THE EFFECTS OF THE NITRIC OXIDE SYNTHASE INHIBITOR 7-NITROINDAZOLE ON THE BEHAVIOUR OF MICE AFTER CHRONIC ETHANOL ADMINISTRATION

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Abstract — The effects of the nitric oxide synthase (NOS) inhibitor 7-nitroindazole (7-NI) on the behaviour of mice after chronic and acute ethanol administration were studied. Male albino mice received ethanol by inhalation for 25 days. The plus-maze and staircase tests were carried out with control, ethanol-intoxicated and ethanol-withdrawn mice (7.5 h after the end of ethanol administration). The administration of NOS inhibitor 7-NI [20.0 mg/kg, intraperitoneally (i.p.)] 60 min or 7.5 h before the plus-maze test induced an anxiolytic effect in control mice. Chronic ethanol administration induced an anxiolytic, and ethanol withdrawal an anxiogenic, effect in mice. The administration of 7-NI (20.0 mg/kg, i.p.) caused behavioural depression in ethanol-intoxicated mice, but had no effect on the behaviour of ethanol-withdrawn mice. 7-NI had no effect on the behaviour of control mice in the staircase test. Chronic ethanol administration increased, and ethanol withdrawal decreased, the locomotor activity of mice in the staircase test. Likewise, in the plus-maze test, administration of 7-NI caused behavioural depression in ethanol-intoxicated mice, but had no effect on the behaviour of ethanol-withdrawn mice. In additional experiments, vehicle or 7-NI (20.0–120.0 mg/kg, i.p.) were administered 30 min before ethanol (3.0 g/kg, i.p.). 7-NI dose-dependently increased the duration of ethanol-induced sleep and inhibited ethanol clearance. On the basis of these data we can propose that the NO system has no major role in behavioural changes caused by ethanol withdrawal. At the same time NOS inhibitors can cause synergistic CNS depression with ethanol.

INTRODUCTION

Several previous authors have proposed that some of the effects of ethanol are mediated through nitric oxide (NO) (for reviews see Lancaster, 1995; Adams and Cicero, 1998) and nitrous oxide (N₂O) in the plus-maze test. Chronic ethanol administration increased, and ethanol withdrawal decreased, the locomotor activity of mice in the staircase test. Likewise, in the plus-maze test, administration of 7-NI caused behavioural depression in ethanol-intoxicated mice, but had no effect on the behaviour of ethanol-withdrawn mice. In additional experiments, vehicle or 7-NI (20.0–120.0 mg/kg, i.p.) were administered 30 min before ethanol (3.0 g/kg, i.p.). 7-NI dose-dependently increased the duration of ethanol-induced sleep and inhibited ethanol clearance. On the basis of these data we can propose that the NO system has no major role in behavioural changes caused by ethanol withdrawal. At the same time NOS inhibitors can cause synergistic CNS depression with ethanol.

MATERIALS AND METHODS

Ethics

The experimental protocol of the present study was approved by the Ethics Committee of the University of Tartu.

Animals

Naive male albino mice (NIH/S strain, Kuopio National Animal Centre, Kuopio, Finland) weighing 36.29 ± 0.57 g (mean ± SEM) were used throughout the study. Mice were maintained under standard housing conditions with water and standard laboratory food (commercial rat pellets Laborf R70; Lactamin, Stockholm, Sweden) being available ad libitum. Mice were housed 14 per cage and exposed to a 12 h:12 h light/dark cycle. Lights were switched on from 07:00 to 19:00. Aspen chips (chip size 4 × 4 × 1 mm, Tapvei, Kortteinen, Finland) were used for bedding. To minimize the effect of stress due to handling, each animal was handled twice a day for 7 days before the start of experiments.

Drugs

7-NI (Sigma, St Louis, MO, USA) was suspended in saline with a few drops of Tween-80. As vehicle, saline with a few drops of Tween-80 was used.

Chronic ethanol administration

For chronic ethanol administration, we used a method modified in our laboratory (Vassiljev et al., 1998a,b, 1999) from the work of Ferko and Bobyock (1977). Thirty mice...
were placed into a plexiglas box (50 × 30 × 25 cm; length × width × height), with standard laboratory food and water available ad libitum. Air was bubbled into two bottles with an ethanol solution with two air pumps (2 × 1 l/min), and the vapour above the solution was passed through the chamber. The bottles with ethanol solution were kept at controlled room temperature (20 ± 1°C). The concentration of ethanol was gradually raised from 28 ml/400 ml (day 1) to 78 ml/400 ml (day 25). The level of alcohol in the air was calculated according to the formula $X_i = p_i/p$, where $X_i$ stands for the molar fraction of ethanol in the air, $p_i$ stands for the partial pressure of ethanol in the air over the solution and $p$ stands for the total pressure (atmospheric pressure). The concentration of ethanol in the air on the first day was 11.68 mg/l and on the last day 32.53 mg/l. Ethanol solution was changed twice a day. After 12 h the concentration of ethanol in solution was decreased by 20%. Over 25 days, blood-ethanol levels increased slowly. To control for blood-ethanol levels, one mouse each was killed on days 7, 14 and 21. Their respective blood-ethanol levels were 0.10, 0.21 and 0.96 mg/ml. Raising blood-ethanol levels slowly resulted in the development of tolerance to the effects of ethanol. During the inhalation period, no mice died and no obvious signs of ethanol intoxication (sedation, ataxia) were observed.

**Study design and drug administration**

The following groups of mice were used. (1) Control mice were kept grouped in their home cages until the behavioural tests. (2) Ethanol-intoxicated mice were kept in the ethanol inhalation box until the behavioural tests. (3) Ethanol-withdrawn mice were removed from the inhalation box and group-housed in new cages until the behavioural tests. From all groups, mice were assigned for vehicle or 7-NI treatment. 7-NI or vehicle were injected intraperitoneally (i.p.) 60 min or 7.5 h before the behavioural experiments. After drug or vehicle injections were carried out, mice were returned to their cages or ethanol inhalation box.

**The plus-maze test**

The plus-maze test was carried out according to Lister (1987). The plus-maze consisted of two open (8 × 17 cm) and two closed arms (8 × 17 × 30 cm), which were connected by a central platform (8 × 8 cm). Mice were placed on the central platform facing an open arm. During 5 min the number of entries made onto the open and into the closed arms and the time spent on the open arms were recorded. On the basis of these data, the percentage of entries made onto the open arms and the percentage of time spent on the open arms were calculated. Chronic ethanol administration (Table 1) caused an anxiolytic effect in the plus-maze test that was evidenced by an increase in the number of entries made onto the open arms, in the percentage of entries made onto the open arms and in the percentage of time spent on the open arms of the plus-maze. Chronic ethanol administration also increased the total number of entries made in the plus-maze test. The administration of 7-NI during ethanol administration at a dose of 20.0 mg/kg caused a decrease in the number of entries made onto the open arms $[F(1,8) = 5.57, P < 0.05]$, in the total number of entries made in the plus-maze $[F(1,8) = 5.83, P < 0.05]$, and in the percentage of entries made onto the open arms $[F(1,8) = 5.46, P < 0.001]$. 7-NI had no effect on the total number of entries made in the plus-maze test under analysis of variance (ANOVA), using group and 7-NI treatment as factors. Post-hoc statistical analysis was made by contrast analysis.

**Acute ethanol administration**

In experiments involving acute ethanol administration, ethanol was injected i.p. at a dose of 3.0 g/kg. Vehicle or 7-NI (at doses of 20.0–120.0 mg/kg) were injected i.p. to different groups of animals 30 min before ethanol. After ethanol administration, ethanol-induced sleep was measured or mice were killed by decapitation for blood-ethanol level determination.

**Measurement of ethanol-induced sleep**

Ethanol-induced sleeping time was measured as the time elapsed between the loss and regaining of the righting reflex. The experimental criterion was that the animal had to regain its righting reflex three times within 1 min.

**Blood-ethanol determination**

Following chronic ethanol administration and ethanol withdrawal, mice were killed by decapitation immediately after the behavioural tests, and trunk blood was collected for blood-ethanol determination. In experiments involving acute ethanol administration and ethanol-induced sleep, mice were killed 6 and 9 h after ethanol administration. Blood-ethanol concentration measurement was carried out by head-space chromatography with n-propanol as internal standard, as reported by Solanky and Wylie (1993).

**Statistical analysis**

The data concerning the behaviour of animals in the plus-maze test underwent analysis of variance (ANOVA), using group and 7-NI treatment as factors. Post-hoc statistical analysis was made by contrast analysis.

**RESULTS**

**The plus-maze test**

ANOVA showed a significant effect of group on the number of entries made onto the open arms $[F(7,34) = 5.46, P < 0.001]$, on the total number of entries $[F(7,34) = 3.70, P < 0.005]$, on the percentage of entries made onto the open arms $[F(7,34) = 4.27, P < 0.005]$ and on the percentage of time spent on the open arms $[F(7,34) = 5.56, P < 0.001]$. In control mice (Table 1), 7-NI at a dose of 20.0 mg/kg, administered 60 min or 7.5 h before the plus-maze test, induced an anxiolytic effect that was evidenced by an increase in the number of entries made onto the open arms $[F(2,13) = 4.24, P < 0.05]$, in the percentage of entries made onto the open arms $[F(2,13) = 7.84, P < 0.01]$ and in the percentage of time spent on the open arms of the plus-maze $[F(2,13) = 4.85, P < 0.05]$. 7-NI had no effect on the total number of entries made in the plus-maze test (Table 1).
in the percentage of entries made onto the open arms \[F(1,8) = 6.59, P < 0.05\] and in the percentage of time spent on the open arms of the plus-maze \[F(1,8) = 5.79, P < 0.05\] (Table 1).

Ethanol withdrawal (Table 1) caused an anxiogenic effect (in comparison with chronic ethanol administration) in the plus-maze test that was evidenced by a decrease in the number of entries made onto the open arms, in the percentage of entries made onto the open arms and in the percentage of time spent on the open arms of the plus-maze. The total number of entries was also decreased in ethanol-withdrawn mice. The administration of 7-NI at a dose of 20.0 mg/kg immediately after the end of ethanol exposure or 6.5 h later had no effect on the behaviour of ethanol-withdrawn mice (Table 1).

### The staircase test

ANOVA showed a significant effect of group on the number of steps \[F(1,34) = 4.48, P < 0.001\] made in the staircase test. 7-NI administered at a dose of 20.0 mg/kg 60 min or 7.5 h before the staircase test had no effect on the number of steps or rearings made by control mice (Table 2). However, when administered 7.5 h before the staircase test, 7-NI induced a tendency towards decreasing the number of rearings (\(P = 0.051\)).

Chronic ethanol administration significantly increased the exploratory activity of mice in the staircase test as evidenced by an increased number of steps. The administration of 7-NI during ethanol administration at a dose of 20.0 mg/kg caused a decrease in the number of steps \[F(1,34) = 11.35, P < 0.05\] and rearings \[F(1,34) = 17.73, P < 0.005\] made in the staircase test, in comparison with ethanol alone (Table 2). Ethanol withdrawal significantly decreased the number of steps made in the staircase test and had no effect on the number of rearings made, in comparison with chronic ethanol treatment. 7-NI administered at a dose of 20.0 mg/kg 60 min or 7.5 h before the staircase test had no effect on the behaviour of ethanol-withdrawn mice (Table 2).

### Blood-ethanol levels after chronic ethanol administration

Immediately after the end of the behavioural experiments (i.e. 10 min after the removal of mice from the inhalation box), the mean (±SEM) blood-ethanol concentration was 1.66 ± 0.20 mg/ml (\(n = 6\)) in the ethanol-intoxicated group. After the end of ethanol administration, blood-ethanol level decreased rapidly and 7.5 h later it was practically zero in all groups withdrawn from ethanol. Contrary to our previous

#### Table 1. The effects of nitric oxide synthase inhibitor 7-nitroindazole (7-NI) (20 mg/kg, i.p.) on the behaviour of control, ethanol-intoxicated and ethanol-withdrawn mice in the plus-maze test

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Entries made onto open arms</th>
<th>Total no. of entries made</th>
<th>% entries made onto open arms</th>
<th>% time spent on open arms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control/vehicle</td>
<td>6</td>
<td>3.83 ± 0.48</td>
<td>17.17 ± 1.17</td>
<td>23.12 ± 3.56</td>
<td>4.39 ± 0.77</td>
</tr>
<tr>
<td>Control/7-NI 60 min before test</td>
<td>5</td>
<td>9.60 ± 2.31*</td>
<td>20.20 ± 3.57</td>
<td>45.75 ± 5.73***</td>
<td>17.20 ± 4.74**</td>
</tr>
<tr>
<td>Control/7-NI 7.5 h before test</td>
<td>5</td>
<td>6.60 ± 1.12</td>
<td>15.20 ± 1.83</td>
<td>42.55 ± 4.24**</td>
<td>12.73 ± 2.84*</td>
</tr>
<tr>
<td>Ethanol/vehicle</td>
<td>6</td>
<td>16.00 ± 2.44****</td>
<td>27.17 ± 2.90***</td>
<td>57.51 ± 3.23***</td>
<td>27.11 ± 3.69****</td>
</tr>
<tr>
<td>Ethanol/7-NI 60 min before test</td>
<td>5</td>
<td>6.75 ± 3.12††††</td>
<td>16.75 ± 2.96††</td>
<td>35.37 ± 9.63†</td>
<td>12.17 ± 5.26†††</td>
</tr>
<tr>
<td>Withdrawn/vehicle</td>
<td>6</td>
<td>6.17 ± 1.33†††‡</td>
<td>15.83 ± 1.78†‡</td>
<td>39.37 ± 6.10†</td>
<td>10.78 ± 2.82††††</td>
</tr>
<tr>
<td>Withdrawn/7-NI 60 min before test</td>
<td>5</td>
<td>8.00 ± 1.79</td>
<td>18.80 ± 2.52</td>
<td>41.71 ± 5.25</td>
<td>11.53 ± 2.27</td>
</tr>
<tr>
<td>Withdrawn/7-NI 7.5 h before test</td>
<td>5</td>
<td>3.60 ± 0.68</td>
<td>11.80 ± 2.35</td>
<td>32.00 ± 3.54</td>
<td>6.47 ± 1.04</td>
</tr>
</tbody>
</table>

#### Table 2. The effects of the nitric oxide synthase inhibitor 7-nitroindazole on the behaviour of control, ethanol-intoxicated and ethanol-withdrawn mice in the staircase test

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>No. of steps made</th>
<th>No. of rearings made</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control/vehicle</td>
<td>6</td>
<td>63.17 ± 6.10</td>
<td>19.17 ± 1.47</td>
</tr>
<tr>
<td>Control/7-NI 60 min before test</td>
<td>5</td>
<td>52.20 ± 3.48</td>
<td>19.60 ± 3.01</td>
</tr>
<tr>
<td>Control/7-NI 7.5 h before test</td>
<td>5</td>
<td>48.60 ± 8.64</td>
<td>13.80 ± 1.83</td>
</tr>
<tr>
<td>Ethanol/vehicle</td>
<td>6</td>
<td>85.17 ± 9.63*</td>
<td>20.33 ± 1.48</td>
</tr>
<tr>
<td>Ethanol/7-NI 60 min before test</td>
<td>5</td>
<td>43.50 ± 3.57††††</td>
<td>8.25 ± 2.78†††</td>
</tr>
<tr>
<td>Withdrawn/vehicle</td>
<td>6</td>
<td>51.50 ± 8.49††††</td>
<td>19.67 ± 3.99</td>
</tr>
<tr>
<td>Withdrawn/7-NI 60 min before test</td>
<td>5</td>
<td>51.40 ± 4.13</td>
<td>20.80 ± 1.39</td>
</tr>
<tr>
<td>Withdrawn/7-NI 7.5 h before test</td>
<td>5</td>
<td>37.60 ± 5.88</td>
<td>17.20 ± 3.60</td>
</tr>
</tbody>
</table>

Details and abbreviations are as in Table 1.

\(n = \) number of animals.

\(*P < 0.05\) vs control/vehicle; ††††\(P < 0.005\), †††\(P < 0.005\) vs control/vehicle (contrast analysis).

7-NI was given i.p. in a dose of 20 mg/kg body wt to control, to chronic ethanol-treated (ethanol) and to ethanol withdrawn (withdrawn) mice as described in the Materials and methods section.
experiments, the administration of 7-NI at a dose of 20.0 mg/kg immediately after the end of ethanol exposure had no effect on blood-ethanol levels 7.5 h later as compared with vehicle-treated mice (data not shown).

_Ethanol-induced sleep and blood-ethanol levels after acute ethanol administration_

Ethanol administered at a dose of 3.0 g/kg (i.p.) induced sleep for 26.25 ± 11.13 min in vehicle-treated mice (Fig. 1A). One-way ANOVA showed a significant effect of 7-NI on the duration of ethanol-induced sleep \(F(4,27) = 3.953, P < 0.05\). Further analysis revealed that 7-NI administered i.p. at doses of 20.0–120.0 mg/kg 30 min before ethanol dose-dependently increased the duration of ethanol-induced sleep (Fig. 1A). The effect was statistically significant at doses of 80.0 and 120.0 mg/kg.

At 5 min after its acute administration, ethanol reached a concentration in the blood of 5.11 ± 0.11 mg/ml. In vehicle-treated mice, blood-ethanol levels decreased rapidly and 6 h later the blood-ethanol level was zero. 7-NI dose-dependently inhibited ethanol clearance. ANOVA showed a significant effect of drug treatment on blood-ethanol levels 6 h (Fig. 1B) and 9 h (Fig. 1C) after acute ethanol administration \(F(4,10) = 166.4, P < 0.001\) and \(F(4,10) = 42.5, P < 0.001\), respectively.

**DISCUSSION**

The administration of 7-NI to control mice significantly increased the percentage of entries made onto the open arms and the percentage of time spent on the open arms of the plus-maze. Since the validation of the plus-maze test in rats (Pellow et al., 1985) and mice (Lister, 1987), it has been repeatedly shown that anxiolytic drugs increase the percentage of time spent on the open arms and anxiogenic drugs decrease it (Rodgers et al., 1992; Dalvi and Rodgers, 1996). Our results also agree with previous studies demonstrating the anxiolytic effect of 7-NI in the plus-maze test (Volke et al., 1997; Yildiz et al., 2000). However, a novel finding of our studies is that the anxiolytic effect of 7-NI is long-lasting and is observed even 7.5 h after its acute administration. This finding is interesting considering the time course of NOS inhibition by 7-NI (MacKenzie et al., 1994). These latter authors reported maximal inhibition of NOS activity in striatum, cerebellum, hippocampus, cerebral cortex, and olfactory bulb 0.5 h after acute i.p. administration of 7-NI with consequent fast recovery of NOS activity. Two hours later, significant inhibition of NOS activity was observed only in cerebellum and hippocampus, and 4 h later, no significant changes were observed in any brain region. One explanation is that inhibition of NOS in the brain triggers a chain of neurochemical reactions causing long-lasting behavioural effects. Another possible explanation is that these changes are partly mediated by other neurotransmitter systems. It has been shown that 7-NI inhibits the activity of MAO in the brain (Desvignes et al., 1999) and increases the release of dopamine and serotonin in the brain (Wegener et al., 2000) and these changes can be observed for at least 2.5 h.

In accordance with numerous data in the literature, chronic ethanol administration induced an anxiolytic, whereas ethanol withdrawal induced an anxiogenic, effect in the plus-maze test (Onaivi et al., 1989; File et al., 1993; Cole et al., 2000). In ethanol-intoxicated mice, administration of 7-NI caused a strong sedative effect that was evidenced by a decrease in the number of entries made onto the open arms and in the total number of entries. As a consequence, the percentage of entries made onto the open arms and the percentage of time spent on the open arms were also decreased. These results contradict those of Ferreira et al. (1999), who reported that 7-NI increased the percentage of open arm entries and time spent on open arms in rats injected with ethanol. This discrepancy can be explained with different routes and regimens of ethanol administration used — Ferreira et al. used acute ethanol administration by i.p. injection whereas we used chronic ethanol administration by inhalation. Therefore, it could be assumed that the administration of 7-NI could cause strong synergistic CNS depression with ethanol. This assumption is supported by our previous data where administration of 7-NI significantly increased the duration of ethanol-induced sleep in rats (Vassiljev et al., 1998b). The administration of 7-NI had no effect on the behaviour of ethanol-withdrawn mice in the plus-maze test.

7-NI did not significantly affect the number of steps or rearings made by control mice in the staircase test.

Chronic ethanol administration increased the number of steps made by mice in the staircase test. In earlier work regarding the staircase test, rearing was considered an index of the anxiety or emotionality, and climbing (the number of steps) an index of exploratory or locomotor activity (Simiand et al., 1984; Thiebot et al., 1984). However, it has also been proposed that changes observed in the number of rearings reflect changes in the level of locomotor activity (Lister, 1990). It is probable that both are indices of exploratory behaviour and both of them depend on the level of anxiety and the level of locomotor activity. It could therefore be concluded that chronic ethanol administration increases the locomotor activity of mice in the staircase test. However, the effects of chronic ethanol administration in the staircase test differ from the effects of acute ethanol administration. It had been reported in the literature that acute ethanol administration reduces the number of rearings at doses that do not influence the number of steps climbed (Pollard and Howard, 1986; Belzung et al., 1988). As in the plus-maze test, the administration of 7-NI to ethanol-intoxicated mice caused a prominent sedative effect that was evidenced by a decrease in the number of steps and rearings made in the staircase test.

Ethanol withdrawal decreased the number of steps made in the staircase test. These results agree with those of Moy et al. (1997), who reported a decrease in locomotor activity in the plus-maze test after alcohol withdrawal. 7-NI had no effect on the behaviour of ethanol-withdrawn mice in the staircase test.

In contrast to our previous data (Vassiljev et al., 1998a), 7-NI at a dose of 20 mg/kg had no effect on ethanol clearance after chronic ethanol administration. Since in our previous work (Vassiljev et al., 1998a), relatively high blood-ethanol levels \(3.47 ± 0.37 \text{ mg/ml}\) were observed at the end of ethanol inhalation, we tested the hypothesis that the inhibitory effect of 7-NI on ethanol pharmacokinetics is more pronounced at high ethanol concentrations. Therefore we carried out additional experiments using acute ethanol administration. In accordance with our experiments with rats (Vassiljev et al., 1998b), the administration of 7-NI to mice before acute ethanol
administration significantly increased the duration of ethanol-induced sleep, an effect probably caused by synergistic CNS depression induced by ethanol and 7-NI. However, 7-NI at a dose of 20 mg/kg had no effect on ethanol pharmacokinetics after acute ethanol administration. Considerably higher doses (40–120 mg/kg) were needed to inhibit ethanol elimination after acute ethanol administration. Therefore, the most probable cause of this discrepancy with our earlier results lies in different strains of mice used. In our previous experiments, we used balb/c mice and in this set of experiments we used NIH/S mice. It is possible that these strains differ in their sensitivity to the effects of 7-NI.

In conclusion, 7-NI had no effect on the behavioural changes caused by ethanol withdrawal in the plus-maze or staircase test. Therefore it can be proposed that NO pathways do not have a major role in the behavioural changes caused by ethanol withdrawal. At the same time, NOS inhibitors can cause synergistic CNS depression with ethanol.

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