千葉大学学位申請論文

Depressive-like behavior in adrenocorticotropic hormone-treated rats blocked by memantine

（副腎皮質刺激ホルモン処置ラットにおけるうつ様行動に対するメマンチンの阻害作用）

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Abstract

Hyperactivity of the hypothalamic pituitary-adrenal (HPA) axis plays a role in the pathophysiology of major depressive disorder (MDD). Recent studies suggest the role of the glutamatergic system in the pathophysiology of MDD, and *N*-methyl-$_{D}$-aspartate (NMDA) receptor antagonists have shown antidepressant effects in both preclinical and clinical studies. However, little is known about the role of adrenocorticotropic hormone (ACTH) specifically in the glutamatergic response to HPA axis activation. Glutamate is an NMDA receptor agonist, and glycine and $_{D}$-serine act as co-agonists. Here, we measured brain concentrations of these amino acids in rats given repeated administration of ACTH (100 μg/rat/day, sc, for 14 days). Further, we also evaluated behavioral effects of ketamine and memantine, non-competitive NMDA antagonists, on immobility time in the forced swimming test and on locomotor activity in ACTH-treated rats. Compared with control rats, glutamine, glycine, $_{L}$-serine, and $_{D}$-serine levels were increased in the hippocampus of ACTH-treated rats; glutamate, glutamine, glycine, $_{L}$-serine, and $_{D}$-serine were increased in the cerebellum; and glutamine and glycine were increased in the frontal cortex and striatum, all with statistical significance. Remarkably, these increases in agonists and co-agonists might have lead to the augmentation of NMDA receptor activity. ACTH treatment increased immobility time in the forced swimming test and decreased locomotor activity in rats. Ketamine (20 mg/kg, ip) did not show any effects in these behavioral tests in ACTH-treated rats. On the contrary, memantine (10 mg/kg, ip) significantly decreased immobility time in the forced swimming test and increased locomotor activity in ACTH-treated rats. These results suggest that depressive-like behaviors by chronic ACTH treatment could be blocked by memantine but not ketamine.

Abbreviation list:

MDD, Major depressive disorder; HPA, hypothalamic pituitary-adrenal; CRH, corticotrophin releasing hormone; ACTH, adrenocorticotropic hormone; 5-HT, 5-hydroxytryptamine; CNS, central nervous system; EAATs, excitatory amino acid transporters; NMDA, *N*-methyl-$_{D}$-aspartate; TFA, trifluoroacetic acid; HPLC, high performance liquid chromatography; ANOVA, one way analysis of variance; GFAP, Glial fibrillary acidic protein; TRH, thyrotropin-releasing hormone; WKY, Wistar Kyoto; SD, Sprague-Dawley; BN, Brown-Norway; MRI, Magnetic resonance imaging; SSRI, selective serotonin reuptake inhibitor.

1. Introduction

Major depressive disorder (MDD), also called major depression, is a chronic recurring illness (Kessler and Wang, 2008, Lopez and Mathers, 2006), between one- and two-thirds of patients do not respond to the first antidepressant prescribed, and treatment-resistant depression represents an area of substantial unmet medical need (Little, 2009, Shelton et al., 2010). One factor thought to play a role in the symptoms of depression is environmental stress-induced hyperactivity of the hypothalamic pituitary-adrenal (HPA) axis (Gillespie and Nemeroff, 2005, Mello et al., 2003, Swaab et al., 2005). Stress initiates the release of corticotropin-releasing hormone (CRH), followed by that of adrenocorticotropic hormone (ACTH), and induces changes in the serotonergic system which involve an increase in the expression of 5-hydroxytryptamine (5-HT) $_{2A}$ receptors (Arango et al., 1990, Arora and Meltzer, 1989, Leonard, 2005, Mann et al., 1986, Pandey et al., 2002). Chronic ACTH treatment increases the expression of 5-HT_{2A} receptor mRNA in the frontal cortex in rats (Kitamura et al., 2008). Moreover, chronic treatment with ACTH disturbs the antidepressant effects of the tricyclic antidepressants 3-(5,6-dihydrobenzo[b][1]benzazepin-11-yl)-*N*,*N*-dimethylpropan-1-amine (imipramine) and 3-(5,6-dihydrobenzo[b][1]benzazepin-11-yl)-*N*-methylpropan-1-amine (desipramine) (Kitamura et al., 2002). ACTH-treated rats might exhibit a severe depressive state, in which the response to tricyclic antidepressants is impaired.

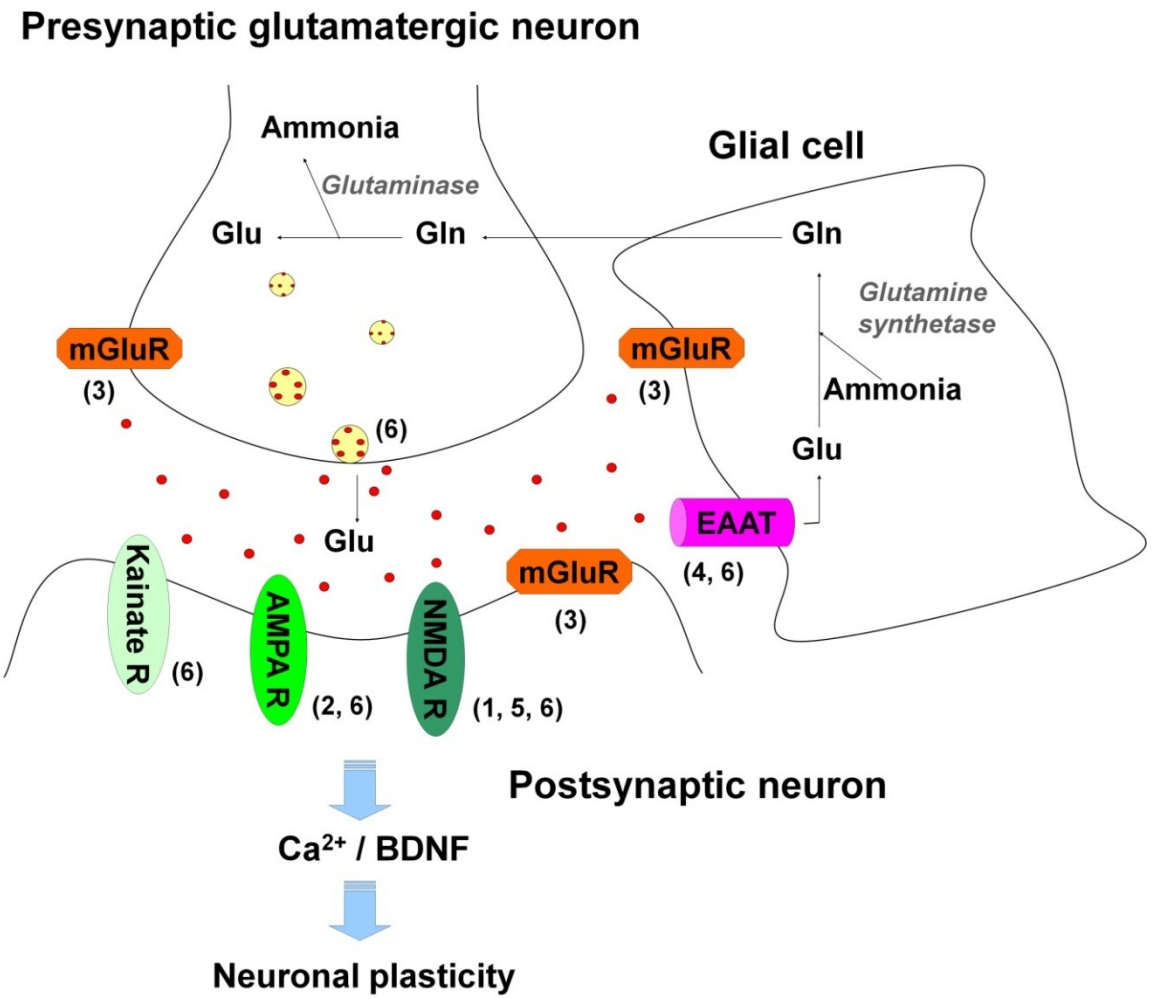

Fig. 1. Major functional components for glutamatergic neurons and potential targets of glutamatergic agents exerting antidepressant-like actions.
Glutaminase hydrolyzes glutamine to glutamate and ammonia in presynaptic neurons. Glutamate is released into the synaptic cleft and stimulates glutamate receptors (kainate receptors, NMDA receptors, AMPA receptors, and mGluRs) in postsynapses, presynapses, and glial cells. Glutamate is taken up by EAATs on glial cells. Glutamine synthetase converts glutamate and ammonia to glutamine, which is transported to presynaptic neurons. Glutamatergic agents are considered to act on the numbered targets in the Figure as follows targets: (1) NMDA receptor antagonists (ketamine, NR2B subunit antagonists, memantine, magnesium, and zinc); (2) positive modulators of AMPA; (3) group I mGluR antagonists, group II mGluR antagonists and agonists, and group III mGluR agonists; (4) EAAT2 enhancer (ceftriaxone); (5) possible indirect NMDA receptor modulator (minocycline); and (6) possible inhibitor of glutamate release, antagonist of NMDA, AMPA, and kainate receptors, and potentiator of glutamate uptake (riluzole). AMPA R, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor; NMDA R, N-methyl-D-aspartate receptor; mGluR, metabotropic glutamate receptor; EAAT, excitatory amino acid transporter; Gln, glutamine; Glu, glutamate; BDNF, brain-derived neurotrophic factor; Ca2+, calcium ion.

L-glutamic acid (glutamate) is accepted as the major excitatory neurotransmitter in the central nervous system (CNS), and glutamine synthesis from glutamate and ammonia occurs

exclusively in glial cells (Hashimoto, 2009, 2011, Tokita et al., 2012) (Fig. 1). Glutamine plays major roles in nitrogen and carbon homeostasis, and in the detoxification of ammonia, in addition to acting as a precursor for the synthesis of glutamate in specialized excitatory neurons (Hashimoto, 2009, 2011, Tokita et al., 2012) (Fig. 1). Glutamate released from presynaptic neurons can interact with postsynaptic glutamate receptors, including *N*-methyl-D-aspartate (NMDA) receptors. The released glutamate is taken up by the surrounding glial cells via excitatory amino acid transporters (EAATs), converted to glutamine, transported back to the presynaptic neurons, and reconverted to glutamate (Hashimoto, 2009, 2011, Tokita et al., 2012) (Fig. 1). There is growing evidence that glutamate levels are altered in blood, cerebrospinal fluid, and brain of patients with MDD (Auer et al., 2000, Block et al., 2009, Hashimoto, 2009, 2011, Hashimoto et al., 2007, Hasler et al., 2007, Sanacora et al., 2004, Tokita et al., 2012). Glutamate released from presynaptic neurons can interact with postsynaptic glutamate receptors such as NMDA receptors. The NMDA receptor has modulatory sites on its subunits (Hashimoto et al., 2005b, Martineau et al., 2006). Glycine is converted to $_{L}$-serine by serine hydroxymethyltransferase, and $_{L}$-serine is converted to $_{D}$-serine by serine racemase (Hashimoto et al., 2005b, Martineau et al., 2006). Endogenous glycine and $_{D}$-serine act as co-agonists on the glycine binding site on the NMDA receptor and co-activate NMDA receptors along with glutamate (Chen et al., 2003, Hashimoto et al., 2005b, Martineau et al., 2006, Yang and Svensson, 2008). The change of glycine and $_{D}$-serine levels in plasma and brain of patients with MDD has been investigated in a few studies, but no unified view has been obtained (Hashimoto et al., 2007, Mitani et al., 2006, Sumiyoshi et al., 2004).

Several clinical studies have reported that NMDA receptor antagonists showed rapid and/or sustained antidepressant effects in patients with treatment-resistant MDD (Berman et al., 2000, Preskorn et al., 2008, Zarate et al., 2006). In preclinical animal models, NMDA

receptor antagonists have been shown to exert antidepressant-like effects (Eby and Eby, 2010, Machado-Vieira et al., 2009, Nowak et al., 2005, Paul and Skolnick, 2003, Skolnick et al., 2009, Szewczyk et al., 2008, Tokita et al., 2012). A non-competitive NMDA receptor antagonist, 2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one (ketamine), decreased the immobility time in the forced swim test in rats (Engin et al., 2009; Garcia et al., 2008a; Garcia et al., 2008b; Li et al., 2010; Yilmaz et al., 2002) and mice (da Silva et al., 2010; Maeng et al., 2008; Rosa et al., 2003). Another non-competitive NMDA receptor antagonist, 3,5-dimethyladamantan-1-amine (memantine), also decreased immobility time in the forced swimming test, which is widely used for the screening of antidepressants, in rats and mice (Almeida et al., 2006, Moryl et al., 1993, Reus et al., 2010, Rogoz et al., 2002). Although confirmation of the antidepressant effects of NMDA receptor antagonists requires further investigation, direct targeting of NMDA receptor complexes may bring about rapid and relatively sustained antidepressant effects (Zarate et al., 2010, Zarate et al., 2006).

Here, we determined the concentrations of amino acids (glutamate, glutamine, glycine, $_{L}$-serine, and $_{D}$-serine) in the frontal cortex, hippocampus, striatum, and cerebellum of rats receiving repeated administration of ACTH. Moreover, we also evaluated behavioral effects of ketamine and memantine on immobility time in the forced swimming test and on locomotor activity in ACTH-treated rats.

2. Materials and methods

2.1. Animals

Male Wistar rats (Japan SLC, Inc., Hamamatsu, Shizuoka, Japan) were used for all experiments at age 9 weeks. The numbers of animals per group were set to have the sufficient statistical power of 80%. All animals were given food and water ad libitum. They were housed in an air-conditioned room at a temperature of 23±2 °C and humidity of 55±10% under a 12:12-h light/dark cycle, with lights on at 7:30 a.m. Rats were acclimated to the environment for 1 week before the experiments began. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Astellas Pharma Inc. The Tsukuba Research Center of Astellas Pharma Inc. has been awarded Accreditation Status by AAALAC International.

2.2. Drugs

Synthesized adrenocorticotropic hormone (Cortrosyn® Z, tetracosactide acetate, ACTH (1-24)) was purchased as a suspension in vials from Daiichi Sankyo Co., Ltd, Tokyo, Japan. Imipramine hydrochloride was purchased from Sigma-Aldrich Co. LLC., St. Louis, MO, USA. Ketamine hydrochloride (ketalar®) was purchased from Daiichi Sankyo Co., Ltd, Tokyo, Japan. Memantine hydrochloride was purchased from Tocris Bioscience, Bristol, UK. Study rats received subcutaneous injection of ACTH suspension at 100 μg in a volume of 200 μL per day for 14 days. Control rats received saline instead of ACTH. Imipramine, ketamine, and memantine were dissolved in saline and intraperitoneally injected at 5 mL/kg body weight. Imipramine was administered at 15 mg/kg, ketamine was administered at 20 mg/kg, and memantine was administered at 10 mg/kg.

2.3. Measurement of amino acids in brain regions

One day after the last administration of ACTH, rats were anaesthetized with 2% isoflurane and then euthanized by exsanguination. The brain was removed and the frontal

cortex, hippocampus, striatum, and cerebellum were dissected on ice. Tissues were frozen in liquid nitrogen and stored at -80 °C until measurement.

Tissues were homogenized in 1.5 mL of methanol (HPLC grade) on ice. The homogenates were centrifuged at 3000g for 6 min at 4 °C, and 20 μL of supernatant was evaporated to dryness at 40 °C. To the residue, 20 μL of H_2O (HPLC grade), 20 μL of 0.1 M borate buffer (pH 8.0), and 60 μL of 50 mM 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F; Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) in CH_3CN (HPLC grade) were added. The reaction mixture was then heated to 60 °C for 2 min, and immediately supplemented with 100 μL of H_2O/acetonitrile (90/10) containing 0.1% trifluoroacetic acid (TFA) to stop the reaction.

Total $_D$- and $_L$-serine levels were measured using a column-switching high performance liquid chromatography (HPLC) system (Shimadzu Corporation, Kyoto, Japan), as previously reported (Fukushima et al., 2004, Hashimoto et al., 2007, Horio et al., 2011, Yamada et al., 2005). Glycine, glutamine, and glutamate were measured using an HPLC system with fluorescence detection as previously reported (Hashimoto et al., 2005a, Horio et al., 2011). A 20 μL aliquot of the resulting solution was injected into the HPLC system. A reversed-phase ODS column (TSKgel ODS-80Ts (Tosoh Corporation, Tokyo, Japan) as Column 1) was used for the separation and quantification of total $_D$- and $_L$-serine, and the gradient elution of the mobile phase was maintained at a constant flow rate of 0.8 mL/min. Mobile phase 1a consisted of H_2O/acetonitrile (90/10) containing 0.1% TFA, and phases 1b and 1c of H_2O/ acetonitrile (10/90) containing 0.1% TFA and acetonitrile, respectively. The time program for gradient elution was as follows: 0–25 min 1a:1b:1c =92:8:0, 25–25.1 min linear gradient from 8% 1b to 100% 1b, 25.1–35 min 1a:1b:1c =0:100:0, 35–35.1 min linear gradient from 0% 1c to 100% 1c, 35.1–40 min 1a:1b:1c =0:0:100, 40–40.1 min linear gradient from 0% 1b to 8% 1b, and 40.1–60 min 1a:1b:1c =92:8:0. The chiral column (Column 2) used for the separation and quantification of $_D$- and $_L$-serine with NBD-F consisted of two Sumichiral OA-2500

columns (S) (Sumika Chemical Analysis Service Ltd., Osaka, Japan), which were connected in tandem. The mobile phase was 15 mM citric acid in methanol. Flow rate was isocratically pumped at 1.0 mL/min, and column temperature was maintained at 35 °C for all columns. Fluorescence detection was performed at 530 nm with an excitation wavelength of 470 nm. Glycine, glutamine, and glutamate were determined using a reversed-phase ODS column (TSKgel ODS-80Ts, Tosoh Corporation, Tokyo, Japan). The gradient elution of the mobile phase was kept at a constant flow rate of 0.8 mL/min. The time program for gradient elution was programmed as follows: 0–50.5 min 1a:1b:1c =95:5:0, 50.5–55.5 min 1a:1b:1c =0:100:0, and 55.5–57 min, 1a:1b:1c =0:0:100. All column temperatures were maintained at 35 °C. Fluorescence detection was performed at 530 nm with an excitation wavelength of 470 nm.

2.4. Forced swimming test

The forced swimming test was performed according to Porsolt's method (Castagne et al., 2011). A rat was placed in an opaque cylinder (20 cm diameter, 40 cm height) containing water (25 cm height, at 24±1 ºC) equipped with a coil current detector near the water surface around the cylinder (MicroAct®, Neuroscience, Inc., Tokyo, Japan). The coil current generated by the movement of magnets attached to both forepaws of a rat was detected as activity of the rat in seeking to escape from the water in the cylinder. The coil currents were sampled and analyzed according to the following parameters. The range of amplified and transformed voltage was set from 0.05 V to 2.5 V. The range of frequency was set from 7 Hz to 15 Hz. The criterion for the splitting of events was set to over 0.2 s. The criterion of a valid event was set to over 0.35 s. In the pre-test session, rats were forced to swim for 15 min one day after the last administration of ACTH in the case of repeatedly ACTH-treated rats. Imipramine, ketamine, and memantine were administered at 15 min after the end of this swimming. In the test session, imipramine and ketamine were administered 5 h and 30 min before the test, or memantine was administered 2 h before the test. Rats were forced to swim

at 24.5 h after the pre-test for 5 min. Immobility time in the test session was obtained as the total time (300 s) minus the time in which the rat sought to escape from the water.

2.5. Locomotor activity

Activity of a rat was measured in a plastic cage (20.5 cm width, 37.5 cm depth, 20.3 cm height) equipped with a passive infrared sensor placed on the board over it (Supermex, Muromachi Kikai Co., Ltd., Tokyo, Japan). The movement of the rat was counted with pulse data obtained from the sensor as locomotor activity. Rats were evaluated one day after the last administration of ACTH. They were placed in plastic cages and their activities were measured for 30 min during exposure to the novel environment, beginning 30 min after the administration of imipramine and ketamine, or 2 h after that of memantine.

2.6. Statistical analysis

All results are expressed as the mean ± SE. Statistical analysis was conducted using two-way analysis of variance (ANOVA) followed by post hoc Bonferroni's multiple comparison test for evaluation of the effect of imipramine, ketamine, and memantine with or without ACTH treatment on immobility time in the forced swimming test and on locomotor activity. Student’s t test was used to evaluate the effect of ACTH on levels of amino acids in brain regions. $P<0.05$ was considered statistically significant. All statistical analyses were conducted using Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) and the PASW Statitics 20 (formerly SPSS Statistics; SPSS, Tokyo, Japan).

3. Results

Amino acid levels were determined in brain regions of ACTH-treated rats (Table 1). Levels of glutamine ($t=8.887$, $P<0.0001$), glycine ($t=6.194$, $P=0.0001$), $_{L}$-serine ($t=4.374$, $P=0.0014$), and $_{D}$-serine ($t=2.585$, $P=0.0272$) were increased in the hippocampus of ACTH-treated rats compared to control rats (Table 1). In the cerebellum, glutamate ($t=4.687$, $P=0.0009$), glutamine ($t=6.606$, $P<0.0001$), glycine ($t=5.967$, $P=0.0001$), $_{L}$-serine ($t=8.802$, $P<0.0001$), and $_{D}$-serine ($t=6.326$, $P<0.0001$) were increased (Table 1). In addition, glutamine and glycine were increased in the frontal cortex and striatum (glutamine in frontal cortex: $t=5.599$, $P=0.0002$, glycine in the frontal cortex: $t=4.042$, $P=0.0024$, glutamine in the striatum: $t=5.631$, $P=0.0002$, glycine in striatum: $t=3.604$, $P=0.0048$) (Table 1). Glutamate/glutamine ratios were decreased in the frontal cortex ($t=3.870$, $P=0.0031$), hippocampus ($t=8.415$, $P<0.0001$), and striatum ($t=6.453$, $P<0.0001$) of ACTH-treated rats (Table 1).

Table 1. Effect of repeated ACTH administration (100 μg/rat/day, sc, 14 days) on levels of glutamate, glutamine, glycine, $_{L}$-serine, and $_{D}$-serine (nmol/ mg tissue) in the frontal cortex, hippocampus, striatum, and cerebellum in rats

	Glutamate	Glutamine	Glycine	$_{L}$-serine	$_{D}$-serine	Glutamate /Glutamine (ratio)
Frontal cortex						
Control	8.922 ± 0.556	3.784 ± 0.227	0.557 ± 0.034	0.5128 ± 0.0273	0.2031 ± 0.0112	2.362 ± 0.077
ACTH	10.186 ± 0.169	5.222 ± 0.121***	0.704 ± 0.014**	0.5675 ± 0.0072	0.2055 ± 0.0026	1.958 ± 0.070**
Hippocampus						
Control	6.999 ± 0.070	3.429 ± 0.048	0.556 ± 0.008	0.4910 ± 0.0115	0.1806 ± 0.0055	2.043 ± 0.034
ACTH	7.235 ± 0.114	4.349 ± 0.092***	0.647 ± 0.012***	0.5493 ± 0.0068**	0.1958 ± 0.0020*	1.666 ± 0.029***
Striatum						
Control	7.391 ± 0.155	4.245 ± 0.164	0.481 ± 0.015	0.5100 ± 0.0152	0.1961 ± 0.0051	1.749 ± 0.046
ACTH	7.279 ± 0.149	5.313 ± 0.095***	0.567 ± 0.018**	0.5480 ± 0.0164	0.1963 ± 0.0060	1.372 ± 0.036***
Cerebellum						
Control	5.296 ± 0.096	3.157 ± 0.058	0.406 ± 0.009	0.3723 ± 0.0093	0.0039 ± 0.0000	1.678 ± 0.021
ACTH	5.837 ± 0.065***	3.614 ± 0.038***	0.475 ± 0.007***	0.4877 ± 0.0093***	0.0047 ± 0.0001***	1.617 ± 0.030

Data are expressed as the mean ± SE. Six animals were used for each group. *p<0.05, **p<0.01, ***p<0.001 compared to control group (Student's t test). ACTH: adrenocorticotropic hormone

The effects of imipramine, ketamine, and memantine with or without ACTH treatment were evaluated in the forced swimming test. A two-way ANOVA revealed the effect of ACTH [$F(1, 64)= 89.40$; $p<0.0001$], drugs [$F(3, 64)= 26.27$; $p<0.0001$], and interaction between drugs and ACTH [$F(3, 64)= 6.618$; $p=0.0006$]. Post hoc analysis showed that acute imipramine treatment significantly ($P<0.0001$) decreased immobility time in the control groups (Fig. 2). Acute ketamine treatment significantly ($P<0.0001$) decreased immobility time in the control groups (Fig. 2). Acute memantine treatment significantly ($P<0.0001$) decreased immobility time in the control groups (Fig. 2). Repeated ACTH treatment significantly ($P<0.01$) increased immobility time compared to the vehicle control group (Fig. 2). However no change was seen with imipramine treatment in the ACTH-treated groups ($P>0.05$) (Fig. 2). No change was also seen with ketamine treatment in the ACTH-treated groups ($P>0.05$) (Fig. 2). Memantine treatment significantly ($P<0.001$) decreased immobility time in the ACTH-treated groups (Fig. 2).

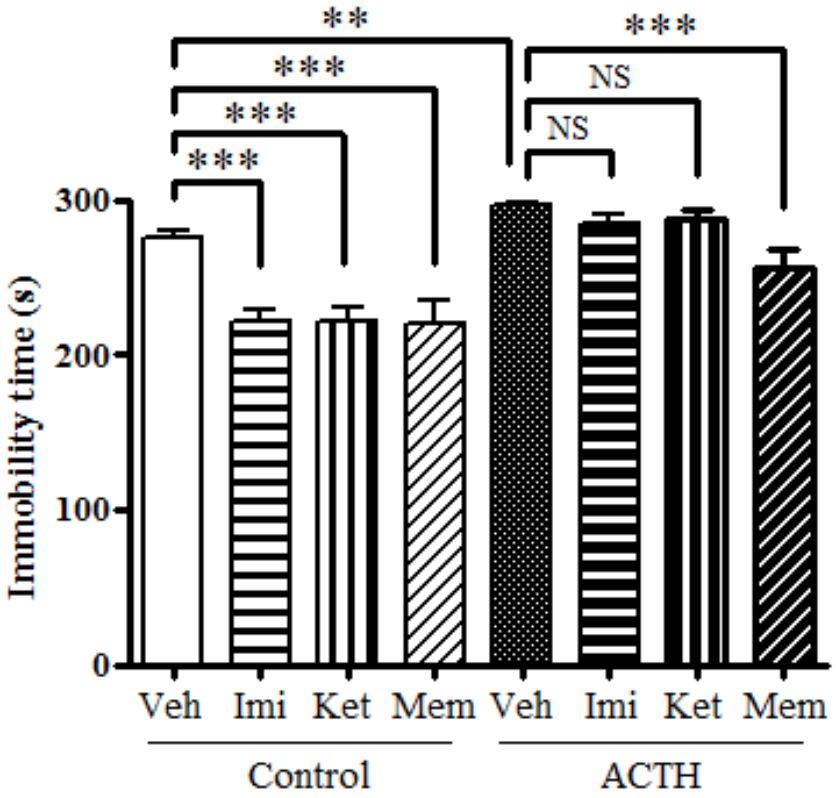

Fig. 2. Effects of imipramine, ketamine, and memantine with or without ACTH treatment on immobility time in the forced swimming test in rats
ACTH (100 μg/rat/day, sc) was injected for 14 days. The following day the forced swimming test was performed. Imipramine (15 mg/kg, ip) and ketamine (20 mg/kg, ip) were administered 15 min after the end of swimming in the pre-test session, 5 h before the test session, and 30 min before the test session. Memantine (10 mg/kg, ip) was administered 15 min after the end of swimming in the pre-test session and 2 h before the test session. Data are expressed as the mean ± SE. Eighteen animals were used for each vehicle group in control and ACTH treatment, and six animals were used for each other group. **p<0.01, ***p<0.001; statistically significant by Two-way ANOVA followed by post-hoc test. NS: not significant. ACTH: adrenocorticotropic hormone. Veh: vehicle. Imi: imipramine. Ket: ketamine. Mem: memantine.

The effects of imipramine, ketamine, and memantine with or without ACTH treatment on locomotor activity were evaluated. A two-way ANOVA revealed no effect of ACTH [$F(1, 88)= 0.2193$; $p=0.6407$] but drugs [$F(3, 88)= 22.58$; $p<0.0001$], and interaction between drugs and ACTH [$F(3, 88)= 26.27$; $p<0.0001$]. Post hoc analysis showed that acute imipramine treatment significantly ($P<0.0001$) decreased locomotor activity in the control groups (Fig. 3). Acute ketamine treatment significantly ($P<0.0001$) decreased locomotor activity in the control groups (Fig. 3). Acute memantine treatment significantly ($P<0.001$) decreased locomotor

activity in the control groups (Fig. 3). ACTH treatment significantly (P<0.0001) decreased locomotor activity compared to the vehicle control group (Fig. 3). Imipramine treatment significantly (P<0.01) decreased locomotor activity in the ACTH-treated groups (Fig. 3). However no change was seen with ketamine treatment in the ACTH-treated groups (P>0.05) (Fig. 3). Memantine treatment significantly (P<0.0001) increased locomotor activity in the ACTH-treated groups (Fig. 3).

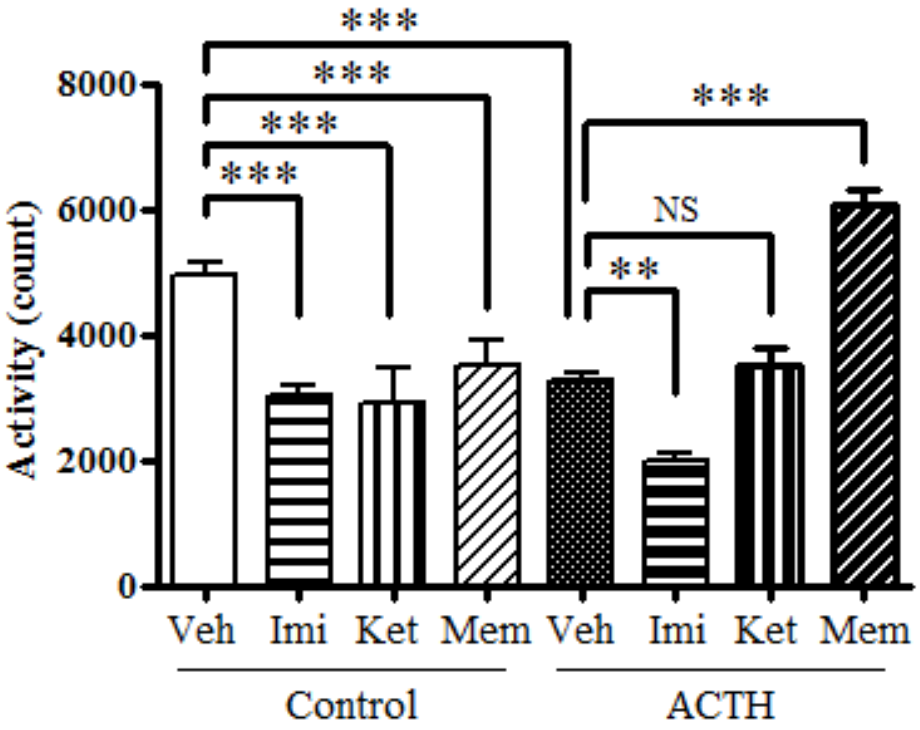

Fig. 3. Effects of imipramine, ketamine, and memantine with or without ACTH treatment on locomotor activity in rats
ACTH (100 μg/rat/day, sc) was injected for 14 days. The following day locomotor activity was measured. Imipramine (15 mg/kg, ip) and ketamine (20 mg/kg, ip) were administered 30 min before the test. Memantine (10 mg/kg, ip) was administered 2 h before the test. Data are expressed as the mean ± SE. Twenty four animals were used for each vehicle group in control and ACTH treatment, and eight animals were used for each other group. **p<0.01, ***p<0.001; statistically significant by Two-way ANOVA followed by post-hoc test. ACTH: adrenocorticotropic hormone. Veh: vehicle. Imi: imipramine. Ket: ketamine. Mem: memantine.

4. Discussion

In this study, we found that glutamate levels were increased in the cerebellum of ACTH-treated rats, that glycine was increased in the frontal cortex, hippocampus, and cerebellum of these rats, and that D-serine was increased in the hippocampus and cerebellum. ACTH treatment increased immobility time in the forced swimming test and decreased locomotor activity in rats. Ketamine did not show any effects in these behavioral tests in ACTH-treated rats. On the contrary, memantine decreased immobility time in the forced swimming test and increased locomotor activity in ACTH-treated rats. These findings suggest that chronic ACTH treatment might change the glutamatergic response blocked by memantine but not ketamine.

Little is known about the role of ACTH specifically in the glutamatergic response to HPA axis activation. A major finding of our present study is that repeated ACTH treatment affected amino acid levels in rat brain since these amino acids are known to affect the neurotransmission via the NMDA receptor (Hashimoto et al., 2007). Glutamate was increased in the cerebellum (Table 1). Glycine was increased in the frontal cortex, hippocampus, striatum, and cerebellum of ACTH-treated rats (Table 1). D-serine was increased in the hippocampus and cerebellum of rats treated with ACTH (Table 1). These elevations of glutamate, glycine, and D-serine are thought to augment the activities of NMDA receptors through their agonist or co-agonist activities. The melanocortin receptor subtypes that ACTH binds to are rarely expressed in brain (Millington, 2006). Further investigation is necessary to clarify the mechanism through that ACTH treatment affected these amino acid levels in brain.

Glutamine is a precursor for the synthesis of glutamate in specialized excitatory neurons (Hashimoto, 2009, Hashimoto et al., 2005b) (Fig. 1). Released glutamate is taken up by glia, where it is converted to glutamine by glutamine synthetase, transported back to the presynaptic neuron, and reconverted to glutamate by glutaminase (Hashimoto, 2009,

Hashimoto et al., 2005b) (Fig. 1). Thus, the glutamate-glutamine cycle plays a role in neuron-glia communication in the synapse (Hashimoto, 2009, Hashimoto et al., 2005b) (Fig. 1). The pathophysiology of MDD likely involves abnormalities in glutamate-glutamine cycling in the brain (Hashimoto, 2009, Valentine and Sanacora, 2009). Chronic ACTH treatment induced an increase in glutamine and decrease in the ratio of glutamate/glutamine in the frontal cortex, hippocampus, and striatum (Table 1). The glutamate-glutamine cycle between neurons and glia cells could have been changed by repeated ACTH treatment. Especially increasing glutamine level might be caused by some kind of glial change, which warrants further investigation about the effect of ACTH treatment on glia. Glial fibrillary acidic protein (GFAP), a specific marker for astrocytes, was reduced in cerebellum of patients with MDD (Fatemi et al., 2004). The expression levels of glutamine synthetase were decreased or unchanged in some brain regions of MDD patients (Beasley et al., 2006, Choudary et al., 2005, Karolewicz et al., 2009, Miguel-Hidalgo et al., 2010). The role of glia cells in the pathophysiology of MDD should be studied more.

Repeated treatment with ACTH induced significant increases in immobility time in the forced swimming test (Fig. 2). Kitamura and colleagues reported that ACTH treatment for 14 days did not affect immobility time in the rat forced swimming test (Kitamura et al., 2002). The reason for this discrepancy between our present data and Kitamura's data is unclear, but may involve differences in breeding conditions during ACTH treatment or in experimental procedures in the forced swimming test. The ACTH-induced increases in immobility time might have been at least in part due to decreases in locomotor activity (Fig. 3). Acute administration of ACTH dose-dependently decreased locomotor activity in rats pre-treated with dexamethasone (Reddy and Kulkarni, 1998). In contrast, intracerebroventricular administration of prepro-thyrotropin releasing hormone (TRH) 178-199, a peptide with ACTH release-inhibiting properties, induced an increase in locomotor activity in rats

(McGivern et al., 1997). Wistar Kyoto (WKY) rats, a line derived from Wistar rats, are a genetic animal model that exhibits depressive behaviors (Malkesman and Weller, 2009). WKY lines exhibited significantly longer immobility time in the forced swimming test and higher levels of plasma ACTH than a control line of Wistar rats (Malkesman et al., 2006). Among WKY, Lewis, and Sprague-Dawley (SD) rats, acute immobilization stress-induced increase in plasma ACTH was most enhanced in WKY rats (Pardon et al., 2002). This strain also exhibited the lowest level of locomotor activity among the three in a novel open-field environment (Pardon et al., 2002), and showed reduced locomotor activity in a novel environment and high ACTH levels in plasma compared to Brown-Norway (BN) rats regardless of age (Tizabi et al., 1992). Furthermore, prepubertal WKY rats exhibited higher levels of anxiety behavior, such as freezing behavior, than controls (Malkesman et al., 2005). These findings may indicate that low locomotor activity is observed in certain exacerbated depressive conditions and connected to disturbance of the HPA axis. Given the difficulty in discriminating depressive or anxiety behavior from generalized motor impairment, the present results point to the possibility that the chronic activation of the HPA axis induced by repeated ACTH treatment produces the depressive-like state and/or motoric change in rats.

The cerebellum is responsible for motor coordination. All amino acids measured in the present study were increased in the cerebellum of rats treated by ACTH (Table 1). Chronic ACTH treatment might modify motoric function in the cerebellum. Magnetic resonance imaging (MRI) studies reported that structural deficits in the cerebellum were associated with depressive symptoms (Escalona et al., 1993, Lin et al., 2012, Liu et al., 2010, Pillay et al., 1997). Notably, the volume of cerebellum was decreased in the MDD patients who had not responded to the treatment with *N*-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine (fluoxetine), a selective serotonin reuptake inhibitor (SSRI) (Pillay et al., 1997). GFAP was reduced in the cerebellum

of patients with MDD (Fatemi et al., 2004). Focusing on the role of the cerebellum may become important in characterizing the pathophysiology of refractory MDD.

We evaluated the effects of imipramine on immobility time in the forced swimming test and on locomotor activity to confirm that an antidepressant works in our behavioral models. In control rats, imipramine decreased immobility time in the forced swimming test (Fig. 2), and also decreased locomotor activity (Fig. 3). A number of experiments have shown that imipramine decreases locomotor activity in rodents (Diniz et al., 2011, Hughes and Pither, 1987, Martin et al., 1982, Meltzer and Fox, 1971, Teixeira et al., 2000). At least, the imipramine-induced decrease in immobility time did not stem from any increase in locomotor activity. Therefore, the decreased immobility time is considered to be due to its antidepressant effect. In ACTH-treated rats, imipramine did not show any change in the forced swimming test (Fig. 2) while imipramine decreased locomotor activity (Fig. 3). The motoric modification by imipramine is thought to have insignificant impact on the immobility time in the forced swimming test. Repeated ACTH treatment for 14 days negated the antidepressant effect of imipramine, as was also reported by Kitamura and colleagues (Kitamura et al., 2002, Kitamura et al., 2008). Acute imipramine treatment reduced immobility time in the forced swimming test in BN and SD rats but not in WKY rats (Lahmame et al., 1997). ACTH-treated rats appear to resemble WKY rats in some respects, including a poor response to imipramine. The levels of amino acids were altered in brain regions of ACTH-treated rats (Table 1). However, further exploration is needed to elucidate a link between changing amino acid levels and masking the antidepressant effect of imipramine.

Ketamine decreased the immobility time in the forced swim test in control rats as is the case with results in several laboratories (Engin et al., 2009; Garcia et al., 2008a; Garcia et al., 2008b; Li et al., 2010; Yilmaz et al., 2002) (Fig. 2), and decreased locomotor activity (Fig. 3). Ketamine was thought to exert its antidepressant-like effect despite its motoric effects.

However, ketamine did not affect immobility time in the forced swimming test in rats after chronic ACTH treatment just as imipramine did not (Fig. 2). The antidepressant-like effect of ketamine seems to have disappeared like that of imipramine. No effect of ketamine was also seen on locomotor activity in ACTH-treated rats although imipramine even show the decreasing effect on locomotor activity after ACTH-treatment (Fig. 3). Chronic ACTH treatment might strongly inhibit the behavioral effects of ketamine, however the reason is unknown.

Consistent with previous observations (Moryl et al., 1993, Reus et al., 2010, Rogoz et al., 2002), we found that memantine decreased immobility time in the forced swimming test in control rats (Fig. 2), and decreased locomotor activity (Fig. 3). The motoric effect of memantine appears to be limited on immobility time in the forced swimming test in control rats. Namely, memantine demonstrated its antidepressant-like effect in control rats as well as imipramine did. Additionally, memantine showed the decreasing effect on immobility time in the forced swimming test in rats treated with ACTH for 14 days where imipramine did not (Fig. 2). However, memantine showed the increasing effect on locomotor activity in ACTH-treated rats (Fig. 3). The memantine-induced activity might reflect the decreasing effect on immobility time in the forced swimming test. Memantine countered the increase in immobility time (Fig. 2) and the decrease in locomotor activity (Fig. 3) induced by ACTH-treatment. Remarkably, memantine increased locomotor activity in ACTH-treated rats while decreasing it in the control rats (Fig. 3). The likely explanation is that memantine improved the motoric disturbance via inhibiting NMDA receptor activation enhanced by increase in levels of agonists and co-agonists for NMDA receptors. That is to say, NMDA receptors have relevant to the motoric disturbance induced by repeated ACTH treatment. Recently, memantine administration showed the increase in locomotor activity in the 7-methoxy-1-methyl-3,4-dihydro-2H-pyrido[3,4-b]indole (harmaline)-treated rats, which are

thought to be a model of transient action tremor with locomotor activity decreased (Iseri et al., 2011). Furthermore, memantine prevented the harmaline-induced neurodegeneration in the cerebellum. Memantine can improve the locomotor deficits via blocking the injury in cerebellum. In the cerebellum, both of agonists and co-agonists for NMDA receptors were increased (Table 1). These findings indicate that activating NMDA receptors in the cerebellum may account for the locomotor deficit in ACTH-treated rats.

Table 2. Summary of behavioral effects of imipramine, ketamine, and memantine with or without ACTH treatment

	Control rats		ACTH-treated rats	
	Immobility time	Activity	Immobility time	Activity
ACTH	n/a	n/a	↑	↓
Imipramine	↓	↓	↔	↓
Ketamine	↓	↓	↔	↔
Memantine	↓	↓	↓	↑

n/a: not applicable.

Two NMDA receptor antagonists acted differently in ACTH-treated rats in spite of the same effect in control rats. Memantine counteracted the effects of ACTH in the forced swimming test and locomotor activity (Table 2). Conversely, the effects of ketamine were cancelled by ACTH treatment (Table 2). Memantine and ketamine can bind at the deep site of NMDA receptors however memantine, but not ketamine, can bind at the superficial site (Kotermanski et al., 2009). Memantine is a strong voltage-dependent antagonist but ketamine is a less voltage-dependent one (Gilling et al., 2009). A randomized, double-blind,

placebo-controlled, three-way crossover trial in healthy male volunteers showed that ketamine significantly increased serum prolactin and cortisol levels, whereas memantine and placebo did not affect hormone levels (Hergovich et al., 2001). Cold water swim stress reduced the antiseizure efficacies of MK-801 and memantine without affecting phencyclidine and ketamine in mice (Deutsch et al., 1997). Adaptive changes in the NMDA receptor complex is considered to occur in response to exposure to stress, however stress does not result in a simple reduction in the number of activated or open channels, but rather may alter their size or charge characteristics (Deutsch et al., 1997). Thus, memantine and ketamine have different properties about the binding with NMDA receptors, effects on hormone levels, and antiseizure effects in a stressful situation. In the present study, ACTH treatment might plasticize NMDA receptors, to which memantine but not ketamine could interact. However, further investigation is necessary.

The cerebellum regulates mood and emotion other than balance and motor control (Baldacara et al., 2008, Konarski et al., 2005, Schmahmann et al., 2007). Co-agonists were also increased in hippocampus and frontal cortex of ACTH-treated rats (Table 1). It is of interest whether ACTH-treated rats have affective abnormality. We have tried to evaluate anhedonia-like behavior with the sucrose preference test in ACTH-treated rats. However, we failed to measure sucrose preference because ACTH-treated rats showed increase in the total consumption of water and sucrose solution (data not shown). Other good methods would be demanded to evaluate affective behavior in ACTH-treated rats. Importantly, the low response rate to existing antidepressants is an area of unmet medical need (Little, 2009, Shelton et al., 2010). Given the interest in functional proteins related to glutamate signal transduction as potential targets for a new generation of antidepressants (Hashimoto, 2009, 2011, Tokita et al., 2012), ACTH-treated animals may be useful to assess glutamatergic effects of other types of compounds with possibly higher efficacy than existing antidepressants.

In summary, this study showed that glutamate levels were increased in the cerebellum of ACTH-treated rats, that glycine was increased in the frontal cortex, hippocampus, and cerebellum of these rats, and that $_{D}$-serine was increased in the hippocampus and cerebellum. ACTH treatment increased immobility time in the forced swimming test and decreased locomotor activity in rats. Ketamine did not show any effects in these behavioral tests in ACTH-treated rats. On the contrary, memantine decreased immobility time in the forced swimming test and increased locomotor activity in ACTH-treated rats. Taken together, these findings suggest that chronic ACTH treatment alters the glutamatergic neurotransmission in rat brain, and that depressive-behaviors after chronic ACTH treatment could be blocked by memantine but not ketamine.

Index

This doctoral dissertation was written based on the following articles published in peer-reviewed scholarly journals.

Main article (an original article):

Tokita K, Fujita Y, Yamaji T, Hashimoto K.

Depressive-like behavior in adrenocorticotropic hormone-treated rats blocked by memantine.

Pharmacol Biochem Behav. 2012;102(2):329-34.

Relevant article (a review article):

Tokita K, Yamaji T, Hashimoto K.

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