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Inhibition of neuronal nitric oxide synthase suppresses the maintenance but not the induction of psychomotor sensitization to MDMA ('Ecstasy') and *p*-chloroamphetamine in mice

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Abstract

Repeated exposure to psychostimulants such as cocaine and amphetamines results in behavioral sensitization, a paradigm thought to be relevant to drug craving and addiction in humans. We have previously shown that the induction, expression, and maintenance of psychomotor sensitization to cocaine, methamphetamine, and methylphenidate (indirect dopamine agonists) are blocked by co-administration of the neuronal nitric oxide synthase (nNOS) inhibitor 7-nitroindazole (7-NI). In the present study, we investigated the effects of 7-NI on the induction, expression, and maintenance of psychomotor sensitization to 3,4-methylenedioxymethamphetamine (MDMA; 'Ecstasy') and *p*-chloroamphetamine (PCA). The following observations are reported: (a) Repeated administration of MDMA (10 mg/kg) and PCA (5 mg/kg) to Swiss Webster mice for six consecutive days caused a 3-fold increase in the psychomotor stimulating effect of the drugs on day 6 compared to day 1. (b) Pretreatment with 7-NI (25 mg/kg) did not affect the induction and expression of sensitization to MDMA and PCA. (c) Pretreatment with 7-NI did, however, suppress the enduring sensitized response to challenge injections of MDMA and PCA which was observed in mice pretreated with vehicle instead of 7-NI. (d) Unlike other psychostimulants, MDMA and PCA treatment did not produce conditioned (context-dependent) hyperlocomotion. These findings, coupled with our previous studies, suggest the following: (a) The induction and expression of psychomotor sensitization to MDMA and PCA are independent of nNOS activity and involve primarily serotonergic transmission. (b) The maintenance of psychomotor sensitization is dependent on intact nNOS activity and involves primarily dopaminergic transmission.

Keywords: Psychostimulants; Hyperlocomotion; Sensitization; Condition locomotion; Cocaine; Methamphetamine; Methylphenidate

Nitric oxide (NO)[1] is considered a retrograde messenger involved in synaptic plasticity and neurotoxicity [1–3]. In brain, NO is generated by the neuronal, inducible, and endothelial isoforms of nitric oxide synthase (nNOS, iNOS, and eNOS, respectively). nNOS and eNOS are calcium-dependent, while iNOS, present primarily in microglia and macrophages, is calcium-independent. There is particular interest in the role of nNOS in central nervous system (CNS) pathology, since stimulation of nNOS is associated primarily with activation of the *N*-methyl-D-aspartate (NMDA) type of glutamate receptors. The increase of calcium influx caused by NMDA receptor activation leads to the binding of calcium to calmodulin, which in turn stimulates nNOS [1–3]. L-Arginine is the substrate of NOS; the conversion of L-arginine to L-citrulline causes the release of NO. Several studies have shown that NO modulates the release of various neurotransmitters such as dopamine (DA), glutamate, and norepinephrine [4–6]. Thus, there is reason to consider NO as a major neuromodulator of synaptic transmission in brain.

Psychostimulants such as cocaine and the amphetamine analogs methamphetamine (METH) and 3,4-methylenedioxymethamphetamine (MDMA; 'Ecstasy') cause an increase in synaptic levels of DA, 5-hydroxytryptamine (5-HT; serotonin), and norepinephrine. Repeated administration of psychostimulants to mice and

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[1] *Abbreviations used:* MDMA, 3,4-methylenedioxymethamphetamine; METH, methamphetamine; PCA, *p*-chloroamphetamine; nNOS, neuronal nitric oxide; NO, nitric oxide; 7-NI, 7-nitroindazole; 5-HT, 5-hydroxytryptamine; DA, dopamine.

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rats causes behavioral sensitization ('reverse tolerance') which is relevant to the development of drug craving and addiction in humans [7,8]. Besides the role of DA in the development of psychomotor sensitization, evidence suggests the involvement of excitatory amino acid transmission [8,9] and nNOS [10] in the effects of psychostimulants. We reported that the relatively selective nNOS inhibitor 7-nitroindazole (7-NI) blocks the induction and expression of psychomotor sensitization caused by repeated administration of cocaine, METH [11], and methylphenidate [12]. Mice lacking in the nNOS gene [homozygous nNOS(−/−)] did not develop psychomotor sensitization following repeated cocaine administration [13] and were also resistant to the rewarding effect of cocaine [14]. Several lines of evidence suggest that nNOS is involved in METH-induced striatal dopaminergic neurotoxicity. 7-NI [15,16] and other nNOS inhibitors [17] attenuated METH-induced dopaminergic neurotoxicity, and nNOS(−/−) mice were protected against such neurotoxic insult [18]. Together, these results suggest that nNOS has a major role in dopamine-mediated effects of psychostimulants.

Although the psychomotor stimulating effect of substituted amphetamines is dependent on dopaminergic and serotonergic transmission, the relative contribution of DA and 5-HT release to the behavioral effects of these drugs may be different. For instance, acute administration of methylphenidate caused primarily increase in DA but not 5-HT efflux [19], while MDMA and PCA are known to release both DA and 5-HT. In rats, the psychomotor stimulating effect of MDMA was modulated by various 5-HT receptor antagonists [20,21] and the neurotoxin 5,7-dihydroxytryptamine [22]. In Swiss mice, (±)MDMA-induced hyperlocomotion, hyperthermia, and lethality were attenuated by the 5-$HT_{2A/2C}$ receptor antagonist ketanserin [23]. Several studies have shown that *p*-chloroamphetamine (PCA) caused the release of both 5-HT and DA [24–26], and it elicits serotonin syndrome and serotonergic neurotoxicity in rodents [27]. Likewise, the locomotor stimulating effects of PCA may be due to the indirect 5-HT agonist properties of the drug [28]. Since serotonergic transmission may contribute more to the behavioral effects of MDMA and PCA compared to those of methylphenidate, cocaine, and METH, we sought to investigate the effect of the nNOS inhibitor 7-NI on the development of locomotor sensitization due to repeated administration of MDMA and PCA to Swiss Webster mice.

Experimental procedure

Drugs

(D/L)PCA-HCl and 7-NI were purchased from Sigma (St. Louis, MO). (±)MDMA–HCl was a gift from the National Institute on Drug Abuse (Bethesda, MD). PCA and MDMA were dissolved in saline (0.9% NaCl) and 7-NI was dissolved in a solution containing dimethyl sulphoxide/propylene glycol/water (1:3:6) (considered as vehicle). All drug injections were administered by intraperitoneal (i.p.) injection in a volume of 0.1 ml/10 g of body weight. The treatments were conducted once a day between 10:00 and 14:00 h.

Animals

Male Swiss Webster mice (8 weeks of age at the beginning of the experiment; 30–32 g; Charles River, Wilmington, MA, USA) were maintained on a 12-h light/dark cycle at a room temperature of 23 °C, and housed in groups of five with free access to food and water for 5–7 days before the experiments began. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, 1996) and approved by the University of Miami Animal Care and Use Committee.

Experiment 1: dose–response studies

To determine a dose–response for the locomotor stimulating effects of MDMA and PCA, mice received four doses of each drug: 0, 5, 10, and 20 mg/kg ($n = 5$ for each dose). Locomotor activity was recorded for 60 min (peak drug effect occurred 20–30 min after i.p. injections).

Experiment 2: effect of 7-NI on the induction and short-term maintenance of psychomotor sensitization to MDMA and PCA

To investigate the effect of 7-NI on MDMA-induced psychomotor sensitization, four groups of mice ($n = 8$ each) received the following injections once daily for six consecutive days: (1) vehicle/saline, (2) vehicle/MDMA (10 mg/kg), (3) 7-NI (25 mg/kg)/saline, and (4) 7-NI/MDMA. The interval between the two injections was 30 min. The dose of MDMA chosen was based upon results of the dose–response studies which showed an intermediate level of locomotion induced by 10 mg/kg MDMA (Fig. 1). The dose of 7-NI was based on our previous studies which had shown that 25 mg/kg 7-NI inhibited about 80% of mouse brain NOS activity [29] and blocked the development of psychomotor sensitization to cocaine and METH [11]. The same schedule was used to investigate the effect of 7-NI on PCA-induced hyperlocomotion. Four groups of mice ($n = 8$ each) received one of the following treatment once a day for six consecutive days: (1) vehicle/saline, (2) vehicle/PCA (5 mg/kg), (3) 7-NI (25 mg/kg)/saline, and (4) 7-NI/PCA. The dose of PCA was chosen based upon results of the dose–response studies (Fig. 1).

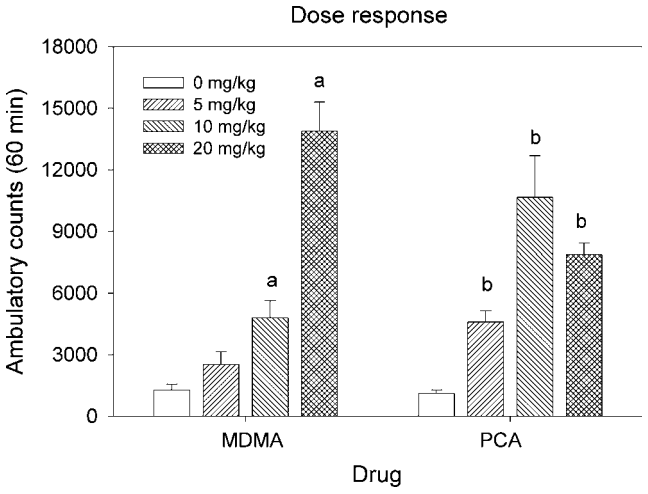

Fig. 1. Dose–response of MDMA- and PCA-induced locomotion. Data represent the means ± SEM ambulatory counts recorded for 60 min. $P < 0.05$ (a) compared to MDMA (0 mg/kg), $P < 0.05$ (b) compared to PCA (0 mg/kg) ($n = 5$, each dose).

On days 1 and 6, the injections were paired with the locomotor activity cages (e.g., novel environment) and locomotor activity was recorded. This paradigm allowed us: (a) to compare between the responses to *acute* and *repeated* drug administration and (b) to determine whether 7-NI influenced the *induction* of psychomotor sensitization. On days 2–5, injections were delivered in the animals' home cage and locomotor activity was not recorded.

Following a 10-day drug-free period (day 16), the short-term maintenance of psychomotor sensitization was investigated. A challenge injection of MDMA (10 mg/kg) was administered to vehicle/saline, vehicle/MDMA, 7-NI/saline, and 7-NI/MDMA pretreated mice, while a challenge injection of PCA (5 mg/kg) was administered to vehicle/saline, vehicle/PCA, 7-NI/saline, and 7-NI/PCA pretreated mice. Routinely, a vehicle injection was administered 30 min before the drug and locomotor activity was recorded for 60 min following the drug administration.

Experiment 3: effect of challenge injections of saline

We have reported that pairing the first and last injections of cocaine, METH [11], and methylphenidate [12] with the test cage (novel environment) caused the development of place-dependent hyperlocomotion (e.g., conditioned response). The conditioned response was determined by the intensity of locomotion induced by a saline injection given to saline and drug pretreated mice in the cage that was previously paired with saline or drug. 7-NI blocked the development of conditioned hyperlocomotion due to cocaine [11] and methylphenidate [12] administration. Accordingly, on day 9 animals received a saline injection and locomotor activity was recorded for 60 min.

Experiment 4: effect of 7-NI on the expression of psychomotor sensitization to MDMA and PCA

Mice pretreated with vehicle/MDMA or vehicle/PCA developed a sensitized response to challenge injections of MDMA or PCA, respectively, on day 16. To investigate whether 7-NI could block the *expression* of the sensitized response, on day 24, the two groups were injected with 7-NI (25 mg/kg) followed by MDMA (10 mg/kg) or PCA (5 mg/kg), and locomotor activity was recorded for 60 min.

Experiment 5: long-term maintenance of psychomotor sensitization to MDMA and PCA

To investigate the long-term maintenance of the sensitized response and the effect of 7-NI pretreatment on the maintenance of sensitization, mice were challenged on days 31 and 61 with MDMA (10 mg/kg) or PCA (5 mg/kg) and locomotor activity was recorded for 60 min.

Measurement of locomotor activity

The locomotor activity cages were standard transparent rectangular rodent cages ($42 \times 24 \times 20$ cm high) and locomotion was monitored by an activity meter (Opto-Varimex-Mini Model B; Columbus Instruments, Columbus, OH). The activity meter consisted of an array of 15 infrared emitter/detector pairs, spaced at 2.65 cm intervals, measuring activity along a single axis of motion. Each emitter and detector were mounted alongside the length of the cage (42 cm long). Both the total counts and the ambulatory counts were recorded and transferred by a counter interface to a computer. The Opto-Varmix-Mini Model B separates counts (beam interruptions) of total activity from counts that correspond to ambulatory (horizontal) activity. This is accomplished by memorizing the location of the last broken beam and blocking additional counts from being scored on front panel counter until a different beam is broken. Subtraction of the ambulatory counts from the total counts provides an index of vertical activity. Based on our previous observations [11,12] and the current results, it appears that the fraction of non-ambulatory counts (25–30% of total counts) increased or decreased in parallel to corresponding changes in ambulatory counts. Because of this relationship, only ambulatory counts are reported. Counts were registered every 10 min for a total of 60 min and results are presented as means ± SEM cumulative horizontal counts recorded. Routinely, each mouse was placed in the test cage for a 30 min habituation period to the new environment prior to the recording of drug-induced locomotor activity.

Statistical analysis

Differences between the responses to various drug treatments at a single time point were analyzed by one-way ANOVA followed by post hoc Newman–Keuls' test. Differences between the responses to various drug treatments at multiple time points were analyzed by two-way ANOVA, drug effect × time (with time as the repeated measure) followed by Bonferroni multiple comparisons post hoc test. A P value of less than 0.05 was considered statistically significant.

Results

Experiment 1: dose–response studies

MDMA (5, 10, and 20 mg/kg) resulted in a dose-dependent increase in locomotion, main effect $F[3,16] = 31.74$; $P < 0.0001$. While 5 mg/kg MDMA had no significant effect on locomotion, 10 and 20 mg/kg caused a significant increase in locomotion compared with the control group ($P < 0.05$; Newman–Keuls' test; Fig. 1). PCA (5, 10, and 20 mg/kg) resulted in a dose-dependent increase in locomotor activity, main effect $F[3,16] = 14.83$; $P \leqslant 0.0001$. The locomotor activity generated after administration of 5, 10, or 20 mg/kg PCA was significantly higher than control (0 mg/kg) ($P < 0.05$; Newman–Keuls' test; Fig. 1). However, 20 mg/kg PCA resulted in less locomotor activity compared to 10 mg/kg (the difference was not statistically significant). This reduced locomotor activity was due to the emergence of the serotonin syndrome, as manifested by head and body weaving, and occasional hind-limb extensions. Since a dose of 10 mg/kg MDMA and 5 mg/kg PCA induced optimal levels of locomotion with no signs of stereotypy or serotonin syndrome, these doses were chosen for subsequent experiments.

Experiment 2: effect of 7-NI on the induction and short-term maintenance of psychomotor sensitization to MDMA and PCA

Fig. 2A depicts the response to acute and repeated administration of MDMA, and the influence of 7-NI on MDMA-induced locomotion. A two-way ANOVA, drug treatment × time (time as the repeated measure) resulted in the following parameters: drug effect: $F[3,84] = 85.41$, $P < 0.0001$; time effect: $F[2,84] = 51.02$, $P < 0.0001$; and interaction: $F[6,84] = 29.73$, $P < 0.0001$. Bonferroni multiple comparisons for drug effect on day 1, e.g., vehicle/MDMA vs. vehicle/saline as well as 7-NI/MDMA vs. 7-NI/saline, yielded significant differences $P < 0.05$ ($t = 3.06$ and 3.27, respectively). No significant differences were observed between vehicle/MDMA and 7-NI/MDMA groups, suggesting that 7-NI did not affect the acute response to MDMA. On day 6, comparison between vehicle/MDMA vs. vehicle/saline as well as 7-NI/MDMA vs. 7-NI/saline yielded significant differences, $P < 0.001$ ($t = 13.45$ and 12.61, respectively). A comparison between MDMA-induced locomotion on days 1 and 6 in the vehicle/MDMA group as well as in the 7-NI/MDMA group showed significant differences, $P < 0.001$ ($t = 9.126$ and 10.23, respectively), suggesting that both groups developed sensitization to the locomotor stimulating effect of

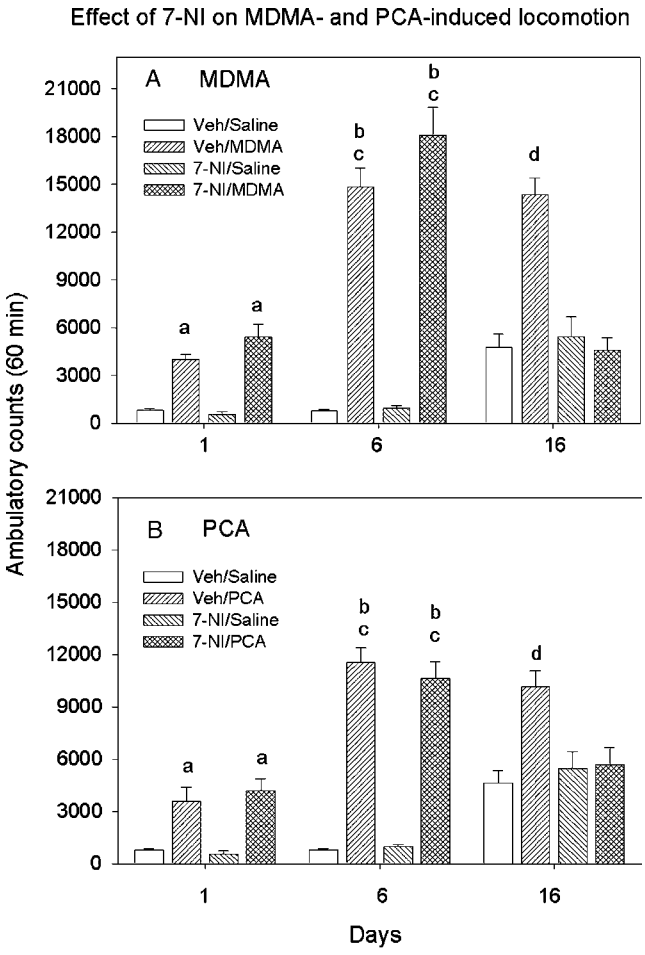

Fig. 2. Effect of 7-NI on MDMA- and PCA-induced locomotion. Mice were treated with vehicle/saline, vehicle/drug [MDMA (10 mg/kg) or PCA (5 mg/kg)], 7-NI (25 mg/kg)/saline, and 7-NI/drug for six consecutive days. Locomotor activity was recorded for 60 min on days 1 and 6. After a 10-day drug-free period (day 16) four groups were challenged with MDMA (10 mg/kg; A), and four groups were challenged with PCA (5 mg/kg; B) and locomotor activity was recorded. The intensity of locomotion in vehicle/drug and 7-NI/drug groups was significantly higher than vehicle/saline and 7-NI/saline groups on day 1, $P < 0.05$ (a), and day 6, $P < 0.001$ (b). Locomotion measured in the vehicle/drug and 7-NI/drug groups on day 6 was significantly higher than on day 1, $P < 0.001$ (c), suggesting the development of sensitization. On day 16, the intensity of locomotion in the vehicle/drug groups was significantly higher than in the vehicle/saline, 7-NI/saline, and 7-NI/drug groups, $P < 0.001$ (d), but no significant difference was found between the vehicle/saline, 7-NI/saline, and 7-NI/drug groups ($n = 8$, each group).

MDMA. Therefore, 7-NI did not affect the induction of sensitization to MDMA. On day 16, after a 10-day drug free period, mice were challenged with MDMA (10 mg/kg). Results show that MDMA-induced locomotion in the vehicle/MDMA group was significantly higher, $P < 0.001$ ($t = 9.12$–10.67) than the three other groups tested, vehicle/saline, 7-NI/saline, and 7-NI/MDMA. These findings suggest that only the vehicle/MDMA group remained sensitized to the locomotor stimulating effect of MDMA, while the 7-NI/MDMA group lost the sensitized response to MDMA challenge (Fig. 2A).

The treatment with vehicle/PCA and 7-NI/PCA yielded similar results. Fig. 2B depicts the response to acute and repeated administration of PCA, and the influence of 7-NI on PCA-induced locomotion. A two-way ANOVA, drug treatment × time (time as the repeated measure) resulted in the following parameters: drug effect: $F[3, 84] = 64.73$, $P < 0.0001$; time effect: $F[2, 84] = 44.22$, $P < 0.0001$; and interaction: $F[6, 84] = 13.89$, $P < 0.0001$. Bonferroni multiple comparisons for the drug effect on day 1, e.g., vehicle/PCA vs. vehicle/saline as well as 7-NI/PCA vs. 7-NI/saline, yielded significant differences, $P < 0.05$ ($t = 2.912$ and 3.401, respectively). Since no significant difference between vehicle/PCA and 7-NI/PCA groups was observed, it appears that 7-NI did not affect the acute response to PCA. On day 6, a comparison between vehicle/PCA vs. vehicle/saline as well as 7-NI/PCA vs. 7-NI/saline yielded significant differences, $P < 0.001$ ($t = 9.054$ and 11.37, respectively). A comparison between PCA-induced locomotion on days 1 and 6 in the vehicle/PCA and 7-NI/PCA groups showed significant differences, $P < 0.001$ ($t = 6.204$ and 6.237, respectively), suggesting that both groups developed sensitization to the locomotor stimulating effect of PCA. Therefore, 7-NI did not affect the induction of sensitization to PCA. On day 16, after a 10-day drug free period, mice were challenged with PCA (5 mg/kg). Results show that PCA-induced locomotion in the vehicle/PCA group was significantly higher, $P < 0.001$ ($t = 6.275$–6.896) than the three other groups tested, vehicle/saline, 7-NI/saline, and 7-NI/PCA. These findings suggest that only the vehicle/PCA group remained sensitized to the locomotor stimulating effect of PCA, while the 7-NI/PCA group lost the sensitized response to PCA challenge (Fig. 2B).

Experiment 3: effect of a challenge injection of saline

Mice were challenged with saline injection three days following the termination of repeated drug administration (day 9) and locomotor activity was recorded. Results in Fig. 3 show that the intensity of locomotion following saline injection was similar in all groups tested. These findings suggest that: (a) pairing MDMA and PCA injections with the test cage did not cause the development of context-dependent hyperlocomotion and

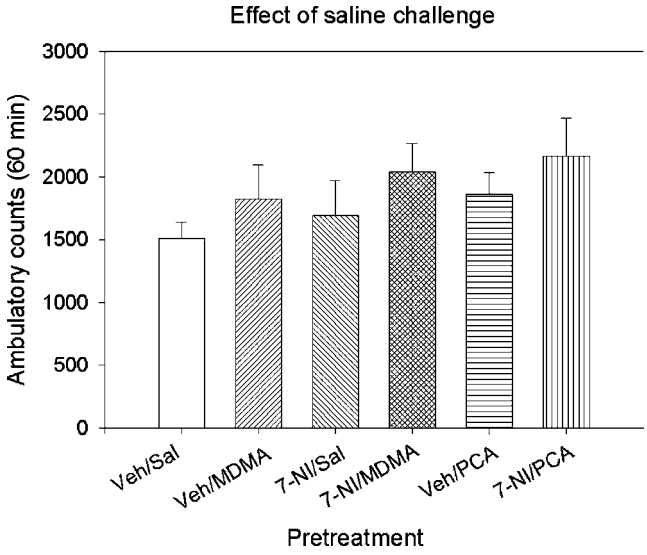

Fig. 3. Effect of saline injection on locomotor activity. Three days following termination of the repeated drug administration (day 9), mice received a saline injection and locomotor activity was recorded for 60 min. No significant difference was found between the various groups ($n = 8$, each) tested $F[5, 42] = 2.112$; $P = 0.0827$, suggesting the absence of conditioned hyperlocomotion.

(b) 7-NI pretreatment did not affect the response to saline injection.

Experiment 4: effect of 7-NI on the expression of psychomotor sensitization to MDMA and PCA

As shown in Figs. 2A and B, mice pretreated with vehicle/MDMA and vehicle/PCA remained sensitized to challenge injections of MDMA and PCA, respectively, on day 16. To investigate if 7-NI could block the expression of sensitization, on day 24, these two groups received 7-NI (25 mg/kg) followed by MDMA (10 mg/kg) or PCA (5 mg/kg). Results in Fig. 4 show that 7-NI did not affect the expression of sensitization, because the intensity of locomotor activity following 7-NI + MDMA (or PCA) administration was not significantly different from the intensity of locomotion which resulted from vehicle + MDMA (or PCA) administration on day 16.

Experiment 5: long-term maintenance of psychomotor sensitization to MDMA and PCA

To further investigate the long-term maintenance of psychomotor sensitization, vehicle/drug (MDMA or PCA), and 7-NI/drug pretreated mice were challenged with MDMA (10 mg/kg) and PCA (5 mg/kg) on days 31 and 61 after the initial drug exposure. Results in Fig. 5A depict the effect of MDMA challenge. Two-way ANOVA resulted in the following parameters: drug effect: $F[1, 28] = 150.2$, $P < 0.0001$; time effect: $F[1, 28] = 30.17$, $P < 0.0001$; and interaction: $F[1, 28] = 25.12$,

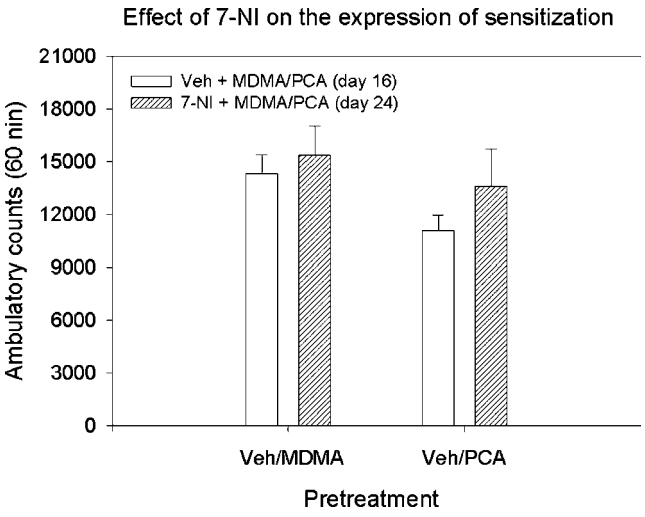

Fig. 4. Effect of 7-NI on the expression of psychomotor sensitization. The vehicle/MDMA ($n = 8$) and vehicle/PCA ($n = 8$) groups that were sensitized on day 16 (Fig. 2) were administered 7-NI (25 mg/kg), followed by MDMA (10 mg/kg) or PCA (5 mg/kg) eight days later (day 24), and locomotor activity was recorded for 60 min. 7-NI affected neither the sensitized response to MDMA ($P = 0.217$) nor PCA ($P = 0.108$), suggesting that the expression of sensitization was not altered.

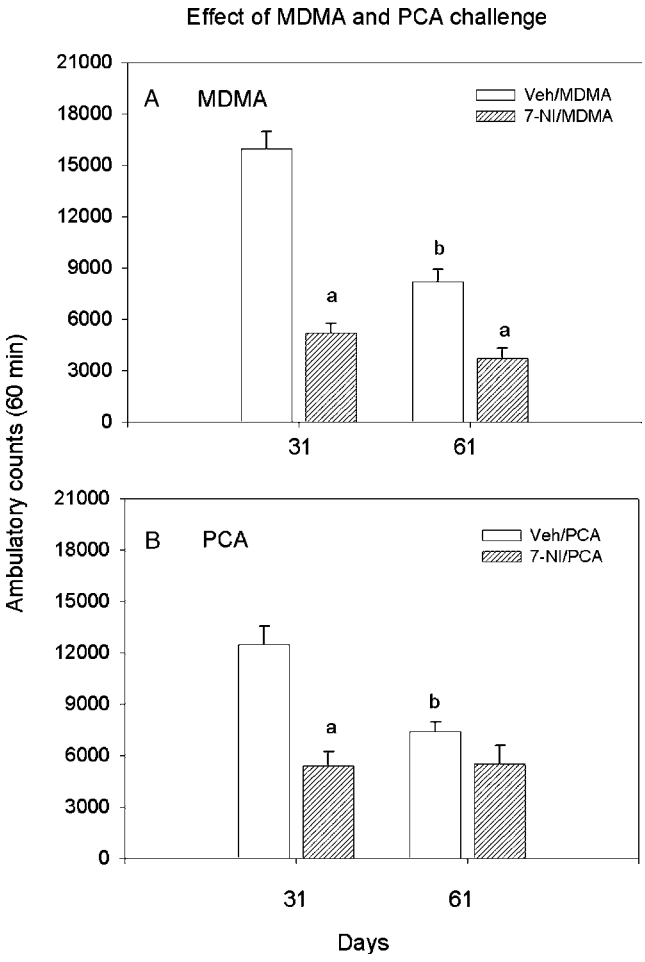

Fig. 5. Effect of MDMA and PCA challenge injections on the maintenance of psychomotor sensitization. On days 31 and 61, the vehicle/MDMA and 7-NI/MDMA groups ($n = 8$ each) were challenged with MDMA (10 mg/kg) (A), and the vehicle/PCA and 7-NI/PCA groups ($n = 8$ each) were challenged with PCA (5 mg/kg) (B). On day 31, the intensity of locomotion in the 7-NI/drug groups was significantly lower than in the vehicle/drug groups $P < 0.001$ (a), suggesting that the sensitized response to the drug was maintained only in the vehicle/drug groups. On day 61, ambulatory activity measured in the 7-NI/MDMA group was still less than the vehicle/MDMA group, $P < 0.01$ (a), but no significant difference was found between the vehicle/PCA and 7-NI/PCA groups. Comparison between the intensity of locomotion on days 31 and 61 (b) showed a significant reduction in locomotor activity both in the vehicle/MDMA group, $P < 0.001$ (A) and in the vehicle/PCA group, $P < 0.01$ (B).

$P < 0.0001$. Bonferroni post hoc test showed significant difference between the vehicle/MDMA and 7-NI/MDMA groups on day 31 ($t = 12.21$; $P < 0.001$) as well on day 61 ($t = 5.121$; $P < 0.001$), suggesting that the 7-NI/MDMA group remained desensitized to MDMA compared to the vehicle/MDMA group. On day 61, however, the response of the vehicle/MDMA group to MDMA was significantly lower than on day 31 ($t = 7.428$; $P < 0.001$), suggesting a reduced responsiveness to MDMA challenge after 2 months.

The results of PCA challenge on days 31 and 61 (Fig. 5B) were similar to the results of MDMA challenge (Fig. 5A). A two-way ANOVA yielded the following parameters: drug effect: $F[1, 28] = 23.38$, $P < 0.0001$; time effect: $F[1, 28] = 7.300$, $P = 0.0116$; and interaction $F[1, 28] = 7.970$, $P = 0.0087$. Bonferroni post hoc test showed a significant difference between the vehicle/PCA and 7-NI/PCA groups on day 31 only ($t = 5.415$; $P < 0.001$), suggesting that the 7-NI/PCA group remained desensitized to the effect of PCA. On day 61, no significant difference was found between the two groups because of the diminished response of the vehicle/PCA group to PCA (day 31 vs. day 61: $t = 3.907$; $P < 0.01$). The diminished effect of PCA on day 61 suggests that the sensitized response was no longer maintained in the vehicle/PCA group 2 months after initial drug treatment.

Discussion

The major finding of the present study is the differential effect of the nNOS inhibitor 7-NI on the induction and expression versus the preservation of psychomotor sensitization caused by MDMA and PCA. While 7-NI did not affect the induction and expression of psychomotor sensitization, the nNOS inhibitor prevented the maintenance of a sensitized response.

We have previously reported that pretreatment with the same dose of 7-NI (25 mg/kg) blocked the induction, expression, and maintenance of psychomotor sensitization caused by the indirect DA agonists, cocaine, METH [11], and methylphenidate [12]. Although METH is a major drug of abuse, METH and particularly

methylphenidate are used for the treatment of attention deficit hyperactive disorders (ADHD) due to their DA agonist effects [30].The objective of the present study was to investigate how the inhibition of nNOS affects the development of psychomotor sensitization to other substituted amphetamines (MDMA and PCA) that exert more serotonergic effects compared to methylphenidate, METH, and cocaine.

Evidence suggests that the behavioral effects of MDMA are governed by both serotonergic and dopaminergic transmission [31,32]. The contribution of 5-HT to the effects of MDMA in mice stems from the following findings: (a) The 5-$HT_{2A/2C}$ receptors antagonist, ketanserin, attenuated (±)MDMA-induced hyperlocomotion, hyperthermia, and lethality in Swiss mice [23]. (b) 5-HT_{1B} knockout mice were less sensitive to the psychomotor stimulating effects of MDMA compared to wild-type mice [33]. (c) High doses of MDMA caused the depletion of both DA and 5-HT in several brain regions of Swiss Webster mice, though the magnitude of dopaminergic neurotoxicity was greater than serotonergic [34–36]. PCA is known to induce the release of both 5-HT and DA [24–26]. In the present study, PCA (20 mg/kg) caused head and body weaving and occasional hind-limb extensions, symptoms that are consistent with PCA-induced serotonin syndrome [27]. Also, PCA-induced hyperthermia and serotonergic neurotoxicity in mice were attenuated by the 5-HT_2 receptor antagonist ritanserin [37]. Taken together, it is likely that the release of both 5-HT and DA contribute to the behavioral effects of MDMA and PCA in mice.

That repeated administration of MDMA and PCA caused sensitization to the psychomotor stimulating effects of the drugs is illustrated by the finding that 10 mg/kg MDMA and 5 mg/kg PCA induced a 3-fold higher locomotor activity on day 6 than on day 1 (Figs. 2A and B). Co-administration of 7-NI did not affect the intensity of MDMA- and PCA-induced locomotion either on day 1 or 6. These findings suggest that 7-NI did not block the *induction* of sensitization to MDMA and PCA. It is unlikely that a higher dose of 7-NI (>25 mg/kg) may have inhibited the induction of sensitization because a single administration of 25 mg/kg 7-NI to Swiss Webster mice inhibited about 80% of brain NOS activity [29]. This dose was sufficient to block the induction and expression of cocaine-, METH- [11], and methylphenidate-induced [12] psychomotor sensitization. Evidence suggesting that 7-NI did not affect the *expression* of MDMA and PCA sensitization stems from the finding that the co-administration of 7-NI and MDMA or PCA to vehicle/MDMA or vehicle/PCA mice, respectively, on day 24 did not affect the sensitized response to these drugs (Fig. 4).

The inhibition of nNOS, however, prevented the *maintenance* of the sensitized response. When mice were challenged with MDMA or PCA 10 days following termination of drug treatment, the 7-NI/MDMA, and the 7-NI/PCA mice did not show a sensitized response to MDMA and PCA challenges, respectively. The response of the 7-NI/MDMA and 7-NI/PCA mice to the challenge injections was not significantly different from control mice (vehicle/saline and 7-NI/saline) that received MDMA or PCA for the first time on day 16 (Figs. 2A and B). However, the mice that did not receive the 7-NI pretreatment were sensitized to the effects of MDMA and PCA on day 16 as they were on day 6. That the co-administration of 7-NI and the drug prevented the long-term maintenance of the sensitized response is evident from the results of challenge injections given on days 31 and 61 following the initial drug exposure (Fig. 5). The 7-NI/MDMA and 7-NI/PCA mice responded to challenge injections of the drug as control mice that received the drug for the *first time* on days 1 or 16. On day 31, the vehicle/MDMA and vehicle/PCA mice were still sensitized to the challenge injection of the drugs (Fig. 5). However, following another month (day 61), the sensitized response to challenge injection of the drug was reduced, suggesting that sensitization dissipated within a period of 2 months from the initial drug exposure.

We have recently shown the significance of the frequency of MDMA injections for the development of psychomotor sensitization to MDMA [36]. A single treatment with MDMA (10 mg/kg) was sufficient to induce psychomotor sensitization to a second MDMA injection only if the injections were administered 5 days apart. When subsequent injections were given 15–30 days apart, the sensitized response to MDMA was no longer apparent. In the present study, the repeated daily injections of vehicle/MDMA and 7-NI/MDMA caused the development of sensitization to MDMA. However, due to the pretreatment with 7-NI, the sensitized response dissipated after 10 days, and the repeated challenge injections given after 15 and 30 days could not reinstate sensitization. Likewise, PCA challenge given to the 7-NI/PCA group on days 31 and 61 could not reinstate sensitization (Fig. 5). This outcome is in accordance with the finding that infrequent PCA injections to Swiss Webster mice did not induce sensitization (Itzhak et al. submitted). Thus, the frequency of MDMA and PCA injections is critical for the induction of psychomotor sensitization.

Further differences between the response to MDMA and PCA versus cocaine, METH, and methylphenidate originate from the conditioned hyperlocomotion experiments. The environment in which the psychostimulant is administered has an important role in the development of a conditioned response. When the administration of a psychostimulant with rewarding properties is paired with a specific environment, this environment becomes a conditioned stimulus and animals respond with heightened locomotor activity upon receiving a

saline injection in this particular environment. This conditioned response is relevant to drug addiction and relapse [38,39]. We have shown that pairing cocaine, METH [11], and methylphenidate [12] injections twice with the same test cages that were used in the present study induced robust conditioned hyperlocomotion that was abolished by pretreatment with 7-NI. Likewise, nNOS (−/−) mice were resistant to cocaine-induced psychomotor sensitization and conditioned hyperlocomotion [13]. However, pairing MDMA and PCA injections with the test cage did not cause the development of conditioned hyperlocomotion, and 7-NI pretreatment did not affect the response to saline injection (Fig. 3). It is likely that the development of the conditioned response is primarily dependent on the incentive value of the drug rather than on the development of psychomotor sensitization per se. For instance, methylphenidate elicited the same rewarding effect in the conditioned place preference paradigm as cocaine [40]. In spite of the development of tolerance (instead of sensitization) to the psychomotor stimulating effect of methylphenidate, conditioned hyperlocomotion still emerged [12]. Thus, the finding that MDMA and PCA did not induce a conditioned response suggests that the incentive value of these drugs is low or absent in Swiss Webster mice.

Results of the present study provide indirect evidence for the specificity of DA/NO interactions with relevance to the effects of psychostimulants. We hypothesize that inhibition of nNOS attenuates psychostimulant effects which are dependent primarily on dopaminergic rather than serotonergic transmission. Besides the finding that inhibition of nNOS blocked the induction and expression of sensitization to the indirect DA agonists, inhibition of nNOS prevented the development of cocaine- [14], nicotine- [41], and alcohol-induced [42] conditioned place preference, a paradigm used to assess the rewarding properties of natural stimuli and drugs of abuse; DA has a major role in the rewarding effects of many abused substances [43]. Studies on dopaminergic neurotoxicity revealed that inhibition [15–17] and deletion [18] of nNOS blocked METH-induced dopaminergic neurotoxicity, as well as MPTP-induced dopaminergic neurotoxicity [44,45]. Although a few studies implicated the role of NO in DA release [4,6] the precise mechanism by which nNOS inhibition attenuates dopamine-mediated effects remains unclear.

Based on the findings described and the present results, we speculate that the development of psychomotor sensitization to MDMA and PCA involves both serotonergic and dopaminergic transmission. Serotonergic transmission may be more critical for the induction and expression of sensitization to MDMA and PCA because 7-NI did not affect these behaviors. However, the maintenance of the sensitized response to these drugs may be dependent primarily on dopaminergic transmission. Neural adaptations in the nucleus accumbens and prefrontal cortex that receive major dopaminergic inputs are thought to be critical for long-lasting sensitization to psychostimulants [46]. The finding that inhibition of nNOS prevented the maintenance of sensitization to MDMA and PCA, coupled with the findings that point to DA/NO interactions, suggest that the preservation of sensitization is primarily dependent on dopaminergic transmission. Thus, an intact nNOS system may be a prerequisite for the development of dopamine-dependent neural adaptation that leads to prolonged psychomotor sensitization.

In summary, the results of the present study support the role of nNOS in neural plasticity that is relevant for the maintenance of behavioral sensitization to psychostimulants.

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