Possible involvement of nitric oxide (NO) signaling pathway in the antidepressant-like effect of MK-801 (dizocilpine), a NMDA receptor antagonist in mouse forced swim test

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L-arginine-nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) is an important signaling pathway involved in depression. With this information, the present study aimed to study the involvement of this signaling pathway in the antidepressant-like action of MK-801 (dizocilpine; N-methyl-d-aspartate receptor antagonist) in the mouse forced-swim test. Total immobility period was recorded in mouse forced swim test for 6 min. MK-801 (5-25 μg/kg, ip) produced a U-shaped curve in reducing the immobility period. The antidepressant-like effect of MK-801 (10 μg/kg, ip) was prevented by pretreatment with L-arginine (750 mg/kg, ip) [substrate for nitric oxide synthase (NOS)]. Pretreatment of mice with 7-nitroindazole (7-NI) (25 mg/kg, ip) [a specific neuronal nitric oxide synthase inhibitor] produced potentiation of the action of subeffective dose of MK-801 (5 μg/kg, ip). In addition, treatment of mice with methylene blue (10 mg/kg, ip) [direct inhibitor of both nitric oxide synthase and soluble guanylate cyclase] potentiated the effect of MK-801 (5 μg/kg, ip) in the forced-swim test. Further, the reduction in the immobility period elicited by MK-801 (10 μg/kg, ip) was also inhibited by pretreatment with sildenafil (5 mg/kg, ip) [phosphodiesterase 5 inhibitor]. The various modulators used in the study and their combination did not produce any changes in locomotor activity per se and in combination with MK-801. MK-801 however, at higher doses (25 μg/kg, ip) produced hyperlocomotion. The results demonstrated the involvement of nitric oxide signaling pathway in the antidepressant-like effect of MK-801 in mouse forced-swim test.

Keywords: Cyclic guanosine monophosphate, Forced-swim test, L-arginine, Nitric oxide, MK-801 (dizocilpine), Phosphodiesterase 5 inhibitor

An abnormality in glutamate function has been implicated in the neural substrate of depressive disorders1,2. Glutamate injected into the nucleus accumbens dose-dependently decrease swimming time in forced-swim test2. An extensive library of noncompetitive N-methyl-d-aspartate (NMDA) antagonists (e.g., MK-801 or dizocilpine, memantine, ketamine) that reduce glutamatergic transmission at the NMDA receptor have demonstrated antidepressant-like effects in animal models, including forced-swim and tail suspension tests, inescapable stressors, and in learned helplessness3. An acute administration of neramexane (novel NMDA receptor antagonist) enhanced the antidepressant-like effect of imipramine (tricyclic antidepressant), fluoxetine (selective serotonin reuptake inhibitor), or venlafaxine (dual reuptake inhibitor of serotonin and norepinephrine)4. Also, Berman et al.5 reported the first placebo-controlled, double-blind study assessing the therapeutic effects of a single infusion dose of ketamine in unipolar depression.

MK-801 [(+)-5-methyl-10, 11-dihydro-5H-dibenzo [a, d] cyclohepten-5, 10-imine maleate)] or dizocilpine, a potent, noncompetitive antagonist of the NMDA receptor6 have been extensively studied for use in treatment of diseases with excitotoxic components, such as stroke, traumatic brain injury, and neurodegenerative diseases such as Huntington's, Alzheimer's, and amyotrophic lateral sclerosis. It has shown effectiveness in protecting neurons in cell culture and animal models of excitotoxic neurodegeneration7,8. Studies have also demonstrated the antidepressant effect of MK-801 in various animal models of despair10. Although the antidepressant-like effect of MK-801 in various animal models of depression have been reported, little is known concerning its possible mechanism of action.

L-arginine-nitric oxide (NO)-cyclic guanosine monophosphate
monophosphate (cGMP) is an important signaling pathway that is reported to be involved in depression\(^\text{11}\). Nitric oxide, a messenger molecule in the brain, synthesized from L-arginine by nitric oxide synthase (NOS), have been implicated in neurotransmission, synaptic plasticity, learning, perception of pain, aggression and depression\(^\text{12}\). Recent evidences have shown that the reduction of nitric oxide levels within the hippocampus can induce antidepressant-like effects, thus implicating endogenous hippocampal nitric oxide in the neurobiology of stress and depression\(^\text{13}\). Several of physiological actions of nitric oxide are mediated through its interaction with the heme iron of soluble guanylate cyclase (sGC), leading to enzyme activation and consequent increase in cGMP\(^\text{14}\). It is proposed that, in response to activation of the NMDA receptor, nitric oxide is synthesized from L-arginine by NOS\(^\text{12,15}\). Various studies have shown the possibility that various NOS inhibitors can be used as a strategy to enhance the antidepressant effect of various drugs including memantine (non-competitive NMDA receptor antagonist)\(^\text{16}\). However, none of the studies have explored the involvement of nitric oxide signaling pathway in the antidepressant-like effect of MK-801. In a recent study, Dhir and Kulkarni\(^\text{17}\) have demonstrated the involvement of L-arginine-nitric oxide-cGMP signalling pathway in mediating the antidepressant activity of venlafaxine in mouse forced-swim test. It was suggested that NOS-inhibitors when administered prior to venlafaxine treatment enhances its antidepressant action. Similarly, the importance of nitric oxide signaling pathway has been elucidated in the antidepressant action of bupropion, a dopamine reuptake inhibitor\(^\text{18}\) or berberine chloride, an alkaloid obtained from \textit{Berberis aristata}\(^\text{19}\). Since nitric oxide pathway is involved in the pathophysiology of depression, the present study has been undertaken to investigate the participation of the L-arginine-NO-cGMP signaling pathway in the antidepressant-like effect of an acute administration of MK-801 in the mouse forced-swim test.

**Experimental Procedure**—

**Forced Swim Test (FST)**—The test procedure was carried out according to the method described by Porsolt\(^\text{20}\) and validated in the laboratory\(^\text{21-25}\). In brief, mice were individually forced to swim inside a rectangular glass jar (25 \(\times\) 12 \(\times\) 25 cm\(^3\) containing 15 cm of water maintained at 23\(^\circ\)-25\(^\circ\) C). After the initial 2-3 min of vigorous activity the animals showed period of immobility by floating with minimum movements. An animal is considered to be immobile whenever it remained floating passively in the water in a slightly hunched but upright position, its nose above the water surface. The total immobility time for the period of 6 min was recorded with the help of stop-watch\(^\text{23}\).

**Measurement of locomotor activity**—

Locomotor activity (ambulations) was measured by using a computerized actophotometer (IMCORP, India). An array of 16 infrared emitter, detector pairs measured animal activity along a single axis of motion, the digital data being displayed on the front panel meters as ambulatory movements. Mice were allowed to acclimatize to the observation chamber for a period of 2 min. Locomotion was expressed in terms of total photobeams counts per 5 min per animal\(^\text{26,27}\).

**Drugs and treatment**—The following drugs were used: MK-801 (Sigma Chemicals, MO, USA), L-arginine (Loba-Chemie, Mumbai, India), methylene blue (S.D.-fine Chem Ltd., Gujarat, India), 7-nitroindazole (7-NI) (Tocris Bioscience, Missouri, USA), and sildenafil (Panacea Biotec, New Delhi, India). All the drugs were dissolved in 0.9% w/v NaCl except 7-nitroindazole which was dissolved in few drops of Tween 80 and volume was adjusted with distilled water. The doses of

**Materials and Methods**

**Animals**—Male albino mice (Laca strain) weighing between 22-30 g bred in Central Animal House (CAH) facility of the Panjab University, Chandigarh, India were used. The animals were housed under standard laboratory conditions and maintained on natural light and dark cycle, and had free access to food and water. Animals were acclimatized to laboratory conditions before the experiment. Each animal was used only once. All the experiments were carried out between 0900 and 1500 hrs. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) and conducted according to the Indian National Science Academy (INSA) Guidelines for the use and care of experimental animals.
the drugs used and time duration were selected according to the previous studies\(^{28}\) and as reported in the literature\(^{16,18,21,29}\). Different doses were administered ip in a fixed volume of 1 ml/100g body weight 30 min before the animals were subjected to test. The possible participation of the L-arginine-NO-cGMP pathway in the antidepressant effect of MK-801 was investigated. Mice were pretreated with L-arginine, a precursor of nitric oxide (750 mg/kg, ip, a dose that does not affect the immobility period as compared to vehicle treatment group), or vehicle. After 30 min, L-arginine, MK-801 (10 \(\mu\)g/kg, ip, a dose active in the forced-swim test and having no effect on the locomotor activity) or vehicle was injected and 30 min later animals were subjected to forced-swim test. In another set of experiments, the synergistic effect of MK-801 (5 \(\mu\)g/kg, ip, a subeffective dose) was investigated with a subeffective dose of 7-nitroindazole (25 mg/kg ip, a specific neuronal nitric oxide synthase inhibitor) or methylene blue (10 mg/kg, ip, an inhibitor of NOS synthase and an inhibitor of sGC). These modulators were administered 30 min before MK-801 or vehicle and 30 min later challenged with forced-swim test. Further in another set, mice received an injection of sildenafil (5 mg/kg, ip, phosphodiesterase 5 inhibitor) or vehicle 30 min before MK-801 (10 \(\mu\)g/kg, ip). After 30 min of MK-801 administration, the animals were subjected to forced-swim test. The same drug treatment schedule was followed while measuring the locomotor activity in mice. Different group of animals were taken for both tests.

**Statistical analysis**—Results are expressed as mean (sec.) ± S.E and the data were analyzed using One-Way or Two-Way Analysis of Variance (ANOVA) where appropriate. If any statistically significant change was found, post-hoc comparisons were performed using a Dunnett’s test. \(P<0.05\) was considered statistically significant.

**Results**

**Effect of pretreatment of MK-801 in mouse Forced Swim test**—MK-801, in a dose range of 10 and 25 \(\mu\)g/kg, ip, produced a decrease in immobility period with respect to vehicle control group in forced-swim test (Fig. 1) \([F=31.681, P<0.001]\). However, the effect was not dose dependent. MK-801 at the lower dose (5 \(\mu\)g/kg, ip) was ineffective in decreasing the immobility period in mice in forced-swim test (Fig. 1). MK-801 (5-25 \(\mu\)g/kg, ip) produced a U-shaped curve in reducing the immobility period in mouse FST. MK-801 (5 and 10 \(\mu\)g/kg, ip) did not alter the locomotor activity in mice (Table 1). However, MK-801 at higher dose (25 \(\mu\)g/kg, ip) increased the locomotor activity as evident from increase in ambulatory movements \([F=5.459, P<0.05]\) (Table 1). A dose of 10 \(\mu\)g/kg, ip (dose effective in forced-swim test and not affecting locomotor activity) or 5 \(\mu\)g/kg, ip (dose ineffective in forced-swim test and also not affecting locomotor activity) was chosen for carrying out drug interaction studies.

**Effect of pretreatment with various nitric oxide modulators on the antidepressant-like activity of MK-801**—Pretreatment with a subeffective dose of L-arginine (750 mg/kg, ip, NO precursor) reversed the antidepressant action of MK-801 (10 \(\mu\)g/kg, ip) as shown by an increase in immobility period compared to MK-801 (10 \(\mu\)g/kg, ip) per se group (Fig. 2A). A two-way ANOVA revealed significant differences of pretreatment \([F(1, 20) = 67.89, P < 0.001]\), treatment \([F(1, 20) = 86.41, P < 0.001]\) and of pretreatment × treatment interaction \([F(1, 20) = 176.09, P < 0.001]\) on immobility period in the forced swim test. Post hoc analyses indicated that the antidepressant-like effect of MK-801 (10 \(\mu\)g/kg, ip) was prevented by pretreatment of animals with L-arginine.

In contrast, 7-nitroindazole (25 mg/kg, ip, a specific nNOS inhibitor) enhanced the antidepressant-like activity of MK-801 (10 \(\mu\)g/kg, ip) compared to MK-801 per se group (Fig. 2A).

**Table 1**—Effect of MK-801 and its interaction with L-arginine, 7-nitroindazole, methylene blue or sildenafil on the mean ambulatory movements in mice

<table>
<thead>
<tr>
<th>Dose ((\mu)g/kg)</th>
<th>Ambulatory Movements (sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>225 ± 10</td>
</tr>
<tr>
<td>MK-801 (5)</td>
<td>250 ± 15</td>
</tr>
<tr>
<td>MK-801 (10)</td>
<td>230 ± 12</td>
</tr>
<tr>
<td>MK-801 (25)</td>
<td>200 ± 10</td>
</tr>
<tr>
<td>L-arginine (750 mg)</td>
<td>280 ± 18</td>
</tr>
<tr>
<td>7-nitroindazole (25)</td>
<td>210 ± 13</td>
</tr>
<tr>
<td>Methylene blue (10)</td>
<td>240 ± 17</td>
</tr>
<tr>
<td>Sildenafil (5)</td>
<td>260 ± 14</td>
</tr>
</tbody>
</table>

**Fig. 1**—Effect of different doses of MK-801 (5, 10, 25 \(\mu\)g/kg, ip) on the mean immobility period in mouse forced-swim test (FST). The values are expressed as mean ± SE (n = 6–8). Data were analyzed by One Way Analysis of Variance (ANOVA) followed by Dunnett’s test. \(^*\) \(P<0.05\) compared with the vehicle-treated control. \(^{*a}\) \(P<0.01\) compared with the MK-801 (10 \(\mu\)g/kg, ip) group.
Values are mean ± SE

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Treatment</th>
<th>Dose (mg/kg, ip)</th>
<th>Mean ambulatory movement</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle</td>
<td>—</td>
<td>215 ± 20.3</td>
</tr>
<tr>
<td>2</td>
<td>MK-801</td>
<td>5 µg/kg</td>
<td>235.5 ± 33.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 µg/kg</td>
<td>245 ± 7.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25 µg/kg</td>
<td>327 ± 13.7*</td>
</tr>
<tr>
<td>3</td>
<td>L-arginine</td>
<td>750</td>
<td>226 ± 11.8</td>
</tr>
<tr>
<td>4</td>
<td>L-arginine</td>
<td>750 + MK-801</td>
<td>218 ± 28.8</td>
</tr>
<tr>
<td>5</td>
<td>7-nitroindazole</td>
<td>25</td>
<td>248 ± 10.0</td>
</tr>
<tr>
<td>6</td>
<td>7-nitroindazole + MK-801</td>
<td>25 + 5 µg/kg</td>
<td>249 ± 23.1</td>
</tr>
<tr>
<td>7</td>
<td>Methylene blue</td>
<td>10</td>
<td>238.3 ± 15.4</td>
</tr>
<tr>
<td>8</td>
<td>Methylene blue + MK-801</td>
<td>10 + 5 µg/kg</td>
<td>219.9 ± 13.6</td>
</tr>
<tr>
<td>9</td>
<td>Sildenafil</td>
<td>5</td>
<td>229.6 ± 19.9</td>
</tr>
<tr>
<td>10</td>
<td>Sildenafil + MK-801</td>
<td>5 + 10 µg/kg</td>
<td>230.6 ± 33.6</td>
</tr>
</tbody>
</table>

*P < 0.001 compared with the vehicle-treated control (One way ANOVA followed by Dunnett’s test)

effect of subeffective dose of MK-801 (5 µg/kg, ip) [F=5.111, P<0.05] (Fig. 2B). A two-way ANOVA revealed significant differences of pretreatment [F (1, 20) = 6.13, P < 0.05], treatment [F (1, 20) = 6.13, P < 0.05] and of pretreatment × treatment interaction [F (1, 20) = 6.55, P < 0.05] on immobility period in the forced swim test. Post hoc analyses indicated that the antidepressant-like effect of MK-801 (5 µg/kg, ip) was enhanced by pretreatment of animals with 7-nitroindazole.

Methylene blue (10 mg/kg ip, direct inhibitor of both NOS and sGC) did not affect the immobility period per se. However, methylene blue significantly enhanced the antidepressant effect of subeffective dose of MK-801 (5 µg/kg, ip) [F= 41.522, P<0.001] (Fig. 2C). A two-way ANOVA revealed significant differences of pretreatment [F (1, 20) = 79.96, P < 0.001], treatment [F (1, 20) = 57.92, P < 0.001] and of pretreatment × treatment interaction [F (1, 20) = 60.72, P < 0.001] on immobility period in the forced swim test. Post hoc analyses indicated that the antidepressant-like effect of MK-801 (5 µg/kg, ip) was enhanced by pretreatment of animals with methylene blue.

Figure 2 D shows the effect of pretreatment with sildenafil (5 mg/kg, ip, a PDE5 inhibitor). Sildenafil did not affect immobility period per se, but pretreatment with sildenafil reversed the
antidepressant effect of MK-801 (10 μg/kg, ip) [F=53.701, P<0.001]. A two-way ANOVA revealed significant differences of pretreatment [F (1, 20) = 48.45, P < 0.001], treatment [F (1, 20) = 15.24, P < 0.001] and of pretreatment × treatment interaction [F (1, 20) = 14.52, P < 0.001] on immobility period in the forced swim test. Post hoc analyses indicated that the antidepressant-like effect of MK-801 (10 μg/kg, ip) was prevented by pretreatment of animals with sildenafil.

Combination of all the nitric oxide modulators with MK-801 did not affect the locomotor activity of mice (Table 1).

**Discussion**

L-arginine- nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) is an important signaling pathway that has been recently implicated in depression. In the present study, the antidepressant-like effect of MK-801 was attenuated by pretreatment with L-arginine and the action of subeffective dose of MK-801 was potentiated by various NOS inhibitors. Another important observation of the study was the reversal of antidepressant-like action of MK-801 by sildenafil (phosphodiesterase 5 inhibitor). The present study for the first time demonstrated the involvement of the NOS inhibitors and NMDA receptor antagonists have elicits depressive episodes. On the contrary, various NOS inhibitors and NMDA receptor antagonists have been reported to possess antidepressant-like behavioral properties. These effects are dose-dependent and stereoselective and can be reversed by co-treatment with the NO precursor, L-arginine. L-arginine is reported to exert a U-shape effect in the forced swim test, doses ranging from 30, 100 to 1000 mg/kg with lower dose causing no alteration, middle dose causing statistically significant reduction, and higher doses causing no alteration in the immobility time respectively. The dose of L-arginine (750 mg/kg, ip) was chosen as it did not affect the immobility period in the forced swim session and also did not alter the locomotor activity.

The fluoxetine suppressed the dependence and development of tolerance to the antinociceptive effect of morphine. Fluoxetine-induced suppression was potentiated by N (G)-nitro-L-arginine methyl ester (L-NAME) and accentuated by L-arginine, thus demonstrating NO modulation of drug effects. Nitroindazole, a specific neuronal NOS inhibitor and methylene blue, a direct inhibitor of both NOS and soluble guanylate cyclase (sGC), reduces the immobility time in FST. The effect is comparable to standard antidepressant like imipramine. These studies argue for the possibility of inhibition of NO synthase could be a strategy to enhance the clinical efficacy of antidepressants. The involvement of nitric oxide signaling pathway has been demonstrated in the antidepressant activity of venlafaxine, a dual reuptake inhibitor of serotonin and norepinephrine, bupropion, a dopamine reuptake inhibitor, and berberine chloride, an alkaloid obtained from Berberis aristata.

In the present study, it was observed that MK-801 produced reduction in immobility period in FST in mice. The dose response curve obtained was infact U-shaped, lower doses (10 μg/kg) decreasing the immobility period more as compared to higher doses (25 μg/kg, MK -801 at lower doses (5 and 10 μg/kg) did not produce any alterations in the locomotor activity while at higher doses (25 μg/kg), it produced hyperlocomotion. However, for further interaction studies, higher dose of MK 801 (25 μg/kg, ip) was discarded and instead lower doses (5 and 10 μg/kg, ip) were used. This biphasic response obtained is in accordance with the results obtained by Belozertsava et al., who have demonstrated similarly the biphasic response with 3-ethyl-2-methyl-quinolin-6-yl) - (4 - methoxy - cyclohexyl) - methanone methanesulfonate, metabotropic glutamate receptor antagonist, in mouse FST. From this, it can be argued that the antidepressant-like effect is not dependent on any alterations in the locomotor activity. Pretreatment of L-arginine resulted in the reversal of the antidepressant action of MK-801 as shown by increased in immobility period. On the contrary, various nitric oxide synthase inhibitors enhanced the
antidepressant action of MK-801 in mouse forced-swim test.

Thus, these results indicate that the inhibition of NO synthesis may underlie the reduction in the immobility time in the FST elicited by MK-801.

Another interesting observation of the present study was the reversal of the antidepressant-like effect of MK-801 by sildenafil, a PDE5 inhibitor. This indicates that MK-801 exerts its effect in the FST probably by decreasing cGMP levels. The intracellular cGMP concentrations are regulated not only by sGC, but also by PDE5, which catalyses the hydrolysis of the second messengers cAMP and cGMP to yield AMP and GMP, respectively. The duration and magnitude of NO-induced cGMP signal is determined by the activity of PDE5. PDE5 is expressed in several brain areas, particularly in the neurons of the Purkinje cell layer in the cerebellum and in the pyramidal neurons of the hippocampus.

Taken together, the present study concluded the involvement of L-arginine-nitric oxide (NO)-cyclic GMP (cGMP) signaling pathway in the antidepressant-like effect of MK-801, a NMDA receptor antagonist in the forced swimming test in mice. Moreover, these findings support the notion that the inhibition of nitric oxide production in the brain may be critical to the action of antidepressants.

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