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Eszopiclone facilitation of the antidepressant efficacy of fluoxetine using a social defeat stress model

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ABSTRACT

This study analyzed the interaction of the sleep aid eszopiclone (ESZ) and antidepressant fluoxetine (FLX) on social defeat stress (SDS) in the mouse. Beta adrenoreceptors, brain-derived neurotrophic factor (BDNF) and cAMP response element binding protein (CREB) expression in the hippocampus and frontal cortex were also analyzed. Subjects were adult male 'intruder' C57/B6 mice that were exposed to a retired 'resident' male breeder ICR mouse in this animal's home cage for a 5 min period for each of 10 consecutive days, and the resident established physical dominance. The following day, all animals were assigned to one of four drug treatment groups, and treatment was given for up to 18 days: vehicle, ESZ only (3 mg/kg), FLX (10 mg/kg) only, or ESZ + FLX. A social interaction test was given on days 1, 5, 10, and 15 of drug treatment to assess SDS. Results showed that the ESZ + FLX group spent less time in avoidance zones during the interaction test at days 1 and 5, and more time in the interaction zone at day 5 compared to defeated mice given vehicle. All drug treatment groups spent more time in the interaction zone compared to defeated mice given vehicle on day 1 as well as day 10. SDS completely dissipated by the fourth interaction test according to both behavioral measures. Neurochemically, SDS did not produce changes in any marker analyzed. This study shows the combination of ESZ and FLX alleviated SDS, but a neurochemical correlate remains elusive.

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1. Introduction

Recent work from Fava et al. (2006) demonstrated the sleep aid and $GABA_A$ receptor agonist, eszopiclone (ESZ; trade name: Lunesta) facilitates the antidepressant efficacy of the commonly prescribed selective serotonin reuptake inhibitor (SSRI) fluoxetine (FLX) in patients clinically diagnosed with MDD. This effect, characterized by a faster onset and greater magnitude of antidepressant action, was demonstrated in MDD patients with insomnia. It has been speculated that eszoplicone-induced improvement of sleep was a contributory factor to the enhancement of the antidepressant response to fluoxetine, although there was also evidence of improvement in non-sleep related depressive behaviors. The neurobiological mechanisms underlying the antidepressant-enhancing effects of eszoplicone have yet to be fully elucidated, although two recent studies demonstrate that eszoplicone, like fluoxetine and other antidepressant drugs, increases neurogenesis in the hippocampal dentate gyrus (Su et al., 2009; Methippara et al., 2010). Interestingly, there have been no reports of whether eszopiclone affects sleep behavior using rodent models.

The social defeat stress behavioral paradigm is a laboratory animal model often used to evaluate antidepressant drug action (Keeney and Hogg, 1999; Beitia et al., 2005; Tsankova et al., 2006; Wilkinson et al., 2009). This model of chronic stress in the mouse is induced through social dominance of an aggressor over an intruder mouse, where the intruder is the subject under study (Miczek, 1979). Social defeat is an ethologically relevant stressor that utilizes the natural establishment of social rank in male rodents. During social defeat an aggressive resident male rodent fights off an intruding male that has entered his territory. As a consequence of social defeat, the intruder male displays subordinate posturing to prevent further attack from the resident male rodent. Following this interaction, subordination is reinforced as the intruder male receives visual, olfactory, and auditory stimuli from the resident male while being separated by a partition (Martinez et al., 2002).

Converging lines of experimental evidence suggest that repeated social defeat can affect reward-related processes (Von Frijtag et al., 2000), increase anhedonia, and induce motivational deficits (Rygula et al., 2005) and affect circadian rhythms of core body temperature and locomotor activity (Keeney et al., 2001). Interestingly, these changes have been shown to be reversible by treatment with the

Abbreviations: (SDS), Social defeat stress; (ESZ), Eszopiclone; (FLX), Fluoxetine.

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antidepressant norepinephreine reuptake inhibitor reboxtine, or the serotonin reuptake inhibitors (SSRI) citalopram (Rygula et al., 2006a, 2006b) or fluoxetine (Berton et al., 2006; Tsankova et al., 2006; Rygula et al., 2006a, 2006b). Thus, this model has been suggested to have clinical relevance to major depressive disorder (MDD) in humans (Rygula et al., 2005). However, there has also been speculation that this model may have relevance to post-traumatic stress disorder (PTSD). Among the symptoms observed in the subordinate male (intruder) are weight loss, increased heart rate, sleep disturbances, increased body temperature (Koolhaas et al., 1997) as well as hypothalamo–pituitary adrenal axis disturbances (Bhatnagar and Vining, 2003) including increased corticosterone levels (Avitsur et al., 2001). Patients diagnosed with PTSD also demonstrate similar types of physiological responses, although the change in stress hormonal levels have been shown to be inconsistent (see review, Fink, 2011). Intruder rats may also display anxiety-like behavior when exposed to novel stressors (Ruis et al., 1999; Von Frijtag et al., 2000; Frank et al., 2006). Therefore, social defeat may also be an appropriate stressor to investigate for the elicitation of physiological responses and behaviors that can be encompassed by PTSD-associated responses to trauma.

This study was designed to analyze the effects of the combination drug treatment eszopiclone (ESZ) with fluoxetine (FLX) in a mouse model of social defeat stress. The primary hypothesis was that eszopiclone will facilitate the antidepressant activity of the SSRI fluoxetine by producing a facilitation of noradrenergic transmission. This hypothesis is based on previous evidence that $GABA_A$ agonists, which is the action of ESZ, facilitate norepinephrine release (Suzdak and Gianutsos, 1985b; Fiber and Etgen, 1998). In addition, repeated treatment of mice with $GABA_A$ agonists have been shown to downregulate beta adrenergic receptors, similar to the effect of antidepressants that block reuptake of norepinphrine (Bartholini, 1985; Suzdak and Gianutsos, 1985b). In the present study, mice were subjected to social defeat stress followed by drug treatment with ESZ, FLX, or a combination of ESZ and FLX in comparison to defeated animals given vehicle and non-defeated controls given the same drug treatments as well as vehicle. We hypothesized that noradrenergic enhancement by eszoplicone would result in downregulation of beta adrenergic receptor binding in cortical and hippocampal tissues. In addition, we hypothesized a decrease of the expression of cyclic AMP response element binding (CREB) protein and brain derived neurotrophic factor (BDNF) in defeated animals given vehicle, which would be restored to control levels by the combination of ESZ and FLX in acute dosing, and FLX in chronic dosing, because antidepressants have been shown to alleviate decreased CREB expression when glucocorticoids are increased (Blom et al., 2002).

2. Methods

2.1. Animals

A total of 232 male C57/Bl6 adult mice served as subjects for this study, and each of these mice served as 'intruders'. Retired male breeder ICR mice served as 'resident' mice that were used as aggressors. All C57/Bl6 mice were ordered from Jackson Laboratories (Bar Harbor, ME), and all resident mice were ordered from Harlan, Inc. (Indianapolis, IN). Importantly, animals were housed on a reverse light–dark cycle, so that the dark cycle existed during the day, and the light cycle during the night. All behavioral testing were performed during the animal's dark cycle.

2.2. Social defeat stress (SDS) procedure

Upon arrival, the resident mice were singly housed in large plastic polycarbonate cages typically used for rats (26 cm × 47.6 cm × 20.3 cm). The intruder mice were initially group housed upon arrival in standard sized mouse cages. A subset of C57/Bl6 mice (3–4) served as screeners for aggression. These mice were placed into a resident's cage and the interaction was recorded. The intruder was exposed to that resident mouse for a 5 min period. During this period, the resident mouse typically established physical dominance of the intruder mouse. In cases where this was not established, the resident mouse was not used as an aggressor in the study.

Once aggressors were screened, SDS was induced every day for the next 10 consecutive days through placement of the intruder mouse into the resident animal's home cage and allowing for a 5 min confrontation, which was terminated by a 3 s pinning of the intruder mouse or the 5 min time period had elapsed. After this daily interaction, the intruder was housed in a standard sized mouse cage (18.4 cm × 29.2 cm × 12.7 cm) with small perforations in the sides of the cage. This cage was placed into the larger cage (a rat-sized cage) of the resident aggressor mouse. Although these two mice could not physically interact, there was sensory contact via the perforations made in the cage and the cage top. This housing arrangement persisted for the 10 consecutive days of SDS induction. In addition, different aggressor mice were used for each social interaction for each intruder mouse so that the intruder mice would not become habituated or adapt to a particular aggressor. Drug treatment began the day after SDS was complete.

2.3. Drug dosages

A 3 mg/kg dose of eszopiclone was used because this dose has been shown to prevent excitotoxicity and neurodegeneration in the hippocampus when given systemically (Fung et al., 2009) and an identical dose has been shown to affect sleep–wake cycle in rats (Gauthier et al., 1997; Gottesmann et al., 1998). For fluoxetine, a dose of 10 mg/kg was used, which was chosen based on past work showing subchronic dosing of 10 mg/kg of fluoxetine sufficiently counteracts the effects of SDS in mice (Berton et al., 2006; Rygula et al., 2006a, 2006b). The compounds were dissolved in 50 mM acetate buffer. Control subjects were injected with an identical vehicle, 50 mM acetate buffer (VEH).

2.4. Drug treatment groups and research design

One day after SDS was complete, drug treatment began, and all drug treatments were administered after SDS was induced. There were a total of eight drug treatment groups, four groups that were socially defeated, and four that were not socially defeated. A total of 6–9 mice served as subjects in each group. The drug treatment groups were as follows: ESZ + FLX, ESZ only, FLX only, and VEH. FLX or VEH was administered every morning (approximately 8 am), and ESZ or VEH was administered at night (approximately 8 pm). The drug treatment times were chosen because in past clinical work by Fava et al. (2006), FLX was administered in the morning, and ESZ was given in the evening. In the present study, FLX was administered in the morning, at the beginning of the active dark cycle for mice as arranged (see above). ESZ was given in the evening, at the beginning of the non-active light cycle. Using a between subjects design, drugs and VEH were administered to different groups of animals for 1, 6, 12, or 18 consecutive days after SDS, and social interaction tests were performed during drug treatments at these same time periods: 1, 5, 10, or 15 days after SDS. This treatment schedule allowed assessment of the time-course of behavioral and neurochemical changes produced by both acute and sub-chronic drug treatment in the SDS model. We chose to administer drug treatment for at least 24 h past the social interaction test to avoid a possible confound of stress induced by the behavioral test on the neurochemical assays performed. An additional group of defeated and non-defeated mice were treated with desipramine (DMI; 10 mg/kg twice daily for 18 days) for use as a positive control for the beta adrenoreceptor binding assay. Chronic treatment with DMI has been shown to produce significant beta receptor downregulation using this treatment regimen (Scott and Crews, 1983;

Sethy et al., 1988; Argenti and D'Mello, 1994). DMI treated mice were not given the social interaction test because all time points were not included in this group.

2.5. Social interaction test (1, 5, 10, or 15 days post-drug treatment)

A social interaction test was used to test for the magnitude of social defeat stress at four different time points after induction after SDS was complete (see Fig. 1). The interaction test was performed approximately 4 h after morning drug treatment. For this interaction test, the intruder mouse was placed into a square locomotor arena measuring 50 cm on a side for 3 min to allow for habituation to the arena. After habituation, the resident aggressor mouse was placed into a perforated Plexiglas box, measuring 10 cm on a side, within the locomotor arena that allowed for sensorial but not physical contact between the two mice, and this interaction lasted for 3 min. During both habituation and interaction, time was recorded in several different defined zones in the arena. Time spent in the social interaction zone (see Fig. 1), located proximal to the perforated Plexiglas box containing the aggressor was recorded as *interaction time*. This dependent measure has often been used in social interaction tests in past work analyzing SDS (e.g., Tsankova et al., 2006). Time spent in the four corner avoidance zones, and the central avoidance zone located directly across from the Plexiglas box (see Fig. 1) were recorded and summed together as *avoidance time*. Avoidance time has not been as often measured in past studies analyzing SDS, although it has been shown to be an effective and sensitive measure for SDS on the interaction test (Lumley et al., 2000; Berton et al., 2006). Both the definition of the specific zones as well as time recorded in each zone was performed using the Any Maze automated behavioral scanning system (Stoelting Co, Wood Dale, IL). Time in the interaction zone and avoidance zone was expressed as a percent of the total time spent on the social interaction test.

2.6. Experimental design

There were three factors in this experimental design, which were all between subjects: morning drug treatment (FLX or VEH), evening drug treatment (ESZ or VEH), and social defeat (social defeat or no defeat). A separate 2×2×2 three-factor ANOVA was used for statistical analysis of the interaction time and avoidance time at each social interaction test. In addition, locomotor activity was measured on each interaction test. The Any Maze software program superimposed a video image grid of lines on to the locomotor arena, and every instance an animal crossed one of these lines an activity count was recorded. The Bonferroni post hoc test ($p = 0.05$) was used to analyze any statistically significant interactions.

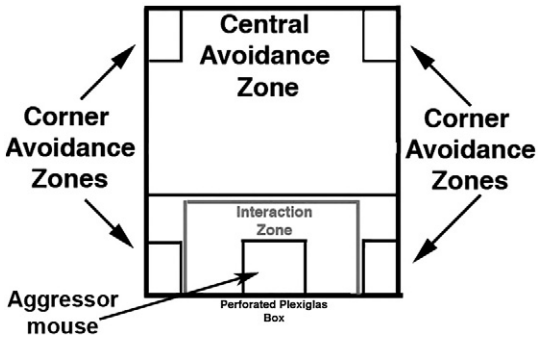

Fig. 1. Depiction of the social interaction test. During the interaction test, after a 3 min habituation, the resident was placed in the perforated Plexiglas box and the intruder was placed in the center of the arena on the line of the central avoidance and interaction zones. All behavioral movements by the intruder were tracked and recorded.

2.7. Biochemical assays

One day following the final day of drug treatment of each treatment interval group, animals were euthanized and brains were dissected, frozen, and stored at −80 °C for biochemical assays as described below, and there was a total N of 6–9 per condition.

2.8. Antidepressant-induced downregulation of beta adrenoceptors

The binding of [125I]iodopindolol (300 pM; Perkin Elmer, Waltham, MA) to beta adrenoceptors was measured in tissues using 25 μM isoproterenol to define non-specific binding as previously described (Ordway et al., 1987). All measurements were performed in triplicate and protein was measured using the bicinchoninic acid method. Data shown are the specific binding of [^{125}I]iodopindolol to beta adrenoceptors, measured as the difference between total and non-specific binding.

2.9. BDNF and CREB measured by quantitative real-time PCR

RNA was isolated from mouse frontal cortex and hippocampus from each treatment group and treatment time interval, using the same mice used in the behavioral experiments and beta adrenoceptor binding experiments above. RNA was reverse transcribed and cDNAs were amplified using standard methods, as described previously (Xiang et al., 2008). Intron-spanning primers were designed for CREB and BDNF genes, and for two reference genes, beta-2 microglobulin (B2M) and TATA box binding protein (TATA). CREB and BDNF gene expressions were normalized with the mean of the gene expressions of the reference genes. For each PCR plate, a standardized dilution series was amplified using a synthetic template of the respective gene. Standards were used to compute actual copy numbers of starting material from each sample on the plate. All samples were run in triplicate and each plate was loaded with cDNAs from one mouse of each treatment and time interval group.

3. Results

3.1. Interaction test 1, day 1 after SDS

As mentioned above, time spent in the interaction zone is the area proximal to the aggressor, and the sum time spent in the avoidance zones are in areas away from the intruder (See Fig. 1). Percent time spent in the interaction zone is presented as a function of drug treatment in Fig. 2(a). A 2×2×2 three-way ANOVA revealed a significant main effect of social defeat ($F_{(1,62)} = 7.32$; $p < 0.01$), a significant two-way interaction of evening drug treatment × social defeat ($F_{(1,62)} = 8.27$; $p < 0.01$), and a significant three-way interaction of morning drug treatment × evening drug treatment × social defeat ($F_{(1,62)} = 4.70$; $p < 0.03$). Post hoc analysis of the significant three-way interaction revealed that the socially defeated group given VEH spent significantly less time in the interaction zone than all other groups, regardless of whether animals were defeated or non-defeated. Importantly, the defeated groups given ESZ + FLX, FLX, and ESZ were equivalent to the VEH-treated non-defeated group, and all spent an increased amount of time spent in the interaction zone compared to the defeated group given VEH. Analysis of the significant two-way interaction of evening drug treatment × social defeat revealed that defeated animals given ESZ treatment spent significantly less percent time in the interaction zone than non-defeated animals given VEH. The significant main effect of social defeat demonstrated that defeated animals spent less time in the interaction zone than non-defeated animals, as expected.

Sum percent time spent in the avoidance zones is presented as a function of drug treatment in Fig. 2(b) for interaction test 1. A 2×2×2 three-way ANOVA revealed a significant main effect of evening drug

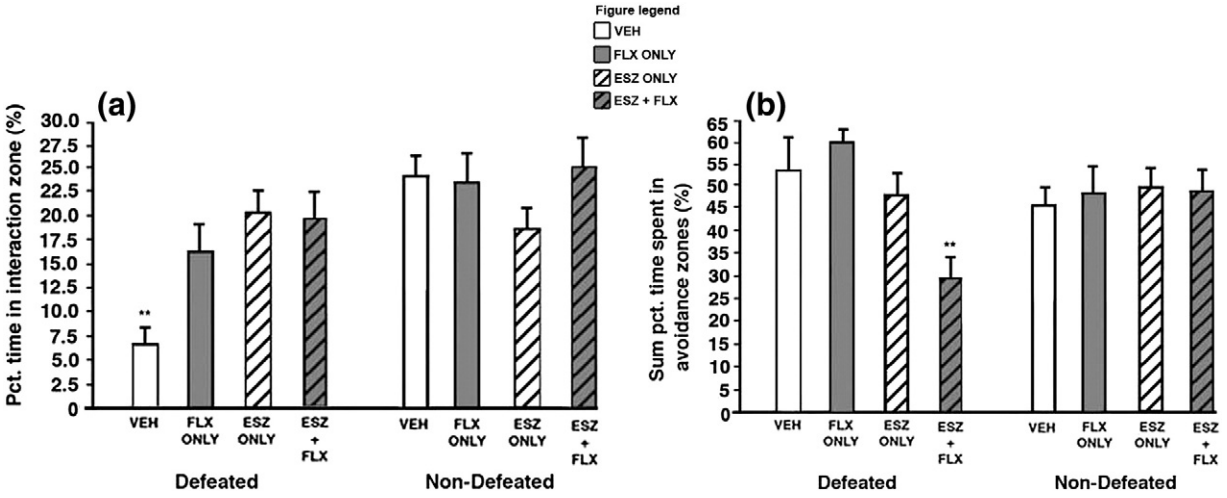

Fig. 2. (a) Percent time spent in the interaction zone is presented as a function of drug treatment and defeated condition for interaction test 1; (b) sum percent time spent in the avoidance zones is presented as a function of drug treatment and defeated condition for interaction test 1. For both figures, a double asterisk (**) indicates this group demonstrates a significantly lower percent time than all other groups (**$p<0.05$).

treatment ($F_{(1,62)}=5.12$; $p<0.05$), significant two-way interactions of morning drug treatment × evening drug treatment interaction ($F_{(1,62)}=4.98$; $p<0.03$), and evening drug treatment × social defeat interaction ($F_{(1,62)}=8.70$; $p<0.01$) and most importantly, a significant three-way interaction of morning drug treatment × evening drug treatment × social defeat interaction ($F_{(1,62)}=4.54$, $p<0.04$). Post hoc analysis of the significant three-way interaction revealed that the defeated group administered ESZ + FLX spent significantly less time in the avoidance zones than all other groups, supporting the hypothesis that this drug cocktail would alleviate defeat-induced social avoidance more effectively than either drug treatment alone. Analysis of the evening drug treatment × social defeat interaction revealed that mice given ESZ, regardless of whether animals were also given FLX or VEH, spent less time in the avoidance zone than animals given VEH for the evening drug treatment. This result demonstrates that ESZ resulted in significant less time spent in the avoidance zone on the first social interaction test at day 1. Likewise, analysis of the morning drug treatment × social defeat interaction revealed that mice given FLX, regardless of whether animals were also given ESZ or VEH, spent less time in the avoidance zone than animals given VEH for the evening drug treatment, and the significant main effect of evening drug treatment revealed that ESZ reduced the amount of time spent in the avoidance zones compared to other treatment groups.

3.2. Interaction test 2, day 5 after SDS

Percent time in the interaction zone is presented as a function of drug treatment in Fig. 3(a) for interaction test 2. A three-way ANOVA revealed significant two-way interactions of morning drug treatment × evening drug treatment ($F_{(1,62)}=11.27$; $p<0.01$), morning × social defeat ($F_{(1,62)}=7.92$, $p<0.01$) and evening drug treatment × social defeat ($F_{(1,62)}=13.66$; $p<0.01$). Analysis of the significant two-way interaction of morning × evening interaction revealed that ESZ + FLX treatment spent significantly more time in the interaction zone than all other groups, defeated groups given FLX, regardless of the co-treatment, spent significantly more time in the interaction zone than non-defeated groups given FLX only or VEH, and defeated groups given ESZ spent significantly more time in the interaction zone than non-defeated groups given ESZ only or VEH. Overall, post hoc analysis making all pairwise comparisons, revealed that the ESZ + FLX defeated group and the VEH treated non-defeated group were equivalent and spent significantly more time in the interaction zone than all other treatment groups.

Percent time in the avoidance zones is presented as a function of drug treatment in Fig. 3(b) for interaction test 2. For the percent avoidance time at interaction test 2, a three-way ANOVA revealed a significant two-way interaction of evening drug treatment × social

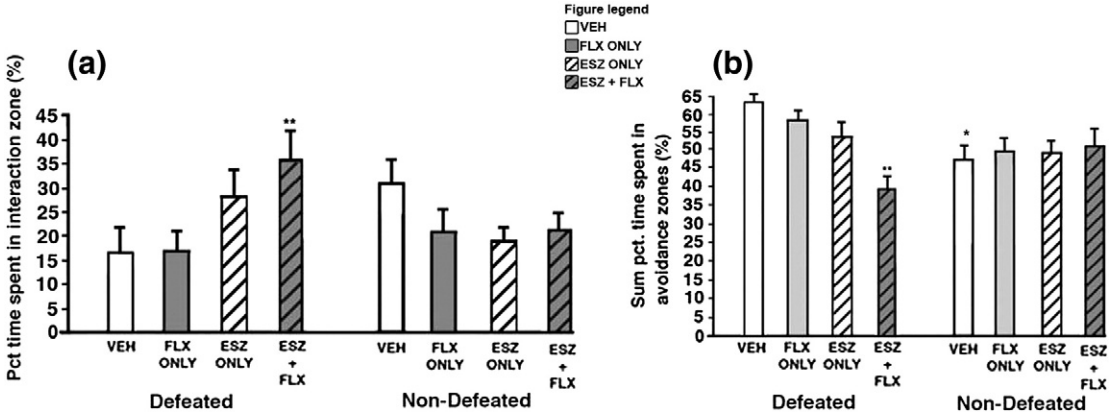

Fig. 3. (a) Percent time in the interaction zone is presented as a function of drug treatment and defeated condition for interaction test 2. A double asterisk (**) indicates that the ESZ + FLX group spent significantly more time in the interaction zone than all other groups; (b) sum percent time in the avoidance zones is presented as a function of drug treatment and defeated condition for interaction test 2. A double asterisk (**) indicates that the ESZ + FLX group spent significantly less time in the avoidance zones than all other groups (**$p<0.05$).

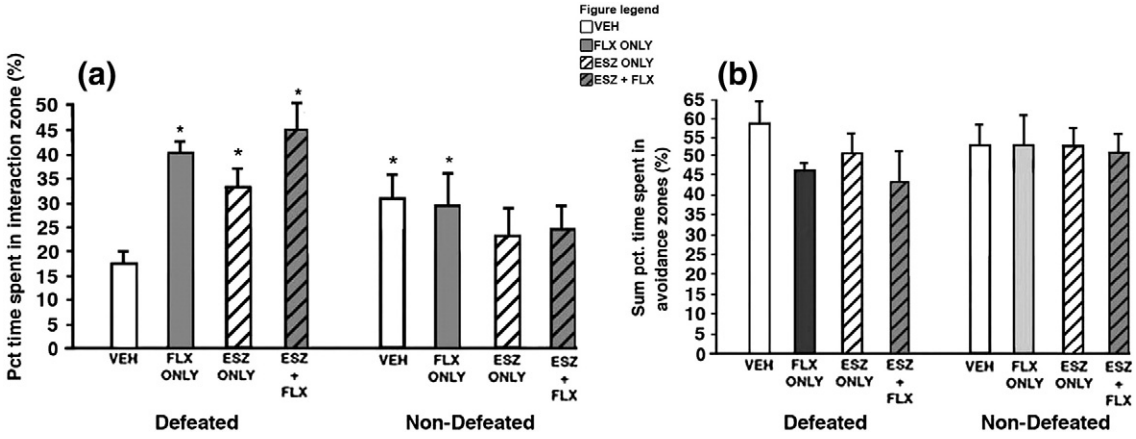

Fig. 4. (a) Percent time in the interaction zone is presented as a function of drug treatment and defeated condition for interaction test 3. A single asterisk (*) indicates defeated groups given FLX, ESZ, or ESZ + FLX as well as the VEH non-defeated group spent significantly more time in the interaction zone than the defeated group given VEH. (b) Sum percent time in the avoidance zones is presented as a function of drug treatment and defeated condition for interaction test 2. There were no significant group differences.

defeat ($F_{(1,62)} = 13.45$; $p < 0.01$) and a significant three-way interaction of morning drug treatment × evening drug treatment × social defeat ($F_{(1,62)} = 4.61$, $p < 0.03$). Post hoc analysis of the significant three-way interaction revealed that the ESZ + FLX defeated group spent less time in the avoidance zone than all other groups, and the defeated group given VEH spent more time in the avoidance zones as compared to the non-defeated group given VEH. This effect again shows that the cocktail of ESZ and FLX appears to be alleviating social defeat on the avoidance measure on interaction test 2, five days post-SDS, and social defeat resulted in more time spent in the avoidance zones in the vehicle-treated group. Analysis of the evening × social defeat interaction revealed that non-defeated groups given ESZ spent significantly more time in the avoidance zones than defeated groups given ESZ.

3.3. Interaction test 3, day 10 after SDS

Percent interaction time is presented as a function of drug treatment in Fig. 4(a). A 2×2×2 ANOVA revealed a significant main effect of morning drug treatment $F_{(1,49)} = 5.95$, $p < 0.019$ and social defeat $F_{(1,47)} = 4.14$; $p < 0.047$ and two significant two-way interactions of morning drug treatment × evening drug treatment $F_{(1,49)} = 4.61$, $p < 0.038$ and evening drug treatment × social defeat $F_{(1,49)} = 4.27$, $p < 0.045$. Mice given FLX, ESZ, or ESZ + FLX treatment spent significantly more time in the interaction zone than animals administered VEH, and socially non-defeated groups given either VEH or FLX spent significantly more time in the interaction zone the defeated group given VEH. Thus, it appears that FLX, ESZ, and ESZ + FLX is increasing the amount of time spent in the interaction zone on this interaction test.

Percent avoidance time is presented as a function of drug treatment in Fig. 4(b). A three-way ANOVA of the percent avoidance time revealed no significant main effects or interactions, demonstrating that on the avoidance measure, the effect of SDS has dissipated 10 days after induction.

3.4. Interaction test 4, day 15 after SDS

Percent interaction time and percent avoidance time are presented as a function of drug treatment in Fig. 5(a) and (b), respectively. There were no significant main effects or interaction at interaction test 4. Thus, SDS appears to have completely dissipated on both the interaction and avoidance measures 15 days after induction.

3.5. Beta receptor binding

Beta adrenoceptor binding in the prefrontal cortex (left panel) and hippocampus (right panel) from the four treatment groups following 1, 6, 12, and 18 days of treatment is shown in Fig. 6. Neither defeat, FLX or ESZ, nor the combination treatment, significantly affected the binding of [^{125}I]iodopindolol to beta adrenoceptors at any of treatment intervals. In the figure, only defeated animals are presented. Desipramine (DMI) binding is presented in Fig. 7. As expected, 18 days of treatment with desipramine (10 mg/kg) significantly reduced beta adrenoceptor binding in the frontal cortex (left panel) and hippocampus (right panel).

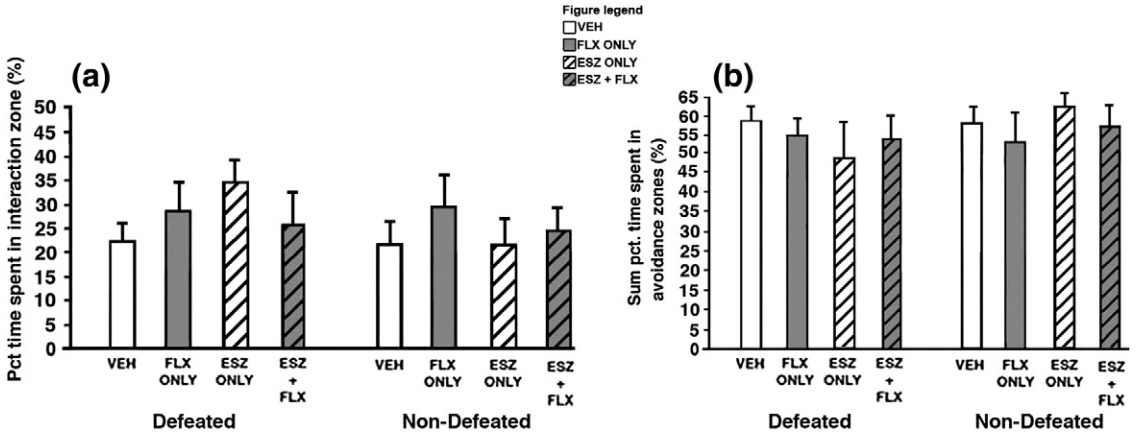

Fig. 5. (a) Percent time in the interaction zone is presented as a function of drug treatment and defeated condition for interaction test 3. (b) Sum percent time in the avoidance zones is presented as a function of drug treatment and defeated condition for interaction test 2. For both measures, there were no significant group differences.

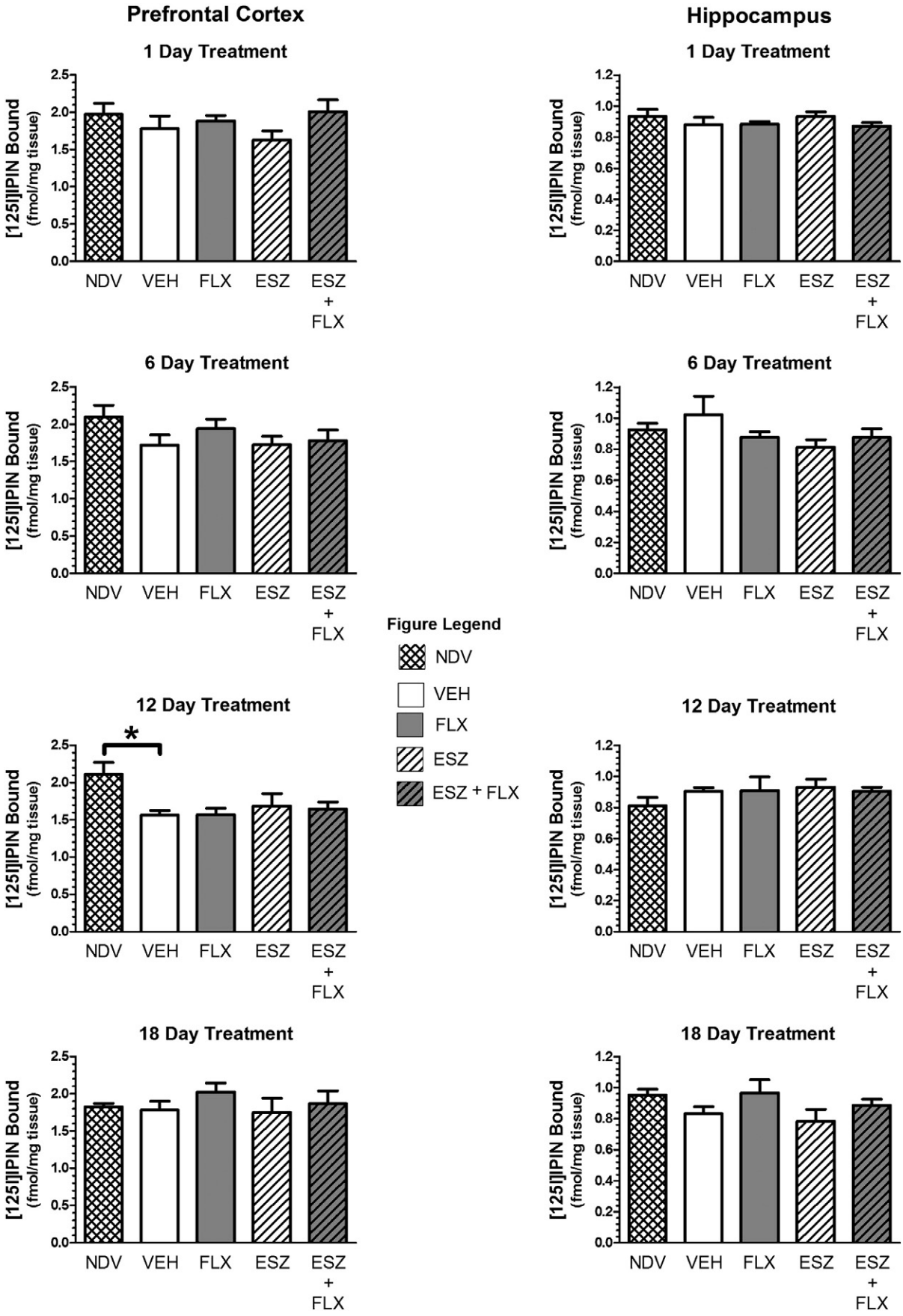

Fig. 6. Beta receptor binding is presented as a function of drug treatment group for 1, 6, 12, and 18 days of drug treatment for the prefrontal cortex (left panel) and hippocampus (right panel). Only socially defeated animals and non-defeated control animals given vehicle (indicated in the figure as NDV) are presented. There were no significant main effects or interactions.

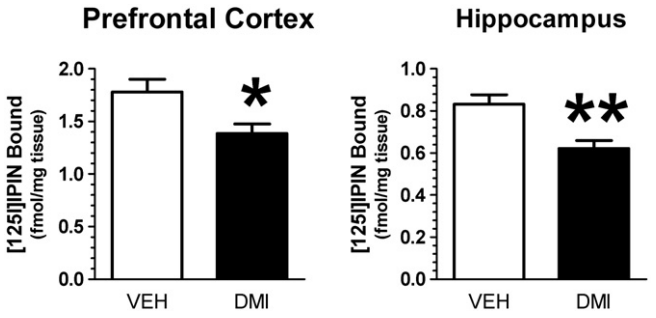

Fig. 7. Beta receptor binding is presented as a function of drug treatment group for 18 days of desipramine (DMI) treatment for the prefrontal cortex (left panel) and hippocampus (right panel). As expected, DMI-treated animals demonstrated significant decreases of beta receptor binding compared to vehicle (VEH)-treated in both the prefrontal cortex and hippocampus (*$p<0.05$).

3.6. BDNF and CREB expression

BDNF and CREB expression are shown in the prefrontal cortex (left panel) and hippocampus (right panel) for the treatment groups following 1, 6, 12, and 18 days of treatment in Fig. 7 for BDNF, and Fig. 8 for CREB (Fig. 9). None of the treatments produced a significant change in BDNF or CREB gene expressions at any of the time points tested. In the figures, only defeated animals are presented. It is noteworthy that this lack of effect of treatments was also observed for the FLX treatment group, which served as a positive control group since FLX has been shown previously to upregulate these gene expressions.

4. Discussion

Behavioral findings of this study demonstrate that treatment with the sleep aid eszopiclone (ESZ) facilitates the antidepressant-like effects of the commonly-prescribed SSRI fluoxetine (FLX) using a social defeat stress paradigm after acute or sub-chronic term treatment contingent upon the dependent measure used to assess antidepressant-like effects. On the percent interaction time measure, acute treatment with any of the compounds appeared to alleviate stress induced by social defeat as measured by time spent in the interaction zone one day post-SDS. However, on percent interaction time at the second interaction test given five days post-SDS, the combination of ESZ + FLX was equivalent to the VEH-treated non-defeated groups, which was similar to the effects observed on the avoidance time measure at interaction tests 1 and 2. The results of interaction test 3 were similar to that of interaction test 1, in that all drug treatments once again alleviated stress induced by social defeat as measured by time spent in the interaction zone. The effects of SDS had completely faded by interaction test 4, where there were no significant effects reported on either measure. On the avoidance time measure, the defeated group administered ESZ + FLX spent less time in the avoidance zones compared to all other defeated groups at interaction tests 1 and 2, demonstrating both an acute and sub-chronic alleviation of stress induced by social defeat as measured by time spent in avoidance zones. The finding that the combination of these drug treatments was effective to alter expression of defeat-induced social avoidance is relatively consistent with clinical findings that have shown ESZ facilitates the antidepressant effects of FLX in patients diagnosed with major depressive disorder after 7 days of co-treatment (Fava et al., 2006). This result is impressive, as this is the first time that ESZ has been shown to facilitate behavioral effects of FLX in a preclinical model. Interestingly, it has been suggested that the 10-day social defeat paradigm mainly produces anxiety, whereas chronic social stress for 20 days leads to depression (Avgustinovich et al., 2003). Thus, the effects observed here may be more relevant to a model of anxiety versus an animal model of depression.

Interestingly, all drug treatments spent more time in the interaction zone on the first social interaction test. Therefore, ESZ or FLX administered alone were equivalent to defeated animals given the ESZ + FLX cocktail as well as the non-defeated groups given the same drug treatments. This appears to indicate that acute treatment with ESZ or FLX is sufficient to alleviate the effects of SDS on this measure, and the combination of these treatments is not synergistic at this time point. Although acute FLX treatment, using twice the dose used in the present study (20 mg/kg), failed to alleviate SDS in mice (Cao et al., 2010), Rygula et al. (2006a, 2006b) have shown a short-term treatment of 7 days of FLX treatment using an identical dose as was used here (10 mg/kg) was sufficient to alleviate some stress-related behaviors due to SDS. There have not been any studies to analyze acute effects of FLX on SDS using the identical dose as was used here thus, this is the first study to show an acute effect using this dose of FLX on SDS in mice. The inconsistency observed here may be related to both dose of FLX and the differences in behavioral methodology.

On the avoidance time measure at the first interaction test, only defeated animals given the ESZ + FLX cocktail spent significantly less time in these zones than all other groups, including non-defeated animals. Taking the results of these two measurements together on interaction test 1 suggests that the defeated group given ESZ or FLX alone may be vacillating more between the interaction and avoidance zones, whereas the group given the ESZ + FLX is spending more of their time perseverating in and around the interaction zone. This finding also suggests that interaction and avoidance times on the social interaction test may be testing fundamentally different aspects of SDS behavioral expression relative to these drug treatments, because each dependent measure appears to interact differently with drug treatment and the time period in between SDS and the social interaction test. Somewhat surprisingly, SDS failed to produce any change in behavior at the social interaction test 15 days post-induction, which is inconsistent with several past studies (Razzoli et al., 2010; Berton et al., 2006; Tsankova et al., 2006). However, there were methodological differences between the present study and these two past studies which may explain these differences. Razzoli et al. and Tsankova et al. (2006) performed the social interaction test in the same context as SDS was induced, whereas a separate context was utilized in the present study. There is a long-standing literature demonstrating the influence of contextual change on extinction and resistance to extinction in rodents (Bouton, et al., 2006). Berton et al. demonstrated that a 10 day SDS paradigm persisted for 28 days. However, this past study conducted the social interaction test in complete darkness, which may have affected interaction behavior, as the present study used normal lighting conditions. These differences between past work and the present study demonstrate the importance of methodology using this paradigm and the drastic effect that small manipulations in methodology can have on conclusions from this behavioral test.

Unfortunately, behavioral changes induced by drug treatments were not paralleled by changes in three biochemical indices commonly shown to be affected by antidepressant drug administration in rodents. The lack of a biochemical correlate of the effect of the combination treatment suggests that the particular biochemical pathways studied do not appear to mediate the behavioral effects of the combination treatment. However, the lack of correlation may be related to a host of factors, including the time period in which measures were taken, brain areas chosen for analysis, and the methods used to analyze gene expressions.

Neither BDNF and CREB expression nor beta adrenoceptor density in the hippocampus or frontal cortex were significantly changed by social defeat stress alone. Haenisch et al. (2009) showed that BDNF expression in the hippocampus decreased by a relatively modest 2-fold change 3 weeks after social defeat stress had been induced. It is important to note that in this past study, repeated social defeats were

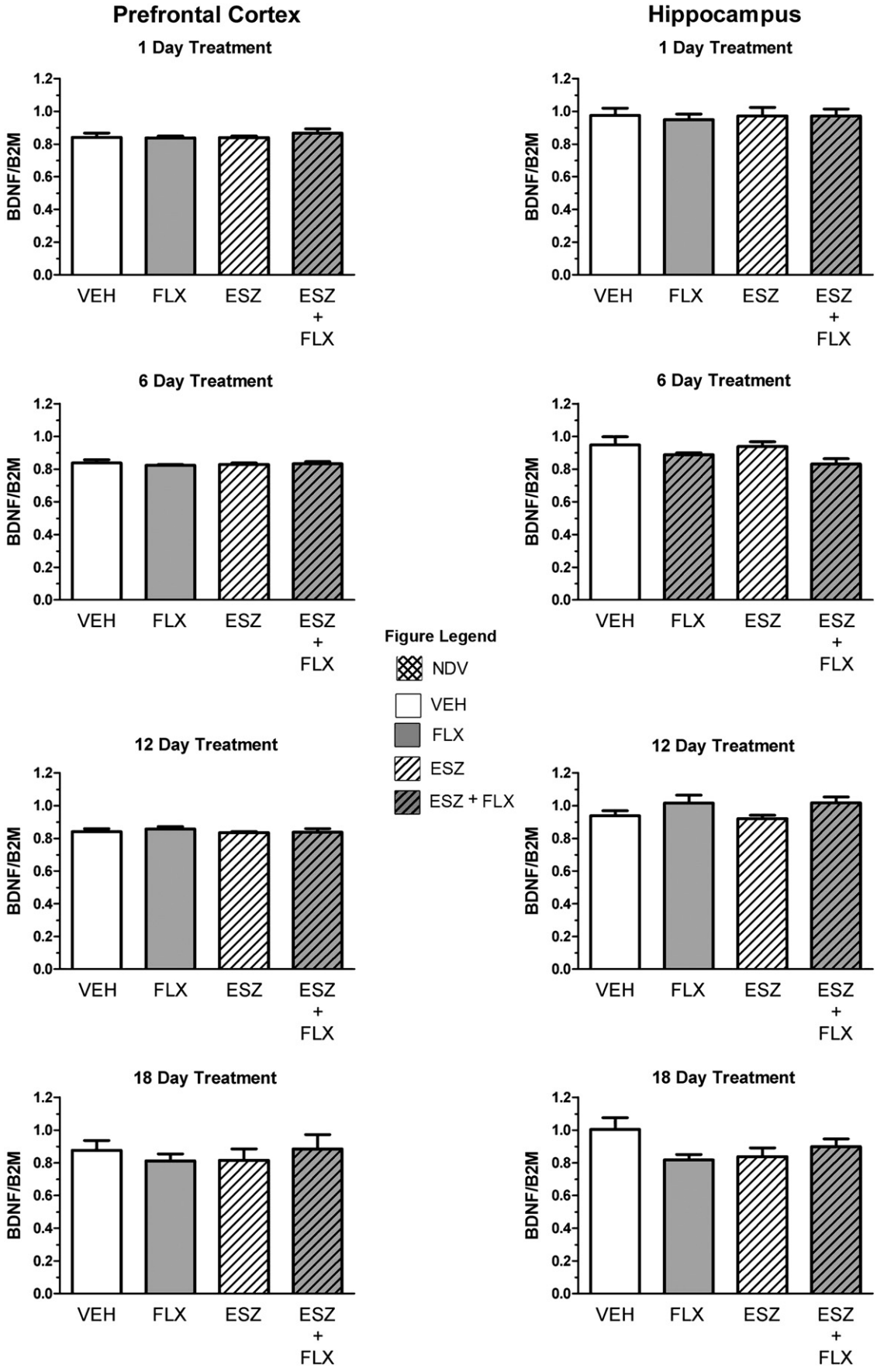

Fig. 8. Brain-derived neurotrophic factor (BDNF) expression is presented as a function of drug treatment group for 1, 6, 12, and 18 days of drug treatment for the prefrontal cortex (left panel) and hippocampus (right panel). Only socially defeated animals and non-defeated control animals given vehicle (indicated in the figure as NDV) are presented. There were no significant main effects or interactions.

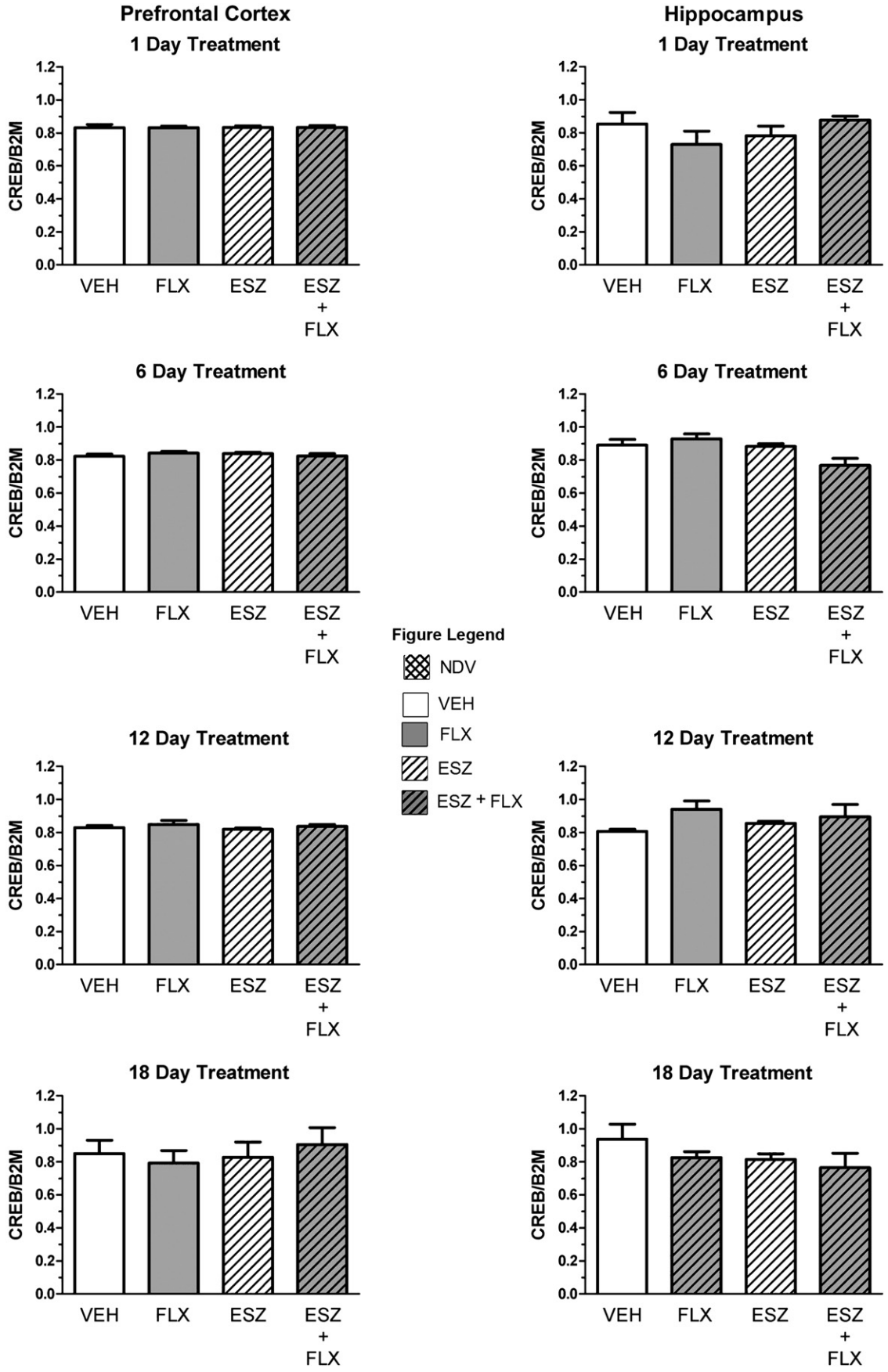

Fig. 9. Cyclic-AMP response element binding protein (CREB) expression is presented as a function of drug treatment group for 1, 6, 12, and 18 days of drug treatment for the prefrontal cortex (left panel) and hippocampus (right panel). Only socially defeated animals and non-defeated control animals given vehicle (indicated in the figure as NDV) are presented. There were no significant main effects or interactions.

given over a 21-day period. Hence, the shorter time course of stress (10 days) in the present study may have led to the lack of effect on BDNF gene expression. SDS in rats did not effect CREB gene expression in the hippocampus (Hollis et al., 2010). We are not aware of a study examining beta adrenoceptor density after SDS, although beta adrenoceptors have been shown to play a role in other forms of stress in rodents (Parale et al., 1987; Edgar et al., 2002; Hasegawa and Saiki, 2002; Claustre et al., 2008). Ultimately, the present data suggest that behaviors exhibited by social defeat in the present study do not appear to be related to changes in BDNF or CREB gene expression or beta adrenoceptor density in the brain areas analyzed.

BDNF and CREB gene expression were also not affected by any of the drug treatments. This is contrary to several reports of upregulation of gene and protein expression of these by antidepressant treatments reported previously (Nibuya et al., 1995; 1996), but consistent with others (Dias et al., 2003; De Foubert et al., 2004). While ESZ, ESZ/FLX, and FLX treatments did not affect radioligand binding to beta adrenoceptors, beta adrenoceptor binding was reduced as expected in the hippocampus and cortex by chronic DMI treatment. It was hypothesized that facilitation of norepinephrine release by ESZ through actions at GABA-A receptors (Suzdak and Gianutsos, 1985a; Suzdak and Gianutsos, 1985b) might be the mechanism by which ESZ facilitates the antidepressant action of FLX. However, the lack of a compensatory regulation of beta adrenoceptors by ESZ does not support this hypothesis. Beta adrenoceptors have not been analyzed in past work using the SDS paradigm, and although DMI produced a significant downregulation of beta adrenoceptor density, there were no other effects of drug treatment or social defeat stress. Thus, it does not appear that beta adrenoceptors play a role in the observed behavioral effects.

In terms of BDNF expression, it should be noted out that our protocol was not exactly the same as those used by other investigators, particularly comparing our study to that of Tsankova et al. (2006) who used very similar methods. This group demonstrated a significant reduction of BDNF gene expression after 10 consecutive days of social defeat that was normalized after 28 days of DMI treatment. Both Tsankova et al. and our group used a similar method to measure gene expression changes, i.e. quantitative real-time PCR. However, some differences are noteworthy. First, Tsankova et al. analyzed the nucleus accumbens, whereas the hippocampus and frontal cortex were analyzed in the present study. The rationale for studying the hippocampus and frontal cortex was based on our hypothesis that the antidepressant-enhancing mechanism of ESZ when combined with FLX was a result of an interaction of the GABA and norepinephrine in the frontal cortex and hippocampus, as has been described for GABA agonists with antidepressant actions (Suzdak and Gianutsos, 1985a, 1985b; Lloyd et al., 1987; Lloyd et al., 1990). Additionally, Tsankova et al. used only one reference gene, GAPDH, while we used two reference genes, B2M and TATA, showing no change in target genes using either reference gene for normalization. Despite this, others have shown upregulations of BDNF gene expression following FLX (for review, see Castrén and Rantamäki, 2010). For most of these studies, non-defeated animals were used, and a different method, in situ hybridization, was used to estimate gene expression levels. In situ hybridization is far more time consuming than quantitative real-time PCR, but may be more sensitive to detecting smaller changes in gene expression.

Further studies are required to understand the biological mechanism of the facilitation of antidepressant action of FLX by ESZ. One obvious possibility is that the prefrontal cortex and hippocampus do not play a role in the behavioral effects observed here in the SDS model. On the other hand, other areas such as the nucleus accumbens may play a more important role in social interaction behaviors. In fact, past studies have shown that social defeat stress alters mesocorticolimbic dopamine release in this region (Tidey and Miczek, 1996) and accumbens BDNF is required for the development of experience-dependent social aversion (Berton et al., 2006). In addition, cAMP response element-binding protein (CREB), which is related to BDNF expression, has been shown to have a decreased binding affinity in the nucleus accumbens in socially defeated mice, and this decrease was reversed by the tricyclic antidepressant imipramine (Wilkinson et al., 2009). Therefore, changes in BDNF expression within the nucleus accumbens or pathways related to this region may be the basis of the behavioral changes observed here.

In conclusion, the present study reports that the sleep aid ESZ appears to facilitate the action of the antidepressant FLX in the SDS paradigm, consistent with recent work in clinical trials (Fava et al., 2006). Although there was no correlation with changes in the neurochemical markers used in the present study, this may be due to a number of factors, including the surprising recent result of Haenisch et al. (2009) that showed changes in BDNF expression do not appear due to SDS until after a 4 week period. Future work will focus on identifying biological markers to investigate the mechanism of the behavioral effects reported.

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