Aripiprazole and haloperidol suppress excessive dopamine release in the amygdala in response to conditioned fear stress, but show contrasting effects on basal dopamine release in methamphetamine-sensitized rats

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ABSTRACT

Although emotional dysfunction in patients with schizophrenia is thought to be associated with poorer outcomes in terms of overall quality of well-being, only a few basic studies have examined the biochemical effect of antipsychotics on emotional function. In this investigation, we examined differences in the effects of aripiprazole and haloperidol on the conditioned fear response in methamphetamine-sensitized and fear-conditioned rats in an *in vivo* microdialysis study. Aripiprazole is the first antipsychotic drug with an action involving partial dopamine D_2 receptor agonism, thus differing from haloperidol, a typical antipsychotic that shows selective dopamine D_2 receptor full antagonism. After exposure to a conditioned stimulus, methamphetamine-sensitized rats exhibited significantly higher dopamine release in the amygdala than unsensitized rats. We considered this hypersensitivity of dopamine release to be a biochemical marker of hypersensitivity and vulnerability to stress in psychosis. In the present study, we found that aripiprazole and haloperidol equally suppressed the marked increase in extracellular dopamine levels in fear-conditioned rats, whereas haloperidol increased and aripiprazole decreased tonic dopamine levels. In conclusion, the effect of an antipsychotic drug is likely to be involved in attenuation of the phasic increase in dopamine associated with the fear response, at least in the amygdala. In addition, the contrasting effects of haloperidol and aripiprazole on tonic dopamine levels in the amygdala are likely due to the difference in their actions (selective dopamine D_2 receptor full antagonist vs. partial agonist, respectively).

Key words:

Dopamine, amygdala, fear conditioning, methamphetamine, antipsychotic agents,

in vivo microdialysis, aripiprazole, haloperidol

1. Introduction

Although it has been suggested that symptoms of emotional dysfunction in patients with schizophrenia are associated with poorer outcomes in terms of overall quality of well-being (Lysaker and Salyers, 2007; Wetherell et al., 2003), no basic study has examined the biochemical effect of antipsychotics on the fear response of a neurotransmitter (i.e. dopamine).

To examine the reaction to emotional stress, conditioned fear stress has been developed as a form of psychological stress based on classical conditioning theory (Fanselow, 1980). Since this method involves no physical invasiveness for imposition of the conditioned stimulus, response can be directly related to emotional changes, thereby making this model suitable for the study of reactions to psychological stress. The amygdala is known to be one of the most potent modulators of the mechanisms responsible for the emotional memory system (LeDoux, 1993a, b). The central nucleus of the amygdala is integral to the acquisition and expression of emotional memory, whereas the basolateral amygdala leads to conditioned or primary reinforcers (Everitt et al., 1999; Simmons et al., 2007). In addition, the amygdala is a site of dopaminergic innervation (Oades and Halliday, 1987), and dopamine afferents are particularly dense within the intercalated cell masses, basolateral nucleus and central nucleus of the amygdala (Asan, 1998; Brinley Reed and McDonald, 1999; Fallon and Moore, 1978; Marowsky et al., 2005).

Methamphetamine-induced sensitization (reverse tolerance phenomenon) in rats has been widely and successfully used as an animal model of stimulant-induced psychosis and schizophrenia in terms of the paranoid psychotic state and its vulnerability to relapse (Robinson and Becker, 1986; Sato et al., 1992; Seiden et al., 1993). This animal model is analogous to human schizophrenia in that the animals show disruption of prepulse inhibition

(Tenn et al., 2005); blocking of sensitization by antipsychotics (Karler et al., 1990); and decreased somal volume, length of spine density, dendrites, and terminals of prefrontal cortical pyramidal neurons in layer II/IIIs (Selemon et al., 2007). Methamphetamine-sensitized animals show significantly higher extracellular dopamine release in the amygdala than unsensitized rats after exposure to a conditioned stimulus (Suzuki et al., 2002). This hypersensitivity of dopamine release is considered to be a biochemical marker of hypersensitivity and vulnerability to stress in psychosis (Suzuki et al., 2002). Interestingly, abnormal responsiveness of central dopamine neurons to stress has been proposed to play a role in the expression and exacerbation of symptoms associated with schizophrenia (Finlay and Zigmond, 1997).

Thus, in order to investigate the pharmacological effect of antipsychotics on the emotional component of psychosis, it would be of great value to compare the effect of the selective dopamine D_2 receptor full antagonist haloperidol with that of the D_2 receptor partial agonist aripiprazole on extracellular dopamine level in the amygdala of methamphetamine-sensitized rats after fear stress.

2. Materials and methods

2-1. Animals

Male Sprague-Dawley rats (Japan CLEA, Tokyo, Japan) weighing 180–190 g at the beginning of the experiment and 290–420 g at the time of microdialysis were used. The animals were kept at constant room temperature (26±2℃ with a 12-h light–dark cycle (dark from 20:00 h) and free access to water and food. All procedures were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Tokyo Women's Medical University School of Medicine Animal Experiments and Ethics Committee.

2-2. Drugs

Methamphetamine hydrochloride was purchased from Dainippon Sumitomo Pharmaceutical (Osaka, Japan); aripiprazole was kindly donated by Otsuka Pharmaceutical Company (Tokushima, Japan); haloperidol was purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals and reagents were the purest available commercially and were purchased from Wako Pure Chemical Industries (Osaka, Japan). Haloperidol was dissolved at 0.5 mg/ml in lactate and physiological saline. Aripiprazole was dissolved at 5 mg/ml in 1 mol/l hydrochloric acid and *N,N*-dimethylformamide and physiological saline. The pH of these solutions was controlled to between 5.0 and 6.0 by addition of 1 mol/l sodium hydroxide solution.

2-3. Methamphetamine sensitization and surgical implantation of cannulae (days 1 – 11)

Methamphetamine was administered to rats using a previously reported method to produce a reverse tolerance model; this method has been confirmed to induce behavioral sensitization upon re-challenge with methamphetamine (Tanaka et al, 1998). Methamphetamine hydrochloride dissolved in saline (2 mg/ml) was injected subcutaneously for 10 days at 2 mg /kg /day (methamphetamine groups; MP). Control groups were given an equivalent volume of physiological saline (saline groups; Sal). Following sensitization on day 11, a guide cannula was inserted using a stereotaxic frame (Model 900, David Kopf Instruments, California, USA) into the left amygdala at a point 2.4 mm posterior and 4.4 mm lateral to the bregma, and at a depth of 7.2 mm from the surface of the bone at the bregma (Paxinos and Watson, 1997). Pentobarbital anesthesia (50 mg/kg, mean body weight at surgery: 330 g) was used during the procedure. For the stereotaxic procedure, an ear bar with dulled tips was used for anchoring to avoid damage to the rats' eardrums. After surgery, the rats were transferred to their individual home cages (opaque sided, 30 cm high, 25 cm wide, 15 cm deep).

2-4. Fear stress conditioning protocol (days 12-14)

Fear conditioning was performed once a day for 3 consecutive days, starting from the day after insertion of the guide cannula. As rats did not show any motor or sensory abnormalities and showed successful fear conditioning, the time course of this method did not influence the study results.

Fear conditioning was performed by transferring the rats from their home cages to stimulation cages (clear sided, height 45 cm, width 22 cm, depth 22 cm) in a soundproof room, and applying an electric foot shock from a floor grid made of stainless steel rods

(diameter, 4 mm, at intervals of 8 mm). A continuous sound of 80 dB for 30 s (conditioned stimulus; CS) was emitted before administration of the electric foot shock at 0.8 mA for 0.5 s (unconditioned stimulus) (fear conditioning groups; FC). The electric foot shock was a constant-current stimulus produced by a shock generator/scrambler (Muromachi Kikai, Tokyo, Japan). Animals in the control groups were exposed to audio stimulation under the same conditions but with no foot shock (sham groups; Sham).

2-5.Microdialysis (day 15)

The day after the conditioning session, microdialysis was begun following insertion of a probe into the left amygdala and intraperitoneal injection of a drug (haloperidol 1 mg/kg, aripiprazole 10 mg/kg, or saline 2 ml/kg in the same volumes) while the animals were anesthetized and unrestrained. The dialysis probe had a membrane length of 2.0 mm, an outer diameter of 0.5 mm, and an MW cutoff of 20,000 Da (AI-12-2; Eicom, Kyoto, Japan). Ringer's solution (147 mM Na^+, 4 mM K^+, 2.3 mM Ca^{2+}, 155.6 mM Cl^-) was used as the perfusate for microdialysis, and samples were collected at a flow rate of 2 μl/min.

Acclimation for 180 min was allowed after the beginning of microdialysis. Then, the pre-CS extracellular dopamine level was measured for 80 min between 180 and 260 min after the start of microdialysis. CS (i.e. sound only, with no foot shock) was then applied to rats in all groups at 260 min after the beginning of microdialysis. The duration of freezing behavior was measured during the 20-min CS application period. Time-based changes in the extracellular dopamine level as the post-CS extracellular dopamine level were measured for 80 min following CS application between 260 and 340 min after the start of microdialysis. The total microdialysis run time was 340 min (acclimation 180 min, sampling of pre-CS extracellular

dopamine levels 80 min, sampling of post-CS extracellular dopamine levels 80 min). As a preliminary experiment had shown that the effect of haloperidol and aripiprazole on dopamine release in the amygdala was prolonged over 340 min, the extracellular dopamine level during the duration of sampling was that under the effect of the drug.

2-6. Measurement of extracellular dopamine levels

Extracellular dopamine levels were measured by high-performance liquid chromatography every 20 min. Samples were collected with an Auto Injector (ESA-20: Eicom). To quantify dopamine on a real-time basis, the samples were placed in a high-performance liquid chromatograph (HITEC-500: Eicom) every 20 min, using a CA-5ODS column (2.1×150 mm; Eicom) with a mobile phase containing 134.49 g/l NaH_2PO_4, 49.40 g/l Na_2HPO_4, 1% methanol, 800 mg/l sodium 1-decanesulfonate, and 50 mg/l EDTA-2 Na. The detector in this system had a graphite working electrode set at +0.45 V relative to an Ag/AgCl reference electrode. Use of the Auto Injector enabled dopamine levels to be measured without sample decomposition or loss through oxidation.

2-7. Histology

At the end of each experiment, the animals were given an overdose of sodium pentobarbital (100 mg/kg) and perfused transcardially with physiological saline, followed by 10% buffered formalin. The brains were post-fixed in 10% buffered formalin for 1 to 10 days and then cryoprotected by immersion in 25% sucrose for 2 days. The location of the microdialysis probe in the amygdala was determined histologically on serial coronal sections (50 μm) stained with cresyl violet. Data that clearly represented preparations extending beyond the range of the

amygdala and including the caudate putamen were excluded. Only data obtained from rats with correctly implanted probes were included in the results (Fig 4A, 4B).

2-8. Grouping

The animals were divided into the following 12 groups: (1) a group given methamphetamine and subjected to fear conditioning (MP/FC); (2) a group given methamphetamine and subjected to sham fear conditioning (MP/Sham); (3) a group given saline and subjected to fear conditioning (Sal/FC); and (4) a group given saline and subjected to sham fear conditioning (Sal/Sham). In addition, each of above 4 groups was divided into the following three groups according to the drug treatment: aripiprazole (APZ), haloperidol (HAL), and saline control (D-).

2-9. Analysis

Pre-CS extracellular dopamine level, post-CS extracellular dopamine level, and freezing duration were analyzed. The duration of freezing, which was defined as an absence of observable movements of skeletal muscles and whiskers (with the exception of movements related to breathing) was measured over the total period of CS application. In addition, freezing behavior was determined using three supplementary parameters: standing on four legs, not lying down, and increased respiratory rate at CS presentation. A preliminary experiment had shown that haloperidol induced catatonia lasting 2-3 hours, whereas aripiprazole did not. As CS was presented at 260 min after drug treatment, extrapyramidal symptoms would have had no effect on the measurement of freezing duration. Pre-CS extracellular dopamine level (in pg /40 μl /20 min) was determined as the mean value from 4 fractions before application of CS. Post-CS extracellular dopamine level was expressed as a

time-based change relative to the pre-CS extracellular dopamine level for each group, after application of CS.

Three-way analysis of variance (ANOVA) for methamphetamine sensitization (two conditions) x fear conditioning (two conditions) x treatment (three conditions) was used for between-group analyses of basal dopamine level and freezing duration. One-way ANOVA with repeated measures across time (using group as the main factor) was used for between-group analyses of the post-CS extracellular dopamine level. The Bonferroni method was used for multiple comparisons and simple main effect testing when the main effect or interaction was significant. $P < 0.05$ was taken as the overall level of significance.

3. Results

Data from 36 rats were excluded because of amygdala catheterization failure or the presence of serious hemorrhaging around the membrane of the microdialysis probe or along the insertion path (27 animals) (Fig. 5A, 5B); or because fear stress conditioning was ineffective (9 animals). Accordingly, data from a total of 84 animals were analyzed, with these animals divided into 12 groups of 7 animals each.

3-1. A preliminary test: Time course of effect of drugs on extracellular dopamine level

Fig.1 illustrates the duration of the effects of aripiprazole and haloperidol on extracellular dopamine level in the amygdala relative to dopamine level prior to the injection of the drugs in naïve rats. The methods of surgical implantation and microdialysis techniques used as well as the measurement of extracellular dopamine are described in the Methods section. The line graphs in Fig. 1 illustrate changes in the relative extracellular dopamine level over time following intraperitoneal injection of a drug (haloperidol 1 mg/kg, aripiprazole 10 mg/kg, or saline 2 ml/kg in the same volume) beginning at 0 min. Extracellular dopamine levels were increased significantly following haloperidol injection compared with the control group ($P < 0.001$, Bonferroni method). This effect was maintained over 420 min and stabilized beginning at 240 min. In contrast, aripiprazole significantly decreased extracellular dopamine levels compared with the control ($P < 0.001$, Bonferroni method). This effect was also maintained over 420 min and stabilized beginning at 20 min.

3-2. Pre-CS extracellular dopamine level

Fig. 2 illustrates the extracellular dopamine levels in the amygdala (pg /40 μl /20 min) before application of CS. The pre-CS extracellular dopamine level was determined as the mean value from 4 samples taken before CS application. Because the interaction between FC x treatment was significant (three-way ANOVA, $F_{2, 72} = 4.657$, $P = 0.013$), treatment was studied by simple main effect testing with each of both FC and Sham. The pre-CS extracellular dopamine level was significantly greater with haloperidol than with the control under both FC and Sham ($P < 0.001$, Bonferroni method). In contrast, the pre-CS extracellular dopamine level was significantly lower with aripiprazole than with the control under both FC and Sham ($P < 0.001$, Bonferroni method). Methamphetamine administration had no significant effect on the extracellular dopamine level (three-way ANOVA, $F_{1, 72} = 0.019$, $P = 0.892$).

3-3. Post-CS extracellular dopamine level

Fig. 3 illustrates the extracellular dopamine level after CS relative to that before CS. The line graphs illustrate changes in the relative extracellular dopamine level with time following application of CS at 0 min. The bar graphs on the upper right illustrate the means of 4 points for the rates at 20, 40, 60 and 80 min. Fig. 3A illustrates the effect of methamphetamine sensitization (D^- groups only). Under fear conditioning in both the MP and Sal groups, the increase in the extracellular dopamine level after CS application was significantly greater than with sham treatment ($P < 0.001$, Bonferroni method). The MP/FC/D- group showed a significantly greater increase than the SAL/FC/D- group ($P < 0.001$, Bonferroni method). Fig. 3B illustrates the effect of haloperidol treatment in the methamphetamine-sensitized groups.

Haloperidol treatment in the MP/FC group resulted in significant suppression of the excessive increase in the extracellular dopamine level (P <0.001,MP/FC/HAL vs. MP/FC/D-, Bonferroni method). Haloperidol treatment also significantly suppressed the increase of dopamine seen in the Sal/FC group (P = 0.004, Sal/FC/HAL vs. Sal/FC/D-, Bonferroni method) (Fig 3C). Fig. 3D illustrates the effect of aripiprazole in the methamphetamine-sensitized group. Aripiprazole significantly suppressed the excessive increase in the extracellular dopamine level seen in the MP/FC/D- group (P < 0.001, MP/FC/APZ vs. MP/FC/D-，Bonferroni method) but resulted in a slight increase of extracellular dopamine level relative to the MP/Sham/D- group (P <0.015, MP/FC/APZ vs. MP/Sham/D-, Bonferroni method). The Sal/FC/APZ groups showed a slight increase in dopamine level comparable to that in the Sal/FC/D- groups (P = 1.000, Sal/FC/APZ vs. Sal/FC/D-. P <0.001, Sal/FC/APZ vs. Sal/sham/D$^-$, Bonferroni method) (Fig 3E).

3-4. Freezing duration

Fig. 4 illustrates the mean freezing time (in seconds) and standard error (±S.E.). Interaction between procedures (MP or Sal x FC or Sham x Drug treatment) for every combination showed no significant effect on freezing time by three-way ANOVA. Only the effect of fear conditioning showed significance (three-way ANOVA, $F_{1, 72}$ = 1060.6, P <0.001). Treatment with either haloperidol or aripiprazole had no significant effect on freezing behavior.

4. Discussion

This is the first study to provide direct neurochemical evidence that antipsychotic agents attenuate an increase in extracellular dopamine level following conditioned fear stimuli in the amygdala of methamphetamine-sensitized rats and that the basal extracellular dopamine level in the amygdala is increased by haloperidol and decreased by aripiprazole. These findings suggest that the common effects to both agents may functionally attenuate the fear response of dopamine in the amygdala, whereas the contrasting effects on the basal extracellular dopamine level in the amygdala may be caused by the difference in their actions (selective dopamine D_2 receptor full antagonist vs. partial agonist).

Previous studies involving systemic injection of dopamine D_2 receptor antagonists (haloperidol, sulpiride, or risperidone) found an increase in the extracellular dopamine level in the nucleus accumbens (Westerink et al., 1996; Meltzer et al., 2003; Li et al., 2005). In contrast, a high dose of the partial agonist aripiprazole (10 mg/kg) decreased the level of extracellular dopamine in the nucleus accumbens but increased dopamine release in the medial prefrontal cortex and hippocampus (Li et al., 2004; Bortolozzi et al., 2007). Because there is no such data for the amygdala, this is therefore the first report of the effects of dopamine D_2 receptor antagonists/agonists on amygdala dopamine release and their persistent effect (Fig.1, Fig. 2). Studies have shown that the ventral tegmental area (A10), nigral (A9), and thalamic (A11) dopaminergic cell groups project to the amygdala (Oades and Halliday, 1987). Furthermore, the mesolimbic and mesocortical dopamine pathways are under tonic inhibition by somatodendritic dopamine D_2 auto receptors (Kalivas, 1993; Mercuri et al., 1997; Kalivas and Duffy, 1991). These facts suggest that the contrasting effects on dopamine release between aripiprazole and haloperidol observed in this study may be attributable to their different actions

as a dopamine D_2 receptor partial agonist and full antagonist, respectively. However, dopamine release in the nucleus accumbens is also inhibited by dopamine D_2 receptors in the medial prefrontal cortex (Chen and Pan, 2000). Thus, it is uncertain which pathway is responsible for modulating tonic dopamine release in this systemic drug injection study. In addition, a recent study has revealed that D_2 receptors of humans are expressed in somewhat lower abundance when compared with the substantia nigra (Aubert et al., 1997). Therefore, the above results cannot be applied directly to humans.

Under the conditions of basal dopamine level mentioned above, both aripiprazole and haloperidol attenuated the marked increase in the extracellular dopamine levels following conditioned fear stress in methamphetamine-sensitized rats (Fig.3B, 3D). Since the effect of these drugs on basal dopamine release in the amygdala lasted for over 420 min (Fig.1), this attenuation of the dopamine fear response must have been caused by these drugs. In a recent microdialysis study, it was found that conditioned fear stimuli activated noradrenaline, dopamine and serotonin metabolism in the amygdala. In contrast, fear stimuli reduced the level of neurotransmitter GABA in the amygdala (Tanaka et al., 2000; Inoue, 1993; Inglis and Moghaddam, 1999; Stork et al., 2002; de Groote and Linthorst, 2007). Specifically, the dopamine increase in response to stress was significantly greater in the amygdala than in the nucleus accumbens and prefrontal cortex (Inglis and Moghaddam, 1999). GABA, serotonin, opioids, excitatory amino acids, and neuropeptides play a modulatory role in the brain aversion system, which includes the neural substrates for fear in the amygdala. Serotonin and GABA attenuate fear-related processes in the amygdala (Amat et al., 1998; Adell et al., 1997; Inoue, 1993; Matsumoto et al., 2005). In contrast, overactivation of dopamine and noradrenaline transmission may exacerbate conditioned fear (Inoue et al., 2000; Borowski and Kokkinidis,

1998; Bissiere et al., 2003; Debiec and LeDoux, 2006). Thus, dopamine in the amygdala seems to be one of the transmitters most potently modulating the mechanisms underlying states of fear and anxiety. There have been some reports of clinical drugs modulating the stress response of neurotransmitters in the amygdala in conjunction with the attenuation of fear behaviour. Acute treatment with the selective serotonin reuptake inhibitor citalopram showed significant dose-dependent increase in extracellular serotonin concentrations in the prefrontal cortex with a reduction in freezing behaviour (Muraki, 2001). The increase in noradrenaline level in the amygdala, which is involved in the provocation of fear, was attenuated by benzodiazepine, thereby reducing negative emotional behaviour (Tanaka et al., 2000). However, no basic study has examined the biochemical effect of antipsychotics on the fear response of dopamine in the amygdala. Thus, this is the first report to address the neurochemical effect of antipsychotic agents on the fear response, as represented biochemically by *in vivo* microdialysis and high performance liquid chromatography. Although aripiprazole and haloperidol both attenuated the fear response of amygdala dopamine, drug treatment did not appear to have any effect on the expression of fear behaviour (Fig. 4). Thus, it remains to be clarified what the attenuation of the fear response of dopamine means.

This dissociation between drug-induced changes in dopamine activity and a lack of corresponding alteration in the expression of fear conditioning is important. The pathway by which freezing behavior is expressed is composed chiefly of glutamatergic neurons in the amygdala that project to the eriaqueductal gray. Dopamine and 5-HT are modulators of fear memory. In addition, although some studies have demonstrated that stimulation of dopamine D_2 receptors reinforces freezing behavior, these studies involved the use of pharmacological substances (acute amphetamine or cocaine administration) (Borowski and Kokkinidis, 1998).

However our results were obtained in animals subjected to psychological stress. Previous studies have shown that clozapine and haloperidol do not affect fear behaviour when injected prior to the expression of fear memory, whereas an acute challenge with selective serotonin reuptake inhibitors reduces fear behaviour (Greba et al., 2001; Inoue et al., 1996; Guarraci et al., 2000; Hashimoto et al., 2009). Together with the above findings, our results suggest that antipsychotics have some ability to functionally attenuate the fear response of dopamine in the amygdala—an ability that could block the later stages of fear conditioning, such as reconsolidation (but not expression). This would be a unique effect of antipsychotics that is unlikely to be shared by antidepressants, which would have a direct anxiolytic effect.

In a study by Nader and LeDoux (1999), systemic treatment with quinpirole, a dopamine D_2 full agonist, blocked the acquisition of second-order fear conditioning, possibly reflecting a blockade of emotional memory recall. It has been suggested that the ability of quinpirole to decrease fear conditioning is because of its action on presynaptic dopaminergic receptors expressed on the cell bodies of dopaminergic neurons in the ventral tegmental area, which decreases dopamine levels in the terminal fields (Nader and LeDoux, 1999). Thus, the dopamine partial agonist aripiprazole could block dopamine transmittance in the amygdala by acting in two ways: Through decrease in the basal extracellular dopamine level and by occupying dopamine D_2 receptors in the amygdala.

One important finding of our study was that aripiprazole blocked a fear-conditioned increase in amygdala dopamine in methamphetamine-sensitized animals only, not in nonsensitized rats (Fig. 3E). In the case of sensitized animals, aripiprazole also showed a slight phasic increase in extracellular dopamine level (Fig. 3D). Therefore, it seems likely that aripiprazole would suppress the dopamine response in physiological degree. However, since

these finding are still preliminary, the mechanism underlying the effect remains unclear.

It is also unclear whether a ceiling effect exists that prohibits any further increase in extracellular dopamine in response to the conditioned stimulus after haloperidol treatment (which in naïve animals causes a marked increase in extracellular dopamine levels). Nevertheless, it is evident that attenuation of the fear response of dopaminergic neuron would be regulated in a differential manner with aripiprazole than with haloperidol. Given the paucity of data in this field, future experiments using other methodologies such as electrophysiological or immunohistological techniques (i.e. c-Fos expression) are needed to replicate our findings using the conditioned fear paradigm.

In conclusion, by using *in vivo* microdialysis, we found that aripiprazole and haloperidol both attenuate the phasic response of extracellular dopamine level in the amygdala, whereas the two drugs showed contrasting effects on tonic extracellular dopamine levels in the amygdala. The latter result may have arisen from the difference in their actions as a dopamine D_2 receptor full antagonist (haloperidol) versus a partial agonist (aripiprazole). Although more study needs to be conducted to elucidate the biochemical mechanisms of fear behaviour, these findings have important implications in the future design and development of drugs to treat emotional dysfunction.

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Figure legends

Fig. 1

Title: Time course of modulation effect of drugs on amygdala dopamine release

Legend: The figure represents the time course of the extracellular dopamine level affected by drug treatment in each of the groups ± S.D. The abscissa represents time in minutes. Drugs were applied at 0 min. One-way ANOVA with repeated measures across time (using three groups as the main factor) was used. The haloperidol treatment group showed a significant increase in the extracellular dopamine level in the amygdala after application ($P < 0.001$, Bonferroni method). Aripiprazole significantly suppressed the extracellular dopamine level after application of conditioned stimulus ($P < 0.001$, Bonferroni method). SAL: Saline injection as a control, HAL: haloperidol injection (1 mg/kg), APZ: aripiprazole injection (10 mg/kg). # = $P < 0.001$ vs. SAL, *** = $P < 0.001$ vs. SAL

Fig. 2

Title: Pre-CS extracellular dopamine level

Legend: Results represent the mean extracellular dopamine level 80 min before application of CS ± S.D. (in pg /40 µl /20 min) and at about 180 min after drug treatment. See the figure for grouping and statistical significance of the results. Three-way ANOVA showed that haloperidol increased the extracellular dopamine level, whereas aripiprazole decreased it significantly. Because the interaction between FC x treatment was significant (three-way ANOVA, $F_{2, 72} = 4.657$, $P = 0.013$), the effect of treatment was studied by simple main effect testing with each of both FC and Sham. # = P<0.001 vs. Sham/D- groups. *** = P<0.001 vs. FC/D- groups.

$$$ = P<0.001 vs. Sham/D- groups. &&& = P<0.001 vs. FC/D- groups. MP: methamphetamine sensitization, Sal: saline injection as a control, FC: fear conditioned, Sham: sham conditioned, D-: Saline injection as a control, HAL: haloperidol injection (1 mg/kg), APZ: aripiprazole injection (10 mg/kg).

Fig. 3 (A-E)

Title: Post-CS extracellular dopamine level

Legend: The ordinate represents the proportionate increase of the extracellular dopamine level above the mean level before CS in each of the groups ± S.D. Bar graphs on the upper right illustrate the mean proportionate increase at 20, 40, 60 and 80 min after CS application. The abscissa represents time in minutes, and conditioned stimulus was applied at 0 min. One-way ANOVA with repeated measures across time (using all 12 groups as the main factor) was used. MP: methamphetamine sensitization, Sal: saline injection as a control, FC: fear conditioned, Sham: sham conditioned, D-: Saline injection as a control, HAL: haloperidol injection (1 mg/kg), APZ: aripiprazole injection (10 mg/kg).

Fig. 3A

Title: Effect of methamphetamine sensitization.

Legend: The methamphetamine sensitization group showed a significant increase in the extracellular dopamine level in the amygdala after application of CS (P<0.001, Bonferroni method). ++ = P<0.001 vs. Sal/Sham/D- and MP/FC/D-, ** =P<0.001 vs. Sal/FC/D-

Fig. 3B

Title: Effect of haloperidol treatment in the “MP” group

Legend: Haloperidol significantly suppressed the increase in the extracellular dopamine level

in the methamphetamine-sensitized group. There was no significant difference between the MP/FC/HAL and sham groups. *** =P<0.001 vs. MP/FC/D-

Fig. 3C

Title: Effect of haloperidol in the “Sal” group.

Legend: Haloperidol also significantly suppressed the increase of dopamine release in the saline group. No significant difference was evident between the Sal/FC/HAL group and sham groups. *** =P<0.001 vs. Sal/FC/D-

Fig. 3D

Title: Effect of aripiprazole in the “MP” group.

Legend: Aripiprazole significantly suppressed the increase in the extracellular dopamine level in the methamphetamine group after application of CS (P<0.001), but showed a slight increase compared to the sham groups. *** =P<0.001 vs. MP/FC/D-, ++ =P =0.015 vs. MP/Sham/D-

Fig. 3E

Title: Effect of aripiprazole in the “Sal” group.

Legend: Aripiprazole showed an increase in the extracellular dopamine level, similar to that in the Sal/FC/D- group (P=1.000). n.s. =P=1.000 vs. Sal/FC/D-, ++ =P<0.001vs. Sal/Sham/D-

Fig. 4

Title: Freezing time duration

Legend: Results represent the mean duration of freezing ± S.D. (in seconds) in a 20-min period following CS administration. Only the effect of FC showed significance in three-way ANOVA (P<0.001, v.s. “MP or Sal” and “HAL, APZ or D-“). Treatment with both haloperidol and aripiprazole had no effect on fear behavior. MP: methamphetamine-sensitized group, Sal:

saline group, FC: fear conditioned group, Sham: sham conditioned, D-: Saline injection as a control, HAL: haloperidol injection (1 mg /kg), APZ: aripiprazole injection (10 mg/kg)

Fig. 5A

Title: Schematic representation of placement of the dialysis probe in the amygdala

Legend: The black bars represent the localization of the dialysis membranes. Data obtained beyond the range of the amygdala and including the caudate putamen were excluded. The numbers indicate anterior-posterior coordinates relative to the bregma, in millimeters. R indicates the right, and L the left hemisphere. Plates are adapted from the atlas of Paxinos and Watson (1997).

Fig. 5B

Title; Micrograph of strip preparation

Legend; This is a typical micrograph of a brain slice preparation stained with cresyl violet. The arrow indicates the area of the probe in the amygdala.

30 April 2009
Dr W.H. Gispen
Editor-in-Chief, European Journal of Pharmacology

Dear Dr Gispen,

Please find enclosed a revised version of our manuscript (#EJP-30252R1) entitled ‘Aripiprazole and haloperidol suppress excessive dopamine release in the amygdala in response to conditioned fear stress, but show contrasting effects on basal dopamine release in methamphetamine-sensitized rats’. We would like to thank the reviewer for his/her suggestions, which have helped us to improve the manuscript.
Our responses to the reviewer’s comments are as follows:

Response to Reviewer
We greatly appreciate your valuable comments and have revised our manuscript accordingly, as follows.

Major points
Comment 1.
The authors indicate in the first paragraph of the discussion that aripiprazole and haloperidol share common effects on fear-conditioning, but have opposing effects on amygdala extracacellular dopamine levels. Lateron they reinforce that point ("Although aripiprazole and haloperidol both attenuated the fear response of amygdalic dopamine, drug treatment did not appear to have any effect on the expression of fear behaviour) (page 16 lines 13-15). The authors should expand on this important dissociation between drug-induced changes in dopamine activity and a lack of corresponding alteration in the expression of fear-conditioning.
Reply to comment 1.
Thank you very much for your thoughtful suggestions, which are indeed relevant. We have considered your suggestion carefully, but there is not sufficient evidence for a correlation between the extracellular dopamine level and conditioned fear behaviour (i.e. fear expression). Therefore, we have mentioned the basic pathway of fear behaviour, and the results of previous behavioural studies using antipsychotic agents and antidepressants. Furthermore, we have mentioned the assumption that antipsychotic agents would have an ability that to block the later stages of fear conditioning, such as reconsolidation (but not expression). We hope that our reply goes some way to addressing your concerns.
Changes to Manuscript
(P16 L17- P17 L9) “This dissociation between drug-induced changes in dopamine activity and a lack of corresponding alteration in the expression of fear conditioning is important. The pathway by which freezing behavior is expressed is composed chiefly of glutamatergic neurons in the amygdala that project to the

periaqueductal gray. Dopamine and 5-HT are modulators of fear memory.
In addition, although some studies have demonstrated that stimulation of dopamine D_2 receptors reinforces freezing behavior, these studies involved the use of pharmacological substances (acute amphetamine or cocaine administration). However our results were obtained in animals subjected to psychological stress. Previous studies have shown that clozapine and haloperidol do not affect fear behaviour when injected prior to the expression of fear memory, whereas an acute challenge with selective serotonin reuptake inhibitors reduces fear behaviour (Greba et al., 2001; Inoue et al., 1996; Guarraci et al., 2000; Hashimoto et al., 2009). Together with the above findings, our results suggest that antipsychotics have some ability to functionally attenuate the fear response of dopamine in the amygdala—an ability that could block the later stages of fear conditioning, such as reconsolidation (but not expression).
This would be a unique effect of antipsychotics that is unlikely to be shared by antidepressants, which would have a direct anxiolytic effect."

Minor points

Comment 2.

page 4 line 22 should read: ...leads to decreases in somal volume and dendritic length and lowers both spine density and the number of terminals.

The authors should mention where (brain region, neuron type) those changes in neuron morphology occur.

Reply to comment 2.

We appreciate your comment and have revised the manuscript accordingly.

Changes to Manuscript

(P4 L2-L3) "and decreased somal volume, length of spine density, dendrites, and terminals of prefrontal cortical pyramidal neurons in layer II/IIIs"

Comment 3.

The statement that D2 receptor mRNA is not present in the ventral tegmental area (page 15 lines 4-6) should be phrased more cautiosly, because D2 receptors are not absent in this region, but are expressed in somewhat lower abundance when compared with the substantia nigra (for example see Aubert et al. J Comp. Neurol. 379:72-87 (1997).

Reply to comment 3.

Thank you for your comment. In keeping with your suggestion, we have revised the text as follows.

Changes to Manuscript

(P15 L4-L7) "In addition, a recent study has revealed that D_2 receptors of humans are expressed somewhat less abundantly than in the substantia nigra. Therefore, the above results cannot be applied directly to humans."

Comment 4.

page 16 line 14 amygdalic should read amygdala

Reply to comment 4.

In accordance with your advice, we have changed ‘amygdalic’ to ‘amygdala’.
Changes to Manuscript
(P16 L14) “amygdala”

Comment 5.
page 17 line 20: what is the meaning of "dopamine-receptor neurons"?
Reply to comment 5.
We apologize for incorrect use of this phrase. We meant “dopaminergic neuron”.
Changes to Manuscript
(P18 L5) “dopaminergic neuron”

Comment 6.
page 28 line 14: "brain strip" should be replacecd by "brain slice".
Reply to comment 6.
In keeping with your suggestion, we have replaced the word “brain strip” with “brain slice”.
Changes to Manuscript
(P29 L15) “brain slice”

We appreciate your considerate review and feedback, and look forward to your reply.

Yours sincerely,

Hidehiro Oshibuchi, MD
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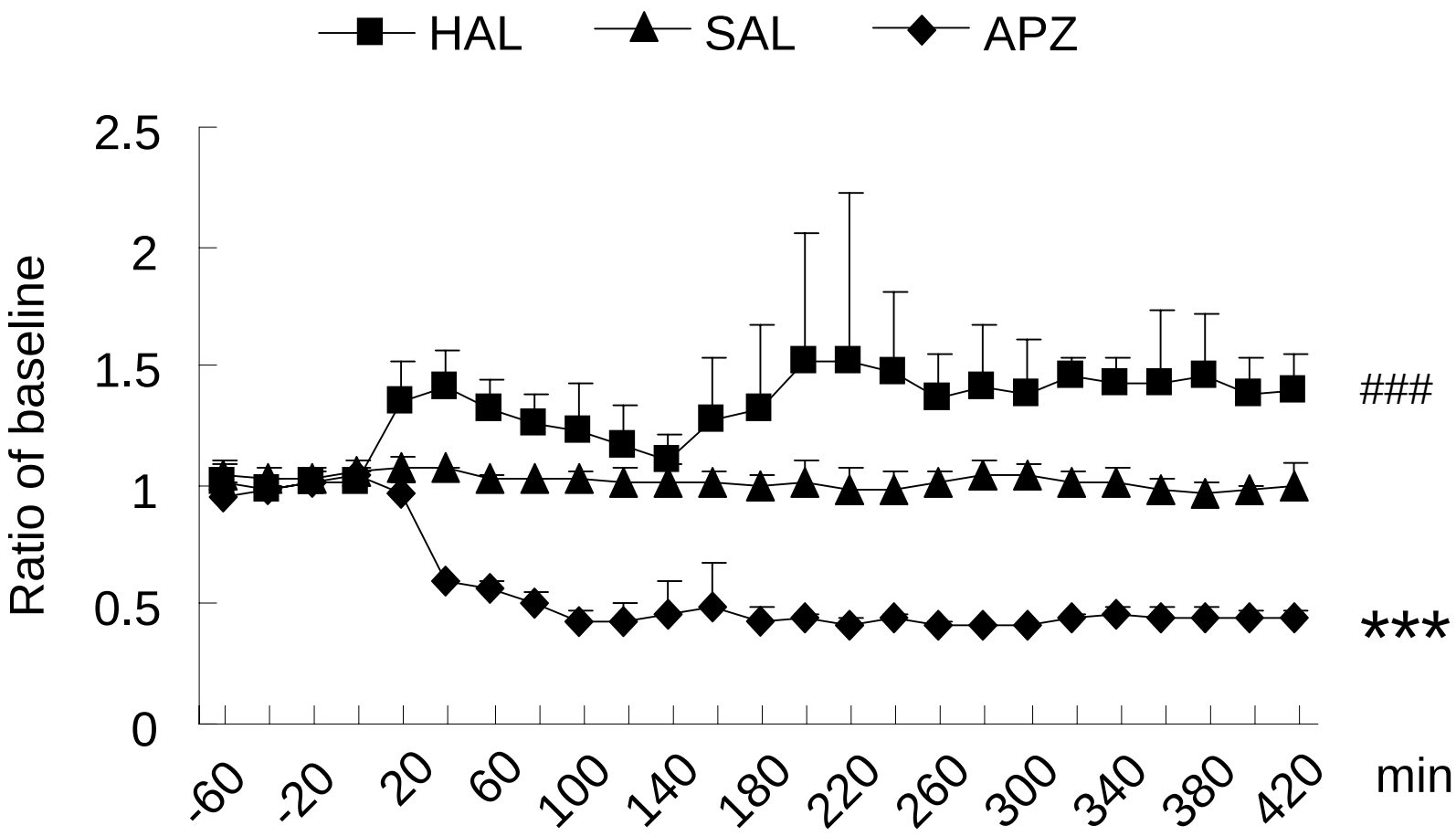
E-mail : hoshibuchi@psy.twmu.ac.jp

Figure 1


HAL
SAL
APZ
Ratio of baseline
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2
1.5
1
0.5
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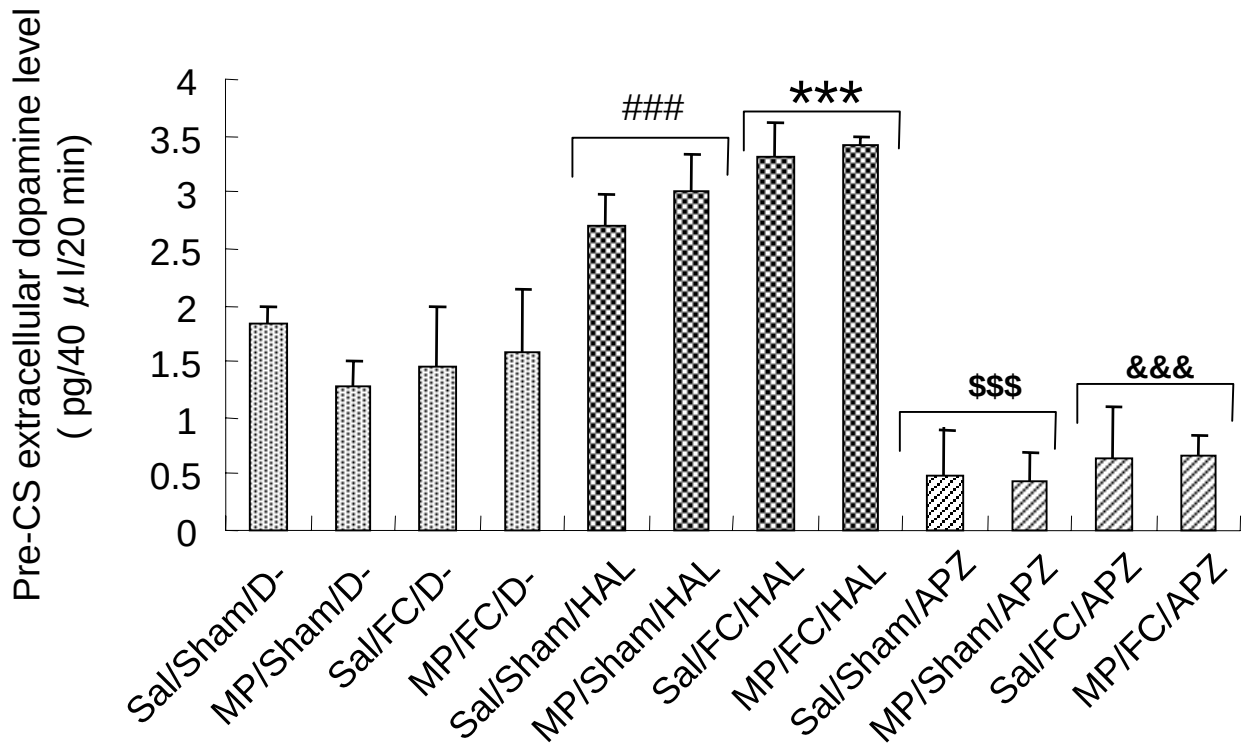
180
220
260
300
340
380
420
min

Figure 2


Pre-CS extracellular dopamine level
(pg/40 μl/20 min)
4
3.5
3
2.5
2
1.5
1
0.5
0
###
***
$$$
&&&
Sal/Sham/D-
MP/Sham/D-
Sal/FC/D-
MP/FC/D-
Sal/Sham/HAL
MP/Sham/HAL
Sal/FC/HAL
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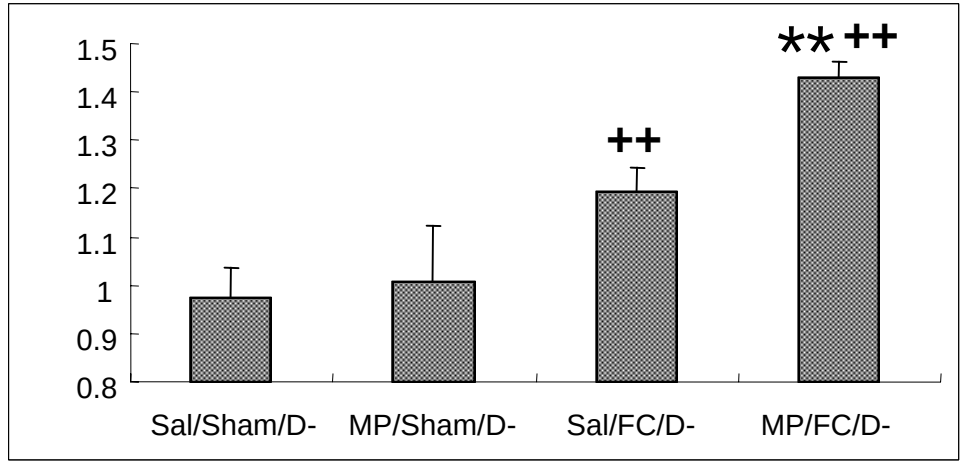
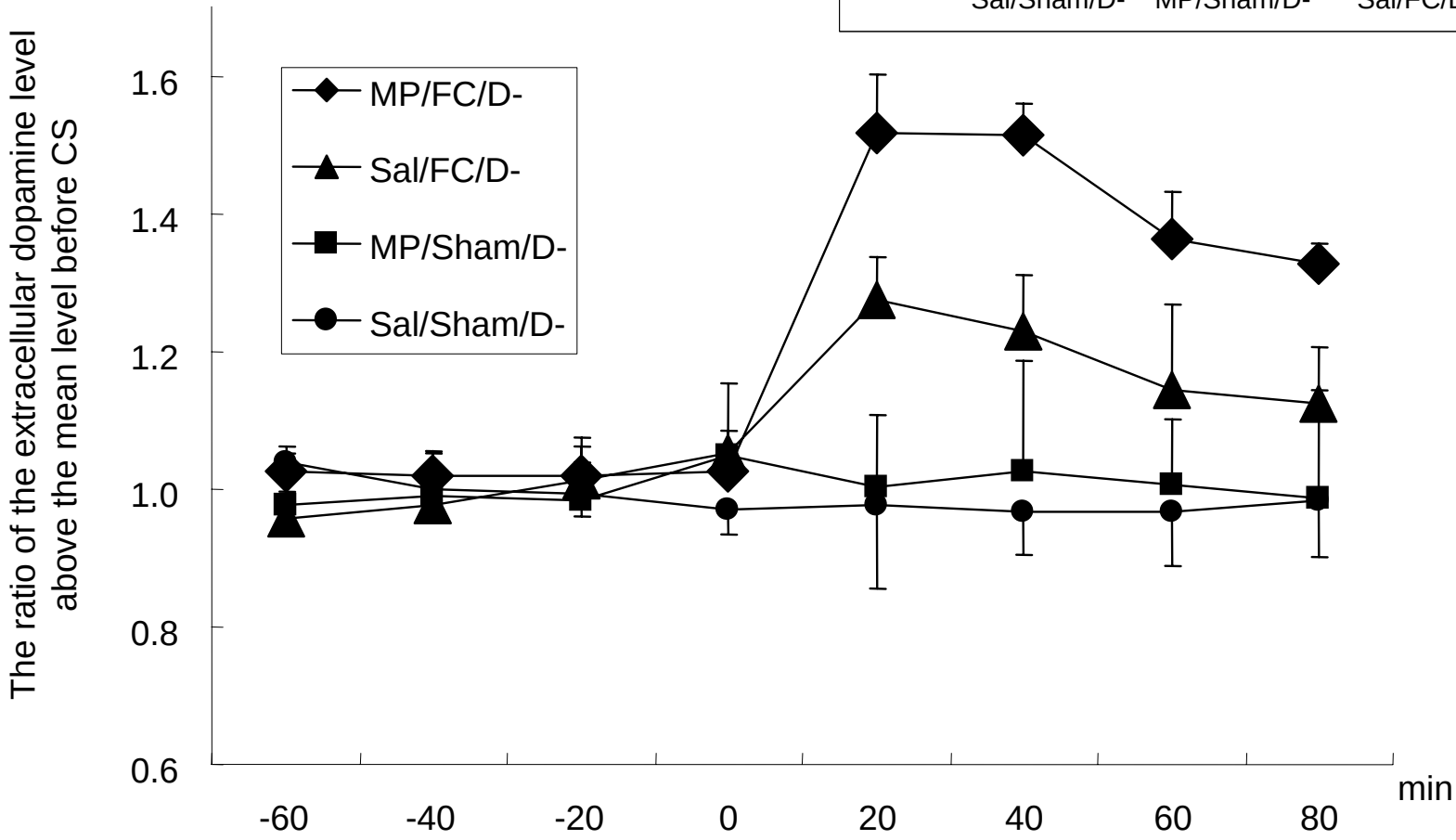
Sal/Sham/APZ
MP/Sham/APZ
Sal/FC/APZ
MP/FC/APZ

Figure 3A


1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
++
**++
Sal/Sham/D-
MP/Sham/D-
Sal/FC/D-
MP/FC/D-


The ratio of the extracellular dopamine level above the mean level before CS
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1.4
1.2
1.0
0.8
0.6
MP/FC/D-
Sal/FC/D-
MP/Sham/D-
Sal/Sham/D-
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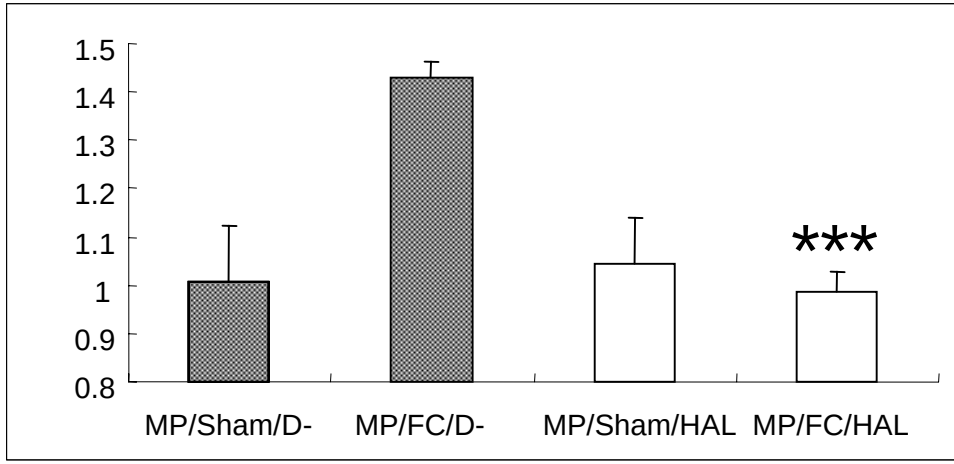
-40
-20
0
20
40
60
80
min

Figure 3B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1.4
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1.2
1.1
1
0.9
0.8
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MP/FC/D-
MP/Sham/HAL
MP/FC/HAL

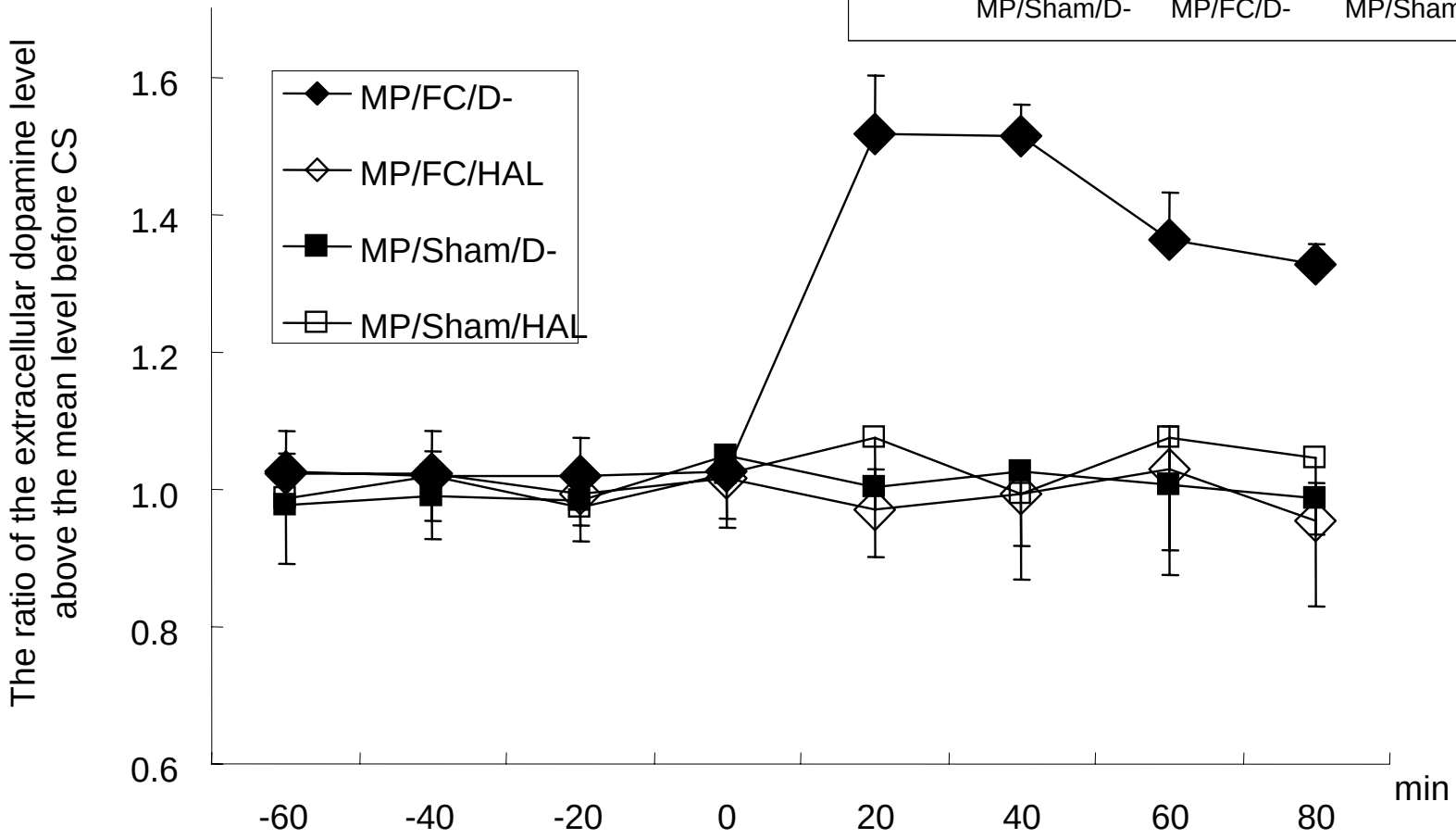
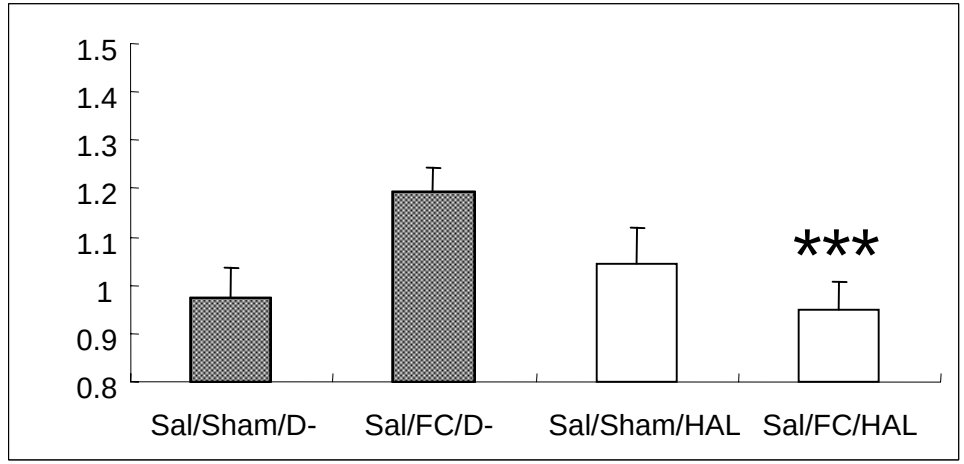

The ratio of the extracellular dopamine level above the mean level before CS
1.6
1.4
1.2
1.0
0.8
0.6
MP/FC/D-
MP/FC/HAL
MP/Sham/D-
MP/Sham/HAL
-60
-40
-20
0
20
40
60
80
min

Figure 3C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1.3
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1
0.9
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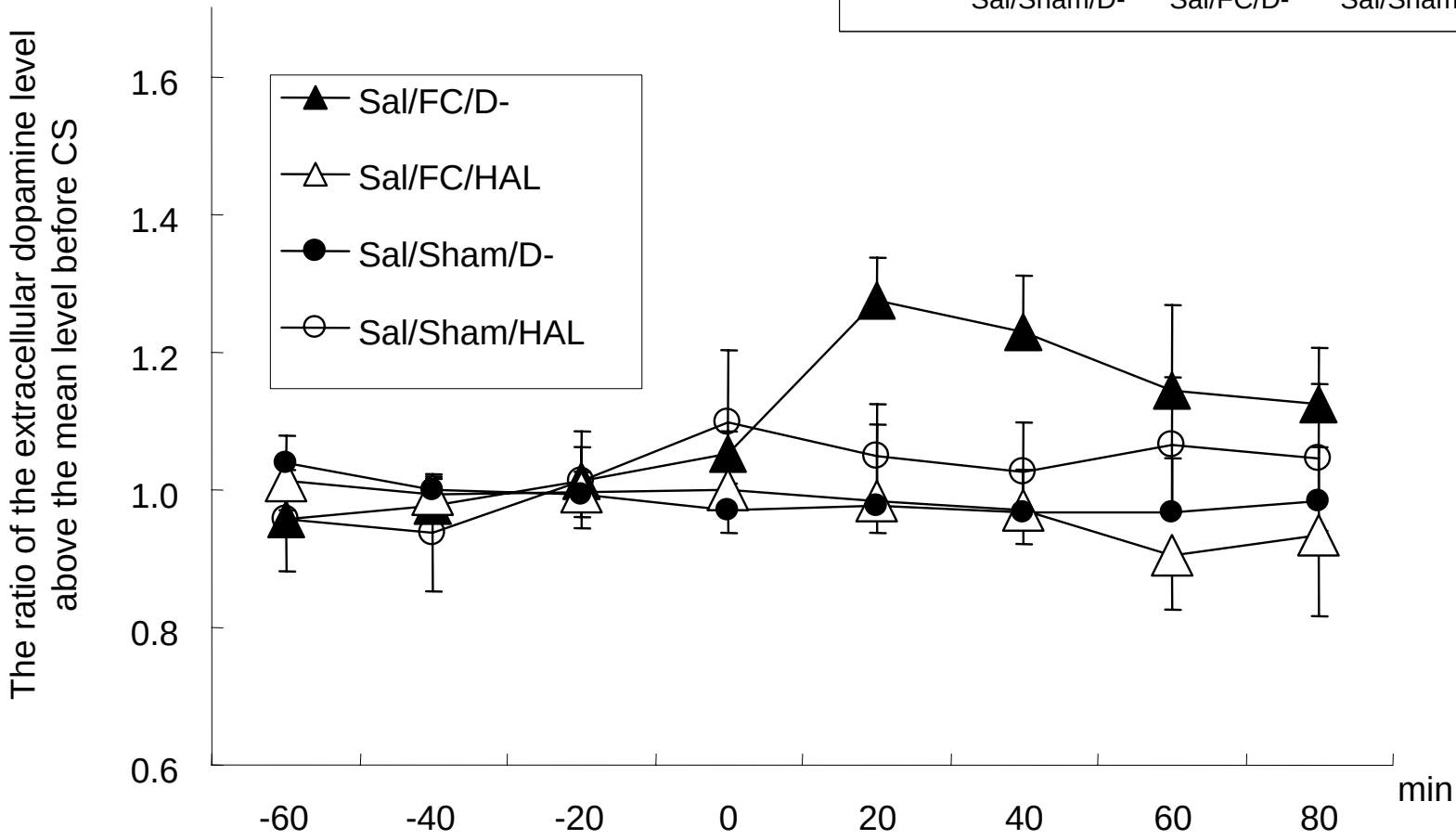
Sal/FC/D-
Sal/Sham/HAL
Sal/FC/HAL


The ratio of the extracellular dopamine level above the mean level before CS
1.6
1.4
1.2
1.0
0.8
0.6
Sal/FC/D-
Sal/FC/HAL
Sal/Sham/D-
Sal/Sham/HAL
-60
-40
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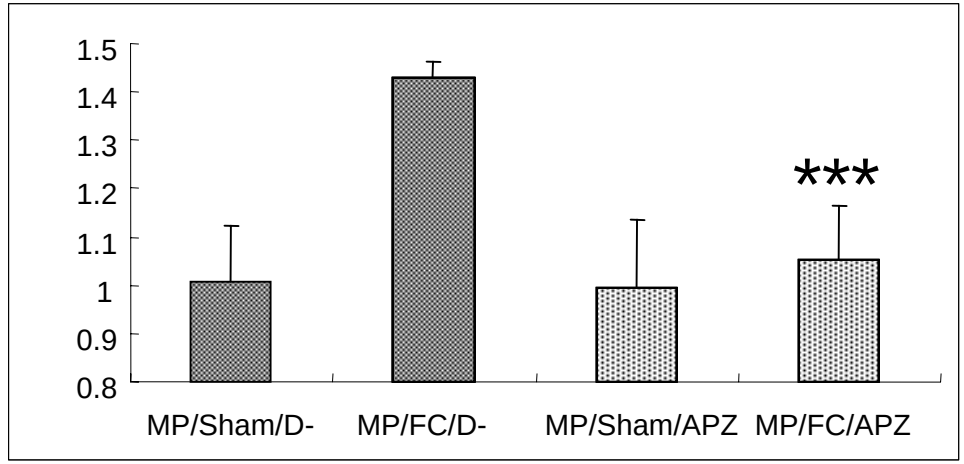
0
20
40
60
80
min

Figure 3D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1.4
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MP/FC/D-
MP/Sham/APZ
MP/FC/APZ

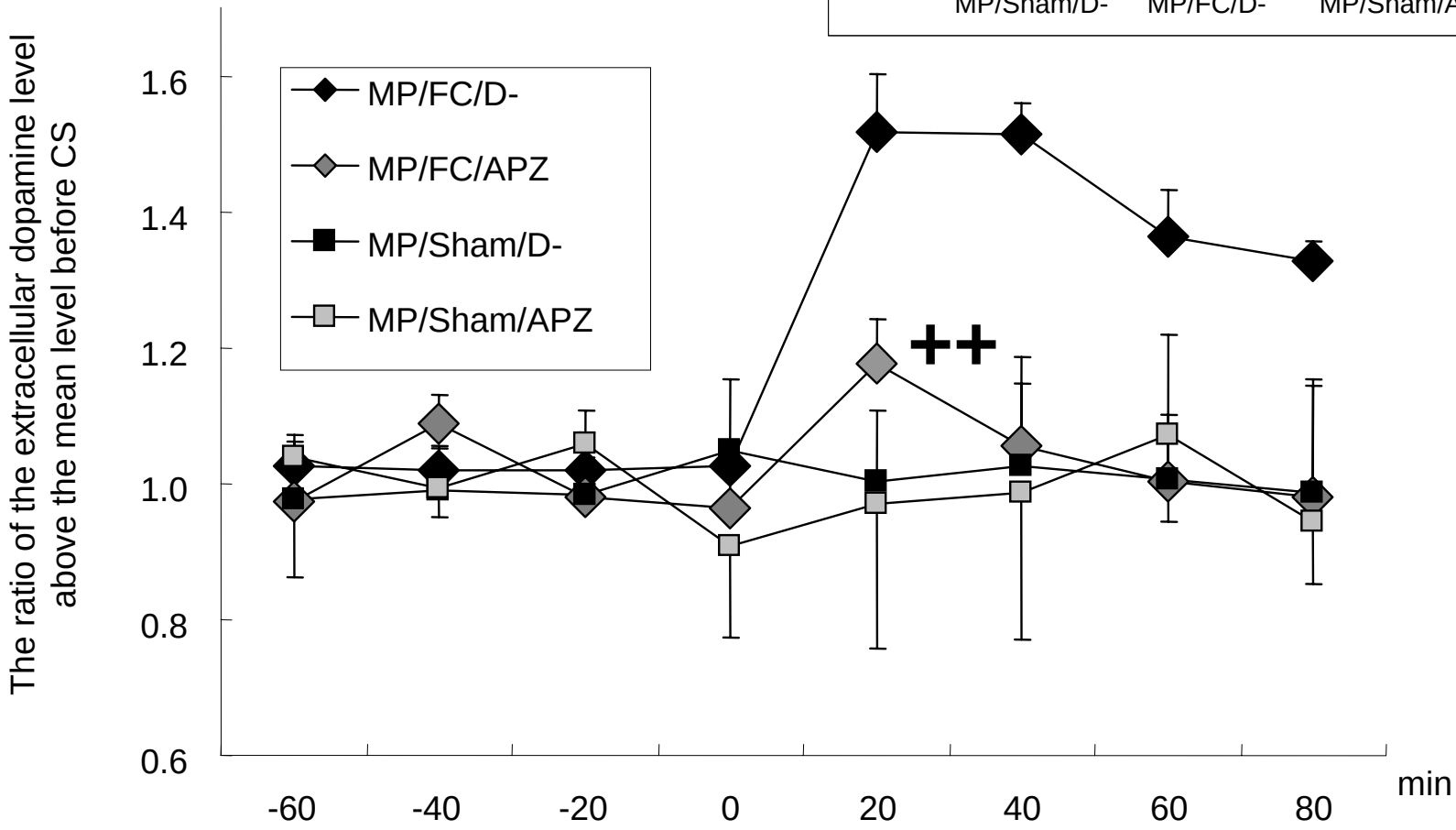
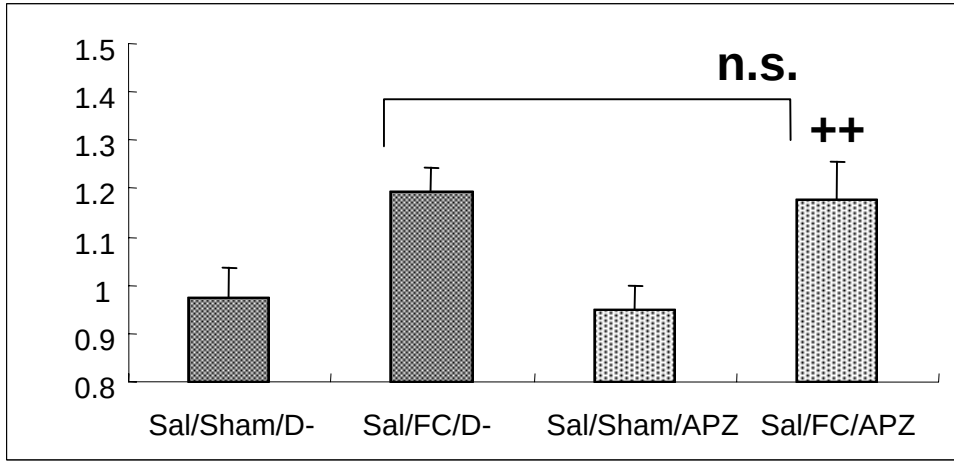

The ratio of the extracellular dopamine level above the mean level before CS
1.6
1.4
1.2
1.0
0.8
0.6
MP/FC/D-
MP/FC/APZ
MP/Sham/D-
MP/Sham/APZ
++
-60
-40
-20
0
20
40
60
80
min

Figure 3E


n.s.
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1.5
1.4
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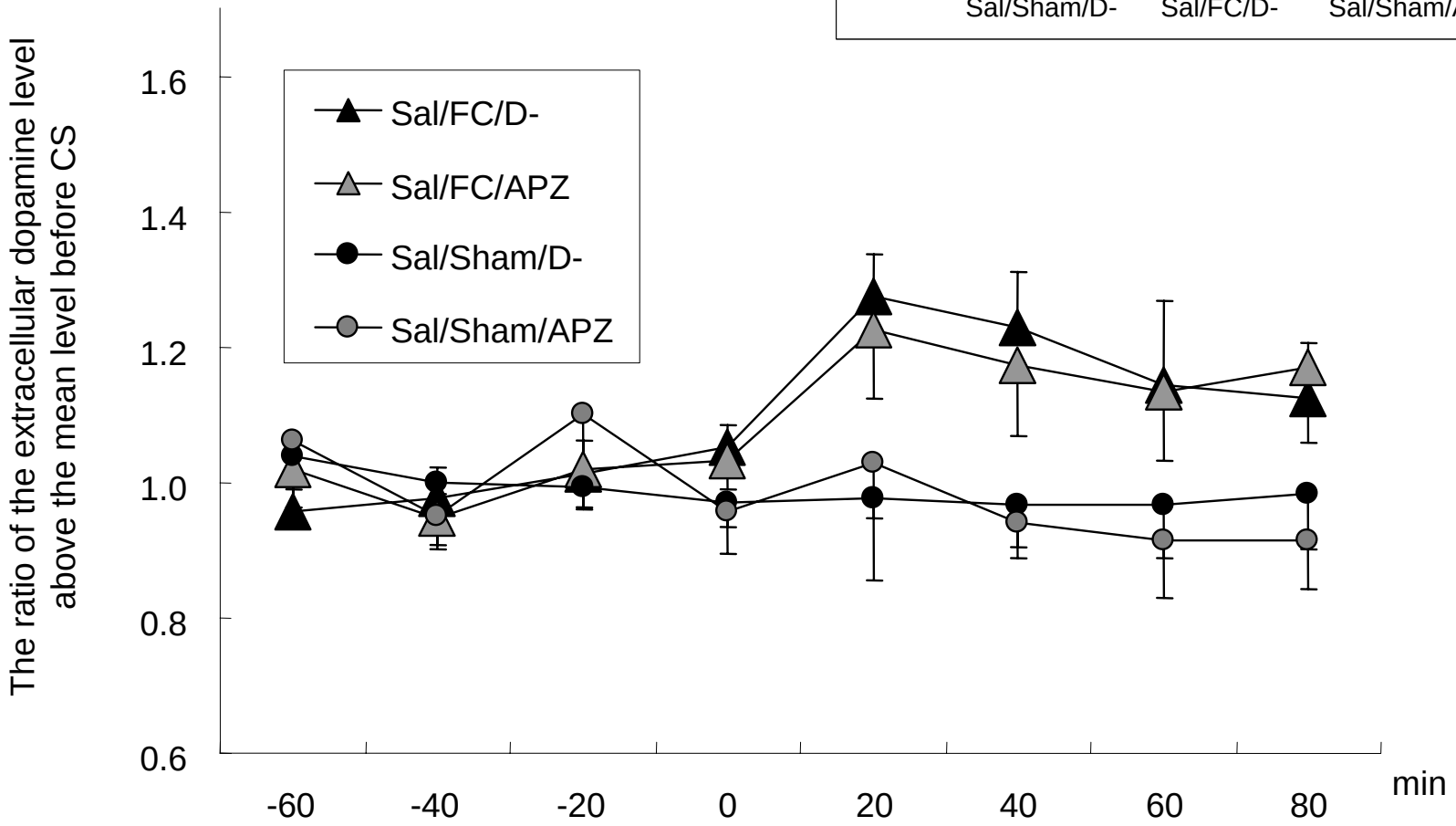
0.9
0.8
Sal/Sham/D-
Sal/FC/D-
Sal/Sham/APZ
Sal/FC/APZ


The ratio of the extracellular dopamine level above the mean level before CS
1.6
1.4
1.2
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Sal/FC/D-
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Sal/Sham/APZ
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-20
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20
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min

Figure 4

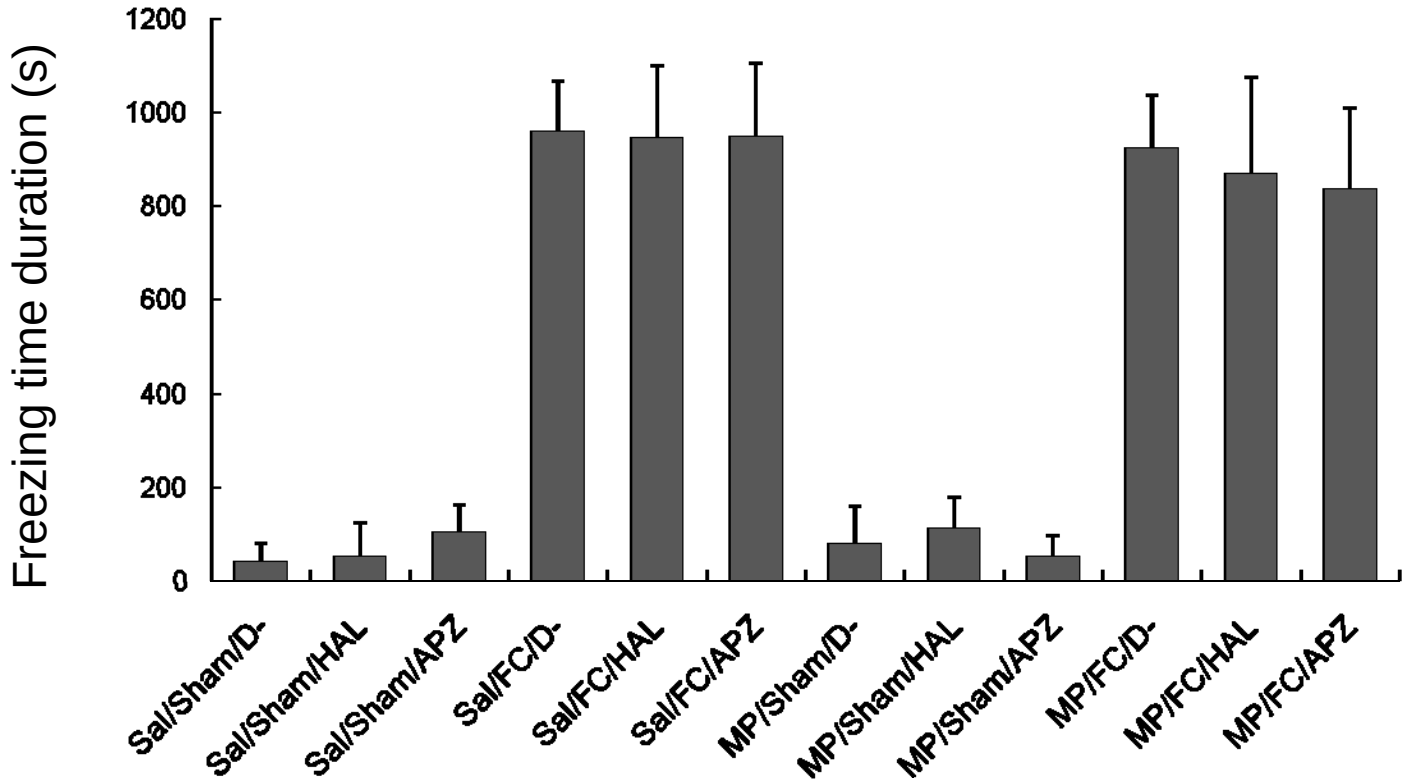
Freezing time duration (s)
1200
1000
800
600
400
200
0
Sal/Sham/D-
Sal/Sham/HAL
Sal/Sham/APZ
Sal/FC/D-
Sal/FC/HAL
Sal/FC/APZ
MP/Sham/D-
MP/Sham/HAL
MP/Sham/APZ
MP/FC/D-
MP/FC/HAL
MP/FC/APZ

Figure 5A

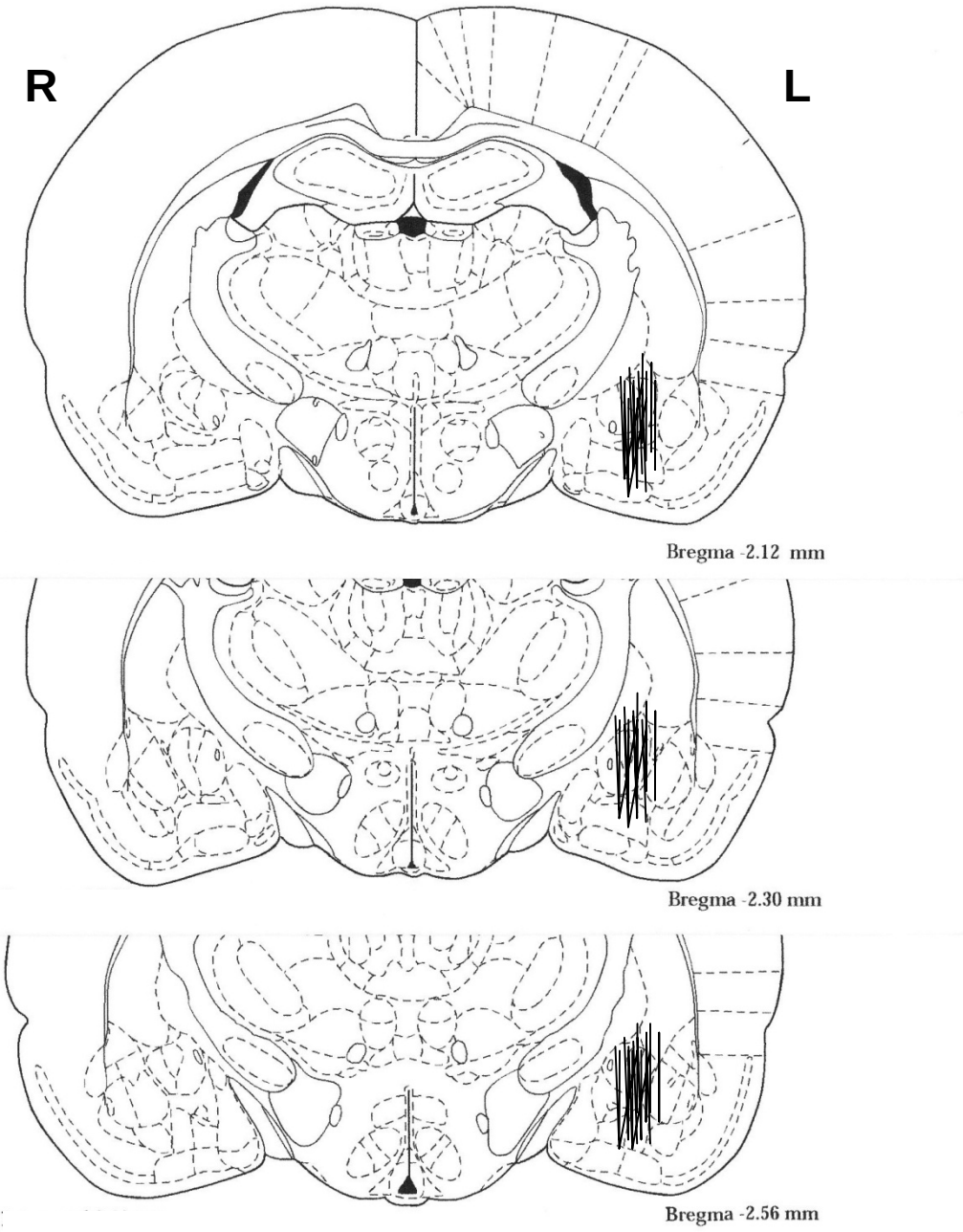

Figure 5B