NOVEL PSYCHOACTIVE DRUGS

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NOVEL PSYCHOACTIVE DRUGS

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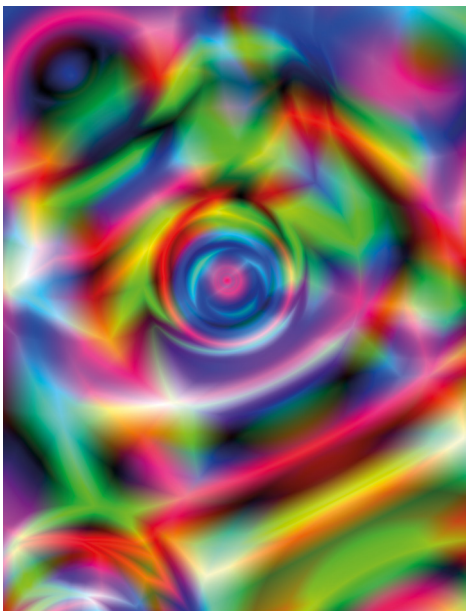

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An increasing number of novel psychoactive drugs are currently available and sold as 'legal highs' or 'research chemicals'. These New Psychoactive Substances (NPS) constitute a broad range of hundreds of natural and synthetic drugs, including synthetic cannabinoids, synthetic cathinones, synthetic opioids and other classes, which use has resulted in a significantly growing number of intoxication and mortality, as reported by emergency and poison centres from all over the world. Definition of "NPS" includes any substance that has recently become available and has been designed purposely to replace illegal drugs, although not necessarily of new synthesis.

Use of NPS is dramatically increased in the last decade and represents a serious risk for the public health. Their ever-evolving chemical structure, the possibility to distribute in real time through the Internet and social networks information about their use and effects have dramatically challenged public health and drug policies internationally. NPS recently attracted great attention, but most are still unregulated and proposed online as legal alternatives to traditional illicit drugs. Unfortunately, this area is still poorly investigated and very limited information are available so far on their nature and potential risks. The phenomenon of NPS requires multi-national and multi-disciplinary collaborations to improve our knowledge on this changing drug

market, to share information and define good practices at a global level. Political and educational efforts are indispensable to regulate this mutable scenario and to inform the public about health consequences of NPS use. Clinicians and emergency staff should be aware that NPS may cause severe health consequences and unexpected adverse effects, and be informed on how to recognized and treat specific intoxication cases.

Considering the widespread use of NPS and paucity of information about their toxicology and pharmacology, this Research Topic will be useful to understand the new trends in the scenario of drug use, abuse and addiction to inform professionals and general public about the health problems caused by NPS and to help drug-control policies to adopt suitable control measures.

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EDITORIAL
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Editorial: Novel Psychoactive Drugs

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Editorial on the Research Topic

Novel Psychoactive Drugs

In the last decade, the trend of drug consumption has completely changed, and an incredibly high number of new psychoactive substances (NPS) have flooded the drug market as legal alternatives to common drugs of abuse. The advent of NPS has contributed to the appearance and growth of a new "drug scenario" characterized by an increased number of intoxicated people presenting with emergencies after consumption of drugs with unknown effects or safety profiles. Indeed, the acute effects of NPS and their long-term side effects are not always known, and safety data regarding their toxicity are often unavailable. Considering that a total of 803 NPS were reported in the period 2009–2017, it is clear that such a situation poses additional challenges for identification, control, and treatment strategies.

We felt that the time has come to deepen and expand our understanding of the peripheral and central actions of NPS and their health consequences. Working as preclinical (LF) and clinical researchers (AW), we have decided to collect leading groups of scientists working in the field to "make the point" of NPS at several levels, from epidemiology to marketing, from clinical to mechanistic studies, including *in vitro* and *in vivo* studies on common (synthetic cannabinoids and cathinones) and less common (2,4-DNP and "hippy crack") psychoactive substances. As a result, the present Research Topic brings together 16 papers (9 original articles and 7 reviews) of excellent quality and broad impact which cover the main classes of NPS and explore further their toxicology and pharmacology, with a particular emphasis given to the role of NPS in modulating brain neurotransmission and behavior.

According to the last World Drug Report 2018 (United Nations publication, Sales No. E.18.XI.9), by the end of 2017 synthetic cannabinoids and synthetic cathinones represented the largest class of NPS, with 251 and 148 compounds detected, respectively. This Research Topic includes 2 original articles on synthetic cannabinoids, 2 original articles on synthetic cathinones, and 2 reviews summarizing the acute and chronic effects of these two classes of NPS. Specifically, Elmore and Baumann from the Designer Drug Research Unit of NIDA-NIH (Baltimore, USA) used the popular synthetic cannabinoid JWH-018 to expand the current knowledge on the relationship between repeated use of synthetic cannabinoids and serotonergic dysregulation. Their study shows that repeated treatment with JWH-018 induces tolerance to its hypothermic and cataleptic effects and produces transient increases in serotonin 5-HT_{1A} receptor responsiveness without affecting 5-HT_{2A} receptor responsiveness in male rats.

To provide further insights on the mechanisms of action underlying the stimulant effects of synthetic cannabinoids, a multidisciplinary Italian study coordinated by Matteo Marti from the University of Ferrara (Italy), showed that the naphthoylindole compound JWH-018 and the unrelated adamantylindazole AKB48 induced psychostimulant effects in male mice through mechanisms mediated by both cannabinoid CB_1 and dopamine receptors and likely facilitated the release of ventral striatal dopamine without affecting the activity of the dopamine transporter (DAT) (Ossato et al.).

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To update the scenario on synthetic cathinones, Tomáš Páleníček and his productive lab located in Prague (Czech Republic) have contributed to this Research Topic with 4 different contributions. The first provides a detailed investigation of pharmacokinetics and bio-distribution of mephedrone (4-methylmethcathinone, MEPH) and its primary metabolite nor-mephedrone (nor-MEPH) to four different substrates, i.e., serum, brain, lungs, and liver (Šíchová et al.). Authors also demonstrated that methylone (3,4-methylenedioxy-N-methylcathinone) and its primary metabolite, nor-methylone, induced alterations in behavior and body temperature changes that are comparable to MDMA (Štefková et al.). Finding that hyperthermic reaction is more pronounced in the group-housed condition relative to individually housed rats confirms the risk for users to suffer from serotonin syndrome especially when the drug is used in crowded conditions. Moreover, they showed that also 3,4-methylenedioxypyrovalerone (MDPV), a potent pyrovalerone cathinone that recreational users substitute for amphetamines, is rapidly absorbed, readily crosses the blood-brain barrier, is excreted primarily as metabolites and, consistent with its primarily dopaminergic mechanism of action, acts as a typical stimulant with modest hyperthermic and psychomimetic properties (Horsley et al.). Finally, authors provided an exhaustive review on synthetic aminoindanes and discussed their therapeutic potential in medical research along with their pharmacology, behavioral effects, pharmacokinetics, and toxicity (Pinterova et al.).

To summarize the last evidence coming from both animal and human studies on these two main classes of NPS, we provided a comprehensive review on epidemiology, pharmacology, central effects and clinical features, legislation, and regulation of both synthetic cannabinoids and synthetic cathinones. We also discussed why they result in adverse medical and psychiatric effects that seem to be higher than those induced by the natural parent compounds, i.e., cathinone and THC (Weinstein et al.).

But the world of NPS is immense, and other drugs besides cathinones and cannabinoids have been studied. Among them are 3,4-dichloromethylphenidate (3,4-CTMP) and ethylphenidate, two drugs closely related to the attention deficit medication methylphenidate (Ritalin®) that have been reported on online user fora to induce effects similar to cocaine. These two NPS were identified from samples obtained from London dance club amnesty bins or samples purchased on the internet and analyzed by Davidson et al. that here described their neurochemical profile in comparison with their parent compound methylphenidate. The authors showed that while 3,4-CTMP increases the release of dopamine more potently than methylphenidate (likely by blocking the DAT), ethylphenidate possesses a weaker effect and increases dopamine release only modestly while both NPS, like methylphenidate, increase noradrenaline efflux.

Over the last few years, MDMA and its phenethylamine derivatives, such as 2,5-dimethoxy-4-bromo-amphetamine hydrobromide (DOB) and para-methoxyamphetamine (PMA), are also increasingly sold through the internet despite their significant toxicity and widely used as substitutes in "ecstasy" tablets. A collaborative study conducted in Milan (Italy) demonstrated that the oxytocin/vasopressin system modulates the rewarding, prosocial, and anxiolytic effects of MDMA, DOB, and PMA in zebra fish, thus providing an important target for the development of new pharmacotherapies for the treatment of affective disorders caused by MDMA and its phenethylamine derivatives (Ponzoni et al.).

Intriguingly, under the umbrella of NPS, recreational users can find not only newly synthetized substances, but also "old" compounds sold as supplements and weight-loss drugs or typically used in clinical setting, two of which have been investigated by *Andrea Petróczi* and her collaborators of the Kingston University (UK). 2,4-dinitrophenol (2,4-DNP), for example, is an effective but highly dangerous fat burner used in the past for obesity treatment but then withdrawn from the market due to an unacceptably high rate of significant adverse effects. Today, DNP has re-emerged within the body-building community and extreme dieters, particularly among young adults, and is sold mostly over the internet under a number of different names as a slimming aid. Using a sequential mixed method design and based on a hypothetical scenario as if 2,4-DNP was a licensed pharmaceutical drug, the authors elegantly discussed the factors men and women may consider before buying a weight-loss drug such as 2,4-DNP (Bleasdale et al.). Another example of "atypical" NPS is nitrous oxide gas, also known as "hippy crack"' or "laughing gas," which is a safe, effective, and inexpensive anesthetic used for the management of labor pain, e.g., to decrease fear and anxiety associated with dental procedures or during childbirth. Yet, it is increasingly consumed recreationally for its euphoric, relaxing, and hallucinogenic effects. Authors here reported for the first time trends, awareness, and perceptions of the use of hippy crack among young adults in England and highlighted a worrying willingness of most respondents to use it in the future coupled with a clear lack of awareness of the serious side effects (e.g., psychosis, myeloneuropathy) this gas may cause (Ehirim et al.).

Considering that some medications (e.g., nitrous oxide) are increasingly abused and that some NPS have been tested clinically (e.g., ketamine), it is not fully unexpected that some NPS, based on their known or predicted pharmacology, might have the potential to be clinically useful in brain disorders. To take stock of the situation, in a collaborative (Malaysia, Germany, Switzerland) review, Hassan et al. revisited the existing literature on several NPS for which the neuropharmacological evaluation has made great progress in recent years, including Kratom, Spice, mephedrone and methylone, dimethyltryptamine and novel serotonergic hallucinogens, ketamine and novel dissociative drugs, GHB and GBL, and 1,4-butanediol.

Fentanyl, fentanyl analogs, and other new synthetic opioids with various chemical structures, such as AH-7921, U-47700, and MT-45, have burst relatively recently onto the illegal drug market as NPS. In this Research Topic, Jolanta Zawilska from the Medical University of Lodz (Poland) provided an updated information on the properties of novel synthetic opioids, with a special emphasis given to their acute toxic effects, and reviewed case reports of fatalities involving these drugs.

With two different contributions, Orsolini et al. and Orsolini et al. have discussed the use of NPS in the context of the

psychopathology and psychopharmacology of the hallucinogen-persisting perception disorder and reviewed the most commonly abused "psychedelic animals" by combining a search of both scientific literature and online psychonauts' experiences.

To conclude the Research Topic, the French group of *Laurent Karila* and colleagues presented an overview of new technologies in the field of addiction and discussed how they can improve assessment of and interventions in addictive disorders (Ferreri et al.).

The field of NPS is too broad to present a comprehensive overview, but the series of papers in this special issue provide an excellent illustration of the relevant topics, which warranted their publication together. They illustrate the rapidly expanding research on NPS encompassing epidemiological analyses, pharmacological mechanisms, and toxic effects. In light of the great interest that this Research Topic has already attracted and of the increasing number of NPS currently on the market, we have decided to edit in 2019 a follow up Frontiers Research Topic on NPS with the ultimate goal of stimulating research and education in the areas of prevention and treatment. Contributions in any aspect related to the complex world of NPS (e.g., intoxication cases, clinical and animal studies, epidemiology analysis, legislative/regulatory considerations) are welcome.

AUTHOR CONTRIBUTIONS

LF and AW contributed equally to this Editorial of the Research Topic on NPS that they edited in 2018.

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ORIGINAL RESEARCH
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Repeated Exposure to the "Spice" Cannabinoid JWH-018 Induces Tolerance and Enhances Responsiveness to 5-HT$_{1A}$ Receptor Stimulation in Male Rats

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Naphthalen-1-yl-(1-pentylindol-3-yl)methanone (JWH-018) is a synthetic compound found in psychoactive "spice" products that activates cannabinoid receptors. Preclinical evidence suggests that exposure to synthetic cannabinoids increases 5-HT$_{2A/2C}$ receptor function in the brain, an effect which might contribute to psychotic symptoms. Here, we hypothesized that repeated exposures to JWH-018 would enhance behavioral responsiveness to the 5-HT$_{2A/2C}$ receptor agonist DOI. Male Sprague-Dawley rats fitted with subcutaneously (sc) temperature transponders received daily injections of JWH-018 (1.0 mg/kg, sc) or its vehicle for seven consecutive days. Body temperature and catalepsy scores were determined at 1, 2, and 4 h post-injection each day. At 1 and 7 days after the final repeated treatment, rats received a challenge injection of either DOI (0.1 mg/kg, sc) or the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT (0.3 mg/kg, sc), then temperature and behavioral responses were assessed. Behaviors induced by DOI included wet dog shakes and back muscle contractions (i.e., skin jerks), while behaviors induced by 8-OH-DPAT included ambulation, forepaw treading, and flat body posture. On the first day of repeated treatment, JWH-018 produced robust hypothermia and catalepsy which lasted up to 4 h, and these effects were significantly blunted by day 7 of treatment. Repeated exposure to JWH-018 did not affect behaviors induced by DOI, but behavioral and hypothermic responses induced by 8-OH-DPAT were significantly augmented 1 day after cessation of JWH-018 treatment. Collectively, our findings show that repeated treatment with JWH-018 produces tolerance to its hypothermic and cataleptic effects, which is accompanied by transient enhancement of 5-HT$_{1A}$ receptor sensitivity *in vivo*.

Keywords: JWH-018, synthetic cannabinoid, serotonin, receptor, spice

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INTRODUCTION

Synthetic cannabinoids are novel psychoactive substances with pharmacological similarity to the phytocannabinoid Δ^9-tetrahydrocannabinol (THC), the main psychoactive ingredient in marijuana. Over the last decade, herbal smoking blends consisting of plant material laced with synthetic cannabinoids (i.e., "spice" products) have emerged in the recreational drug marketplace. Analytical investigations of the first spice products revealed that a primary psychoactive

component was naphthalen-1-yl-(1-pentylindol-3-yl)methanone, also known as JWH-018 (1, 2). JWH-018 and many of its structural analogs were found in spice products during 2010 through 2013, and JWH-018 is still present on the street today (3, 4). JWH-018 is a potent agonist at cannabinoid type-1 (CB_1) and cannabinoid type-2 (CB_2) receptors, which displays at least threefold higher binding affinity than THC at both receptors (5, 6). When administered to mice, JWH-018 produces effects consistent with other CB_1 receptor agonists, including hypothermia, analgesia, reduced motor activity, and catalepsy (5, 6). In drug discrimination studies, JWH-018 fully substitutes for the THC stimulus cue in both mice and rats (7–9).

Several lines of clinical evidence support a relationship between heavy cannabis use and risk for development of psychosis and schizophrenia (10–13). Although the precise underpinnings of schizophrenia are not fully understood, dysregulation of brain serotonin (5-HT) systems has been implicated in certain psychotic symptoms, such as paranoia and hallucinations (14–16). Preclinical studies in rodents show that exposure to CB_1 receptor agonists can influence 5-HT receptor responsiveness *in vivo*. For example, Darmani reported that acute pretreatment with various cannabinoids, including THC and the potent cannabinoid receptor agonist (6aR,10aR)-9-(hydroxymethyl)-6,6-dimethyl-3-(2-methyloctan-2-yl)-6H,6aH,7H,10H,10aH-benzo[c]isochromen-1-ol (HU-210), inhibits behavioral effects of the 5-$HT_{2A/2C}$ agonist 2,5-dimethoxy-4-iodoamphetamine (DOI) in mice (17). By contrast, Hill et al. found that repeated treatment with HU-210 for 12 days enhances wet dog shakes induced by DOI in rats (18). Franklin et al. found that 7-day exposure to the cannabinoid agonist 2-[(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl) cyclohexyl]-5-(2-methyloctan-2-yl)phenol (CP 55,940) enhances cortisoterone release induced by DOI in rats, and this effect is accompanied by upregulation of 5-HT_{2A} receptors in the hypothalamus (19). Recent evidence suggests a direct interaction between CB_1 and 5-HT_{2A} receptors in rat brain. In particular, Viñals et al. showed that CB_1 and 5-HT_{2A} receptor heteromers are present in hippocampus and other brain regions related to memory formation, and these heteromers are necessary for the amnesic effects of THC, but not its analgesic effects (20).

Despite the continued misuse of synthetic cannabinoids by humans, little is known about the functional consequences of repeated administration of JWH-018 or related substances found in spice products. Given the emerging evidence for interactions between cannabinoid and 5-HT systems in the brain, we sought to determine the effects of repeated treatment with JWH-018 on the behavioral responsiveness to selective 5-HT receptor agonists. Specifically, male Sprague-Dawley rats were treated for seven consecutive days with JWH-018, then challenged with DOI or the 5-HT_{1A} receptor agonist 8-hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT) at 1 day and 7 days after the last JWH-018 treatment. Body temperatures and catalepsy scores were determined during the repeated dosing regimen of JWH-018, while body temperatures and agonist-induced behaviors were measured following challenge doses of 5-HT drugs. We hypothesized that repeated exposure to JWH-018 would enhance subsequent behavioral responsiveness to DOI in rats [e.g., see Ref. (18)]. Such increases in 5-$HT_{2A/2C}$ activity produced by cannabinoid exposure could contribute to adverse psychiatric symptoms associated with cannabinoid use.

MATERIALS AND METHODS

Drugs and Reagents

Naphthalen-1-yl-(1-pentylindol-3-yl)methanone (JWH-018) was obtained from Cayman Chemical (Ann Arbor, MI, USA). (−)-2,5-Dimethoxy-4-iodoamphetamine HCl (DOI) and (+)-8- hydroxy-2-(dipropylamino)tetralin HBr (8-OH-DPAT) were obtained from Sigma Aldrich (St. Louis, MO, USA). 5-(4-Chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide HCl (rimonabant) was obtained from the pharmacy at the National Institute on Drug Abuse (NIDA), Intramural Research Program (IRP). JWH-018 and rimonabant were dissolved into a 1:1:18 mix of dimethyl sulfoxide:Tween 80:sterile saline, whereas other drugs were dissolved in sterile saline. All injections were administered at a volume of 1.0 mL/kg.

Animals and Surgery

Male Sprague-Dawley rats (Envigo, Frederick, MD, USA) weighing 250–300 g were double-housed (lights on: 7:00 a.m.–7:00 p.m.) under conditions of controlled temperature (22 ± 2°C) and humidity (45 ± 5%) with free access to food and water. Experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Vivarium facilities were fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and study procedures were approved by the NIDA IRP Animal Care and Use Committee. After 2 weeks of acclimation to the vivarium, rats were subjected to surgical procedures and subsequently used for experiments. Rats were rapidly anesthetized with isoflurane using a drop jar which contained a raised floor above a gauze pad saturated with 5 mL of isoflurane. Once fully anesthetized, each rat received a surgically implanted IPTT-300 transponder (Bio Medic Data Systems, Seaford, DE, USA) to facilitate the non-invasive measurement of body temperature via a portable radio frequency reader system (handheld reader). The transponders were 14 mm × 2 mm cylinders implanted subcutaneously (sc) posterior to the shoulder blades *via* a sterile guide needle. Animals were individually housed postoperatively and allowed 7–10 days for recovery.

Acute JWH-018 Administration and Rimonabant Antagonism

As a first step in our study, we examined the dose–response effects of acute JWH-018 administration in a cohort of 12 rats. Rats were tested once per week for three consecutive weeks. On test day, rats were moved to the testing room in their home cages and given 1 h to acclimate. Feeding trays were removed, and wire lids were placed atop the cages. Rats received sc injections of JWH-018 (0.1, 0.3, or 1.0 mg/kg) or its vehicle. Immediately before injection, and at various times thereafter (0.25, 0.5, 0.75, 1, 1.5, 2, and 4 h post-injection), body temperature was measured using the handheld reader, and animals were observed for 90 s to assess behaviors. Observers were not blind to the drug treatment condition. Rats

were assigned a catalepsy score based on three behaviors: immobility (absence of movement), flattened body posture, and splayed limbs (limbs spread out away from the center of the body). Each behavior was given a numerical score of 1 for "behavior absent," 2 for "behavior present," or 3 for "behavior continuous/intense"; the three scores were summed to provide a single value ranging from 3 to 9 at each time point.

Once dose–response experiments were completed, we next tested the effect of pretreatment with the CB_1 receptor antagonist rimonabant on the responses induced by JWH-018 in a cohort of 12 rats. Rats were tested once per week for three consecutive weeks. Rats were pretreated with either 1.0 mg/kg of the CB_1 receptor antagonist rimonabant or its vehicle 30 min before injection with either 1.0 mg/kg JWH-018 or its vehicle. Body temperature measurements and behavior scoring were carried out as described previously for acute dose–response experiments.

Repeated Dosing with JWH-018

Results from the acute dose–response experiments demonstrated that 1.0 mg/kg JWH-018 produced robust hypothermia and catalepsy. Thus, this dose was used for the repeated injection experiments carried out in a group of 32 rats. The repeated dosing with JWH-018 or its vehicle was carried out in the vivarium. Rats fitted with surgically implanted sc temperature transponders received a single sc injection of either 1.0 mg/kg JWH-018 or its vehicle, and were returned to their home cages. Immediately before injection, and at 1, 2, and 4 h post-injection, body temperature was measured using the handheld reader, and animals were observed for 90 s. During the observation period, behaviors were scored using the catalepsy scale as detailed above in the Section "Acute JWH-018 Administration and Rimonabant Antagonism." The JWH-018 injection procedure was repeated daily for seven consecutive days.

Challenge Injection with Serotonergic Agonists

One day after the last repeated treatment with JWH-018 or vehicle (i.e., day 8, or day 1 of withdrawal), rats were moved to the testing room in their home cages and given 1 h to acclimate. Feeding trays were removed, and wire lids were placed atop the cages. One cohort of 16 rats received 0.1 mg/kg of DOI, whereas another cohort of 16 rats received 0.3 mg/kg of 8-OH-DPAT. The doses of DOI and 8-OH-DPAT were based on preliminary dose–response experiments, which identified drug doses evoking robust behavioral changes that were less than maximal (data not shown). The specific non-contingent behaviors induced by DOI were wet dog shakes and back muscle contractions (i.e., skin jerks). Both behaviors are known to be mediated by $5\text{-}HT_{2A}$ receptors in rats (21–23). The numbers of wet dog shakes and skin jerks present during the observation period were tallied. Wet dog shakes were defined as a rapid and sudden rotation of the head, neck, and shoulders from one side to the other, analogous to the way a wet dog may shake to dry itself. Skin jerks were defined as brief paraspinal muscle contractions of the back muscles in a tail to head direction. Specific non-contingent behaviors induced by 8-OH-DPAT were locomotion in the horizontal plane (i.e., ambulation), forepaw treading, and flattened body posture, components of the 5-HT behavioral syndrome known to be mediated by $5\text{-}HT_{1A}$ receptors (24, 25). Possible scores for each behavior were 0 (behavior absent), 1 (behavior present), or 2 (behavior intense or continuous). At the end of the observation period, the scores for the three behaviors were summed to produce a 5-HT syndrome score for each time point.

After acute serotonergic drug challenge, body temperatures were measured using the handheld reader at 0.25, 0.5, 0.75, 1, 1.25, 1.5, and 2 h post-injection, and behavior scores were given at each time point as appropriate for the treatment received (i.e., wet dog shakes and skin jerks for DOI treatment, and serotonin syndrome scores for 8-OH-DPAT treatment). The acute challenge procedure with DOI and 8-OH-DPAT was repeated 1 week after the last repeated JWH-018 treatment.

Data Analysis and Statistics

Data were tabulated, analyzed, and graphically depicted using GraphPad Prism (version 5.02; GraphPad Software, Inc., La Jolla, CA, USA). Time-course temperature data were analyzed using a two-way analysis of variance (treatment × time), followed by a Bonferroni *post hoc* test to determine significance between group means at specific time points. Mean temperature data from the DOI and 8-OH-DPAT experiments were evaluated by two-tailed *t*-tests. Catalepsy data from the acute dose–response, rimonabant antagonism and repeated treatments were analyzed by Kruskal–Wallis test (non-parametric), followed by Dunn's multiple comparison test to determine significance between group means. Summed behavioral score data from the DOI and 8-OH-DPAT challenge experiments were analyzed using a Mann–Whitney test (non-parametric) comparing effects of repeated JWH-018 versus vehicle pretreatments. Statistical analyses were performed on data from all 7 days of the JWH-018 repeated administration experiment, however, **Figure 3** only shows data from selected days to make the graphs easier to interpret. $p < 0.05$ was considered the minimal criterion for statistical significance.

RESULTS

Effects of Acute JWH-018 Administration

The left panel of **Figure 1** illustrates the effect of acute JWH-018 administration on core body temperature in male rats. JWH-018 produced a dose-related change in core temperature ($F_{3,256} = 111.1$, $p < 0.0001$), with significant reductions compared to vehicle control after the 1.0 mg/kg dose at 0.5, 0.75, 1, 1.5, and 2 h post-injection. A maximum decrease of ~3°C was observed at 1 h after the 1.0 mg/kg dose. It is worth noting that 0.1 mg/kg JWH-018 caused a noticeable, albeit non-significant, increase in temperature for the first 2 h, suggesting biphasic dose–response effects of the drug on body temperature. As seen in the right panel of **Figure 1**, JWH-018 dose-dependently increased the summed catalepsy behavioral score (Kruskal–Wallis statistic 28.53, $p < 0.0001$). Dunn's test revealed that significant increases from vehicle control were present following the 1.0 mg/kg dose.

The left panel of **Figure 2** shows that pretreatment with 1.0 mg/kg of the CB_1 receptor antagonist rimonabant significantly altered the hypothermic effect of 1.0 mg/kg JWH-018 ($F_{3,256} = 56.79$, $p < 0.0001$). Rats treated with rimonabant/

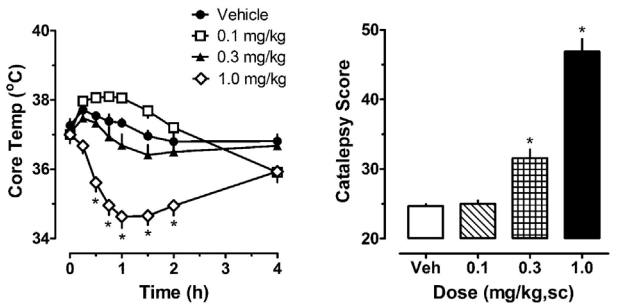

FIGURE 1 | Core temperature measures and summed catalepsy scores for rats receiving acute subcutaneous injections of 0.1, 0.3, and 1.0 mg/kg JWH-018 or its vehicle. Core temperature and behavioral score were recorded at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, and 4 h post-injection, as described in the Section "Acute JWH-018 Administration and Rimonabant Antagonism." Data are mean ± SEM for $N = 9$ rats per group. *Represents significant effects when compared to the corresponding vehicle-treated group for temperature (Bonferroni, $p < 0.05$) and catalepsy (Dunn's, $p < 0.05$).

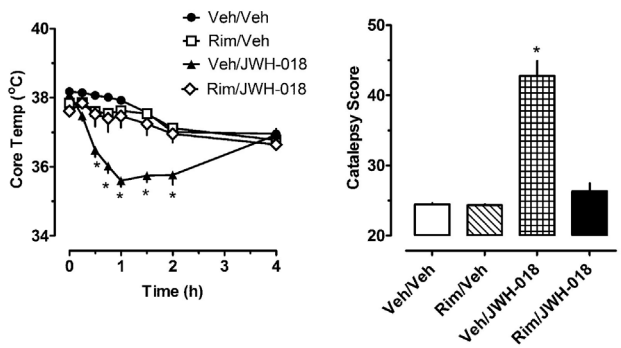

FIGURE 2 | Core temperature measures and summed catalepsy scores for rats receiving either subcutaneous (sc) vehicle (VEH) or 1.0 mg/kg JWH-018 (JWH), 30 min after pretreatment with either sc vehicle (VEH) or 1.0 mg/kg rimonabant (RIM). Core temperature and behavioral score were recorded at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, and 4 h post-injection, as described in the Section "Acute JWH-018 Administration and Rimonabant Antagonism." Data are mean ± SEM for $N = 9$ rats per group. *Represents significant effects when compared to the corresponding vehicle/vehicle-treated group for temperature (Bonferroni, $p < 0.05$) and catalepsy (Dunn's, $p < 0.05$).

JWH-018 were not significantly different from rats treated with vehicle/vehicle, whereas the vehicle/JWH-018 group displayed decreased body temperature that was significantly different from all other groups. Vehicle/JWH-108 rats had significantly decreased body temperature at the 0.5, 0.75, 1, 1.5, and 2 h time points. Likewise, the right panel of **Figure 2** shows that rats treated with vehicle/JWH-018 had significantly higher catalepsy scores when compared to all other groups (Kruskal–Wallis statistic 22.32, $p < 0.0001$).

Effects of Repeated JWH-018 Administration

The left panel of **Figure 3** depicts the effects of 1.0 mg/kg JWH-018 or its vehicle on body temperature on days 1, 3, 5, and 7 of repeated treatment. Vehicle administration did not significantly alter body temperature from preinjection values on day 1 of treatment, or during the 7-day treatment regimen ($F_{6,420} = 0.645$, NS). Because vehicle administration did not affect body temperature over the course of repeated injections, we compared the effects of JWH-018 treatments across days to those of vehicle treatment on day 1. Using this analysis, JWH-018 caused significant hypothermia when compared to vehicle ($F_{7,480} = 22.331$, $p < 0.0001$), but the temperature responses changed over the course of treatment. On day 1 of JWH-018 exposure, temperature was significantly reduced from vehicle at the 1, 2, and 4 h timepoints. By day 3 of treatment, hypothermia was observed only at the 1 h timepoint, and on days 6 and 7, no reduction in temperature was observed.

The right panel of **Figure 3** depicts the effects of 1.0 mg/kg JWH-018 or its vehicle on summed catalepsy scores on days 1, 3, 5, and 7 of repeated treatment. Vehicle administration did not significantly alter summed catalepsy scores on day 1 of treatment, and there was no change in scores for vehicle-treated rats over the 7-day treatment regimen. Since vehicle administration did not change catalepsy scores over the course of treatment, we compared the effects of JWH-018 treatment across days to

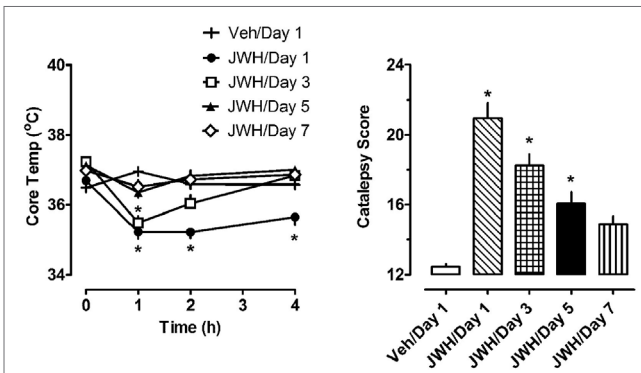

FIGURE 3 | Core temperature measures and summed catalepsy scores for rats receiving either subcutaneous vehicle (VEH) or 1.0 mg/kg JWH-018 (JWH) once daily for seven consecutive days. Core temperature and behavioral score were recorded at 0, 1, 2, and 4 h post-injection each day for 7 days, as described in the Section "Repeated dosing with JWH-018." Data are mean ± SEM for $N = 9$ rats per group. *Represents significant effects when compared to the vehicle group from day 1 of treatment for temperature (Bonferroni, $p < 0.05$) and catalepsy (Dunn's, $p < 0.05$).

the effects of vehicle treatment on day 1. Using this analysis, JWH-018 increased catalepsy scores compared to vehicle (Kruskal–Wallis statistic 63.82, $p < 0.0001$), but the response was attenuated over the course of treatment. Dunn's test demonstrated that summed catalepsy scores were significantly different when compared to vehicle on days 1 through 6, but not on day 7.

Effects of Serotonergic Challenge with DOI and 8-OH-DPAT

Figure 4 depicts the effects of the 5-$HT_{2A/2C}$ receptor agonist DOI on wet dog shakes and skin jerks in rats given the daily regimen of 1.0 mg/kg JWH-018 or its vehicle for 7 days. Rats received

0.1 mg/kg DOI at 1 and 7 days after the last repeated JWH-018 injection. The data demonstrate that there were no significant differences between the pretreatment groups for induction of wet dog shakes (left panel) (Mann–Whitney = 29.50, $p < 0.833$) or skin jerks (right panel) (Mann–Whitney = 18.50, $p < 0.171$) at 1 day after cessation of JWH-018 administration. Similar non-significant effects between pretreatment groups were observed at day 7. DOI did not significantly affect core body temperature in rats pretreated with JWH-018 or vehicle at either test day (data not shown).

A separate cohort of rats was given 0.3 mg/kg of the 5-HT_{1A} agonist 8-OH-DPAT at 1 and 7 days after the daily regimen of 1.0 mg/kg JWH-018 or its vehicle. Specific behaviors and hypothermic responses to 8-OH-DPAT were measured. The left panel of **Figure 5** shows that there was a small yet significant enhancement of 5-HT syndrome score for the JWH-018 pretreated animals at 1 day after the last repeated treatment (Mann–Whitney = 9.50, $p < 0.019$), but this effect disappeared at 7 days (Mann–Whitney = 29.00, $p < 0.791$). The right panel of **Figure 5** shows that mean hypothermic responses produced by 8-OH-DPAT were slightly lower in JWH-018 pretreated rats, but this effect was not significantly different between pretreatment groups day 1 ($t = 1.854$, df = 14, $p < 0.085$) or at day 7 ($t = 1.925$, df = 14, $p < 0.075$).

Because there were trends for enhanced hypothermic responses to 8-OH-DPAT in rats pretreated with JWH-018 (e.g., $p < 0.07$), we evaluated the raw time-course data for temperature responses in this experiment. **Figure 6** illustrates that rats exposed to JWH-018 displayed enhanced hypothermic responses to 8-OH-DPAT when compared to vehicle-pretreated rats at day 1 after cessation of repeated treatments ($F_{1,126} = 17.74$, $p < 0.001$). *Post hoc* tests revealed that temperature was significantly decreased in the JWH-018 group compared to the vehicle group at 1.25, 1.5, 1.75, and 2 h time points after injection of 8-OH-DPAT. The enhanced responsiveness to 8-OH-DPAT in the JWH-018 group was still evident at 7 days after cessation of treatment ($F_{1,126} = 23.26$ $p < 0.001$), though *post hoc* tests found no differences between pretreatment groups at any time point.

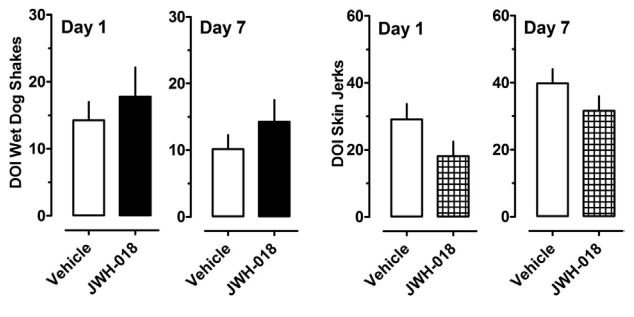

FIGURE 4 | Summed scores for wet dog shakes and back muscle crawls (skin jerks) induced by a subcutaneous challenge injection of 0.1 mg/kg DOI at 1 and 7 days after cessation of repeated JWH-018 treatment. Behavioral scores were recorded at 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, and 2 h post-injection, as described in the Section "Challenge injection with serotonergic agonists." Data are mean ± SEM for $N = 9$ rats per group.

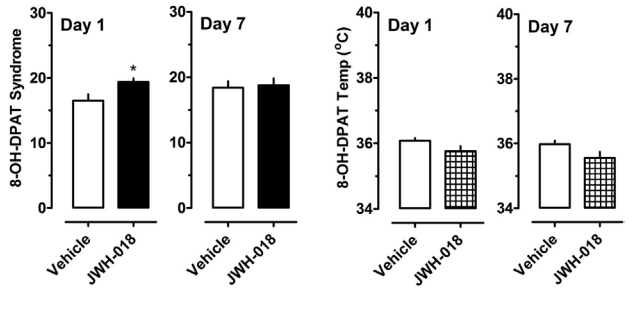

FIGURE 5 | Summed scores for serotonin syndrome behaviors and mean temperature recordings induced by a subcutaneous challenge injection of 0.3 mg/kg 8-OH-DPAT at 1 and 7 days after cessation of repeated JWH-018 treatment. Behavioral scores and core temperatures were recorded at 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, and 2 h post-injection, as described in the Section "Challenge injection with serotonergic agonists." Data are mean ± SEM for $N = 9$ rats per group. *Represents significant effects when compared to the group that received repeated vehicle treatment (Mann–Whitney, $p < 0.05$).

DISCUSSION

The psychiatric literature supports a strong relationship between heavy cannabis use and risk for subsequent psychosis and schizophrenia (12). In addition, misuse of synthetic cannabinoids such as JWH-018 and its analogs is associated with induction of more severe psychotic symptoms when compared to the effects of marijuana (26, 27). Previous studies in rats demonstrate that exposure to synthetic cannabinoids can induce enhanced sensitivity to 5-$HT_{2A/2C}$ receptor activation (18) and upregulation of 5-$HT_{2A/2C}$ receptors in specific brain regions (28, 29). The aim of the present study was to use the popular synthetic cannabinoid JWH-018 to further explore the relationship between repeated cannabinoid exposure and serotonergic dysregulation. JWH-018 is a potent non-selective cannabinoid receptor agonist that was found in the first generation of spice products (1, 2). The present experiments yielded three primary findings. First, in contrast to the results of others [e.g., see

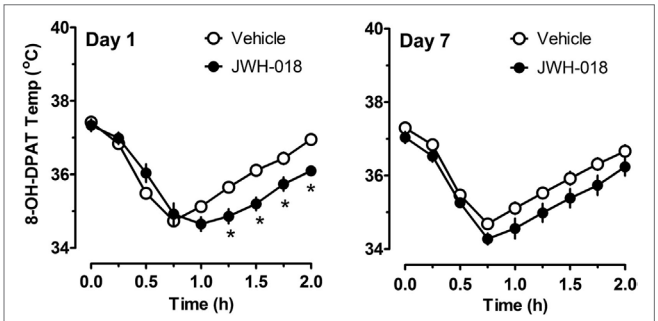

FIGURE 6 | Time-course of core body temperature changes induced by a subcutaneous challenge injection of 0.3 mg/kg 8-OH-DPAT at 1 and 7 days after cessation of repeated JWH-018 treatment. Temperatures were recorded at 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, and 2 h post-injection, as described in the Section "Challenge injection with serotonergic agonists." Data are mean ± SEM for N = 9 rats per group. *Represents significant effects when compared to vehicle-pretreatment group at specific time points (Bonferroni, $p < 0.05$).

Ref. (18)], we detected no significant difference in responsiveness to the 5-$HT_{2A/2C}$ receptor agonist DOI between rats pretreated with synthetic cannabinoids compared to those pretreated with vehicle. Second, we found a modest and significant enhancement of sensitivity to behavioral and hypothermic effects induced by 8-OH-DPAT in rats exposed to repeated injections of JWH-018. Finally, our data show that rats receiving daily injections of JWH-018 develop profound tolerance to its hypothermic and cataleptic effects, such that these effects are nearly absent after 7 days of treatment.

In our experiments, male rats were subjected to seven consecutive days of JWH-018 injections, then given a challenge dose of either the 5-$HT_{2A/2C}$ agonist DOI or the 5-HT_{1A} agonist 8-OH-DPAT at 1 and 7 days after cessation of the repeated dosing regimen. Typical behavioral responses to DOI administration in rats are wet dog shakes (analogous to the head twitch response in mice) and back muscle contractions, also known as skin jerks (21–23). These responses are accepted as specific indicators of 5-HT_{2A} receptor activation since the effects are blocked by selective 5-HT_{2A} receptor antagonists. We found no significant difference in the number of wet dog shakes or skin jerks induced by DOI between the cannabinoid-treated and vehicle-treated groups at either time point. Our findings differ from those of Hill et al., who reported that 12 days of HU-210 administration in rats increases DOI-induced wet dog shakes but decreases skin jerks (18). It is noteworthy that we observed trends for augmented wet dog shakes and attenuated skin jerks in rats exposed to JWH-018, but these effects did not reach significance, perhaps due to variability in the behavioral data. We also administered a submaximal dose of 0.1 mg/kg DOI for our experiments, whereas Hill et al. administered a 10-fold higher dose. Hill et al. theorized that the differential effects of HU-210 on the two behaviors induced by DOI could be due to region-specific changes in 5-HT_{2A} receptors caused by the cannabinoids. This hypothesis was later supported by the work of Franklin et al., who found that 7-day administration of CP 55,940 increased DOI-induced prolactin release, while producing no change in brain levels of 5-HT_{2A} receptor mRNA (19). It is well known that HU-210 displays a much longer time course of action when compared to other synthetic cannabinoids, including JWH-018, and may bind pseudo-irreversibly to the CB_1 receptor. Hruba and McMahon found that rhesus monkeys trained to discriminate THC from vehicle continued to emit drug-appropriate responses for 48 h after administration of HU-210, while such responses to THC and CP 55,940 ceased after 5 h. The same study found that rimonabant treatment increased the ED_{50} values of THC and CP 55,940 discrimination by 12.5 fold, while only causing a 3.8-fold increase for HU-210 (30). Thus, the discrepancies between our results and those of Hill et al. could be due to the use of different cannabinoid agonists for the repeated treatment regimen.

Darmani administered a range of doses of THC, HU-210 and CP 55,940 to mice, followed by an injection of DOI 20 min later, and found that the cannabinoids dose-dependently reduce DOI-induced behaviors (17). Our study used a repeated cannabinoid administration paradigm followed by the administration of DOI after 1 and 7 days of withdrawal, so this may help to explain the differences between our results and those of Darmani. The present findings in rats show that administration of CB_1 agonists causes considerable catalepsy (see **Figures 1–3**), so it seems possible that suppression of motor activity caused by acute cannabinoids could influence subsequent behavioral effects of 5-HT_{2A} receptor agonists. We purposefully designed our experiments to examine the responsiveness to 5-HT agonists at 1 and 7 days after the acute effects of cannabinoid administration had subsided.

We found a modest yet significant increase in the behavioral and hypothermic effects induced by 8-OH-DPAT in rats receiving repeated JWH-018 treatments when compared to those receiving repeated vehicle treatments. The augmented sensitivity to 8-OH-DPAT resolved by 7 days after cannabinoid exposure. In a previous study, Hill et al. found that repeated injections of HU-210 for 12 consecutive days reduce the hypothermic and corticosterone responses produced by 8-OH-DPAT in vehicle-treated animals (18). Both hypothermia and corticosterone release are presumably mediated by 5-HT_{1A} receptors in the brain (31), thus Hill et al. found that repeated administration of HU-210 decreases 5-HT_{1A} activity in response to agonism, whereas we found the exact opposite in rats exposed to JWH-018. It seems possible that discrepancies between our results and those of Hill et al. could be due to the use of different cannabinoid agonists, as noted above. On the other hand, Zavitsanou et al. demonstrated that repeated injections of HU-210 increase 5-HT_{1A} receptor density and mRNA levels in the hippocampus and amygdala of male rats (32), a finding consistent with the possibility of enhanced 5-HT_{1A} receptor responsiveness after cannabinoid exposure. Our data demonstrating an increase in 5-HT_{1A} receptor sensitivity after exposure to JWH-018 is a unique finding, and its relationship to the development of psychiatric symptoms following cannabinoid exposure warrants further study. Future research should determine whether 5-HT_{1A} upregulation occurs after repeated exposure to other synthetic cannabinoids. Importantly, and in contrast to existing findings using other cannabinoid compounds, our data show that repeated exposure to JWH-018 does not induce robust alterations in 5-HT_{2A} receptor responsiveness, but increases 5-HT_{1A} responsiveness.

In addition to assessing changes in serotonergic activity after cannabinoid exposure, one of the secondary aims of our study was to examine pharmacological responses to repeated JWH-018 injections. Rats in our study had implantable temperature transponders to facilitate the non-invasive measurement of body temperature. JWH-018 was shown to dose-dependently cause hypothermia and catalepsy, both of which were reversed by rimonabant (see **Figure 2**). The present data showing acute decreases in body temperature after JWH-018 administration in rats are consistent with previous findings from our laboratory and others, which show dose-related hypothermic effects of JWH-018 as assessed by radiotelemetry or rectal probes to measure core temperatures (33–36). As the repeated injection procedure progressed in our study, rats began to develop tolerance to both the hypothermic and cataleptic effects produced by JWH-018. By day 5 of repeated treatments, the effects of JWH-018 became submaximal at all time points, and continued to decrease in the two remaining days. By day 7 of repeated treatments, the temperature and cataleptic effects JWH-018 were not significantly

different from vehicle-treated animals. Previous studies in mice have shown that repeated daily injections of THC or synthetic cannabinoids produce behavioral tolerance due to downregulation and desensitization of CB_1 receptors (37). Likewise, acute JWH-018 is reported to induce downregulation of CB_1 receptors in cultured neurons by a mechanism involving rapid receptor internalization (38).

The experiments of Tai et al. showed that mice develop tolerance to the hypothermic effects of JWH-018, but not the locomotor suppressing effects (39). The apparently contradictory findings between our results and those of Tai et al. may be due to species-specific differences between rats and mice. Tai et al. also showed that mice repeatedly exposed to THC develop a cross tolerance to the effects of JWH-018. The development of tolerance to cannabis is well documented, and the demonstration of tolerance to JWH-018 could have important clinical implications (40, 41). Dose escalation in human THC users is often observed as a means to overcome cannabis tolerance, but this phenomenon likely will not cause acute bodily harm. By contrast, dose escalation with JWH-018 or other potent synthetic cannabinoids could be more dangerous. Typical adverse effects arising from synthetic cannabinoid use are tachycardia, agitation, and nausea; more serious adverse events include seizures, acute kidney injury, new onset psychosis, severe cardiac crisis, and death (27, 42). Further research is required to determine if such dose escalation occurs in humans who use synthetic cannabinoids.

To summarize, we found that repeated treatment with the synthetic cannabinoid JWH-018 does not lead to significant changes in 5-HT_{2A} receptor responsiveness in rats, but produces transient increases in 5-HT_{1A} receptor responsiveness. These findings, unlike data generated using other synthetic cannabinoids, do not support the contention that exposure to cannabinoid receptor agonists universally leads to an increase in 5-HT_{2A} receptor responsiveness, suggesting that alteration of 5-HT_{2A} neurotransmission may not be responsible for the link between cannabinoid exposure and the subsequent development of psychotic symptoms. On the other hand, rats in our experiments developed tolerance to both hypothermia and catalepsy produced by JWH-018 after several consecutive days of treatment, findings which differ from prior work in mice suggesting that tolerance only develops to hypothermic effects. Synthetic cannabinoid tolerance in humans could potentially lead to dose escalation, which could be more dangerous with synthetic cannabinoids when compared to marijuana.

ETHICS STATEMENT

Experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Vivarium facilities were fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and study procedures were approved by the NIDA Intramural Research Program Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

JE and MB were responsible for experiment design, statistical analysis, and manuscript writing. JE collected the data.

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Psychostimulant Effect of the Synthetic Cannabinoid JWH-018 and AKB48: Behavioral, Neurochemical, and Dopamine Transporter Scan Imaging Studies in Mice

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JWH-018 and AKB48 are two synthetic cannabinoids (SCBs) belonging to different structural classes and illegally marketed as incense, herbal preparations, or chemical supply for theirs psychoactive cannabis-like effects. Clinical reports from emergency room reported psychomotor agitation as one of the most frequent effects in people assuming SCBs. This study aimed to investigate the psychostimulant properties of JWH-018 and AKB48 in male CD-1 mice and to compare their behavioral and biochemical effects with those caused by cocaine and amphetamine. *In vivo* studies showed that JWH-018 and AKB48, as cocaine and amphetamine, facilitated spontaneous locomotion in mice. These effects were prevented by CB_1 receptor blockade and dopamine (DA) $D_{1/5}$ and $D_{2/3}$ receptors inhibition. SPECT-CT studies on dopamine transporter (DAT) revealed that, as cocaine and amphetamine, JWH-018 and AKB48 decreased the [^{123}I]-FP-CIT binding in the mouse striatum. Conversely, *in vitro* competition binding studies revealed that, unlike cocaine and amphetamine, JWH-018 and AKB48 did not bind to mouse or human DAT. Moreover, microdialysis studies showed that the systemic administration of JWH-018, AKB48, cocaine, and amphetamine stimulated DA release in the nucleus accumbens (NAc) shell of freely moving mice. Finally, unlike amphetamine and cocaine, JWH-018 and AKB48 did not induce any changes on spontaneous [^{3}H]-DA efflux from murine striatal synaptosomes.

Abbreviations: AKB48, *N*-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide; DA, dopamine; DAT, dopamine transporter; JWH-018, naphthalen-1-yl-(1-pentylindol-3-yl)methanone; HAL, 4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one; haloperidol; GBR 12783, 1-(2-benzhydryloxyethyl)-4-[(E)-3-phenylprop-2-enyl] piperazine;dihydrochloride; NAc shell, nucleus accumbens shell; [^{123}I]FP-CIT, (^{123}I-2β-carbomethoxy-3β-(4-iodophenyl)-*N*-(3-fluoropropyl)nortropane); SCH23390, 8-chloro-3-methyl-5-phenyl-1,2,4,5-tetrahydro-3-benzazepin-7-ol.

The present results suggest that SCBs facilitate striatal DA release possibly with different mechanisms than cocaine and amphetamine. Furthermore, they demonstrate, for the first time, that JWH-018 and AKB48 induce a psychostimulant effect in mice possibly by increasing NAc DA release. These data, according to clinical reports, outline the potential psychostimulant action of SCBs highlighting their possible danger to human health.

Keywords: AKB48, cocaine, dopamine transporter, microdialysis, SPECT-CT imaging, JWH-018, synthetic cannabinoids, psychostimulants

INTRODUCTION

According to the European Drug Report, 100 new abused substances have been detected for the first time in 2016 (1). Recent literature reported that an incredibly huge number of synthetic cannabinoids (SCBs) has been detected and commonly abused in the US, Europe, and Australia as Marijuana substitutes (2). Indeed, they are not preferred over cannabis but recreationally used to circumvent legal, work- and cost-related obstacles.

The consumption of SCBs can cause adverse events that directly jeopardize the subjects' lives or promote harmful consequences as agitation, tachycardia, sudden cardiac arrest, and seizures along with liver and kidney failure. Suicide and self-injury have also been reported in individuals consuming SCBs (3).

JWH-018 (1-pentyl-3-(1-naphthoyl)indole) and AKB48 (*N*-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide), respectively, classified as naphthoylindoles and adamantylindazoles, have been seized in different countries (4, 5). *In vitro* binding studies shown that JWH-018 and AKB48 display nanomolar affinity for both CD-1 murine and human CB_1 and CB_2 receptors, presenting a slight preference for CB_2 receptors (6, 7). In particular, in CD-1 murine preparation, AKB48 and JWH-018 displayed a similar affinity for CB_1 receptors [Ki = 5.34 and 5.82 nM, respectively; (6)], while AKB48 showed a slightly higher affinity than JWH-018 [Ki = 9.53 and 3.24 nM, respectively; (6)] for human CB_1 receptors. Based on these findings, it seems likely that, compared to other SCBs, the two compounds might induce similar or higher *in vivo* effects.

CB_1 receptors are highly expressed as limbic regions, such as the ventral tegmental area (VTA), the nucleus accumbens (NAc), ventral pallidum and prefrontal cortex (PFC). SCBs probably act in these brain regions by modulating reward, addiction, and cognitive functions (8). In line with this view, several rodent studies showed that these compounds, similar to other drugs of abuse, affect the mesolimbic dopaminergic transmission (7, 9, 10) and influence conditioned behaviors (11, 12).

It has been reported that SCBs may have atypical side effects, often larger and more negative than those of natural cannabinoids. For example, as detected by National Poison Data System that tracks US poison control calls, agitation is the most common adverse effect of SCBs consumption observed in humans (3), while other reported side effects are irritability, sadness, restlessness, aggression, combativeness, and psychomotor agitation (13–15). Differently, high doses of Δ^9-THC or cannabis intoxication can cause, among other adverse events, xerostomia, injected conjunctivae, tachycardia, and psychotic effects (including hallucinations and paranoia) (14). Extreme agitation, irritability physical violence, convulsions, and nephrotoxicity have also been reported after SCBs consumption (16). Preclinical data have reported that JWH-018 (17), AKB48 (7) and other SCBs (7) increase, in a narrow range of doses, spontaneous locomotion in mice. This behavioral effect resembles the psychostimulant action of cocaine (18–22) and amphetamine (23–25). Moreover, previous *in vivo* microdialysis studies demonstrated that JWH-018, at the dose of 0.25 mg/kg i.p. [but not at lower (0.125 mg/kg i.p.) or higher (0.5 mg/kg i.p.) doses], increases dopamine (DA) transmission in the NAc shell but not in the NAc core and in the mPFC (9). Similar pharmacological properties were displayed by subsequent chemical generations of SCBs (7, 10, 26). However, the mechanism of action of JWH-018, AKB48, and their analogs is still not completely understood.

This study, by combining different experimental approaches, such as *in vitro* (binding), *in vivo* (behavioral tests, imaging and microdialysis) and *ex vivo* (synaptosome) ones, aimed at clarifying how these SCBs modulate dopaminergic signaling and whether these putative effects could be relevant for their locomotion facilitating properties. In particular, the effects of JWH-018 and AKB48 have been compared to those induced by cocaine and amphetamine, two psychostimulant drugs affecting the dopamine transporter (DAT) in a different way. Indeed, while cocaine acts as a DAT blocker by directly binding to DAT and, thus, preventing the translocation of DA, amphetamine competes with DA for binding to the empty transporter, leading to the reverse transport (efflux) of DA from the intracellular compartment to the synaptic cleft, thus exerting indirect effects [e.g., it reverses the action of VMAT2; (27)]. In view of the results obtained, the involvement of CB_1 receptor- and the D_1/D_2 receptor-mediated mechanisms in the behavioral effects induced by JWH-018 and AKB48 has also been evaluated.

MATERIALS AND METHODS

Animals

Male ICR (CD-1®) mice, 25–30 g (Harlan Italy; S. Pietro al Natisone, Italy), were group-housed (8–10 mice per cage; floor area per animal was 80 cm^2; minimum enclosure height was 12 cm) on a reverse12:12-h light-dark cycle, temperature of 20–22°C, and humidity of 45–55%; and were provided *ad libitum* access to food (Diet 4RF25 GLP; Mucedola, Settimo Milanese, Milan, Italy) and water. The experimental protocols performed in this study were in accordance with the new European

Communities Council Directive of September 2010 (2010/63/EU) a revision of the Directive 86/609/EEC and were approved by the Italian Ministry of Health and by the Ethical Committee of the University of Ferrara and of the University of Cagliari (*microdialysis studies*). Moreover, adequate measures were taken to minimize the number of animals used and their pain and discomfort.

Drug Preparation and Dose Selection

Amphetamine sulfate, cocaine, ketamine hydrochloride, JWH-018, and AKB48 were purchased from LGC Standards (LGC Standards S.r.L., Sesto San Giovanni, Milan, Italy), xylazine hydrochloride from Sigma-Aldrich (St. Louis, MO, USA) and GBR 12783 dihydrochloride, AM-251, SCH23390, and haloperidol from Tocris (Bristol, United Kingdom).

For *in vivo* behavioral studies, all compounds (JWH-018, AKB48, amphetamine sulfate, cocaine hydrochloride, AM-251, SCH23390, and haloperidol) were initially dissolved in absolute ethanol and Tween 80 and then diluted to the final volume with saline (0.9% NaCl; final ethanol or Tween 80 concentration = 2%) The ethanol, Tween 80, and saline solution were also used as vehicle. Drugs were administered by intraperitoneal injection in a volume of 4 μl/g. The used doses of JWH-018 (0.3 and 1 mg/kg i.p.) and AKB48 (0.3 and 1 mg/kg i.p.) were chosen based on previous studies (6, 7, 9, 10).

For *in vitro* release experiments, JWH-018 and AKB48 were dissolved in absolute ethanol (ethanol = vehicle; maximum concentration = 0.04% v/v). The used concentrations of JWH-018, AKB48, cocaine, and amphetamine were chosen on the basis of previous studies (7, 17, 28, 29). Moreover, for *in vivo* DaTSCAN, imaging studies, the [^{123}I]-FP-CIT (^{123}I-2β-carbomethoxy-3β-(4-iodophenyl)-*N*-(3-fluoropropyl)nortropane, [^{123}I]-IDaTSCAN) was purchased from GE Healthcare B.V. Den Dolech 2 NL-5612 AZ, Eindhoven, The Netherlands (specific activity 2.5–4.5 × 10^{14} Bq/mmol at the date and time of calibration; radiochemical purity >97%).

Spontaneous Locomotor Activity

The spontaneous locomotor activity was measured by using the ANY-maze video tracking system (Ugo Basile, application version 4.99 g Beta). The mouse was placed in a square plastic cage (60 cm × 60 cm) located in a sound- and light-attenuated room and motor activity was monitored for 240 min. Four mice were monitored in parallel in each experiment. Parameters measured were distance traveled (meter), total time in the peripheral zone (seconds), total time in the central zone (seconds), and immobility time (seconds; the animal was considered immobile when 95% of his image remained in the same place for at least 2 s). Parameters were analyzed every 15 min for a maximum of 240 min and to avoid mice olfactory cues, cages were carefully cleaned with a dilute (5%) ethanol solution and washed with water between animal trials. All experiments were performed between 9:00 a.m. and 1:00 p.m.

In Vivo DaTSCAN, Imaging Studies

SPECT-CT studies have been performed using a YAP(S)PET scanner (30–33). The spatial resolution of the system was verified for ^{123}I, using a NEMA NU 4-2008 phantom (34) with hot rods ranging from 1 to 5 mm. 18 CD-1 male mice were divided into six different groups (three mice per treatment). During the scanning procedure, each mouse was previously anesthetized by intramuscular injections of a mixture of ketamine and xilazine (respectively, 100 and 20 mg/kg), and submitted to a pretreatment (by intraperitoneal injection) with vehicle (see drug preparation and animal dose determination), cocaine (20 mg/kg), amphetamine sulfate (10 mg/kg), JWH-018 (1 mg/kg), or AKB48 (1 mg/kg). A control group (i.e., naïve untreated mice) was also included in the study. Thirty minutes after drug administration, all mice were submitted to an intravenous injection with a solution of [^{123}I]-DaTSCAN (15–20 MBq, ≤200 μl). The body temperature of the animals was maintained at 37°C during the imaging sessions and under the cage, between imaging sessions, using a heating lamp. The SPECT–CT whole-body images were acquired at 1 h and 30 min after [^{123}I]-FP-CIT injection, with the initial tomographic acquisition starting nearly 15 min after the injection. Each SPECT-CT whole-body acquisition consisted of one bed positions (36 mm), 60 min, 128 views over 360 (35). The used energy window is 119–219 keV and the images were reconstructed by using the iterative EM-ML algorithm, including the collimator response. CT images have been acquired, using the digital X-ray imaging system integrated into the YAP(S)PET scanner (36). Acquisition parameters for X-ray projections were X-ray tube voltage = 35 keV, anode current = 1 mA, exposure = 1 s, 64 views over 360, and magnification factor = 1.2. Subtraction of dark noise contribution and flat field corrections was accomplished to obtain final images. The CT data were reconstructed by using the FDK algorithm. Amide software (37) has been used for images' registration, visualization and analysis. The size of the ROIs was voxels (100 mm^3 volume), corresponding to entire striatum. These ROIs were used as a template. To avoid the variability of the slice selection and to gain statistical power, the entire striatum volume for the analysis was used. The template was positioned manually (without changing the size and form of the ROIs) on the SPECT images with the backing of anatomical information from LONI MAP 2003 MRI mouse atlas (38, 39). For analysis of striatal [^{123}I]-FP-CIT binding, two consecutive horizontal slices (total thickness approximately 4 mm) with the highest striatal binding were selected. The landmarks for positioning were the intra-orbital glands, striatum, and the borders of the brain. Striatal binding ratios are expressed as average activity per unit volume [Bq/mm^3], each value has been calculated as the ratio between the activity inside the ROI and the ROI volume, normalized for injected activity and for mouse brain weight.

[^{3}H]-WIN 35,428 Competition Binding Experiments

Competition binding experiments were carried out incubating 8 nM [^{3}H]-WIN 35,428 (specific activity 84 Ci/mmol; Perkin Elmer, Boston, MA, USA) with CHO membranes transfected with human DAT (Perkin Elmer) or mouse striatal synaptosomes with different concentration of the examined compounds for 120 min at 4°C. Non-specific binding was determined in the presence of 1 μM GBR 12783. At the end of the incubation time, bound and free radioactivity were separated by filtering

the assay mixture through Whatman GF/B glass fiber filters in a Brandel cell harvester (Brandel, Unterföhring, Germany). Filter bound radioactivity was counted in a Perkin Elmer 2810TR scintillation counter (Perkin Elmer).

In Vivo Brain Microdialysis Studies

Male ICR (CD-1®) mice, 25–30 g (ENVIGO. Harlan Italy; S. Pietro al Natisone, Italy) were anesthetized with Isoflurane (3%; 200 ml/min) and implanted with vertical dialysis probe (1 mm dialyzing portion) prepared with AN69 fibers (Hospal Dasco, Bologna, Italy) in the NAc shell (A + 1.4, L 0.4 from bregma, V-4.8 from dura) according to the mouse brain atlas by Paxinos and Franklin (40). On the day following surgery, probes were perfused with Ringer's solution (147 mM NaCl, 4 mM KCl, 2.2 mM $CaCl_2$) at a constant rate of 1 μl/min. Dialyzate samples (10 μl) were injected into an HPLC equipped with a reverse phase column (C8 3.5 um, Waters, USA) and a coulometric detector (ESA, Coulochem II) to quantify DA. The first electrode of the detector was set at +130 mV (oxidation) and the second at −175 mV (reduction). The composition of the mobile phase was as follows: 50 mM NaH_2PO_4, 0.1 mM Na_2-EDTA, 0.5 mM n-octyl sodium sulfate, 15% (v/v) methanol, pH 5.5. The sensitivity of the assay for DA was 5 fmol/sample. At the end of each experiment, animals were sacrificed and their brains removed and stored in formalin (8%) for histological examination to verify the correct placement of the microdialysis probe.

Striatal Synaptosome Preparation

On the day of the experiment, the animal was euthanized, the brain was rapidly removed, and both striata isolated. Thereafter, a crude synaptosomal (P2) fraction was prepared as follows: the striata were suspended in ice-cold buffered sucrose solution (0.32 M, pH 7.4) and homogenized. The homogenate was centrifuged (10 min, 2,100 *g*, 4°C) to remove nuclei and debris. The supernatant was further centrifuged at 13,500 *g* for 20 min at 4°C. For [^{3}H]-WIN 35,428 binding experiments, the P2 pellet was resuspended in 50 mM Tris–HCl, 100 mM NaCl, pH 7.4. For [^{3}H]-DA release experiments, the P2 pellet was then resuspended in 5 ml of Kreb's solution (mM: NaCl 118; KCl 4.4; $CaCl_2$ 1.2; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25; glucose 10), gassed 20 min with a mixture of 95% O_2 plus 5% CO_2 containing [^{3}H]-DA (50 nM; Perkin Elmer, Monza, Italy), disodium EDTA (0.03 mM), and ascorbic acid (0.05 mM; to prevent [^{3}H]-DA degradation).

Spontaneous [^{3}H]-DA Release

After synaptosomal preparation, 0.5 ml aliquots of the suspension were distributed on microporous filters placed at the bottom of a set of parallel superfusion chambers maintained at 37°C and perfused with aerated (95% O_2/5% CO_2) Kreb's solution (0.3 ml/min). After 30 min of superfusion to equilibrate the system, 5-min fractions were collected from the 30th to the 75th min (nine samples). When required, after the collection of three basal samples, amphetamine (10 μM), cocaine (100 nM), JWH-018 (100 nM, 1 μM), AKB48 (100 nM, 1 μM), and vehicle were added to the perfusion solution in order to evaluate their effects on spontaneous [^{3}H]-DA release. At the end of the experiment, the radioactivity of the samples and filters was determined by liquid scintillation spectrometry (LS1800 Beckman). In view of the results obtained, in a separate set of experiments, [^{3}H]-DA uptake was also evaluated.

[^{3}H]-DA Uptake Experiments

After synaptosomal preparation, the suspension was maintained under a light and continuous oxygenation (95% O_2, 5% CO_2) for 20 min at 37°C. Thereafter, 0.5 ml aliquots of striatal synaptosomal suspension were prepared. When required the selective DA reuptake blocker GBR 12783 (100 nM, Sigma-Aldrich, USA), cocaine (100 nM), amphetamine (1 μM), JWH-018 and AKB048 (100 nM, 1 μM), and vehicle were added and after 5 min the synaptosomes were incubated for 10 min with 50 nM [^{3}H]-DA. After this period, the reaction was stopped by filtration through microporus nylon filters (0.45 μm, 13 mm; Analytical Technology, Brugherio, Italy). The filters were then washed with 1 ml ice-cold Kreb's solution and the radioactivity accumulated on synaptosomes was extracted by eluting two times with 1 ml of warm NaOH (1 N) and then determined by liquid scintillation spectrometer. Non-specific uptake was measured by following the same procedure at 0°C.

RESULTS

Studies on Spontaneous Locomotor Activity in Mice

The acute i.p. administration of JWH-018 (0.3 mg/kg), amphetamine (10 mg/kg), and cocaine (20 mg/kg) induced long-lasting increases in the total distance traveled (i.e., spontaneous locomotion) by the mice, while AKB48 (1 mg/kg) facilitated the spontaneous locomotion only in the first 15 min after the injection [**Figure 1A**; significant effect of treatment ($F_{4,560} = 64.65$, $p < 0.0001$), time ($F_{15,560} = 120.40$, $p < 0.0001$), and time × treatment interaction ($F_{60,560} = 4.628$, $p < 0.0001$)]. In particular, the effects of JWH-018 or cocaine lasted 90 min, while amphetamine increased the mouse spontaneous locomotion also from 135 to 210 min after drug administration.

JWH-018, amphetamine, and cocaine reduced the immobility time in mice, while AKB48 increased it 30 min after the drug administration [**Figure 1B**; significant effect of treatment ($F_{4,560} = 199.3$, $p < 0.0001$), time ($F_{15,560} = 79.13$, $p < 0.0001$), and time × treatment interaction ($F_{60,560} = 10.39$, $p < 0.0001$)]. Differently to mice treated with cocaine and amphetamine, JWH-018- and AKB48-injected animals spent more time in the central zone [**Figure 1D**; significant effect of treatment ($F_{4,560} = 70.37$, $p < 0.0001$), time ($F_{15,560} = 32.48$, $p < 0.0001$), and time × treatment interaction ($F_{60,560} = 12.24$, $p < 0.0001$)] than in the peripheral area of the cage [**Figure 1C**; significant effect of treatment ($F_{4,560} = 9.751$, $p < 0.0001$), time ($F_{15,560} = 13.33$, $p < 0.0001$), and time × treatment interaction ($F_{60,560} = 4.394$, $p < 0.0001$)].

The facilitation of spontaneous locomotion induced by JWH-018 (0.3 mg/kg) and AKB48 (1 mg/kg) was prevented by a pretreatment with AM 251 [1 mg/kg i.p.; **Figure 1E**: significant effect of treatment ($F_{3,56} = 13.74$, $p < 0.0001$), time ($F_{1,56} = 31.88$,

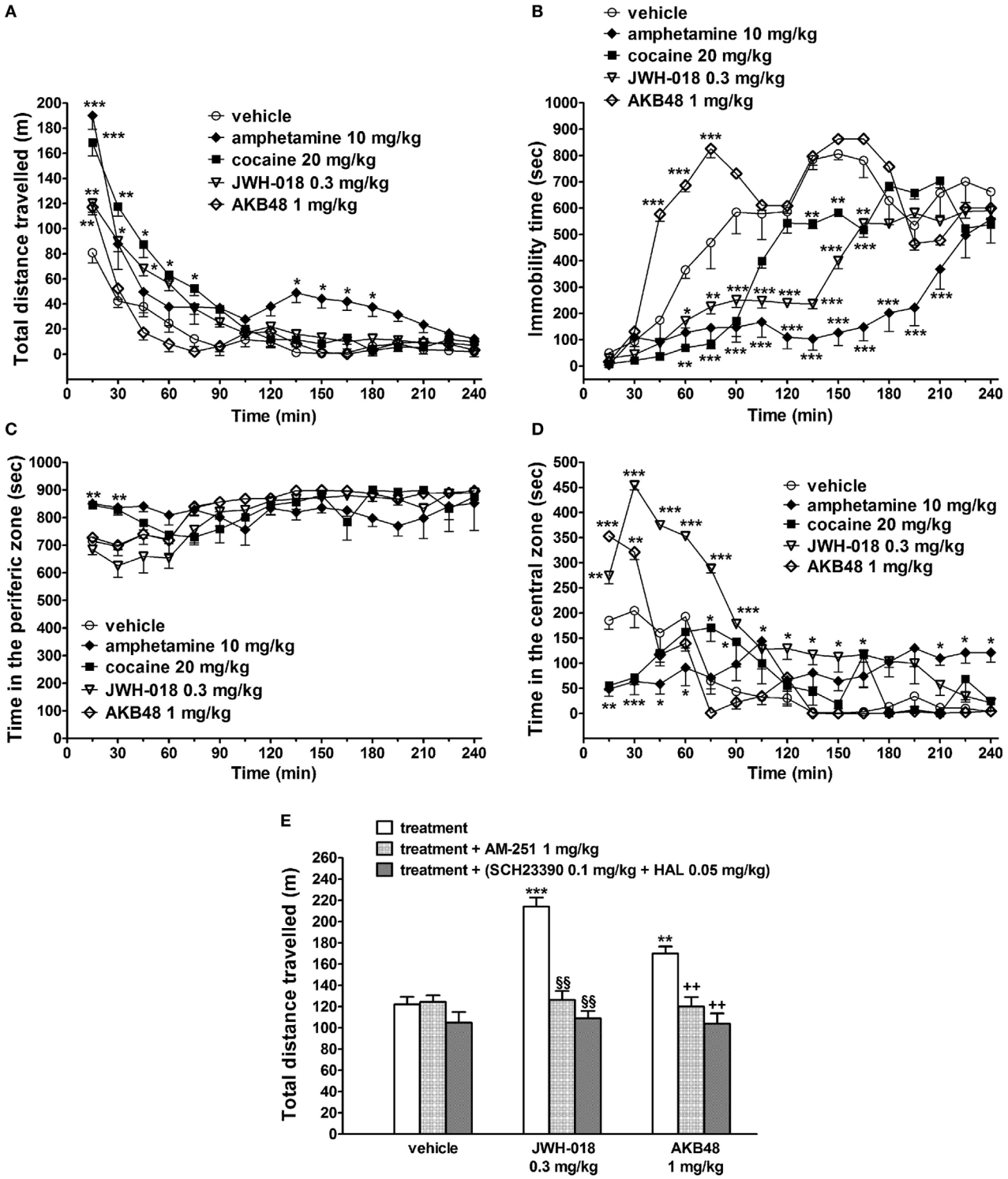

FIGURE 1 | Effect of the systemic administration of vehicle, amphetamine (10 mg/kg i.p.), cocaine (20 mg/kg i.p.), JWH-018 (0.3 mg/kg i.p.), and AKB48 (1 mg/kg i.p.) on the total distance traveled **(A)**, on the immobility time **(B)**, and on the total time spent in the peripheral and central area **(C,D)** of the mouse. Interaction of JWH-018 and AKB48 with the selective CB_1 receptor antagonist AM 251 [6 mg/kg, i.p.; **(E)**], the D_1 receptor antagonist SCH23390 [0.1 mg/kg i.p.; **(E)**], and the D_2 receptor antagonist haloperidol [HAL; 0.05 mg/kg i.p.; **(E)**]. AM 251, and SCH23390 + HAL were administered 20 min before synthetic cannabinoids injection. Data are expressed as meters (total distance traveled) and as seconds (immobility time; time in the peripheral and central zone). Data represent the mean ± SEM of eight determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparisons **(A–D)** or by one-way ANOVA followed by Tukey's test **(E)**. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus vehicle; $^{§§}p < 0.01$ versus JWH-018; $^{++}p < 0.01$ versus AKB48.

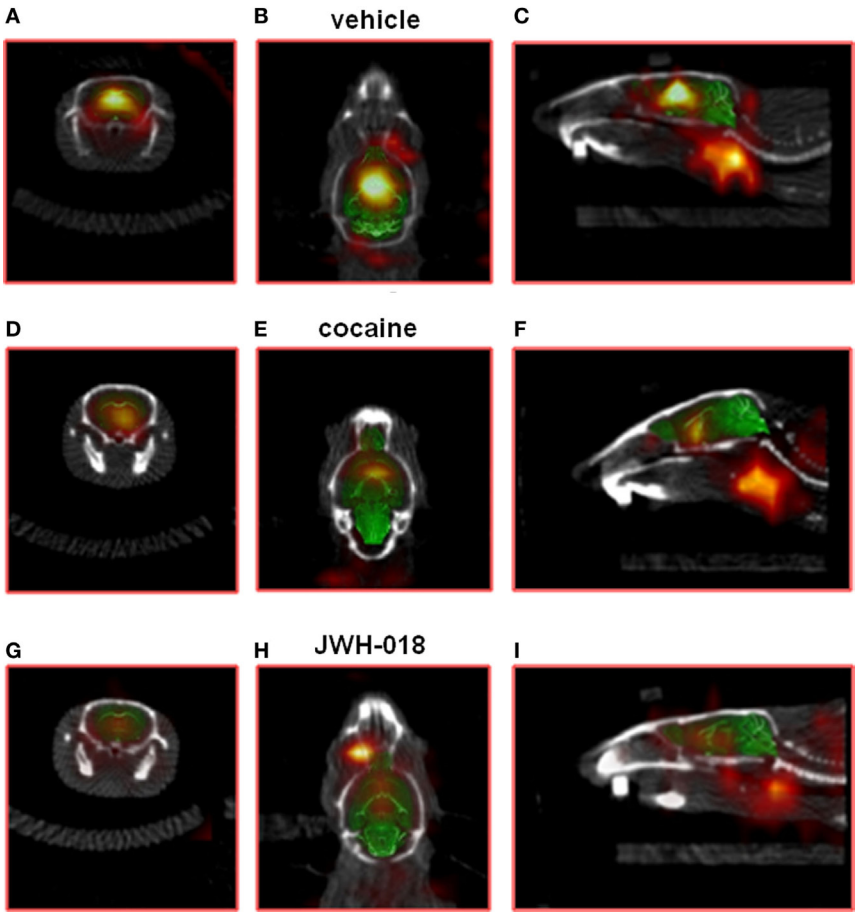

FIGURE 2 | Sample slice from a [^{123}I]-FP-CIT SPECT/CT image of a vehicle [**(A–C)**; respectively, coronal, transverse, and sagittal plan], cocaine [**(D–F)**; respectively, coronal, transverse, and sagittal plan], and JWH-018 [**(G–I)**; respectively, coronal, transverse, and sagittal plan] treated mice. ROIs for the striatum.

$p < 0.0001$), and time × treatment interaction ($F_{3,56} = 17.59$, $p < 0.0001$)] or by the coadministration of SCH23390 (0.1 mg/kg i.p.) and haloperidol [0.05 mg/kg i.p.; **Figure 1E**: significant effect of treatment ($F_{3,56} = 13.74$, $p < 0.0001$), time ($F_{1,56} = 31.88$, $p < 0.0001$), and time × treatment interaction ($F_{3,56} = 17.59$, $p < 0.0001$)]. AM 251, SCH23390, and haloperidol by themselves did not alter the spontaneous locomotion in mice (**Figure 1E**).

In Vivo DaTSCAN, Imaging Studies

Intense, symmetrical [^{123}I]-FP-CIT binding was observed in the striatum of control mice (*images not shown*). Vehicle injection did not change [^{123}I]-FP-CIT binding in the striatum of mice (**Figures 2A–C**). The acute systemic injection of cocaine (20 mg/kg i.p.; **Figures 2D–F**) or amphetamine (10 mg/kg i.p.; *images not shown*) induced significant decreases of the [^{123}I]-CIT binding in the striatum of mice (reduction of ~40 and ~25%, respectively; **Figure 3**). Similarly, the administration of JWH-018 (1 mg/kg i.p.; **Figures 2G–I**) or AKB48 (1 mg/kg i.p.; *images not shown*) decreased the [^{123}I]-FP-CIT binding in the striatum of mice (reduction of ~39 and ~42%, respectively; **Figure 3**); these effects were comparable to those caused by the administration of cocaine (**Figure 3**).

Competition Binding Experiments on Mice and Human DAT

Competition binding experiments with the reference compound GBR 12783 revealed that it displays a similar affinity for human and mouse DAT (**Table 1**). As expected, cocaine showed affinity for DAT in the nanomolar range, with Ki values of 174 and 193 nM in CHO membranes transfected with human DAT or mouse striatal synaptosomes, respectively. Amphetamine bound human and mouse DAT with affinity values of 554 and 622 nM, respectively. Interestingly, the SCBs JWH-018 and AKB48 were able to bind human DAT with affinity values of 7,183 and 4,588 nM, respectively (**Table 1**).

In Vivo Microdialysis Study

Basal NAc shell extracellular DA levels were 15 ± 5 fmol/10 µl sample. Systemic administration of amphetamine (10 mg/kg i.p.), cocaine (20 mg/kg i.p.), JWH-018 (0.3 mg/kg i.p.), and AKB48 (0.3 mg/kg i.p.) significantly increased NAc shell extracellular DA levels in the awake and freely moving mice (**Figures 4A–D**). Interestingly, JWH-018 or AKB48 had a

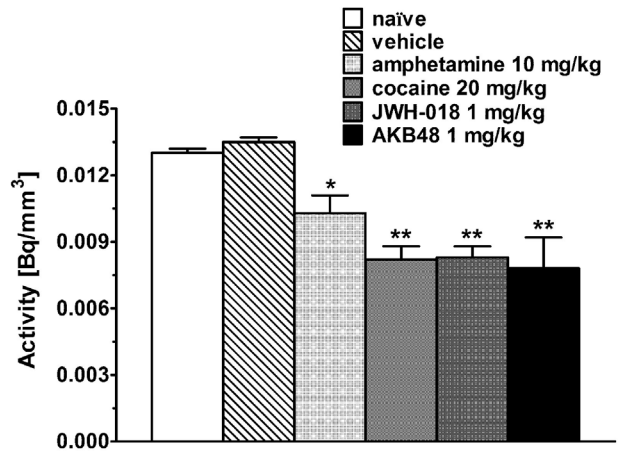

FIGURE 3 | Striatal uptake of [^{123}I]-FP-CIT in control mice (naïve) and in mice after the administration of vehicle, amphetamine (10 mg/kg), cocaine (20 mg/kg), JWH-018 (1 mg/kg), and AKB48 (1 mg/kg). Means and SDs are shown. Significance levels are $^{*}p < 0.05$, $^{**}p < 0.01$.

TABLE 1 | Affinity values of GBR 12783, cocaine, amphetamine, JWH-018, and AKB48 to DAT obtained from [^{3}H]-WIN 35,428 competition binding experiments in human CHO membranes transfected with DAT and in mouse striatal synaptosomes.

Compounds	hDAT-CHO membranes Ki (nM)	Mouse striatal synaptosomes Ki (nM)
GBR 12783	1.93 ± 0.14	1.72 ± 0.11
Cocaine	174 ± 13	193 ± 16
Amphetamine	554 ± 47	622 ± 53
JWH-018	7,183 ± 528	>10,000
AKB48	4,588 ± 326	>10,000

Data are expressed as mean ± SEM.

different profile of action. In fact, JWH-018 induced a long-lasting increase of NAc shell extracellular DA levels (~150% of baseline values; **Figure 4C**), while AKB48 caused a rapid and significant increase in extracellular DA levels in the NAc shell of mice, reaching a peak value (~150% of baseline values) 40 min (**Figure 4D**) after its administration.

Effects of Cocaine, Amphetamine, JWH-018, and AKB48 on Spontaneous [^{3}H]-DA Release in Striatal Synaptosomes

In synaptosomes from mouse striatum, spontaneous [^{3}H]-DA efflux tended to decrease during the collection period (from 30 to 75 min from the start of perfusion, **Figure 5**). As expected, the perfusion with amphetamine (10 μM), or cocaine (100 nM), induced a significant increase in spontaneous [^{3}H]-DA efflux from mouse striatal synaptosomes (**Figures 5A–C**). On the other hand, JWH-018 and AKB48 (100 nM and 1 μM) did not affect spontaneous [^{3}H]-DA efflux from striatal synaptosomes (**Figures 5B,C**, respectively).

Effects of Cocaine, Amphetamine, JWH-018, and AKB48 on [^{3}H]-DA Uptake

As shown in **Figure 6**, cocaine (1 μM) and amphetamine (1 μM) reduced [^{3}H]-DA uptake in mouse striatal synaptosomes in the order of 50 and 40%, respectively. At 20 nM, GBR 12783 produced a similar inhibition of [^{3}H]-DA uptake as found with 100 nM of cocaine. On the contrary, JWH-018 and AKB48 were ineffective on [^{3}H]-DA uptake at the tested concentrations (100 nM and 1 μM, **Figure 6**).

DISCUSSION

The present multidisciplinary study, for the first time, directly compared the effects of JWH-018 and AKB48, with those of cocaine and amphetamine, to provide further insights on the mechanism of action possibly underlying the psychomotor stimulant effects of SCBs.

The behavioral studies, first, showed that JWH-018 (0.3 mg/kg) e AKB48 (1 mg/kg) facilitated spontaneous locomotion in mice through CB_1 receptor- and DA-dependent mechanisms. In fact, the motor facilitation induced by the two SCBs was prevented by the CB_1 receptor antagonist AM-251 as well as by the simultaneous blockade of DA D_1 and D_2 receptors. The SCBs-induced motor facilitation probably occurs in a narrow range of doses since SCBs mainly inhibited both spontaneous and stimulated motor activity in CD-1 mice (6, 7, 10, 41, 42). Motor impairment is one of the main behavioral effects observed after systemic administration of cannabinoid receptor agonists (43, 44), and it has been associated with the stimulation of cerebellum and basal ganglia CB_1 receptors (43, 45, 46). However, preclinical studies reported that cannabinoid receptor agonists time- and dose-dependently modulated rodent spontaneous locomotion in a biphasic fashion, with a facilitation and an inhibition at low and high doses, respectively. This biphasic effect has been displayed by the endocannabinoid anandamide (47), Δ^9-THC (41, 48) along with the synthetic compounds WIN 55,212-2 (44), JWH-018-R (17), 5 F-ADBINACA, AB-FUBINACA, and STS-135 (42), suggesting that it is typical of the cannabinoid system and not of a single molecule class (43).

Although the acute administration of either JWH-018 (0.3 mg/kg) or AKB48 (1 mg/kg) induced a prompt facilitation of mouse spontaneous locomotion, the profile of action of the two compounds is different. In particular, while the effect of JWH-018 is long-lasting, AKB48 only induces a transitory (15 min) increase, after which the inhibitory effect of the compound prevails, as evidenced by the significant increase in the animal's immobility time (**Figure 1B**). These diverse profiles are probably due to the different doses of JWH-018 (0.3 mg/kg) and AKB48 (1 mg/kg) used, and to their pharmacokinetic, rather than pharmacodynamics, properties (*see also below*). It seems likely that the steric hindrance of the adamantly group of AKB48 delays the passage through the blood–brain barrier or limits a quick bond to CB_1 receptors. Furthermore, although JWH-018 [Ki = 5.82 nM; (6)] and AKB48 [Ki = 5.34 nM; (7)] show similar nanomolar affinity for CD-1 mouse CB_1 receptor, their *in vivo* behavioral responses are quantitatively different, being JWH-018 more effective (7).

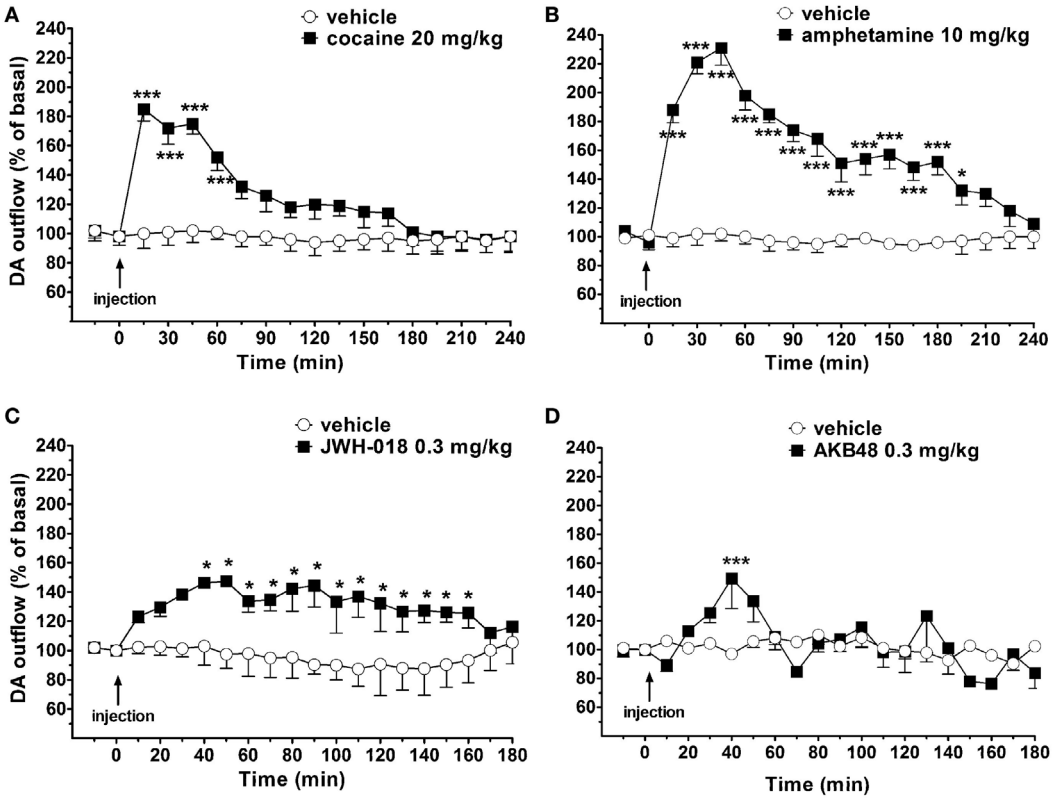

FIGURE 4 | Effect of the systemic administration of cocaine [20 mg/kg i.p.; **(A)**], amphetamine [10 mg/kg i.p.; **(B)**], JWH-018 [0.3 mg/kg i.p.; **(C)**], and AKB48 [0.3 mg/kg i.p.; **(D)**] on dopamine (DA) transmission in the nucleus accumbens (NAc) shell of mice. Results are expressed as mean ± SEM of change in DA extracellular levels expressed as the percentage of basal values. $^{*}p < 0.05$, $^{***}p < 0.001$ versus vehicle (NAc shell $n = 13$) (two-way ANOVA, Tukey's HSD *post hoc*).

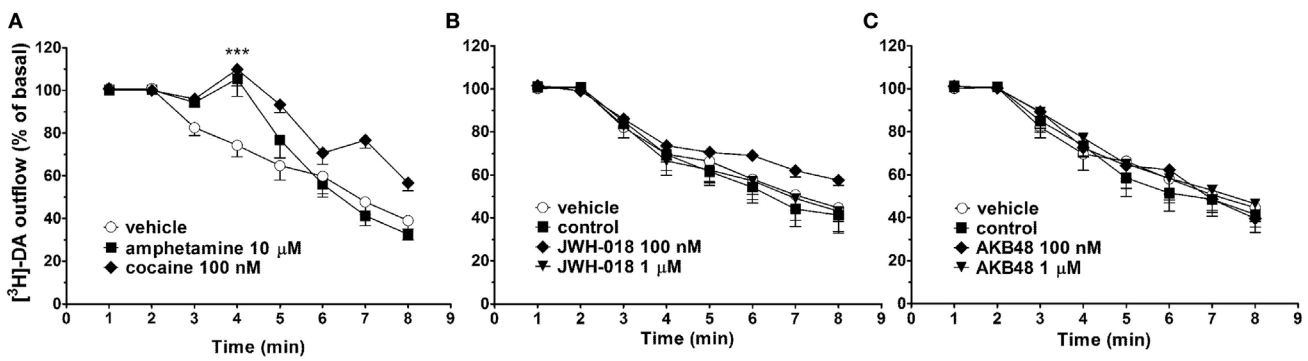

FIGURE 5 | Effect of cocaine [100 nM; **(A)**], amphetamine [10 μM; **(A)**], JWH-018 [100 nM and 1 μM, **(B)**], and AKB48 [100 nM and 1 μM, **(C)**] on spontaneous [^{3}H]-dopamine (DA) efflux from striatal synaptosomes obtained from CD-1 mice. Data are expressed as percentage of basal values and represent the mean ± SEM of 4–6 repetitions for each treatment. $^{***}p < 0.001$ significantly different from the respective control group according to ANOVA followed by Newman–Keuls test for multiple comparisons.

Normally, rodents tend to move in the perimeter of an arena (i.e., thigmotaxis), thus, spending there more time than in the center of the apparatus. As from an ethological point of view, a mouse that spends more time in an open space is less concerned about being attacked by predators. In fact, the animal's occupancy of the peripheral areas, either in corners or near the walls, has been identified as an index of "timidity" (49) or "anxiety" (50, 51). The present behavioral data also demonstrate that JWH-018 and AKB48 qualitatively increase the mouse spontaneous motor activity (total distance traveled) in a similar way to cocaine (18–22) and amphetamine (23–25). However, in respect to cocaine and amphetamine, the two SCBs displayed a different behavioral

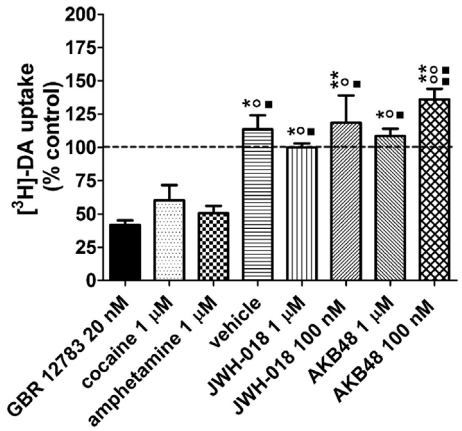

FIGURE 6 | Effects of cocaine (1 μM), the selective dopamine (DA) reuptake blocker GBR 12783 (20 nM), amphetamine (1 μM), JWH-018 (1 μM, 100 nM), and AKB48 (1 μM, 100 nM) on [^{3}H]-DA uptake in striatal synaptosomes from CD-1 mice. The drugs were added to synaptosomes 5 min before [^{3}H]-DA and uptake was measured for 10 min at 37°C. A same volume of drug vehicle (Kreb's solution or ethanol) was added 5 min before [^{3}H]-DA incubation in the control/vehicle groups, respectively. The effect of the treatments on [^{3}H]-DA uptake is expressed as percent of control values, i.e., tritium content measured in untreated synaptosomal aliquots, always assayed in parallel (100 ± 3%, $n = 4$; indicated by a dashed line). Unspecific uptake was measured at 0°C. Each treatment bar represents the mean ± SEM of four determinations ran in duplicate. $^{**}p < 0.01$, $^{*}p < 0.05$ significantly different from GBR 12783 20 nM; $^{\circ\circ}p < 0.01$, $^{\circ}p < 0.05$ significantly different from amphetamine 1 μM; $\blacksquare\blacksquare p < 0.01$, $\blacksquare p < 0.05$ significantly different from cocaine 100 nM according to one-way ANOVA followed by Newman–Keuls test for multiple comparisons.

profile as assessed by evaluating the mouse arena's exploration. In fact, unlike the two psychostimulants, SCBs increase the animal's standing time at the center of the arena, suggesting an "anxiolytic-like" profile in the open field context (52, 53). This behavior, unusual for the mouse, suggests that the administration of SCBs may cause a reduction in the danger perception (54). This finding is in line with previous data demonstrating that CB_1 receptor agonists, at least at low doses, induced anxiolytic effects in rodents (52, 55–57). However, it cannot be ruled out that the motor stimulation effect associated with motor sensory impairment caused by JWH-018 and AKB48 (7, 41) may lead the mouse to a loss of sensory contact with the walls of the box and to the consequent disoriented movements into the open space of the arena. In fact, spatial information collected by tactile sensations and integrated in visual control in rodents play a pivotal role of spatial orientation (58, 59). Conversely, cocaine and amphetamine increase the time spent in the peripheral arena, suggesting an "anxiogenic-like" effect, which is typical of stimulant substances promoting catecholaminergic transmission (60, 61). This anxiogenic-like behavior causes greater alertness and attention in the mouse by promoting the combat and flight behavior that is typical of non-predatory animals, such as the mouse (54).

As reported above, JWH-018- and AKB48-induced increases in motor activity were prevented by pretreatment with SCH23390 ($D_{1/5}$ receptor antagonist) and haloperidol ($D_{2/3}$ receptor antagonist), thus suggesting that increased DA transmission underlies the SCBs motor-stimulant properties. This is consistent with the implication of dopaminergic mechanisms in the motor-stimulant properties of amphetamine and cocaine (19, 62, 63). In view of this, along with the different behavioral profile of action of the compounds under investigation, *in vivo* and *in vitro* experiments have been performed in order to evaluate their effects on dopaminergic system. Interestingly, *in vivo* DaTSCAN imaging studies demonstrated that, similarly to amphetamine and cocaine, either JWH-018 or AKB48 administration decreased the [^{123}I]-FP-CIT binding to DAT in mice striatum. In consideration of this finding, *in vitro* experiments have been performed to verify the possible direct interaction between the two SBCs and DAT. In fact, previous data proposed that both cannabinoid agonists and antagonists inhibit DAT activity *via* molecular targets other than CB_1 receptors (64). The present *in vitro* competition binding experiments clearly indicated that, unlike cocaine and amphetamine, JWH-018 and AKB48 did not bind to DAT expressed in mouse striatal nerve terminals, while they showed only a low affinity (micrometer range) for human DAT in CHO transfect cell membranes. The affinity values of cocaine and amphetamine for human DAT, observed in this present study, are in line with literature data (65, 66). Despite various paper reported the affinity values of GBR 12783, cocaine, and amphetamine in rat striatum, this is the first study, to our knowledge, reporting [^{3}H]-WIN 35,428 competition binding experiments of these compounds in mouse striatal synaptosomes, where they show affinity values similar to those found on human DAT. In line with the binding results, this study also demonstrates that, in contrast to cocaine and amphetamine, neither JWH-018 nor AKB48, at the concentration tested, significantly affected [^{3}H]-DA uptake from murine striatal synaptosomes. This is in apparent contrast with some literature data showing that cannabinoids significantly reduces DA uptake in striatal nerve terminals or slices (64, 67, 68). However, in line with the present results, a previous study (69) failed to observe alterations of DA uptake following treatment of mouse striatal synaptosomes with some SCBs. Although other possibility cannot be definitely ruled out, it seems likely that these discrepancies could be due to the different experimental conditions used in the reported studies (i.e., different cannabinoid receptor agonists, different drug concentrations, different DA concentration, and time of incubation).

Taking into account the above *in vitro* results, the possibility that the observed JWH-018- or AKB48-induced reduction of [^{123}I]-FP-CIT signal in the mice striatum is due to a direct interaction between the SCBs and DAT seems unlikely. A logical alternative explanation is that JWH-018 or AKB48 systemic administration induces an increase in the levels of endogenous DA which, in turn, competes with [^{123}I]-FP-CIT for DAT. This hypothesis is supported by the present *in vivo* microdialysis results, showing that the systemic administration of a low dose of JWH-018 (0.3 mg/kg) or AKB48 (0.3 mg/kg) stimulated extracellular DA levels in the NAc shell of freely moving mice. In particular, either JWH-018 or AKB48 caused a maximal increase to ~150% of baseline DA concentrations. However, in line with the drug behavioral profile, the effect of JWH-018 was long-lasting, while the effect of AKB48 was transient. As expected, either cocaine or amphetamine also increased DA extracellular levels and their

effects were significantly higher than those of the two SCBs. It is well established that the mechanism of action of these classes of drugs is different. Indeed, classical psychostimulants, as cocaine and amphetamine, increase DA neurotransmission by inhibiting the DAT activity in DA nigrostriatal and mesolimbic neuronal terminals; in particular the psychostimulant-induced increase in DA neurotransmission is mainly due to DA reuptake inhibition, an enhancement of DA release or to a combination of the two mechanisms (70–78). On the contrary, SCBs increase NAc shell DA release mainly through indirect CB_1 receptor-mediated mechanisms. In fact, while CB_1 receptors are not expressed on midbrain DA neurons (79), CB_1 receptor activation closely modulates DA neuronal activity, through modulation of local circuitry in the midbrain (80). In mesolimbic DA pathway, CB_1 receptors are located in axon terminals forming either inhibitory or excitatory-type synapses with dopaminergic as well as non-dopaminergic, putative GABAergic, neurons in the VTA, and systemic administration of CB_1 receptor agonists enhances the bursting activity of VTA DA neurons, many of which project to the NAc shell (81). It has been reported that, by reducing the activity of GABAergic terminals, cannabinoids can facilitate dopaminergic activity through suppression of inhibitory input onto $GABA_A$ or $GABA_B$ receptors on DA neurons (80). In line with this, *ex vivo* whole cell patch clamp recordings from rat VTA DA neurons showed that JWH-018 decreases $GABA_A$-mediated post-synaptic currents, suggesting that the stimulation of DA release observed *in vivo* might result from a disinhibition of DA neurons (26, 82, 83). The different mechanisms underlying the SBCs- or psychostimulants-induced DA release are confirmed by the present *in vitro* studies on striatum, including NAc, nerve ending. In fact, accordingly to their direct or indirect inhibitory modulation of DAT activity and DA-releasing effects, either amphetamine or cocaine significantly increased [^{3}H]-DA efflux from mouse striatal synaptosomes. In this context, it is worth noting that under the present experimental conditions (i.e., 0.3 ml/min flow rate) DA levels in the perfusate have been reported to represent the net consequence of [^{3}H]-DA release and reuptake (84). Differently, JWH-018 and AKB48 did not induce any effects on spontaneous [^{3}H]-DA efflux from murine striatal synaptosomes. These findings are in line with previous data showing that the CB_1/CB_2 cannabinoid receptor agonists WIN 55,212-2 and CP 55,940 had no effects on basal and electrically evoked DA release in the corpus striatum and the NAc slices (85). The lack of a presynaptic effect on terminals of nigrostriatal and mesolimbic dopaminergic neurons is also in accord with the absence of CB_1 receptor on dopaminergic terminals (*see above*). Taken together, these findings indicate that, at least at the concentration tested, the two SCBs did not affect the DAT activity, leading to hypothesize that their inhibitory effects on the [^{123}I]-FP-CIT binding to DAT in the mice striatum could be a consequence of an increase in endogenous DA levels.

CONCLUSION

The present data demonstrate, for the first time, that JWH-018 and AKB48 induce psychostimulant effects in mice possibly related to the facilitation of NAc DA release induced by the two compounds. Although the motor activation induced by the tested SCBs or the two classical psychostimulants involve dopaminergic mechanisms, it seems likely that the two classes of compound recruit different neurochemical pathways in mouse nigrostriatal and mesolimbic regions. These data, according to clinical reports, outline the potential psychostimulant action of SCBs highlighting their possible danger to human health (16, 86–89).

ETHICS STATEMENT

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in the studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. In particular, the experimental protocols performed in this study were in accordance with the new European Communities Council Directive of September 2010 (2010/63/EU) a revision of the Directive 86/609/EEC and were approved by the Italian Ministry of Health and by the Ethical Committee of the University of Ferrara and of the University of Cagliari (microdialysis studies).

AUTHOR CONTRIBUTIONS

Substantial contributions to the conception (AO, MM, LF, and MDL) or design of the work (AO, MM, LF, MDL, and KV); or the acquisition (AO, LU, SBilel, IC, GD, MP, GP, MDL, FV, and SBeggiato), analysis (AB, CR, PB, FD-G, and GS), or interpretation of data for the work (AO, MM, LF, and MDL); drafting the work or revising it critically for important intellectual content (AO, LU, SBilel, IC, GD, MP, GP, MDL, AB, FV, CR, SBeggiato, LF, KV, PB, GSF, F-DG, and MM); final approval of the version to be published (AO, LU, SBilel, IC, GD, MP, GP, MDL, AB, FV, CR, SBeggiato, LF, KV, PB, GF, FD-G, and MM); and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved (AO, LU, SBilel, IC, GD, MP, GP, MDL, AB, FV, CR, SBeggiato, LF, KV, PB, GS, FD-G, and MM).

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Mephedrone (4-Methylmethcathinone): Acute Behavioral Effects, Hyperthermic, and Pharmacokinetic Profile in Rats

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Mephedrone (MEPH) is a synthetic cathinone derivative with effects that mimic MDMA and/or cocaine. Our study in male Wistar rats provides detailed investigations of MEPH's and its primary metabolite nor-mephedrone's (nor-MEPH) pharmacokinetics and bio-distribution to four different substrates (serum, brain, lungs, and liver), as well as comparative analysis of their effects on locomotion [open field test (OFT)] and sensorimotor gating [prepulse inhibition of acoustic startle reaction (PPI ASR)]. Furthermore, in order to mimic the crowded condition where MEPH is typically taken (e.g., clubs), the acute effect of MEPH on thermoregulation in singly- and group-housed rats was evaluated. Pharmacokinetics of MEPH and nor-MEPH after MEPH (5 mg/kg, sc.) were analyzed over 8 h using liquid chromatography with mass spectrometry. MEPH (2.5, 5, or 20 mg/kg, sc.) and nor-MEPH (5 mg/kg, sc.) were administered 5 or 40 min before the behavioral testing in the OFT and PPI ASR; locomotion and its spatial distribution, ASR, habituation and PPI itself were quantified. The effect of MEPH on rectal temperature was measured after 5 and 20 mg/kg, sc. Both MEPH and nor-MEPH were detected in all substrates, with the highest levels detected in lungs. Mean brain: serum ratios were 1:1.19 (MEPH) and 1:1.91 (nor-MEPH), maximum concentrations were observed at 30 min; at 2 and 4 h after administration, nor-MEPH concentrations were higher compared to the parent drug. While neither of the drugs disrupted PPI, both increased locomotion and affected its spatial distribution. The effects of MEPH were dose dependent, rapid, and short-lasting, and the intensity of locomotor stimulant effects was comparable between MEPH and nor-MEPH. Despite the disappearance of behavioral effects within 40 min after administration, MEPH induced rectal temperature elevations that persisted for 3 h even in singly housed rats. To conclude, we observed a robust, short-lasting, and most likely synergistic stimulatory effect of both drugs which corresponded to brain pharmacokinetics. The dissociation between the duration of behavioral and hyperthermic effects is indicative of the possible contribution

of nor-MEPH or other biologically active metabolites. This temporal dissociation may be related to the risk of prolonged somatic toxicity when stimulatory effects are no longer present.

Keywords: mephedrone, 4-methylmethcathinone, nor-mephedrone, pharmacokinetics, open field, prepulse inhibition, thermoregulation, Wistar rat

INTRODUCTION

Mephedrone (4-methylmethcathinone, 4-MMC; MEPH, hereafter), a synthetic derivative of cathinone was first synthetized in 1929 with the aim of developing this compound for therapeutic purposes (1). At the turn of the twenty-first century MEPH was rediscovered by recreational users (as a so-called "new psychoactive substance": NPS) and owing to its psychoactive effects, it became widely used as party drug known under the street name "meow meow" (2, 3). Based on users' reports, MEPH's effects are very similar to amphetamine, to 3,4-methylenedioxymethamphetamine (MDMA) and to cocaine, or their combination (4–6). MEPH's effects are rapid and of relatively short duration depending on the administration route (intranasal: ~30 min, oral: ~2–3 h) (7, 8), resulting in a tendency for recreational users to re-dose, as is the case with cocaine (9, 10). Prolonged and/or poly-drug use [including "slamming"—intravenous injection of MEPH combined with other drugs (11)] may be associated with adverse psychological (e.g., paranoia, depression, panic attacks), cardiovascular, or renal effects (12, 13). Furthermore, at least 90 deaths have been documented where MEPH alone (or its combination with other psychoactive compounds) was implicated (14–17). In 2010, MEPH was classified as a controlled substance in some European countries, and 2 years later in the USA (7). Despite its ban, it has remained a popular recreational drug to this day (18, 19).

Mephedrone acts as non-selective monoamine uptake inhibitor and releaser with dopamine transporter: serotonin transporter (DAT: SERT) inhibition ratio being 1.4, which led authors to label MEPH as mixed MDMA-cocaine-like compound (20, 21). However, while MEPH's uptake of dopamine (DA) is roughly equivalent to that of serotonin (5-HT), it is (such as MDMA or cathinone) several times more potent at nor-epinephrine transporter (NET) with NET: DAT ratio being approximately 13 (20). MEPH is also active on vesicular monoamine transporters 2, where its activity is approximately 10 times less potent than MDMA (22). Partly contrasting the transporter studies, according to *in vivo* microdialysis studies in nucleus accumbens (NAcc), MEPH had approximately twofold greater effect on 5-HT than DA release (23, 24). Furthermore, MEPH also has some activity at serotonin 5-HT_{2A}, noradrenaline $\alpha_{1,2}$ and trace amine associated receptor ($TAAR_1$). Affinity for DAT together with its high blood–brain barrier permeability (twofold greater than amphetamine and MDMA) (20) and direct effects on DA in NAcc make MEPH a compound with high addictive potential, which is confirmed by users (10, 20, 25, 26) and by animal studies (27–29). Its strong affinity for NET then might be indicative of cardiovascular toxicity (7).

Mayer et al. (30), using *in vitro* assays, showed that the phase I metabolites 4-methylcathinone (nor-mephedrone (nor-MEPH) hereafter), 4-hydroxytolylmephedrone (4-OH-MEPH) and dihydromephedrone also have measureable activity at DAT, NET, and SERT, although of these, only nor-MEPH and 4-OH-MEPH at a range meaningful for behavioral tests. Therefore, bioactive metabolites can also contribute to MEPH's effects. However, this was previously confirmed only for nor-MEPH, which displayed *in vivo* behavioral stimulatory activity (30).

In rodent models, MEPH administration leads to dose-dependent increases in locomotion [reviewed in Ref. (7)]. The intensity and duration of these changes is comparable to those observed after the same dose of MDMA, but lesser than amphetamine's effects (23, 24). MEPH's effect on sensorimotor gating has only been evaluated in a chronic administration paradigm by Shortall et al. (31); in order to mimic weekend type recreational use of drugs, they administered MEPH (1, 4, or 10 mg/kg) twice a week on two consecutive days for 3 weeks and tested prepulse inhibition of acoustic startle reaction [PPI ASR; a behavioral operationalization of sensorimotor gating (32)]; 30 min (min) after the final injection; this yielded no disruptive effect. On the other hand, related drugs, such as MDMA, amphetamine, cocaine, also cathinone itself, and methylone, have shown some disruptive effects in this paradigm (33–39). No information currently exists on MEPH's acute effect nor the effects of its metabolites on PPI.

Studies of MEPH effects on thermoregulation are inconsistent in their results; both hyperthermic (Sprague-Dawley rats (24, 27)) and hypothermic (40) responses have been documented. Alteration of body temperature is an effect that is dose- and environment-dependent in the case of MDMA and related compounds [e.g., Ref. (38, 39, 41, 42)]. In two of our previous studies, we have found that serotonergic compounds, along with severe hyperthermia, can induce profound sweating, particularly when rats are housed in cages in groups (38, 41). Group-housing mimics the crowded conditions in clubs where drugs, such as MDMA and MEPH are typically used. It is generally known that the hyperthermia associated with the use of these compounds is one of the key preceding conditions of neurotoxicity as well as of acute somatic toxicity related to serotonin syndrome (43). Therefore detailed examination of dose-related interactions with environmental conditions (such as crowding) is necessary in order to elucidate inconsistencies in MEPH's effects on thermoregulation.

Our main intention was to enrich current knowledge of MEPH by detailed description of the temporal characteristics of its behavioral effects in relation to its pharmacokinetics and bio-distribution and to investigate effects of its major active metabolite nor-MEPH. To describe the temporal profile of behavioral changes, two testing-onsets (5 or 40 min after drug administration) were used to register both peak and prolonged drug effects. Stimulatory locomotor effects, exploration and/or anxiogenic/anxiolytic potential were tested in the open field test (OFT) and the effects on sensorimotor gating were measured in

PPI ASR. Alongside this, pharmacokinetic profile of MEPH and nor-MEPH in brain and serum, and their bio-distribution to liver and lungs were established, over 8 h. To evaluate MEPH's effects on thermoregulation under crowded and isolated environmental conditions, rectal temperatures were measured over 8 h in groups of five rats versus rats housed alone.

MATERIALS AND METHODS

Animals

Male outbred Wistar rats (VELAZ, Czech Republic) weighing approximately 180–250 g were housed in pairs under controlled conditions (light/dark arrangement: 12/12 hours, temperature: 22 ± 2°C, humidity: 30–70%) with *ad libitum* water and standard diet. In each study, rats acclimatized to the laboratory facility for seven days, with tests performed in the seven days following. Therefore, testing/sampling occurred when rats were approximately 10–11 weeks old (adult) and they were in the laboratory for approximately 10–14 days in total. During the acclimatization period, rats were handled four times and weighed twice. Experiments and measurements were conducted in the light phase of the cycle (between 07:00 and 15:00 h). Experimental groups consisted of 10 individuals, each rat was tested only once, with the exception that to reduce the number of animals used, rats treated by MEPH/nor-MEPH in behavioral studies were subsequently used for pharmacokinetic sampling. Hence, only eight additional rats were needed (for 30 min post-drug administration samples).

Drugs and Chemicals

Mephedrone was purchased *via* the internet and subsequently purified and converted to MEPH hydrochloride by Alfarma s.r.o. (Czech Republic). The resulting MEPH was certified to be of 99.18% purity (analyzed by infrared spectroscopy) and also served as a reference standard for pharmacokinetic analyses using liquid chromatography. Nor-MEPH was synthesized at the Department of Organic Chemistry, Faculty of Chemical Technology (University of Chemistry and Technology Prague, Czech Republic) at a purity of 99.18%. Internal standards MEPH-D7.HCl and nor-MEPH-D7.HCl for quantitative liquid chromatography/mass spectrometry (LC/MS) assays were synthesized at the Department of Organic Chemistry, Faculty of Chemical Technology (University of Chemistry and Technology Prague, Czech Republic). Extraction columns (Bond Elut Certify 50 mg/3 ml) were supplied by Labicom s.r.o., Olomouc. Other chemicals used for laboratory purposes were of analytical grade purity. MEPH was stored in dry and dark place and dissolved in physiological saline (0.9% NaCl) immediately before experiments.

Dosage

The doses for subcutaneous (sc.) administration were estimated with respect to the amounts usually used by humans, reported potency/affinity at transporters and based on our previous studies with related compounds especially MDMA, MDAI, and related ring-substituted cathinone methylone (35, 38, 39, 44, 45). Furthermore, we set these doses with the intention to mimic the dosage comparable to human use and intermediate—high dose with expected strong acute effect, but non-lethal toxicity. Finally, the doses were also adequately adjusted for interspecies differences according the formula suggested by Reagan-Shaw et al. (46). All substances were dissolved in vehicle (0.9% physiological saline) at a volume of 2 ml/kg administered sc. (for comparability with our previous studies). Rats used for pharmacokinetic sampling were treated by MEPH 5 mg/kg. MEPH 5 or 20 mg/kg was used in the temperature monitoring study, and MEPH 2.5, 5, or 20 mg/kg and nor-MEPH 5 mg/kg were used in behavioral tests. As vehicle controls (VEH) animals were treated with an equivalent volume of 0.9% physiological saline.

Pharmacokinetics

For pharmacokinetics, rats were administered MEPH (5 mg/kg sc.) and subsequently decapitated after 30, 60, 120, 240, or 480 min (n = 8/experimental group). Sera, brain, liver, and lung tissues were collected and stored at −20°C until analysis.

Determination of MEPH and Nor-MEPH Levels in Serum and Tissue Samples Using LC/HRMS

Serum Pretreatment

0.2 ml of rat serum was fortified with the internal standard MEPH-D7 and nor-MEPH-D7 in methanolic solution (in an amount with respect to the levels of MEPH/nor-MEPH in assayed samples) and 0.5 ml of a 0.1 M phosphate buffer (pH 6) in a labeled tube.

Tissue Pretreatment

250 mg of tissue (brain, lung, liver) was homogenized with 5 ml methanol and the internal standard MEPH-D7 and nor-MEPH-D7 (in an amount with respect to the MEPH/nor-MEPH levels in samples). Each specimen was then ultrasonicated for 20 min and after supernatant separation by centrifugation, the supernatant was transferred into a clean labeled tube and evaporated to dryness. The residue was reconstituted in 0.1 M phosphate buffer (pH 6). For solid-phase extraction (SPE) of MEPH/nor-MEPH, a pretreated sample of serum or tissue, along with the buffer and internal standard, was loaded onto a Bond Elut Certify cartridge previously conditioned with 0.5 ml of 0.1 M phosphate buffer (pH 6). After application of each pretreated sample, the cartridge was washed with 0.5 ml of distilled water, 0.5 ml of 0.1 M HCl and 0.5 ml of CH_3OH/H_2O (1/1, v/v) and then air-dried for 5 min. The analytes were eluted three times with 0.5 ml of a freshly prepared mixture of dichloromethane/2-propanol/ammonium hydroxide (25%), 80/20/4, v/v/v. The eluate was gently evaporated to dryness under a stream of air at 40°C and then dissolved into mobile phase for LC/HRMS analysis.

LC/HRMS Conditions

The analyses were performed using Dionex Ultimate 3000 UHPLC coupled to an Exactive Plus-Orbitrap MS (ThermoFisher Scientific, Bremen, Germany) equipped with a HESI-II source. The chromatographic analyses of the serum and tissue samples were performed using a Kinetex PFP 100 A (50 × 2.1 mm, 2.6 mm) and Security Guard Cartridge PFP 4 × 2.0 mm (Phenomenex) with a flow rate of 400 ml/min, and gradient elution with 10 mM

ammonium formate in 0.1% of formic acid as the mobile phase B. Gradient 0 min 5%, 4 min 45% B, 5–6 min held at 95%. The MS conditions were as follows: full MS in scan range of 50–500 *m/z* with positive electrospray ionization, resolution of 70000 FWHM (full width at half-maximum, scan speed 3 Hz), spray voltage of 3 kV, and an ion transfer capillary temperature of 320°C.

Behavior: Open Field and PPI

Open Field

The OFT was performed in accordance with our previous studies (38, 47). An empty black square arena (68 cm × 68 cm × 30 cm) was used, which was virtually divided into a 5 × 5 grid of identical squares; 16 squares were located near the arena walls (comprising the peripheral zone), and 9 squares were situated centrally (comprising the central zone). Rats were placed individually into the center of the arena 5 or 40 min after the drug administration (testing-onset) and their behavior was recorded for 30 min (nor-MEPH-treated rats were tested at the 5 min testing-onset only). The software EthoVision Color Pro v. 3.1.1 (Noldus, Netherlands) was used to capture the raw data used in the calculation of the following dependent variables: trajectory length (cm; corrected for deviations of <3 cm) and its temporal dynamics in 5 min intervals; thigmotaxis ($\sum f_{\text{peripheral zones}}/\sum f_{\text{all zones}}$, where f = frequency of appearance in the zone) reflects the probability of appearance in the peripheral zone; T_{center} reflects time spent centrally ($\sum \text{time}_{\text{centralzones}}$).

Prepulse Inhibition

Prepulse inhibition was evaluated in two identical startle chambers (SR-LAB, San Diego Instruments, CA, USA) each consisting of a sound-proof, evenly lit, ventilated enclosure with a Plexiglas stabilimeter (8.7 cm inner diameter). The experimental design was adopted from our previous studies [e.g., Ref. (38, 41, 47)]. Briefly, 2 days before testing, rats were acclimatized to the startle chamber with a drug-free 5 min pre-training procedure consisting of 5 pulse alone stimuli (115 dB/20 ms) presented over background white noise (75 dB). Startle data were not recorded for acclimatization. On the test day, the testing session was initiated 5 or 40 min after drug administration (only 5 min for nor-MEPH). The test session consisted of 72 trials in total with an inter-trial interval (ITI) of 4–20 s (mean ITI: 12.27 s). After 5 min exposure to a continuous 75 dB background white noise, six 125 dB/40 ms duration pulse alone trials were delivered to establish baseline ASR (for later calculation of habituation). Following this, 60 trials of the following were presented in a pseudorandom order: (A) pulse alone: 40 ms/125 dB; (B) prepulse–pulse: 20 ms/83 dB or 20 ms/91 dB prepulse with a variable (30, 60, or 120 ms) inter-stimulus interval (ISI: mean = 70 ms), then 40 ms/125 dB pulse; (C) 60 ms no stimulus. Finally, six pulse alone trials were delivered. Habituation was expressed as the percentage reduction in ASR from the initial six baseline trials, to the final six trials. PPI was calculated as follows: [100 − (mean prepulse − pulse trials/mean pulse alone trials) × 100]. Mean ASR was obtained from pulse alone trials. All measures were derived from the average of the area under the curve in arbitrary units (AVG). Animals with a mean ASR (AVG) response lower than 10 were excluded from analyses as non-responders.

Body Temperature

To evaluate the possible interactive effect of drugs and environmental conditions, we measured rectal temperatures in rats housed singly or in groups of five per cage. In total, 13 measurements were conducted as follows: three drug-free hourly measurements (07:00–09:00 h) followed by administration of (MEPH 5 or 20 mg/kg or VEH) at 09:00 h, then four 30 min measurements (09:30–11:00 h), and finally six hourly measurements (12:00–17:00 h). A digital thermometer was used; each rat was briefly (max. 10 s) immobilized in a Plexiglas tube during the procedure. Rats were kept under controlled laboratory conditions (temperature: 22 ± 2°C, humidity: 30–70%) in the experimental room throughout the study (which was where all temperature measurements were taken).

Statistics

All statistical analyses were performed using the data analysis software system STATISTICA version 9.1. [StatSoft, Inc. (2010)]. Tests used a default alpha set at $p = 0.05$, two tailed. Behavioral and thermoregulation studies used factorial designs; therefore, analysis of variance (ANOVA) or analysis of covariance (ANCOVA) were used. Where these yielded significant main effects involving a factor with >2 levels or significant interactions, pair-wise *post hoc* comparisons were conducted using Newman–Keuls tests.

Behavioral Data (OFT and PPI)

Open field test spatial distribution (thigmotaxis and T_{center}) and PPI parameters (habituation, ASR, and PPI) were each analyzed using a 2 × 4 factorial ANOVA with testing-onset (5 or 40 min) and drug treatment (VEH or MEPH 2.5, 5, and 20 mg/kg sc.) as between subjects factors. In the case of significant main effects on ASR or habituation, the significant factor was included as a covariate in subsequent analysis of PPI data (using ANCOVA). The temporal pattern of locomotor activity in the OFT (trajectory length in 5 min blocks) was analyzed using a 2 × 4 × 6 mixed factorial ANOVA with testing-onset and drug treatment as between subjects factors, and time blocks (6 × 5 min) as a within-subjects factor.

Additional analyses to compare the potency of nor-MEPH to MEPH were analyzed using one-way ANOVA with five drug treatment levels (VEH or nor-MEPH 5 mg/kg or MEPH 2.5, 5, and 20 mg/kg sc.) as a between-subjects factor. For the OFT, the temporal pattern of locomotor activity was analyzed using a 5 × 6 mixed factorial ANOVA with drug treatment as a between subjects factor and 5 min time blocks as a within subjects factor. Only data from the 5 min testing-onset were used in this analysis (because data for the 40 min testing-onset were not available for all drug treatments).

Body Temperature

Data were analyzed using 3 × 2 × 13 mixed factorial design with drug treatment (VEH or MEPH 5 or 20 mg/kg) and home-cage

condition (singly- or group housed) as between subjects factors and time (13 measurements) as a within subjects factor.

RESULTS

Pharmacokinetics

The maximum mean MEPH serum concentration (826.2 ng/ml) was attained within 30 min. Influx into the brain was not evidently delayed compared to serum; maximum mean concentration in the brain tissue (767 ng/g) was also attained by 30 min after the dose. MEPH robustly accumulated in lung: concentration at 30 min was 1,044.5 ng/g, exceeding concentrations in sera, brain, and liver. Four hours after administration, the levels in sera and all tissues were almost undetectable (**Figure 1A**).

The maximum mean nor-MEPH (metabolized from MEPH *in vivo*; recall that nor-MEPH itself was not administered in pharmacokinetic studies) serum concentration of 351.9 ng/ml was attained within 1 h of treatment. The maximum mean concentration in the brain (197.1 ng/g) was also evident at 30 min. Nor-MEPH accumulated in lung tissue with a maximum mean concentration of 382.9 ng/g observed at 30 min. Six hours after administration, nor-MEPH was only slightly above the level of detection in all tissues and plasma (**Figure 1B**).

Mean brain: serum ratio was 1:1.19 for MEPH and 1:1.91 for nor-MEPH throughout the whole temporal observation.

Behavior

Open Field Test

Analysis of locomotion revealed a main effect of drug treatment [F (3, 72) = 24.754, $p < 0.001$], testing-onset [F (1, 72) = 72.042, $p < 0.001$] as well as blocks [F (5, 360) = 101.67, $p < 0.001$]. All interactions were significant, including the three-way drug × testing-onset × blocks interaction [minimum F (15, 360) = 2.979, $p < 0.001$]. The three-way interaction was explored further; at the 5 min testing-onset, while the normal pattern of locomotor habituation (i.e., a progressive decrease in activity over the session) was evident in all groups, *post hoc* tests showed that all MEPH-treated rats were hyperactive (compared to VEH) across the six time blocks ($p < 0.001$) (**Figure 2A**). At the 40 min testing-onset, elevated activity was no longer present ($p > 0.05$), although rats still showed normal locomotor habituation (**Figure 2B**). Additional analysis of total locomotion including nor-MEPH (5 min testing-onset) confirmed a significant main effect of drug treatment [F (4, 45) = 27.699, $p < 0.001$], blocks [F (5, 225) = 50.171, $p < 0.001$], and their interaction [F (20, 225) = 3.350, $p < 0.001$]. *Post hoc* tests showed that nor-MEPH 5 mg/kg rats displayed elevated activity (compared to VEH) across all six time blocks ($p < 0.001$) (**Figure 2A**). For typical trajectory patterns induced by the treatments see **Figure 2C**.

The effects of drug treatment, testing-onset, and their interaction were each significant for both T_{center} [minimum F (3, 72) = 5.385, $p < 0.01$] and for thigmotaxis [minimum F (3, 72) = 6.792, $p < 0.001$]. Additional one-way ANOVA analyses with nor-MEPH confirmed an effect of drug treatment on T_{center} [F (4, 45) = 26.845, $p < 0.001$] and thigmotaxis [F (4, 45) = 48.704, $p < 0.001$]. *Post hoc* tests showed that the 5-min testing-onset, MEPH 2.5 and 5 mg/kg-treated rats spent more time in the center ($p < 0.001$) compared to VEH. Thigmotaxis was reduced after MEPH 5 mg/kg and nor-MEPH 5 mg/kg ($p < 0.001$), and increased after MEPH 20 mg/kg ($p < 0.001$) (**Figures 3A,B**). No such significant effects were observed at the 40 min testing-onset (data not shown). Finally, MEPH 5 mg/kg treated rats spent more time in the center ($p < 0.001$) and exhibited lower thigmotaxis ($p < 0.001$) at the 5 min compared to 40 min testing-onset; this pattern was absent in the rest of the groups (data not shown).

Prepulse Inhibition

Acoustic startle reaction was not affected by drug treatment or testing-onset, or their interaction [maximum F (1, 72) = 3.322, $p > 0.05$; see **Table 1**]. Analysis of habituation data revealed a main effect of drug treatment [F (3, 72) = 3.345, $p < 0.05$]; *post hoc* tests revealed reduced habituation in MEPH 2.5 mg/kg rats compared to VEH ($p < 0.05$); the other MEPH doses did not differ from VEH. There was also a significant main effect of testing-onset [F (1, 72) = 6.405, $p < 0.05$] manifested as

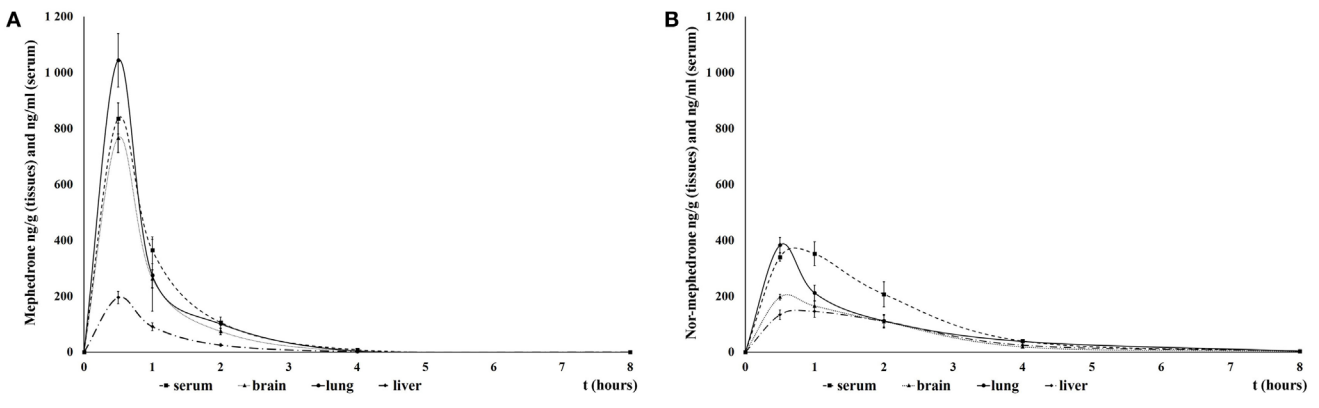

FIGURE 1 | Mean mephedrone (MEPH) **(A)** and its metabolite nor-mephedrone **(B)** levels in serum, brain, lungs, and liver over 6 h after application of MEPH 5 mg/kg sc. Error bars display ±1 SEM.

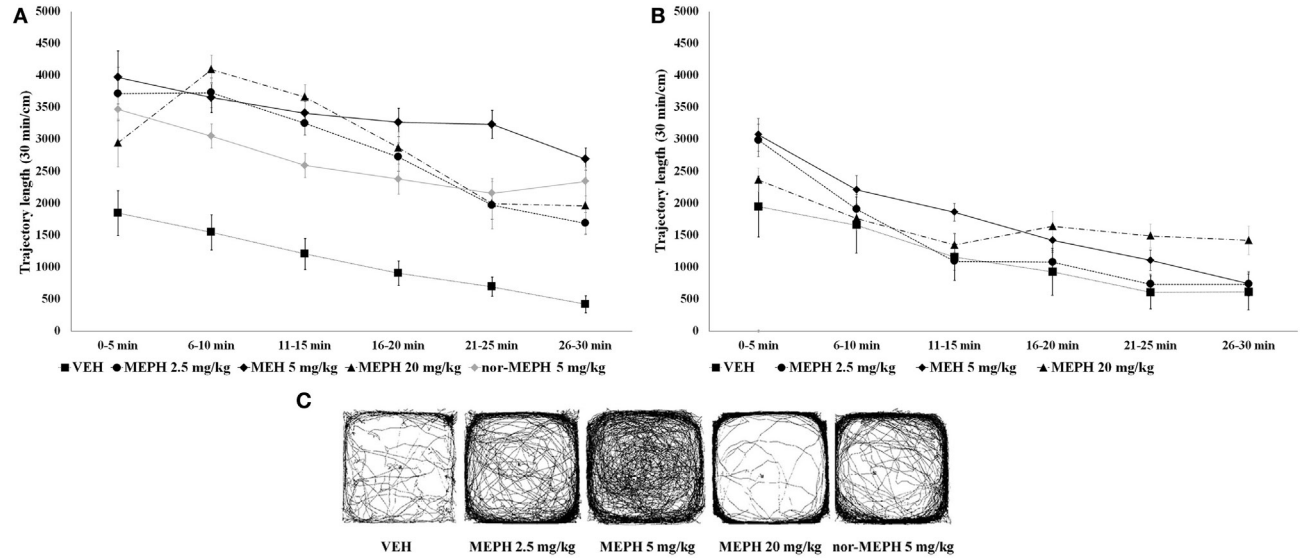

FIGURE 2 | Open field test (OFT): mean trajectory length (divided into 5-min blocks) by testing-onsets [5 and 40 min; **(A)** and **(B)**, respectively] and drug treatments [vehicle controls (VEH), mephedrone (MEPH) 2.5, 5, and 20 mg/kg and nor-mephedrone (nor-MEPH 5 mg/kg)]. Compared to VEH, significant hyperactivity ($p < 0.001$ for all drug groups and in all time blocks) was present at the 5-min testing-onset **(A)**, however the treatment effects were no longer significant at the 40 min testing-onset **(B)**. Error bars display ±1 SEM. Picture inserts below **(C)** show typical trajectory patterns induced by the treatment in animals with 5-min testing-onset.

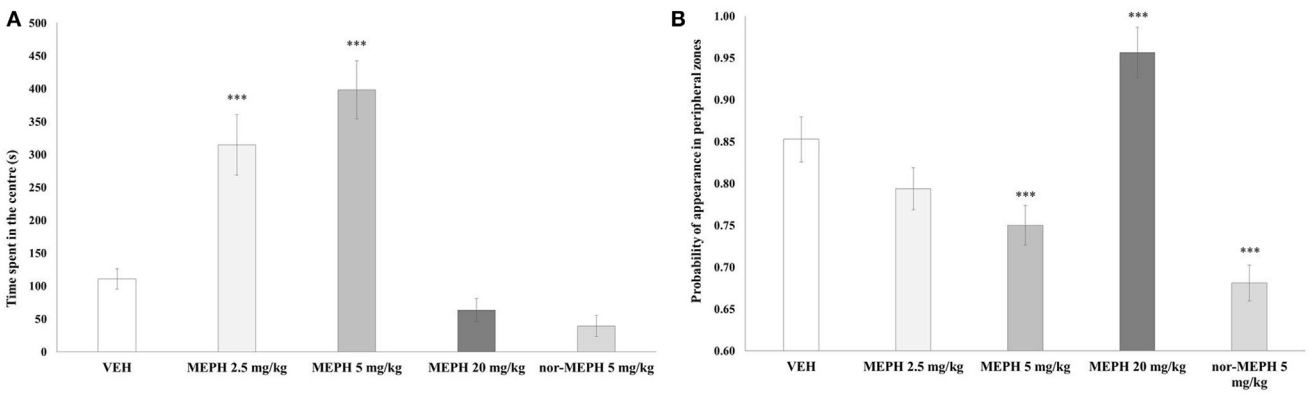

FIGURE 3 | Mean time spent in the arena center [T_{center}, **(A)**] and mean probability of appearance in peripheral zones [thigmotaxis, **(B)**] after vehicle controls (VEH), mephedrone (MEPH) 2.5, 5, and 20 mg/kg, and nor-mephedrone (nor-MEPH) 5 mg/kg administered at the 5-min testing-onset. MEPH 2.5 and 5 mg/kg-treated rats spent significantly more time in the central zones compared to VEH, and thigmotaxis was decreased by MEPH 5 mg/kg and nor-MEPH 5 mg/kg, and increased by MEPH 20 mg/kg. Error bars display ±1 SEM. ***$p < 0.001$ compared to VEH.

reduced habituation at the 5 min testing-onset compared to 40 min. The drug treatment × testing-onset interaction was not significant.

Since there were significant effects of drug treatment and testing-onset on habituation, it was included as a covariate in PPI analyses. PPI was not affected by the drug treatment or testing-onset, while their interaction was significant [$F(3, 71) = 3.483$, $p < 0.05$]. At the 40 min testing-onset, means suggested some disruption of PPI (MEPH 5 and 20 mg/kg); however, *post hoc* tests comparisons showed that differences from VEH were only marginal ($p = 0.062$, $p = 0.081$, respectively). There were no clear differences in means (eye-balling the data) at 5 min that seemed likely to account for the significant interaction; since a further one-way ANOVA was planned to explore effects of MEPH (alongside nor-MEPH) on PPI, further *post-hoc* tests on the 5 min testing-onset data were not conducted at this time. This additional one-way ANOVA showed no significant effect of treatment (MEPH or nor-MEPH) on PPI at the 5 min testing-onset [$F(4, 45) = 0.696$, $p > 0.05$]; therefore, the marginal effects at 40 min must explain the previous interaction. Similarly, there was no effect of MEPH

TABLE 1 | Mean values of acoustic startle reaction (ASR) amplitude and percentage of prepulse inhibition (PPI) after vehicle controls (VEH), mephedrone (MEPH), and nor-mephedrone (nor-MEPH) by testing-onsets (5 and 40 min).

Measure	Testing-onsets (min)	Drug treatment				
		VEH	MEPH 2.5 mg/kg	MEPH 5 mg/kg	MEPH 20 mg/kg	nor-MEPH 5 mg/kg
ASR	5	104.5 (14.3)	117.5 (17.4)	155.5 (32.7)	110.5 (14.2)	72.1 (11.5)
	40	137.2 (20.0)	140.8 (26.4)	144.6 (22.3)	173.9 (24.8)	–
% PPI	5	36.8 (5.4)	32.8 (5.6)	31.1 (6.2)	31.3 (4.1)	30.2 (6.5)
	40	41.3 (3.7)	41.1 (2.1)	25.1 (7.5)	28.4 (3.3)	–

Numbers represent means and SEMs are shown in brackets. Differences between testing-onsets and drug treatments were non-significant.

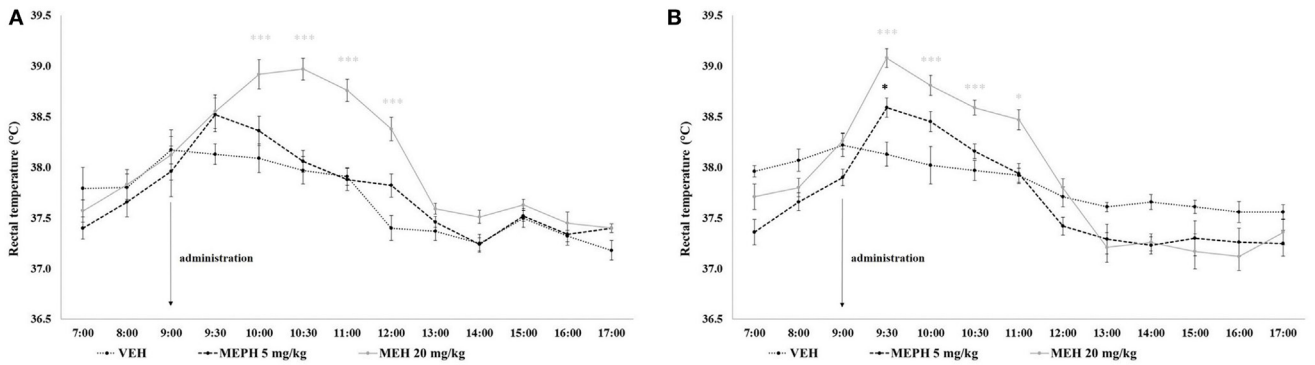

FIGURE 4 | Mean rectal temperature (°C) over 10 h after vehicle control (VEH), and mephedrone (MEPH) 5 and 20 mg/kg treatments for rats housed singly **(A)** or in groups of five **(B)**. Substances were administered at 09:00 h. Temperatures of rats treated by 5 mg/kg did not differ from VEH, except for the short-term elevation in the first 30 min after the administration in group-housed rats. The increase induced by 20 kg/kg was maintained from 10:00 to 12:00 h in singly housed rats and from 09:30 to 11:00 h in group-housed rats. Error bars display ±1 SEM. $^{*}p < 0.05$, $^{***}p < 0.001$ compared to VEH.

or nor-MEPH on ASR [$F(4, 45) = 2.454$, $p > 0.05$] or habituation [$F(4, 45) = 1.912$, $p > 0.05$] at the 5-min testing-onset.

Body Temperature

Rectal temperature was significantly affected by drug treatment [$F(2, 54) = 9.409$, $p < 0.001$] and time [$F(12, 648) = 124.560$, $p < 0.001$] but not home-cage condition [$F(1, 54) = 0.127$, $p > 0.05$]. All interactions were significant including the three-way drug treatment × time × home-cage interaction [minimum $F(12, 648) = 2.406$, $p < 0.010$]. *Post-hoc* tests revealed no significant differences between MEPH 5 mg/kg and VEH groups, except the elevation (~0.5°C) which occurred in the first 30 min after administration in group-housed rats ($p < 0.05$). Compared to VEH, MEPH 20 mg/kg induced modest elevation (~0.4°C) in singly-housed rats that appeared in the first 30 min after administration; however, it became statistically significant 30 min later and the effect was maintained for the next 2 h (~1°C; minimum $p < 0.001$). In group-housed rats, the elevation became significant within first 30 min and remained increased for next 2 h (~1°C; minimum $p < 0.001$)—**Figure 4**.

DISCUSSION

Mephedrone quickly peaked in the serum and was rapidly incorporated into all tissues, with lungs showing the highest concentrations and liver the lowest. MEPH was almost undetectable in serum and tissue by 4 h after its administration. Nor-MEPH had a similar profile; however the concentrations of nor-MEPH decreased more gradually in comparison to the parent drug (with MEPH, a steep decrement occurred immediately after the peak). Therefore, compared to MEPH, the elimination of nor-MEPH was slightly delayed. Acute administration of both compounds resulted in dose-dependent stimulatory effects, disrupted habituation, and altered the spatial distribution of locomotor behavior in the open field; however, there was no significant effect on PPI. MEPH induced dose- and environment-dependent increases in rectal temperature (of up to ~1°C) in both group-housed rats (as expected), but also in singly housed rats, where temperature remained elevated for 3 h after administration of the highest MEPH dose.

Pharmacokinetics

In their study with iv. administration, Aarde et al. (29) showed that MEPH peaked in the brain within 2 min; since the most pronounced locomotor effects in our study were present within 5–10 min of administration, it is likely that the peak concentration in serum also occurred earlier than suggested by our pharmacokinetic study (where the first measurement was at 30 min after the sc. administration). As expected, we detected the highest serum levels of both compounds in our dataset slightly

earlier compared to oral administration, where MEPH peaked in serum within 45 min–1.5 h after administration (48). The speed of crossing the blood–brain barrier by MEPH implied by our current results was consistent with Aarde et al. (29); as shown by others (20), MEPH easily crosses blood–brain barrier and, thus, influx into brain and lung tissues is most likely due to its lipophilic profile. This finding is also consistent with the pharmacokinetics of another ring-substituted cathinone, methylone (39) as well as with the phenethylamines 2C-B and PMMA, aminoindanes such as MDAI where highest tissue concentrations were detected in lungs and brains (41, 49, 50). Not surprisingly, since nor-MEPH is not the only one major metabolite, it reached lower overall serum and tissue levels than the parent drug and the slope of its elimination was less steep, resulting in higher serum and brain concentrations compared to MEPH 3 h after its administration. One possible explanation could be the slightly higher polarity of nor-MEPH leading to slower crossing of the blood–brain barrier (30) and, theoretically, nor-MEPH may, therefore, be responsible for some delayed or prolonged effects of MEPH.

Behavioral Effects: Open Field and PPI

In line with pharmacokinetics, locomotor stimulant effects declined quickly, so MEPH and nor-MEPH lacked any significant stimulatory effects 40 min after administration. The rapid action of MEPH observed here is in line with other rodent studies (23, 28, 30) and reports from human users (10). Since MEPH and nor-MEPH have both been shown to act on DAT (23, 30), it is most likely the underlying cause of these effects (51). MEPH and nor-MEPH seemed to be behaviorally equipotent. The fact that the effects lasted a very short time (due to fast kinetics) may increase the likelihood of re-dosing by humans and, together with its strongly reinforcing effects (shown in self-administration studies), indicates highly addictive characteristics (10).

Spatial characteristics of the trajectory after MEPH showed bi-directional effects dependent on the dose used. While increased exploration of the central zones following lower doses might imply decreased anxiety, increased thigmotaxis following the highest dose could suggest the opposite (52, 53). Compared to our findings, studies measuring anxiety using the elevated plus-maze (EPM) revealed contradictory results including either increased anxiety after acute treatment with low doses [0.25–10 mg/kg (54)], or no effect after sub-chronic MEPH treatment with very high doses (30 mg/kg twice a day) (55, 56). Direct comparison of anxiety measures in the OFT versus EPM, however, may be difficult. While some authors report a good comparability (57) others have questioned this (58). In our study, spatial trajectory characteristics may be also affected by other mechanisms, such as increased stereotyped behaviors (e.g., circling the perimeter of the arena) such as was also observed in our previous studies with other related compounds (38, 41, 47).

In accordance with previous research (31), we did not see any significant effect of acute MEPH or nor-MEPH on PPI. When our data are compared with similar data sets from phenethylamines, cathinones and aminoindanes performed in our laboratory, it is evident that that the more serotonergic the drug is [e.g., according to their DAT: SERT inhibition ratios (20)], the more pronounced the disruptive effect on PPI. While MDMA, PMMA, and MDAI significantly disrupted PPI at the lowest doses used (35, 38, 41), which have mild-to-moderate stimulatory effects and do not induce stereotyped circling in the OFT, amphetamine and MDPV was effective only at the highest dose used where stereotyped behaviors were also evident [(37); unpublished observation Horsley et al.]. MEPH has also shown some activity at 5-HT_{2A} receptor (20), however, it is not clear whether it acts as agonist or antagonist. In relation to this, disruption of PPI is typically seen after administration of various 5-HT_{2A} agonists, serotonergic hallucinogens, such as LSD, mescaline, psilocybin, 2C-B or DOI, etc., and it is known that antagonists at this receptor can reinstate normal PPI (37, 59–63). Similarly, MDMA-induced PPI deficits in rats can be also normalized by 5-HT2A antagonists (64, 65), therefore suggesting a role for this receptor subtype in PPI; if MEPH acts as an antagonist at 5-HT2A receptors, this might theoretically be protective against psychomimesis.

Temperature

The hypothesis that MEPH, such as other cathinones (7), has a potency to alter thermoregulation was supported by evidence in our study. It is in line with reports of recreational users suffering from adverse effects related to altered peripheral thermoregulation, such as cold-blue fingers, hot flushes, and/or intensive sweating (9, 26). Likewise comparable preclinical studies [for review, see Green et al. (7)], we observed significant hyperthermia in both singly housed as well as group-housed rats under normal room temperature (22 ± 2°C). In contrast to our expectations, the temperature increase was almost identical (~1°C) in both groups but had slightly longer duration in singly housed rats. A possible explanation might be the faster onset of the temperature increase in the group-housed animals, where aggregation of animals in one cage would increase the microclimate temperature and in turn increase the speed of metabolism. The persistence of the temperature increase (3 h in singly housed rats), surprisingly, did not correspond with the rapid pharmacokinetic and locomotor profile of MEPH. Therefore additional factors, such as other active metabolite/s, may contribute to this prolonged effect and may indicate a potential for prolonged somatic drug toxicity, as in the case of toxic MDMA metabolites (66). In general, thermoregulation is mainly affected by drugs that primarily target serotonergic system [e.g., MDMA, PMMA, or MDAI (38, 41, 67)]. Dopaminergic stimulants may also increase body temperature (by increasing the behavioral activity), but effects are not as robust as with serotonergics (7). Direct comparisons of MEPH with other related cathinones, methylone 20 mg/kg sc., and MDPV 2 mg/kg sc. tested in our laboratory shows that the temperature increase was similar [(39); unpublished observation Horsley et al.]. This is of interest since the stimulant activity relative to the potency of the drug (DAT inhibition) should be approximately the same; however, the inhibition of SERT is much lower compared to DAT, and in the case of the lower MPDV dose would be approximately five times less effective (inhibiting SERT) than with MEPH or methylone (20). Taken together with the fact that the temperature increase was more prolonged in singly- than in group-housed rats and that it did not exceed 40°C, we suggest that increases in the overall behavioral activity relevant to dopaminergic stimulation are responsible for the hyperthermia observed. However,

against this interpretation, locomotor activation disappeared within 40 min of administration which is not consistent with the prolonged temperature increases. Further experiments will be needed in order to explain these discrepancies.

CONCLUSION

To conclude, both MEPH and nor-MEPH had rapid kinetics with accumulation in lungs and behaved as short-acting, potent stimulants with low capacity to disrupt sensorimotor gating. Dissociation between the duration of behavioral and hyperthermic effects may be due to the presence of another active metabolite with slower pharmacokinetic profile and may be indicative of prolonged risk of somatic toxicity even though acute stimulant-like effects have already worn off.

ETHICS STATEMENT

All procedures were conducted in accordance with the principles of laboratory animal care of the National Committee for the Care and Use of Laboratory Animals (Czech Republic), and according to Guidelines of the European Union (86/609/EU). The protocol was approved by the National Committee for the Care and Use of Laboratory Animals (Czech Republic) under the number: MEYSCR-27527/2012-31.

AUTHOR CONTRIBUTIONS

All authors made a substantial contribution to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work. All authors were involved in drafting the work or revising it critically for important intellectual contents. All authors gave final approval for the current version of the work to be published. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Pharmacokinetic, Ambulatory, and Hyperthermic Effects of 3,4-Methylenedioxy-*N*-Methylcathinone (Methylone) in Rats

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Methylone (3,4-methylenedioxy-*N*-methylcathinone) is a synthetic cathinone analog of the recreational drug ecstasy. Although it is marketed to recreational users as relatively safe, fatalities due to hyperthermia, serotonin syndrome, and multi-organ system failure have been reported. Since psychopharmacological data remain scarce, we have focused our research on pharmacokinetics, and on a detailed evaluation of temporal effects of methylone and its metabolite nor-methylone on behavior and body temperature in rats. Methylone [5, 10, 20, and 40 mg/kg subcutaneously (s.c.)] and nor-methylone (10 mg/kg s.c.) were used in adolescent male Wistar rats across three behavioral/physiological procedures and in two temporal windows from administration (15 and 60 min) in order to test: locomotor effects in the open field, sensorimotor gating in the test of prepulse inhibition (PPI), and effects on rectal temperature in individually and group-housed rats. Serum and brain pharmacokinetics after 10 mg/kg s.c. over 8 h were analyzed using liquid chromatography mass spectrometry. Serum and brain levels of methylone and nor-methylone peaked at 30 min after administration, both drugs readily penetrated the brain with serum: brain ratio 1:7.97. Methylone dose-dependently increased overall locomotion. It also decrease the amount of time spent in the center of open field arena in dose 20 mg/kg and additionally this dose induced stereotyped circling around the arena walls. The maximum of effects corresponded to the peak of its brain concentrations. Nor-methylone had approximately the same behavioral potency. Methylone also has weak potency to disturb PPI. Behavioral testing was not performed with 40 mg/kg, because it was surprisingly lethal to some animals. Methylone 10 and 20 mg/kg s.c. induced hyperthermic reaction which was more pronounced in group-housed condition relative to individually housed rats. To conclude, methylone increased exploration and/or decreased anxiety in the open field arena and with nor-methylone had short duration of action with effects typical for mixed indirect dopamine–serotonin agonists such as 3,4-metyhlenedioxymethamphetamine

(MDMA) or amphetamine. Given the fact that the toxicity was even higher than the known for MDMA and that it can cause hyperthermia it possess a threat to users with the risk for serotonin syndrome especially when used in crowded conditions.

Keywords: methylone, bk-3,4-metyhlenedioxymethamphetamine, nor-methylone, novel psychoactive substances, cathinones, behavior, pharmacokinetics, metabolites

INTRODUCTION

Methylone (3,4-methylenedioxy-*N*-methylcathinone, also known as MDMC, bk-MDMA, M1) belongs to the group of new psychoactive substances called synthetic cathinones often also termed as β-keto amphetamines or the new generation of designer phenethylamines (1). This β-keto analog of 3,4-methylenedioxymethamphetamine (MDMA; "ecstasy") was first synthetized in 1996 as an antidepressant and anti-Parkinsonian agent (2) but was never used for therapeutic purposes; instead, it gained popularity as a recreational "legal high" owing to its MDMA-cocaine-like effects (3). Methylone users describe their subjective experience as feeling stimulated, with a great need to socialize, spiritual, and empathic connection. Methylone first appeared in 2004 on the illicit drug market (in the Netherlands) and quickly became commonly available and easily obtainable (4), leading to extensive abuse worldwide (5). European Monitoring Center for Drugs and Drug Addiction and Europol have monitored methylone since 2005 (6) and starting in 2011, methylone was reclassified as Schedule I under the Controlled Substance Act in the Unites States (7). In the United Kingdom, methylone has been illegal since 2010. There have been a number of reports of methylone toxicity and even fatal overdoses have been registered. The causes of death include hyperthermia where body temperature elevated up to 41.7°C as a core symptom of serotonin syndrome, metabolic acidosis, and multi-organ system failure (8, 9). Even though the popularity of methylone among users as well as its availability on the gray/black market is widespread, the relevant scientific data are still relatively scarce, and there are no published data on behavioral effects of nor-methylone. Therefore, we investigated effects of these substances on behavior, pharmacokinetics, and body temperature.

Methylone only differs from MDMA by the presence of a ketone at the benzylic position. Based on their structural similarity, and, in turn, similar mechanism of action, comparable effects on behavior, and neurochemistry could be postulated (10). *In vitro* neuropharmacological studies in rat's brain, synaptosomes reported methylone as a non-selective inhibitor of the dopamine, norepinephrine, and serotonin transporters (DAT, NET, and SERT, respectively). Further, methylone *via* blocking re-uptake evokes high-releasing activity of all monoamines (dopamine, norepinephrine, and serotonin) (10, 11). The ratio of DAT:SERT inhibition is 3.3 which suggests that methylone has a high-abuse potential similar to cocaine (DAT:SERT ratio 3.1) (3, 12) as has been also confirmed in behavioral tests (13, 14). On the other hand, in discrimination studies, methylone substituted for MDMA indicating a similar profile (and subjective effects) to this serotonergic compound (15). Thus far, behavioral research has shown that methylone increases locomotor activity, an effect which was inhibited by both dopamine (D2) or serotonin (5-HT_{2A}) receptor antagonists (16–19). Repeated administration of methylone, similar to acute effects of other MDMA-like compounds, induced hyperthermia in rats: an effect typically mediated by serotonin and typically associated with acute toxicity of sertonergic drugs (20–22). On the other hand, simultaneous study of Javadi-Paydar et al. (19) showed no change in body temperature in rats treated by methylone. Methylone is the subject of extensive metabolism in the liver at cytochrome P450 (isoenzymes CYP2D6, CYP1A2, CYP2B6, and CYP2C19) with major primary metabolite nor-methylone, which are subsequently excreted to urine unchanged or in their conjugated forms (23, 24). Other metabolites (dihydroxymethcathinone, *N*-hydroxy-methylone, and dihydro-methylone) were also detected and to date no information about the biological activity of these have been published (23).

Our primary intention was to describe in detail temporal profile of methylone's effects in behavioral tests alongside pharmacokinetics and the effects on body temperature to evaluate its eventual serotonergic toxicity related to hyperthermia. An added value of our study was in the evaluation of the effects of nor-methylone as the primary metabolite in the same series of behavioral tasks. Finally, since we have performed series of experiments in our laboratory with related cathinones this allows us to make indirect comparisons between those (21, 25–27).

In the behavioral study, to test its effects on locomotion, exploratory activity, anxiety, and stereoytypy the open field test was used, further on effects on sensorimotor gating indicative of its psychomimetic potential were tested in the test of prepulse inhibition of acoustic startle response (PPI ASR) (28). To cover the peak effects as well as possible late onset of changes in these tests, we tested both paradigms in two temporal windows (15 and 60 min) after drug administration. To link the behavioral data to serum and brain levels of methylone, the samples for pharmacokinetics were collected from animals involved in behavioral experiments. According to its structural and pharmacological similarity with MDMA and cocaine, we hypothesized that it will have similar behavioral profile (stimulatory and disruptive) but shorter duration of action. Finally, as it has been shown previously for other compounds, environmental factors, especially an effect of "individually/group-housed" rats (e.g., people dancing in a crowded clubs) significantly increase the risk of hyperthermic reaction, we also tested the effect of isolation and aggregation on hyperthermic effects of methylone.

MATERIALS AND METHODS

Animals

All animals were male Wistar rats (Hannover breed, obtained from Konárovice, Czech Republic) weighing 200–250 g and aged

8 weeks at the start of testing. Rats were housed two per cage under controlled temperature (22 ± 2°C) and humidity (30–70%) with food pellets and water freely available. Lights were on from 6:00 to 18:00 h and all experiments were carried out between 7:00 and 13:00 h, except the temperature study where the test lasted until 17:00 h. Animals were allowed 7–10 days to habituate to laboratory conditions before being used in experiments, during which they were weighed twice and handled four times. All behavioral experiments were conducted in the same standard conditions (temperature and humidity) as in the animal housing facility. Each experimental group for behavioral and temperature studies included 10 animals and each animal was tested only once with the exception that (to reduce animal use) rats from behavioral experiments were used for subsequent pharmacokinetic sampling [for pharmacokinetic studies, $n = 8$ (for methylone) and $n = 5$ (for nor-methylone) per experimental group]. All procedures were conducted in accordance with the principles of laboratory animal care of the National Committee for the Care and Use of Laboratory Animals (Czech Republic), and according to Guidelines of the European Union (86/609/EU). The protocol was approved by the National Committee for the Care and Use of Laboratory Animals (Czech Republic) under the number: MEYSCR-27527/2012-31.

Drugs and Chemicals

3,4-Methylenedioxy-*N*-methylcathinone (methylone) was purchased *via* the internet and subsequently purified and converted to a hydrochloride (HCl) by Alfarma s.r.o. (Czech Republic). The resulting methylone was certified to be of 99.18% purity (analyzed by infrared spectroscopy) and also served as a reference standard for pharmacokinetic analyses using liquid chromatography. Nor-methylone was synthesized in the Forensic Laboratory of Biologically Active Substances (University of Chemistry and Technology Prague, Czech Republic) in a purity of 99.18%. Internal standards for quantitative liquid chromatography/mass spectrometry (LC/MS) assays were deuterated MDA-D2. HCl with the purity 99.7% (Lipomed, Inc., Switzerland). Reference standards for confirmation of metabolites by LC/HRMS (high-resolution mass spectrometry) and gas chromatography/mass spectrometry were synthesized with purity within 97.5–89.3% (Institute of Chemical Technology, Department of Organic Chemistry, Prague). β-Glucuronidase type HP-2 from Helix Pomatia, EC 3.2.1.31 (184,973 U/ml) was purchased from Sigma-Aldrich, Prague. Extraction columns Bond Elut Certify 50 mg/3 ml were supplied by Labio s.r.o., Olomouc. Other chemicals used for laboratory purposes were of analytical grade purity. Methylone was stored in dry and dark place and dissolved in physiological saline (0.9% NaCl) immediately before experiments.

Dosage

The methylone doses used in the present study were estimated according to the reported usage by humans and according to our previous studies with entactogens MDMA, para-methoxymethamphetamine (PMMA), and 2C-B (4-bromo-2,5-dimethoxyphenylethylamine). Doses were selected to range from those that: (1) at the lower end are close to those used by humans, to (2) higher doses that might produce significant acute non-lethal toxicity, and (3) with respect to our previous analogous experiments with MDMA, PMMA, and 2C-B (21, 29–31). The treatment range for methylone was set to be 5, 10, 20, and 40 mg/kg, nor-methylone at 10 mg/kg for behavioral experiments. In behavioral experiments (open field, PPI ASR) nor-methylone was tested only in 15 min testing onset. Doses for pharmacokinetic [10 mg/kg subcutaneously (s.c.)] and temperature experiments (10 and 20 mg/kg s.c.) were selected according to the inherent sensitivity of the analytical LC/HRMS procedure utilized and according to the effectiveness in behavioral tasks (effects body temperature). For the pharmacokinetic study, a single bolus of methylone 10 mg/kg s.c. was administered, subsequently animals were decapitated after 30, 60, 120, 240, or 480 min. Additional pharmacokinetic data with the same design were also obtained for nor-methylone 10 mg/kg s.c. Separated sera and whole brains were kept at −20°C until the toxicological analyses. Both drugs were dissolved in a volume of 2 ml/kg and administered s.c. (for comparability with previous studies) in all cases.

Pharmacokinetic Analyses

Determination of Methylone and Nor-Methylone Levels in Serum and Brain Sample Using LC/HRMS

Serum Pre-Treatment

0.2 ml of rat serum was fortified with the internal standard mephedrone-D7 and nor-mephedrone-D7 in methanolic solution (in an amount with respect to the level of methylone and nor-methylone in assayed samples) and 0.5 ml of a 0.1 M phosphate buffer (pH 6) in a labeled tube.

Brain Pre-Treatment

250 mg of brain was homogenized with 5 ml methanol and the internal standard methylone-D3 (in an amount with respect to the methylone levels in samples). The specimen was then ultrasonicated for 20 min and after supernatant separation by centrifugation, the supernatant was transferred into a clean labeled tube and evaporated to dryness. The residue was reconstituted in a 0.1 M phosphate buffer (pH 6). Solid phase extraction of methylone in pre-treated samples: a pre-treated sample of serum or brain with the buffer and internal standard was loaded onto a Bond Elut Certify cartridge previously conditioned with 0.5 ml of a 0.1 M phosphate buffer (pH 6). After application of a pre-treated sample, the cartridge was washed with 0.5 ml of distilled water, 0.5 ml of 0.1 M HCl, and 0.5 ml of CH_3OH/H_2O (1/1, v/v) and then dried by air for 5 min. The analyses were eluted three times with 0.5 ml of a freshly prepared mixture of dichloromethane/2-propanol/ammonium hydroxide (25%), 80/20/4, v/v/v. The eluate was gently evaporated to dryness under a stream of air at 40°C and then dissolved into mobile phase for LC/HRMS analysis.

Determination of 4-Hydroxy-3-Methoxymethcathinone Metabolite in Serum

4-hydroxy-3-methoxymethcathinone (4-OH-3-MeO-MC) was identified in rat serum samples according its exact mass. The calculated $[M + H^+]$ m/z for 4-OH-3-MeO-MC ($C_{11}H_{16}NO_3$)

was 210.1125. Any peak at the same *m*/*z* was found in blank rat sera.

LC/HRMS Conditions

The analyses were performed using Dionex Ultimate 3000 UHPLC coupled to an Exactive Plus-Orbitrap MS (ThermoFisher Scientific, Bremen, Germany) equipped with an HESI-II source. The chromatographic analyses of serum and tissue samples were performed using a Kinetex PFP 100 A (50 mm × 2.1 mm, 2.6 mm) and Security Guard Cartridge PFP 4 mm × 2.0 mm (Phenomenex) with a flow rate of 400 ml/min, gradient elution with 10 mM ammonium formate in 0.1% of formic acid as the mobile phase B. Gradient 0 min 5%, 4 min 45% B, and 5–6 min hold at 95%. The MS conditions were: full MS in a scan range of 50–500 *m*/*z* with positive electrospray ionization, resolution of 70,000 FWHM (scan speed 3 Hz), spray voltage of 3 kV, and ion transfer capillary temperature of 320°C.

Behavioral Procedures

Open Field

Open field testing was conducted in a temperature 22 ± 2°C, sound-proof, and evenly lit chamber with low levels of light intensity. The open field apparatus comprised a black square plastic open field arena (68 cm × 68 cm) with walls (30 cm high). At the beginning of each test, the rat was placed individually into the center of arena 15 or 60 min after drug administration and allowed to move about the arena freely for 30 min. The apparatus was cleaned with 50% ethanolic solution to avoid odors after each test. Behavioral activity was registered by an automatic video tracking system (EthoVision Color Pro v. 3.1.1, Noldus, the Netherlands).

Dependent variables were (i) total locomotor activity over 30 min, (ii) locomotor activity in 5 min intervals, (iii) time spent in the center of the arena and (T_{center}), and (iv) thigmotaxis (i.e., likelihood of appearance in the periphery). For evaluation of time spent in the center and thigmotaxis the arena was virtually divided into 5 × 5 grid of identical square zones with 16 being located on the periphery and 9 centrally. Time spent in the center of the arena is the sum of time spent in the nine central zones ($T_{center} = \Sigma time_{1-9}$). Thigmotaxis indicates probability of appearances in peripheral zones (*f*; the total number of appearances of the animal in each zones) and is calculated as $\Sigma f_{peripheral\ zones}$ divided by $\Sigma f_{all\ zones}$.

PPI of ASR

Prepulse inhibition took place in startle chambers (SR-LAB, San Diego Instruments, CA, USA), each containing sound-proof and evenly lit enclosure, high-frequency loudspeaker (produced background noise at 75 dB and all acoustic stimuli), and Plexiglas stabilimeter (8.7 cm inner diameter). A piezoelectric accelerometer detected amplitudes of the startle responses which were digitized for subsequent analysis. 15 or 60 min prior to test rats were administered with methylone, nor-methylone, or vehicle. The experimental design was according to previous studies (21, 27, 29) and consisted of acclimatization and two sessions.

Acclimatization performed 2 days before test, drug-free rats were habituated in 5 min session with five presentations of pulse alone stimuli (115 dB/20 ms) over background white noise (75 dB). Startle data were not recorded for acclimatization.

The test started with a habituation period lasting 5 min in the startle chamber in which a 75 dB background white noise was continuously presented. The PPI test followed with 72 trials in all with an inter-trial interval (ITI) of 4–20 s (mean ITI: 12.27 s). Six 125 dB/40 ms duration pulse alone trials were then delivered to establish baseline ASR. Following this, 60 trials of the following were presented in a pseudorandom order: (A) pulse alone: 40 ms 125 dB; (B) prepulse-pulse: 20 ms 83 or 91 dB prepulse, a variable (30, 60, or 120 ms) inter-stimulus interval (mean 70 ms), then 40 ms 125 dB pulse; and (C) 60 ms no stimulus. Finally, six pulse alone trials were delivered. Habituation was calculated by the percentage reduction in ASR from the initial six baseline trials, to the final six trials. The PPI was calculated as [100 − (mean response for the prepulse − pulse trial/startle response for the single pulse trials) × 100].

Rectal Temperature

Rats were divided into two groups: rats housed individually and five animals per cage. These two conditions compared isolated and group-housed conditions and their interaction of drug on body temperature. Rectal temperature was measured using a digital thermometer; every temperature measurement lasted 10 s and rat was momentarily immobilized in a Plexiglas tube. The first measurements were taken every hour at 7:00 until 9:00 h and were taken under drug-free conditions. Methylone or vehicle was administered at 9:00 h and temperature was recorded every half hour until 11:00 h. Thereafter, temperature was recorded at hourly intervals until 17:00 h.

Design and Statistical Analysis

All statistical analyses were conducted using IBM SPSS version 22. For the open field, PPI, and temperature analyses, factorial designs for later analysis with analysis of variance (ANOVA) were used.

Significant main effects and interactions ANOVAs were followed with pairwise comparisons using independent *t*-tests. For repeated measures ANOVAs, where Mauchly's test of sphericity was significant (and Mauchley's $W < 0.75$), Greenhouse–Geisser corrected statistics are reported. For independent *t*-tests, where Levene's test for equality of variances was significant, statistics corrected for unequal variances are given $p < 0.05$ (two tailed) was considered the minimal criterion for statistical significance. For multiple comparisons, *t*-tests were used with Bonferroni correction. Nor-methylone was not included in ANOVA analyses (only one time of administration was tested, 15 min) and data were analyzed using additional independent *t*-test.

RESULTS

Pharmacokinetics of Methylone and Nor-Methylone in Serum and Brain Tissue

For methylone, maximum brain and serum concentration were attained within 30 min after the drug administration. The influx into the brain was not delayed and the concentration

of methylone in brain was approximately five times higher than serum levels throughout the experiment (serum:brain ratio during the peak was 1:4.54; **Figure 1A**). Serum levels of nor-methylone were also quantified after methylone administration; they peaked 30 min later than methylone (1 h after administration) and reached about 20% of methylone levels (350 ng/ml). The second most abundant metabolite identified in the serum was 4-OH-MeO-MC (4-hydroxy-3-methoxymethcathinone) (**Figures 1C,D**), quantification was not possible because of lack of reference standard at the time of analysis. 4-OH-MeO-MC peaked quickly at 30 min after administration of methylone, the peak had bigger area under the curve and then quickly disappeared and nor-methylone became the most abundant at later time points evaluated. After nor-methylone administration, the maximum serum concentrations were reached between 30 min and 1 h, the brain peak appeared at 30 min; the maximum levels were approximately one half when compared with methylone and serum:brain ratio during the peak was 1:7.97 (**Figure 1B**).

Acute Toxicity

Rats, treated with methylone 40 mg/kg, were tested only in 15 min testing onset in PPI because after 2 h after administration seven rats no longer produced much behavior only lying on the floor. After 5 h rats began moving around the home cage again, however, mortality occurred within 24 h after injection in six rats. In open field testing, only one rat died within 24 h after administration of methylone (40 mg/kg). Behavioral testing at 60 min after administration was not performed since 40 mg/kg was lethal to some animals.

Locomotor Activity in the Open Field

Trajectory length was evaluated using 4 × 2 × 6 mixed factorial ANOVAs with drug treatment (methylone at 5, 10, or 20 mg/kg versus vehicle) and time of administration (15 and 60 min) as independent factor, and blocks (5 min interval) as a repeated measures factor. Mauchly's test of sphericity was significant and Greenhause–Geisser correction are presented for repeated measures, Mauchly's $W(14) = 0.21$, $p < 0.001$. Degrees of freedom were rounded to whole number for presentational purposes.

Analyses produced significant main effects of drug treatment [$F_{(3, 71)} = 22.43$, $p < 0.001$], time of administration [$F_{(1, 71)} = 50.68$, $p < 0.001$], and blocks [$F_{(3, 211)} = 188.43$, $p < 0.001$]. In addition, there was a significant time of administration × drug treatment interaction [$F_{(3, 71)} = 8.37$, $p < 0.001$] and a significant time of administration × blocks interaction [$F_{(3, 211)} = 6.81$, $p < 0.001$] no other interactions were observed (**Figure 2**).

Since no interaction between drug treatment × blocks was observed further pairwise comparisons using independent *t*-tests were used to explore the significant on total trajectory

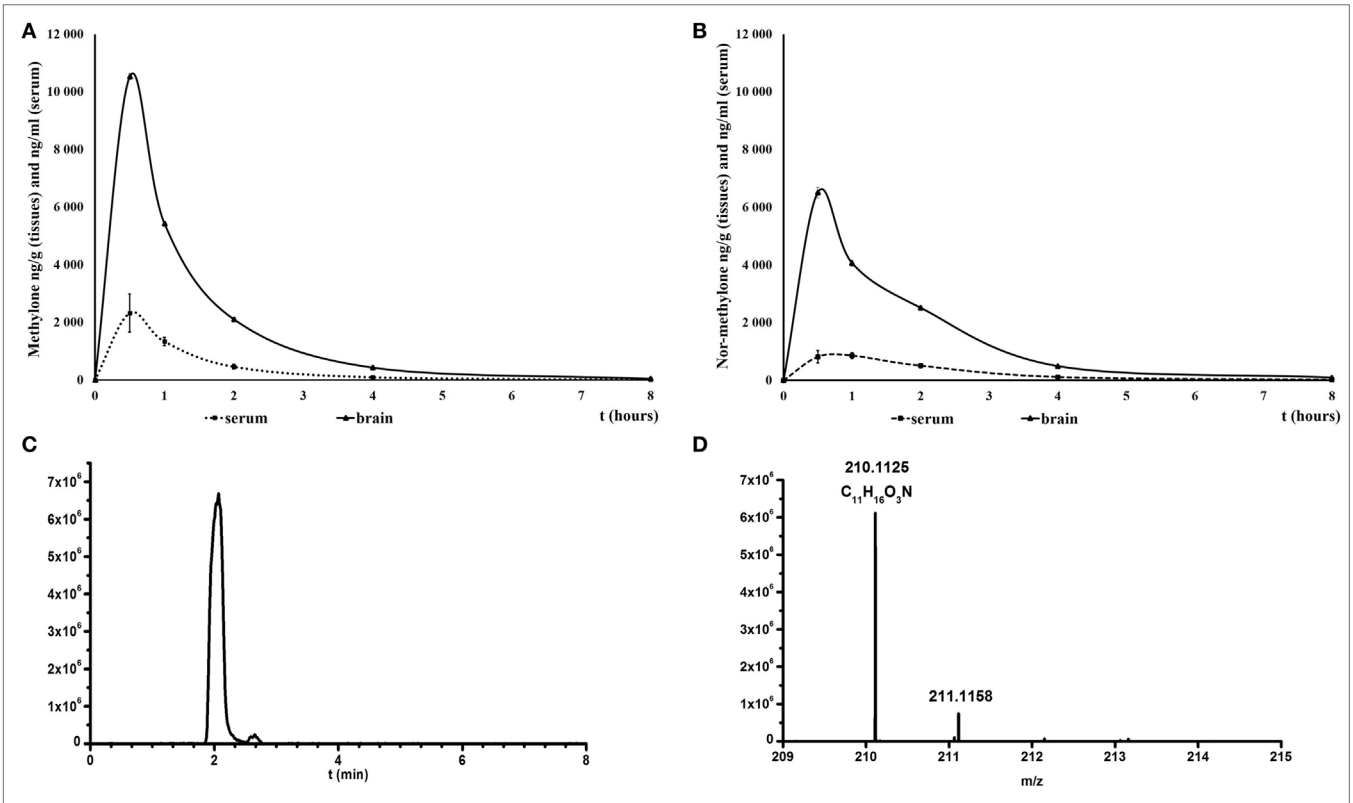

FIGURE 1 | Mean concentrations of methylone **(A)** and nor-methylone **(B)** in serum (nanogram per milliliter) and brain (nanogram per gram) over 8 h after subcutaneously administration of methylone 10 mg/kg and nor-methylone 10 mg/kg, respectively. Symbols represent means and vertical bars SEMs. Second panel represents extracted ion chromatogram of 4-OH-3-MeOH-MC taken at *m/z* 210.1125 in rat serum **(C)** and the measured [M + H$^+$] *m/z* in full spectrum **(D)**.

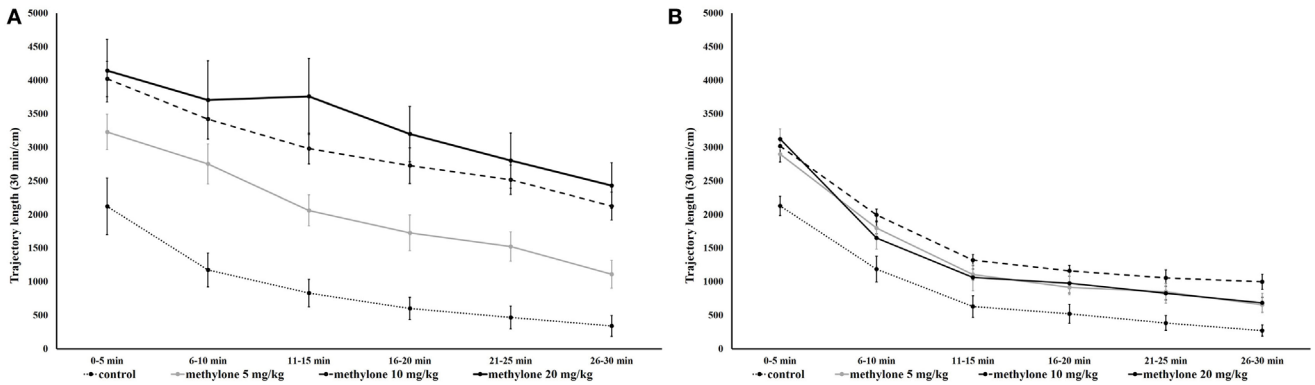

FIGURE 2 | Mean traveled distance within 5-min interval 15 min **(A)** and 60 min **(B)** after administration of methylone (5, 10, and 20 mg/kg). Symbols represent means and vertical bars SEMs.

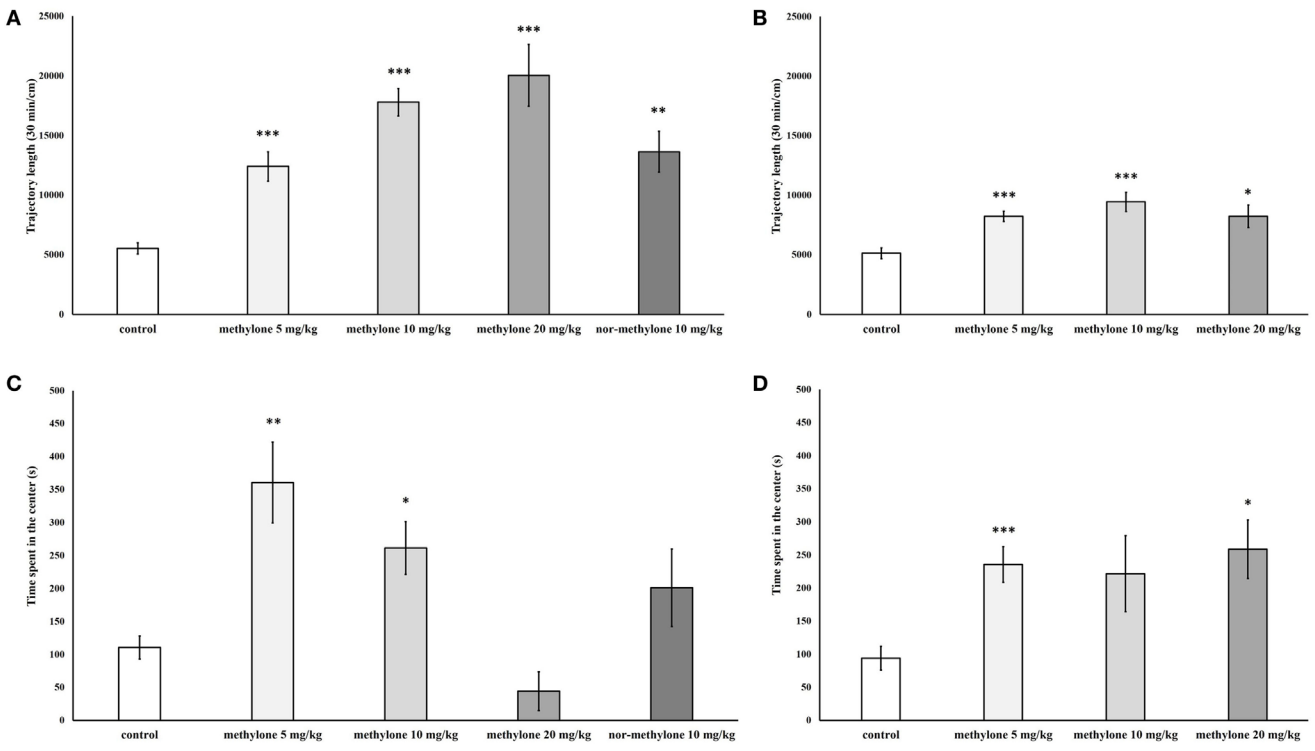

FIGURE 3 | Total locomotion measured 15 min **(A)** and 60 min **(B)** after methylone (5, 10, and 20 mg/kg) and nor-methylone (10 mg/kg) administration. Second panel represents mean T_{center} measured 15 min **(C)** and 60 min **(D)** after methylone (5, 10, and 20 mg/kg) and nor-methylone (10 mg/kg) administration. Columns represent means and vertical bars SEMs. ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$.

length; these revealed that compared with vehicle all three doses of methylone significantly increased locomotion at 15 min [minimum $t(13) = 5.17$, $p < 0.001$; **Figure 3A**] as well as at 60 min [minimum $t(12) = 2.99$, $p < 0.05$; **Figure 3B**]. The increase at 60 min was much less pronounced.

Nor-methylone 10 mg/kg compared with vehicle significantly increased total locomotion at 15 min after administration [$t(18) = 4.57$, $p < 0.01$], by contrast, compared nor-methylone to methylone 10 mg/kg there was no significant difference (**Figure 3A**).

Thigmotaxis and Time Spent in the Center Part of the Apparatus

Thigmotaxis and T_{center} of arena were each analyzed with 4 × 2 ANOVAs with drug treatment and time of administration as independent factors.

T_{center}

There was only a significant main effect of drug treatment [$F_{(3, 71)} = 9.82$, $p < 0.001$] and a significant interaction of time of administration × drug treatment [$F_{(3, 71)} = 6.37$, $p < 0.001$].

Independent *t*-tests showed that methylone at all doses and both times of administration, except 20 mg/kg 15 min and 10 mg/kg at 60 min, significantly increased T_{center} [minimum $t(11) = 3.44$, $p < 0.05$]. Nor-methylone had no effect on T_{center} (**Figures 3C,D**).

Thigmotaxis

The main effect of time of administration [$F_{(1, 71)} = 22.15$, $p < 0.001$], drug treatment [$F_{(3, 71)} = 7.38$, $p < 0.001$] as well as their interaction [$F_{(3, 71)} = 14.89$, $p < 0.001$] were significant.

Methylone 20 mg/kg at 15 min before measurement significantly increased thigmotaxis [$t(18) = 7.93$, $p < 0.001$]. In contrast, at 60 min 5 and 20 mg/kg decreased it [minimum $t(18) = 2.68$, $p < 0.05$], while nor-methylone had no effect on this parameter (**Table 1**).

Prepulse Inhibition

Habituation, ASR, and PPI data were each analyzed with 4 × 2 independent ANOVAs with drug treatment (methylone at 5, 10, and 20 mg/kg versus vehicle) and time of administration (15 and 60 min) as independent factors.

Habituation data showed a significant main effect of time of administration on habituation [$F_{(1, 72)} = 8.17$, $p < 0.01$] but no interaction was observed. Independent *t*-tests revealed that methylone compared with vehicle did not affect habituation at any of the doses tested (**Table 2**).

Acoustic startle response data showed a significant main effect of drug treatment [$F_{(3, 72)} = 2.83$, $p < 0.05$] and again no interaction was detected. Independent *t*-tests revealed that methylone compared with vehicle did not affect ASR at any of the doses tested (**Table 2**).

Independent ANOVA showed a significant main effect of drug treatment on PPI [$F_{(3, 72)} = 2.88$, $p < 0.05$], no other interactions were observed. Subsequent independent *t*-test revealed a trend to decrease for 20 mg/kg at 15 min, compared with control, $t(18) = 1.91$, $p = 0.1$ (one-tailed). Nor-methylone did not differ from vehicle (**Figure 4**).

The Effect of Methylone on Body Temperature

The effect of methylone on body temperature was analyzed using 3 × 2 × 13 mixed factorial ANOVAs with drug treatment

TABLE 1 | Mean thigmotaxis measured 15 and 60 min after methylone (5, 10, and 20 mg/kg) and nor-methylone (10 mg/kg) administration.

		Drug treatment				
Measure	**Admin time**	**Vehicle**	**5 mg/kg**	**10 mg/kg**	**20 mg/kg**	**Nor-methylone**
Thigmotaxis	15 min	0.82 (0.01)	0.78 (0.02)	0.83 (0.02)	0.97 (0.02)	0.84 (0.04)
	60 min	0.81 (0.01)	0.75 (0.02)	0.82 (0.03)	0.74 (0.02)	xxx

Numbers represent means and in brackets are shown SEMs.

TABLE 2 | The effect of methylone (5, 10, and 20 mg/kg) and nor-methylone (10 mg/kg) on acoustic startle response (ASR) and habituation.

		Drug treatment				
Measure	**Admin time**	**Vehicle**	**5 mg/kg**	**10 mg/kg**	**20 mg/kg**	**Nor-methylone**
ASR (*arbitrary units*)	15 min	183.4 (60.1)	79.9 (12)	237.2 (36)	188.5 (23.4)	172 (33)
	60 min	157.5 (36.2)	125 (26.5)	157.2 (34.5)	145 (17.2)	xxx
Percentage habituation	15 min	40.4 (10.9)	25.7 (13.4)	19.4 (9)	35.7 (8)	35.6 (7.1)
	60 min	67.1 (6.1)	50.8 (8.4)	43.3 (8.6)	47.2 (6.9)	xxx

Numbers represent means and in brackets are shown SEMs.

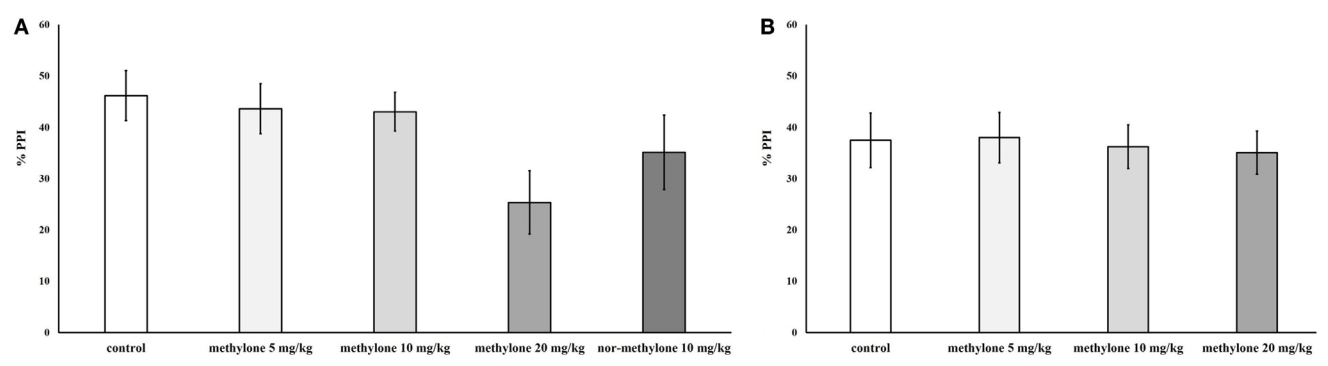

FIGURE 4 | Mean percentage prepulse inhibition of methylone (5, 10, and 20 mg/kg) and nor-methylone (10 mg/kg) 15 min **(A)** and 60 min **(B)** after administration. Columns represent means and vertical bars SEMs.

(methylone at 10 and 20 mg/kg versus vehicle) and home-cage conditions (individually and group-housed rats) as independent factors, and time as a repeated measures factor. Mauchly's test of sphericity was significant and Greenhouse–Geisser correction are presented for repeated measures, Mauchly's $W(44) = 0.05$, $p < 0.001$. Although temperature data before drug administration were significantly different from vehicle, these data were averaged for individual treatment and subtracted from temperature data after drug administration.

Temperature data showed a significant main effect of drug treatment [$F_{(2, 54)} = 5.29$, $p < 0.05$], home-cage conditions [$F_{(1, 54)} = 4.41$, $p < 0.05$], and time [$F_{(5, 289)} = 161.58$, $p < 0.001$]. The interaction of drug treatment × time [$F_{(11, 289)} = 6.87$, $p < 0.001$] and the three-way interaction of drug treatment × time × home-cage conditions [$F_{(11, 289)} = 4.3$, $p < 0.001$] were significant.

Independent *t*-tests revealed that under individually conditions, methylone significantly increased body temperature half an hour (9.30 h) after administration for both doses (10 and 20 mg/kg), an effect that was maintained until 13.00 for 10 mg/kg groups, minimum $t(18) = 2.15$, $p < 0.05$, and to 14.00 in the case of 20 mg/kg, minimum $t(18) = 2.07$, $p = 0.05$, **Figure 5A**.

In rats housed under group-housed condition, the temperature started to increase at 30 min after methylone administration after each of the doses. Methylone 10 mg/kg significantly increased body temperature from 9.30 to 10.30 h, minimum $t(18) = 2.6$, $p < 0.05$. At 20 mg/kg dose, temperature maintained elevated until 11.00, minimum $t(18) = 2.46$, $p < 0.05$, **Figure 5B**.

DISCUSSION

The main findings were as follows: methylone (i) had fast pharmacokinetics with a peak at 30 min, readily crossed the blood–brain barrier and reached levels approximately five times higher in the brain tissue (compared with serum); the major metabolite nor-methylone peaked in the brain at 30 min after methylone administration; (ii) showed marked stimulant effects at 15 min after administration which significantly diminished when tested 1 h after administration; (iii) methylone has relatively weak potency to disrupt PPI; and (iv) methylone significantly increased rectal temperature in individually as well as group-housed rats. When nor-methylone was administered alone, even though it reached approximately 1/2 and 1/3 of the serum and brain levels compared with methylone, it had comparable stimulant potency to methylone.

Pharmacokinetics

Compared with our data, Elmore et al. (32) found the peak serum of methylone levels even earlier at 15 min using the same route of administration. Interestingly, Lopez-Arnau et al. (33) found maximum plasma levels at 30 min after oral administration of methylone in rats which is indicative of a very fast gastrointestinal absorption. Additionally, only our experiments indicate a very fast and effective crossing of blood–brain barrier as methylone levels in the brain were more than five times those in serum. The incorporation of methylone into the brain may be associated with high lipophilicity, as we have already suggested for other compounds, e.g., PMMA or MDAI (5,6-methylenedioxy-2-aminoindane) (21, 27). Similarly, as methylone, its metabolite nor-methylone showed similar serum:brain ratio. The other important and major metabolites 4-OH-MeO-MC (4-hydroxy-3-methoxymethcathinone) (1, 34) were also detected in serum and brain. The rapid decrease of its dominance in the analytical spectrum at 60 min may be related to its fast conjugation, which would explain its lower plasmatic concentrations compared with nor-methylone. Even though we did not perform enzymatic hydrolysis, we might assume that the rapid decrease of its levels might be related to its fast conjugation with glucuronic and/or sulfuric acid that is typical for fenolic metabolites (35). Compared with MDMA where peak MDMA concentrations are achieved within 1 h after subcutaneous or oral administration both methylone and its metabolite nor-methylone showed a more rapid kinetic profile (36, 37) which is in line with the reported shorter duration of effects in humans (and might lead to more frequent re-dosing by users).

Acute Toxicity

According to our knowledge, there is no evidence about determination of lethal methylone dose in animals. In our

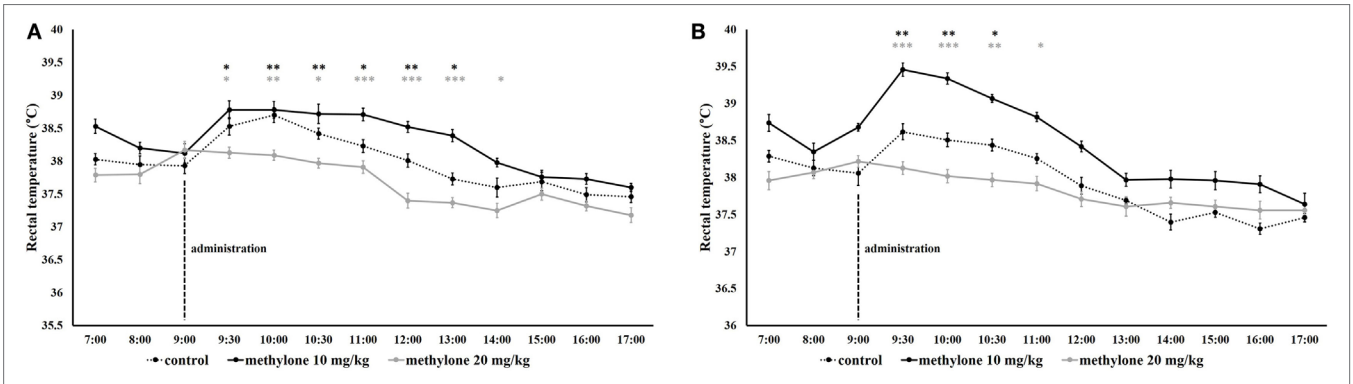

FIGURE 5 | The effect of methylone on rectal temperature in individually **(A)** and group-housed **(B)** rats. Vertical lines represented administration of methylone (10 and 20 mg/kg or vehicle). Symbols represent means and vertical bars SEMs. ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$, gray asterisks refer to methylone (10 mg/kg) versus vehicle comparison, black asterisk methylone (20 mg/kg) versus vehicle comparison.

study, we obtained unexpected findings on the lethal effects of the highest dose of methylone (40 mg/kg) in the rats. The symptoms observed in this case (i.e., hyperventilation, seizures) were similar to symptoms detected in MDAI (27) and may be associated with serotonin syndrome, mainly hyperthermia which is one of the core symptom caused by 5-HT release (38).

Open Field

In accordance with its kinetic profile, the overall locomotor stimulatory activity was more pronounced 15 min after administration and was also comparable with other studies in rats (16, 19) and mice (17, 18). In mice, after methylone 30 mg/kg, locomotor activity was lower compared with 10 mg/kg (18) indicative of an inverted U shaped curve of locomotor effects. This inverted U shaped locomotor curve is also typical for most of the stimulants and characteristically linked to an increase in stereotyped behavior (e.g., circling) (21, 39). It is well established that the stimulatory versus hallucinogenic potency of cathinones and other related compounds is related to their DAT:SERT inhibition ratio. As stated above, methylone has been reported to have similar DAT:SERT inhibition ratio to cocaine (3, 12), and in contrast to other related cathinones, e.g., mephedrone, naphyrone, and methylenedioxypyrovalerone (MDPV) methylone has lower selectivity over DAT making it less stimulatory (3, 40). As reported in comparable behavioral studies of our currently submitted manuscripts, its stimulatory potency is slightly lower compared with mephedrone and much less potent compared with MDPV (unpublished observations Horsley et al. and Sichova et al.).

According to the temporal and spatial patterns in locomotor activity, methylone disrupted habituation, increased exploration, and stimulated activity at lower doses, however, high doses induced stereotyped behavior. In this respect, methylone behaves in a very similar manner to other stimulants and entactogens tested in identical (or near-identical) paradigms in our laboratory (21, 31).

Prepulse Inhibition

Methylone has a relatively weak potency to disturb sensorimotor processing. In line with this, our recent experiments with mephedrone, nor-mephedrone, and MDPV showed comparable weak or negative effects on PPI in rats (unpublished observations Horsley et al. and Sichova et al.). Interestingly amphetamine, which is approximately 10 times more potent in disrupting PPI in rodents, in humans also failed to have disruptive effect on PPI (41). However, this might be related to the fact that it was used in much lower dose (0.45 mg/kg) in humans compared with rodents (typically 1–4 mg/kg). On the contrary drugs affecting mainly SERT, e.g., MDMA, PMMA, or MDAI seem to have much stronger ability to disrupt sensorimotor gating in animals as well as in humans (26, 31, 42). Since PPI is typically used as a model of psychotomimetic potential in animals with translational validity, we may conclude that methylone has only mild psychotomimetic effects. Apart from PPI, and similarly like with the open field, the habituation to startle was attenuated during the peak of methylone effect (i.e., in 15 min time of administration). This can be theoretically also related to the overall stimulatory effect or to anxiety, since with the highest dose also the decreased time spent in the center was present.

Temperature

As expected and in accordance with previous studies with methylone (22, 43, 44), MDMA (45, 46) as well as our comparable studies with phenethylamine PMMA, and aminoindane MDAI (21, 27) the hyperthermic reaction was more pronounced in group-housed condition where it increased up to 1.5°C. The temperature increase was rapid and was not associated with visible perspiration as has been described with PMMA and MDAI (21, 27). In animals housed separately, the increase in temperature lasted for a 1 h longer compared with animals housed in groups. This is surprising since the opposite would be expected. This might be explained by accelerated metabolism due to higher increase in body temperature (cca 1°C) in animals housed in groups. Serotonergic drugs have more pronounced hyperthermic effects compared with drugs with dopaminergic actions. Since serotonin is a critical neuromodulator involved in the thermoregulation, with 5-HT2A receptors being a key mechanism responsible for hyperthermia (47). It is therefore very probable that this is also the case for methylone where the 5-HT2A receptor is stimulated *via* indirect mechanisms related to the increased serotonergic tone (44). On the other hand, in study of Javadi-Paydar et al. (19) was shown that mean body temperature did not vary more than 0.5°C from baseline temperature after methylone application. These findings of different (negative) results could be caused by methodological differences, where they measured temperature using radiotelemetry with lower doses of methylone than us.

Also in some of the cathinones, e.g., mephedrone have been also reported to induce hypothermia in rats but not mice (43, 48, 49). This effect is typically stimulated by activity at 5-HT1A receptors, and sometimes drugs that induce serotonin release might have biphasic effects on temperature or bidirectional depending on pharmacodynamics and the stimulation of these receptors (50).

Since here rats in group-housed conditions exhibited greater elevations in temperature (than under individually housed rats), this provides more support for the idea that environmental conditions that are crowded and/or hot (e.g., people dancing in a crowded clubs) increase the risk of hyperthermia and acute toxicity associated with methylone (51).

CONCLUSION

Methylone and its primary metabolite, nor-methylone induced behavioral, and temperature changes that are comparable with MDMA and other related stimulants, however, our results indicate it has a weaker capacity to disrupt PPI than MDMA and other stimulants. Since we have observed lethal toxicity in our study and that several deaths have been also associated with methylone in humans, its toxicity should not be underestimated,

especially when hyperthermic reaction appears in a crowded environments.

ETHICS STATEMENT

All procedures were conducted in accordance with the principles of laboratory animal care of the National Committee for the Care and Use of Laboratory Animals (Czech Republic), and according to Guidelines of the European Union (86/609/EU). The protocol was approved by the National Committee for the Care and Use of Laboratory Animals (Czech Republic) under the number: MEYSCR-27527/2012-31.

AUTHOR CONTRIBUTIONS

All authors made a significant contribution to this study, read, revised, and gave final approval for the current version of the work to be published.

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Behavioural, Pharmacokinetic, Metabolic, and Hyperthermic Profile of 3,4-Methylenedioxypyrovalerone (MDPV) in the Wistar Rat

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3,4-methylenedioxypyrovalerone (MDPV) is a potent pyrovalerone cathinone that is substituted for amphetamines by recreational users. We report a comprehensive and detailed description of the effects of subcutaneous MDPV (1–4 mg/kg) on pharmacokinetics, biodistribution and metabolism, acute effects on thermoregulation under isolated and aggregated conditions, locomotion (open field) and sensory gating (prepulse inhibition, PPI). All studies used male Wistar rats. Pharmacokinetics after single dose of 2 mg/kg MDPV was measured over 6 h in serum, brain and lungs. The biotransformation study recorded 24 h urinary levels of MDPV and its metabolites after 4 mg/kg. The effect of 2 mg/kg and 4 mg/kg on body temperature (°C) was measured over 12 h in group- vs. individually-housed rats. In the open field, locomotion (cm) and its spatial distribution were assessed. In PPI, acoustic startle response (ASR), habituation, and PPI were measured (AVG amplitudes). In behavioural experiments, 1, 2, or 4 mg/kg MDPV was administered 15 or 60 min prior to testing. Thermoregulation and behavioural data were analysed using factorial analysis of variance (ANOVA). Peak concentrations of MDPV in sera, lung and brain tissue were reached in under 30 min. While negligible levels of metabolites were detected in tissues, the major metabolites in urine were demethylenyl-MDPV and demethylenyl-methyl-MDPV at levels three-four times higher than the parent drug. We also established a MDPV brain/serum ratio ~2 lasting for ~120 min, consistent with our behavioural observations of locomotor activation and disrupted spatial distribution of behaviour as well as moderate increases in body temperature (exacerbated in group-housed animals). Finally, 4 mg/kg induced stereotypy in the open field and transiently disrupted PPI. Our findings, along with previous research suggest that MDPV is rapidly absorbed, readily crosses the blood-brain barrier and is excreted primarily as metabolites. MDPV acts as a typical stimulant

with modest hyperthermic and psychomimetic properties, consistent with a primarily dopaminergic mechanism of action. Since no specific signs of acute toxicity were observed, even at the highest doses used, clinical care and harm-reduction guidance should be in line with that available for other stimulants and cathinones.

Keywords: 3 4-methylenedioxypyrovalerone, behaviour, hyperthermia, locomotion, MDPV, pharmacokinetics, sensory gating, wistar rat

INTRODUCTION

Background and Context

"Novel psychoactive substances" (NPSs) are synthetic compounds that produce similar subjective psychological and behavioural effects to pre-existing illicit recreational drugs, however, they may circumvent legal controls owing to differences in their chemical structures [1]. In recent years, NPSs have proliferated across the globe [2, 3] as a result of their perceived "legality," widespread availability [4] and of changes in socio-cultural attitudes where "sensible" recreational drug-taking has become increasingly normalised [5]. Unsurprisingly, rates of NPS dependence and toxic reactions/fatalities have risen simultaneously ([6, 7]) resulting in the increasing prominence of NPS (mis)use on public health agendas [8]. "Bath salts" is the colloquial street name for a class of NPSs where the principle psychoactive ingredient is a synthetic derivative of [9, 10]. Despite their common origin, cathinone derivatives have heterogeneous effects and mechanisms of action and it remains difficult to reliably predict the behavioural and physiological effects of NPSs based on their structural similarities [11]. Since controlled studies are not usually possible in humans, animal studies provide useful indicators of effects *in vivo* [12]. Studying effects (on an NPS-by-NPS basis) in animal models and *in vitro* continues to be a central approach oriented to obtaining knowledge with translational relevance to humans (e.g., harm reduction, clinical care). Here, we present data pertinent to further understanding of the acute pharmacokinetic, metabolic, behavioural, and thermoregulatory effects of the high-potency pyrovalerone cathinone 3,4-methylenedioxypyrovalerone (MDPV).

Mechanisms of Action

MDPV belongs to a sub-class of stimulating cathinones "pyrrolidinophenones" characterised by a complex chemical structure containing a pyrrolidine ring and an alkyl side-chain extending from the α-carbon [13]. Pyrrolidinophenones' stimulant effects reflect their inhibition of monoamine transporter sites where they are highly selective for the dopamine and norepinephrine transporter (DAT and NET), with particularly powerful effects on DAT, and only a negligible effect on the serotonin transporter (SERT) ([4, 14–20], but see [21]). To date, no evidence has been found (in rodents, or humans) that pyrrolidinophenones act as transporter substrate "releasers" [4, 14–16, 20] nor is there evidence that MDPV is active at non-transporter sites [16, 20]. Data from human embryonic kidney (HEK) cells expressing human transporters *in vitro* show a DAT/SERT inhibition ratio ranging from 100 to 300 [20, 22] and in rat brain synaptosomes, a ratio of 806 [18] for MDPV, values which far exceed those for other psychostimulants (e.g., amphetamine and cocaine), as well as most other pyrrolidinophenones [18, 20, 22]. MDPV is also much more effective at DAT than NET inhibition than other psychostimulants, e.g., for cocaine it is 50 times more potent at inhibiting DAT and ten times more potent at inhibiting NET [14, 18, 23, 24].

Pharmacokinetics and Metabolism

Recreational users report that the desirable subjective effects of MDPV include euphoria, sensory and physical stimulation [25, 26], similar to amphetamine (to which MDPV is chemically related; [1, 7, 27]). Recreational users typically ingest MDPV orally or by insufflation [4, 25]. Typical low-moderate active dosages for human recreational use range between 3 and 10 mg [25] with reported onset of subjective effects within 30 min and a duration of 120–210 min, with after-effects up to 8 h, [25, 28]. *In vivo* studies in rats that examined the concentrations of MDPV in plasma after subcutaneous (sc.) 0.5–3 mg/kg MDPV showed maximal plasma concentrations 10–20 min after administration, which then declined rapidly with an estimated elimination half-life of ∼80 min [29, 30]. Only one previous study, however, has reported on the penetration of MDPV into the brain (after 1 mg/kg sc.), showing peak concentrations by 25 min in Sprague-Dawley rats [30].

In vitro studies of human liver microsomes have shown that MDPV is metabolised first to 3,4-dihydroxypyrovalerone (demethylenyl-MDPV) via *O*-demethylenation of its 3,4-methylenedioxy ring, and then to 4-hydroxy-3-methoxypyrovalerone (demethylenyl-methyl-MDPV) by *O*-methylation; these phase II metabolites are excreted in urine in conjugated form ([31], but see [32]). More recently it has been shown, that in rats, concentrations of demethylenyl-MDPV and demethylenyl-methyl-MDPV in plasma peak more slowly than MDPV, at around 3–4 h [29]. Demethylenyl-methyl-MDPV is the most abundant urinary metabolite in both species, humans and rats [32].

Toxicity and Thermoregulation

Human data on MDPV and pyrrolidinophenone toxicity is often confounded because "bath salts" commonly contain multiple cathinones and unknown contaminants [1, 7, 33]. However, since MDPV has been implicated more frequently in cases of serious intoxication [7, 34, 35] and confirmed in a number of fatalities [7, 36–39] than other cathinones, it may have higher risk of acute toxicity. Acute toxic effects of MDPV specifically involve over-stimulation of the cardiovascular

system and the central nervous system (CNS), resulting in agitation, hyperthermia, and tachycardia [13, 26], and in one fatal case (where urine levels of MDPV were 670 ng/mL), coagulopathy, acidosis, rhabdomyolysis and anoxic brain injury were observed [38]. Psychiatric symptoms such as paranoia, delirium and hallucinations may be present, and serotonin syndrome, deterioration of muscle tissue and kidney failure have also been reported [26, 28, 37, 40–42]. Prominent in cathinone-related toxidrome (in humans) is hyperthermia; however, prior research on the effects of MDPV specifically on thermoregulation in rodents has been inconsistent. MDPV can elevate body temperature under conditions of higher ambient temperatures or social interaction, [21, 43, 44], however effects can be negligible, even under such conditions [45].

Behavioural Effects

MDPV is a highly reinforcing psychostimulant with notable addictive potential [20, 22, 46–51]. Increased extracellular dopamine (DA), particularly in the nucleus accumbens (NAcc), is an indicator of the reinforcement potential of drugs, and the accompanying locomotor response itself can be used as a marker of this. MDPV's stimulatory effects are most likely mediated by striatal extracellular DA ([29, 30], see [4]). MDPV has been shown to increase both locomotion in rodents ([14, 45, 52–55], see also [50], for a recent review) as well as increased NAcc DA [4, 14, 29]. Moreover, locomotor stimulation positively correlates with MDPV concentrations in the striatum [30], in plasma, and with extracellular DA concentrations [56] in the NAcc [4, 29]. The open field test is sensitive to stimulatory/sedative effects of centrally acting drugs, as well as having some sensitivity to emotionality (manifested as changes in the temporo-spatial characteristics of locomotor behaviour). Although effects of MDPV have been characterised in a number of other locomotor tests (activity cage, running wheel and rotorod), there is only one study of its effects in the open field using (Sprague-Dawley) rats, which showed 0.5 mg/kg intraperitoneally (ip.) increased locomotion. In mice, lower doses (1–3 mg/kg) MDPV increased locomotion and exploration were reported, with ataxia, hyperactivity and stereotypies emerging at doses 3–30 mg/kg [18, 43, 49, 53, 57]. Locomotor stimulation at lower doses and gross motor effects/stereotypies at higher doses are consistent with the inverted "U" curvilinear dose response identified for MDPV [45, 58].

Prepulse inhibition (PPI) of the acoustic startle response (ASR) is a behavioural operationalisation of sensorimotor gating that reflects pre-attentional filtering of redundant information [59]. PPI is useful in assessing the psychomimetic properties of drugs and is a behavioural endophenotype of psychosis [60]. Stimulants and cathinones can disrupt PPI, but usually only at higher doses ([61–68], but see [69]). As already described, MDPV use (in humans) can, like other cathinones, result in psychotic symptoms such as hallucinations, however to date, MDPV has not been tested in PPI, nor have any other pyrrolidinophenones.

Aims and Predictions

The aim of the present manuscript is to provide a detailed characterisation of the effects of subcutaneous (sc.) MDPV in the range 1–4 mg/kg with regard to pharmacokinetics (in sera, brain and lung), biotransformation (urine and sera), acute systemic toxicity (thermoregulation), locomotion (open field), and sensory gating (PPI). The study was performed as a part of an experimental series performed in our laboratory enabling direct comparisons with other NPSs belonging to cathinones, phenethylamines as well as aminoindanes (e.g., [66, 70, 71]). Based on these findings and methods as well as more recent ones presented by others [22, 31, 72], we expected MDPV to peak within first hour after administration in serum and brain, and due to its lipophilicity to accumulate into lung tissue. Additionally, levels of major metabolites were evaluated in tissues, sera as well as in urine. In order to simulate the typical environmental situations in which human MDPV use occurs, we measured body temperature under isolated and aggregated conditions with the prediction that aggregation will lead to more pronounced hyperthermic effects. In order to evaluate the characteristic inverted U locomotor dose-response curve we recorded open field activity at three MDPV doses at two testing-onsets following drug administration. Finally, we expected that MDPV, owing to its powerful effects on DAT, will disrupt PPI more potently than other ring-substituted cathinones tested in our laboratory.

METHOD

Design

For the pharmacokinetic study, samples of blood, whole brains and lungs were collected 30, 60, 120, 240, or 480 min after administration of 2 mg/kg MDPV. Concentrations of MDPV in sera and tissues were calculated as ng/ml or ng/g. The brain/serum ratio was calculated as mean brain concentration /mean serum concentration per sampling time point. The biotransformation study screened for MDPV metabolites and quantified MDPV and major metabolites in urine, sera (ng/mL) and tissues (ng/g) collected over 24 h after 4 mg/kg MDPV.

Open field and PPI experiments used a 4 × 3 factorial design with MDPV treatment (1, 2, 4 mg/kg or vehicle) and testing-onset (15 or 60 min) as independent factors. For the open field, trajectory length (cm, corrected for 3 cm deviations), thigmotaxis (measured as $\Sigma f_{\text{peripheral zones}} / \Sigma f_{\text{all zones}}$, where f = frequency of line crossings) and T_{centre} (calculated as Σ_{time} in the central zones) were measured. A repeated measures factor was also included in the open field experimental design in order to measure trajectory length over six 5 min time bins (4 × 3 × 6 mixed design). In the PPI experiment, all measures were derived from average startle amplitudes (AVG), and were as follows: % habituation (percentage reduction in ASR from six baseline trials, to the final six trials), ASR (mean ASR was derived from pulse alone trials) and % PPI (calculated as: [100–(mean prepulse–pulse trials/mean pulse alone trials)*100]) The thermoregulation study measured rectal temperature (°C) and used a 3 × 2 × 13 mixed factorial design with MDPV treatment (2, 4 mg/kg or VEH) and home-cage condition (group or individually caged) as independent factors and measurement time points as a repeated measures factor.

Animals

Male outbred Wistar rats (Velaz, Czech Republic) weighing 180–250 g were housed in pairs at 22 ± 2°C on a 12/12 h light/dark cycle and with *ad libitum* water and standard diet. Rats were acclimatised for 7–10 days prior to testing, during which they were weighed twice and handled four times. All tests were conducted under standard conditions: humidity 30–70% and temperature 22 ± 2°C. All studies were carried out in accordance with the principles of laboratory animal care of the National Committee for the Care and Use of Laboratory Animals (Czech Republic), and according to Guidelines of the European Union (86/609/EU). The protocol was approved by the National Committee for the Care and Use of Laboratory Animals (Czech Republic) under the number: MEYSCR-27527/2012-31.

Across all experiments, 193 rats were used. For open field and PPI experiments, each had a total sample size of 60 ($n = 10$). Naïve rats were used for each experiment with the exception that (to reduce animal use) 40 rats from the open field were used for pharmacokinetic sampling ($n = 8$). For thermoregulation studies, $N = 60$ ($n = 10$). For the biotransformation study the total number of rats was three. As previously described, data from vehicle (VEH) control animals for PPI, open field and temperature studies were collected twice per annum as part of a series of standardised NPS studies undertaken in our laboratory (e.g., see [73]).

MDPV Doses

MDPV and other analytical standards were synthesised in-house at the Department of Organic Chemistry, University of Chemistry and Technology, Prague. In all studies, MDPV was dissolved in physiological saline in a volume of 2 ml/kg, and was always administered sc. as a single bolus. MDPV has been shown to be behaviourally active in locomotor tests in rodents at between 0.5 and 30 mg/kg across ip. and sc. routes. Based on previous behavioural research 1, 2, and 4 mg/kg doses were selected for behavioural testing with the expectation that 1 and 2 mg/kg would produce mild stimulatory effects similar to those sought by people. 4 mg/kg was selected to represent more extreme use, with stereotyped behaviours expected. Pharmacokinetic, biotransformation and thermoregulation studies tested 2 and/or 4 mg/kg were tested; these higher doses were chosen in order to increase the likelihood of detectable effects. The dose rage tested was similar to that used by Novellas et al. [30] where they argued that doses up to 3 mg/kg were within a range used by consumers.

Pharmacokinetics: Determination of MDPV in Serum and Tissues

After completing behavioural testing in the open field, rats were decapitated at 30, 60, 120, 240, or 480 min after administration of 2 mg/kg MDPV, and blood, whole brains and lungs were collected. Separated sera and tissues were stored at −20°C until the toxicological analyses. Sample preparation, LC and MS conditions, method of validation and calibration were based on Meyer et al. [32] and similar to other previously published methods (e.g., [31, 72]). For a full description of pharmacokinetic methods, please refer to Horsley et al. [73].

Biotransformation Study: Determination of MDPV and Its Main Metabolites in Urine

Rats were administered 4 mg/kg MDPV, were placed individually into metabolic cages (Harvard Apparatus, USA) and their 24-h urine fractions were collected. During collection urine was maintained below 4°C throughout. Samples were subsequently stored at −40°C until analysis. The methods used to the screening of MDPV metabolites, and quantification of MDPV and its metabolites has been described more fully in Horsley et al. [73].

Systemic Toxicity: Thermoregulation

Rats were housed singly vs. five to a home-cage and rectal temperature was measured for 10 s using a digital thermometer. The first three measurements were drug-free, and were taken hourly, 07:00–09:00 h (inclusive) whereupon 2, 4 mg/kg MDPV or VEH was administered. Thereafter, observations were at 0.5 h intervals 09.30–11.00 h, before resuming hourly measurements 12.00–17.00 h. For a fuller description, see e.g., Páleníček et al. [70, 71].

Behaviour: Open Field and PPI

Open Field

Rats were administered MDPV at 1, 2, 4 mg/kg or VEH, and then 15 or 60 min post-administration they were placed individually into the centre of the open field apparatus (a 68 × 68 × 30 cm square black plastic arena) and their behaviour was video-recorded for 30 min. Ethovision Colour-Pro version 3.1.1, (Noldus, Netherlands) was used for behavioural capture and pre-processing. During pre-processing, the arena was virtually divided into 5 × 5 identical square zones with 16 located around the periphery and 9 centrally in order to derive thigmotaxis and T_{centre} variables (see section Design for calculations). The procedures used were the same as those used previously, e.g., Páleníček et al. [70, 71].

PPI

Two days before test, rats were acclimatised to the startle chamber (SR-LAB, San Diego Instruments, California, USA) with a drug-free five min pre-exposure to five pulse alone stimuli (115 dB/20 ms) over 75 dB continuous white noise. On the test day, 15 or 60 min prior to testing, rats were administered 1, 2, 4 mg/kg MDPV or VEH, placed into the startle chamber and acclimatised for five min to a continuous 75 dB white noise. They were then presented with six 125 dB/40 ms duration pulse alone trials, followed by 60 pseudorandomised trials of the following: (A) pulse alone: 40 ms 125 dB; (B) prepulse-pulse: 20 ms 83 dB or 91 dB prepulse, a variable (30, 60 or 120 ms) inter-stimulus interval (ISI: mean 70 ms), then 40 ms 125 dB pulse; (C) 60 ms no stimulus. Finally, six pulse alone trials were delivered. There were 72 trials in total with inter-trial intervals (ITIs) of 4– 20 s (mean ITI = 12.27 s). A fuller description can be found in our previously published work (e.g., [70, 71]).

Statistics

Statistical analyses of behavioural and thermoregulation data were conducted using IBM SPSS version 22. Unless stated

otherwise, default alpha was set at $p = 0.05$, and tests were two tailed. For open field, PPI, and temperature analyses we used factorial analysis of variance (ANOVA). For repeated measured ANOVAs, where Mauchly's test of sphericity was significant Greenhouse-Geisser corrections were used. Planned pairwise comparisons (to follow up significant main effects and interactions involving MDPV) used independent *t*-tests. In order to limit inflation of type 1 error the number of comparisons was restricted to those necessary to test the primary hypotheses (MDPV vs. VEH, no between dose comparisons were made), and in the thermoregulation study, comparisons were focused on early time points with observable mean differences. Where Levene's test for equality of variance was significant, corrected statistics are presented. Corrected degrees of freedom are rounded to the nearest whole number for presentational purposes.

RESULTS

Pharmacokinetics, Metabolism, and Hyperthermic Response

MDPV in Serum and Tissue After 2 mg/kg MDPV

Maximal MDPV (**Figure 1**) concentrations in sera were likely attained before the first measurement, since we saw the highest levels at 30 min (140 ng/mL). The same applied to tissues where influx was not detectably delayed compared to serum since maximal concentrations were also observed at 30 min: 263.97 ng/g in brain, and 531.58 ng/g in lung. Until 2 h, the brain/serum ratio was between 1.8 and 2.5, and at 4–6 h the brain/serum ratio was ∼4 however, by this time levels in serum and tissue were very low, and almost undetectable by 6 h.

MDPV and Metabolites in Urine, Sera, and Tissues After 4 mg/kg MDPV

MDPV itself was present in urine but was extracted primarily as metabolites. We adopted the nomenclature of Meyer et al. [32] for the most abundant substances detected which were 4-hydroxy-3-methoxypyrovalerone (demethylenyl-methyl-MDPV), followed by 3,4-dihydroxypyrovalerone (demethylenyl-MDPV) (see **Table 1** and **Figure 2** for quantification and structures). All other metabolites were in a minority.

In tissues, the metabolites demethylenyl-MDPV, demethylenyl-methyl-MDPV, demethylenyl-oxo-MDPV, and oxo-MDPV) were present at very low levels. A maximum value of 8 ng/ml demethylenyl-methyl-MDPV in the brain was observed, and 27 ng/ml in the lungs). In serum demethylenyl-oxo-MDPV was present at low levels (maximum value 23.8 ng/ml), and oxo-MDPV was almost undetectable (maximum value 2.8 ng/ml).

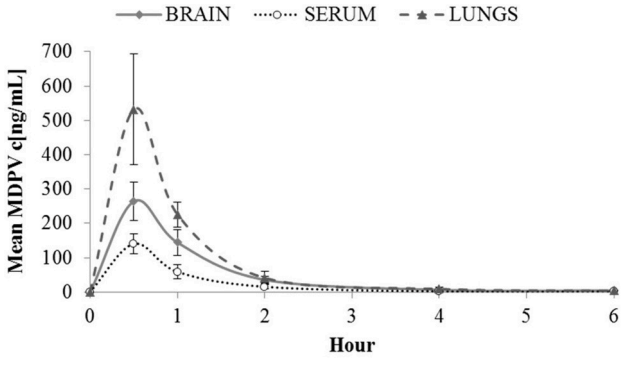

FIGURE 1 | Mean MDPV concentrations (c[ng/mL]) in brain, serum and lungs observed over 6 h at the following time points 0.5, 1, 2, 4, and 6 h after sc. administration of 2 mg/kg of MDPV. At 6 h, all tissue values were <4. For graphical purposes, at 6 h a mean of 4 and a standard deviation of 1 is used. Likewise at 6 h, all serum values were <1 (so a mean of 1, and standard deviation of 0.25 are used). Error bars show ±1 standard deviation.

Rectal Temperature After 2 or 4 mg/kg MDPV

There were significant main effects on rectal temperature of drug and time, minimum $F_{(2, 54)} = 44.08$, $p = 0.0001$, but no main effect of home-cage, $F_{(1, 54)} = 0.19$, $p = 0.66$. All interactions, including the three-way drug x home-cage x time interaction were significant, minimum $F_{(7, 383)} = 2.79$, $p = 0.001$. **Figure 3** shows that the groups had equivalent body temperatures at the 8.00 h baseline temperature measurement, maximum $t_{(38)} = 0.41$, $p = 0.68$. VEH rats' temperatures remained steady at about 37.7–38°C, throughout the temporal observation, irrespective of housing condition.

After 2 or 4 mg/kg temperature increased in both individually- and (more dramatically in) group-housed rats, peaking at 9.30 h and then diminishing. After 2 mg/kg, temperature was significantly elevated in group- and individually-housed rats until 12.00 noon, minimum $t_{(18)} = 2.32$, $p = 0.03$ (with some fluctuating differences thereafter). Compared to 2 mg/kg the increase in temperature after 4 mg/kg was longer-lasting: significant elevations were seen in group- and individually-housed rats at all-time measurements up until 14.00 h (except 10.00 h, individually-housed), minimum $t_{(18)} = 1.86$, $p = 0.04$, one tailed (with some fluctuating differences thereafter).

Behaviour: Open Field and PPI

Locomotor Stimulation in the Open Field

The main effect of testing-onset and the bins x testing-onset interaction were not significant, maximum $F_{(3, 183)} = 2.12$, $p = 0.11$. All other main effects and interactions, including the three-way drug × testing-onset × bins interaction were significant, minimum $F_{(8, 183)} = 3.23$, $p = 0.001$.

TABLE 1 | The urinary metabolites of phase II were screened using UHPLC-QTOF(MS) and the following glucuronides were confirmed: glucuronide demethylenyl-methyl-MDPV and glucuronide demethylenyl-MDPV.

	Concentration in urine (ng/mL)		
	Demethylenyl-MDPV	**Demethylenyl-methyl-MDPV**	**MDPV**
Urine 1	704	398	122
Urine 2	586	790	227
Urine 3	284	145	74

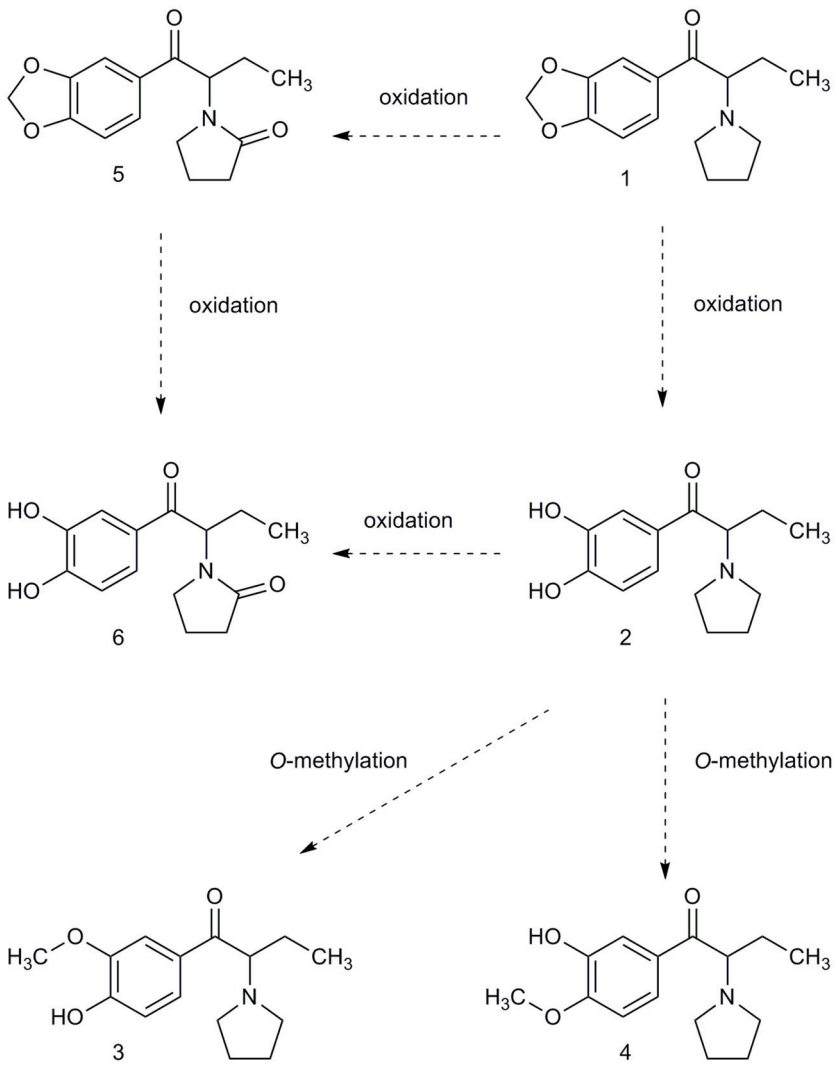

FIGURE 2 | The metabolites of phase I were determined using LC-MS/MS and synthetized metabolite standards. Phase II metabolites of compound (2) and (6) were confirmed using UHPLC-QTOF(MS) as glucuronide demethylenyl-methyl-MDPV and glucuronide demethylenyl-MDPV.

At both 15 and 60 min testing-onsets the 4 mg/kg treated rats were indistinguishable from VEH (except in bin 1, 60 min), maximum $t_{(13)} = 1.69$, $p = 0.11$). At both testing-onsets, rats showed a normal pattern of locomotor habituation (progressively diminishing activity over the session) which was not disturbed by any dose MDPV. However, 1 and 2 mg/kg treated rats were significantly more active than VEH at both the 15 min testing-onset, minimum $t_{(11)} = 2.98$, $p = 0.00$ (**Figure 4A**), and at the 60 min testing-onset after 1 mg/kg (bins 4–6: minimum $t_{(18)} = 2.54$, $p = 0.02$), and after 2 mg/kg (bins 2–5: minimum $t_{(18)} = 2.11$, $p = 0.05$ (**Figure 4B**).

Spatial Distribution of Locomotor Behaviour in the Open Field

There was no main effect of testing-onset on T_{centre}, $F_{(1, 72)} = 0.00$, $p = 0.95$, but the main effect for drug, and drug x testing-onset interaction were significant, minimum $F_{(3, 72)} = 7.13$, $p = 0.00$, **Figure 5A**. At 15 min 1 mg/kg-treated rats spent significantly more time in the centre (than VEH), and 4 mg/kg –treated rat significantly less, minimum $t_{(10)} = 3.92$, $p = 0.00$, with 2 mg/kg having no significant effect, $t_{(12)} = 0.47$, $p = 0.64$. At 60 min, 1 and 2 mg/kg increased time in the centre, minimum $t_{(18)} = 2.26$, $p = 0.04$, with 4 mg/kg having no effect, $t_{(18)} = 1.87$, $p = 0.07$.

There was no main effect of testing-onset on thigmotaxis, $F_{(1, 72)} = 2.76$, $p = 0.10$, however there was a significant main effect of drug, and drug x testing-onset interaction, minimum $F_{(3, 72)} = 5.77$, $p = 0.00$, **Figure 5B**. At 15 min, 2 and 4 mg/kg increased, and 1 mg/kg decreased thigmotaxis, minimum $t_{(10)} = 2.27$, $p = 0.05$. At 60 min, 4 mg/kg again increased thigmotaxis, $t_{(18)} = 2.55$, $p = 0.02$, but 1 and 2 mg/kg had no significant effect, maximum $t_{(18)} = 0.77$, $p = 0.45$.

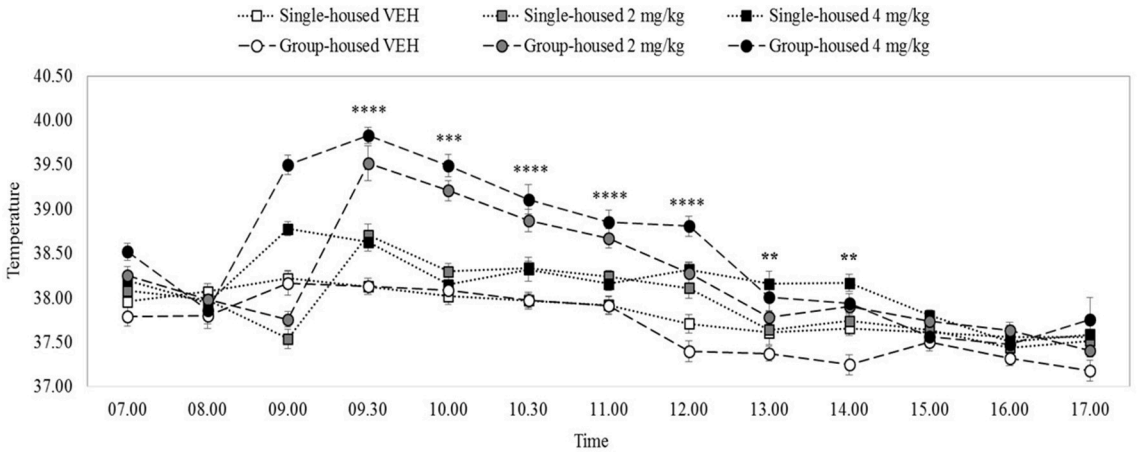

FIGURE 3 | Mean rectal temperature (°C) over 10 h in rats ($n = 10$) housed individually (solid lines) or in groups of five (dashed lines). 4 mg/kg MDPV (black markers), 2 mg/kg MDPV (grey markers) or VEH (white markers) was administered sc. at 09.00 (black dotted vertical line). Error bars show ±1 standard error of the mean. Asterisks indicate significant differences from VEH at minimum $p < 0.05$.

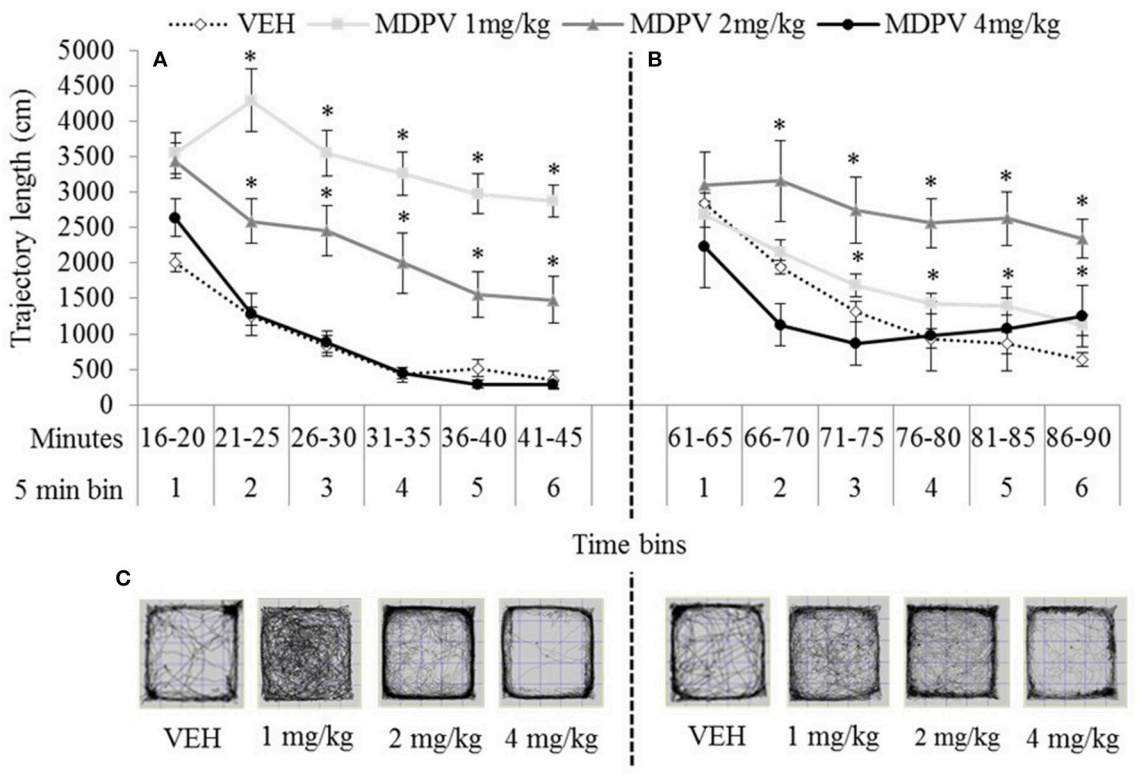

FIGURE 4 | Mean trajectory length (cm/5 min over 30 min) tested 15 min **(A)** or 60 min **(B)** after drug administration: sc. MDPV 1 mg/kg (light grey), 2 mg/kg (mid-grey) or 4 mg/kg (black) vs. VEH (white). Shown alongside 5 min bins (on the x axis) are minutes elapsed since drug administration. Error bars show ±1 standard error of the mean. Asterisks indicate significant differences from VEH for the 1 and 2 mg/kg groups, at minimum $p < 0.05$. Example trajectory patterns are shown in **(C)**.

Prepulse Inhibition

Habituation and ASR data showed no significant main effects or interactions, maximum $F_{(3, 72)} = 2.25$, $p = 0.24$. PPI data showed no main effect of the drug treatment, $F_{(3, 72)} = 2.21$, $p = 0.09$, but the main effect of testing-onset, and the drug treatment × testing-onset interaction were significant, minimum $F_{(3, 72)} = 2.73$, $p = 0.05$, **Figure 6**. In the 15 min testing-onset group, 4 mg/kg significantly disrupted PPI, $t_{(18)} = 2.70$, $p = 0.02$, and 1 and 2 mg/kg had a marginally significant effect, maximum $t_{(18)} = 1.95$, $p = 0.07$. At 60 min testing-onset, none of the

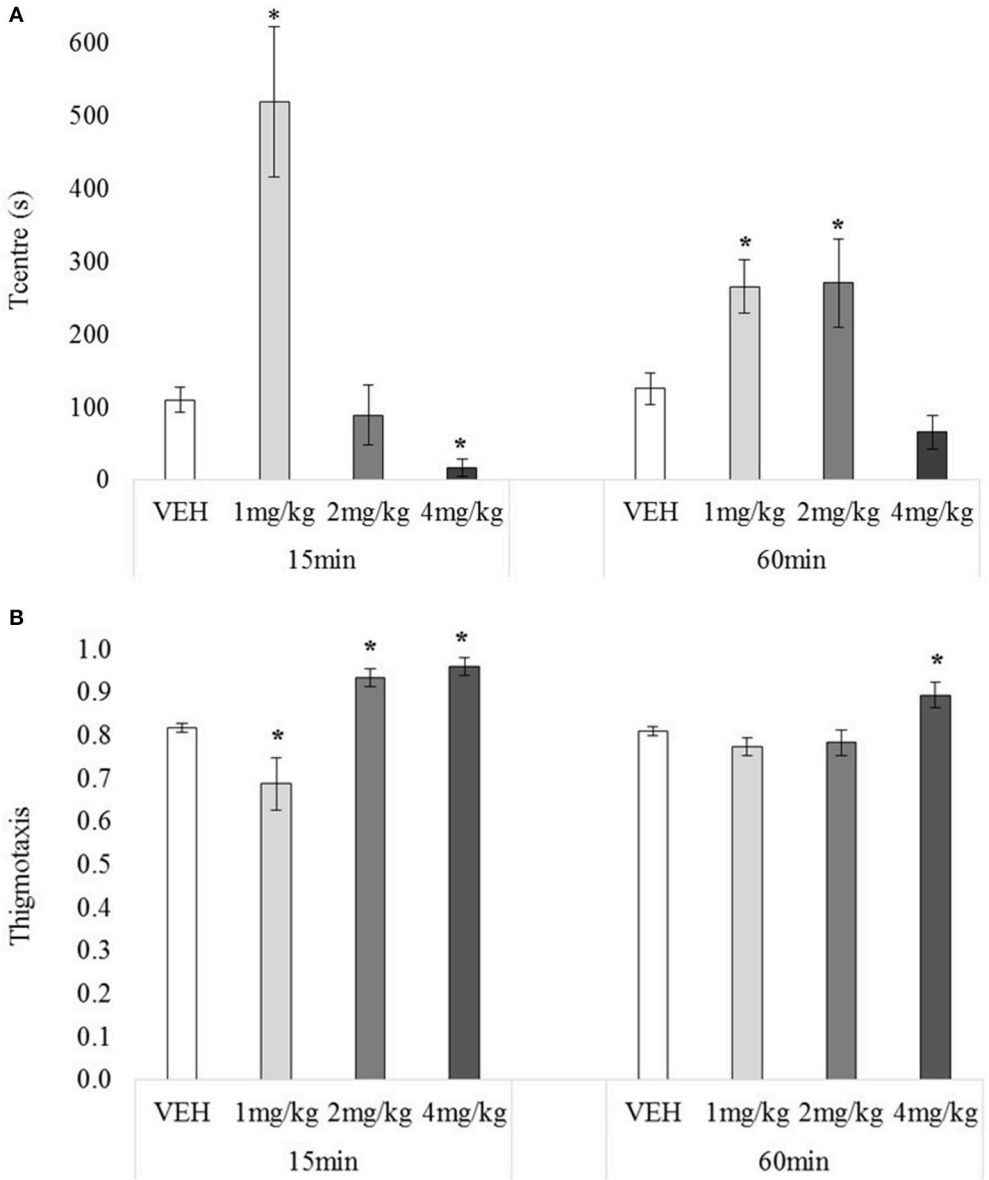

FIGURE 5 | Mean T_{centre} **(A)** and mean thigmotaxis (**B**) over 30 min tested 15 or 60 min after drug administration: sc. MDPV 1 mg/kg (light grey), 2 mg/kg (mid-grey) or 4 mg/kg (dark grey) vs. VEH (white). Error bars show ±1 standard error of the mean. Asterisks indicate significant differences from VEH at minimum $p < 0.05$.

MDPV doses significantly affected PPI, maximum $t_{(18)} = 1.67$, $p = 0.11$.

DISCUSSION

As expected, our pharmacokinetic findings were in line with previous results for serum and brain, and we reported new data on lungs; after 2 mg/kg, MDPV reaches its peak concentrations in serum and tissues in under 30 min and declines rapidly thereafter. Our biotransformation study detected a number of metabolites in sera and tissues, however at very low levels. In urine, concentrations of metabolites exceeded those of MDPV itself, and we confirmed that demethylenyl-methyl-MDPV the most abundant. As previous studies have shown we found a small increase in temperature in individually-housed rats, which was exacerbated by crowded cage conditions. In line with existing findings, our behavioural effects were systematically related to drug concentrations/dose, and showed good correspondence with the known pharmacokinetic time-course for MDPV. As expected, in the open field, lower doses generally stimulated locomotion and exploration, whereas the higher dose initially induced stereotypy/gross suppression of motor behaviour, consistent with stimulant-typical psychomimesis. We presented additional original data which supported this: 4 mg/kg can induce transient psychomimesis shown as disrupted PPI.

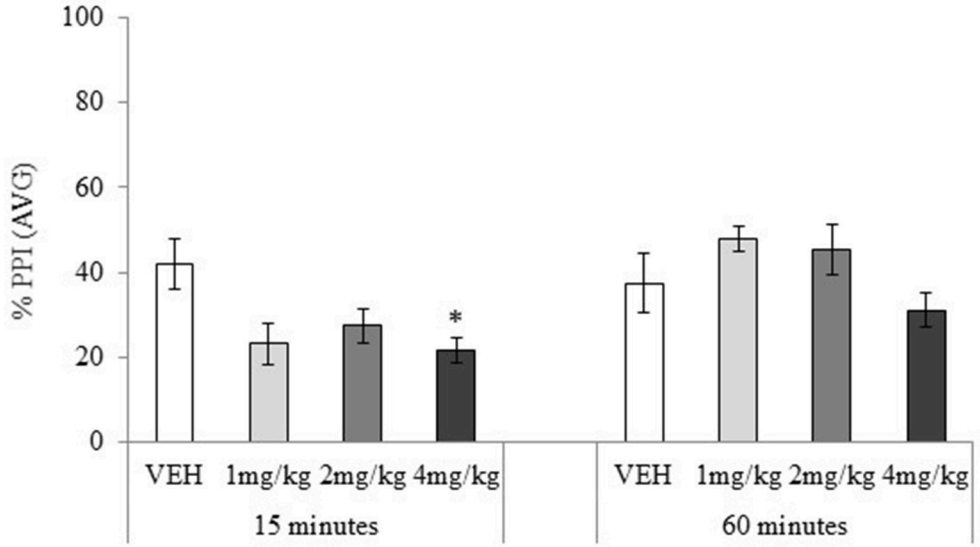

FIGURE 6 | Mean percentage (%) prepulse inhibition (AVG amplitude) tested 15 or 60 min after drug administration: sc. MDPV 1 mg/kg (light grey), 2 mg/kg (mid-grey) or 4 mg/kg (dark grey) vs. VEH (white). Error bars show ±1 standard error of the mean. Asterisks indicate significant differences from VEH at minimum $p < 0.05$.

Pharmacokinetics and Metabolism

The highest concentrations of MDPV in serum, brain, and lung tissue were recorded at 30 min followed by a rapid decline, suggesting that in all cases, the actual peaks were prior to our first measurement. Previous pharmacokinetic data in rats (which were not published at the time of our data collection) showed peak plasma concentrations of MDPV at 10–20 min post-administration [29, 30]. It is therefore almost certain that our value of 140 ng/mL for peak serum concentration is an underestimation. Even so, concentrations in this range in the plasma of human MDPV users are more typical of cases of acute intoxication and overdose (in excess of 50–300 ng/mL) than of recreational use where milder effects are associated with much lower blood levels at ∼10– 50 ng/mL [74].

Novellas et al. [30] reported that MDPV reached the striatum of the brain around 5 min after sc. MDPV (1 mg/kg) in rats and peaked 20–25 min after that, suggesting that our observed peak brain concentration value at 30 min (263.97 ng/g) was likely close to the actual value. From 30 min and the following 2 h, the brain concentration was approximately twice that in the sera. This is consistent with the brain/serum ratio of 2.21 reported by Novellas et al. [30], and reflects MDPV's lipid solubility and capacity to cross the blood-brain barrier. Evidence suggests a kinetic profile of fast onset and relatively short duration, consistent with our short-lasting peak behavioural effects, and with estimates of the onset and duration of subjective effects in humans [25, 28]. MDPV's short duration may have a causal role in re-dosing and escalating use (shown in human and rodent studies), and together with fast penetration of the brain [30] these characteristics are indicative of MDPV's addictive potential [36].

We observed levels of MDPV that were approximately four times higher in lungs than in sera. Rapid transition of substances (such as drugs) from blood to lungs is characteristic of parenterally administered cationic compounds with a lipophilic profile [75]. The rapidity and magnitude of accumulation is partly a consequence of the relatively large volume of blood flowing to the lungs in compared to other organs, such as the brain, where molecules become trapped by lysosomes and mitochondria and accumulate in lung tissue as gradually eluting pools [75]. In consequence, accumulation in, and elimination from lung tissue is usually rapid, which was supported by our data, where lung concentrations of MDPV declined more quickly than in the brain. The relevance (if any) of drug accumulation in the lungs for MDPV toxicity is unclear; pyrrolidinophenone toxicity primarily involve CNS and cardiovascular related problems [13, 56].

As expected, MDPV itself was detected in urine at only very low concentrations, instead MDPV was detected as two major metabolites demethylenyl-MDPV and demethylenyl-methyl-MDPV. These urinary metabolites were at concentrations approximately three to four times higher than the parent drug. Other metabolites were detected in urine, serum, brain and lung but again at very low concentrations. Taken together with pharmacokinetic findings here and previous studies [30–32, 72], MDPV appears to be cleared from sera and tissues relatively rapidly (here almost undetectable by 6 h), and by 24 h only very small amounts of MDPV in the form of metabolites remain in the body the majority having been excreted in urine. Toxicological tests that seek to confirm MDPV in blood or tissues after 6 h should search for a range of MDPV metabolites rather than MDPV itself and use the most sensitive methods available. Our sample size for the biotransformation study was small, and there was variability in the data, both of which may have contributed to the observation of lower concentrations, and few metabolites than other studies have shown previously.

Thermoregulation

As in our pharmacokinetic study, we found peak temperatures at our earliest measurement time-point (30 min post-administration), therefore, we cannot exclude the possibility that temperatures were increased prior to this. MDPV increased body temperature for several hours up to a maximum of ∼0.5°C in rats housed individually at ∼22°C. This small increase is in agreement with previous work using individually-housed Wistar rats where MDPV's effects on body temperature (at a similar dose range) were negligible even at 30°C ambient temperatures [45]. A number of findings have been published now that demonstrate that environmental crowding and hotter ambient temperatures can exacerbate drug-induced hyperthermia [70, 73, 76, 77]. We found longer-lasting increases in temperature in group-housed rats, up to 2°C. MDPV increases brain and body temperature in mice at high ambient temperatures and under conditions of social interaction [21, 43, 44], perhaps due to combined DAT-mediated hyperactivity and NET-mediated sympathomimetic effects [20, 22], although recent research has also implicated 5-HT [21]. If environmental/social conditions can potentiate both locomotor and temperature effects simultaneously, these circumstances might increase the risk of toxic reactions. On the other hand, although MDPV toxicity and fatalities in humans have been documented [7, 34–39, 41], they remain relatively uncommon given the prevalence of cathinone use. Moreover, the doses tested here equate to very high human doses (in excess of ∼20 mg, [78]), that are in the range where adverse reactions would be expected [25].

Locomotor Behaviour

As expected, the locomotor stimulant action of MDPV across the three doses (1, 2, and 4 mg/kg) was consistent with the inverted "U" behavioural dose-response of dopaminergic agonists (such that progressively larger doses resulted in progressively increasing, then decreasing activity). In our behavioural studies, the 15 min testing-onset synchronised with peak serum and brain concentrations as well as their initial decline. Effects over testing-onsets showed that locomotor effects related to declining systemic levels of MDPV, e.g., effects of 1 mg/kg at 15 min were equivalent to residual effects of 2 mg/kg at 60 min. Other pyrrolidine-containing synthetic cathinone derivatives, α-pyrrolidinopropiobutiophenone (α-PBP), and α-pyrrolidinopentiophenone (α-PVP), as well as MDPV, show this inverted "U" curvilinear relationship with locomotor stimulation in rats ([14, 45, 52, 58] but see [53] for effects in mice). The normal pattern of locomotor habituation was not disturbed by lower doses of MDPV, however at 4 mg/kg (which induced some hypolocomotion at 15 min) by 80–90 min post-drug administration, locomotion appeared to be increasing. Had testing continued, hyperlocomotion may have been observed as brain drug concentrations diminished, as previously reported [45, 52]. MDPV's potent stimulatory effects most likely relate to its potent DAT inhibition, coupled with its lack of capacity to increase extracellular 5-HT which can attenuate DA-induced locomotor effects [4].

Rats usually avoid the aversive centre of the open field (which is brighter more and open) preferring to spend time in the periphery (next to the arena walls). The spatial characteristics of locomotor behaviour in this paradigm can provide some indication of drug effects on emotionality with increased exploration of the centre suggesting decreased emotionality (anxiolytic-like effects) and *vice versa*. [79–81]. In our experience, effects on T_{centre} and thigmotaxis are usually (but not always) the inverse of one another, however, whilst related, T_{centre} may be more sensitive to effects on emotionality, and thigmotaxis to locomotor stereotypy. MDPV has been shown previously to increase exploration in mice [53], as well as induce stereotypy (at a range of doses) in rats and mice [30, 45, 53, 72]. Here, as the dose/systemic levels of MDPV declined below ∼2 mg/kg there was increased exploration of the centre (unconfounded by thigmotaxis), possibly indicative of anxiolytic effects (perhaps equating to positive affect or euphoria in humans at acute lower doses; [25]). At ∼2 mg/kg or more, there was only increased thigmotaxis indicative of locomotor activation and/or emerging stereotypy. Stimulant-typical stereotypies emerge in a systematic manner [30, 72, 82], which was shown here, and, with MDPV, are reversible with the typical antipsychotic (a DA D2 antagonist), haloperidol (e.g., [30]), which is indicative of its DA-ergic origin and psychomimetic potential.

Sensorimotor Gating and Psychomimesis (PPI)

Only 4 mg/kg MDPV disrupted PPI significantly and only transiently so (at the 15 min testing-onset), when plasma levels of MDPV were at their peak, and peak brain concentration was developing. By 60 min, MDPV levels in sera and brain had declined by ∼30–40% to around 50 and 150 ng/mL respectively, and PPI was no longer significantly disturbed. A specific disruption of PPI (i.e., in the absence of significant baseline drug effects on habituation or ASR that might confound interpretation) reflects dysfunctional sensorimotor gating, and is an index of the psychomimetic properties of drugs [60]. Evidence to date has shown that other (ring-substituted) cathinones and stimulants can disrupt PPI, but usually only at higher doses [61–69]. Although different cathinones and stimulants operate via different DA-ergic mechanisms (i.e., DAT inhibition and/or non-exocytotic efflux) the net result is an increase extracellular DA. 5-HT-ergic compounds more reliably disturb PPI (e.g., [73, 83, 84]), however it would appear that hyperdopaminergia alone is sufficient to disrupt PPI, since MDPV has negligible effects on 5-HT [14, 85]. Moreover, cathinone-induced PPI deficits and stimulant-typical psychomimesis can be reversed with typical and atypical antipsychotics [30, 86] which have DA D2 receptor antagonism in common [87], suggesting a role for this receptor subtype specifically [63]. Our findings indicate that the duration of psychological symptoms is likely to be short, which suggests that in cases of acute MDPV intoxication, adverse psychological effects may pass relatively quickly.

CONCLUSIONS

In conclusion, we confirmed MDPV's typical DA-ergic psychostimulant drug profile: at lower doses, it modestly

increased locomotion and exploration; at higher doses it produced stimulant-typical psychomimetic and gross motor effects/stereotypy. Behavioural findings have relevance to recreational users who, in striving for stimulatory effects may take large amounts of MDPV, unaware that this will only delay rather than increase stimulation (which is modest, in any case) and induce adverse psychological and physical effects instead, with risks of toxicity (especially if taken in combination with other drugs) increasing with dose. However, our lowest dose of MDPV, 1 mg/kg, has been calculated as equivalent to 10 mg human dose and our highest dose is equivalent to 30 mg or more [78]. Thus even our lowest dose, whilst within the range used by people, was nevertheless a high dose, in the range associated with unwanted effects [25]. Responsible and occasional use of sensible doses of MDPV alone is probably relatively low-risk for most healthy people. However, users should be aware that MDPV's acute effects may create interactions between their activity levels, environmental and social circumstances and potentiate temperature dysregulation. Moreover, it is important, in a world where NPSs proliferate [3, 36] and where "sensible" recreational drug-taking is increasingly normalised [4, 5] that even where acute use appears low risk, the possible effects of long-term, chronic or binge use of MDPV on psychological and physical health are kept in mind. These are simply, as yet, unknown, and data will only emerge in years to come. The short-lasting behavioural effects and pharmacokinetics shown here are consistent with existing evidence that MDPV has characteristics that encourage risky use (such as re-dosing, binge or poly-drug use, 36) as well as significant addictive potential.

AUTHOR CONTRIBUTIONS

All authors made a substantial contribution to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work. All authors were involved in drafting the work or revising it critically for important intellectual contents. All authors gave final approval for the current version of the work to be published. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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MINI REVIEW
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Synthetic Aminoindanes: A Summary of Existing Knowledge

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Objectives: Aminoindanes ("bath salts," a class of novel psychoactive substances, NPSs) increased rapidly in popularity on the recreational drug market, particularly after mephedrone and other synthetic cathinones were banned in the UK in 2010. Novel aminoindanes continue to emerge, but relatively little is known about their effects and risks. Their history, chemistry, pharmacology, behavioral effects, pharmacokinetics, and toxicity are reviewed in this paper.

Methods: Scientific literature was searched on ISI Web of Knowledge: Web of Science (WoS) during June and July 2017, using English language terms: aminoindanes such as 5,6-methylenedioxy-2-aminoindane (MDAI), 5-iodo-2-aminoindane (5-IAI), 2-aminoindane (2-AI), 5,6-methylenedioxy-*N*-methyl-2-aminoindane (MDMAI), and 5-methoxy-6-methyl-2-aminoindane (MMAI). WoS was selected as it searches several databases simultaneously and has quality criteria for inclusion. For typical use and effects, Erowid, PsychonautWiki, Bluelight, and Drugs-Forum were searched; for legal status and epidemiology, the European Information System and Database on New Drugs (EDND) was used.

Results: Aminoindanes were first synthesized for medical use, e.g., as anti-Parkinsonian drugs and later as a potential compound facilitating psychotherapy; however, they are now widely substituted for ecstasy. Their mechanisms of action (primarily *via* serotonin) mean that they may pose a significant risk of serotonin syndrome at high doses or when combined with other drugs. Fatally toxic effects have been observed both in the laboratory in animal studies and in clinic, where deaths related with aminoindanes have been reported.

Conclusion: Greater knowledge about aminoindanes is urgently required to decrease risks of fatal intoxication, and appropriate legislation is needed to protect public health without impeding research.

Keywords: aminoindanes, 5,6-methylenedioxy-2-aminoindane, 5-iodo-2-aminoindane, 2-aminoindane, 5,6-methylenedioxy-*N*-methyl-2-aminoindane, 5-methoxy-6-methyl-2-aminoindane

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INTRODUCTION

During the past decade, there has been a dramatic increase in the number and variety of novel psychoactive substances (NPSs) available on the illicit and gray drug markets (particularly *via* the Internet and "dark web"). In 2014, the number of NPSs boomed with 101 new compounds detected. In 2016, approximately one new NPS per week was identified, and the European Monitoring Centre

for Drugs and Drug Addiction (EMCDDA) was monitoring more than 620 NPSs (1). One of the first NPSs that became widely used recreationally was the cathinone derivative mephedrone (4-MMC, 4-methylmethcathinone), marketed at the time as a "legal" substitute for ecstasy (MDMA, 3,4-methylenedioxymethamphetamine) and cocaine, sharing effects of both (2). Mephedrone and other cathinones such as methylone (βk-MDMA, 3,4-methylenedioxy-*N*-methcathinone) and butylone (βk-MBDB, β-keto-*N*-methylbenzodioxolylbutanamine) were initially sold as, e.g., "bath salts" or "plant food," labeled "not for human consumption." Mephedrone became very popular due to its low price, high purity, and "legality" and, in the UK, it rapidly became as widespread as cocaine (3). In 2009 and 2010, the UK government placed piperazine derivatives, mephedrone, and other related cathinones under legal control (4), which resulted in their immediate replacement with new structural analogs and with a new class of NPSs: synthetic aminoindanes. One of the first was 5,6-methylenedioxy-2-aminoindane (MDAI), which claimed to be a "legal," non-neurotoxic analog of MDMA, with strong empathogenic and weaker stimulatory effects (5). Aminoindanes such as MDAI, 5,6-methylenedioxy-*N*-methyl-2-aminoindane (MDMAI), 5-iodo-2-aminoindane (5-IAI), 2-aminoindane (2-AI), 5-methoxy-6-methyl-2-aminoindane (MMAI), and 5-methoxy-2-aminoindane (MEAI) represent a relatively new generation of NPS. Cases of acute toxicity, including fatal poisoning, have been reported with their use (6). Only minimal reliable information on aminoindanes exists at present and, owing to their increasing popularity, the present brief review is timely. The paper summarizes the history of their creation, therapeutic potential in medical research and subsequent discovery by recreational drug users, their pharmacology, behavioral effects, pharmacokinetics, and toxicity.

METHOD

ISI Web of Knowledge: Web of Science (WoS) was searched during June and July 2017. WoS was selected because it simultaneously searches other databases such as PubMed and ScienceDirect, and includes quality criteria for inclusion (e.g., peer review). Keywords (used separately and in combination) were as follows: aminoindane, MDAI, 5,6-methylenedioxy-2-aminoindane, 5-IAI, 5-Iodo-2-aminoindane, 2-aminoindane, MDMAI, 5,6-Methylenedioxy-N-methyl-2-aminoindane, MMAI, 5-Methoxy-6-methyl-2-aminoindane. Full empirical/review articles containing relevant information about aminoindanes written in the English language were included; no date limits applied (**Figure 1**). When suitable articles were found, citation searches were also conducted. For subjective effects, typical use and doses, Erowid,[1] PsychonautWiki,[2] Bluelight,[3] and Drugs-Forum[4] (Internet discussion fora and wikis) were searched using the same search terms as above. Information about legal status and availability of aminoindanes in the European Union (EU), the EDND was consulted *via* the senior author, Dr. Palenicek, through the "Working group: Early warning system on new drugs," National Monitoring Centre for Drugs and Addiction, Czech Republic.

RESULTS

Chemistry

2-aminoindane is an amphetamine (AMPH) analog with a rigid conformation due to a bridge between the α-carbon and the aromatic ring (8). In the 1990s, Nichols et al. synthesized cyclic analogs of 3,4-methylenedioxyamphetamine (MDA), MDMA, 3-Methoxy-4-methylamphetamine (MMA), and *p*-iodoamphetamine (PIA) containing the 2-AI compound. Their procedures for synthesizing aminoindanes were well described (9–13). NPSs synthesized from the substances listed above are MDAI, MDMAI, MMAI, and 5-IAI (**Table 1**); all of these are psychoactive and their presence on the market has been confirmed in confiscated samples of "legal highs" (14). The EU Early Warning System[5] and the United Nations Office on Drugs and Crime (UNODC) Early Warning Advisory (EWA) on New Psychoactive Substances[6] have reported additional novel substances with an aminoindane structure, such as NM-2AI (*N*-methyl-2-aminoindane), 1-AI (1-aminoindane), and a fenfluramine analog ETAI (*N*-ethyl-5-trifluoromethyl-2-aminoindane); however, there is currently no scientific information available about these compounds.

Origins of Aminoindanes in Pharmacological Research

Owing to an amino group, aminoindanes are potentially vasoactive and bronchodilatory, which was the main focus for their initial development (15, 16). Since the chemical structure of aminoindanes is similar to that of AMPHs (owing to the presence of the phenethylamine skeleton), there was a strong assumption that aminoindanes would have the same bronchodilatory effect as ephedrine. Therefore, Levin et al. (17) evaluated the bronchodilatory and toxic effects of 2-AI and its N-substituted derivatives in the rat. 2-AI hydrochloride given intravenously showed less toxicity than AMPH hydrochloride, and 2-AI derivatives were more effective bronchodilators as compared with L-ephedrine. Aminoindanes have also been studied for their analgesic potency (comparable to morphine sulfate)—potency to increase blood pressure, respiration, and spinal reflexes (18, 19).

Based on Kier's receptor mapping technique (20)—a drug discovery method where the distance between oxotremorines's heteroatoms and dopamine's heteroatoms in reported conformations is similar—Martin et al. (21) designed and synthesized a series of aminoindanes with the intention to invent an anti-Parkinsonian drug. Although none of the resulting substances antagonized Parkinsonian-like symptoms (in a model of oxotremorine-induced tremors) nor showed any dopaminergic properties in mice, some of the molecules

[1]https://www.erowid.org/.
[2]https://psychonautwiki.org/.
[3]http://www.bluelight.org/.
[4]https://drugs-forum.com/.
[5]http://www.emcdda.europa.eu/themes/new-drugs/early-warning.
[6]https://www.unodc.org/LSS/Home/NPS.

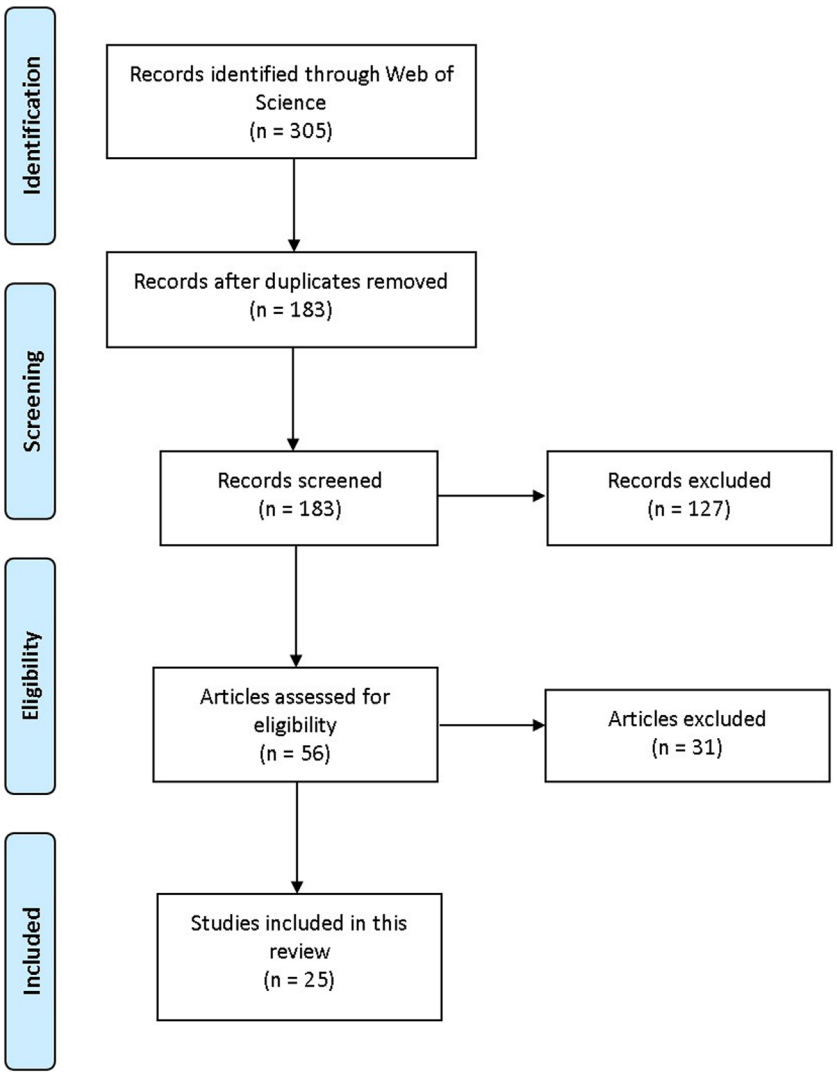


FIGURE 1 | PRISMA flowchart visualization of the search and selection process. Adapted from Moher et al. (7).

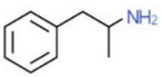
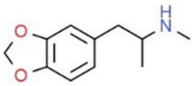
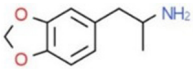
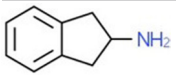
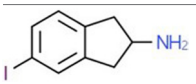
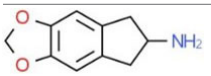
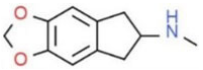
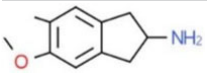
showed monoamine oxidase (MAO) inhibition and analgesic activity. Therefore, they investigated molecules with higher MAO-inhibiting potential *in vivo* and identified a candidate molecule N-methyl-5-methoxy-1-indanamine in mice. The authors concluded that the size of amine substituent and position of methoxyl substitution are most important for their biological activity (22).

Kalir et al. (23) examined the inhibitory action of substances containing aminoindanes on brain mitochondrial MAO type A and B, to ascertain MAO B inhibitors' anti-Parkinsonian potential. Two irreversible, selective-type MAO B inhibitors were identified: AGN-1133 (*N*-methyl-*N*-2-propynyl-1-indanamine hydrochloride) and AGN-1135 (*N*-propargyl-1R-aminoindane). AGN-1135 showed greater selectivity *in vitro* and *in vivo*, with no central nervous system, cardiovascular, or sympathomimetic effects and was eventually patented as a Parkinson's disease treatment (US patent no. 5457133A; US patent no. 5387612A; US patent no. 5453446A), known as rasagiline (24). The key difference between rasagiline and its analog selegiline is that rasagiline's major metabolite is aminoindane, whereas selegiline metabolizes to L-amphetamine and L-methamphetamine (24, 25). Therefore, no AMPH-like adverse effects are seen after rasagiline.

Aminoindanes–A Unique Drug Class with Entactogenic Properties

Contemporary research has focused on the psychoactive effects of substituted 2-AIs (9–13, 26–31). In their earlier work, Nichols et al. (32) proposed a new class of therapeutic psychoactive substances "entactogens," which were neither hallucinogens nor psychostimulants; instead, they facilitated communication and introspection, and were argued to be valuable agents in psychotherapy and potentially powerful tools for understanding the neurochemistry of emotion (27). To begin with, entactogens included MDMA, MDA, and 3,4-methylenedioxy-*N*-ethylamphetamine

TABLE 1 | Chemical structures and names, International Union of Pure and Applied Chemistry (IUPAC) names of amphetamine, MDMA, MDA, 2-aminoindane, and its derivatives with psychoactive effects.

Structure	Name	Chemical name	IUPAC name
	Amphetamine, AMPH, Speed	Alpha-methylphenethylamine	l-phenylpropan-2-amine
	MDMA, Ecstasy, Molly, X, XTC	3,4-methylenedioxymethamphetamine	l-(1,3-benzodioxol-5-yl)-*N*-methylpropan-2-amine
	MDA	3,4-methylenedioxyamphetamine	l-(1,3-benzodioxol-5-yl)propan-2-amine
	2-AI	2-aminoindane	2,3-dihydro-1H-inden-2-amine
	5-IAI	5-Iodo-2-aminoindane	5-iodo-2,3-dihydro-1H-inden-2-amine
	MDAI	5,6-methylenedioxy-2-aminoindane	6,7-dihydro-5H-cyclopenta[f][1,3]benzodioxol-6-amine
	MDMAI	5,6-methylenedioxy-2-methylaminoindane	N-methyl-6,7-dihydro-5H-cyclopenta[f][1,3] benzodioxol-6-amine
	MMAI	5-methoxy-6-methyl-2-aminoindane	5-methoxy-6-methyl-2,3-dihydro-1H-inden-2-amine

(MDEA), and later, related novel compounds such as 1,3-benzodioxolyl-*N*-methylbutanamine (MBDB) and MDAI were synthesized. Since MDMA and its analogs (MDA, MDEA) had been widely abused by recreational drug users and serotonergic neurotoxicity was identified, Nichols et al. refocused on the preparation of non-neurotoxic analogs of MDMA. The result was the description, for the first time, of these novel "entactogenic" compounds (27, 32).

Nichols et al. (9) described effects of MDAI on catecholamines and serotonin (5-HT), measured metabolite levels, and determined the affinity (K_D) and number of binding sites (B_{max}) for 5-HT transporter (SERT) (in rat brain cortical resp. hippocampal homogenates) measured 1 week after subcutaneous (s.c.) administration of 40 mg/kg MDAI. After 1 week of recovery, there were no significant changes in levels of any of the measured neurotransmitters or SERT compared with controls; by contrast, significant reductions in the neurotransmitter levels and SERT were induced by MDMA. No changes in K_D and B_{max} were observed, indicating no detectable 5-HT neurotoxicity or 5-HT terminal degeneration. However, drug discrimination experiments with MDMA-trained rats showed that MDAI fully substitutes for MDMA and that MDAI and MDMAI were observed to completely substitute for another MDMA-like drug MBDB (10). It was concluded that both drugs have MDMA-like behavioral pharmacology but without lasting 5-HT neurotoxicity following an acute, very high dose. However, the effects of chronic administration of MDAI (most drugs, whether for medical or recreational purposes are taken on multiple occasions) were not investigated until much later, and research on the chronic effects of aminoindanes, including MDAI, is still lacking.

A study on *in vitro* monoamine reuptake inhibition (using rats' synaptosomes) identified MDAI as a highly potent inhibitor of 5-HT and dopamine (DA) reuptake rather than causing non-vesicular DA release. 5-IAI and MMAI were subsequently evaluated, both of them increased non-vesicular release of 5-HT, DA, and norepinephrine (NE), but MMAI had 100- and 50-fold selectivity for 5-HT over DA and NE uptake inhibition, indicating that it is a very selective serotonergic releaser (28). In the monoamine reuptake transporter inhibition test performed on HEK 293 (human embryonic kidney 293) cells, MDAI's ability to preferentially inhibit the NE transporter (NET) and SERT over the DA transporter (DAT) was confirmed, with an approximately twofold lower potency compared with MDMA. The other aminoindane tested, 5-IAI, showed a similar pattern/ratio of inhibitory action at NET/SERT/DAT. 2-AI selectively inhibited just NET, and for SERT and DAT it has low potency. Apart from inhibitory actions on transporter molecules, aminoindanes have been shown to cause transporter-mediated release (reverse transport) of monoamines: MDAI released 5-HT and NE, 5-IAI released 5-HT and DA, and 2-AI released NE and DA (33).

The pharmacokinetics of MDAI in Wistar rats have been described in our recently published paper (34). Tissue samples were collected after a single bolus of MDAI (10 mg/kg, s.c.) at intervals of 30, 60, 120, 240, and 480 min after administration. Separated sera, whole brains, livers, and lungs were analyzed. MDAI showed fast and high influx into the brain; the drug

was accumulated in lungs where the concentration exceeded the concentration in the brain by approximately 30% (~30 vs. 18 μg/g, respectively) indicating its high-lipid solubility (34). When compared with s.c. MDMA in Sprague-Dawley rats (35), the kinetic profile of MDAI is much faster and its storage profile is similar to PMMA or 2C-B (36, 37). These results can be associated with potential selective MDAI neurotoxicity, exacerbated by combination with other drugs (6).

Subjective Effects and Acute Behavioral Studies

Very little is known about acute behavioral effects of aminoindanes in animal studies. We described acute behavior in Wistar rats after MDAI administration. Three different s.c. doses of MDAI (5, 10, 20, and 40 mg/kg) administered (at two testing onsets 15 respectively 60 min) prior to open field test (OFT) and prepulse inhibition test (PPI) were examined to evaluate effects on locomotor activity and sensorimotor gating. At all doses used, MDAI showed a disruptive effect on sensorimotor gating and, most evidently, at testing onset 15 min. The same disruptive effect on PPI can be seen after MDMA, AMPH or other psychoactive drugs (37), and it is related to changes in sensory filtering of information due to manipulation with DA and 5-HT levels in brain (38). These changes may alter information processing and induce a schizophrenic state (39). MDAI increased trajectory length in a dose-related manner, but not dramatically. MDAI has short-acting, slightly stimulatory and anxiolytic effects (34). In another animal model, in Swiss-Webster mice, Gatch et al. (40) examined the effect of MDAI [1, 3, 10, and 30 mg/kg; administered intraperitoneally (i.p.)] on locomotor activity. Lower MDAI doses produced a rapid onset of locomotor depression and at higher doses, a slower onset of locomotor stimulation was observed, but it was longer lasting. These findings suggest that although MDAI affects DA and stimulation, this is not a strong effect. This can lead users to combine MDAI with other drugs with stimulatory potency.

Since no clinical trial has yet been performed in humans with recreational aminoindanes, information about subjective effects and health risks comes from subjective personal experiences shared on drug website platforms, wikis, and discussion fora. Based on users' reports on PsychonautWiki, Erowid, Drugs-Forum, and Bluelight, MDAI and 5-IAI effects are mainly euphoria, empathy, stimulation (not the case with MDAI), and cognitive enhancement. The adverse effects described by users include dehydration, increased perspiration, anxiety, depression, panic attacks, and tachycardia. Several routes of administration have been reported from insufflation, oral ingestion to rectal application. The latter has the fastest onset of effects. Smoking and injecting have not been described (6, 41, 42). The onset of subjective psychoactive effects is reported to be around 30 min and their peak varies from 45 min up to 3 h after being taken orally. The wide time-window for peak effects after oral use could be caused by different product purities (6, 43): administration routes and factors influencing absorption (e.g., with oral consumption, food in the digestive tract). Users' "recommended" dose for a mild MDAI effect is 100–150 mg, for 2-AI it is 10–20 mg orally (43, 44). The doses of 5-IAI in trip reports are approximately 100 mg orally for a mild effect (45).

Toxicity and Health Risks

Palenicek et al. (34) examined an acute toxicity including median lethal dose (LD_{50}). The highest dose of MDAI (40 mg/kg) showed 50% greater locomotion activity compared with 20 mg/kg during the onset of its action; however, animals rapidly began to hyperventilate and showed signs of serotonin syndrome (intense perspiration, copious salivation, and seizures). In total, 100% of the rats died within 15 min of administration. This was unexpected, since Nichols et al. (9) had previously used this dose and route, and did not report adverse effects or fatalities. While for s.c. administration the LD_{50} was 28.3 mg/kg and i.v. 35 mg/kg, for oral administration all rats survived 40 mg/kg (34). The autopsy and histologic evaluation of tissues of deceased animals confirmed serotonin syndrome as a causal factor in death, with disseminated intravascular coagulopathy and brain edema implicated. Gatch et al. (40) tested MDAI at 100 mg/kg, with a similar outcome to Palenicek et al. (34): this dose was lethal for all mice. Experiments on thermoregulation clearly showed that MDAI dramatically increased body temperature accompanied by profound perspiration, particularly when administered to rats housed in groups. This, along with the other findings from this study, suggests a potentially higher risk of serotonergic toxicity when the drug is used by humans in settings such as clubs or rave/dance parties, where ambient temperatures are increased due to crowding.

Since recreational users take these ecstasy-like drugs frequently in the environment of rave/dance parties for euphoric and entactogenic effects but also to enhance their abilities to dance for long periods, many users desire stimulatory effects. However, in the case of aminoindanes, where primary activity is on the 5-HT system, stimulation is limited. This often leads users to consume aminoindanes in larger doses (to increase the DA release) or in drug cocktails with stimulants such as AMPH, cocaine, or MDMA to potentiate the stimulatory properties of the drug. In these combinations, when 5-HT-ergic substances potentiate DA-ergic substances, an unexpected neurotoxicity and cardiotoxicity may occur (6, 31). Tormey and Moore (46) reported a steady increase in deaths in Ireland from 9 in 2004 to 47 in 2009 from the drug category that includes NPSs (but also includes substances such as solvents); by contrast, their data for cocaine, stimulants, and hallucinogen deaths suggest a peak in 2007, followed by a decline (which would be accounted for if "classic" drugs were being replaced by NPSs). MDAI has been related to renal failure, acute respiratory distress syndrome, hepatic failure, and increased risk of primary pulmonary hypertension or valvular heart disease (47). Furthermore, MDAI-related deaths have been reported: a 17-year-old woman died of cardiac arrest with postmortem toxicological tests detecting MDAI at a concentration of 26.3 mg/L and an ethanol concentration of 14 mg/dL. No other drugs or metabolites were detected. In the other two deaths (men aged 35 and 28), the postmortem toxicology showed MDAI along with AMPHs, MDMA, lignocaine, etc., and ethanol (6). A 27-year-old man was successfully resuscitated by paramedics but died in hospital the following

day, with edema of the brain and lungs, aspiration pneumonia, blood-congested internal organs (and MDAI concentrations of 38 μg/L in peripheral blood and 1800 μg/L in urine 6 h before death) (48). Two 5-IAI and 2-AI fatalities were reported between 2010 and 2012, with one case each (49).

Legal Status

At the time of writing, only a few aminoindanes are controlled in some parts of the EU. 2-AI is controlled in Croatia, Denmark, Estonia, Finland, Hungary, Lithuania, Poland, and Portugal. MDAI is controlled in Cyprus, Czech Republic, Denmark, Estonia, Finland, Hungary, Italy, Lithuania, Portugal, and Sweden. 5-IAI is controlled in Finland, Hungary, Lithuania, and Portugal. For instance, the UK has not specifically restricted aminoindanes yet (4).

CONCLUSION

Although there are some existing studies focusing on MDAI, more research should be performed on the behavioral effects and toxicity of this substance. As we have shown in this review, fatal intoxications connected with MDAI have been reported and animal studies provided evidence of its potentially deadly toxicity due to serotonin syndrome. Furthermore, there is lack of information about toxicity, pharmacokinetics, and behavioral effects of the other aminoindanes. An important issue is also the legal status of these substances, since just a few EU countries control aminoindanes. This may increase the probability of their recreational use and, in turn, the incidence of acute toxicity.

AUTHOR CONTRIBUTIONS

NP contributed to the writing of the paper, searched for relevant literature, and composed the main idea for the study. RH and TP discussed the content of manuscript, added comments and critical feedback, and contributed to the final version of the paper.

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REVIEW
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Synthetic Cathinone and Cannabinoid Designer Drugs Pose a Major Risk for Public Health

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As part of an increasing worldwide use of designer drugs, recent use of compounds containing cathinones and synthetic cannabinoids is especially prevalent. Here, we reviewed current literature on the prevalence, epidemiology, bio-behavioral effects, and detection of these compounds. Gender differences and clinical effects will also be examined. Chronic use of synthetic cathinone compounds can have major effects on the central nervous system and can induce acute psychosis, hypomania, paranoid ideation, and delusions, similar to the effects of other better-known amphetamine-type stimulants. Synthetic cannabinoid products have effects that are somewhat similar to those of natural cannabis but more potent and long-lasting than THC. Some of these compounds are potent and dangerous, having been linked to psychosis, mania, and suicidal ideation. Novel compounds are developed rapidly and new screening techniques are needed to detect them as well as a rigorous regulation and legislation reinforcement to prevent their distribution and use. Given the rapid increase in the use of synthetic cathinones and cannabinoid designer drugs, their potential for dependence and abuse, and harmful medical and psychiatric effects, there is a need for research and education in the areas of prevention and treatment.

Keywords: synthetic drugs, cathinones, cannabis, amphetamine, new psychoactive drugs

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INTRODUCTION

Over the past decade, there has been a worldwide increase in the opportunity for use and consumption of Novel Psychoactive Substances (NPS) – which produce "legal highs" (1–8). Usually mimicking the psychoactive effects of illicit drugs of abuse, these "designer" drugs vary widely in composition, and are mainly sold online and from street retailers. Some of the drugs are advertised as being legal and are often labeled as "not for human consumption" or "licensed by the Ministry of Health," to bypass—legislation enforcement. Based on their pharmacological mechanisms and psychoactive properties, designer drugs fall into four major categories: (i) amphetamine-like stimulants, which include cathinones derivatives and piperazine derivatives that are sold as substitutes for "ecstasy," (ii) synthetic cannabinoids, (iii) hallucinogenic/dissociative agents, and (iv) opioid-like compounds (9). While new compounds are appearing relentlessly on the drug market, information regarding their potential toxicity is scarce and the number of emergencies related to their use is increasing (10, 11). Given the public health threat that is growing in complexity, a multidisciplinary coordinated effort is required to elucidate the acute and chronic effects of synthetic drugs of abuse (12). According to

the World Drug Report 2016 (13), the majority of the substances reported for the first time between 2012 and 2014 were synthetic cannabinoids, followed by synthetic cathinones that have been steadily increasing since their first appearance (2010). Synthetic cathinones and synthetic cannabinoids have been studied more extensively than other classes of NPS (e.g., new hallucinogens and opioid-like compounds) over the past 10 years. These two classes of substances are the most popular, both induce central and peripheral serious effects and activate the brain reward system thus showing to possess abuse potential (14, 15). Since they pose a major health hazard potential, this review will focus on these two classes of NPS.

Synthetic cathinones are psychostimulants related to the naturally occurring parent compound cathinone (16, 17), a monoamine alkaloid found in the khat plant (*Catha edulis*), a flowering plant native to the Horn of Africa and the Arabian Peninsula that is being chewed in these areas for thousands of years. Cathinone is a psychoactive substance known to cause excitement, loss of appetite, and euphoria. As members of the phenethylamine class of drugs, they are structurally and pharmacologically similar to amphetamine and 3,4-methylenedioxymethamphetamine (MDMA). The most commonly used drugs in this class have been 4-methylmethcathinone (mephedrone), 3,4-methylenedioxy-*N*-methylcathinone (methylone), and 4-methylenedioxypyrovalerone (MDPV), although MDPV and mephedrone are no longer prevalent (16, 17). Reports of abuse of cathinone derivatives date back to the 1990s, when mephedrone was the first designer drug of this class (18). Mephedrone is the most widely abused synthetic cathinone in Europe, and MDPV and methylone are the most frequently abused synthetic cathinones in the United States (7). These drugs have been commonly referred to as "bath salts," "plant food," or "fertilizer," because they were at times disguised and commonly included in products that were labeled and sold as such (7, 9). New analogs, legal to possess, at least until they are formally banned, are frequently introduced, and it has been estimated that nearly 250 new analogs are produced each year (19). These drugs are consumed by oral ingestion, inhalation, and snorting. Notably, the recent trend to supplement use of more conventional psychostimulants, such as amphetamine and cocaine, with mephedrone, may lead to serious psychotic, neurological, cardiovascular, and sexual health consequences (4).

Synthetic cannabinoid receptor agonists, which often mimic the effects of marijuana, are added to herbs, such as *Damiana* leaves, and sold under different brand names, such as "Spice" or Mr. Nice Guy in Europe since 2006 (20, 21). These products are also called "K2," "herbal incense," "Cloud 9," "Mojo," and with many other names (22). Advertised as "exotic incense blends which release a rich aroma," Spice, and Spice-like preparations in Europe have been found to contain different substances with various chemical structures, including those (i) based on over 450 compounds originally produced for medical research by John W. Huffman (e.g., JWH-018), (ii) HU-210 developed by Raphael Mechoulam at the Hebrew University, and (iii) the cyclohexylphenol ("CP") cannabinoids developed at Pfizer Pharmaceuticals (23). AM-2201, an indole derivative, which differs from JWH-018 by a fluorine atom in the pentyl chain, is commonly found in Korea and has nanomolar affinity for cannabinoid receptors (24). After the appearance on the drug market of over 100 compounds that activate cannabinoid receptors, new compounds with different chemical structures that directly or indirectly stimulate the cannabinoid CB1 receptors are expected. The perceived harmfulness of synthetic cannabinoids among secondary school students (twelfth grade) increased between 2012 (the first year of measurement) and 2014, which may have contributed to the decline in use (9). This review is divided into the following categories: epidemiology of use, pharmacology, neuropsychiatric findings, other medical conditions, and regulation.

EPIDEMIOLOGY

Synthetic Cathinones

Masticating khat leaves has been a social habit among Saudi Arabian and East African cultures, for several centuries. Cathinone is the main psychoactive ingredient that was detected in the leaves of the *C. edulis*. In the Middle East and in East Africa khat use is still common, more among men than women, although the gap is narrowing (25). Yet, although both sexes typically report to use khat to upkeep of tradition, men's use is more frequently associated to recreational purposes, while women often report to use it to treat headache or lose weight (26).

Recent surveys have considered the problem of synthetic cathinones use in the United States. In an online survey of 113 participants, who reported use of synthetic cathinones, respondents were males, with age range of 18–24 years, and Caucasian holding college education (27). Their use in the past year was low ($\leq$10 days), but recurrent. The intranasal route of administration was most frequently reported, and its effects were similar to those of cocaine and amphetamines. Synthetic cathinones increased sexual desire and sexual risk-taking behavior. More than half of the responders met DSM-V diagnostic criteria for a substance-related disorder. Self-reported prevalence of the use of synthetic cathinones was less than that of marijuana, cocaine, *Salvia divinorum*, synthetic cannabinoids, methamphetamine, and MDMA. In another survey, reaching over 2,300 students at a large university in the Southeastern United States, 1.07% of respondents endorsed ever using synthetic cathinones, and those who did were more often men than women, Hispanics, and Native American rather than Caucasian students, and athletes more than non-athletes (27).

Studies of synthetic cathinones use in Europe have also been informative. In Hungary, there has been a shift among street drug users from the use of heroin to mephedrone injection, potentially increasing the risk of severe psychiatric symptom profile and increased possibility of dependence (28). Among 1,000 individuals who completed a survey in schools and universities in Scotland (49.8% males and 50.2% females), 20.3% reported previous use mephedrone, with 23.4% reporting single use and 4.4% daily use (29). In a survey of 249 NPS users interviewed in open public places, clubs, and discotheques in Slovenia, 67.9% of the respondents endorsed having tried either synthetic cathinones or amphetamines (30). Of those who reported using synthetic cathinones or amphetamines, most reported having used

3-methylmethcathinone first, 43.0% had first used methylone, and 37.3% had first used mephedrone. Users have associated the new drugs with high risks and favored traditional drugs. A report on the occurrence and trends of new synthetic drugs in Sweden included participants who were 13–63 (median 20) years of age (31). The report described a widespread use of NPS in adolescents and young male adults (79%), in incidents of drug intoxications reported by emergency departments and intensive care units. Of the 189 blood and urine samples that were examined in the laboratory, 156 (83%) samples were found positive for NPS. More than 50 new synthetic drugs were detected. These included synthetic cannabinoid receptor agonists ("Spice"), piperazines, substituted phenethylamines, synthetic cathinones, hallucinogenic tryptamines, piperidines, opioid-related substances, ketamine and related substances, and γ-aminobutyric acid analogs. About half of the cases involved multiple drug intoxications, making it hard to link the clinical presentations with one specific substance.

Synthetic Cannabinoids

Synthetic cannabinoid users are usually men in their twenties, who use also other drugs. In a study of adult marijuana and tobacco users, the 42 respondents were male young adults, high school graduates, who also smoked tobacco and cannabis (86% smoked cannabis on five or more days per week) (32). A very high proportion (91%) were familiar with synthetic cannabinoid products, half (50%) smoked them previously, and a minority (24%) used them over the last month. Common reasons for use included expecting a strong "high" while avoiding detection by urine toxicology. The main side effects were difficulties in thinking, anxiety, dry mouth, and headache. Students also used synthetic cannabinoids out of curiosity and for feeling "high" (33). Eleven adolescents experienced euphoria and negative effects on memory, and nine reported negative mood changes (34).

Several informative surveys of synthetic cannabinoids use have involved college students. In a study of 852 college students in the United States, 69 (8%) reported using the drugs at least once, and more common use in males and in the first or second year of college (35). A survey of students from a local health clinic and a US University found 9, 5, and 3% lifetime, past-year, and past 30-day use of synthetic cannabinoids, respectively (36). In Rhode Island, 1,080 young (18–25 years old) participants were surveyed between January 2012 and July 2013, and 9% reported use of synthetic cannabinoids in the last month. Synthetic cannabinoid users were predominantly males who did not attend school, smoked cigarettes, were binge alcohol drinkers, who used marijuana daily, as well as other recreational drugs (37). A survey of over 3,100 college students in North Carolina and Virginia showed that lifetime prevalence of synthetic cannabinoids was 7.6% at college entry and 6.6% first use during college (38). During the fourth year of college, lifetime synthetic cannabinoids use was reported by 17.0%. The "Monitoring the Future study," a nationally representative sample of high school seniors between 2011 and 2013 included almost 12,000 participants of mean age 18 (39). In this sample, 10% reported any recent use and 3% reported frequent use (over six times) of synthetic cannabinoids. Females were less likely to use synthetic cannabinoids, and going out 4–7 evenings per week increased likelihood of drug use. Several factors were identified as increasing the risk for synthetic cannabinoid use, mainly high frequency of lifetime marijuana use, lifetime use of alcohol, cigarettes, and other illicit drugs. Among 396 patients entering residential treatment for Substance Use Disorder in the United States, 150 reported using synthetic cannabinoids in their lifetime. Motives for drug use were curiosity (91%), "feeling good" or "getting high" (89%), relaxation (71%), and "getting high" while avoiding detection (71%) (40). According to the 2009–2013 US National Survey of Drug Use and Health, the gender gap in the self-reported use of synthetic cannabinoids is smaller compared to other categories of NPS among non-institutionalized individuals aged 12–34 years old (41).

In an anonymous online survey among almost 15,000 participants in the UK, 2,513 (17%) reported use of synthetic cannabinoids. Among them, 41% reported use over the last 12 months (42). Almost all synthetic cannabinoids users (99%) used natural cannabis at least once. Synthetic cannabinoids had a shorter effect and faster time to peak effect than natural cannabis. Most users (93%) preferred natural cannabis to synthetic cannabinoids. Natural cannabis had greater pleasurable effects when "high" and allowed better function after use. Synthetic cannabinoids had negative effects such as hangover, and paranoia. In an anonymous follow-up online survey of drug use with over 22,000 respondents, use of synthetic cannabinoids was estimated as 30 times as more risky than natural cannabis (43).

In Australia, a sample of 316 synthetic cannabinoid users (77% male, mean age 27 years) reported mean synthetic cannabinoid use of 6 months, 35% reported weekly use and 7% reported daily use (44). Reasons for first use included: curiosity (50%), legality (39%), availability (23%), recreational effects (20%), therapeutic effects (9%), avoidance of detection by standard drug screening tests (8%), and aid for the reduction or cessation of cannabis use. In a further study of over 1,100 students (mean age 14.9 years) from secondary schools in Australia, 2.4% had ever used synthetic cannabis and 0.4% had used synthetic stimulants (45). Users also had an episode of binge drinking in the past 6 months, used tobacco and had higher levels of psychological distress and lower perceived self-efficacy to resist peer pressure than non-users, but did not differ from users of other illicit drugs.

Comparative Epidemiology

In a survey covering the years 2009 to 2012 in the United States, synthetic cathinone and synthetic cannabis exposures totaled 7,467 and 11,561, respectively (46), with increases in the use of both from 2009 to 2011. Synthetic cathinone use increased from none reported in 2009, to 298 in 2010, and 6,062 in 2011. By comparison, there were 14 reported synthetic cannabis exposures in 2009, 2,821 in 2010, and 6,255 in 2011. The number of those who were first-time exposed to synthetic cathinones was lower in 2012 (1,007) than in 2011 (2,027), while the number of first-time exposures to synthetic cannabis was higher in 2012 (2,389) than in 2011 (1,888) possibly reflecting a shift from synthetic cathinone use toward the use of synthetic cannabinoids. Most exposures occurred in the Midwest and Southeastern US (64.8% of synthetic cathinone and 58% of synthetic cannabis exposures). Males comprised 69% ($n = 5,153$) of synthetic cathinone users and 74% ($n = 8,505$) of synthetic cannabis users. Use of

synthetic cathinones was highest in individuals 20–29 years of age ($n = 2{,}943$), while use of synthetic cannabinoids was highest for younger respondents, i.e., individuals 13–19 years of age ($n = 5{,}349$). A recent study involving 1,740 young adults recruited in US nightlife scenes (18–40 years old, mean age 26.4) showed that use of mephedrone and synthetic cannabinoids adults in US nightlife scenes is lower than in EU nightlife scenes (47). Specifically, 8.2% used synthetic cannabinoids, 1.1% used mephedrone. Gay and bisexual men reported more frequent use of mephedrone and more frequent use of synthetic cannabinoid use in individuals with Latin origin. In conclusion, synthetic cannabis emerged first with overall more reported exposures than synthetic cathinone. In 2012, synthetic cathinone abuse declined while synthetic cannabis abuse increased. Young men intentionally abusing synthetic cannabinoids *via* inhalation make up the majority of users.

DRUG PHARMACOLOGY, CENTRAL EFFECTS, AND CLINICAL FEATURES

Synthetic Cathinones

Synthetic cathinones represent a broad class of pharmacologically active compounds that induce numerous effects with different mechanisms of action. As such, each case of synthetic cathinone intoxication should be evaluated separately, at least until preclinical research will provide structure–activity relationships for each compound. Similar to the action of other psychostimulants, synthetic cathinones have an effect on plasma membrane transporters of the monoamine neurotransmitters, dopamine, norepinephrine, and serotonin (48). Mephedrone and methylone, but not MDPV, act as non-selective transporter agonists, thereby promoting release of all of these neurotransmitters. Conversely, MDPV acts as a potent blocker at catecholamine transporters with little effect at the serotonin transporter (48). Mephedrone or methylone administered to rats increase extracellular concentrations of dopamine and serotonin in the brain, similar to the effects of MDMA (48). Synthetic cathinones elicit locomotor stimulation in rodents similar to other psychstimulants. The enhanced dopamine transmission by synthetic cathinones presents a high potential for addiction that may result in adverse effects (49). See **Table 1** for behavioral and pharmacological effects of synthetic cathinones in rodents.

TABLE 1 | Studies investigating the behavioral effects of synthetic cathinones.

Animals	Synthetic cathinone tested	Main findings	Reference
Male ICR mice	α-PBP, α-PPP, α-PVP MDPV MEPH Methylone 3-FMC, 4-FMC, 4-MePPP	Motor stimulation Decreased motor coordination Ataxia	(50–52)
Male Sprague-Dawley rats	α-PVP MDPV MEPH Methcathinone Methylone R-MEPH, 4-MEC	Significantly lower ICSS threshold	(53–55)
Male rats (Wistar, Sprague-Dawley)	α-PVT BMAPN Buphedrone MACHP Methylone MPDV 4-MEC, 4-MePPP	Sustain IVSA behavior	(56–63)
Male mice (CD-1, ICR, C57BL/6J or Swiss Webster)	α-PBP, α-PVP, α-PVT BMAPN Buphedrone MACHP, MDPV MEPH, MAOP Methylone PIPP	Induce CPP	(58–62, 64, 65)
Male and female (MDPV) Sprague-Dawley rats	MDPV R-MEPH 4-MEC	Induce CPP	(55, 66–68)
Male Sprague-Dawley rats	α-PBP, α-PVP, α-PVT Methcathinone Pentedrone Pentylone 3-FMC, 4-MePPP, 4-MEC	Substitute for the discriminative stimulus effects of METH in a DD paradigm	(61, 65, 69, 70)

α-PBP, α-pyrrolidinopropiobutiophenone; α-PVP, alpha-pyrrolidinovalerophenone α-PVT, alpha-pyrrolidinopentiothiophenone; buphedrone, [2-(methylamino)-1-phenylbutan-1-one, α-methylamino-butyrophenone]; BMAPN, 2-(methylamino)-1-(naphthalen-2-yl) propan-1-one; CPP, conditioned place preference; DD, drug discrimination; ICSS, intracranial self-stimulation; IVSA, intravenous self-administration; MACHP, [1] 2-cyclohexyl-2-(methylamino)-1-phenylethanone; MAOP, [2] 2-(methylamino)-1-phenyloctan-1-one; MEPH, mephedrone; METH, metamphetamine; MDPV, methylenedioxyphyrovalerone; PIPP, f α-piperidinopropiophenone; R-MEPH, R-mephedrone; 3-FMC, 3-fluoromethcathinone; 4-FMC, 4-fluoromethcathinone; 4-MEC, 4-methylethcathinone; 4-MePPP, 4-methyl-alpha-pyrrolidinopropiophenone.

Acute and Chronic Side Effects

Acute administration of low doses of synthetic cathinones produces euphoria and increases alertness, but high doses or chronic use can result in serious adverse effects, such as hallucinations, delirium, hyperthermia, and tachycardia (70). Repeated use of synthetic cathinones is associated with paranoia and hallucinations and some patients developed "excited delirium," a syndrome with symptoms of extreme agitation and violent behavior (70). The symptoms included dehydration, muscle damage and renal failure that may result in multi-organ failure and death. A 40-year-old male, who had no previous psychiatric history developed "excited delirium" after ingesting "bath salts" with psychosis and violence (71). Forty-three postmortem cases with detected synthetic cathinones had the following associated causes of death: driving under the influence of drugs (17 cases), domestic violence (2 cases), suicide (4 cases), overdose (12 cases), accidents (6 cases), drug-facilitated assault (1 case), and homicide (1 case) (72). The highest measured MDPV and methylone concentration was detected in a case of suicide by hanging and in a driver, respectively. A single case of death following methylone ingestion was reported in France (73).

Cardiovascular effects (tachycardia, hypertension) and hallucinations are the most recurrent medical complications of synthetic cathinone use. The most frequently reported unwanted

clinical effects among cases reported to Texas poison centers during 2010–2011 were tachycardia (45.9%), agitation (39.2%), hypertension (21.0%), hallucinations (17.7%), and confusion (13.0%) (74). A study of "bath-salt" exposure conducted in two poison centers in the United States found primarily neurological and cardiovascular effects. Drugs effects included agitation, combative behavior, tachycardia, hallucinations, paranoia, confusion, chest pain, myoclonus, hypertension, mydriasis, elevations in creatine phosphokinase, hypokalemia, blurred vision, and death (8). Signs of severe toxicity, such as hyperthermia, metabolic acidosis and prolonged rhabdomyolysis, indicative of high serotonergic activity, were also reported (75). A single case described cardiovascular manifestations, including tachycardia, hypertension, myocardial infarction, arrhythmias, and cardiac arrest (76).

Seizures and Withdrawal

Synthetic cathinone exposure has also resulted in many cases of seizures in the pediatric population. The American Association of Poison Control Centers database was used to examine synthetic cathinone exposures in children and youth below 20 years of age between 2010 and 2013 (77). There were 1,328 cases of pediatric synthetic cathinone exposures, of which 73 cases presented seizures complications: 37 (50.7%) involved a single seizure, 29 (39.7%) involved multiple seizures, and seven (9.6%) developed epilepsy. Fever and acidosis were associated with single seizures, multiple seizures, and status epilepsy. There were no correlations between seizure activity and electrolyte abnormalities, hallucinations and/or delusions, tachycardia, or hypertension. The most commonly co-ingested substances were tetrahydrocannabinol, alcohol, and opioids. Finally, in Italy, a baby born to a woman who was a chronic consumer of 4-methylethcathinone showed symptoms of neonatal withdrawal syndrome (78). The newborn presented with increased jitteriness and irritability, high-pitched crying, limbs hypertonia and brisk tendon reflexes (78).

Regrettably, there is little information regarding the pharmacokinetics of synthetic cathinones in preclinical or clinical studies. Studies conducted so far have demonstrated that, upon systemic administration, synthetic cathinones are metabolized in several phase I compounds, some of which have been found to be substrates at monoamine transporters when assessed *in vitro* or to exert neurochemical actions *in vivo* (79, 80). The resulting metabolites can also partially undergo phase II metabolism (81). The presence of active metabolites could account, at least in part, for the diversified effects of these drugs.

Synthetic Cannabinoids

Unlike Δ9-tetrahydrocannabinol (THC), synthetic cannabinoids are extremely potent, highly efficacious, full agonists of the cannabinoid receptors (82, 83), including CB1 receptors in the brain (84–89). There is substantial variability in the molecular constituents of different compounds, between assortment of the same product, and even within a package (20). In addition to synthetic cannabinoids, Spice drugs may contain preservatives, additives, fatty acids, amides, esters, the benzodiazepine phenazepam and *O*-desmethyltramadol, an active metabolite of the opioid medication tramadol (90–92). Studies in rodents have indicated that most synthetic cannabinoids produce effects and toxicity that, overall, are similar to those of THC and include hypothermia, analgesia, hypo-locomotion, and akinesia (93). Yet, most effects are more potent and long-lasting than THC. See **Table 2** for motor and reward-related behavioral effects of synthetic cannabinoids in rodents.

Psychosis

Cannabis use has potential for inducing psychosis [for recent reviews, see Ref. (107, 108)], and it would be reasonable to expect synthetic cannabinoids to have the same effect. Because of their high potency and the fact that synthetic cannabinoids act as full cannabinoid receptor agonists, even short or occasional use of these synthetic compounds can produce unwanted effects, such as insomnia, memory impairment, headaches, dizziness, and delusions. Moreover, unlike natural cannabis, synthetic cannabinoids contain no cannabidiol that may be protective against psychosis. Cannabidiol antagonizes the psychotomimetic and other psychotropic effects of THC although the mechanisms underlying its therapeutic effect are still not clear (109). Compared with natural cannabis, synthetic cannabinoids may cause more frequent and more severe unwanted negative effects, and may have high-risk for psychosis especially in young users (110). Case reports have documented psychosis (111, 112), mania (113), and suicidal ideations (114) in synthetic cannabinoid users.

Brain Imaging Studies

Although brain imaging studies have pointed to abnormalities in cerebral perfusion, deficits in brain volume and white-matter pathways (115), brain imaging has scarcely been applied to understand the neural correlates of synthetic cannabis use. A comparison of 20 male patients who had used synthetic cannabinoids with 20 healthy male controls indicated that drug users had smaller gray-matter volume in the thalamus and the cerebellum (116). A single case study of a 23-year-old patient reported severe withdrawal syndrome upon voluntary abstinence from "Spice Gold." Craving, affective symptoms and a range of somatic complaints were reported, but these were resolved after several days of monitored abstinence (117). In this patient, dopamine D_2 and D_3 receptor availability was 20% lower in the striatum and in extra-striatal regions with respect to healthy control participants, but returned to control values with detoxification. Brain imaging studies suggest that synthetic cannabinoid use can produce remarkable changes in the brain, but they are still preliminary, and the extent and duration of the neural sequelae of synthetic cannabinoid use remains to be determined.

Effects on Driving

Reports concerning driving under the influence of synthetic cannabinoids also reflect their impact on the nervous system. One report from the United States indicated that drivers under the influence of synthetic cannabinoids had slow and slurred speech, and poor coordination (118). A survey in Germany found behavioral deficits that were moderate except for worsening of paranoia in one case (119). The symptoms were similar to the effects of cannabinoid agonists but could also be a result of alcohol or other

TABLE 2 | Studies investigating the behavioral effects of synthetic cannabinoids.

Animals	Synthetic cannabinoid tested	Main findings	Reference
Male mice (C57BL/6J, Swiss Webster)	"Buzz" (5.4% JWH-018) JWH-018 JWH-073	Induces a dose-related tetrad effects similar to marijuana/THC	(94, 95)
Male Swiss Webster/ICR mice	AB-FUBINACA AM-2201 APINACA/AKB-48 JWH-018, JWH-073, JWH-200, JWH-203 JWH-250 PB-22 (QUPIC) UR-144, XLR-11, 5F-PB-22	Decrease locomotor activity Induce catalepsy	(96–99)
Male ICR mice	JWH-018	Significantly impairs sensorimotor functions Induced convulsions, myoclonia and hyperreflexia (at high doses)	(14, 99)
Male Sprague-Dawley rats/C57BL/6 mice	JWH-018	Sustains IVSA behavior	(100)
Male mice (ICR)	JWH-073, JWH-081, JWH-210	Induce CPP	(101)
Male Sprague-Dawley rats	JWH-175	Induces CPP	(102)
Male ND4 Swiss–Webster/ICR mice	AB-CHMINACA AB-PINACA ADBICA ADB-PINACA FUBIMINA JWH-018, JWH-122, JWH-210 RCS-4, THJ-2201	Fully substitute for THC in a DD paradigm	(98, 103)
Male and female (JWH-018) Sprague-Dawley rats	AB-FUBINACA AM-2201 APINACA/AKB-48 JWH-018, JWH-073, JWH-200, JWH-203, JWH-250 PB-22/QUPIC UR-144, XLR-11, 5F-PB-22	Fully substitute for THC in a DD paradigm	(96, 97, 104)
Male adolescent rhesus monkeys	JWH-018	Shows discriminative stimulus effects; dose-dependently increases drug-lever responding and decreased response rate	(105)
Female and male adult rhesus monkeys	AM-2201 JWH JWH	Substitute for the discriminative stimulus effects of Δ9-THC	(106)

AB-CHMINACA, N-[1-amino-3-methyl-oxobutan-2-yl]-1-[cyclohexylmethyl]-1H-indazole-3-carboxamide; AB-FUBINACA, N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1H-indazole-3-carboxamide; AB-PINACA, N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-pentyl-1H-indazole-3-carboxamide; ADBICA, N-(1-amino-3,3-dimethyl-1-oxobutan-2-yl)-1-pentyl-1H-indole-3-carboxamide; ADB-PINACA, N-[1-(aminocarbonyl)-2,2-dimethylpropyl]-1-pentyl-1H-indazole-3-carboxamide; AM-2201, [1-(5-fluoropentyl)-1H-indol-3-yl]-1-naphthalen-methanone; APINACA/AKB-48, N-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide; CPP, conditioned place preference; DD, drug discrimination; FUBIMINA, (1-(5-fluoropentyl)-1H-benzo[d]imadazol-2-yl)(naphthalen-1-yl)methanone; ICSS, intracranial self-stimulation; IVSA, intravenous self-administration; JWH-018,1-pentyl-3-(1-naphthoyl) indole; JWH-073, naphthalen-1-yl-(1-butylindol-3-yl)methanone; JWH-081, 4-methoxynaphthalen-1-yl-(1-pentylindol-3-yl)methanone; JWH-175, (1-pentylindol-3-yl) naphthalen-1-ylmethane; JWH-200, [1-[2-(morpholinyl)ethyl]-1H-indol-3-yl]-1-naphthalenyl-methanone; JWH-203, (2-(2-chlorophenyl)-1-(1-phentyl)-1H-indol-3-yl)-methanone; JWH-210, 4-ethylnaphthalen-1-yl-(1-pentylindol-3-yl)methanone; JWH-250, 2-(2-methoxyphenyl)-1-(1-pentylindol-3-yl)methanone; PB-22/QUPIC, quinolin-8-yl 1-pentyl-1H-indole-3-carboxylate; RCS-4, 2-(4-methoxyphenyl)-1-(1-pentyl-indol-3-yl)methanone; THJ-2201, [1-(5-Fluoropentyl)-1H-indazol-3-yl](naphthalen-1-yl)methanone; UR-144, (1-pentylindol-3-yl)(2,2,3,3-tetramethylcyclopropyl)methanone; XLR-11, 5F-UR-144[1-(5-fluoro-pentyl)-1H-indol-3-yl](2,2,3,3-tetramethylcyclopropyl)methanone; 5F-PB-22, quinolin-8-yl-1-(5-fluoropentyl)-1H-indole-3-carboxylate.

drugs detected by blood analysis. In several case reports sedating effects and impairment of fine motor skills were noted (120). In Poland, a single case showed that use of a synthetic-containing product caused effects and impairment similar to THC (121). Very few cases of synthetic cannabinoids were detected in the blood of drivers in Norway (122, 123).

Health Hazards and Withdrawal

Synthetic cannabinoid use has been associated with serious hazardous health effects on multiple systems, and with death (124, 125). For example, among 3,572 calls related to synthetic cannabinoid use to call centers in the United States, 2,961 had a medical outcome, 11.3% callers had a major adverse effect, and 15 deaths were reported (126). The most common side effects are tachycardia, agitation, irritability, confusion, dizziness, drowsiness, hallucinations, delusions, hypertension, nausea, vomiting, vertigo and chest pain (127). Central nervous system effects range from headache to coma and included seizures, myoclonus, catatonic stupor, cerebral ischemia, and encephalopathy (128–131). Case reports have documented

cardiac complications, ranging from chest pain (132) to myocardial infarction (133, 134), and cardiac arrest (135–137). Cases of acute kidney damage and renal failure following use of synthetic cannabinoids have also been reported (138). Dyspnea, rhabdomyolysis, diaphoresis, and hypokalemia, which are not commonly reported by cannabis users, have been associated with synthetic cannabinoid use (22). Case reports have also described respiratory depression following synthetic cannabinoids use (139) and, with chronic use, also pulmonary complications and pneumonia (140, 141). Rare cases of cannabinoid-induced hyperemesis syndrome were described which included repeated nausea and vomiting, abdominal pain, and a compulsion to take hot showers (142, 143).

Prolonged habitual use of synthetic cannabinoids resulted in withdrawal syndrome in case reports and in a study of 47 patients admitted to detoxification services (144, 145). The symptoms were similar to those of withdrawal from THC, including anxiety, myalgia, chills, anorexia, mood swings and tachycardia, but were more severe and did not seem to improve with the administration of THC (144, 146). The differences in presentation may reflect the inclusion of extraneous compounds, including amphetamine-like stimulants.

Noteworthy, differently from marijuana, while THC is metabolized to one active metabolite only, metabolism of new synthetic cannabinoids leads to the generation of pharmacologically active metabolites that remain biologically active and hold high affinity for the cannabinoid CB1 receptor (147, 148). As for synthetic cathinones, these active metabolites may prolong the psychotropic effects of the parent compound thus contributing to its toxicity.

REGULATION AND LEGISLATION

Responding to the rapid appearance of NPS with molecular structures that have not been covered by legislation, the governments of several countries have recognized the need for new mechanisms of control with accelerated ways to curtail the free sale and distribution of these substances (149). In Europe, since 1997 three levels of control were introduced: Early Warning System, risk assessments of newly emerged substances performed by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) scientific committee and European Council decisions advocating new legislations (132). The possession, use, and synthesis of synthetic cathinones became subject to legal classification in Europe in 2010 (150) and in the United States in 2011 (149). Some countries such as Denmark, the UK, and Israel (151) opted for "temporary bans" of new psychoactive substances considered to pose danger to public health during which a risk assessment of a particular compound could be performed thus facilitating its subsequent inclusion into the Dangerous Drugs Ordinance. Other countries such as New Zealand, Ireland, Poland, and Romania (152) chose "pre-market approval" regulation regime for synthetic drugs that pose low health risk on the base of preclinical and clinical evidence. The effectiveness of these legal measures and regulations on the selling and marketing of the new psychoactive substances has still to be assessed.

CONCLUSION

Synthetic cathinones and synthetic cannabinoids became increasingly popular despite the potential harms associated with their use. Synthetic cathinones have similar clinical effects to amphetamines and MDMA whereas synthetic cannabinoids are high-potency, full agonists at cannabinoid receptors and induce THC-like effects, but more potent and enduring. Both classes of substances have various adverse health effects. Synthetic cathinones cause anxiety, agitation, panic, dysphoria, psychosis, and bizarre behavior whereas synthetic cannabinoids cause agitation, irritability, confusion, hallucinations, delusions, psychosis, and death (as well as other health problems illustrated above in the text and in **Tables 1** and **2**).

Chronic use of synthetic cathinones and synthetic cannabinoids results in adverse medical and psychiatric effects that seem to be higher than those induced by the natural parent compounds (i.e., cathinone and THC). In comparison with other known amphetamines, synthetic cathinones such as MEPH and MDPV exhibit a pharmacological profile that is more typical of methamphetamine and cocaine, respectively, while methylone shows a pharmacological profile that more closely resembles MDMA; yet, clinical toxicology of synthetic cathinones is not yet fully characterized. Synthetic cannabinoids show higher toxicity compared with natural cannabinoids, and their long-term effects are still to be investigated. In view of the increasing demand for these substances and their severe associated risks, more rigorous research on the effects of synthetic cathinones and synthetic cannabinoids is urgently required in order to understand their pharmacological effects and assist clinicians in managing adverse events.

These two classes of synthetic drugs are composed of pharmacologically diversified compounds with multiple mechanisms of action. Preclinical studies are urgently needed to elucidate their single action in both the brain and the periphery as well as their synergistic effects and long-term consequences. At clinical level, development of combined and integrated pharmacological and psychological approaches to treat intoxication symptoms is necessary. Treatment protocols currently available for the better-known parental drugs need to be adapted to face with the increasing number of intoxications reported after the use of synthetic cathinones and synthetic cannabinoids.

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AW and LF contributed substantially to the conception and design of the review. All the authors contributed to further drafts of the manuscript, critically revised, and approved the final version of the manuscript.

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In Vitro Neurochemical Assessment of Methylphenidate and Its "Legal High" Analogs 3,4-CTMP and Ethylphenidate in Rat Nucleus Accumbens and Bed Nucleus of the Stria Terminalis

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3,4-dichloromethylphenidate (3,4-CTMP) and ethylphenidate are new psychoactive substances and analogs of the attention deficit medication methylphenidate. Both drugs have been reported on online user fora to induce effects similar to cocaine. In the UK, 3,4-CTMP appeared on the drug market in 2013 and ethylphenidate has been sold since 2010. We aimed to explore the neurochemical effects of these drugs on brain dopamine and noradrenaline efflux. 3,4-CTMP and ethylphenidate, purchased from online vendors, were analyzed using gas chromatography and mass spectroscopy to confirm their identity. Drugs were then tested in adolescent male rat brain slices of the nucleus accumbens and stria terminalis for effects on dopamine and noradrenaline efflux respectively. Fast cyclic voltammetry was used to measure transmitter release. Methylphenidate (10 μM) increased evoked dopamine and noradrenaline efflux by 4- and 2-fold, respectively. 3,4-CTMP (0.1 and 1 μM) increased evoked dopamine and noradrenaline efflux by ∼6-fold and 2-fold, respectively. Ethylphenidate (1 μM) doubled evoked dopamine and noradrenaline efflux in both cases. 3,4-CTMP's effect on dopamine efflux was greater than that of methylphenidate, but ethylphenidate appears to be a weaker dopamine transporter inhibitor. Experiments using the dopamine D_2 antagonist haloperidol, the noradrenaline α_2 receptor antagonist yohimbine, the dopamine transporter inhibitor GBR12909 and the noradrenaline transporter inhibitor desipramine confirmed that we were measuring dopamine in the accumbens and noradrenaline in the ventral BNST. All three psychostimulant drugs, through their effects on dopamine efflux, may have addictive liability although the effect of 3,4-CTMP on dopamine suggests that it might be most addictive and ethylphenidate least addictive.

Keywords: novel psychoactive substance, dopamine, noradrenaline, voltammetry, brain slice, adolescent

INTRODUCTION

Concern has arisen over the past few years about "legal highs" or new psychoactive substances (NPS). Some of these NPS produce similar effects to cocaine, amphetamine, or MDMA (Ecstasy) and have changed the landscape of the UK drug scene by offering drug users the opportunity to use drugs without the risk of prosecution. The most prominent NPS is mephedrone, which was banned in the UK in 2010. The number of legal highs being sold on the Internet is increasing year-on-year. Deluca et al. (1) carried out a European-wide web-mapping project to quickly identify NPS use in Europe. By monitoring drug fora and websites, which provide information on legal highs between the drug communities, the authors uncovered 414 substances/products. The number of NPS discovered is now thought to be closer to 1000 (EMCDDA).

Many of these NPS have been associated with emergency room visits (2–4) and drug related deaths (5), it is therefore important to learn about the pharmacology of these substances, for which very little is known. We have recently identified 2 NPS from samples obtained from London dance club amnesty bins or samples purchased on the internet and examined these drugs, 3,4-CTMP and ethylphenidate, using neurochemical assays. 3,4-CTMP is an NPS that became available for purchase on the Internet in January 2013. Ethylphenidate is an NPS that has been sold since 2010. Both drugs are closely related to the attention deficit medication methylphenidate (Ritalin®; **Figure 1**).

Drugs such as cocaine, amphetamine, and MDMA increase dopamine concentration in the rodent nucleus accumbens (6, 7) making the accumbens an ideal location to investigate the addictive liability of NPS. Imaging studies of the human brain have also shown that increased dopamine levels in the striatum as a result of drug use are associated with the euphoric effects of drugs (8). The nucleus accumbens and bed nucleus of the stria terminalis are both part of the extended amygdala (9), which is thought to integrate brain arousal-stress systems with hedonic processing systems. Stress induced reinstatement of some drugs of abuse in animal models appear to be partly dependent on the activation of noradrenaline in the BNST (10, 11). The ventral BNST is highly innervated by noradrenergic neurons and has very few dopamine or 5-HT terminals (12–14). This means measured transmitter release from the ventral BNST is directly attributable to noradrenaline. The ventral BNST is therefore ideal for studying drug effects on the noradrenergic system ((15–19)).

Here we examined 3,4-CTMP and ethylphenidate on dopamine and noradrenaline efflux in rat brain slices. We examined dopamine efflux as this would help us determined the likely abuse liability of these drugs. We examined noradrenaline efflux, as a drug that increases noradrenaline levels will likely be a vasoconstrictor and cause hypertension (20). Thus, these data also give us some idea of the possible cardiotoxicity of the drug.

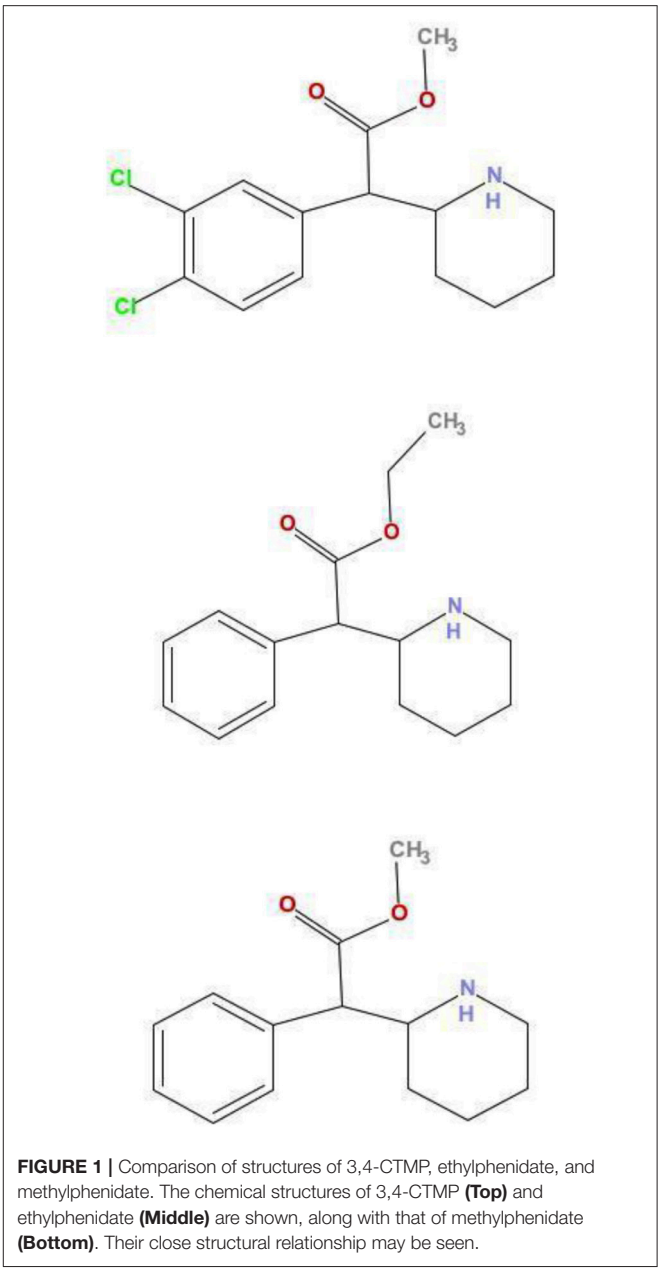

FIGURE 1 | Comparison of structures of 3,4-CTMP, ethylphenidate, and methylphenidate. The chemical structures of 3,4-CTMP **(Top)** and ethylphenidate **(Middle)** are shown, along with that of methylphenidate **(Bottom)**. Their close structural relationship may be seen.

METHODS

The two NPS were found in samples from dance club amnesty bins in London (October—December 2012) and also from subsequent test purchases from internet sites. They were shown to be pure samples using gas chromatography with mass spectroscopy (GCMS) and nuclear magnetic resonance spectroscopy (NMR, data not shown). 3,4-CTMP was found in one amnesty bin sample while ethylphenidate was found in 27 samples often in combination with other active compounds. Three samples of each drug were purchased from different websites and all were found to be essentially pure.

Animals and Dissection

Brain slices used in all experiments were obtained from 8 week old male Wistar rats (Charles River Laboratories). Animals were housed 4 per cage, kept on a 12/12-h light/dark cycle and had food and water available *ad libitum*. Rats were killed

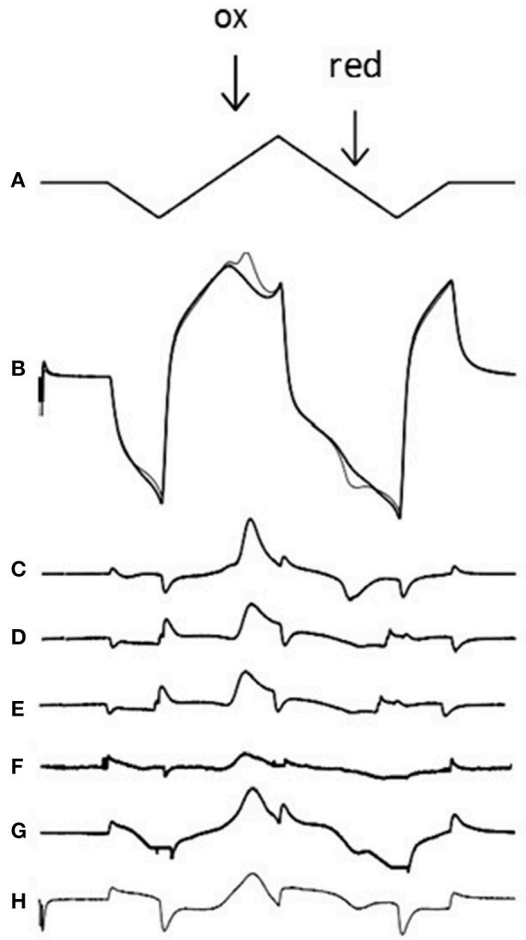

FIGURE 2 | Voltammetric technique and voltammograms for exogenous and endogenous amines. **(A)** The applied voltage to the carbon electrode (0 to −1 to +1.4 to −1 and back to 0 V at 480 V/s which takes 20 ms). **(B)** The charging current at the electrode in the absence (thick line) and presence (thin line) of 5 μM dopamine; note the dopamine oxidation peak (ox; 0.6 V) where dopamine releases 2 electrons, creating a Faradaic current. Note also the reduction peak (red; −0.2 V) where dopamine-o-quinone is reduced back to dopamine, using 2 electrons. If one subtracts the signal in the presence of dopamine from the signal in the absence of dopamine one is left with a voltammogram **(C)**. Voltammograms are also shown for 5 μM noradrenaline **(D)**, stimulated dopamine in the accumbens **(E)**, and stimulated noradrenaline in the BNST **(F)**. The voltammogram from the apparent ethylphenidate (30 μM) evoked increase in basal accumbens dopamine (see **Figure 3C**) is shown in **(G)**, but the voltammogram from the calibration of the electrode in 30 μM ethylphenidate (see **Figure 3D**) reveals that this drug is electroactive **(H)**. In each case the oxidation peak is clearly seen. Note that we often get small non-biological artifacts when the voltage changes direction. Neither methylphenidate nor 3,4-CTMP were found to be electroactive (data not shown).

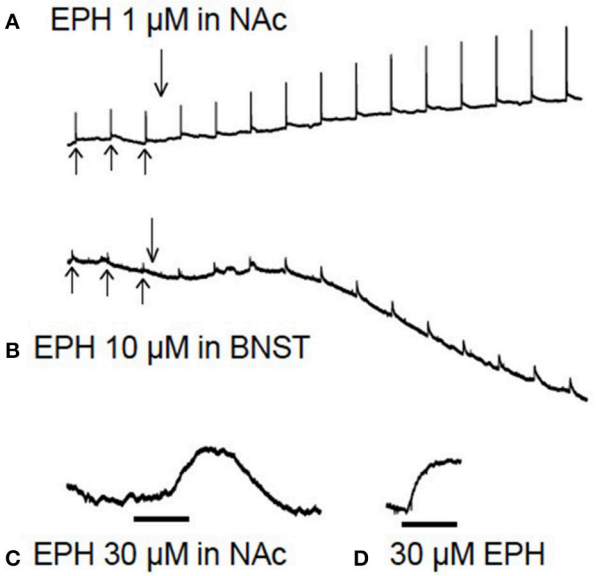

FIGURE 3 | Raw data from brain slice experiments. In **(A,B)**, after 3 baseline electrical stimulations (upward arrows), the drug is added (downward arrow) for 60 min while stimulations continue every 5 min. In the lower traces **(C,D)** there are no electrical stimulations. **(A)** Shows evoked dopamine efflux in the nucleus accumbens (NAc) after application of 1 μM ethylphenidate (EPH), note the increase in electrically evoked dopamine. **(B)** Application of EPH (10 μM) in the bed nucleus of the stria terminalis (BNST), note the increase in stimulated noradrenaline efflux and the apparent increase in background levels of transmitter 10 min after drug application. **(C)** Application of 30 μM EPH (black bar = 10 min) in the NAc appeared to increase basal dopamine levels (there is no electrical stimulation in this trace), but on testing 30 μM EPH at the carbon electrode in a calibration it was found that this drug is electroactive **(D)**, although not nearly as electroactive as dopamine or noradrenaline.

by cervical dislocation, without anesthesia, as anesthetics are known to affect neurotransmitter levels, particularly monoamine neurotransmitters. Schedule 1 euthanasia procedures were done in accordance with the relevant regulations under the UK Animals (Scientific Procedures) Act 1986.

The brain was cut to ∼+3 and −5 mm vs. bregma, which left a block including the nucleus accumbens and bed nucleus of the stria terminalis (BNST; (21)). Ice-cold aCSF was applied to the brain throughout the dissection. The posterior surface of the brain slice was glued to the chuck of a vibratome (Campden Instruments, Loughborough, UK). The brain and chuck were submerged in ice-cold aCSF and 400 μm coronal slices were taken to include the nucleus accumbens and BNST, ∼+2.2 to −0.3 vs. bregma. Sections were then transferred to a slice saver and suspended on a plastic mesh in aCSF at room temperature, while continually bubbled with 95%O_2/5%CO_2.

Chemicals and aCSF

Salts, glucose, haloperidol, yohimbine, GBR12909, and desipramine were bought from Sigma Aldrich (UK). Concentrations used were based on previous studies (15, 16). Artificial cerebrospinal fluid (aCSF) was prepared daily. The composition of aCSF was: (mM): NaCl (126.0), KCl (2.0), KH_2PO_4 (1.4), $MgSO_4$ (2.0), $NaHCO_3$ (26.0), $CaCl_2$ (2.4), (+)glucose (10.0), bubbled continually with 95% O_2/5% CO_2. 3,4-dichloromethylphenidate (3,4-CTMP) and ethylphenidate were initially dissolved in deionized water to 10 mM, then diluted with aCSF.

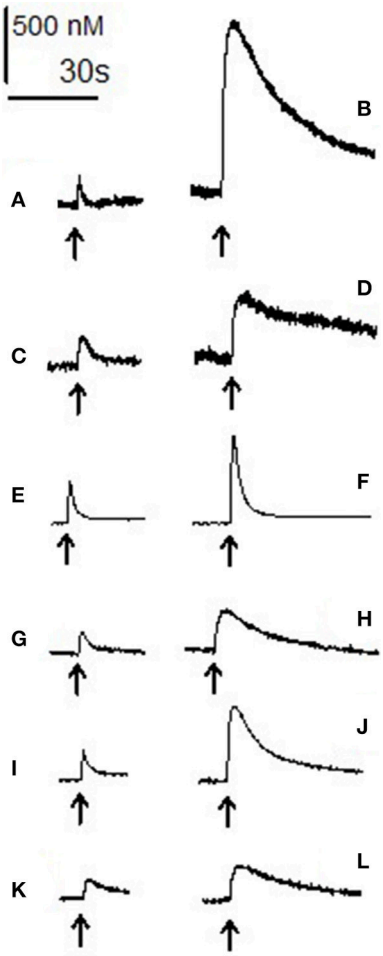

FIGURE 4 | Electrically evoked dopamine and noradrenaline: raw data. Each trace shows the increase in amine level after electrical stimulation (arrow) and its subsequent reuptake in the absence (left) and presence (right) of the three psychostimulant drugs. **(A)** Baseline stimulation in accumbens and **(B)** stimulation after 60 min of 10 μM 3,4-CTMP. **(C)** Baseline evoked efflux in the BNST and **(D)** after 60 min of 10 μM 3,4-CTMP. **(E)** Baseline evoked efflux in the accumbens and **(F)** after 60 min of 10 μM ethylphenidate, **(G)** baseline evoked efflux in the BNST and **(H)** after 60 min of 10 μM ethylphenidate. **(I)** Baseline evoked efflux in the accumbens and **(J)** after 60 min of 10 μM methylphenidate, **(K)** baseline evoked efflux in the BNST and **(L)** after 60 min of 10 μM methylphenidate. All traces are scaled such that the bars denoting concentration and time are accurate for each trace. The 2 traces in each pair (**A** and **B; C** and **D; E** and **F; G** and **H; I** and **J; K** and **L**) were taken from the same brain slice.

Fast Cyclic Voltammetry

Fast cyclic voltammetry (FCV) is an electrochemical technique that takes advantage of the electroactive properties of the monoamine neurotransmitters. It can be used to quantitatively measure neurotransmitter concentrations, and how these concentrations differ in the presence of drugs, in real-time (22). A triangular voltage waveform is applied to a carbon fiber microelectrode (**Figure 4A**), which oxidizes dopamine and noradrenaline at ∼600 mV. Calibrations of electrodes in a known concentration of dopamine or noradrenaline allow the recorded Faradaic current to be converted into the relevant neurotransmitter concentration (**Figure 4**).

Using a Millar Voltammetric Analyser (PD Systems, West Molesey, UK) we sampled dopamine or noradrenaline levels at 8 Hz. Changes in the sampled signal were captured using a CED1401 micro3 analog-to-digital converter (Cambridge Electronic Design (CED), UK), displayed using Spike2 v7.1 data capturing software.

Electrical Stimulation Protocol

Bipolar tungsten electrodes, with their tips 400 μm apart, were used to locally stimulate either the core of the nucleus accumbens or the ventral BNST (immediately below the anterior commissure). In most experiments pseudo-one pulse stimulation was used to avoid the activation of autoreceptors (23), which occurs ∼500 ms after striatal dopamine release (24, 25). A train of 10 × 1 ms 10 mA pulses at 100 Hz was applied every 5 min using a Neurolog NL800 stimulus isolator (Warner Instruments, Hamden, CT, USA) under computer control (Spike, CED). In experiments examining antagonists we used longer stimulation trains (10 pulses at 10 Hz, 900 ms train duration). Longer stimulation trains are useful in examining autoreceptor antagonists as described above. In the ventral BNST it has been found that stimulation frequencies of 10 or 20 Hz were best to see autoreceptor antagonist effects (16).

Experimental Protocol

To begin an experiment, slices were transferred from the slice saver to a laminar flow recording chamber that was supplied with aCSF via gravity feed, at a rate of 100 ml/h. Slices were left to equilibrate in the recording chamber for 30 min before starting stimulation of the slice, and the tips of both stimulating and recording electrodes were placed in the appropriate location in the brain to record monoamine release in eitherthe nucleus accumbens or ventral BNST. Recording took place from the beginning of this 30 min period as large spontaneous release events of dopamine can occur, which is indicative of poor slice health (26), and on such occasions (5–10%) the experiments were terminated.

Carbon Fiber Microelectrodes

Carbon fiber microelectrodes were constructed by inserting a single carbon fiber (Goodfellow Cambridge Ltd, UK), 7 μm in diameter, into a 10 cm long borosilicate glass capillary tube. The capillary tube was then pulled using an electrode puller (P-30, Sutter instruments Co, USA) and the exposed carbon fiber was cut to ∼70 μm under a microscope using a scalpel. Microelectrodes were backfilled with a saline solution before a length of copper wire was inserted into the end so it could be connected to the head-stage. A Ag/AgCl reference electrode and a steel wire auxiliary electrode were also connected to the head-stage and positioned within the recording chamber fluid, well away from the slice. Carbon fiber electrodes were calibrated using 5 or 10 μM dopamine or noradrenaline in aCSF. We also tested 3,4-CTMP and ethylphenidate in calibrations, to determine whether they would contribute to our signals (**Figures 3C,D**). Some drugs

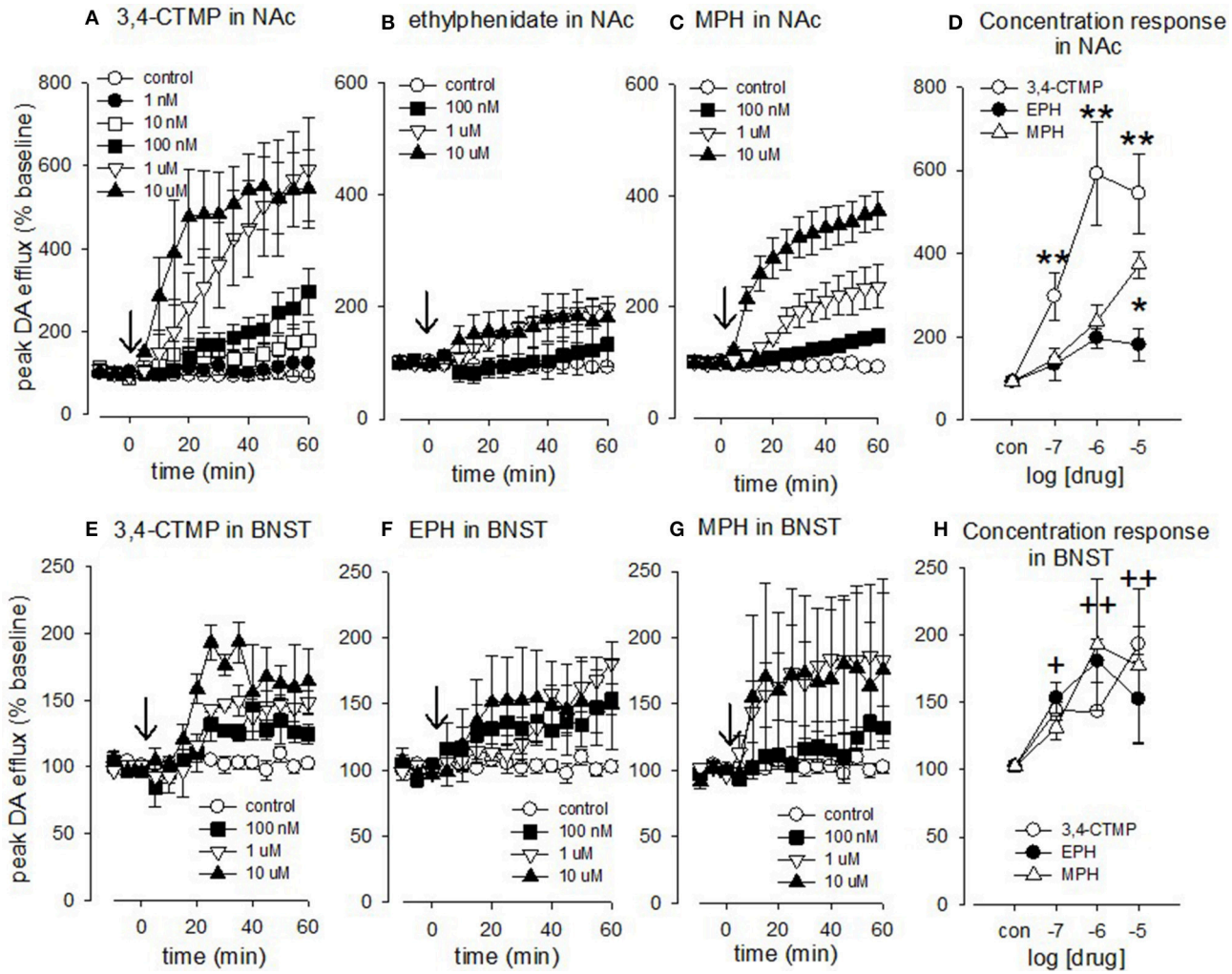

FIGURE 5 | Effect of methylphenidate, 3,4-CTMP and ethylphenidate in the nucleus accumbens (NAc) and bed nucleus of the stria terminalis (BNST) slice. After 3 baseline electrical stimulations (10 pulses at 100 Hz) methylphenidate, 3,4-CTMP or ethylphenidate (EPH) was added at a single concentration (arrow). The drug was left on for 60 min and stimulations continued at 5 min intervals. **(A)** Effect of 3,4-CTMP in the accumbens. **(B)** Effect of ethylphenidate in the accumbens. **(C)** Effect of methylphenidate in the accumbens **(D)** Comparison of methylphenidate, 3,4-CTMP and ethylphenidate in the accumbens using a two-way ANOVA. Asterisks denote difference between drugs, **(E)** Effect of 3,4-CTMP in the BNST. **(F)** Effect of ethylphenidate in the BNST. **(G)** Effect of methylphenidate in the BNST. **(H)** Comparison of methylphenidate, 3,4-CTMP and ethylphenidate in the BNST using a two-way ANOVA; there was no significant difference between the three drugs, but there was a significant effect of drug concentrations as denoted by the cross (+). Values are means ± SEM, $n = 3$–8. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{+}p < 0.05$, $^{++}p < 0.005$.

are electroactive themselves and/or can foul electrodes ((27); **Figure 4**).

Data Acquisition and Analysis

The current at the carbon electrode was sampled at 50,000 Hz by the computer and stored on one channel using Spike7. Another channel recorded changes at the dopamine or noradrenaline oxidation potential (600 mV). Peak height of transmitter efflux was calculated. An exponential curve was also fitted to the reuptake phase of the signal after each stimulation, using Spike7. This calculated the time-constants for the exponential decay phase of transmitter reuptake. The time-constant has been shown to be an appropriate method to accurately measure monoamine reuptake (28).

Dopamine or noradrenaline efflux was electrically evoked every 5 min and peak height was measured. Aggregate reuptake data are not shown as on some occasions, especially in the BNST and most often when drugs were present, the transmitter signal did not fall to basal levels in an exponential fashion, making modeling of reuptake difficult. In addition, it has been shown that peak height correlates well with reuptake effects (28) and so we only show peak height effects here. Baseline (before drug application) reuptake time-constants are given in the results section (Baseline Efflux Data). Once three steady baseline efflux events had been obtained, the drug was added for 60 min. See

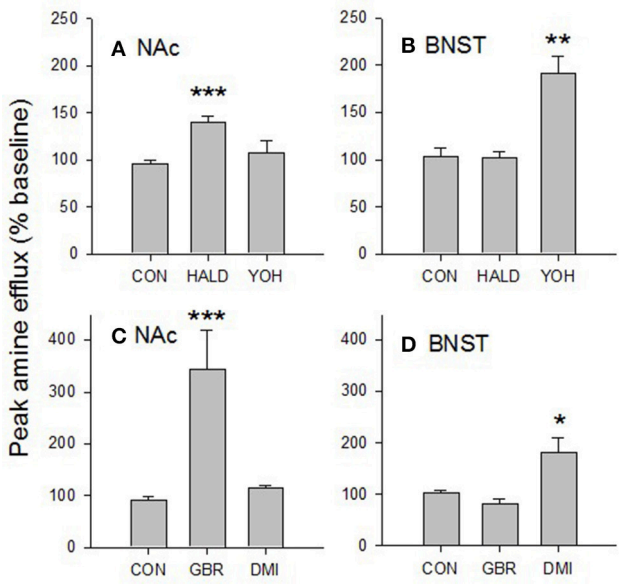

FIGURE 6 | Characterization of amine released in the BNST and NAc. The dopamine D_2 receptor antagonist, haloperidol (1 μM) increased electrically evoked amine efflux in the accumbens **(A)** but not the BNST. Similarly, the noradrenergic α_2 receptor antagonist, yohimbine (1 μM), increased amine efflux in the BNST **(B)** but not the accumbens. The selective dopamine transporter inhibitor GBR12909 (200 nM) increased amine efflux in the accumbens **(C)** but not the BNST while the selective noradrenaline transporter inhibitor desipramine (50 nM) increased amine levels in the BNST **(D)** but not the accumbens. Taken together these data suggest that the amine we are measuring in the accumbens is dopamine, while that in the ventral BNST is noradrenaline. Values are means SEM taken at peak drug effect. Data were analyzed by one-way ANOVA. ***$p < 0.001$ vs. control; **$p < 0.01$ vs. control; *$p < 0.05$ vs. control. CON, control; HALD, haloperidol; YOH, yohimbine; GBR, GBR12909; DMI, desipramine. $N = 2$–8.

Figures 3A,B for example experiments. Data was converted to a percentage of mean baseline data for statistical analysis.

Statistical analysis of differences between the two drugs on evoked transmitter release was carried out using two-way ANOVA with Tukey's *post-hoc* multiple comparisons test (drug X concentration). The data using antagonists or GBR12909/desipramine were analyzed by one-way AONOVA. For all analysis we used the peak effect of the drugs at each concentration. Data is presented as mean ± standard error of the mean (SEM), with a significance level set at $p < 0.05$.

RESULTS

Baseline Efflux Data

We used local electrical stimulation to evoke both dopamine and noradrenaline efflux in the accumbens and BNST respectively. Baseline (non-drug) dopamine efflux had a peak height of 419 ± 50 nM with decay phase time-constant of 1.23 ± 0.08 s ($n = 66$). Baseline noradrenaline efflux had a peak height of 169 ± 19 nM with decay phase time-constant of 1.84 ± 0.16 s ($n = 43$). See **Figure 2** for example efflux events.

Effect of Methylphenidate, 3,4-CTMP, and Ethylphenidate on Amine Efflux in the Accumbens

Using a two-way ANOVA we compared the effects of methylphenidate, 3.4-CTMP and ethylphenidate on electrically evoked dopamine efflux in the accumbens (**Figures 5A–D**). There was a main effect of drug [$F_{(2, 62)} = 38.929$, $p < 0.001$] a main effect of drug concentration [$F_{(3, 62)} = 43.756$, $P < 0.001$] and a drug X concentration interaction [$F_{(6, 62)} = 8.67$, $p < 0.01$]. Within the methylphenidate treated slices both 1 and 10 μM caused a significant increase in dopamine efflux ($p < 0.05$). Within the 3,4-CTMP treated slices, 100 nM caused an increase in evoked dopamine levels vs. controls ($p < 0.05$) while both 1 and 10 μM 3,4-CTMP increased evoked dopamine levels vs. both control and 100 nM ($p < 0.05$). Ethylphenidate did not significantly increase evoked dopamine levels at any concentration (all $p > 0.1$).

3,4-CTMP increased dopamine levels to a greater extent than both methylphenidate and ethylphenidate at 100 nM ($p < 0.05$ vs. both), 1 μM ($p < 0.001$ vs. both), and 10 μM ($p < 0.001$ vs. ethylphenidate and $p < 0.05$ vs. methylphenidate) while methylphenidate increased dopamine levels to a greater extent than ethylphenidate at 10 μM ($p < 0.001$).

Effect of Methylphenidate, 3,4-CTMP, and Ethylphenidate on Amine Efflux in the BNST

Using a two-way ANOVA we compared the effects of methylphenidate, 3.4-CTMP and ethylphenidate on electrically evoked noradrenaline efflux in the BNST (**Figures 5E–H**). There was no main effect of drug [$F_{(2, 54)} = 0.0781$, $p = 0.925$] and no drug X concentration interaction [$F_{(6, 54)} = 0.926$, $p = 0.486$] but there was a main effect of drug concentration [$F_{(3, 54)} = 13.933$, $P < 0.001$, 10 μM ($p < 0.001$), 1 μM ($p < 0.001$), and 0.1 μM ($p < 0.05$)] all increased evoked noradrenaline efflux vs. controls.

Characterization of Amine Efflux in Accumbens and BNST

Using a one-way ANOVA we compared the effects of haloperidol and yohimbine in the accumbens and BNST. In the accumbens there was a significant effect of drug [$F_{(2, 18)} = 10.598$, $p < 0.001$; **Figure 6A**]. Tukey's test revealed that amine efflux was greater in the accumbens after haloperidol vs. control ($p < 0.001$) and was nearly greater after haloperidol vs. yohimbine ($p = 0.056$). There was no significant effect of yohimbine vs. control ($p = 0.659$).

In the BNST there was also a significant effect of drug [$F_{(2, 9)} = 12.477$, $p < 0.005$; **Figure 6B**]. Yohimbine increased amine efflux vs. haloperidol ($p < 0.05$) and control ($p < 0.01$). There was no significant difference in the effect of haloperidol vs. control ($p = 0.999$).

Again using a one-way ANOVA we tested the effects of GBR12909 and desipramine on amine efflux in the accumbens and BNST. In the accumbens there was a significant effect of drug [$F_{(2, 12)} = 20.073$, $p < 0.001$; **Figure 6C**]. Tukey's test revealed that GBR12909 had a significant effect on amine efflux

vs. control ($p < 0.001$) and desipramine ($p < 0.005$). There was no significant effect of desipramine vs. control ($p = 876$).

In the BNST there was also a significant effect of drug [$F_{(2, 13)} = 6.26$, $p < 0.005$; **Figure 6D**]. Tukey's test showed that desipramine had a greater effect on amine efflux vs. control ($p < 0.05$) and GBR12909 ($p < 0.05$). There was no effect on amine efflux of GBR12909 vs. control ($p = 0.813$).

DISCUSSION

The majority of drugs of abuse increase dopamine efflux in the nucleus accumbens (6) by either directly acting on the dopaminergic terminals or indirectly at the ventral tegmental area. Both of the NPS studied here are structurally related to methylphenidate (Ritalin), a treatment for attention deficit hyperactivity disorder (ADHD). Methylphenidate increases dopamine levels by blocking the dopamine transporter (DAT) (29). This potential for abuse has been exploited by legal high vendors, with methylphenidate's analogs 3,4-CTMP and ethylphenidate being offered for sale on many websites.

There is a paucity of published research surrounding 3,4-CTMP, which was developed and tested in the 1990's as a potential treatment for cocaine abuse (30). It was suggested that some methylphenidate analogs could be used as a potential substitution therapy in the treatment of cocaine abuse, but 3,4-CTMP did not fall into this category. We have found 3,4-CTMP to dose-dependently increases electrically evoked dopamine release; 1 and 10 μM increased stimulated efflux ∼6-fold. The most likely mechanism of action, through which 3,4-CTMP increases dopamine efflux, is by DAT inhibition. Methylphenidate has been shown to partially block the DAT (29), and a study using rotating disk electrode voltammetry in rat striatal suspensions proposed that halogenation of the aromatic ring of methylphenidate with chlorine increases a compounds affinity to the DAT (31). In the case of 3,4-CTMP, this halogenation has occurred twice (at the 3′ and 4′ positions).

Previous work from this lab has examined the effects of cocaine on accumbens dopamine efflux (32, 33). The present data suggest that 3,4-CTMP is slightly more potent than cocaine at increasing peak dopamine efflux in the rat accumbens slice. The potential increased potency of 3,4-CTMP vs. cocaine is supported by the findings of Deutsch et al. (30),who carried out ligand binding studies in rat striatal preparations to determine 3,4-CTMP's ability to inhibit [3H]WIN 35,428, a DAT inhibitor, as well as to look at the uptake of [3H]dopamine into rat striatal synaptosomes. They found that 3,4-CTMP was eight times more potent than methylphenidate at inhibiting WIN,35428 binding. Drug discrimination studies also suggest that 3,4-CTMP is much more potent than methylphenidate (34). We have found 3,4-CTMP to increase dopamine levels more potently than methylphenidate (**Figure 5D**).

We also found 3,4-CTMP to increase electrically evoked noradrenaline efflux, with 10 μM resulting in an ∼2-fold increase in the BNST. We have been unable to find any published data relating to the effect of 3,4-CTMP on noradrenaline levels, but methylphenidate does block the noradrenaline transporter (NET; (35)). Our data suggest that 3,4-CTMP may be have abuse liability, and that its effects are "cocaine-like" rather than "amphetamine-like," as it increases stimulated dopamine release, but not basal (unstimulated) dopamine levels (36). Similarly, we found no evidence for methylphenidate or ethylphendiate to cause reverse transport of dopamine (data not shown).

There is a lack of published research on the effects of ethylphenidate on monoamine release. Ethylphenidate was first identified in two overdose victims who had co-administered large quantities of methylphenidate and ethanol (37), which resulted in the formation of the previously unknown metabolite of these two substances, ethylphenidate. The formation of ethylphenidate, in rat liver preparations incubated with methylphenidate and ethanol, showed a carboxylase- dependant trans-esterification process (38) that is thought to be analogous to the formation of cocaethylene; the metabolite produced with co- administration of cocaine and ethanol (39). The augmented effects of co-administration of methylphenidate and ethanol on mouse locomotion and brain levels of ethylphenidate have been examined (40, 41). Co-administration of these drugs has also been tested in humans where ethylphenidate was shown to be produced along with increased subjective highs (42).

Internet drug fora have provided some useful information on the effects of ethylphenidate, with some reports suggesting that it produces a less jittery, more euphoric high than methylphenidate[1,2]. Additionally, multiple user reports confirm euphoria associated with ethylphenidate, as well as less locomotor stimulation, increased music appreciation and abstract thinking.

We found ethylphenidate to increases electrically evoked dopamine efflux by ∼2-fold. The likely mechanism of action is by blocking the DAT; the same mechanism of action as methylphenidate (43). In previous ligand binding studies, ethylphenidate has been shown to bind to the dopamine transporter with ∼50% less binding potency than methylphenidate (29). Ethylphenidate also increased locomotor activity in mice by 20% less than methylphenidate (44, 45). Taken together these studies suggest that ethylphenidate is not as potent as methylphenidate at increasing dopamine levels, consistent with the present study (**Figure 5D**).

CONCLUSION

This study explored the neurochemical profile of the NPS methylphenidate analogs 3,4-CTMP and ethylphenidate as well as the parent compound methylphenidate. 3,4-CTMP was more potent than methylphenidate, increasing dopamine release ∼6-fold, presumably caused by blockade of the DAT. Ethylphenidate has a weaker effect, modestly increasing stimulated dopamine release by ∼2-fold. All 3 drugs increased noradrenaline efflux ∼2-fold. At the concentrations tested, no drug increased basal levels of dopamine or noradrenaline, that is, they did

[1] Drugs Forum – legal high user blog. Available online at: http://www.drugs-forum.com/forum/showthread.php?t=137752 (Accessed July, 16 2013)

[2] Drugs Forum: Ethylphenidate. Available at: http://www.drugs-forum.com/forum/showthread.php?t=134578&page=2 (Accessed June, 05 2013)

not cause reverse transport as one might expect with an amphetamine-like drug. The effects of each drug on dopamine indicate that they have the potential to be addictive, especially 3,4-CTMP.

ETHICS STATEMENT

This work was approved by the ethics committee at St George's University of London. Because only *in vitro* work was carried out and animals were killed by schedule 1 methods, no UK Home Office License was needed. Thus this work was done in accordance with the UK animal scientific procedures act.

AUTHOR CONTRIBUTIONS

CD conceived of the work, undertook some experiments, did the statistics and wrote most of the manuscript. CR and VB undertook most of the experiments and helped analyse data. JR helped write the manuscript and conceived some of the work and analysed the NPS structures using mass spec.

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frontiers in Psychiatry

ORIGINAL RESEARCH
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The Non-Peptide Arginine-Vasopressin v_{1a} Selective Receptor Antagonist, SR49059, Blocks the Rewarding, Prosocial, and Anxiolytic Effects of 3,4-Methylenedioxymethamphetamine and Its Derivatives in Zebra Fish

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3,4-Methylenedioxymethamphetamine (MDMA) and its derivatives, 2,5-dimethoxy-4-bromo-amphetamine hydrobromide (DOB) and *para*-methoxyamphetamine (PMA), are recreational drugs whose pharmacological effects have recently been attributed to serotonin $5HT_{2A/C}$ receptors. However, there is growing evidence that the oxytocin (OT)/vasopressin system can modulate some the effects of MDMA. In this study, MDMA (2.5–10 mg/kg), DOB (0.5 mg/kg), or PMA (0.005, 0.1, or 0.25 mg/kg) were administered intramuscularly to adult zebra fish, alone or in combination with the V_{1a} vasopressin antagonist, SR49059 (0.01–1 ng/kg), before carrying out conditioned place preference (CPP), social preference, novel tank diving, and light–dark tests in order to evaluate subsequent rewarding, social, and emotional-like behavior. The combination of SR49059 and each drug progressively blocked: (1) rewarding behavior as measured by CPP in terms of time spent in drug-paired compartment; (2) prosocial effects measured on the basis of the time spent in the proximity of a nacre fish picture; and (3) anxiolytic effects in terms of the time spent in the upper half of the novel tank and in the white compartment of the tank used for the light–dark test. Antagonism was obtained at SR49059 doses which, when given alone, did not change motor function. In comparison with a control group, receiving vehicle alone, there was a three to five times increase in the brain release of isotocin (the analog of OT in fish) after treatment with the most active doses of MDMA (10 mg/kg), DOB (0.5 mg/kg), and PMA (0.1 mg/kg) as evaluated by means of bioanalytical reversed-phase high-performance liquid chromatography. Taken together, these findings show that the OT/vasopressin system is involved in the rewarding, prosocial, and anxiolytic effects of MDMA, DOB, and PMA in zebra fish and underline the association between this system and the behavioral alterations associated with disorders related to substance abuse.

Keywords: phenethylamines, zebra fish, isotocin, social preference, hallucinogens, novel tank diving test, light–dark test

INTRODUCTION

New psychoactive substances are available in various formulations and are mainly used as legal substitutes for traditional drugs of abuse. One of the largest and most important groups are psychostimulants, which affect a range of behavioral patterns in humans (1) and laboratory animal models (2, 3). It has been demonstrated that the repeated administration of psychostimulants to rodents (2, 4–8) and humans (9) can lead to addiction, induce changes in emotional states such as fear, anxiety, and depression, interfere with social behavior, and cause cognitive impairment. It has also been found that the repeated administration of cocaine and methamphetamine are anxiogenic in mice performing the elevated plus maze task (2), lead to cognitive deficit in rats when using the novel object recognition test, and induce depressive-like behavior as evaluated by the forced swimming task (4). The repeated administration of 3,4-methylenedioxymethamphetamine (MDMA) decreases social investigation and increases anxiety-like behavior (5). Rats that are prenatally exposed to (6), or neonatally treated with methamphetamine (P11-P15) (7), and subsequently given a subthreshold dose of methamphetamine in adulthood show impaired working memory. Although no working memory deficit has yet been documented in rats prenatally exposed to MDMA in the Morris water maze when a fixed platform schedule is used, they do show perseverative behavior using a cued platform schedule (8). Finally, the repeated use of MDMA by humans has been associated with sleep, mood, and anxiety disturbances, increased impulsiveness, memory deficits and attention problems, which may persist for up to 2 years after cessation (9).

Increasing attention has been given to MDMA and its phenetylamine derivatives over the last few years because, although illegal, they are sold openly through internet websites (10–15) despite their significant toxicity (13, 16, 17). It is known that MDMA is prosocial and enhances empathy in humans (18), but it evokes hyperlocomotion and anxiety in rodents (5, 19, 20) and, depending on the dose, can have an anxiogenic or anxiolytic effect on mice, rats, and zebra fish (21–23). However, there has been controversy concerning its effects on anxiety in humans as reduced anxiety has been observed in a clinical setting (24), whereas Schifano (25) found a high level of anxiety after chronic use.

Among the MDMA derivatives, 2,5-dimethoxy-4-bromo-amphetamine hydrobromide (DOB) and *para*-methoxyamphetamine (PMA) are widely used as substitutes in "ecstasy" tablets because of their similarity to MDMA (26).

2,5-Dimethoxy-4-bromo-amphetamine hydrobromide was first synthesized by Shulgin and Shulgin (27), and its recreational use increased in the mid-1980s as it was best alternative to LSD and psilocybin. Its psychoactive effects are mediated by $5HT_{2A/2C}$ receptor interactions within the central nervous system (28–30) and are similar to those of other hallucinogenic phenylalkylamines such as mescaline (30, 31). At an oral dose of 2 mg, it is emotionally stimulating and enhances perceptions without giving rise to perceptual distortions or hallucinations (32); however, the uncontrolled illegal use of higher doses may cause hallucinations, panic, vasospasms, coma, and even death (29–32). DOB-related fatal and non-fatal intoxication has also been reported (32–36).

Para-methoxyamphetamine is cheaper and more readily available than MDMA, which it was designed to replace. However, as suggested by its street name of "death," PMA poisoning has been reported in various countries (37, 38). It is stronger than MDMA, but users tend to take more because it takes longer to act (39). In humans, it induces life-threatening hyperthermia, rhabdomyolysis, breathing difficulties, and acute renal failure (40); in rats, its acute administration increases dopamine (DA) and 5HT release in the striatum, nucleus accumbens (NAc) and frontal cortex (41). Despite their well-documented toxicity, MDMA and hallucinogens such as psylocybin, have recently been proposed as new treatments for alcohol addiction, post-traumatic stress disorder, anxiety, and depression (27, 42).

Zebra fish (*Danio rerio*) is a valuable model for high-throughput drug discovery and screening and can be used to investigate some aspects of neuropsychiatric disorders, including hallucinogen-evoked states (43). It has been found that MDMA and hallucinogens such as salvinorin A are rewarding for zebra fish undergoing the conditioned place preference (CPP) test, a widely used means of evaluating rewarding effects of different compounds (44, 45). In addition, the innate tendency of zebra fish to form shoals has often been used to examine the effects of drugs on social preference, and it has been shown that LSD, ibogaine, PCP, MK801, and MDMA markedly decrease shoal cohesion (44). It is also possible to assess anxiety by analyzing the habituation of zebra fish to novelty (46) and their response to brightly lit environments (47). The novel tank test has shown that zebra fish exposed to various doses of MDMA, LSD, mescaline, ibogaine, phencyclidine, and ketamine show anxiolytic-like responses: i.e., they spend a longer time in the upper half of the tank and there is a reduction in the latency to get to the top of the tank (48–52). The light–dark test has shown no change in zebra fish exposed to LSD (52) or psylocybin (43), but an anxiolytic effect in those exposed to ibogaine (49).

The recently discovered, dose-dependent rewarding, anxiolytic, and prosocial effects of MDMA, DOB, and PMA on zebra fish (23, 53) can be completely blocked by ritanserin, thus suggesting the involvement of $5\text{-}HT_{2A/C}$ receptors. However, there is growing evidence that the neuropeptide oxytocin (OT) can modulate drug-related reward and may act as a pharmacological treatment of drug dependence (54). Accordingly, use of the CPP or self-administration paradigm has shown that peripheral OT injections in mice, or intracerebroventricular (ICV) OT microinjections into mouse NAc or subthalamic nucleus, attenuate methamphetamine-induced reward (55). It is also interesting to note that the prosocial effect of MDMA has been related to central OT release in both rat and human studies (56, 57).

Abbreviations: 5 $HT_{2A/C}$: serotonin receptor type 5, subtype A and C; AVP, arginine-vasopressin system; AVT: arginine vasotocin; DOB: 2,5-dimethoxy-4-bromo-amphetamine hydrobromide; IM: intramuscularly; IT: isotocin; MDMA: 3,4-methylenedioxymethamphetamine; OT: oxytocin; NAc: nucleus accumbens; PMA, *para*-methoxyamphetamine; SR49059: ((2S)-1-[[(2R,3S) 5-chloro-3(2-chlorophenyl)-1-[(3,4-dimethoxyphenyl) sulfonyl]-2,3-dihydro-3-hydroxy-1H-indol-2-l]carbonyl]-2-pyrrolidine carboxamide); V_{1a}: vasopressin receptor, type1a; V_1b: vasopressin receptor, type1b.

The characteristic changes in rat adjacent lying and anogenital sniffing induced by MDMA, vasopressin (AVP), and OT can be reversed by pretreatment with SR49059 (56), thus indicating that OT and AVP may directly act on V_{1a} vasopressin receptors to induce prosocial effects. At the same time, MDMA may indirectly stimulate V_{1a} vasopressin receptors as a result of serotonin-induced OT and/or AVP release in the hypothalamus (58).

It has emerged that nonapeptides of the vasotocin family are key regulators of social behavior in a wide range of vertebrate species: AVP and OT in mammals, and arginine vasotocin (AVT) and isotocin (IT) in teleosts (59). In order to investigate the mechanism(s) underlying the activity of amphetamine derivatives and the possibility that some of the effects of MDMA, DOB, and PMA are influenced by IT and AVT, we tested the effects of the selective V_{1a} vasopressin receptor antagonist, SR49059, on various aspects of behavior that can be successfully evaluated in zebra fish: i.e., reward, anxiety, and social behavior. We also investigated the changes in IT release within the brain after treatment with these drugs.

MATERIALS AND METHODS

Animals

Adult wild-type short-finned zebra fish (*D. rerio*) of different genetic backgrounds (weight 0.4–1 g; age 6 and 12 months) were obtained from a local aquarium supplier (Aquarium Center, Milan, Italy). The males and females were distinguished as previously described (60, 61) and were used in a 1:1 ratio in all of the experiments. The fish were kept at a temperature of 28°C using a 14-h (from 8.00 am to 10.00 pm) light and 10-h dark cycle and were fed daily with brine shrimp and flake fish food [tropical fish food, Consorzio G5, Casatenovo (LC), Italy]. The tank water consists of deionized H2O and sea salts (0.6 g/10 L of water; Instant Ocean, Aquarium Systems, Sarrebourg, France). Each home tanks contained about 30 fish each and were constantly filtered and aerated. All of the fish were drug naive, and each fish was only used once. The experiments were started one month after the fish arrived in the laboratory in order to minimize stress, and they were habituated to the experimental apparatus for 1 h a day during the week preceding the experiments. During the experiments, the observer was blinded to the treatment allocation and sat 2 m from the tank. Behavioral testing was carried out between 9.00 am and 2.00 pm, using 10 fish per group. All of the tests were video-recorded (Canon Digital MV900) for subsequent video-aided analysis by trained observers who were also blinded to the treatments. The experiments were carried out in accordance with European Community Council Directive No. 86/609/EEC, and the subsequent Italian law governing the protection of animals used for experimental and other scientific purposes. The experimental protocol was approved in accordance with Italian Governmental Decree Nos. 18/2013 and 34/2017. Every effort was made to minimize the number of animals used and their discomfort.

Drugs

Figure 1 shows the chemical structures of MDMA, DOB, and PMA (Sigma-Aldrich, St. Louis, MO, USA), which were dissolved in sterile saline at doses of, respectively, 10, 0.5, and 0.1 mg/kg and administered intramuscularly (IM) 5 min before each test. The doses were chosen on the basis of their known ability to maximize the drugs' rewarding, social, and anxiolytic effects (23, 53). Given the unavailability of specific antagonists of zebra fish IT/AVT receptor subtypes and selective OT antagonists, we used (2S)-1-[[(2R,3S)5-chloro-3(2-chlorophenyl)-1-[(3,4-dimethoxyphenyl) sulfonyl]-2,3-dihydro-3hydroxy-1H-indol-2-l]carbonyl]-2-pyrrolidine carboxamide (SR49059; Sigma-Aldrich, St. Louis, MO, USA), the most selective antagonist of human and rat vasopressin V_{1a} receptors (60), at doses of 0.01, 0.1, and 1 mg/kg, which are known to block the prosocial effects of IT and AVT in zebra fish (62). When multiple treatments were required, the drug solutions were put into the same syringe in order to avoid unnecessary tissue trauma. The drug doses were calculated as salts, and all of the drugs were freshly prepared on a daily basis. The control group received saline 2 μL/g.

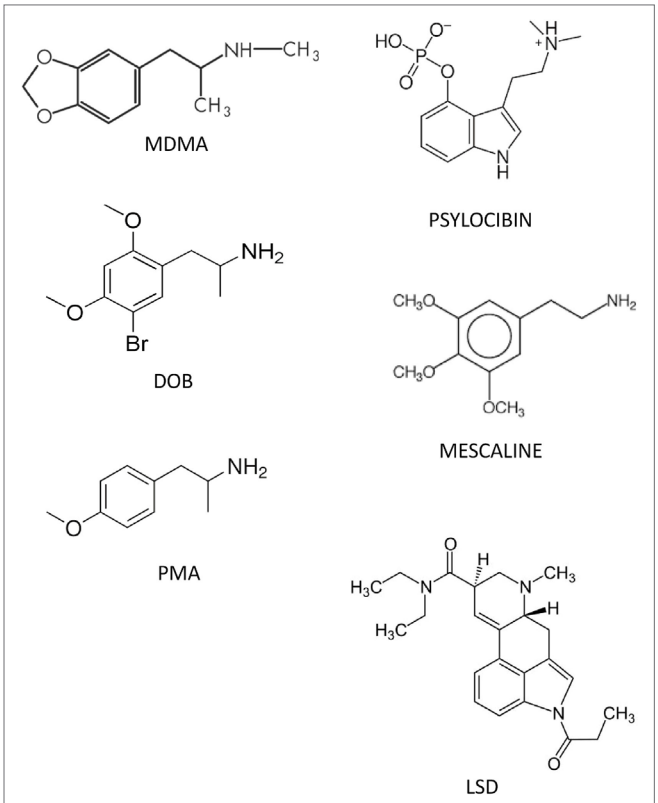

FIGURE 1 | Structures of some of the phenetylamines used in the study and the main serotonergic hallucinogens.

Treatments

The injections were given along the posterior axis of the caudal musculature (61), in the area below the caudal fin on the left side of each fish, using a Hamilton syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland).

Conditioned Place Preference

The fish were tested in a 10 cm × 20 cm × 15 cm tank divided into two 10 cm × 10 cm halves, one of which had three black

polka dots on the bottom; the two halves were separated by a perforated wall that allows the fish to pass from one side to the other, albeit in a slightly obstructed manner as previously described (45) (**Figure 2**). During the week before the start of the CPP test, the fish were given subcutaneous injections of colored dyes (Sigma-Aldrich) so that they could be easily distinguished, as suggested by Cheung et al. (63). On the first day, after the fish had been introduced to the tank, baseline preference was established by recording the percentage of time spent in one side or the other during a 15-min trial (the preconditioning phase). After 6 h, the fish were intramuscularly injected with the different drugs, and then confined to the least preferred side for 30 min. After 24 h, the fish receiving the vehicle were confined to the opposite compartment for 30 min. The drug-texture pairings were always counterbalanced. On the third day (postconditioning phase), the fish were allowed to pass freely from one side of the tank to the other for 15 min, and the time spent in each half was recorded

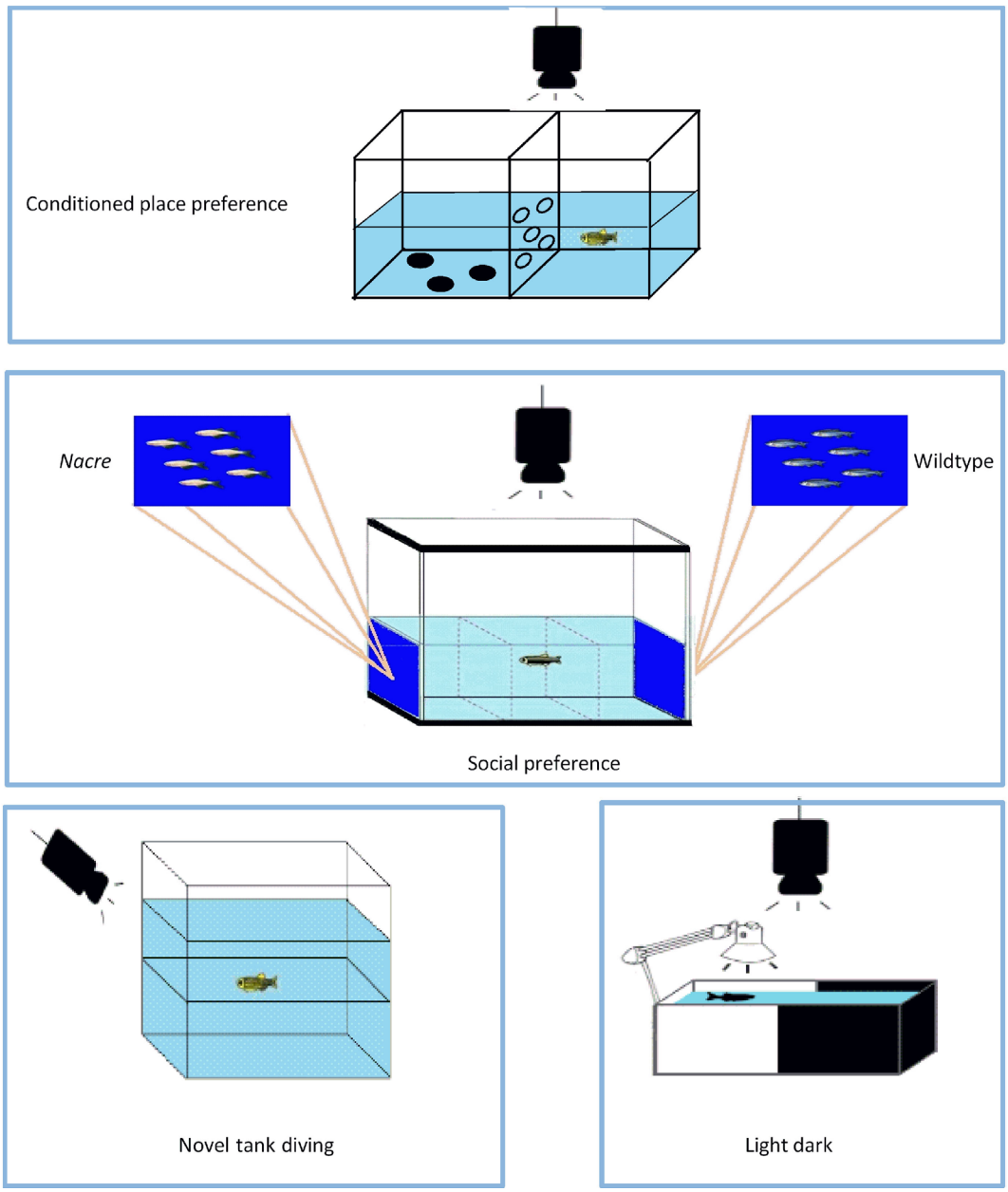

FIGURE 2 | The apparatuses used in the study. The conditioned place preference test apparatus was used to evaluate the rewarding effects of 3,4-methylenedioxymethamphetamine (MDMA), 2,5-dimethoxy-4-bromo-amphetamine hydrobromide (DOB), and *para*-methoxyamphetamine (PMA). The social preference test apparatus was used to evaluate shoaling behavior. Anxiety-like behavior was studied using the novel tank diving and light–dark tests. For further explanation, see Material and Methods.

offline. The rewarding or aversive effects of the drugs on the fish were determined by subtracting the baseline values from the final values.

Social Behavior

The shoaling preference test was carried out as previously described (23) in a glass tank that was 122 cm long, 55 cm high, and 32 cm wide and divided into three equal compartments (**Figure 2**). There was a picture of six 3 cm long nacre fish on a blue background on the left side of the tank and an unaltered picture of six zebra fish [taken from Ref. (64)] on the right side of the tank, and the lateral compartments (or stimulus areas) were separated from the central compartment. The glass tank was divided into three zones of equal volume: a left preference area, a central no-preference area, and a right preference area. The tank had two 250 W halogen lamps above either side of the tank, the light of which reflected off two sheets of Teflon positioned at an angle of 45° on the top of the tank in order to ensure that the whole of the tank received even, full-spectrum lighting. The depth of the water was 25 cm. At the beginning of the experimental trials, thin sheets of opaque plastic were placed on either side of the central compartment to act as temporary visual barriers and, immediately after undergoing drug treatment, each fish was placed in the central compartment and allowed 5 min to acclimatize. The opaque barriers were then removed and, when a fish swam parallel to one of the shoal members, it was assumed it had recognized the stimulus shoal (65). The fish were given up to 15 min to recognize both stimuli and, if they failed to do so, the test was postponed until the following week: only 0.1% of the fish failed to recognize both stimuli within the first 5 min. Shoaling preference was quantified by recording the total time each fish spent in proximity to each stimulus shoal within a period of 5 min. The data were expressed as the difference (Δ) between the time spent close to the nacre and wild-type fish pictures.

Novel Tank Diving Test

The novel tank diving test of Egan et al. (66) was used to evaluate the anxiety-like behavior evoked by novelty. After a 1-h period of acclimatization in the experimental room, each fish was gently transferred to a transparent 1.5-L tank that had a line drawn on the outside walls midway between the surface of the water and the bottom of the tank (**Figure 2**). Zebra fish typically have vertical exploratory behaviors that gradually tend to increase over time (44, 66). The time spent in the upper and lower halves during the first 5 min was recorded, together with the number of times the fish moved from the lower to the upper half.

Light–Dark Test

In order to investigate anxiety-related behaviors further, the fish underwent a previously described light/dark test (23). The test apparatus consisted of a rectangular half-black, half-white acrylic tank (20 cm × 10 cm × 15 cm) separated by a gray divider (**Figure 2**), and filled with water to a depth of 13 cm. The room was lit overhead (250 lux), and a 9-watt lamp was positioned above the white half of the tank in order to ensure further constant, uniform and shadow-free lighting. After being individually placed in the white compartment and allowed to acclimatize for 5 min, the divider was removed and the fish were given 5 min to swim freely between the compartments. The analyzed parameters were the time spent in the white compartment and the number of crossings between the two compartments, and the data were expressed as the difference between the time spent in each compartment. Greater exploration of the white compartment reflects a low state of anxiety.

Brain IT Assay

The brain IT assay was restricted to the fish receiving the maximum active doses of the compounds alone, and those receiving MDMA combined with SR49059. Five minutes after being intramuscularly injected with vehicle, MDMA 10 mg/kg, PMA 0.1 mg/kg, DOB 0.5 mg/kg alone, or MDMA 10 mg/kg + SR49059 1 mg/kg, the fish were sacrificed by means of an overdose of tricaine solution (500–1,000 mg/L), and their whole brains were removed within 2 min of death. Each brain was weighed, immediately frozen on dry ice, and then stored at −80°C until analysis. IT concentrations were measured by means of bio-analytical, reversed-phase high-performance liquid chromatography with fluorescence detection. Solid-phase extraction and peptide derivatization were carried out as described in Ref. (67). The chromatographic equipment consisted of an Agilent 1100 series system (Agilent Technologies, Inc., Santa Clara, CA, USA) and Ascentis Express C_{18} column (4.6 mm × 150 mm, particle size 2.7 μm) operating in isocratic mode using acetonitrile and 0.05 M $Na_2HPO_4.H_2O$ (80:20 v/v) as mobile phase (0.4 mL/min). The eluent was monitored at 530 nm (470 nm excitation).

Statistical Analysis

The data are expressed as mean values ± the standard error of the mean (SEM). Between-group differences were assessed using one-way analysis of variance (ANOVA) for repeated measures followed by Tukey's *post hoc* test. All of the statistical analyses were made using Prism 6 software (GraphPad Inc., La Jolla, CA, USA).

RESULTS

Conditioned Place Preference

During the preconditioning phase, a simple paired *t* test revealed no significant difference between the time spent in the polka dot chamber (432.8 ± 15.3 s) and dot-free compartment (469.2 ± 14.84 s) ($t_{128} = 0.236$, $p = 0.81$). One-way ANOVA revealed between-group differences after treatment with MDMA, PMA, or DOB alone or in combination with SR49059: MDMA ($F_{3,36} = 4.82$, $p = 0.01$), DOB ($F_{3,36} = 6.94$, $p = 0.003$), PMA ($F_{3,36} = 5.57$, $p = 0.007$) (**Figure 3**). *Post hoc* analysis showed a significant increase in postconditioning time after treatment with MDMA 10 mg/kg, DOB 0.5 mg/kg, or PMA 0.1 mg/kg, whereas SR49059 completely blocked MDMA-, DOB-, and PMA-induced CPP. SR49059 antagonism was obtained at doses that did not affect CPP, except for the highest dose that induced a rewarding effect ($F_{3,36} = 8.00$, $p = 0.0003$) (**Table 1**).

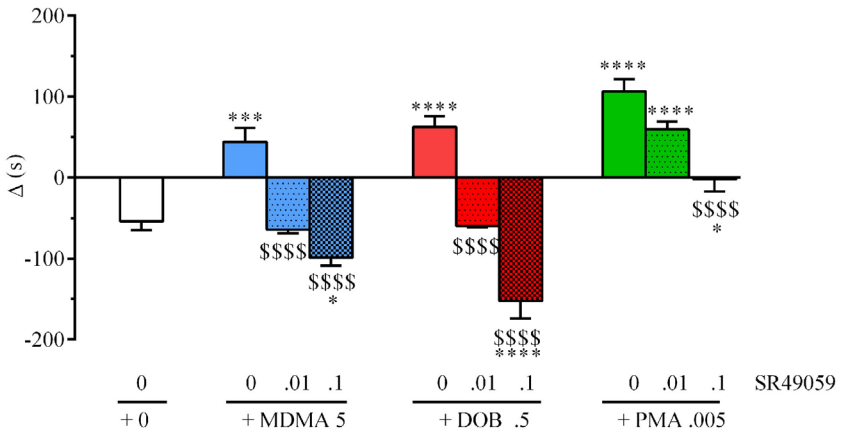

FIGURE 3 | SR49050 blocks 3,4-methylenedioxymethamphetamine (MDMA)-, 2,5-dimethoxy-4-bromo-amphetamine hydrobromide (DOB)-, and *para*-methoxyamphetamine (PMA)-induced conditioned place preference (CPP). Mean values ± SEM of differences in the time spent (s) by each zebra fish in the drug-paired compartment before and after conditioning. Each drug (mg/kg) plus SR49059 (ng/kg) or vehicle was administered intramuscularly (IM) immediately before the conditioning session. $n = 10$ fish per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the vehicle group (0 + 0); $^{\$}p < 0.05$, $^{\$\$\$}p < 0.001$ vs. the corresponding drug alone (Tukey's *post hoc* test).

TABLE 1 | Effect of SR40059 on different behaviors in zebra fish.

Treatment	Dose (ng/kg)	CPP (postconditioning–preconditioning) (s)
Vehicle	0	28.48 ± 46.01
SR49059	0.01	−50.33 ± 42.06
SR49059	0.1	95.2 ± 23.83
SR49059	1	183.3 ± 0.91*,$
Treatment	**Dose (ng/kg)**	**Social preference (time)**
Vehicle	0	−54.25 ± 10.90
SR49059	0.01	−103.70 ± 4.25
SR49059	0.1	−49.50 ± 17.00

Mean values ± SEM.
SR49059 was given intramuscularly 5 min before the start of each test (n = 10 for each dose).
$^{*}p < 0.05$ vs. corresponding vehicle; $^{\$}p < 0.01$ vs. SR49059 (one-way ANOVA, Tukey's test).

Social Preference

There were significant between-group differences in the time spent by each fish near the con-specific or nacre picture when SR49059 or vehicle were given in combination with the different drugs: MDMA ($F_{3, 36} = 29.82$, $p < 0.0001$), DOB ($F_{3, 36} = 39.67$, $p < 0.0001$), and PMA ($F_{3, 36} = 30.58$, $p < 0.0001$) (**Figure 4**). *Post hoc* comparisons showed that all of the drugs given alone significantly increased the time spent near the nacre picture in comparison with the vehicle group. SR49059 blocked the social preference induced by MDMA, DOB, or PMA in a dose-dependent manner. SR49059 antagonism was obtained at doses that did not affect social preference ($F_{3, 36} = 1.14$, $p = 0.36$) (**Table 1**).

Novel Tank Diving Test

There were significant between-group differences in the time spent in the upper half of the novel tank during the 5 min after treatment (**Figure 5A**): MDMA ($F_{3, 36} = 42.99$, $p < 0.0001$), DOB ($F_{3, 36} = 25.45$, $p < 0.0001$), and PMA ($F_{3, 36} = 54.81$, $p < 0.0001$). *Post hoc* analysis showed that treatment with the drugs alone significantly increased the time spent in the upper half in comparison with vehicle group, but this behavior was blocked by the coadministration of SR49059. Acute treatment with MDMA, DOB, and PMA decreased the number of transitions from the lower to the upper half of the tank during the 5 min after treatment: MDMA ($F_{3, 36} = 24.54$, $p < 0.0001$), DOB ($F_{3, 36} = 22.43$, $p < 0.0001$), and PMA ($F_{3, 36} = 35.76$, $p = 0.0001$) (**Figure 5B**). SR49059 antagonized the decrease in the number of transitions induced by the drugs, except for those induced by the highest dose of PMA. SR49059 antagonism was obtained at doses that did not affect the time spent in the upper and lower halves of the tank ($F_{3, 36} = 0.56$, $p = 0.64$) or the number of transitions ($F_{3, 36} = 0.33$, $p = 0.81$) (**Table 2**).

Light–Dark Test

The difference in the time spent in the white and black compartments after treatment revealed between-group differences in emotional-like behavior: MDMA ($F_{3, 36} = 19.304$, $p < 0.0001$), DOB ($F_{3, 36} = 13.06$, $p < 0.0001$), and PMA ($F_{3, 36} = 19.37$, $p < 0.0001$) (**Figure 6A**). *Post hoc* analysis showed that treatment with the drugs alone significantly increased the time spent in the white compartment in comparison with the vehicle group, but the addition of SR49059 reduced this time in a dose-dependent manner. The increased time spent in the light–dark compartment was not due to motor impairment as there was no change in the number of transitions from one compartment to the other: MDMA ($F_{3, 36} = 0.42$, $p = 0.74$), DOB ($F_{3, 36} = 0.77$, $p = 0.52$), and PMA ($F_{3, 36} = 1.9$, $p = 0.14$) (**Figure 6B**). When given alone, SR49049 did not affect either parameter (time: $F_{2, 27} = 1.83$, $p = 0.18$; transitions: $F_{2, 27} = 2.29$, $p = 0.12$) (**Table 2**).

Brain IT Levels

The drugs significantly increased brain IT levels in comparison with the vehicle group ($F_{4, 25} = 13.88$, $p < 0.0001$) (**Figure 7**),

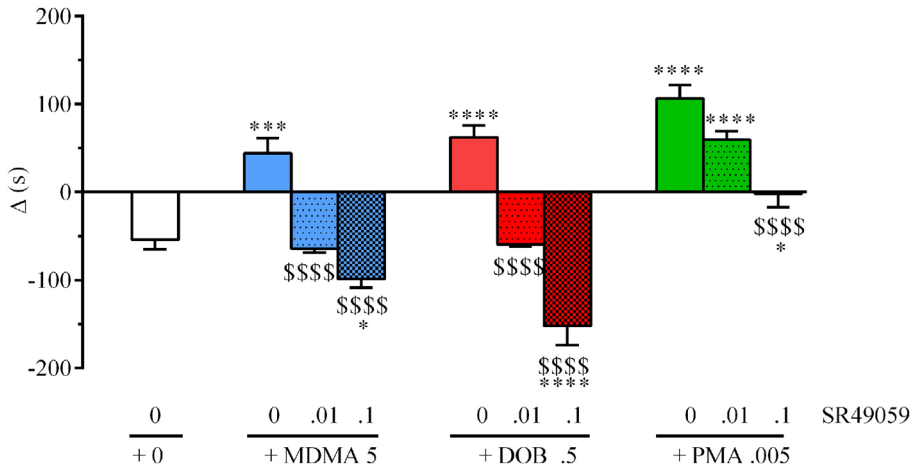

FIGURE 4 | SR49050 dose-dependently antagonizes 3,4-methylenedioxymethamphetamine (MDMA)-, 2,5-dimethoxy-4-bromo-amphetamine hydrobromide (DOB)-, and *para*-methoxyamphetamine (PMA)-induced social preference in zebra fish. Mean values ± SEM of the differences in the time spent (s) in the compartment near to the nacre picture and the time spent near to the WT picture (Δ). The combination of SR49059 (ng/kg) or vehicle and each drug (mg/kg) was given intramuscularly (IM) immediately before the test. $n = 10$ fish per group. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. saline (0 + 0); $^{\$\$\$\$}p < 0.001$ vs. the corresponding drug alone (Tukey's test).

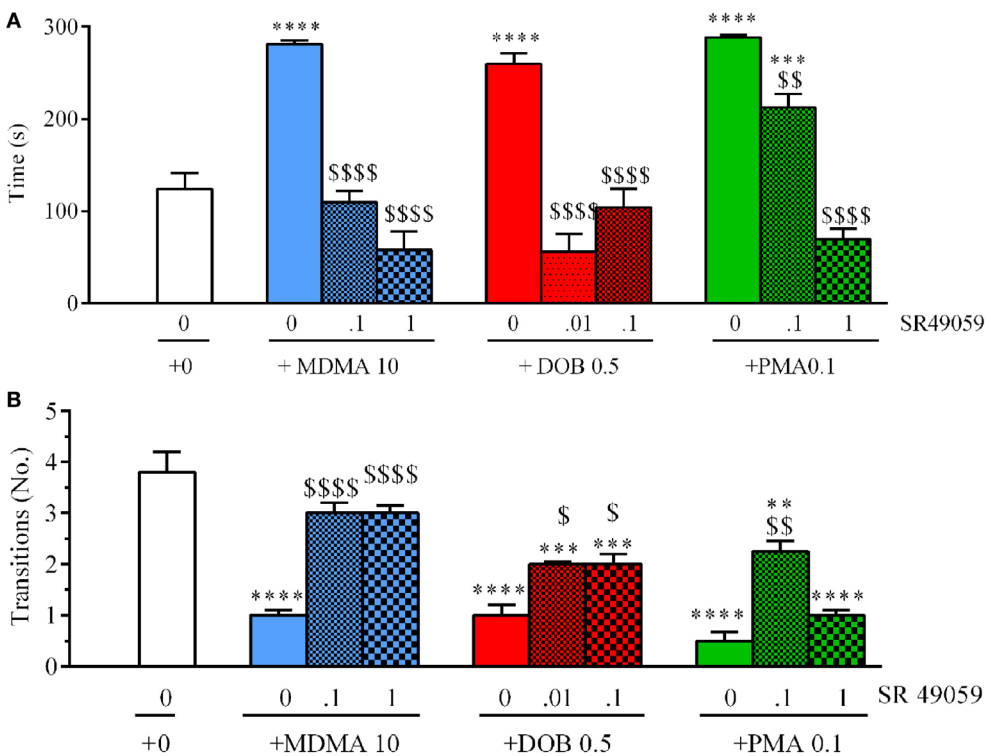

FIGURE 5 | SR49050 dose-dependently reduces the anxiolytic effect induced by 3,4-methylenedioxymethamphetamine (MDMA), DOB, or *para*-methoxyamphetamine (PMA) in the novel tank diving test. The time spent in the upper half of the tank **(A)** and the number of transitions from the lower half to the upper half **(B)** were recorded during the 5-min sessions. The combination of SR49059 (ng/kg) or vehicle and each drug (mg/kg) was given intramuscularly (IM) immediately before the test. Mean values ± SEM; $n = 10$ fish per group. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. the corresponding saline group (0 + 0); $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$\$}p < 0.0001$ vs. the corresponding drug alone (Tukey's *post hoc* test).

whereas the coadministration of SR49059 and MDMA significantly reduced the MDMA-induced increase.

DISCUSSION

This study investigated the modulatory role of V_{1a}-like subtype receptors on MDMA-, DOB-, and PMA-induced rewarding, prosocial, and anxiolytic effects in zebra fish. The selective antagonist of vasopressin V_1a subtype receptors, SR49059, reduced the effects induced by all of the tested drugs (which were associated with increased IT concentrations in the brain), whereas SR49059 completely blocked the brain IT release induced by MDMA.

It has been previously shown that SR49059 blocks the prosocial and anxiolytic effects induced by the injection of neurohypophyseal OT/AVP hormones and their teleost fish homologs IT/AVT (60). AVT receptors have been identified in non-mammalian vertebrates such as teleosts, and it has been shown that they are involved in water balance, osmotic homeostasis, sociality, aggression and sexual behavior (68, 69). Although teleost fish receptors have not yet been fully characterized, like mammalian OT and V_{1a}/V_1b receptor subtypes, AVT and IT receptors may act through a phosopholipase C/inositol 1,4,5-trisphosphate intracellular signaling pathway (70). It has been previously shown (71) that SR49059 is a more selective and potent antagonist of V_1a than V_{1b} receptors, but its affinity for V_{1A} and OT receptors is similar at least in mice (Ki = 0.94 ± 22 and 13.2 ± 19, respectively). However, further studies are needed to investigate its affinity for zebra fish IT/AVT receptors.

TABLE 2 | Effect of SR40059 on anxiety-like behavior in zebra fish.

Treatment	Dose (ng/kg)	Novel tank diving test (s)	Transitions to upper half (No.)
Vehicle	0	123.80 ± 17.40	3.87 ± 1.40
SR49059	0.01	61.80 ± 23.40	4.00 ± 1.90
SR49059	0.1	102.32 ± 4.86	4.86 ± 1.80
SR49059	1	94.00 ± 37.70	2.25 ± 0.95
Treatment	**Dose (ng/kg)**	**Light–dark test (s)**	**Transitions (No.)**
Vehicle	0	−122.70 ± 17.59	58.59 ± 4.19
SR49059	0.01	−74.25 ± 22.31	66.50 ± 16.60
SR49059	0.1	−74.50 ± 4.85	37.5 ± 9.30

Mean values ± SEM (n = 10 for each treatment).

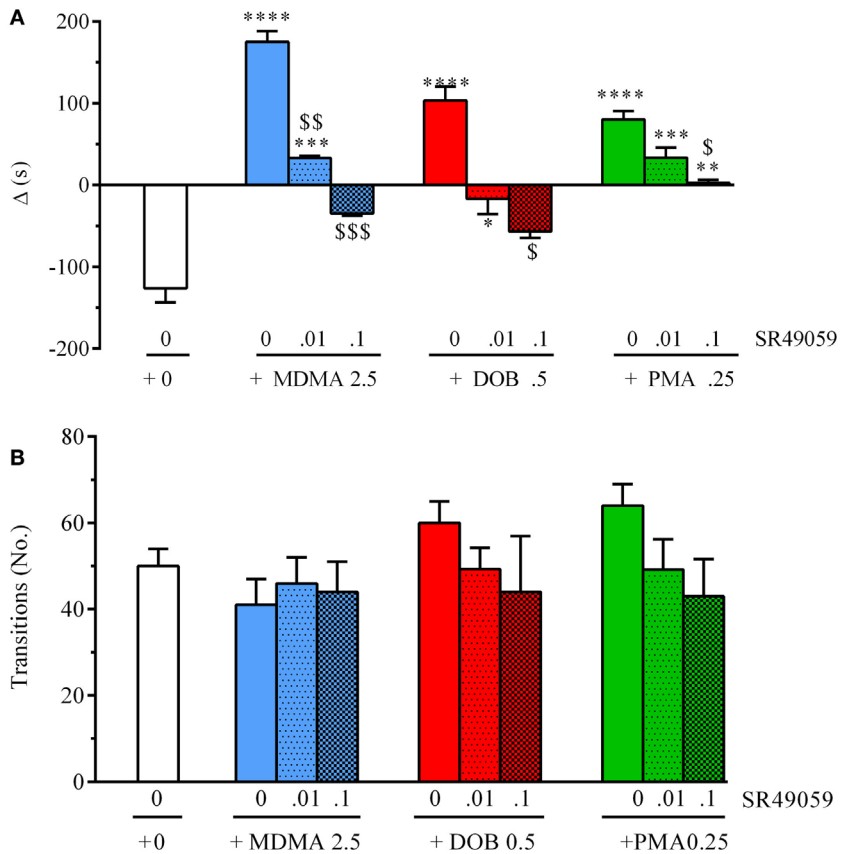

FIGURE 6 | SR49050 dose-dependently blocks the anxiolytic effect induced by 3,4-methylenedioxymethamphetamine (MDMA), 2,5-dimethoxy-4-bromo-amphetamine hydrobromide (DOB), and *para*-methoxyamphetamine (PMA) in the light dark test. Mean values ± SEM of the differences (Δ) in the time spent in the light and dark compartments **(A)** and the number of transitions between them **(B)** during the 5-min sessions. The combination of SR49059 (ng/kg) or vehicle and each drug (mg/kg) was given intramuscularly (IM) immediately before each test. n = 10 fish per group. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs. the corresponding saline group (0 + 0); $^{\$}p$ < 0.05, $^{\$\$}p$ < 0.01, $^{\$\$\$}p$ < 0.001 vs. the corresponding drug alone (Tukey's test).

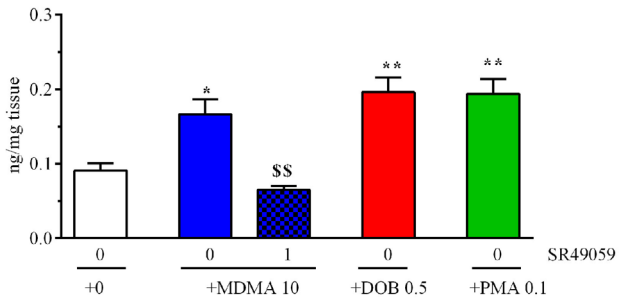

FIGURE 7 | 3,4-Methylenedioxymethamphetamine (MDMA), 2,5-dimethoxy-4-bromo-amphetamine hydrobromide (DOB), or *para*-methoxyamphetamine (PMA) significantly increased cerebral IT levels 5 min after treatment. Mean values ± SEM of three to four samples per group. The combination of SR49059 (1 ng/kg) and MDMA (mg/kg) significantly reduced IT levels. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. the corresponding saline group (0 + 0); $^{\$\$}p < 0.01$ vs. MDMA alone (Tukey's test).

Our findings show that IT/AVT receptors are involved in MDMA-, PMA-, DOB-induced reward in zebra fish, as shown by the reduction in CPP when SR49059 was coadministered with the drugs. Previous studies of the interactions between OT-like systems and the rewarding effects of drugs have found that OT receptor density or mRNA expression change differently depending on the dose (72) and the considered brain area (73–81). The OT antagonist atosiban reduces MDMA-induced drug discrimination in rats, and the OT analog carbetocin partially generalizes to the MDMA training cue (82), thus suggesting that OT receptor activation is a major factor in the subjective effects of MDMA. It is known that OT modulates DA turnover, and it has been shown that OT receptors functionally interact with DA D_2 receptors in the NAc, one of the most important brain areas for reward (83). On the basis of these findings, there is increasing interest in using OT to treat alcohol and nicotine dependence (84, 85). A significant rewarding effect has been observed when a high dose of SR49059 is administered alone. As suggested by Baracz et al. (54) who found that the OT receptor antagonist desGly-NH2,d(CH2)5[D-Tyr2,Thr4] had a similar effect on rats undergoing the CPP test, a tonic level of endogenous neurohyphophyseal hormones could be a contributory factor, but more studies are needed to clarify the mechanism.

Our social preference test findings show that the IT/AVT system also modulates the social behavior of zebra fish as the coadministration of SR49059 with MDMA, DOB, or PMA significantly blocked the increased sociability induced by the drugs alone. Drug abuse is closely associated with social contexts in which OT plays an important role. No data are available concerning PMA and DOB, but the prosocial effects of MDMA have been associated with central OT release in rat and human studies (18). MDMA increases plasma OT levels in human MDMA users at dance parties and in humans given MDMA in placebo-controlled laboratory experiments, and this increase has been related to increased subjective feelings of sociability (57, 86). The increased OT levels have been attributed to the ability of MDMA to release serotonin via hypothalamic 5-HT terminals apposed to OT-containing perikarya (87). The 5-HT_{1A} antagonist WAY 100,635 reduces the increase in plasma OT levels and the adjacent lying behavior of rats induced by MDMA (88). In line with this, the selective OT receptor antagonist L-3668999 abolishes the prosocial effects of MDMA in mice, and quantitative analyses of brain proteome have revealed changes in 21 proteins associated with sociability (89). In addition, the MDMA-induced increase in rodent prosocial behavior is prevented by the same dose of WAY 100,635 and the 5-$HT_{2B/2C}$ receptor antagonist SB 206553 (90), thus suggesting that the prosocial effect of MDMA in rodents may be mediated by OT release as a result of 5-HT_{1A} and 5-$HT_{2B/2C}$ receptor interactions. The involvement of 5-$HT_{2A/2C}$ serotonin receptors in the social preferences of zebra fish has recently been confirmed by the blockade obtained using ritanserin coadministered with MDMA, DOB, or PMA (23). Treatment with the vasopressin V_1a antagonist SR49059 attenuates the increase in adjacent lying elicited in rats by MDMA, OT, and AVP, thus suggesting that a common mechanism mediated by V_1a receptors underlies their prosocial behavioral effects (56). It has also been shown that the ICV administration of OT reverses the social deficits observed in OT receptor knockout mice and that this is also probably due to the functional activity of OT at V_1a receptors (91). It is thought that the prosocial effects of OT are at least partially mediated by its ability to alleviate anxiety. We used both the novel tank diving test and the light–dark test to evaluate emotional-like behavior as previous studies have shown that one test alone does not adequately capture the information necessary to interpret the results (92, 93). In both paradigms, our findings indicate that SR49059 reduced the anxiolytic effects induced by MDMA, DOB, and PMA. The reduced diving and bottom dwelling observed in our tank diving experiments after this treatment is in line with the typical top dwelling responses induced by MDMA dissolved in tank water (40 and 80 mg/L) (44). The possibility that the SR49059-induced blockade of the anxiolytic effects of the drugs was due to altered locomotion can be excluded because the number of transitions from one level to the other by the control and treated zebra fish was similar.

The biochemical evaluation of brain IT levels after MDMA, PMA, and DOB treatment revealed a significant increase in comparison with the vehicle group, when using the highest active dose of each drug: one of the limitations of this study is that IT levels were not evaluated after increasing drug doses. However, it was found that the coadministration of SR49059 significantly blocked IT release, thus supporting the involvement of the IT/AVT system in the drug-elicited effects. Further studies are now needed to evaluate IT release after the combined administration of SR49059 and DOB/PMA, and after treatment with increasing doses of each compound. The only previously published study of brain nonapeptide levels in zebra fish (67) evaluated the relationship between social status and the IT/AVT system in different brain areas, and found that nonapeptide levels rapidly increased after an acute social interaction. Our data concerning MDMA are in line with those of Forsling et al. (94), who found that the presence of MDMA is associated with a dose-dependent increase in OT and AVP release in isolated rat hypothalami.

The mechanisms underlying the MDMA-, DOB-, and PMA-induced increase in brain IT levels, and the SR49059-induced

blockade of this effect of MDMA, are not clear. SR49059 has more affinity for V_{1a} than V_{1b} or V_2 receptors, and a weak affinity for OT receptors (71). Our findings suggest that MDMA (and perhaps also DOB and PMA) indirectly stimulates V_{1a} receptors as a result of serotonin-induced OT/IT and/or AVP/AVT release in the hypothalamus, as previously suggested by Jørgensen et al. (58). It has been reported that 5-HT, serotonin precursors, serotonin releasers, serotonin reuptake inhibitors and serotonin receptor agonists stimulate the release of vasopressin and OT into peripheral blood (58). Furthermore, 5-HT stimulates vasopressin secretion via 5-HT_{2A} and 5-HT_{2C} receptors, whereas 5-HT_{1A} receptors are also involved in OT secretion (58). In zebra fish, 5-HT_{1A}-like, 5-HT_{1B} and 5-HT_2 receptors have been found in homologous regions in the brain, including the hypothalamus (95, 96), where it has been shown that neuropeptides and 5-HT receptors are associated with fear and anxiety (97–99). The decrease of IT operated by SR49059 in combination with MDMA appears difficult to be explained with a simple effect through V_{1a} receptors. It can be argued that SR49059 acts directly to inhibit IT release through 5-HT receptors. Experiments using 5HT receptor antagonists on IT release could better explain the mechanism. In line of our findings, SR49059 partially reversed MDMA-induced increases in adjacent lying, a measure of social behavior in rats (56).

In conclusion, our findings show for the first time that there is an interaction between IT/OT and the rewarding, social, and anxiolytic effects of PMA and DOB in zebra fish. Taken together, they suggest that the IT/OT system may be an important target for the development of new pharmacotherapies for the treatment of the misuse of MDMA and its phenetylamine derivatives and related affective disorders (sociability, anxiety). Although further studies are required to clarify the mechanism underlying the effects of the OT system on drug abuse, the findings of this study support the view that that the zebra fish model is a highly sensitive means of screening new OT-like agonists and antagonists.

ETHICS STATEMENT

Experimental procedures were carried out in accordance with the European Community Council Directive No. 86/609/EEC and the subsequent Italian Law on the Protection of animals used for experimental and other scientific reasons. The experimental protocol was approved by the Italian Governmental Decree No. 18/2013 and 34/2017.

AUTHOR CONTRIBUTIONS

All of the authors meet all of the criteria for authorship and significantly contributed to the research described in this article. MS and DB developed and planned the experiment. LP performed the behavioral experiments. GB performed the HPLC experiments. MS and DB were responsible for all of the statistical analyses. MS and DB wrote the manuscript.

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Would You Use It With a Seal of Approval? Important Attributes of 2,4-Dinitrophenol (2,4-DNP) as a Hypothetical Pharmaceutical Product

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Background: 2,4-Dinitrophenol (2,4-DNP) is an effective but highly dangerous fat burner, not licensed for human consumption. Death cases reported for 2,4-DNP overdose, particularly among young adults, have raised concerns about the ineffective regulatory control, lack of education and risks associated with impurity, and the unknown concentration of 2,4-DNP purchased on the Internet.

Methods: Using a sequential mixed method design and based on a hypothetical scenario as if 2,4-DNP was a licensed pharmaceutical drug, first we conducted a qualitative study to explore what product attributes people consider when buying a weight-loss aid. Focus group interviews with six females and three males (mean age = 21.6 ± 1.8 years) were audiorecorded, transcribed verbatim, and subjected to thematic analysis. Sixteen attributes were identified for the Best–Worst Scale (BWS) in the quantitative survey with 106 participants (64% female, mean age = 27.1 ± 11.9 years), focusing on 2,4-DNP. Demographics, weight satisfaction, and risk for eating disorder data were collected.

Results: In contrast to experienced users such as bodybuilders, our study participants approached 2,4-DNP cautiously. Attributes of 2,4-DNP as a hypothetical weight-loss drug comprised a range of desirable and avoidable features. Of the 16 selected attributes, BWS suggested that long-term side effects were the most and branding was the least important attribute. Effectiveness and short-term side effects were also essential. Those in the >25 year group showed least concerns for legality. Neutral BWS scores for cost, treatment, degree of lifestyle changes required, and specificity required for the hypothetical weight-loss drug to be effective were likely caused by disagreement about their importance among the participants, not indifference.

Conclusion: With advances in research, 2,4-DNP as a pharmaceutical drug in the future for treating neurodegenerative diseases and potentially for weight loss is not inconceivable. Caution is warranted for interpreting the BWS scores. Owing to the difference in what data represent at individual vs. population levels, with pooled data, the method correctly identifies attributes by which most people are satisfied but misrepresents attributes that are individually very important but not universally agreed. Whilst this may be an advantage in marketing applications, it limits the utility of BWS as a research tool.

Keywords: diet pill, fat burner, 2,4-dinitrophenol, DNP, weight loss, bodybuilding, eating disorder

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INTRODUCTION

Due to increased concerns of body weight and image, along with the widespread use of the Internet and social media platforms, the already considerable market of weight-loss drugs and supplements grows rapidly (1, 2). Within this market, various products are available which include substances that could pose health hazards to users. Like any other market where effective regulatory control is lacking (3), the supplement market is also open to unethical practice whereby products could contain unlicensed ingredients (4, 5) contaminated with controlled substances and/or deliberately spiked with potent controlled substances to increase effectiveness [e.g., Ref. (6–12)]. The adulterants in the latter being unconventional and dose set to produce the desired effect, deliberate dietary supplement fraud poses greater health risks than trace contamination from lack of quality control (13). In addition, regulatory effort to curb economically motivated fraud is further challenged by the readily available cross-border retail options on the Internet (14). Drugs that are withdrawn before marketing and thus not licensed for human consumption such as melanotan II or Cardarine (also known as Endurobol or GW-501516) (15–17) and/or reintroduced after being officially withdrawn decades ago [e.g., Ref. (18, 19)] remain available on the black market and easily obtainable *via* the Internet.

Renaissance of 2,4-DNP

One example of substances not licensed for human consumption is 2,4-dinitrophenol (2,4-DNP), which is an effective but highly dangerous fat burner. Currently, 2,4-DNP has industrial use but it is not licensed for human consumption and its sale as such is prohibited around the world. Despite the danger, 2,4-DNP has re-emerged within the bodybuilding community and extreme dieters, particularly among young adults.

History of 2,4-DNP as a Weight-Loss Drug

The attractiveness of 2,4-DNP arises from the fat-burning effects without the need of dietary control (20). 2,4-DNP was used in diet pills for obesity treatment between 1933 and 1938 under brand names of Dinitriso, Nitromet, Dinitrenal, and Alpha Dinitrophenol. Owing to its severe side effects, diet pills containing 2,4-DNP were withdrawn from the market in 1938 (21, 22). Over 100,000 people were prescribed the drug, with claims of increasing metabolism by up to 50% at a harmless dose (21, 22). However, it was disputed whether the drug was as effective and harmless as evidence suggested; alongside DNP's release to the public, warnings of the potential toxicity of the compound were issued by the council on Pharmacy and Chemistry (23). In 1938, there was enough evidence collected to suggest that DNP had potential lethal adverse effects and posed a threat to public health. As a result, 2,4-DNP was subsequently banned by the Federal Food, Drug, and Cosmetic Act (24). Efforts of regulatory bodies to protect the public from harm associated with 2,4-DNP are counterbalanced by the ease of access and availability through Internet retailers (2, 19), and by a plethora of online discussion forums that share experiences among users and readily offer guidance and advice on what and how to use to chemically boost athletic performance or to achieve the desired appearance (25).

Function

2,4-DNP is an organic compound which is chemically manufactured in two forms (see **Figure 1**). The product is the result from the hydrolysis of 1-chloro-2,4-dinitrobenze (26). The compound was initially used to manufacture products such as explosives and dyes. It was later discovered that it behaved as a protonophore in the human body which allowed the movement of protons from the mitochondrial intermembrane space across the inner mitochondrial membrane, acting as a strong uncoupler within oxidative phosphorylation (27).

The outcome of this process causes the breakdown of carbohydrates and fats (21). This allows energy from cellular respiration to be released as heat rather than being stored as adenosine triphosphate, resulting in reduced fat stores and a rise in body temperature (27). This was highly attractive as there was no recorded effect on proteins or nitrogen excretion (21). Past research has proposed the argument that DNP is ineffective, with some evidence suggesting that DNP has little effect on the rate of weight loss at a therapeutic dose in comparison to dietary restriction (28). In some cases, DNP at a therapeutic level only increased metabolic rate by <15% with an outcome of no weight loss, with some patients gaining weight due to increased appetite (29).

Recently, the novel central anti-obesity mechanism of the action of 2,4-DNP has been evidenced which offers new avenues for targeting obesity with 2,4-DNP and other mitochondrial uncoupling agents (30–32). The emerging evidence has suggested that mitochondrial uncoupling proteins (UCPs) have key roles in neuronal plasticity and resistance to metabolic and oxidative stress. UCPs are induced by activities such as caloric restriction and exercise. 2,4-DNP recreates a similar pathway to these activities. The resulting outcome has the potential to protect neurons against dysfunction and degeneration. This may lead to a possible product containing low doses of 2,4-DNP to improve diseases such as Alzheimer's and Parkinson's disease. A major concern would be to certify that the low dose of 2,4-DNP was therapeutic (33). Notably, research in these areas is primarily driven by finding new ways of targeting mitochondrial coupling and uncoupling, in which 2,4-DNP is used as a proof-of-concept drug rather than repurposing 2,4-DNP specifically.

Toxicity

These adverse side effects ranged from short-term nausea, vomiting, and increased pulse rate, to increasingly severe long-term

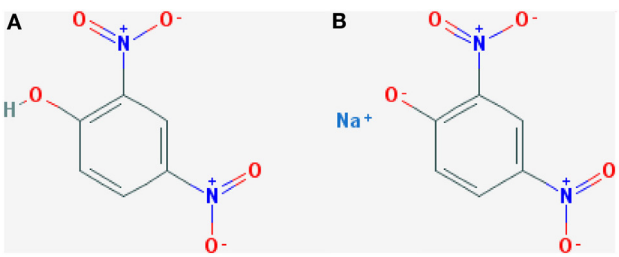

FIGURE 1 | Chemical structure of 2,4-DNP produced in two forms: **(A)** 2,4-Dinitrophenol and **(B)** sodium dinitrophenolate. Taken from Petróczi et al. (19).

effects such as cataracts, hepatotoxicity, and death (34, 35). The issue surrounding DNP is that the adverse side effects may be triggered at various doses, whether considered therapeutic or not. This is because the tolerance to the drug is different for each individual; therefore, doses that may be considered "safe" for some may be lethal to others. There has also been evidence of a person's tolerance altering over time, allowing previously tolerated doses to become lethal (36).

Access

As 2,4-DNP is currently illegal for human consumption, there is concern over those accessing the drug whilst it is unregulated. Although manufacturers have the potential to contaminate or be inconsistent with the purity of their product, increasing its potential to cause harm, research has shown that this is not a major concern (19).

Motivation

2,4-DNP is used knowingly and willingly by obese individuals (37) and those desiring extreme outcomes, such as achieving unnaturally low body fat in bodybuilding (38), compensating for overeating among bulimia sufferers (39) or motivated by a rapid and effective weight loss and used 2,4-DNP as shortcut (19, 40). The drug is often taken in cycles, and information regarding dosage and personal programs is readily made available by bodybuilders for others in the industry to follow (25). Although this is concerning, bodybuilders appear to control the dose and follow a program to reduce the potential lethal effects (19). However, there is increasing concern of usage within vulnerable groups such as those with eating disorders or young and naïve users who are not fully informed about the drug. People's willingness to take the risk with 2,4-DNP—whilst remaining low among the general population—has shown to increase considerably if there is a relatively large amount of weight gain within a short period of time (40).

Regulatory Efforts

Until recently when 2,4-DNP resurfaced in the gym-going population as a potent fat burner and more attention has been drawn to this substance, thanks to numerous media reports of DNP overdose deaths, law enforcement and regulatory bodies were largely unaware that protecting the public by controlling sales and access in the Internet era is an impossible task (19). Because 2,4-DNP—and many similar drugs—is easily available on the uncontrollable global online drug market, efforts of protecting the public are more likely to be effective if preventive and harm-reduction measures target the potential users, not solely focusing on controlling sales and the suppliers.

In fact, having dedicated agencies [FDA in the US, Food Standard Agency (FSA) in the UK, EFSA at the EU level] and regulatory policies in place means that perceived safety could backfire. Consumers of dietary supplements may make their decision in a false sense of safety about the products. In fact, research has shown that a significant proportion of physically active people rely on label information when making decision about supplements but only half were concerned about the quality of the information (41). It is recognized that providing more information does not automatically lead to a greater decision-making autonomy or better behavioral choices (42), nor are people sufficiently health-literate to be able to make the right decision amidst the complexity of evidence for pros and cons (43). Furthermore, preliminary results, with regard to 2,4-dinitrophenol (DNP—used as fat burner weight-loss product), suggest that willingness is inversely related with the severity of the health consequences (40). As such, willingness to take risks with DNP despite health warnings appears to be influenced more by the desired goal (the magnitude of weight people wished to lose) and having past experience with similar products than general risk-taking propensity or other psychosocial factors.

Due to the market increase *via* the internet, cases of 2,4-DNP misuse are being seen globally in countries such as America, England, and China (20, 44). The FSA has had to communicate repeatedly to publicly raise awareness about the potentially lethal and permanent effects of 2,4-DNP, in an attempt to combat the rising health complications and deaths caused by the drug by reducing those interested in the drug, alongside warning the current users.

Alarmingly, the plethora of personal accounts of successful 2,4-DNP use in bodybuilding websites, discussion boards, and forums (25) gives some the untrue impression that 2,4-DNP is suitable for human consumption and may be considered a "safe" product if used "properly." Discounting the idea that 2,4-DNP poses danger because it may be disguised within slimming pills, research has shown that customers buy the product knowingly (19). However, 2,4-DNP poses serious health risks to new or naïve users who are not experienced with performance- and image-enhancing drugs or conscientious of dosage, and lack of knowledge on the extent of potential harmful effects (19). This has contributed to an increasing number of mortalities caused by 2,4-DNP (5, 37, 39, 44–46). It is also important to note that although isolated cases of treatments for 2,4-DNP overdoses have been reported with varying degrees of effectiveness [see (47)], there is to date no established cure or treatment for a 2,4-DNP overdose.

Looking forward, the future of 2,4-DNP as a pharmaceutical drug is not inconceivable. Although the use of 2,4-DNP is controversial, there might be a use for 2,4-DNP for treating extremely overweight or morbidly obese individuals in a clinically controlled environment.

The benefits in entertaining such hypothetical case are twofold: 2,4-DNP is an effective compound for weight loss and its use under a controlled clinical setting would require appropriate understanding of the unique toxicity profile of 2,4-DNP and training for clinical staff. The latter then would carry over to successfully treating accidental overdose and preventing tragic deaths. Media reports and medical case studies fail to portray how 2,4-DNP users feel and think about the drug. With a few exceptions [e.g., Ref. (19, 25, 40, 48)], thought processes and rationalization of 2,4-DNP use have not been adequately captured. Understanding people's motives for taking risk with 2,4-DNP as well as understanding how they negotiate the risks and where they draw the line is vital for devising meaningful prevention and harm-reduction strategies [see Presentation S1 in Supplementary Material for an example of a real-world case study (49)].

AIMS

One concern around the re-emerged 2,4-DNP is that despite official warnings and media reports, 2,4-DNP is continuing to show activity within the weight-loss community and it seems to be increasing in popularity. One plausible approach to mitigate risks against the unintended overdose is to produce 2,4-DNP as a weight-loss drug again to ensure quality control and safety of this—otherwise still dangerous—drug. This controversial proposition could be rationalized on the premise that regulating production and distribution, while still making the drug available, could reduce harm from questionable quality and uncertainty around concentration. Equally, it can be argued that this approach would still be too dangerous, and instead regulatory efforts should focus on improving control over the illegal supply. Regardless of the route taken for regulation, education with harm prevention and reduction in mind is warranted, and the more we understand the motives and barriers behind using 2,4-DNP (and similarly risky substances) the better we can tailor health education to the users' wants, needs, and motives. Often, successful health education for prevention and harm reduction requires taking a holistic approach and addressing the problem in a broader context. In the present study, the key research question is not restricted to 2,4-DNP specifically, but rather, 2,4-DNP is used as a controversial example.

Thus, using a hypothetical scenario, in this study we set out to explore whether producing the drug with proper quality control and advice on safe use would (1) increase willingness to use and (2) reduce harm from 2,4-DNP and investigated what factors people would consider important in buying 2,4-DNP if it would be a licensed pharmaceutical drug.

Alongside this, we also investigated whether demographic details (age, gender, educational level) and health condition (disordered eating) influence the importance of these factors. This will give an indication of who may be at a higher risk of purchasing unsafe weight-loss substances such as 2,4-DNP.

MATERIALS AND METHODS

Based on the exploratory nature of the research questions, a sequential mixed method design (50) was used within the current study. Specifically, the research undertook two phases: first, we conducted two focus group interviews which served as an elicitation for the survey content, followed by a quantitative survey. Focus groups concentrated on the factors young people would consider before buying weight-loss supplements and drugs, such as 2,4-DNP. The results from the elicitation phase contributed toward a self-reported survey which was composed of 31 closed questions and investigated what factors would be considered most or least important in the possible scenario of 2,4-DNP as a licensed weight-loss drug.

Ethical Considerations

The study was approved by the Research Ethics Committee of the Faculty of Science, Computing and Engineering, Kingston University, under the delegated approval scheme. Participation in the study was voluntary and anonymous. Participants were fully informed about the aim of the study and conditions of participation. Consent was implied by voluntary participation in the focus group and/or by completing and returning the survey. Focus group participants also gave written informed consent to the use of their demographic information such as age and gender, purpose for weight loss and past experience with weight-loss products—with anonymity preserved—for scientific purposes and academic dissemination. Consent was obtained prior to the focus group, and participants were asked to complete the "personal information sheet" which contained questions about the above demographic details. Participants received no compensation.

Qualitative Phase: Focus Group Interviews

Participants and Sampling

Following institutional ethical approval, convenience sampling was used to recruit (in person) university students between 18 and 30 years of age *via* personal networks. The focus groups contained six females and three males with a mean age of 21.56 ± 1.71 years. In the focus groups, only two participants had previously used weight-loss substances both of which were female and with their main concern being appearance (see **Table 1**).

The age distribution in the first focus group was slightly more spread (ranging from 20 to 25 years) than in the second group (age range of 19–21 years). Both groups were mixed in terms of gender and involvement in sport and exercise but only the first group included participants with the experience of using a weight-loss product.

Process

Participants were given documents providing a brief background on the topic (participants' prior knowledge of 2,4-DNP was not assessed in the current study), a consent form, and a personal information sheet to gather the demographics of the focus groups (i.e., gender, age, purpose for weight loss, and whether participants had taken weight-loss substances—see **Table 1**). Participants were informed about the purpose of the study, the voluntary nature of participation, and the confidential nature of the focus groups. Focus groups lasted between 30 and 45 minutes were audiorecorded and transcribed verbatim by the first author.

Focus Groups

Semi-structured focus groups were used to collect information on topics surrounding weight-loss drugs and substances in terms of possible benefits, negatives, outcomes and specifically

TABLE 1 | Focus groups demographics.

Focus group	Gender	Age	Purpose for weight loss	Experience with weight-loss substances in the past
Group 1 M1	Male	23	Fitness/sport	No
Group 1 F1	Female	20	Appearance	Yes
Group 1 F2	Female	21	Appearance	Yes
Group 1 F3	Female	25	Appearance	No
Group 1 F4	Female	23	Health	No
Group 2 M1	Male	21	N/A	No
Group 2 M2	Male	21	Fitness/sport	No
Group 2 F1	Female	21	Fitness/sport	No
Group 2 F2	Female	19	Fitness/sport	No

the factors considered when buying weight-loss drugs such as 2,4-DNP. Based on recommendations within the literature (51), each focus group consisted of four to five participants. Participants were asked to consider the scenario of 2,4-DNP as a possible weight-loss drug and what factors they or others may consider before buying the products. The focus group interview matrix, alongside the questions, is presented in Presentation S2 in Supplementary Material.

Data Analysis

Focus group transcripts were analysed using a thematic analysis. Following Braun and Clarke's (52) procedures, transcripts were read and re-read to promote content familiarity. Data were then analysed *via* a process of line-by-line coding to allow themes (i.e., the factors considered when buying weight-loss drugs such as 2,4-DNP) to emerge. Once identified, themes were labelled and grouped together to create higher-order themes. Finally, the data were revisited to ensure that each theme was appropriately represented. Following the second focus group, a satisfactory level of saturation regarding important attributes of 2,4-DNP was reached.

Quantitative Phase: Questionnaire Study

Participants and Recruitment

In line with the qualitative phase, individuals over 18 years of age were recruited for the survey phase using convenience and snowballing sampling techniques. Apart from the age limit of 18 and over, no specific inclusion/exclusion criteria were set for this phase of the research. The questionnaire was made available online using a closed survey platform (SurveyMonkey) and as a hard paper copy. The content of the two surveys was identical. This allowed participants to be recruited online *via* social media and in person.

Measures

Attributes of 2,4-DNP as a Hypothetical Weight-Loss Drug

Desirable attributes of a 2,4-DNP as a hypothetical weight-loss drug were identified using the Best–Worst Scale (BWS) technique which involves choice modelling. In this method, multiple options are provided in several iterations but only the best and the worst option are selected in each case. This method is a multiple-choice extension of the paired comparison method, which is scale-free and forces participants to make a selective choice among the issues under consideration (53). The attractiveness of the method in market research is that respondents are forced to trade off the most desirable features against the "would-be-nice-to-have" attributes, which resembles real-life decision making. For example, when having an ethically produced premium quality product at a low price is not possible, customers must make a choice of which attribute is more important to them (e.g., ethics, quality, or price). Similarly, an ideal weight-loss product would be highly effective but also pleasant and free of side effects but this may not be possible in real life.

In the current study, the BWS was formed around the 16 factors produced from the two focus groups (see **Table 2**). The template design was produced within Datagame (a gamification tool for online surveys, https://datagame.io/) (54) and was set to produce 20 unique sets of four of the 16 factors in each question without repeats. In the questionnaire, each attribute appears five times. In this survey, the BWS was embedded in a hypothetical scenario. The scenario provided a brief background of 2,4-DNP and its current use in society alongside its potential dangers. The hypothetical situation specified that a pharmaceutical company is considering reintroducing 2,4-DNP on the weight-loss drug market and want to explore what customers think about 2,4-DNP using a market survey. Participants are asked to place themselves as a participant in this market research and to consider what factors they felt were most or least important. The scenario and the full BWS survey are presented in Presentation S2 in Supplementary Material.

Eating Behaviour and Disordered Eating

To assess participants' at risk status for disordered eating, six questions from the EAT-26 test [Part C (55)] were used. EAT-26 is an established screening measure (not a diagnostic tool) to determine a possible eating disorder or a person who may be at risk. Section C of the test comprises six questions: (1) *Gone on eating binges where you feel that you may not be able to stop?* (Defined as eating much more than most people would under the same circumstances and feeling that eating is out of control.); (2) *Ever made yourself sick (vomited) to control your weight or shape?* (3) *Ever used laxatives, diet pills, or diuretics (water pills) to control your weight or shape?* (4) *Exercised more than 60 min a day to lose or to control your weight?* (5) *Lost 20 pounds or more in the past 6 months?* (6) *Have you ever been treated for an eating disorder?* The first four questions were rated as Never/Once a month or less/two to three times a month/Once a week/two to six times a week/once a day or more whereas the last two questions were answered as Yes/No. The EAT-26, both the belief section and the behavioural aspects, is one of the most widely used screening tools for identifying high-risk individuals for referral to clinical evaluation, consistently showing good psychometric properties (56, 57). It has been noted that beliefs manifest to a larger extent than behavioural symptoms, suggesting that beliefs are the precursors for developing disordered eating (58, 59). Those who report behavioural symptoms respond to the belief items congruently, but the opposite is not necessarily the case (i.e., beliefs can present without behavioural symptoms).

Satisfaction With Weight

Satisfaction with weight was recorded with three progressive questions. First, participants were asked if they were happy with their current weight (Yes/No) and whether they wanted to lose weight (Yes/No). In case the answer was yes to the weight-loss goal, the main reason behind this goal was further explored. To facilitate statistical analysis, closed question format questions with pre-set answers were used (e.g., Appearance/Health/Fitness/Other, with open text option).

Demographics

Demographic information we collected included age, gender, ethnicity, employment status, and highest completed education level. The categories within the highest completed education level included GCSE, A-Level/B.Tech, Undergraduate level 4, Undergraduate level 5, Degree, Postgraduate, and other. Ethnicity

categories were based on the categories recommended by the Office for National Statistics (60): White, Mixed/multiple ethnic groups, Asian/Asian British, Black/African/Caribbean/Black British, or other. Finally, employment status was split into five categories: unemployed, student, part-time, full-time, or other.

Data Analysis

Following the selection count method [e.g., used in Ref. (61–63)], we used simple count data analysis (i.e., the scoring was based on how many times an attribute is ranked as "most important" and "least important"). Previously, Marley and Louviere (64) showed that this simple calculation is a close and suitable approximation of the true scale values obtainable from multinomial logit analyses. When an attribute is selected as most important, a score of 1 was given, and when an attribute is selected as least important, a score of −1 is given. To obtain the BWS score for each item at the individual level, the difference between "most" and "least" rankings was taken, which resulted in a rank between +5 and −5. The aggregated BWS scores for the 16 attributes for the sample were obtained by calculating the average time that each attribute was mentioned. In this scale, a score of +5 indicates a "very important" attribute and a score of −5 indicates that the attribute is "not important at all." By coding "least" important as a negative value and "most desired" as a positive value allows for calculating not only the preference counts but also to calculate the mean [and standard deviation (SD)] without the need to note whether the counts come from the most or the least preferred choices.

Descriptive data are reported as median, mean and SD, frequencies, and/or percentages. We dichotomised age (18–25 years and >25 years) and at-risk status for eating disorder (i.e., having at least two affirmative answers of the six screening questions). Two affirmative answers were used instead of the traditional "having an affirmative answer" because the relative high proportion of athletes in the sample might routinely control their weight for sport reasons. Comparisons between two groups were tested using a factorial ANOVA. The association between categorical variables was tested using chi-squared statistics with Fisher's exact significance. Statistical significance was set at $p < 0.05$ and tested two-tailed unless specified otherwise. Excel version 2016 for windows and IBM SPSS Statistics 22 were used for data entry and statistical analysis.

RESULTS

Qualitative Phase: Focus Group Interviews

Focus group interviews yielded 16 themes which reflected the characteristics and factors considered when buying weight-loss drugs such as 2,4-DNP. Themes, theme explanations, and supporting evidence are presented in **Table 2**. The themes on drug characteristics were used as attributes for the BWS in the survey.

It is important to note that despite an agreement in relation to the importance of drug characteristics, participants did not always agree on how important each attribute was or the reasons why they felt it was important. For instance, participants agreed that the cost of a drug (such as 2,4-DNP) was important but some participants felt that a high price point would prevent or discourage them from buying it. As one 21-year-old female with experience of using weight-loss substances explained: "*I think the price [of 2,4-DNP] would hinder me, as it would probably cost ridiculous amounts of money, I think that would be the point where I'd be like no I don't want it that bad*" (Focus Group 1—F2). By contrast, other participants felt that the price would not prevent them from buying the drug and that they may actually choose a more expensive option, if they believed it would be more effective. As another 21-year-old female participant stated: "*I know myself if I went to buy a drug and there was one that cost 50p and one that cost £10, I would probably be like the £10 one is more effective*" (Focus Group 2—F1). The following quote captures these contracting views surrounding the importance of cost:

> You get people that are like if it's like £30 cheaper then its not going to make a huge amount of difference and you get people at the other end of the scale who go well it's the most expensive so it must be the best. (Focus Group 1—M1)

In addition to cost, participants also differed in their views regarding the preferred administration (i.e., formulation) and how the drug is taken (i.e., treatment). For instance, some participants felt that there was a stigma associated with taking pills for weight loss: "*I think there's a lot of stigma around taking pills though…like if you're talking to someone and saying you're taking pills for weight loss…their immediate reaction would be like are you sure, where did you get them from, are they legit kind of thing, I probably would go for a shake*" (Focus Group 1—F2), whilst other participants favoured the simplicity and efficiency of pills and favoured this over other formulations. The following quote from a 19-year-old female illustrates this point:

> I don't like putting things in water, it's too much effort and it tastes horrible so as soon as I can take it in a tablet and its just done in a couple of seconds for me that is ideal, it would be things like powder and suppository that would be a massive no! (Focus Group 2—F2)

Building on this point, although the majority of participants preferred taking drugs orally (*via* pills or shakes), some participants felt that injecting drugs were favourable especially if it resulted in a reduced dosage. As one participant explained:

> I would prefer an injection if it was less often… just because I wouldn't have to remember every day, I would be happy to go to my doctor if it was like once a week… especially if you can just go to the nurse and like get it (Focus Group 2—F1)

Quantitative Phase: Questionnaire

The survey sample consisted of 106 individuals (64% female). The mean age of the sample was 27.08 ± 11.92 years with the majority ($n = 81$) being between 18 and 25 years of age. For this reason, age groups were divided as 18–25 and over 25. Sixty-five percent of the participants were students.

Attributes of 2,4-DNP as a Hypothetical Weight-Loss Drug

The relative importance of the 16 attributes for 2,4-DNP as a hypothetical weight-loss drug is depicted in **Figure 2** (depicting

TABLE 2 | Themes, theme explanations, and supporting evidence for the factors considered when buying weight-loss drugs such as 2,4-DNP.

Theme	Theme explanation	Supporting evidence
Accessibility	How easy is it to access the drug (e.g., online, pharmacy, prescription-only, over the counter)	"I think more people would be willing to buy it if it [2,4-DNP] were more readily available but I think for the sake of safety that you should buy it over the counter" (Focus Group 2—F1)
Effectiveness	How well the drug achieves the desired results (i.e., weight loss)	"I think if a drug is effective, and they are really good at marketing that, it would beat all the other factors, I mean you will always find a way to store it, or find a way to take it if it's that important to you" (Focus Group 2—F1)
Degree of lifestyle change required	The extent to which individuals have to change their lifestyle for a drug to be effective (i.e., increasing exercise, water intake, managing diet)	"I definitely would want to do a bit of research, does it say it only works with something else, like do you have to drink a lot of water every day to make sure it works? Do you actually still have to do exercise? Or do you drop the weight by sitting on the sofa still?" (Focus Group 2—M2)
Adherence required	The period of time in which you have to take a drug for and the dose required	"If it's something like weight loss, it's gonna be quite a long time before you really notice a difference so having to remember to take it every 4–5 h would become a bit of a chore" (Focus Group 2—M2)
Dosage	How often a drug needs to be taken (e.g., once a week/several times a day)	"If it's like a tablet, I have to take at a specific time of the day, even like several times a day, I'm not good at that so it would probably be like, maybe if like it was only effective if you like take it exactly at like 5 h intervals, then I would just be like that is never going to happen" (Focus Group 2—F1)
Short-term side effects	Temporary negative effects (e.g., headaches, mild rashes, pain) as a consequence of the drug	"If it's small stuff you could live with like not driving a car, or even a rash as long as it's not painful, people would be more inclined to just do it anyway…whereas if it's something that actively stops you from doing something or causes a lot of pain and discomfort I think that's when people would be like no, it's not worth it" (Focus Group 2—M2)
Long-term side effects	Severe negative effects that become permanent/irreversible (e.g., chronic migraines, blindness) as a consequence of the drug	"I would be on board, if it was very temporary and I would lose weight as a result of it. I could use this drug for x amount of time, but I can't do this, then I'd probably be willing to put my life on the side and lose weight, and then find my life again, but side effects that I would really would be a no for me, would be if I got really ill, or a danger that I become really ill… if I'm going to go blind or something" (Focus Group 2—F1)
Cost	The price of the drug	"I think the price would hinder me, as it [2,4-DNP] would probably cost ridiculous amounts of money. I think that would be at that point where I'd be like no I don't want it that bad" (Focus Group 1—F2)
Formulation	The physical state of the drug (e.g., pill, liquid, powder form)	"I think there's a lot of stigma around taking pills…like if you're talking to someone and saying you're taking pills for weight loss…their immediate reaction would be like are you sure, where did you get them from, are they legit kind of thing, I probably would go for a shake" (Focus Group 1—F2)
Specificity	If the drug targets a specific/localised area of the body or is generalised across the whole body	"Everyone has bits of their body where they have more fat than other parts of their body and if someone takes it and they start to lose weight like on their bum but not their stomach they might not take it anymore so, yeah, it [the drug] needs to target where" (Focus Group 2—M2)
Legality	Whether the use of a drug is within the law or not	"I think young people and athletes are the ones who are gonna buy something illegal… so, if someone just wants to lose a few kilos they're not gonna look into anything illegal, I think they'll just go to the pharmacy" (Focus Group 2—M1)
Reviews and experiences	Other people's opinions and experiences of using a weight-loss drug.	"I have gone on the internet and researched so many things, drug control and stuff like that, and going on forums, other people's experiences versus like science, and stuff like that helps, reading peoples experiences online and checking things, and obviously being careful and reading a tonne of things rather than just one website" (Focus Group 1—F3)
Branding	The extent to which a brand is known or recognisable for certain products	"I think it [branding] is important as not everyone knows a lot about drugs, so you just see brands and go "oh I've heard of that before" it must be better… I'll probably buy whatever is more familiar even though it's more expensive" (Focus Group 2—F2)
Interactions with other substances	If the drug interacts with and impacts on other medications or substances (i.e., stops other medication working)	"[You need to consider] other medications…because they can sometimes have like negative effects when drugs are combining with other drugs" (Focus Group 2—M2)
Treatment	How the drug is taken (e.g., orally, injections, suppositories)	"I don't like putting things in water, it's too much effort and it tastes horrible so as soon as I can take it in a tablet and it's just done in like a couple of seconds, for me that's ideal, it'd be things like powder or suppository that would be like a massive no" (Focus Group 2—F2)
Storage and preparation	How the drug needs to be stored (e.g., in the fridge) and prepared (e.g., needs to be dissolved)	"I'd probably be less inclined to take it, if it [storage and preparation] was complicated, yeah like if there was some sort of complicated process to it" (Focus Group 1—F2)

the average times an attribute was selected as most and least important) and **Table 3** (summarising the outcome of the item count methods). Stratified analyses of the average times an attribute was selected as most and least by age group, gender, and at risk for disordered eating status are shown in **Table 4**.

Based on the survey results, the most important attributes for such a drug were long-term side effects, followed by effectiveness and short-term side effects, with branding, formulation, and route of administration (formulation) being the least important. Drug interactions, user reviews, cost, treatment, lifestyle changes required, drug target specificity, access, and duration of the treatment were placed in the middle region (BWS scores between +1 and −1).

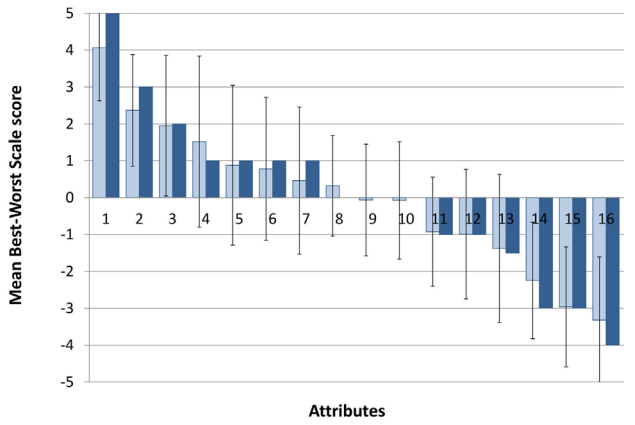

FIGURE 2 | Aggregated Best–Worst Scale scores. Dark blue, median score; light blue, mean score; error bars represent standard deviation. Attributes on the *x*-axis are (1) long-term side effects, (2) effectiveness, (3) short-term side effects, (4) legality, (5) interactions with other substances, (6) reviews and experiences of others, (7) cost, (8) treatment, (9) degree of lifestyle change required, (10) specificity, (11) accessibility, (12) adherence required, (13) dosage, (14) formulation, (15) storage and preparation, and (16) branding.

Table 4 and **Figure 3** offer a more detailed analysis of the BWS choices. Taken together, the results indicated that across both gender age groups, the highest scoring factor was long-term side effects (LT side effects) and the lowest scoring factor was branding. The female group ages 18–25 also rated short-term side effects as important. Both younger age groups and females over 25 selected effectiveness as an important factor and storage and preparation as unimportant. The older age groups and females between 18 and 25 years of age ranked form as unimportant. The older age groups showed a higher concern in terms of legality, in comparison to 18–25 males and females. Statistically significant differences were only found for age effect on accessibility ($p < 0.001$) and legality ($p = 0.029$), and gender effect on branding ($p = 0.026$) and formulation ($p = 0.002$). The latter also showed a significant interaction effect between gender and at-risk status ($p = 0.035$). These are marked in **Table 4**, along with the corresponding test statistics.

In stratified analysis by reasons for weight loss, the data showed a statistically significant difference in ranking dosage and long-term side effects between those who were satisfied with weight and those who were not. Those satisfied with body weight scored dosage ($p = 0.040$) and long-term side effects ($p = 0.019$) with a significantly lower rank in comparison to those unsatisfied with body weight. Alongside this, there was a significant difference in long-term side effect ranks between those who want to lose weight and those that do not. Those wanting to lose weight scored a significantly higher importance ($p = 0.003$) towards long-term side effects than those who do not want to lose weight. The data show that those who selected health as the main reason for weight loss scored effectiveness differently from those who chose appearance ($p = 0.014$) or fitness ($p = 0.012$). Those who chose health for main reason for weight loss scored effectiveness significantly lower than those who chose the appearance or fitness. The only significant result within eating behaviour was those who were not at risk ranked branding significantly lower than those at risk.

TABLE 3 | Attribute Best–Worst Scale counts, interval scale difference scores, and (pseudo-)ratio scale.

Attributes	Number of times ranked 5× as most important	Number of times ranked 5× as least important	Number of times ranked as most important (MI)	Number of times ranked as least important (LI)	Diff MI–LI	Rank (most important to least important)	Ratio $\ln\sqrt{(MI/LI)}$
Accessibility	0	0	22	119	−97	11	−0.84
Adherence	0	1	132	31	−101	12	0.72
Branding	0	34	8	246	−238	14	−1.71
Cost	1	0	109	61	48	7	0.29
Dosage	1	1	30	180	−150	13	−0.90
Drug Specificity	6	0	60	68	−8	10	−0.06
Effectiveness	**0**	**4**	**252**	**6**	**246**	**2**	**1.87**
Form	**3**	**0**	**3**	**355**	**−352**	**16**	**−2.39**
Interactions (with medicines)	10	1	143	50	93	5	0.53
Legality	0	0	201	40	161	4	0.81
Lifestyle change	5	0	58	65	−7	9	−0.06
Long-term (LT) side effects	**0**	**0**	**439**	**2**	**437**	**1**	**2.70**
Reviews and experiences	0	0	132	50	82	6	0.49
Short-term (ST) side effects	5	0	226	21	205	3	1.19
Storage and preparation	**0**	**17**	**3**	**317**	**−314**	**15**	**−2.33**
Treatment	0	0	71	37	34	8	0.33

TABLE 4 | Ranked means Best–Worst Scale attribute scores within 2,4-DNP scenario by age and gender.

Attributes	18–25 years male (n = 31)	18–25 years female (n = 46)	Over 25 years male (n = 7)	Over 25 years female (n = 22)	Not at risk for DE male (n = 29)	Not at risk for DE female (n = 39)	At risk for DE male (n = 9)	At risk for DE female (n = 29)
Accessibility	−1.23[a]	−1.20[a]	0.41[a]	−0.57[a]	−0.32	−0.89	−1.78	−1.00
Adherence	−0.90	−1.02	0.0	−1.36	−0.72	−0.89	−0.78	−1.45
Branding	−2.90[b]	−3.43[b]	−2.57[b]	−3.91[b]	−2.72	−4.03	−3.22	−3.00
Cost	0.61	0.64	−0.43	−0.09	0.97	0.26	−0.11	0.59
Dosage	−1.42	−1.13	−1.0	−1.95	−1.38	−1.56	−1.22	−1.17
Drug Specificity	−0.16	−0.33	−0.14	0.62	−0.07	0.14	−0.44	−0.24
Effectiveness	2.45	2.41	2.00	2.25	2.31	2.21	2.56	2.57
Form	−1.68	−2.63	−2.14	−2.27	−2.07[c]	−2.44[c]	−0.78[c]	−2.62[c]
Interactions with other substances	0.97	1.07	−0.14	0.82	0.72	1.03	0.88	0.93
Legality	0.68[d]	1.61[d]	2.57[d]	2.18[d]	1.00	1.97	1.11	1.55
Lifestyle change	−0.2	0.22	−1.14	−0.14	−0.50	0.11	0.00	0.10
Long-term (LT) side effects	3.68	4.07	4.14	4.59	3.59	4.21	4.33	4.28
Reviews and experiences	0.73	0.83	0.14	0.95	0.71	0.87	0.33	0.86
Short-term (ST) side effects	2.10	1.73	0.71	2.59	1.86	2.03	1.78	2.00
Storage and preparation	−2.74	−2.96	−2.43	−3.45	−2.59	−3.08	−3.00	−3.17
Treatment	0.35	0.43	−0.14	0.18	0.17	0.46	0.56	0.21

Statistical significance (at $p < 0.05$) is marked.
[a]*Main effect: age [F(1,101) = 13.957, p < 0.001].*
[b]*Main effect: gender [F(1,102) = 5.080, p = 0.026].*
[c]*Main effect: gender [F(1,102) = 10.267, p = 0.002]; interaction effect: [F(1,102) = 4.580, p = 0.035].*
[d]*Main effect: age [F(1,102) = 4.883, p = 0.029].*

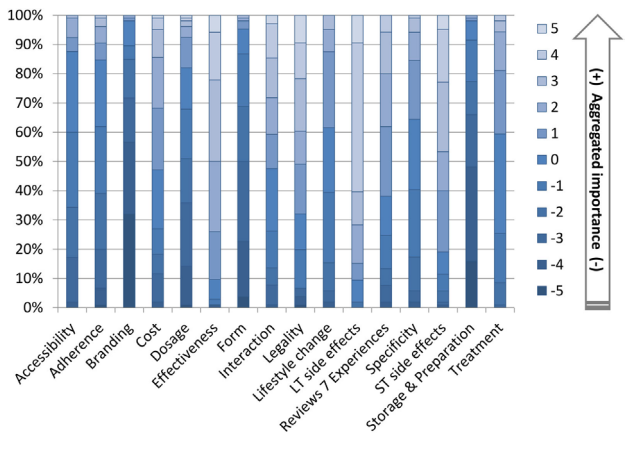

FIGURE 3 | Best–Worst Scale choice counts [n (100%) = 106].

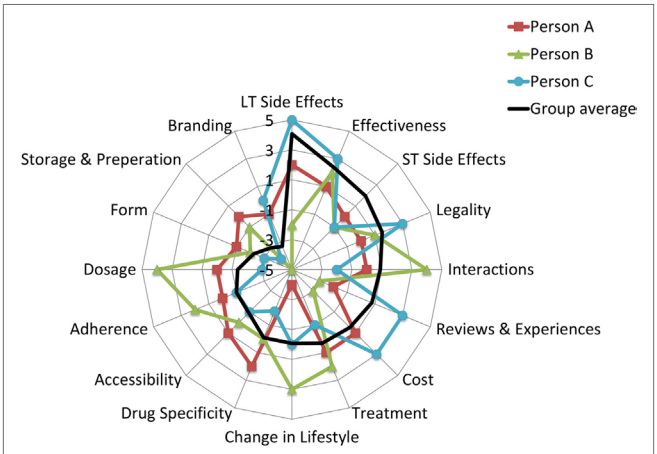

FIGURE 4 | An illustrative example of personal preferences for attributes. Person A: a 21-year-old male who is happy with his current weight and he does not want to lose weight, at risk for disordered eating score is 1/6; Person B is an 18-year-old male who is happy with his current weight and he does not want to lose weight, at risk for disordered eating score is 0/6; Person C is a 20-year-old female, who is not happy with the current weight and she wants to lose weight, at risk for disordered eating score is 4/6. Black line represents the average BWS score for the group.

Juxtaposing qualitative and quantitative data, the overall picture emerges from the detailed analyses suggesting that attributes everyone agreed upon as important or as unimportant scored accordingly on the BWS scale (close to 5 and close to −5) but those attributes where participants in the focus group disagreed scored as "neutral" (neither important or unimportant). Using three participants from the set of 106, **Figure 4** illustrates these notable differences at the individual level.

As the black line (representing the sample average) shows, the attributes were arranged in the order of importance, going from not important (−5) to very important (+5). Overlaying individual scores on the sample average highlight the contrasting views on attributes such as cost, change in lifestyle, and dosage. By contrast, the patterns of scores are fairly consistent across the participants for long-term health effects, effectiveness, and branding.

Eating Behaviour

The 18–25 age group showed a higher risk of developing a potential eating disorder than those aged over 25 years of age. Specifically, within the 18–25 age group, under 10% of males and 20% of females were classified as being a non-risk. By contrast, within the over 25-age group, over 30% were classified as a non-risk.

Participants who selected appearance or fitness for the purpose of weight loss are at a higher risk of a potential eating disorder than those who chose health. These results may show some bias, as only a small number (14% of overall response) of participants were represented within the health category.

Satisfaction With and Intention to Lose Weight

Satisfaction with weight was significantly associated with gender ($\chi^2 = 6.076$, $p = 0.016$) and age ($\chi^2 = 6.114$, $p = 0.017$). Satisfaction with current weight and "at-risk" status for developing eating disorder only reached the level of statistical significance if one-tailed test statistics were considered ($\chi^2 = 3.992$, $p = 0.067/p = 0.036$ one-tailed).

Intention to lose weight showed statistically significant association with gender ($\chi^2 = 9.206$, $p = 0.003$) but not age ($\chi^2 = 1.718$, $p = 0.252$). Similar to the satisfaction with current weight, "at-risk" status for developing an eating disorder only reached the level of statistical significance for the intention to lose weight if one-tailed test statistics were considered ($\chi^2 = 4.402$, $p = 0.053/p = 0.028$ one-tailed).

Using a stratified sample (**Figure 5**), the data indicate a significant dissatisfaction in weight from the over 25 age groups with 68.2% of females (25+ years) being unhappy with current weight and 6 of the 7 males over 25 years felt the same. Age played a significant role in weight satisfaction for males ($\chi^2 = 8.808$, $p = 0.003$). The 18–25 age groups indicated more weight satisfaction in comparison to the over 25 age group. The female 18–25 age group still indicated slight dissatisfaction, with 58.7% being unhappy with body weight, in contrast to 74.2% of males aged 18–25 showing satisfaction of weight. In terms of losing weight, over 70% of males and females over 25 along with females between 18 and 25 years of age expressed desire. By contrast, only 41.9% of males between 18 and 25 years of age wanted to lose weight.

The data showed a substantial difference in weight satisfaction in different ethnic groups. Those identified with white ethnic group were the most dissatisfied with their weight, with 57% being unhappy with their current weight. The mixed/multiple ethnic group showed a balanced response with a 50:50 ratio, whereas both the black/African/Caribbean/Black British and Asian/Asian British groups show less dissatisfaction with weight, with 66.7 (Black) and 77.8% (Asian) being satisfied with their current weight, respectively.

Stratifying the sample by "at-risk" status for disordered eating showed only one statistically significant association. Among males, at-risk status and weight satisfaction were associated ($\chi^2 = 4.508$, $p = 0.037$). Regardless of the at-risk status, 60–65% of the females were dissatisfied with their current weight and 70–75% wanted to lose weight. Over 70% of the at-risk male group was satisfied with their weight but 51.5% still wanted to lose weight.

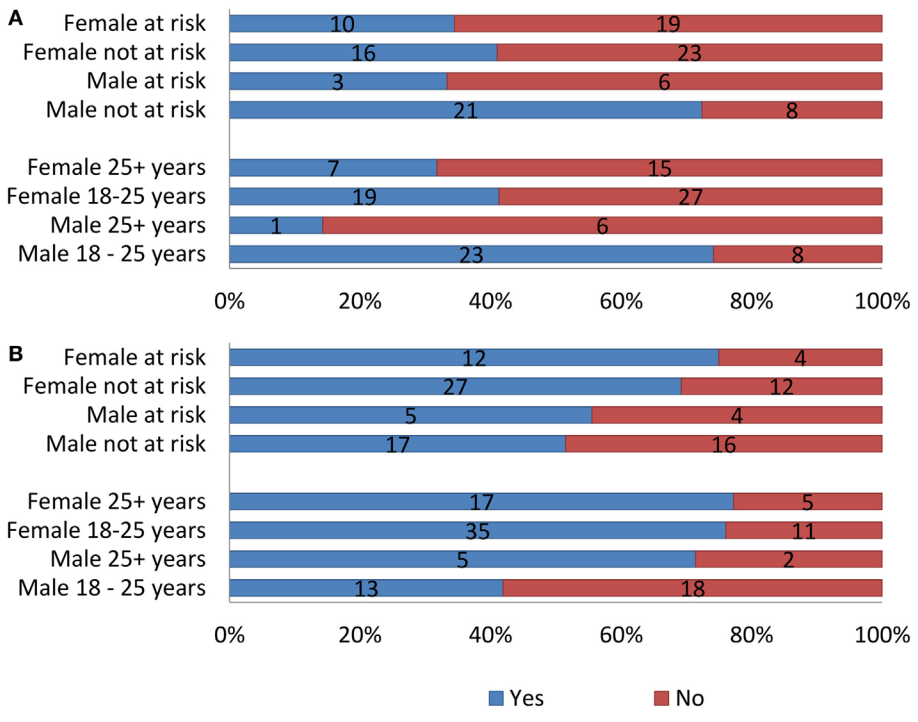

FIGURE 5 | **(A)** Satisfaction with current weight. **(B)** Intention to lose weight in the stratified sample by age, gender, and at risk for developing eating disorder.

Reason for Weight Loss

Age and at-risk status for developing eating disorder were independent of the reasons for weight loss. Gender, however, showed statistically significant association with reasons for weight loss ($\chi^2 = 8.958$, $p = 0.031$), with appearance dominating the reasons for females. Frequency counts in the stratified sample (**Figure 6**) show that the most prevalent reason for weight loss among females over 25 is appearance with 80% choosing this option. In females between 18 and 25 years of age, the results are more varied with appearance coming top (57.1%) followed by fitness (28.6%) and then by health (14.3%). In males aged 18–25, the most common reason for weight loss was fitness and appearance (3/7 both) followed by health (1/7). Males over 25 years of age showed a very similar pattern. Overall, women showed a greater concern towards appearance than men and men showed a greater concern over fitness than women.

Generally, at-risk status for both genders is manifested in similar patterns. Not-at-risk females indicated health reasons as most important for wanting to lose weight as opposed to those at risk who put appearance first. Among males, both "at-risk" and "not-at-risk" groups ranked appearance and fitness as equally important. There was no significant association between age, gender, or at-risk status.

DISCUSSION

2,4-DNP is present in the Internet black market and increasingly gaining attention, not only in the hard core bodybuilding community but also among the members of the general public. This project aimed to look into the factors people may consider before buying a weight-loss drug such as 2,4-DNP. In order to give an indication of who is more susceptible to buy 2,4-DNP and to inform public health policies, we also explored how the importance of these attributes was influenced by personal factors such as age, gender, weight satisfaction, and the desire to lose weight.

The overall results are in line with the previous study using a hypothetical scenario with 2,4-DNP (40). Participants in the study by Hoxha and Petróczi (40) were comparable in terms of age and body consciousness, and the study also evidenced inverse relationship between the severity of the health consequences (side effects) and willingness to take risks with 2,4-DNP as an unlicensed industry chemical. Our study indicated that this relationship is not affected by having a seal of approval for human consumption or quality-controlled pharmaceutical production, or branding. The effect of the drug on the body and lifestyle—both on the desirable and on the avoidable spectrum—appeared to be the most influential factor for considering 2,4-DNP.

Women showed a greater concern towards appearance than men but less concern over fitness. This is in line with the existing literature which indicates that exposure to media images depicting a thin-ideal body type relates to body image concerns within women (65, 66). This may indicate why females are more concerned over body weight in accordance to appearance and explain the increasing number of deaths in young females due to black market slimming pills. Notably, however, 2,4-DNP users are predominantly males (19, 25) which might be due to the fact that 2,4-DNP is considered a controversial drug even within bodybuilding and among those who otherwise use a wide range of performance- and image-enhancing substances (19, 48).

In contrast to females, the male participants showed a greater concern towards fitness, especially within the younger 18–25 age group. There is increasing research indicating men and boys are becoming more concerned with body image and are undergoing more peer pressure to become slender and muscular [e.g., (67)].

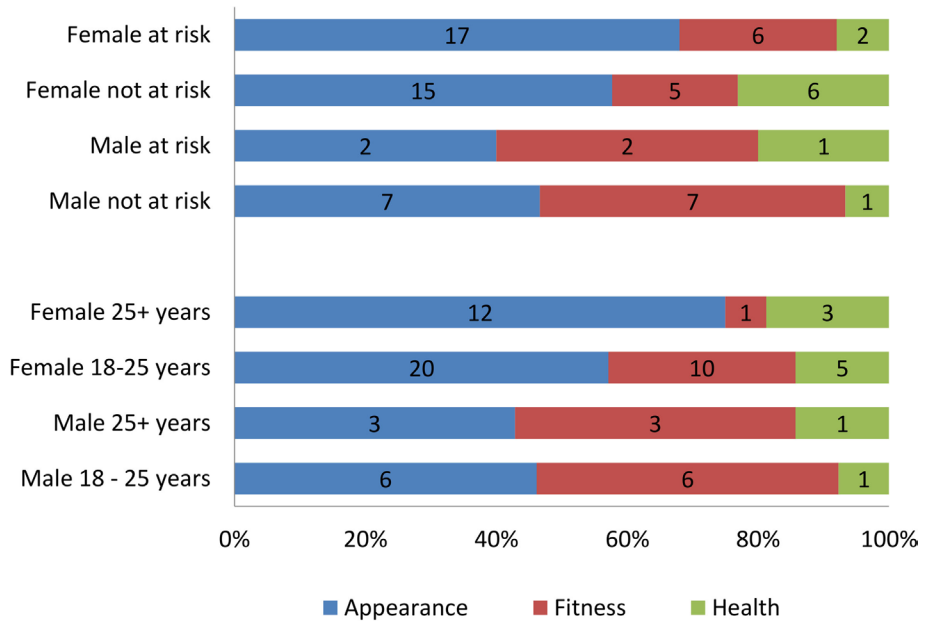

FIGURE 6 | The main reason for wanting to lose weight in the stratified sample by age, gender, and at risk for developing eating disorder.

This may be one reason why 2,4-DNP is prevalent within the bodybuilding community (48).

Notably, the approach to manage the risks with 2,4-DNP differed between the current study and of Hoxha and Petróczi (40), and the studies in which the primary focus was on bodybuilders' lived experiences and rationalisations (19, 25, 48). Contrary to the general views about risks and unpleasant side effects expressed in the present study was avoidance (i.e., not doing it if ….), bodybuilders' approach to 2,4-DNP-related risks was to have control over as many aspects as possible (19, 48). One plausible reason for the observed differences is the use of a situation, which does not translate directly to nor can be interpreted as actual behaviour. The other, more likely, reason is the qualitative difference in the target population. Bodybuilders approached 2,4-DNP from the position that using drugs to achieve the desired body shape is normal but 2,4-DNP is one extreme measure whereas members of the general public showed a conservative approach to using drugs in the first place.

Interestingly, having in-depth knowledge and understanding of the drug, which was pertinent in all bodybuilding-focused studies (19, 25, 48) did not feature among the attributes of 2,4-DNP as a hypothetical pharmaceutical drug although some reference was made to the mode of action in the focus groups.

Attributes

The most common factors considered before buying weight-loss products were accessibility, reviews, experiences, adherence (course), treatment, short-term side effects, long-term side effects, effectiveness, storage, preparation, dosage, change in lifestyle, cost, interactions with other substances, drug specificity, legality, branding, and form of drug.

The highest and lowest ranking factors across the different age and gender groups were long-term side effects and branding. The long-term side effects were expected to receive a high mark of importance as people tend to avoid harm especially with the severity and length of effects being unknown. Both young (male and female) age groups and females over 25 years of age selected effectiveness as a very important factor, indicating that these groups may have a higher interest with weight loss.

The older age groups showed a higher concern in terms of legality in comparison to those who are younger. These findings suggest that younger people may be more willing to take risks to achieve their desired physique and as a result are more likely to buy illegal weight-loss drugs. This may go some way to explaining why the majority of mortalities due to 2,4-DNP are among young people (44).

Those wanting to lose weight scored significantly higher in terms of importance regarding long-term side effects than those who did not want to lose weight. Interestingly, this potentially contradicts other research as it may be assumed that those wanting to lose weight would have greater-risk willingness, so would see a reduced concern towards potential hazards. However, this potentially could be because those who wanted to lose weight related more to DNP scenarios, than to those who did not want to lose weight and may have given a more realistic consideration to the potential harms of weight-loss drugs.

Those who chose health as the main reason for weight loss scored effectiveness significantly lower. This might be because losing a significant amount of weight in a short period can be considered unhealthy. Therefore, those who are health conscious wouldn't be as concerned with how effective the drug was. Also, it may be assumed that those choosing health over appearance and fitness are less likely to purchase a weight-loss product due to the potential hazards and so wouldn't consider effectiveness as important as side effects.

Judging from the quantitative BWS scoring alone, it would appear that some attributes (e.g., cost, treatment, degree of lifestyle changes required, and specificity of the hypothetical weight-loss drug) are neither important nor unimportant when in fact any one of these factors alone would stop a person taking 2,4-DNP. The rationale for using BWS instead of scaled responses was to capture the relative importance of attributes if a risky substance such as 2,4-DNP would be manufactured and sold as a pharmaceutical product, and indirectly to shed the light on what aspects are the most important to potential consumers of 2,4-DNP. The latter would help to address public health concerns about 2,4-DNP and devise effective preventive and/or harm-reduction strategies.

Best–Worst Scale method (also known as maximum difference scaling) originates from consumer research exploring relative preferences. The BWS model is a multichoice extension of the scale-free paired comparison where respondents are not asked to assess the absolute importance of an issue on some arbitrary scale but presented as a trade-off choice (i.e., would you rather have option A or option B). Because of this characteristic, BWS is thought to resemble the actual cognitive process by which consumers make product choices (68). In subsequent applications, outside marketing showed that the BWS method adequately captures abstract values and value systems (61, 69–71) and is suitable to assess a wide range of issues such as health care (72, 73), education (74, 75), and sport (71, 76).

Since its conception more than 25 years ago, limitations of a direct preference assessment with BWS have started to emerge [e.g., 77, 78], showing a clear discrepancy between consumers' declared relative importance of an attribute such as packaging or calorie information and the attributes' actual influence on purchasing. This limitation, however, is not linked to the BWS but rather caused by the discrepancy between declared preferences and behaviour, the latter being influences by a host of other—temporary—factors. The issue our results highlighted is different and likely caused by the information process between individual vs. aggregated levels. BWS is thought to model the thought process of a single individual; thus, BWS score is reflective of what this person explicitly expresses for preference. When the data are aggregated across the sample to represent the population, individual differences are lost in the process because extreme polar views on the same attribute cancel each other out. The result of this process is a strong agreement about attributes that all individuals believe are important and attributes that are considered less important. Attributes that are in fact critically important but without agreement in how these attributes should manifest (e.g., formulation and route of administration of a drug in our study, or the level of lifestyle changes required to make the drug work) falsely manifest as neither important nor unimportant. From the practical point of view, BWS results could be quite

useful for marketing purposes because it makes sense to provide features in a product that all individuals deem important and avoid those that are considered less important. However, this is a serious limitation for applying BWS as a research tool because the richness of the data is lost in translation from the individuals to the group. Similar caution has been made by Krucien et al. (79) finding that, in comparison with the discrete choice experiment (DCE) method, BWS method yields lower quality data for developing Health Utility Indices. Based on a systematic review, Whitty and Oliveira Gonçalves (80) suggest that profile-case BWS and DCE are equally robust measures but they might tap into different constructs. Multiprofile BWS might be concordant with DCE outcomes but this observation was based on a single study, thus requiring further verification. Unfortunately, these observations are not directly applicable to our present study because we used object-case BWS (81, 82). Object-case BWS, while successfully addressing some concerns associated with rating scales having ties (i.e., everything very important and socially desirable responding), BWS lacks accuracy and discriminatory power to compare respondents with differing preferences (81, 82). Furthermore, it must be noted that BWS scores alone do not offer any insight into how important the entire choice scenario to the respondent and how important each attribute is (81, 82). The combination of our quantitative and qualitative results offers support to this observation. In our study, we partially addressed the first aspect by including and analysing BWS in the context of weight satisfaction, weight-loss goals, and disordered eating. To address the latter aspect, an additional ranking scale is required prompting respondents to not only rank attributes in a forced choice setting but also evaluate the importance of each of them. Including this additional information could address the issue we highlighted, namely the misrepresentation of some attributes as "neutral" by including weighting to each attribute. Future studies using BWS for research are also recommended to incorporate methods that afford individual-level analyses. Triangulating the individual and aggregated BWS results with qualitative and/or behavioural data could provide useful insights into the method and facilitate further development.

Limitations

The questionnaire consisted of 31 questions, with some sections such as the DNP scenario and BWS containing a considerable amount of information resulting in a lengthy survey. It was mentioned by a few participants that the survey was too long, with at least 26 participants taking over 10 min to complete the survey. This may have led to some questions being answered superficially, reducing the accuracy of the results.

The majority of participants were young females which inadvertently led to females aged between 18 and 25 years to be over-represented within the data. Given that females are more conscious about weight, talk more about weights (83), and more likely to use weight-management clinics and weight-loss products (84), this characteristic of the sample may not be a limitation but a true reflection of the weight-loss product market.

Lastly, the eating behaviour scale showed some discrepancies, as the tool defines a person at risk of an eating disorder with a score of 1 or higher. However, the survey collected information from a high proportion of young people, mainly students. In this population, many are highly active within sport and require high levels of exercise to control weight and remain competitive. Therefore, many athletes scored at least a 1 due to controlling their weight by exercise. Alongside these, many athletes may experience eating binges more frequently due to this high activity, in which their body needs to quickly replace depleted energy stores.

CONCLUSION

Due to the range of side effects, which vary in severity depending on a person's tolerance, 2,4-DNP has remained illegal for human consumption since its ban by the FDA in 1938. Facilitated by easy access to the substance *via* the Internet, legislation cannot curb its use by the general public which raises public health concerns. Despite numerous warnings to make the public aware of the dangers from using 2,4-DNP, the drug is still showing activity within the weight-loss community.

With advance in research, 2,4-DNP as a licensed pharmaceutical drug in the future for treating neurodegenerative diseases with chronic micro-dosing and potentially for aiding weight loss is not inconceivable. However, owing to the media reports of deaths and irresponsible marketing, supply, and use, 2,4-DNP has a reputation of being very risky and rightly so. Participants in this study exhibited a reassuringly cautious and conservative approach to a risky drug like 2,4-DNP. Focusing on young adults, we showed that those most interested in weight loss are females predominantly 18–25 years of age and indicated that both males and females under 25 years exhibited a higher risk for disordered eating. Due to the rising body pressure effects on these age groups and with a reduced concern towards legality, this group of young people are at risk of becoming susceptible to different weight-loss products, including 2,4-DNP. Vast differences in social group norms (e.g., bodybuilders, athletes) around using pharmaceutical aids to weight loss were noted. There is little doubt that the market for such products exists and current control policies are inadequate; thus there is a need for finding new ways for prevention and harm reduction. Failing to control the risk through supply and access, public health policies should consider pragmatic solutions for controlling 2,4-DNP-related harm *via* education as well as research into the possibility of making 2,4-DNP a safer drug by controlling purity and quality as well as efforts to mitigate against side effects.

However, our results expand beyond 2,4-DNP and speak for young adults' approach to using pharmaceutical products for achieving a "desired" body, which provide useful insights for public health policies. Caution is warranted for interpreting the BWS scores. Our combined qualitative and quantitative results showed that the BWS method is capable of correctly identifying attributes most people feel the same way but misrepresents attributes that are individually very important but not agreed upon as unimportant or insignificant. This feature of the BWS method is very suitable for marketing purposes but outcomes should be interpreted cautiously in research applications.

ETHICS STATEMENT

The study was approved by the Research Ethics Committee of the Faculty of Science, Computing and Engineering, Kingston University, under the delegated approval scheme. Participation in the study was voluntary and anonymous. Participants were fully informed about the aim of the study and conditions of participation. Consent was implied by voluntary participation in the focus group and/or by completing and returning the survey.

AUTHOR CONTRIBUTIONS

AP conceived the study and developed the research protocol with EB. EB collected data and analyzed the data with AP and ST. All authors contributed equally to drafting the manuscript and have read and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fpsyt.2018.00124/full#supplementary-material.

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ORIGINAL RESEARCH
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No Laughing Matter: Presence, Consumption Trends, Drug Awareness, and Perceptions of "Hippy Crack" (Nitrous Oxide) among Young Adults in England

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In clinical settings, nitrous oxide gas is a safe anesthetic used during childbirth, in dentistry, and to relieve anxiety in emergencies. Colloquially known as "hippy crack"' or "laughing gas," it is increasingly taken recreationally for its euphoric and relaxing effects and hallucinogenic properties. Using a self-reported survey, we gathered quantitative and qualitative information on users and non-users of hippy crack among a young population regarding: consumption patterns, knowledge, risk awareness and intentions toward future abuse. Quantitative responses from a total of 140 participants were analyzed for frequencies and relationships, whereas qualitative data were evaluated *via* identifying the reoccurring themes. Overall, 77.1% (n = 108) had heard of hippy crack and 27.9% (n = 39) admitted to past-year use. Prior users mostly indicated intended future use, had an average low number of past-year uses but some with > 20 occasions, had a varied number of inhalations per occasion (often 1–10) with an effect lasting up to 5 min, and a majority preferred social rather than lone use. For non-users, 79.2% said they would take hippy crack with the vast majority (94%) preferring a social setting. The results show a concerning gap between available evidence and awareness of side effects. Despite serious reported side effects, including psychosis and myeloneuropathy—especially on the young developing brain—only a minority (29.3%) was aware of any side effects. In contrast, in a hypothetical scenario depicting a first social encounter with hippy crack, the qualitative responses were in contrast to qualitative outcomes revealing that participants would try (n = 30)/not try (n = 25) it, would feel under pressure to try it (n = 6) with only 11 opting to exit the situation. In summary, this first report of trends and perceptions of the use of hippy crack among young adults in the England highlights a lack of concern with side effects, coupled to a willingness to partake. Because typical users are young with risks to the still developing brain, education about the nitrous oxide abuse is warranted to prevent impaired brain development. Further studies to investigate the possible effects of nitrous oxide on the developing brain in young adults would advance meaningful prevention.

Keywords: hippy crack, nitrous oxide, laughing gas, prevalence, legal high, novel psychoactive substances, harm-reduction

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INTRODUCTION

Hippy crack or laughing gas (nitrous oxide, N_2O) is used clinically as a safe anesthetic, allowing pain relief during childbirth, in dentistry, and to relieve anxiety in emergencies (1, 2). Nitrous oxide in gaseous form, once inhaled, dissolves in the bloodstream, reaching the brain within seconds. Being more water soluble than oxygen, it will diffuse more rapidly across the alveolar basement membrane, resulting in rapid entry into the bloodstream causing dilution of the volume of the gaseous contents (e.g., oxygen) of the alveolus (3, 4). In consequence, diminished oxygen levels (hypoxia) *via* the decrease in alveolar oxygen tension can subsequently decrease oxygen delivery to the brain. A laboratory simulation confirmed that nitrous oxide displaces oxygen in a closed space leading to asphyxia (5), which occurs when inadequate amounts of oxygen are supplied to the tissues and organs.

Furthermore, studies involving the use of nitrous oxide show it causes vitamin B12 (cobalamin) deficiency (6). Nitrous oxide irreversibly binds to the cobalt ion within vitamin B12, causing the inactivation (2). Vitamin B12 deficiency has been correlated with demyelination of nerve cells (7). In addition to nitrous oxide-mediated consequences of hypoxia and vitamin B12 deficiency, hippy crack leads to an increase in homocysteine, a *N*-methyl-D-aspartame agonist associated with oxidative stress and mitochondrial disruption *via* intracellular calcium release (2, 8). Abuse of hippy crack has been reported to cause fatalities (5, 9–12), and—in association with low or low-normal levels of vitamin B12—to psychiatric effects such as psychosis, peripheral neuropathy and other medical effects relating to blood flow (13–18). Extreme case reports have involved subacute combined spinal cord degeneration and ataxia following nitrous oxide abuse (19–21).

Brain development from age 18 to 25 years specifically involves the rewiring-process within the prefrontal-cortex, which develops last (22). The prefrontal-cortex role involves, obtaining information from all of the senses and coordinating thoughts and actions, giving an individual the ability to utilize good judgment when presented with difficult life situations *via* abstract thought (22). The rewiring-process involves dendritic pruning, cutting and eradicating unused synapses (23) and myelination (22), involving the accumulation of the myelin sheath, the myelin-forming cells (Schwann cells), increasing the speed, and propagation of impulse conduction across the brain (23). Vitamin B12 deficiency—a result of repeated nitrous oxide intake—will likely impair these processes through multiple effects especially defective myelination.

However, hippy crack remains popular among young people and—with ease of accessibility—is increasingly being taken recreationally (2). According to the Global Drug Survey (24), 91% of users used once or less per month and 64% of users take of up to five balloons per session (25). The following year, 4% of users reported symptoms consistent with nerve damage (24). Serious short-term reversible effects have been reported with case reports describing use of up to 100 cartridges per day over several months (26). In the United Kingdom, legislation has been introduced to ban psychoactive substances and to prevent supply of these substances for human consumption. Nitrous oxide was specifically included in the "Guidance to Retailers" accompanying the Psychoactive Substances Act (27) along with other legal highs/novel psychoactive substances. A recent report reveals nitrous oxide is the seventh most popular drug worldwide and there is increasing use of nitrous oxide in the UK with a 38.6% lifetime prevalence by 2014 (24, 28). However, no in depth study has looked into the effects on the developing brain from 18 to 25, an age group possibly more likely to recreationally consume this drug. The overuse of hippy crack may slow down if not reverse the progress of the developing brain *via* effects on vitamin B 12. What remains unknown is how much is too much and how this level compares to the typical recurrent use less than monthly and up to five balloons per occasion.

Thus, it is timely to explore how popular hippy crack is among young people (18–25 years), as well as to decipher the consumption pattern of the drug taken recreationally, in addition to measuring people's actual knowledge of the drug and what it does. Using self-reported surveys, we aimed to gather quantitative and qualitative information on users and non-users of hippy crack among a young population regarding: consumption patterns, knowledge, risk awareness, and intentions toward future abuse.

MATERIALS AND METHODS

A survey approach was used to explore how aware young people are of the harmful effects of the drug itself. Furthermore, exploring young peoples' susceptibility to using the drug as to whether it is a case of simple enjoyment or whether recreational use is more popular as a social activity, bringing light to how people are likely to behave in a given situation. In addition, we employed a qualitative approach using a hypothetical scenario depicting a plausible first social setting encounter to investigate attitudes toward potential first use.

Participants

Data were collected from 140 participants in south west London during January and February 2017. Participants were recruited randomly using a combination of hard copy or electronic copy *via* email ($n = 130$) within Kingston University and online *via* survey monkey ($n = 10$). The content of the surveys was identical (details of the full survey appear in Annex 1). Participants were in the age range of 18–25 years with 94 females, 40 males, and 6 not declaring gender. The study was approved by the delegated approval scheme of the Faculty Research Ethics Committee of the Faculty of Science, Engineering and Computing, Kingston University.

Measures

The five primary outcome measures include: presence, knowledge of the drug, hippy crack intake, behavior, and risk. A range of secondary outcome measures were also assessed to help determine the consumption pattern of hippy crack for 18–25 year olds. Response options in closed questions ranged from 1 to 10 and 1 to 5 (on various answers capturing willingness, likelihood, agreement and risk perception), with some only offering dichotomous response options (yes and no). In addition, a hypothetical scenario was used to assess participants approach to trying hippy

crack to allow participants to mentally put themselves in a situation in which they are presented with hippy crack. The quantitative scenario question was complemented with an open answer option, which prompted participants to elaborate on their scaled quantitative response. The full survey is detailed in Annex 1.

Scenario-Based First Encounter

A social encounter with encouragement to take hippy crack based on a scenario was used to elicit attitudes toward potential use. A gender-neutral name (Alex) was used to avoid gender bias and allow respondents from both genders to identify with the protagonist. The scenario question also explores perceptions of risk (Annex 1).

Data Analysis

For quantitative data, descriptive statistics was used to obtain mean, highest and the lowest value and the SD. Chi square test was used to test relationship between categorical responses (e.g., between two groups/variables such as gender and whether they have taken hippy crack). IBM SPSS statistics version 23 was utilized. The open question was analyzed manually using thematic analysis to identify repeated words/phrases ("peer-pressure," "knowledge," and "risk") and potentially emerging new themes between individuals in addition to observing frequency of the reoccurrence of that phrase.

RESULTS

Quantitative Survey

The majority of participants were female (67.1%, $n = 94$) with gender not declared for 4.3% ($n = 6$), with ages between 18 and 25 years (mean age of 21 ± 1.70), and 94.6% were students ($n = 135$). The majority (77.1%, $n = 108$) had heard of the drug hippy crack and 27.9% ($n = 39$) had taken hippy crack in the past 12 months. Hippy crack consumption was more popular among males at 39.0% ($n = 16$) compared to females at 24.7% ($n = 23$) but did not reach statistical significance [χ^2 (2) = 5.31, $p = 0.07$]. Non-users in the past year comprised of 75.3% ($n = 70$) females and 61.0% ($n = 25$) males, with participants over the age of 20 being more likely to take hippy crack. The group aged 18 ($n = 9$) had no report of taking hippy crack. There was no statistically significant association between age and consumption of hippy crack [χ^2 (7) = 9.68, $p = 0.208$].

Outcomes: Hippy Crack Users

Of the 39 participants reporting previous experience of using hippy crack, the majority ($n = 27$) took it on more than one occasion during the past year. Notably, only 7 users had taken it at or exceeding 10 occasions reinforcing the majority of respondents being light users in line with previous reports (6) (**Figure 1**). The amount taken on each occasion varied with 46.2% ($n = 18$) partaking once or twice in one sitting but for the majority this extends to ≥3 intakes and even greater than 20 intakes (**Figure 2**). Furthermore, users are more inclined to take hippy crack among friends, 97.43% ($n = 38$) than on their own. Most users (87.2%, $n = 34$) reported an effect after one or two intakes that lasts for up to 1 min (**Figure 3**). Most users reported the ease of acquisition of hippy crack (57.15%, $n = 28$). Many past users, indicated that they would take hippy crack again in the next 3 months (scale = 1 absolutely not, 5 definitely, average 3.38, most common = 5). Those over the age of 20 were more likely to reuse hippy crack, with participants aged 21 years being most likely to retake hippy crack (46.7%, $n = 7$). A statistically significant association was found between age and likelihood to retake hippy crack [χ^2 (24) = 79.442, $p = 0.001$].

Outcomes: Non-Users of Hippy Crack

For non-users ($n = 101$) of hippy crack in the past 12 months, the likelihood of trying hippy crack in the next 3 months was very probable (79.2%, $n = 80$), with the mean scale value being 4.49 ± 1.11 (where 1 means very unlikely and 5 means very likely). Of the non-users, the majority (76.2%, $n = 77$), would have hippy crack one or two times, with a minority (4.0%), indicating using hippy crack 5–10 times with no one

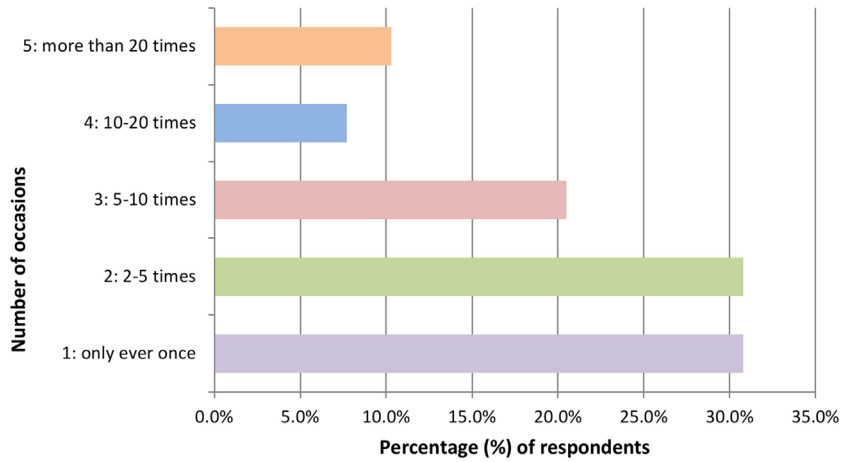

FIGURE 1 | Number of occasions hippy crack was taken in the past year ($n = 39$ responses).

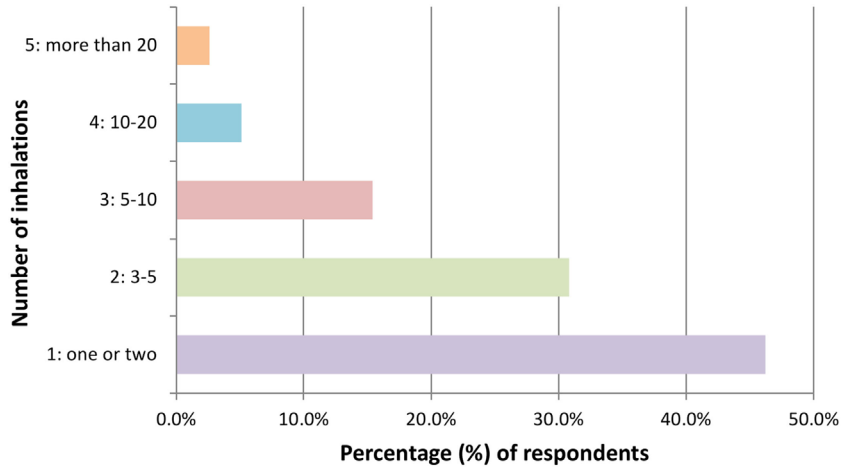

FIGURE 2 | Quantities (inhalations) of hippy crack inhaled in one sitting ($n = 39$ responses).

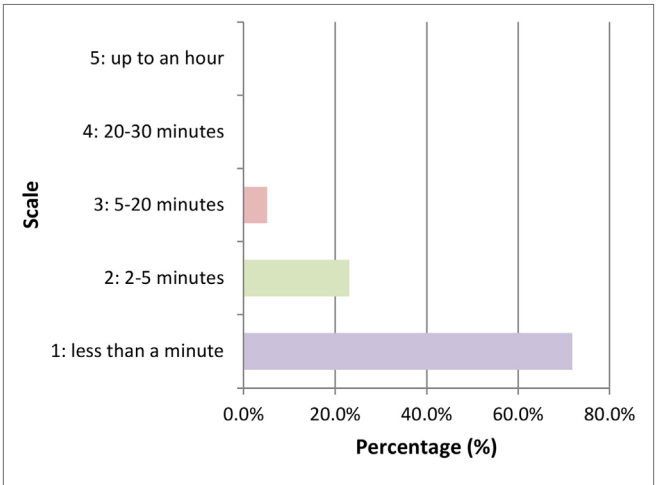

FIGURE 3 | Duration of the effect after inhalation of hippy crack ($n = 39$ responses).

considering over 10 times (**Figure 4**). Many non-users also reported perceived ease of supply with 37.62% ($n = 38$) reporting it as "very easy." In addition the majority (93.9%, $n = 92$) would be more comfortable taking hippy crack among friends than on their own.

Harm Awareness and Reduction

Perceptions of the level of harmful effects of hippy crack varied widely with a full scale response between 1 (not harmful at all) and 10 (extremely harmful) with the mean scale value being 5.31 ± 3.01 (**Figure 5**). Additionally, the majority (91.6%, $n = 99$) of those who had heard of hippy crack were not aware of any side effects associated with hippy crack use. Views regarding how much hippy crack should be consumed in one setting were diverse with a full range from 1 to 10 (the maximum given) (**Figure 6**).

A clear message was received regarding the importance of teaching young people about the harmful effects of hippy crack. The majority (60%, $n = 86$), chose the scale value of 10 (extremely important) as to how important it is to educate young people about the effects of hippy crack, with the mean scale value of 8.43 ± 2.47 emphasizing a willingness to learn about the risks.

Qualitative Scenario Setting

The scenario setting extension to the self-report was designed to encompass wider views based on a plausible first encounter with hippy crack. The approach taken was to elicit attitudes toward the offer of hippy crack, along with awareness of risks of consumption in confined spaces. Themes emerging, from the qualitative responses to the scenario question are shown in **Table 1**, which is further split into content subgroups and frequency, showing how often content is repeated.

In-depth analysis of the qualitative responses revealed three overarching themes: intention to use hippy crack, knowledge and acting on knowledge, and susceptibility to risks.

Taking Hippy Crack

Those who have not taken hippy crack and answered no, were actually more likely to have taken hippy crack if offered, the word "*try*" and "*experience*" were most common ("*try it just for experience*") in responses, which could be due to lack of knowledge, therefore, less skeptical/less cautious and more likely to try hippy crack, even if it's just due to curiosity ("*go for it man has nothing to lose, it's not the things we have done in life that we think about when we die, it's the things we didn't do that haunts us.*"). A few participants stated if effects were euphoric enough chances of use may increase ("*depends on if the affect was deemed worthy enough*"), moreover, this could lead to a higher consumption pattern.

Some people stated that they would try just the one or at the very least a small amount ("*Try maybe half a balloon just to be sure*"). However, taking even a small amount may lead to them taking more once under the influence, still leaving them at risk, so they are still susceptible to harm. Furthermore, for those willing to take in the car with windows down, it is more likely, they

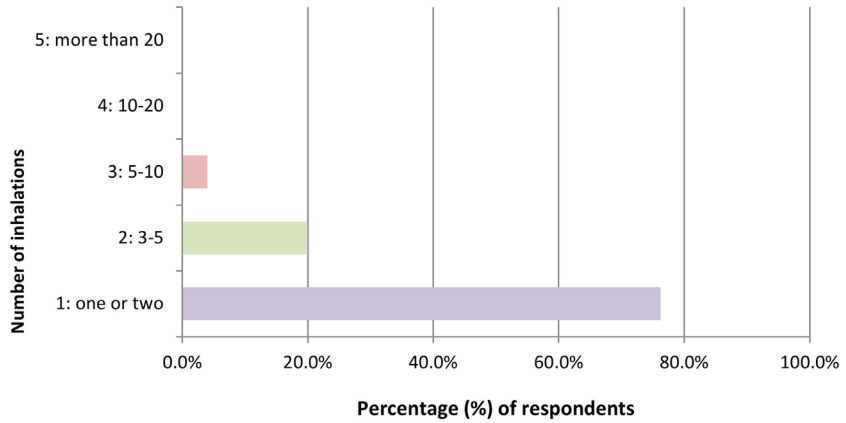

FIGURE 4 | Number of inhalations of hippy crack that would be taken in one sitting if one has never taken hippy crack (n = 101 responses).

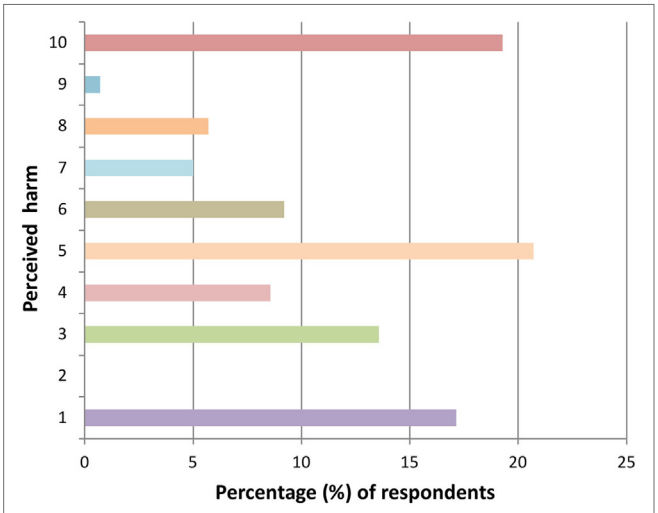

FIGURE 5 | Perceived harm from using hippy crack (10 = extremely harmful) (n = 140 responses).

could be persuaded to inhale hippy crack with the car windows up once under the effects of the drug, as judgment may be altered, momentarily, friends may close the windows in a fit of laughter thus leaving them more susceptible to danger.

As the scenario question is set as Alex's first encounter—some responses were more cautious, as to whether they would take hippy crack as it would be their first try ("*Sounds too much for the first try*"). However, if one was more experienced/taken hippy crack before, is it more likely that they would have taken hippy crack once offered, perhaps due to feeling less hesitant as according to results. There are some participants that stated that they would still go along with them/sit in the car, even though they would be releasing nitrous-oxide into the car (a closed space), consequently they could still be susceptible to harmful effects of hippy crack, moreover them being cautious, still leaves them vulnerable ("*wouldn't take it but go along with them bearing in mind that it could cause side effects*").

Knowledge of Hippy Crack

Uncertainty because of lack of information showed to be quite common ("*I think there needs to be more awareness on all drugs at this age, it is very common for young people to take drugs not being aware if the risks involve*d"). People could also be dissuaded due to the little knowledge they've obtained from the scenario question ("*never intentionally take drugs, knowing it could have an effect on me*"), suggesting knowledge of hippy crack can indeed have an effect on one's consumption pattern. Some participants said they would not do it and leave, however, others, instead of just leaving, would also warn their friends of possible dangers involved in taking hippy-cark ("*inform my friends of side effects*") (**Table 1**). This suggests that the more people that are informed, the more likely they would share this information with others among their peer group, increasing knowledge and awareness of hippy crack, further suggesting that informing people of risks could result in them and those around them being less susceptible to risks/side-effects, overuse of hippy crack and at the very least making people take hippy crack in a safer way ("*Tell my friends not to roll up the windows*"), possibly even reducing consumption pattern. A number of participants suggested taking hippy crack in a safer way by taking it outside ("*open environment*"), which could lower possible risks leaving them less susceptible to side-effects. Further emphasizing that knowledge of the drug could be beneficial itself as it could alter the way it is taken.

Susceptibility to Risks Involved with Hippy Crack

Furthermore, some people do not see the importance of the harmful side effects hippy crack may cause, neglecting information/warnings given in the scenario question ("*go for it, there's no strong evidence to suggest its damaging to you moreover, the high only last a few seconds*"). Even though effects of hippy crack were reported to last a few seconds, people may not be aware of hippy crack causing a lack of B12, increasing levels of homocysteine in the blood, which causes more harm, as it can take up to days to clear. Additionally, due lack of knowledge, people may not view hippy crack as something worth being cautious

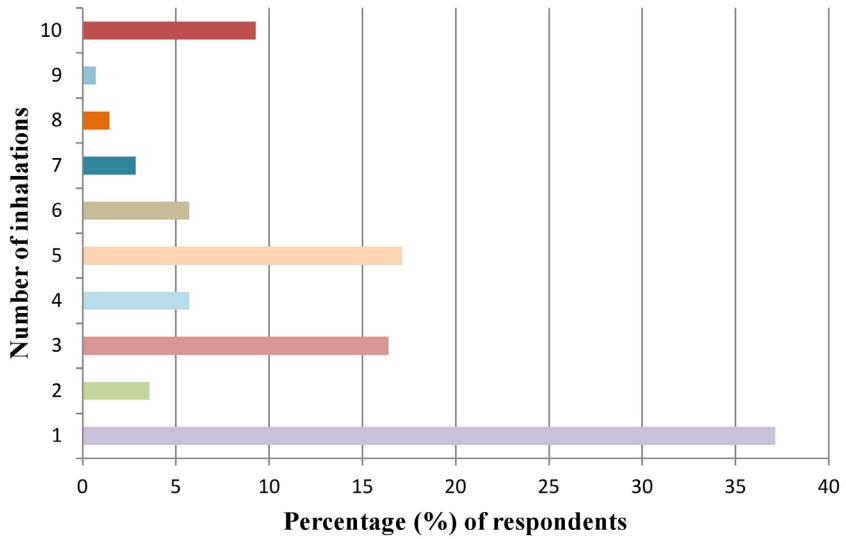

FIGURE 6 | Number of inhalations perceived as enough hippy crack for a single occasion (n = 140 responses).

TABLE 1 | Summary of qualitative results, behavior of those offered hippy crack, susceptibility to risks, knowledge of hippy crack.

Category (themes)	Content	Frequency
Behavior of those offered hippy crack	Would take hippy crack just to try it	30
	Experience	9
	Feeling of being under (peer pressure)	6
	Intake of hippy crack dependent on level of comfort	4
	Go home/leave	11
	Simply would not take hippy crack	25
Susceptibility to risks involved with hippy crack	Inhale in the car	8
	Sit in the car even though they stated that they would not inhale hippy crack	5
Knowledge of hippy crack	Inhaling outside the car	26
	Just inhale hippy crack *via* balloon in the house	3
	Inform friends of possible dangers involved with inhaling hippy crack	21
	Change their minds due to known information	28

over and may have a relaxed attitude toward the drug ("*yolo, you only live once so why not*"). This lack of awareness of risk could increase consumption pattern leaving them more susceptible to side-effects/risks.

DISCUSSION

This is the first study to capture a wide range of key characteristics of both users but also potential future users to inform future strategies to educate, avoid harm and prepare young people for the risks involved in the regular abuse of this seemingly harmless legal high. At a time when little is known about the effects of hippy crack on the developing brain, it is very concerning that the majority of participants showed little or no knowledge of serious side effects associated with this drug. Previous studies have largely focused on quantitative data such as prevalence without gaining a fuller appreciation for the behavior and future intentions of young people.

The current study used convenience sampling whereas prior study used a slightly older age range and targeted recruitment (purposive sampling). Thus, the key findings of the current study are complimentary in highlighting, in particular, the desire to partake of hippy crack for both non-users and last-year users and, the lack of awareness of potential side-effects and the desire to be educated on the effects of hippy crack. It is notable, that despite the introduction of legislation by UK Government, this report testifies to the relative ease in which young people assert that they can access hippy crack. A further complication arose in 2017 when—owing to its medicinal properties—a UK judge dismissed a case of festival attendees possessing hippy crack (29).

This study aimed to assess the widespread abuse of hippy crack along with the issues related to its "silent" toxicity profile. Increased understanding of attitudes to—and involvement in—hippy crack abuse among young adults is importance to health issues—both physical and mental—along with fatalities. The lack of awareness of the associated serious side-effects lends perspective to the need for increased education of young people, their guardians and health workers. Key findings of this study included the paucity of understanding of the toxic effects of hippy crack alongside a widespread desire for education for harm-reduction. For example, in severe cases of hippy crack-induced vitamin B12 deficiency, serious symptoms could be reversed with administration of vitamin B12 in a clinical setting (30). With appropriate education and risk awareness, users

would be empowered to protect their health—both mental and physical.

Previous research has ascertained the prevalence of use across six nations with the United Kingdom leading for lifetime, last year and past month use (38.6, 20.5, and 7.7%, respectively) (28), which is slightly below the past-year use (20.5 vs. 27.9%) identified in the current study. It identified the main sources of purchase (internet), routes of administration (by mouth *via* balloon), and location of use (house parties). The authors further focused on adverse effects last-year users had experienced along with their health concerns. Furthermore, inhalants use among adolescents and young children is a growing public health concern (31, 32). Evidence from previous research also suggests that inhalants, including nitrous oxide, are often the first category of substances to be abused by adolescents and thus considered a 'gateway drug' (33–35); and that the growing popularity of e-cigarettes among adolescents increases the relative risk for using nitrous oxide (36).

This study has limitations in that the results are restricted to a relative small sample size and the restrictions associated with self-report measures. Participants could not give a verbal confirmation when using number scales as answers. The closed questions (selection of yes or no) can be considered to be limited in terms of data, which in turn could have affected the precision of the overall data obtained. However, by using the scenario/open question a reasonable idea as to how people would behave in a given situation, in addition to, helping to understand a little more about how people may view the drug itself, in terms of the harm the drug can cause and also how they consider risks associated with the drug.

CONCLUSION

These results provide considerable insights into the level of use, awareness and perceptions of hippy crack in young people in England. The reported use (number of occasions and amounts taken), coupled to the apparent willingness of most respondents to use it in the future, is overshadowed by the clear lack of awareness of the serious side effects it may cause. Holistically this report emphasizes the growing need to educate people on the dangers involved upon consumption of hippy crack especially as it appears that the new Psychoactive Substances Act is failing to curb abuse. A drive toward harm-reduction is necessary through dissipating the widespread belief that this "legal high" is without side effects. Furthermore, empirical studies should be designed to address the effects of hippy crack on the developing brain—based on the reported considerable molecular and structural changes that result from frequent use.

ETHICS STATEMENT

The study was approved by the Faculty Research Ethics Committee of the Faculty of Science, Engineering and Computing, Kingston University under the delegated approval scheme. To ensure complete anonymity, implied consenting process was used. At the start of the data collection, participants were informed about the study, the use of data, the data collection process and they gave consent by voluntarily completing and returning the survey.

AUTHOR CONTRIBUTIONS

AP and EE made substantial contributions to conception and interpretation; drafting and critically revising the work for important intellectual content, final approval and agreement to be accountable for all aspect of the work. DN made substantial contributions to drafting and critically revising the work for important intellectual content, final approval, and agreement to be accountable for all aspect of the work.

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ANNEX 1. SURVEY QUESTIONS

1. Gender ☐ male ☐ female ☐ neither ☐ other ☐
2. Age in years ☐
3. Student: yes or no
4. Have you heard of hippy crack before this questionnaire? yes or no
5. Have you taken hippy crack recreationally in the past 12 months? yes or no

If you have answered **yes (have taken hippy crack)** answer questions **1–13**

If you have answered **no (haven't taken hippy crack)** answer questions **14–17**

All, answer questions from 18–22

If you answered YES

6. If you have taken hippy crack before, would you take hippy crack again within the next 3 months?
   Scale 1–5
   1: absolutely not
   5: definitely
7. If you wanted to, how easy would it to obtain hippy crack?
   Scale 1–5
   1: very easy
   5: very difficult
8. How many times would you say you have taken hippy crack in the past 12 months?
   Scale 1–5
   1: only ever once
   2: 2–5 times
   3: 5–10 times
   4: 10–20 times
   5: more than 20 times
9. In your opinion, would you say you more likely to take hippy crack on your own or among friends?
   ☐ On my own
   ☐ Among friends
10. How much hippy crack would you inhale in one go/sitting?
    Scale 1–5
    1: one or two
    2: 3–5
    3: 5–10
    4: 10–20
    5: more than 20
11. After inhalation of hippy crack, how much would you say it affected you?
    Scale 1–10
    1: not at all
    10: completely affected
12. How many times would you say you would take hippy crack before it has an effect on you?
    Scale 1–5
    1: one or two
    2: 3–5
    3: 5–10
    4: 10–20
    5: more than 20
13. What was the duration of the effect?
    Scale 1–5
    1: less than a minute
    2: 2–5 min
    3: 5–20 min
    4: 20–30 min
    5: up to an hour

If you answered NO (haven't taken hippy crack)

14. If you haven't taken hippy crack, what is the likelihood for you to take hippy crack in the next 3 months?
    Scale 1–5
    1: very likely
    5: very unlikely
15. If you would want hippy crack, how easy would it be for you in your opinion to obtain hippy crack?
    Scale 1–5
    1: very easy
    5: very difficult
16. If you have never taken hippy crack before, in your opinion, would you be more comfortable taking hippy crack on your own or among friends?
    ☐ On my own
    ☐ Among friends
17. If you have never taken hippy crack before and it was your first time taking hippy crack, how many times, in your opinion, would you take hippy crack in one sitting?
    Scale 1–5
    1: one or two
    2: 3–5
    3: 5–10
    4: 10–20
    5: more than 20

All, answer questions

18. In your opinion, how harmful would you say hippy crack is?
    Scale 1–10
    1: not harmful at all
    10: extremely harmful
19. Are you aware of any of the side effects of hippy crack? yes or no
20. In your opinion, how much would you say is too much hippy crack in one go?
    Scale 1–10
    1: one is enough
    10: should be no limit
21. Alex is at a house party with friends, it has reached 1 am and everyone has now settled down and is listening to music, when one of Alex's friends brings out a whippet canisters (filled with nitrous oxide) and a small box of balloons.

Everyone has already filled their balloons with nitrous oxide. One friend suggested that they should inhale hippy crack in the car, rolling up all the windows as it is a closed space and would be more fun as it will give have a greater euphoric effect.

However, Alex remembers in a previous lecture that informed all of them how hippy crack could possibly effect the brain and possible risks upon inhalation, Alex also remembers that there is a greater risk if it was inhaled in a closed space.

A balloon filled with nitrous oxide is handed to Alex and although some friends stay behind inhaling with the balloon Alex's best friend heads out to the car inviting and pulling Alex along. Bearing in mind that this is Alex's first time inhaling hippy crack, if you were Alex what would you do?

.....
.....
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.....
.....
.....

22. In your opinion, how important would you say it is to educate young people (16–25) about the effects of hippy crack?
Scale 1–10
1: not important at all
10: extremely important

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REVIEW
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Novel Psychoactive Substances—Recent Progress on Neuropharmacological Mechanisms of Action for Selected Drugs

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A feature of human culture is that we can learn to consume chemical compounds, derived from natural plants or synthetic fabrication, for their psychoactive effects. These drugs change the mental state and/or the behavioral performance of an individual and can be instrumentalized for various purposes. After the emergence of a novel psychoactive substance (NPS) and a period of experimental consumption, personal and medical benefits and harm potential of the NPS can be estimated on evidence base. This may lead to a legal classification of the NPS, which may range from limited medical use, controlled availability up to a complete ban of the drug form publically accepted use. With these measures, however, a drug does not disappear, but frequently continues to be used, which eventually allows an even better estimate of the drug's properties. Thus, only in rare cases, there is a final verdict that is no more questioned. Instead, the view on a drug can change from tolerable to harmful but may also involve the new establishment of a desired medical application to a previously harmful drug. Here, we provide a summary review on a number of NPS for which the neuropharmacological evaluation has made important progress in recent years. They include mitragynine ("Kratom"), synthetic cannabinoids (e.g., "Spice"), dimethyltryptamine and novel serotonergic hallucinogens, the cathinones mephedrone and methylone, ketamine and novel dissociative drugs, γ-hydroxybutyrate, γ-butyrolactone, and 1,4-butanediol. This review shows not only emerging harm potentials but also some potential medical applications.

Keywords: Kratom, synthetic cannabinoids, dimethyltryptamine, serotonergic hallucinogens, mephedrone, ketamine, γ-hydroxybutyrate

INTRODUCTION

It appears to be a human trait to constantly seek for new psychoactive substances and to explore potential use of them. As long as human record keeping dates back, humans consume psychoactive plant preparations. Since centuries they isolated single compounds yielding "natural drugs," while since decades synthetic chemistry allowed the innovation of completely new compounds that are not

available from natural resources (1, 2). Despite the risk of being toxic upon single or chronic consumption, there are constantly new drugs that find their way into drug-taking communities (3).

Novel stimuli and novel information about the external world have an incentive salience and maintain seeking behavior in animals and in humans (4, 5). The search for novel external stimuli may translate to novel "mental states," as an experience of new interoceptive states. Human brains generate distinct working modes that are subjectively perceived as mental states. This is at the neurobiological side believed to be organized by the summatory tonic activity of modulatory transmitter systems. Mental states can determine how an organism perceives, processes, and stores external and internal information. It also affects how efficiently behavior is generated (6–8). Mental states change spontaneously or as a consequence of environmental influences, thereby some mental states are perceived as more pleasurable and useful for goal-directed behavior than others. The rewarding value of novelty may, thus, be expanded to novel mental states, which have never been incurred by natural means. Psychoactive substances can induce and maintain a desired mental state. Some of them may also provoke novel mental states. Only a few of the well-established psychoactive substances induce "euphoria" or a sense of "well-being," which directly reinforces drug-seeking and consumption behaviors. Most psychoactive substances, however, induce mental states that are primarily useful for other purposes. In that, they exert complex reinforcing effects during drug instrumentalization (6–10). Thus, the mental state that is induced by a psychoactive drug and for which humans develop a memory (11) may facilitate other behaviors with positive or negative reinforcing outcome, such as the facilitation of social interaction, mating behavior, coping with stress, and cognitive enhancement (9, 12–16). When a new drug is discovered and experimentally used, the new user may not only judge the novelty and emotional impact of the newly experienced mental state but subsequently decide for what purposes this new mental state may serve (17, 18). Once a new drug is made available, an experimental consumption starts that determines individual subjective effects as well as context and possibility of instrumentalization. This may not only work in humans but also for a newly experienced psychoactive drug in animals (19).

A major factor that fuels continuous search for new psychoactive drugs is the need to replace existing ones in routine use. Once a long known drug has been criminalized and banned, availability of the drug becomes limited. Legal control imposes punishment on drug possession, trade, and use, which limits its instrumentalization for frequently performed behavior, such as coping with stress (20). If the drug was useful for this behavior, e.g., to better relax after stressful work, users may start looking for a legal replacement of the banned drug and, thus, be motivated for testing new drugs (21).

Novel psychoactive substances (NPSs) had been defined by the United Nations as new narcotic or psychotropic drugs that are not controlled by the United Nations' 1961 Single Convention on Narcotic Drugs or by Psychotropic Substances Conventions (22). NPSs are by definition those psychoactive drugs used for intoxication which are not already prohibited by UN Single Convention on Narcotic Drugs or Misuse of Drugs Act (23), thereby "novel" does not necessarily mean that the drug has been developed completely new recently. It may also refer to substances that have lately become popular and/or more widely available, constituting a reason of current or potential public health concerns (24).

The way of a NPS in society, from its introduction, experimental use, instrumentalization, habitual abuse, up to its legal control, depends essentially on the relationship of adverse side effects and potential medical use. The adverse side effects are those effects of the drug that threaten the physiological integrity and behavioral repertoire of the whole organism, beyond the desired psychoactive action. Many known psychoactive drugs are strong toxins and harm the user. This can occur after acute consumption or after chronic intake (3). Humans establish cultural rules for the consumption and the control of side effects of psychoactive drugs (25). This keeps even highly dangerous drugs, such as alcohol, legal and limits their harm potential when incorporated in cultural activities (26). But establishing those initially "non-written" rules requires a certain amount of experience and a user/non-user discourse. One result of this discourse is the possibility of legal control, and a "written down" law on where, when, and how a psychoactive substance can be used. Drugs can be labeled as addictive drugs and made illicit. However, many new substances are at the same time tested for a potential beneficial application, e.g., to treat pain, or even as substitutes for well-known addictive drugs, thereby the verdict may be that a NPS might have some addiction- and harm potential but also beneficial effects, which may actually dominate the use profile. There is occasionally also a reversal of the discourse decision in that addictive drugs may receive an additional medical application, e.g., ketamine, which was discussed as an abuse drug (27, 28), but is now also considered as a useful treatment for depression (29).

Newly introduced psychoactive substances do not usually arise from controlled pharmacodevelopment. In that, these drugs initially have the status of a "legal high" and virtually everybody is allowed to possess, distribute, and consume them. Only when after consumption accidents with physical- and/or behavioral impairments occur, or in the worst case drug fatalities, a NPS can be classified and legally controlled or its medical use defined (21). However, the drug discourse requires evidence, ideally scientific, arising from controlled experimentation. This evidence should go well beyond the accumulation of single cases. Quite naturally, during the information collecting period, the NPS is used, and thus, not brand new anymore. What is new afterward is the certainty with which sufficiently reliable statements about the drug can be made (30).

It has to be admitted that the legal status discourse is in practice way more complicated and also culturally selective, which shall not be the focus of this review that rather focuses on the neurobiological evidence that feeds into this discourse. In this article, we review the state of knowledge on a number of NPS for which now a considerable penetration of society has developed in distinct cultural or geographical regions and for which sufficient evidence has been gathered to allow for evidence-based statements. This should provide a comprehensive overview on some of the currently most relevant NPS, thereby the choice of substances discussed was driven by the perceived progress in the understanding of their neuropharmacological action by the

authors. In that, the review does not provide a complete coverage of all currently available NPS.

KRATOM AND MITRAGYNINE

Mitragyna speciosa Korth. (*M. speciosa*), from the Rubiaceae family, is a tropical medicinal plant native to Southeast Asia (31, 32). In Malaysia, *M. speciosa* leaves are known as Ketum or Biak (31, 32), and in Thailand as Kratom (33, 34). *M. speciosa* has been historically used in Southeast Asia as a stimulant drug and in its traditional context as a remedy for various symptoms (33, 34). Previous studies mainly described the traditional uses of Kratom among rural folk, peasants, and laborers in Southeast Asia (33, 35, 36). More recently, studies on Kratom use are emerging from Europe and the US (37–40). They suggest that Kratom is now also used outside its traditional context. In the West, it is still considered a "safe" herbal drug with potential medicinal application (38, 39, 41, 42).

In Southeast Asia, manual laborers commonly chew fresh Kratom leaves and ingest brewed Kratom tea/juice to reduce fatigue, promote work desire, and enhance physical tolerance to debilitating work (32, 33, 43, 44). Kratom leaves are also used as an opium substitute to treat morphine addiction in Malaysia and Thailand (31, 45). Because of its unique healing properties, rural inhabitants use Kratom leaves to treat various medical conditions such as cough, fever, pain, diarrhea, diabetes, and hypertension (32, 44, 46). However, Kratom is potentially addictive and chronic users find it difficult to refrain from prolonged Kratom use (33, 36, 43). The common side effects of long-term use include constipation, weight loss, hyperpigmentation, dehydration, fatigue, insomnia, and increased urination (33, 36, 46). The majority of Kratom users believe its use is not as harmful as those of other illicit substances, such as methamphetamine and heroin, and that Kratom dependence carries little or no health risks (45–47). So far, there have been no deleterious incidents directly related to Kratom use in Southeast Asia. Only one study from Thailand has reported Kratom poisoning cases, with palpitation, seizure, and nausea. However, these effects may have been arisen from coadministration of other illicit substances (48).

Regular users are more likely to increase the quantity and frequency of Kratom use over time. In Thailand, traditional users often chew fresh or powdered Kratom leaves (33, 44). In Malaysia, Kratom users commonly ingest brewed Kratom tea/juice (25, 36, 47, 49). In the US and in Europe, Kratom is primarily used as a natural alternative to self-medicate for chronic pain and as an opioid withdrawal treatment (37, 50, 51). Kratom is marketed as a "legal high" and can be easily obtained in different forms, such as powder extracts, tablets, capsule, or liquids, through the Internet (38, 52). As a consequence of the rise in Kratom mortality and toxicity cases in the West, regulatory agencies have begun to view Kratom negatively (39, 53, 54). The US Drug Enforcement Administration intends to regulate Kratom use in the US (51). However, it appears that most, if not all of the Kratom-induced medical complications in the West were triggered by the use of adulterated Kratom products (53, 54).

About 40 alkaloids have been identified in *M. speciosa* leaves. The alkaloid content in the leaves varies, depending on geographical location and season of harvest (55). Mitragynine and 7-hydroxymitragynine are the principal psychoactive constituents of *M. speciosa* and were shown to induce morphine-like effects in animal models (31, 55, 56). The synthesis of the mitragynine was reported by Takayama et al. (57) and later by Ma and colleagues (58, 59). However, a synthesis of mitragynine is with 18–23 steps rather laborious, time-consuming, not economical, and has only a low yield of 3–13% (60). Thus, direct isolation of mitragynine from the leaves is much more efficient and cost-effective.

A comprehensive pharmacokinetic description of mitragynine in rats was provided by Parthasarathy et al. (61) after intravenous (i.v.) and oral administration. The blood concentration peaked at 1.2 h with 2.3 μg/mL followed by biphasic elimination with a half-life of 2.9 h and a clearance of 0.09 L/h/kg after administration of 1.5 mg/kg mitragynine (i.v.). The volume of distribution was rather small with 0.79 L/kg, suggesting that mitragynine is not widely distributed into tissue compartments (62). The oral absorption of mitragynine was shown to be lengthy and incomplete, with an absolute oral bioavailability of around 3%. Several studies revealed that after oral application of 20–50 mg/kg mitragynine, a volume distribution of 37–89 L/kg and clearance of 1.6–7 L/h (per kg) was reached (61–63), which supports the low bioavailability and poor absorption of mitragynine.

Mitragynine is a lipophilic alkaloid with poor water solubility (64). Mitragynine has a biphasic metabolism. The first phase produces seven identified metabolites, thereby mitragynine is processed through hydrolysis of methyl ester in position 16 and O-demethylation of the 9-methoxy- and of the 17-methoxy groups (65). The second phase involves further oxidation to carboxylic acids or reduction to alcohols and the combinations of some steps *via* the intermediate aldehydes. Four metabolites were additionally conjugated to glucuronides and to sulfates in rats and humans (65). Abuse of mitragynine and related compounds can be detected through gas chromatography or liquid chromatography with mass spectrometry, respectively (65–67).

Mitragynine shows the highest affinity to κ-opioid receptors followed by μ- and δ-opioid receptors (68). It acts as a receptor agonist at μ-opioid receptors and possibly as an antagonist at κ-opioid receptors (56, 69–71). At cellular level, mitragynine blocks neuronal Ca^{2+} channels (72). It was also found to inhibit forskolin-stimulated cyclic adenosine monophosphate (cAMP) formation *in vitro* in an opiate receptor-dependent way (73, 74). A study by Fakurazi et al. (75) showed that repeated exposure to mitragynine attenuated the expression of cAMP and cAMP response element-binding protein.

Mitragynine was extensively investigated for its antinociceptive effects. A study by Reanmongkol et al. (76) found prolonged antinociceptive effects in the hot plate test, but not in the tail flick test. Another study showed prolonged antinociceptive effect in both tests (77). Intraperitoneal administration also yielded positive antinociceptive results in the hot plate, formalin-, and acetic-acid tests (78). Mitragynine's antinociceptive effects were comparable to those of oxycodone suggesting an abuse potential (79, 80).

In animal models, mitragynine induces anxiolytic effects after acute treatment in several test paradigms (81). This may be mediated by its effects on Fos expression in dorsal raphe nucleus (82), and the activation of δ-opioid receptors (83). Withdrawal from

chronic mitragynine induces anxiety-related behavior in rats (84). There have been conflicting reports of mitragynine affecting cognitive function. Apryani et al. (85) found that mitragynine i.p. administration can impair object location memory in mice. Another study, however, showed no impairment of short-term memory in the Y-maze task. The mice, however, were given *M. speciosa* extract through oral administration (86). In rats, a study showed an increase in learning ability when given *M. speciosa* extract in a passive- and an active avoidance task. However, mitragynine alone did not have significant effects on long-term memory consolidation in both tasks (87). A recent study using a passive avoidance task showed independent impairments of learning, memory consolidation, as well as memory retrieval after acute mitragynine administration at a dose ≥10 mg/kg (i.p.) in rats. In parallel, mitragynine-treated rats showed a disrupted low frequency rhythm (delta and theta) in the electroencephalogram (EEG), which may account for the learning and memory impairments (84).

Chronic administration of mitragynine at a dose of ≥10 mg/kg (i.p.) may cause addiction-like behaviors in animal models (56, 84, 88). Mitragynine (15 mg/kg, i.p.) shows discriminative stimulus properties in rats. It fully substituted for a morphine (5 mg/kg) stimulus, and partially for a cocaine cue (10 mg/kg, i.p.) (89). Thus, mitragynine likely possess both opioid and psychostimulant effects. Mitragynine at doses ≥10 mg/kg (i.p.) shows rewarding properties in rodents as measured by conditioned place preference. These effects are opiate receptor dependent and can be blocked by the opiate receptor antagonist naloxone (56). Subchronic administration of mitragynine increased the expression of dopamine transporter- and dopamine (DA) receptor-regulating factor mRNA in the limbic system of the brain (84) indicating a critical role of DA in the rewarding effects of mitragynine, thereby the dose of mitragynine may be crucial, given that addiction-like behaviors were only observed at doses ≥10 mg/kg (i.p.) in rodents. Those are considerably higher than reported maximum doses of mitragynine consumed by humans, which are usually in the range of <3 mg/kg (p.o.) per day.

Altogether, Kratom and its main psychoactive ingredient mitragynine are drugs that are widely used in Southeast Asia with an increasing appearance in Western countries. Experimental studies in animals have now shown that mitragynine has an addictive potential, however, only at higher doses. Human users in countries of frequent use with a traditional context report a rather low daily consumption with only mild side effects. Kratom and mitragynine can be instrumentalized to enhance physical work power and endurance. A major reason for Kratom consumption is its reported efficacy to replace opiates in chronic users. This makes the Kratom plant preparation and also the isolated compound mitragynine interesting options to treat opiate addiction.

SYNTHETIC CANNABINOIDS

The abuse of herbal preparations spiked with synthetic cannabinoids is still increasing. A hallmark of this consumption is the use of an inhomogeneous group of substances that occur on the market with different names, such as Spice, Spice gold, diamond-spice, chill X, abyss, Pandora's box, exodus, annihilation, fire, smoke, sence, chillX, chillys, highdi's, earth impact, and many more (90, 91). Synthetic cannabinoid preparations are frequently mislabeled as research chemicals, herbal incenses, or as legal highs, including the explicit warning that it is not for human consumption (92–97). The first evidence of synthetic cannabinoid use as a recreational drug appeared in 2004 (98). However, a wide spread use of synthetic cannabinoids did not emerge until 2008. In 2012, the lifetime prevalence for "Spice" consumption was already at 7% among the 15- to 18-year olds (99–101), thereby the coabuse of synthetic- and natural cannabinoids is common (102–109).

Research in the active ingredients of synthetic cannabinoids such as Spice and their neuropharmacological action has revealed several hundred compounds that are artificially added to a carrier medium of herbal origin (110). The synthetic compounds usually display a high affinity for cannabinoid receptors (CB-R), which reaches far beyond that of natural cannabinoids (100, 111). Compared to the partial agonist, Δ9-tetrahydrocannabinol (THC), synthetic cannabinoids can act as agonists, neutral antagonists, or inverse agonists at the CB-R1 (110–112). Synthetic cannabinoid preparations also lack the naturally occurring cannabidiol, which is present in cannabis preparations and which is supposed to antagonize some of the psychotogenic effects of THC (113, 114).

A gram of herbal preparation can contain up to 200 mg of a synthetic cannabinoid. However, the variability in substance composition and amount between one package and another is high and largely unpredictable. Additional ingredients have been found and may include, e.g., clenbuterol, which may be responsible for the frequently observed sympathomimetic manifestations of an intoxication with synthetic cannabinoids, or tocopherol. The latter is usually added to blur chemical detection (113–115). Occasionally, some investigated herbal preparations did not contain any pharmacologically active synthetic cannabinoids, but only psychoactive compounds from plant-derived carrier material, such as mitragynine (116–120).

Users report that synthetic cannabinoids can cause psychotropic effects that are qualitatively similar, but much more intense, than those of cannabis. As such, synthetic cannabinoids may cause THC-like effects including alterations of mood, sleep, perception/wakefulness, body temperature, and cardiovascular function (121–123). Additional diffuse effects, which are different from cannabis, include palpitations, tachycardia, and unspecific effects in the electrocardiogram (110, 124, 125). Harmful somatic effects comprise gastrointestinal and renal defects (91, 126–128). Neuropsychiatric symptoms were reported, such as psychosis, panic and anxiety attacks, agitation, and aggressive behavior (106, 107, 129, 130). A psychosis induced by synthetic cannabinoids manifests by delusions, acoustic and visual hallucinations, and paranoia. Neurological symptoms may include seizures, dystonia, and tremors. Other frequently reported side effects are nausea, vomiting, diaphoresis, and respiratory depression (131–141). Use of synthetic cannabinoids may have fatal consequences. Reported single cases mention coronary ischemic events and suicide caused by an extreme anxiety attack (138, 139).

The active compound of the preparation "Spice" was first described in 2009, following the detection of formerly non-declared, synthetic CB-R1 agonists (141, 142). Synthetic

cannabinoids were originally developed for research purposes in the 1970s with the goal of better understanding the endogenous cannabinoid pathways and to develop pharmacotherapies for conditions such as cancer-associated pain (108). Synthetic cannabinoids may contain aminoalkyl-indoles of the JWH series, which was first synthesized by the chemist J. W. Huffmann. Major ingredients of herbal preparations in the past included the aminoalkyl-indoles, JWH-018, JWH-073, JWH-019, JWH-250, and the cyclohexylphenols, CP-47,497-C6, CP-47,497, and CP-47,497-C8. These compounds are lipid-soluble, non-polar, and typically contain 20–26 carbon atoms. However, there are at least 100 chemically related compounds currently known (122, 143–146). While some of them have been legally controlled on individual level, recent legislation in Germany now considers the lead structures and attempts to control whole drug classes. It is expected that this will make it more difficult to simply replace single banned compounds by their substituted analogs in the synthetic cannabinoid preparations (122, 147–153).

At the current stage, one may conclude that synthetic cannabinoids constitute dangerous psychoactive drug preparations with a rather chimeric nature (154). It is not a single compound, but draws from a plethora of already available synthetic cannabinoids that are unsystematically mixed and brought on a plant carrier material, that may even by itself have psychoactive effects. This strategy of drug preparation paved the way into the perception as a natural and perfectly "legal high" by consumers. The natural claim is now clearly rejected by the understanding that most psychoactive effects are brought about by purely synthetic compounds added to a natural carrier. Given the strong cannabinoid-like effects of synthetic cannabinoids, which are now increasingly understood, single substances have been legally banned. But this has done little damage to the unique drug design of synthetic cannabinoid preparations in that single disallowed compounds were almost immediately replaced by substituted analogs that had not been banned yet. The now emerging control of whole substance classes will most likely put an end to this strategy and help to reduce harm that is clearly associated with synthetic cannabinoid consumption.

DIMETHYLTRYPTAMINE

N-*N*-dimethyltryptamine (DMT) is an indole alkaloid found in plants and animals. It has been proposed that the endogenous DMT may act as a neurotransmitter. DMT is a natural psychedelic substance and has similar effects as other serotonergic hallucinogens such as lysergic acid diethylamide (LSD), psilocybin, and mescaline. DMT is one of the ingredients used in various shamanic preparations, such as ayahuasca, hoasca, or yagé in South America and is used as a recreational drug in Europe and North America (155). DMT rich plants belong to genera such as *Phalaris*, *Delosperma*, *Acacia*, *Desmodium*, *Mimosa*, *Virola*, and *Psychotria*. When DMT is ingested at high concentrations, the user experiences episodic visual hallucinations (155, 156). The recreational use of DMT has been rising for its acclaimed self-perceived benefits. Capsules, known as pharmahuasca, became available containing DMT as a free base together with some monoamine oxidase inhibitors (MAOIs), such as synthetic harmaline, or plant-based MAOIs such as Harmala alkaloids (157, 158). The MAOIs inhibit the otherwise rapid metabolization of DMT and, thus, allow for the hallucinogenic effects when the drug is taken orally.

Endogenous DMT can be found in the human brain and other tissues of the body such as blood, urine, cerebral spinal fluid (155, 156, 159), and the pineal gland (156, 160). Synthesis of endogenous DMT begins with the decarboxylation of tryptophan to tryptamine. *N*-methyltryptamine (NMT) and DMT are the products of methyl group additions to tryptamine by the enzyme indolethylamine-*N*-methyltransferase (160). DMT levels were found to increase under stress in the rodent brain and adrenal gland (161). This can activate trace amine-associated receptors and serotonin receptors (5-HT-Rs), such as the 5-HT_{1A}-Rs, 5-HT_{2A}-Rs, and the 5-HT_{2C}-Rs (159, 162). It was suggested that endogenous DMT has a role in cellular protective mechanisms (155).

Exogenous DMT is metabolized by MAO and peroxidases leading to the metabolites NMT, 6-hydroxy-DMT, 6-OH-DMT-*N*-oxide, DMT-*N*-oxide, and indole-3-acetic acid (160). The pharmacokinetics of DMT shows a rapid onset of action within 5–30 min. This is followed by an intense modification of the mental state lasting for approximately 4 h (163). The routes of DMT administration are *via* smoking or snorting. For the hallucinogenic or psychedelic effects to occur, an oral formulation must contain MAOIs to prolong the half-life of DMT in the body. MAOIs block the enzyme in the stomach after which DMT is able to be absorbed through the stomach lining into the blood stream. An oral dosing of DMT, e.g., *via* ayahuasca, produces both behavioral and neuroendocrinological effects, such as a decrease in locomotor activity, cognitive impairments, sympathomimetic effects, increased prolactin, and cortisol levels (164, 165). DMT also interacts with various ionotropic and metabotropic receptors in the glutamate, DA, and acetylcholine systems. The subjective effects of exogenous DMT are primarily mediated by 5-HT_{2A}-Rs. 5-HT_{2C}-Rs play little or no role (166, 167). Glutamatergic mGluR2 receptors might have modulatory effects in DMT action (167). DMT does not affect DA receptors but may alter DA levels in the brain with subsequent neurochemical and behavioral effects.

Chronic DMT induces tolerance for some behavioral and subjective effects. However, it failed to elicit tolerance to the disruption of responding maintained on a fixed-ratio schedule of food reinforcement (168, 169). DMT yields similar discriminative stimulus effects as the serotonergic hallucinogens 2,5-dimethoxy-4-methylamphetamine (DOM) and LSD. Furthermore, DMT fully substituted in DOM-trained rats and for LSD in rats and pigeons (170, 171).

Beside its sought-after acute effects, DMT can cause considerable side effects. The ingestion of DMT may induce intense fear, paranoia, anxiety, grief, and depression, which may result in physical harm to the user or others (157). There have been no serious adverse events reported on long-term use of DMT apart from the acute cardiovascular effects. Single and repeated administrations of DMT produce marked changes in the cardiovascular system (172). In fact, DMT has been reported to act as neuroprotective agent, working *via* Sigma-1 receptor (Sig-1R) activation (173–177). Sig-1Rs activate the antioxidant response elements (176). Hence, DMT may function as an indirect antioxidant. Frecska et al. (177)

have suggested that peripheral synthesis of DMT, consumptions of DMT-containing plant material, or systemic administration of DMT can trigger endogenous central nervous system pathways that produce psychedelic experiences. At the same time, it may serve mechanisms such as neuroprotection and neuroregeneration. Interestingly, ayahuasca and DMT mixtures have been proposed as a treatment for psychiatric disorders. Symptoms of schizophrenia, such as delusions and hallucinations, have been assumed to involve activation of 5-HT_{2A}-Rs along with changes in the DA system (166, 178). Endogenous DMT has been reported to be increased in schizophrenic patients during psychotic episodes (179) indicating that the endogenous DMT signaling pathway might be a treatment target for schizophrenia. Based on animal models and on clinical studies in humans, DMT has potential antidepressant and anxiolytic effects (180), possibly mediated by a 5-HT_{1A}-R agonistic action (181). Further therapeutic applications include the treatment of cancer and inflammations. DMT has been shown to increase immune system activity (165, 182). Sig-1R activation can reduce pro-inflammatory cytokines and enhance the production of the anti-inflammatory cytokine IL-10 (183).

In conclusion, DMT is a naturally occurring psychoactive compound found in various plants. It is now understood that its main psychoactive effects are mediated by 5-HT_{2A}-R activation. Endogenous DMT may play a role in the immunoregulation in peripheral and brain tissues. Preliminary evidence now suggests a possible therapeutic use of DMT.

NOVEL SEROTONERGIC HALLUCINOGENS

Since thousands of years, indigene cultures in North and South America have used plants and mushrooms containing serotonergic hallucinogens for shamanic rituals and religious ceremonies (184). The most famous examples are (1) *Psilocybe* mushrooms containing psilocybin, which were used as Teonanacatl ("god's flesh") by the Aztecs, (2) the cactus *Lophophora williamsii* enclosing mescaline and applied as Peyote or Peyotl by Mexican and North American indigene cultures, and (3) a brew of *Banisteriopsis caapi* and *Psychotria viridis* called Ayahuasca utilized by Amazonian indigene cultures containing the psychedelic ingredient DMT together with harmala alkaloids acting as MAOIs inhibitors and preventing the metabolism of DMT (185).

Classical serotonergic hallucinogens usually have either a tryptamine or phenylethylamine basic structure (186). Typical tryptamines, such as psilocybin and its psychoactive metabolite psilocin, 5-methoxy-*N*,*N*-dimethyltryptamine (5-MeO-DMT), and bufotenine, resemble in their structure the neurotransmitter 5-HT, while the phenylethylamine mescaline has a similar basic structure as the neurotransmitter DA and as amphetamines. In addition, ergoline alkaloids such as the naturally occurring D-lysergic acid amide, also called ergine—the psychoactive compound of *Turbina corymbosa*, *Argyreia nervosa*, and *Ipomea tricolor*—and the semi-synthetic LSD (Delysid®), have a tryptamine backbone as well (186).

It was suggested that the term "hallucinogens" may be a misnomer as these drugs not necessarily produce real hallucinations, at least when applied at typical doses, but many other emotional, perceptual, cognitive, and behavioral effects. It was suggested that "psychotomimetics" might be the more appropriate term for them (186). However, all 5-HT hallucinogens have in common that they induce altered states of consciousness (186, 187). According to Hollister (188), the psychoactive effects of classical serotonergic hallucinogens usually include (1) somatic symptoms: dizziness, weakness, tremors, nausea, drowsiness, paresthesia, and blurred vision; (2) perceptual symptoms: altered shapes and colors, difficulty in focusing on objects, sharpened sense of hearing, and rarely synesthesia; and (3) psychic symptoms: alterations in mood (happy, sad, or irritable at varying times), tension, distorted time sense, difficulty in expressing thoughts, depersonalization, dream-like feelings, and visual hallucinations.

All tryptamine- and phenylethylamine-based hallucinogens share the agonistic mechanism of action at postsynaptic 5-HT_{2A}-Rs and 5-HT_{2C}-Rs, where they act as partial, mixed-partial, or full agonists (186, 189). In animals and humans, 5-HT_{2A}-R antagonists such as ketanserin are able to block most of the behavioral and psychotropic effects of psilocybin, mescaline, DOI, and LSD, indicating that the 5-HT_{2A}-R agonism is necessary for the induction of psychedelic effects (189–194). However, some of these drugs show a strong affinity to 5-HT_{1A}-Rs and other 5-HT receptor subtypes as well as to DA D2-Rs. These additional mechanisms are likely to contribute to the specific psychotropic effects of each compound (189, 191, 195). A decade ago, it has been proposed that only 5-HT_{2A}-Rs coupled to metabotropic mGluR2 mediate the psychotogenic effects of 5-HT hallucinogens (196)—a position that has been questioned recently (197). At the neuronal level, 5-HT hallucinogens, such as psilocin, LSD, and DMT, directly activate 5-HT_{2A}-Rs located on cortical pyramidal neurons. In addition, they increase extracellular glutamate levels in the prefrontal cortex through stimulation of postsynaptic 5-HT_{2A}-Rs located on large glutamatergic pyramidal cells in deep cortical layers V and VI. This glutamate release leads to an activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors and *N*-methyl-D-aspartic acid (NMDA) receptors on cortical pyramidal neurons (187).

Historically, LSD was probably one of the first NPS of the hallucinogen class as it was a semi-synthetic compound whose psychedelic effects have only accidentally been discovered by its inventor Albert Hofmann in 1943 (198). The next, even though less accidental, producer of NPS hallucinogens was Alexander T. Shulgin, who synthesized hundreds of novel hallucinogenic tryptamines and phenylethylamines in his home laboratory. He described the synthesis of these compounds and also their psychotomimetic effects experienced in self-experiments in detail in his books PIHKAL and TIHKAL (199, 200). He created several dimethoxy-substituted phenylethylamines, such as DOM, 2,5-dimethoxy-4-bromoamphetamine (DOB), 2,5-dimethoxy-4-iodoamphetamine (DOI), and 2,5-dimethoxy-4-ethylamphetamine (DOET), which all display strong hallucinogenic properties. These drugs usually have much longer durations of action (12–30 h) and are much more potent agonists at 5-HT_{2A}-Rs (50- to 175-fold) compared to their related phenylethylamine derivative mescaline (duration of action: 4–8 h) (189, 199, 200). Also, another novel class of substituted

dimethoxyphenethylamines—the "2C psychedelics"—was invented by Shulgin, which mostly contains methoxy groups at positions 2 and 5 of a benzene ring together with lipophilic substituents (often halogens) at position 4. The most famous exponent of this class is 2,5-dimethoxy-4-bromophenethylamine (2C-B, "nexus," "bromo"), which was initially marketed as a legal surrogate of MDMA ("ecstasy") in the late 80s before it was finally scheduled by the UN Commission on Narcotic Drugs in March 2001 (201). Dozens of 2C-B analogs, such as 2C-I, 2C-C, 2C-F, 2C-E, and 2C-N, have later been sold as "research chemical" or "legal highs" *via* the Internet. Because their structure can be easily changed without losing their psychoactive properties, 2C drugs have, thus, often been referred as a typical class of designer drugs (201). 2C drugs commonly do not only act as 5-HT_{2A}-R and 5-HT_{1A}-R agonists but also as monoamine transporter inhibitors (195). Consequently, these compounds have not only hallucinogenic properties but also slight stimulating and empathogenic/entactogenic effects sometimes mimicking the effects of the prototypical empathogen MDMA (199). Shulgin also described novel ergolines such as *N*-allyl-nor-lysergic acid diethylamide (AL-LAD), *N*-ethyl-nor-lysergic acid diethylamide (ETH-LAD), and *N*-propyl-nor-lysergic acid diethylamide (PRO-LAD) (200). These LSD-analogs are as potent as LSD (potency relative to LSD in human: AL-LAD: 110%, ETH-LAD: 140%, PRO-LAD: 90%), but AL-LAD and PRO-LAD have shorter duration of action (6–8 h) as ETH-LAD and LSD (both: 8–12 h) (189, 200). Finally, Shulgin synthesized a large number of novel tryptamines, such as 4-hydroxy-*N*-methyl-*N*-ethyl-tryptamine (4-HO-MET), 5-methoxy-diisopropyltryptamine (5-MeO-DIPT), and alpha-ethyltryptamine (alpha-ET), which are mostly hallucinogenic, but with some exceptions (e.g., alpha-ET has pronounced empathogenic effects) (200). Shulgins books PIHKAL and TIHKAL served as cook books for a generation of illegal drug laboratories. His dimethoxyphenethylamines, 2C drugs, and novel ergolines and tryptamines are still circulating as NPS, although they have been created at least 20 years ago. However, their human toxicology and their consequences are still unknown as they are neither used frequently nor purely enough in order to systematically investigate their chronic effects in recreational users.

In the last decade, a substantial amount of new serotonergic hallucinogens appeared on the drug markets. As their number grows each day, it is simply not possible to list them exhaustively here. Thus, only some prototypical exponents of each class will be discussed. Again the main classes are either tryptamines and related ergolines or substituted phenethylamines but also some new classes such as benzodifurans and aminoindanes occurred (202–205). Novel tryptamines such as alpha-methyltryptamine (AMT), *N*,*N*-diallyl-5-methoxytryptamine (5-MeO-DALT) have multiple serotonergic actions including strong affinity for the 5-HT_{2A}-R, but can also act as monoamine transporter substrates. They combine hallucinogenic effects with stimulant and empathogenic features (203, 205). Novel ergolines such as 1-propionyl-lysergic acid diethylamide (1P-LSD) and lysergic acid 2,4-dimethylazetidide (LSZ) are LSD-analogs mainly interacting with 5-HT_{2A}-R and 5-HT_{1A}-R subtypes. They are slightly more potent as LSD and have a comparable duration of action. They are also mostly marketed as blotters (202, 205). *N*-2-methoxybenzyl derivatives of 2,5-dimethoxy-substituted phenethylamines also called NBOMe drugs, such as 25B-NBOMe, 25C-NBOMe, 25I-NBOMe, 25T2-NBOMe, and mescaline-NBOMe, are highly potent 5-HT_{2A}-R full agonists. In addition, they show a high-binding affinity to the 5-HT_{1A}-R, to adrenergic α1A and α2A, and histamine H1 receptors. Some derivatives also possess low-to-moderate affinity to DA D2- and D3-Rs. Several NBOMe drugs show higher affinity, higher activation potency, and higher activation efficacy at 5-HT_{2A}-Rs than LSD. Anecdotal user reports consider them as very strong hallucinogens (195, 205, 206). Benzodifurans, the so-called "fly drugs," such as 2C-B-FLY, 3C-Bromo-Dragonfly, and TFMFly, are a group of ring-substituted phenethylamines that are structurally related to MDMA. Unlike MDMA, benzodifurans commonly display a high affinity for 5-HT_{1A}-Rs, 5-HT_{2A}-Rs, 5-HT_{2B}-Rs, and 5-HT_{2C}-Rs, but show only little action at monoamine transporters (195, 205). Aminoindanes, such as 5-iodo-2-aminoindane (5-IAI), are usually 5-HT and noradrenaline (NA) releasers that have been sold as a legal surrogate for MDMA (203, 205). At least 5-IAI was recently demonstrated to show a strong affinity for 5-HT_{1A}-Rs and 5-HT_{2A}-Rs, thus, indicating that aminoindanes can not only be empathogens, but they can also display hallucinogenic properties (207).

At the moment, systematic investigations on the prevalence of novel serotonergic hallucinogens are rare. In the global drug survey of 2012, 11.3% of the respondents, mainly regular drug users, reported to have used a 2C drug at least once during their lifetime and that 2C-B was the most common one (8.4%). Moreover, 2.6% of respondents reported to have used 25B-NBOMe, 25C-NBOMe, or 25I-NBOMe at least once, while 25I-NBOMe (2.0%) was the most popular derivate. The most common drug source for NBOMes was the Internet (41.7%). For comparison, 39.4% of the respondents in this survey had used LSD and 43.1% "magic mushrooms" at least once during lifetime (206). A recent representative survey in the US ($N = 213.076$) revealed that the lifetime prevalence of novel hallucinogenic drugs was generally low: NBOMes, 0.015%; 2C drugs, 0.195%; dimethoxyphenethylamines, 0.019%; novel tryptamines, 1.060% (primarily DMT) (208). It should be noted that DMT was the only hallucinogenic NPS that was systematically asked for but that participants were given the opportunity to type in the names of NPS they used, indicating that these numbers are likely underestimated (208).

Data from the European Drug Emergencies Network have recently shown that, compared to all other investigated drugs, novel tryptamine users have the highest risk [odds ratio (OR) = 12.4] to be treated for psychosis-like symptoms in an emergency care unit, while also LSD use was significantly associated with an increased psychosis risk (OR = 3.1) (209). Overall frequencies for the development of acute psychosis following experimentally administered LSD range between 0.08 and 4.6%, while patients having a psychiatric disorder before LSD intake displayed the highest risk (185). However, if 5-HT hallucinogens can also induce long-lasting psychotic disorders is still controversially discussed (185). Beyond acute psychotic reactions including hallucinations, ego impairment, and

paranoia, also "bad trips," panic attacks, confusion, agitation, aggression, and disorientation are common acute psychiatric side effects of classical and novel serotonergic hallucinogens (185, 203, 205, 210). Moreover, also nausea and vomiting, serotonin syndrome including hyperthermia, liver and kidney failures, and cardiovascular complications have been reported for serotonergic hallucinogens. The acute toxicity of high potency dimethoxyphenylethylamines, NBOMEs, and 2C drugs seems to be considerably increased compared to classical hallucinogens. High potency compounds have been associated with a number of life-threatening conditions, such as rhabdomyolysis, seizures, vasoconstriction/hypertension, tachycardia, pulmonary edema, and serotonin syndrome with hyperthermia and organ failures, sometimes with fatal outcome (210–214).

Chronic side effects of hallucinogens can include panic disorder and a hallucinogen persisting perception disorder (HPPD, "flashback") (185). In fact, 60% of LSD users know "flashbacks" and 4% of users report sustained HPPD of putative clinical significance (215). Also, MDMA users are at risk to develop HPPD (216). It is highly likely that potent novel serotonergic hallucinogens bear a strong risk to induce HPPD too. Changes of 5-HT_{2A}-R function in the visual cortex were claimed to be responsible for HPPD (185, 216). In general, 5-HT-Rs show considerable plasticity after exposure to serotonergic drugs. Accordingly, due to post-transcriptional mechanisms, 5-HT_{2A}-Rs show a rapid and long-lasting downregulation in response to 5-HT agonists (217–219). Specifically, LSD, 2-bromo-LSD, and DOI selectively reduce 5-HT_{2A}-R density without affecting 5-HT_{2C}-Rs (220). Furthermore, hallucinogens acting at 5-HT_{2A}-Rs show strong behavioral tolerance coinciding with a robust decrease in 5-HT_{2A}-Rs. This might explain the strong tolerance effect of 5-HT hallucinogens (221). Recently, it was shown that 5-HT hallucinogens can also reduce either 5-HT_{2A}-R binding sites or glutamate-binding sites and that tolerance effects were correlated with changes in both binding sites (222).

High potency 5-HT hallucinogens—specifically if they have a long duration of action—are probably neurotoxic due to their sustained activation of 5-HT_{2A}-Rs that can induce apoptosis in neurons (223). Neurotoxic effects have been shown not only for DOI (224, 225) and 5-MeO-DIPT (226) but also for chronic low doses of LSD (227) and repeated high doses of MDMA (223–225). Thus, it is likely that all long-acting dimethoxyphenylethylamines, 2C drugs, NBOMes, tryptamines, and ergolines with strong agonistic actions at 5-HT_{2A}-Rs have a neurotoxic potential.

In conclusion, beyond LSD, mescaline, and psilocybin, a vast amount of new serotonergic hallucinogens appeared on the drug market during the last decades. Their distribution has strongly increased and will likely further increase in the future due to their easy availability on the Internet. Alarmingly, little is known about the acute and chronic effects of novel 5-HT hallucinogenic drugs in human users. The neuropsychiatric long-term consequences of regular intake of such compounds are completely unclear. However, it is becoming increasingly apparent that high potency drugs with very strong affinities to 5-HT_{2A}-Rs and long durations of action bear a considerable risk for negative health effects and fatalities.

MEPHEDRONE AND METHYLONE

Dozens of research chemicals with a cathinone basic structure appeared as "legal highs" on the drug marked. However, an exhaustive discussion of all of them is not possible here due to space restrictions (228). Thus, in this section, the two generic compounds, mephedrone and methylone, are discussed as important examples.

Mephedrone (4-methylmethcathinone) is a substituted cathinone homolog of ephedrine first described in 1929 (229, 230). Mephedrone has a ring-substituted cathinone structure which is related to the phenethylamine family, to which also drugs such as amphetamine, MDMA, and methamphetamine belong to (231). As a hydrochloride salt, mephedrone is a water soluble white, yellow, beige, or brown powder. In the European market, it is sold under different names such as Meow Meow, Bubbles, Mef, MMC Hammer, and many more (231). Mephedrone is available on the Internet, or from street dealers. On Internet sources, mephedrone is often marketed as bath salt, plant fertilizer, or research chemical (232, 233).

Mephedrone was first identified as an abused drug by European authorities in 2007 (234, 235). By 2010, mephedrone use spread, and the drug was found in many European countries (236). The use of mephedrone increased rapidly in the club scene and soon reached the level MDMA and cocaine use, reaching a life-time use in Europe among the 15- to 24-year olds of 6% by 2010 (236, 237). Mephedrone is frequently used together with other synthetic cathinones, such as methylone, butylone, or ethylcathinone (236). The predominant user populations are teenagers and young adults (238), thereby use of new psychoactive cathinones is highly correlated with binge-drinking habits in young adults (239).

Mephedrone can be consumed by different routes. In an oral preparation, mephedrone powder is rolled up in cigarette paper (bombing). Furthermore, intranasal, intramuscular, intravenous (slamming), and rectal routes of administration have been reported (240). Mephedrone is also mixed with other drugs, such as heroin, alcohol, cocaine, MDMA, or cannabis (235, 241). Consumption usually takes place in a social context at home, at rave parties, clubs, or music festivals. Mephedrone binge consumption has been reported to last for up to 9 h with a new dose all 0.5–2 h (231). Intranasal mephedrone elicits rapid effects within minutes. They reach a peak level in less than 30 min and last for up to 1 h. Orally applied mephedrone powder or tablets induce psychoactive effects in 45–120 min which may last for 2–4 h, thereby a sequence of first intranasal snorting followed by repeated oral ingestion has been reported, in order to achieve both, fast and long-lasting effects (231, 240, 242, 243). The sought-after psychoactive effects of mephedrone comprise an elevated mood, the feeling of an intense euphoria, a sense of well-being, increased self-esteem, motor excitation, reduced tiredness, increased alertness and concentration, talkativeness, empathy, disinhibition, and a mild sexual stimulation (231, 244, 245).

A high dose and/or chronic consumption of mephedrone have been associated with significant adverse effects. Those include cardiovascular, gastrointestinal, and neurological side effects

(233, 246). Well-described effects are also jaw clenching, reduced appetite, increased body temperature, increased sweating, abnormal vision, dilated pupils, headaches, tachycardia, palpitations, hypertension, arrhythmias, chest pain, nausea, bruxism, teeth grinding (bruxism), rhabdomyolysis, and renal failure (247). An important dangerous side effect is the significant hyponatremia. This is similar to that shown after acute MDMA consumption. It is supposed to be induced by a combination of sweating, electrolyte loss, and antidiuretic hormone secretion (247). The intranasal application of mephedrone is associated with a significant nasal irritation. Mephedrone addiction is often associated with intravenous drug use that is also found to be linked to an increased risk of using other addictive drugs (248). Intravenous mephedrone injections often result in vein blockages, leading to localized infections, blisters, abscesses, scabs, lumps, gangrenous tissue, blood clots, and large necroses at the injection site (249). Major adverse psychiatric effects associated with mephedrone use include agitation, anxiety, dysphoria, depression, insomnia, hallucinations, paranoia, delusions, aggressive behavior, as well as suicidal ideation and suicidal action. Cognitive impairments affect short-term memory and attention span (250). Psychotic effects predominantly occur after a high mephedrone dose, after binge consumption in one session, and in users with an individual vulnerability for psychiatric disorders (251–253). Fatalities resulting from mephedrone use have been reported worldwide now (254). They are related to hyponatremia and brain edema (255–257). However, the lethal dose (LD_{50}) is not known yet (258).

Accumulating evidence suggests that mephedrone has a clear addiction potential (246, 259, 260). The abuse potential for intranasally consumed mephedrone was suggested to be comparable with that of cocaine or methamphetamine (246). Among regular users, about 50% reported an addiction to the drug (261) and about 25% admitted mephedrone-related craving (262). Mephedrone withdrawal effects include tiredness, insomnia, impaired concentration, irritability, tremor, temperature dysregulation, palpitations, headaches, depression, anxiety, and paranoia (235, 244, 260).

Virtually all synthetic cathinones are considered to inhibit the monoamine uptake in the brain, thereby mephedrone acts as a substrate for the transporter proteins and evokes a reverse neurotransmitter transport and, thus, neurotransmitters release (231, 244, 263).

Synthetic cathinones including mephedrone are now classified as illicit substances in many countries (231). However, since the legal ban of single substances came in place, various second-generation analogs have appeared, including 4-methyl-*N*-ethylcathinone (4-MEC). The consumption may in the long term only effectively be limited when whole substance classes, i.e., with a cathinone lead structure, are legally controlled (231).

Methylone (3,4-methylenedioxymethcathinone) is a substituted cathinone methylated on the amine group of the ketophenethylamine backbone. It has a chemical structure similar to that of MDMA by a methylenedioxy ring attached to the aromatic ring (264). Methylone was first synthesized in 1996 as a potential antidepressant and anti-Parkinson agent (265), which, however, never made it into pharmacotherapy. Instead, it emerged on the street market under different names, such as Ease, Explosion, M1, MDMC, and bk-MDMA (231, 246). Methylone was marketed initially in a liquid solution as a vanilla-scented room odorizer. Following its introduction in 2004, methylone could be purchased in the Internet and in headshops (266), where it was sold in powder form and as tablets (267). Methylone use has been reported to be high in the club scene (261) and in addicts on substitution therapy (267).

Similar to other cathinones, methylone can be administered by different routes, such as orally, intranasally, intravenously, sublingually, or rectally. The most popular route is the oral administration. A common application pattern is to start with a large "boosting" dose and then maintain effects by smaller "bumping" doses (268, 269). The onset of the desired psychoactive effects of methylone is usually 15–60 min after oral administration. These effects last approximately 30–45 min (268). They have been described as an amphetamine-like stimulation with calm euphoria, happiness, thought acceleration, alertness, restlessness, reduced fatigue, and increased locomotor activity. They might also involve MDMA-like entactogenic effects with a strong sense of emotional openness, enhanced empathy, and reduced fear (270). A methylone high can be from moderate to extreme euphoria with tingling sensation (231, 268).

The adverse effects of methylone include anxiety and psychosis with derealization, depersonalization, hallucinations, and suicidal ideation. Cognitive impairments affect the short-term memory (258). Furthermore, methylone may induce seizures and hyponatremia, similar to that induced by MDMA. Methylone may also induce a hyperthermia (271). This is believed to be a major cause for the fatal consequences of a methylone overdose (272). Other factors in fatal overdose can be cardiac events, metabolic acidosis, rhabdomyolysis, acute renal failure, intravascular coagulation, and a serotonin syndrome (273–276).

Accumulating evidence suggests a considerable addictive potential of methylone (231, 277). Much like mephedrone, methylone acts as a monoamine reuptake blocker that leads to a profound hyperactivity of DA, 5-HT, and NA in the brain and periphery (263). In particular, dopaminergic and serotonergic adaptations in the brain may drive the addiction potential of psychostimulant drugs (278, 279). The use and abuse of the substance emerged with considerable side effects around the world (268). The legal ban of methylone started in 2007 with now an increasing number of countries controlling it (231).

KETAMINE AND NOVEL DISSOCIATIVE DRUGS

(±)Ketamine (±2-chlorophenyl-2-methylamino-cyclohexanone) is a non-competitive antagonist of the NMDA receptor (27). It has been widely used in clinical settings as an anesthetic agent and in veterinary medicine. However, ketamine is also recreationally consumed in entertainment settings for its hallucinogenic, mood enhancing, and reinforcing properties by young club goers (28, 280–282). Ketamine is a derivative of phencyclidine (PCP), which was discovered as anesthetic in 1956 and became a popular street drug during 1960s (280). Ketamine is regulated in many countries due to its abuse potential as a psychotropic substance

(282). A significant number of studies have demonstrated that ketamine has a short-acting antidepressant effect and is increasingly used to treat therapy-refractory major depression and pain (29, 283–285). Although ketamine is viewed as a safe substance in medical settings, its recreational use is reported to impose adverse effects on users by producing neurological and peripheral toxicity (286, 287).

Ketamine can be administered through intravenous, intramuscular, smoking, and snorting routes (288). Apparently, snorting or intranasal use is the main route of ketamine consumption among recreational users (289). Ketamine produces dose-dependent effects. Lower doses are associated with a feeling of relaxation. At higher doses, ketamine induces a dream-like state called a "*k-hole*." This experience is akin to dissociative anesthetic characteristics (290, 291). Chronic ketamine use is reported to induce schizophrenia-like positive and negative symptoms, including hallucinations, detachment, delusion, auditory, and verbal hallucinations (292). A major concern of ketamine use is that people drive under the influence of the drug (293). Ketamine can impair cognitive functioning, such as executive and memory function, as well as attentional control (294, 295). Ketamine users are also more vulnerable to HIV infections. The use of the drug is reported to enhance sexual experience and predispose users to engage in unprotected sex (296, 297). Ketamine-related mortality has increased 10-fold in the UK from 1999 to 2008 (287), while in Australia 40% of party drug users were tested positive for ketamine use (281).

Some of the most common complaints of ketamine use include chest pain, palpitations, and tachycardia (298). However, these symptoms are often transient (286). Abdominal pain and urinary tract symptoms, such as suprapubic pain, dysuria, and hematuria, are common symptoms of chronic regular ketamine use (299–301). Findings from clinical case studies have shown that ketamine use can decrease bladder volume, bladder wall thickening, mucosal enhancement, dilation of ureter, and cause perivesical inflammation (302, 303). The renal toxicity of ketamine is due to the direct toxic action of ketamine and its metabolites (288).

Fatigue, poor appetite, drowsiness, craving, anxiety, sleeping problems, and dysphoria are common physical and psychological side effects of ketamine use (304, 305). Currently, there is no specific treatment for ketamine users presenting with peripheral toxicity. However, it was reported that cessation from ketamine abuse may lead to a recovery from organ damage (28). Despite its abuse potential and reported side effects, ketamine has promising medicinal properties. Currently, it is used to treat therapy-refractory depression (306), although the antidepressant effect of a single infusion only last for some days. Despite that development, which moved the drug increasingly out of the drug abuse focus, proper prevention strategies for young club goers engaged in recreational ketamine use are still warranted. Moreover, addiction experts warned recently that psychiatrist should not underestimate the addictive potential of ketamine when treating depressive patients with the drug (307).

Dissociative anesthetics such as PCP and ketamine are non-medically used since more than 60 years (280). Importantly, "dissociative anesthetics" are originally defined as substances inducing a general form of anesthesia characterized by analgesia, amnesia, and cataplexy, but with minimal effect on respiratory function (308). Today, the term "dissociative drugs" includes the family of dissociative anesthetics but is not restricted to them. It more generally denotes hallucinogenic drugs inducing dissociative states, including sensory alterations and hallucinations as well as dream-like states or trance (280). More than 14 known derivatives of PCP have been marketed for non-medical but also illicit use already between the late 1960s and the 1990s. However, with the advent of online drug shops selling "legal highs," novel dissociative drugs appeared too. Starting with the first dissociative, 4-MeO-PCP in 2008, thenceforth at least 12 novel dissociative drugs appeared on the drug marked, which were unknown in the scientific literature prior to their introduction to the drug market (280). In the meantime, the most common agents, methoxetamine (MXE), diphenidine, methoxphenidine (MXP), 3-MeO-PCP, and 4-MeO-PCP, have reached widespread use in Europe and North America.

PCP, ketamine, and its novel derivatives belong to the chemical class of arylcyclohexylamines, which have in common that they act as non-competitive antagonists at the PCP-binding site of the NMDA receptor (280). Beyond their high affinity for NMDA receptors, some of the arylcyclohexylamines have shown agonistic actions at DA receptors (e.g., D2 receptors) and inhibitory effects at DA transporters, agonistic effects at μ-opioid and σ-1 receptors, as well as antagonistic actions at both nicotinic and muscarinic acetylcholine receptors. It is plausible that the specific receptor profile of each compound mediates its characteristic psychotropic effects (280). Beyond the desired dissociative acute effects, these drugs exert a number of severe and sometimes fatal side effects. Following MXE ingestion, users were confused, agitated, hallucinating, and unresponsive. The somatic and neurological adverse effects included tachycardia, hypertension, ataxia, mydriasis, nystagmus, seizures, leukocytosis, massive rhabdomyolysis, hepatic failure, onset of acute renal failure, sinus bradycardia, elevated creatinine kinase, and hyponatremia (210). Several fatalities have been reported each for MXE, MXP, 3-MeO-PCP, and 4-MeO-PCP (210, 240). According to anecdotal reports, MXE and MXP seem to have stronger empathogenic and euphorigenic properties than PCP and ketamine (210).

Novel dissociative drugs from the arylcyclohexylamine class, such as MXE, have been sold as a "legal" and "bladder friendly" alternative to ketamine. However, animal studies have shown that MXE and likely all arylcyclohexylamines are in fact equally toxic for the bladder and the kidneys as ketamine when applied chronically (240). Further chronic side effects of novel arylcyclohexylamines have not been investigated yet, but it is likely that the total class might have an addictive potential similar to that of ketamine and PCP (309, 310). This seems to be specifically high in adolescents and young adults (311). Moreover, like PCP and ketamine, all arylcyclohexylamines with a strong action at the NMDA receptor may impair memory function (310) and induce psychotic symptoms after acute and chronic consumption (312, 313).

γ-HYDROXYBUTYRATE, γ-BUTYROLACTONE, AND 1,4-BUTANEDIOL

γ-Hydroxybutyrate (GHB, or sodium oxybate), γ-butyrolactone (GBL), and 1,4-butanediol (1,4-BD) are potent central depressant agents with a broad spectrum of subjective, behavioral, and neuropharmacological effects in humans. These drugs are used clinically for the treatment of neuropsychiatric disorders such as narcolepsy, alcohol withdrawal, and fibromyalgia but also instrumentalized illicitly for hedonic purposes (314).

GBL and 1,4-BD are rapidly metabolized endogenously to GHB. The psychoactive effects of the drug result from this conversion (315, 316). GHB is an endogenous short-chain fatty acid. It is biosynthetically derived from γ-aminobutyric acid (GABA) which occurs naturally in the mammalian brain, mainly in the hypothalamus and the basal ganglia (317, 318). The molecule binds to GABA-B receptors (319) and to specific GHB receptors (320). Due to the presence of endogenous GHB in the brain, specific G-protein-coupled GHB receptors, and the specificity of the GHB antagonist NCS-382, GHB is considered to be a neurotransmitter (321). While physiological concentrations of GHB seem to be insufficient to stimulate GABA-B receptors, the subjective and behavioral effects of the exogenously applied drug, and thus GBL and 1,4-BD, result from direct stimulation of these receptors (322). Moreover, GHB has extensive downstream effects on DA, 5-HT, NA, glutamate, and acetylcholine transmission (323). GHB, GBL, and 1,4-BD are well absorbed orally in humans. Peak plasma concentrations are reached within 25–60 min, with a half-life of 20–60 min (324, 325). All compounds are metabolized to water and carbon dioxide through the citric acid cycle (326).

In humans, the spectrum of the subjective effects of these compounds ranges from euphoria, stimulation, and disinhibition in oral doses of 10–25 mg/kg (327–329), toward heavy sedation and loss of consciousness at oral doses of 35–70 mg/kg (324, 330). A seemingly paradoxical pattern of concomitant sedation and stimulation was described in several reports (327, 328).

GHB, GBL, and 1,4-BD strongly influence behaviors related to core autonomic functions, such as the control of food intake, sexual behavior, and sleep–wake regulation (314). GHB was reported to normalize dysfunctional food intake behavior and body weight in preclinical and in clinical studies (331–334). It was effective in the treatment of binge-eating disorder (335). Confirming subjective reports from illicit GHB users (336–338), the drug was experimentally shown to have prosocial (328), and prosexual effects in healthy male subjects (339). Moreover, GHB and its precursors have a unique effect on sleep–wake regulation (340). Since GHB improves sleep and daytime vigilance, it is used as standard treatment for disorders of the sleep–wake cycle, such as narcolepsy and fibromyalgia (341–343).

Neuropharmacological studies with GHB, GBL, and 1,4-BD are scarce and were until recently limited to early EEG investigations. Resting state EEG studies showed a paradoxical EEG-behavioral dissociation with the occurrence of increased delta and theta oscillations, during wake states, which usually occur during sleep (344, 345). Moreover, increased nocturnal slow wave sleep under the influence of GHB was demonstrated (346). A recent EEG study showed increased current source density of theta oscillations in the posterior cingulate cortex and alpha oscillations in the anterior cingulate cortex (ACC) under 20 and 35 mg/kg GHB in healthy male subjects (347). In the first functional neuroimaging study with GHB, 35 mg/kg of the drug increased regional cerebral blood perfusion in the ACC and the insula, both of which correlated with increased subjective ratings of emotion and body awareness (348). Moreover, the drug increased the susceptibility of the mesolimbic reward system, resulting in an increased sexual arousal after the presentation of erotic but also neutral pictures of persons. This effect correlated with an increased activity in the nucleus accumbens and the ACC (349).

The euphoric, prosocial, and prosexual effects of GHB, GBL, and 1,4-BD are instrumentalized illicitly, mostly by members of urban subcultures (314). Internationally, GHB, GBL, and 1,4-BD are mainly used as recreational drugs by young adults aged 20–29 years (349, 350). Reliable prevalence data are difficult to obtain (351). However, the prevalence of GHB, GBL, and 1,4-BD seem low compared to other drugs of abuse and are estimated at about 4.3% in Europe (349). After GHB was used in a deadly case of drug-facilitated sexual assault in the USA in the year 2000, the drug was internationally banned (352). However, a recent meta-analysis showed that GHB is very infrequently used as a date rape drug (353).

The development of addiction after illicit use of these drugs was estimated for about 4–21% of illicit users (351). Both addiction and withdrawal can be severe and in extreme cases lead to psychosis, delirium, and death (314). Interestingly, the development of addiction after medical use of GHB is at a very low rate with an estimated risk of about 0.015% (351).

Internationally, GHB is approved for the treatment of narcolepsy with cataplexy. In a recent meta-analysis, it was confirmed to be effective in treating major, clinically relevant narcolepsy symptoms and sleep architecture impairments in patients (354). Another clinical indication is the treatment of alcohol withdrawal, for which GHB is used since two decades in Italy and Austria (355). Moreover, several randomized controlled trials showed a therapeutic effect of GHB on clinical course and life quality in patients suffering from fibromyalgia (343, 356–358). Other neuropsychiatric disorders in which GHB showed therapeutic effects are binge-eating disorders (335), schizophrenia (359), Parkinson's disease (360), and cluster headache (361, 362), mostly by regulating homeostatic dysbalances, as well as improving sleep and pain symptoms. Because disrupted homeostatic processes including food intake, sexual behavior, and the sleep–wake cycle frequently occur in major depressive disorder, GHB was proposed as an experimental therapeutic in this condition (363, 364). However, therapeutic use of the drug is limited by side effects, such as nausea, vomiting, altered consciousness, and nocturnal O_2 desaturations (357, 365–367).

In conclusion, GHB and partially its precursors GBL and 1,4-BD have undeniable caveats such as limiting side effects and abuse liability. These, however, seem to be outweighed by a unique spectrum of clinically relevant psychopharmacological effects, which warrant further studies in neuropsychiatric conditions such as major depressive disorder following a personalized treatment paradigm (368).

CONCLUSION

The amount of evidence on the psychoactive drugs discussed shows that many of them are not really novel anymore. For most of them a classification in terms of their use and harm potential has been made. In fact, most of them are already legally controlled or banned in certain countries. An important feature of this process is that it appears socio-geographically biased. Even in a globalized world, new psychoactive drugs emerge and spread in a regionally bound way. This brings about that evidence on their use, instrumentalization, and abuse accumulates often only regionally. Also, the drug may for a long time not spread beyond the socio-geographic boundaries. However, this does not mean that it is not eventually "discovered" by other societies making use of the drug for a new and essentially different purpose. The scientific challenge is then to use locally gathered knowledge to be prepared for a drug that is novel in a certain culture and establish a judgment on its harm potential and/or medical use on a rather global scale. It also means to delineate future research needs for those drugs that are brand new as a psychoactive drug. This review shows that despite accumulating evidence, for many of those NPSs, a final classification is still in progress and gathering of more evidence is pivotal.

AUTHOR CONTRIBUTIONS

CM and ZH planned the review article and contributed to several chapters. CM prepared the final version of the manuscript. CM, ZH, OB, DS, SN, BK, ES, JK, and BQ wrote single chapters of the manuscript.

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REVIEW
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An Expanding World of Novel Psychoactive Substances: Opioids

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The abuse of novel psychoactive substances (NPS) has been increasing dramatically worldwide since late 2000s. By the end of 2015, more than 560 NPS had been reported to the European Monitoring Centre for Drugs and Drug Addiction. Although the most popular compounds are synthetic cannabinoids and psychostimulatory derivatives of cathinone (so-called β-keto-amphetamines), novel synthetic opioids have recently emerged on the recreational drug market. They include fentanyl (a potent narcotic analgesic) and its analogs (e.g., acetylfentanyl, acryloylfentanyl, carfentanil, α-methylfentanyl, 3-methylfentanyl, furanylfentanyl, 4-fluorobutyrylfentanyl, 4-methoxybutyrylfentanyl, 4-chloroisobutyrylfentanyl, 4-fluoroisobutyrylfentanyl, tetrahydrofuranylfentanyl, cyclopentylfentanyl, and ocfentanil) and compounds with different chemical structures, such as AH-7921, MT-45, and U-47700. This survey provides an overview of the pharmacological properties, pattern of use, and desired and unwanted effects of the above-listed novel opioids. Special emphasis is given to cases of non-fatal and lethal intoxication involving these compounds.

Keywords: novel psychoactive substances, opioids, fentanyls, MT-45, AH-7921, U-47700, toxicity, naloxone

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INTRODUCTION

The last decade has seen a worldwide surge in the recreational use of novel psychoactive substances (NPS). Although various products are labeled with warnings "not for human consumption," they are intended to mimic the psychoactive effects of illicit drugs of abuse. Between 2008 and 2015, a total of 644 NPS were reported by 102 countries to the United Nations Office for Drugs and Crime (1) and by the end of 2015, a total of 561 of NPS had been notified to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) (2). While NPS can be purchased online, from head shops or drug dealers, buying drugs through the internet, both from freely accessible websites, and more recently, from the so-called dark web, has become increasingly popular (3, 4).

Novel psychoactive substances are mainly of synthetic origin (e.g., derivatives and analogs of existing controlled drugs and analogs of pharmaceutical products) and comprise different drug classes, including, among others, synthetic cannabinoids, synthetic cathinones, phenethylamines, piperazines, ketamine- and phencyclidine-type substances, tryptamines, benzofurans, and opioids (5). Although the most popular of these have been cannabinoids and designer cathinones, recent years have seen the appearance of novel synthetic opioids on the recreational drug market (1, 2, 6). By analogy to other NPS groups, the primary motivation for using designer opioids is pleasure and enjoyment. In addition, the use of opioids is markedly motivated by habit, addiction, and coping with life challenges (7). Designer opioids pose an especially serious concern for public health as they are endowed with a high potency and are often sold under the guise of heroin to unsuspecting users (8). Many of them are derivatives of therapeutically used drugs, namely fentanyl. However, new synthetic

opioids such as AH-7921, MT-45, and U-47700, with structures distinct from those of known therapeutic or recreational drugs, have also emerged. They are used on their own or in combination with other opioids. The aim of the current contribution is to present updated information on the properties of novel synthetic opioids. Special attention is given to the acute toxic effects exerted by this group of NPS.

METHODS

A literature search was performed on two representative databases (PubMed and Google Scholar) and various governmental and institutional websites, using the following keywords alone or in combination: NPS, synthetic opioid, fentanyl, illicit fentanyls, names of particular designer analogs of fentanyl, MT-45, AH-7921, U-47700, toxicity, and naloxone. Only those articles that had abstracts available in the English language were included. All articles were screened from their abstracts to determine their relevance in the framework of the current review.

FENTANYL, CARFENTANIL, AND NON-PHARMACEUTICAL FENTANYLS (NPF)

Fentanyl, *N*-phenyl-*N*-[1-(2-phenylethyl)piperidin-4-yl]propanamide, was first synthesized by Paul Janssen and his research team from Janssen Pharmaceutical (Belgium) in 1960 as an opioid analgesic agent. It was introduced into medical practice as an intravenous anesthetic under the trade name of Sublimaze in 1960s (9). The drug is a potent agonist of μ-opioid receptors, with an activity 50–100 times higher than morphine. Fentanyl quickly crosses the blood–brain barrier due to its high lipid solubility; it has a rapid onset and short duration of action. The drug is used as a narcotic analgesic supplement in general and regional anesthesia as well as in management of severe chronic pain and postoperative pain (9). Fentanyl pharmaceutical products are available in forms of oral transmucosal lozenges (Actiq®), buccal tablets (Fentora™), sublingual tablets (Abstral®), sublingual spray (Subsys™), nasal spray (Lazanda®), transdermal patches (Duragesic® and generics), and injectable formulations. Fentanyl-containing transdermal patches are used to treat patients with chronic pain who require continuous opioid analgesia. The recommended serum concentration is 1–2 ng/mL for analgesia and 10–20 ng/mL for anesthesia (9, 10).

In addition to analgesia, fentanyl and its analogs (hereafter fentanyls) depress the respiratory system, constrict the pupils, and produce drowsiness and euphoria, the latter being less pronounced than with heroin and morphine (9, 10). The most common side effects include nausea, dizziness, vomiting, fatigue, headache, and constipation. Repeated use of fentanyls leads to the development of tolerance and dependence. Characteristic withdrawal symptoms include sweating, anxiety, diarrhea, bone pain, abdominal cramps, and shivers or "goose flesh" (9, 10). Due to the narrow therapeutic index, the use of fentanyls in the recreational drug scene is exceptionally dangerous, especially in opioid intolerant users. High doses might result in death due to respiratory arrest and pulmonary edema. Importantly, serious interactions can occur when fentanyls are mixed with heroin, cocaine, alcohol, and other CNS depressants, in particular benzodiazepines [e.g., Ref. (10, 11)]. The most common therapies administered to patients intoxicated with fentanyls include naloxone, oxygen, intubation, and intravenous fluids (10).

As fentanyl and its analogs are endowed with high abuse liability, dependence potential and toxicity (see below), all fentanyls approved for medical use are internationally controlled, as well as several compounds from this group that have never been developed into pharmaceutical products (12) (**Table 1**).

The first documented large-scale illicit use of fentanyl (street names: "China White," "Synthetic Heroin," "Drop Dead," "Flatline," "Lethal Injection," "Apache," "China Girl," "Chinatown," "Dance Fever," "Great Bear," "Poison," and "Tango & Cash") was in the USA, mainly in California, between 1979 and 1988 (13–15). Fentanyl exposures reported to the American Association of Poison Control Centers increased from 300 in 2010 to 1,724 in 2011, and since then have remained steadily high (1,632 in 2012, 1,486 in 2013, and 1,418 in 2014) (16). In March 2015, the United States Drug Enforcement Administration (DEA) issued a nationwide warning of fentanyl laced in heroin causing significant health problems across the USA (16). Between 2009 and 2014, there were at least 1,019 drug poisoning deaths in Canada where postmortem toxicological screening indicated the presence of fentanyl. More than half of these deaths occurred in the latter 2 years, 2013 and 2014 (17). In Europe, the first cases of fatal intoxication with fentanyl were reported in Sweden (18). Later on, illicit fentanyl use became a serious health problem in Estonia, with an estimated number of 1,100 deaths during 2005–2013 (19). Outside of Estonia, 180 fentanyl-related deaths were reported in Sweden (2006–2013), 160 in Germany (2007–2011), 70 in the UK (2007–2012), 40 in Finland (2008–2010), and five in Greece (2005–2011) (19). According to epidemiological data, the increase in use of fentanyls in Europe and in the USA was largely associated with the low availability, low purity, and/or high price of heroin, features that were at least partially linked to the imposition of Taliban control on opium production (10, 19).

Among various fentanyl-containing products that are available on prescription, abuse of transdermal fentanyl patches has received increasing attention in recent years. These patches can be misused in a variety of ways: (1) they can be placed in a glass containers, heated, and smoked, (2) gel contents removed from the patches can be injected or ingested, (3) fentanyl in patches can be scratched and smoked, (4) patches are simmered in a small volume of water and the obtained solution is injected intravenously, (5) frozen patches are cut into pieces and then

TABLE 1 | Fentanyl and its analogs controlled under the 1961 Single Convention on Narcotic Drugs (12).

Year	Compound
1964	Fentanyl
1980	Sufentanil
1984	Alfentanil
1988	α-Methyl-thiofentanyl, β-hydroxyfentanyl, β-hydroxy-3-methylfentanyl, 3-methylfentanyl, *para*-fluorofentanyl, thiofentanyl
2016	Acetylfentanyl

chewed, placed under the tongue, or in the cheek cavity for drug absorption through the oral mucosa or inserted into the rectum [e.g., Ref. (20–31)]. It has been reported that chewing a fentanyl patch could quickly decrease the user's level of consciousness and result in the intrabronchial aspiration of the patch, a clinical feature that intensifies fentanyl-induced breathing problems (26).

Carfentanil [methyl 1-(2-phenylethyl)-4-(*N*-propanoylanilino)piperidine-4-carboxylate; carfentanil, 4-carbomethoxyfentanyl], a fentanyl analog, was first synthesized by chemists at Janssen Pharmaceutical in 1974 and marked under the brand name Wildnil. The drug is a very potent agonist of opioid receptors, with the rank order of potency: $\mu >>> \delta > \kappa$. Binding studies found the calculated Ki values for human opioid receptors to be 0.024 nM (μ_1), 3.3 nM (δ), and 43 nM (κ). By comparison, Ki values for fentanyl were 1.9 nM (μ_1), 153 nM (δ), and 197 nM (κ).[1] It is estimated that the clinical potency of carfentanil is 10,000 times that of morphine, 4,000 times that of heroin, and 100 times that of fentanyl, making it one of the most potent known and the most potent commercially used opioids. The drug is approved to be used only by veterinarians as a tranquilizing agent for large wildlife animals, such as elephants and bears, for examination and procedures. The first confirmed case of a human being poisoned with carfentanil was published in 2010 (32). A 42-year old veterinarian was accidentally splashed in the eyes and mouth with a dart containing 1.5 mg carfentanil citrate and 50 mg xylazine. Despite immediate decontamination, the man became drowsy within 2 min. The patient was administered 100 mg parenterally of naltrexone and transported to the hospital, where he fully recovered (32). Recently, alarming reports from the USA and Canada show that carfentanil has been increasingly laced with or disguised as heroin. The drug has already been connected to hundreds of overdose cases, many of them fatal [e.g., Ref. (33–37)].

Several fentanyl analogs are clandestinely synthesized for recreational use (10, 12, 19, 38–40). These compounds have been developed by modification or replacement of the fentanyl's propionyl chain or by replacement of its ethylphenyl moiety. The obtained analogs have been further modified by substitution with fluoro-, chloro-, or methoxy- groups at the *N*-phenyl ring. Examples of fentanyls that have not been approved for medical use, the so-called NPF, are listed below (see also **Figure 1**).

- acetylfentanyl (*N*-phenyl-*N*-[1-(2-phenylethyl)piperidin-4-yl] acetamide; acetyl fentanyl, desmethyl fentanyl, MCV 4848, NIH 10485),
- acryloylfentanyl (*N*-phenyl-*N*-[1-(2-phenylethyl)piperidin-4-yl] prop-2-enamide, acrylfentanyl, acryloyl-F, Acr-F, ACF),
- α-methylfentanyl (*N*-phenyl-*N*-[1-(1-phenyl-2-propanyl) piperidin-4-yl]propanamide),
- 3-methylfentanyl (*N*-[3-methyl-1-(2-phenylethyl)piperidin-4-yl]-*N*-phenylpropanamide; mefentanyl, 3-MF),
- butyrylfentanyl (*N*-phenyl-*N*-[1-(2-phenylethyl)piperidin-4-yl]butanamide; butyl fentanyl; BF),
- 4-methoxybutyrylfentanyl (*N*-(4-methoxyphenyl)-*N*-[1-(2-phenylethyl)piperidin-4-yl]butanamide; 4-MeO-BF),
- 4-fluorobutyrylfentanyl (*N*-(4-fluorophenyl)-*N*-[1-(2-phenylethyl)piperidin-4-yl]butanamide; 4-FBF),
- 4-fluoroisobutyrylfentanyl (*N*-(4-fluorophenyl)-2-methyl-*N*-[1-(2-phenylethyl)piperidin-4-yl]propanamide; 4F-iBF),
- 4-chloroisobutyrylfentanyl (*N*-(4-chlororophenyl)-2-methyl-*N*-[1-(2-phenylethyl)piperidin-4-yl]propanamide; 4F-iBF),
- furanylfentanyl (*N*-phenyl-*N*-[1-(2-phenylethyl)piperidin-4-yl]-2-furancarboxamide; furafentanyl),
- cyclopentylfentanyl (*N*-(1-phenylethylpiperidin-4-yl)-*N*-phenylcyclopentanecarboxamide; CP-F),
- tetrahydrofuranylfentanyl (*N*-(1-phenethylpiperidin-4-yl)-*N*-phenyltetrahydrofuran-2-carboxamide; tetrahydrofuranfentanyl, THF-F), and
- ocfentanil (*N*-(2-fluorophenyl)-2-methoxy-*N*-[1-(2-phenylethyl)piperidin-4-yl]acetamide; ocfentanil, A-3217).

The reported doses and duration of action of fentanyls in comparison to morphine and heroin[2] are presented in **Table 2**.

As in the case of other NPS groups, new designer opioids quickly replace the scheduled ones. For example, following the ban of acryloylfentanyl in 2016, four new fentanyls, i.e., 4-chloroisobutyrylfentanyl, 4-fluoroisobutyrylfentanyl, tetrahydrofuranylfentanyl, and cyclopentylfentanyl, appeared on the Swedish drug market (38).

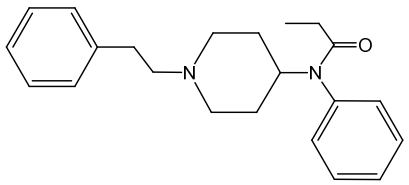
Commonly, fentanyls are sold as powders, nasal sprays, liquids, or in tablet forms. Clandestine opioids are often up mixed with heroin ("fake heroin") to masquerade heroin, included in cocaine products or black tar heroin, or pressed into counterfeit prescription pills (12, 40, 41).

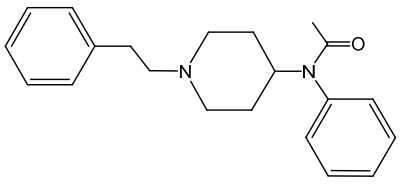
The first designer fentanyls were α-methylfentanyl and its more potent and dangerous successor, 3-methylfentanyl; the two substances which appeared on the illicit drug market in California in 1978 and 1984, respectively, laced in heroin products or as a heroin substitute [reviewed in Ref. (19)]. The slang terms for these two fentanyls include "China white," "China girl," "Persian white," "egg white," "crocodile," and "synthetic heroin." Importantly, 3-methylfentanyl is one of the most potent opioids that has been widely sold on the black market; its *cis*-(+)-isomer is approximately 7,000 times more potent than morphine, and the *trans*-(±)-isomer is about 1,000 times as potent (42). Numerous fatalities involving 3-methylfentanyl were reported in Estonia in the period 2004 to 2008. In the majority of cases, fentanyl was detected in blood samples together with 3-methylfentanyl [e.g., Ref. (43, 44)].

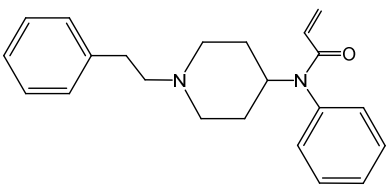
Three other illicit compounds from this class, acetylfentanyl, butyrylfentanyl, and 4-fluorobutyrylfentanyl, were first notified by the European Early Warning System in 2014. They were typically seized in powder form or tablet form, and, to a lower extent, in liquids and in capsules (31, 45, 46). As acetylfentanyl is often the first synthetic opioid used by those who want to try new opioids, it is colloquially called "the first Apostle of extinction" (47). Acetylfentanyl is five to 15 times more potent than heroin,

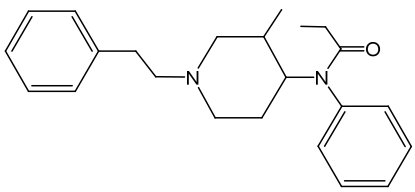
[1] https://www.bindingdb.org/jsp/dbsearch/PrimarySearch_ki.jsp?energyterm=kJ/mole&tag=lidki&monomerid=50012477&column=KI&startPg=0&Increment=50&submit=Search.

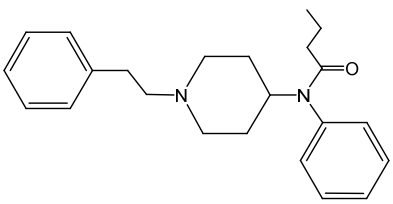
[2] http://drugs.tripsit.me/category/opioid.

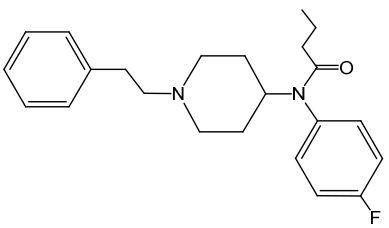


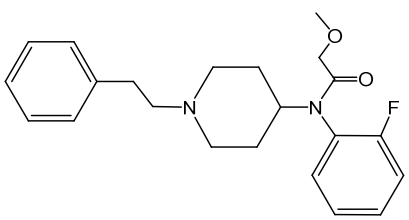












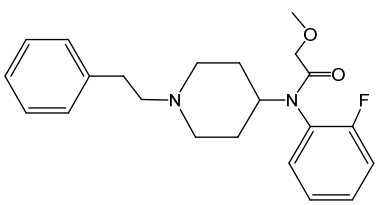


FIGURE 1 | Chemical structures of fentanyl and its analogs.

80 times more potent than morphine and 15 times less active than fentanyl (45, 48, 49), whereas the potency of butyrylfentanyl was found to be seven times higher than that of morphine and 13 times lower than fentanyl (46, 49, 50). Acetylfentanyl, butyrylfentanyl, and 4-fluorobutyrylfentanyl are typically administered orally, nasally (using sprays), by snorting, smoking, and by intravenous injection (19, 45, 46). By 2014, Germany, Poland, Sweden, and the UK had reported eight acute intoxications and 32 deaths associated with acetylfentanyl to EMCDDA (45). Furthermore, since 2012, acetylfentanyl has been found to be involved in at least 12 deaths in Russia, three in Japan, and more than 50 in the USA (51–59). From April to November 2015, 14 analytically confirmed intoxications with fentanyls (including one fatal) were reported to the Swedish STRIDA project (59). The concentrations of drugs in biological samples were as follows: acetylfentanyl ($n = 8$)—serum 0.6–51.6 (mean 18.3) ng/mL, urine 2.4–3,180 (mean 939) ng/mL; 4-methoxybutyrylfentanyl ($n = 3$)—serum 1.3–11 (mean 5.1) ng/mL, urine 15.8–1,000 (mean 348) ng/mL; and furanylfentanyl ($n = 2$)—serum 4.4 and 148 ng/mL, urine 1,779 and 1,430 ng/mL. Most of the cases were also positive for not only other drugs, mainly benzodiazepines, but also synthetic cathinones, amphetamine, cocaine, and other opioids (oxycodone, tramadol, and fentanyl) (59). Case reports of fatal intoxication involving acetylfentanyl are summarized in **Table 3**.

TABLE 2 | Doses and duration of action of synthetic opioids (see text foonote 2).

Route of administration	Dose			Action		
	Light	Common	Strong	Onset	Duration	After-effects
Morphine						
	5–10 mg	**15–20 mg**	**>30 mg**			
Insufflation				10–30 min	4–5 h	1–12 h
Intravenous/intramuscular				0–1 min	2–4 h	1–12 h
Heroin						
Insufflation	7.5–20 mg	20–35 mg	35–50 mg	10–15 min	3–6 h	1–24 h
Smoked	5–15 mg	15–25 mg		5–10 min	3–5 h	1–24 h
Intravenous		5–10 mg	8–15 mg	0–5 min	4–5 h	1–24 h
Fentanyl						
Intranasal	10–25 μg	25–50 μg	50–75 μg			
Transdermal	12.5 μg/h	25–50 μg/h	50–100 μg/h	2–4 h	48–72 h	
Buccal				15–30 min	1–4 h	
Insufflated				15–30 min	1–4 h	
Acetylfentanyl						
Oral	1–3 mg	3–5 mg	5–7 mg	Minutes	Hours	1–8 h
Acryloylfentanyl						
Insufflation	5–12.5 μg	12.5–25 μg	25–47.5 μg	1–5 min	10–30 min	1–2 h
Butyrylfentanyl						
Oral	0.4–0.8 mg	0.8–1.5 mg	1.5–3 mg	15–30 min	3–4 h	1–4 h
4-Fluorobutyrylfentanyl						
Insufflation	0.3 mg	0.6–0.9 mg	0.9–1.2 mg	Minutes	30–60 min	
4-Methoxybutyrylfentanyl						
Oral				5–15 min	45–120 min	1–2 h
Insufflation				1–2 min	30–75 min	1–2 h
Furanylfentanyl						
Oral	0.3–0.5 mg	0.5–0.9 mg	0.9–1.6 mg			
Insufflation	0.2–0.4 mg	0.4–0.8 mg	0.8–1.6 mg	1–10 min	1–3 h	1–3 h
AH-7921						
Oral	5–10 mg	10–25 mg	>25 mg	15–45 min	6–8 h	1–6 h
U-47700						
	5–7.5 mg	**7.5–15 mg**	**15–25 mg**			
Oral				15 min	5–7 h	1–4 h
Insufflation				15 min	3–4 h	1–4 h
Intravenous				0–1 min	1–2 h	1–4 h
MT-45						
Oral	30–45 mg	45–60 mg	>60 mg	30–45 min	4–6 h	2–3 h

Cole and coworkers present a history of an 18-year-old heroin abuser who had overdosed butyrylfentanyl (75). The man was found unconscious with labored breathing and taken to the emergency department (ED), where he was treated intravenously with 0.4 mg of naloxone. The patient developed a pulmonary edema, acute lung injury, and diffuse alveolar hemorrhage. The man stated that he had snorted what he believed to be acetylfentanyl, which he had purchased over the internet (75). In 2015, the DEA reported 40 confirmed fatalities associated with butyrylfentanyl from three states: Maryland (one), New York (37), and Oregon (one) (50). Other documented and published cases of fatal butyrylfentanyl overdose (60–62) are presented in **Table 3**. Two analytically confirmed cases of fatal intoxication with 4-fluorobutyrylfentanyl were recently reported in Poland by Rojkiewicz and coworkers (63) (see **Table 3**).

The first report of a furanylfentanyl-induced intoxication was recorded in December 2015 in the USA. From December 2015 through September 2016, a total of 494 forensic cases of furanylfentanyl, including 128 confirmed fatalities, were reported to the DEA (76). During a 4-day period (July 15–18, 2016), 43 patients in British Columbia, Canada, were diagnosed as intoxicated with crack cocaine contaminated with furanylfentanyl. Most of them were men, with an average age of 42 years (range 18–63) (77). A series of furanylfentanyl-related deaths that occurred in Sweden between 2015 and 2016 (64) are summarized in **Table 3**.

Ocfentanil was developed in early 1990s in an attempt to obtain an analgesic with less cardiovascular and respiratory side effects than morphine, but it has never been approved for medical use. The drug has recently been detected in the hidden market as an adulterant of heroin (78). Deaths involving ocfentanil were

TABLE 3 | Case reports of fatalities involving novel synthetic opioids.

Gender/ age	Case data	Toxicological findings	Ref.
Acetylfentanyl			
M/32	A deceased was found dead in the bed in a supine position. Snorting at least 12 h before death. Insufflation straws were found in his bag and in the drawers of a chest. At autopsy: pulmonary edema with mild to severe intraalveolar hemorrhage.	Acetylfentanyl was detected in heart blood, urine and gastric contents.	(52)
M/early 30s	A deceased was found at home, not breathing. A small plastic bag with a pale brown white powder and a syringe with a small amount of liquid were found at the scene. Acetylfentanyl and 4-methoxy-PV8 were detected in both the powder and the liquid. At autopsy: congested lungs, petechiae on eyelid conjunctiva, capsula cordis and pleura, fluidity of the heart blood, and two very recent forearm needle marks. History of habitual "bath salt" use.	Acetylfentanyl: femoral blood, 153 ng/mL; urine, 240 ng/mL; gastric contents, 880 ng/mL. 4-MethoxyPV8: femoral blood, 389 ng/mL; urine, 245 ng/mL; gastric contents, 500 ng/mL. Additionally in femoral blood: 7-aminonitrazepam (200 ng/mL), phenobarbital (7,700 ng/mL), methylphenidate (30 ng/mL), chlorpromazine, and risperidone.	(53)
M/24	A deceased was found unresponsive with uncapped syringe and rubber tourniquet. At autopsy: pulmonary congestion and edema, three recent punctures in left forearm. History of heroin abuse, with two previous overdoses.	Acetylfentanyl: peripheral blood, 260 ng/mL; heart blood, 250 ng/mL; vitreous humor, 240 ng/mL; urine, 2,600 ng/mL.	(55)
M/28	A deceased was found in the bathroom with a tourniquet secured around his arm and a syringe nearby. At autopsy: marked pulmonary and cerebral edema and needle track marks. History of illicit drug abuse.	Acetylfentanyl: subclavian blood, 235 ng/mL; vitreous humor, 131 ng/mL; urine, 234 ng/mL; liver, 2,400 ng/g.	(57)
M/20	A deceased was found dead at home. History of illicit drug abuse.	Acetylfentanyl: heart blood, 285 ng/mL; femoral blood, 192 ng/mL; urine, 3,420 ng/mL; liver, 1,100 ng/g; brain, 620 ng/g. Additionally in heart blood: methoxetamine and fluoxetine.	(58)
F/50	A deceased was found unresponsive in bed. History of bilateral knee replacement, chronic pain, depression and seizures, prescription drug abuse, and ethanol abuse.	Acetylfentanyl: heart blood, 219 ng/mL; femoral blood, 255 ng/mL; urine, 2,720 ng/mL. Additionally in heart blood: venfalaxine, nordiazepam, and chlordiazepoxide.	(58)
Butyrylfentanyl			
M/23	A deceased was found unresponsive in the bathroom. A tray with traces of white powder and a tube were found in the bedroom. At autopsy: cerebral edema and small amounts of residual white powder in the nose. History of drug use.	Butyrylfentanyl: peripheral blood, 66 ng/mL; heart blood, 39 ng/mL; liver, 57 ng/g; kidney, 160 ng/g, muscle, 100 ng/g.	(60)
F/53	A deceased was found unresponsive in the bathroom. At autopsy: edematous and congested lungs. History of smoking, prescription drug abuse, and psychiatric disorder hospitalization.	Butyrylfentanyl: peripheral blood, 99 ng/mL; heart blood, 220 ng/mL; vitreous humor, 32 ng/mL; bile, 260 ng/mL; urine, 64 ng/mL; gastric contents, 590 ng/mL; brain, 93 ng/g; liver, 41 ng/g.	(61)
Butyrylfentanyl and acetylfentanyl			
F/49	A deceased was found unresponsive and not breathing on the bed. At autopsy: edematous and congested lungs. History of anxiety, bipolar disorder, and two previous suicide attempts.	Acetylfentanyl: peripheral blood, 21 ng/mL; heart blood, 95 ng/mL; vitreous humor, 68 ng/mL; bile, 330 ng/mL; urine, 8 ng/mL; gastric contents, 28,000 ng/mL; brain, 200 ng/g; liver, 160 ng/g. Butyrylfentanyl: peripheral blood, 3.7 ng/mL; heart blood, 9.2 ng/mL; vitreous humor, 9.8 ng/mL; bile, 49 ng/mL; urine, 2 ng/mL; gastric contents, 4,000 ng/mL; brain, 63 ng/g; liver, 39 ng/g Additionally in peripheral blood: alprazolam, 40 ng/mL and ethanol, 0.11 g/dL.	(61)
M/44	A deceased was found unresponsive on the bathroom floor. A box with drug paraphernalia (used syringes, aluminum foil with black residue, scissors, and alcohol wipes) was found elsewhere. At autopsy: pulmonary edema and congestion, evidence of subacute and chronic intravenous drug use in the antecubital fosse, forearms, left wrist, and ankles. History of heroin use.	Butyrylfentanyl: peripheral blood, 58 ng/mL; heart blood, 97 ng/mL; vitreous humor, 40 ng/mL; urine 670 ng/mL; gastric contents, 170 mg/mL; liver, 320 ng/g. Acetylfentanyl: peripheral blood, 38 ng/mL; heart blood, 32 ng/mL; vitreous humor, 38 ng/mL; urine, 690 ng/mL; gastric contents, <170 mg/mL; liver, 110 ng/g.	(62)
4-Fluorobutyrylfentanyl			
M/26	A deceased was found dead at home. History of drug abuse.	4-Fluorobutyrylfentanyl: blood, 91 ng/mL; urine, 200 ng/mL; liver, 902 ng/g; kidney, 136 ng/g.	(63)
F/25	A deceased was found dead at home. History of occasional drug and novel psychoactive substances use.	4-Fluorobutyrylfentanyl: blood, 112 ng/mL; urine, 414 ng/mL; liver, 411 ng/g; kidney, 197 ng/g.	(63)

(*Continued*)

TABLE 3 | Continued

Gender/ age	Case data	Toxicological findings	Ref.
Furanylfentanyl			
M/26	A deceased was found dead in the bathroom. A tourniquet was found around his arm and a used needle next to the body. At autopsy: brain edema and pulmonary edema. History of drug abuse.	Blood (ng/mL): furanylfentanyl, 1.05; Δ^9-tetrahydrocannabinol (THC), 0.63; mirtazapine, 74.1; desmethylnitrazapine, 31.7; pregabalin, 6,032; buprenorphine, 2.01; norbuprenorphine, 2.86; clonazepam, 21.1; 7-aminoclonazepam, 624. Urine (ng/mL): buprenorphine, 30; norbuprenorphine, 180.	(64)
M/36	A deceased was found lying on the floor of the bathroom. At autopsy: pulmonary edema and froth in the airways. History of drug abuse.	Blood (ng/mL): furanylfentanyl, 7.66; pregabalin, 14,815.	(64)
M/37	A deceased was found lying in the ditch, with a body temperature of 25°C. An empty strip of zopiclone was found nearby. Resuscitation for 35 min was unsuccessful. At autopsy: generalized visceral congestion.	Blood (ng/mL): furanylfentanyl, 0.95; carbamazepine, 9,524; venlafaxine, 9,480; alimemazine, 317; promethazine, 63.5; desmethylpromethazine, 106; methyphenidate, 28.6; ritalinic acid, 762; acetaminophen, 8,466; pregabalin, 33,862; amphetamine, 116; 7-aminoclonazepam, 95.	(64)
M/26	A deceased was found dead on the couch. A used needle, a spoon, and a suspected drug were found at the scene. At autopsy: brain edema and pulmonary edema. History of drug abuse.	Blood (ng/mL): furanylfentanyl, 0.43.	(64)
M/26	A deceased was found dead in his apartment. Three nasal sprays suspected to contain fentanyl were found at the scene. At autopsy: pulmonary edema and froth in the airways. History of drug abuse.	Blood (ng/mL): furanylfentanyl, 0.78; carbamazepine, 14,815; pregabalin, 28,481; gabapentin, 94,937; norbuprenorphine, 1.37; fentanyl, 0.4; alprazolam, 42.2; alimemazine, 211; desmethylalimemazine, 211; diazepam, 31.6; methylphenidate, 4.2; ritalinic acid, 232. Urine (ng/mL): buprenorphine, 6; norbuprenorphine, 30.	(64)
M/27	A deceased was found dead in an apartment shared by drug abusers. History of suicide attempts.	Blood (ng/mL): furanylfentanyl, 1.16.	(64)
M/24	A deceased was found dead on the couch. Drug paraphernalia were found nearby. At autopsy: congested and edematous lungs. History of drug abuse and recent treatment in an addiction center.	Blood (ng/mL): furanylfentanyl, 0.4; fentanyl, 1.27. Urine (ng/mL): fentanyl, 150.	(64)
Ocfentanil			
M/16	A deceased was found dead at home, seated and leaning forward on the toilet. Drug paraphernalia, brown powder in a small zip-locked plastic bag lying on a card with a straw were found at the scene. History of illicit drug abuse and depression.	Ocfentanil: femoral blood, 15.3 ng/mL; heart blood, 23.3 ng/mL; vitreous humor, 12.5 ng/mL; urine 6.0 ng/mL; bile, 13.7 ng/mL; liver, 31.2 ng/g; kidney, 51.2 ng/g; brain, 37.9 ng/g; nose mucus membrane, 2,999 ng/swab. Additionally in peripheral blood: acetaminophen, 45 µg/mL; caffeine, 230 ng/mL.	(65)
M/24	A deceased was found dead in his apartment. Drug paraphernalia, plastic zipper bag with brown powder, identified as ocfentanil, were found at the scene. At autopsy: lung congestion and edema, brain congestion and edema. History of illicit drug use.	Ocfentanil: peripheral blood, 9.1 ng/mL; heart blood, 27.9 ng/mL; urine, 480 ng/mL. Additionally in peripheral blood: citalopram (130 ng/mL); quetiapine (<10 ng/mL), THC (2.8 ng/mL), and carboxy-THC (<5 ng/mL).	(66)
AH-7921			
M/early 20 s	A deceased, victim of a minor traffic accident, was discharged from hospital the following day with a prescription for 30 mg codeine/400 mg acetaminophen. He ingested six tablets and some powder from zip-lock bags marked 3-methylmetcathinon (3-MMC) and 4-fluoromethamphetamine (4-FMA) bought on the internet. Soon after ingestion, when lying on the floor, he began to snore and was unresponsive. At autopsy: pulmonary edema.	Peripheral blood: AH-7921, 430 ng/mL; 2-FMA, 6.9 ng/mL; 3-MMC, 2.1 ng/mL; codeine, 420 ng/mL; acetaminophen, 18,700 ng/mL.	(67)
F/young	A deceased was found dead at home. Used needles and small plastic bags labeled "AH-7921" and "etizolam" were found in waste bins. At autopsy: needle marks in various stages of healing on the right cubital fossa.	Peripheral blood: AH-7921, 330 ng/mL; methoxetamine, 64 ng/mL; etizolam, 270 ng/mL; phenazepam, 1,330 ng/mL; 7-aminonitrazepam, 43 ng/mL; diazepam, 46 ng/mL; oxazepam, 18 ng/mL; nordiazepam 73 ng/mL.	(67)
M/19	A deceased was found dead on the bed. Frosty substance around the mouth. At autopsy: pulmonary congestion and edema.	AH-7921: peripheral blood, 6,600 ng/mL; heart blood, 3,900 ng/mL; urine, 6,000 ng/mL; bile, 17,000 ng/mL, liver, 26,000 ng/g; kidney, 7,200 ng/g; brain, 7,700 ng/g.	(68)
F/22	A deceased was found dead in the bedroom of her apartment. A plastic bag labeled "AH-7921" was found in the apartment. At autopsy: cerebral edema with increased intracranial pressure, the internal organs full of blood. History of drug abuse and AH-7921 use.	AH-7921: femoral blood, 450 ng/mL; heart blood, 480 ng/mL; urine, 760 ng/mL; vitreous humor, 190 ng/mL; stomach content, 40 µg/mL; liver, 530 ng/g.	(69)

(*Continued*)

TABLE 3 | Continued

Gender/ age	Case data	Toxicological findings	Ref.
U-47700			
M/20	A deceased was found dead with a syringe clutched in his hand. Drug paraphernalia were located to his proximity. History of drug abuse.	Blood: U-47700, 382 ng/mL; amphetamine, 12 ng/mL.	(70)
M/39	A deceased was found unresponsive lying on the sofa; a syringe was found on the floor. History of ordering designer drugs on the internet.	Blood: U-47700, 217 ng/mL; mephedrone, 22 ng/mL.	(70)
M/25	A deceased was found unresponsive with symptoms of pulmonary edema. A white powder, determined to be U-47700, was found at the scene. History of polydrug abuse.	Blood: U-47700, 334 ng/mL.	(70)
M/23	A deceased was found on the bathroom floor with a ligature around his arm. Syringe and a pocket containing a powdery substance labeled "U-47700" were found at the scene. History of drug abuse.	Blood: U-47700, 252 ng/mL; citalopram, 43 ng/mL.	(70)
M/29	A deceased was complaining of a headache the day of his death and suddenly collapsed. At the autopsy: pulmonary edema and brain edema.	Blood: U-47700, 453 ng/mL.	(70)
M/29	A deceased was found unresponsive with the evidence of pulmonary edema. A rolled-up 10 dollar bill with a residue of white powder and series of packets with white powder were found at the scene. History of drug abuse.	Blood: U-47700, 242 ng/mL; carboxy-THC, 5.3 ng/mL.	(70)
M/26	A deceased was found dead at home. Five syringes, benadryl and etizolam pills, diphenhydramine tables, and three glass dropper bottles were found at the scene. History of drug abuse.	Blood: U-47700, 103 ng/mL; diphenhydramine, 694 ng/mL.	(70)
M/21	A deceased was found dead at home with an injection site in the right arm containing a needle. History of drug abuse.	Blood: U-47700, 299 ng/mL; tramadol < 250 ng/mL; alprazolam, 47 ng/mL; lorazepam, 11 ng/mL; 3-methoxyphencyclidine, 180 ng/mL.	(70)
M/24	A deceased was found unconscious and unresponsive at home. History of "U-47700" abuse.	Blood: U-47700, 487 ng/mL; etizolam, 86 ng/mL; chlorpheniramine < 250 ng/mL; diphenhydramine, 250 ng/mL.	(70)
M/23	A deceased was found dead sitting up in a chair.	Blood: U-47700, 311 ng/mL; oxycodone, 11 ng/mL; venlafaxine, 2,600 ng/mL; *O*-desmethylvenlafaxine, 380 ng/mL.	(70)
M/24	A deceased was found unresponsive with a syringe in his arm. History of drug abuse.	Blood: U-47700, 59 ng/mL.	(70)
M/46	A deceased was snorting a compound from an envelope labeled "U-47700." At autopsy: pulmonary congestion and edema.	Peripheral blood: U-4770, 190 ng/mL; alprazolam, 120 ng/mL; doxylamine, 300 ng/mL; diphenhydramine, 140 ng/mL; carboxy-THC, 2.4 ng/mL. Urine: U-47700, 360 ng/mL. Liver: U-47700, 1,700 ng/g.	(71)
Not provided	A deceased was found on the bed. At autopsy: pulmonary congestion.	U-47700: femoral blood, 525 ng/mL; heart blood, 1,347 ng/mL; urine, 1,393 ng/mL; kidney, 270 ng/g; liver, 430 ng/g; lung, 320 ng/g; brain, 97 ng/g. Additionally in blood: diphenidine (ca. 1.7 ng/mL); methoxphenidine (ca. 26 ng/mL); ibuprofen (ca. 1.8 µg/mL); and naloxone (1.9 ng/mL).	(72)
Not provided	A deceased was found on the bed. At autopsy: pulmonary congestion.	U-47700: femoral blood, 819 ng/mL; heart blood, 1,043 ng/mL; urine, 1,848 ng/mL; kidney, 140 ng/g; liver, 3,100 ng/g; lung, 240 ng/g; brain, 110 ng/g. Additionally in blood: diphenhydramine (ca. 45 ng/mL) and methylphenidate (ca. 2.5 ng/mL).	(72)
U-47700 and furanylfentanyl or fentanyl or butyrylfentanyl			
M/36	A deceased was found unresponsive in the bathroom with a syringe cup in his mouth. History of drug abuse.	Blood: U-47700, 135 ng/mL; furanylfentanyl, 26 ng/mL.	(70)
M/33	History of heroin and cocaine abuse.	Blood: U-47700, 167 ng/mL; furanylfentanyl, 56 ng/mL; morphine, 48 ng/mL.	(70)
M/29	A deceased was found unresponsive.	Blood: U-47700, 490 ng/mL; furanylfentanyl, 76 ng/mL.	(70)
M/40	History of heroin/opioid abuse.	Blood: U-47700, 105 ng/mL; furanylfentanyl, 2.5 ng/mL.	(70)
M/36	At autopsy: pulmonary edema. History of drug abuse and experimentation with substances purchased over the internet.	Blood: U-47700, 13.8 ng/mL; fentanyl, 10.9 ng/mL. Urine: U-47700, 71 ng/mL.	(73)

(*Continued*)

TABLE 3 | Continued

Gender/ age	Case data	Toxicological findings	Ref.
M/18	A deceased was found unresponsive in the bed. Syringes and two white powders, determined to be butyrylfentanyl and U-47700, were found at the scene. History of drug abuse.	Blood: U-47700, 17 ng/mL; butyrylfentanyl, 26 ng/mL; ethanol, 0.03 g/dL.	(70)
MT-45			
M/24	A deceased was found dead sitting on the chair in front of the desk. An e-cigarette with unknown fluid, drug paraphernalia, and several bags of white powder labeled "Methoxphenidine," "Methoxmetamine," and "MT-45" were found at the scene. At autopsy: brain edema, hemorrhagic pulmonary edema, and hyperemia of the internal organs. History of amphetamine abuse.	MT-45: femoral blood, 660 ng/mL; heart blood, 1,300 ng/mL; urine, 370 ng/mL; vitreous humor, 260 ng/mL; gastric content, 49 μg/mL; liver, 24 μg/g. Also in femoral blood: lidocaine and two synthetic cannabinoids—PB-22 and 5 F-APINACA.	(69)
M/35	A deceased was found dead at home. Drug paraphernalia (scale, spoon, pipe, and lighter) and two packets of white powder, one testing positive for MT-45 and the other for etizolam, were found at home. At autopsy: pulmonary congestion and edema. History of substance abuse.	Peripheral blood: MT-45, 520 ng/mL; etizolam, 35 ng/mL; diphenhydramine, 220 ng/mL	(74)

reported in Belgium (65) and in Switzerland (66), and the cases are summarized in **Table 3**.

Another new synthetic analog of fentanyl, i.e., acryloylfentanyl, has been recently identified in a few European countries: Denmark, Estonia, Finland, Latvia, and Sweden (12, 39). The compound has been typically seized in liquid or in a tablet form and less frequently as a powder or in capsule form (12, 79). Acryloylfentanyl was the most common fentanyl derivative in Sweden from the end of January 2016 (a time when 4-methoxybutyrylfentanyl and furanylfentanyl were banned) to September 2016, after which time it became scheduled as narcotic (12, 38, 39). Limited data indicate that the drug is taken nasally (using a nasal solution or by snorting), orally, and by intravenous injection (12, 39). Twenty-one acute intoxications associated with acryloylfentanyl were reported by Sweden to the EMCDDA; all of them occurred between March and August 2016. In the analytically confirmed cases, the concentration of acryloylfentanyl in serum ($n = 8$) ranged from 0.5 to 2.1 (mean 1.0) ng/mL, and in urine ($n = 9$) from 1.8 to 196 (mean 63) ng/mL (38). Clinical symptoms were generally consistent with the opioid toxidrome. They predominantly included unconsciousness, respiratory depression, and miosis, and less commonly, tachycardia, vomiting/nausea, restlessness/anxiety, low oxygen saturation, dizziness, hypertension, chest pain, cyanosis, blurred vision, constipation, somnolence, hallucinations, and high body temperature (12, 38, 39). Forty-two deaths associated with acryloylfentanyl have been reported in Europe: one in Denmark, one in Finland, one in Latvia, and 39 in Sweden, all of them occurred between April and September 2016. The presence of acryloylfentanyl in biological samples taken from the deceased was confirmed in 40 cases. While acrylyolfentanyl was the only substance detected in two fatal cases, samples from the remaining cases included benzodiazepines and their metabolites, ethanol, antidepressants, antipsychotics, "Z"-drugs, pregabalin, and, to a lesser extent, Δ^9-tetrahydrocannabinol (THC), synthetic cathinones, synthetic cannabinoids, amphetamine, methylenedioxymethamphetamine (ecstasy), gabapentin, and opioids (12, 39).

NEW GENERATION OF SYNTHETIC OPIOIDS: AH-7921, U-47700, AND MT-45

Since 2010, new potent synthetic opioids with chemical structures different from fentanyl, i.e., AH-7921, U-47700, and MT-45, have appeared on the recreational drug market. Their chemical structures are presented in **Figure 2**. Based on user reports and clinical data, the desired and adverse effects of these compounds resemble those of classical opioids. The reported doses and duration of action of these drugs are presented in **Table 2**.

AH-7921

AH-7921, 3,4-dichloro-*N*-{[1-(dimethylamino)cyclohexyl] methyl}benzamide, was invented in mid-1970s by researchers from Aston University in Birmingham (UK) and the pharmaceutical company Allen & Hanburys Ltd. as a potent opioid analgesic agent. However, due to its abuse potential and toxicity AH-7921 has never been developed into a medicine. The compound acts as an agonist of μ-opioid receptors, although at high doses it can also stimulate κ-opioid receptors. In animal studies, AH-7921 produced typical morphine-like actions, i.e., antinociception, respiratory depression, sedation, miosis, inhibition of gut propulsion, and lowered body temperature, with a potency almost equipotent to that of morphine (80, 81).

AH-7921 was first identified in Europe in a sample purchased from an internet retailer in July 2012 (81). The following year, the compound was found in Japan in "legal highs" products containing synthetic cannabinoids and cathinones (82). AH-7921 is sold as a free base and as a hydrochloride salt in a white/off-white powder form (81). It should be emphasized that AH-7921 is also sold or discussed on user websites and public media under the alternative name *Doxylam*. This name could be easily confused with that of doxylamine, a popular antihistamine drug with sedative–hypnotic properties that is present in several over-the-counter medicines; the unintentional use of AH-7921/doxylam for the treatment of allergy or as a hypnotic might have serious health consequences (81).

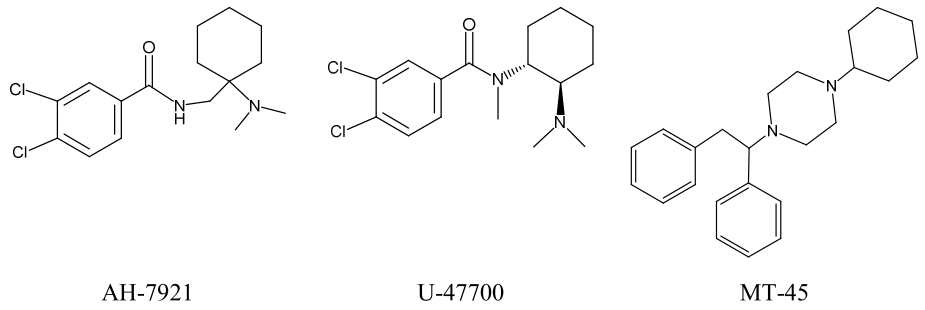


FIGURE 2 | Chemical structures of novel synthetic opioids.

There is limited information available on the routes of administration and the doses of AH-7921 used. The compound is taken orally, nasally, by smoking, and, less commonly, by intravenous injection (81). By 2014, six non-fatal intoxications associated with AH-7921 had been reported by Sweden to the EMCDDA; five of these were analytically confirmed (81). The clinical symptoms included tachycardia, hypertension, and seizures. The first death associated with AH-7921 use was reported by Norway in December 2012 (81). The next year, a total of 16 cases of fatal intoxications involving AH-7921 were reported by Sweden (10), UK (three), Norway (two), and USA (one). The AH-7921 concentrations in these postmortem blood samples were found to be in the range from 0.03 to 0.99 μg/g (Sweden), 0.05, 0.58, and 4.46 mg/L (UK), 0.33 and 0.43 mg/L (Norway), and 9.1 mg/L (USA) (67–69, 81, 83). In most cases, other psychoactive compounds, mainly benzodiazepines but also amphetamines, synthetic cathinones, codeine, acetaminophen, and methoxetamine, were also detected (67–69, 81, 83). The case reports of fatalities involving AH-7421 are presented in **Table 3**.

U-47700

U-47700, 3,4-dichloro-*N*-[2-(dimethyloamino)cyclohexyl]-*N*-methylbenzamide; "Fake morphine," "U4," a structural isomer of AH-7921, is a selective μ-opioid receptor agonist developed in 1970s by the chemist Jacob Szmuszkovicz from the Upjohn Company in a search for non-addicting analgesics (84). U-47700 produces morphine-like effects in animals (85). Preclinical studies found U-47700 to be approximately 7.5 times more potent than morphine and about 10 times less potent than fentanyl (85). The compound has not been studied in humans and no pharmacokinetic data exist.

U-47700 was first identified in Sweden in October 2014, and then found in seized powders, tablets, and liquids in various European countries and in the USA (86). The compound is gaining popularity on drug user forums as a legal alternative to morphine/heroin and is typically sold as a white powder. There is limited information available on the routes of administration and the doses of U-47700 used. It is taken orally, nasally, intrarectally, by smoking, intravenous injection, or by combinations of these routes. According to user reports, U-47700 acts longer than AH-7921 (see **Table 2**).

During 2016, a significant number of U-47700 acute intoxication cases were reported in the USA. Clinical symptoms included respiratory depression, cyanosis, miosis, depressed level of consciousness, drowsiness, tachycardia, nausea, anxiety, and abdominal pain. In most cases, the symptoms were reversed by intravenous injection of naloxone. A 22-year-old man with a history of heroin abuse was found unconscious, apneic, and cyanotic, respiring at four breaths per minute, with a blood pressure of 138/88 mmHg and a pulse of 134 per minute. He was comatose with a Glasgow Coma Scale (GCS) of 3 (87). After recovery by an injection of naloxone, the patient reported using the opioid agonist U-47700, which he had acquired over the internet for recreational purposes (87). A 23-year-old woman insufflated and injected a drug called "U4" and within minutes became unresponsive (88). Paramedics found her cyanotic, respiring at four breaths per minute and with an oxygen saturation percentage in the 60s. Her chest X-ray revealed mild congestion consistent with pulmonary edema. Toxicological analysis of her serum and urine samples detected U-47700 at concentrations of 228 and 393 ng/mL, respectively. In addition, one U-477000 metabolite was found in her serum and four in her urine (88). A 41-year old woman presented to ED with pinpoint pupils for a depressed level of consciousness (89). The patient reported that she had ingested three tablets of what she believed to be Norco, which had been illicitly purchased, to relieve chronic back pain. Toxicological analysis of serum samples identified the following compounds (in ng/mL): acetaminophen, 10,033; benzoylecgonine, 46.6; fentanyl, 15.2; gabapentin, 351; hydrocodone, 107.7; sertraline, 15.7; and U-47700, 7.6 (89). A 29-year-old man was found unresponsive after intravenous injection of U-47700. He spontaneously regained consciousness. Concentrations of U-47700 and phenazepam in serum samples were 240 ng/mL and 1.4 mg/mL, respectively (90). Domanski and coworkers (91) described the case of a 26-year-old man and 24-year-old woman who consumed alcohol and insufflated a powdered substance named U-47700 purchased on the internet that they believed to be a "synthetic cocaine." Approximately 3 h after use, the man was found with agonal breathing; he was cyanotic, with oxygen saturation of 50%, GCS of 3, and pinpoint pupils. The chest X-ray showed bilateral pulmonary consolidation. At the hospital, the patient was sedated with propofol. The female partner reported

that after insufflation of U-47700 she had been feeling "cool and relaxed," then had fallen asleep and awoken about 3 h later with symptoms of anxiety, nausea, drowsiness, and abdominal pain. Urine samples of both patients were positive for U-47700 (91). Four cases of acute intoxication with U-47700 were reported by Fleming and coworkers (92). One patient presented to ED with cyanosis, miosis, CNS and respiratory depression, and sinus tachycardia. Another one was euphoric, but suffered from nausea, anxiety, abdominal pain, and shivering. Both patients believed they had purchased "synthetic cocaine" from the internet and insufflated the powder substance. Toxicological analysis revealed the presence of U-47700 (urine, case one) and ethanol in blood samples of both patients (92). In the third case, a patient went into cardiac arrest and was administered naloxone. U-47700 was detected in his urine at a concentration of 224 ng/mL (92). In the last case, U-47700 (140 ng/mL) was found in a patient's urine sample (92). The case reports of analytically confirmed deaths involving U-47700 (70–73, 93) are summarized in **Table 3**.

MT-45

MT-45, 1-cyclohexyl-4-(1,2-diphenylethyl)piperazine (also known as IC-6, CDEP, and NSC 299236), was developed in 1970s by Dainippon Pharmaceuticals Co. in Japan as an alternative to morphine for analgesia (94). The free amine of MT-45 is a colorless solid, while the dihydrochloride salt of MT-45 is an off-white solid. MT-45 is usually sold as a white or off-white powder. The compound exists in two enantiomer forms. Racemic MT-45 and the *S*-MT-45 enantiomer exert opioid-like analgesic effects in animals, with the *S*-MT-45 being more potent than morphine. Data from studies performed on mice suggest that MT-45 may have a dependence potential in humans. The pharmacological activity of MT-45 is complex and involves stimulation of δ- and κ-opioid receptors, but also includes interactions with non-opioid molecular targets that are currently not fully understood (95, 96).

There is limited information available on the routes of administration and the doses of MT-45 used. The compound is typically administered orally or by nasal insufflations, although snorting MT-45 causes an intolerable level of irritation in some users, and inhalation, while intravenous or intramuscular injection and rectal insertion are less common (95, 96). The tentative single doses and durations of MT-45 action as reported by users are presented in **Table 2**.

MT-45 was first reported to the EMCDDA by Sweden in December 2013. The next year, Helander and coworkers published data from nine non-fatal intoxication cases associated with MT-45 that had been reported from November 2013 to February 2014 within the Swedish STRIDA project (97). All patients were men aged 17–32 years. In four cases, MT-45 was the sole compound identified in blood and urine samples, while one or several psychoactive substances (carboxy-THC, pyrazolam, flubromazepam, dextromethorphan, methiopropamine, 3-methoxyphencyclidine, and 3-methylmethcathinone) were detected together with MT-45 in the urine of five patients. The MT-45 concentration in blood was in the range from 6 to 157 (mean 60) ng/mL (97). The majority of patients presented clinical symptoms of opioid intoxication: a decreased level of consciousness or coma (seven cases), respiratory depression or cyanosis (seven cases), and miosis (three cases). Neurological disturbances such as paresthesia in the hands and feet, hand weakness, balance disturbances, vision impairments, and hearing impairment or loss were reported in four cases (97). Unusual symptoms of intoxication that probably involved MT-45 were observed in three Swedish men aged 23–34 years: loss and depigmentation of hair that was most apparent on the eyelashes and eyebrows, widespread folliculitis and dermatitis, painful intertriginous dermatitis, and elevated liver enzymes (98). Two of the men also had lines of discoloration across the nails of the fingers and toes. One patient reported loss of smell and taste. Two patients suffered from tremors and coldness for months. The symptoms gradually disappeared over time. Notably, in the acute phase of intoxication all patients showed eye symptoms of redness, dryness, and irritation; two of them subsequently developed severe bilateral secondary cataracts requiring surgery. A blood test demonstrated the presence of MT-45 at concentrations: 280, 122, and 22 ng/mL (98). However, as the clinical symptoms resembled the toxic actions of chemiotherapeutic agents (98), it is possible that they could indicate the presence of nitrogen mustards, reagents used in the synthesis of MT-45, in poorly purified batches (99).

Twenty-eight deaths associated with MT-45 have been reported by Sweden, all of which were analytically confirmed. These deaths occurred within a short period time, from November 2013 to April 2014; the deceased were male, aged between 19 and 59 years, and a female aged 23 (95). The concentration of MT-45 in postmortem femoral blood ranged from 0.006 to 1.9 μg/g. In 24 of them, MT-45 was found in combination with at least one other psychoactive substance, including anxiolytic/hypnotics (benzodiazepines), antidepressants (fluoxetine, sertraline, mirtazapine, and venlafaxine), antipsychotic drugs (olanzapine, quetiapine, and levomepromazine), antiepileptic drugs (gabapentin, lamotrigine, and carbamazepine), opioids (morphine, codeine, tramadol, fentanyl, and hydrocodone), ethylphenidate, methiopropamine, 2-aminoindane, amphetamine, THC, APBP, and ethanol (95). Very recent case reports of fatal intoxication involving MT-45 (69, 74) are summarized in **Table 3**.

THE ROLE OF NALOXONE IN TREATING OPIOID NPS-INDUCED INTOXICATION

Naloxone is a short-acting semisynthetic competitive opioid receptor antagonist with the highest affinity for μ-receptor, though it also blocks δ- and κ-receptors. It is a standard drug for treatment of opioid overdose (100, 101). Naloxone rapidly reverses the clinical signs of opioid overdose, life-treating respiratory depression in particular, and its timely administration is crucial for reducing opioid-linked mortality. Notably, to reduce harms associated with opioids use "… a number of countries have recently adopted policies and procedures that allow medical staff to distribute naloxone to first reponders (e.g., police and firemen) and to people dependent on opioids" (100). In addition to reversing opioid toxidrome, naloxone may induce withdrawal symptoms in a dependent patient who is under the influence of opioids. The drug can be administered by intravenous, intramuscular, subcutaneous, and intranasal routes. The initial dose should

be between 0.4 and 2 mg for adults and 0.01 mg/kg body weight in children, given intravenously. If the intravenous route is not available, then intramuscular and subcutaneous administration should be considered. Intranasal delivery may require a higher dose of 4 mg. The dose may be repeated at 2–3-min intervals until the patient is breathing at a rate greater than 10 breaths/minute (100, 101).

CONCLUDING REMARKS

Over the last decade, a significant change has been seen in the use and availability of recreational drugs in various parts of the world, with an increasing number of NPS being observed. While the majority of NPS are designer cannabinoids and psychostimulants, a range of different synthetic opioids have recently appeared on the illicit drug market, namely analogs of fentanyl and compounds with various chemical structures, such as AH-7921, U-47700, and MT-45.

No general population or targeted surveys on the prevalence of illicit use of fentanyls and other novel synthetic opioids were found during this review. Information on the use of these drugs is mostly limited to discussions on user websites. It appears that they are predominantly used by individuals who use illicit opioids, such as heroin and/or prescription opioids, and to a lower extent, by individuals interested in exploring the effects of psychoactive substances, so-called psychonauts. The desired effects are similar to those experienced with heroin: relaxation and euphoria often followed by a sedated, dream-like state.

Vast majority of synthetic opioids was originally synthesized by pharmaceutical companies in a search for effective analgesic drugs with lower adverse effects than morphine. However, due to their toxicity or abuse potential, they were never approved for human medical use. These compounds are opioid receptor agonists which in general are far more potent than morphine. Their effects on humans are largely identical to those of the opioid toxidrome. It should be emphasized that conventional drug tests will not detect synthetic opioids. The growing number of acute intoxication cases, often associated with multidrug abuse, indicates that these drugs should be considered as posing a serious threat to public health. Broad pharmacological, toxicological, and forensic research of these compounds is necessary in order to establish their pharmacokinetic properties, long-term effects, and effective detection methods.

AUTHOR CONTRIBUTIONS

JZ prepared and wrote the manuscript.

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The "Endless Trip" among the NPS Users: Psychopathology and Psychopharmacology in the Hallucinogen-Persisting Perception Disorder. A Systematic Review

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Hallucinogen-persisting perception disorder (HPPD) is a syndrome characterized by prolonged or reoccurring perceptual symptoms, reminiscent of acute hallucinogen effects. HPPD was associated with a broader range of LSD (lysergic acid diethylamide)-like substances, cannabis, methylenedioxymethamphetamine (MDMA), psilocybin, mescaline, and psychostimulants. The recent emergence of novel psychoactive substances (NPS) posed a critical concern regarding the new onset of psychiatric symptoms/syndromes, including cases of HPPD. Symptomatology mainly comprises visual disorders (i.e., geometric pseudo-hallucinations, haloes, flashes of colors/lights, motion-perception deficits, afterimages, micropsia, more acute awareness of floaters, etc.), even though depressive symptoms and thought disorders may be comorbidly present. Although HPPD was first described in 1954, it was just established as a fully syndrome in 2000, with the revised fourth version of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR). HPPD neural substrates, risk factors, and aetiopathogenesys still largely remain unknown and under investigation, and many questions about its pharmacological targets remain unanswered too. A critical mini review on psychopathological bases, etiological hypothesis, and psychopharmacological approaches toward HPPD, including the association with some novel substances, are provided here, by means of a literature search on PubMed/Medline, Google Scholar, and Scopus databases without time restrictions, by using a specific set of keywords. Pharmacological and clinical issues are considered, and practical psychopharmacological recommendations and clinical guidelines are suggested.

Keywords: hallucinogen-persisting perception disorder, novel psychoactive substances, hallucinogens, hallucinations, flashbacks, palinopsia

INTRODUCTION

Hallucinogen-persisting perception disorder (HPPD) is a long-lasting and potentially permanent syndrome characterized by a spontaneous recurrence of perceptual/visual disturbances which are reminiscent of those generated while a subject was intoxicated with hallucinogens. According to

the Fifth Version of Diagnostic and Statistical Manual of Mental Disorders (DSM-5), HPPD is defined as the following criteria (1):

(A) following cessation of use of a hallucinogen, the reexperiencing of one or more of the perceptual symptoms that were experienced while intoxicated with the hallucinogens (e.g., geometric hallucinations, false perceptions of movement in the peripheral visual fields, flashes of color, intensified colors, trial images of moving objects, positive after images, haloes around objects, macropsia, and micropsia);
(B) the symptoms in criterion (A) cause clinically significant distress or impairment in social, occupational, or other important areas of functioning;
(C) the symptoms are not due to a general medical condition (e.g., anatomical lesions and infections of the brain, visual epilepsies) and are not better accounted for by another mental disorder (e.g., delirium, dementia, schizophrenia) or hypnopompic hallucinations.

Before diagnosing an HPPD, post-traumatic stress disorder, depersonalization, derealization, and hallucinogen-induced psychotic mood or anxiety disorders should be excluded (2). Moreover, other causes of visual disturbances should be investigated and excluded, such as anatomical lesions, brain infections, epilepsy, schizophrenia, delirium state, or hypnopompic hallucinations (2). Furthermore, an association between the first intake, frequency, and quantity of drug taken and the likelihood of developing an HPPD has not been demonstrated, as has been the onset of the disorder following a single hallucinogenic experience (3).

Epidemiology

Overall, prevalence of HPPD has been generally considered low (2). However, limited publications suggested that chronic visual disturbances may be relatively common among hallucinogens' users. It has often been assumed that HPPD may be a severe clinical manifestation of the drug-induced visual changes (3–5). While the probability of flashbacks occurring in the wake of hallucinogen use may vary from 5 to 50% among hallucinogens' users (6, 7), the probability of an HPPD being manifested is lower (3).

Historical Background

Hallucinogen-persisting perception disorder was first described in 1954 (8). Subsequent observations have been then described (3, 4, 8–12). Horowitz (10) first introduced the term *flashbacks*, referring to recurrent and spontaneous perceptual distortions and unbidden images. When these "flashbacks" present as recurrent, but without a current acute, or chronic hallucinogen intake, the disturbance is referred to as HPPD. Horowitz (10) classified also three types of visual flashbacks: (a) *perceptual distortions* (e.g., seeing haloes around objects); (b) *heightened imagery* (e.g., visual experiences as much more vivid and dominant in one's thoughts); and (c) *recurrent unbidden* images (e.g., subjects see objects that are not there). HPPD has been introduced under the diagnosis of *Post- hallucinogen Perception Disorder* in 1987 within the DSM-III-R (13). Subsequently, the DSM-IV-TR (14) recognized the syndrome as *Hallucinogen-Persisting Perception Disorder (Flashbacks)* (code 292.89) (15). The disorder was confirmed as nosological entity as well in the DSM-5 (1).

Phenomenology

Hallucinogen-persisting perception disorder is characterized by a plethora of visual disturbances (e.g., geometric imagery, afterimages, false perceptions of movement in the peripheral fields, flashes of light, etc.) (3) (**Table 1**), including pseudohallucinations. It has been also associated with a LSD (lysergic acid diethylamide)-like dysphoria, panic attacks, and depressive symptomatology. Visual disturbances may be episodic, stress or substance-induced, or persistent. However, the episodes may last

TABLE 1 | Main clinical and psychopathological characteristics in HPPD.

Psychopathological and clinical features	Description
Teleopsia	Objects are perceived much further away than they actually are
Pelopsia	Objects are perceived nearer than their actual size
Macropsia	Objects are perceived larger than their actual size
Micropsia	Objects are perceived smaller than their actual size
Criticism/egodystonic psychosis	Patient manifests criticism toward own thoughts and perceptual disturbances, as well as experiencing perceptual disorders perceived as inconsistent with one's self concept or ego state
Depersonalization	A state in which some individual feels that either he/she him/herself or the outside world is unreal
Derealization	A state in which an individual feels a detachment within the self-regarding one's mind or body or being a detached observer of oneself (e.g., Feeling like being inside a transparent bubble)
Feeling of body being light or heavy	
Visual trailing	Transient disturbance of visual motion perception of unknown origin (i.e., subject perceives a series of discrete stationary images trailing in the wake of otherwise normally moving objects)
Haloes around objects	A geometric shape, usually in the form of a disk, circle, ring, or rayed structure around an object really present
Afterimages/palinopsia	An image that continues to appear in one's visual field after the exposure to the original image has ceased
Other visual disturbances	Flashes of color Intensified colors Colored images Geometric imagery False perception of movement of images in the peripheral-field

HPPD, hallucinogen-persisting perception disorder.

for 5 years or more and the symptomatology is disliked by patients (10). While a *flashback* is usually reported to be infrequent and episodic, HPPD is usually persisting and long-lasting. Moreover, some HPPD subjects report that adaptation to the dark takes significantly longer compared with the general population (16). Moreover, HPPD has been associated with abnormal results for tests of visual function, suggesting disinhibition in the processing of visual information (4, 16). The subject does not develop any paranoid misinterpretation related to and does not believe that their own visual hallucinogenic experiences currently occur (3). Therefore, it has been supposed that there is involvement of the primary visual cortex, the first cortical area responsible for geometric processing of visual input (17). Further data coming from clinical, psychological, and neurophysiological sources may suggest specific physiological changes in the visual system function implicated in the onset of hallucinations after the intake of psychedelics/hallucinogens (5). **Table 1** summarizes all clinical and psychopathological characteristics associated with an HPPD.

Aetiopathogenesys

The pathogenesis of HPPD is currently unknown, even though it has been frequently reported to be associated with the intake of LSD (4, 16, 41). However, HPPD has also been reported following the consumption of all substances with hallucinogenic properties which possess pharmacological and clinical effects resembling those experienced with LSD by serotoninergic 5-HT2A (42), such as cannabis (18, 43, 44), 3,4-methylenedioxymethamphetamine (MDMA, aka "ecstasy") (45–47), and the recently marketed novel psychoactive substances (NPS). In fact, the advent of NPS facilitated the onset of new psychopathologies and new clinical manifestations, including cases of HPPD, particularly following the intake of synthetic cannabinoids (SCs) and other new synthetic psychedelics and hallucinogens, which facilitated the reoccurrence of this disorder, by posing a new clinical concern to clinicians (19, 20, 48, 49).

Types of HPPD

Two types of HPPD have been proposed here, accordingly to Lev-Ran (40). *Type-1 HPPD*, consistent with the definition of flashback provided by the ICD-10 (50), is characterized by brief reexperiences of altered perception, mood, and/or consciousness, as previously experienced during a hallucinogenic intoxication. Symptomatology may be pleasurable and even controllable. They may appear days to months after the hallucinogen-induced experience. The subject is usually aware of the unreality of their own experience. The perception of time may be altered. Visual perceptions usually comprise perceived increased color intensity, dimensionality, vibrancy, illusory changes, and movements of a perceived object. *Type-1 HPPD* comprises the *flashbacks* definition, while *type-2 HPPD* has been used to indicate the HPPD definition elaborated by Abraham (3) as well proposed in the DSM-5. The symptoms usually include palinopsia (afterimages effects), the occurrence of haloes, trails, akinetopsia, visual snows, etc. Sounds and other perceptions are usually not affected. Visual phenomena have been reported to be uncontrollable and disturbing. Symptomatology may be accompanied by depersonalization, derealization, anxiety, and depression (3).

The present systematic mini review aims at providing an overview of HPPD, by specifically focusing on both clinical manifestations and psychopharmacological approaches, in general, and among NPS users.

MATERIALS AND METHODS

Search Sources and Strategies

A critical mini review was conducted, following the methods recommended by the Cochrane Collaboration (51) and the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (52). Searches were carried out by using PubMed/Medline, Google Scholar, and Scopus. We combined the search strategy of free text terms and exploded MESH headings for the topics of HPPD and NPS as follows: ((*Hallucinogen Persisting Perception Disorder [*Title/Abstract] OR *HPPD [*Title/Abstract])) OR ((*Hallucinogen Persisting Perception Disorder [*Title/Abstract] OR *HPPD [*Title/Abstract])) and ((*novel psychoactive substances* [Title/Abstract]) OR (*NPS*[Title/Abstract])). All articles through August 15, 2017 without time restriction were selected. In addition, the authors performed further secondary searches by using the reference listing of all eligible papers.

Study Selection

We considered studies about HPPD, and whenever available, evaluating the relationship between HPPD and NPS. The authors examined all titles/abstracts and obtained full texts of potentially relevant papers. Two reviewers (LO and DP), independently and in duplicate, read the papers and selected papers according to the inclusion criteria. Duplicate publications were excluded. All the articles identified by the data sources, reporting original data related to HPPD in general and, more specifically, among NPS users, were considered in the present review. All experimental and observational study designs, including case reports and case series, were included as limited data have been published so far. Narrative and systematic reviews, letters to the editor, and book chapters were excluded, even though they were used for retrieving further secondary searches. To be included in the present review, studies were required to meet the following criteria: (a) empirical and peer-reviewed study; (b) at least an abstract with estimates and/or full results available/complete; (c) investigate HPPD in general and more specifically among NPS users; (d) human studies; and (e) provide data on psychopathological features and/or psychopharmacological treatments in these cases.

Data Extraction and Management

LO and DP independently extracted the data on participant characteristics, intervention details, and outcomes measures. Disagreements were resolved by discussion and consensus with a third member of the team (DDB). Data were collected using an *ad hoc* developed data extraction spreadsheet.

Characteristics of Included Studies

The set of keywords initially generated 260 results (**Figure 1**). A total of 31 papers were excluded because of duplicates; 7 papers were excluded because they did not provide relevant data useful

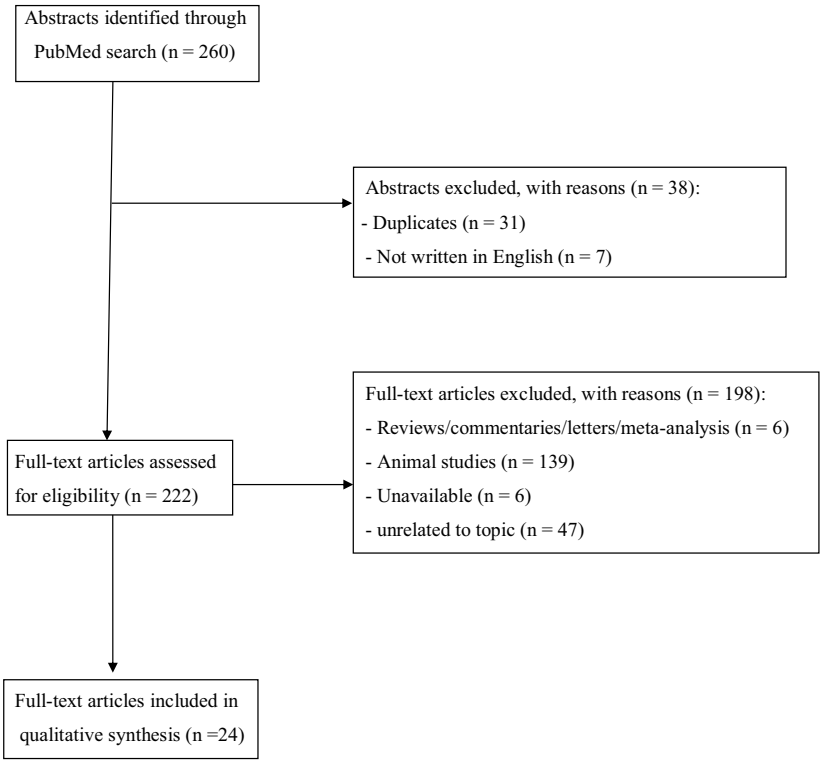

FIGURE 1 | Selection of retrieved studies.

for the aims of our papers (due to the lack of an English abstract). Of the remaining 222 studies, further 186 studies were excluded because they did not meet the inclusion criteria or because they were non-human studies. Of the remaining 36 papers, 6 papers were excluded because they were reviews, letters to editors, or metanalyses; however, 6 papers were not included here due to the lack of an available full text or an abstract useful for collecting relevant data. Finally, a total of 24 papers were included and accounted for in our analysis. **Table 2** shows the main characteristics (study design, sample size, main outcomes, and findings) of all studies reviewed here.

RESULTS

Studies on Psychopharmacotherapy of HPPD

An observational study recruited 21 HPPD subjects who were treated with benzodiazepines and/or phenothiazines (3). Among subjects receiving benzodiazepines, eight out of nine reported a reduction in intensity/frequency of visual disorders. Most (11 out of 12) phenothiazine-treated subjects described an exacerbation of HPPD (3). A case series reported three cases of HPPD, treated with risperidone, who presented a worsening of visual perceptions and panic symptomatology (21). Lerner et al. (22) described two LSD-induced HPPD male subjects who reported an improvement of symptomatology following naltrexone (50 mg daily) treatment (22). Another case report described a 22-old-year male who developed HPPD after 8-month discontinuation of LSD (22). The subject significantly improved after sertraline (100 mg/daily) treatment (23). An open-label pilot study recruited eight HPPD drug-free patients who were consecutively treated with 0.025 mg of clonidine, three times a day, for 2 months, in order to evaluate the efficacy of drug in treating persistent visual disturbances associated with the intake of LSD (24). LSD-related flashbacks demonstrated a good response to clonidine (24). A study described two HPPD outpatients who efficaciously responded to clonazepam (25). An HPPD patient with a comorbid depressive symptomatology and a prior history of cannabis, ecstasy and LSD abuse, clinically responded to 6 mg daily of reboxetine (26). An open-label study recruited 16 drug-free patients affected with HPPD with anxiety features for at least 3 months who received 2 mg daily of clonazepam for 2 months (27). Subjects reported a significant relief of anxiety and HPPD symptomatology with only mild symptomatology during the clonazepam treatment, suggesting the efficacy of clonazepam in these cases (27). Espiard et al. (18) presented a case report of an HPPD patient with a previous mixed intoxication with psilocybin and cannabis. Perceptual disorders appeared after a single psilocybin consumption. The subject re-experienced the symptomatology the following day during another cannabis snort. Moreover, symptomatology was recurrent daily with an attenuation after discontinuation until 6 months after he had stopped cannabis. Initially, he received amisulpride (100 mg/daily) treatment, subsequently stopped due to sedative effects. Then he started with olanzapine (5 mg/daily) which caused an exacerbation of symptomatology. Finally,

TABLE 2 | Summary of all included studies.

Study	Study design	Sample characteristics	Substance implicated	Psychopharmacological treatment (dosage)	Summary of findings
(3)	Observational study	21 HPPD	LSD	BZDs (N/A) Phenothiazines (N/A)	An improvement was observed among HPPD subjects following the use of BDZs; while phenothiazine worsened HPPD
(18)	Case report	1 M, 18 years, student with a history of anxiety disorder	Cannabis and psilocybin	Amisulpride (100 mg daily) Olanzapine (5 mg daily) Risperidone (2 mg daily) Sertraline (150 mg daily)	Combination of risperidone and sertraline ameliorated HPPD symptomatology after 6 months of treatment
(19)	Case reports	2 HPPD (DSM-IV-TR criteria): 1. M, 26 years, college student who developed HPPD with recurrent panic attacks after discontinuation of SC intake 2. M, 24 yr, who developed HPPD experienced with anxiety features after discontinuation of SC intake	SC	Clonazepam (1 mg/daily)	Clonazepam improved HPPD symptomatology
(20)	Case report	M, 18 years, with a history of heavy daily use of cannabis and SC who experienced HPPD	SC	Clonazepam (6 mg/daily)	For 3 years after SC consumption, the patient occasionally reexperienced the same symptoms developed during acute intoxication. These symptoms appeared during heavy cannabis consumption or in periods of boredom and inactivity
(21)	Case series	3 HPPD (DSM-IV criteria): • Case 1: F (first LSD usage: 14 years; at 21 years, first acute onset of an LSD-like euphoria and persistent visual distortions, e.g., trails of objects, particles in air, round objects) • Case 2: M, 22 years, college student (first LSD usage: 15–18 years; at 20 years, first acute onset of persistent visual symptoms of afterimages, trailing of stimuli, orange/blue haloes around objects) • Case 3: M, 40 years, married, builder (first LSD usage: 18 years; at 18 years, first acute onset of dots on a blank wall, intensification of lights, trails of his hand, anxiety, depression)	LSD	RIS: • 2–3 mg/daily • 1–6 mg/daily • 1–2 mg/daily	RIS worsened LSD-like panic and visual symptoms
(22)	Case series	2 HPPD (DSM-IV criteria)	LSD	Naltrexone (50 mg/daily)	Naltrexone caused a dramatic improvement in HPPD symptomatology. The remission was sustained also after discontinuation of naltrexone
(23)	Case report	M, 22 years who developed HPPD after an 8-month history of LSD abuse	LSD	Sertraline (100 mg/daily)	Sertraline determined initially an exacerbation of HPPD symptomatology, then it attenuated symptoms after 1 month's administration
(24)	Observational study	8 HPPD	LSD	Clonidine (0.025 mg for 3 times/daily) for 2 months	Clonidine may alleviate LSD-related flashbacks
(25)	Case series	2 HPPD outpatients	LSD	Clonazepam	Clonazepam was efficacious in reducing HPPD symptomatology
(26)	Case report	1 HPPD with comorbid MDE	MDMA, LSD, and cannabis	Reboxetine (6 mg/daily)	Reboxetine did not exacerbates visual disturbances either recurrence of depressive features
(27)	Observational study	16 HPPD with anxiety features	LSD	Clonazepam (2 mg/daily)	Clonazepam was efficacious in attenuating both anxiety and HPPD symptomatology

(*Continued*)

TABLE 2 | Continued

Study	Study design	Sample characteristics	Substance implicated	Psychopharmacological treatment (dosage)	Summary of findings
(28)	Case report	F, 33 years with HPPD	LSD	Sertraline (200 mg daily) Citalopram (20–30 mg daily) Fluoxetine (20 mg daily) Risperidone (0.5–1 mg daily) Lamotrigine (100–200 mg daily)	Lamotrigine reduced almost completely visual disturbances of HPPD
(29)	Case report	M, 36 years with HPPD	LSD, cannabis, alcohol, cocaine	Clonidine Lamotrigine (200 mg/daily)	Clonidine did not improve symptomatology; while lamotrigine was associated with a significant symptomatology improvement
(30)	Case report	F, 38 years with HPPD (DSM-5 criteria)	LSD	Risperidone (0.5 mg/daily)	Significant reduction in the frequency and intensity of panic attacks and perceptual disturbances within 3–4 weeks with low dosages of risperidone
(31)	Case report	M, 30 years, presented to the emergency department after surviving two subsequent suicide attempts by hanging, with a previous history of bipolar disorder and who developed HPPD	Cannabis LSD PCP Cocaine	Citalopram (40 mg/daily) Lamotrigine (50 mg/daily) Mirtazapine (15 mg/daily)	Patient poorly responded to treatment and was found to have committed suicide
(32)	Web-based survey	626 hallucinogens' users	Cannabis MDMA Psilocybin LSD Ketamine	N/A	Long-term perceptual disturbances were mainly reported among LSD users
(33)	Web-based survey	3139 hallucinogens' users	Several hallucinogens (including cannabis, MDMA, psilocybin, LSD, ketamine, *Salvia divinorum*)	N/A	LSD appeared to be the most robust predictor of HPPD
(34)	Case reports	2 HPPD (DSM-5 criteria): 1. M, 24 years, university student 2. F, 25 years, university student	LSD	N/A	Both cases reported the appearance of visual disturbances that were not originally experienced during LSD intoxication
(35)	Case–control study	12 inpatients with schizophrenia and HPPD vs. 14 inpatients with schizophrenia without HPPD (DSM-IV-TR criteria)	LSD Cannabis MDMA	N/A	No significant differences have been found between two groups in sociodemographic and clinical features. Individuals with schizophrenia and HPPD reported the ability to identify specific precursory cues for the appearance of HPPD-associated perceptual disturbances
(36)	Case–control study	4 HC vs. 1 M, 23 years, HPPD patient	Cannabis	N/A	Cannabinoids may have a direct effect on the retina and retinal pigment epithelium function which may be involved in perceptual disturbances experienced in cannabis-induced HPPD
(37)	Case–control study	37 inpatients with schizophrenia and HPPD vs. 43 inpatients with schizophrenia without HPPD (DSM-IV-TR criteria)	LSD	N/A	No significant differences found between two groups in sociodemographic features. Individuals with schizophrenia and HPPD reported lower general psychopathology and negative symptoms scores compared with individuals without HPPD

(*Continued*)

TABLE 2 | Continued

Study	Study design	Sample characteristics	Substance implicated	Psychopharmacological treatment (dosage)	Summary of findings
(38)	Case report	M, 26 years, university student who developed AIWS and HPPD (DSM-5 criteria)	LSD Cannabis Alcohol	N/A	The patient refused any psychotropic treatment and after 1 year of psychiatric follow-up visual disturbances completely disappeared
(39)	Survey	23 out of 67 completed the survey (2 HC; 19 who reported persisting perceptual disturbances triggered or worsened by past drug use) 6 out of 19 with co- diagnosis of HPPD, 3 with persistent migraine aura, 2 psychotic disorders, 1 PTSD and 3 anxiety disorder, 2 depression, 2 hypochondriasis and 3 dissociative disorders HPPD	Various hallucinogenic and non-hallucinogenic drugs	N/A	Many perceptual symptoms reported were not first experienced while intoxicated and are partially associated with pre-existing psychiatric comorbidity
(40)	Observational study	40 patients who sought psychiatric consultation for SUD with a previous LSD intake who developed HPPD	LSD	N/A	Subjects with type-2 HPPD significantly more likely reported lifetime use of SC, stimulants and inhalants than type-1 HPPD (who reported more likely alcohol)

HPPD, hallucinogen-persisting perception disorder; DSM, Diagnostic and Statistical Manual; F, female; M, male; LSD, lysergic acid diethylamide; yr, years; RIS, risperidone; HC, healthy controls; MDE, major depressive episode; SC, synthetic cannabinoids; PCP, phencyclidine; SUD, substance use disorders; N/A, not available; AIWS, Alice in Wonderland Syndrome.

he was treated with risperidone (2 mg/daily). Then, it was coadministered with sertraline (150 mg/daily) for the treatment of persisting dysphoric mood and recurrent anxiety-like symptoms. After 6 months of combination risperidone and sertraline, HPPD disappeared (18). A 33-year-old female developed an HPPD following the recreational use/abuse of LSD for a year at the age of 18 (28). Approximately, 2–3 weeks after the last drug intake, the subject developed persistent visual disturbances (a sort of *attenuated flashbacks*), like those experienced during an acute LSD intoxication, which lasted for over 13 years, with a little change in intensity and frequency. Despite the patient receiving several psychopharmacological treatments (e.g., sertraline, citalopram, fluoxetine, risperidone, etc.), only a year-long trial of lamotrigine (100–200 mg/day) improved abnormal visual perceptions. In addition, no significant cognitive deficits (i.e., memory functioning, attention span, visual-construction, and frontal-executive functioning) were detected, but an underperformance in the phasic attention. A follow-up after 19 months showed a continued improvement in attention performance. Brain magnetic resonance imaging scans, electroencephalograms, median nerve somatosensory, and visual evoked potential tests were all reported normal (28). A case report described a 36-year-old HPPD subject who experienced persistent visual disturbances after recreational use of LSD, cannabis, alcohol, and cocaine (29). The patient was initially treated with a 3-month course of clonidine without any improvement; then, he was treated with lamotrigine (200 mg daily) for a 7-month period with a significant improvement of his visual disturbances and overall mental well-being (29). A case report described a woman who experienced panic attacks and persistent "re-experience phenomena" characterized with flashbacks to the first trip with LSD. She responded to 0.5 mg daily of risperidone (30). A case report described an HPPD patient with a history of bipolar disorder, a previous consumption of LSD, cannabis, cocaine, and phencyclidine (PCP) (30). The patient, even though treated with lamotrigine, citalopram, and mirtazapine, committed suicide, after the hospitalization (31).

Studies on the Clinical Manifestation and Psychopathology in HPPD

A web-based questionnaire study investigated users' perceptions of the benefits/harms of hallucinogens' intake, including the occurrence and prevalence of flashback phenomena and/or HPPD (32). Only 10% of the sample recruited reported long-term perceptual changes, which they would "rather not have but could live with," while 1% reported changes that "drove them mad" (32). Of these, 39% reported them following the use of LSD, 11% after psilocybin, 9% after MDMA and cannabis, 4% ketamine, and 1% alcohol. Despite 22% of subjects reported having experienced a "flashback", only 3% of subjects experienced a "negative" flashback (32). A web-based questionnaire investigated abnormal visual experiences among 3139 hallucinogens' users (33). Each participant had their drug-use history (i.e., classical serotonergic hallucinogens, including LSD, psilocybin, dimethyltryptamine, etc.; NMDA antagonists, including ketamine and dextromethorphan; MDMA, anticholinergic-containing Datura plants; cannabis; *Salvia Divinorum*, etc.); psychiatric and neurological history; and their visual experiences investigated. Several drugs with

hallucinogenic properties have been statistically associated with unusual visual experiences, even though LSD seemed to be the most frequently reported among the HPPD-like cases (33). Lerner et al. (19) reported two case reports with a prior history of LSD intake, who experienced new visual disturbances, not previously appeared during the first LSD intoxication, after totally stopping substance use (34). A pilot study recruited 26 inpatients affected with schizophrenia and concomitant self-reported past LSD use who presented with HPPD ($n = 12$) or without HPPD ($n = 14$) (35). No significant differences in demographic, clinical features, adverse effects, and response to medications have been reported between the two groups. Furthermore, the authors reported that patients with schizophrenia and concomitant HPPD were able to distinguish HPPD symptoms from hallucinations related to their own psychotic state (35). A case-control study recruited a cannabis-user who developed HPPD and four healthy controls in order to evaluate the differences in their ophthalmological state (36). Ophthalmological examination did not report clinically significant abnormal values for the HPPD patient or for any healthy controls, even though the HPPD subject reported a slightly reduced fast oscillation rate, a diminished standing potential of the slow oscillations, and a light peak within normal range resulting in higher Arden ratios (36). Another study by Lev-Ran et al. (37) compared the characteristics of schizophrenic patients with a prior LSD consumption who developed HPPD ($n = 37$) with those who did not develop HPPD ($n = 43$). No significant differences in sociodemographic characteristics were reported between the two groups. Individuals with schizophrenia and HPPD showed lower scores for negative symptoms ($p < 0.001$), general psychopathology ($p = 0.02$), and total symptoms ($p < 0.001$), as measured with Positive and Negative Symptomatology Scale (PANSS), as compared with those reported in the group of schizophrenics without HPPD (37). A case report described a patient with a prior history of cannabis, alcohol, and LSD sporadic recreational consumption, who developed LSD-induced "Alice in Wonderland Syndrome" and HPPD after discontinuation of all substances (38). A questionnaire specifically identified prevalence and characteristics of self-reported altered perception experiences in hallucinogens' users (39). The survey concluded that HPPD may be due to a subtle overactivation of predominantly neural visual pathways which in turn may worsen anxiety after ingestion of arousal-altering drugs (39). A study explored triggers associated with type-1 and type-2 HPPD following the use of LSD, by recruiting 40 outpatients affected with HPPD (40). The findings reported differences in terms of visual disturbances and triggers between the two HPPD types. The most common types of visual disturbances were slow movement of still objects (among type-1 HPPD) and trailing phenomena (among type-2 HPPD). Furthermore, type-1 HPPD subjects were more likely to experience disturbances in a dark environment (40).

Studies on NPS and HPPD

A case series described two cases of HPPD induced by SCs (19). A recent case report described a patient with a previous history of heavy daily use of cannabis admitted to an Addiction Treatment Unit who reexperienced visual hallucinations and disturbances, like those who experienced during acute consumption of SC, in the days following SC consumption (20).

DISCUSSION

Several case reports of reoccurring or prolonged persistent visual perceptual disturbances (HPPD) have been described occurring within a certain time frame after cessation of some hallucinogenic drugs (2, 53, 54).

The present critical mini review presents several limitations. First, given the paucity of double-blind, placebo-controlled/case-control studies, we included case reports and case series as well, and studies with small sample size which may greatly limit the generalizability of findings reviewed here. Second, methodological strategies (sample size, study design, diagnostic criteria, etc.) may vary greatly in several studies retrieved. Most studies included here investigated HPPD cases following the intake of LSD, even though other isolated cases described incidents following the intake of other serotonergic hallucinogens, and cannabis. Furthermore, most studies here retrieved do not specifically distinguish between the two types of HPPD, as previously discussed, by limiting the complete understanding of the clinical symptomatology and manifestation.

The recent wave of novel substances available on the market, includes several novel cannabimimetics, novel hallucinogens (e.g., NBOMe compounds), and new LSD derivatives (48, 49). With regard to these substances, the prevalence of HPPD among NPS users is difficult to detect, due to the limited number of published reports, mainly due to the lack of knowledge among the clinicians and the consequent difficulty in the diagnosis (48). However, the specific pharmacological profile of some psychostimulants and hallucinogens may likely determine an increasing number of NPS-induced HPPD cases (48, 49). In fact, despite several studies reporting a higher occurrence of HPPD following the consumption of LSD, HPPD has been associated with a broader range of substances, e.g., natural and synthetic serotonergic 5-HT_{2A} receptor hallucinogens (44, 47, 55–57), including the most recent new synthetic psychostimulants and hallucinogens (19, 20, 48, 49). In particular, the intake of SCs, which are CB_1 receptor full agonists and possess indole-derived structures, which may itself facilitates the 5-HT_{2A} receptor dysfunction (48, 58, 59), has been associated with the onset of perceptual disorders, even after a total discontinuation, as it has been supposed that SCs may provoke a partial/total reminiscence of the previous perceptual experience in predisposed and susceptible NPS users (20, 60, 61).

Furthermore, the pharmacotherapy of HPPD may be different from study to study, as only few studies have been published so far and any recommendations are based almost entirely on non-controlled studies of small patient populations or single case reports (3, 18–31). Risperidone is a highly potent antagonist of both postsynaptic 5-HT_2 and dopamine D_2 receptors (62). As 5-HT_2 receptor antagonists are effective in treating hallucinations among schizophrenic patients, it has been previously supposed that risperidone would be efficacious in treating HPPD (18, 30). However, most studies reviewed here reported a worsening of symptomatology, particularly visual disturbances and an abrupt onset of panic attacks after the intake of risperidone among LSD users (21, 28). The symptomatology tended to return to baseline levels when risperidone was discontinued (21, 28). A case report described an improvement in HPPD symptomatology with a

combination of sertraline and risperidone (18). Some studies described a mild efficacy of clonidine (24, 25) or completely ineffective (28). Several studies suggested prescribing clonazepam as an effective treatment in reducing persistent perceptual disturbances (19, 20, 25, 27). In fact, it has been hypothesized that high-potency benzodiazepines (e.g., clonazepam), which as well as possessing serotoninergic activity, may be superior to low-potency benzodiazepines (27). Moreover, a case report suggested that reboxetine made a good improvement both in visual disturbances and depressive symptomatology (26). Reboxetine is an α-2-adrenoceptor modulating the effect on both noradrenaline and serotonin release which may affect sympathetic activity, hence facilitating the improvement of HPPD symptomatology (26). Other studies suggest lamotrigine as efficacious in ameliorating HPPD symptomatology (28, 29). Lamotrigine acts by blocking sodium and voltage-gated calcium channels and inhibiting glutamate-mediated excitatory neurotransmission, thereby suggesting its potential use in the treatment of HPPD (28).

Serotonin neurotransmission has been hypothesized to be involved in the aetiopathogenesys of both acute and persisting LSD- and SC-induced perceptual disturbances (61). The main mechanism supposed to be implicated consists of a vulnerability/predisposition of psychedelics' consumers to continue centrally processing visual imagery after the visualization has been totally eradicated from the visual field (23). Persisting visual disorders may be explained by a reversible (or irreversible) "dysfunction" in the cortical serotonergic inhibitory inter-neurons with GABA-ergic outputs (63). The anandamidergic system has been also implicated by involving the areas of visual information processing (64, 65).

Further studies should be implemented in order to better clarify the role of the NPS, particularly the new psychedelics and psychostimulants with LSD-like properties in the pathogenesis and etiology of new HPPD cases.

AUTHOR CONTRIBUTIONS

LO and FS conceived the topic of the manuscript, while LO, AG and GP carried out the main analysis. JC and DB assisted in either screening of the studies or preparation of the attachments. FS served as study reviewer. FS served as senior study reviewer. All the coauthors substantially contributed to the present piece of work before approving it for final submission.

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MINI REVIEW
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Psychedelic Fauna for Psychonaut Hunters: A Mini-Review

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Currently different classes of psychoactive substances are *easily* available for abuse, including several hundred novel psychoactive substances (NPS). Some of these drugs occur naturally in plants and animals or are chemically modified from plant or animal compounds and have been abused by humans over millennia. Recently, the occurrence of a new "drug culture" (e.g., psychonauts) who consume a great variety of NPS with hallucinogenic/psychedelic properties, facilitated the development of a new "psychedelic trend" toward the consumption of substances contained in some species of animals ("psychedelic fauna"). The present review aims at providing an overview of the most commonly abused "psychedelic animals," by combining a dual search strategy coming from online psychonauts' experiences and English literature searches on the PubMed/Medline Google Scholar databases. A multilingual qualitative assessment on a range of websites and online resources was performed in order to identify a list of animals who possess some psychoactive properties and could be abused by humans for recreational purposes. Several species are implicated (i.e., ants, amphibians, fish). Routes of administration depend on the animal, substance included, metabolism, toxicity and individual, social and cultural variability. Online purchase and access are easy through tourism-related search strategies ("*frog trip,*" "*help of charmer snake,*" "*religious trip*").

Keywords: psychedelic animals, psychedelics, NPS, novel psychoactive substances, hallucinogens, psychedelic fauna

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INTRODUCTION

Humans have used a range of naturally occurring psychoactive substances to modify their minds, for recreational/mystic/spiritual/religious/psychedelic purposes, over millennia (1). Many psychotropic chemicals, widely distributed in plants and animals, were discovered by ancient hunter-gatherers prior to the Neolithic agricultural revolution (2). Humans have learnt how to cultivate/modify/exploit these chemicals and pass on this cultural knowledge to others (1–3). Most commonly abused natural drugs and, nowadays, novel psychoactive substances (NPS), cause changes in brain systems that alter consciousness or affect moods/emotions in some way (1–6). Moreover, ritualistic/spiritual use of these psychoactive substances has a long history among ancient tribes/shamanic communities (7–9), by suggesting some evolutionary benefits (mainly in terms of an increased chance in the survival of a species) related to the historical spread of plant- and/or animal-derived compounds possessing psychoactive properties, mostly entheogens/hallucinogens

(3, 10, 11). For example, many psychotropic substances originally were taken by humans due to their bactericidal and/or antiparasitic effects (12). Within this context, some psychoactive compounds have been progressively taken by humans, despite some toxicological effects, as their intake might determine some evolutionary benefit, in terms of increasing the survival of a species and, then, increasing the reproducibility rate (1–6). Moreover, some psychoactive substances, naturally occurring in "fauna," used for ritualistic and religious purposes over the millennia, could be taken by humans, even though they may have some toxicological effect, as the advantages in taking them would exceed the disadvantages, from an evolutionary perspective. Therefore, the spread of these psychoactive substances, naturally occurring in "fauna," may be facilitated by some "*cultural*" processes. In fact, some drugs come with a "*cultural reputation*" for giving pleasure or relief of physical or emotional pain (13). Subsequently, in the light of the recent NPS phenomenon, the "*reputation*" of these recreational drugs in animals has also been amplified by the virtual dissemination through the Internet and social networks (3, 5, 11, 14). Moreover, the current generation of drug users (i.e., psychonauts) belong to a new sub-culture, which seems to resemble more to the shamanic communities (3, 15), by specifically possessing an "*attractiveness*" toward such entheogens/psychedelic/hallucinogenic substances, also included in some animals and/or parts of them.

Despite many psychoactive substances/NPS have been easily found in plant sources (11), a variety of animal sources of psychoactive substances appear to be equally abused, potent and risky. The term "*psychoactive fauna*" comes from the Greek noun *psuchè* ("*life breath*," "*spirit*," "*soul*," "*mind*"), the Latin adjective *activus* ("*active*"), and *Fauna*, the name of the Roman goddess of fertility (16). "*Psychoactive fauna*" is currently used to denote the group of animals whose body parts or excretions contain one or more substances which, in a sufficiently high dose, have the potential to alter the user's state of consciousness (17). These compounds, naturally occurring in "fauna," may be as well considered NPS, as the "novelty," as previously defined, does not necessarily mean "new" psychoactive compounds, but all individual drugs in pure form or in complex preparations that are not yet scheduled under the Single Convention on Narcotic Drugs (1961) or the Convention on Psychotropic Substances (1971) (5).

As the recent spread and interest toward the "natural" NPS by the psychonauts' community, the present mini-review aims at providing an overview of the presence of some substances with psychoactive/psychedelic properties in fauna, by identifying their potential human abuse/misuse, their pharmacological and clinical effects on humans, in order to better qualify another category of NPS.

MATERIALS AND METHODS

A mini-review was carried out by using the PubMed/Medline and Google Scholar databases. However, given the limitation of peer-reviewed data published so far, a preliminary nonparticipant multilingual qualitative study of a list of prodrug websites and other online resources (i.e., e-newsgroups, chat-rooms, bulletin boards, and e-newsletters) was conducted in order to obtain a list of potentially representatives of the "*psychedelic fauna*." A systematic Internet search was conducted on Duckduckgo® and Google® which included the following keywords: "*animal's name*" and/or possible acronyms, street names etc. plus "*to buy*," "*experience*," "*trip*," "*legal high*," "*psychedelic*," "*hallucinogen*," "*psychoactive*." The first 5 pages recorded per search term and search engine were consequently analyzed and selected only if relevant in terms of information and data provided regarding to the "*psychedelic fauna*." Within the time frame January–July 2017, data were collected from 12 unique prodrug websites. Confidentiality measures applied to the dataset included storage in an online, password-protected computer and removal of screen pseudonyms, URLs, country and city identifiers. Some 2,900 fora threads were screened. After removal of those Web pages, which were either duplicates or non-relevant to the aims of the study, 268 fora threads, were analyzed and used to identify four main species implicated. Ethical approval for the study has been sought and granted by the Department of Pharmacy Ethics Committee at the University of Hertfordshire (December 15, 2010, reference code PHAEC/10-42), with a further extension of the approval granted in November 2013.

Then, we combined the search strategy of free text terms and exploded MESH headings for the topics of Psychedelic Fauna and Novel Psychoactive Substances as following: ((((*Psychedelic* OR *hallucinogenic* OR *psychoactive) substances) [*Title/Abstract]) AND ((various name of *Animals*) *[*Title/Abstract])), as previously identified with the above-mentioned online search. Secondary searches were performed using the reference list of included articles and relevant systematic reviews. All articles published in English without time restriction were selected. Studies published through to 15 September 2017 were included. We considered studies describing some psychoactive/psychedelic/hallucinogenic effects following the intake of some animals (or parts of them), through different routes of administration, by humans. Working independently and in duplicate, two reviewers (LO and MC) read the papers and determined whether they provided data on psychedelic fauna. To be included in the present review, studies were required to meet the following criteria: (a) empirical and peer-reviewed study; (b) at least an abstract with estimates and/or full results published in English; (c) investigate psychoactive/psychedelic/hallucinogenic properties of some animals. Studies evaluating the intake of psychedelics/hallucinogens/other psychoactive substances by animals were properly excluded as not pertinent with the aims of the present paper. Moreover, studies mainly focused on intoxications rather than psychedelic experiences following the intake of some animals were also excluded from this review. As non-systematic review, reviews, letters to editors and meta-analyses were as well considered for retrieving data. LO and MC, independently extracted the data. Disagreements were resolved by discussion and consensus with a third member of the team (DP). Data were collected using an *ad-hoc* developed data extraction spreadsheet. The present comprehensive review

TABLE 1 | Summary of results.

Species	Origin	Psychopharmacological effects	References
HALLUCINOGENIC FISH			
a) Clown fish and damselfish (sp. *Abudefduf septemfasciatus*; commonly called "*Banded sergeant*") b) Rabbitfish (sp. *Siganus argenteus*; sp. *Siganus corallinus*; sp. *Siganus luridus*; sp. *Siganus rivulatus*; sp. *Siganus spinus*) c) Sea bream (sp. *Sarpa salpa*, commonly called '*Salema*') d) Sea chub (sp. *Kyphosus inerascens*; sp. *Kyphosus vaigiensis*; *Kyphosus bigibbus*) e) Surgeon fish (sp. *Acanthurus triostegus*, commonly called '*Convict surgeonfish*') f) Goatfish (sp. *Mulloidichthys flavolineatus*; sp. *Upeneus taeniopterus*) g) Mullet (sp. *Migil cephalus*; sp. *Neomyxus leuciscus*) h) Groupers (sp. *Epinephelus corallicola*, commonly called '*Coral grouper*')	South Africa and Hawaiian and Norfolk Islands in the Pacific Ocean. The *Sarpa salpa* originates from temperate and tropical areas, from the Atlantic coast of Africa extending to the Mediterranean Sea, particularly near to Spanish coasts; occasionally found around the British coastline.	Fish contain hallucinogenic substances. **If ingested raw:** may induce hallucinatory and onyroid effects such as vivid/ terrifying auditory and visual hallucinations, dizziness, loss of equilibrium, lack of motor coordination and mental depression, terror and nightmares, itching, burning of the throat, muscular weakness, rarely abdominal distress. **If orally ingested:** "*Sarpa salpa*" fish may produce vivid auditory and visual hallucinations.	(18–22)
Sea chubs from the genus *Kyphosus*, supposed to be *K. Fuscus* or more likely *K. Vaigiensis*	Norfolk Island, between Australia and New Zealand	Hallucinations and '*dreadful nightmares*'	(18)
Urolophus jamaicensis species	The Caribbean and Colombia	Entheogen/intoxicant/inebriating and aphrodisiac effects originating from stingrays' venom	(23)
Siganus spinus	Waters around Réunion, South Atlantic	Psychedelic effects	(24)
Mulloides flavolineatus	Hawaii	Psychedelic effects	(24)
Tetraodontidae include puffers, balloon fish, blowfish, bubble fish, globefish, swellfish, toadfish, and toadies.	Tropical regions of South America, Africa and South East Asia	**If orally ingested:** poisonous puffer fish can cause a slight numbness of the lips and tongue, followed by increasing paresthesia in the face and extremities, sensations of lightness or floating. Headache, epigastric pain/nausea/diarrhea and/or vomiting may also occur. Reeling or difficulty in walking have been reported. The second stage of intoxication includes increasing paralysis, respiratory distress, altered speech, dyspnea, cyanosis, and hypotension. Whilst paralysis increases, convulsions, mental impairment, cardiac arrhythmia and death may occur.	(25, 26)
Fugu	Japan	Stimulant and aphrodisiac effects **If orally ingesting a non-lethal dose (i.e., <8** μg per kg body weight)**:** tingling in the lips, fingers, and toes, and extremities may occur. **If orally ingesting a lethal dose (i.e., >8** μg per kg body weight)**:** numbness, anesthesia, paresthesia, abdominal pain, nausea, and vomiting, muscle paralysis and respiratory insufficiency may occur.	(27, 28)
Somniosus microcephalus	North Atlantic and Artic Oceans	**If orally ingested:** may cause diarrhea/ vomiting/ hallucinations/ numbness. It may cause a state of near-death for several days, while the subject remains conscious.	(29)
Sea sponges such as *Smenospongia aurea*	Caribbean Sea	Psychedelic effects	(30–32)

(Continued)

TABLE 1 | Continued

Species	Origin	Psychopharmacological effects	References
Sea sponges such as *S. echina*	Caribbean Sea	Psychedelic effects	(30–32)
Sea sponges such as *erongula rigida*	Western Atlantic Ocean: Florida, Gulf of Mexico, and the Caribbean	Psychedelic effects	(30–32)
PSYCHEDELIC AMPHIBIANS			
Bufo alvarius and *Bufo marinus*	Mesoamerica	Used as ritual intoxicants owing to their viscous milky-white venom that contains bufotenin and bufotoxin. **If orally ingested:** *Bufo* toad venom can be fatal. Single deep inhalations of vaporized venom can produce intense and transient psychoactive effects mainly auditory and visual hallucinations.	(33)
Bufo marinus	North America	*B. Marinus* venom was used as "*Zombie's powder*". **If orally ingested:** *Bufo* toad venom can be fatal. Single deep inhalations of vaporized venom can produce intense and transient psychoactive effects mainly auditory and visual hallucinations.	(34, 35)
B. Alvarius	The Sonoran Desert an area of California across the southern half of Arizona and South Mexico.	It contains the enzyme O-methyl-transferase, which converts bufotenin (5-OH-DMT) to the potent hallucinogen 5-MeO-DMT. The skin also contains bufotenin analogs. **If orally ingested:** *Bufo* toad venom can be fatal. Single deep inhalations of vaporized venom can produce intense and transient psychoactive effects mainly auditory and visual hallucinations.	(36, 37)
Phillomedusa bicolor	The Peruvian and Brazilian Amazon	**Buccal absorption** of opioid peptides scraped from the skin may induce rapid pulse/incontinence/vomiting, a state of listlessness and euphoria.	(37, 38)
PSYCHEDELIC ANTS			
Red harvester ants (e.g., *Pogonomyrmex californicus*)	South and South Central California	**Oral ingestion** of live ants may cause hallucinogenic and/or mind-altering effects.	(39, 40)

provides summary of data collected on three main categories of animals (fish, amphibians and ants), as illustrated in **Table 1**.

RESULTS

"Hallucinogenic" Fish

Certain species of fish, particularly coming from South Africa, in the Hawaiin and Norfolk Islands in the Pacific Ocean, have been demonstrated to contain hallucinogenic substances which may give a "*fishing trip*" like that produced by lysergic acid diethylamide (LSD) intake (34). Toxic fish species belonging to eight families have been implicated: (a) Clown fish and damselfish (sp. Abudefduf septemfasciatus; commonly called "*Banded sergeant*"); (b) Rabbitfish (sp. *Siganus argenteus*; sp. *Siganus corallinus*; sp. *Siganus luridus*; sp. *Siganus rivulatus*; sp. *Siganus spinus*); (c) Sea bream (sp. *Sarpa salpa*, commonly called "*Salema*"); (d) Sea chub (sp. *Kyphosus inerascens*; sp. *Kyphosus vaigiensis*; *Kyphosus bigibbus*); (e) Surgeon fish (sp. *Acanthurus triostegus*, commonly called "*Convict surgeonfish*"); (f) Goatfish (sp. *Mulloidichthys flavolineatus*; sp. *Upeneus taeniopterus*); (g) Mullet (sp. *Migil cephalus*; sp. *Neomyxus leuciscus*); (h) Groupers (sp. *Epinephelus corallicola*, commonly called "*Coral grouper*") (18).

Several hallucinatory and onyroid experiences, also called "*Ichthyoallyeinotoxic or hallucinatory mullet poisoning*" or "*ichthyoallyeinotoxism,*" have been reported after ingestion of the above-mentioned fish as raw (19–21). The effects of eating Ichthyoallyeinotoxic raw fish may include vivid/terrifying auditory and visual hallucinations, dizziness, loss of equilibrium, lack of motor coordination and mental depression, terror and nightmares, itching, burning of the throat, muscular weakness, rarely abdominal distress (19–21). Symptomatology may occur within a few minutes to 2 h after consumption, and may last for up to 24 h (18, 19). The first symptoms usually comprise imbalance, loss of coordination and a generalized malaise, followed by delirium, visual and/or auditory hallucinations (mainly zooptic), depression and nightmares (18, 19, 22, 41). However, there is no clear evidence of an intentional recreational use of these toxins for their "dream-inducing" properties, as most cases have been described occurring due to an accidental intoxication (18).

The "*Sarpa salpa*" (aka "*salema porgy*," "*dream fish*" or "*nightmare fish*") may produce vivid auditory and visual hallucinations if orally ingested (18, 20). *Sarpa salpa* is easily recognized by its gold stripes running along its side. It usually inhabits temperate and tropical areas, from the Atlantic coast of Africa to the Mediterranean Sea, particularly near to Spanish

coasts; whilst it is occasionally found around the British coastline (22). It belongs to the Sparidae family and represents a popular dish across many Mediterranean countries (22). It may cause vivid hallucinations in few minutes after ingestion, which may last for days (18). It originally became a recreational drug during the Roman Empire in which it was commonly called as "*the fish that makes dreams*" in Arabic (18). Anecdotal online trip reports described that *"[…] the subjective effects are evident the next day after eating, they had vivid nightmares (I like a giant black dog chasing me through a forest as a kid sometimes after eating some of these usually just fried whole, after being scaled and gutted on each side in butter), more lethargy than any psychedelia and with excessive consumption (usually with beer) people often have slurred speech and slow/reduced reflexes […]"* (42).

Other species commonly claimed to be capable of producing hallucinations include several species of sea chub from the genus *Kyphosus*, supposed to be *K. fuseus* or more likely *K. vaigiensis*, which may cause "*dreadful nightmares*" (18). Furthermore, some Caribbean natives, particularly Mayan tribes during the pre-Colombian period, were and are usual consumers of the *Urolophus jamaicensis* species with their stingrays' venom for their entheogen/intoxicant/inebriating and aphrodisiac properties (23). Moreover, *Siganus spinus* (aka "*the fish that inebriates*"), in the waters around Réunion, and *Mulloides flavolineatus* (formerly *Mulloidichthys samoensis*), called "*the chief of ghosts*" in Hawaii, have been consumed due to their psychedelic properties (24).

Another hallucinogenic fish group is represented by the *Tetraodontidae* fish which include puffers, balloon fish, blowfish, bubble fish, globefish, swellfish, toadfish, and toadies (25). The name derived by the tetrodotoxin (TTX) that is a particularly potent neurotoxin, which specifically blocks voltage-gated sodium channels on the surface of nerve membranes (43). Its use has been historically documented as a pain-killer, for rheumatism/arthritis/neurological pain, as aphrodisiac/inebriant and essential component used during the preparation process of the "*zombie drug*" (34). TTX is commonly present in the gonads, liver, intestines, and skin of pufferfish (43). The first symptomatology consists in a slight numbness of the lips and tongue, appearing between 20 min to 3 h after eating poisonous pufferfish. Subsequently, an increasing paresthesia in the face and extremities, which may be followed by sensations of lightness or floating, appears. Headache, epigastric pain/nausea/diarrhea and/or vomiting may occur. Occasionally, some reeling or difficulty in walking have been described. The second stage of the intoxication is an increasing paralysis. Many victims are unable to move; even sitting may be difficult. There is an increasing respiratory distress, altered speech, dyspnea, cyanosis, and hypotension. Whilst paralysis increases, convulsions, mental impairment and cardiac arrhythmia may occur. Although completely paralyzed, subject may be conscious and completely lucid until shortly before dying. Death usually occurs within 4 to 6 h, with a known range of about 20 min to 8 h (26). Puffer fish toxins have been described by psychonauts as causing "*local paralysis, apparently, a feeling similar to lidocaine*", "*a tingly feeling on the tongue after eating the meticulously prepared puffer-fish*" *and for a few people there is no recreation value and plenty of risk for overdose*" (30). A few online anecdotal reports described the substance "*Zombinol*", a fictional substance, to refer to what Wade Davis wrote in his book "*The Serpent and the Rainbow*" (44) which has been supposed to be TTX, the deadly pufferfish toxin (45, 46).

An example of fish containing TTX is *Takifugu* and related genera (27). *Fugu* fish is eaten in Japan and is famous for being deadly if prepared incorrectly. The goal of preparing *Fugu* is not to remove the drug, but to reduce its levels in the animal, so it is enjoyable to the person eating it. It has powerful stimulant and aphrodisiac effects. The trick in preparing *Fugu* is to remove just enough of the organs that contain the nerve toxin TTX. When a non-lethal dose is consumed, it causes tingling in the lips, fingers, and toes, and extremities. Whilst after a time (or with a larger dose), the toxin can cause numbness and anesthesia, paresthesia along with abdominal pain, nausea, and vomiting until muscle paralysis and respiratory insufficiency (28).

Another hallucinogenic fish is *Somniosus microcephalus*. It contains in its flesh trimethylamine oxidase which is converted into trimethylamine when eaten (29). After its oral intake, subjects described the onset of a "*shark sick*" comprising diarrhea/vomiting/hallucinations/numbness. Despite it not always being lethal, it may cause a state of near-death for several days, while the subject remains conscious (29).

Moreover, as previously discussed by Shulgin and Shulgin (1997), who wrote about "marine tryptamines" (i.e., 5-Bromo-DMT and 5,6-dibromo-DMT), there are several sea sponges like *Smenospongia aura*, *S. echina* and *Verongula rigida* which have been demonstrated to have some psychedelic activity (30–32).

"Psychedelic" Amphibians

Overall, it has been well documented that amphibian skin may contain a large range of biological active alkaloids, most possessing a unique pharmacological and therapeutic profile (31). Amongst alkaloids identified are: steroidal *salamandarines* (from salamanders); *batrachotoxins* (a potent and selective activator and ligand for Sodium Channels); *histrionicotoxins* (potent not-competitive blockers and ligands for nicotine receptor channels); *epibatidine* (with a potent anti-nociceptive activity at nicotinic receptors); the neotropical poison frogs (*dendrobatidae*); the *pumiliotoxin* (with myo-/cardio-tonic activity) contained in some anuran genera from Dendrobatidae, Mantellidae, Bufonidae and Myobatrachidae families; several izidines (pyrrolizidines, indolizidines, quinolizidines and legmizidines), pyrrolidinesm piperidines and various tricyclics (related in structures to the coccinellines), and spiropyrrolizidines, pseudophrynamine, and the tryptamine bufotenin (47–49).

Furthermore, high levels of amines, including serotonin, histamine and tyramine, have been found in the skin of various toads and frogs and synthesized by the amphibian itself due to their irritant properties on buccal tissue which is used as chemical defense (50). In addition, high levels of vasoactive peptides such as bradykinin, sauvagine, physaelaemin, caerulein, bombesin, dermorphins, etc., have been presumably used as defense against predators and microorganisms (51).

Amongst the amphibians, *Bufo alvarius* and *Bufo marinus*, morphologically similar, possess prominent parathyroid glands

that secrete a viscous milky-white venom (52) containing two cardiac glucosides, namely bufotenin and bufotoxin (33). Some anthropologists described ancient peoples of Mesoamerica who used these toads as a ritual intoxicant (7, 53, 54). Furthermore, it has been assumed to be a specific psychoactive ingredient in *B. Marinus* venom, used as "*Zombie's powder*", known during the pre-Columbian period in North-America (34, 35). Toad venom may be sometimes found in some Chinese products used in traditional Chinese medicine, such as "*chan su*," sold as topical aphrodisiacs (aka "*stone*," "*love stone*," "*rock hard*") (55). *B. alvarius*, the Sonoran Desert toad, is a semi-aquatic amphibian found only in the Sonoran Desert, an area of California across the southern half of Arizona and South Mexico (36). *B. Alvarius* contains the enzyme O-methyl-transferase, which converts bufotenin (5-OH-DMT) to the potent hallucinogen 5-methoxydimethyl-tryptamine (5-MeO-DMT) (36). The skin contains some indolealkylamines and their metabolites belonging to the common series of 5-hydroxy-indolealkylamines (e.g., bufotenin) and to the less common series of 5-methoxyindolealkylamines (e.g., O-methyl-bufotenin) (37, 56, 57). In addition, its skin contains also sulfur-containing indolealkylamines (e.g., bufoviridine). Consuming *Bufo* toad venom orally, through licking or eating, is ineffective and potentially risky, being associated sometimes with fatalities, as the venom was evolved to be a defensive poison to deter predators from eating the toads. Furthermore, many species of frogs and toads produce other venoms or skin irritants that should not be ingested orally. Single deep inhalations of vaporized venom proved powerfully psychoactive effects within 15 s. Consistent with the known effects of 5-MeO-DMT, the intoxication is intense and short-lived, marked by auditory and visual hallucinations. The strongest effects dissipated after 5 min, but residual changes in perception persisted for up to 1 h (35, 36).

Another specie is *Phillomedusa bicolor,* which is a large green nocturnal frog that lives in the trees of the Peruvian and Brazilian Amazon. Adult frogs secrete a material which is used by the native Mayoruna Indians as a hunting aid (37). Skin secretions are rich in vasoactive, opioid peptides and a peptide called "adenoregulin" (58). Peptides tested acted as potent *mu* opioid agonists on isolated organ preparations (59). Skin secretions, previously scraped from a live frog and stored dry on a stick mixed with saliva, for buccal absorption, may induce a plethora of symptoms including rapid pulse/incontinence/vomiting, followed by the onset of a state of listlessness (which may last some days) that proceeds with a euphoric state (38).

Psychedelic Ants

It has been as well documented that ants have been used in both curative and preventative medicine, for treating common illnesses (e.g., paralysis, gastrointestinal illnesses, severe colds, pain, arthritis, and gynecological disorders), frequently swallowed live as an emetic or bitten the exterior of the ants' body (60). Behind the medicine practice, ants seemed to play a significant role during some initiatory/ritual/esoteric activities. For example, ants have a prominent role during the antinic or "*ant ordeal*" of the Luiseiio, a rite which followed temporally the ritual of *Datura* drinking (39). The ingestion of live ants may cause hallucinogenic and/or mind-altering effects, as described in discussions on the shamanistic behavior or medical knowledge of native people (39). Indian people ate harvester ants after 3 days of abstaining from food, water, and sex and avoiding contact with blood, as a ritualistic hallucinogen (40). They ate balls of moistened eagle down with about 5 ants inside each (38, 60). The dose was regulated, from dozens to ninety or so balls, and the ant feeding stopped when the eyes of the subject turned red, they became lethargic and refused more (40). To obtain some shamanistic powers, the ants would be eaten in a similar manner, every summer, until the powers were obtained (40). Ant ingestion persisted through the Mission Period (1800–1878), but these practices appeared to have been abandoned during the last century (40). However, most studies were confounded by the simultaneous employment of techniques such as fasting, sleep deprivation, and the concomitant use of *Datura* and/or other psychotropic substances.

However, it has been speculated that the ants used in vision quests may have belonged to the yellow honey ant or other species of the *Myrmacomecocystus* genus, which do not contain any known psychoactive substances (39). *Red harvester ants* (e.g., *Pogonomyrmex californicus*), largely used in religious and medical practices, were as well reported to be taken by native people of Southern and South-central California, as hallucinogens (40). Their venom contains many kinds of proteins, enzymes, histamines, and other chemicals. It has been reported to be 5 and 8–10 times more toxic than, respectively, Oriental Homet and honeybee venoms (61). The doses employed in visionary contexts by California Indians were clearly within the range of pharmacological activity, representing approximately 35% of a lethal dose for an individual with a body weight of 45.5 kg (40). Moreover, it contains formic acid and polypeptide kinins which induce pain/inflammation/hypotension. Some kinins own nicotinic cholinergic activity that may be responsible for the induction of hallucinations (40).

DISCUSSION

The present paper specifically focused on invertebrates and vertebrates, such as ants, fish, and amphibians and their possible hallucinogenic properties and their relative and potential human recreational consumption and/or abuse. Specifically, the above-mentioned "*psychoactive fauna*" (17) have been reviewed, focusing on their first intake according to a historical perspective, their chemical/pharmacological profile, potential mechanisms of action and desired/adverse effects. Despite limited literature and scientific evidence being available to date, as most studies more specifically focused on psychedelic/hallucinogenic drugs contained in some plants and plant-derived, a plethora of data coming from the online platform and psychonauts' fora and/or blogs have been collected, according to a nonparticipant netnographic methodological approach, about the "*psychedelic animals*" (15) and subsequently compared with published literature, following a mini-review approach.

Substances influencing mood and thinking processes have been known to humanity at least from early Neolithic

times in all known cultures (8). Entheogens are typically psychedelics/hallucinogens, and, by definition, imply the use of psychoactive substances for religious/spiritual reasons (9, 62, 63). Entheogens generate transcendental feelings or hallucinatory experiences, which may be enhanced by ritualistic environments e.g., darkness, chanting, spiritual discourse. Shamans throughout the world incorporate entheogens in their arsenal of techniques to connect with the spirit world (64).

Nowadays, many different classes of psychoactive substances are available for abuse, including several hundred NPS (5, 6, 65). Some of these drugs are entirely synthetic and others occur naturally in plants (or in animals), or are chemically modified from plant or animal compounds (11). Moreover, the current drug culture, i.e., psychonauts, consume a great variety of NPS with hallucinogenic/psychedelic properties, including those contained in some species of animals (3, 15). Specifically, there is a subcategory of e-psychonauts, as previously studied, who are particularly interested in taking only "natural" NPS, like plants and animals (3, 6, 11, 15).

"*Psychedelic fauna*" appears to be divided in two groups: the producers and the consumers. Producers represent animal species able to produce psychoactive chemicals, usually by converting precursors which they consume, into a new substance able to be "psychoactive" to humans. Consumers comprise any animal which takes psychoactive chemicals and store them unchanged (42). In the present mini-review we focused on the "producers," supposed to be taken by the psychonauts' communities due to their supposed psychedelic/hallucinogenic properties. Data were collected and summarized for three main species (i.e., fish, amphibian and ants), even though the recreational and psychedelic intake of other animals by psychonauts has been as well documented (30, 34, 42).

Amongst fish, despite the toxic agents implicated remaining unknown, some authors suggested that their hallucinogenic properties may derive from alkaloids of the indole group, dimethyltryptamine (DMT) which naturally occur in certain types of microalgae (*Caulerpaceae* family) that fish usually ingest and phytoplankton and which are probably present in the head of some fish (18, 19).

The metabolic source of TTX, included in the *Tetraodontidae* fish (e.g., puffer-, balloon-, blow-, bubble-, globe-fish, etc.) is uncertain. No algal source has been identified. TTX was recently assumed to be a metabolic product of the host. However, recent reports of the production of TTX/anhydro-TTX by several bacterial species, including strains of the family *Vibrionaceae*, *Pseudomonas* sp., and *Photo bacterium phosphoreum*, point toward a bacterial origin of this family of toxins. These are relatively common marine bacteria that are often associated with some marine animals. An association between TTX-producing bacteria and higher organisms appears to offer clear advantages to both partners. The bacteria get a safe place to live, eat, and reproduce; the hosts use the toxin for predation or defense or both (25, 26, 43).

Whilst amongst amphibians, the tryptamine *bufotenin*, isolated in the skin of some species of toads, has been implicated to determine hallucinogenic effects after the intake of some amphibians, as abovementioned (47–49, 51). In fact, its name originates from the *Bufo* genus of toads that secrete bufotoxins from their parotid glands (33, 52). Bufotenin is chemically like the psychedelic psilocin (4-HO-DMT), 5-MeO-DMT, and DMT (36, 56, 57).

Furthermore, some potentially hallucinogenic and/or mind-altering substances have been isolated and demonstrated to be effective from ant toxins (39, 40, 61), even though formally detected specific substances have not been implicated yet.

In conclusion, the recent re-emergence of a specific "appealing"/"attractiveness" toward psychedelic/hallucinogenic substances by psychonauts (3, 5, 15), particularly plant-derived as previously investigated (11), is gradually opening to the development and spread of a new "psychedelic trend," including "*psychedelic fauna*" as well. Further evaluation/isolation/identification of substances potentially psychedelic/hallucinogenic from some species of animals as well as further netnographic studies, specifically investigating the psychonauts' preferences and interest toward the "psychedelic fauna" should be carried out, to better understand this new NPS trend.

AUTHOR CONTRIBUTIONS

LO, MC, and DP: conceived the topic of the manuscript, while LO, MC, and DD carried out the main analysis; DP and AG: assisted in either screening of the studies or preparation of the attachments; JC: served as study reviewer; FS: served as senior study reviewer. All the coauthors substantially contributed to the present piece of work before approving it for final submission.

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REVIEW
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e-Addictology: An Overview of New Technologies for Assessing and Intervening in Addictive Behaviors

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Background: New technologies can profoundly change the way we understand psychiatric pathologies and addictive disorders. New concepts are emerging with the development of more accurate means of collecting live data, computerized questionnaires, and the use of passive data. *Digital phenotyping*, a paradigmatic example, refers to the use of computerized measurement tools to *capture* the characteristics of different psychiatric disorders. Similarly, machine learning–a form of artificial intelligence–can improve the classification of patients based on patterns that clinicians have not always considered in the past. Remote or automated interventions (web-based or smartphone-based apps), as well as virtual reality and neurofeedback, are already available or under development.

Objective: These recent changes have the potential to disrupt practices, as well as practitioners' beliefs, ethics and representations, and may even call into question their professional culture. However, the impact of new technologies on health professionals' practice in addictive disorder care has yet to be determined. In the present paper, we therefore present an overview of new technology in the field of addiction medicine.

Method: Using the keywords [e-health], [m-health], [computer], [mobile], [smartphone], [wearable], [digital], [machine learning], [ecological momentary assessment], [biofeedback] and [virtual reality], we searched the PubMed database for the most representative articles in the field of assessment and interventions in substance use disorders.

Results: We screened 595 abstracts and analyzed 92 articles, dividing them into seven categories: e-health program and web-based interventions, machine learning, computerized adaptive testing, wearable devices and digital phenotyping, ecological momentary assessment, biofeedback, and virtual reality.

Conclusion: This overview shows that new technologies can improve assessment and interventions in the field of addictive disorders. The precise role of connected devices, artificial intelligence and remote monitoring remains to be defined. If they are to be used effectively, these tools must be explained and adapted to the different profiles of physicians and patients. The involvement of patients, caregivers and other health professionals is essential to their design and assessment.

Keywords: addictive medicine, digital phenotype, ecological momentary assessment, virtual reality, wearable devices, machine learning

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INTRODUCTION

Addictive disorders are common, but only a small minority of patients receives adequate treatment (1). Diagnosis, early detection of at-risk patients, and the daily monitoring of symptoms and treatments (including self-management) are major issues in addictive medicine and public health. New technologies (smartphone, computers, biomarkers) and the parallel expansion of medical information technology and artificial intelligence have brought about a paradigm shift, resulting in more personalized and predictive medicine (2). These new tools are spurring practitioners to think about addictive disorders in different ways that may ultimately modify their practices. If behavior disorders can be captured by mathematical or computer models, and if diseases can be predicted and relapses detected earlier by machines or smartphones, what role will be left for healthcare teams to play?

These new technologies are also revolutionizing research, insofar as data collection methods can now be classified as either *active* or *passive* (3). The collection of active (or live) data, which refers to all self-evaluation procedures that can be implemented on a computer or smartphone, requires input from the patient, whereas passive data (heart rate, motion detection, sound or light sensor, number of calls sent, duration of calls, etc.) are collected via *background tasks*. Sometimes, patients do not even know when data are being collected, allowing the observer's influence to be reduced to a minimum.

New technologies also appear to be important in the field of treatment. The expansion of web-based and smartphone-based interventions holds out the prospect of having a coach or therapist in the pocket (4). The democratization of virtual reality and the development of neurofeedback methods also appears useful in addictive disorders.

There are many issues in addictive medicine: pathology screening in patients who sometimes minimize their consumption; active treatment of craving using cognitive behavioral therapy (CBT)-driven techniques applied remotely and in real time; and the strengthening of cooperation between patients and clinicians by facilitating the links between them. The tools currently under development look set to bring many concrete improvements. They may also improve our understanding of the underlying mechanisms of various addictions.

In this article, we conducted an overview of the various innovative technologies related to assessment and intervention in addictive disorders, describing various concepts, applied to addictive disorders, as this new approach could profoundly change the therapeutic relationship, patient assessment and the nature of interventions.

METHOD

We conducted a narrative review using the MeSH terms and keywords [e-health], [m-health], [computer], [mobile], [smartphone], [wearable], [digital], [machine learning], [ecological momentary assessment], [biofeedback] and [virtual reality], and we searched the PubMed database for studies in the field of substance use disorders (SUDs). AB, FF, and SM screened 595 abstracts, and 92 articles were then analyzed and divided into seven categories, the first five for evaluation and the last two for treatment: 1. e-health applications and web-based interventions, 2. Machine learning, 3. Computerized adaptive testing (CAT), 4. Wearable devices and digital phenotyping, 5. Ecological momentary assessment (EMA) and ecological momentary intervention (EMI), 6. Biofeedback and neurofeedback, and 7. Virtual reality. These categories were chosen because they correspond to the most innovative topics and most reported in most studies on new technologies in psychiatry.

CONCEPTUAL OVERVIEW

A brief description of the concepts underlying new technologies in the field of mental health is summarized in **Table 1**.

OVERVIEW OF TECHNOLOGICAL INNOVATION STRATEGIES IN SUBSTANCE USE DISORDERS

e-Health Applications and Web-Based Interventions

The term *e-health* was originally defined by John Mitchell in 1999 as a "new term needed to describe the combined use of electronic communication and information technology in the health sector. The use in the health sector of digital data—transmitted, stored, and retrieved electronically—for clinical, educational, and administrative purposes, both at the local site and at a distance" (5). This term now covers a broader reality (6), as it includes any device or computer software relating to health, centered around two fields:

1. *Telehealth*, in other words, health mediated by telecommunications tools (telemedicine, telemonitoring and mobile health);
2. *Robotics*, defined as a set of techniques using automatic machines or robots, that includes both medical robotics itself (e.g., robot surgeons) and the use of programs based on artificial intelligence.

It is at the interface of these two fields that new Clinical Decision Support Systems (CDSS) have been developed, defined as computer applications "whose aim is to provide clinicians with information describing the clinical situation of a patient in useful time and place as well as appropriate knowledge … to improve the quality of care and the health of patients" (17). Although e-health technology in the area of SUDs is still at a relatively early stage, several projects are worthy of interest, as these new technologies allow realtime evaluation to be combined with an interventional dimension. Several e-health solutions [i.e., A-CHESS (18)] improve self-management by providing self-assessment modules and reminders and also allow for rapid contact with a support service to ensure swift responses in case of need. Other types of software [ORION (19, 20), D-ARIANNA (21, 22), Steering Clear program (23)] optimize behavioral risk quantification (overdose for Orion, binge-drinking for D-ARIANNA, drink-driving for Steering Clear) via scalar self-assessment modules. These programs deliver rapid intervention

TABLE 1 | Summary of concepts.

Concept	Description	Reference
e-health	Combined use of electronic communication and information technology in the health sector. This includes telehealth (health mediated by telecommunications tools: telemedicine, telemonitoring and mobile health/ m-health) and robotics (techniques using automatic machines or robots, including machine learning)	(5, 6)
Clinical Decision Support System	Computer-based tool supporting the decision-making process, in order to facilitate organizational processes and provide clinicians with information about patients' clinical status and the knowledge they need to improve quality of care and patient health	(7)
Machine learning	Scientific discipline that focuses on how computers learn from data, using statistics to learn relationships from data, and computer science to accurately detect classification patterns via efficient computing algorithms	(8)
Computerized adaptive testing, CAT	A computer-administered test in which each item or set of items is selected according to the test taker's responses to the previous ones	(9)
Ecological momentary assessment	Smartphone-based evaluation of symptoms from day to day in patient's usual environment, free from recall biases, as the patient self-assesses "*right then, not later; right there, not elsewhere*"	(10, 11)
Ecological momentary intervention	Smartphone-based intervention involving the delivery of psychoeducation, advice or recommendations about how to behave according to the patient's immediate environment	(12–14)
Digital phenotyping	Moment-by-moment quantification of the individual-level human phenotype using passive data (GPS, accelerometer, voice, call and text logs, screen use) from digital devices (smartphone, wearable devices)	(15)
Biofeedback or neurofeedback	Painless, noninvasive procedure that consists in capturing biometric data (EEG, ECG, EMG, skin conductance, temperature) and feeding them back to patients in real time so that they gradually learn (through positive reinforcement) how to promote brain activity corresponding to the therapeutic target using CBT techniques and relaxation	(16)

in the form of guidelines, tips, motivational techniques and people to contact. Finally some programs [e.g., JITAI (24)] are intended to prevent relapse by providing regular monitoring and individualized coping strategies. A recent review (25) showed that computer-based alcohol interventions are generally effective in reducing alcohol consumption. Longer, multisession interventions are more effective than shorter or single-session interventions. *Other programs* provide individually tailored clinical content in a multimedia format (26, 27) to promote psychoeducation. *In the field of smoking cessation, meta-analysis showed the interest of web-based interventions* (28).

The US Food and Drug Administration (FDA) recently authorized a smartphone-based e-health program for prescription: the Pear® reSET application, which uses CBT, psychoeducation, social connection and self-assessment (craving, mood, etc.) to treat different types of SUDs (except for opioid dependence). The good results (40.3% adherence to abstinence in reSET users vs. 17.6% in control group) nevertheless need to be replicated (data should be available in 2018) (29). A description of these technologies can be found in **Table 2**.

TABLE 2 | Smartphone- and web-based e-health interventions.

Concept	Description
A-CHESS	The Addiction-Comprehensive Health Enhancement Support System includes both static content (e.g., audio-guided relaxation) and interactive features (e.g., if a patient is near a high-risk location such as a familiar bar, a GPS-initiated alert asks the patient if s/he really wants to be there)
ORION	The Overdose RIsk InfOrmatioN project was set up to develop and pilot an e-health psychoeducational tool that provides information about the risks of having a drug overdose
D-ARIANNA	The Digital-Alcohol RIsk Alertness Notifying Network for Adolescents and young adults was set up to develop and pilot an e-health psychoeducational tool that provide information to adolescents and young adults about the risks of binge drinking
Steering Clear	Steering Clear of Driving After Drinking is a tailored e-health intervention that aims to reduce repeat offending by first-time convicted drink driving offenders via an online program
JITAI	The Just-In-Time Adaptive Intervention framework could be used to design a mobile app that carries out in-the-moment monitoring of triggers for lapsing, and delivers personalized coping strategies to prevent lapses from occurring
reSET	reSET is a digital therapeutic system designed to be used as an adjuvant to standard outpatient therapy for treating SUDs. It combines patient-facing interventions and assessments via a mobile device, with clinician-facing dashboards and data analytics on the back end

Machine Learning

Machine learning is the subfield of artificial intelligence that gives "computers the ability to learn without being explicitly programmed" [Arthur Samuel, 1959 (30)]. It uses a different kind of classification process: *Supervised* classification seeks to automatically identify rules from databases constituted of "examples" (classically, these are cases that have already been validated, such as a well-established diagnosis), while with *unsupervised* classification, the collected data are not labeled, and the objective of the software is to group them into clusters so that the closest and most similar ones are placed in the same cluster, whereas those that are further apart are placed in separate clusters. This method makes it possible to find new structures. The coupling of machine learning with complementary examinations (MRI, EEG) reveals patterns that allow patients to be divided into different groups, which could be useful for screening or for describing groups with a particular phenotype (e.g., patients at

risk of relapse). These techniques will make it possible in the near future to improve addictologists' predictive skills, as is already the case for the prediction of psychotic transition in patients in an at-risk mental state (31) and in the field of mood disorders (32, 33). In addictive medicine, one study (34) has already shown that the risk of alcohol relapse can be predicted with 77% accuracy by analyzing clinical data (e.g., demographics, alcohol use, dependence severity, craving, health-related quality of life, and psychological measures at baseline), while the probability of treatment success can be predicted with an AUC between 0.793 and 0.820 (35) using clinical data (10 patient characteristics, 3 treatment characteristics, principal source of referral, summary of type of problematic substance and mental health problem). Chih et al. (36) used a Bayesian network model to predict relapse, based on responses to 2934 A-CHESS weekly surveys provided by 152 alcohol-dependent individuals who had recently completed residential treatment. It showed good predictability, with an AUC between 0.829 (cross-validation) and 0.912 (external validation). Mumtaz et al. (37) developed a machine learning method that utilized resting-state EEG-derived features as input data to distinguish patients with alcohol use disorder from healthy controls and to perform automatic screening. Results showed that interhemispheric coherences between brain regions differed significantly between the study groups, with high classification efficiency (accuracy = 80.8, sensitivity = 82.5, specificity = 80; $F = 0.78$). The authors concluded that EEG data can be used as objective markers for screening patients with alcohol use disorder.

In the field of addictology research, these techniques are now being used to identify behavioral biomarkers predictive of the use of substances such as cocaine (38) or heroin (39).

Computerized Adaptive Testing

Computerized Adaptive Testing (CAT) (40) has been developed to *mimic* clinicians. It uses a limited form of artificial intelligence to automatically adapt questionnaire items to the answers provided by the patient to previous items, using a large database of possible answers/questions. More specifically, after the first general questions, an algorithm adapts the subsequent items according to the patient's initial answers. Complementary questions enhance the accuracy of the evaluation. The advantages of this type of testing are therefore an improvement in performance and a reduction in test duration, which is important, as the difficulties reported by patients mostly concern the amount of time spent completing the scales and the repetitiveness of the questions. This method, first developed by Fliege in 2005 and replicated by Gibbons (41), has been validated in depression disorders (42) with the CAT-DI. In the field of addictions, Pilkonis (43) demonstrated the validity of this method using the Patient-Reported Outcomes Measurement Information System (PROMIS), which includes five item banks for alcohol use. PROMIS CATs has been shown to be efficient and makes it feasible to use a comprehensive health status profile in a substance use treatment setting, providing important prognostic information regarding abstinence and drinking severity. Versions of this tool can also be used to rapidly explore common comorbidities with SUDs, such as the CAT-SS for suicidal behaviors (44) or the CAT-ANX for anxiety (45). Some related tests are now being developed using virtual *avatars* of psychiatrists who can converse directly with patients, known as *embodied conversational agents* (46).

Wearable Devices and Digital Phenotyping

John Torous and Lisa Gualtieri recently recalled the potential worth of connected objects in the field of mental health (47), as many devices now include multiple sensors such as accelerometers, heart rate sensors, sleep trackers, skin conductance sensors, and light sensors. The potential to gather real-time physiological data from fitness trackers, with the addition of symptom surveys from smartwatches, is attractive, and there is increasing interest in using real-time patient data as biomarkers of illness (48).

John Torous et al. have developed the concept of the *digital signature* or *digital phenotype* of pathology. These terms refer to the capture by computerized measurement tools of specific characteristics of psychiatric disorders (49, 50). Some behaviors or symptoms can be objectified and quantified by computer tools, constituting an *e-semiotics*. Sensor miniaturization and the ubiquitous use of smartphones make it possible to collect a large amount of data that psychiatrists had never been able to access before. Models based on these new *semiological signs* are beginning to emerge (51, 52). This collection uses *passive* data, for which no intervention is necessary, as they are collected in *background tasks*, sometimes without the patient realizing it. The objective is to reduce the influence of the observer as much as possible. This detection may involve both a mobile phone and its onboard sensors (GPS, accelerometer, verbal flow detector, etc.) or else connected wearable objects that enable biometric monitoring in real time. For example, it is now possible to use heart rate variability (HRV) to distinguish an alcohol-dependent patient from a nondependent chronic alcohol user. Defined as the degree of fluctuation in the interval between two cardiac contractions, HRV is dependent on the autonomic nervous system and is markedly decreased in dependent patients (53). The links between HRV and addictive disorders, alcohol dependence in particular, are now quite well known (54). It is an interesting biomarker, albeit not very specific, as changes in HRV are also encountered in mood disorders and posttraumatic stress disorder (PTSD) (55). In addictology, there has not yet been any research on specific digital phenotypes for different types of substance use, but more and more researchers are interested in coupling EMA with GPS (56, 57) or biosensor data. Activity-space analysis, which examines motion in different contexts, and EMA, which captures microlevel contextual changes as individuals move through their day can, for instance, improve understanding of drinking contexts in alcohol studies. Better identification of drug-using contexts can trigger the implementation of targeted interventions to prevent acting out.

Wearable biosensors have been developed to:

- Study physiological change during opioid use (decrease in locomotion and increase in skin temperature are consistently detected) (58);

- Monitor real-time drug use (59) or alcohol consumption (60), possibly through the detection of a metabolite (ethyl glucuronide) in human sweat (61);
- Monitor and study autonomic nervous system activity via electrodermal activity, 3-axis acceleration, ECG and temperature, in order to detect arousal events and automatically send therapeutic and empathetic messages to the patient using CBT (62).

These sensors can be coupled with biofeedback systems (cf. 2.6).

EMA and EMI

Classic data collection relied on a conventional interview format where the psychiatrist observes, questions, and evaluates the patient in order to form an opinion about that patient's diagnosis, consumption, and the consequences of that consumption. EMA is the evaluation of symptoms day to day, in the patient's usual environment, free from recall biases as the patient self-assesses *right then, not later; right there, not elsewhere* (10, 11). This new method using active data (supplied by the patient) profoundly modifies the assessment procedure by introducing a computerized third party between doctor and patient. The use of dedicated smartphone apps allows patients to keep an accurate diary of their symptoms, behaviors, or consumption. Studies have shown that EMA apps are just as reliable as the scales usually used for psychiatric disorders (49, 63), with excellent acceptability, and possibly even a preference for this medium. Several studies [for a review, see in Ref. (13, 64)] have allowed SUDs to be assessed in real time:

- Alcohol use (12);
- Relationship between alcohol use and PTSD symptom intensity (65);
- Relationship between alcohol use and mood (66, 67);
- Real-time illicit drug use (68, 69);
- Opioid craving (70);
- The context or state of mind in which a patient consumes (71);
- Effects of stress on relapse (72);
- Effects of Topiramate on alcoholic craving (73);
- Physical, interpersonal, or legal consequences of SUD (74).

Moreover, this repeated evaluation of symptoms over time may in itself have a therapeutic effect. A study conducted among patients with bipolar disorder (75) found that it potentially limits manic or hypomanic episodes, and we can assume that repeated assessment of addictive behaviors also has a therapeutic effect. The feeling of intrusion generated by self-report questionnaires is seldom reported in these studies, indicating good acceptability (76). Compliance is variable and depends on the patient's type of consumption (77). For example, cannabis users are the least compliant, and a study is currently ongoing (78).

Smartphone-based intervention can take the form of either a virtual coach (79) applying CBT therapies or else a Screening, Brief Intervention, and Referral to Treatment (SBIRT) program (80, 81) that evaluates the patient quickly and offers appropriate care. Mobile phones afford the possibility of undertaking EMIs, that is, targeted, one-off interventions if a patient risks relapse or consumption. These targeted interventions can take many forms (12–14):

- SMS messages;
- Psychoeducation information;
- Realtime coping strategies;
- Motivational messages;
- Behavioral change promotion (82).

These tools construct a genuinely protective network (22) around the patient, who can benefit at any time from a healthcare platform offering information on possible care (including emergency care).

Biofeedback and Neurofeedback

Developed in the 1970s (16), biofeedback is a painless, noninvasive procedure that consists in capturing biometric data (EEG, ECG, EMG, skin conductance, temperature) and feeding them back to the patient in real time. The objective of neurofeedback, the name given to biofeedback measuring brain activity (by EEG or real-time fMRI), is to model the patient's brain activity in real time as an image (video game type) or a sound. Based on CBT techniques and relaxation, patients gradually learn (through positive reinforcement) to promote brain activity corresponding to the therapeutic target. When activity in a desirable frequency band increases, the symbol modeling the brain activity changes (e.g., the video game moves faster), whereas when activity in an unfavorable band increases, the symbol changes in the opposite direction (e.g., the video game slows down). Patients gradually *learn* the newbrain wave, taking a wave corresponding to what is observed in healthy individuals as their model. Biofeedback research is currently focused on a variety of clinical issues (83), and several studies have examined the treatment of addictions by neurofeedback (84–87), especially for opiates and alcohol. One therapeutic hypothesis is that striatal cue reactivity to alcohol stimuli is reduced after neurofeedback training (88). A clinical study is in progress to compare neurofeedback training for alcohol dependence with classic treatment (89).

Virtual Reality

Until recently, virtual reality was limited by its cost and by the quality of the multimedia content. There has been a recent democratization of these systems (PS4 VR, Occulus Rift, etc.) concomitant with the video game industry's growing interest in this technology. Decreasing costs and increasing power are making it useful for performing neuropsychological (cognition, emotions, and behavior) assessments in real-time (90). Both the environment and the perceptual stimuli can be manipulated to trigger pathological behaviors or sensations (e.g., craving) as well as to evaluate behavioral responses to a given situation that can elicit distress, allowing patients to learn how to cope with their problems better. Many studies are therefore underway, with a focus on environmental trigger disorders (anxiety disorders in particular), but also on depression. Eichenberg and Wolters (91) conducted a descriptive review of virtual reality studies prior to 2012 that was subsequently complemented with studies up to 2015 by Valmaggia et al. (92). These reviews showed that the most commonly treated disorders are anxiety disorders, eating

disorders, schizophrenia (distress associated with hallucinations or delusions), and PTSD. Virtual reality therapy has good acceptability (93) and has been shown to be more effective in comparisons with patients receiving standard treatment or wait list control groups. Its results are similar to those of conventional CBT or *in vivo* exposure. Neither review contained data on SUDs, but several recent studies have been conducted in alcohol addiction care, online gaming addiction (94, 95), and opioid use (96). Concerning alcohol dependence treatment, a study featuring a combination of relaxation, presentation of a high-risk situation, and presentation of an aversive situation (97) highlighted a neurobiological imbalance (high sensitivity to stimuli) in the limbic system in individuals with alcohol dependence. This protocol could potentially have a regulating effect on limbic circuits. Several studies (98–100) examining craving triggers and control have found that patients with alcohol dependency report extremely high levels of craving immediately upon exposure to a virtual environment with alcohol cues, regardless of social pressure, social drinkers' alcohol use is strongly influenced by their social environment. The use of virtual reality in the treatment of SUDs therefore involves exposure to the stimulus that induces craving, either via situational cues (social environment) or via the implementation of alcohol-based cues, allowing patients' coping skills to be tested in real time. Finally, as in surgery, virtual reality can be used for training purposes to enhance screening or intervention methods for caregivers who may be confronted with SUDs (101).

DISCUSSION

There is a constantly growing body of knowledge in addictive medicine, and it is becoming increasingly complicated to handle all these data on a daily basis. In addition, more validated tools are needed to optimize the management the complexities of addiction. In the present overview, we discuss the major concepts related to new technologies that may well provide solutions in the field of addiction evaluation, diagnosis, or therapy within the near future. E-psychiatry is already booming, and some even talk about a *digital mental health revolution* (102). The acceptability of these technologies must therefore be assessed at different levels. It is generally based on several major criteria: usability (device's flexibility and ease of learning), utility (technology's contribution), satisfaction and reliability (including accuracy, effectiveness and efficiency). Cost, although fundamental, is a secondary consideration. Finally, the concept of risk impinges on acceptability and constitutes an important dimension of medical reasoning. It must therefore be taken into account when these technologies are being assessed (impact of false positives or false negatives, ethical issues).

In the present overview, we showed that these tools could prove extremely useful. The improved communication between healthcare providers via web-based, computer-based, or smartphone-based interventions can facilitate the management of a range of psychiatric or behavioral disorders. Some software (CAT) can support (and sometimes replace) clinicians in screening and monitoring. The recent development of digital phenotyping, where a computer collates the clinical characteristics of a mental state (sometimes with greater accuracy than a clinician), and the possibility of doing so remotely, will undoubtedly modify current practice. Most authors advocate the use of passive rather than active data in the context of disorders for which there is some anosognosia (e.g., bipolar disorder, SUDs), as this type of automatically generated data makes it possible to limit biases and the feeling of intrusion that EMA and self-report questionnaires can generate (especially if they are to be filled out regularly or appear in pop-ups).

Confidence in e-health among patients with addictions and healthcare professionals is a major issue (103). Studies have highlighted good acceptability and patient compliance (except for patients with a cannabis addiction), as there is no feeling of being *observed*. Using a smartphone seems less stigmatizing than using a specific device (e.g., connected bracelet). Machine learning is revolutionizing fundamental research by allowing for better classification of patients, based not only on clinical data, but also on biological or neuroimaging-derived data. It is becoming reasonable to talk about genuinely complementary examinations in behavioral studies. Finally, these new technologies are enabling the development of new therapies, including biofeedback and virtual reality, which focus on craving control and the learning of coping skills.

Although there is mounting evidence that e-addictology offers new opportunities for treatment, there is a lack of randomized controlled trials. Available studies have several limitations, including small sample sizes, heterogeneous study samples, only short-term follow up, and difficulty in determining whether the treatment effects were restricted to the studied addiction or could be generalized to other types of addiction. For example, smartphone apps are steadily increasing in number, but most are of a commercial nature and not truly efficient for patients and practitioners (104). There is also a gap between patients' assessments and evidence-based medicine: a *like* is not a statistical difference. Users may thus be exposed to dangerous recommendations, and in any case, the overall agreement between guidelines and their content is often very low (105). Another concern is liability in the case of the malfunction of products, sensors, software or in security (hacking of data for example). There are also potential errors in false-positives and false-negatives. Data must therefore be objectively analyzed to avoid the use of tools with low validity and reliability. Physicians obviously need to be involved in the development and evaluation of these tools.

There is also a dearth of information regarding the cost-effectiveness of e-health tools and services (106). The lack of reimbursement schemes is equally problematic. Drug users live in more precarious conditions than the general population in terms of housing, social protection, and resources. The use of these new technologies may therefore be out of the reach of people with low incomes and/or limited computer literacy.

Lastly, the development of connected health technologies raises many ethical issues, the most important probably being the protection and ownership of personal and health data. The issues of confidentiality and transparency regarding data use have yet to be resolved. Very few patients are currently willing for their data to be shared with private companies (107).

These changes call for a shift in thinking and in ways of doing things, and we can still ask what technological help addictologists would welcome and for which tasks? Can they bear comparison with certain technologies that sometimes seem to exhibit greater predictive accuracy? Or can their role as physicians be better asserted through these devices? A study (108) that used a scenario-based methodology (evaluating the predictive value of medical imaging and passive vs. active data collection) to explore the acceptability of these new technologies among 515 French psychiatrists (including addictologists) revealed considerable disparity in acceptability, depending on the psychiatrists' profiles. Addictologists ($n = 34$), were among those who best accepted these new technologies, deeming that they could usefully support the therapeutic relationship, and who did not feel at all threatened by these devices.

CONCLUSION

Active data obtained from EMA can provide a means of assessing addictive behaviors (intrusion, sleep, etc.) and gaining an idea of their severity. The advantages of passive data gathering through smartphones, biosensors or connected objects, artificial intelligence, and the remote monitoring of patients with psychiatric pathologies have yet to be defined, and the question of data security will soon become central. In order to prevent data from being used for nonmedical purposes, we believe it is essential that physicians take up this issue and make recommendations on the subject. Important ethical considerations are hampering the acceptance of these technologies. If they are to be used, these new tools must therefore be explained and adapted to physician and patient profiles, all the while taking account of the risks inherent to their use (data piracy, false positives, etc.). Patients, caregivers, and other health professionals need to be involved in the design and evaluation of these new tools.

AUTHOR CONTRIBUTIONS

FF and AB: both authors contributed equally to this work, screening and abstract analysis, writing, conceptual framework. SM: supervised development of work, helped in data interpretation and manuscript evaluation. LK: helped to evaluate and edit the manuscript.

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