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The effects of chronic valproate and diazepam in a mouse model of posttraumatic stress disorder

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Abstract

To better understand neurochemical and psychopharmacological aspects of post-traumatic stress disorder (PTSD), it is necessary establish an animal model of PTSD in which behavioral changes persist for a long time after the initial traumatization. The present study aimed to characterize long-term behavioral alterations in male ICR mice as an animal model of PTSD consisting of a 2-day foot shock (0.8 mA, 10 s) followed by 3 weekly situational reminders (SR), and to evaluate the effects of repeated administration of valproate and diazepam on behavioral deficits of this animal model. The results showed that the aversive procedure induced several long-term behavioral deficiencies: increased freezing behavior and anxiety level, reduced time spent in an aversive like context. Repeated treatment with valproate (100–400 mg/kg, i.p.) induced a dose-dependent reduction of these behavioral changes. In contrast, diazepam at a low dose (0.25 mg/kg) but not at a high dose (4 mg/kg) reduced the behavioral deficiencies. These results demonstrate that exposure to intense foot shock associated with repeated situational reminders elicits long-term disturbances that last about 4 weeks after the foot shock exposure. These behavioral deficits can be ameliorated by repeated administration of valproate or diazepam at some special dose ranges.

Keywords: Post-traumatic stress disorder; Animal model; Valproate; Diazepam

1. Introduction

Post-traumatic stress disorder (PTSD) is recognized to be a development of long-lasting symptoms following exposure to life threatening experience to which the immediate reaction is intense fear, helplessness, or horror. Resulting symptoms include persistent re-experiencing of the traumatic event (flash backs, intrusive recollections, recurrent nightmares, etc.), psychic numbing, persistent avoidance, and hyper-arousal (American Psychiatric Association, 1994). PTSD manifests a high co-morbidity with anxiety disorders.

In animal studies, long-lasting changes in synaptic plasticity in the medial prefrontal cortex has been implicated in the pathophysiology of PTSD (Herry and Garcia, 2002), in addition to the stress vulnerability of the hippocampus, possibly induced by elevation of glucocorticoid levels (Holsboer, 2001), alteration of neurotrophic factors levels, (Nibuya et al., 1995; Smith et al., 1995), and changes in serotonergic (Heim and Nemeroff, 2001) and noradrenergic neurotransmission (Geracioti et al., 2001).

With regard to the complexity to conduct prospective studies of PTSD in humans, numerous animal models have been developed using different types of traumatic events (for review, see Foa et al., 1992; Yehuda and Antelman, 1993; Rasmusson and Charney, 1997), such as inescapable electric foot shock (Servatius et al., 1995; Pynoos et al., 1996), social confrontations (Stam et al., 2000), under-water trauma (Richter-Levin, 1998; Wang et al., 2000), and exposure to a predator (Cohen et al., 2003). Various behavioral tests and bio-physiologic indices have been performed in such animal models of PTSD. Although PTSD is characterized by a long-lasting symptomatology (American Psychiatric Association, 1994), behavioral alterations in these animal models are

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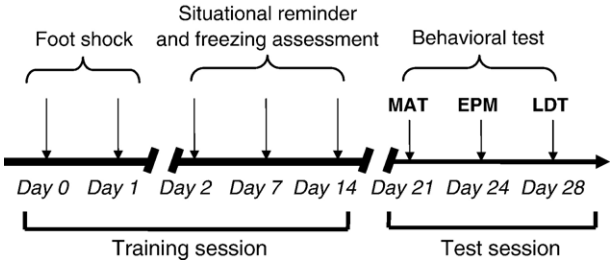

Fig. 1. Experimental schedule. Animals were exposed to electric foot shocks (0.8 mA, 10 s) followed by 3 weekly exposures to situational reminder (SR). After the training session, animals were submitted to testing session composed of 3 different behavioral tests: motor activity test (MAT), elevated plus-maze test (EPM) and light–dark transition test (LDT).

observed rather early after the traumatic paradigm and long-lasting effects of trauma are somewhat difficult to be induced in animals (Maier, 2001). Moreover, it has been proposed that the temporal persistence of PTSD results from re-experiencing (Brewin and Holmes, 2003). However, in animals, the concept of re-experiencing is difficult to address. Exposure to contextual cues present during an aversive stressful situation may induce re-experiencing of the traumatic event (Wagner, 1981; Maier, 2001; Gisquet-Verrier et al., 2004). Indeed, it has been demonstrated that exposure to an environment associated previously to foot shock is capable of extending behavioral effects of the foot shock (Maier, 2001). The effectiveness of the environment does not reduce with multiple exposures, suggesting that environmental cues are themselves stressors. The effects of the foot shock decline with time, while multiple exposures to situational reminders induce over time a progressive increase in the magnitude of the startle reflex (Pynoos et al., 1996). Moreover, situational reminders could parallel situations in human in which traumatized individuals are often exposed to reminders of the trauma but not to the traumatic events (Brunet, 1996).

Valproate, an antiepileptic drug and mood stabilizer (Bowden et al., 1994), has been clinically applied in treatment of PTSD, especially the symptom of hyper-arousal (Fesler, 1991; Ford, 1996; Clark et al., 1999; Otte et al., 2004). Valproate induced modulation of the GABA levels in the brain (Gould and Manji, 2002; Richard et al., 2003), corticotrophin-releasing factor neuronal systems (Stout et al., 2001; Gilmor et al., 2003) and extracellular signal-regulated kinase signaling pathway (Hao et al., 2004). There are, however, no reports about the therapeutic effects of valproate with pre-clinical experiments in a PTSD animal model and its exact molecular mechanisms in the treatment of PTSD are primarily unknown. Moreover, benzodiazepines such as diazepam are also used in short-term treatment of PTSD, but its effects of choric treatment are not affirmative (Gelpin et al., 1996; Cates et al., 2004; Pitman and Delahanty, 2005).

In this study, we treated male mice with inescapable electric foot shock followed by 3 weekly situational reminders to make a behavioral animal model of PTSD and elucidated the effects of valproate and diazepam on fear and anxiety-related behavior in this mouse model.

2. Materials and methods

2.1. Animals

Male ICR mice (Japan SLC, Shizuoka, Japan) were obtained at the age of 8 weeks and housed 5 per cage on a 12-h L:D cycle (lights on: 0730–1930) at 25±1 °C. Food and water were available ad libitum. Animals have at least 5 days habituations to the housing condition before experiments and then received a 2-day period of consecutive aversive foot shocks followed by 3 weekly situational reminders. During a 5-day habituation period, mice were handled once daily. Handling consisted of holding the animal with gloved

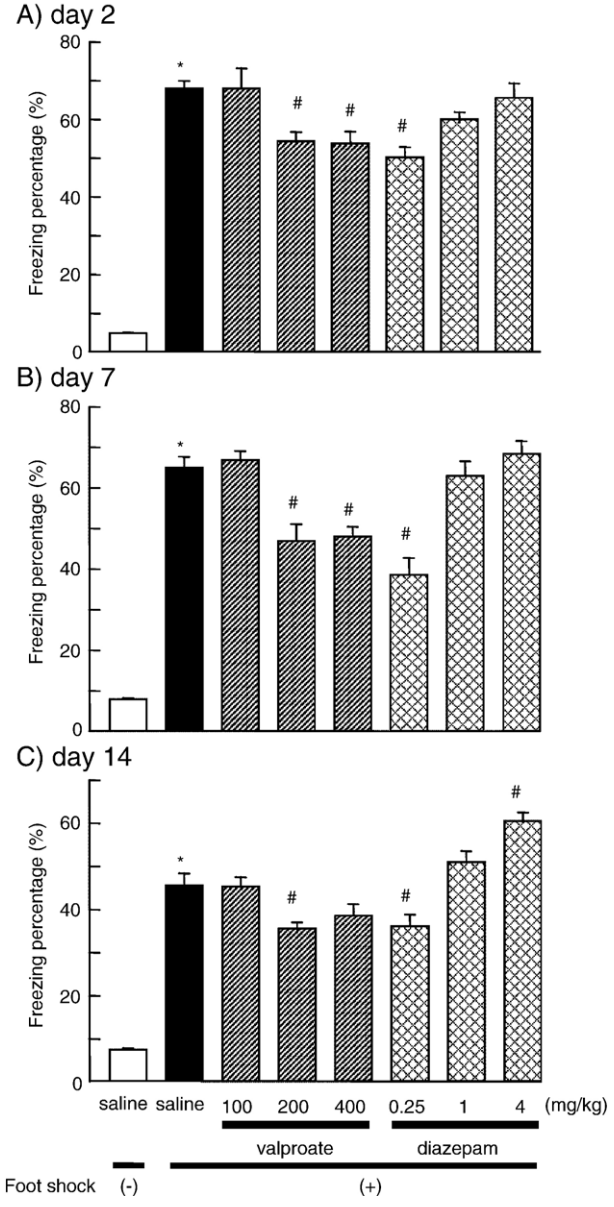

Fig. 2. Effects of valproate and diazepam on freezing behavior in mice after exposure to aversive procedure. The percentage of freezing behavior was determined on days 2 (A), 7 (B), and 14 (C). Daily administrations of valproate and diazepam were started from the first day of training session. The effect on the situational reminder was elucidated 30 min after the treatment. Each column represents the mean percentage of freezing with S.E.M. *$p<0.05$ compared with foot shock (−) group. #$p<0.05$ compared with saline-treated foot shock (+) group.

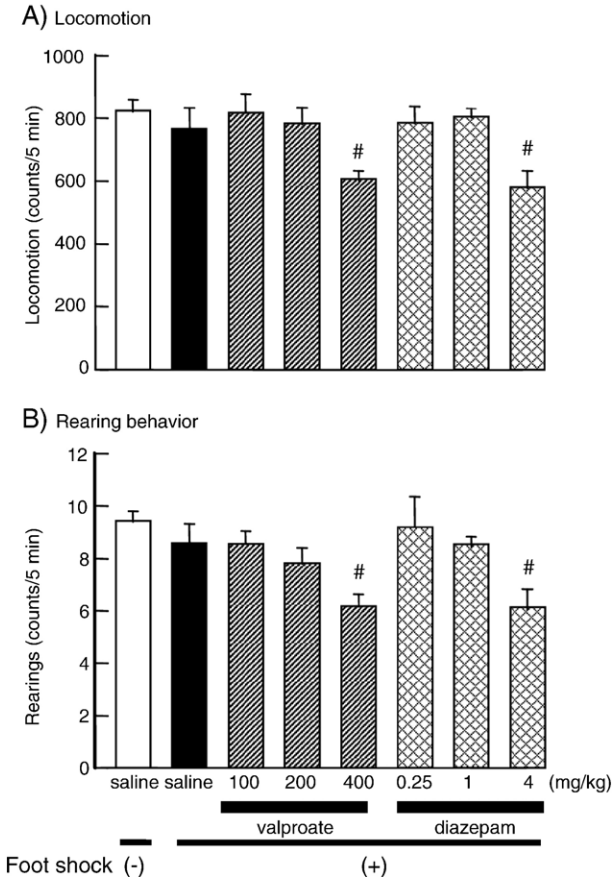

Fig. 3. Effects of valproate and diazepam on motor activity of mice exposed to the aversive event. On day 21 after aversive procedure, the locomotion (A) and rearing behavior (B) were measured. Daily administrations of valproate and diazepam were started from the first day of training session. The effect on the motor activity was elucidated 30 min after the treatment. Each column represents the mean with S.E.M. #$p<0.05$ compared with saline-treated foot shock (+) group.

hands for 2 min. The experiments were performed during the light phase from 9 AM to 5 PM. All tested mice were 9–13 weeks old during the whole testing course. All experiments were conducted in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and had the approval of the Institutional Animal Use and Care Committee of University of Toyama.

2.2. Drugs and administration

The drugs used were valproate (Sigma Chem., St. Louis, MO) and diazepam (Cercine® injection; Takeda Pharmaceutical Co., Osaka, Japan). Valproate was dissolved in saline and diazepam was diluted with saline to reach the proper concentrations. The drugs or vehicle were injected i.p. once daily from days 2 to 28 during the situational reminder training session and testing session 30 min before each experiment.

2.3. Behavioral experiments

2.3.1. Training session of aversive procedure

All the experiments were carried out in a sound proof testing room with a constant illumination where the animals were habituated for 2 h in their home cages before the start of experiments. Animals were randomized to 8 groups: unstressed, vehicle control-, valproate (100, 200, and 400 mg/kg)-, and diazepam (0.25, 1, and 4 mg/kg)-treated groups ($n=10$ in each group). For the training session, we used a Plexiglass chamber (300×300×350 mm) with a stainless steel grid floor (4 mm diameter, 9 mm interval). A total of 15 intermittent inescapable electric foot shocks (intensity: 0.8 mA, interval: 10 s, duration: 10 s) were delivered through the grid floor by an isolated shock generator (Muromachi Kikai, Tokyo, Japan). In the training session, animals were exposed to an aversive procedure by modifying the method reported by Pynoos et al. (1996) and Masuo et al. (1997). Briefly, the animals received inescapable electric foot shocks on day 0 and day 1 followed by 3 weekly re-exposure to the same chamber (as a situational reminder) without foot shock on day 2, day 7 and day 14 (Fig. 1). Each animal was placed in the chamber. After a 2-min adaptation period, the inescapable foot shocks were delivered for a total 5 min. Control animals were placed in the same chamber for 5 min, but were not subjected to electric foot shocks. All the animals were exposed to reminders of the situation for 5 min on days 2, 7, and 14 without foot shock. This was achieved by placing the animal in the same chamber where the foot shocks were delivered. The duration of freezing behavior of mice were recorded by video camera and analyzed automatically by SMART® system (Panlab, S.L., Barcelona, Spain). Freezing was defined as the absence of movement other than breathing, and thresholds were selected via the software of high correlation with human observers.

2.3.2. Behavioral tests session

As depicted in Fig. 1, behavioral assessments were conducted after the aversive training procedure. Behavior was video-recorded for offline scoring.

2.3.3. Motor activity test

On day 21 post-foot shock, motor activity was assessed for 5 min using Scanet SV-10® system (Toyo Sangyo Co. Ltd., Toyama, Japan). This system was equipped with 144 pairs of photo-sensors see at a 5-mm interval, covering a measurement area of 480×300 mm (Asakura et al., 1994). Prior to the start of

Table 1
The effects of repeated administrations of valproate and diazepam on the motor activity in mice

Drugs	Dose (mg/kg)	Motor activity (counts/5 min)	
		Locomotion (counts/5 min)	Rearings (counts/5 min)
Control		825.3±64.8	7.0±1.0
Valproate	100	854.4±89.5	7.5±3.8
	200	770.3±76.6	6.5±2.2
	400	445.6±91.7**	2.9±0.6**
Diazepam	0.25	1028.0±91.5	9.9±1.6
	1	850.0±82.3	4.6±0.7
	4	441.7±98.5**	1.0±0.5**

Each value represent the mean±S.E.M ($n=10$). **$p<0.01$ compared with control group.

the recording, the animals (one animal per testing cage) were placed in Plexiglas cages (400 × 200 × 200 mm) to which they were not habituated. Locomotion and rearing behavior were recorded in a personal computer (PC980 1-RX, NEC Co. Japan).

2.3.4. Elevated plus maze test

On day 24 post-foot shocks, an elevated plus maze test was conducted by slightly modifying the method reported by Pellow et al. (1985). Briefly, the wooden apparatus consisted of two open arms (500 × 100 mm) altering at right angles with two arms enclosed by 400 mm high walls. The four arms delimited a central area of 10 cm^2. The whole apparatus was placed 60 cm above the floor. The test began with the placing of the animal in the center of the maze with its head facing a closed arm. The time spent and visits in open and closed arms during a 5-min observation period were recorded and a four paws criterion was used for arm entries.

2.3.5. Light–dark transition test

The apparatus, modified from that of Costall et al. (1989), consisted of a Plexiglas chamber subdivided into two compartments: a dark compartment (300 × 300 × 350 mm high, the same scale, color and odor with the chamber where foot shocks were delivered) and a light one (450 × 300 × 350 mm high, totally different from the dark one) illuminated by a white bulb (60 W), set 40 cm above the floor. The compartments were connected by a small divider (50 × 50 mm). On day 28, each animal was placed into the light compartment facing the wall opposite to the divider. The latency of the first entry into the dark compartment, the number of transitions, and the time spent in each compartment were assessed for 5 min.

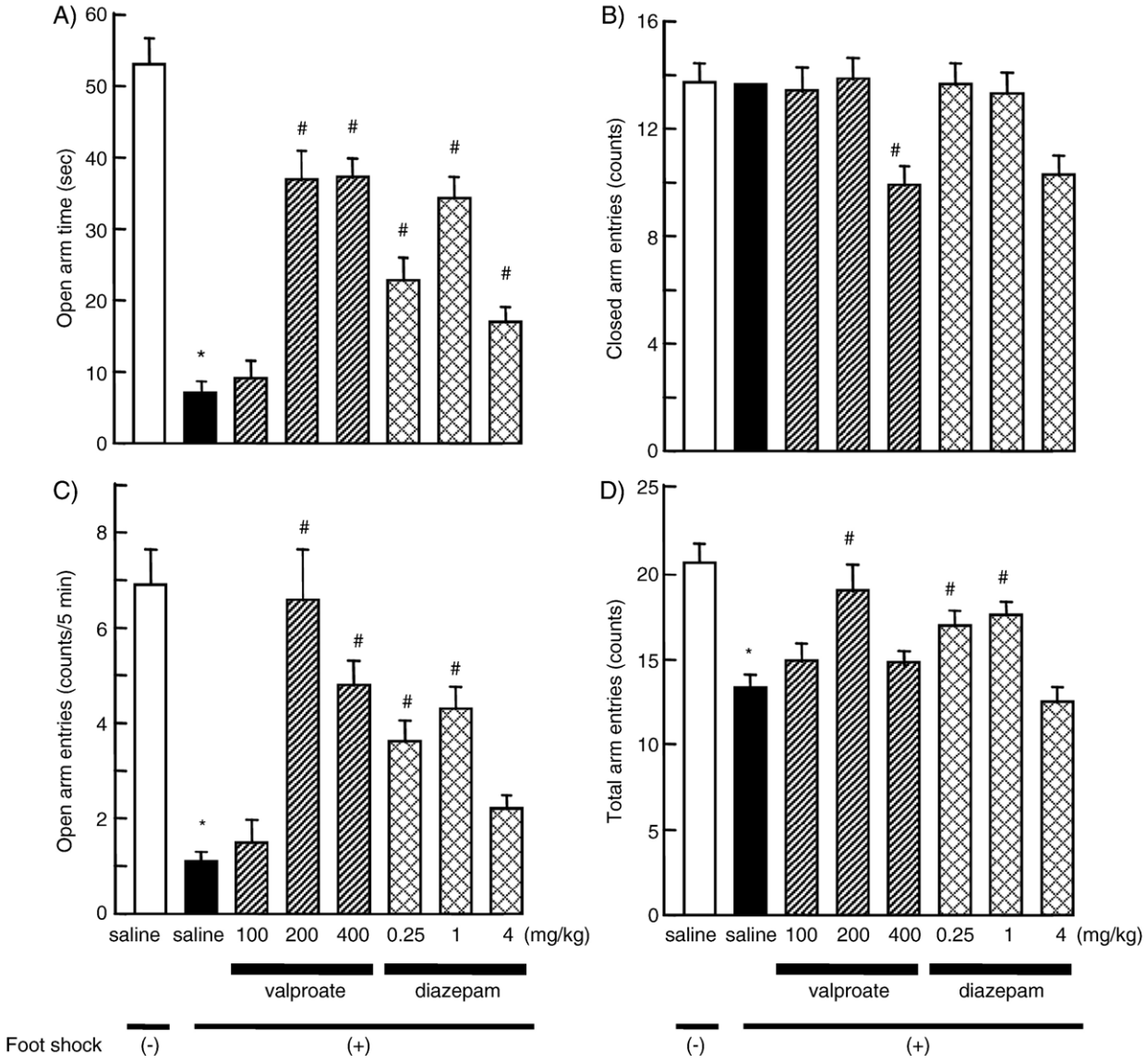

Fig. 4. Effects of valproate and diazepam on elevated-plus maze test performance of stressed mice. On day 24 after aversive procedure, the animals received the elevated-plus maze test for 5 min. The time spent in open arms (A), number of closed arm entries (B), open arm entries (C), and total arm entries (D) were recorded. Daily administration of valproate and diazepam were started from the first day of training session. The effects on the plus-maze performance were elucidated 30 min after the treatment. Each column represents the mean with S.E.M. *$p < 0.05$ compared with foot shock (−) group. #$p < 0.05$ compared with saline-treated foot shock (+) group.

Table 2
The effects of repeated administrations of valproate and diazepam on the behavioral response in the elevated plus-maze test

Drugs	Dose (mg/kg)	Open arm time (sec)	Open arm entries (counts/5 min)	Total arm entries (counts)
Control		70.2±8.8	16.5±2.3	34.2±3.2
Valproate	100	69.1±9.6	18.8±2.7	35.2±3.2
	200	79.5±9.4	22.2±2.1	48.0±3.9
	400	142.2±20.1**	42.3±5.5**	72.8±3.6**
Diazepam	0.25	95.6±16.8	33.0±5.1*	58.5±5.5*
	1	137.7±26.2	33.2±6.1*	49.7±9.2
	4	128.3±43.9	7.7±3.2	14.2±5.1

Each value represent the mean±S.E.M ($n=6$). *$p<0.05$; ** $p<0.01$ compared with control group.

2.4. Statistical analysis

The data are presented as the mean±S.E.M. Statistics were performed using one way analysis of variance (ANOVA) or two way repeated measurement analysis of variance (RM ANOVA) with treatment (vehicle, valproate and diazepam) and time (day 2, 7 and 14) as between factors followed by a Student's Newman–Keuls test for the statistical evaluation. For all tests, differences with $p<0.05$ were considered significant.

3. Results

3.1. Freezing behavior

The effects of valproate and diazepam in the freezing behavior in mice were shown in Fig. 2. On days 2, 7, and 14 after the end of aversive procedure, the percentage of freezing behavior was increased significantly [day 2: $F(7,72)=50.72$, $p<0.01$; day 7: $F(7,72)=43.96$, $p<0.01$; day14: $F(7,72)=42.35$, $p<0.01$]. This result indicated a persistent fear response of mice to the context although the freezing percentage decreased on day 14. Repeated administration of valproate at doses of 100, 200, and 400 mg/kg produced a dose-dependent reduction in freezing behavior (200 mg/kg: $p<0.05$; 400 mg/kg: $p<0.05$). In contrast, while repeated treatment with a low dose of diazepam (0.25 mg/kg) significantly reduced the freezing behavior ($p<0.05$), such effects were not observed at high doses of diazepam (1 and 4 mg/kg) and at 4 mg/kg, diazepam even potentiated the freezing behavior induced by foot shock ($p<0.01$).

3.2. Motor activity

The effects of valproate and diazepam on motor activity in mice with aversive experience were shown in Fig. 3. The aversive procedure did not significantly modify the motor activity in the mice under non-stressed conditions [$F(7,72)=3.65$, $p=0.96$]. At the high dose used in the present study, valproate (400 mg/kg) and diazepam (4 mg/kg) had a sedative like activity and decreased the locomotion and rearing number of the mice ($p<0.05$). As shown in Table 1, repeated administrations of 400 mg/kg valproate and 4 mg/kg diazepam significantly reduced the motor activity in unstressed mice. Valproate at 100–200 mg/kg or diazepam at 0.25–1 mg/kg did not influence the motor activity but at higher doses, valproate (400 mg/kg) and diazepam (4 mg/kg) showed sedative-like effects ($p<0.01$).

3.3. Elevated plus maze

As shown in Fig. 4, the time spent in the open arms and the number of open arm entries were significantly reduced in the saline control animals which had been exposed to the aversive procedure [$F(7,72)_{time}=32.18$, $p<0.001$ and $F(7,72)_{number}=14.48$, $p<0.01$]. Repeated administrations of valproate at doses of 200 and 400 mg/kg and diazepam at doses of 0.25 and 1 mg/kg significantly increased the time spent and the number of entries into the open arms ($p<0.05$), while the effect of diazepam on these indices were

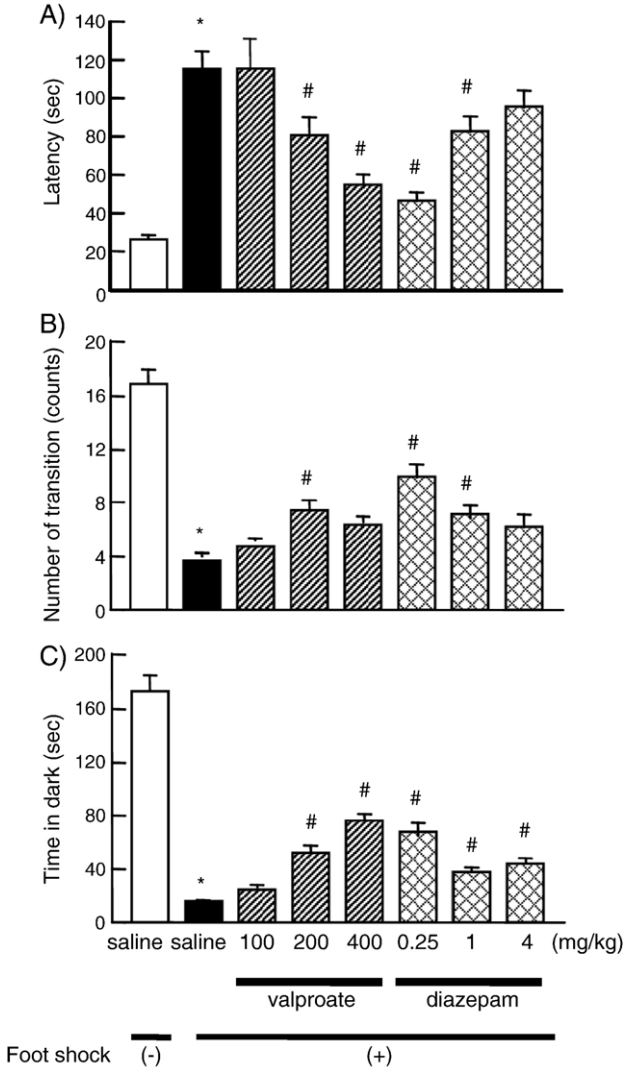

Fig. 5. Effects of valproate and diazepam on performance of stressed mice in light–dark transition test. On day 28 after aversive procedure, the animals received the light–dark transition test for 5 min. The latency to enter the dark chamber (A), number of transition (B), and time spent in dark chamber (C) were recorded. Daily administration of valproate and diazepam were started from the first day of training session. The effect on the light–dark transition performance was elucidated 30 min after the treatment. Each column represents the mean with S.E.M. *$p<0.05$ compared with foot shock (−) group. #$p<0.05$ compared with saline-treated foot shock (+) group.

reduced at a high dose (4 mg/kg). In the unstressed animals, repeated administrations of valproate (400 mg/kg) and diazepam (0.25 and 1 mg/kg) significantly increased the number of open arm entries compared with the control group (Table 2) [$F(6,35)=2.04$, $p<0.01$].

3.4. Light–dark transition

Animals which had been previously exposed to foot shocks and SRs exhibited an increase of the latency to escape from the light compartment to the dark compartment and the time spent in that compartment (Fig. 5) [$F(7,72)=14.91$, $p<0.01$]. This result showed that the animals still avoided the aversive-like compartment, indicating that they exhibited a fear response to the context associating with traumatic events. Repeated administrations of valproate (200 mg/kg) and diazepam (0.25 mg/kg) significantly improved this behavior induced by the aversive procedure (valproate: $p<0.04$; diazepam: $p<0.01$).

4. Discussion

The present study was performed to evaluate the long-term effects of an aversive procedure on behavioral parameters in mice and the therapeutic potential of valproate and diazepam administration in this animal model. Our results demonstrated that a 2-day foot shocks administration followed by repeated SRs induced behavioral alterations, which last about 4 weeks after the initially traumatic event and 2 weeks after the last SR. Moreover, we found that valproate, a mood stabilizing and antiepileptic drug, reduced those behavioral deficits observed in this animal model, whereas diazepam, a typical anxiolytic benzodiazepine receptor agonist, showed an interesting dose effect curve compared with that of valproate.

Previous studies have assessed long-lasting avoidance (from 2 h up to 2 weeks) of stressful situations such as the elevated plus maze in shocked animals (Steetnbergen et al., 1991; Grahn et al., 1995; Koba et al., 2001). In this study, we demonstrated that the animals submitted to foot shocks and SRs not only exhibited long-term and increased anxiety level but also induced a specific avoidance to the context associated with the aversive foot shock. Moreover, the aversive procedure had no influence on the motor activity of the animals in the motor activity test or the number of closed arm entries, an index of the anxiety-independent motor activity, in the elevated plus maze test, (Ramos et al., 1997; Salome et al., 2002). Our findings are in accordance with the studies of Pynoos et al. (1996) showing that a foot shock associated to SRs did not affect the motor activity of male mice in an open field test which was performed 3 to 6 weeks after the stress. However, the present results are in contrast to those of Van Dijken et al. (1992) and Van den Berg et al. (1998) describing a hypoactivity in male rats in the open field test, respectively 28 and 15 days after one or five exposures to electric foot shock. Further experiments are necessary to confirm the alterations of motor activity in such conditions.

In the light–dark transition test, animals generally spend more time in the dark compartment than in the light one. However, in the present study, 28 days after the initially traumatic foot shocks and 14 days after the last re-experiencing to the situational reminder, the stressed mice still avoided the dark compartment associated with the aversive procedure. These results indicate that the animals still exhibit a fear response to the aversive-like context although the freezing percentage, one of important indices of fear responses for rodent, decreased significantly 2 weeks after the foot shock.

In our study, although most of the animals exposed to the aversive procedure exhibited behavioral deficiencies, a large inter-individual variance was observed, indicating that there were still some animals showing a lower stressful reaction to the traumatic event or they process a better resilience than most of the animals exposed to the traumatic event. This result is in accordance with the fact that only a vulnerable sub-population of individuals exposed to a traumatic event will develop into PTSD syndromes. Antiepileptic drugs were introduced into the psychiatric pharmacopoeia for the treatment of manic-depressive illness (as a mood stabilizer) and to decrease the frequency of impulsive or violent behaviors. With the development of the kindling model as a possible pathophysiological abnormality underlying mood oscillation, these medications were found to have anti-kindling properties, offering a possible explanation for their pharmacological effects. Kindling phenomena have been demonstrated in limbic structures such as the amygdala (Cullen and Goddard, 1975; Adamec, 1990) that are implicated in stress response, fear, and potentially in PTSD symptoms. Thus it has been suggested that after exposure to traumatic events, limbic structure like the amygdala may become kindled or sensitized as a result of exaggerated noradrenergic input from locus ceruleus (Post et al., 1997), producing exaggerated fear responses, mood instability, anger, and aggression. Consequently, drugs with anticonvulsant and anti-kindling effects have been considered a potential treatment for PTSD. Another candidate drugs for PTSD treatment are benzodiazepine anxiolytics since PTSD manifests a high co-morbidity with anxiety disorder. Although benzodiazepines may be a logical choice for the treatment of PTSD, there is little empirical support for their efficacy for specific PTSD symptoms. Furthermore, several factors suggest that initiation of benzodiazepine treatment in PTSD might require careful consideration, because of the risk of abuse and of the possibility that withdrawal from benzodiazepine can exacerbate PTSD symptoms (Risse et al., 1990).

In the present study, valproate at doses of 200 and 400 mg/kg significantly decreased freezing behavior, shortened the latency to enter the aversive-like context and increased the time spent in the aversive-like context. These results showed that valproate alleviated the fear feeling of the stressed animal to the context associated with the traumatic event. The increased entries into open arms in the elevated plus maze test also indicate that valproate reduced the anxiety level increased by the aversive procedure. The effect of valproate apparently reduced when animals were treated with a high dose of the compound (400 mg/kg). Considering the results that valproate at this dose significantly reduced spontaneous motor activity in the motor activity test and closed arms entries in the elevated plus maze test and decreased latency to enter the aversive like context in the light–dark transition test, it is likely that the weakened effect of the compound on freezing behavior is due to its sedative like property.

At low dose (0.25 mg/kg), diazepam reduced the freezing behavior, but it, at higher doses (1 and 4 mg/kg), failed to affect the behavior. Moreover, after repeated daily administration for 14 days, diazepam at 4 mg/kg even exacerbates the freezing behavior induced by foot shocks. Similar to valproate, the high dose of diazepam showed a sedative like effect as indicated by the decreased motor activity and reduced entries in closed arms. Diazepam (0.25 and 1 mg/kg) decreased the anxiety level of stressed animals in the elevated plus maze. The higher dose of diazepam (4 mg/kg) showed a poor effect with the reason of sedative like effects. In the light–dark transition test, diazepam at dose of 0.25 and 1 mg/kg ameliorate the avoidance behavior, but at 4 mg/kg dose, diazepam did not show such activity.

The reason why diazepam showed such a blurry dose-effect curve is not clear. One possibility is that there might be a critical range of benzodiazepine receptor activity in modulating changes in emotionality. Further experiments are necessary to clarify the relationships between the benzodiazepine receptor system and the regulation of emotionality under stressful condition and the exact molecular mechanisms underlying the behavioral effects of valproate and diazepam. In animal studies, long-lasting change in synaptic plasticity in the medial prefrontal cortex has been implicated in the pathophysiology of PTSD, in addition to the stress vulnerability of the hippocampus, possibly induced by elevated glucocorticoid levels, altered levels of neurotrophic factors, and by changes in serotonergic and noradrenergic neurotransmission. Selective serotonin reuptake inhibitors (SSRIs) have been reported to show clinical efficacy in the treatment of PTSD and can ameliorate the above-mentioned alterations. Since after chronic administration of valproate and diazepam, the behavioral deficiencies in this animal model also can be reversed at some specific dose range, the animal model described in this paper may provide a proper way to explore the neural basis for how other drugs alleviate PTSD symptoms and to better understand neuropathological changes in PTSD.

In conclusion, the present results indicated that aversive foot shocks followed by repeated reminders should be a reliable long-lasting animal model for PTSD. Moreover, the repeated administration of valproate and diazepam showed a therapeutic effect in this animal model within some certain dose range.

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