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Original Research

Ameliorating Effect of Piperine on NO-cGMP Pathway in Stress Induced Depression

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Abstract

Depression has become a common illness among individuals of every age group. Among numerous factors held responsible for depression stress is most vital. Behind the specified disorder various hypothesis has been laid out where Nitric Oxide is emerging target to treat stress induced depression. Antidepressant potential of piperine in stressed and unstressed condition was evaluated using tail suspension test and forced swim test whereas locomotor activity was evaluated by actophotometer. Results of the present study indicate the potential of antidepressant effect of piperine in stress. Methylene blue potentiated the effect of sub-effective dose of PP and SB-203580 enhanced effect of Piperine in stressed mice with no array on locomotor activity with direct influence on Nitric oxide. Piperine produced significant changes in Nitric oxide level which is pathophysiologic mediator(s) of depression, which validate the action of piperine on depression symptoms.

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INTRODUCTION

Depression is a debilitating illness with an increasing morbidity and mortality. Furthermore, world health organization revealed that depression is the fourth leading cause of disability worldwide, exceeded by lower respiratory infections, perinatal conditions and HIV/AIDS (World Health Organization, 2001). Clinical studies showed elevated plasma nitrate levels and increased nitric oxide synthase (NOS) expression in the hippocampus of depressed patients (De Oliveira *et al*., 2000, 2008).

Stress has long been observed to play a role in the etiology of neurodegenerative diseases and mental disorders (Esch *et al*., 2002). Restrain stress induces a generalized increase in the production of Nitric Oxide (NO) and cause anxious behavior in rodents (Sevgi *et al*., 2006). Immobilization-induced stress has been observed to significantly increase an expression of Nitric Oxide Synthases (NOS) in rodents (Madrigal *et al.,* 2002; Tsuchiya *et al.,* 1997).

Nitric oxide (NO), which is an important neurotransmitter in the nervous system, (Baranano *et al.,* 2001) is synthesized from L-arginine aminoacid by the NOS enzyme (Schuman and Madison, 1994). Nitric oxide, an intercellular messenger in the brain, plays an important role in various physiological and pathological processes (Gow *et al*., 2004). It is a short lived, lipophilicmolecule generated from L-arginine, by various NADPH-dependent enzymes called NOS. There are three NOS isoforms in the NOS family, termed neuronal NOS, inducible NOS, and endothelial NOS (Michel & Feron, 1997). It plays an important role in regulating many behavioural, cognitive and emotional processes such as learning, aggression, locomotion, anxiety and depression (Dzoljic *et al*., 1997; Harkin *et al*., 1999; Holscher 1997; Nelson *et al*., 1995; Wiley *et al*., 1995). In recent studies, inhibition of NOS enzyme elicited antidepressant-like behavioural effects in several animal experiments (Harkin *et al*., 1999; Jefferys and Funder, 1996; Da Silva *et al*., 2000; Yildiz *et al*., 2000a,b) and this effect was reversed by NOS substrate L-arginine suggesting that NO plays an important role in these behavioural responses (Harkin *et al*., 1999; Jefferys and Funder, 1996; Yildiz *et al*., 2000a,b). Further, NOS activity is involved in the mechanism of action of several antidepressants. For example, the selective serotonin reuptake inhibitor paroxetine inhibits in vitro NOS activity and decreases plasma nitrite and nitrate levels significantly in depressed patients (Finkel *et al*., 1996), whereas chronic therapy with imipramine or citalopram did not change NOS activity in the examined brain regions (cortex, hippocampus or cerebellum) (Jopek *et al*., 1999). Furthermore, Wegener *et al*., 2003 showed that, serotonergic antidepressants; paroxetine, citalopram and tianeptine and mixed serotonergic–noradrenergic antidepressant; imipramine decreased hippocampal NOS activity in vitro in rats although they don't have direct effects on NOS under clinically relevant conditions. It seems that there are controversial results for the effects of different antidepressants on NOS activity but the actions

on NOS are common to a variety of structurally dissimilar serotonergic antidepressants. Further, iNOS-derived NO activates an endogenous NO-sensitive guanylyl cyclase, resulting in increased levels of cGMP (Snyder and Bredt, 1991; Nagao *et al*., 2003; André *et al*., 2005).

Immobilization is one of the best explored models of stress in rodents as this model combines both emotional (escape reaction) and physiological (muscle work) stress (Bhattacharya and Bhattacharya, 1982). Forced immobilization is one of the best explored models of stress in rodents. This model combines emotional stress (escape reaction) and physiological stress (muscle work), resulting in both restricted mobility and aggression. As painful stimuli are not directly involved in restraint stress, this form of stress is probably more akin to physiological stress (Bhattacharya and Bhattachatyya, 1982). Stress initiates a series of underlying mechanisms and cascades and one of the several implicated chemicals that is elevated after stress is NO (Esch *et al.,* 2002). Immobilization stress as long as 6h is well reported to activate the release of TNF- α through activation of NF-kappa B (Madrigal *et al*., 2002). TNF-α activates the mitogen-activated protein kinase (MAPK) pathways p42/p44 MAPK, JNK/SAPK, and p38, the last of which is responsible for interleukin-6 production (Paola *et al*., 1999). Thus, immobilization stress is hypothesized to involve activation of p38 MAPkinase and consequently induce the symptoms of depression and increase the duration of immobility in relevant behavioral models of depression like FST and TST. This immobilization stress-induced depression has been reported y earlier studies too (Sevgi *et al*., 2006).

Selective serotonin re-uptake inhibitors are believed to exert their clinical antidepressant effects by blocking the re-uptake of serotonin at the synapse, resulting in an elevation of extracellular serotonin concentrations in brain. Fluoxetine is one of the most currently used antidepressant among this group of drugs. Fluoxetine prevented the stress-induced deficit in the grooming behaviour in the splash test. Fluoxetine also significantly decreased the attack frequency when compared to the stressed control group in the resident-intruder test. These results support the assumption that NOS inhibitors can be a new class of antidepressant drugs possibly acting on neuronal NOS (Mutlu *et al.,* 2009). Further, NOS inhibitors being a class of drugs, acting on enzyme level may prove to be better agents, devoid of any long term changes in cellular biochemistry and bring behavioral stigmas like dependence or withdrawal syndromes.

MATERIAL AND METHODS

Swiss albino mice (22–30 g) were employed in the study. Animals were procured from DRDE, Gwalior, India. Animals were housed under laboratory conditions with alternating light and dark cycles of 12 h each. They had free access to food and water. The animals were acclimatized to the laboratory conditions before behavioral experiments. The experimental protocol was approved by the Institutional Animal Ethics Committee with registration number 1546/PO/a/11/CPCSEA and care of the animals

Fluoxetine was obtained from Cadila Pharmaceuticals, Ahmedabad. Piperine, Methylene Blue and SB-203580 were obtained from sigma Chemicals.

Tail suspension test was performed according to the method described by Steru *et al*. (1985) and Forced swim test was proposed as a model to test antidepressant activity by Porsolt *et al*. for evaluating potential antidepressants. The total duration of immobility was observed (Bhutani *et al*., 2008).

The effect of various treatments on locomotor activity was observed in actophotometer (Inco, Ambala, India). The locomotor activity scores for each animal were recorded for a period of 10 min (Gilhotra and Dhingra, 2009).

For nitrite estimation, blood was withdrawn from tail vein of mice immediately before setting the animal free and subjecting it to behavioral tests in all the groups. The sampling procedure was completed during immobilization to avoid the extra stress incurred upon mice during an altogether a new procedure of mouse immobilization for handling the tail of mice. Plasma was separated using cooling centrifuge at 2500 r.p.m. for 10 min. It was stored in refrigerator and processed for nitrite estimation within 24 hrs. Plasma nitrite was measured by spectrophotometric assay based on Griess reaction (Green *et al*., 1982, Gilhotra and Dhingra, 2009).

Twenty two groups of mice were employed in the study. Each group consisted of 6 mice. Stress was produced in them by immobilizing for 6h. Mice subjected to immobilization were called as stressed mice and mice not subjected to immobilization were called as unstressed mice and has been mentioned accordingly. Behavioral testing was performed carefully in a stepwise manner i.e. mice in each group were subjected to three tests (Dunn *et al.,* 2005): (a) Tail Suspension Test; then a 6 min rest in home cage, there after (b) locomotor activity test in actophotometer, again followed by 6 min rest, and then (c) Forced Swim Test. All the drugs were administered intraperitoneally (i.p.) 30 min before the behavioral testing in unstressed group and immediately before immobilization in stressed group. When combinations of the drugs were employed, pretreatments were administered 15 min before the administration of the other drug. For nitrite estimation blood samples were collected before subjecting the mice to behavioral testing. All statistical analysis has been done using one-way analysis of variance (ANOVA) followed by Tukey's test in the Graph Pad Instat (GPIS) package, version 3.05.

RESULT

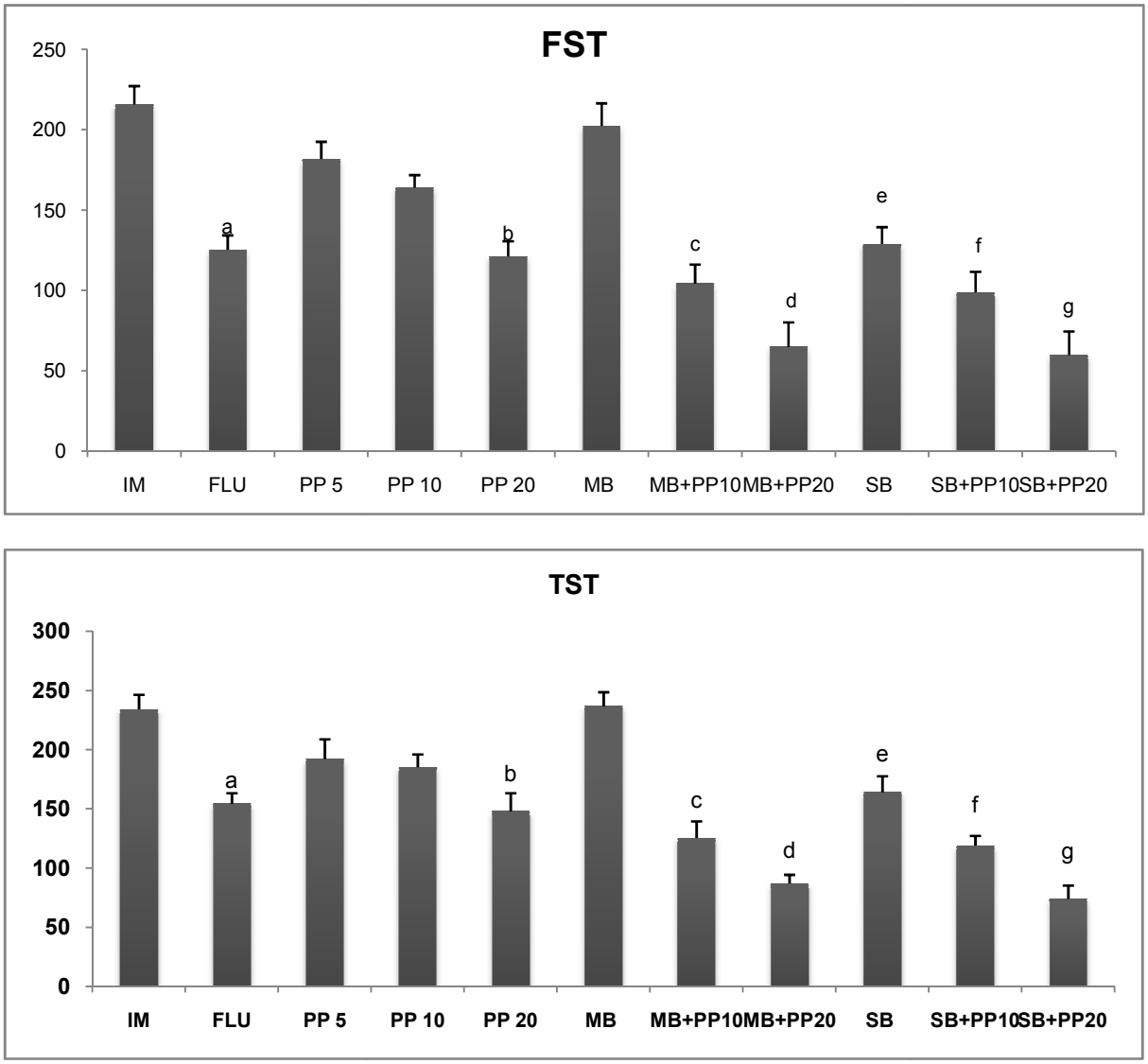
Study showed that immobilization stress has marked effect on depression and piperine decreased immobility time in stressed condition at 10 mg/kg and 20 mg/kg but in unstressed condition piperine has no significant effect in immobility time (Table 1). This indicated significant antidepressant effect of piperine in stressed condition.

Different treatments provided to stressed group showed different effects. Methylene blue (15mg/kg) has no significant effect on immobility time as compared to immobilized group but combination of methylene blue (15mg/kg) and subeffective dose PP (10mg/kg) and effective dose (20mg/kg) significantly decreased immobility time in TST and FST (Figure 1).

Table 1: Effect of piperine on Immobility time in stressed as well as unstressed mice expressed in seconds in Forced swim test as well as Tail Suspension Test

Treatment	Dose	Duration of Immobility (sec) (Mean ± SE)	
		Forced swim test	Tail suspension test
VEH(UnS)	10ml	144.7 ± 7.2	188.5 ± 10.6
IM	10ml	215 ± 11.3[a]	234.2 ± 12.3[a]
PP U	5	139.3 ± 8.5	174.3 ± 8.5
PP U	10	143.0 ± 13.1	175.0 ± 11.1
PP U	20	147.9 ± 10.3	182.9 ± 7.3
PP S	5	182.0 ± 10.5	192.3 ± 16.5
PP S	10	164.0 ± 7.8[b]	185.2 ± 10.8[b]
PP S	20	121.2 ± 9.3[c]	148.0 ± 15.3[c]

In Tail Suspension Test. n=6 in each group. Values are expressed as Mean ± S.E. Data was analyzed by one way ANOVA followed by Tukey's Post Hoc Test. In Forced Swim test F(7,40)= 40.90; p<0.0001,a=p<0.001 significant difference from unstressed group,b==p<0.001 significant difference from immobilized group,c=p<0.001 significant difference from immobilized group. In Tail Suspension Test F(7,40)=24.39;p<0.001,a=p<0.001 significant difference from unstressed group,b==p<0.001 significant difference from immobilized group,c=p<0.001 significant difference from immobilized group. **UnS**: unstressed ,**IM**: Immobilization, **PP**: Piperine. Doses mentioned are in mg/kg



Values are expressed as mean ± S.E. Data was analyzed by one way ANOVA followed by Tukey's Post Hoc Test,in FST F (10, 55) = 19.60, TST F(10,55)= 19.3; p < 0.0001, a = p < 0.001 significant difference from immobilized group, b=p < 0.001 significant difference from immobilized group, c = p < 0.05 significant difference from PP (10mg/kg) treated group, d = p < 0.05 significant difference from PP (20mg/kg)treated group, e = p<0.001 significant from immobilized group, f = p< 0.05 significant from PP (10mg/kg) treated group, g = p < 0.05significant from PP (20mg/kg) treated group. **UnS**: unstressed ,**IM**: Immobilization, **PP**: Piperine, **MB**: Methylene blue, **SB**: SB-203580. Doses mentioned are in mg/kg

Figure 1: Effect of different treatmens on immobility time on stressed mice in Forced swim test(FST) as well as Tail Suspension Test (TST), n=6 in each group.

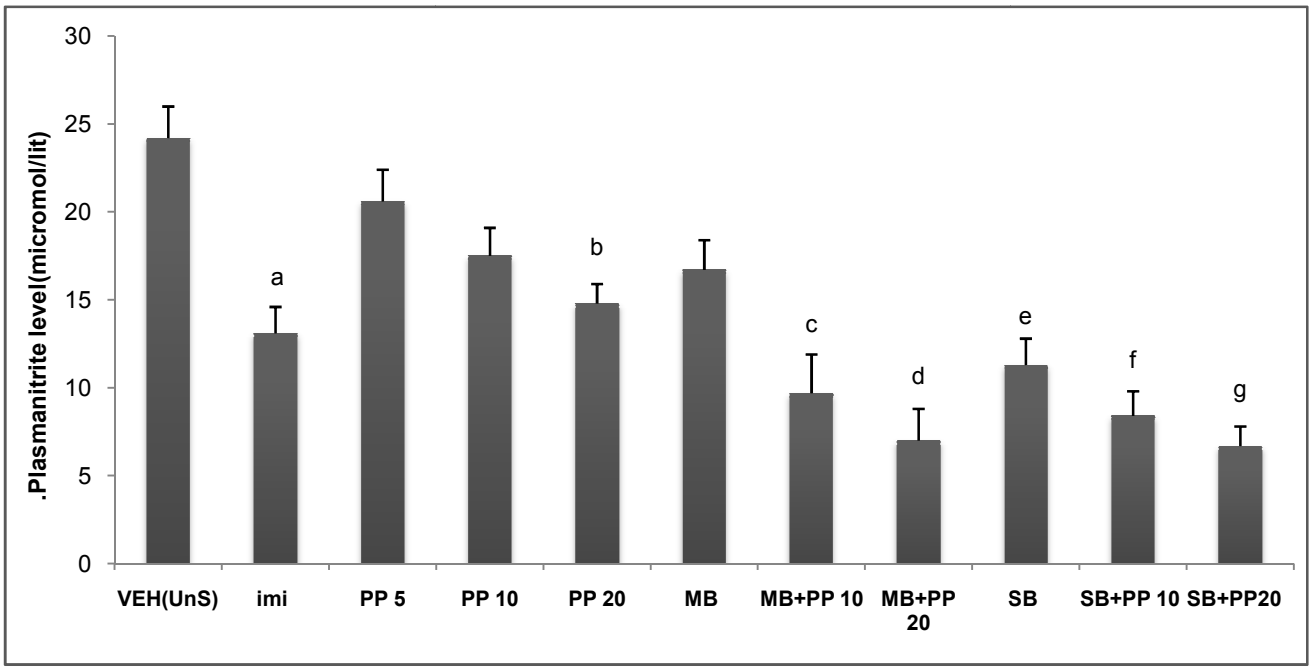
As shown in figure 2 SB-203580 significantly decreased immobility time in stressed group. A significant decrease in immobility time was observed when administered in combination with PP (20mg/kg). Table 2 shows that there is no significant change in locomotor activity as compared to vehicle treated group and immobilized group with all treatments.

Treatments on unstressed group have no significant change in plasma nitrite level. As shown in figure 2 Fluoxetine (15mg/kg), PP (20 mg/kg) significantly decreased plasmanitrite levels as compared to immobilized group. Combination of Methylene blue(15mg/kg) and Methylene blue (10,20 mg/kg) significantly decreased plasma nitrite levels as compared to PP (10mg/kg) treated group. A significant reduced levels has been also observed in SB-203580 (1mg/kg) in combination with PP(10,20 mg/kg) as compared to per se SB-203580(1 mg/kg) .

Table 2: The effect of different treatments on locomotor activity in stressed mice (n=6)

Treatment	Dose (mg/kg, i.p)	Locomotor activity counts (Mean ± SEM)
IM	6h	156.1 ± 13.0
FLU	15	137.1 ± 16
PP	5	154.3 ± 15
PP	10	137.5 ± 11
PP	20	119.2 ± 10
MB	15	149.3 ± 19.6
MB + PP	15+10	138.5 ± 16.7
MB + PP	15+20	159.3 ± 15.8
SB	1	141.6 ± 10
SB + PP	1+10	155.3 ± 14
SB + PP	1+20	143.8 ± 18.5

Effect of different treatments on locomotor activity in stressed mice expressed in Locomotor activity counts in test for locomotor activity. **UnS**: unstressed, **IM**: Immobilization, **PP**: Piperine, **MB**: Methylene blue, **SB**: SB-203580. Doses mentioned are in mg/kg.



Values are expressed as Mean ± S.E. Data was analyzed by one way ANOVA followed by Tukey's Post Hoc Test, F (10, 55) = 19.3; p < 0.0001, a = p < 0.001 significant difference from immobilized group, b = p < 0.01 significant difference from immobilized group, c = p <0.05 significant difference from PP(10mg/kg) treated group, d = p < 0.05 significant difference from PP (20mg/kg) treated group, e = p <0.001 significant from immobilized group, f = p < 0.05 significant from PP (10mg/kg) treated group, g = p < 0.05significant from PP (20mg/kg) treated group. **UnS**: unstressed ,**IM**: Immobilization, **PP**: Piperine, **MB**: Methylene blue, **SB**: SB-203580. Doses mentioned are in mg/kg.

Figure 2: The effect of different treatments on plasma nitrite levels (µmol/L) in stressed mice (n=6)

DISCUSSION

The results of the present study indicate the potential of antidepressant effect of piperine in stressed mice, exposed to 6 h immobilization stress. MB, an inhibitor of cGMP, a downstream component of NO signaling potentiated the effect of sub-effective dose of PP. Further, SB-203580, a potent inhibitor of p38MAPkinase, an upstream component of iNOS formation after stress, is also observed to enhance the effect of PP in stressed mice.

Piperine, being an inhibitor of iNOS mRNA expression has been successful to prevent the immobilization stress-induced increase in plasma nitrite levels in stressed mice. Similarly, SB-203580 *per se* has decreased plasma nitrite levels and produced antidepressant effect in stressed mice. cGMP is a second messenger in neuronal cell–cell communication and in cell–cell signaling from between presynaptic fibres as well as between postsynaptic structures (Southam and Garthwaite, 1993). The present study showed that MB, an inhibitor of cGMP, significantly produced antidepressant effect in unstressed mice. An important finding of the present study is that MB enhanced the antidepressant effect of PP in stressed mice at a dose, previously reported to produce antidepressant effect (Eroğlu and Cağlayan, 1997). The possible mechanism of MB-enhanced antidepressant effect of PP can be explained by its influence on NO–cGMP signaling pathway, thereby, preventing the further downstream signaling of nitriergic stimulus induced by immobilization stress. The antidepressant effect of MB *per*

se as well as pretreatment combination with PP in unstressed mice indicates only the effect produced by *per se* treatment of MB.

Mice were subjected to testing for induction of depression by using two most commonly used and reliable models like Tail suspension test and Forced swim test. Pathophysiologic indicator of immobilization stress-induced depression was measured in form of plasma nitrite, a stable metabolic product of nitric oxide, which has been implicated in underlying pathology of depression. The salient findings of the study can be summarized saying Mice subjected to FST and TST experience the immobility, however, there was no change in plasma nitrite levels in these mice.

Mice, subjected to 6 h immobilization stress experienced enhanced depression as indicated by an increase in duration of immobility in behavioral paradigms. This increase in duration of immobility was accompanied by an increase in plasma nitrite levels in these stressed mice.

Administration of Piperine, in unstressed mice did not produce any change in duration of immobility in mice, whereas, in stressed mice, Piperine served to decrease the immobility time as well as plasma nitrite levels.

Methylene blue, a direct inhibitor of NO and its downstream component; cGMP markedly produced antidepressant activity in unstressed mice and decreased plasma nitrite levels. On the other hand, in stressed mice, MB *per se* could not exert any significant antidepressant effect. However, MB served to enhance the effect of Piperine in stressed mice.

SB-203580, an inhibitor of p38MAPkinase, served to produce a significant antidepressant effect in stressed mice, but not in unstressed mice. Similarly, it decreased plasma nitrite in stressed mice, but not in unstressed mice. Further, SB-203580 served to enhance the effect of Piperine in stressed mice and could not produce any change in observed effect of Piperine alone in unstressed mice.

CONCLUSIONS

These results obtained by these treatments administered in the present study can be attributed to their effects on depression since they had no any effects on locomotor activity. Further, they also served to directly influence the plasma nitrite levels, an indicator of NO production and a pathophysiologic mediator(s) of depression, which further serve to validate the action of treatments on depression symptoms rather than on locomotor aspect involved in behavior of mice in both the behavioral paradigms used in the present study.

Conflict of Interest

Conflict of interest none declared.

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