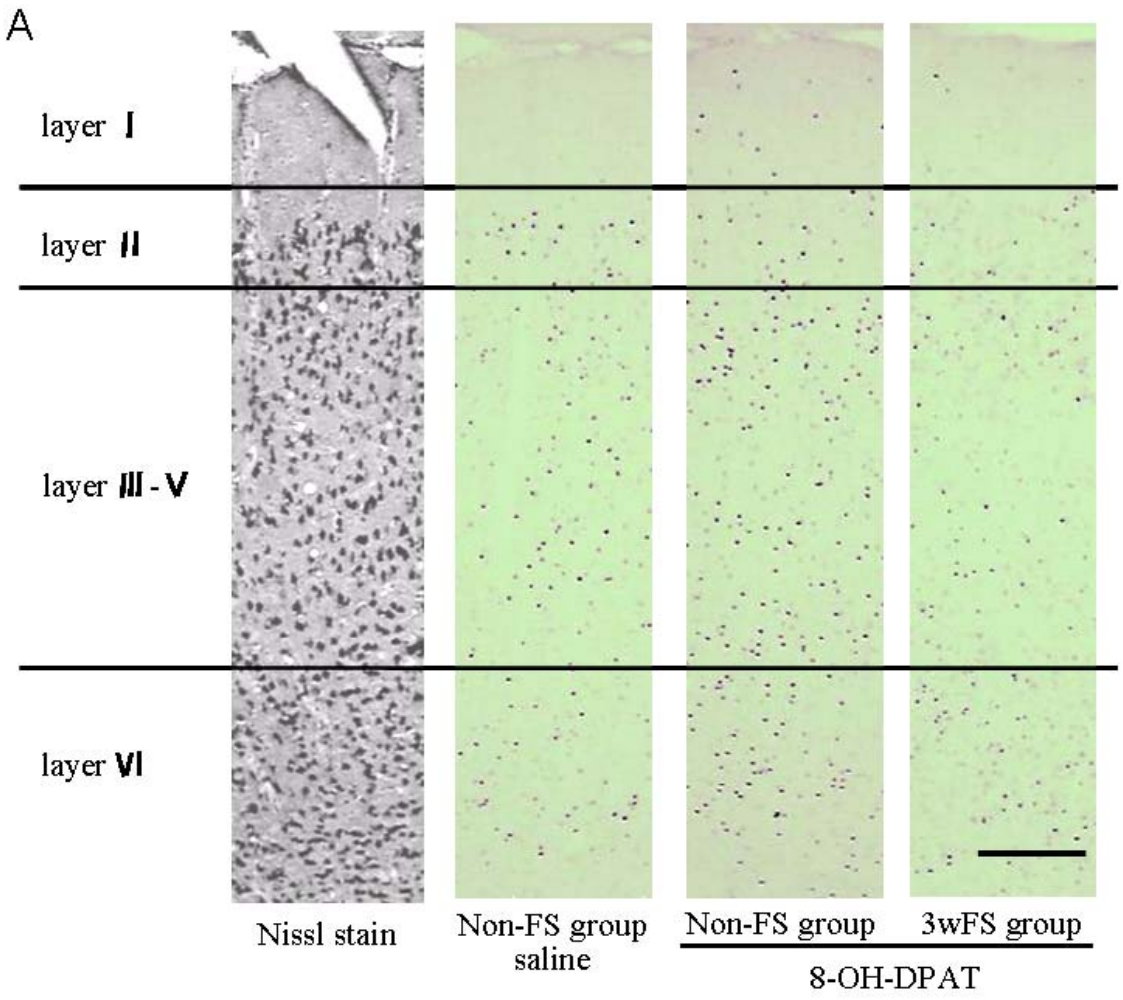
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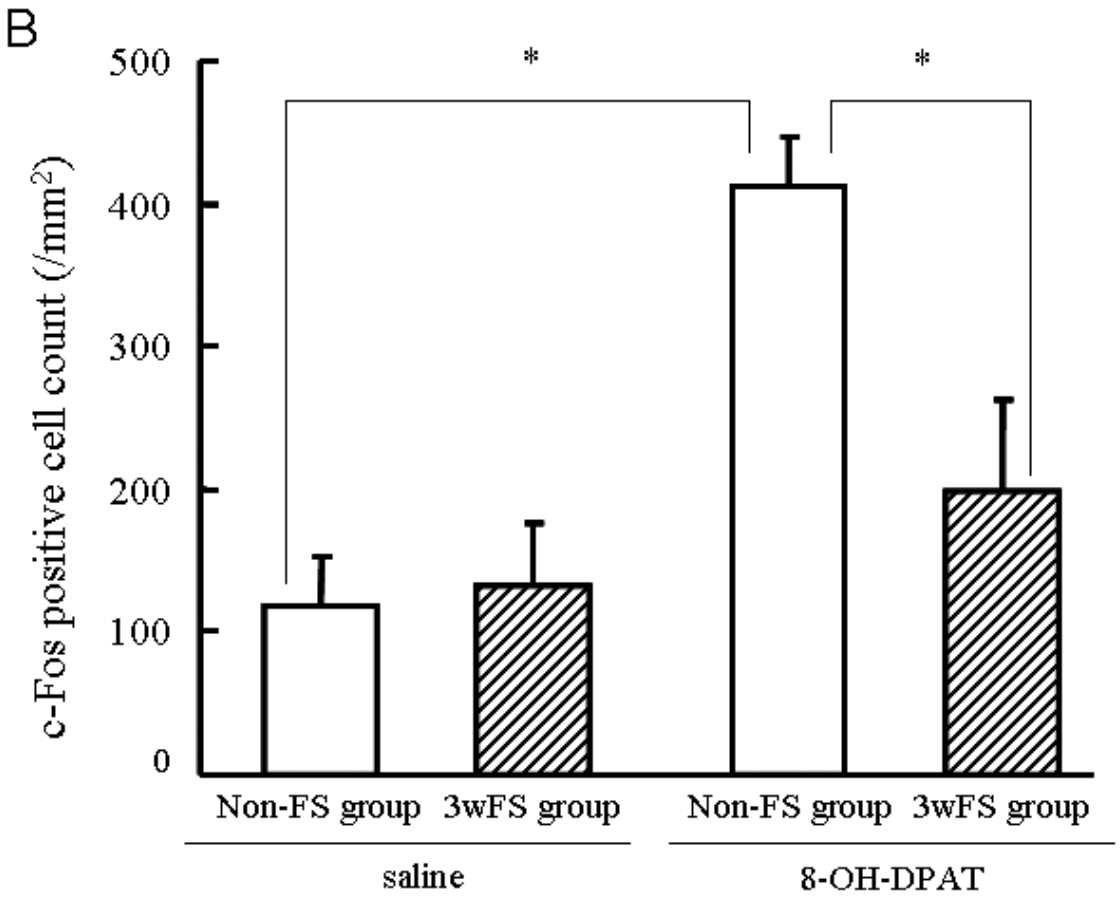
layer II
layer III - V
layer VI
Nissl stain
Non-FS group
saline
Non-FS group
3wFS group
8-OH-DPAT



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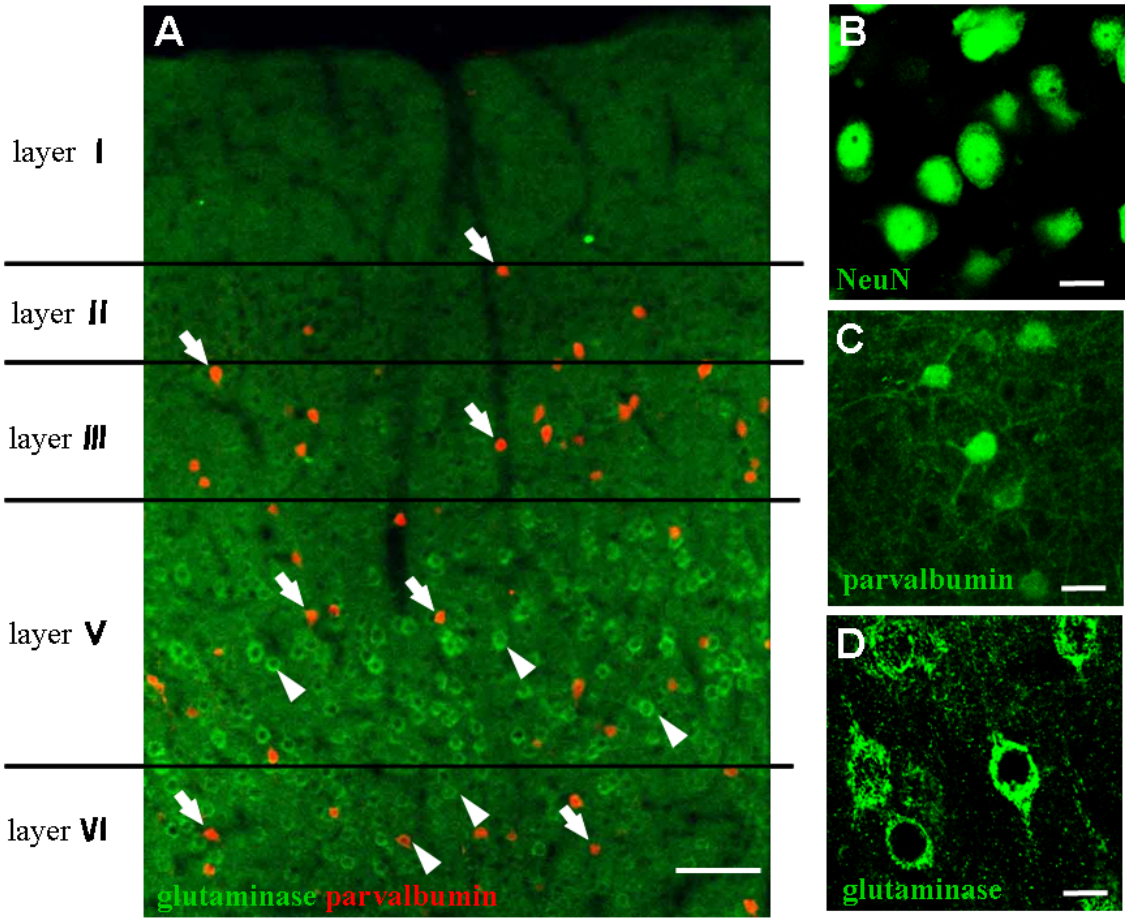
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layer I
layer II
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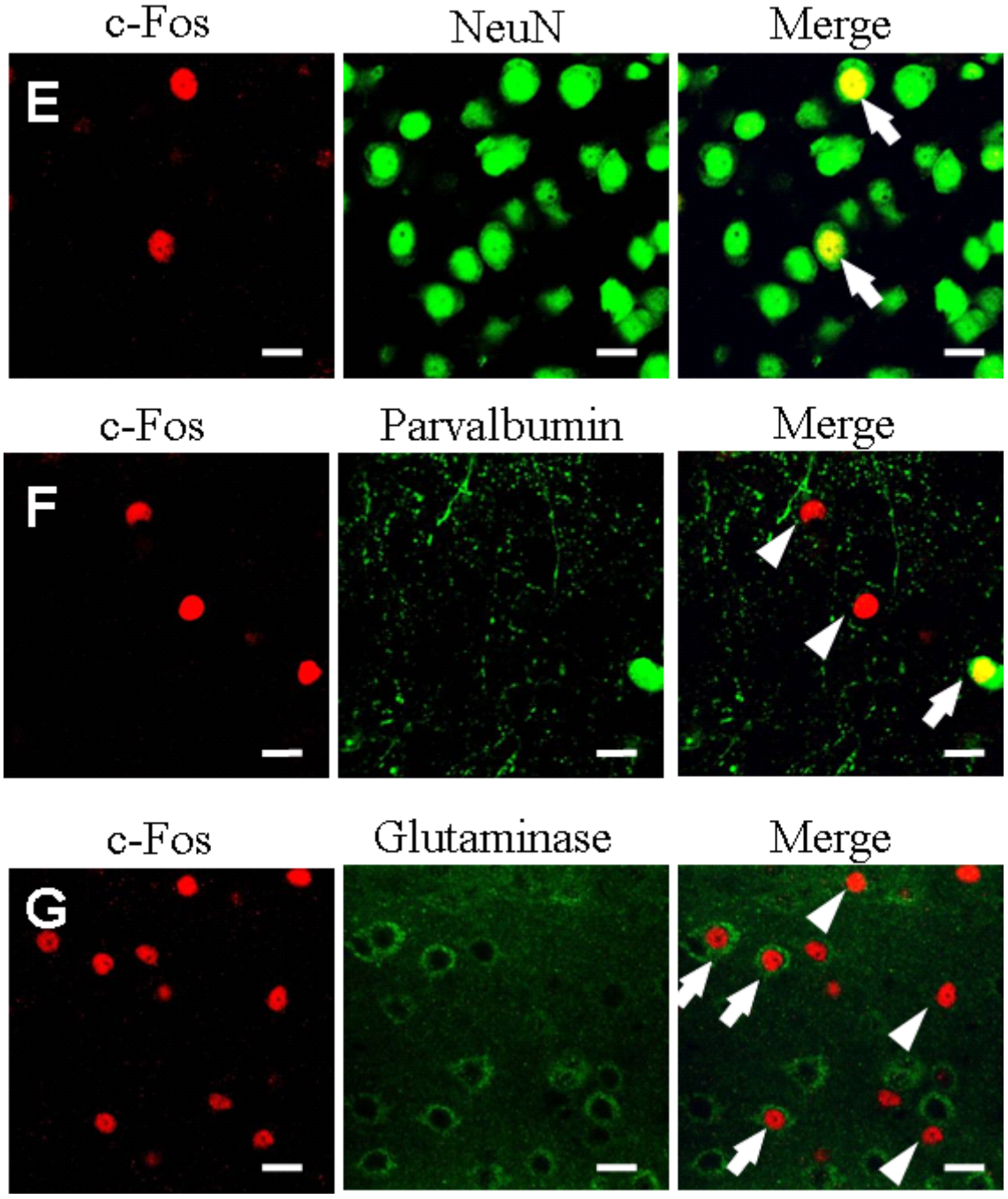
layer V
layer VI
glutaminase parvalbumin
B
NeuN
C
parvalbumin
D
glutaminase


c-Fos
NeuN
Merge
E
c-Fos
Parvalbumin
Merge
F
c-Fos
Glutaminase
Merge
G

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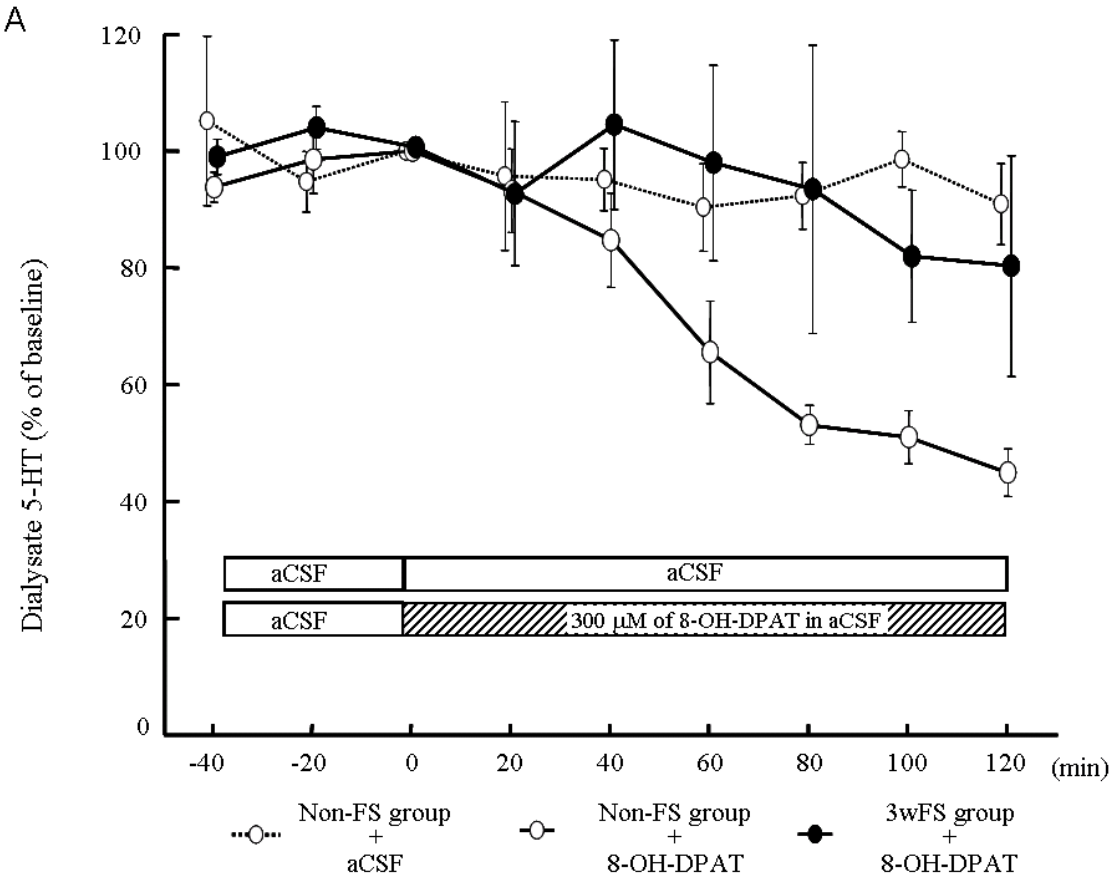
A
120
100
80
60
40
20
0
Dialysate 5-HT (% of baseline)
-40
-20
0
20
40
60
80
100
120
(min)
aCSF
aCSF
aCSF
300 μM of 8-OH-DPAT in aCSF
Non-FS group + aCSF
Non-FS group + 8-OH-DPAT
3wFS group + 8-OH-DPAT

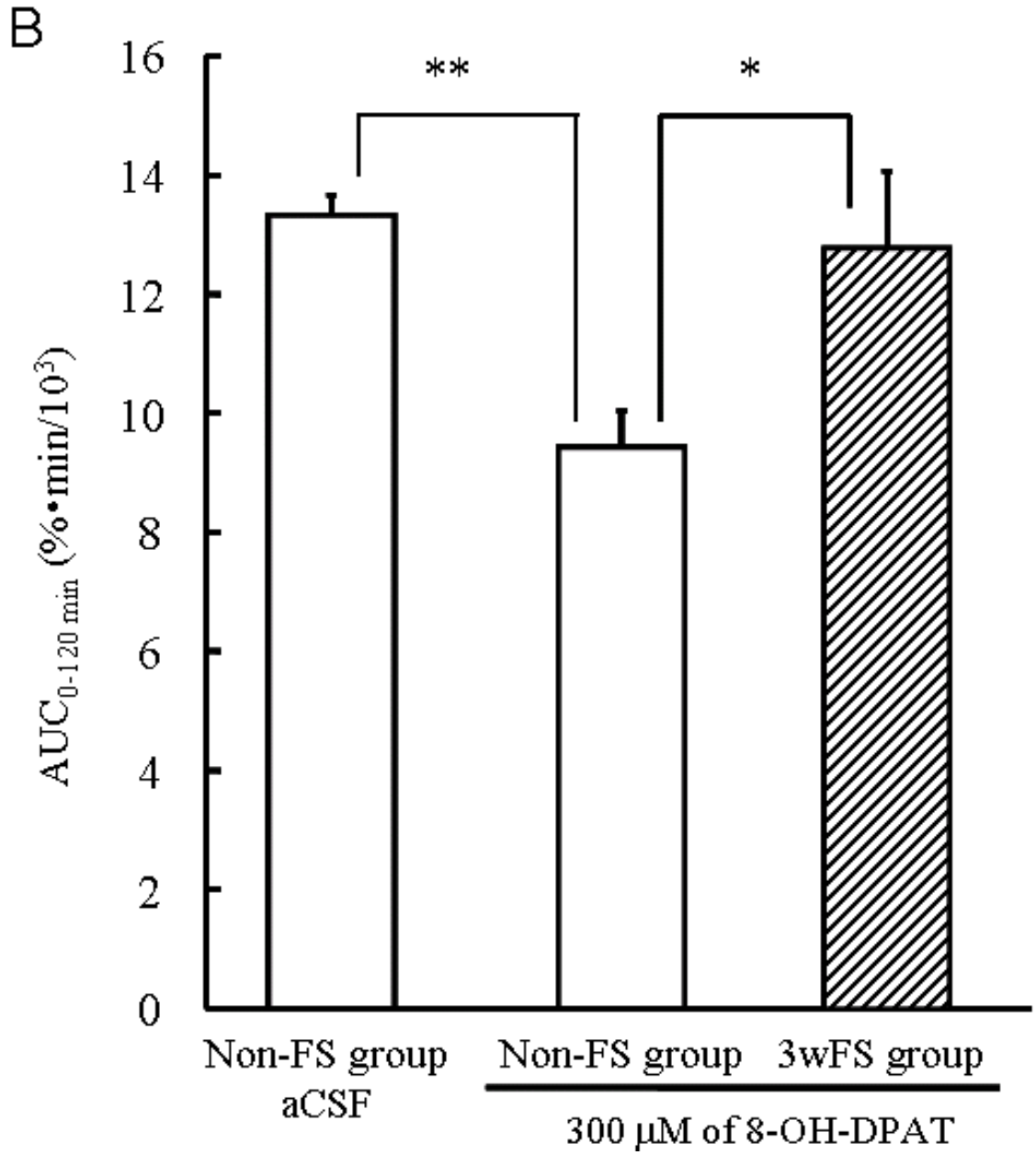

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Table 1

Positive c-Fos cell counts (/mm2) in each area

	Saline		8-OH-DPAT	
	Non-FS group	3wFS group	Non-FS group	3wFS group
Secondary motor cortex	147 ± 28	119 ± 41	554 ± 81	458 ± 78
Somatosensory cortex	151 ± 87	133 ± 64	575 ± 99	411 ± 108
Central amygdala	79 ± 36	81 ± 26	322 ± 62	350 ± 98

Values are the mean ± S.E.M. of total cell counts.

Table 2

Counted of c-Fos and its co-localization with NeuN, parvalbumin, and glutaminase

marker	c-Fos singlelabeled	c-Fos and marker double labeled
NeuN	79	77
Parvalbumin	182	28
Glutaminase	200	130

Table 3

The effects of early postnatal stress on 5-HT syndrome induced by 8-OH-DPAT

	1 mg/kg of 8-OH-DPAT		3 mg/kg of 8-OH-DPAT		Main effect of FS (2-way ANOVA)	Main effect of 8-OH-DPAT (2-way ANOVA)	8-OH-DPAT 1 mg/kg v.s. 3 mg/kg
	Non-FS group	3wFS group	Non-FS group	3wFS group			
Forepaw treading	8.9±1.61	8.0±1.58	12.0±0.82	12.7±0.94	N.S.	F(1,21)=9.2 P=0.0064	P<0.01
Straub tail	4.9±1.12	2.6±0.68	11.0±1.93	9.6±1.02	N.S.	F(1,21)=21.4 P=0.0001	P<0.01
Flat body posture	11.0±2.16	15.0±1.30	17.8±1.08	18.0±0.72	N.S.	F(1,21)=10.9 P=0.0035	P<0.01
Hind limb abduction	1.4±0.57	1.6±1.17	3.2±1.22	4.1±1.82	N.S.	N.S.	N.S.
Head weaving	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	N.S.	N.S.	N.S.
Back ward movement	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	N.S.	N.S.	N.S.

Each value represents the mean of summed behavioral score ± S.E.M.