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ORIGINAL ARTICLE

Changes in dopamine, serotonin and their metabolites in brain microdialysates from rats following exposure to new psychoactive drugs

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Abstract New psychoactive drugs (NPDs), or so-called "designer drugs" are chemically transformed compounds of traditional drugs of abuse for the purpose of evading crackdown. The abuse of NPDs is a significant social problem and threatens public health; however, few studies on their effects on the central nervous system have been conducted. Microdialysis is a useful in vivo sampling technique in neurochemistry because it enables monitoring of synaptic release of neurotransmitters by drug exposure or other stimuli in real time. Dopamine (DA) and serotonin (5-HT) are important neurotransmitters associated with drug abuse and addiction. In this study, changes of DA, 5-HT and their metabolites in brain microdialysates from rats following exposure to selected 11 NPDs (MPA, 5-APDB, PCA, α-PVT, AB-PINACA, QUPIC, 5-fluoropentyl-3-pyridinoylindole, AMT, NMT, 4-OH-DET and desoxy-D2PM, 0.3, 1 and 3 mg/kg, consecutively, intraperitoneally) were investigated using a validated liquid chromatography –tandem mass spectrometry method. Most NPDs affected the extracellular levels of DA, 5-HT and/or their metabolites, showing consistent changes depending on the groups of chemical structures, such as amphetamines, synthetic cannabinoids and tryptamines. Significant DA and/or 5-HT increases were observed for all the amphetamine analogues. Weak fluctuations of DA and/or 5-HT concentrations were observed following exposure to synthetic cannabinoids and more severe fluctuations were shown by the tryptamines. The current results could be used as the preliminary data for further research concerning monoamine neurotransmitter-related mechanisms of NPDs. Moreover, the understanding gained from this research could be helpful to monitor the liability of NPD abuse and addiction.

Keywords New psychoactive drugs · Synthetic cannabinoids · Dopamine · Serotonin · Microdialysis · LC–MS/MS

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Introduction

New psychoactive drugs (NPDs), or so-called "designer drugs", are chemically transformed compounds of traditional drugs of abuse for the purpose of evading crackdown. These compounds are distributed under the names of 'research chemicals', 'bath salts' and 'plant food' etc. and have been mainly dealt on the Internet markets; thus, the NPDs also get the nickname of 'internet drugs' [1]. The NPDs not only produce similar psychoactive effects as existing drugs of abuse but also could be used in less invasive manners (e.g., smoking, insufflating or ingesting orally rather than injecting). Recently, more and more NPDs have rapidly appeared and spread globally. According to the European Union (EU) Early Warning

System (EWS), 418 NPDs appeared during 2005–2014, and in particular, in 2014, 101 NPDs were notified. In addition, between 2008 and 2013, the quantity of confiscated NPDs increased sevenfold [2].

Microdialysis is a useful sampling technique of an in vivo experiment concerning the neurochemical effects of drug exposure and other stimuli, by collecting extracellular fluid in the brain [3–5]. This approach enables monitoring of the synaptic release of neurotransmitters, such as catecholamines, amino acid neurotransmitters, and acetylcholine, in real time in different brain parts in awake, freely-moving animals and to determine the changes of these neurotransmitters during a prolonged sampling time (e.g., up to several days) [6, 7]. However, the limited sample size (20–30 μL), analytical interference, such as inorganic salts in fluid, and low basal concentrations (picomolar range) of neurotransmitters, demand sophisticated analytical methods with high sensitivity and selectivity [8–10].

The neurotransmitters are related to various neuropsychiatric symptoms, such as anxiety, affective regulation, learning ability, pain, regulation of body temperature and so on [11, 12]. Dopamine (DA) and serotonin (5-hydroxytryptamine, 5-HT) are important neurotransmitters in both the central nervous system (CNS) and the peripheral nervous system. The imbalance of DA and 5-HT contributes to neuropsychiatric disorders, such as Parkinson's disease, epilepsy, Alzheimer's disease, depression, stress, schizophrenia and drug addiction [11, 13, 14]. First-generation NPDs stimulate or inhibit the dopaminergic and/or serotonergic system. For instance, 3,4-methylenedioxymethamphetamine (MDMA), also known as ecstasy, produces not only a psychostimulant effect but also an empathogenic effect [15] and clearly increases 5-HT and DA levels in areas of rat brain, such as the hippocampus and the caudate-putamen [16]. 4-Methylmethcathinone (mephedrone) belongs to the group, cathinones, as its name suggests, and it is known to produce higher addictive effects than cocaine, based on user experience [1]. When mephedrone was injected at 1 and 3 mg/kg to rats, DA and 5-HT levels significantly increased in the nucleus accumbens [17].

Even though a lot of NPDs has been appearing quickly, previous studies on the dopaminergic and serotonergic effects of the NPDs were conducted for only a limited numbers of drugs. In this study, changes in DA, 5-HT and their metabolites (Fig. 1) in brain microdialysates from rats following exposure to 11 selected NPDs (Table 1), the candidates for legislation to Narcotics Control Law in Korea in 2015, were simultaneously investigated using sensitive and selective liquid chromatography–tandem mass spectrometry (LC–MS/MS) developed and fully validated in our previous study [18].

Materials and methods

Reagents and drugs

DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), DA-d_4, DOPAC-d_5, HVA-d_5, 5-HIAA-d_5 and ascorbic acid were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Also, to prepare the artificial cerebrospinal fluid (aCSF), 1.0-M phosphoric acid solution, sodium phosphate dibasic, magnesium chloride hexahydrate, calcium chloride dehydrate, potassium chloride and sodium chloride were also purchased from Sigma-Aldrich. 5-HT-d_4 was purchased from TLC PharmaChem (Vaughan, Ontario, Canada). Saline, Tween 80 and dimethyl sulfoxide (DMSO) were purchased from JW Pharmaceutical (Seoul, Republic of Korea), Sigma-Aldrich and PanReac AppliChem (Darmstadt, Germany), respectively, for the preparation of the NPD solutions and vehicles of microdialysis. Methanol was of LC grade and purchased from Fisher Scientific (Leics, UK). All NPDs were synthesized and provided from Kyunghee University (Seoul, Republic of Korea).

Preparation of standards

The aCSF (pH 7.4) consisted of 0.8-mM magnesium chloride hexahydrate, 1.4-mM calcium chloride dehydrate, 3.0-mM potassium chloride and 150-mM sodium chloride in 10-mM phosphate buffer. All analytical stock solutions (1 mg/mL) were prepared in 1-mM ascorbic acid in water and methanol (1:1, v/v) and stored at −80 °C. A working mixture standard solution of DA, 5-HT, DOPAC, HVA and 5-HIAA (10 μg/mL for each) and a working mixture internal standard solution (DA-d_4 30 ng/mL; 5-HT-d_4 20 ng/mL; DOPAC-d_5 5 μg/mL; HVA-d_5 800 ng/mL; 5-HIAA-d_5 500 ng/mL) were prepared in aCSF from stock solutions, immediately before analysis.

Animals

Male Sprague–Dawely (SD) rats (Daehan Animal, Seoul, Republic of Korea) weighing 270–320 g were used for the animal study. The rats were kept in the laboratory animal facility with a 12 h light/dark cycle. Food and water were freely available.

Microdialysis

Microdialysis was conducted according to the previous study with minor modification [19]. Rats were anesthetized by sodium pentobarbital [50 mg/kg, intraperitoneally (i.p.)] and then microdialysis probe guide cannula (CMA 11;

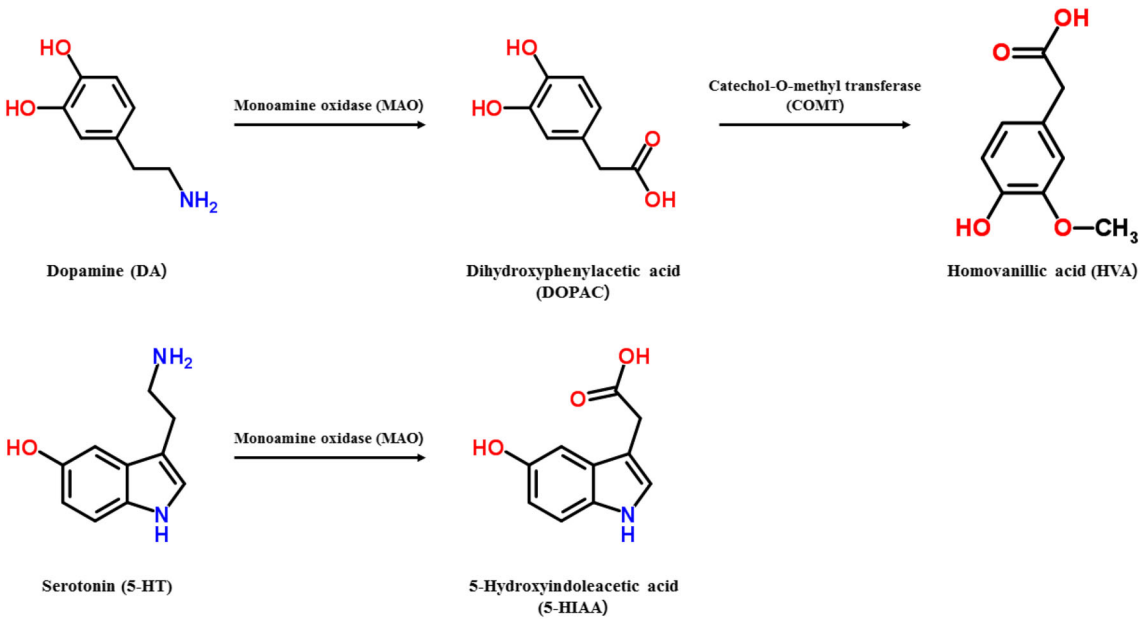


Fig. 1 Main metabolism pathways of dopamine (DA) and serotonin (5-HT)

CMA Microdialysis AB, Kista, Sweden) were stereotaxically implanted into the rats' brains. The rats were allowed to recover from surgery for 6 days. In each case, a microdialysis probe (membrane length, 2 mm; cut-off, 6 kDa; CMA Microdialysis AB) was inserted into the nucleus accumbens shell (AP + 1.7 mm, ML + 0.8 mm, from bregma; DV-6.0 mm, from skull) through the guide cannula of unanesthetized rats and the aCSF was perfused at a rate of 1.5 µl/min (CMA 100, microinjection pump) at least during 2 h for stabilization. Six baseline samples were collected following microdialysates every 20 min for 2 h. Then, the NPDs were administered by intraperitoneal injection every hour with increasing doses (0.3, 1 and 3 mg/kg) gradually and microdialysates were collected at 20-min intervals. α-PVT, 5-APDB, MPA, PCA, 4-HO-DET, AMT, NMT and desoxy-D2PM were dissolved in saline, and 5-fluoropentyl-3-pyridinoylindole, AB-PINACA and QUPIC were prepared in a mixture solution of DMSO/Tween 80/saline (5:5:90, v/v/v). The schedule for microdialysate collection from rats during the administration of vehicle or NPDs is shown in Fig. 2. To confirm the location of the microdialysis probe, rats were sacrificed and brains were prepared for histological verification on completion of the microdialysis experiment.

Sample preparation

Microdialysates (25 µL each) collected from rats were mixed with 5 µL each of the internal standard solution. The calibrators were prepared with 25 µL of aCSF including each analyte and mixed with 5 µL of internal standard solution. The sample preparation was conducted on ice bath to prevent degradation.

LC–MS/MS analysis

The analysis of microdialysates was conducted using a fully validated LC–MS/MS, as described in our previous study using a 1260 Infinity LC system and 6460 triple quadrupole MS/MS (Agilent Technologies, Santa Clara, CA, USA) coupled with a 1260 Infinity extra binary pump and degasser (Agilent Technologies) [18]. Separation was conducted with the Atlantis T3 column (100 × 2.1 mm i.d., particle size 3 µm; Waters, Milford, MA, USA) after on-line sample enrichment with the XBridge BEH HILIC Sentry Guard Cartridge 130 Å (20 × 4.6 mm i.d., particle size 3.5 µm; Waters). The mobile phases (A 5-mM ammonium formate/0.1 % formic acid in water; B 0.1 % formic acid in acetonitrile) were flowed through both of the enrichment and separation columns by gradient condition as follows: 0–1.0 min, 5 % B; 1.0–6.5 min, 5–90 % B; 6.5–7.5 min, 90 % B; 7.5–7.6 min, 90–5 % B; 7.6–11.5 min, 5 % B.

The MS/MS system was operated using the multiple reaction monitoring (MRM) mode (Table 2) and polarity-switching electrospray ionization (ESI). The MS/MS conditions were optimized as follows: drying gas temperature, 350 °C; drying gas flow, 10 L/min; nebulization pressure, 35 psi; capillary voltage, 4.5 kV; temperature of sheath gas, 250 °C; and sheath gas flow, 5 L/min. The limits of quantification for DA, 5-HT, DOPAC, HVA and 5-HIAA were 0.1, 0.025, 2.5, 25 and 0.5 ng/mL, respectively [18].

Table 1 Selected new psychoactive drugs tested in the present study

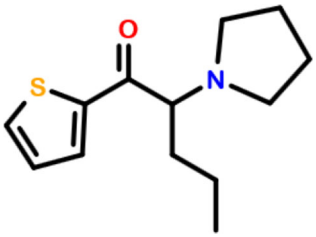
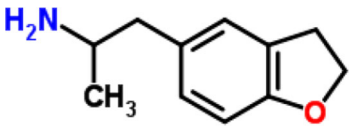
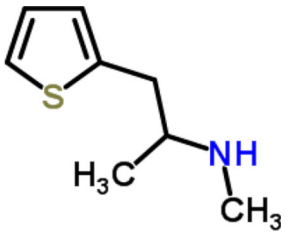
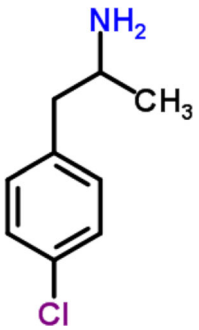
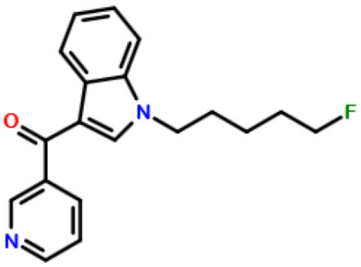
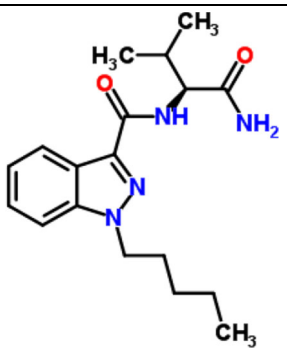
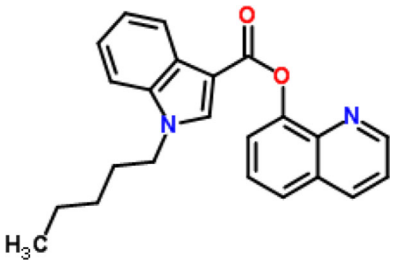
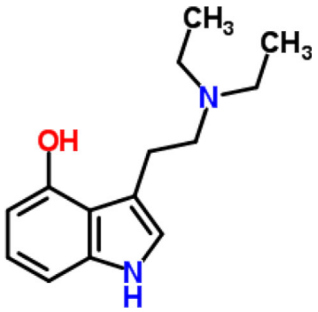
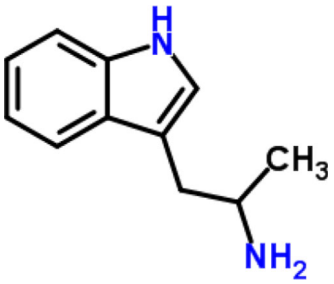
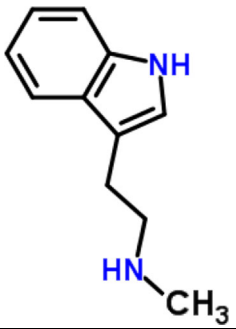
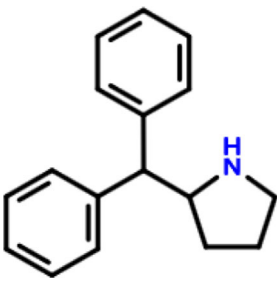
Class	Compound name [formal name]	Chemical structure
Amphetamines (extended)	α-PVT [α-pyrrolidinopentiothiophenone]	
	5-APDB [5-(2-aminopropyl)-2,3-dihydrobenzofuran]	
	MPA [methiopropamine]	
	PCA [*p*-chloroamphetamine]	
Synthetic cannabinoids	5-Fluoropentyl-3-pyridinoylindole	
	AB-PINACA [(*S*)-*N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-pentyl-1*H*-indazole-3-carboxamide]	

Table 1 continued

	PB-22 or QUPIC [quinolin-8-yl 1-pentyl-1*H*-indole-3-carboxylate]	
Tryptamines	4-HO-DET or CZ-74 [4-hydroxy-diethyltryptamine]	
	AMT [α--methyltryptamine]	
	NMT [*N*-methyltryptamine]	
Etc.	Desoxy-D2PM [2-diphenylmethylpyrrolidine]	

Chemical structures were originated from Chemspider (http://www.chemspider.com/)

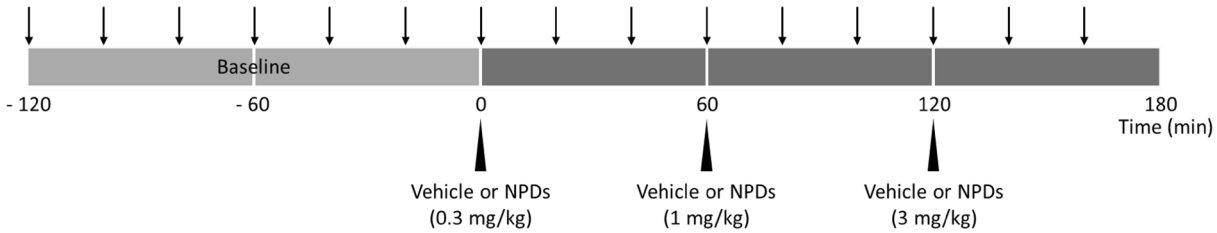


Fig. 2 Time points (↓) starting microdialysate collection from rats during the administration of vehicle or new psychoactive drugs (NPDs)

Table 2 Multiple reaction monitoring (MRM) transitions, retention times and other conditions for each analyte and each internal standard

Compound	MRM transition, *m/z* (CE, V)		tR (min)	Ionization polarity
	Quantifier	Qualifier		
5-HT	177.1 → 160 (9)	177.1 → 115 (32)	3.7	+ESI
DA	154.1 → 137.1 (8)	154.1 → 90.9 (26)	2.7	+ESI
5-HIAA	192.1 → 146.1 (16)	192.1 → 91.2 (42)	5.2	+ESI
HVA	180.9 → 136.9 (2)	–	5.5	−ESI
DOPAC	166.9 → 123.1 (8)	–	4.9	−ESI
5-HT-d_4	181 → 164.1 (8)	–	3.7	+ESI
DA-d_4	157.9 → 141 (6)	–	2.7	+ESI
5-HIAA-d_5	197 → 149.9 (14)	–	5.2	+ESI
HVA-d_5	186.2 → 142.1 (3)	–	5.5	−ESI
DOPAC-d_5	172.7 → 128.8 (8)	–	4.9	−ESI

CE collision energy, *tR* retention time, *ESI* electrospray ionization

+ positive ionization mode, − negative ionization mode

Data processing and statistical analysis

Analytical data was processed using the MassHunter software (B. 04. 00, Agilent Technologies). The baseline values were defined as those with less than 15 % of the coefficient of variation of 3 consecutive quantitative results before the administration of drugs or vehicle. The changes in the levels of DA, 5-HT and their metabolites were expressed as a percentage of their concentration in a microdialysate obtained from each rat administered with a drug against the baseline value, adjusting with those of vehicles at the same time points. Statistical evaluation was performed by one-way analysis of variance (ANOVA) for repeated measures followed by Bonferroni post hoc testing.

Results and discussion

α-PVT, 5-APDB, MPA and PCA belong to a large family of amphetamine compounds. It was previously reported that both traditional (e.g., amphetamine, methamphetamine etc.) [20] and novel (e.g., camfeamine, methylphenyl-amphetamines, MPA, aminopropylbenzofurans, etc.) amphetamine derivatives [21] provoke the release and reuptake inhibition of DA and/or 5-HT to varying degrees. There have been no studies on the effects of α-PVT, 5-APDB and MPA on changes in DA, 5-HT and their metabolites using microdialysis. α-PVT is one of the newly identified synthetic cathinones with a pyrrolidine ring and only very little information is available regarding its effects on the changes in neurotransmitter release. Our results demonstrated that α-PVT exposure markedly increased the level of DA (Fig. 3a). Kaizaki et al. [22] reported that 25 mg/kg oral ingestion of α-pyrrolidinovalerophenone (α-PVP), one of the pyrrolidinophenones, significantly increased the extracellular level of DA in the striatum of mice. The authors concluded that the rapid increase in DA concentration would be mediated by the stimulation of DA_1 and DA_2 receptors. Recently, it was also suggested that two pyrrolidinophenones, α-PVP and 3,4-methylenedioxypyrovalerone (MDPV) are potent dopamine transporter (DAT) reuptake inhibitors, which were produced by a longer alkyl chain length on the α-carbon [23, 24]. Accordingly, it is presumed that a DA increase in the present study occurs following exposure to α-PVT, the pyrrolidinophenone with the same alkyl chain length as α-PVP and MDPV, due to a strong inhibition of DAT.

5-APDB was originally synthesized for research purposes to study the neurochemical effects of analogues of 3,4-methylenedioxymethamphetamine (MDMA) [25]. In the current study, the exposure of 5-APDB provoked the

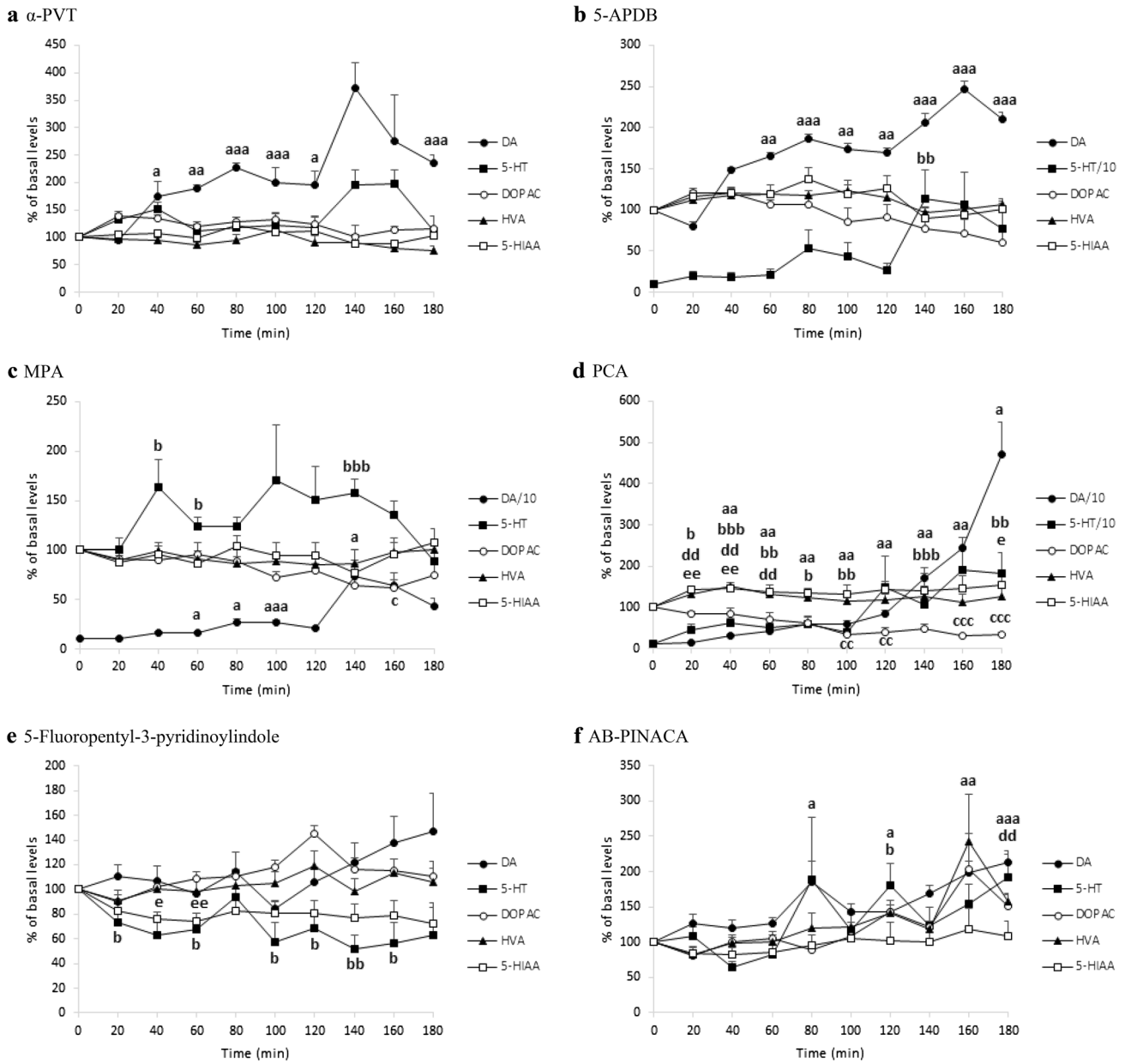


Fig. 3 Changes in the levels of DA, 5-HT and their metabolites in microdialysates collected from rats following exposure to NPDs. The *vertical bar* represents standard error of the mean obtained from four to six animal experiments. DA, $^{a}P < 0.1$, $^{aa}P < 0.05$ or $^{aaa}P < 0.01$; 5-HT, $^{b}P < 0.1$, $^{bb}P < 0.05$ or $^{bbb}P < 0.01$; DOPAC, $^{c}P < 0.1$, $^{cc}P < 0.05$ or $^{ccc}P < 0.01$; HVA, $^{d}P < 0.1$, $^{dd}P < 0.05$ or $^{ddd}P < 0.01$; 5-HIAA, $^{e}P < 0.1$ or $^{ee}P < 0.05$

significant increase of both DA and 5-HT concentrations. In particular, the DA level tended to increase in proportion to the dose of 5-APDB (Fig. 3b). 5-APDB is one of the dihydrobenzofuran analogues of 3,4-methylenedioxyamphetamine (MDA). A previous study reported that 5-APDB did not significantly affect DAT while it inhibited the serotonin transporter (SERT) more potently than both MDMA and MDA in HEK 293 cells expressing the transporters, and the DAT/SERT inhibition ratio of 5-APDB was even lower than those of MDMA and MDA. In addition, 5-APDB induced both DA and 5-HT release following its exposure (100 μM) in HEK 293 cells [26]. Another previous study reported that 5-APDB was more selective to the 5-HT reuptake carrier than other neurotransmitters, such as DA [25]. In our results, the increase in 5-HT was more considerable than that of DA; however, severe variations were observed.

MPA was first synthesized by Blicke et al. for research purposes in 1942 and it started to appear as a "legal high" on the Internet from 2010 [21]. It was reported that MPA

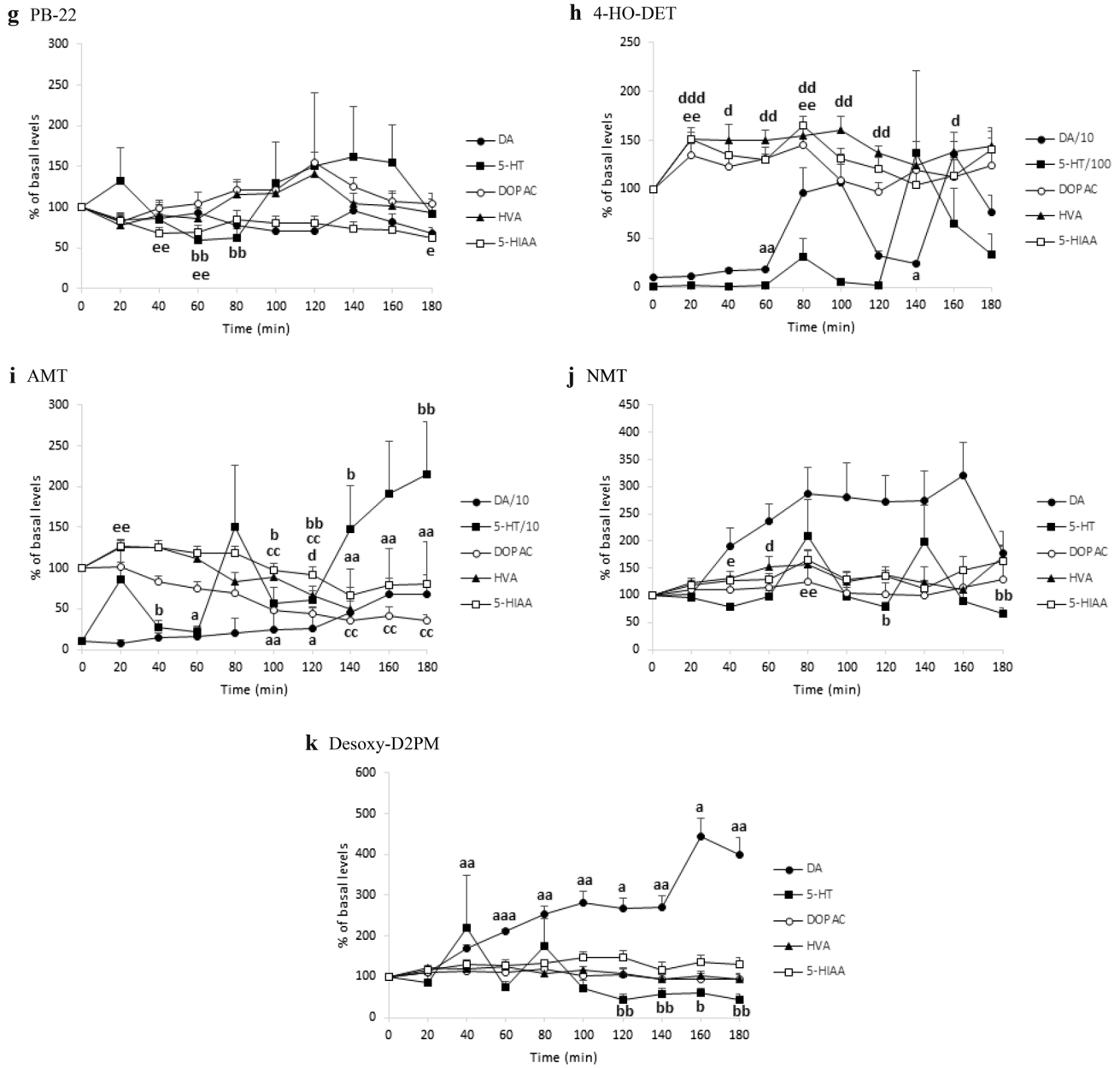


Fig. 3 continued

acted as an inhibitor of DAT but that it was not as potent as amphetamine [27]. Based on this knowledge, it was presumed that MPA could prevent DA reuptake and increase the extracellular level. As expected, our results showed that MPA increased DA and 5-HT levels with serious variation among animals; however, the concentrations of their metabolites were not significantly changed by exposure to MPA. (Fig. 3c).

The administration of PCA provoked significant alterations in the concentrations of DA, 5-HT and their metabolites. The levels of DA and 5-HT markedly increased in a dose-dependent manner, while DOPAC, one metabolite of DA, significantly decreased, and HVA, another metabolite, slightly increased. The metabolite of 5-HT, 5-HIAA, also show a tendency of increase following exposure to PCA (Fig. 3d). Many studies regarding the effects of PCA on the central changes of monoamines and/or their metabolites were conducted by other research groups but their results were inconsistent. A previous study reported that PCA behaved as a potent inhibitor of DA and 5-HT uptake in whole-brain synaptosomes from male SD rats and showed significant changes in the concentration of DA, 5-HT and their metabolites in the in vivo microdialysis experiments [28]. PCA (10 mg/kg) induced the

increase of the extracellular concentration of DA but a decrease of DOPAC in the striatum [28]. On the other hand, a decrease in the level of both 5-HT and 5-HIAA was observed [28], which is in contrast to the results of our study. However, other previous studies demonstrated that the cortical extracellular 5-HT levels were increased in C57Bl6 male mice (7 mg/kg, i.p) [29] and that the concentrations of both DA and 5-HT were significantly reduced in mouse striatum (male NIH-Swiss) following the ingestion of a neurotoxic dose (15 mg/kg, twice, 6-h interval, i.p.) [30]. Murnane et al. [31] also reported a significant decrease in the concentrations of DA, 5-HT, DOPAC, HVA and 5-HIAA in mouse striatum tissue. In spite of the exposure to the same drug, the monoamine levels were diametrically different, depending on the dose, administration method and/or animal species. Most of researches demonstrated that PCA affected the neurotransmitter systems but did not agree if it was up- or down-regulated.

5-Fluoropentyl-3-pyridinoylindole, AB-PINACA and PB-22 are synthetic cannabinoids and few studies have been conducted regarding their effects on monoamine neurotransmission. In our study, the changes in the levels of DA, 5-HT and their metabolites were not as considerable as amphetamine-related compounds tested but fluctuations in the DA and/or the 5-HT concentrations were observed (Fig. 3e–g). Previous studies on the DA-stimulating properties of other synthetic cannabinoids such as JWH-018 [32], JWH-250 [33], JWH-073 [33] and 5F-PB-22 [34] demonstrated that these drugs increased extracellular DA levels in the nucleus accumbens shell of mice or rats but not for all tested doses.

The effects of three tryptamines, 4-HO-DET, AMT and NMT on monoamine neurotransmitters were also investigated. The levels of DA and/or 5-HT severely fluctuated by the administration of the tryptamines with large variations among the animals while those of their metabolites were slightly or little changed. 4-HO-DET increased the concentration of HVA across all doses, and that of 5-HIAA immediately increased after the administration of 0.3 and 1 mg/kg. Distinctively, AMT gradually increased the concentrations of DA while it decreased the concentrations of its metabolites, DOPAC and HVA, in a dose- and time-dependent manner. The levels of HVA were below the limit of quantification at 160 and 180 min. NMT also affected the release of DA, 5-HT and their metabolites in spite of a little statistical significance. In a previous study, both AMT and NMT showed DAT and SERT-mediated releasing properties in rat brain synaptosomes [35, 36]. It was also reported that AMT acted as a potent inhibitor of DA and 5-HT reuptake into the rat brain synaptosome and showed that the releasing activity of the monoamines was similar to methamphetamine. A methoxylated tryptamine with a primary amine group, 5-methoxy-α-methyltryptamine (5-MeO-AMT) stimulates DA and 5-HT release from the rat brain synaptosome while other methoxylated tryptamines with a tertiary amine group, such as *N,N*-dipropyltryptamine (DPT), 5-methoxy-*N,N*-diisopropyltryptamine (5-MeO-DIPT), 5-methoxy-*N,N*-methylisopropyltrypatmine (5-MeO-MIPT), 5-methoxy-*N,N*-dimethyltryptamine (5-MeO-DMT) and 5-methoxy-*N,N*-diallyltryptamine (5-MeO-DALT), did not show any monoamine-releasing effect [35, 36]. Arunotayanun et al. [37] discovered that AMT expressed high affinity to cloned 5-HT receptors (5-HT_{1A}, 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}) and SERT, which implies that this drug could also affect 5-HT release. No previous reports regarding the effects of 4-HO-DET on the monoamine neurotransmitter system were found. 3-[2-(Dimethylamino)ethyl]-4-indolol (4-HO-DMT) increased the extracellular levels of DA and/or 5-HT in the mesoaccumbens and/or mesocortical pathway in a previous animal study of male Wistar rats [38].

The exposure of desoxy-D2PM remarkably caused the gradual increase in the level of DA; in contrast, the 5-HT level fluctuated after the ingestion of 0.3 and 1 mg/kg and then significantly decreased after the ingestion of 3 mg/kg. Their metabolites were not significantly changed. The effects of desoxy-D2PM on monoamine neurotransmitters have not been reported elsewhere. Consistent with our results, its structural analogues, such as desoxypipradrol (2-DPMP) and diphenyl-2-pyrrolidinemethanol (D2PM), showed potent DAT inhibition [27, 39], stimulated the DA efflux in rat brain and a transporter-mediated assay, and inhibited DA reuptake dose-dependently [39, 40] in previous studies. However, both compounds did not affect 5-HT release [39], SERT inhibition [27, 39] or 5-HT reuptake inhibition [39].

To take advantage of the loopholes in the law, NPDs with diverse chemical structures appear quickly, but scientific understanding of their effects on the CNS is not able to keep up with their appearance. The current study was conducted to rapidly monitor the changes of monoamine neurotransmitters induced by 11 selected NPDs. Most NPDs affected the extracellular levels of DA, 5-HT and/or their metabolites, showing consistent changes depending on groups of chemical structures, such as amphetamines, synthetic cannabinoids and tryptamines. These consistencies could be observed because the animal experiments were carried out in a system under the same conditions. As such, the results can now be used as the preliminary data for further research concerning the monoamine neurotransmitter-related mechanisms of NPDs, in spite of the limitations of drug dose, administration routes and intervals or animal species used. Moreover, this understanding could be helpful to monitor the liability of their abuse and addiction.

Conclusions

DA and 5-HT are important neurotransmitters associated with drug abuse and addiction. In this study, changes in DA, 5-HT and their metabolites in brain microdialysates from awake rats following exposure to 11 selected NPDs were investigated using a validated LC–MS/MS method. Most NPDs up- and/or down-regulated the extracellular levels of DA, 5-HT and/or their metabolites, which implies that they disturb the CNS. The results will be useful not only for further studies of neurotoxicity of NPDs in neuroscience and forensic science but also to legislate for their regulation with scientific evidence.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants performed by any of the authors. All experiments were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee at Daegu Haany University.

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