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Original article

Antidepressants with different mechanisms of action show different chronopharmacological profiles in the tail suspension test in mice

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Abstract

The circadian system regulates sleep/wake cycles, metabolism, mood, and other functions. It also influences medication efficacy. In this study, we studied the chronopharmacological profiles of antidepressants with various modes of action. We also investigated the effects of dosing time on the pharmacological activity of several antidepressants acting on serotonergic, noradrenergic, and/or dopaminergic neurons. C57BL/6 mice were intraperitoneally administered fluoxetine, imipramine, venlafaxine, or bupropion at 08:00 h (morning), 14:00 h (mid-day), 20:00 h (evening), or 02:00 h (mid-night). Antidepressant activity was evaluated by the tail suspension test. All antidepressants reduced immobility, and their activities varied according to the dosing time. Fluoxetine and imipramine induced relatively strong rhythms with high amplitudes. Their maximal effects were observed in the morning and evening, respectively. Venlafaxine and bupropion induced weak rhythms with maximal effects in the evening and dawn, respectively. These results suggest that the antidepressant activity is associated with circadian fluctuation, and antidepressants with different modes of action have different chronopharmacological profiles. They affect locomotor activity in animals placed in novel (unfamiliar) environments. Fluoxetine, imipramine, and venlafaxine reduced locomotor activity, whereas bupropion increased it. The effects on locomotor activity also vary with circadian rhythm, and the tested drugs showed a maximal effect during the light phase. The peak time was different from that in TST. Plasma and brain levels of all drugs were slightly

higher in the morning than in the evening. The dosing time dependency of the antidepressant activity did not correlate with the sedative/stimulatory activity or tissue drug level. Therefore, these latter two factors may have only a small impact on circadian antidepressant activity fluctuations. The relative activity of the serotonergic, noradrenergic, and dopaminergic systems may determine the chronopharmacological profiles of each drug. These results suggest the possibility that drug therapy be optimized by considering the dosing time when the antidepressant activity is high and other pharmacological activities leading to adverse effects are low. Further studies using animal models of depression and in clinical settings are necessary to confirm the effects of dosing time on depressed subjects.

Keywords: antidepressant; chronopharmacology; dopamine; noradrenaline; serotonin; tail suspension test.

Introduction

The circadian clock governs almost all physiological functions. Its disruption leads to various physical and psychological disorders. Depression is one of the most common conditions associated with circadian rhythm. Chronotype is correlated with depression. Individuals with an eveningness orientation are prone to depression and are unresponsive to antidepressant therapy (Au and Reece 2017; Merikanto et al. 2013; McGlashan et al. 2018). Depressive symptoms show diurnal variations (Moffoot et al. 1994). Patients with depression show reduced circadian body temperature rhythm (Avery et al. 1982). Circadian rhythm is also associated with depression therapy. Biological rhythm modification by sleep deprivation, sleep phase advance, and/or bright light treatment have antidepressant effects in > 60% of all patients (Wehr et al. 1979; Bicakova-Rocher et al. 1996; Voderholzer et al. 2003). Understanding the relationship between circadian rhythm and depression may help elucidate the pathogenesis of this condition and development of effective therapies for it.

Antidepressant activity is influenced by circadian rhythm. Several antidepressants show diurnal activity in clinical settings and animal models. Lofepramine and clomipramine most effectively ameliorate depression when they are administered at midnight (00:00 h) and midday (12:20 h), respectively (Philipp and Marneros 1978; Nagayama et al. 1991; Nagayama 1999). The sedation and xerostomia caused by amitriptyline administration are more severe following morning than evening treatments (Nakano and Hollister 1983). On the

other hand, the effects of fluoxetine do not differ between morning and evening administration (Usher et al. 1991). Timing for the maximal efficacy differs among the drugs. In animal studies, amitriptyline and fluvoxamine were most effective in the early dark phase (Ushijima et al. 2005), whereas nomifensine, milnacipran, and imipramine worked best in the light phase (Borsini et al. 1990; Kawai et al. 2018a, 2018b). A behavioral test suggested that the serotonergic and noradrenergic activities of milnacipran may contribute toward its overall antidepressant activity in the morning and evening, respectively (Kawai et al. 2018a). The dosing time-dependent antidepressant activity of these drugs could be explained by the circadian fluctuation of monoaminergic neuron activity (Ushijima et al. 2005; Kawai et al. 2018a). Earlier results suggested that the chronopharmacological profiles of antidepressants depend on their modes of action. To the best of our knowledge, however, no study to date has yet compared the chronopharmacological profiles of various antidepressants administered under the same experimental conditions.

A forced swim test (FST) and tail suspension test (TST) are often used to analyze antidepressant activity in mice. TST and FST are validated tests having high predictive validity (Cryan et al. 2005). Both tests record the time a mouse is immobilized in an unpleasant and inescapable situation. In TST, the mouse is hung by its tail, whereas in FST, it is placed in a water tank. In TST and FST, the mouse struggles to escape but assumes an immobile posture after several minutes. Pretreatment with an antidepressant decreases the

duration of immobility (Porsolt et al. 1978; Steru et al. 1985). Neither FST nor TST induce depression. However, these tests could predict the antidepressant activities of the compounds. Many of the clinically potent antidepressants reduce the immobility time, whereas a wide range of non-antidepressant compounds including anxiolytics such as benzodiazepines do not alter the immobility time in these tests. Although psychostimulants can induce false-positive responses, these tests discriminate between these effects and antidepressant activity by estimating the locomotor activity in novel environment (Steru et al. 1985; Slattery and Cryan 2012). Many of the currently available antidepressants reduce immobility time in TST/FST and locomotor activity in novel environment, whereas psychostimulants increase locomotor activity. As the pathogenesis of depression and the mechanism of action of antidepressants are not thoroughly understood, there is a possibility that unknown factor(s) cause a false result in TST and FST. However, by combining the analysis of the effects on the general activity, TST and FST can provide reliable data to highlight the antidepressant property of the drugs (Slattery and Cryan 2012).

Despite their similarities, a major difference between TST and FST is drug responsiveness (Cryan et al. 2005). FST cannot detect selective serotonin reuptake inhibitor (SSRI) antidepressant activity, whereas TST can. Certain atypical agents reduce immobility in FST but not in TST. Cryan et al. reported that TST was relatively more sensitive than FST (Cryan et al. 2005). Although these tests have insufficient face and constructive validity, they

are useful and effective with a high predictive validity for screening and analysis of antidepressant activity.

In this study, we investigated the dosing time-dependent antidepressant activity of several drugs with various modes of action. We tested the SSRI fluoxetine and the tricyclic antidepressant imipramine. They are commonly used in both preclinical studies and clinical settings. We also used the serotonin and noradrenaline reuptake inhibitor (SNRI) venlafaxine and the noradrenaline and dopamine reuptake inhibitor (NDRI) bupropion. Both compounds are used in clinical research. Venlafaxine showed superior therapeutic efficacy in several meta-analyses (Einarson et al. 1999; Anderson 2001; Cipriani et al. 2009). We used TST to evaluate the activity of antidepressants with various modes of action at different times of the day. TST is usually conducted 30–60 min after drug administration and the duration of the test session is usually 6 min (Cryan et al. 2005; Steru et al. 1985). As TST is generally validated by this protocol, we conducted the test according to this protocol. We have elaborated on the circadian activity rhythms of these drugs and discussed the relative differences in their chronopharmacological profiles.

Materials and methods

Chemicals

Ethylenediamine-*N,N,N',N'*-tetraacetic acid (EDTA) was obtained from Dojindo (Kumamoto, Japan). Fluoxetine, imipramine, venlafaxine, bupropion, and other chemicals were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Animals

Male adult C57BL/6NCrSlc mice (20–25 g) were obtained from Japan SLC (Shizuoka, Japan), and housed four mice per cage in an air-conditioned room at 24 ± 2°C with a 12 h/12 h light/dark cycle (lights on at 07:00 h). The mice had free access to food and water. The animals were maintained and treated in accordance with the general recommendations of animal protection legislation in Japan. All procedures were approved by the Institutional Animal Care and Use Committee of Josai International University (approval nos. 69 and 80).

The experimental schedule is shown in Figure 1. The time of the day is expressed as the zeitgeber time (ZT), which is defined in terms of hours after the 07:00 h light onset. The mice were intraperitoneally administered with fluoxetine (30 mg kg^{-1} as a hydrochloride salt), imipramine (30 mg kg^{-1} as a hydrochloride salt), venlafaxine (30 mg kg^{-1} as a hydrochloride salt), or bupropion (20 mg kg^{-1} as a hydrochloride salt) in the morning (08:00 h, ZT1), mid-day (14:00 h, ZT7), evening (20:00 h, ZT13), or mid-night (02:00 h, ZT19). These doses

were set because each drug reduced immobility up to 50 ± 10% against the respective controls in the TST. As these drugs showed more reduction in immobility at a higher dose in the preliminary experiments, their anti-immobility activities were not saturated at this dose. Therefore, the doses were selected to detect anti-immobility activity and diurnal fluctuation efficiently. All drugs were dissolved in saline. The control mice were administered with plain saline. All behavioral tests and sample collections were conducted 1 h after treatment.

Tail suspension test (TST)

The modified method of Steru et al. (1985) was used for TST. The mice were intraperitoneally administered with an antidepressant at ZT1, ZT7, ZT13, or ZT19. One hour after treatment, the mice were hung individually with paper adhesive tape on a bar 35 cm above the table. The adhesive tape was placed 2–3 cm above the tip of the tail. The mice were hung for 6 min. The duration of immobility was measured and recorded by trained observers. The mice were considered immobile when they showed no body movement during the test. Immobility reduction was regarded as an antidepressant activity.

Measurement of locomotor activity

The mice were intraperitoneally administered an antidepressant at ZT1, ZT7, ZT13, or ZT19. One hour after treatment, they were individually placed in a novel plastic field (40 cm × 20

cm × 20 cm) equipped with an infrared sensor (Supermex, Muromachi Kikai, Tokyo, Japan), which monitored locomotor activity for 30 min. In this system, the sensor detects the radiated body heat of the animal and counts the body movement in the *x*-, *y*-, and *z*-axes (Masuo et al. 1997). The signals from the sensor were processed by the CompACT AMS software (Muromachi Kikai, Tokyo, Japan). This system has been widely used to detect the locomotor activity of laboratory animals in various studies, including chronobiological studies (Satoh et al. 2006; Kohsaka et al. 2014).

Analysis of plasma and brain antidepressant levels

The mice were intraperitoneally administered an antidepressant at ZT1 or at ZT13. One hour after treatment, they were anesthetized with pentobarbital, and the blood and whole brain samples were drawn from them. The blood sample was removed from the postcaval vein with a heparinized syringe and centrifuged at 1,200 *g* at 4 °C for 10 min to separate the plasma. The samples were immediately frozen in liquid nitrogen and stored at − 80 °C until use.

Tissue analyses were performed according to the methods of Wong et al. (1994) and Tournel et al. (2001) with modifications. Citalopram was added to 0.2 mL plasma at 1 nmol sample^{-1} and used as an internal standard. The samples were alkalinized with 0.02 mL of 8 M NaOH and extracted with *n*-hexane/2-propanol (95/5). The organic layer was extracted with 0.1 mL of 50 mM phosphoric acid. The extracts were then passed through a 0.45-μm filter.

Some of the filtrates were analyzed by HPLC as described below. The brains were homogenized in a Polytron (Kinematica, Switzerland) using five volumes of 0.2 M perchloric acid containing 0.1 mM EDTA. Citalopram was added at 1 nmol sample^{-1} and used as an internal standard. The homogenate was centrifuged at 20,000 *g* at 4 °C for 15 min. The supernatant was alkalinized with 0.2 mL of 8 M NaOH. The brain samples were then extracted and filtered in the same way as the plasma samples. The standard samples used to prepare the HPLC calibration curves were obtained from naïve mice. Appropriate quantities of authentic compounds were added to the plasma and brain, and the treated tissues were processed as described above.

The antidepressants were analyzed by HPLC-UV. The LC-10AD/SPD-10A system (Shimadzu, Kyoto, Japan) was used. The separation was performed on a Gemini ODS (2.1 mm i.d. × 150 mm, Phenomenex, Torrance, CA, USA) fitted with a precolumn (SecurityGuard, Phenomenex, Torrance, CA, USA) at 40 °C. The eluents were 70% of 50 mM sodium phosphate buffer (pH 3.8), 30% acetonitrile to analyze fluoxetine, imipramine, and desipramine, 80% of 50 mM sodium phosphate buffer (pH 3.8), and 20% acetonitrile to analyze venlafaxine and bupropion. The flow rate was maintained at 0.2 mL min^{-1}. The compounds were detected at 200 nm.

Statistical analysis

The data are expressed as means ± SEM. The effects of dosing time and drug treatment were evaluated by two-way ANOVA (Figures 2, 4, and 5). Intra-day fluctuations were determined by one-way ANOVA (Figures 2, 4, 6, and 7). The differences in fluctuation among the drugs were assessed by two-way ANOVA (Figure 3 and 5). Post-hoc pairwise comparison with control was conducted by Dunnett test (Figure 5). Circadian rhythms were identified by Cosinor analysis (Figure 3, 5, and Table 1) with a single Cosinor method according to Tong (1976) and Cornelissen (2014) by setting the period length as 24 h. The significance level was set to $P < 0.05$. In Cosinor analysis, a regression cosine curve based on a data time series is obtained and three curve parameters are calculated. Acrophase is the time at which the regression curve is at a maximum. Mesor is the mean regression curve level. Amplitude is the regression curve height from the mesor at the acrophase.

All statistical analyses were performed with R (The R Foundation for Statistical Computing, Vienna, Austria) and EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R. EZR is a modified version of the R Commander, which adds statistical functions and is frequently used in biostatistics (Kanda 2013).

Results

Effects of dosing time on antidepressant activity in TST

As shown in Figure 2, all drugs reduced immobility in the TST. Two-way ANOVA detected an interaction effect between drug treatment and dosing time for imipramine ($F_{3,96}$ = 3.702, P = 0.014). The interaction effect in fluoxetine also showed a low P value but it was not significant ($F_{3,94}$ = 2.371, P = 0.075). The main effect of drug treatment was detected for all drugs (fluoxetine: $F_{1,94}$ = 71.408, P < 0.001; imipramine: $F_{1,96}$ = 39.863, P < 0.001; venlafaxine: $F_{1,72}$ = 61.620, P < 0.001; and bupropion: $F_{1,72}$ = 116.471, P < 0.001). The main effect of dosing time was not detected.

To evaluate the intra-day fluctuations of the antidepressant activity, relative immobility (% of each control) was measured by one-way ANOVA (Figure 2). Fluoxetine and imipramine showed significant fluctuations (fluoxetine: $F_{3,46}$ = 2.951, P = 0.042; imipramine: $F_{3,48}$ = 5.264, P = 0.003). Venlafaxine also showed a low P value ($F_{3,36}$ = 2.260, P = 0.098). Differences in fluctuation among drugs were calculated by two-way ANOVA. The main effects of the drug and dosing time and the interaction effect of these factors were detected (drug: $F_{3,166}$ = 6.133, P < 0.001; dosing time: $F_{3,166}$ = 3.653, P = 0.014; drug × dosing time: $F_{9,166}$ = 2.992, P = 0.002). These results indicate that the four drugs differ in terms of the intra-day rhythmicity of their antidepressant activity.

Figure 3 shows the Cosinor analyses of the circadian rhythmicity of antidepressant

activity for each drug. The antidepressant activity was calculated as the immobility reduction rate (% reduction relative to each control). Fluoxetine, imipramine, and venlafaxine showed significant circadian rhythms with peaks at ZT3.9, ZT10.2, and ZT9.3, respectively, as shown in Table 1 (fluoxetine: $F_{2,47}$ = 4.258, P = 0.020; imipramine: $F_{2,49}$ = 4.923, P = 0.011; venlafaxine: $F_{2,37}$ = 3.407, P = 0.044). Fluoxetine and imipramine showed relatively large circadian rhythms with amplitudes of 17.9 and 18.2 points (39% and 57% of each mesor), respectively.

Effect of dosing time on the locomotor activity

Antidepressants may also present with pharmacological effects on the locomotor activity. To analyze whether this effect causes the chronopharmacological activity in TST, we analyzed the effects of the four drugs on mouse locomotor activity using the same treatment schedule for the TST. As the dosing time-dependent difference of antidepressant activity was large between ZT1 and ZT13 in all four tested drugs (Figure 2), the effect on locomotor activity was analyzed at these ZTs. As shown in Figure 4, fluoxetine, imipramine, and venlafaxine reduced the locomotor activity, whereas bupropion increased it. Two-way ANOVA detected no interaction effect between drug treatment and dosing time or the main effect of dosing time. The main effect of drug treatment was detected in fluoxetine, imipramine, and venlafaxine (fluoxetine: $F_{1,20}$ = 235.654, P < 0.001; imipramine: $F_{1,20}$ = 35.633, P < 0.001; venlafaxine:

$F_{1,20}$ = 19.159, P < 0.001).

To measure the intra-day fluctuation in the locomotor activity, the relative locomotion (% of each control) was assessed by one-way ANOVA. Bupropion showed significant differences among time points ($F_{1,10}$ = 5.725, P = 0.038). The other drugs did not present with significant temporal differences.

To analyze the circadian rhythm of the effects on locomotor activity, tests were conducted at four different ZTs, and circadian rhythm of the activity was analyzed (Figure 5). Figure 5A shows the effect of each drug on locomotor activity. Two-way ANOVA detected main effect of dosing time and drug treatment (dosing time: $F_{3,80}$ = 24.826, P < 0.001; drug: $F_{4,80}$ = 56.170, P < 0.001). Post-hoc Dunnett test detected difference against control in fluoxetine, imipramine, and bupropion (fluoxetine: P < 0.001; imipramine: P < 0.001; bupropion: P = 0.006). Figure 5B shows the relative locomotor activity (% of respective control) and regression cosine curve for each drug. Two-way ANOVA detected main effect of dosing time and drug treatment (dosing time: $F_{3,64}$ = 5.687, P = 0.002; drug: $F_{3,64}$ = 76.290, P < 0.001). Circadian rhythm parameters calculated by Cosinor analysis are summarized in Table 1.

Effect of dosing time on plasma and brain drug levels

To analyze the possibility that the difference in tissue drug levels among different ZTs causes

the chronopharmacological activity observed in TST, we analyzed the plasma and brain drug levels after administration using the same treatment schedule for TST. As the dosing time-dependent difference in antidepressant activity was large between ZT1 and ZT13 in all four tested drugs (Figure 2), tissue drug levels were analyzed at these ZTs. Desipramine, an imipramine metabolite, has antidepressant activity and has comparable potency as that of imipramine itself (Tatsumi et al. 1997). Therefore, desipramine levels were also measured in imipramine-treated mice. The results are shown in Figures 6 and 7. The differences between ZT1 and ZT13 were small and not significant in all four drugs.

The metabolites norfluoxetine, *O*-desmethylvenlafaxine, and hydroxybupropion are also pharmacologically active (Tatsumi et al. 1997; Wong et al. 1993; Wong et al. 1995; Holliday and Benfield 1995; Martin et al. 1990). Nevertheless, they were not quantified in the present study because we did not have the authentic chemicals. There was a large unidentified peak in the HPLC chromatogram for venlafaxine (data not shown). This unknown metabolite may contribute to the pharmacological activity of its parent compound. However, the area of this peak between ZT1 and ZT13 differed so little that this compound is not expected to influence the circadian rhythm of the antidepressant activity. No other major peaks were detected in the HPLC analyses of fluoxetine and bupropion (data not shown). Therefore, the metabolites of fluoxetine, venlafaxine, and bupropion might have little effect on the chronopharmacological profiles of their parent compounds.

Discussion

In this study, we analyzed the dosing time-dependent activity of four antidepressants. Antidepressants with different modes of action differ in terms of their chronopharmacological profiles. Fluoxetine, venlafaxine, imipramine, and bupropion showed maximal antidepressant activity in the morning (ZT3.9), afternoon (ZT9.3), afternoon (ZT10.2), and pre-dawn (ZT23.5), respectively. These results suggest that therapeutic efficacy could be improved by drug dosing time and combinations. For example, treatment with fluoxetine in the morning and venlafaxine in the afternoon might maximize drug efficacy.

Various factors interfere with behavioral tests. In TST and FST, the pharmacological effects of drugs on general activity could confound the results. Stimulants, such as amphetamine reduce immobility (false positive), whereas sedatives may increase it (false negative) (Steru et al. 1985; Slattery and Cryan 2012). In the present study, the pharmacological effects of the drugs on general activity were evaluated via locomotor activity in a novel environment. As shown in Figure 4 and 5, fluoxetine, imipramine, and venlafaxine could reduce locomotor activity. These effects on locomotion might possibly increase the immobility in TST. However, these drugs reduced immobility in the TST (Figure 2). The effects on locomotor activity cannot cause these positive results in the TST. Furthermore, from the chronopharmacological point of view, the acrophases of activity in TST and locomotor activity are different according to the respective drugs (Table 1). The effects on

locomotor activity were not likely to cause the chronopharmacological effects in TST. The action of bupropion was different from the other three drugs. Bupropion tended to increase the locomotor activity (Figure 4 and 5). If this effect on the locomotor activity was responsible for the reduction of immobility in TST, the locomotor activity and reduction of immobility must show a similar acrophase. However, these two activities show different circadian profiles (Figure 3 and 5). These results suggest that the effect on general activity had little influence on the TST in the present study. The results of TST may reflect the specific antidepressant activities of these drugs.

Despite their different chronopharmacological profiles in TST, the tested drugs had similar profiles in locomotor activity analysis. Fluoxetine, imipramine, and venlafaxine showed maximal reduction of locomotor activity during the light phase. Comparing the antidepressant activity rhythm and sedative/stimulatory activity rhythm (Figure 3 and 5, and Table 1), fluoxetine showed its maximal pharmacological effects during the early light phase for both activities. However, the other drugs show maximal effect at different timing for these activities. The peak times of respective effects were different for 6–8 h. The antidepressant and sedative/stimulatory activities have independent rhythmicity, and acrophases of these activities are different in the respective drugs. These results suggest the possibility that drug therapy could be optimized by choosing the dosing time when antidepressant activity is high and other adverse effects are low. Such chronotherapy has not yet been reported for

antidepressants but has been proposed for anti-cancer drugs. The adverse effects of anti-cancer drugs show circadian fluctuations and can be reduced by considering dosing time (Lévi et al. 1997). Chronochemotherapy with oxaliplatin, fluorouracil, and folinic acid improves efficacy and reduces toxicity by controlling the drug dosing times (Lévi et al. 1997). Correlations among the rhythms of antidepressants and their other activities merit further study.

The control mice did not show obvious circadian phase-dependent response in TST (Figure 2). Although the locomotor activity of control mice at ZT19 was high and that at ZT7 was low (Figure 5), there was no difference in the locomotor activity between ZT1 and ZT13 (Figure 4 and 5). These results are consistent with previous studies (Jones and King 2001; Gomes et al. 2009; Huynh et al. 2011; Ago et al. 2014; Ota et al. 2015; Richetto et al. 2019). Mice are nocturnal and show circadian activity rhythm with high activity during nights in their home cage. However, their responses against experimental interventions are often unaltered between morning and evening. Non-treated or vehicle-treated naïve animals show circadian phase-dependent responses in some behavioral tests, but not in other behavioral tests including locomotor activity test and forced swim test (Jones and King 2001; Huynh et al. 2011; Ago et al. 2014; Richetto et al. 2019). As for locomotor activity in a novel environment, most studies have reported higher activity during the light phase or no circadian phase effect. Richetto et al. (2018) observed locomotor activity in C57BL mice with open

field test for 1 h during early light or early dark phase, and revealed that the ambulation score during first 30 min of the test was not different between the early light and early dark phase, and the ambulation score during the latter half of the test was higher in the early light phase. Some other studies observed locomotor activity for a shorter duration and detected no circadian phase-dependency (Jones and King 2001; Huynh et al. 2011). Since we observed locomotor activity for 30 min in a novel environment in the present study, our results on the locomotor activity at ZT1 and ZT13 are consistent with these previous reports.

Most of the previous studies conducted open field test in the morning (early light phase) and/or evening (early dark phase), and these tests could not detect any obvious difference between different ZTs (Jones and King 2001; Huynh et al. 2011; Ago et al. 2014). In the present study, we conducted tests at four-time points and revealed that the exploratory locomotor activity in a novel environment shows circadian phase-dependent response with a peak during the late dark phase (Figure 5). This rhythm is different from the rhythm of home cage activity that shows a peak during the early dark phase. Although the mechanism is unclear, the response to the novel environment may be highest during the late dark phase, and this response may be large enough to mask the circadian effect of home cage activity.

Differences in the plasma and brain antidepressant levels among different ZTs could lead to dosing time-dependent pharmacological effects of behavioral tests. A previous study indicated that the brain level of imipramine was higher in the evening treatment than that in

the morning treatment in Wistar rats, and reduction of locomotor activity was higher in the evening treatment (Lemmer and Holle 1991; Lemmer et al. 1986). Chronopharmacological effects on locomotor activity might be due to differences in the brain-drug level. The results of our study shown in Figures 4 and 7 also indicated the same correlation between brain drug level and sedative activity in fluoxetine, imipramine, and venlafaxine. Both brain drug levels and sedative activities showed slightly higher mean value at ZT1 than at ZT13, but they were not significant. Although the peak timing and amplitude were different between rats (the previous study) and mice (the present study), and the reason for this difference remains to be elucidated, the chronopharmacological effects of these drugs on locomotor activity might be dependent on the dosing time-dependent difference of the brain drug levels. For antidepressant activity, however, the brain and plasma drug levels could not explain the chronopharmacological effects. Differences in the plasma and brain drug levels between ZT1 and ZT13 were too small to account for the difference in anti-immobility activity in the TST. Ushijima et al. reported chronopharmacological analysis of fluvoxamine in ICR mice and showed that the pharmacokinetic parameters of fluvoxamine were different between morning and evening treatments. However, the brain drug levels at the time when the pharmacological test was conducted were not different between morning and evening treatments (Ushijima et al. 2005). They concluded that the chronopharmacokinetics of fluvoxamine did not affect the chronopharmacological antidepressant activity of the drug. We consider that the dosing

time-dependent difference in tissue drug levels after treatment at different ZTs could affect the chronopharmacological effects of the drug on locomotor activity, but not on antidepressant activity.

As all the four tested drugs are highly lipophilic, they could distribute to the brain within 1 h and exert antidepressant activity in TST (Cryan et al. 2005; Lemmer and Holle 1991; Holladay et al. 1998; Alvarez et al. 1998; Poleszak et al. 2015; Yoo et al. 1996). Compared to other drugs, fluoxetine showed high brain distribution after administration (Figures 6 and 7), which is consistent with previous studies (Holladay et al. 1998; Alvarez et al. 1998). This high brain distribution might be due to drug efflux pumps that are expressed at the blood-brain barrier. The drug efflux pump, P-glycoprotein, differentially affects the transport of antidepressants. Antidepressants, such as imipramine and venlafaxine, are substrates of P-glycoprotein and are effluxed from the brain by this pump, while other antidepressants, including fluoxetine, are not (O'Brien et al. 2013; Uhr et al. 2003). This effect contributes to the relatively high brain distribution of fluoxetine. High brain drug levels may cause different pharmacological effects from other drugs, especially after chronic treatment. However, in the present study, the high brain level of fluoxetine did not show any special effect on pharmacological tests. Fluoxetine showed equipotent activity with imipramine and venlafaxine (Figure 2), and the brain levels of fluoxetine were not different between the administration at ZT1 and ZT13 (Figure 7). Thus, the brain distribution of drugs

may not be an important factor that determines circadian fluctuation of antidepressant activity.

We considered that circadian fluctuation of the monoaminergic system may lead to the chronopharmacological effects of antidepressant activity. The brain monoamine levels and expression levels of their transporters and receptors show circadian rhythms (Nagayama 1999; Ushijima et al. 2005; Kawai et al. 2018a; Quay 1968; Kafka et al. 1983; Bhaskaran and Radha 1984; Akiyoshi et al. 1989; Castaneda et al. 2004). The brain serotonin levels and serotonin receptor binding reach peak during the light phase (Quay 1968; Bhaskaran and Radha 1984; Akiyoshi et al. 1989), while those of noradrenaline and dopamine reach maxima in the dark phase (Kawai et al. 2018a; Kafka et al. 1983; Bhaskaran and Radha 1984; Castaneda et al. 2004). This difference may lead to the different chronopharmacological profiles of antidepressants. Fluoxetine inhibits serotonin transporter without interacting with other transporters (Tatsumi et al. 1997; Horst et al. 1998). This inhibition causes an increase in serotonin level in the synaptic cleft, and increased serotonin enhances the neural transmission of the system via interaction with postsynaptic receptors, which finally results in antidepressant effects. As serotonin level and receptor binding are high in the light phase, the serotonergic neuronal transmission may be enhanced by fluoxetine more efficiently than in the dark phase. In contrast, the antidepressant effects via noradrenergic or dopaminergic neurons might be higher in the dark phase, because noradrenergic and dopaminergic neurons

show activity peak in the dark phase. Imipramine and venlafaxine potentiate serotonergic and noradrenergic systems, while bupropion potentiates dopaminergic and noradrenergic systems (Tatsumi et al. 1997; Horst et al. 1998). Therefore, these drugs might show a different activity rhythm from fluoxetine. The results of TST suggest that drug interactions with the serotonergic, noradrenergic, and dopaminergic systems produce different chronopharmacological profiles in which potent antidepressant activity occurs at different time points.

The precise mechanism determining the antidepressant chronopharmacological profile remains to be elucidated. Previous studies have reported that diurnal variations in serotonin transporter activity and monoamine level may be a part of the mechanism underlying the chronopharmacological antidepressant activity (Ushijima et al. 2005; Kawai et al. 2018a). However, other receptors may also add to circadian fluctuations in antidepressant activity. Nonselective agents such as imipramine interact with not only monoamine transporters, but also multiple receptors including α-adrenergic, histaminergic, and cholinergic receptors (Richelson and Nelson 1984; Mochizuki et al. 2002). Recently developed antidepressants interact with various receptors without inhibiting monoamine transporters. Noradrenaline and specific serotonergic antidepressant (NaSSA) inhibits α_1-adrenergic receptor and several 5HT receptors (De Boer et al. 1995; Anttila and Leinonen 2001). Agomelatine and ketamine interact with melatonin receptors and glutamate receptors,

respectively (Bourin et al. 2004; Dutta et al. 2015). Direct interactions with these receptors could affect chronopharmacological antidepressant activity. In particular, $5HT_2$, α_2-adrenergic, dopamine D_2, and glutamatergic receptors are considered to play important roles in antidepressant activity (Wong et al. 1994; Richelson and Nelson 1984; De Boer et al. 1995; Peroutka and Snyder 1980; Muguruza et al. 2013; Basso et al. 2005; Murrough et al. 2017; Williams and Schatzberg 2016). Circadian fluctuations of these receptors could be major factors determining the chronopharmacological profile of antidepressants. Chronobiological analyses of these factors may clarify the mechanisms underlying the chronopharmacological activity of antidepressants.

There are inconsistencies among studies in terms of the reported chronopharmacological profiles of different antidepressants. The present study indicated that the SSRI fluoxetine showed maximal antidepressant activity in the morning. A previous study, however, indicated that the SSRI fluvoxamine showed maximal antidepressant activity early at night in the FST in ICR mice (Ushijima et al. 2005). In the present study, the SNRI venlafaxine showed peak antidepressant activity in the afternoon, whereas the SNRI milnacipran showed potent antidepressant activity in the morning in the FST in Wistar-Hannover rats (Kawai et al. 2018a). The tricyclic antidepressant imipramine showed maximal antidepressant activity in the morning in rats (Kawai et al. 2018b). In contrast, imipramine and amitriptyline showed maximal antidepressant activity in the evening in

C57BL/6 mice (present study) and ICR mice (Ushijima et al. 2005), respectively. The present study failed to identify the reason for these inconsistent results. Nevertheless, differences in species (mice or rats), strain (C57BL/6 or ICR), behavioral test (TST or FST), and the drug might influence the chronopharmacological effects of antidepressants. Different strains have varying sensitivities to these behavioral tests (Liu and Gershenfeld 2001; Lucki et al. 2001). TST and FST use a similar paradigm but may show different behavioral and biochemical results. Therefore, the physiological mechanisms underlying the anti-immobility activity in these tests are different (Bai et al. 2001; Renard t al. 2003). Different results for the TST and FST suggest that patients with varying depressive symptoms may show unequal dosing time-dependencies for antidepressant treatment. This discrepancy must be considered in coordination with the administration of chronopharmacological depression therapy.

The present study had some limitations. It is difficult to predict the chronopharmacological profiles of the antidepressants in clinical settings based on the results of mouse TST. Fluoxetine showed a circadian activity rhythm here; however, in a clinical setting, its efficacy did not differ between morning and evening (Usher et al. 1991). Differences between mouse and humans and between normal and depressive conditions must be considered. Depressed patients present with various disturbances in the circadian rhythm (Avery et al. 1982; Bicakova-Rocher et al. 1996), which might alter drug dosing time-dependent responses in certain cases. Chronopharmacological profiles may vary between

normal mice and depressed patients. Since TST is highly predictive (Cryan et al. 2005), the present study may disclose the relationship among the chronopharmacological profiles of various antidepressants and their modes of action. However, it is unclear whether mouse TST predicts chronopharmacological effects in clinical settings. Further studies with animal models of depression and in clinical settings are necessary to confirm the effect of dosing time on depressed subjects and to develop a chronopharmacological antidepressant therapy protocol.

In the present study, we revealed the differences in chronopharmacological profile among various antidepressants. Each drug showed a circadian activity rhythm that was specific to the antidepressant activity. Fluoxetine, imipramine/venlafaxine, and bupropion showed maximal antidepressant activity in the early light phase, late light phase, and late dark phase, respectively. Relative activity levels in serotonergic, noradrenergic, and dopaminergic systems might be important factors determining the drug chronopharmacological profile. The results of the present study suggest that antidepressant efficacy could be improved by considering the dosing time. Therapeutic efficacy of these drugs might be optimized by administering them when antidepressant activity is high and side effects are low. Further analysis with other antidepressants and an animal depression model may elucidate the mechanism of chronopharmacological action and the potential value of chronopharmacological antidepressant therapy.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Table 1. Summary of Cosionr analysis of antiimmobility activity and locomotor activity.

	Fluoxetine	**Imipramine**	**Venlafaxine**	**Bupropion**
Antiimmobility activity				
Acrophase (ZT)	3.9	10.3	9.3	23.5
Mesor	45.8	31.9	36.0	53.4
Amplitude	17.9	18.2	10.5	5.2
p	0.020	0.011	0.044	0.464
R^2	0.153	0.167	0.156	0.041
Locomotor activity				
Acrophase (ZT)	15.6	14.0	15.4	7.2
Mesor	31.9	62.3	86.1	130.0
Amplitude	15.3	9.9	22.7	1.0
p	< 0.001	0.506	0.032	0.990
R^2	0.565	0.077	0.332	0.001

Figure Legends

Figure 1. Experimental schedule. White and black bars represent the light and dark periods, respectively. Mice were administered a drug at ZT1, ZT7, ZT13, or ZT19. Behavioral tests or sample collections were conducted 1 h after treatment.

Figure 2. Effects of the antidepressants on immobility in TST at ZT1, ZT7, ZT13, and ZT19. Immobility after administration of fluoxetine (A), imipramine (B), venlafaxine (C), and bupropion (D) in TST is shown. Left panels: immobility scores for the control (open column) and antidepressant (closed column) groups. Right panels: immobility relative to each control (% of control). Horizontal white and black bars at the top of each graph represent the light and dark periods, respectively. Data are shown as the mean + SEM (n = 10-13). $^{\S}P < 0.05$ for the interaction effect between drug treatment and dosing time, $^{\dagger\dagger}P < 0.01$ for the main effect of drug treatment as determined by two-way ANOVA; $^{*}P < 0.05$, $^{**}P < 0.01$ by one-way ANOVA.

Figure 3. Cosinor analysis of intra-day fluctuations in antidepressant activity. Immobility reduction rates (% reduction in immobility relative to each control) by fluoxetine (A), imipramine (B), venlafaxine (C), and bupropion (D) in the TST are shown. The diamond with error bars represents the means ± SEM of the TST data at ZT1, ZT7, ZT13, and ZT19. The

line represents the regression cosine curve calculated by Cosinor analysis. Horizontal white and black bars at the top of each graph represent the light and dark periods, respectively.

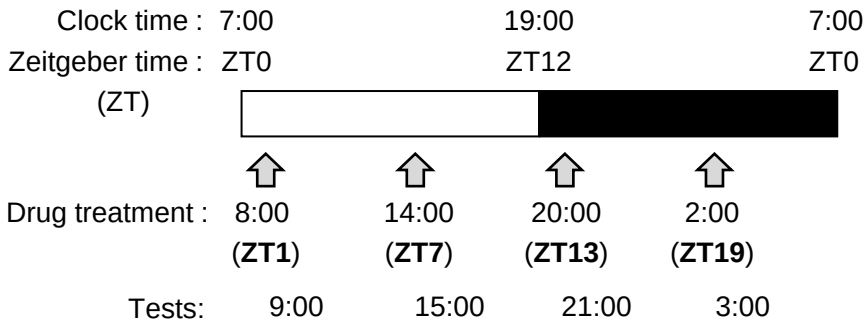
Figure 4. Effect of the antidepressants on the locomotor activity at ZT1 and ZT13. Locomotor activities after administration of fluoxetine (A), imipramine (B), venlafaxine (C), and bupropion (D) are shown. Left panels: locomotion in the control (open column) and antidepressant (closed column) groups. Right panels: locomotion relative to each control (% of control). Data are shown as the means + SEM (n = 6). $^{\dagger\dagger}P < 0.01$ for the main effect of drug treatment by two-way ANOVA; $^{*}P < 0.05$ by one-way ANOVA.

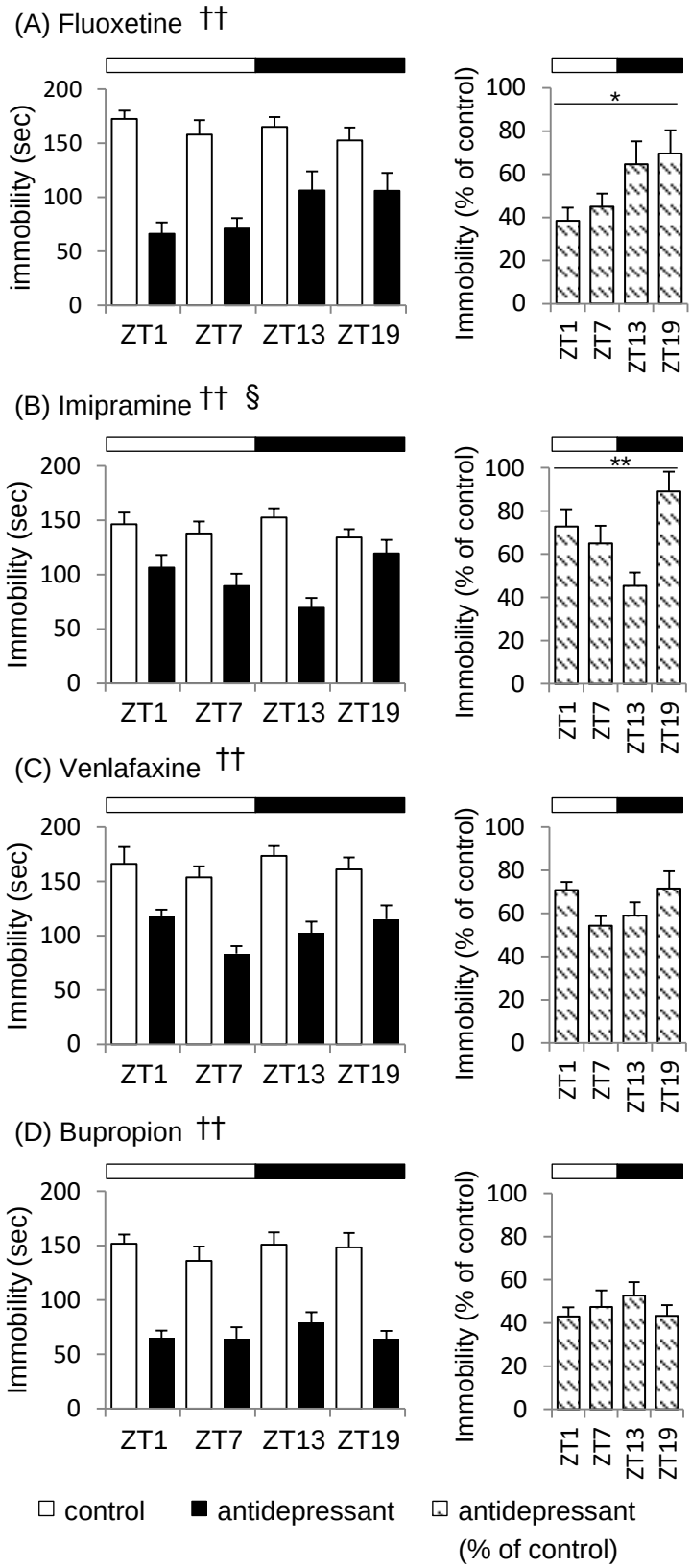
Figure 5. The circadian fluctuation of the effect of antidepressants on locomotor activity. (A) Locomotor activities after administration of antidepressants and (B) locomotion relative to control at respective ZT (% of control). Plot with error bars represents the mean ± SEM of the observed data, and the line represents the regression cosine curve calculated by Cosinor analysis. Horizontal white and black bars at the top of each graph represent the light and dark periods, respectively. Data are shown as the means ± SEM (n = 5). $^{\dagger\dagger}P < 0.01$ for the main effect of dosing time or drug treatment by two-way ANOVA.

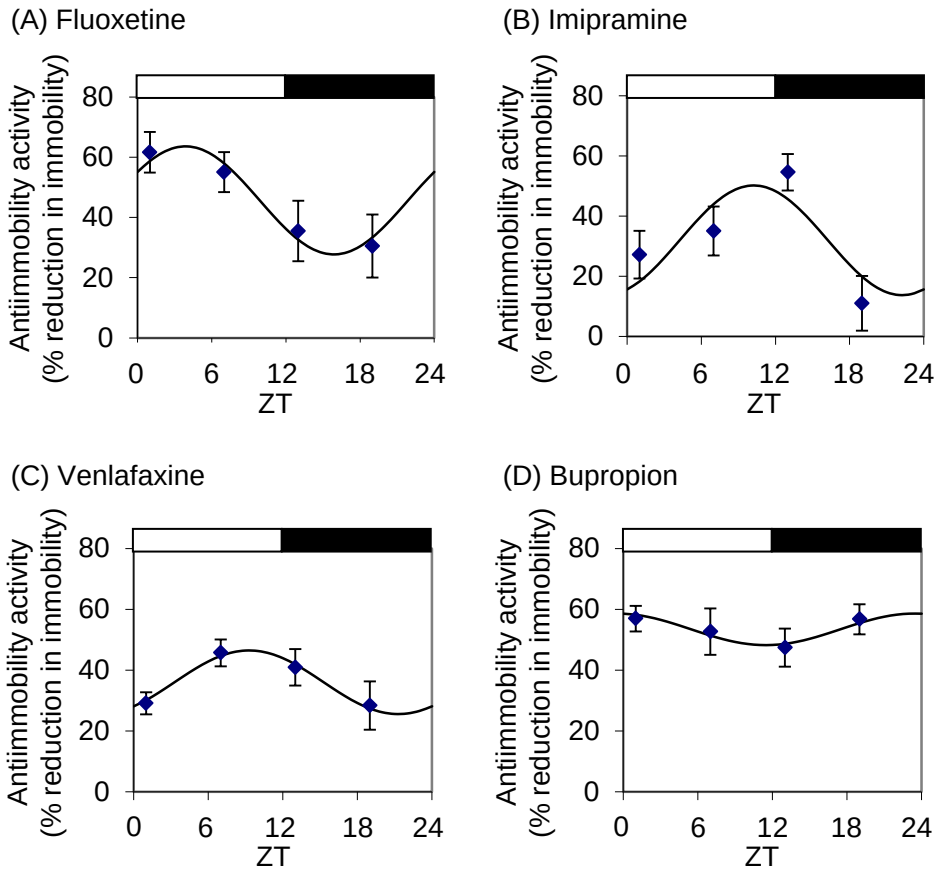
Figure 6. Plasma antidepressant levels at ZT1 and ZT13. Plasma antidepressant levels were

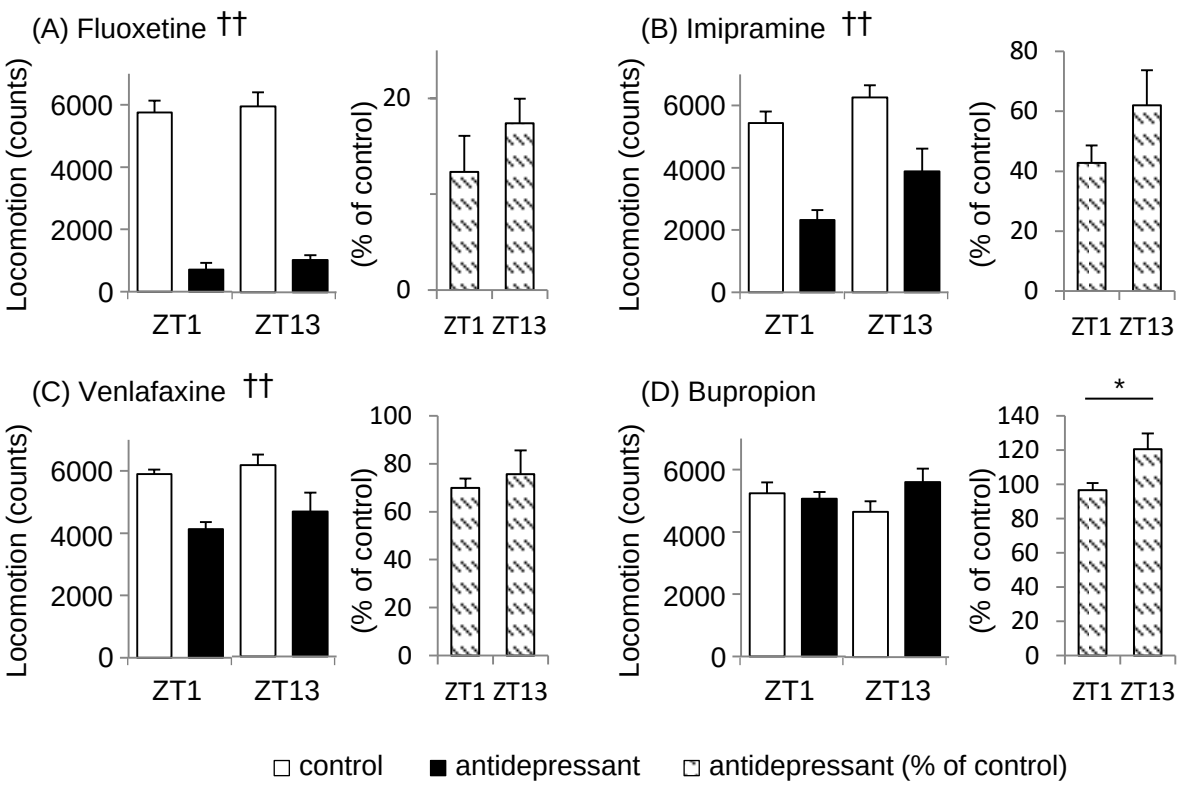
measured after the administration of fluoxetine (A), imipramine (B), venlafaxine (C), and bupropion (D) at ZT1 and ZT13. Data are shown as the means + SEM (n = 4). No significant differences were detected by one-way ANOVA.

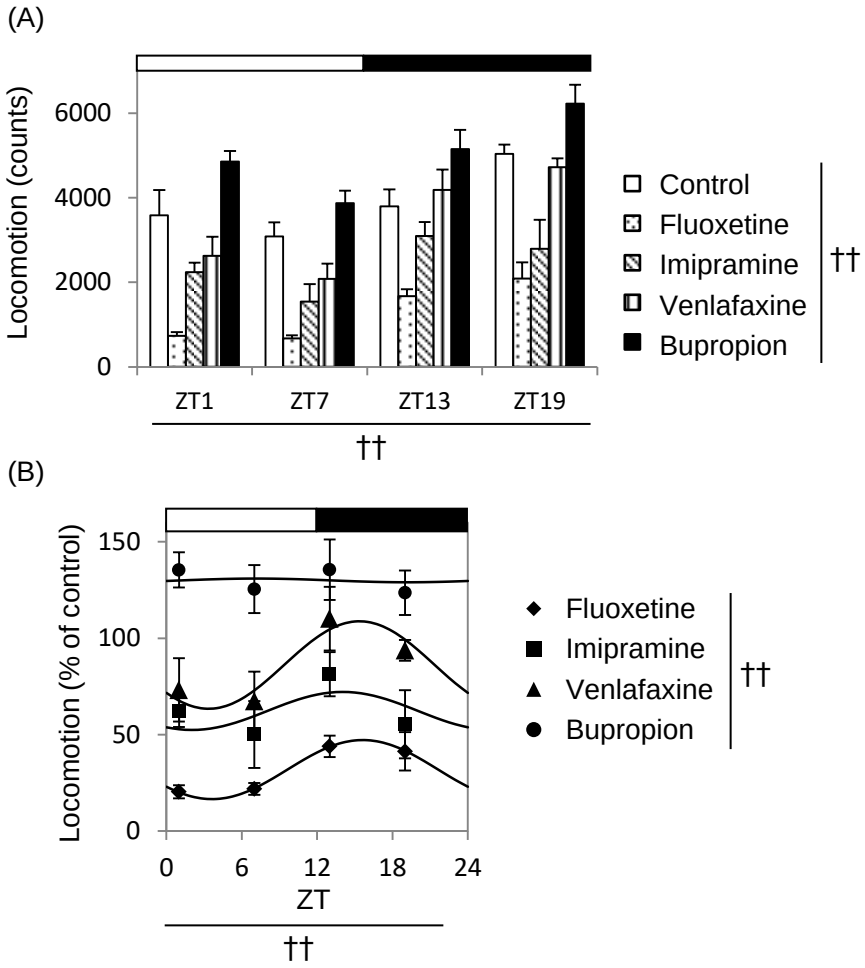
Figure 7. Brain antidepressant levels at ZT1 and ZT13. Brain antidepressant levels were measured after the administration of fluoxetine (A), imipramine (B), venlafaxine (C), and bupropion (D) at ZT1 and ZT13. Data are shown as the means + SEM (n = 4). No significant differences were detected by one-way ANOVA.

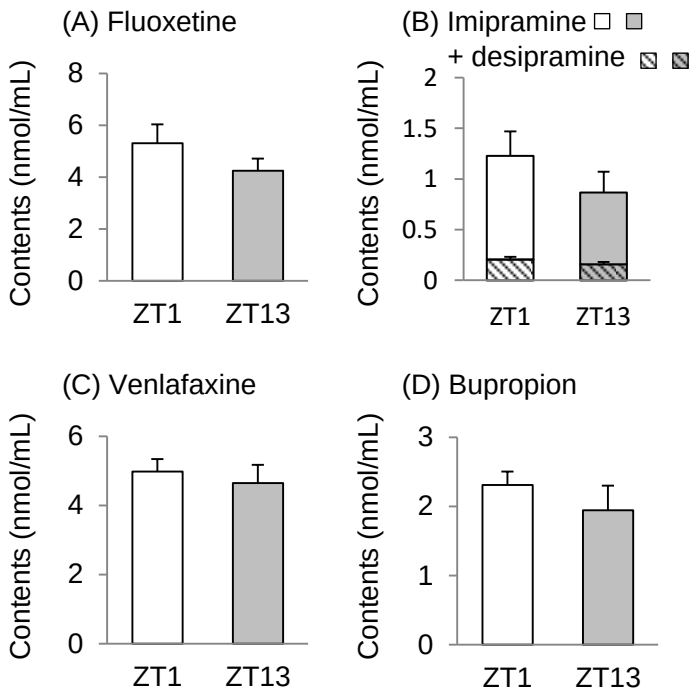

Clock time : 7:00 19:00 7:00
Zeitgeber time : ZT0 ZT12 ZT0
(ZT)
Drug treatment : 8:00 (ZT1) 14:00 (ZT7) 20:00 (ZT13) 2:00 (ZT19)
Tests: 9:00 15:00 21:00 3:00

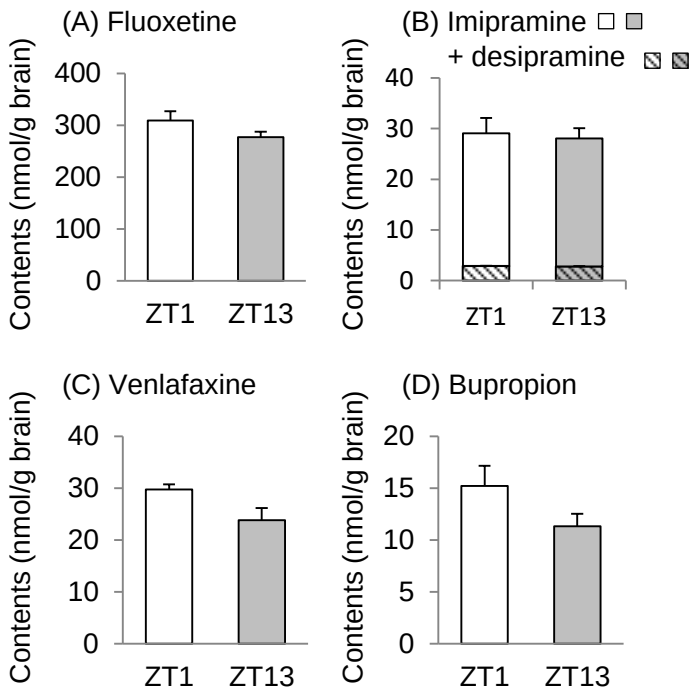

(A) Fluoxetine ††
immobility (sec)
200
150
100
50
0
ZT1 ZT7 ZT13 ZT19
Immobility (% of control)
100
80
60
40
20
0
*
ZT1 ZT7 ZT13 ZT19
(B) Imipramine †† §
Immobility (sec)
200
150
100
50
0
ZT1 ZT7 ZT13 ZT19
Immobility (% of control)
100
80
60
40
20
0
**
ZT1 ZT7 ZT13 ZT19
(C) Venlafaxine ††
Immobility (sec)
200
150
100
50
0
ZT1 ZT7 ZT13 ZT19
Immobility (% of control)
100
80
60
40
20
0
ZT1 ZT7 ZT13 ZT19
(D) Bupropion ††
Immobility (sec)
200
150
100
50
0
ZT1 ZT7 ZT13 ZT19
Immobility (% of control)
100
80
60
40
20
0
ZT1 ZT7 ZT13 ZT19
□ control
■ antidepressant
◪ antidepressant (% of control)


(A) Fluoxetine
(B) Imipramine
(C) Venlafaxine
(D) Bupropion
Antiimmobility activity
(% reduction in immobility)
0
20
40
60
80
0
6
12
18
24
ZT


(A) Fluoxetine ††
Locomotion (counts)
6000
4000
2000
0
ZT1
ZT13
(% of control)
20
0
ZT1 ZT13
(B) Imipramine ††
Locomotion (counts)
6000
4000
2000
0
ZT1
ZT13
(% of control)
80
60
40
20
0
ZT1 ZT13
(C) Venlafaxine ††
Locomotion (counts)
6000
4000
2000
0
ZT1
ZT13
(% of control)
100
80
60
40
20
0
ZT1 ZT13
(D) Bupropion
*
Locomotion (counts)
6000
4000
2000
0
ZT1
ZT13
(% of control)
140
120
100
80
60
40
20
0
ZT1 ZT13
□ control
■ antidepressant
◳ antidepressant (% of control)


(A)
Locomotion (counts)
6000
4000
2000
0
ZT1
ZT7
ZT13
ZT19
††
Control
Fluoxetine
Imipramine
Venlafaxine
Bupropion
††
(B)
Locomotion (% of control)
150
100
50
0
0
6
12
18
24
ZT
††
Fluoxetine
Imipramine
Venlafaxine
Bupropion
††


(A) Fluoxetine
Contents (nmol/mL)
8
6
4
2
0
ZT1
ZT13
(B) Imipramine
+ desipramine
Contents (nmol/mL)
2
1.5
1
0.5
0
ZT1
ZT13
(C) Venlafaxine
Contents (nmol/mL)
6
4
2
0
ZT1
ZT13
(D) Bupropion
Contents (nmol/mL)
3
2
1
0
ZT1
ZT13


(A) Fluoxetine
Contents (nmol/g brain)
400
300
200
100
0
ZT1
ZT13
(B) Imipramine
+ desipramine
Contents (nmol/g brain)
40
30
20
10
0
ZT1
ZT13
(C) Venlafaxine
Contents (nmol/g brain)
40
30
20
10
0
ZT1
ZT13
(D) Bupropion
Contents (nmol/g brain)
20
15
10
5
0
ZT1
ZT13