JPET Fast Forward. Published on August 3, 2005 as DOI: 10.1124/jpet.105.088849



**Title page**

# Chronopharmacological study of antidepressants in forced swimming test of mice

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**Running title page**

**a)** Influence of dosing time on antiimmobilizing effect

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**c)** Text pages, 25 pages

Tables, 3 tables

Figures, 5 figures

References, 38 items

Abstract, 240 words

Introduction, 456 words

Discussion, 1021 words

**d) Abbreviations:**

ANOVA, analysis of variance; FST, forced swimming test; NONMEM, nonlinear mixed effect model; SERT, serotonin transporter; SSRI, selective serotonin reuptake inhibitor; Ke, elimination rate constant; Vd, volume of distribution.

**e)** A recommended section assignment: Behavioral Pharmacology.

**Abstract**

The influence of dosing time on the antiimmobility effect of antidepressants and mechanisms underlying this phenomenon were investigated in mice. In the forced swimming test (FST), the immobility time of mice treated with amitriptyline (15 mg/kg) and fluvoxamine (30 mg/kg) showed a significant 24-hr rhythm. The antiimmobility effect of fluvoxamine in FST was potent at the early part of the dark phase without increasing locomotor activity. Concerning pharmacokinetics, although Ke of fluvoxamine was about 1.3-fold higher in mice injected with fluvoxamine at 21:00 than at 9:00, no dosing time-dependence was demonstrated for either plasma or brain fluvoxamine concentration at 0.5 hr after the drug injection. On the other hand, SERT mRNA expression and 5-HT uptake activity in the mouse midbrain showed significant time-dependent changes with higher levels during the dark phase and lower levels during the light phase. These results suggest that the reuptake of 5-HT might be more increased during the dark phase. Since the reuptake of 5-HT is inhibited almost completely by injection with 30 mg/kg of fluvoxamine at any time, the extracellular 5-HT level may be more increased by the injection of fluvoxamine at the early part of the dark phase. The present results suggest that the antiimmobility effect of fluvoxamine in FST increases depending on dosing time. Furthermore, the time-dependent change of SERT mRNA expression and uptake activity in the midbrain are suggested to be the mechanism underlying the 24-hr rhythm of antiimmobility effect of fluvoxamine.

## Introduction

A large number of physiological rhythmic variables are apparent in the central nervous system, in hormone secretion and so on (Kafka et al., 1981; Thomson et al., 1980). Also, many drugs vary in potency and/or toxicity according to the time in the circadian cycle when they are administered (Ohdo et al., 1995, 1996, 2001; Frederickson et al., 1977; Walker and Owasoyo, 1974). However, several drugs cause alterations in the 24-hr rhythms of biochemical, physiological and behavioral processes (Akiyama et al., 1999; Horikawa et al., 2000) depending on the dosing time. It is thus very important to consider the administration schedule of drugs in pharmacotherapy.

The major theory of depressive disorder, the monoamine hypothesis, proposes that decreasing the levels of one or more of the brain monoamine neurotransmitters, such as serotonin (5-HT), noradrenaline (NA) or dopamine, can produce such diseases. A refinement of this hypothesis is that depressive illness may arise, specifically, from decreased brain 5-HT function (Coppen, 1967) and a number of factors have been proposed to account for a reduced functionality of the 5-HT system in depression. The 5-HT system has formed the basis of investigation into currently available antidepressants and still represents an area of drug development. The forced swimming test (FST) is a behavioral model developed to predict the efficacy of antidepressants in humans. Many classes of antidepressants decrease the immobility time in FST, including tricyclic antidepressants, monoamine oxidase inhibitors and atypical antidepressants. However, 5-HT uptake inhibitors have sometimes failed to produce positive effects in FST (Detke et al., 1997), although they have therapeutic efficacy

in depressive patients.

The serotonin transporter (SERT), a member of the $Na^+/Cl^-$ dependent transporter family, plays a key role in central serotonergic neurotransmission (Rudnick, 1977). SERT is the target of antidepressant drugs, in particular SSRIs (Thomas et al., 1987; Dechant and Clissold, 1991), which enhance 5-HT neurotransmission by increasing the extracellular 5-HT level (Invernizzi et al., 1995). SERT mRNA expression is detected several brain areas; the brain stem, hippocampus and frontal cortex. In particular, the midbrain raphe complex showes higher expression levels than other areas (Bengel et al., 1997). Concerning the circadian change of SERT expression, [3H]-labeled imipramine binding sites showed markedly increased in suprachiasmatic nuclei of the anterior hypothalamus during the dark phase (Wirz-Justice et al., 1983).

Chronopharmacological studies of psychotropic drugs have been reported using animals s, and trials for clinical application considering the dosing-time have already been undertaken (Nagayama, 1993). However, the mechanisms underlying the phenomenon in which the activity of psychotropic drugs, including antidepressants, varies depending on the dosing schedule are still unclear. The purpose of this study was to examine the 24-hr rhythm of antidepressant activity in FST. The mechanisms underlying this phenomenon were also investigated from the perspectives of pharmacokinetics and pharmacodynamics.

## Methods

### Animals

5-week-old male ICR mice were purchased from Charles River Japan (Kanagawa, Japan). Mice were housed 8-10 per cage under standardized light- dark cycle condition (lights on at 7:00, lights off at 19:00) at a room temperature of 24 ± 1 $^{o}$C and humidity of 60 ± 10 % with food and water *ad libitum*. All mice were exposed to the light-dark cycle for 1 week before the experiments. During dark periods, dim red light was used to minimize the light-stimuli to mice.

### Drugs and treatment procedures

The following drugs were used; amitriptyline hydrochloride (Sigma, St Louis, MO, USA) and fluvoxamine maleate (Solvay Pharmaceuticals Ltd., Weesp, Netherlands). Both drugs were dissolved in sterilized saline and the volume of injection was 0.01 mL/g of body weight. In preliminary experiments, we examined whether the antiimmobility effect of antidepressants in forced swimming test (FST) showed any dose-dependency. This experiment was performed at 9:00, and the dosages were 5, 15 and 30 mg/kg for amitriptyline, and 15, 30 and 60 mg/kg for fluvoxamine.

To investigate the influence of dosing time on antiimmobility effect, groups of 6-12 mice were injected intraperitoneally with saline, amitriptyline (15 mg/kg) or fluvoxamine (30 mg/kg) at 9:00, 13:00, 17:00, 21:00, 1:00 or 5:00. FST was performed at 30 min after the drug injection. To study the influence of fluvoxamine on locomotor activity, groups of 5-6 mice were injected intraperitoneally with saline or fluvoxamine (30 mg/kg) at 9:00 or 21:00. To

study the influence of dosing time on pharmacokinetics of fluvoxamine, groups of 4-8 mice were injected intraperitoneally with fluvoxamine (30 mg/kg) at 9:00 or 21:00. Blood and whole brain samples were obtained at 0.25, 0.5, 1, 2 or 4 hr after the drug injection and blood samples were centrifuged at 3,000 rpm for 15 min to get the plasma. Both plasma and brain samples were stored at -80 $^{o}$C until assay. To clarify the time-dependent changes of serotonin transporter (SERT) mRNA expression and 5-HT uptake activity in mouse midbrains, whole brain samples were removed from groups of 5 mice and the midbrain separated at 9:00 or 21:00. For assay of 5-HT activity, crude synaptosomes were prepared at each time and all samples were stored at -80 $^{o}$C until assay.

**Forced swimming test**

The procedure was performed based on a previous report (Porsolt et al., 1977). Briefly, mice were placed individually into plastic cylinders (height: 25 cm, diameter: 10 cm) containing 10 cm of water, maintained at 21-23 $^{o}$C, and left there for 5 min. A mouse was judged to be immobile when it floated in an upright position, and made only small movements to keep its head above water. The duration of immobility was recorded during the 5 min testing period.

**Monitoring locomotor activity**

To assess locomotor activity, mice were housed individually in plastic cages (24 cm × 16 cm × 12 cm) and their locomotor activity was measured by area sensors (model NS-AS01) with a thermal radiation detector. The sensors were set at 15 cm height from the cage floor. The data were stored on a personal computer. Activity counts (number of movements) were

recorded at 1 min intervals. Activity was recorded 15 min before and 30 min after injection. For visualization of locomotor activity, activity counts were calculated using a 3-min window.

**Determination of fluvoxamine concentration in plasma and brain**

Measurement of plasma fluvoxamine concentration was performed as described previously (Wong SHY et al., 1994). 200 μL of plasma sample spiked with solution of clomipramine, as the internal standard, was alkalinized by addition of 200 μL of 1N NaOH, followed by 1 mL of n-hexane:3-methylbutanol (99:1), and then was shaken for 30 min and centrifuged at 10,000 rpm for 10 min. The organic layer was transferred to another tube for acid back-extraction with 100 μL of 0.05 % phosphoric acid, which was then shaken for 20 min and centrifuged at 10,000 rpm for 10 min. The top organic layer was discarded, and 25 μL of the remaining aqueous layer was injected into the HPLC system for analysis. To determine the brain fluvoxamine concentration, mouse whole brain was homogenized in 1 mL of methanol and centrifuged at 12,000 rpm for 15 min. The supernatant spiked with clomipramine solution was alkalinized by addition of 1 mL of 1N NaOH, followed by 4 mL of hexane:isoamyl alcohol (99:1), and then was shaken for 30 min and centrifuged at 4,500 rpm for 10 min. The organic layer was transferred to another tube for acid back-extraction with 200 μL of 0.05 % phosphoric acid, which was then shaken for 20 min and centrifuged at 4,500 rpm for 10 min. The top organic layer was discarded, and 25 μL of the remaining aqueous layer was injected into the HPLC system for analysis. The HPLC apparatus consisted of a LC-10AS Liquid Chromatograph (Shimadzu, Kyoto, Japan), a 5 μm particle size (4.0 mm I.D. × 10 mm) CAPCELL PAK C18 MG S-5 guard column (Shiseido, Tokyo, Japan), a 5 μm

JPET Fast Forward. Published on August 3, 2005 as DOI: 10.1124/jpet.105.088849
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particle size (4.6 mm I.D. × 250 mm) CAPCELL PAK C18 MG analytical column (Shiseido), a SPD-10A UV-Vis Detector (Shimadzu), set at 245 nm, and a C-R7A plus Chromatopac (Shimadzu). The column temperature was maintained at room temperature. The mobile phase was 50 mM $KH_2PO_4$ (pH 4.7 with KOH) and acetonitrile (60:40, v/v). The mobile phase was filtered and degassed prior to use and the flow rate was 1 mL/min.

**RNA extraction and RT-PCR**

Total RNA was extracted from mouse midbrains by using Trizol® solution (Gibco BRL, California, USA). Reverse transcription of RNA and cDNA amplification was performed with a one-step RT-PCR system (Invitrogen, California, USA). The following oligodeoxynucleotide primers were used: sense primer, 5'-ATCATAGCCTG GGCGCTCTAC-3' and antisense primer, 5'-CATGTAGCCAAGCACCGTGAA-3' for SERT (GenBank accession number, AF013604) and sense primer, 5'-GACCTC AACTACATGGTCTACA-3' and antisense primer, 5'-ACTCCACGACATACTCAGCAC-3' for GAPDH (GenBank accession number, M32599). The PCR product was electrophoresed through a 3 % agarose gel. The amounts of RT-PCR products of each mRNA were quantified using Kodak 1D image analysis software and normalized against GAPDH.

**Preparation of crude synaptosome and 5-HT uptake assay**

Crude synaptosome was prepared as described previously (Gray and Whittaker, 1962). Midbrain samples were homogenized by 0.32 M sucrose and centrifuged at 1,000 rpm at 4 $^oC$ for 10 min. The pellet was discarded and the supernatant was centrifuged at 12,000 rpm at 4 $^oC$ for 20 min. The supernatant was discarded and the pellet was resuspended in the original

volume of 0.32 M sucrose used as crude synaptosome. 5-HT uptake assay was performed as described previously (Snyder and Coyle, 1969). Crude synaptosome (final concentration 0.25 mg protein) was preincubated at 37 $^{o}$C for 5 min with Krebs-HEPES buffer (127 mM NaCl, 5 mM KCl, 1.3 mM $NaH_2SO_4$, 15 mM HEPES, 10 mM glucose and 1.2 mM $MgSO_4$). The buffer was gassed with oxygen for 30 min prior to use. Then [3H]-labeled 5-HT (final concentration 50 nM; Amercham, Buckinghamshire, UK) was added and incubated at 37 $^{o}$C for 5 min followed by rapid filtration under vacuum (GF/B filters; Whatman, Maidstone, UK). The filters were washed three times with cold Krebs-HEPES buffer, dried and placed in ACSII Scintillation Cocktail (Amersham). Nonspecific uptake was calculated from data obtained incubation at 0 $^{o}$C.

**Statistical analysis**

One-way ANOVA, Two-way ANOVA, repeated measure ANOVA and Scheffe's test were applied for the multiple comparison. The Student's t-test was used for independent comparison between two groups. The 5 % level of probability was considered to be significant. The population pharmacokinetics parameters were calculated on an HP-9000 series 700 (Yokogawa-Hewlett Packard Ltd., Tokyo, Japan) with the NONMEM program (version IV, level 1.1), following the one-compartment model (PREDPP program, subroutines ADVAN1 and TRANS1). Since pharmacokinetic parameters were analyzed by NONMEM, statistical significance was performed by $\chi$2-test. NONMEM is widely used for fragmentary data such as a limited number of samples in clinical practice and drug concentrations in animal tissues.

## Results

### Dose-dependent manner of antiimmobility effect of antidepressants

The immobility time in the forced swimming test (FST) is shown in Fig. 1a and 1b. This experiment was performed at 9:00. The antiimmobility effect showed dose-dependency for both antidepressants and the immobility time was significantly decreased at doses of 15 and 30 mg/kg of amitriptyline ($P< 0.01$), and 30 and 60 mg/kg of fluvoxamine ($P< 0.05$ and $P< 0.01$, respectively). Therefore, 15 mg/kg of amitriptyline and 30 mg/kg of fluvoxamine were selected in present study.

### Influence of dosing time on the antiimmobility effect of antidepressants

The results are shown in Fig. 2a and 2b. There was no significant interaction between the drug-injection and the dosing-time in Fig. 2a and 2b. As for the effect of the drug-injection, the immobility times of mice treated with amitriptyline and fluvoxamine were significantly decreased compared with those of mice treated with saline ($Df$ =1, $F$ =225.49, $P$ <0.01 for Fig. 2a; $Df$ =1, $F$ =83.19, $P$ <0.01 for Fig. 2b). The multiple comparison test was performed separated by dosing-time, and the immobility times of mice treated with amitriptyline and fluvoxamine were significantly decreased compared with mice treated with saline at any six different times ($P$ <0.01 at 9:00, 13:00, 17:00, 21:00, 1:00 and 5:00 for Fig. 2a; $P$ <0.05 at 17:00 and $P$ <0.01 at 9:00, 13:00, 21:00, 1:00 and 5:00 for Fig. 2b). As for the effect of dosing-time, the immobility time showed a significant time-dependent change ($Df$ =5, $F$ =4.00, $P$ <0.01 for Fig. 2a; $Df$ =5, $F$ =6.60, $P$ <0.01 for Fig. 2b). The multiple comparison test was performed separated by saline or antidepressants, and the immobility time of mice treated

with saline was decreased slightly during the dark phase but showed no significant 24-hr rhythm. In contrast, the immobility time of mice treated with amitriptyline and fluvoxamine showed a significant 24-hr rhythm ($Df$ =5, $F$ =3.29, $P$ <0.05 for amitriptyline group; $Df$ =5, $F$ =6.27, $P$ <0.01 for fluvoxamine group). The antiimmobility effect of antidepressants in FST was potent at the early part of the dark phase and weak during the light phase. In particular, immobility time when treated with fluvoxamine at 21:00 was significantly decreased compared with 9:00, 13:00 and 17:00 ($P$ <0.01).

**Influence of dosing time of fluvoxamine on locomotor activity**

The results are shown in Fig. 3a and 3b. The basal locomotor activity was much higher at 21:00 (Fig. 3b), when antidepressants appeared to inhibit immobility most, than at 9:00 (Fig. 3a). The locomotor activity was slightly increased immediately after injection at 9:00, but not at 21:00. However, the activity counts after injection of fluvoxamine were not significantly different from those of saline at both 9:00 and 21:00. Therefore, fluvoxamine showed no dosing time-dependent difference effect on locomotor activity.

**Influence of dosing time on fluvoxamine pharmacokinetics**

The time courses of fluvoxamine concentration in plasma and brain are shown in Tables 1 and 2, and Figs. 4a and 4b. There was no significant interaction between the time after the injection and the dosing-time. As for the effect of dosing-time, plasma concentration showed a significant time-dependent change ($Df$ =1, $F$ =5.46, $P$ <0.05 for Fig. 4a), but brain concentration showed no significant time-dependent change. The multiple comparison test was performed separated by the time after the injection, and plasma concentration at 1 hr after

the injection at 9:00 was significantly higher than that at 21:00 ($P$ <0.05, Fig. 4a). Calculating pharmacokinetic parameters, the elimination rate constant (Ke) of fluvoxamine was 1.06 or 1.40 when injected at 9:00 or 21:00, respectively. It was about 1.3-fold higher in mice injected at 21:00 than at 9:00, and these two values showed significant difference ($P$ <0.01). However, Vd of fluvoxamine was not different between injection at 9:00 and at 21:00.

**Time-dependent change of SERT mRNA expression and 5-HT uptake activity in mice midbrain**

SERT mRNA expression levels and 5-HT uptake activity in mice midbrains are shown in Fig. 5a and Fig. 5b. The relative expression levels were normalized against GAPDH as an internal standard. The mRNA expression of SERT that SSRIs bind specifically was significantly higher at 21:00 than at 9:00 in mouse midbrains ($P$ <0.05). The 5-HT uptake activity was measured by an assay system using crude synaptosome. Preparation of crude synaptomes from mouse midbrains was performed at 9:00 or 21:00. Associated with SERT mRNA expression level, 5-HT uptake activity in mouse midbrains was also significantly higher at 21:00 than at 9:00 ($P$ <0.01).

**Discussion**

In the present study, the immobility times of mice treated with amitriptyline (15 mg/kg) and fluvoxamine (30 mg/kg) were decreased compared with that of mice treated with saline. And the immobility times of mice treated with amitriptyline and fluvoxamine in the forced swimming test (FST) showed significant 24-hr rhythms. From these results, the antiimmobility effect of antidepressants was potent at the early part of the dark phase and weak during the light phase. According to other reports, melatonin receptor antagonist, luzindole (N-0774), reduces the immobility time in FST pronouncedly in the middle of the dark phase rather than in the light phase (Dubocovich et al., 1990). Behavioral profiles and responses in several tests vary under diurnal and nocturnal conditions (Kelliher et al., 2000; Bertoglio and Carobrez, 2002).

In FST, antidepressant effects could be clearly distinguished from the effects of psychostimulants which markedly increased locomotor activity (Porsolt et al., 1978). Although the locomotor activity was increased during the dark phase rather than during the light phase, the immobility time of mice treated with saline showed no significant 24-hr change. Furthermore, since fluvoxamine had no effect on the locomotor activity of mice at 9:00 and 21:00, fluvoxamine seemed to decrease the immobility time in FST without increasing locomotor activity. FST was developed by Porsolt in rats and mice, and is the most widely used tool for preclinically assessing antidepressant activity. However, FST is considered to be insensitive in evaluating the effect of SSRIs, since it is less effective for SSRIs than drugs inhibiting noradrenaline reuptake such as amitriptyline (Lucki, 1997). The

present results suggest that FST is more sensitive to SSRIs by considering the dosing time. Then, we investigated the mechanisms underlying the dosing-time dependent antiimmobility effect of fluvoxamine from the perspectives of pharmacokinetics and pharmacodynamics.

First, we examined the influence of dosing time on fluvoxamine pharmacokinetics. In the preset study, the plasma fluvoxamine concentration at 1 hr after injection at 9:00 was higher than that at 21:00. The elimination rate constant (Ke) was about 1.3-fold higher in mice injected at 21:00 than at 9:00. The major metabolic pathway of fluvoxamine is oxidative demethylation and oxidative deamination in the liver (Overmars et al., 1983), and fluvoxamine is metabolized by CYP1A2 and CYP2D6 in human (Carrillo et al., 1996; Spigset et al., 1997). The expression of CYP1A2 mRNA is observed in mice (Dey et al., 1999), but 24-hr rhythms of mRNA expression and enzyme activity of mouse CYP1A2 have not been clarified yet. Although the exact mechanism underlying the dosing time-dependence of fluvoxamine pharmacokinetics is not clear, a significant 24-hr rhythm is demonstrated for hepatic blood flow (Lemmer and Nold, 1991). Therefore, this factor may be considered to be one of the mechanisms underlying the time-dependence change of fluvoxamine pharmacokinetics. Nevertheless, both plasma and brain fluvoxamine concentrations at 0.5 hr after the drug injection, when FST was performed, showed no significant difference between 9:00 and 21:00. Consequently, the dosing time-dependence in the antiimmobility effect of fluvoxamine does not seem to be related to the pharmacokinetics of the drug.

Secondly, we investigated the mechanism from the perspective of pharmacodynamics, focused on the mRNA expression and uptake activity of serotonin transporter (SERT)

associated with the reuptake of 5-HT from the synapse cleft. Since high expression levels of SERT mRNA are detected in the mouse midbrain raphe complex including dorsal and median raphe nuclei, it may be better to examine the mouse midbrain in detail. However, since only a small volume of RNA is obtained from each raphe nucleus, the whole midbrain complex was used in the present study. Expression levels of SERT mRNA in mouse midbrains showed a significant time-dependent change with higher level at 21:00 and lower level at 9:00. Also, 5-HT uptake activity in crude synaptosome prepared from mouse midbrains showed a significant time-dependent change associated with mRNA expression. These results suggest that the reuptake of 5-HT from the synapse cleft may be more increased during the dark phase than during the light phase.

According to previous reports, the extracellular 5-HT level in the dorsal raphe nucleus in freely moving rats is higher during the dark phase than during the light phase (Portas et al., 1998). And 5-HT neurotransmission in brain slices prepared from the rat hypothalamus shows time-dependent difference between the dark phase and the light phase (Biler et al., 1989). Furthermore, it is supposed that the reuptake of 5-HT from the synapse cleft is inhibited almost completely by injection with 30 mg/kg of fluvoxamine at any time (Claassen et al., 197). After all, extracellular 5-HT levels could be more increased by the injection of fluvoxamine at 21:00 than at 9:00. Therefore, the time-dependent change of extracellular 5-HT levels may contribute to the antiimmobility effect of fluvoxamine in FST. Since the antiimmobility effect of amitriptyline showed a significant 24-hr rhythm, expression of norepinephrine transporter (NET) or activity of MAO enzyme might show a time-dependent

change. However, since we focused on the mechanisms underlying the dosing time-dependent antiimmobility effect of fluvoxamine in the present study, we did not measure expression of NET or activity of MAO. Further study may be necessary to investigate whether expression of NET or activity of MAO shows a significant 24-hr rhythm.

The time-dependent change of SERT mRNA expression might be affected by circadian change of neuronal and/or hormonal signals in the transcriptional stage. However, adrenalectomy does not affect [3H]-citalopram binding in the rat midbrain (Kulikov et al., 1997) nor [3H]-imipramine binding in the frontal cortex (Arora and Meltzer, 1986). On the other hand, [3H]-paroxetine binding to the rat cortical membrane under restriction conditions significantly reduces without any change of transporter affinity (Zhou et al., 1996). The exact mechanisms underlying the time-dependent change of SERT mRNA expression should be clarified.

In the present study, the antiimmobility effect of fluvoxamine in FST was increased depending on dosing time. In other words, the sensitivity of FST to SSRIs was improved by considering the dosing time. Furthermore, time-dependent change of SERT mRNA expression and uptake activity in the midbrain, probably producing the rhythmicity of extracellular 5-HT levels, is suggested to be the mechanism underlying 24-hr rhythm of antiimmobility effect of fluvoxamine in FST.

JPET Fast Forward. Published on August 3, 2005 as DOI: 10.1124/jpet.105.088849
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**Acknowledgements**

We are indebted to Solvay Pharmaceuticals Ltd., (Weesp, Netherlands) for supplying the fluvoxamine used in this study.

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**Legends of figures**

**Fig. 1.** Dose-dependencies of antiimmobility effects of amitriptyline (**a**) and fluvoxamine (**b**). This experiment was performed at 9:00. The immobility time was significantly decreased at doses of 15 and 30 mg/kg of amitriptyline, and 30 and 60 mg/kg of fluvoxamine. Each value is mean with S.E. of 8-12 mice. *, $P$ <0.05, **, $P$ <0.01 when compared with saline group.

**Fig. 2.** Influence of dosing time on immobility time of mice injected with amitriptyline (**a**, 15 mg/kg i.p.) or fluvoxamine (**b**, 30 mg/kg i.p) at six different times in forced swimming test. Each value is the mean with S.E. of 6-12 mice. The immobility time of mice injected with amitriptyline and fluvoxamine showed a significant 24-hr rhythm ($F$ =3.29, $P$ <0.05 for amitriptyline group, $F$ =6.27, $P$ <0.01 for fluvoxamine group). *, $P$ <0.05, **, $P$ <0.01 when compared with saline group at the corresponding dosing time. ##, $P$ <0.01 when compared with 21:00 of fluvoxamine group. saline,○; amitriptyline,■; fluvoxamine,●.

**Fig. 3.** Influence of dosing time on locomotor activity after saline or fluvoxamine (30 mg/kg i.p.) injection at 9:00 (a) or 21:00 (b). The activity counts (number of movements) were calculated using a moving average with a 3-min window. Each value is the mean with S.E. of 5-6 mice. No significant difference between two groups at either dosing time. saline,○; fluvoxamine,●.

**Fig. 4.** Influence of dosing time on plasma (a) and brain (b) fluvoxamine concentrations after fluvoxamine (30 mg/kg, i.p.) injection at 9:00 (○) or 21:00 (●). Each value is the mean with S.E. of 4-8 mice. *, $P$ <0.05 when compared between two groups.

**Fig. 5.** Time-dependent changes of SERT mRNA level (a) and 5-HT uptake activity (b) in mouse midbrains. The relative expression levels were normalized against GAPDH. Each value is the mean with S.E. of 5 mice. Both mRNA expression and 5-HT uptake activity were significantly higher at 21:00 than at 9:00. *, $P$ <0.05, **, $P$ <0.01 when compared between two groups. Open column shows 9:00 group, shadowed column shows 21:00 group.

## Tables

TABLE 1

Influence of dosing time on plasma fluvoxamine concentration after fluvoxamine injection at 9:00 or 21:00. Each value is the mean with S.E. of 6-8 observations.

| Time after drug injection | Injection time | | Statistical significant |
|---|---|---|---|
| | 9:00 | 21:00 | |
| | ng/ml | | |
| 0.25 hr | 1557.13 ± 72.95 | 1569.78 ±105.75 | N.S. |
| 0.5 hr | 1181.84 ± 77.79 | 1009.73 ± 119.89 | N.S. |
| 1 hr | 930.78 ±125.92 | 581.45 ± 33.62 | $p<0.05$ |
| 2 hr | 231.44 ± 73.33 | 153.49 ± 7.86 | N.S. |
| 4 hr | 25.95 ± 1.87 | N.D. | |

N.D.; not detected, N.S.; not significant

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TABLE 2

Influence of dosing time on brain fluvoxamine concentration after fluvoxamine injection at 9:00 or 21:00. Each value is the mean with S.E. of 4-8 observations.

| Time after drug injection | Injection time | |
|---|---|---|
| | 9:00 | 21:00 |
| | μg/g tissue | |
| 0.25 hr | 14.74 ± 3.48 | 16.51 ±3.78 |
| 0.5 hr | 13.86 ± 0.91 | 11.16 ± 1.17 |
| 1 hr | 9.54 ±1.67 | 6.41 ± 0.46 |
| 2 hr | 1.89 ± 0.14 | 1.38 ± 0.14 |
| 4 hr | 0.32 ± 0.01 | N.D. |

Statistical analysis by two-way ANOVA. As for the effect of the doing-time, brain concentration showed no significant time-dependent change.

N.D.; not detected

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TABLE 3

Final estimates of population pharmacokinetic parameters.

| | Mean | 95 % C.I. |
|---|---|---|
| Ke (1/hr) = $\theta_1 \times \theta_3^{CRH}$ | | |
| $\theta_1$ | 1.06 | (0.90, 1.22) |
| $\theta_3$ | 1.32 | (1.13, 1.51) |
| $\omega_{Ke\ (1/hr)}$ | 0.0462 | (0, 0.11) |
| Vd (L) = $\theta_2$ | | |
| $\theta_2$ | 13.8 | (12.5, 15.1) |
| $\omega_{Vd\ (L)}$ | 6.1 | (0, 12.7) |
| $\sigma$ (ng/mL) | $4.46 \times 10^{-8}$ | (0, 0.035) |

CRH: 9:00 = 0, 21:00 = 1
95%C.I. = 95 % confidence intervals of the mean

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**Figure 1.**

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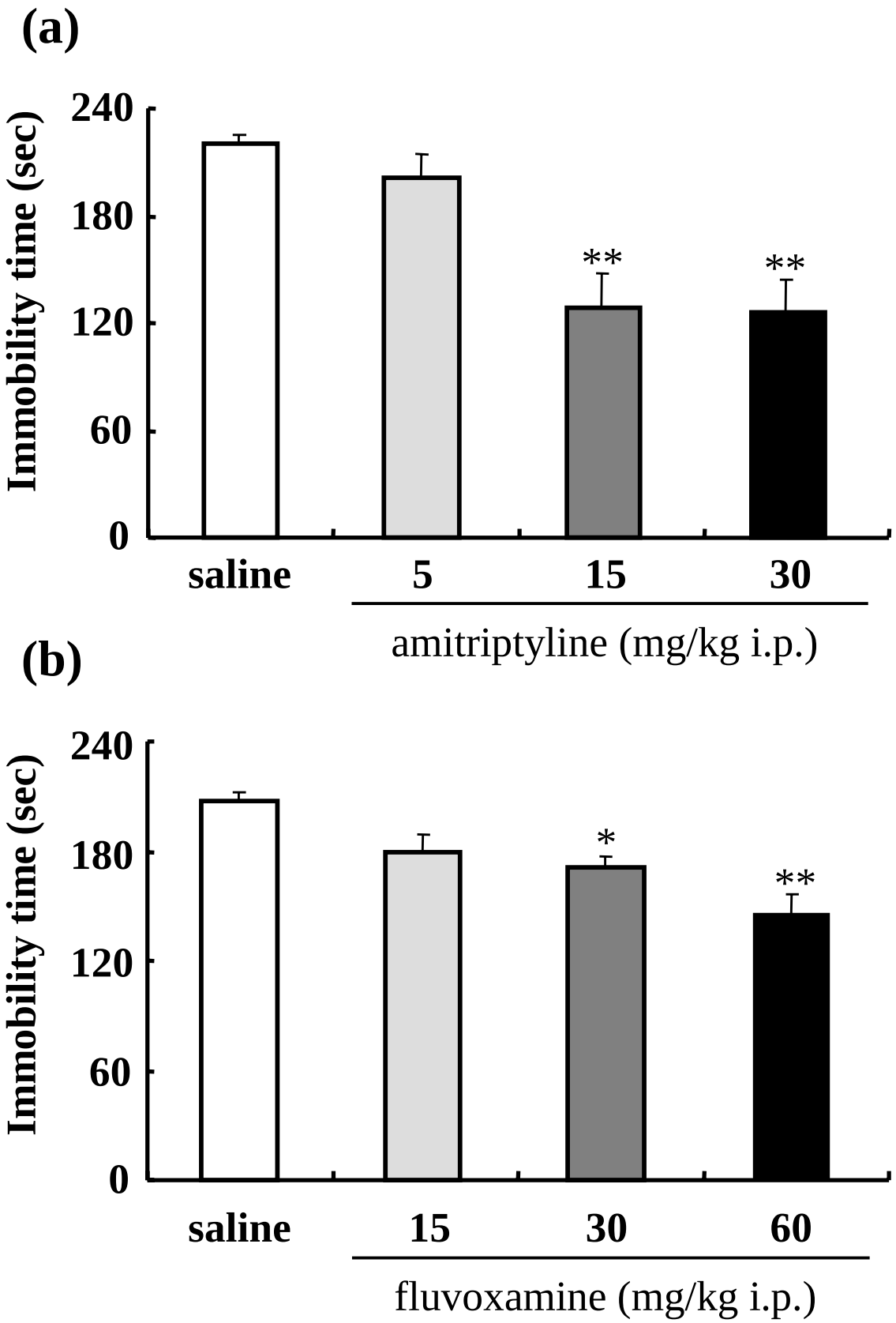

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**Figure 2.**

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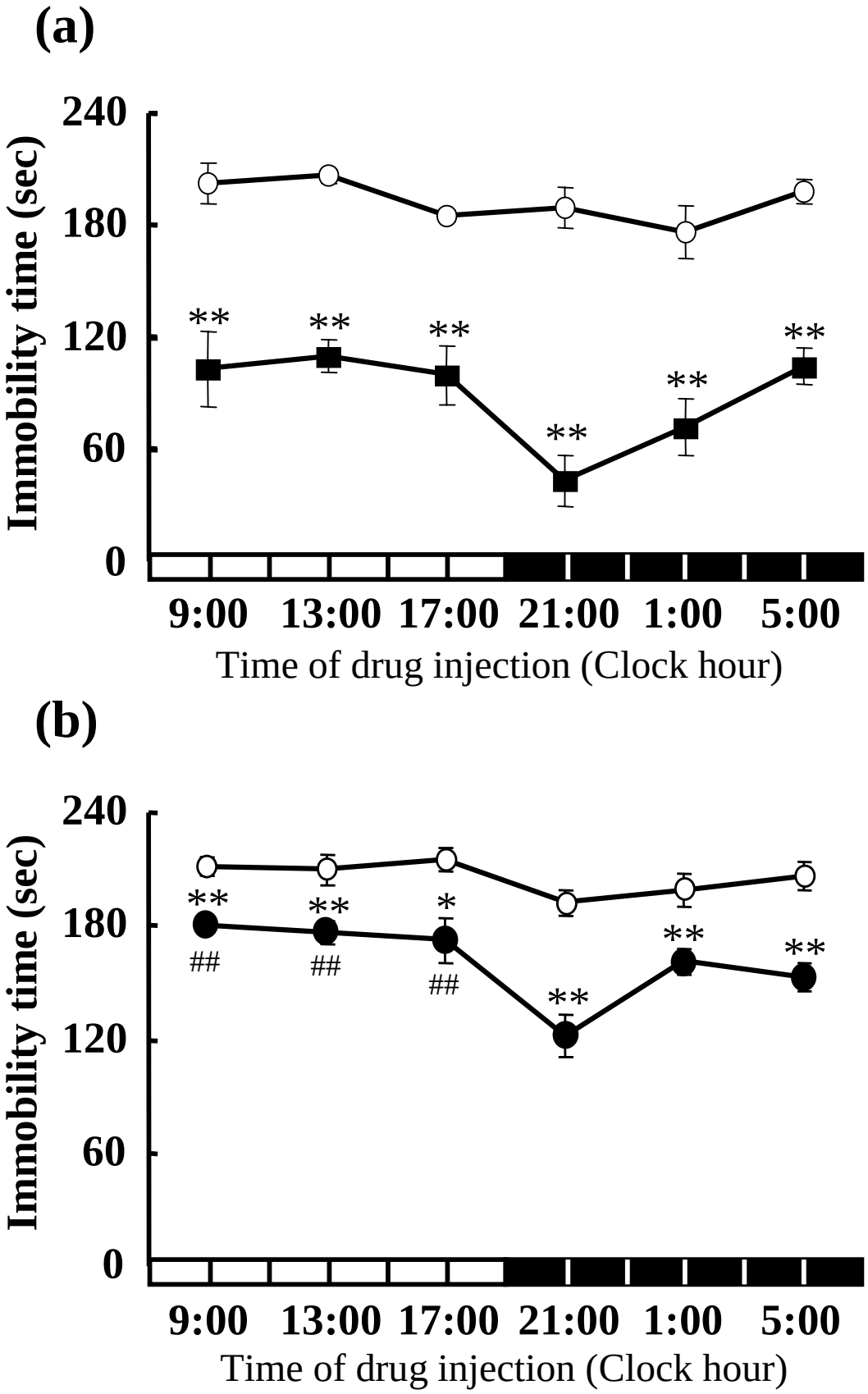

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**Figure 3.**

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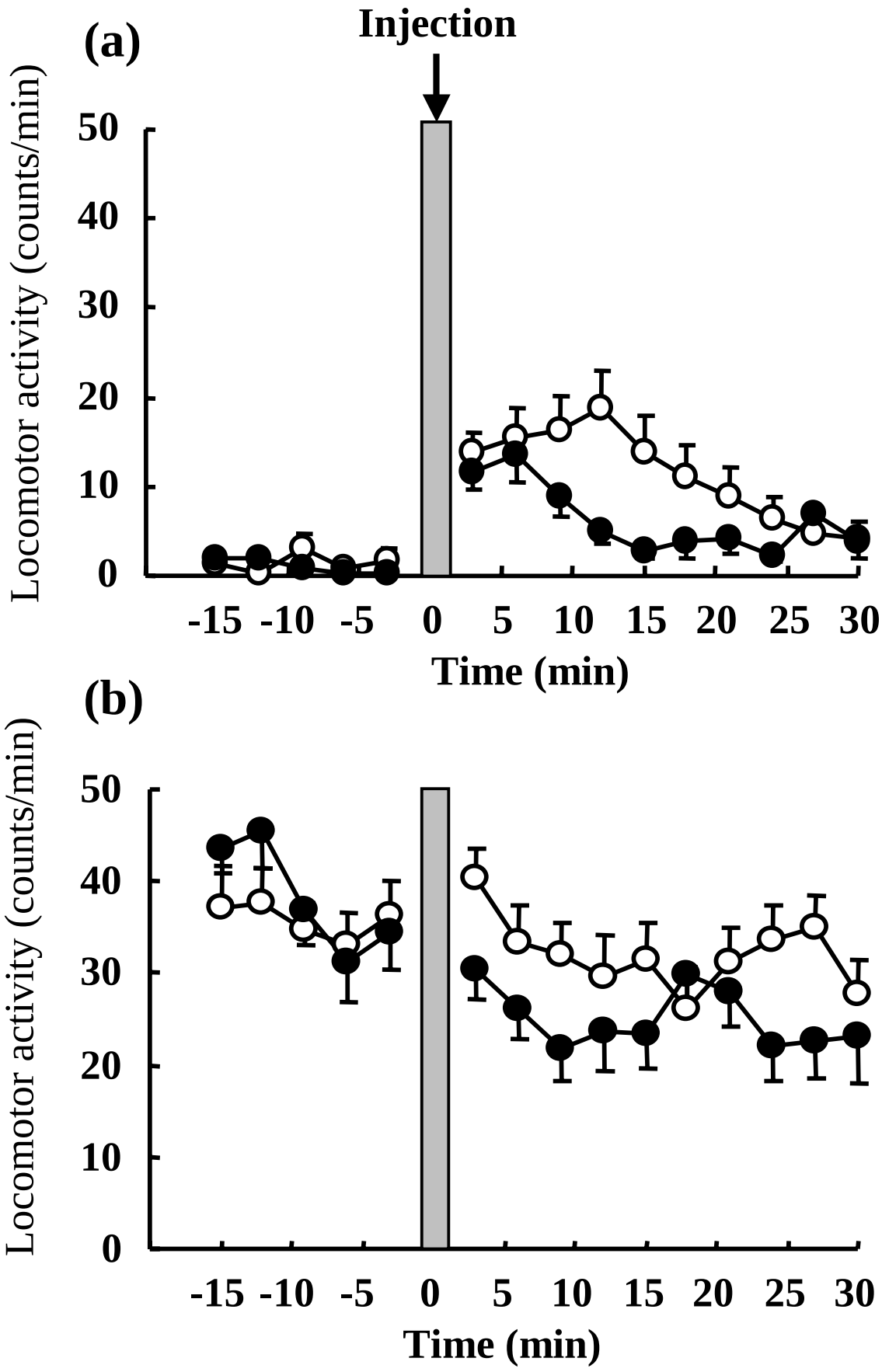

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**Figure 4.**

**JPET #88849**

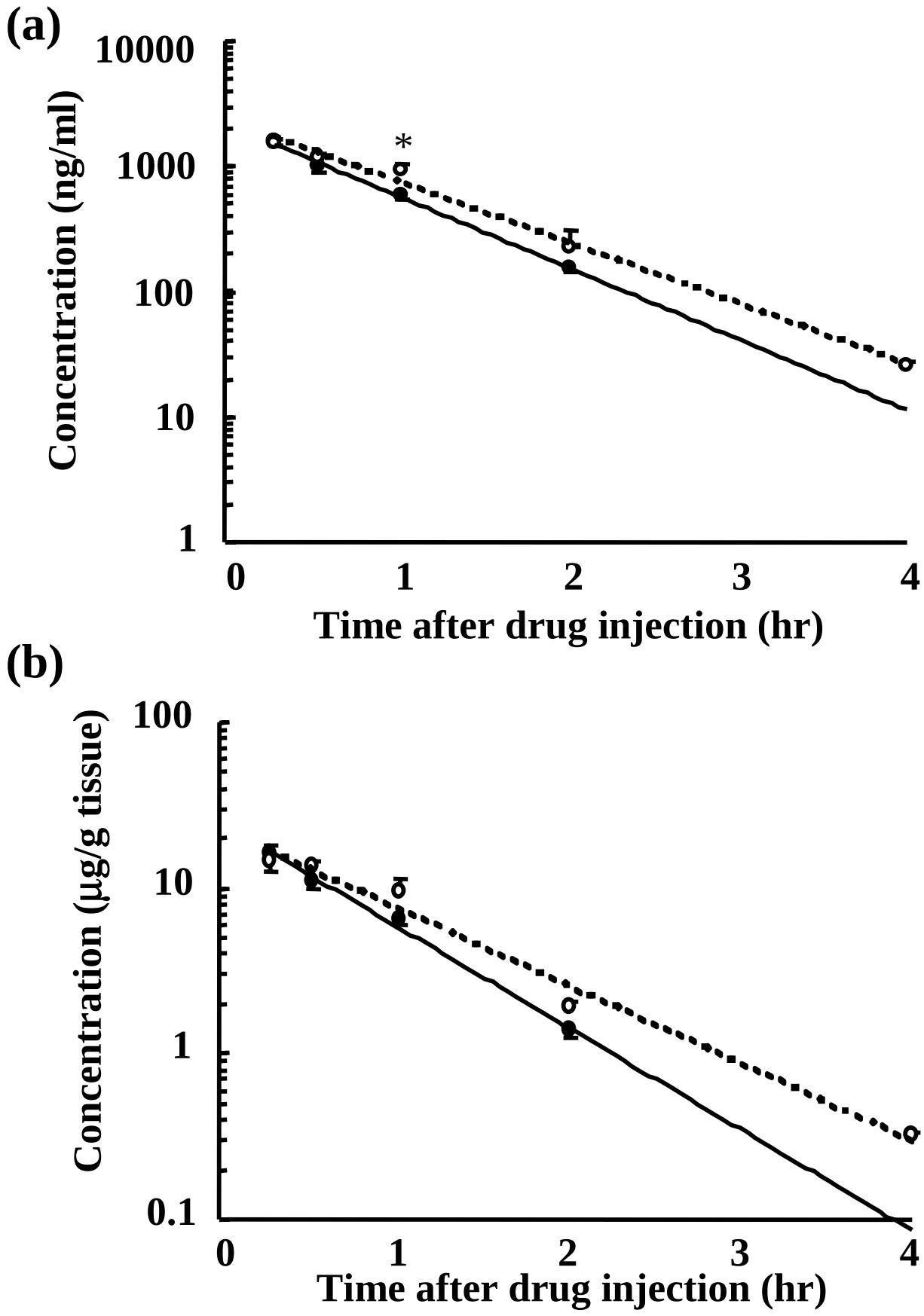

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**Figure 5.**

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