

Comparative studies on behavioral neurochemical effects of *N*, α -diethylphenethylamine and methamphetamine in rats

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Abstract One objective was to compare the psychostimulating effects of *N*, α -diethylphenethylamine (NADEP) with those of methamphetamine (METH) in experiments on behavioral activities of rats. Another objective was to compare concentrations of neurotransmitters dopamine (DA), serotonin (5-HT), and their metabolites such as dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA) in brain tissues (striatum and frontal cortex) carried out after administration of NADEP and METH in rats. The animals were treated with NADEP and METH for 6 consecutive days (5 mg/kg per day, respectively), and the scores of stereotypy were measured on the first, second, fourth, and sixth day. The increase of the stereotypy score was observed in the NADEP-treated group, but it was less than that after treatment with METH. NADEP administration (5 and 10 mg/kg) resulted in a significant increase of DA and 5-HT and a decrease of DOPAC and HVA in the striatum tissues, which were collected 1 h after administration, but the changes of the compounds were less than those after treatment with METH (5 mg/kg). Unlike the METH-treated group, the changed DA, 5-HT, DOPAC, and HVA levels of the NADEP-treated group (5 and

10 mg/kg) were soon recovered within 6 h after administration. Both NADEP and METH had no significant effect on the 5-HIAA concentrations of the brain tissues. These results suggest that NADEP has significant psychostimulatory effects, though the effects were less than those of METH. Thus, NADEP should be carefully monitored to avoid abuse as a psychoactive drug.

Keywords *N*, α -Diethylphenethylamine · Methamphetamine · Stereotypy · Neurochemical effects · Pharmacological study · Dopamine

Introduction

Amphetamines (AMPs) exert their behavioral effects by modulating several key neurotransmitters in the brain, including dopamine (DA) and serotonin (5-HT) [1]. The major neural systems affected by AMPs are largely implicated in the brain's reward circuitry. Moreover, neurotransmitters involved in various reward pathways of the brain appear to be the primary targets of AMPs [2]. The fact that AMPs influence neurotransmitter activity specifically in regions implicated in reward, including the striatum, frontal cortex, the nucleus accumbens, and the ventral striatum, provides insight into the behavioral consequences of the drug, such as the stereotyped onset of euphoria [3]. The most widely studied neurotransmitter involved in AMP action is DA, the "reward neurotransmitter," that is highly active in numerous reward pathways of the brain. Various studies have shown that in the specific regions, AMPs increase the concentrations of DA in the synaptic cleft, thereby enhancing the response of the postsynaptic neuron [4, 5]. Effects on endocytosis, uptake inhibition, and monoamine oxidase inhibition are generally known as the

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mechanisms by which AMPs affect dopamine concentrations [6–8].

AMPs have also been found to exert similar effects on 5-HT. It has been reported that AMPs increase the post-synaptic concentrations of 5-HT by inhibition of uptake [9]. Another report suggested that AMPs may indirectly alter the behavior of glutamatergic pathways, which are strongly correlated with increased excitability at the level of the synapse, extending from the ventral tegmental to the frontal cortex [10]. The proposed ability of AMPs to increase the excitability of glutamatergic pathways was considered significant for 5-HT-mediated addiction [11].

N, α -Diethylphenethylamine (NADEP) is a β -phenethylamine derivative with a similar chemical structure to methamphetamine (METH). NADEP and its analogs were patented as psychoactive substances by Knoll Pharmaceuticals [12], and NADEP was also reported as a potential designer drug analog of amphetamines [13]. However, it was not used as a commercially available medicine or designer drug until recently. Apart from being used as a research chemical in laboratories, it first appeared in a powder seized during drug trafficking in South Korea in December 2011 [14]. Shortly after that case, NADEP was also found in the pre-workout supplement branded as “Craze” sold worldwide [15–17]. For its abuse potential, it had been classified as a controlled substance by the Misuse of Drugs Act of 1971 in the United Kingdom [18]. However, it is not yet regulated in many countries. Its analogs, *N,N*-dimethylamphetamine, *N*-ethylamphetamine, and α -ethylphenethylamine were reported to have stimulating properties, weaker than methamphetamine [19–22], but a systematic study on the neurophysiological effects of NADEP has not yet been reported.

Daily taking of a pre-workout supplement containing psychoactive ingredients could cause short-term and/or long-term neurotoxicity. Furthermore, not only chemical similarity, but also pharmacological properties should also be considered for regulation of the chemical as a controlled substance. Thus, in this study, we performed comparative *in vivo* experiments using rats on NADEP and METH, the most frequently abused AMP derivative.

Materials and methods

Chemicals

The authentic NADEP-HCl (93.3 % purity, racemic mixture) identified previously [14] was used for this study. METH-HCl (99.0 % purity, *d*-form) was selected from those seized by the police agency for drug trafficking, and was used for the present study. DA, DA-*d*₄, 5-HT,

dihydroxyphenylacetic acid (DOPAC), DOPAC-*d*₅, homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA), acetic acid, and perchloric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other common chemicals used were of the highest purity commercially available.

Animals

Male Sprague–Dawley rats (8 weeks), weighing 280–310 g, were housed in conditions of constant temperature (24 °C) and controlled light (12/12 h lighting), and given food and water *ad libitum*. The numbers of the rats used was five per group for stereotypy behavior tests, and six per group for the analysis of neurochemicals.

Drug administration

Drug dosage for systematic administration was calculated as mg of drug/kg of body weight. The drugs were injected intraperitoneally (*i.p.*), and the volume of injections was adjusted to 0.1 ml/100 g of body weight.

Stereotypy behavior test

METH or NADEP were injected *i.p.* into rats ($n = 5$) with a daily dosage of 5 mg/kg, and the same volume of saline was injected into another group ($n = 5$) as a negative control. The rats were returned to their home cage, where food and water were removed for 30 min following the injection. The resultant behavior was recorded in 10 min intervals for 2 h, using modifications of the rating scale suggested by Naylor and Olley in 1972 [23] as follows: 0, normal behavior (moving forward and grooming); 1, explorative behavior (forward locomotion, head movements, and rearing); 2, continuous sniffing; 3, small and compulsive head-neck movements; and 4, licking or biting the wires of the cage. Drugs and saline were injected everyday for 6 consecutive days at a constant time, and the tests were carried out on the first, second, fourth, and sixth day.

Brain sample preparation

Rats received injections of NADEP (5 or 10 mg/kg), METH (5 mg/kg), or saline *i.p.*, at 1 h or 6 h before brain tissue sampling. After decapitation, striatum and frontal cortex tissues were removed and weighed. Tissue samples were immediately flash frozen with liquid nitrogen, and stored at approximately -70 °C until analysis. The tissue samples were homogenized on wet ice with cold 0.1 % formic acid using a Kontes microgrinder, which contained internal standards (ISs) (for DA, 5-HT and 5-HIAA, DA-*d*₄; and for DOPAC and HVA, DOPAC-*d*₅) at a final

concentration of 10 ng/g, at a ratio of 1: 0.01 (mg: ml). Homogenized samples were centrifuged at 2,500 rpm for 10 min at 4 °C. The supernatant was then collected and filtered through a Millipore hydrophilic micro-filter (Billerica, MA, USA) for liquid chromatography–tandem mass spectrometry (LC–MS–MS) analysis.

Preparation of stock solutions and calibration standards

To make a blank matrix, the whole brain tissues were homogenized in distilled water, and the supernatant was collected after centrifugation. DA, 5-HT, and their metabolites are known to be unstable at room temperature, so the supernatant was kept at room temperature for 15 days before confirming by LC–MS–MS that none of them remained. Then, formic acid was added to the supernatant to prepare 0.1 % formic acid solution, which was used as a blank solution.

Solutions of analytes were prepared as a stock (1 mg/ml) with 0.1 % formic acid and then diluted. Standard solutions were prepared by spiking a prepared blank solution to the following amounts, but the added volumes were less than 10 % of the total volume. The final concentrations for the standard curve were 6, 12, 60, 100, 200, 300, 600, 1,080, and 1,200 ng/g.

LC–MS–MS analysis

An Agilent 1290 LC coupled to an MDS Sciex API 4000 Qtrap MS/MS system (AB SCIEX, Framingham, MA, USA) was used for the quantitative analysis of the neurochemicals. Chromatographic separation was carried out using a Capcell Core PFP (100 × 2.1 mm i.d., particle

size 2.7 μm) column purchased from Shiseido (Tokyo, Japan). The analytes were separated using a binary gradient system with 1 % (v/v) acetic acid in water (A) and 1 % (v/v) acetic acid in acetonitrile (B). The initial condition was 100 % A, and the equilibrium time was 10 min. The gradient profile started at 100 % A, which was decreased linearly to 70 % A until 2.6 min, and then decreased to 5 % A at 2.7 min, where it was held for another 0.6 min. Eluent A was then increased to 100 % at 4.31 min, where it was held for another 6.7 min to re-equilibrate the system. The flow rate of the mobile phase was set at 0.3 ml/min, and the column temperature was maintained at 30 °C. The mass spectrometer was operated in electrospray ionization (ESI) positive ion mode for DA, 5-HT, and 5-HIAA and in negative ion mode for DOPAC and HVA. Other conditions were: curtain gas, 20 psi; collisionally activated dissociation, medium; heated nebulizer temperature, 400 °C; nebulizing gas, 50 psi; and heater gas, 50 psi. Detection of ions was performed in multiple reaction monitoring (MRM) mode with two transitions for each analyte. MRM transitions, retention times, and conditions are shown in Table 1. Data acquisition and quantification were performed using Analyst Software version 1.6 (AB SCIEX).

Results and discussion

Method validation

The calibration curve was linear at 6–1200 ng/g for DA ($r^2 = 0.998$, $y = -0.635 + 0.166x$), 5-HT ($r^2 = 0.998$, $y = 3.71 + 0.217x$), DOPAC ($r^2 = 0.995$, $y = 0.823$

Table 1 Multiple reaction monitoring transitions, retention times, and conditions of each analyte and the internal standards

	Precursor ion (<i>m/z</i>)	Product ion (<i>m/z</i>)	RT (min)	DP (V)	EP (V)	CE (V)	CXP (V)
DA	154	<i>137</i>	1.85	46	10	15	10
		91		46	10	35	6
5-HT	177	<i>160</i>	3.09	56	10	15	12
		115		56	10	37	8
DOPAC	167	<i>123</i>	2.79	−40	−10	−12	−7
		95		−55	−10	−28	−5
HVA	181	<i>136</i>	3.36	−55	−10	−10	−9
		122		−55	−10	−22	−1
5-HIAA	192	<i>146</i>	3.26	76	10	21	12
		91		76	10	49	6
DA- <i>d</i> ₄	158	<i>141</i>	1.83	41	10	15	10
		95		41	10	35	6
DOPAC- <i>d</i> ₅	172	<i>128</i>	2.77	−60	−10	−10	−7
		127		−60	−10	−30	−9

Italic values indicate the transitions that were used for quantification
RT retention time, *DP* declustering potential, *EP* entrance potential, *CE* collision energy, *CXP* collision cell exit potential, *DA* dopamine, *5-HT* serotonin, *DOPAC* dihydroxyphenylacetic acid, *HVA* homovanillic acid, *5-HIAA* 5-hydroxyindoleacetic acid

Table 2 Precision, accuracy, limits of detection (LOD), and limits of quantification (LOQ) at low (6 ng/g) and high (1,200 ng/g) concentrations of each analyte

Analyte concentration (ng/g)	Precision (% RSD)	Accuracy (% RE)	LOD (ng/g)	LOQ (ng/g)
DA	6	7.5	0.3	1
	1,200	6.2		
5-HT	6	8.3	0.3	1
	1,200	2.2		
DOPAC	6	7.6	0.6	2
	1,200	5.1		
HVA	6	11.9	0.6	2
	1,200	6.5		
5-HIAA	6	6.4	0.6	2
	1,200	4.8		

RSD relative standard deviation, RE relative error

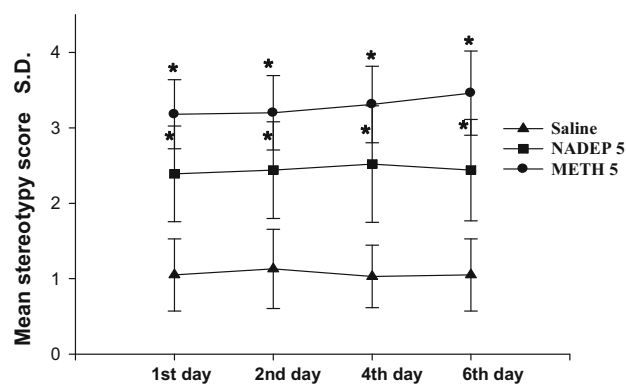
Table 3 Matrix effects for each analyte and internal standard ($n = 5$ for each test)

Analyte	Matrix effect (%)			
	Low (6 ng/g)		High (1,200 ng/g)	
	Mean	SD	Mean	SD
DA	108	4.2	106	3.5
5-HT	112	5.3	107	4.1
DOPAC	107	5.1	118	2.1
HVA	114	4.3	105	3.8
5-HIAA	108	3.9	104	6.2
DA- d_4	89	6.1	97	2.1
DOPAC- d_5	96	3.3	91	2.7

SD standard deviation

+ 0.264x), HVA ($r^2 = 0.997$, $y = -0.152 + 0.0242x$), and 5-HIAA ($r^2 = 0.998$, $y = -0.675 + 0.0395x$). Precision and accuracy were determined by replicated analysis ($n = 5$) of blank matrices spiked with low (6 ng/g) and high (1,200 ng/g) concentrations of each analyte. Limit of detection (LOD) and limit of quantification (LOQ) were determined using assays of the blank matrix. The value of LOD was the concentration that gives a signal-to-noise ratio = 3. The LOQ can be defined as the lowest concentration that gives a signal-to-noise ratio = 10 with precision not greater than 15 %. Matrix effect was determined by comparing the absolute peak area for each analyte reference standard with that of the reference standard spiked into the blank matrix just before instrumental analysis at low and high concentrations ($n = 5$).

The precisions at low and high concentrations of the analytes were in the range of 2.2–11.9 %, while the accuracies in terms of their bias ranged from –8.1 % to

**Fig. 1** Effects of *N*, α -diethylphenethylamine (NADEP) and methamphetamine (METH) on rat stereotypy, measured on the first, second, fourth, and sixth day ($n = 5$ for each test). The data represents the mean stereotypy score of the group \pm standard deviation (SD), as scored by the modified method of Naylor and Olley [23]. NADEP 5 or METH 5 indicates the intraperitoneal administration of 5 mg drug/kg of rat body weight. The values marked with “asterisk” indicate significant differences from “saline” values (as determined by One-way ANOVA with Holm-Sidak post hoc test, $P < 0.05$)

5.6 %. The LODs were 0.3 ng/g for DA and 5-HT and 0.6 ng/g for DOPAC, HVA, and 5-HIAA. The obtained LOQs were 1 ng/g for DA and 5-HT and 2 ng/g for DOPAC, HVA, and 5-HIAA (Table 2).

The mean matrix effect values of the DA, 5-HT, DOPAC, HVA, and 5-HIAA were 107–114 % and 104–118 % at low and high concentrations, respectively, and those of ISs were 89 % and 96 %, and 97 % and 91 % at low and high concentrations, respectively (Table 3).

Behavioral effects of *N*, α -diethylphenethylamine or methamphetamine in rats

Tests of the spontaneous motor activity of rats and mice are widely used in the initial stages of evaluating psychotropic drugs [24]. In rats, an acute injection of AMPs initially produces an increase in the incidence of forward locomotion, head movements, sniffing and rearing (hyperactivity), and a concomitant decrease in the incidence of other behaviors, such as grooming. With higher doses, the initial hyperactivity is soon followed by stereotyped behavior. During the stereotypy phase, the rat assumes a crouched posture and engages in continuous or nearly continuous repetitive head-neck movements, sniffing, licking, or biting. The intensity and duration of the focused stereotyped behavior increase with increasing doses of AMPs [25–27].

In spite of administration of equal doses, the stereotypy scores of the NADEP rats were within 2 and 3, while the METH rats were within 3 and 4. However, the scores of rats treated with NADEP or METH were significantly higher than the saline control score. We assumed that stress

Table 4 Effects of *N,α*-diethylphenethylamine (NADEP) or methamphetamine (METH) administration on concentrations of monoamines and their metabolites in the striatum and the frontal cortex samples of rats collected 1 h and 6 h after administration (*n* = 6 animals for each group)

Sample	Analyte	Administered compound	% of saline treatment				
			1 h after administration		6 h after administration		
			Mean	SD	Mean	SD	
Striatum	DA	NADEP 5	131 ^a	6.9	100	4.6	
		NADEP 10	149 ^a	8.7	100	6.3	
		METH 5	177 ^a	5.4	118 ^a	5.1	
	5-HT	NADEP 5	111 ^a	13.3	103	10.5	
		NADEP 10	125 ^a	14.0	101	9.6	
		METH 5	146 ^a	13.3	108	12.6	
	DOPAC	NADEP 5	91.2	11.6	99.5	6.6	
		NADEP 10	84.9 ^a	6.1	98.8	9.7	
		METH 5	67.8 ^a	5.7	85.7 ^a	7.5	
	HVA	NADEP 5	80.1 ^a	8.7	98.9	5.8	
		NADEP 10	71.3 ^a	7.3	98.0	3.9	
		METH 5	63.4 ^a	8.6	82.2 ^a	5.1	
	5-HIAA	NADEP 5	97.0	5.5	99.9	6.6	
		NADEP 10	95.2	4.7	100	8.1	
		METH 5	93.9	4.4	100	6.3	
	Frontal cortex	DA	NADEP 5	122 ^a	12.0	100	11.6
			NADEP 10	137 ^a	9.9	99.8	8.4
			METH 5	182 ^a	15.1	123 ^a	9.9
5-HT		NADEP 5	108	8.4	101	5.8	
		NADEP 10	117 ^a	5.5	101	6.6	
		METH 5	127 ^a	6.8	117 ^a	6.3	
DOPAC		NADEP 5	95.9	6.2	99.8	8.2	
		NADEP 10	86.0 ^a	8.1	99.3	8.1	
		METH 5	81.0 ^a	7.7	83.6 ^a	8.8	
HVA		NADEP 5	93.1	5.9	101	10.8	
		NADEP 10	87.3 ^a	6.8	99.4	12.1	
		METH 5	77.3 ^a	8.0	88.7 ^a	9.2	
5-HIAA		NADEP 5	96.0	4.6	100	5.6	
		NADEP 10	95.1	6.5	98.6	6.2	
		METH 5	97.1	7.0	97.2	4.7	

NADEP 5 or METH 5 indicates that 5 mg of drug/kg of rat body weight was administered intraperitoneally; NADEP 10 indicates the administration of 10 mg/kg of NADEP

^a Significant difference from “saline” values ($P < 0.05$; One-way ANOVA with Holm-Sidak post hoc test)

received during i.p. injections of saline, or difficulty of the experimenter in distinguishing the difference between “normal behavior” and “explorative behavior” led to the stereotypy score of approximately “1” for the saline treated group. NADEP at 5 mg/kg and METH at 5 mg/kg were administered daily for 6 days, but differences in behavior between days were not observed (Fig. 1).

Effects of *N,α*-diethylphenethylamine or methamphetamine administration on the levels of monoamines and their metabolites in brain regions of rats

DA and 5-HT levels increased both in the striatum and in the frontal cortex 1 h after the administration of NADEP

and METH. In contrast, DOPAC and HVA levels decreased after the administration (Table 4). When 5 mg/kg of NADEP was administered, DA and 5-HT levels in the striatum increased up to 131 % and 111 % of those observed after saline administration, respectively. When 10 mg/kg NADEP was administered, the mean DA and 5-HT levels were 149 % and 125 % of those after saline administration, respectively. Decreases of DOPAC and HVA showed patterns contrary to the levels of DA and 5-HT. These results indicated that NADEP has a weaker, but similar psychoactivity as compared with METH.

Six hours after the administration of 5 mg/kg METH, the DA and 5-HT levels were still increased, and DOPAC and HVA levels decreased. However, the monoamine levels 6 h after NADEP administration showed little

differences as compared to the control group administered with saline. This means that psychoactivity induced by NADEP administration lasts for a shorter time than that of METH administration.

Abuse potential of *N*, α -diethylphenethylamine

AMPs reliably produce focused stereotypy behaviors (hypolocomotion accompanied by intense sniffing/licking/biting of a restricted area of the environment) in rats, which are mediated by increased concentrations of DA in the striatum [26, 28]. As mentioned earlier, both vesicular and newly synthesized DA and 5-HT appear to be released by AMPs into the striatal extracellular compartment. AMPs also decrease DA oxidative metabolism by inhibiting monoamine oxidase (MAO), thereby inhibiting depletion of its main substrate, the newly synthesized DA, which has been suggested as an additional mechanism [6–8].

According to the results of our experiments, both the behavioral and neurochemical effects of NADEP were similar to METH, the strongest of AMPs, though the effects of NADEP were weaker and lasted only for a short time (Table 4). However, considering that *d*-form AMPs shows much stronger stimulant effects than the *l*-form, pure *d*-NADEP may have stronger psychoactivity than the racemic mixture of NADEP used in this study.

In vivo tests using rats cannot completely represent the human tests because of the biological differences between the species. However, in vitro tests cannot represent the whole complexity of human beings; in vivo tests may be a more suitable method.

Although there has been no clinical study in humans yet, some users reviewed that “CRAZE™” has several psychostimulatory effects [29]. Other phenethylamines of dendrobium extract in the supplements labelled as “CRAZE” could also have caused the stimulatory side effect [15]. However, results of this study strongly suggest that NADEP may have contributed to the psychostimulant effect of the pre-workout supplement.

Conclusions

In the present study, we found that NADEP clearly has weaker and shorter lasting effects, but that it has similar behavioral and neurochemical effects to those of its structural analog, METH. NADEP caused focused stereotypy behavior, increased DA and 5-HT levels, and decreased DOPAC and HVA levels in the striatum and frontal cortex of rats. However, NADEP’s stereotypy score, increase in DA and 5-HT levels, and decrease in DOPAC and HVA levels were all smaller than those of METH. This means that NADEP is not as potent as METH, but it still

can be abused as an alternative for METH. Thus, it should be regulated as a controlled substance to avoid inappropriate abuse.

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Conflict of interest There is no financial or other relationship that could lead to a conflict of interest.

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