NOVEL PSYCHOACTIVE DRUGS - THE SAGA CONTINUES...

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NOVEL PSYCHOACTIVE DRUGS - THE SAGA CONTINUES...

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Table of Contents

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EDITORIAL
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Editorial: Novel Psychoactive Drugs—The Saga Continues…

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Editorial on the Research Topic

Novel Psychoactive Drugs–The Saga Continues…

This Research Topic has been planned, organized, and edited as a follow-up of the first Frontiers Research Topic on Novel Psychoactive Substances (NPS, https://www.frontiersin.org/research-topics/5249/novel-psychoactive-drugs) that by the end of 2020 has collected almost 134,000 visits. This 2nd Research Topic collects 15 articles, namely 11 original articles (four animal and seven human studies) and four reviews, and covers the main classes of NPS, including "old" drugs with renewed interest, such as ayahuasca.

Among the wide world of NPS, synthetic cannabinoids and cathinones continue to be among the most widely used NPS worldwide. In a preclinical study, Bilel et al. provide a pharmacological and behavioral characterization of the effects of a new *synthetic cannabinoid* belonging to the 3rd generation, AKB48 [APINACA, N-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide], in rats. Besides the classic "tetrad," the battery of behavioral tests included motor, sensorimotor, neurochemical and cardiorespiratory responses, place preference conditioning, and pre-pulse inhibition tests. All behavioral and neurochemical effects were fully prevented by the selective cannabinoid CB1 receptor antagonist/inverse agonist AM251 and blood concentrations of AKB48 were monitored and correlated with behavioral measurements. In two different clinical studies, Cohen, Mama et al. have first assessed the performance on executive function and emotional processing tasks in chronic *synthetic cannabinoids* users by using computerized neurocognitive function tests (i.e., the N-back task, Go/No-Go task, Wisconsin Sorting Card-like Task, and emotional face recognition task) and questionnaires of depression, anxiety and schizotypal traits and symptoms. The authors have reported significant impairments in mental flexibility and executive functions in synthetic cannabinoids users, along with elevated depressive and anxiety symptoms and schizotypal traits. Secondly, Cohen, Rosenzweig et al. have explored schizotypy measures and the personality characteristics of chronic synthetic cannabinoids users. They were reported to differ from natural cannabis users and non-users on dimensions of specific personality traits and schizotypy measures that may indicate psychotic proneness. Specifically, synthetic cannabinoids users have displayed higher scores of neuroticism and lower scores of agreeableness and extraversion compared with natural cannabis users and non-users, and lower levels of conscientiousness relative to non-users.

In a very elegant study, Zwartsen et al. combined integrated measurements (microelectrode arrays recordings of neuronal activity) with single target assays (monoamine reuptake transporter inhibition) to investigate the acute effects of several *synthetic cathinones* [4-methylethcathinone (4-MEC), 3-methylmethcathinone (3-MMC), 4-MMC, methylone, pentedrone, a-pyrrolidinovalerophenone (α-PVP), and 3,4-methylenedioxypyrovalerone (MDPV)]. The

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authors showed that inhibition of monoamine reuptake transporters is the most sensitive target for this set of synthetic cathinones, although the inhibition of neuronal activity at concentrations relevant for recreational exposure suggests additional targets for some cathinones. As many other NPS, synthetic cathinones are often used concomitantly with other drugs, which can lead to acute toxicities and fatalities. Kapitány-Fövény et al. compared opioid dependent patients under opioid substitution treatment with and without a history of synthetic cathinones use during therapy and found that use of synthetic cathinones during opioid substitution treatment (i) was associated with poorer treatment outcomes (including less adaptive strategies to cope with negative life events) and enhanced risk for reduced treatment retention, and (ii) might be characterized by more severe psychiatric symptoms and amotivation to change substance use among opioid dependent patients. In a first article, Papaseit, Pérez-Mañá et al. focused on the interactions between the synthetic cathinone mephedrone (4-methylmethcathinone, 4MMC) and alcohol, i.e., the most common two-drug combination reported among NPS recreational users in nightclubs, music festivals, and rave parties. The authors have found that alcohol increases the cardiovascular effects and abuse liability of mephedrone while mephedrone reduced the drunkenness and sedation produced by alcohol. Importantly, their combination induced a more intense feeling of euphoria and well-being in comparison to the two drugs alone.

In a second contribution, Papaseit, Olesti et al. conducted an observational study in a real-life setting of recreational use to assess pharmacokinetics and acute effects of 2,5-dimethoxy-4-ethylphenethylamine (2C-E), a psychedelic *phenylethylamine* with a chemical structure similar to mescaline. The authors have described severe alterations in perceptions, hallucinations, and euphoric-mood, even at low-moderate doses, which display high inter-individual variability and marked similarities with the psychedelic-like effects of other serotonin-acting drugs. Another synthetic phenethylamine with psychedelic and entactogenic effects, i.e., the compound 4-iodo-2,5-dimethoxy-N-(2-methoxybenzyl)phenethylamine (25I-NBOMe) better known as "N-Bomb," was the object of a preclinical study by Miliano et al., which has described how this NPS alters the brain dopaminergic transmission, induces sensorimotor modifications, impairs the startle amplitude, and inhibits the prepulse inhibition in rats.

The role of dopamine in the molecular and neurobiological responses to stimulant NPS has been elegantly investigated by Loi et al. that used a combined *in vitro*, *in vivo*, and *in silico* approach to investigate the central mechanisms of action of desoxypipradrol, also known as 2-diphenylmethyl*piperidine* (2-DPMP). The study has demonstrated that 2-DPMP is a potent stimulant that directly interacts with the brain's rewards system mainly through molecular rearrangements toward an outward-facing conformation of the presynaptic dopamine transporter (DAT), which suggested a cocaine-type effect. Using the open-web crawling/navigating software NPS.Finder®, Arillotta et al. have identified a large number (426) of unknown *opioids* with a possible recreational/misuse potential, including 176 very potent fentanyl analogs not listed in either International or European NPS databases. The study not only highlights a strong interest by psychonauts toward opioid drugs, but also confirms the utility of psychonaut fora/platforms to better understand the online situation regarding opioids use and misuse. As last original article contribution, the double-blind, randomized and placebo-controlled clinical trial by Zeifman et al. have examined for the first time the impact of ayahuasca (a brew containing N,N-dimethyltryptamine and beta-carboline alkaloids) on suicidality in individuals with treatment-resistant depression, to test their hypothesis that ayahuasca would lead to decreases in suicidality, and reported promising, although limited and mixed, results.

In the first of the four reviews, Zawilska et al. have provided a comprehensive and updated overview of the history of NBOMe derivatives, a specific set of psychedelic *phenylalkylamines*, their central and peripheral effects, pattern of use and metabolism. Commonly observed adverse effects were reported and cases of non-fatal and lethal intoxications involving these compounds were discussed. Importantly, being the analysis of NBOMes in biological materials particularly challenging, the analytical methods most commonly used for detection and identification of NBOMes and their metabolites were presented. Then, Donnadieu-Rigole et al. described complications related to drugs used to improve sexual performance and/or to promote disinhibition (a phenomenon better known as "*chemsex*"), showing how use of these drugs can be dramatically associated with high-risk sexual behaviors. Specifically, the authors have reviewed complications related to the use of cathinones, methamphetamines, gamma-butyrolactone/gamma-hydroxybutyrate (GBL/GHB), ketamine, cocaine and speed in parties, sex meetings and homosexual/heterosexual practices, soliciting specific prevention, and intervention strategies. In their systematic review, Segawa et al. have provided an overview of Virtual Reality (VR), i.e., head mounted devices, in the assessment of cue reactivity (craving, psychophysiological response, and attention to cue) and treatment of addictive disorders, i.e., intervention in nicotine, cocaine, alcohol and cannabis addiction, and gambling. Current evidence suggests the VR provides benefits in the assessment and treatment of substance use disorders and gambling. Yet, contrary to craving provocation in VR that is effective across addiction disorders, treatments based exclusively on virtual exposure to drug related cues showed heterogeneous results. Finally, the review by Orsolini et al. examined the main clinical and psychopathological features of the psychoses induced by synthetic cannabinoids and cathinones and their therapeutic strategies, and underlie the main differences with the "classical" psychoses. Importantly, the authors have provided further insight on therapeutic strategies and practical guidelines for managing patients affected with synthetic/chemical NPS-induced psychoses.

Overall, we feel that the present Research Topic provides an interesting and valuable picture of the current state-of-the-art in the field that contributes to our understanding of the pharmacological and toxicological effects of NPS and their

evolution on the market, and will likely help clinicians and emergency staff in managing intoxications symptoms.

AUTHOR CONTRIBUTIONS

AW and LF contributed equally to this Editorial of the 2nd Research Topic on NPS that they edited in 2020. Both authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Pharmacological and Behavioral Effects of the Synthetic Cannabinoid AKB48 in Rats

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AKB48 is a designer drug belonging to the indazole synthetic cannabinoids class, illegally sold as herbal blend, incense, or research chemicals for their psychoactive cannabis-like effects. In the present study, we investigated the *in vivo* pharmacological and behavioral effects of AKB48 in male rats and measured the pharmacodynamic effects of AKB48 and simultaneously determined its plasma pharmacokinetic. AKB48 at low doses preferentially stimulated dopamine release in the nucleus accumbens shell (0.25 mg/kg) and impaired visual sensorimotor responses (0.3 mg/kg) without affecting acoustic and tactile reflexes, which are reduced only to the highest dose tested (3 mg/kg). Increasing doses (0.5 mg/kg) of AKB48 impaired place preference and induced hypolocomotion in rats. At the highest dose (3 mg/kg), AKB48 induced hypothermia, analgesia, and catalepsy; inhibited the startle/pre-pulse inhibition test; and caused cardiorespiratory changes characterized by bradycardia and mild bradipnea and SpO2 reduction. All behavioral and neurochemical effects were fully prevented by the selective CB_1 receptor antagonist/inverse agonist AM251. AKB48 plasma concentrations rose linearly with increasing dose and were correlated with changes in the somatosensory, hypothermic, analgesic, and cataleptic responses in rats. For the first time, this study shows the pharmacological and behavioral effects of AKB48 in rats, correlating them to the plasma levels of the synthetic cannabinoid.

Chemical Compound Studied in This Article: AKB48 (PubChem CID: 57404063); AM251 (PubChem CID: 2125).

Keywords: AKB48, AM251, conditioned place preference (CPP), sensorimotor responses, synthetic cannabinoids, microdialysis, cardiorespiratory changes, prepulse inhibition (PPI)

Abbreviations: AM251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide; AKB48, N-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide; CPP, conditioned place preference; DA, dopamine; NAc, shell Nucleus Accumbens shell; NAc, core Nucleus Accumbens core; mPFC, medial prefrontal cortex; PPI, Prepulse Inhibition; JWH-018, Naphthalen-1-yl-(1-pentylindol-3-yl)methanone.

INTRODUCTION

The New Psychoactive Substances (NPS) comprise a large number of drugs, that are classified based on their chemical structures into different classes such as synthetic cannabinoids, cathinones, opioids, and benzodiazepines, and are widely used because of their easy availability on the Internet (Wood et al., 2014; Orsolini et al., 2015, 2017; Miliano et al., 2016, 2018; Corkery et al., 2017; EMCDDA, 2018). The synthetic cannabinoids (SCBs) are the most popular of the NPS, representing the largest group of substances currently monitored by the EU Early Warning System. Their development on the illicit drug market is likely a response to the popularity of cannabis in many countries. The SCBs mimic the effects of Δ^9-THC binding in the brain, although they are becoming increasingly chemically different (EMCDDA, 2019).

AKB48 [APINACA, N-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide] is a synthetic cannabinoid identified for the first time in 2012 in an herbal mixture in Japan (Uchiyama et al., 2012). It belongs to the third generation of cannabinoids, but it cannot be classified among alkylindoles and cyclohexylphenols (De Luca et al., 2015; Smith et al., 2015; Miliano et al., 2016; De Luca and Fattore, 2018) because of its different chemical structure coming from the increasing demand to synthetize new compounds in order to avoid controls. In particular, AKB48 differs from earlier JWH-type SCBs by having an adamantyl group connected to an indazole moiety through a carboxamide linkage (Canazza et al., 2016).

AKB48 is often added to ready-to-smoke herbal mixtures to enhance psychoactive effects, but as reported recently in forums (e.g., Drugs-forum), a new trend is coming where users buy pure powder and vaporize it to try several synthetic cannabinoids and find out the best one for them. According to users' opinions, the minimal dose of AKB48 required to vaporize it is about 1 mg, and the onset is gradual until a very pleasant body high, amazing mood, and large smile, until 4 h afterward. On the other hand, the most common adverse effect observed after AKB48 administration is agitation (White, 2017), while other reported side effects are irritability, restlessness, sadness, combativeness, aggression and psychomotor impairments, as reported for SCBs (Brewer and Collins, 2014). Toxicological and forensic analysis revealed AKB48 presence in seized products (Uchiyama et al., 2012; Odoardi et al., 2016) or in biological fluids of people subjected to toxicological control (Karinen et al., 2015; Vikingsson et al., 2015).

From a pharmacodynamics point of view, AKB48 binds in nanomolar concentrations at CB_1 and CB_2 human (Uchiyama et al., 2013; Canazza et al., 2016) and mice (Canazza et al., 2016) cannabinoid receptors, suggesting that it could induce similar or higher *in vivo* effects as other SCBs. Preclinical data have reported that AKB48, such as other cannabinoid agonists, induces the typical "tetrad" effect in mice, characterized by hypolocomotion, catalepsy, hypothermia, and acute mechanical and thermal analgesia (Canazza et al., 2016). Moreover, AKB48 causes important alterations of sensorimotor responses (visual, acoustic, and tactile), induces neurological alterations (seizures, hyperreflexia, and myoclonias), and promotes spontaneous aggressive responses in mice by activating CB_1 receptors (Canazza et al., 2016). It was recently shown that AKB48 induces psychostimulant effects in mice through CB_1 receptor- and dopamine (DA)-dependent mechanisms. In fact, the motor facilitation induced by AKB48 was prevented by the CB_1 receptor antagonist AM251, as well as the simultaneous blockade of DA D_1 and D_2 receptors (Ossato et al., 2017). Moreover, it was shown that AKB48 and its fluorinated derivative, 5F-AKB48, facilitated extracellular DA release in the nucleus accumbens shell of mice (Canazza et al., 2016; Ossato et al., 2017) and rats (De Luca et al., 2016), suggesting its potential positive involvement in rewarding mechanisms (Gatch and Forster, 2015; Miliano et al., 2016; Ossato et al., 2017), as already established for other synthetic cannabimimetics including JWH-018 (De Luca et al., 2015), JWH-250, and JWH-073 (Ossato et al., 2016).

The metabolism of AKB48 has been identified using a hepatocyte model (Gandhi et al., 2013) and human liver microsomal incubation (Holm et al., 2015). In particular, AKB48 was metabolized in 11 major metabolites, including monohydroxylated, dihydroxylated, trihydroxylated, and mono- and dihydroxylated glucuronide conjugates and dihydroxylated with ketone formation at the N-pentyl side chain (Gandhi et al., 2013).

Despite the presence of these *in vitro* and *in vivo* studies, there is poor preclinical *in vivo* evidence on the addictive properties and pharmaco-toxicological effects of AKB48 in rats. Therefore, the present study aimed to investigate the acute effect of AKB48 on body temperature, acute mechanical and thermal analgesia, motor activity, sensorimotor responses (to visual, acoustic, and tactile stimulation), startle/pre-pulse inhibition tests, conditioned place preference, and modulation of DA release in the mesoaccumbal pathway in adult rats. Also, the effect of AKB48 on cardiorespiratory parameters (hearth rate, breath rate, and SpO2 saturation) was determined. Moreover, to correlate its pharmacological effects with its blood levels, we measured somatosensory responses, body temperature, and mechanical analgesia at timed intervals post-injection, while simultaneously obtaining serial blood specimens for analysis of AKB48 using liquid chromatography tandem mass spectrometry (LC-MS/MS).

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Envigo, Italy) weighing 275–300 g were housed in groups of six per cage, at a constant temperature (22 ± 2°C), humidity (60%), and light/dark cycle (lights on from 08:00 to 20:00 h). Tap water and standard laboratory rodent chow (Mucedola, Settimo Milanese, Italy) were provided *ad libitum* in the home cage. All animal experiments were carried out in accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research according to Italian (D.L. 116/92 and 152/06) and European Council directives (609/86 and 63/2010) and in compliance with the approved animal policies by the Ethical Committee for Animal Experiments (CESA, University of Cagliari) and the Italian Ministry of Health (Aut. n°162/2016-PR). All animals

were handled once daily for 5 min for 5 consecutive d before experimentation began. We made every effort to minimize pain and suffering, and to reduce the number of animals used.

Drug Preparation and Dose Selection

AKB48, purchased from LGC Standards S.r.l (Milan, Italy), was dissolved in 2% EtOH, 2% Tween 80, and 96% saline and administered intraperitoneally (i.p.; 3.0 mL/kg) at different doses (0.1–3.0 mg/kg i.p.). AM251 (Sigma-Aldrich, Milano, Italy) was dissolved in a solution composed of 10% DMSO, 0.1% Tween 80, and 89.9% distilled water. Drugs were administered 20 min before the beginning of the test. AM251 was administered 20 min before AKB48 injection. The doses of AKB48 and AM251 were selected on the basis of our previous work (Canazza et al., 2016; De Luca et al., 2016).

Behavioral Studies

The effects of AKB48 were investigated using a battery of behavioral tests widely used in studies of safety pharmacology for the preclinical characterization of NPS in rodents (Ossato et al., 2015, 2018; Vigolo et al., 2015; Canazza et al., 2016; Fantinati et al., 2017; Marti et al., 2019), which in particular have been validated to describe the effects of synthetic cannabinoids in mice and rats (De Luca et al., 2015; Vigolo et al., 2015; Ossato et al., 2015, 2016; Canazza et al., 2016, 2017). To reduce the number of animals used, the behavior of rats was evaluated in four consecutive experimental sections. Moreover, to reduce the stress induced by manipulation, and to confirm the stability and reproducibility over time of the responses of our tests, animals were trained twice per week for 2 weeks before the pharmacological treatment. All experiments were performed between 8:30 a.m. and 2:00 p.m. Experiments were conducted in blind by trained observers working in pairs (Ossato et al., 2016). The behavior of rats (sensorimotor responses) was videotaped and analyzed offline by a different trained operator that gives test scores.

Sensorimotor Studies

We studied the voluntary and involuntary sensorimotor responses resulting from different rat reactions to visual, acoustic, and tactile stimuli (Marti et al., 2019).

Evaluation of the visual response

Visual response was verified by two behavioral tests that evaluated the ability of the rat to capture visual information even when stationary (the visual object response) or when moving (the visual placing response). The visual object response test was used to evaluate the ability of the rat to see an object approaching from the front or the side, inducing the animal to shift or turn its head or to retreat (modified from Ossato et al., 2015; Marti et al., 2019). For the frontal visual response, a white horizontal bar was moved frontally to the rat's head; the maneuver was repeated three times. For the lateral visual response, a small dentist's mirror was moved into the rat's field of view in a horizontal arc until the stimulus was between the rat's eyes. The procedure was conducted bilaterally and was repeated three times. The score assigned was a value of 1 if there was a reflection in the rat movement or 0 if not. The total value was calculated by adding the scores obtained in the frontal with that obtained in the lateral visual object response (overall score 9). Evaluation of the visual object response was measured at 0, 10, 30, 60, 120, and 180 min post-injection. The visual placing response test was performed using a tail suspension-modified apparatus able to bring the rat toward the floor at a constant speed of 10 cm/sec (modified from Ossato et al., 2015; Marti et al., 2019). The downward movement of the rat was videotaped. Frame-by-frame analysis allowed us to evaluate the beginning of the reaction of the rat while it was close to the floor. When the rat started the reaction, an electronic ruler evaluated the perpendicular distance in millimeters between the eyes of the rat to the floor. The naïve rats perceived the floor and prepared for contact at a distance of about 27 $\pm$ 4.5 mm. Evaluation of the visual placing response was measured at 0, 15, 35, 65, 125, and 190 min post-injection.

Evaluation of acoustic responses

Acoustic response measures the reflex of the rat in reply to an acoustic stimulus produced behind the animal. Four acoustic stimuli of different intensities and frequencies were tested (see Marti et al., 2019). Each sound test was repeated three times, giving a value of 1 if there was a response and 0 if not present, for a total score of 3 for each sound. The acoustic total score was calculated by adding scores obtained in the four tests (overall score 12). Evaluation of the visual object response was measured at 0, 10, 30, 60, 120, and 180 min post-injection.

Evaluation of tactile responses

Tactile responses were verified through vibrissae, pinna, and corneal reflexes (Marti et al., 2019), and data were expressed as the sum of these parameters. The vibrissae reflex was evaluated by touching the vibrissae (right and left) with a thin hypodermic needle once per side, giving a value of 1 if there was a reflex (turning of the head to the side of touch or vibrissae movement) or 0 if not present (overall score 2). Evaluation of the vibrissae reflex was measured at 0, 10, 30, 60, 120, and 180 min post-injection. The pinna reflex was assessed by touching the pavilions (left and right) with a thin hypodermic needle, the interior pavilions first. This test was repeated twice per side, giving a value of 1 if there was a reflex and 0 if not present (overall score 4). Evaluation of the pinna reflex was measured at 0, 10, 30, 60, 120, and 180 min post-injection. The corneal reflex was assessed by gently touching the cornea with a thin hypodermic needle and evaluating the response, assigning a value of 1 if the rat moved only its head, 2 if it only closed its eyelid, and 3 if it closed its lid and moved its head. The procedure was conducted bilaterally (overall score 6) and was measured at 0, 10, 30, 60, 120, and 180 min post-injection.

The "Tetrad" Paradigm for Screening Cannabinoid-Like Effects

Evaluation of core and surface body temperature

To better assess the effects of the ligands on thermoregulation, we measured both changes in the core (rectal) and surface (ventral fur) temperature. The core temperature was evaluated by a probe (1 mm diameter) that was gently inserted, after lubrication with liquid Vaseline, into the rectum of the rat (to about 2 cm) and

left in position until the stabilization of the temperature (about 10 sec; De Luca et al., 2015; Vigolo et al., 2015). The probe was connected to a Cole Parmer digital thermometer, model 8402. The surface temperature was measured by a Microlife FR 1DZ1 digital infrared thermometer, placed 1 cm from the surface of the abdomen of the rat (Vigolo et al., 2015). Core and surface body temperatures were measured at 0, 15, 35, 65, 125, and 190 min.

Evaluation of pain induced by mechanical stimulation of the tail

Acute mechanical nociception was evaluated using the tail pinch tests (modified by Vigolo et al., 2015). A special rigid probe connected to a digital dynamometer (ZP-50N, IMADA, Japan) was gently placed on the tail (in the distal portion), and progressive pressure was applied. When the rat flicked its tail, the pressure was stopped, and the digital instrument saved the maximum peak of weight supported (g/force). A cut-off (500 g/force) was set to avoid tissue damage. The test was repeated three times, and the final value was calculated with the average of three obtained scores. Acute mechanical nociception was measured at 0, 20, 40, 70, 140, and 195 min post-injection.

Evaluation of catalepsy in the bar test

In the bar test, the rat's forelimbs were placed on a bar made of plastic (height 6 cm). The time spent on the bar was measured (immobility cut-off: 20 sec), and akinesia was calculated as the total time spent on the bar after three consecutive trials (total maximal time of catalepsy: 60 s; De Luca et al., 2015; Canazza et al., 2016). The bar test was performed at 0, 20, 40, 70, 140, and 195 min post-injection.

Open field test and gross behavior test

An open field, used to measure locomotor activity, consisted of a wooden chamber (45 cm high) with a circular base (75 cm diameter). The floor was divided into 12 sections of similar area by two concentric circles and radial segments. The apparatus was placed in a sound-proof room, illuminated by a white 80 W lamp placed 200 cm over the center of the arena. Rat behavior in the test sessions was videotaped, analyzed, and scored. The following parameters were measured: time spent in the central or peripheral area, locomotor activity, and number of rearing reactions. Each of the behavioral parameters was scored manually by a tally counting method. The behavioral parameters scored were head twitches, wet dog shakes, grooming, licking, number of defecations, and tail rigidity. In the open field test, 21 male Sprague-Dawley rats were used and divided into three groups (n = 7 per group) and received different doses of AKB48 according to the CPP protocol. After treatment, the animals were leaved undisturbed for 10 min in the arena and then observed for 30 min.

Conditioned Place Preference Paradigm

The CPP chambers consisted of two equally sized compartments interconnected by a guillotine door, adopting a classical conditioning procedure that has been successfully used to assess the rewarding properties of several drugs of abuse. The compartments are differentiated by both visual and tactile cues: the color of the walls in each compartment (white or black) and the texture of the floors (wooden flat or metal wired). The box was placed in a dimly illuminated room. Place conditioning morning sessions ran from 8:30 a.m. to noon, while afternoon sessions ran from 2:30 p.m. to 6 p.m.

Experiment 1: effect of AKB48 on conditioned place preference

According to the conditioned place preference paradigm, on day 0 (pre-test), rats freely explored the two compartments for 15 min, and the time spent in each compartment during the exploratory period was measured. Rats that spent 60–70% of the total time on one side were excluded from the experiment. We used an unbiased-like protocol and assigned the drug-paired compartment randomly. On days 1–3 (conditioning phase), male Sprague-Dawley rats (n = 24) were divided into three groups (n = 8 per group) and given injections of AKB48 (0.1 or 0.5 mg/kg) or vehicle twice daily (9:00 a.m. to 7:00 p.m.) and confined to one compartment for 30 min for 3 days. During these conditioning trials, the animals developed an association between the subjective state produced by the drug and the environmental cues present in the compartment in which they received the drug. On day 4 (test day), rats were allowed to explore the two compartments freely for 15 min, and the time spent in each compartment during the exploratory period was measured.

Experiment 2: effect of the CB_1 antagonist AM251 on AKB48-induced activity on the CPP test

To evaluate if the aversive effect obtained with the higher dose of AKB48 (0.5 mg/kg) was mediated by activation of CB_1 cannabinoid receptors, male Sprague-Dawley rats (n = 32) were divided into four groups (n = 8 per group). Group 1 received AKB48 vehicle in both compartments and served as a control. Group 2 was conditioned in one of the two compartments with 0.5 mg/kg of AKB48, and Group 3 was conditioned with AM251 (1 mg/kg). The fourth group received a combination of AM251 and AKB48. CB_1 antagonist was injected 20 min before the agonist AKB48, which was given 10 min prior to the test.

In vivo Brain Microdialysis Studies

Male Sprague-Dawley rats were anaesthetized with isoflurane gas, and maintained under anaesthesia using a breathing tube under a scavenging system while placed in a stereotaxic apparatus and implanted with vertical dialysis probes (1.5 or 3 mm dialyzing portion for NAc or mPFC, respectively) in the NAc shell (A + 2.2, L + 1.0 from bregma, V-7.8 from dura) or core (A + 1.4; L + 1.6 from bregma; V-7.6 from dura) or in the mPFC (A + 3.7, L + 0.8 from bregma, V-5.0 from dura), according to the rat brain atlas (Paxinos and Watson, 1998).

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 μm, Waters, United States) 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: 50 mM NaH2PO4, 0.1 mM Na2-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 the 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.

Startle and Pre-pulse Inhibition Analysis

As previously reported (Marti et al., 2019), rats were tested for acoustic startle reactivity in startle chambers (Ugo Basile apparatus, Milan, Italy) consisting of a sound-attenuated, lighted, and ventilated enclosure holding a transparent non-restrictive Perspex® cage (modified version for rats 200 × 90 × 80 mm). A loudspeaker mounted laterally by the holder produced all acoustic stimuli. Peaks and amplitudes of the startle response were detected by a load cell. At the onset of the startling stimulus, 300 ms readings were recorded, and the wave amplitude evoked by the movement of the rat startle response was measured. Acoustic startle test sessions consisted of startle trials (pulse-alone) and pre-pulse trials (pre-pulse + pulse). The pulse-alone trial consisted of a 40 ms 120 dB pulse. Pre-pulse + pulse trial sequences consisted of a 20 ms acoustic pre-pulse, 80 ms delay, and then a 40 ms, 120 dB startle pulse (100 ms onset–onset). There was an average of 15 s (range = from 9 to 21 s) between the trials. Each startle session began with a 10 min acclimation period with a 65 dB broadband white noise that was present continuously throughout the session. The test session contained 40 trials composed by pulse-alone and pre-pulse + pulse trials (with three different pre-pulses of 68, 75, and 85 dB) presented in a pseudorandomized order. Rats were placed in the startle chambers 5 min after treatment with AKB48. The entire startle/PPI test lasted 20 min. The pre-pulse inhibition (PPI) was expressed as the percentage decrease in the amplitude of the startle reactivity caused by the presentation of the pre-pulse. AKB48 (0.1–3 mg/kg i.p.) was administered intraperitoneally, and startle/PPI responses were recorded 15 min (including the 10 min acclimation period) after drug injections.

Cardiorespiratory Analysis

To monitor cardiorespiratory parameters in awake and freely moving rats with no invasive instruments and minimal handling, a collar with a sensor was applied to continuously detect heart rate, breath rate, and oxygen saturation, with a frequency of 15 Hz (Ossato et al., 2018; Foti et al., 2019). During the experiment, the rat was allowed to freely move in a cage (40 × 40 × 30 cm) with no access to food and water while being monitored by the sensor collar through MouseOx Plus (STARR Life Sciences® Corp., Oakmont, PA, United States) software. In the first hour of acclimation, a fake collar similar to the real one used in the test but with no sensor was used to minimize the potential stress during the experiment. Then, the real collar (with sensor) was replaced, and baseline parameters were monitored for 60 min. Subsequently, AKB48 (0.1–3 mg/kg) or vehicle was administered, and data were recorded for 180 min.

AKB48 Pharmacokinetic Studies and Behavioral Correlation

To correlate the pharmacological effects of AKB48 with its blood levels, we measured somatosensory responses (visual, acoustic, and tactile), body temperature, and mechanical analgesia in rats at timed intervals post-AKB48 injection (for behavioral tests, see before), while simultaneously obtaining serial blood specimens for analysis of AKB48.

Surgical Procedures and Blood Collection

Sixteen male Sprague-Dawley rats were used in the study. Rats were anaesthetized with Equitesin [3 mL/kg intraperitoneal (ip); chloral hydrate 2.1 g, sodium pentobarbital 0.46 g, MgSO 1.06 g, propylene glycol 21.4 mL, ethanol (90%) 5.7 mL, H2O 3 mL] and implanted in the right jugular vein with a catheter, consisting of medical-grade tubing (Silastic, Dow Corning Corporation, MI, United States) according to the technique previously described (Lecca et al., 2006). A stable fixation in the mid-scapular region of the back was embedded by a polypropylene mesh (Evolution, BULEV, weight 48 g/mq, Dipromed, Italy). During the recovery period, at least 7 days after surgery, the catheters were flushed daily with 0.1 mL of gentamicin (40 mg/mL) and with heparinized saline (heparin 250 U/mL in 0.9% sterile saline). Fifteen days after recovery from surgery, rats were trained in handling, behavioral tests, and withdrawing blood from the catheter. Four groups of rats ($n = 4$) randomly received intraperitoneal injection of a single dose of AKB48 (0.1, 0.3, 0.5, or 3.0 mg/kg), and behavioral tests were conducted as previously reported in safety pharmacology studies (see before). Blood specimens (300 μL) were withdrawn via catheters immediately before the behavioral measurements ($T = 0$) and at 20, 40, 70, 140, and 195 min after drug injection. Blood specimens were collected into 1–mL vials containing sodium fluoride (4 mg/mL of blood) as preservative and anticoagulant. After each blood withdrawal, an equal volume of saline solution was infused via the intravenous catheter to maintain volume and osmotic homeostasis.

Chemicals

N-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide (AKB48) and JWH-209-D9 were purchased from LGC Standards (Milan, Italy). Water, chloroform, formic acid, and methanol were purchased from 3V-Chemicals (Rome, Italy). Ammonium formate was purchased from Agilent (Agilent Technologies, Santa Clara, CA, United States). All reagents and solvents were of LC/MS grade.

Sample Preparation

A dispersive liquid-liquid microextraction (DLLME) was performed for sample purification. Three hundred microliter of blood samples were spiked with 10 μL of JWH-209 D9 as an internal standard, achieving the final concentration of 20 ng/mL, and deproteinized with 500 μL of methanol. The sample was centrifuged at 10,000 rpm for 10 min, and 500 μL surnatant was transferred into a 15 mL conical tube containing 1 mL of water, 0.2 g of NaCl, and 100 μL of carbonate buffer, pH 9. In order to obtain the formation of the cloudy solution, 350 μL of a mixture, chloroform/methanol 1:2.5, respectively the extractant and the

disperser solvent, was rapidly added to obtain the formation of a turbid mixture. The sample was sonicated for 2 min and then centrifuged at 4000 rpm for 5 min to sediment the fine droplets of the extractant phase at the bottom of the tube. The sediment phase (about 50 ± 5 μL) was transferred into a vial, evaporated under a gentle nitrogen stream, reconstituted in 20 μL of methanol and 80 μL of water with 0.1% formic acid, and 10 μL were injected in the UHPLC-MS/MS system.

Instrumental Analysis

The analytical method (UHPLC-MS/MS) and its validation are described in detail elsewhere (Odoardi et al., 2016). N-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide (AKB48) and JWH-209-D9 were purchased from LGC Standards (Milan, Italy). Water, chloroform, formic acid, and methanol were purchased from 3V-Chemicals (Rome, Italy). Ammonium formate was purchased from Agilent (Agilent Technologies, Santa Clara, CA, United States). All reagents and solvents were of LC/MS grade. Chromatography was performed using an Agilent 1290 Infinity system, equipped with a binary pump with integrated vacuum degasser, high-performance well-plate autosampler, and thermostatted column compartment modules. The detection system was an Agilent 6460 triple-quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, United States) with a Jet-Stream electrospray ionization source. The column was a superficially porous Kinetex C18 column (2.6 μm, 100 × 2.1 mm, Phenomenex, Bologna, Italy). The column temperature was set at 40°C, and the injection volume was 10 μL. The mobile phases used were: (A) 5 mM ammonium formate containing 0.1% formic acid and (B) methanol with 0.1% of formic acid. The mobile phase gradient was from 45% to 100% B within 12 min, plus 3 min of equilibration, for cannabinoids analysis, and from 0 to 90% B within 11 min, plus 3 min of equilibration for stimulants. The flow rate was set to 400 μL/min. The eluate was introduced into the mass spectrometer by means of electrospray ionization (ESI) in the positive mode. The optimized MS parameters were as follows: capillary voltage was set to 4000 V, the ion source was heated up to 350°C, and nitrogen was used as nebulizing and collision gas at 12 L/min and 40 psi, respectively; EM voltage was set to + 1000 V, and nozzle voltage to 2000 V. The detector operated in Multiple Reaction Monitoring (MRM) mode. Transitions selected for AKB48 were 384 → 135, 107, and 93.

Behavioral Analysis

To correlate the pharmacological effects of AKB48 with its blood levels, we measured somatosensory responses (visual, acoustic and tactile), body temperature, mechanical analgesia, and catalepsy (as reported before) at each blood withdrawal. Sixteen rats were used in the study. Blood samples were collected immediately after behavioral testing.

Data and Statistical Analysis

In sensorimotor response experiments, data are expressed in arbitrary units (visual object response, acoustic response, overall tactile response) and percentage of baseline (visual placing response). Core and surface temperature values are expressed as the difference between control temperature (before injection) and temperature following drug administration (Δ°C). Antinociception (tail pinch tests) and catalepsy (bar test) are calculated as percent of maximal possible effect {EMax% = [(test – control latency)/(cut-off time – control)] × 100}. Data are expressed in absolute values [seconds (sec) in time spent in the open field arena, meters (m) for distance traveled, number of head shakes, amount of grooming, number of wet dog shakes, amount of defecation, tail rigidity, and amount of licking]. In microdialysis experiments, data are expressed as percentage of DA basal values. The amount of PPI was calculated as a percentage score for each pre-pulse + pulse trial type:% PPI = 100 - {[(startle response for prepulse + pulse trial)/(startle response for pulse-alone trial)] × 100}. Startle magnitude was calculated as the average response to all pulse-alone trials. Changes in heart rate, breath rate, and SpO_2 saturation, expressed as heartbeats/minute (bpm), breath rates/minute (brpm), and% oxygen blood saturation, respectively, are expressed as percentage of basal values. Concentration of AKB48 in plasma samples was reported as μg/L.

All the numerical data are given as mean ± SEM. Data were analyzed by utilizing repeated measures ANOVA. Results from treatments showing significant overall changes were subjected to *post hoc* Tukey tests with significance of $p < 0.05$. The statistical analysis of the effects of the individual substances in different concentrations over time and that of antagonism studies in histograms were performed by two- or three-way ANOVA followed by Bonferroni's test for multiple comparisons. The analysis of the total average effect induced by treatments was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. Relationships between AKB48 plasma concentrations and behavioral (sensorimotor responses and catalepsy) and physiological (body temperature and mechanical analgesia) changes were assessed using a Pearson's correlation analysis. The statistical analysis was performed using Prism software (GraphPad Prism, United States).

RESULTS

Evaluation of the Visual Object Response

Visual object response did not change in vehicle-treated rats over 180 min observation (**Figure 1A**). Systemic administration of AKB48 (0.1–3.0 mg/kg i.p.) dose-dependently reduced the visual object response in rats. At 0.3 mg/kg, the effect was transient, while the effect caused at the higher doses (0.5 and 3.0 mg/kg i.p.) persisted up to 180 min (**Figure 1A**); effect of treatment [$F_{(4,\ 210)} = 59.29$, $p < 0.0001$], time [$F_{(5,\ 210)} = 11.85$, $p < 0.0001$] and time × treatment interaction [$F_{(20,\ 210)} = 2.95$, $p < 0.0001$]. The inhibition of visual object response induced by the highest dose of AKB48 (3 mg/kg i.p.) was prevented by the pre-treatment with AM251 (1 mg/kg i.p., **Figure 1B**); effect of treatment [$F_{(1,\ 28)} = 15.86$, $p = 0.0004$], antagonist [$F_{(1,\ 28)} = 4.222$, $p = 0.0493$], and interaction [$F_{(1,\ 28)} = 14.35$, $p = 0.0007$], which alone did not alter the visual object response in rats.

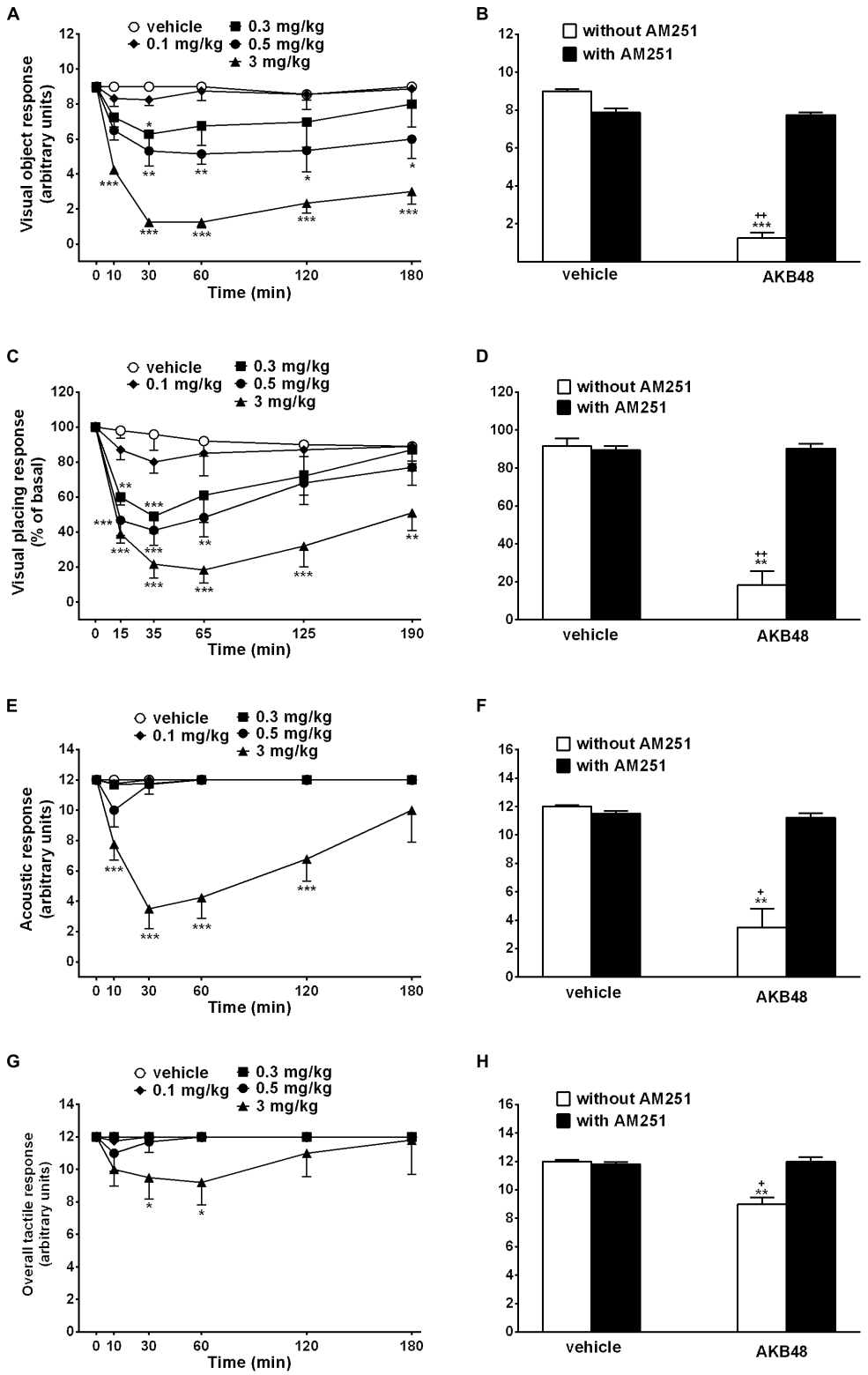

FIGURE 1 | Effect of the systemic administration of AKB48 (0.1–3 mg/kg i.p.) on the visual object **(A)**, the visual placing **(C)**, the acoustic **(E)** and the overall tactile responses in rats. Interaction of AKB48 (3 mg/kg) with the selective CB_1 receptor antagonist AM251 (1 mg/kg, i.p.) were reported in **B,D,F,H**). Data are expressed (see Materials and Methods) as arbitrary units **(A,B,E–H)** or% of basal **(C,D)** and represent the mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose response curve at different times **(A,C,E,G)** and for the interaction with AM251 **(B,D,F,H)**. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. vehicle and $^{+}p < 0.05$, $^{++}p < 0.01$ vs. AM251 + AKB48.

Evaluation of the Visual Placing Response

Visual placing response slightly decreased in vehicle-treated rats over 190 min observation (∼10% of reduction at 190 min; **Figure 1C**), and the effect was similar to that observed in naïve untreated animals (data not shown). Systemic administration of AKB48 reduced the visual placing response in rats, and the effect caused at 3 mg/kg i.p. persisted up to 190 min (**Figure 1C**); effect of treatment [$F_{(4,\ 210)} = 31.68$, $p < 0.0001$], time [$F_{(5,\ 210)} = 15.63$, $p < 0.0001$], and time × treatment interaction [$F_{(20,\ 210)} = 2.077$, $p = 0.0058$]. Visual impairment induced by AKB48 was prevented by the pre-treatment with AM251 (1 mg/kg i.p., **Figure 1D**): effect of treatment [$F_{(1,\ 28)} = 5.234$, $p = 0.0299$], antagonist [$F_{(1,\ 28)} = 4.671$, $p = 0.0394$], and interaction [$F_{(1,\ 28)} = 5.663$, $p = 0.0244$], which alone did not alter the visual placing response.

Evaluation of the Acoustic Response

Acoustic response did not change in vehicle-treated rats over 180 min observation (**Figure 1E**). Systemic administration of AKB48 reduced the acoustic response only at the highest dose, and the effect persisted up to 120 min (**Figure 1E**); effect of treatment [$F_{(4,\ 210)} = 55.63$, $p < 0.0001$], time [$F_{(5,\ 210)} = 5.59$, $p < 0.0001$], and time × treatment interaction [$F_{(20,\ 210)} = 5.108$, $p < 0.0001$]. The inhibition of acoustic response induced by AKB48 (3 mg/kg i.p.) was prevented by the pre-treatment with AM251 (1 mg/kg i.p., **Figure 1F**): effect of treatment [$F_{(1,\ 28)} = 7.507$, $p = 0.0106$], antagonist [$F_{(1,\ 28)} = 3.804$, $p = 0.0612$], and interaction [$F_{(1,\ 28)} = 5.985$, $p = 0.0210$], which alone did not alter the acoustic response in mice (data not shown).

Evaluation of the Tactile Response

The overall tactile response (vibrissae, corneal, and pinna) did not change in vehicle-treated mice over 180 min observation (**Figure 1G**). Systemic administration of AKB48 only at the highest dose reduced the tactile responses in rats (∼23% of reduction), and the effect persisted up to 60 min (**Figure 1G**); effect of treatment [$F_{(4,\ 210)} = 5.161$, $p = 0.0006$], time [$F_{(5,\ 210)} = 0.9673$, $p = 0.4388$], and time × treatment interaction [$F_{(20,\ 210)} = 0.6782$, $p = 0.8454$]. The inhibition of the overall tactile response induced by AKB48 (3 mg/kg i.p.) was prevented by the pre-treatment with AM251 (1 mg/kg i.p., **Figure 1H**); effect of treatment [$F_{(1,\ 28)} = 27.83$, $p < 0.0001$], antagonist [$F_{(1,\ 28)} = 21.56$, $p < 0.0001$], and interaction [$F_{(1,\ 28)} = 22.18$, $p < 0.0001$], which alone did not alter the acoustic response in mice (data not shown).

Evaluation of Core and Surface Body Temperature

Body temperature did not change in vehicle-treated rats over 190 min observation. Systemic administration of AKB48 reduced core body temperature only at 3 mg/kg, and the effect was evident only at 65 min (**Figure 2A**); effect of treatment [$F_{(4,\ 175)} = 2.214$, $p = 0.0694$], time [$F_{(4,\ 175)} = 0.08992$, $p = 0.9855$], and time × treatment interaction [$F_{(16,\ 175)} = 0.4359$, $p = 0.9711$]. The hypothermia induced by AKB48 (3 mg/kg i.p.) was prevented by the pre-treatment with AM251 (1 mg/kg i.p., **Figure 2B**); effect of treatment [$F_{(1,\ 28)} = 13.33$, $p = 0.0011$], antagonist [$F_{(1,\ 28)} = 15.49$, $p = 0.0005$], and interaction [$F_{(1,\ 28)} = 14.86$, $p = 0.0006$], which alone did not alter the acoustic response. AKB48 did not affect surface body temperature [$F_{(4,\ 175)} = 5.129$, $p = 0.0006$], time [$F_{(4,\ 175)} = 2.757$, $p = 0.0295$], and time × treatment interaction [$F_{16,\ 175)} = 0.1627$, $p > 0.9999$].

Evaluation of Pain Induced by a Mechanical Stimulus

The threshold to acute mechanical pain stimulus did not change in vehicle-treated rats over 195 min observation (**Figure 2C**). Systemic administration of AKB48 slightly increased the threshold to acute mechanical pain stimulus in rats in the tail pinch test (**Figure 2C**); effect of treatment [$F_{(4,\ 175)} = 7.266$, $p < 0.0001$), time [$F_{(4,\ 175)} = 0.6093$, $p = 0.6565$], and time × treatment interaction [$F_{(16,\ 175)} = 0.2989$, $p = 0.9962$]. The effects were prevented by the pre-treatment with AM 251 (1 mg/kg i.p.; **Figure 2D**); significant effect of treatment [$F_{(1,\ 28)} = 4.439$, $p = 0.0442$], antagonist [$F_{(1,\ 28)} = 2.873$, $p = 0.1012$], and interaction [$F_{(1,\ 28)} = 3.994$, $p = 0.0554$], which alone did not alter the threshold to acute mechanical pain stimuli.

Evaluation of Catalepsy in the Bar Test

The time spent on the bar did not change in vehicle-treated rats over 195 min observation (**Figure 2E**). Systemic administration of AKB48 slightly increased the time spent on the bar (**Figure 2E**); effect of treatment [$F_{(4,\ 175)} = 32.19$, $p < 0.0001$], time [$F_{(4,\ 175)} = 9.843$, $p < 0.0001$], and time × treatment interaction [$F_{(16,\ 175)} = 3.197$, $p < 0.0001$]. The effect was prevented by the pre-treatment with AM 251 (1 mg/kg i.p.; **Figure 2F**); significant effect of treatment [$F_{(1,\ 28)} = 47.09$, $p < 0.0001$], antagonist [$F_{(1,\ 28)} = 41.21$, $p < 0.0001$], and interaction [$F_{(1,\ 28)} = 41.21$, $p < 0.0001$], which alone did not induce catalepsy in rats.

Evaluation of Gross Behavior and Spontaneous Locomotion

Systemic administration with AKB48 (0.1 and 0.5 mg/kg i.p.) induced a significant increase of tail rigidity (**Figure 3A**) [$F_{(2,\ 21)} = 59.9$, $p < 0.0001$] and a decrease of licking (**Figure 3B**) [$F_{(2,\ 21)} = 9.12$, $p < 0.001$] for both doses tested. However, drug exposure did not affect wet dog shaking (**Figure 3C**) [$F_{(2,\ 21)} = 0.27$, $p > 0.05$], head shaking (**Figure 3D**) [$F_{(2,\ 21)} = 0.28$, $p > 0.05$], amount of defecation (**Figure 3E**) [$F_{(2,\ 21)} = 0.04$, $p > 0.05$], and grooming (**Figure 3F**) [$F_{(2,\ 21)} = 0.72$, $p > 0.05$]. Systemic administration with AKB48 (0.1 and 0.5 mg/kg i.p.) reduced spontaneous locomotor activity (**Figure 4**). In particular, AKB48 at 0.5 mg/kg reduced the distance traveled in the central area (**Figure 4A**) [$F_{(2,\ 18)} = 4.08$, $p < 0.05$], the total distance traveled (**Figure 4B**) [$F_{(2,18)} = 4.31$, $p < 0.05$], and the time spent in the central area (**Figure 4C**) [$F_{(2,\ 18)} = 3.63$, $p < 0.05$]. On the other hand, treatment with AKB48 had no effect on rearing (**Figure 4D**) [$F_{(2,\ 18)} = 0.44$, $p > 0.05$], even if there was a slight decrease.

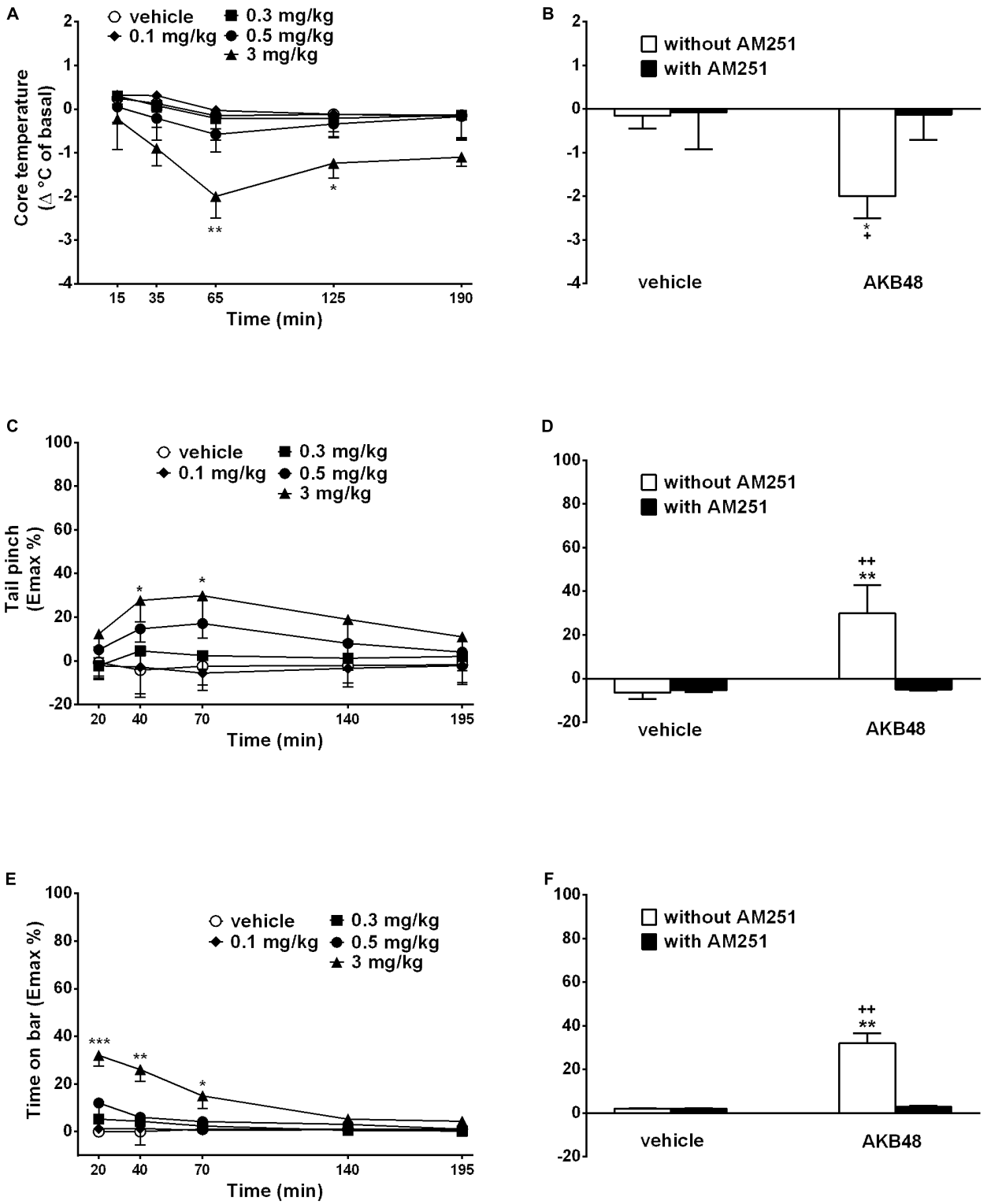

FIGURE 2 | Effect of the systemic administration (0.1–3 mg/kg i.p.) of AKB48 on the rat core temperature **(A)**, on the tail pinch test **(C)** and on the bar test **(E)**. Interaction of AKB48 (3 mg/kg) with the selective CB_1 receptor antagonist AM251 (1 mg/kg) were reported in **(B,D,F)**. Data are expressed (see section Materials and Methods) as the difference between control temperature (before injection) and temperature following drug administration (Δ°C of basal) and the percentage of maximum effect (Emax %; tail pinch and bar tests) and represent the mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose response curve of each test at different times **(A,C,E)** and for the interaction with AM251 **(B,D,F)**. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. vehicle and $^{+}p < 0.05$, $^{++}p < 0.01$ vs. AM251 + AKB48.

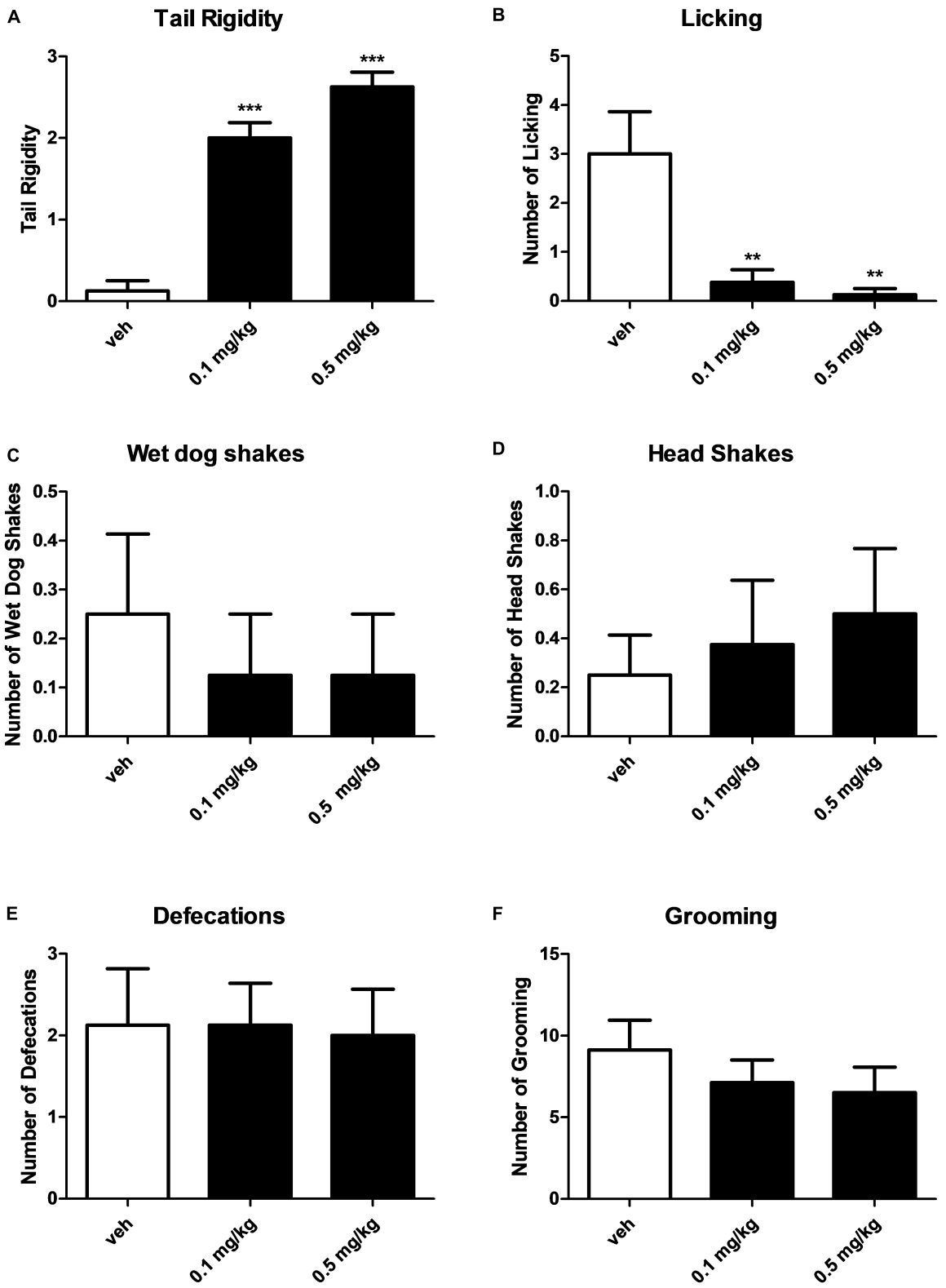

FIGURE 3 | Effect of treatment with AKB48 (0.1 and 0.5 mg/kg, i.p.) on tail rigidity **(A)**, licking **(B)**, wet dog shakes **(C)**, head shakes **(D)**, defecation **(E)**, and grooming behavior **(F)** in rats. Data are expressed (see Materials and Methods) as arbitrary units **(A)** or absolute values **(B–F)** and represent the mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by one-way ANOVA followed by the Bonferroni's test for multiple comparisons. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. vehicle.

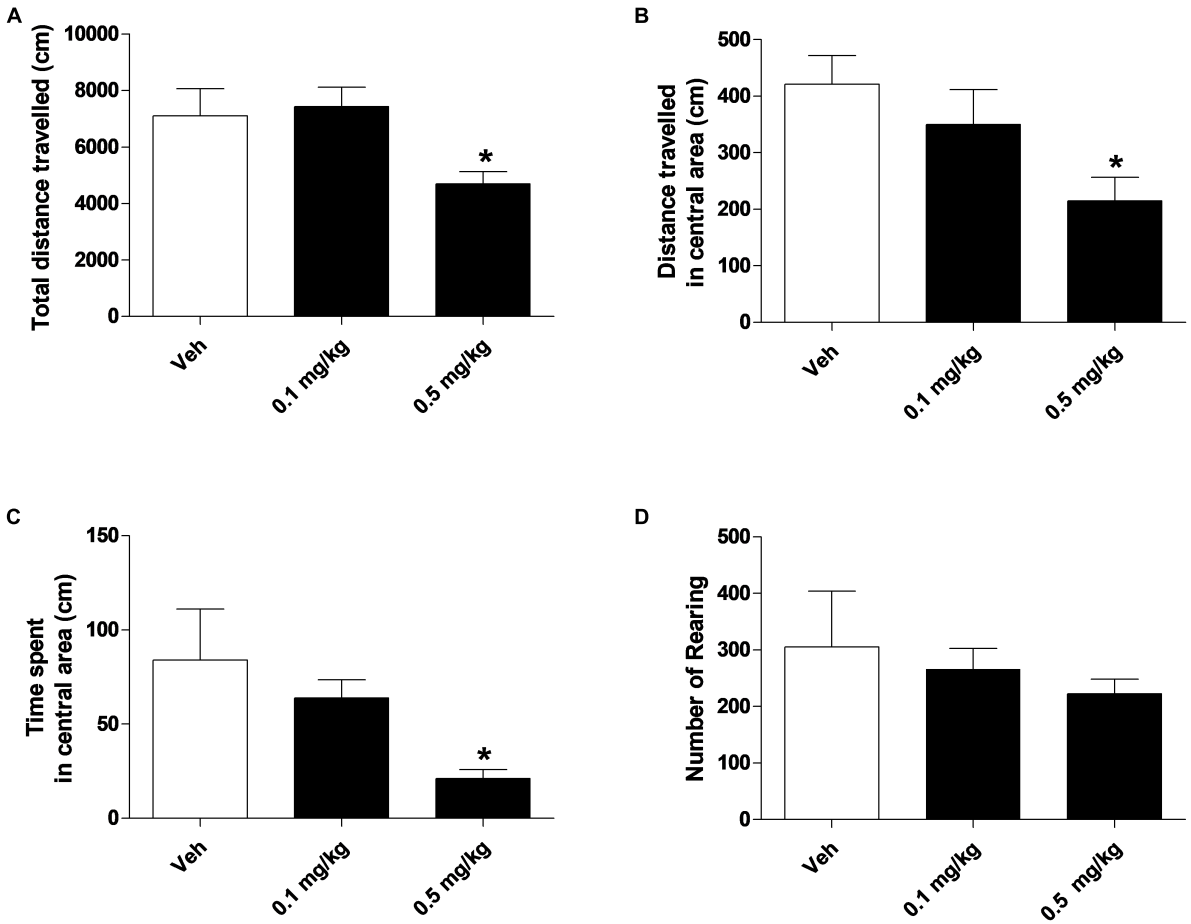

FIGURE 4 | Effect of treatment with AKB48 (0.1 e 0.5 mg/kg, i.p.) on overall total distance traveled **(A)**, total distance traveled and time spent in the central zone **(B,C)** and number of rearings **(D)** in rats. Data are expressed (see section Materials and Methods) as absolute values (cm **A,B**; sec **C**; n° of rearing **D**) and represent the mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by one-way ANOVA followed by the Bonferroni's test for multiple comparisons. $^{*}p < 0.05$ vs. vehicle.

Evaluation of the Conditioned Place Preference

Systemic administration with AKB48 at 0.5 mg/kg induced a significant aversive effect on conditioned place preference (**Figure 5A**) [$F_{(2,\ 21)} = 4.13$, $p < 0.05$]. The lower dose of 0.1 mg/kg was ineffective. The aversive effect was blocked by administration of AM251 at 1 mg/kg i.p. (**Figure 5B**) [$F_{(3,\ 26)} = 3.22$, $p < 0.05$].

Effect of AKB48 Administration on DA Transmission in the NAc Shell and Core, and in the mPFC of Rats

Rat basal values of DA, expressed as fmoles/10 μL sample (mean ± SEM), were: NAc shell 49 ± 5 (n = 14), NAc core 48 ± 4 (n = 9), and mPFC 14 ± 4 (n = 13). In this experiment, we evaluated the effect of three doses of AKB48 on extracellular DA levels in NAc shell (0.125, 0.25, and 0.5 mg/kg i.p.) and two doses in NAc core and mPFC (0.125 and 0.25 mg/kg i.p.). As shown in **Figure 6**, this synthetic cannabinoid increased DA levels preferentially in the NAc shell (**Figure 6A**) as compared to the NAc core (**Figure 6B**) and mPFC (**Figure 6C**) when administered at 0.25 mg/kg i.p.; lower or higher doses were ineffective in the NAc shell. No significant effects were observed in the NAc core and mPFC. Three-way ANOVA showed a main effect of treatment [$F_{(2,\ 24)} = 5.53$; $^{*}p < 0.05$] and time [$F_{(18,\ 432)} = 1.651$; $^{*}p < 0.05$]. In animals implanted in the NAc shell, two-way ANOVA showed a main effect of treatment [$F_{(3,\ 10)} = 6.126$; $^{*}p < 0.05$]. Tukey's *post hoc* tests showed a larger increase of dialyzate DA in the NAc shell after 0.25 mg/kg i.p. of AKB48, revealing differences at the 20 and 40 min samples compared to basal values (**Figure 6A**). In animals implanted in the NAc core, two-way ANOVA showed a main effect of time [$F_{(18,\ 108)} = 3.24$; $^{*}p < 0.0001$] and a significant time × treatment interaction [$F_{(36,\ 108)} = 3.97$; $^{*}p < 0.0001$]. Tukey's *post hoc* tests showed a larger increase of dialyzate DA in the NAc core after 0.25 mg/kg i.p. of AKB-48 and after 0.125 mg/kg i.p., revealing differences with respect to basal values (**Figure 6B**). In animals implanted in mPFC, two-way ANOVA showed no significant effects (**Figure 6C**).

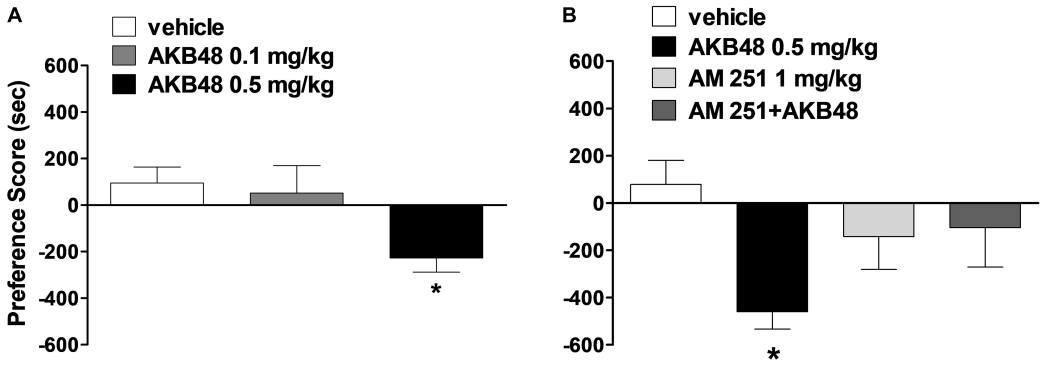

FIGURE 5 | Effect of treatment with AKB48 (0.1 e 0.5 mg/kg, i.p.) on preference place conditioning **(A)**. Interaction of AKB48 (0.5 mg/kg) with the selective CB_1 receptor antagonist AM 251 (1 mg/kg) on the on preference place conditioning of the rat **(B)**. In each experimental group Δ Time (preference score) was obtained by subtracting the time spent in the drug-paired compartment to that spent in the other compartment. Data are expressed (see section Materials and Methods) as Δ Time and represent the mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by one-way ANOVA followed by the Bonferroni's test for multiple comparisons. *$p < 0.05$ vs. vehicle.

Startle/Pre-pulse Inhibition Studies

Vehicle injection did not change startle/PPI response in rats, and the effect was similar in naïve untreated animals (data not shown). Administration of AKB48 impaired the startle amplitude in rats (about ∼50% inhibition, **Figure 7A**) [$F_{(4,\ 45)} = 3.579$; $P = 0.0129$] at the higher dose tested (3 mg/kg) at 15 min after drug administration. Moreover, AKB48 inhibited the PPI in rats at 68 [$F_{(4,\ 45)} = 4.154$; $P = 0.006$] and 75 dB [$F_{(4,\ 45)} = 3.445$; $P = 0.0154$] of pre-pulse intensity (**Figure 7B**). The inhibitory effect of AKB48 on startle was prevented by AM251 (1 mg/kg i.p.; **Figure 7C**) [$F_{(3,\ 36)} = 7.735$; $P = 0.0004$], which alone did not modify the startle response. AM251 also prevented an inhibitory effect of AKB48 on PPI in rats at 68 [$F_{(3,\ 36)} = 5.007$; $P = 0.0053$] and 75 dB [$F_{(3,\ 36)} = 6.837$; $P = 0.0009$] of pre-pulse intensity (**Figure 7D**).

Cardiorespiratory and Blood Pressure Analysis

Systemic administration of AKB48 affected cardiorespiratory parameters in rats (**Figure 8**). The basal heart rate (430 ± 15 bpm), breath rate (92 ± 8.3 brpm), and SpO_2 saturation (99.4 ± 1.3%) did not change in vehicle-treated rats over the 3 h observation (**Figure 8A**). Systemic administration of AKB48 only at the highest dose tested (3.0 mg/kg i.p.) decreased the heart rate of rats (**Figure 8A**) [$F_{(5,\ 30)} = 5.514$; $p = 0.0010$]. The effect was significant after 30 min from drug injection (∼36% of reduction); it lasted about 90 min and disappeared at 180 min.

Basal breath rate activity was also reduced by highest dose of AKB48 (**Figure 8A**) [$F_{(5,\ 30)} = 2.054$; $p = 0.0994$]. The effect was significant after 60 min from drug injection (∼30% of reduction) and disappeared at 120 min. Basal SpO_2 saturation was transiently decreased by the highest dose of AKB48 (**Figure 8A**) [$F_{(5,\ 30)} = 8.227$; $p < 0.0001$]. The effect was significant after 60 min from drug injection (∼10% of reduction) and disappeared at 120 min. The CB_1 receptor antagonist AM251 at 1 mg/kg did not affect the cardiorespiratory parameters and completely prevented the effects of AKB48 at 3 mg/kg (**Figure 8B**).

AKB48 Pharmacokinetic Studies and Behavioral Correlation

AKB48 peak plasma concentration was reached after 30 min at low doses (0.1 and 0.3 mg/kg) and after 60 min at higher doses. Mean values obtained ranged from 4 to 52 ng/mL. After a low decrease following the peak concentration, plasmatic concentrations remained quite stable for the following 120 min. Plasma time-concentration profiles for AKB48 were significantly affected by dose [$F_{(3,\ 72)} = 32.5$, $p < 0.0001$], time [$F_{(5,\ 72)} = 11.74$, $p < 0.0001$] and time treatment interaction [$F_{(15,\ 72)} = 2.153$, $p = 0.0162$], with concentrations rising linearly as dose increased (**Figure 9A**). AKB48 concentrations after a dose of 0.5 mg/kg were significantly greater than those after 0.1 mg/kg at 40 and 70 min post-injection, whereas concentrations after 3 mg/kg were greater than those after 0.1 and 0.3 mg/kg up to 195 min post-injection. Because we measured pharmacodynamic and pharmacokinetic endpoints from the same rats, we were able to examine relationships between somatosensory responses (visual, acoustic and tactile), body temperature, mechanical analgesia, catalepsy and AKB48 concentrations in plasma. The correlation findings are depicted in **Figure 9**. Visual object response (**Figure 9B**; Pearson's $r = –0.9433$, $P < 0.0001$), visual placing response (**Figure 9C**; Pearson's $r = –0.8838$, $P < 0.0001$), acoustic response (**Figure 9D**; Pearson's $r = –0.79.45$, $P < 0.0001$), overall tactile response (**Figure 9**; Pearson's $r = –0.6553$, $P < 0.0001$), body temperature (**Figure 9F**; Pearson's $r = –0.741$, $P < 0.0001$), mechanical analgesia (**Figure 9G**; Pearson's $r = 0.911$, $P < 0.0001$), catalepsy (**Figure 9H**; Pearson's $r = 0.5279$, $P < 0.0001$) were significantly correlated to AKB48 plasma concentrations.

DISCUSSION

This is the first study showing through a battery of behavioral tests, the effects caused by the third-generation synthetic cannabinoid AKB48 on "tetrad," sensorimotor, motor,

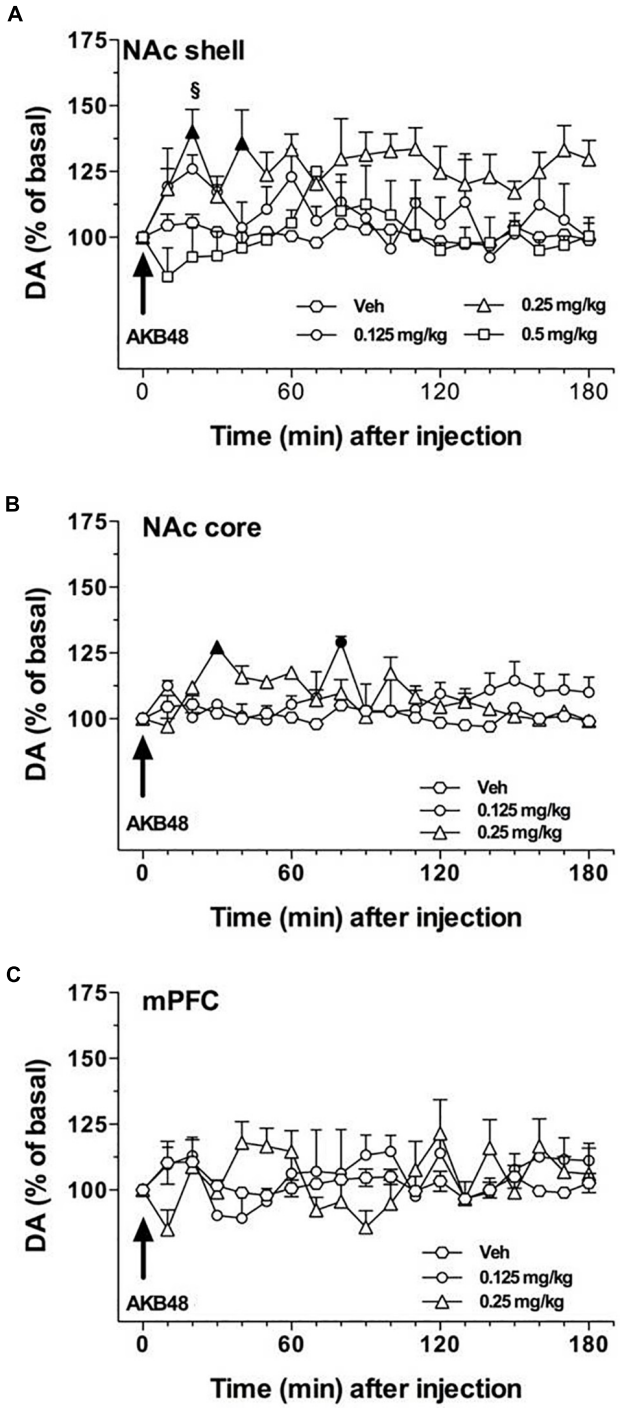

FIGURE 6 | Effect of AKB48 administration on DA transmission in the NAc shell **(A)**, NAc core **(B)**, and mPFC **(C)**. Results are expressed as mean ± SEM of change in DA extracellular levels expressed as the percentage of basal values. The arrow indicates the start of AKB48 i.p. injection at the dose of 0.125 mg/kg (*circles*), 0.25 mg/kg (*triangles*), 0.5 mg/kg (*squares*) or vehicle (*diamonds*) in the NAc shell **(A)**, NAc core **(B)**, and mPFC **(C)**. Statistical analysis was performed by Three-way or two-way ANOVA followed by the Tukey's HSD *post hoc* test for multiple comparisons. Solid symbol: $p < 0.05$ with respect to basal values; § $p < 0.05$ vs. NAc core group; $^{*}p < 0.05$ vs. mPFC group (NAc shell $n = 11$; NAc core $n = 10$; mPFC $n = 13$).

neurochemical, cardiorespiratory responses, place preference conditioning, and pre-pulse inhibition tests in adult rats. Moreover, AKB48 concentrations in the blood of rats were also monitored and correlated with behavioral measurements.

Consistent with a previous study in mice (Canazza et al., 2016), we showed that the administration of increasing doses of AKB48 causes the progressive onset of different pharmacobehavioral effects in rats. In particular, AKB48 at low doses (0.1–0.3 mg/kg) mainly inhibits visual sensorimotor responses and preferentially facilitates the release of DA in the NAc shell; increasing the doses of AKB48 (0.5 mg/kg), hypokinesia and place aversion are then observed. At the higher dose (3 mg/kg), cardiorespiratory alterations (bradycardia, bradypnea, and SpO2 reduction), analgesia, hypothermia, reduction of acoustic and tactile sensorimotor responses, and alteration of sensory gating (**Figure 10**) are observed.

All these behavioral and neurochemical effects were fully dependent on CB_1 receptor stimulation since they are completely prevented by the administration of the selective CB_1 receptor antagonist/inverse agonist AM251, as previously reported in the mouse model (Canazza et al., 2016). AKB48 in the range-doses tested (0.1–3.0 mg/kg) reproduced the typical "tetrad" in rats, characterized by hypothermia (at 3 mg/kg), analgesia (at 3 mg/kg), catalepsy (at 3 mg/kg), and hypolocomotion (at 0.5 mg/kg). These findings are in line with previous studies showing the effectiveness of different SCBs based on indole and indazole scaffolds in inducing the overall "tetrad" effect (De Luca et al., 2015), hypothermia, and catalepsy (Carlier et al., 2018; Elmore and Baumann, 2018) or hypothermia (Banister et al., 2015a,b, 2016; Schindler et al., 2017) in rats.

AKB48 is less active than JWH-018 in inducing the "tetrad" effect in the rat (De Luca et al., 2015). From a chemical structural point of view, AKB48 differs from naphthoylindole SCBs (JWH-type) by having an adamantyl group connected to an indazole moiety through a carboxamide linkage (Uchiyama et al., 2012). The presence of the adamantil group rather than the indazolic structure could cause its lower efficacy and potency *in vivo*. In fact, indazole synthetic cannabinoids 5F-AMB, MDMB-FUBINACA (Banister et al., 2016), AB-FUBINACA, and AB-PINACA (Banister et al., 2015a) cause hypothermia in the rat in a range of concentrations similar to those of indolic compounds such as JWH-018 and AM-2201 (Banister et al., 2015b). It appears evident that the presence of an adamantyl group linked to the main structure of the SCBs causes a loss of power and duration of action of the SCBs on the hypothermic effect (Banister et al., 2015b).

Besides this hypothesis, it is also possible that the lower responses could be related to the biotransformation of AKB48, as well as others SCBs, into glucuronitated or monohydroxylated metabolites that can act as neutral antagonists at CB_1 receptors, dampening the overall activity of the parent drug (Brents et al., 2012; Seely et al., 2012).

Unlike previous studies demonstrating that the analgesic effect on mechanical pain stimuli provoked by JWH-type compounds precede or match the motor impairment (De Luca et al., 2015; Vigolo et al., 2015; Ossato et al., 2016; Canazza et al., 2017), AKB48 firstly induces hypolocomotion (0.5 mg/kg i.p.) and then

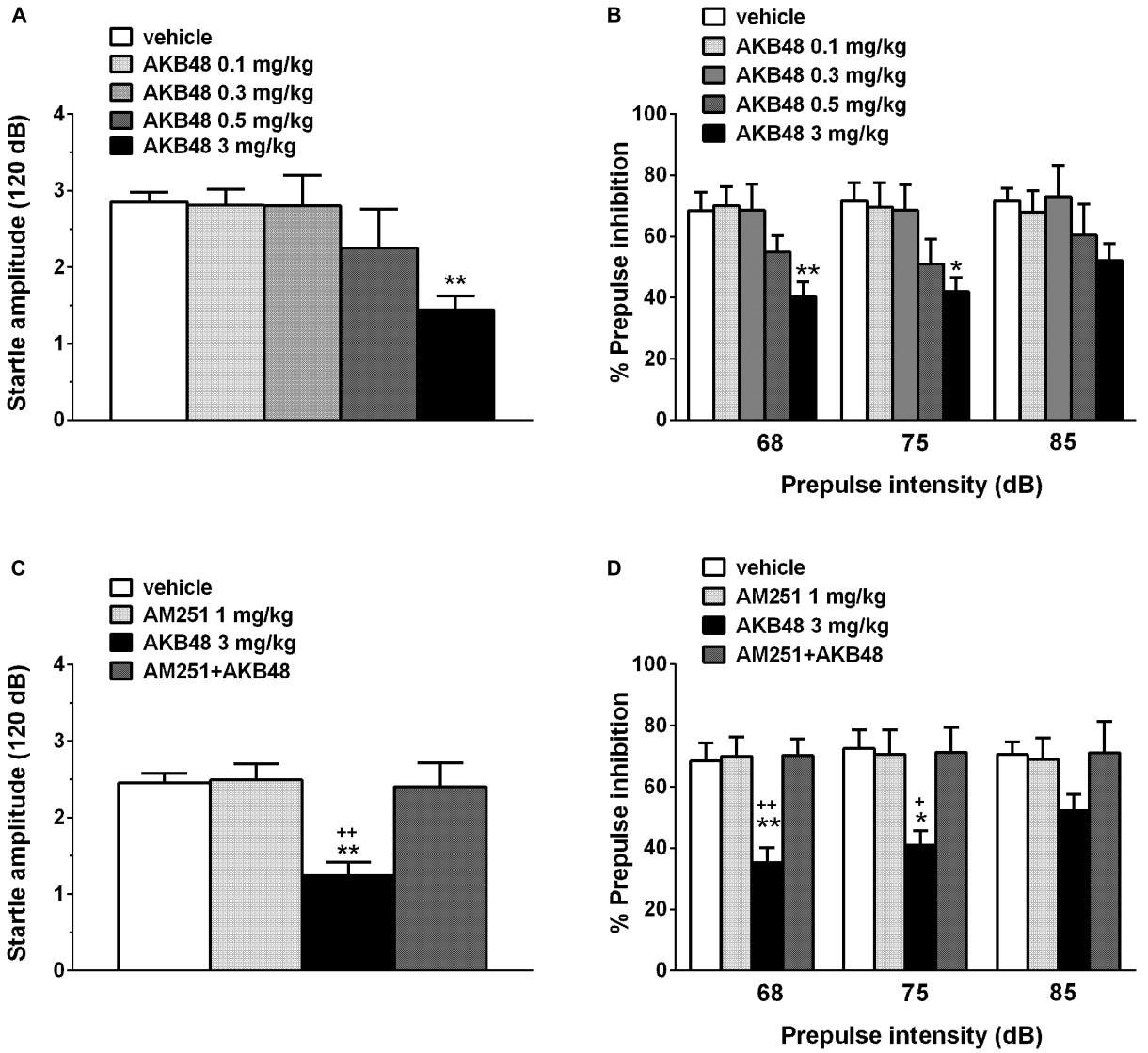

FIGURE 7 | Effect of the systemic administration of AKB48 (0.1–3 mg/kg i.p.) on startle amplitude **(A)** and pre-pulse inhibition (PPI; **B**) in the rat. Effects on PPI are shown for the three prepulse intensities (68, 75, and 85 dB), 15 min after treatment. Effect of AM251 (1 mg/kg i.p.; injected on startle amplitude **(C)** and on AKB48-inhibited PPI **(D)** in the rat was also reported. Data are expressed (see section Materials and Methods) as absolute values (dB; **A,C**) and percentage decrease in the amplitude of the startle reactivity caused by presentation of the pre-pulse (% PPI; **B,D**) and values represent mean ± SEM of 10 animals for each treatment. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's test for multiple comparisons. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. vehicle and $^{+}p < 0.05$, $^{++}p < 0.01$ vs. AM251 + AKB48.

starts to become an analgesic (3 mg/kg i.p.). This responsiveness is in line with previous studies in mice (Canazza et al., 2016) and is in agreement with evidence showing that small modifications of the molecular structure of SCBs induce consistent disparities among potencies and efficacies of *in vivo* effects (Wiley et al., 1998, 2014; Ossato et al., 2016). In our experimental conditions, the possibility that the acute analgesic effect induced by AKB48 and/or its metabolites (Gandhi et al., 2013; Holm et al., 2015) is due to the activation of peripheral CB_2 receptors (Guindon and Hohmann, 2008) and should be ruled out since their analgesic effects are fully prevented by the administration of the selective CB_1 receptor antagonist/inverse agonist AM251. As reported by others (Ossato et al., 2015, 2016; Canazza et al., 2016, 2017) AKB48 at lower doses (0.3 mg/kg) greatly impairs visual sensorimotor responses in rats and reduces acoustic and tactile reflexes at a dose 10 times higher (3 mg/kg). Reig and Silberberg have demonstrated in their recent study that visual information in rodents is elaborated in a subpopulation of neurons selectively localized in the dorsomedial striatum (Reig and Silberberg, 2014), in which CB_1 receptors are expressed (Tsou et al., 1998; Marsicano and Lutz, 1999). Even though in our study we cannot reveal which brain areas and neural mechanisms are involved in the reduction of visual response of the rat, it is possible to hypothesize that AKB48 could stimulate CB_1 receptors expressed

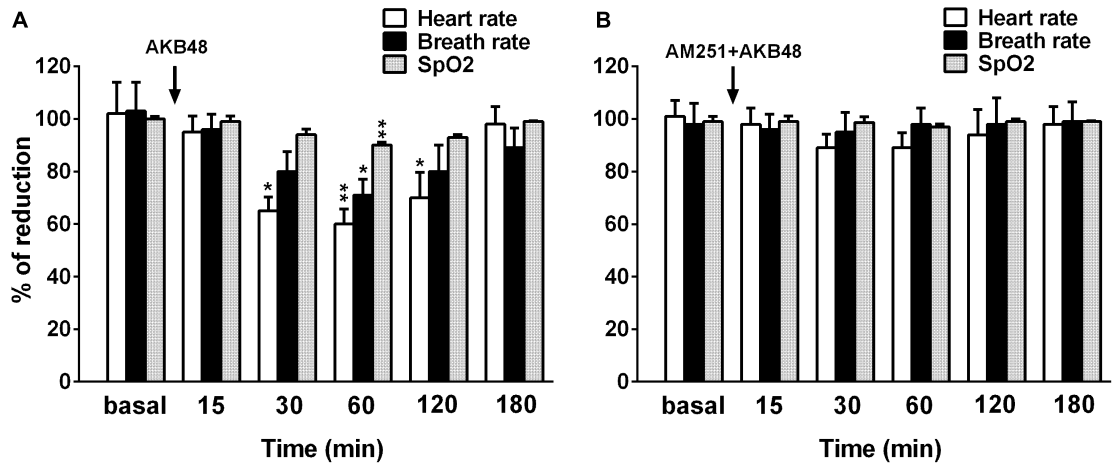

FIGURE 8 | Effect of the systemic administration of AKB48 (3 mg/kg i.p.) on heart rate, breath rate and oxygen arterial saturation in rats **(A)**. Effect of AM251 on AKB48-reduced cardiorespiratory parameters in the rat was reported in **(B)**. For **(A,B)**, data are expressed as the percentage of basal value (heart and breath rate) and as a percentage of oxygen blood saturation (% SpO_2 saturation) and represent the mean ± SEM of 6 animals for each treatment. Statistical analysis was performed by one-way ANOVA followed by the Bonferroni's test for multiple comparisons. $^*p < 0.05$, $^{**}p < 0.01$ vs. vehicle.

in thalamocortical-striatal visual circuitry (Tsou et al., 1998; Marsicano and Lutz, 1999; Dasilva et al., 2012; Yoneda et al., 2013), and cause an impairment of the visual function in rats.

It is interesting to note that AKB48 impairs visual sensorimotor responses in rats at a low dose (0.3 mg/kg) that does not cause hypolocomotion (open field studies). These findings reveal that effects induced by AKB48 on visual sensorimotor responses and motor activity are mediated by separate processes and suggest that a decrease in sensory responsiveness does not reflect a disruption of motor function (Ossato et al., 2015).

Our study also demonstrates that AKB48 impairs the acoustic startle response in rats through selective stimulation of CB_1 receptors. This interpretation is in accordance with previous studies that have proved the effectiveness of acute administration of Δ^9-THC (Malone and Taylor, 2006; Nagai et al., 2006; Ossato et al., 2015), CP 55940 (Mansbach et al., 1996; Martin et al., 2003), WIN-55,212-2 (Bortolato et al., 2005), JWH-018 (Ossato et al., 2015), JWH-250, and JWH-073 (Ossato et al., 2016) in reducing the acoustic startle reflex in rodents. A recent study on acoustic startle reflex showed that this mechanism is induced by the activation of three serially connected structures that involve the activation of the dorsal cochlear nucleus (Gomez-Nieto et al., 2014). In addition, a study of Tzounopoulos and colleagues showed that CB_1 receptors are expressed on the presynaptic terminals of parallel fibers in the dorsal cochlear nucleus (Tzounopoulos et al., 2007). Thus it is possible to speculate that AKB48 could impair the acoustic startle reflex in rats by stimulating CB_1 receptors located in the dorsal cochlear nucleus.

A common feature of cannabinoid drugs is to induce in both humans (Kedzior and Martin-Iverson, 2006) and animals (Peres et al., 2016) a reduction in PPI of the acoustic startle reflex, which is considered an operational measure of the sensory gating (or filtering) that is severely impaired in schizophrenia patients (Javitt and Zukin, 1991). Our study demonstrates that AKB48 impairs the PPI response in rats by the selective stimulation of CB1 receptors. This finding is in agreement with previous studies that have demonstrated the effectiveness of acute administration of Δ9-THC (Malone and Taylor, 2006; Nagai et al., 2006), CP 55940 (Mansbach et al., 1996; Martin et al., 2003), and WIN 55,212-2 (Schneider and Koch, 2002; Wegener et al., 2008) in reducing PPI in rodents. These findings extend to these synthetic compounds the ability to induce information processing deficits and sensory disturbances that may account for their psychotic effects in humans (Every-Palmer, 2010; Every-Palmer, 2011). The acute administration of AKB48 (0.5 mg/kg ip) reduced the locomotor activity and induced aversion in the place preference task. According to our results, place conditioning tests in rodents showed that high doses of Δ9-THC or synthetic cannabinoid agonists such as WIN 55212-2 and HU210 caused a significant place aversion (CPA), counteracted by the CB1 receptor antagonist/inverse agonist SR 141716A (Chaperon et al., 1998; Cheer et al., 2000; Valjent and Maldonado, 2000; Tzschentke, 2007). Similarly, in our test, the aversive effect of high doses of AKB48 was abolished by treatment with the selective CB1 antagonist AM251.

The observation of aversive effects in the CPP paradigm is in line with the inability of AKB48, when administered at the dose of 0.5 mg/kg ip, to increase DA transmission in the NAc shell thus confirming a lack of rewarding properties of high doses of AKB48 analogs (De Luca et al., 2015). However, as demonstrated in this study, an acute administration of 0.25 mg/kg, but not 0.125 mg/kg, affected DA signaling in the NAc shell but not in the NAc core nor mPFC. It is well-established that a selective increase of extracellular DA in the NAc shell, estimated by *in vivo* brain microdialysis in rodents, is a common feature of drugs abused by humans (Di Chiara and Imperato, 1988), including cannabinoids (Tanda et al., 1997; Sidhpura and Parsons, 2011). Therefore, a complete pharmacological and toxicological characterization

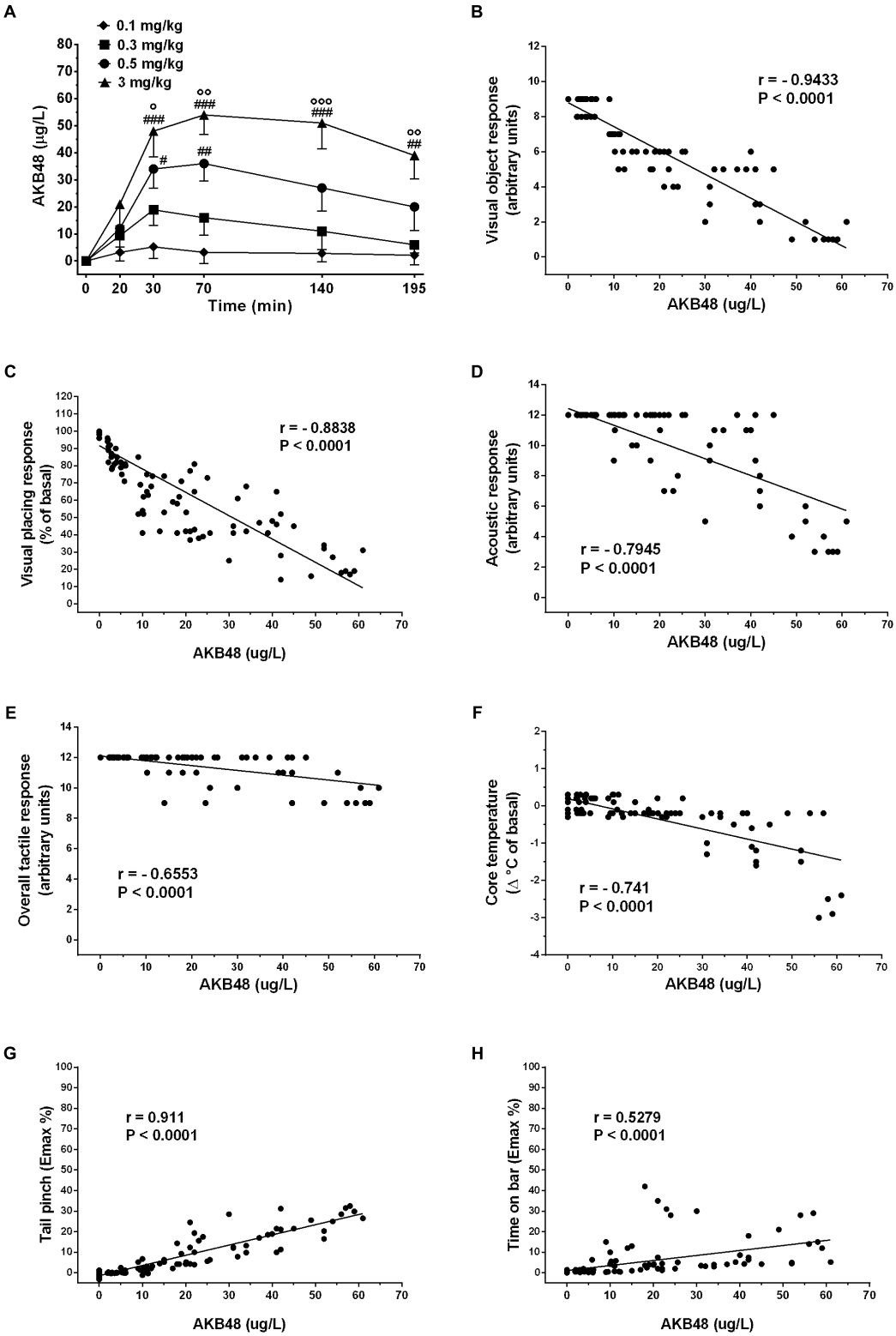

FIGURE 9 | Time-concentration profiles for AKB48 in rats **(A)**. Rats fitted with indwelling jugular catheters received AKB48 doses of 0.1, 0.3, 0.5, or 3.0 mg/kg i.p. at time zero. Blood samples were withdrawn via the catheters at 20, 40, 70, 140, and 195 min after AKB48 injection, and plasma specimens were assayed for analytes using LC-MS/MS. Data are expressed as absolute values (µg/L) and represent the mean ± SEM of 4 rats/group. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose response curve of each test at different times. Correlations between plasma

(Continued)

FIGURE 9 | Continued
concentrations of AKB48 vs. visual object responses **(B)**, visual placing responses **(C)**, acoustic responses **(D)**, overall tactile responses **(E)**, body core temperatures **(F)**, mechanical analgesia **(G)** and catalepsy **(H)**. Each point in the correlation graphs is plotted by correlating the AKB48 concentrations (μg/L) in blood samples against the behavioral effects observed in rats taken at the same time period. Statistical analysis and correlation were performed by Pearson's test. Pearson's *r*- and *P*-values are shown. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ vs. AKB48 at 0.1 mg/kg; $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$, and $^{\circ\circ\circ}p < 0.001$ vs. AKB48 at 0.3 mg/kg.

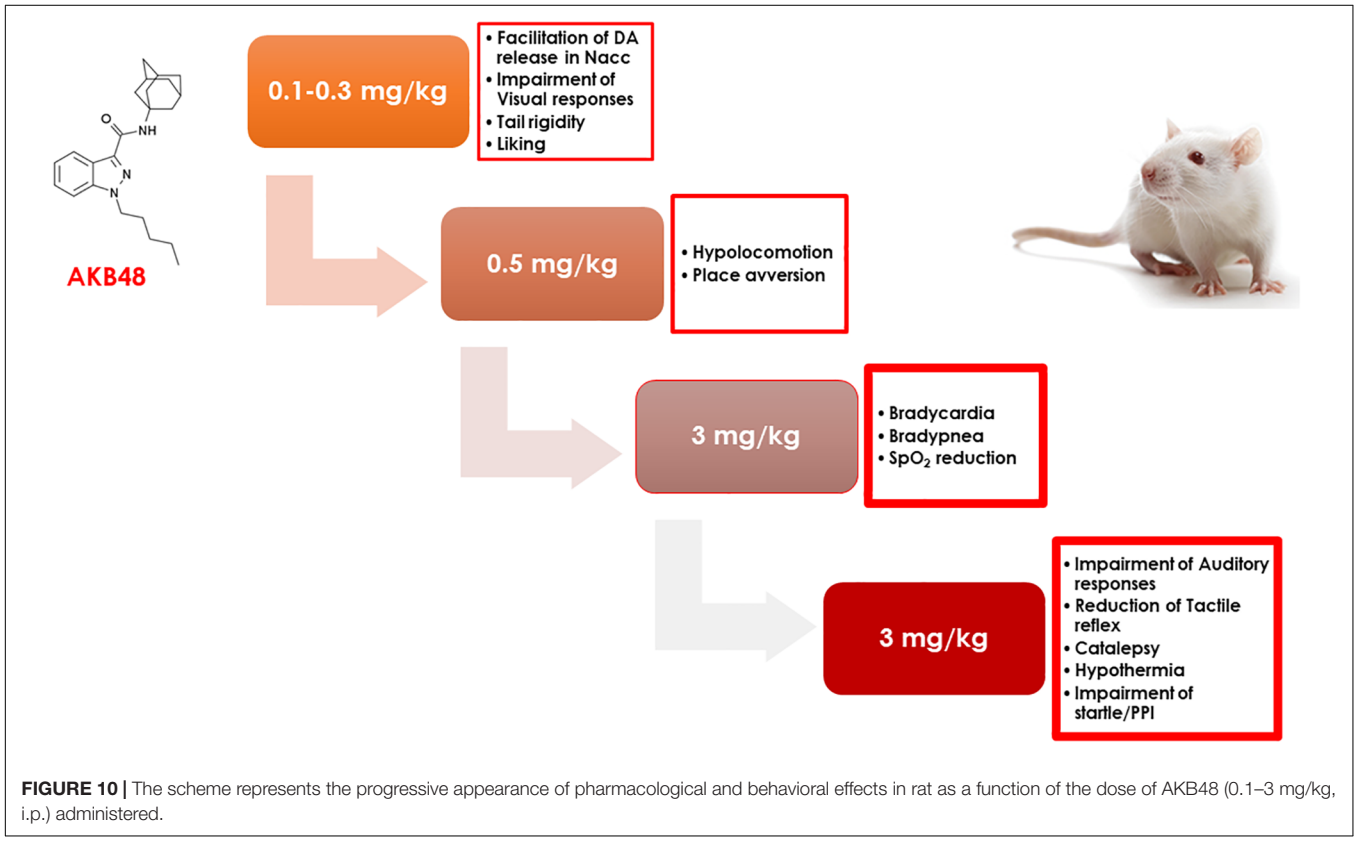

FIGURE 10 | The scheme represents the progressive appearance of pharmacological and behavioral effects in rat as a function of the dose of AKB48 (0.1–3 mg/kg, i.p.) administered.

of NPS generally comprises the measurement of DA levels in specific brain areas (i.e., NAc shell/core/mPFC). For this reason, although already performed in mice (Canazza et al., 2016) and demonstrated for different generations of synthetic cannabinoids (De Luca et al., 2015, 2016), the present study displays a set of microdialysis experiments after the administration of AKB48. We observed that AKB48 selectively stimulated NAc shell DA at the same dose of JWH-018 (De Luca et al., 2015), the prototypical compound of the first generation of SCBs. However, the chemical hindrance of the adamantyl group (**Figure 10**) may be the reason for a lower (max increase of about 40%, at 20 and 40 min) and less-lasting increase of DA with respect to values observed after the same dose of JWH-018 (0.25 mg/kg ip) (max increase of about 60%, from 20 to 70 min) (De Luca et al., 2015; Miliano et al., 2016).

The present study discloses the possible rewarding properties of AKB48, as well as the peculiar pharmacological profile of SCBs. Indeed, in the response of NAc shell DA, we observed an inverted U-shaped dose-response curve at a dose enclosed between an extremely narrow range of doses (0.125–0.5 mg/kg ip). Nethertheless, few and contrasting studies specifically investigated the relationship between cannabinoid effects in CPP and dopaminergic release in specific rewarding areas (Polissidis et al., 2009; Tampus et al., 2015). Polissidis et al. (2009) demonstrated that a stimulatory low dose (0.1 mg/kg) of the cannabinoid CB1 receptor agonist WIN 55,212-2 on motor activity was not accompanied by place preference, but enhanced dopaminergic activity in the nucleus accumbens shell of rats. Ossato et al. (2017) also demonstrated that the transitory (15 min) psychostimulant effect of AKB48 in mice, facilitating spontaneous locomotion, is mediated by activation of both CB1 and D1/5 and D2/3 dopaminergic receptors, resulting in an increased NAc DA release. In addition, as regards to the synthetic cannabinoid JWH-018, high doses (1 and 3 mg/kg) that induced CB1 receptor-dependent behavioral effects in rats (such as catalepsy and hypomotility) have no effect on DA release in the NAc shell (De Luca et al., 2015), but induced place aversion (Hyatt and Fantegrossi, 2014). Interestingly, only the 0.25 mg/kg dose increased DA release in the NAc shell. On the other side, a lower (0.125 mg/kg) and higher (0.50 mg/kg) doses were ineffective (De Luca et al., 2015). Similarly to JWH-018, the doses of AKB48 that evoke an increase in dopamine signaling are lower than those that induced, for example, hypolocomotion or aversive effects.

Taking this into consideration, it could be speculated that low doses of AKB48 exhibit conditioned rewarding effects, that, for higher doses seems to be masked by the prevalent aversive and anxiolytic-like properties. In line with this reasoning, the increased tail rigidity and the reduction of amount of licking could be interpreted as an index of anxiety and related-behaviors. In fact, some of the most established indicators of negative emotional behavior (fear response or anxiety) in the open field test are low ambulation and increased tail rigidity (Sestakova et al., 2013).

The present study shows for the first time that AKB48, at the highest dose tested (3 mg/kg), induces bradycardia in rats through CB1 receptor activation. This action is consistent with previous studies showing the cardiovascular depressive effects induced by different SCBs in rats (Banister et al., 2015a,b, 2016) and is possibly due to the mechanism of sympathoinhibition and enhancement of cardiac vagal tone mediated by CB1-receptors (Schmid et al., 2003). Cardiac alterations, such as palpitations, chest pain, bradycardia, tachycardia, arrhythmias, hypotension, syncope, and ECG changes like T-wave inversion, represent one of the main adverse effects associated with SC use in humans (Hermanns-Clausen et al., 2016; McIlroy et al., 2016; Von Der Haar et al., 2016; Sud et al., 2018). Some literature also describes cases of death by myocardial infarction or cardiac arrest directly attributed to synthetic cannabinoid use (Mir et al., 2011; Ibrahim et al., 2014). Further studies will be carried out to verify if the administration of SCBs can cause direct damage to heart tissue.

In addition to bradycardia, AKB48 induces a CB1 receptor-mediated respiratory depression, as shown by a decrease in respiratory rate (inhibition of about 40% of basal breath rate values) and hypoxia (reduction of about 15% of basal SpO2 values). Recent data suggest that SCBs induced acute respiratory depression (Alon and Saint-Fleur, 2017). This effect was most likely not a consequence of cardiovascular depression, since cardiovascular depression usually leads to a stimulation of the central ventilatory drive, and consequently, to an increase in respiratory frequency (Schmid et al., 2003). The cannabinoids inhibited respiration probably by acting directly in the central nervous system (Pfitzer et al., 2004). However, an additional effect at the periphery cannot be ruled out since cannabinoids could play an important role in the functioning of different peripheral receptors involved in respiratory regulation (e.g., pulmonary stretch receptors, chemo- and baroreceptors). They could also have a direct action on the bronchi altering the airway resistance (Calignano et al., 2000).

Finally, the present study firstly reported a plasma pharmacokinetic profile for AKB48 in rats monitored for about 3 h and correlating plasma levels of AKB48 with different behavioral measurements. As shown in **Figure 9**, a correlation was observed between plasma concentration and sensorimotor (visual, acoustic and tactile) responses, mechanical analgesia, core temperature and catalepsy. This preclinical study is aimed to extend pharmaco-toxicolgical features of AKB48 in the animal model, however the results obtained could not be correlated with clinical studies due to limited number of samples and information reported in cases of intoxication with this SCB in human.

CONCLUSION

The present study discloses, for the first time, the overall progressive pharmacological and behavioral effects induced by the progressive administration of adamantylindazole AKB48 in rats (**Figure 10**), highlighting its ability to primarily disrupt visual sensimotor responses and facilitate DA release in the NAc shell. With increasing doses, hypokinesia and place aversion were registered. Finally, at higher doses, a reduction of cardiorespiratory signs (bradycardia, bradypnea, and spO2); acoustic and tactile sensimotor responses; and analgesia, hypothermia, and catalepsy were observed.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethical Committee for Animal Experiments (CESA, University of Cagliari) and the Italian Ministry of Health (Aut. n°162/2016-PR).

AUTHOR CONTRIBUTIONS

MM, MD, MN, and FD-G contributed conception and design of the study. SB, MT, RA, SS, LS, SS-R, and AF performed experimental sections. SB, SS, and CM organized the database. MT, RA, and CM performed the statistical analysis. MM wrote the first draft of the manuscript. SB, SS, LS, RC, PF, SS-R, CM, GS, MN, and MD wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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The Impact of Ayahuasca on Suicidality: Results From a Randomized Controlled Trial

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Suicide is a major public health problem. Given increasing suicide rates and limitations surrounding current interventions, there is an urgent need for innovative interventions for suicidality. Although ayahuasca has been shown to target mental health concerns associated with suicidality (i.e., depression and hopelessness), research has not yet explored the impact of ayahuasca on suicidality. Therefore, we conducted secondary analyses of a randomized placebo-controlled trial in which individuals with treatment-resistant depression were administered one dose of ayahuasca (n = 14) or placebo (n = 15). Suicidality was assessed by a trained psychiatrist at baseline, as well as 1 day, 2 days, and 7 days after the intervention. A fixed-effects linear mixed model, as well as between and within-groups Cohen's d effect sizes were used to examine changes in suicidality. Controlling for baseline suicidality, we found a significant effect for time ($p < .05$). The effect of the intervention (i.e., ayahuasca vs. placebo) trended toward significance ($p = .088$). At all time points, we found medium between-group effect sizes (i.e., ayahuasca vs. placebo; day 1 Cohen's d = 0.58; day 2 d = 0.56; day 7 d = 0.67), as well as large within-group (ayahuasca; day 1 Cohen's d = 1.33; day 2 d = 1.42; day 7 d = 1.19) effect sizes, for decreases in suicidality. Conclusions: This research is the first to explore the impact of ayahuasca on suicidality. The findings suggest that ayahuasca may show potential as an intervention for suicidality. We highlight important limitations of the study, potential mechanisms, and future directions for research on ayahuasca as an intervention for suicidality.

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INTRODUCTION

Suicide is a public health issue of major concern: it is a leading cause of premature death, accounting for nearly one million deaths annually (World Health Organization, 2014). For every completed suicide, it is estimated that 20–30 suicide attempts occur (Wasserman, 2001). Furthermore, suicide rates have been increasing within the United States (Curtin et al., 2016).

Suicide occurs most commonly among individuals with major depressive disorder (MDD) (Cavanagh et al., 2003; World Health Organization, 2014) and individuals with comorbid MDD

and borderline personality disorder (BPD) exhibit especially heightened levels of suicidality (Soloff et al., 2000; Galione and Zimmerman, 2010; Perugi et al., 2013; Zeng et al., 2015). Given the drastic consequences of suicide and suicide attempts, effective suicide interventions are of great importance.

A number of interventions are effective for treating suicidality (i.e., suicide attempts, suicide planning, and suicidal ideation; for a review, see Zalsman et al., 2016), including electroconvulsive therapy, psychotherapy (e.g., cognitive behavior therapy, dialectical behavior therapy), and pharmacological interventions (e.g., antidepressants, lithium, clozapine). However, there remain a number of important limitations surrounding current interventions for suicidality, including (a) non-immediate effects (e.g., weeks to months; Griffiths et al., 2014), (b) limited treatment availability (Bateman, 2012), (c) negative side-effects (e.g., increased suicidality with antidepressant use among adolescents; Vitiello and Ordóñez, 2016), (d) the need for ongoing administration (Kellner et al., 2005), and (e) high rates of non-responsiveness (Stone et al., 2009; Pompili et al., 2010). Individuals who do not respond to conventional interventions (i.e., individuals with treatment-resistant depression) show especially heightened levels of suicidality (Nelsen and Dunner, 1995; Malhi et al., 2005; Souery et al., 2007) and are, therefore, especially in need of novel interventions for suicidality.

Novel Interventions for Suicidality

One novel intervention that has recently received attention for the treatment of suicidality is ketamine, a dissociative that acts as an antagonist of N-methylD-aspartate (NMDA). A recent meta-analysis (k = 10; N = 167; Wilkinson et al., 2018) of randomized controlled trials on the impact of a single dose of ketamine (vs. saline or midazolam) on suicidality found medium to large between-group effect sizes both 1 day [effect size (ES) = 0.85], 2 (ES = 0.85), and 7 days (ES = 0.61) after administration. Importantly, this meta-analysis suggests that the impact of ketamine on suicidality may begin to decrease within a week after administration. Moreover, there is no evidence that the antisuicidal effects of ketamine are long-lasting (Zalsman et al., 2016; Dadiomov and Lee, 2019) and there are significant concerns surrounding repeated administration of ketamine, including the potential for abuse and cognitive impairment (Schak et al., 2016; Strong and Kabbaj, 2018). Thus, there is a need for identifying alternative novel interventions for suicidality with less potential for abuse and a longer-lasting impact on suicidality.

One potentially promising novel intervention for suicidality, which has shown promise for a wide range of mental health concerns (for a review, see dos Santos et al., 2018) are psychedelics. Psychedelics are a class of pharmacological agents, including psilocybin and ayahuasca (a brew which contains N,N-dimethyltryptamine and beta-carboline alkaloids), that induce changes in affect, cognition, and perception, as well as non-ordinary states of consciousness at high doses (Griffiths et al., 2006; Hamill et al., 2019).

Cross-sectional and longitudinal research indicates that lifetime use of psychedelics is associated with lower levels of suicidality. For instance, among adult males (N = 190,000), lifetime psychedelic use was associated with lower levels of past-year suicide ideation, planning, and attempts (Hendricks et al., 2015). Furthermore, within a community-based cohort of marginalized women, lifetime psychedelic use was predictive of reduced risk of suicidality (Argento et al., 2017), as well as buffered the relationship between opioid use and suicidality (Argento et al., 2018). However, given that these studies were non-experimental, they leave open the question of whether these effects are due to factors associated with psychedelic use, such as personality, or whether *administration* of psychedelics leads to decreases in suicidality.

To date, only a single study has experimentally explored the impact of psychedelics on suicidality. Carhart-Harris and colleagues (2018) conducted an open-label trial in which individuals with treatment-resistant MDD received two doses of psilocybin with psychological support. Results indicated significant decreases in self-reported suicidality 1 and 2 weeks after the intervention. This study was limited by reliance upon self-reported suicidality and the open-label design. Accordingly, additional experimental research on the impact of psychedelics on suicidality, using clinician assessed suicidality and a placebo-controlled design, is necessary.

Additional support for the impact of psychedelics on suicidality comes from clinical research indicating that interventions that include administration of psilocybin (Carhart-Harris et al., 2016; Griffiths et al., 2016; Ross et al., 2016; Carhart-Harris et al., 2018) and ayahuasca (Santos et al., 2007; Thomas et al., 2013; Osório et al., 2015; Sanches et al., 2016; Palhano-Fontes et al., 2019; Uthaug et al., 2018) lead to acute and sustained reductions in mental health concerns associated with suicidality, such as depression and hopelessness (Brown et al., 2000; Nock et al., 2009). For instance, among individuals with treatment-resistant MDD, a recent randomized placebo-controlled trial showed large decreases in depressive symptoms 1, 2, and 7 days administration of ayahuasca (Palhano-Fontes et al., 2019). However, extant research suggests that suicidality can occur independent from depressive symptoms (Brent et al., 2005; Brent, 2010; Brent and Mann, 2010; Dutta et al., 2017; Batterham et al., 2019) and decreases in depressive symptoms are not always associated with decreases in suicidality (Christensen et al., 2013). For instance, compared with placebo, even first-line interventions for depression (i.e., selective serotonin reuptake inhibitors; SSRIs) lead to limited to no decreases in suicidality (intent to treat ES = –0.04–0.20; Näslund et al., 2018). Therefore, there is a need for research on the impact of ayahuasca directly on suicidality.

In sum, suicide is an increasingly problematic mental health concern and there are important limitations surrounding current interventions for suicidality. Lifetime psychedelic use is associated with lower levels of suicidality. Furthermore, ayahuasca and psilocybin have shown promise as interventions for a wide range of mental health issues associated with suicidality. However, research has not yet explored whether the administration of ayahuasca leads to reductions in suicidality. In order to fill this gap in the literature, we conducted secondary analyses of data from a randomized placebo-controlled trial, in which individuals with treatment-resistant MDD were administered a single dose of ayahuasca or placebo (see primary analysis: Palhano-Fontes et al., 2019). We hypothesized that ayahuasca would lead to decreases

in suicidality that are sustained (i.e., from 1 to 7 days after the intervention). We also conducted exploratory analyses in order to determine whether changes in suicidality were associated with changes in non-suicide-related depressive symptoms.

METHODS

Procedures

We conducted secondary analyses of a double-blind, parallel-arm, randomized placebo-controlled trial for individuals with treatment-resistant MDD (for primary outcomes, see Palhano-Fontes et al., 2019). Participants were recruited *via* referral from outpatient psychiatric units and advertisement. Interested participants received a full clinical assessment by a psychiatrist in order to determine eligibility. To be eligible to participate in the study, participants needed to be: between ages 18 and 60, meeting criteria for a unipolar MDD, which was assessed using the Portuguese version (Del-Ben et al., 2001) of the Structured Clinical Interview for DSM-IV (SCID-IV; First et al., 1997), and treatment-resistant (i.e., inadequate response to 2 or more antidepressant medications from different classes; Conway et al., 2017). Exclusion criteria for the study included: prior experience using psychedelics, current medical disease, pregnancy, imminent suicidal risk, or use of substances of abuse, current or previous neurological disorders, and personal or family history of schizophrenia, bipolar affective disorder, mania, or hypomania. Eligible participants were randomly assigned (1:1) to either the ayahuasca or placebo group, randomized in blocks of 10. Investigators and participants were blind to the treatment condition. Blindness was enhanced through the exclusion of individuals with past experience with ayahuasca, the use of an active placebo, as well as through randomly assigning participants to different assessors following the intervention.

Antidepressant medication was discontinued prior to intervention (average 2 weeks, dependant on the half-life of the antidepressant) and for 7 days post-intervention. Daily benzodiazepine use was permitted, excluding during the acute phases of the intervention. On the morning of the intervention, participants were reminded with information regarding potential experiences and strategies for dealing with difficult experiences during the ayahuasca inebriation. Participants were also instructed to focus on their bodies, thoughts, and emotions. The intervention occurred in a quiet and dimly lit environment with a bed and a recliner. Participants listened to a predefined music playlist throughout the intervention.

Within an individual setting, participants were administered a single 1 ml/kg dose of either ayahuasca (mean ± S.D.; 0.36 ± 0.01 mg/ml of N, N-DMT, 1.86 ± 0.11 mg/ml of harmine, 0.24 ± 0.03 mg/ml of harmaline, and 1.20 ± 0.05 mg/ml of tetrahydroharmine) or placebo (per 1 ml of water: 0.1 g of yeast, 0.02 g of zinc sulfate, and 0.02 g of citric acid). The placebo was designed to imitate the bitter/sour taste, brownish color, and gastrointestinal distress often present during the effects of ayahuasca. During the session, two investigators remained next door to provide support when needed. Sessions lasted approximately 8 h, after which participants were permitted to return home. Following the intervention, four participants opted to remain as inpatients throughout the 7-day period. Suicidality was assessed at (a) baseline, (b) 1 day, (c) 2 days, and (d) 7 days after the intervention. The study adhered to recommended clinical guidelines for safely conducting psychedelic administration (Johnson et al., 2008), it occurred at the Onofre Lopes University Hospital (HUOL), Natal-RN, Brazil, and was approved by the University Hospital Research ethics committee. For additional details regarding study procedures, including a CONSORT diagram of the trial profile, as well as the impact of ayahuasca on depressive symptoms, not including analyses of suicidality, see Palhano-Fontes and colleagues (2019).

MEASURES

Montgomery-Åsberg Depression Rating Scale—The Montgomery-Åsberg Depression Rating Scale (MADRS; Montgomery and Asberg, 1979) is a 10-item, clinician-administered measure of depression severity. The measure includes one item (item 10; MADRS-suicidality item; MADRS-SI) that assesses current suicidality. MADRS-SI is rated on a scale from 0 to 6. The ratings are as follows: 0 ("Enjoys life or takes it as it comes."), 2 ("Weary of life. Only fleeting suicidal thoughts."), 4 ("Probably better off dead. Suicidal thoughts are common, and suicide is considered as a possible solution but without specific plans or intention."), and 6 ("Explicit plans for suicide when there is an opportunity. Active preparations for suicide."). Odd ratings (i.e., 1, 3, and 5) may be used but are not specifically defined. Past research has defined clinically significant suicidality as MADRS-SI ≥ 4 (Ballard et al., 2015). Assessment of suicidality using the MADRS-SI is common in suicidality research (e.g., Price et al., 2009; Larkin and Beautrais, 2011; Perroud et al., 2011; Price et al., 2014; Ballard et al., 2015) and is considered a valid approach for the assessment of suicidality (Desseilles et al., 2012). In line with past research (e.g., Price et al., 2014; Ballard et al., 2015), we used the sum of the remaining nine items of the MARDS to measure non-suicide-related depressive symptoms (MADRS-total$_{nonSI}$).

Statistical Analysis

We used a modified intention-to-treat analysis, in which all participants that received the intervention (i.e., ayahuasca or placebo) were included in analyses. We ran a fixed-effects linear mixed model, examining MARDS-SI scores 1 day, 2 days, and 7 days after the intervention, with baseline MADRS-SI scores as a covariate. We used an unstructured covariance structure and estimated missing data (1 and 2 days after the intervention two participants failed to attend assessments) with restricted maximum-likelihood estimation. We evaluated the main effects of time and intervention, as well as a time x intervention interaction. For MADRS-SI and MADRS-total$_{nonSI}$ scores, we calculated between-group Cohen's *d* effect sizes by dividing estimated marginal means (1 day, 2 days, and 7 days after the intervention) for each group by pooled standard deviations. We also calculated within-group Cohen's *d* effect sizes by dividing change scores (time point—baseline) at each time point (1 day, 2 days, and 7 days after the intervention) by the standard deviation of the change

score. For within-group effect sizes, missing values were not imputed. For the relationship between changes in MADRS-SI and MADRS-total$_{nonSI}$ scores 7 days after the intervention, within both the ayahuasca and placebo groups, we calculated Pearson correlation coefficients. We set the alpha level indicating significance at $p < 0.05$, two-tailed. All analyses were conducted using IBM SPSS Statistics (Version 25).

RESULTS

Demographics

Participant mean age was 42.04 (*SD* = 11.66). The majority of participants were female (72%), Caucasian (59%), unemployed (52%), had a lifetime suicide attempt (55%), and a comorbid personality disorder (76%). Nine individuals (31%) had a diagnosis of BPD. At baseline, participant mean MADRS-SI score was 2.35 (*SD* = 1.91). For additional details regarding participants' characteristics by condition, see **Table 1**. For individual participant details related to the presence of a personality disorder and MADRS-SI at each time point, see **Table 2**.

TABLE 1 | Sample characteristics and treatment history by study condition.

Variable	Ayahuasca group (*n* = 14)	Placebo group (*n* = 15)
Age *M* (*SD*)	39.71 (11.26)	44.2 (11.98)
Sex-Female	11	5
Ethnicity		
Black	1	0
Pardo	4	7
Caucasian	9	8
Education		
Incomplete Primary Education	5	4
Completed Primary Education	1	2
Completed Secondary Education	4	7
Completed Undergraduate Education	1	0
Completed Postgraduate Education	3	2
Employment status		
Employed-working	3	3
Employed-leave of absence	2	4
Unemployed	7	8
Student	2	0
Presence of personality disorder	10	12
Borderline personality disorder	5	4
Dependant personality disorder	1	0
Histrionic personality disorder	4	7
Narcissistic personality disorder	1	0
Schizoid personality disorder	0	1
Cluster B (undefined)	1	0
Depression duration (years)		
Baseline MADRS-SI *M* (*SD*)	3.36 (1.65)	1.40 (1.68)
Lifetime suicide attempt	10	6
Number of failed antidepressant medications *M* (*SD*)	3.93 (1.44)	3.80 (1.90)
History of psychotherapy	11	12
History of electroconvulsive therapy	1	1
Inpatient during the intervention	2	2

Cluster B personality disorders include antisocial personality disorder, borderline personality disorder, histrionic personality disorder, and narcissistic personality disorder.

TABLE 2 | Participant clinical characteristics.

Condition	Personality disorder	Suicidality (MARDS-SI)			
		Baseline	1 day post	2 days post	7 days post
A1	Histrionic	4	4	4	4
A2	Histrionic	2	0	0	0
A3	Borderline	4	3	*	2
A4	Histrionic + Dependent	3	0	0	0
A5	Cluster B (undefined)	0	0	0	0
A6	None	3	0	0	0
A7	Borderline + Narcissistic	5	1	1	1
A8	Histrionic	4	2	0	2
A9	None	5	1	0	0
A10	None	0	0	0	0
A11	Borderline	5	0	0	0
A12	None	4	3	3	0
A13	Borderline	4	2	0	0
A14	Borderline	4	0	1	5
P1	Schizoid	2	0	0	0
P2	Histrionic	0	0	0	0
P3	Borderline	5	2	2	2
P4	None	0	4	0	2
P5	Borderline	3	*	3	3
P6	Histrionic	1	0	0	0
P7	None	0	0	0	0
P8	Borderline	3	1	1	1
P9	None	2	1	1	1
P10	Histrionic	0	0	0	0
P11	Histrionic	0	0	0	0
P12	Histrionic	0	0	0	2
P13	Histrionic	4	*	*	4
P14	Borderline	1	2	1	5
P15	Histrionic	0	3	0	0

*MADRS-SI, Montgomery-Asberg Depression Rating Scale-Suicidality Item; A, ayahuasca; P, placebo; *, Failed to attend assessment.*

Clinical Response

For changes in suicidality (MADRS-SI) by group, see **Figure 1**. Results of the linear mixed model showed a significant effect for time, $F(2,25.72 = 3.38; p < .05)$ and a trend toward significance for the intervention (i.e., ayahuasca vs. placebo), $F(1,27.44 = 3.13; p = .088)$. The interaction between time and intervention was not significant, $F(3,25.72 = .395; p = .678)$.

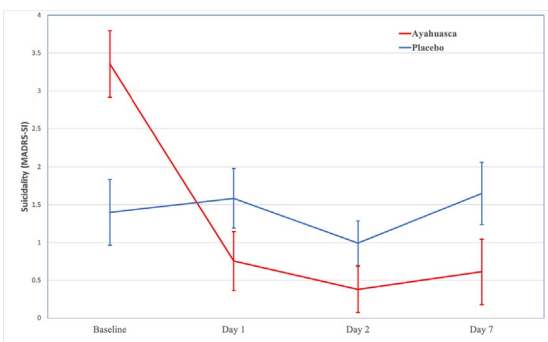

FIGURE 1 | Changes in suicidality (MADRS-SI) over time. Baseline values are means (± standard error of the mean) for MADRS-SI. Day 1, Day 2, and Day 7 values are estimated marginal means (±standard error of the mean) for MADRS-SI.

We found medium between-group (ayahuasca vs. placebo) effect sizes for decreases in MADRS-SI 1 day (Cohen's d = 0.58; 95% CI –1.32–0.17), 2 days (Cohen's d = 0.56; 95% CI –1.30–0.18), and 7 days (Cohen's d = 0.67; 95% CI –1.42–0.08) after the intervention (see **Table 3**). Within the ayahuasca group, we found large within-group effect sizes for decreases in MADRS-SI 1 day (Cohen's d = 1.33; 95% CI 1.25–3.18; n = 14), 2 days (Cohen's d = 1.42; 95% CI 1.50–3.74; n = 13), and 7 days (Cohen's d = 1.19; 95% CI 1.21–3.50; n = 14) after the intervention. Within the placebo group, we found small within-group effect sizes for decreases in MADRS-SI 1 day (Cohen's d = 0.00; 95% CI –1.09–1.63; n = 13) and 7 days (Cohen's d = 0.04; 95% CI –0.90–1.04; n = 15) after the intervention, as well as a medium effect size 2 days (Cohen's d = 0.64; 95% CI 0.06–1.23; n = 14) after the intervention (see **Table 4**). Overall, these effect sizes suggest that ayahuasca leads to decreases in suicidality that are sustained from 1 to 7 days after administration. For between and within-group effect sizes for changes in non-suicide-related depressive symptoms (MADRS-total$_{nonSI}$), see **Tables 3** and **4**, respectively.

Association Between Changes in Suicidality and Depressive Symptoms

Seven days after the intervention, within the ayahuasca group, the association between changes in changes in suicidality (MADRS-SI) and changes in non-suicide-related depressive symptoms (MADRS-total$_{nonSI}$) approached significance, r = .53, p = .053. Within the placebo group, the association between changes in suicidality (MADRS-SI) and changes in non-suicide-related depressive symptoms (MADRS-total$_{nonSI}$) was not significant, r = .16, p = .579.

TABLE 3 | Between-group effect sizes (Cohen's d) for suicidality (MADRS-SI) and non-suicide-related depressive symptoms (MADRS-total$_{nonSI}$)

	Day 1	Day 2	Day 7
MADRS-SI			
Cohen's *d*	0.58	0.56	0.67
95% CI	–1.32–0.17	–1.30–0.18	–1.42–0.08
MADRS-total$_{nonSI}$			
Cohen's *d*	0.59	0.65	1.55
95% CI	–1.33–0.16	–1.40–0.10	–2.38–0.72

DISCUSSION

Given the limitations surrounding current interventions, there is an urgent need for innovative interventions for suicidality (Research Prioritization Task Force, 2014). To date, experimental research had not yet directly explored the impact of ayahuasca on suicidality. Therefore, this study aimed to fill this gap by being the first to explore the impact of ayahuasca on suicidality.

We hypothesized that ayahuasca would lead to decreases in suicidality that are sustained (i.e., from 1 to 7 days after the intervention). Our results are mixed. Although across groups there was a significant decrease in suicidality over time, the effect for the treatment group (i.e., ayahuasca vs. placebo) trended toward but did not reach significance. There are a number of potential explanations for these results. First, compared with placebo, ayahuasca may not lead to significant decreases in suicidality. Given that research has not yet explored the direct impact of ayahuasca on suicidality, this is a plausible explanation. Alternatively, given that large effects may not be detected by statistical tests, especially within small sample sizes (Greenland et al., 2016), our study was likely underpowered to detect the impact of ayahuasca (compared with placebo) on suicidality. Therefore, consideration of effect sizes is essential for the interpretation of our findings. In support of this possibility, we found medium between-group effect sizes for decreases in suicidality at all time points. Furthermore, within the ayahuasca group, we found large effect sizes for decreases in suicidality at all time points. These findings are in line with past research on the impact of psilocybin on suicidality (Carhart-Harris et al., 2018), as well as cross-sectional (Hendricks et al., 2015) and longitudinal (Argento et al., 2017) research indicating that lifetime use of psychedelics is associated with reduced levels of suicidality and decreased risk of becoming suicidal. Furthermore, these results are in line with

TABLE 4 | Mean scores and within-group effect sizes (Cohen's d) for suicidality (MADRS-SI) and non-suicide-related depressive symptoms (MADRS-total$_{nonSI}$).

	Baseline		Day 1		Day 2		Day 7	
	Aya	Pla	Aya	Pla	Aya	Pla	Aya	Pla
n	14	15	14	13	13	14	14	15
MADRS-SI								
Mean	3.36	1.40	1.14	1.00	0.69	0.57	1.00	1.33
SD	1.65	1.68	1.41	1.35	1.32	0.94	1.66	1.63
Cohen's d	–	–	1.33	0.00	1.42	0.64	1.19	0.04
95% CI	–	–	1.25–3.18	–1.09–1.63	1.50–3.74	0.06–1.23	1.21–3.50	–0.90–1.04
MADRS-total$_{nonSI}$								
Mean	32.79	28.73	15.57	15.00	13.54	13.86	14.64	22.20
SD	4.93	5.08	12.68	11.16	10.07	10.04	8.64	9.87
Cohen's d	–	–	1.46	1.61	2.17	1.57	2.20	0.91
95% CI	–	–	10.42–24.02	8.06–17.78	14.03–24.89	8.94–19.35	13.38–22.92	2.55–0.51

Aya, ayahuasca; Pla, placebo.

past research indicating that the administration of ayahuasca is associated with improvement in mental health concerns associated with suicidality (e.g., depression and hopelessness; Santos et al., 2007; Palhano-Fontes et al., 2019). Interestingly, 7 days after the intervention, between and within-group effect sizes for decreases in non-suicide-related depressive symptoms were larger than those found for suicidality, which may suggest that ayahuasca has a greater impact on non-suicide-related depressive symptoms than suicidality. Alternatively, these results may be due to a floor effect as a result of low levels of baseline suicidality. Nonetheless, overall, these results suggest that the therapeutic benefits of ayahuasca may extend to suicidality and that investigation of the impact on ayahuasca on suicidality using a larger sample is warranted.

These findings are also important as they indicate that ayahuasca may have a fast-acting impact on suicidality (i.e., as soon as 1 day after the intervention). Given that the time between the emergence of suicidality and suicide can be very short (Deisenhammer et al., 2009), there is a need for fast-acting interventions for suicidality. Currently, recommended interventions for suicidality are limited by the duration of time they take to be effective. For instance, individuals with MDD that are treated with antidepressants remain at high risk of suicide for at least 10–14 days after treatment begins (Jick et al., 2004; Simon et al., 2006). Furthermore, compared with nonsuicidal individuals, individuals with moderate to high levels of suicidality show slower responses to antidepressants (Baldessarini et al., 2006). Similarly, among individuals receiving ECT three times a week, suicidality often persists for 1–2 weeks after intervention (Kellner et al., 2005). Similar to the fast-acting effects of ketamine on suicidality (Bartoli et al., 2017), ayahuasca may also show promise as a fast-acting intervention for suicidality.

We found similar medium between-group and large within-group effect sizes for decreases in suicidality at all time points, with the largest between-group effect size 7 days after the intervention. These results suggest that the impact of ayahuasca on suicidality may last beyond the acute and post-acute effects of ayahuasca. These findings are in line with past research indicating that ayahuasca (e.g., Sampedro et al., 2017; Sanches et al., 2016; Palhano-Fontes et al., 2019); and psilocybin (e.g., Griffiths et al., 2016; Ross et al., 2016; Carhart-Harris et al., 2018) lead to mental health improvements that last beyond their acute effects. These results are especially important in light of the need for ongoing treatment in interventions for suicidality. For instance, ECT (Tanney, 1986; Prudic and Sackeim, 1999; Kellner et al., 2005) and traditional interventions for suicidality require ongoing administration in order to maintain their antisuicidal effects (Valuck et al., 2009). Similarly, research suggests that ketamine also requires repeated administration in order to maintain its efficacy (Zalsman et al., 2016; Dadiomov and Lee, 2019), which is problematic given the potential for cognitive impairment and abuse with repeated administration of ketamine (Schak et al., 2016; Strong and Kabbaj, 2018). Importantly, ayahuasca is associated with a low abuse and dependence potential (Hamill et al., 2019). Therefore, these findings suggest that ayahuasca may show promise as an intervention for suicidality that does not require repeated administration. Additional research with longer-term follow-up will be necessary to determine the long-term impact of ayahuasca on suicidality.

Interestingly, within the ayahuasca group, the relationship between changes in suicidality and changes in non-suicide-related depressive symptoms approached significance, with a large effect size (i.e., $r = .53$). These findings suggest that the impact of ayahuasca on suicidality may, in part, be due to its impact on non-suicide-related depressive symptoms or mechanisms overlapping both non-suicide-related depressive symptoms and suicidality. Research suggests that suicide functions as a means of escaping intense emotional distress (Baumeister, 1990; Shneidman, 1998). Extant research indicates that psychedelics in general, and ayahuasca in particular, leads to decreases in emotional distress (for a review, see dos Santos et al., 2018). Similarly, a recent study found that the administration of ayahuasca led to decreases in emotion dysregulation, within a community sample and among individuals with BPD traits (Domínguez-Clavé et al., 2019). Similarly, among males in a community sample, lifetime use of psychedelics was associated with lower levels of emotion dysregulation (Thiessen et al., 2018). One particular means through which ayahuasca may decrease emotion dysregulation is *via* increased mindfulness-related capacities (e.g., acceptance and decentering), which have been shown to increase after administration of ayahuasca (Thomas et al., 2013; Soler et al., 2016; Sampedro et al., 2017; Domínguez-Clavé et al., 2019; Soler et al., 2018; Uthaug et al., 2018). Neurobiological research similarly suggests that ayahuasca may impact suicidality *via* decreases in emotion dysregulation. For instance, among individuals with MDD, a single dose of ayahuasca led to increased blood flow in regions of the brain associated with emotion regulation (e.g., left nucleus accumbens, right insula and left subgenual area; Sanches et al., 2016). Furthermore, research has found that administering psychedelics to rats promotes neuroplasticity (Ly et al., 2018), and markers of neuroplasticity (Nichols and Sanders-Bush, 2002), within the prefrontal cortex, a region of the brain implicated in emotion dysregulation (Vollenweider and Kometer, 2010) and suicidality (Ding et al., 2015). Therefore, the impact of ayahuasca on suicidality may be accounted for by its impact on psychological and neurobiological mechanisms associated with emotion dysregulation. Additional research is necessary in order to understand the mechanisms that account for the impact of ayahuasca on suicidality.

Ayahuasca and Borderline Personality Disorder

One psychiatric disorder that ayahuasca may show promise as an intervention for is BPD, a severe psychiatric disorder associated with especially high rates of suicide (i.e., 3%–10%; Links 2009) and suicide attempts (i.e., 60%–78%; Links 2009). Importantly, there is limited evidence for the efficacy of treating BPD with pharmacological agents and a pressing need for innovative pharmacological interventions for BPD (Chanen, 2015; Starcevic and Janca, 2018). Interestingly, among individuals with BPD traits, a recent study found that the administration of ayahuasca led to decreases in components of emotion dysregulation (Domínguez-Clavé et al., 2019), which is considered the core

dysfunction in BPD (Linehan, 1993; Carpenter and Trull, 2013). However, they did not include a sample of individuals that met diagnostic criteria for BPD and the impact of ayahuasca on suicidality was not assessed. The present study was the first clinical trial with psychedelics to report including individuals with BPD. It is noteworthy that no serious adverse events occurred among individuals with BPD. Furthermore, while all five individuals with BPD that received ayahuasca showed clinically significant suicidality at baseline (i.e., MADRS-SI ≥ 4), none (i.e., 0%) reported clinically significant suicidality 1 and 2 days after administration and only 1 (i.e., 20%) reported clinically significant suicidality 7 days after administration (see **Table 2**). Given the limited number of individuals with BPD included in the sample, these results must be interpreted with caution. However, given the pressing need for innovative pharmacological interventions for BPD, the wide-ranging mental health concerns for which psychedelics have shown promise, and our results related to the impact of ayahuasca on suicidality, additional research exploring the safety, tolerability, and clinical utility of ayahuasca as an intervention for BPD may be warranted.

Strengths, Limitations, and Future Direction

This study includes a number of strengths and limitations that are important to consider. The study utilized a double-blind randomized placebo-controlled design, a gold-standard for suicide research (Zalsman et al., 2016). Furthermore, in order to increase blinding, the study only included individuals without past experience with psychedelics and used an active placebo designed to imitate characteristics of ayahuasca. Additionally, the sample used in the present study showed severe psychiatric comorbidity, with the majority of the sample (76%) meeting criteria for a comorbid DSM-IV axis II disorder. Furthermore, all individuals had treatment-resistant MDD and some had failed to respond to as many as 10 interventions. The impact of ayahuasca on suicidality among individuals with such high rates of non-responsiveness to conventional interventions is especially promising.

The primary limitation surrounding the present study is that, despite the use of randomization, suicidality among those in the placebo group was low. Therefore, although, we controlled for baseline differences in suicidality, the possibility that our results may be influenced by the placebo effect or regression to the mean, cannot be ruled out. Nonetheless, given the large within-group effects we found, we suggest that additional placebo-controlled research on the impact of ayahuasca on suicidality will be important. Relatedly, our study is limited by the use of a small sample size, which limits the extent to which inferential statistics are able to identify significant changes. Furthermore, due to the small sample size, potentially important covariates (e.g., use of benzodiazepines) were not included in our analyses. Future research would benefit from studies with larger samples that are better powered to detect the impact of ayahuasca on suicidality. Second, due to safety concerns, the study did not include individuals with imminent suicide risk. Accordingly, it is difficult to determine from the present study whether ayahuasca would lead to reductions in suicidality among individuals with higher levels of suicidality. Past research on pharmacological interventions for suicidality has employed similar exclusion criteria (e.g., Price et al., 2009) and the suicidality exclusion criteria employed in this study were less strict than past studies on pharmacological interventions for suicidality (e.g., MADRS-SI > 4; Ballard et al., 2015). Future research would benefit from exploring the impact of ayahuasca on individuals with higher levels of suicidality. Third, we did not conduct a qualitative analysis of participants descriptions of *why* ayahuasca impacted suicidality. Future research would benefit from using a mixed-methods approach, as well as analysis of potential mediators of the impact of ayahuasca on suicidality. Finally, similar to all research on psychedelics, due to the psychological experience induced by ayahuasca, ensuring that participants are blind to treatment condition is difficult (Barnby and Mehta, 2018). In order to improve blinding procedures in psychedelic research, future research should continue to develop increasingly convincing placebos.

This study was the first to explore the impact of ayahuasca on suicidality and our findings suggest that ayahuasca may show promise as a fast-acting and innovative intervention for suicidality. Given important limitations of our study (e.g., small sample size, low levels of baseline suicidality), additional research will be necessary in order to determine the long-term impact of ayahuasca on suicidality, as well as the safety, tolerability, and clinical outcomes associated with administration of ayahuasca among highly suicidal individuals.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Onofre Lopes University Hospital (HUOL), Natal-RN, Brazil, University Hospital Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FP-F, JH, EN, JM-O, and DA contributed to study design and conception. FP-F and DA coordinated data acquisition. RZ, FP-F, and DA analyzed data and interpreted the results. RZ was responsible for the first draft of the manuscript. All authors read, critically revised, and approved the manuscript.

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REVIEW
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The Bridge Between Classical and "Synthetic"/Chemical Psychoses: Towards a Clinical, Psychopathological, and Therapeutic Perspective

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The critical spread and dissemination of novel psychoactive substances (NPS), particularly among the most vulnerable youngsters, may pose a further concern about the psychotic trajectories related to the intake of new synthetic drugs. The psychopathological pattern of the "new psychoses" appears to be extremely different from the classical presentation. Therefore, clinicians need more data on these new synthetic psychoses and recommendations on how to manage them. The present mini-review aims at deepening both the clinical, psychopathological features of synthetic/chemical NPS-induced psychoses and their therapeutic strategies, according to the different NPS classes implicated, by underlining the main differences with the "classical" psychoses. A comprehensive review was conducted using the PubMed/Medline database by combining the search strategy of free-text terms and exploding a range of MESH headings relating to the topics of novel psychoactive substances and synthetic/chemical psychoses as follows: {(*Novel Psychoactive Substances*[Title/Abstract]) AND *Psychosis*[Title/Abstract])} and for each NPS categories as well, focusing on synthetic cannabinoids and cathinones, without time and/or language restrictions. Finally, an overview of the main clinical and psychopathological features between classical versus NPS-induced chemical/synthetic psychoses is provided for clinicians working with dual disorders and addiction psychiatry. Further insight is given here on therapeutic strategies and practical guidelines for managing patients affected with synthetic/chemical NPS-induced psychoses.

Keywords: psychosis, synthetic psychosis, chemical psychosis, novel psychoactive substances, NPS

INTRODUCTION

During the last decade, the "traditional" drug panorama has been gradually "reshaped" and integrated, even though not totally replaced, by the appearance of "new/novel psychoactive substances" (NPS) which are either newly created or existing substances which are now being used in "novel" modalities (1–3). The clinical, toxicological, and psychopathological effects of NPS have not been completely

investigated and discovered through modelling; hence, clinical and psychopathological/psychiatric concerns have arisen among clinicians/professionals working in dual diagnosis, drug addiction, and mental health area (1, 2, 4–6). The recent published report from the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) described more than 730 NPS notified to the agency by the end of 2018, with 55 new compounds reported for the first time in Europe only in 2018 (3). Of these, 51% were synthetic cannabinoids (SC), 24% synthetic cathinones/"bath salts," 5% benzodiazepines, 2% synthetic opioids, and 18% other substances (i.e., tryptamines, phenethylamines, arylcyclohexylamines, psychostimulants, etc.) (3).

SC/"Spice drugs," mainly marketed as nail polish remover, deodorizers, incense, and potpourri, labeled as "natural"/"legal" alternatives to cannabis, have been documented to be dramatically associated with extremely more severe adverse health effects compared to "classical" cannabis (1, 7). SC are mainly consumed by inhalation, typically smoked together with a dried herbal material onto which they are previously sprayed during the production phase with chemical additives, or by e-cigarettes, or sold in tablets, etc. (1, 7). SC are mainly sold and purchased in "smart-shops" and on online drug marketplaces (7). Among these adverse effects typically described are: cases of fatalities, cardiac dysrhythmias, seizures, liver toxicity, kidney failure, hypothermia, hypertension, myocardial infarction, cardiac arrest, acute tubular necrosis, interstitial nephritis, rhabdomyolysis, nausea, vomiting, cognitive deficits, memory loss, sedation, catatonia, agitation, irritability, sympathomimetic syndrome, subarachnoid hemorrhage, cerebrovascular accidents, hematuria, bloody noses, bleeding gums, internal hemorrhage, hemoptysis, panic attacks, anxiety, altered mental status, coma, and a peculiar "synthetic" psychosis, designated "Spiceophrenia" (1, 7). Moreover, animal studies demonstrated that the administration of SC to adolescent rodents or non-human primates may determine the onset of a schizophrenia-like phenotype in adulthood (8–11).

Being beta-keto-phenethylamines, synthetic cathinones (i.e., ethylone and methylone) are structurally similar to amphetamines [i.e., 3,4-methyl-enedioxy-methamphetamine (MDMA), methamphetamine, etc.] and catecholamines, with subtle differences which modify their chemical, pharmacokinetic, and pharmacodynamic properties (1). Some cathinones are analogues of pyrovalerone, e.g., 3,4-methylenedioxypyrovalerone (MDPV), naphyrone, 3, 4-methylenedioxy-α-pyrrolidinobutiophenone (MDPBP), and α-pyrrolidinovalerophenone (α-PVP) (12). Different synthetic cathinones may exert different effects and potency levels on the dopaminergic, noradrenergic, and serotoninergic pathways, even though all usually own sympathomimetic and/or amphetamine-like effects (1). They are typically sold as pills, capsules, and powders, commonly insufflated (snorted/sniffed), orally consumed by "bombing" (swallowing the powder wrapped in a cigarette paper), mixed in a drink, or injected intravenously (13). Synthetic cathinones are generally known as "bath salts" in the USA and as "plant food" in Europe (1). Several motivations have been identified in their consumption and "appeal"/attractiveness among NPS and drug consumers, such as attaining feelings of euphoria/stimulation, increased energy, mood improvement, increasing empathy/openness, reaching a more mental clarity, experiencing vivid hallucinations, and increasing libido. Synthetic cathinone users seem to frequently report hyperthermia, rhabdomyolysis, renal failure, seizures, as well cardiac, psychiatric, and other neurological signs, in addition to the onset of several levels of agitation, ranging from mild agitation to severe psychosis (14). Moreover, mood instability and paranoid ideation have been described among chronic users (15–17). Furthermore, most synthetic cathinone consumers described tolerance, dependence, and withdrawal syndrome (1).

The class of synthetic hallucinogens/psychedelics includes all substances able to alter consciousness (i.e., synthetic lysergamides, tryptamines, and phenethylamines) by distorting time/motion/color/sound perceptions of consumers and/or altering the perception of "self" and/or stimulating some sensory/perceptual disturbances (1, 18, 19). Hallucinogens are usually consumed orally, occasionally through small blotter paper portions (i.e., "tabs") held in the mouth to allow absorption through the oral mucosa. Moreover, it has been reported as route of administration by insufflation, smoking, rectal, and injection (intravenous and intramuscular) (1, 18, 19). The route of administration may influence the effects, their onset, and duration (1, 18, 19). Hypertension, tachycardia, hyperthermia, dizziness, sleeplessness, loss of appetite, xerostomia, sweating, impulsiveness, fast and labile emotional modifications (from fear, anxiety to euphoria), numbness, weakness, and tremors have been reported as frequent short-term effects. While long-term effects may comprise persistent psychosis (i.e., visual disturbances, disorganized thinking, and paranoia), mood fluctuations, and/or onset of a hallucinogen-persisting perception disorder (HPPD) (1, 5, 19).

Synthetic/novel psychostimulants comprise several NPS classes, i.e., piperazines (compounds labeled as "legal" alternatives to ecstasy, mainly identified as cutting agents in some ecstasy pills) and "amphetamine-type stimulants" (ATS) (1, 3). ATS, e.g., PMA (4-methoxyamphetamine/"Dr. Death"), PMMA (4-methoxymeth-amphetamine), 4-MTA (4-methylthioampheta mine/"flatliners"), DMA (2,5-dimethoxyamphetamine), aminorex derivatives (4,4″-DMAR); diclofensine, methiopropamine, etc., have been marketed as para-substituted methoxy drugs pharmacologically and clinically comparable to amphetamines (1, 3, 18). ATS are commonly supplied as tablets/crystals/powder, sometimes mixed with other substances (20). They are usually swallowed/smoked/snorted, and their common "street names" include "crystal meth" (aka "ice")/"speed"/"crystal," etc. (20). Obsessive delusions, paranoia, hallucinations, suicidal ideation, anxiety, insomnia, depression, and psychotic symptoms have been commonly reported following intake of ATS (20, 21).

Overall, NPS have been largely associated with several clinical and psychiatric symptomatology which may vary from the occurrence of an acute transient psychotic episode to more complex psychopathological patterns, depending on the substance implicated (and its pharmacological profile), the frequency, intensity and route of administration, and vulnerability and individual characteristics of NPS consumers (1, 4–6, 22, 24) (as shown in **Table 1**). The psychopathological pattern of NPS-induced "new/synthetic psychoses" appears to be extremely different from the classical presentation, by introducing a new interesting field of research from a

psychopathological and phenomenological point of view. More specifically, NPS mainly implicated in the onset of psychotic patterns are represented by SC, synthetic cathinones, psychostimulants, and some psychedelics/hallucinogenic NPS (1, 6, 19). However, as synthetic cannabinoids and cathinones are the most representative NPS groups, the present review mainly focused on these two categories in collecting, analyzing, and critically discussing data so far published about NPS-induced synthetic/chemical psychoses by underlining differences with "classical" psychosis.

TABLE 1 | Pharmacological and clinical features of the main NPS classes.

Type of NPS category	Description	Neurotransmitter/pathways/receptors implicated	Clinical manifestations
Psychostimulants	This category includes all compounds derived from psychostimulants (i.e., amphetamine, methamphetamine, MDMA, and cocaine). Some synthetic psychostimulants include the biochemical family of synthetic cathinones (see below) and novel stimulants, aminorex derivatives, ATS such as diclofensine, methiopropamine, 4,4′-DMAR, NBOMe-series, 2C-series, etc.	Traditionally, psychostimulants primarily target monoaminergic systems, leading to increased extracellular levels of SER, DA, and/or NA.	*Desired effects*: • Euphoria • Social disinhibition • Extroversion • Increased energy • Appetite suppressant • "High" *Untoward effects*: • Serotoninergic syndrome • Psychosis • Paranoid ideation • Impulsivity • Mania • Agitation • Cardiovascular symptoms • Hyperthermia
Synthetic cathinones ("bath salts")	This category includes analogues of naturally occurring cathinones such as *Catha edulis* (Khat). Synthetic cathinones are classified into: a) Substrates of DAT, SERT, and NAT (pharmacological profile similar to MDMA: i.e., butylone, ethylone, 4-MEC, etc.) b) Selective substrates of DAT (pharmacological profile similar to amphetamine and methamphetamine, i.e., methcathinone, flephedrone, etc.) c) Non-substrates of transporter inhibitors (i.e., MDPV, etc.) Some synthetic cathinones include mephedrone ("meow meow"), methedrone, methylone, 3-MMC, 2-MeOMC, 4-MeO-a-PVP, 4-MeO-PBP, 4-MeO-PV9, 4-MPD, 4F-PV8, 4F-PV9, 4F-PVP, a-PBT, a-PHP, a-PVT, dibutylone, DL-4662, Ethylone, MDPPP, MOPPP, NEB, Pentedrone, PV-8 (crystals), etc.	The mechanism by which synthetic cathinones exhibit an effect is similar to other stimulants through functionally changing monoamine transporters. They inhibit monoamine transporters, leading to increased extracellular levels of SER, DA, and NA. They exhibit a sympathomimetic activity.	*Desired effects*: • Euphoria • Hypervigilance • Increased energy • Openness • Empathy • Increased libido • Appetite suppression *Untoward effects*: Excited delirium Hallucinations Agitation Aggressiveness Paranoid ideation Exacerbation of mood disorders
Synthetic cannabinoids (synthetic cannabimimetics, synthetic cannabinoid receptor agonists, "Spice drugs," "legal cannabis alternative")	A wide range of brands of herbal products containing synthetic cannabinoids have been available on the market. They include, but are not limited to, Spice, Black Mamba, Annihilation, and Amsterdam Gold. New brands continue to emerge. Brand names are sometimes reminiscent of street names of strains of cannabis. Synthetic cannabinoids may be classified into seven major structural groups: • naphthoyndoles (e.g. JWH-018, JWH-073, etc.); • naphthylmethylindoles; • naphthoylpyrroles; • naphthylmethylindenes phenylacetylindoles (i.e. benzoylindoles, e.g. JWH-250); • cyclohexylphenols (e.g. CP 47,497 • homologues of CP 47,497)\ • classical cannabinoids (e.g. HU-210).	They act on the endocannabinoid system, particularly on: • CB_1 (total agonism) • CB_2 (total agonism)	*Desired effects*: • Relaxation • Analgesia • "High" • Sedation • Euphoria • Anxiolysis

(Continued)

TABLE 1 | Continued

Type of NPS category	Description	Neurotransmitter/pathways/receptors implicated	Clinical manifestations
			Untoward effects: • Paranoid ideation • Brief psychotic episode • Persistent psychotic disorder/"Spiceophrenia" • Cognitive impairment • Anxiety/agitation • Dyscontrol of impulses • Dysphoria • Manic symptoms • Worsening of preexisting bipolar disorder and/or psychosis and/or schizophrenia • Seizures • Tachycardia, hypertension, mydriasis, hyperglycemia, hypokalemia, dyspnea, tachypnea, nausea, vomiting • Stroke, encephalopathy, renal failure • Serotoninergic syndrome
Dissociatives	The compounds included in this category produce dissociative effects similar to phencyclidine (PCP or "angel dust"), ketamine ("Special K" or "K" or "Ket"), methoxetamine etc. Some of NPS included in this category comprise 3-MeO-PCP, ethylketamine, 3-HO-PCP, 4-MeO-PCP, Diphenidine, Methoxphenidine, etc.	Dissociative drugs act mainly as uncompetitive antagonists, through open channel blockade, of the glutamate ionotropic NMDA receptor. Dissociatives affect numerous other receptors (i.e., DA, opioid, 5-HT, adrenergic, nicotinic, muscarinic, and adenosine receptors) and ionic channels, leading to their occasional informal description of being neurochemically "dirty drugs."	*Desired effects*: • Dissociation/depersonalization (at high dosages) • Analgesia • "High" (at low dosages) • Euphoria (at low dosages) • "Out-of-body" or "near-death" experiences • Weightlessness *Untoward effects*: • Headache • Psychosis • Reference ideation • Hallucinations • Flashbacks • Nausea • Dizziness • Paranoia • Anxiety • Cognitive impairment • Serotonergic syndrome (possible)
Hallucinogens (psychedelics/ psychotomimetics/ entheogens)	A category of substances developed as analogues of "classical" hallucinogens (i.e., LSD, psilocybin). They can be further divided into three subgroups: • Phenethylamines (e.g., mescaline, Bromo-DragonFLY, etc.) • Tryptamines (e.g., psilocybin, 5-MeO-DALT, etc.) • Lysergamines (e.g., LSD, AL-LAD, etc.) Novel tryptamines recently marketed include 5-MeO-DMT, 5-MeO-DIPT, 4-HO-DALT, 5-MeO-AMT, DET, etc. Among the novel derivatives of "classical" psychedelic phenethylamines/MDMA-like drugs, we include the following: 2C-molecules (e.g., 2-CB/Nexus, 2C-I, 2C-E, etc.), PMA/Dr Death, Bromodragonfly/B-fly, 25C-NBOMe/N-bomb/Pandora, etc. Some hallucinogens also include herbs/plants such as *Salvia divinorum*, Mescaline, or other herb-related compounds previously used in traditional shamanic practices.	All three subgroups share a common mechanism on serotonergic system, represented by the agonism/partial agonism at the 5-HT_{2A} receptor (activation), 5-HT_{1A}, 5-HT_{2C}. However, the different hallucinogenic/psychedelic drugs may differently interact with other neurotransmitters, i.e., NMDA receptors, σ-receptors, μ-opioid receptors, and muscarinic receptors, apart from causing serotonin and dopamine reuptake inhibition at their transporters.	*Desired effects*: • Perceptual restructuralization • Oceanic boundlessness • Visual hallucinations • Alterations in sensory perception • Distortion in body image • Depersonalization • Entactogenic feelings • Euphoria

(Continued)

TABLE 1 | Continued

Type of NPS category	Description	Neurotransmitter/pathways/receptors implicated	Clinical manifestations
			Untoward effects: • Anxiety • Agitation • Psychosis • Hallucinations • Serotonergic syndrome (possible)
Sedatives/analgesics	This category includes the synthetic/derivatives opioids (i.e., AH-7921, IC-26, MT-45, nortilidine, W15, W18, U-47700, lefetamine, etc.) and designer/synthetic benzodiazepines (i.e., etizolam, flubromazepam, flubromazolam, phenazepam, pyrazolam).	They may act on opioid system and/or GABA system.	*Desired effects*: • Analgesia • Sedation • Emotional *Untoward effects*: • Lack of consciousness • Somnolence/sedation • Respiratory depression • Coma and death

ATS, amphetamine-type stimulants; SER, serotonin; DA, dopamine; NA, noradrenalin; SERT, serotoninergic transporter; DAT, dopamine transporter; NAT, noradrenergic transporter; NMDA, N-methyl-D-aspartate.

MATERIAL AND METHODS

Search Sources and Strategies

A mini-review was conducted by analyzing and collecting clinical data (only human studies) on cases of NPS-induced psychosis, by using PubMed/Medline and focusing on synthetic cannabinoids and cathinones. We combined the search strategy of free-text terms and exploded MESH headings for the topics of "Novel Psychoactive Substances" and "Synthetic/Chemical Psychoses" as follows: {(*Novel Psychoactive Substances*[Title/Abstract]) AND *Psychosis*[Title/Abstract])} and for each NPS category (synthetic cannabinoids and synthetic cathinones) as well, without time/language restrictions. All studies published up to 30 June 2019 were included. In addition, secondary searches were performed using the reference listing of all eligible as well as relevant articles and consultation with experts in the field and or manual searches.

Study Selection, Data Extraction, and Management

We considered studies evaluating the relationships between NPS and synthetic psychosis. We examined all titles and abstracts and obtained full texts of potentially relevant papers. Working independently and in duplicate, two reviewers (LO and SC) read the papers and determined whether they met inclusion criteria. Duplicate publications were excluded. All experimental and observational study designs, including case reports, case series and surveys, were retrieved. 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 published in English; c) human studies; and d) provide data on synthetic psychosis induced by NPS. LO and SC independently extracted the data on participant characteristics, intervention details, and outcome measures. Disagreements were resolved by discussion and consensus with a third member of the team (GDP). Data were collected using an *ad hoc* developed data extraction spreadsheet.

Characteristics of Included Studies

The set of keywords initially generated 402 results. A total of 123 papers were excluded because of duplication; seven papers were excluded through lack of an english abstract. Of the remaining 272 studies, 145 studies were excluded because they did not meet the inclusion criteria or because they were animal studies. Of the remaining 127 papers, 37 papers were excluded because they were reviews, letters to editors, or meta-analyses, while seven 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 83 papers were included and accounted for in our analysis. **Table 2** clearly explains the main characteristics (study design, sample size, main outcomes, and findings) of all studies retrieved.

RESULTS

Data on NPS-Induced Psychosis in General

A cross-sectional survey carried out as a retrospective review of electronic discharge letters of all patients discharged from general adult psychiatric wards in the Royal Edinburgh Hospital recruited 483 admissions, 86 of which had NPS involvement reported. Among NPS users, the diagnosis of drug-induced psychosis was significantly higher ($p < .001$, OR = 18.7) compared to non-NPS users (25). The European Drug Emergencies Network (Euro-DEN) collected data on presentations to emergency department (ED) with acute recreational drug and/or NPS intoxication in 16 sentinel centers in 10 European countries, reporting psychosis

TABLE 2 | Summary of included studies.

Reference	Study design (country)	Sample features (size, age, ethnicity, sex)	Psychiatric history	NPS involved	Symptomatology	Previous substance use history	Treatment [drug name, dosage, duration (if available)]
Studies on NPS-induced psychosis in general (unspecified or multiple NPS involved)							
Stanley et al. (25)	Cross-sectional survey (UK)	86/388, 36.1 ± 9.4; NA, 60 M and 26 F	Personal psychiatric history of schizophrenia, schizoaffective disorder, and personality disorders	NPS unspecified	Psychosis	NPS users present significantly higher levels of cannabis, alcohol, and substitute opiate use	General adult psychiatric ward (NA, NA, NA)
Vallersnes et al. (26)	Retrospective cohort study—European Drug Emergencies Network (Euro-DEN, several countries)	5,529, 24-38, NA, 79.3% M	NA	MDPV (27.3%), tryptamines (57.1%), methylphenidate (23.1%), SC (15.4%), mephedrone (5.7%), methedrone (3.3%)	Psychosis, significantly associated with agitation (63.2%; $p < .001$); hallucinations (43.7%, $p < .001$) and anxiety (37.1%, $p < .001$)	NA	ED (NA, NA, NA)
Liakoni et al. (27)	Retrospective cohort study (Switzerland)	157,328, 16–74, NA, 68% M	NA	2 cases with NPS (2C-P and methylone)	Psychosis	NA	ED (NA, NA, NA)
Studies on synthetic cannabinoid-induced psychosis							
Every-Palmer (28)	Case report (New Zealand)	NA, NA, NA, NA	Personal psychiatric history of psychotic disorder ($n = 5$)	"Aroma" blend, containing CP-47,497 and/or JWH-018 (SC)	Mental states deteriorated significantly after use including sudden re-emergence of florid psychosis, predominantly agitation, disorganization, and delusional beliefs ($n = 5$)	NA	NA
Müller et al. (29)	Case report (Germany)	1, 25, NA, M	Personal psychiatric history of cannabis-induced recurrent psychotic episodes associated with a strong family history of paranoid schizophrenia	Spice (SC)	Anxiety, psychotic symptoms with feelings of manipulation, worsening of symptomatology (imperative voices and paranoid hallucinations)	Cannabis	NA
Rodgman and Kinzie (30)	Case report (USA)	3, NA, NA, NA	NA	"Mojo" (SC)	Acute psychosis	NA	Beta-blockers (NA), low dose of BDZs (NA)
Sobolevsky et al. (31)	Case report (Russia)	3, 22 ± 1, NA, 2 M and 1 F	NA	Tropical Synergy (SC) containing JWH-018, CP 49,497	Anxiety, tachycardia, paranoia, hallucinations, short-term memory, and sense of time impaired	NA	NA
Vearrier and Osterhoudt (32)	Case report (USA)	1, 17, NA, F	None	JWH-018 (SC)	Violent and "crazy" visual hallucinations, lower extremities numbness, muscle twitches, elevated pulse, dilated pupils, anxiety	Occasionally abuse alcohol and cannabis	ED, LOR (2 mg ev, once)
Benford and Caplan (33)	Case report (USA)	1, 20, NA, M	None	Spice (SC)	Severe anxiety and paranoia, auditory and visual hallucinations, halted speech	Cannabis	ED

(Continued)

TABLE 2 | Continued

Reference	Study design (country)	Sample features (size, age, ethnicity, sex)	Psychiatric history	NPS involved	Symptomatology	Previous substance use history	Treatment [drug name, dosage, duration (if available)]
Castellanos et al. (34)	Case series (USA)	11, 15–19, NA, 10 M and 1 F	Personal psychiatric history of ADHD (*n* = 3); ADHD + learning disorder + MDD (*n* = 1); none (*n* = 7)	SC	Euphoria, irritability, anxiety, numbness, memory impairment, auditory and visual hallucinations, paranoia, palpitations, muscle tremors, weakness, blackouts, restlessness, stimulation	Cannabis and alcohol use (*n* = 10)	NA
Every-Palmer (35)	Case series (New Zealand)	9/15, 18–65, NA, M	Personal psychiatric history of schizophrenia (*n* = 10), schizoaffective disorder (*n* = 4), bipolar disorder with psychotic features (*n* = 1)	JWH-018 (SC)	Excited psychotic symptoms, relapse of psychosis (agitation, delusions and disorganization after SC intake), anxiety, paranoia	Cannabis	NA
Forrester et al. (36)	Retrospective cohort study (USA)—Texas Poison Center Network (2010)	464, 12–67, NA, 343 M and 118 F	NA	SC	Agitation (18.5%), hallucinations (10.8%), drowsiness (18.5%), vomiting, nausea, tachycardia	Alcohol, cocaine, acetaminophen, hydrocodone, chlorpeniramin, dextromethorphan, carisoprodol	Hospitalization, ev fluids, BDZs, oxygen, antiemetics, AC (NA)
Hurst et al. (37)	Case series (USA)	10, 21–25, NA, M	None	SC	Auditory hallucinations, paranoid delusions, blocked thoughts, disorganized speech and behavior, suicidal ideation, alogia, ideation, insomnia, flatted affect, psychomotor retardation and/or agitation, anxiety	Concurrent cannabis or alcohol use (*n* = 8)	Hospitalizations (*n* = 7), not specified AP (*n* = 10) (NA)
Johnson et al. (2011)	Case report (USA)	1, 23, Caucasian, M	None	Spice (SC) smoked 96 and 48 h before admission	Paranoia, disorganized thoughts, nonsensical speech, delusion	Earlier cannabis use over 3 years ago	Spontaneous remission after 24 h
Locatelli et al. (39)	Retrospective cohort study (Italy)—Pavia Poison Centre and National Early Warning System for Drugs	17, 14–55, Caucasian, NA	NA	"Spice" (*n* = 1), "N-Joy" (*n* = 6), "Forest Green" (*n* = 3), "Jungle Mystic Incense" (*n* = 5), JWH (*n* = 2), all SC	Agitation, confusion, hallucinations, dyspnea, coma, seizure, mydriasis, xerostomia, vertical nystagmus, psychomotor agitation, vomiting	NA	BDZs (NA), symptomatic treatment
McCain et al. (40)	Retrospective cohort study (USA)—Arkansas Poison and Drug Information Center (March–August 2010)	6/9, NA, NA, NA	NA	SC	Agitation/irritability (50%), hallucinations (33.3%), confusion (66.7%), nausea (33.3%), mydriasis (33.3%), pallor (33.3%), tachycardia (100%), hypokalemia (100%)	NA	BDZs (NA), supportive infusive therapy
Simmons et al. (41)	Case report (USA)	a) 1, 25, NA, M b) 1, 21, NA, M c) 1, 19, NA, M	None	"Spice" (SC) containing JWH-018, JWH-073	a) Seizure, tachycardia, acidosis, unresponsiveness to verbal stimuli, "eye-crossed and flailing arms", mydriasis b) Hypertension, unresponsiveness, agitation, c) Delusions, paranoia, short-term memory impairment, bizarre behavior, muscle spasm, depressed breathing	None	BDZs (LOR, 4 mg, NA), APs (HALO, 5 mg, NA), supportive infusive therapy and observation

(Continued)

TABLE 2 | Continued

Reference	Study design (country)	Sample features (size, age, ethnicity, sex)	Psychiatric history	NPS involved	Symptomatology	Previous substance use history	Treatment [drug name, dosage, duration (if available)]
Young et al. (42)	Case report (USA)	1, 17, NA, M	None	"K9" smoking (SC)	Hallucinations, dizziness, tachycardia, chest pressure that lasted for 3 days, difficulty breathing, lightheadedness	Occasional alcohol use	NA
McGuiness and Newell (43)	Case report (USA)	1, 18, NA, F	NA	SC	Paranoia, chest pain, hyperventilation, nausea, panic attack, ideas of reference	NA	NA
Tung et al. (44)	Case report (Hong Kong)	1, 36, Chinese, M	Family psychiatric history of SMI (unspecified) and SUD	"K2" (SC) daily for 4 weeks before admission	Agitation, profuse seating, tachycardia, delusion, elevated blood pressure, irritability, insomnia, restlessness, accelerated speech	Previous abuse history of multiple psychoactive substances (codeine, heroin, and other unspecified)	Hospitalization; spontaneous remission
Van der Veer and Friday (45)	Case report (USA)	3, 20–30, NA, M	Personal psychiatric history: none (n = 1), PTSD (n = 1), amphetamine-induced brief psychotic episodes (n = 1)	"Spice" (SC) 3–4 weeks before admission	Persistent psychosis after SC intake, disorganized speech, poverty of thought, loosening of associations, paranoia, delusion, aggressiveness, inappropriate affect, suicidality, "Capgras delusions"	Previous cannabis use	Hospitalization; HALO (n = 2, NA, NA), RIS (n = 1, NA, NA)
Bebarta et al. (46)	Case report (USA)	3, 19–23, NA, 2 M and 1 F	NA	"Spice" (n = 1) and "Space" (n = 2), all containing SC	Paranoia, hallucinations, agitation, tachycardia, hyperglycemia, mild drowsiness, short-term memory impairment, hyperglycemia, anxiety, agitation, breathing difficulty, hyperventilation, tachycardia, injected sclera	None	ED observation; LOR (2 mg, NA)
Cohen et al. (47)	Case report (USA)	3, 16–18, NA, 2 M and 1 F	NA	SC	Catatonia, tachycardia, vertical nystagmus, agitation, aggression restlessness, tachycardia, hyperventilation, disorientation, slowed speech	NA	NA
Hoyte et al. (48)	Retrospective Cohort study (USA)—National Poison Data System (January–October 2010)	1898, 20 (median), NA, 74.3% M	NA	"K2" and "Spice Gold", all containing SC	Hallucinations, delusions (9.4%)	NA	BDZs (n = 16%, NA, NA)
Oluwabusi et al. (49)	Case report (USA)	2, 16–17, Hispanic, M	None	"K2" and "Spice", all containing SC	New-onset psychosis, insomnia, hyperactivity, anxiety, paranoid and grandiose delusions, musical auditory hallucinations, colorful visual hallucinations, low mood, agitation, irritability, pressure of speech, flight of ideas, disorganized behavior, thought broadcasting, somatic preoccupation, mild cognitive impairment	NA	Hospitalization, ARI (n = 1, 20 mg daily, NA), OLA (n = 1, 15 mg daily, NA)

(Continued)

TABLE 2 | Continued

Reference	Study design (country)	Sample features (size, age, ethnicity, sex)	Psychiatric history	NPS involved	Symptomatology	Previous substance use history	Treatment [drug name, dosage, duration (if available)]
Peglow et al. (50)	Case report (USA)	1, 59, Latin, M	PTSD	Smoking two joints of "Spice" (SC) daily for the previous 3 weeks before admission	New-onset psychosis, visual hallucinations, disorganized and bizarre behavior, consciousness disorder, flashbacks of trauma	Alcohol, heroin, cocaine, cannabis discontinued 3 years before	Hospitalization, BZDs (NA, NA, NA), gabapentin (400 mg QID), hydroxyzine (25 mg TID), ARI (10 mg qD), benztropine (1 mg BID), BUP (150 mg BID)
Vandrey et al. (51)	Internet-based survey (13 different countries)	391, 26 ± 9, 90% Caucasian, 83% M	NA	"Spice" (SC)	Hallucinations (28%)	Alcohol (92%) Cannabis (84%)	NA
Barratt et al. (52)	Online questionnaire using purposive sampling strategy (Australia)	316, 23–34, NA, 77% M	NA	SC	Paranoia (18%) Psychosis (4%)	Cannabis (96%)	NA
Berry-Cabán etal. (53)	Case report (USA)	1, 20, Hispanic, M	None	"Spice" (SC)	New-onset psychosis, uncommunicative, unable to follow commands, combative	Alcohol, "plant food"	Hospitalization, LOR (2 mg, three administrations during hospitalization), HALO (5 mg once), RIS (1 mg daily, NA)
Chan et al. (54)	Case-report (UK)	1, 21, NA, M	None	6-APB (SC), metabolites of both THC and JWH-122 (SC)	Delusions ("trying to read his mind", "looking at him in a funny way"), agitation, suicidal and homicidal tendencies, paranoia, low mood	Cannabis	Hospitalization, diazepam (4–10 mg, NA)
Hermanns-Clausen et al. (55)	Retrospective cohort study (Germany)—Database of the Poisons Information Center Frelburg (2008–2011)	29, 14–30, NA, 25 M and 4 F	NA	SC	Mild visual or auditory hallucinations, tachycardia, drowsiness, paresthesia, abdominal pain, mild hypoglycemia, mild electrolyte imbalance, short-term hypothermia, delirium, agitation, confusion, rhabdomyolysis, etc.	NA	Supportive care, potassium supplementation, BDZs (NA)
Papanti et al. (7)	Case report (Italy)	1, 18, Caucasian, M	None	"Bonzai" herbal blend (SC)	Anxiety, insomnia, palpitations, ideas of reference, somatic and visual hallucinations, autoscopy, agitation, behavioral dyscontrol	None	Hospitalization with diazepam 5 mg ev, then, bromazepam 3 mg daily and OLA 5 mg daily for 4 weeks
Glue et al. (56)	Case series (New Zealand)	17, 26.1 ± 10, NA, 10 M and 7 F	Personal psychiatric history of recurrences of preexisting affective disorders (n = 9/17)	"K2" (SC)	Paranoia, thought disorder, disorganized behavior, anxiety, depression, suicidal ideation	NA	Hospitalization, ADs (NA, NA), APs (NA, NA)

(Continued)

TABLE 2 | Continued

Reference	Study design (country)	Sample features (size, age, ethnicity, sex)	Psychiatric history	NPS involved	Symptomatology	Previous substance use history	Treatment [drug name, dosage, duration (if available)]
Leibu et al. (57)	Case report (USA)	1, 36, African-American, M	Personal psychiatric history of schizophrenia and cannabis dependence	"K2" (SC) 3 times smoked weeks before admission	Worsening paranoia, illogical speech and auditory hallucinations	Previous cannabis use	Hospitalization, ECT, CLO (225 mg BID, NA)
Bozkurt et al. (58)	Survey on sociodemographic and clinical data (Turkey)	158, 26.1 ± 7.1, NA, 94.9% M	Personal psychiatric history of comorbid psychiatric disorders (7%)	"Bonzai" (SC) in 70.3% "Jamaika" (SC) in 21.5%	Hallucinations (40.4%), delusions (16%)	Cannabis	NA
Celofiga et al. (59)	Case report (Slovenia)	4, 21–35, NA, M	Personal psychiatric history of schizophrenia	AM-2201 (SC)	Delusions, hallucinations (n = 2), psychosis relapse	None	None (n = 1), increased BDZs (n = 3)
Haro et al. (60)	Case report (Spain)	1, 18, NA, F	NA	SC for at least 6 months	New-onset psychosis, violent behavior, talking to self, visual hallucinations	Cannabis	ARI (15 mg daily, 2 months), LOR (NA, NA), Biperiden (NA, NA)
Meijer et al. (61)	Case report (USA)	1, 26, NA, M	Personal psychiatric history of ADHD stable with lisdexamfetamine	"Black Diamond" (SC)	New-onset psychosis, paranoid delusions	NA	Amputation
Smith and Roberts (62)	Case report (USA)	1, 17, NA, M	None	"K2" daily for 2 months before admission	Auditory and visual hallucinations, catatonia, disorganized thought process, new-onset psychosis	NA	Hospitalization, LOR (2 mg daily, NA), OLA (NA, NA), ECT (6 sessions, NA)
Durand et al. (63)	Case report (USA)	1, 23, NA, M	Family psychiatric history of schizophrenia	"Mr. Nice Guy" (SC) sporadically for 6 months	New-onset psychosis, persecutory delusions	Cannabis	Hospitalization; HALO (10 mg TID, NA), VA (500–1,000 mg, NA), LOR (2 mg TID, NA)
Schwartz et al. (64)	Case series (USA)	7, 16–30, NA, 3 M and 4 F	None	"Crazy Clown" (SC)	Delirium, new-onset psychosis, aggressive behaviors	1 subject with cocaine use	ICU, intubation (n = 3), no treatment (n = 3), normal saline (n = 1)
Ustundag et al. (65)	Case report (Turkey)	1, 18, NA, M	None	SC for 6 months	Talking to self and laughing, new-onset psychosis, delusions, manic symptoms	Volatile substances	OLA (10–20 mg daily, NA), VA (500–1,500 mg daily, NA), QUE (200–400 mg daily, NA)
Sönmez and Köşger (66)	Case report (Turkey)	1, 31, NA, M	None	"Bonsai" (SC) 3 times a week for >6 months	Anger, insomnia, delusions, new-onset psychosis	None	Hospitalization; OLA (NA)
Rahmani et al. (67)	Case report (USA)	a) 1, 17, Caucasian, M b) 1, 17, Caucasian, M	a) Strong family psychiatric history b) Strong family psychiatry history	"Spice" (SC)	a) Delusions, visual hallucinations, new-onset psychosis b) Paranoia, disorganized thought and behavior, new-onset psychosis	a) Cannabis, LSD, psilocybin, mushrooms, bath salts, oxycodone b) Cannabis, LSD, ecstasy, benzodiazepines	a) Hospitalization, CLO (NA), metoprolol (NA) b) Hospitalization, CLO (NA)

(Continued)

TABLE 2 | Continued

Reference	Study design (country)	Sample features (size, age, ethnicity, sex)	Psychiatric history	NPS involved	Symptomatology	Previous substance use history	Treatment [drug name, dosage, duration (if available)]
Tyndall et al. (68)	Retrospective cohort study (USA)	35, 14–58, NA, 88.6% M	NA	SC	Hallucinations (6%)	NA	Hospitalization (28%)
Altintas et al. (69)	Cross-sectional study (Turkey)	50/81, 25.9 ± 5.5, NA, M	Strong family psychiatric history and personal psychiatric history of substance use disorder (36%)	SC	Paranoia, disorganized behavior, suicide thoughts, anxiety, visual and auditory hallucinations	Cannabis	NA
Khan et al. (70)	Case report (USA)	a) 1, 21, African-American, M b) 1, 17, Caucasian, M	a) Personal psychiatric history of untreated childhood ADHD b) None	a) Continuous heavy use of "Kush" for the previous 18 months b) 2-week "Spice" binge	Catatonia, incongruent affect and paraphasic errors, flat affect, blank stare, echolalia, muscle rigidity, bradykinesias, psychosis, euphoria, grandiosity, paranoia, decreased sleep, disorganization	a) Occasional previous cannabis use b) Periodic cannabis smoking for 1 year prior	a) Hospitalization; ARI (25 mg daily, NA) b) Hospitalization; VA (100 mg qHS, NA), LOR (2 mg BID, NA), OLA (NA, NA)
Hermanns-Clausen et al. (71)	Retrospective cohort study (Germany)—Poisons Information Center (2011–2014) and Institute of Forensic Medicine of the University Medical Center Freiburg	22/46, 12–25, NA, 18 M and 4 F	NA	SC	Visual hallucinations (23%), perceptual disturbance (9.1%), retrograde amnesia (4.5%), tachycardia (82%), nausea (78%), somnolence (55%), mydriasis (45%), restlessness (13.6%), agitation (13.6%),	Alcohol (n = 1)	BDZs (n = 2, NA), metoprolol (n = 3, NA), metoclopramide (n = 2, NA), ondansetron (n = 2, NA), diphenhydramine (n = 2, NA), lamotrigine (n = 1, NA)
Bassir Nia et al. (72)	Retrospective cohort study (USA)	82/676, NA, NA, NA	NA	SC ± cannabis	Subject with SC use only were significantly associated with more psychotic symptoms (OR = 4.44), more psychotic disorder diagnosis (OR = 6.74), and greater agitation (OR = 4.39) and aggression (OR = 3.10), followed by subjects with SC and concomitant cannabis use (OR = 3.61, OR = 4.78, and OR = 2.51, respectively)	NA	APs, MS, AD (NA, NA)
Roberto et al. (73)	Case report (USA)	1, 18, African American, M	None	"K2" and "Spice" daily for 3–4 weeks	Insomnia, paranoid ideation, agitation, elated mood, beliefs that others were inserting thoughts into his head ("thought insertion" and "thought broadcasting"), pacing, bizarre delusional thoughts with thought derailment, disorganized behavior, decreased short-term memory	Alcohol and cannabis	Hospitalization; RIS (5 mg daily, NA), LOR (2 mg, NA)

(Continued)

TABLE 2 | Continued

Reference	Study design (country)	Sample features (size, age, ethnicity, sex)	Psychiatric history	NPS involved	Symptomatology	Previous substance use history	Treatment [drug name, dosage, duration (if available)]
Samaan et al. (74)	Case report (USA)	1, 18, Hispanic, M	None	SC	Acute-onset auditory hallucinations, paranoid delusions, panic attacks, palpation, shortness of breath, diaphoresis, chest tightness, hand numbness, impulsivity, aggressiveness, agitation, suicidal thoughts	Cannabis	NA
Ozer et al. (75)	Case report (Turkey)	1, 17, NA, M	None	Inhaled "Bonsai" (SC) for 10 days before admission	Capgras syndrome, persecutory delusions and auditory hallucinations	NA	Hospitalization; OLA (10 mg daily, 3 months)
Shalit et al. (76)	Retrospective cohort study (Israel)	60, 30.46 ± 7.83, NA, 86% M	NA	SC	Higher PANSS score in SC users vs. cannabis users No differences in major psychiatric diagnoses between SC vs. cannabis users	Cannabis (73.3%)	Hospitalization
Waugh et al. (77)	Retrospective cohort study (UK)—National Poisons Information Service telephone enquiry records (2007–2014)	510, 12–78, NA, 80.8% M	NA	"Black Mamba" (SC) in 20.3% "Pandora's Box" (SC) in 15% "Clockwork Orange" (SC) in 6.2%	Hallucinations (4.6%), paranoia (1.2%)	9% previous use of other substances	NA
Zorlu et al. (78)	Case–control (Turkey)	22 SC users vs. 18 HC, 26.5 ± 5.2, NA, NA	NA	SC	Long-term use of SC associated with white matter abnormalities and disturbed brain connectivity associated with an increased vulnerability to psychosis	NA	NA
Babi et al. (79)	Case report (USA)	1, 40, NA, M	None	SC	Acute psychosis, new-onset refractory status epilepticus	None	LOR ev (NA, NA), VA (NA, NA), levetiracetam (NA, NA), lacosamide (NA, NA)
Manseau et al. (80)	Retrospective cohort study (USA)	110, 33 (median), non-white (90%), 95.5% M	Personal psychiatric history of primary psychotic disorder diagnosis (40%)	SC	Acute psychotic symptoms (70%), agitation	Alcohol (39.1%), cannabis (35.5%), cocaine (24.5%)	Inpatient admission (34.5%), unspecified APs
Monte et al. (81)	Retrospective cohort study (USA)—Toxic Registry (2010–2015)	353, 25 (median), NA, 84% M	NA	SC	Delirium and toxic psychosis (41.4%), hallucinations (7.1%)	3.4% previous cannabis use whilst 5.7% previous use of other substances	BDZs (37%) and unspecified APs (10%) most frequently prescribed
Kassai et al. (82)	Qualitative research (Hungary)	6, 20–27, NA, M	NA	SC	Paranoia, difficulty socializing, increased egoism, self-neglect, switch off brain, inability to sleep, feeling under control, sweating	NA	NA

(Continued)

TABLE 2 | Continued

Reference	Study design (country)	Sample features (size, age, ethnicity, sex)	Psychiatric history	NPS involved	Symptomatology	Previous substance use history	Treatment [drug name, dosage, duration (if available)]
Van Hout and Hearne (83)	Online survey (UK)	6, NA, NA, 3 M and 3 F	NA	SC	Agitation, restlessness, fear, paranoia, aggression, severe dissociation, chest pain, aches and pains, palpitations, nausea, sweating, vomiting	NA	NA
Welter et al. (84)	Prospective pilot study (Germany)	24/332, 23.52 ± 5.29, NA, 71.4% M	Personal psychiatric history of schizophrenia, schizoaffective disorder and schizophrenic disorder (71.4%)	SC ± cannabis	SC use was 2 times higher among psychotic patients vs. non-psychotic patients (p = .033). Delusion of persecution, hallucinations, blunted affect, motor retardation, disorganization or worsening of preexistent psychotic symptoms	Cannabis	NA
Bonaccorso et al. (85)	Case series (UK)	a) 1, 28, Caucasian, M b) 1, 32, Caucasian, F c) 1, 20, Black-Caribbean, M d) 1, 39, Asian British, M	a) Personal psychiatric history of paranoid schizophrenia b) Personal psychiatric history of schizoaffective disorder, poly-substance misuse (cocaine and heroin) c) Personal psychiatric history of first psychotic episode post poly-substance misuse d) Personal psychiatric history of bipolar disorder	SC	Intense exacerbations of positive symptoms, psychomotor agitation, sexual disinhibition, verbal/physical aggression, poor responses to medications	Previous poly-substance misuse (variable)	a) RIS-LAI (37.5 mg fortnightly, NA), OLA (10 mg daily, NA) pregabalin (100 mg daily, NA) b) ARI (30 mg daily, NA), lithium carbonate (800 mg daily, NA) c) HALO-LAI (50 mg monthly, NA), HALO (10 mg daily per os, NA) d) ARI-LAI (400 mg monthly, NA)
Sweet et al. (86)	Case report (USA)	1, 47, African-American, M	None	SC	Bizarre behavior, delusions, hallucinations, agitation, aggressiveness	NA	OLA (10 mg daily, NA), HALO (10 mg im, NA), LORA (2 mg, NA), dipgenhydramine (50 mg, NA)
Skryabin and Vinnikova (87)	Single-center analysis cohort study (Russia)	46, 23.2 ± 3.5 (mean), NA, M	NA	SC	Delirium, tactile hallucinations, auditory verbal hallucinations, Kandinsky–Clerambault's syndrome delusion of influence, automatisms, anxiety, paranoia, delusional mood, agitation, delusional persecutory ideas, thought insertion or withdrawal, etc.	Cannabis, alcohol	NA
Studies on synthetic cathinone-induced psychosis							
Antonowicz et al. (88)	Case report (USA)	a) 1, 27, NA, F b) 1, 32, NA, M	None	"Powdered Rush" containing MDPV	a) Tachycardia, diaphoresis, paranoid psychosis, disorganized though process, poor memory, insomnia b) Hypertension, tachycardia, disorganized behavior, paranoid psychosis, insomnia	Previous history of opiate dependence	a) Hospitalization, RIS (0.5 mg BID, NA) b) Hospitalization, refusal of any medications

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TABLE 2 | Continued

Reference	Study design (country)	Sample features (size, age, ethnicity, sex)	Psychiatric history	NPS involved	Symptomatology	Previous substance use history	Treatment [drug name, dosage, duration (if available)]
Derungs et al. (89)	Case report (Germany)	1, 31, NA, M	NA	Naphyrone powder ingestion	Blurred vision, restlessness, anxiety, dysphoria, hallucinations, insomnia, paranoia	Regular MDMA, alcohol and benzodiazepine abuse	NA
Penders and Gestring (90)	Case report (USA)	3, NA, NA, 2 M and 1 F	None	MDPV	Hyperactivity, anger, confusion, hallucinations, paranoia, anxiety, fearful, insomnia	NA	Hospitalization, RIS (*n* = 2, 0.5 mg orally BID, NA), HALO (*n* = 1, 1 mg orally BID, NA)
Striebel and Pierre (91)	Case report (USA)	1, 22, NA, M	None	MDPV	Anxiety, hallucinations, chest pain, diaphoresis, nausea, tachycardia	Regular cannabis use	Hospitalization, supportive care, LOR (NA, NA)
Adebamiro and Perazella (92)	Case report (USA)	1, 26, NA, M	NA	Synthetic cathinones	Diaphoresis, kidney dysfunction, confusion, paranoia, agitation, hallucinations, hypertension, tachycardia	NA	Hospitalization
Kasick et al. (93)	Case report (USA)	a) 1, 38, Caucasian, M b) 1, 26, Caucasian, M	None	"Arctic Blast" and "Posh Aromatherapy Bath Salts" containing synthetic cathinones	a) Snakes hallucinations, aggressiveness, tachycardia, hyperthermia, agitation, alertness, anxiety, paranoia b) Auditory hallucinations, feelings of detachment, derealization, paranoia, suicidal ideation, confusion, delirium, tremors, hyperreflexia, myoclonus, amnestic effects	NA	a) Hospitalization, LOR (13 mg IV, NA) and supportive therapy ev, then HALO (5 mg IM, NA) b) Hospitalization, LOR (5 mg IV, NA), RIS (0.5 mg daily, NA)
Lajoie and Rich (94)	Case report (USA)	1, 50, NA, M	None	MDPV	Agitation, hallucinations, psychosis, suicidal ideation, chest pain, skin lacerations, tachycardia	History of methamphetamine abuse	Hospitalization, LOR (NA, NA), OLA (NA, NA)
Thornton et al. (95)	Case report USA)	1, 23, NA, M	Personal psychiatric history of psychotic disorder not better specified	MDPV	Bizarre behavior, suicidality, visual, tactile and auditory hallucinations, agitation, tachycardia, hyperthermia	NA	Hospitalization, LOR (6 mg daily, NA), droperidol (2.4 mg daily, NA)
Gunderson et al. (96)	Case report (USA)	1, 20, NA, M	Personal psychiatric history of MDD	"Infinity", "TranQuility," "Cool Wave," "White Lightning" containing synthetic cathinones + diphenhydramine	Irritability, weight loss, insomnia, hallucinations "seeing different things, including people walking around his yard and house dressed all in white, having sex in his yard, chanting, etc."), suicidal ideation, paranoid psychosis, defensive homicidality	Previous cocaine, alcohol and opioid dependence in stable remission Previous cannabis and SC consumption	Hospitalization
Khan et al. (97)	Case report (USA)	1, 19, NA, F	None	"Ivory Wave" containing synthetic cathinones	Severe agitation, aggressive behavior, psychosis, poisoning delusion, auditory and visual hallucinations, vernal aggressiveness, labile mood, tachycardia, hypertension, diaphoresis, mydriasis	None	Hospitalization, LOR (NA, NA) and OLA (NA, NA)

(Continued)

TABLE 2 | Continued

Reference	Study design (country)	Sample features (size, age, ethnicity, sex)	Psychiatric history	NPS involved	Symptomatology	Previous substance use history	Treatment [drug name, dosage, duration (if available)]
Mangewala et al. (98)	Case report (USA)	1, 15, NA, M	None	MDPV	Agitation, paranoid psychosis, barricading himself in his home, aggressiveness towards health professionals, Capgras syndrome	Previous cannabis use	Hospitalization, LOR (0.5 mg BID, NA), OLA (7.5 mg daily, NA)
Stoica and Felthous (99)	Case report (USA)	1, 30, Caucasian, M	Personal psychiatric history of bipolar affective disorder and schizophrenia associated with a family psychiatric history of schizophrenia and bipolar disorder	Synthetic cathinones	Suicidal threats and gestures, auditory hallucinations, euphoria, increased energy level, insomnia, paranoid delusion ("he thought his neighbors were at his door, watching for him"), homicide ideation towards his neighbors	Previous heroin, cannabinoids, BDZs, LSD, and methamphetamine abuse	Hospitalization, VA (250 mg BID, NA), trazodone (100 mg daily, NA)
Winder et al. (100)	Case report (USA)	1, 33, NA, M	None	MDPV and mephedrone	Diaphoresis, hyperthermia, tachycardia, agitation, dysphoria, euphoria, hallucinations, insomnia, paranoia	Prior methamphetamine, opioids, alcohol, benzodiazepines abuse	Hospitalization, LOR (NA, NA), QUE (NA, NA), unspecified AD
Bertol et al. (101)	Case report (Italy)	1, 27, Caucasian, M	Personal psychiatric history of bipolar disorder	MDPV ev ± 3-methyl-methcathinone ± pentedrone	Mydriasis, unresponsiveness, agitation, delirium, visual, tactile and auditory hallucinations, persistent sleeplessness, euphoria, micro-zoopsia	Previous alcohol dependence	Hospitalization, diazepam (NA), chlorpromazine (NA), ARI (NA)
Stevenson et al. (102)	Case report (UK)	1, NA, NA, M	Personal psychiatric history of drug-induced psychosis	3-MeO-PCP and MDPV	Vivid hallucinations, bizarre ideas, violence and aggressiveness, murder attempt	History of regular recreational use of several illicit drugs	NA
Crespi (103)	Case report (USA)	1, 17, NA, F	None	"Flakka" (Alpha-PVP)	Altered mental status, agitation, psychotic behaviors, cognitive deterioration, bizarre behavior	NA	Hospitalization, LOR (NA, NA) and OLA (10 mg BID, NA)
Dolengevich-Segal et al. (104)	Case report (Spain)	1, 25, NA, M	Personal psychiatric history of ADHD, antisocial behavior and early substance abuse in adolescence	Mephedrone in the context of ChemSex	Delusional paranoid ideation with an intense emotional and behavioral impact, visual hallucinations involving human forms and cellphone lights, suicide ideation, psychomotor restlessness, feeling of being "controlled," suspiciousness, cenesthopathy (i.e., "insects crawling under the skin")	Cocaine, alcohol, ketamine, GHB, MDMA, methamphetamine	Hospitalization, PALI (6 mg daily, NA) zonisamide (300 mg daily, NA), pregabalin (75 mg daily, NA)
Romanek et al. (105)	Retrospective single-center study (Germany)	81, 17–49, NA, 64% M	NA	Synthetic cathinones various	10 patients manifested prolonged psychosis	NA	NA

(Continued)

TABLE 2 | Continued

Reference	Study design (country)	Sample features (size, age, ethnicity, sex)	Psychiatric history	NPS involved	Symptomatology	Previous substance use history	Treatment [drug name, dosage, duration (if available)]
Richman et al. (106)	Case report (USA)	1, 20, North African descent immigrated to USA, M	None	"Flakka"	Anger, mystic delusions "believing to be the embodiment of a prophet"), aggressiveness, impulsivity, hyper-religiosity, command auditory hallucinations, visual hallucinations of serpents, suicide and homicidal ideation, mood lability, disorganized behavior, echopraxia, echolalia, stupor, staring	NA	Hospitalization, LOR (2–3 mg TID, NA), then clonazepam (2 mg BID, NA), ARI (10–25 mg daily, NA), then QUE (800 mg daily, NA)
Simonato et al. (107)	Case report (Italy)	1, 28, Caucasian, M	Personal psychiatric history of cannabis and skunk-induced psychotic episodes	"Flakka"	Depressive symptoms, lack of energy, visual hallucinations, panic attack, hyperthermia, sexual arousal, insomnia, Ekbom syndrome, persecutory delusion	Previous cannabis and skunk consume	Hospitalization, BUP (150 mg daily, NA)

NA, not available data; SC, synthetic cannabinoids; M, male, F female; BDZ, benzodiazepine; ED, emergency department; LOR, lorazepam, ev endovenous; ADHD, attention/deficit hyperactivity disorder; MDD, major depressive disorder; AC, anticonvulsants; AP, antipsychotics; HALO, haloperidol; RIS, risperidone; PTSD, post-traumatic stress disorder; ARI, aripiprazole; BUP, bupropion; OLA, olanzapine; ECT, electroconvulsant therapy; CLO, clozapine; VA, valproic acid; ICU, intensive care unit; QUE, quetiapine; ARI-LAI, aripiprazole long-acting injection; OR, odds ratio; PANSS, Positive and Negative Schizophrenia Scale; RIS-LAI, risperidone long-acting injection; HALO-LAI, haloperidol long-acting injection; 3-MeO-PCP, 3-methoxyphencyclidine; MDPV, methylene-dioxy-pyrovalerone; PALI, paliperidone.

varying from 3% to 16.3% (26). A retrospective analysis of cases presenting to the ED of the University Hospital of Bern, Switzerland, with symptoms/signs consistent with acute toxicity of recreational and/or NPS use identified two intoxications with NPS: one with methylone and one with 2C-P; 71 cases of psychoses even though the authors do not clearly describe clinical presentation related to the NPS identified (27).

Data on Synthetic Cannabinoids

A case series described some instances of SC-induced reemergence of florid psychotic symptomatology among five forensic patients who took a mixture of SC (like "K2" and JWH-018) contained in "Aroma" blend (28). Psychopathological patterns comprised the onset of psychomotor agitation, disorganized behavior, and ideation, delusional beliefs (grandiose and paranoid) in previously stable patients with a personal history of severe mental illness (SMI) (28). No data are provided about pharmacotherapy and/or specific/suggested treatment (28).

Müller et al. (29) described a 25-year-old man, with a previous history of recurrent psychotic episodes triggered by cannabis consumption and stable on monotherapy with amisulpride (800 mg daily), who presented to hospital with increased levels of anxiety, feelings of manipulation, and psychotic symptoms after smoking "spice" drugs on three different occasions (3 g each). The subject reported the feeling of being controlled and manipulated through a chip which he thought was implanted in his abdomen several years before together with the onset of imperative voices and paranoid hallucinations, symptoms he never had before (29). no data are provided about pharmacotherapy and/or specific/suggested treatment (29).

Another case report described three cases of "Mojo psychosis," a mixture of SC (30). No further details on psychopathological presentation have been reported by the authors (30).

A study evaluating urine metabolism of SC described three cases of new-onset psychosis following the intake of a mixture blend named "Tropical synergy" containing several SC (31). No further data are provided on psychopathological pattern and/or about pharmacotherapy and/or specific/suggested treatment (31).

Vearrier and Osterhoudt (32) described a case of an adolescent girl who arrived at the ED agitated, violent, and uncontrollable after inhaling a "K2." She described a previous history of alcohol and cannabis intake. Her psychopathological pattern comprised the onset of visual hallucinations, anxiety, restlessness, tachycardia, higher blood pressure, muscle fasciculation, hypokalemia, and mydriasis. She was given lorazepam 2 mg intravenously, with a good remission (32).

Benford and Caplan (33) reported a case of a 20-year-old honor college student who presented to the ED with severe anxiety/paranoia and auditory/visual hallucinations after smoking "Spice" drugs. Psychopathological pattern comprised the onset of increasing levels of anxiety, paranoia, and both auditory and visual hallucinations. The subject refused voluntary psychiatric admission. No data are provided about pharmacotherapy and/or specific/suggested treatment (33).

A case series described 11 Hispanic adolescents admitted to the South Miami Hospital Addiction Treatment Center in Miami-Dade County, Florida, after smoking SC and developing a new-onset psychosis (34). Subjects reported using Spice drugs more than three times in their lifetime (8 out of 11 subjects), while 4 out of 11 reported smoking SC multiple times per day. Psychopathological patterns comprised mood fluctuations, altered cognition and perception (i.e., visual and auditory), memory difficulty, euphoria, sometimes irritability and anxiety, and paranoid thoughts (34). In addition, subjects reported tachycardia, restlessness, appetite changes, muscle fasciculation, tremors, and weakness. No data are provided about pharmacotherapy and/or specific/suggested treatment (34).

A cohort explorative study recruiting subjects from a Regional Forensic and Rehabilitation service in New Zealand reported a SC-induced psychotic recrudescence in 15 vulnerable individuals previously affected with SMI (35). The psychopathological patterns comprised the onset of psychomotor agitation, disorganized behavior, and ideation, delusions after smoking SC. All subjects reported a previous psychiatric history of psychotic disorder and had been compulsorily treated with therapeutic doses of antipsychotics (unspecified) and, in five cases, together with mood stabilizers (unspecified). No data are provided about pharmacotherapy and/or specific/suggested treatment following SC-induced psychosis (35).

Forrester et al. (36) retrospectively collected data on SC exposures referred to Texas Poison Centers in 2010 by identifying new-onset psychoses following SC intake. Psychopathological patterns comprised the onset of psychomotor agitation and hallucinations. In addition, tachycardia, hypertension, nausea, vomiting, drowsiness, and multiple neurological symptoms were reported. No further information is provided regarding the psychopathology and phenomenology of SC-induced psychoses. No clear data are provided about specific/suggested treatment following SC-induced psychosis, intravenous fluids, benzodiazepines, oxygen, and antiemetics being reported, among the most commonly prescribed medications and, in around 3% of subjects, the use of sedatives and anticonvulsants (36).

Hurst et al. (37) described 10 cases of otherwise healthy men, admitted to the psychiatry ward at the San Diego Naval Medical Center between August and December 2010, who experienced a SC-induced new-onset psychosis. The psychopathological patterns were characterized by auditory and visual hallucinations, disorganized behavior and speech, suicidal ideation, alogia, insomnia, psychomotor agitation and/or retardation, and higher levels of anxiety. No clear data are provided about specific/suggested treatment following SC-induced psychosis, use of antipsychotics (unspecified) being reported in 10 patients (36).

A case report of a 23-year-old high-functioning Caucasian Navy corpsman who developed a SC-induced psychosis was reported by Johnson et al. (38). The psychopathological pattern comprised the onset of nonsensical speech, paranoia of being videotaped, disorganized behavior and speech, tangential thoughts, delusional ideation (i.e., the feeling of own "mind expanded" and the ability to "comprehend infinity," and so on) without perceptual distortions. The psychotic symptoms of the subject spontaneously resolved after abstinence from SC about 24 h after initial presentation. No data are provided about psychopharmacology and/or specific treatment of SC-induced psychosis (38).

Data collected retrospectively by the Italian database of the Pavia Poison Centre reported 17 cases of SC users who exhibited various psychiatric and clinical patterns, including tachycardia, agitation/anxiety, confusion, hallucinations, mydriasis, paresthesia, palpitations, drowsiness, xerostomia, syncope, seizures, vertigo, tremor, hypertonia, coma, etc. (39). The authors reported symptomatic treatment and benzodiazepines for treating neuroexcitatory effects (39). No further details regarding psychopathological pattern of SC-induced psychosis were reported by Locatelli et al. (39).

McCain et al. (40) retrospectively described six cases of SC-induced psychopathological/clinical pattern referred to the Arkansas Poison and Drug Information Center. Psychopathological patterns comprised agitation, irritability, and hallucinations. Benzodiazepines, intravenous fluids, antiemetics, and potassium supplementation were prescribed, among treatments, without details about dosage(s) and/or treatment duration (40).

Simmons et al. (41) described three cases of young adults referred to an ED after "Spice" intake (JWH-018 and JWH-073) who exhibited anticholinergic and sympathomimetic clinical effects and a new-onset SC-induced psychosis. Benzodiazepines (intravenous lorazepam, 4 mg), antipsychotics (haloperidol, 5 mg), supportive therapy, and observation were among the treatments prescribed (41).

A 17-year-old adolescent boy without significant previous psychiatric and medical history presented to an ED reporting "pounding in his chest," constant chest pressure, and a new-onset SC-induced psychosis after smoking a SC, called K9 (42). No data are provided about pharmacotherapy and/or specific/suggested treatment (42).

Other case reports of SC-induced new-onset psychoses have been reported by McGuinness and Newell (43). Psychopathological patterns comprised aggressiveness, paranoia, short-term memory deficits, and increasing levels of anxiety. No data are provided about pharmacotherapy and/or specific/suggested treatment (43).

Tung et al. (44) described a 36-year-old male real-estate agent with a previous polysubstance abuse (heroin, codeine, and cannabis) with a family psychiatric history of SMI (unspecified) and substance use disorder (SUD) who presented an episode of acute psychotic disorder characterized by florid persecutory delusion, auditory hallucinations, disorganized behavior, irritability, aggressiveness, and agitation. No further data are provided about pharmacotherapy and/or specific/suggested treatment (44).

Van der Veer and Friday (45) described three patients who presented with severe, persisting psychotic symptoms after regular SC use for 3–4 weeks prior to admission. Psychopathological patterns comprised disorganized speech and behavior, paranoia, bizarre delusions, suicidality, aggressiveness, poverty of thoughts, loosening of associations, poor attention/concentration, and

inappropriate affect; onset of Capgras delusions was reported in one case. Two out of three patients did not have a previous psychiatric history, while one reported a post-traumatic stress disorder (PTSD). All three patients required hospitalization and were treated with antipsychotic medications (haloperidol in two cases and risperidone in one case). No further data are provided regarding dosage(s) and/or treatment duration (45).

Bebarta et al. (46) described three cases of "Spice" use in military members followed by onset of psychotic symptomatology. Psychopathological patterns comprised the onset of paranoia, aggressiveness, agitation, and visual hallucinations. Regarding the treatment, intravenous lorazepam 2 mg was reported for managing agitation, with resolution of symptomatology (46).

Cohen et al. (47) reported three cases of adolescents who presented with a new-onset SC-induced psychosis characterized by catatonia, psychomotor agitation, and aggressiveness. No further data are provided about pharmacotherapy and/or specific/suggested treatment (47).

A retrospective series of exposures to SC coming from the US National Poison Data System between January and October 2010 were analyzed by Hoyte et al. (48) by identifying new-onset psychosis induced by SC products. No further data are provided either regarding further specific psychopharmacological treatment beyond benzodiazepines or about psychopathological patterns of SC-induced psychosis (48).

Two adolescents who manifested a new-onset SC-induced psychosis have been reported by Oluwabusi et al. (49). Both patients reported a family psychiatric history for schizophrenia and/or bipolar disorder. Psychopathological pattern comprised disorganized behavior, paranoid delusions, insomnia, hyperactivity, anxiety, musical auditory hallucinations, mood lability, irritability, euphoria, pressure of speech, flights of ideas, paranoia, and grandiose delusions. Regarding treatment, one subject was firstly stabilized on quetiapine and then changed to aripiprazole (20 mg daily) after the onset of an acute dystonic reaction, while the other one was prescribed olanzapine (15 mg daily bedtime) (49).

Another case report has been described of a new-onset SC-induced psychosis in a 59-year-old Latino male with a history of PTSD and polysubstance abuse (heroin, cocaine, cannabis, and alcohol) (50). His psychopathological pattern comprised visual hallucinations and disorganized and bizarre behavior. He was treated with benzodiazepines, gabapentin (400 mg QID), hydroxyzine (25 mg TID), aripiprazole (10 mg qD), benztropine (1 mg BID), and bupropion (150 mg BID) (50).

An Internet-based survey reported the onset of psychotic symptomatology in 28% of a sample (n = 168) of SC users recruited from 13 different countries (51), while an Australian online survey (n = 316) reported paranoia (18%) and psychosis (4%) (52). No further data are provided about pharmacotherapy and/or specific/suggested treatment in both surveys (51, 52).

Berry-Cabán et al. (53) described a 20-year-old Hispanic male who presented to an ED due to an altered mental status and delirium following SC intake. Initially, the subject was uncommunicative, awake then non-compliant, combative, and aggressive; hence, clinicians applied physical restraint and firstly administered 2 mg/ml parental lorazepam for managing increasing levels of agitation. During hospitalization, he was additionally treated with further 4 mg of lorazepam, 5 mg of haloperidol, and 25 mg of diphenhydramine. His psychopathological pattern comprised agitation, aggressiveness, disorganized speech and behavior, hallucinations, and paranoid ideation. He was discharged with a prescription of risperidone 1 mg daily. No further data on treatment duration has been provided (53).

Chan et al. (54) described a 21-year-old man without a previous psychiatric history who presented to an ED due to the onset of increasing levels of psychomotor agitation and paranoid thoughts after consuming 0.4 g of 6-APB and smoking cannabis over a 2-day period. Psychopathological pattern comprised agitation, paranoia, the belief that others could read his mind, self-harm, and suicidality. He was treated with diazepam (4–10 mg daily) for managing agitation during hospitalization. No further data have been provided regarding further treatments to be prescribed and/or treatment duration (54).

A retrospective cohort study targeting individuals seeking emergency treatment after SC intake and selected from the database of the Freiburg Poisons Information Center between September 2008 and February 2011 reported a plethora of physical symptoms and psychotic presentation (55). Psychopathological patterns included visual and auditory hallucinations, confusion, agitation, delirium, restlessness, and psychotic symptoms. Supportive care, talk down strategies, intravenous fluids, potassium supplementation, and benzodiazepines were prescribed. No further data have been provided regarding specific psychopharmacotherapy and/or treatment duration and/or dosage(s) (55).

A case report of an Italian male who consumed "Bonzai," a mixture of SC, has been presented by Papanti et al. (7). The subject did not have any psychiatric history and his psychopathological pattern was characterized by increasing levels of anxiety, insomnia, ideas of reference, somatic and visual hallucinations, autoscopy, and aggressiveness. He was initially treated with diazepam 5 mg intravenously and was rehydrated, and then he was prescribed olanzapine 5 mg daily and bromazepam 3 mg daily for 4 weeks (7).

Glue et al. (56) retrospectively collected data on 17 patients hospitalized in an acute psychiatric ward, located in New Zealand, following the use of a SC ("K2") and described their clinical and psychopathological features. Nine out of 17 patients had recurrences of preexisting disorders and four presented with new psychotic symptoms. Psychopathological patterns comprised paranoia, thought disorder, disorganized behavior and speech, anxiety, mood lability, and intense suicidal thinking and/or behavior. Antidepressants and antipsychotics (unspecified) were prescribed, without specifying treatment duration and/or dosage(s) (56).

Leibu et al. (57) described a case of a 36-year-old African-American man with a previous long-lasting psychiatric history of schizophrenia who presented with severe and life-threatening catatonia after consuming SC. Catatonia was successfully treated with electroconvulsive therapy, while psychotic symptoms have been managed with 225 mg BID clozapine (57). No further data are available regarding treatment duration (57).

A Turkish survey carried out on a sample of 158 patients who were admitted to Bakirkoy Research and Training Hospital for Psychiatry, Neurology and Neurosurgery, Alcohol and Drug Research, Treatment and Training Center (AMATEM) revealed a new onset of hallucinations and delusions among SC consumers (58). Psychopathological patterns comprised increasing levels of anxiety (75.6%), irritability (18.6%), insomnia (61.5%), hallucinations (40.4%), and delusions (16%). No data have been provided regarding specific psychopharmacological treatment of SC-induced psychosis (58).

A case series of four paranoid schizophrenic patients who took SC showed a worsening of psychotic symptomatology (59). All subjects were hospitalized and were previously prescribed specific medications for their psychiatric disorder (i.e., haloperidol decanoate, quetiapine, risperidone, diazepam, clozapine, lorazepam, olanzapine, clonazepam, and fluoxetine). Psychopathological patterns comprised cognitive deficits, anhedonia, dysphoria, avolition, blunted affect, incoherent speech, thought blocking, severe agitation, increasing levels of anxiety, and so on. The subjects were furtherly treated by increasing benzodiazepine dosages (lorazepam and/or diazepam) (59).

Haro et al. (60) described a 19-year-old Hispanic woman who developed a psychotic episode after consuming SC. Her psychopathological pattern comprised self-references when eating or walking, visual hallucinations, soliloquy, laughter forfeit, catatonia, depersonalization, and derealization. After 2 months of abstinence from SC and treatment with aripiprazole (15 mg daily), lorazepam, and biperiden, psychotic symptomatology partially resolved (60).

Meijer et al. (61) described a case of bilateral upper-extremity self-mutilation and new-onset SC-induced psychosis in a healthy 26-year-old man presented to an ED, with a previous history of attention deficit hyperactivity disorder (ADHD), treated with lisdexamfetamine dimesylate. His psychopathological pattern comprised the onset of paranoid delusions causing him to feel his hands were going to harm him, then he placed them on the stove and attempted to burn them off to "get the devil out of him." No further data have been provided regarding the psychopharmacological treatment following the discharge (61).

A 17-year-old male who developed catatonia and new-onset SC-induced psychosis was reported by Smith and Roberts (62). His psychopathological pattern comprised confusion, bizarre behavior, mutism, rigidity, excitatory catatonia, auditory and visual hallucinations, and disorganized thoughts. Lorazepam 2 mg intramuscular was administered for managing agitation, followed by six sessions of electroconvulsive therapy and olanzapine. No further data have been provided regarding dosage(s) and/or treatment duration (62).

Durand et al. (63) described a case of new-onset SC-induced psychosis associated with severe rhabdomyolysis after consuming SC in a 23-year-old man without prior psychiatric history. His psychopathological pattern comprised altered mental status, severe agitation, acute psychosis, irritability, persecutory delusions, and impulsiveness. Intravenous lorazepam every 6 h was prescribed for managing agitation, then haloperidol 10 mg TID, valproic acid 500–1,000 mg, and lorazepam 2 mg TID. No further data on treatment duration has been provided (63).

Schwartz et al. (64) reported seven cases who presented simultaneously at a hospital in Georgia (USA), after smoking a mixture of SC contained in a blend named "Crazy Clown" during a party. Psychopathological patterns comprised a new onset of SC-induced psychosis, aggressiveness, extreme agitation, and increasing levels of anxiety. Regarding the treatment, two patients were admitted to the intensive care unit due to persistent hypertension and tachycardia and mental status alterations and were intubated. One patient presented with cardiac arrest and was resuscitated by paramedics and underwent successful balloon angioplasty; the other subjects were not treated due to non-compliance and/or spontaneous remission of symptomatology. No further data have been provided regarding specific pharmacological treatment (64).

Ustundag et al. (65) described a case of SC-induced mania with psychotic symptoms in an 18-year-old single boy who developed an increasing speech, spending money, a great deal of interest in religion and insomnia, mystic delusions, irritability, euphoria, mood lability, and without hallucinations after consuming SC. Olanzapine (from 10 to 20 mg daily), valproic acid (from 500 to 1,500 mg daily), quetiapine (from 200 to 400 mg daily), and lorazepam (from 0,5 to 1 mg daily) were prescribed. No further data on treatment duration are available (65).

Sönmez and Köşger (66) described a 31-year-old man who developed a new-onset SC-induced psychosis after consuming a mixture of SC called "Bonsai" three times a week for more than 6 months and was treated with olanzapine.

Rahmani et al. (67) described two cases with a strong family psychiatric history who developed a SC-induced psychosis following the intake of "Spice." Psychopathological pattern comprised the onset of paranoia, bizarre and disorganized behavior and speech, and visual hallucinations. No further data have been provided regarding treatment duration and/or dosage(s) of clozapine prescribed in both cases (67).

An observational case series ($n = 35$) retrospectively collected laboratory analysis of patients presenting to an ED with a documented suspicion of SC intake and described a new onset of SC-induced psychosis (68). Psychopathological patterns comprised altered mental status (61%), hallucinations (6%), and seizures (40%). Five patients were ventilated and intubated. No further data on specific psychopharmacological treatment has been provided (68).

A cross-sectional study recruiting 81 male patients diagnosed with SC-induced psychotic disorder ($n = 50$) or with schizophrenia ($n = 31$) who were concurrently hospitalized described a higher rate of suicidal ideation, involuntary hospitalization, as well as similar clinical picture with schizophrenia by inducing paranoia, disorganized behavior, visual and auditory hallucinations, and suicidal thoughts, not only in vulnerable subjects but also in subjects without a previous history of psychosis (69). Furthermore, verbal learning, short-term memory and working memory, executive functions, abstract ability, and decision-making and attention functions were reported to have been impaired among SC-induced psychotic subjects (69).

Khan et al. (70) described two cases of SC-induced catatonic state associated with psychosis, psychomotor alterations, speech and behavior disorganization, flatted affect, alogia in a 17-year-old male with no psychiatric history who presented to the ED with psychosis after a 2-week Spice binge and in a 21-year-old male who reported a history of childhood ADHD with progressive isolation and negative symptoms after heavy SC consumption. The first case was treated with aripiprazole 7.5 mg daily and lorazepam (2 mg BID), then olanzapine (unspecified dosage) and valproic acid (up to 100 mg qHS) were added while aripiprazole was tapered off, while the second case was treated with lorazepam for managing catatonia and firstly risperidone (1–2 mg BID), then switched to aripiprazole (5 mg daily, titrated up to 25 mg daily) (70).

A retrospective observational case series of patients (n = 22) presenting to an ED with analytically confirmed SC intake were described from a pharmacological, toxicological, and clinical point of view by Hermanns-Clausen et al. (71). Psychopathological patterns comprised restlessness, agitation, and visual hallucinations. Treatment was mainly supportive, benzodiazepines (unspecified), metoprolol, and antiemetics. No further data have been provided regarding dosage(s) and treatment duration (71).

A retrospective chart study (n = 594) evaluating all patients who were admitted to a dual diagnosis psychiatric unit at Mount Sinai Beth Israel in New York City compared SC users vs. cannabis users by reporting more psychotic symptoms (p = .012) and more agitation (p < .001) among SC consumers (72). Among medications, more antipsychotics alone (31.4% vs. 19.6%) or a combination antipsychotic plus mood stabilizers/antidepressants (57.1% vs. 33.5%) were prescribed among SC-only users compared to cannabis-only users (72).

A case report described an 18-year-old antipsychotic-naive African-American male without past psychiatric history admitted after presenting to the psychiatric emergency room with a first-time psychotic episode after a prolonged SC ingestion *via* inhalation (73). The subject was treated with intermittent doses of lorazepam 2 mg orally and stabilized over 1.5 weeks on oral risperidone (5 mg daily) (73).

An 18-year-old Hispanic male admitted to the ED after 5 days of acute-onset auditory hallucinations, paranoid delusions, and panic attacks with a previous history of cannabis abuse and more recently of SC, purchased from Internet blog websites, has been described by Samaan et al. (74). No further data about specific psychopharmacological treatment of SC-induced psychosis has been provided (74).

Ozer et al. (75) described a case of an adolescent with a new-onset psychosis following inhalation of a mixture of SC called "Bonsai." Olanzapine (10 mg daily) was prescribed and the subject was followed up 3 months after discharge without a recurrence of psychotic symptoms (75).

A cohort study reported, among SC consumers, higher psychotic levels compared to cannabis users (76). No further data about specific psychopharmacological treatment of SC-induced psychosis has been provided (76).

A retrospective cohort study recruiting data from the UK National Poisons Information Service reported hallucinations and paranoia as consequences of SC intake (77). No further data about specific psychopharmacological treatment of SC-induced psychosis has been provided (77).

A case–control neuroimaging study comparing SC users vs. healthy controls, using magnetic resonance imaging, reported that chronic SC use was associated with dose-dependent downregulation of CB_1 receptors, lower fractional anisotropy (FA) values in the left inferior fronto-occipital fasciculus (IFOF), and inferior longitudinal fasciculus (ILF) in the left temporal lobe which might be associated with increased risk of the development of psychosis (78). No further data about specific psychopharmacological treatment of SC-induced psychosis has been provided (78).

A case report described a patient who developed acute psychosis, aggressiveness (e.g., homicidal and violent behavior towards staff), and new-onset refractory status epilepticus necessitating emergent neurological life support and prolonged admission to an intensive care unit following SC consumption (79). The patient was treated with intravenous lorazepam, valproic acid, levetiracetam, and lacosamide. Following a 3-week hospitalization in the intensive care unit, the patient was discharged and followed up at 1 month. No further data on dosage(s) and/or treatment duration are available (79).

A cohort study systematically described the clinical characteristics of 100 SC users in a large sample from an urban public hospital in New York City by reporting a high rate of acute psychotic symptoms, particularly among the already socially vulnerable and psychiatrically ill population of the sample (80). Most SC users (73.7%) were prescribed an antipsychotic medication on discharge. No further data on dosage(s) and treatment duration (80).

Monte et al. (81) collected data from the US ToxIC (2010–2015) and found that among 353 SC consumers, about 40% had SC-induced delirium and toxic psychosis.

A qualitative study of six SC users in Hungary revealed the onset of paranoia and a synthetic psychosis among participants (82). Similarly, an online survey reported the onset of agitation, paranoia, and other psychotic symptomatology following SC intake (83). No further data are available regarding specific psychopharmacological treatment of SC-induced psychosis in both studies (82, 83).

A prospective pilot study recruiting 332 patients with cannabis and/or SC use evaluated the psychosis-inducing potential of cannabis vs. SC, reporting more severe psychotic symptoms among SC vs. cannabis users (84). No further data about specific psychopharmacological treatment of SC-induced psychosis has been provided (84).

A case series described four patients taking SC and presenting to a UK acute psychiatric unit with a SC-induced psychosis by reporting mixed hallucinations, persecutory delusions and thought disorganization, physical and verbal aggressiveness, and sexual disinhibition (85). All subjects recruited were treated with benzodiazepines and antipsychotic drugs, as shown in **Table 2**.

A 47-year-old African-American man presented for involuntary inpatient psychiatric admission after being brought in by police secondary to bizarre behavior/hallucinations/agitation/delusions following SC intake (86). He was initially

treated with olanzapine (10 mg daily), then switched to haloperidol intramuscularly and lorazepam due to aggressiveness and increasing levels of agitation towards clinicians. The patient was observed and he refused any follow-up and/or medication after discharge (86).

A single-center cohort study evaluating hospitalized SC-induced psychotic patients in a Russian hospital identified specific clinical variants of psychoses among SC users (87). No further data about specific psychopharmacological treatment of SC-induced psychosis has been provided (87).

Data on Synthetic Cathinones

Antonowicz et al. (88) described two cases of paranoid synthetic psychosis in a 27-year-old female and in a 32-year-old male who consumed MDPV. Psychopathological patterns comprised the onset of paranoid ideation, disorganized thought process, insomnia, and agitation. The first subject was successfully treated with low doses of risperidone, while the second case refused any medications (88).

Penders and Gestring (90) described the onset of a hallucinatory delirium following use of MDPV in three cases who presented to an ED of a tertiary care hospital in the USA. Two cases were successfully managed with risperidone (0.5 mg orally BID), while the third one was treated with haloperidol (1 mg orally BID) (90).

Striebel and Pierre (91) described a 22-year-old man without any previous psychiatric history who used cannabis regularly to manage his Crohn's disease and who developed a new-onset MDPV-induced psychosis. His psychopathological pattern comprised the onset of hallucinations, altered perceptions, "seeing things move that shouldn't be moving," feeling an earthquake, and agitation. He was treated with lorazepam and supportive care (91).

Lajoie and Rich (94) described a 50-year-old male with a previous history of methamphetamine dependence who developed a MDPV-induced psychosis characterized by self-mutilation, suicidality, agitation, increasing levels of panic attack, and auditory hallucinations. He required sedation with olanzapine and lorazepam, for managing agitation. No further data on dosage(s) and treatment duration have been provided (94).

Thornton et al. (95) reported a 23-year-old man with a previous psychiatric history who presented to an ED after developing a bizarre and disorganized behavior, suicidality, visual, tactile, and auditory hallucinations, and agitation following the intake of MDPV. He was treated with 6 mg of lorazepam and 2.4 mg of droperidol intravenously over 90 min in order to manage the agitation. He was also taking aripiprazole, valproic acid, lithium, quetiapine, and clonazepam for his psychiatric disorder. No further data on treatment duration and/or dosage(s) are available (95).

Winder et al. (100) described MDPV-induced paranoid psychosis in a 33-year-old male veteran without a previous psychiatric history. He was treated with as-needed doses of quetiapine and lorazepam for paranoid ideation, agitation, and anxiety. Symptomatology resolved within 12 h after admission. He was then started on an antidepressant (unspecified) for residual symptoms of depressed mood, anhedonia, and hopelessness. No further data on treatment duration and/or specific psychopharmacological treatment for MDPV-induced psychosis are available (100).

Bertol et al. (101) described a case of mixed MDPV and benzodiazepine intoxication in a 27-year-old chronic abuser with a previous SUD and a psychiatric history who developed severe agitation, disorganized and bizarre behavior, suicidality, and visual, tactile, and auditory hallucinations. He was managed with diazepam intravenously due to agitation and then he started chlorpromazine due to sleeplessness and aripiprazole for managing psychotic symptoms. No further data on treatment duration and/or dosage(s) are available (101).

Mangewala et al. (98) reported a psychotic onset (i.e., delirium, agitation, paranoia, and hallucinations) in a 16-year-old adolescent man without a previous personal and/or family psychiatric history who abused synthetic cathinones. He was treated with a combination of olanzapine (from 5 to 7.5 mg daily) and lorazepam (0.5 mg BID) (98).

Derungs et al. (89) reported a case of paranoia and psychosis induced by Naphyrone in a 31-year-old man. Psychopathological pattern comprised agitation, restlessness, insomnia, anxiety, and hallucinations. No further details have been provided regarding a specific psychopharmacological treatment of synthetic psychosis (89).

Two cases of severe intoxication delirium with paranoid psychosis and hallucinations following "bath salts" consumption are described by Kasick et al. (93).

Khan et al. (97) described a case of a 19-year-old female, without a family and/or personal psychiatric history, who developed a paranoid psychosis after consuming synthetic cathinones. She was treated with olanzapine and lorazepam. No further data on dosage(s) and/or treatment duration have been provided (97).

Adebamiro and Perazella (92) reported a case of a new-onset psychosis associated with renal and cardiovascular dysfunction following "bath salts" intoxication. The subject was treated with supportive care and hospitalized (92).

Gunderson et al. (96) described a case of paranoid psychosis induced by "bath salts" and diphenhydramine in a subject with a previous psychiatric history of major depressive disorder (MDD).

Stoica and Felthous (99) described a case of acute psychosis in a 30-year-old subject with a psychiatric diagnosis of bipolar affective disorder and schizophrenia following the intake of synthetic cathinones. Psychopathological pattern comprised suicidality, homicidal tendencies, euphoria, alertness, paranoid delusion, and increased levels of energy. The subject was hospitalized and started initially on valproic acid (250 mg orally BID) and trazodone (100 mg daily at night) for managing impulse dyscontrol and insomnia. No further data are available on treatment duration (99).

A case report described a psychotic onset, associated with the attempted murder of his father, in a man with a history of regular recreational use of a wide range of illicit drugs between 14 and 20 years following the intake of 3-methoxyphencyclidine (3-MeO-PCP) and MDPV (102). The patient experienced vivid hallucinations (auditory, visual, and tactile), panoramic visual hallucinations ("full brown eyes closed visuals" and imperative

voices saying "kill your father"), and bizarre ideas (e.g., "using house as a base for super heroes"), without other symptoms of schizophrenia such as delusional beliefs or thoughts disorder (102).

Several case reports of alpha-PVP (aka "Flakka")-induced psychosis have been described (103, 106, 107). Crespi et al. (103) described a 17-year-old who developed a Flakka-induced prolonged psychosis characterized by altered mental status, agitation, auditory hallucinations, disorganized behavior, and psychotic symptoms. She was managed with olanzapine and lorazepam (103). A 20-year-old male who ingested one tablet of Flakka developed a psychosis characterized by agitation, aggressiveness, hyper-religiosity, command auditory and visual hallucinations of serpents, suicidality, homicidal tendencies, and delusions. He was initially managed with 2 mg of lorazepam intramuscularly for agitation, then he was prescribed oral lorazepam 2 mg TID and aripiprazole 10 mg daily for 3 weeks, then titrated lorazepam 3 mg TID and aripiprazole 24 mg daily. After modest improvement, he was switched from lorazepam to clonazepam (2 mg BID) only for 3 days and then restarted on lorazepam, while aripiprazole was modified with quetiapine (800 mg daily), with marked improvement of psychotic symptoms and catatonia (106). Simonato et al. (107) reported a case of "Flakka-induced" psychosis successfully treated with bupropion.

A case of psychosis onset following the use of mephedrone in the context of ChemSex has been reported by Dolengevich-Segal et al. (104), as described in **Table 2**.

A cohort study reported the major complication of cathinone use as a prolonged psychosis, with a high proportion of cases among MDPV and methylone consumers (107). No further details have been provided regarding a specific psychopharmacological treatment of synthetic psychosis (105).

DISCUSSION

Overall, NPS use seems to exert stronger and more persistent and severe effects among subjects with SMI than in healthy subjects (i.e., without a previous psychiatric history), mostly due to activity in the dopaminergic system, implicated in managing behavior and thought processes and in determining psychosis, or in the serotonergic, noradrenergic, and/or glutamatergic systems (4, 108, 109) (as illustrated in **Table 1**). NPS may exert severe dissociative states, confusion in previously psychotic patients, relapse or worsening of a preexisting psychosis, persistent worsening of psychotic symptoms course, or the onset of a new severe psychotic symptomatology among healthy subjects (110, 111). In fact, the emergence of a psychotic symptomatology is commonly associated with a plethora of neurotransmission changes, e.g., increased central dopamine levels, cannabinoid CB_1 receptor activation, 5-HT_{2A} receptor activation, decreased activity in *N*-methyl--aspartate receptors, and k-opioid receptor activation. NPS may interfere at these neurobiological levels and facilitate the imbalance of several neurotransmitters and receptors (as illustrated in **Table 1**). For this reason, NPS use may really determine severe psychiatric symptoms, also in individuals not previously affected by a mental disorder. It is still unclear how the frequency of use (continuous vs. discontinued), the intensity of consumption (low, medium, or heavy), and the dosages (low vs. high dosages) may or not influence the development of a specific psychopathological pattern. Therefore, the advent of NPS has posed further clinical concern as not only are their clinical, toxicological and safety profiles often completely unknown (1) but they may also cause the onset of new psychopathological entities or the reemergence of "forgotten" psychopathological patterns, such as HPPD (4, 5).

Furthermore, the subtle gap/bridge between a "classical" vs. a "synthetic" psychosis is still the subject of clinical concern, as it seems the psychopathological, phenomenological, and clinical features of these two entities remain completely undefinable and clear. Moreover, this "gap" may have also meaningful effects in the choice of the best treatment and in defining the best outcome(s) and prognosis.

In addition, even though generically classified as NPS, not all NPS possess the same pharmacological and clinical profiles; hence, from a psychopathological point of view, clinicians may observe several types of NPS-induced "synthetic psychoses," depending on the substance involved. Similarly, a specific NPS may cause different psychopathological effects in vulnerable vs. non-vulnerable individuals (as shown in **Table 2**). With regard to this, the model of a substance-related exogenous psychosis (SREP) and its toxic subtype (aka "lysergic psychoma") may be helpful in shedding light to clinicians on differentiating classical/endogenous versus synthetic (NPS-induced) psychoses, from a clinical perspective. Unlike "classical/endogenous" psychosis, SREP is characterized by the following features: a) qualitative and quantitative consciousness alterations (i.e., crepuscular state and onyroid state); b) ego disorders (i.e., somatosensory/allopsychic); c) sensorial–perceptual disorders (i.e., visual, auditory, and coenesthetic); d) egodystonia (i.e., behaviors not coherent with self-image); e) mood swings; f) hyper-presentation of the time/"concrete" psychoses, i.e., alteration of the experienced space; g) modification of body perception; h) anhedonia/apathy/negative symptoms; and i) dyscontrol impulsivity and self-/hetero-aggressiveness (112) (as shown in **Table 3**). "Lysergic psychoma" is a phenomenological construct characterized by the perception of an "extraneous/foreign" body in one's own mind, in which the residual critical ego takes position against the intoxicated part of one's own self (113). It is a syndrome characterized by a clear egodystonic experience in

TABLE 3 | Classical psychosis vs. synthetic psychosis: psychopathological and phenomenological profile.

Endogenous psychosis (classical psychosis)	Exogenous psychosis (synthetic psychosis)
Lucid consciousness	Crepuscular consciousness
Thought disorders	Paraphrenia
Loss of contact with reality due to an ontological insecurity of the self	Loss of contact with reality due to an instability of the object ("floating world")
Primary, metaphysic, systematic delusion	Secondary, common, episodic delusion
Hallucinations	Pseudo-hallucinations
Transcendental ego	Empiric ego
Poor/absent insight, passivity	Present insight, activity
Bizarre and inexplicable behaviors	Aggressiveness and impulse dyscontrol
Apathy, anedonia, flattened affectivity	Overexcited, excessive affectivity

which a subject clearly feels and observes this "foreign entity" as an unusual experience, out of own control, accompanied by hallucinations (mainly visual and kinaesthetic) and delusional perceptions (and thoughts) which are completely resisted by the subject who tries to stem them (114). This psychotic experience is often described as self-limiting, intense, and brief and appears to spontaneously resolve after substance discontinuation, as also observed in several studies examined here (see **Table 2**). However, chronic, persistent NPS use, mainly at high dosages, may cause a complex, persistent, and long-lasting psychopathological pattern, defined as "synthetic psychosis," a paraphrenic syndrome due to a NPS-induced mental automatism which causes a psychotic trajectory (114). Furthermore, the pharmacodynamics and pharmacological profile of each NPS seem to be responsible for the psychopathological and clinical manifestation of each NPS-induced synthetic psychosis (as shown in **Tables 1**, **2**, and **4**) (1, 19, 115–117). Although there is still a need to clearly discriminate and characterize specific psychopathological and pathognomonic patterns depending on the specific NPS classes involved, as previously stated and shown in **Table 2**, an attempt had been made here to provide a critical summary of studies in order to describe some NPS-induced psychopathological clusters (**Table 4**).

Overall, after an acute or repeated consumption of SC, neurological toxidromes have been described, such as mental status changes, panic attacks, agitation, aggressiveness, memory distortions, depersonalization, dissociation, catatonia, recurrent psychotic episodes (e.g., delusional thoughts and paranoia), and auditory and/or visual hallucinations (1, 7, 96, 116–121). Psychosis developing in the context of a SC intake and/or intoxication is often described as endoformic (e.g., verbal hallucinations, kinesthetic automatisms, and delusion of grandeur or influence can be present), long-lasting (from 10–14 days to 4–6 weeks), and gradually self-resolve with the persistence of asthenic-depressive symptomatology and cognitive deterioration for more than 4–8 weeks (7, 38, 44, 87). However, several findings documented here show that SC-induced psychoses may persist even in those subjects without a previous history of mental illness and may induce the development of a schizophrenia-like symptomatology, named "Spiceophrenia" (7, 25, 111, 122–125). In addition, there is evidence of a worsening/recrudescence of a mental health disorder (i.e., mainly an affective and/or a psychotic disorder) in those subjects with a preexisting mental condition (29, 34, 35, 50, 56–59, 61, 67, 69, 80, 84, 85, 111, 122). Some studies reported that SC may influence psychiatric course and prognosis, depending on the first age of SC exposure, psychiatric vulnerability/predisposition, a history of a childhood trauma or other traumatic experiences, and specific genetic factors (67, 69, 124). Furthermore, SC have been supposed to determine a more severe psychosis, accompanied with agitation and significant sympathomimetic effects, compared to "classical" cannabis as SC are more potent full receptor agonists at cannabinoid receptors and do not contain cannabidiol, which possesses anxiolytic and antipsychotic properties (1, 7, 70, 126). Despite the evidence presented here, some authors maintain that it is difficult to clearly prove a causal linkage between SC intake and the onset of an *ex novo* psychosis in psychosis-prone/vulnerable subjects and/or the exacerbation of a prodromal psychotic syndrome/appearance of basic symptoms (127). From a therapeutic perspective, benzodiazepines are useful for managing

TABLE 4 | Clinical variants of NPS-induced psychosis.

Synthetic psychosis with predominant delirium symptoms induced by NPS mainly acting on GABA, Ach, DA, SER, NA, and GLU pathways	• Delirium • Tactile and auditory verbal hallucinations • Kandisnky–Clerambault's syndrome (delusion of influence, automatism)
Synthetic psychosis with predominant dissociative reactions induced by NPS mainly acting on GLU pathways	• Dissociation, derealization • Somatopsychic depersonalization • Illusions, perception distortions • Bodily and kinesthetic hallucinations • "Near-death" experiences • Demonic possession experiences • Affective blunting, anhedonia, anesthesia • Ekbom syndrome (delusions of infestation, delusional parasitosis) • Capgras syndrome (delusional belief that someone known has been replaced by an imposter, delusional misidentification syndrome) • Cotard syndrome (delusional belief to be already dead, to not exist, to be putrefied, to have lost own blood or internal organs)
Synthetic psychosis with predominant paranoia and auditory hallucinations induced by NPS mainly acting on DA pathways	• Paranoia thought • Auditory hallucinations • Delusions of reference, persecution, grandeur, and jealousy • Hypomanic states • Aggressiveness and irritability • Dysphoria, anxiety, and panic
Synthetic psychosis with predominant hallucinatory symptoms induced by NPS mainly acting on SER pathways	• Anxiety, irrational fear, and psychic hyperesthesia • Acute verbal hallucinosis ± sensual delusion • Visual hallucinations with vivid colors associated with intense emotional experiences (either positive or negative) • (Eventually kinesthetic and/or tactile hallucinations) • Auditory, olfactory, and gustatory hallucinations uncommon • (Eventually paranoid delusions, with religious content) • (Eventually hypomanic state, suicidal thoughts, and depression)
Synthetic psychosis with predominant affective-delusional symptoms induced by NPS mainly acting on SER and DA pathways	• Sensation of fear • Delusional ideas of interpretation and reference • Persecutory, philosophical, and esoteric delusion • In some cases, manioform statements
Synthetic psychosis with predominant mental automatism induced by NPS mainly acting on mixed pathways	• Emotional tension • Severe anxiety and confusion • Delusional mood, agitation, and delusional persecutory ideas • Mental, motor, and senestopathic automatisms • Thought broadcasting, thought insertion, or withdrawal • Speech automatism • Kandinsky–Clerambault's syndrome with verbal hallucinations, delusion of influence and persecutory delusion, as well as automatism

GABA, gamma-aminobutyric acid; Ach, acetylcholine/cholinergic; DA, dopaminergic; SER, serotoninergic; Glu, glutammatergic; NA, noradrenergic.

anxiety, agitation, and seizure risk, together with a supportive/symptomatic therapy (1, 7, 30, 32, 36, 39, 41, 46, 48, 59–61, 71, 79, 81). Olanzapine, clozapine, quetiapine, and aripiprazole represent the main prescribed antipsychotic treatments in SC-induced psychoses (1, 7, 37, 50, 57, 60, 62, 65–67, 70, 75, 85, 86), while haloperidol, risperidone, and paliperidone have been prescribed/used in isolated cases (45, 53, 63, 73, 85).

In contrast, published findings reported that synthetic cathinones may cause variable effects in patients with SMI, such as acute psychosis, agitation, violent behavior, confusion, disorientation, insomnia, suicidality, mood lability, and instability in patients affected with bipolar disorder and/or substance poly abuse, while tangential thought process, disorganized speech and behavior, paranoid delusions, and auditory and visual hallucinations are demonstrated in patients affected with schizophrenia, associated with amnesia surrounding their psychotic breaks (88, 90, 91, 93, 94, 96, 100–102, 104, 106, 128). Treatment should be single-minded on managing agitation and psychosis and in supporting renal perfusion. Sedation may be required if the patient is markedly agitated and at risk of self-harm or harm other patients or health professionals (1, 92, 95, 97, 98, 103, 104). In particular, benzodiazepines should be preferred to manage physical violence, to decrease tachycardia and blood hypertension, to prevent seizures, and to reduce muscle hyperactivity, rhabdomyolysis, and renal failure (1). Use of antipsychotics alone should be considered as second-line strategy for managing agitation due to the higher risk in lowering seizure threshold and the higher risk in contributing to the acute toxicity of synthetic cathinones (1, 88, 97, 98, 106). Alternatively, anticonvulsants/mood stabilizers have been suggested (99).

The present review has several limitations which may restrict the generalizability of the findings presented here, e.g., most studies considered here have mainly focused on synthetic cannabinoids which represent the main representative NPS group, but do not necessarily represent all NPS-induced synthetic psychoses. In addition, most studies are represented by case reports, which, on the one hand, may represent a good tool/vehicle to provide more detailed psychopathological and clinical features on NPS consumption and NPS-induced psychosis, but it may not be greatly generalizable to a more representative clinical sample. Most studies do not report if NPS intake is acute or chronic and single or repeated, or do not clearly specify NPS dosage, route of administration, comorbid use of other substances, and so on. Furthermore, the present overview does not possess the scientific robustness of a systematic literature review as most studies are poorly comparable with heterogeneous outcomes and measures. Finally, from a therapeutic point of view, not all studies here considered provide data on therapeutic management and/or strategies. Moreover, those studies providing therapeutic management do not always report dosage(s) and/or treatment duration.

CONCLUSION

The constantly and rapidly developing NPS drug scenario represents a challenge for public health, especially so for the field of addiction and mental health. Indeed, NPS consumption is usually associated with an imbalance of a plethora of neurotransmitter pathways and brain receptors, and, hence, correlated to a huge repertory of psychopathological trajectories. Vulnerable individuals (e.g., children, adolescents, and subjects affected with a psychiatric disorder) may be more prone to develop and/or worsen a psychopathological condition, particularly a psychosis which may have peculiar features, from a psychopathological and phenomenological point of view. Due to a great range of medical and psychopathological disturbances associated with NPS intake, it is detrimental for mental health professionals not to be ignorant about the effects, safety, and toxicity profile of NPS so far known, especially the most widespread ones discussed here, e.g., SC and synthetic cathinones. Indeed, further researches should better investigate their clinical and pharmacological knowledge so that better personalized management and treatment strategies/guidelines can be written and disseminated.

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All authors listed have made a substantial direct and intellectual contribution to the work and approved it for publication. LO and SC collected, analyzed and interpreted data. LO, SC, together with JC and DP drafted the article. DD and FS revised it critically for important intellectual contents.

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Neurochemical and Behavioral Profiling in Male and Female Rats of the Psychedelic Agent 25I-NBOMe

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4-Iodo-2,5-dimethoxy-*N*-(2-methoxybenzyl)phenethylamine (25I-NBOMe), commonly called "N-Bomb," is a synthetic phenethylamine with psychedelic and entactogenic effects; it was available on the Internet both as a legal alternative to lysergic acid diethylamide (LSD) and as a surrogate of 3,4-methylenedioxy-methamphetamine (MDMA), but now it has been scheduled among controlled substances. 25I-NBOMe acts as full agonist on serotonergic 5-HT2A receptors. Users are often unaware of ingesting fake LSD, and several cases of intoxication and fatalities have been reported. In humans, overdoses of "N-Bomb" can cause tachycardia, hypertension, seizures, and agitation. Preclinical studies have not yet widely investigated the rewarding properties and behavioral effects of this compound in both sexes. Therefore, by *in vivo* microdialysis, we evaluated the effects of 25I-NBOMe on dopaminergic (DA) and serotonergic (5-HT) transmissions in the nucleus accumbens (NAc) shell and core, and the medial prefrontal cortex (mPFC) of male and female rats. Moreover, we investigated the effect of 25I-NBOMe on sensorimotor modifications as well as body temperature, nociception, and startle/prepulse inhibition (PPI). We showed that administration of 25I-NBOMe affects DA transmission in the NAc shell in both sexes, although showing different patterns; moreover, this compound causes impaired visual responses in both sexes, whereas core temperature is heavily affected in females, and the highest dose tested exerts an analgesic effect prominent in male rats. Indeed, this drug is able to impair the startle amplitude with the same extent in both sexes and inhibits the PPI in male and female rats. Our study fills the gap of knowledge on the behavioral effects of 25I-NBOMe and the risks associated with its ingestion; it focuses the attention on sex differences that might be useful to understand the trend of consumption as well as to recognize and treat intoxication and overdose symptoms.

Keywords: novel psychoactive substances, sex differences, dopamine, serotonin, behavior

INTRODUCTION

The appearance of new psychoactive substances (NPS) is changing the trend in drug use worldwide (Orsolini et al., 2015; Miliano et al., 2016; UNODC, 2016; Corkery et al., 2017; Orsolini et al., 2017), which includes also their safely perceived purchase on the Internet (Miliano et al., 2018). As a matter of fact, in the last decades the classical drugs of abuse are being progressively substituted by their "legal synthetic alternatives" or simply co-abused with these powerful substances. In 2014, phenethylamines were the second most used class of NPS, after synthetic cannabinoids (UNODC, 2014; UNODC, 2016), while now the trend is moving to synthetic opioids (EMCDDA, 2019). These compounds are usually abused for their psychedelic and entactogenic effects mainly by people who attend electronic dance music, parties at nightclubs, and festivals (Palamar et al., 2016; Schifano et al., 2016; Palamar et al., 2017) in order to reach a "dissociative state" from reality, which unfortunately might lead to severe clinical issues (Schifano et al., 2019). Phenethylamines are a large family of compounds that are molecular variants of the core compounds, i.e., amphetamines, 3,4-methylenedioxy-methamphetamine (MDMA), etc. (Le Roux et al., 2015). The *N*-benzylmethoxy derivatives of the 2C hallucinogens (i.e., 2C-I, 2C-B, and 2C-C), commonly called NBOMes (4-Iodo-2,5-dimethoxy-*N*-(2-methoxybenzyl)phenethylamine), are probably the most famous; they used to be marketed as a legal lysergic acid diethylamide (LSD), with aliases such as "Smiles" and "N-bombs." They act as full agonist on human and rat 5-HT2A receptors (Ki = 0.044 nM and Ki = 0.087 nM, respectively), with high affinity (Braden et al., 2006). As a consequence, low doses of the order of 50 μg are able to produce psychoactive effects (Kyriakou et al., 2015; Suzuki et al., 2015). They are usually ingested sublingually, orally, by insufflations, rarely intravenously, and it seems to be active at doses as low as 50–250 μg, but the typical dose range is 500–800 μg (Halberstadt and Geyer, 2014). The duration of action depends on the route of administration, ranging from 4–6 h (insufflation) to 6–10 h (sublingual). According to analysis performed in seizures of these products, 25I-NBOMe seems to be the most present compound. The central effects of these substances are due to the activation of 5-HT2A receptors, which are heavily expressed in cortical and forebrain areas, various brainstem nuclei, and the hippocampus (Cornea-Hébert et al., 2002), but side effects are both central and peripheral. Indeed, overdoses of "N-Bomb" can cause several toxicological effects such as tachycardia, hypertension, seizures, and agitation persisting for up to 3 days (Hill et al., 2013; Kelly et al., 2013; Rose et al., 2013; Stellpflug et al., 2014; Hieger et al., 2015). Several intoxication cases and some fatalities have been reported after the recreational use of 25-NBOMes (Walterscheid et al., 2014; Andreasen et al., 2015; Laskowski et al., 2015; Kueppers and Cooke, 2015; Shanks et al., 2015; Suzuki et al., 2015; Adamowicz et al., 2016; Morini et al., 2017). Therefore, all the 2C *N*-benzyl-methoxy derivatives were banned and scheduled as illegal drugs in many countries, such as Italy, USA, Canada, Russia, Sweden, and China (https://www.erowid.org/chemicals/2ci_nbome/2ci_nbome_law.shtml). Furthermore, the recurring use of these potent 5-HT2A agonists may contribute to develop seizures and the serotonin syndrome (Bosak et al., 2013), an excessive serotonergic activation that results in specific clinical signs, such as tremor, diarrhea, and delirium, neuromuscular rigidity, and hyperthermia in life-threatening cases (Boyer and Shannon, 2005). Despite the widespread use of these compounds, and the effects reported in humans, there is a lack of knowledge about their behavioral or toxicological effects. The serotonergic psychedelic effects of this compound have been confirmed by behavioral responses such as head twitch in C57BL/6J mice (NBOMe 0.1–1 mg/kg, s.c.) (Halberstadt and Geyer, 2014), wet dog shakes, and back muscle contraction (0.01–3 mg/kg, s.c.) in rats (Elmore et al., 2018), and all these effects were prevented by the administration of the selective 5-HT2A antagonist M100907 (Halberstadt and Geyer, 2014; Elmore et al., 2018). Indeed, 25I-NBOMe time-dependently and dose-dependently decreased locomotor activity in mice (Eshleman et al., 2014; Gatch et al., 2017) and showed full substitution of LSD in rats drug discrimination and more than 50% of appropriate responding in MDMA-trained rats (Eshleman et al., 2014). The MDMA-like action of 25I-NBOMe has been also confirmed *in vitro* since it acts inhibiting the monoamine reuptake transporters with different IC_{50} (hSERT, IC_{50} = 4.3 μM; hDAT, IC_{50} = 75 μM; hNET, IC_{50} = 19 μM) in HEK 293 cells (Zwartsen et al., 2017). Recently, it has been demonstrated that this compound increases dopamine (DA) and serotonin (5-HT) in the frontal cortex of male Wistar–Han rats when administered at 3 mg/kg subcutaneously (Herian et al., 2019). Moreover, DA levels in mice synaptosomal striatal fractions were increased by *in vitro* administration of this substance, and male mice showed an increased conditioned place preference when injected with 25I-NBOMe (0.3 mg/kg, i.p.) (Jeon et al., 2019). Conversely, male Sprague–Dawley rats did not press the active lever to obtain an infusion of 25I-NBOMe (Jeon et al., 2019). Collectively, the results available so far suggest a putative abuse liability of this compound, but further investigations on the neurochemical and behavioral effects seem to be necessary. Therefore, the aim of this study was to evaluate the effect of 25I-NBOMe on DA and 5-HT transmissions, performing *in vivo* microdialysis in three terminal areas strongly involved in reward and drug seeking [nucleus accumbens (NAc) shell and core and medial prefrontal cortex (mPFC)]. Moreover, several behavioral tests were performed to assess sensorimotor impairment as well as the risk of developing hyperthermia and have an altered nociceptive response under the effect of 25I-NBOMe. Additionally, due to the possibility to develop drug use-related psychotic disorders, we included an analysis of the acoustic startle reflex (prepulse inhibition, PPI) that is considered a marker of vulnerability for neuropsychiatric disorders (Siegel et al., 2013; Marti et al., 2019). Moreover, sex differences in drug addiction behavior have been extensively reported in both humans and rodents (Becker et al., 1982; Jackson et al., 2006; Zhao and Becker, 2010; Cummings et al., 2014; Fattore et al., 2014). The reason of such great differences is due to

Abbreviations: DA, Dopamine/dopaminergic; DOI, (±)-2,5-Dimethoxy-4-iodoamphetamine; HPLC, High-performance liquid chromatography; 25I-NBOMe, 4-Iodo-2,5-dimethoxy-*N*-(2-methoxybenzyl)phenethylamine; LSD, Lysergic acid diethylamide; MDMA, 3,4-Methylenedioxy-methamphetamine; mPFC, Medial prefrontal cortex; NAc, Nucleus accumbens; NPS, Novel psychoactive substances; PPI, Prepulse inhibition; 5-HT, Serotonin/serotonergic.

dimorphisms in the anatomy of the reward brain circuits (Walker et al., 2012), differences in the intrinsic properties of DA neurons (Melis et al., 2013), as well as ovarian hormone fluctuations (Becker and Hu, 2008; Castelli et al., 2014).Considering the lack of data about the effects of this compound on females, we decided to perform the entire experimental study in both male and female rats in order to underline possible sex differences. In addition, in order to try to explain why adolescent girls seem to be more susceptible at intense negative psychoactive effects of MDMA (Liechti et al., 2001), and generally more vulnerable to develop hallucinogen dependence (Wu et al., 2009; Wu et al., 2010), in an initial stage of the study, we investigate the relationship among different estrous cycle phases and extracellular DA and 5-HT levels in response to acute 25I-NBOMe administration.

MATERIALS AND METHODS

Animals

Male and female Sprague–Dawley rats, weighing 275–300 g (Harlan Italy), were used for *in vivo* microdialysis and behavioral tests. Rats were housed four per cage, in standard plastic cages with wood chip bedding, at temperature of 22 ± 2°C and 60% humidity and under a 12-h light/dark cycle (lights on from 7.00 a.m.). Tap water and standard laboratory rodent chow (Mucedola, Settimo Milanese, Italy) were provided *ad libitum* in the home cage. All animal experiments were carried out in accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research according to Italian (D.L. 116/92 and 152/06) and European Council directives (609/86 and 63/2010) and in compliance with the approved animal policies by the Ethical Committee for Animal Experiments (CESA, University of Cagliari) and the Italian Ministry of Health (Aut. N. 162/2016- PR; Aut. N.352/2015-PR). All animals were handled once daily for 5 min for five consecutive days before the beginning of the behavioral tests. We made all efforts to minimize pain and suffering and to reduce the number of animals used.

Substances and Doses

25I-NBOMe was purchased from LGC Standards S.r.l. (Milan, Italy), dissolved in 2% EtOH, 2% Tween 80, and 96% saline, and administered intraperitoneally (3 ml/kg) at different doses. A wider range of doses of 25I-NBOMe (0.1, 0.3, 0.5, and 1.0 mg/kg, i.p.) were chosen for behavioral tests in order to assess which was the lower effective dose both in male and female rats. The most effective doses (0.3 and 1.0 mg/kg, i.p.) were chosen for microdialysis experiments in order to minimize the number of rats.

Determination of the Estrous Cycle Phases in Female Rats

Before starting experiments, the estrous cycle of female rats was monitored every day for 15 days, collecting vaginal smears in the early morning (between 8:00 and 9:00 a.m.). Vaginal secretion was collected by flushing into the vagina and out (two to three times) 20 µl of saline (NaCl 0.9%). The pipette tip was inserted gently and not deeply in order to avoid cervical stimulation (Cora et al., 2015). One vaginal fluid drop per rat was placed on glass slides. Unstained material was observed by bright-field microscopy, with ×20 and ×40 objective lenses (Marcondes et al., 2002). Observing the cytology of vaginal smear, it is possible to discriminate three cell types, and it is well established that the proportion among them corresponds to a particular phase of estrous cycle in rodents (Goldman et al., 2007; Cora et al., 2015). In this way, we were able to determine the following: proestrus (predominant nucleated epithelial cells); estrus (anucleuated cornified cells); metestrus (leukocytes, cornified, and nucleated epithelial cells in the same proportion); and diestrus (predominant little round leukocytes) (data not shown).

In Vivo Microdialysis Studies

Surgery

Male and female Sprague–Dawley rats (275–300 g; Harlan, Italy) were anaesthetized with isoflurane gas and maintained under anesthesia using a breathing tube under a scavenging system while placed in a stereotaxic apparatus and implanted with vertical dialysis probes prepared as previously described (De Luca et al., 2015) with 1.5 or 3 mm dialyzing portion for NAc or mPFC, respectively. According to the rat brain atlas of Paxinos and Watson (1998), animals were implanted in the NAc shell (A +2.2, L +1.0 from bregma; V −7.8 from dura) or core (A +1.4, L +1.6 from bregma; V−7.6 from dura), or in the mPFC (A +3.7, L +0.8 from bregma; V −5.0 from dura).

Analytical Procedure

On the day following surgery, animals were connected to an infusion pump and probes were perfused with Ringer's solution (147 mM NaCl, 4 mM KCl, and 2.2 mM $CaCl_2$) at a constant rate of 1 µl/min. After a washout of 1 h, dialysate samples (20 µl) were collected every 20 min and injected into an HPLC equipped with a reversed-phase column (C8 3.5 um, Waters, USA) and a colorimetric detector (ESA, Coulochem II; ESA-CDS software) to quantify DA and 5-HT. The electrodes of the analytical cell were set at +125 mV (oxidation) and −175 mV (reduction) to detect dopamine and at −175 mV (oxidation) and +220 mV (reduction) to detect serotonin. The mobile phase contained 50 mM NaH_2PO_4, 0.1 mM Na_2EDTA, 0.5 mM n-octyl sulfate, and 15% (*v*/*v*) methanol to evaluate dopamine concentration and 22% (*v*/*v*) methanol for serotonin detection (the pH of mobile phase was adjusted with Na_2HPO_4 to 5.5 and 5.7 for dopamine and serotonin, respectively). The sensitivity of the assay for DA/5-HT was 5 fmol/sample. Basal dialysate were collected until DA and 5-HT levels did not differ more than 10% in three consecutive samples. The average value was considered as the basal levels of DA/5-HT. The animals were treated with saline or 25I-NBOMe and monoamine levels were monitored for 2 h after the treatment. At the end of the 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.

Statistical Analysis of Microdialysis Experiments

All the numerical data are given as mean ± SEM. Data were analyzed by utilizing one-way ANOVA or repeated measures ANOVA (two-way and three-way). Results from treatments showing significant overall changes were subjected to Tukey's tests for *post hoc* comparisons, with significance at $p < 0.05$.

Behavioral Studies

The differential effects of 25I-NBOMe were investigated using a battery of behavioral tests widely used in studies of "safety-pharmacology" for the preclinical characterization of NPS in rodents (De Luca et al., 2015; Vigolo et al., 2015; Ossato et al., 2015; Ossato et al., 2016; Canazza et al., 2016; Canazza et al., 2017; Giannotti et al., 2017; Fantinati et al., 2017; Marti et al., 2019). To reduce the animal's stress induced by manipulation, and to confirm the stability and reproducibility over time of the responses of our tests, animals were trained two times per week for 2 weeks before the pharmacological treatment. All experiments were performed between 8:30 a.m. and 2:00 p.m. Experiments were conducted in blind by trained observers working together in pairs (Ossato et al., 2016). The behavior of rats (sensorimotor responses) was videotaped and analyzed off-line by a different trained operator that gives test scores. The behavioral tests were performed in a consecutive manner using six rats per treatment, according to the following sequence: measures of visual object responses (frontal and lateral views), overall tactile response (pinna, vibrissae, and corneal reflexes), acoustic response, measures of core body temperature (rectal measurement), visual placing response, and determination of the mechanical (tail pinch) acute pain. Despite the repetition of tests during the time, no changes in parameters such as body core temperature and responses to noxious stimuli, which are sensitive to stressful situations (Adriaan Bouwknecht et al., 2007; Kozlov et al., 2015), have been observed in naive animals and in saline/vehicle-treated animals. The startle and prepulse inhibition studies, instead, were performed on another cohort of rats using seven animals per treatment.

Sensorimotor Studies

We studied the voluntary and involuntary sensorimotor responses resulting from different rat reactions to visual, acoustic, and tactile stimuli (Ossato et al., 2015; Marti et al., 2019).

Evaluation of the Visual Response

Visual response was verified by two behavioral tests, which evaluated the ability of the rat to capture visual information even when the animal is stationary (the visual object response) or when it is moving (the visual placing response). Visual object response test was used to assess the ability of the rat to see an object approaching from the front or side that causes the animal to move or turn the head or withdraw it (Marti et al., 2019). For the frontal visual response, a white horizontal bar was moved frontally to the rat head and the maneuver was repeated three times. For the lateral visual response, a small dentist's mirror was moved into the rat's field of view in a horizontal arc, until the stimulus was between the rat's eyes. The procedure was conducted bilaterally and was repeated three times. The score assigned was a value of 1 if there was a reflection in the rat movement or 0 if not. The total value was calculated by adding the scores obtained in the frontal with that obtained in the lateral visual object response (overall score, 9). Evaluation of the visual object response was measured at 0, 5, 30, and 60 min post-injection. Visual placing response test is performed using a tail suspension modified apparatus able to bring down the rat towards the floor at a constant speed of 10 cm/s (Marti et al., 2019). A camera videotapes the downward movement of the rat. The analysis frame by frame allows evaluating the beginning of the reaction of the rat while it is close to the floor. When the rat starts the reaction, an electronic ruler evaluates the perpendicular distance in millimeters between the eyes of the rat to the floor. The naive rats perceive the floor and it prepares to contact at a distance of about 27 ± 4.5 mm. Evaluation of the visual placing response was measured at 0, 15, 40, and 70 min post-injection.

Evaluation of Acoustic Response

Acoustic response measures the reflex of the rat in replay to an acoustic stimulus produced behind the animal. In particular, four acoustic stimuli of different intensities and frequencies were tested (Marti et al., 2019). Each sound test was repeated three times, giving a value of 1 if there was a response and 0 if not present, for a total score of 3 for each sound. The acoustic total score was calculated by adding scores obtained in the four tests (overall score, 12). Evaluation of the visual object response was measured at 0, 10, 30, and 60 min post-injection.

Evaluation of Tactile Response

The overall tactile response in the rat was verified through vibrissae, pinna, and corneal reflexes (modified from Ossato et al., 2018; Marti et al., 2019). Vibrissae reflex was evaluated by touching vibrissae (right and left) with a thin hypodermic needle once for side, giving a value of 1 if there was a reflex (turning of the head to the side of touch or vibrissae movement) or 0 if not present (overall score, 2). Evaluation of the vibrissae reflex was measured at 0, 5, 30, and 60 min post-injection. Pinna reflex was assessed by touching pavilions (left and right) with a thin hypodermic needle. First, the interior pavilions and then the external. This test was repeated twice for side, giving a value of 1 if there was a reflex and 0 if not present (overall score, 4). Evaluation of the pinna reflex was measured at 0, 5, 30, and 60 min post-injection. Corneal reflex was assessed gently touching the cornea of the rat with a thin hypodermic needle and evaluating the response, assigning a value of 1 if the rat moved only the head, 2 if it only closed the eyelid, and 3 if it closed the lid and moved the head. The procedure was conducted bilaterally (overall score, 6) and was measured at 0, 5, 30, and 60 min post-injection.

Evaluation of Core and Surface Body Temperature

To assess the effects of 25I-NBOMe on thermoregulation, we measured both changes in the core (rectal) and surface (ventral fur) temperature. The core temperature was evaluated by a probe (1-mm diameter) that was gently inserted, after lubrication with liquid vaseline, into the rectum of the rat (to about 2 cm) and left in position until the stabilization of the temperature (about 10 s; Marti et al., 2019). The probe was connected to a Cole Parmer digital thermometer, model 8402. The surface temperature was measured by a Microlife FR 1DZ1 digital infrared thermometer,

placed at 1 cm from the surface of the abdomen of the rat (Marti et al., 2019). Core and surface rat body temperatures were measured at 0, 10, 35, and 65 min.

Evaluation of Pain Induced by a Mechanical Stimulation of Tail

Acute mechanical nociception was evaluated using the tail and hind paw pinch tests (modified by (Vigolo et al., 2015). A special rigid probe connected to a digital dynamometer (ZP-50N, IMADA, Japan) was gently placed on the tail (in the distal portion) or the hind paw of the rat and a progressive pressure was applied. When the rat flicked its tail or removed the hind paw, the pressure was stopped and the digital instrument saved the maximum peak of weight supported (g/force). A cutoff (500 g/force) was set to avoid tissue damage. The test was repeated three times and the final value was calculated with the average of three obtained scores. Acute mechanical nociception was measured at 0, 15, 40, and 70 min post-injection.

Startle and Prepulse Inhibition Analysis

Startle and prepulse inhibition studies were performed as previously reported (Marti et al., 2019). Male and female rats were tested for acoustic startle reactivity in startle chambers (Ugo Basile apparatus, Milan, Italy) consisting of a sound-attenuated, lighted, and ventilated enclosure holding a transparent non-restrictive Perspex® cage (modified version for rats 200 × 90 × 80 mm). A loudspeaker mounted laterally the holder produced all acoustic stimuli. Peak and amplitudes of the startle response were detected by a load cell. At the onset of the startling stimulus, 300-ms readings were recorded and the wave amplitude evoked by the movement of the rat startle response was measured. Acoustic startle test sessions consisted of startle trials (pulse-alone) and prepulse trials (prepulse + pulse). The pulse-alone trial consisted of a 40-ms 120-dB pulse. Prepulse + pulse trials sequence consisted of a 20-ms acoustic prepulse, 80-ms delay, and then a 40-ms 120-dB startle pulse (100-ms onset–onset). There was an average of 15 s (range = 9–21 s) between the trials. Each startle session began with a 10-min acclimation period with a 65-dB broadband white noise that was present continuously throughout the session. The test session contained 40 trials composed by pulse-alone and prepulse + pulse trials (with three different prepulses of 68, 75, and 85 dB) presented in a pseudorandomized order. Male and female rats were placed in the startle chambers 5 min after treatment with 25I-NBOMe. The entire startle/PPI test lasted 20 min. The amount of PPI was expressed as the percentage decrease in the amplitude of the startle reactivity caused by the presentation of the prepulse (% PPI). 25I-NBOMe (0.1–1 mg/kg, i.p.) was administered intraperitoneally and startle/PPI responses were recorded 30 min (including the 10-min acclimation period) after drug injections.

Statistical Analysis of Behavioral Tests

Core and surface temperature values are expressed as the difference between control temperature (before injection) and temperature following drug administration (Δ°C). Antinociception (tail pinch tests) is calculated as percent of maximal possible effect $\{E_{Max\%} = [(\text{test} - \text{control latency})/(\text{cut-off time} - \text{control})] \times 100\}$. Data are expressed in absolute values, Δ°C (core and surface temperature), $E_{max\%}$ (tail pinch tests), and arbitrary units (tail rigidity). In sensorimotor response experiments, data are expressed in arbitrary units (visual objects response, acoustic response, vibrissae, corneal, and pinna reflex) and percentage of baseline (visual placing response). The statistical analyses of the effects of the individual substances in different concentrations over time and that of antagonism studies in histograms were performed by ANOVA (two-way) followed by Bonferroni's test for multiple comparisons. The statistical analysis was performed with the program Prism software (GraphPad Prism, USA). The amount of PPI was calculated as a percentage score for each prepulse + pulse trial type: %PPI = 100 − {[(startle response for prepulse + pulse trial)/(startle response for pulse-alone trial)] × 100}. Startle magnitude was calculated as the average response to all pulse-alone trials. All the numerical data are given as mean ± SEM. Data were analyzed by utilizing repeated measures ANOVA. The statistical analysis was performed with the program Prism software (GraphPad Prism, USA).

RESULTS

Evaluation of Estrous Cycle

Initially, we evaluated the estrous cycle phase prior to the microdialysis experiment, as described in *Materials and Methods*. Three-way ANOVA was performed comparing proestrus–estrus phase with metestrus–diestrus, and no significant differences have been observed. For this reason, we decided to not show those data and combine the female data across the estrous cycle for simplicity.

Dopamine and Serotonin Basal Levels

Rat basal values of DA, expressed as fmol/20 µl sample (mean ± SEM), were in males: NAc shell 48 ± 9 (N = 7), NAc core 50 ± 13 (N = 5), mPFC 19 ± 3 (N = 8); in females: NAc shell 53 ± 6 (N = 23), NAc core 56 ± 4 (N = 18), mPFC 22 ± 2 (N = 19) (see **Figures 1A**). Rat basal values of 5-HT, expressed as fmol/20 µl sample (mean ± SEM), were in males: NAc shell 10 ± 1 (N = 11), NAc core 8 ± 2 (N = 8), mPFC 12 ± 4 (N = 10); in females: NAc shell 15 ± 2 (n = 23), NAc core 17 ± 3 (n = 18), mPFC 18 ± 3 (n = 21) (see **Figure 1B**). One-way ANOVA was performed for each terminal area, revealing no sex differences in both DA and 5-HT basal outputs (as shown in **Figures 1A**, **B**).

Effect of 25I-NBOMe Administration on DA Transmission in the NAc Shell and Core and in the mPFC

Males

In this experiment, we evaluated the effect of two doses of 25I-NBOMe (0.3 and 1.0 mg/kg, i.p.) on extracellular DA levels in NAc shell and core and the mPFC (**Figures 2A–C**). As shown in **Figure 2**, this phenethylamine affects DA transmission to a small extent only in NAc shell and core, but not in mPFC in male rats. Three-way ANOVA showed a main effect of treatment ($F_{2,30}$ = 6.50,

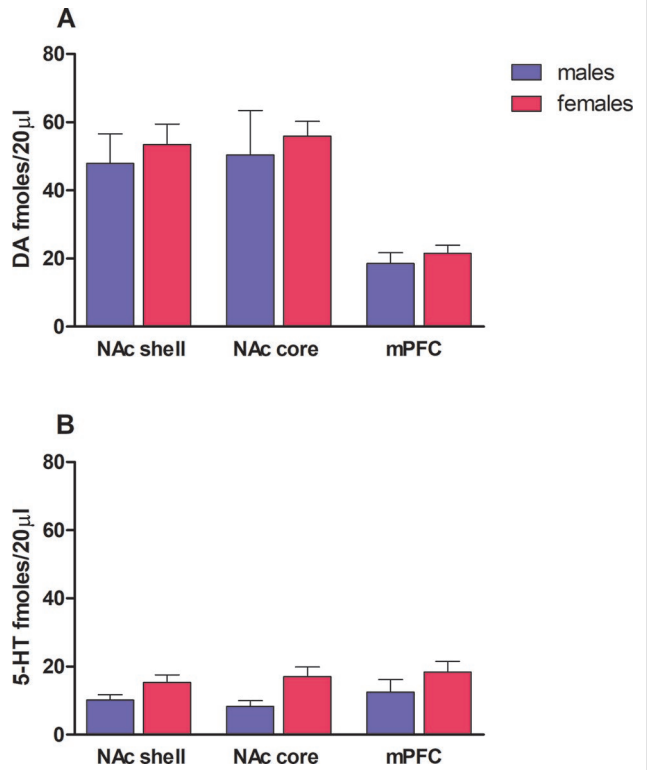

FIGURE 1 | Rat dopamine **(A)** and serotonin **(B)** basal levels in both male (*blue bars*) and female (*magenta bars*) rats. Results are expressed as the mean ± SEM of change in DA/5-HT extracellular levels expressed as fmol/20 µl sample in nucleus accumbens (NAc) shell, NAc core, and mPFC.

$^*p < 0.05$) and time × area interaction ($F_{12,180} = 2.18$, $^*p < 0.05$). In animals implanted in NAc shell, two-way ANOVA showed a main effect of treatment ($F_{2,12} = 7.79$, $^*p < 0.01$) and time ($F_{6,72} = 3.62$,$^*p < 0.01$). Tukey's *post hoc* tests showed a larger increase of dialysate DA in the NAc shell after 25I-NBOMe 0.3 mg/kg, i.p., revealing differences at the 20-min sample with respect to basal values (**Figure 2A**). In animals implanted in NAc core, two-way ANOVA showed a main effect of time ($F_{6,12} = 3.10$, $^*p < 0.05$). Tukey's *post hoc* tests showed a larger increase of dialysate DA in the NAc core, revealing differences at the 40-min sample with respect to basal values (**Figure 2B**). In animals implanted in mPFC, two-way ANOVA showed no significant effects (**Figure 2C**).

Females

In this experiment, we evaluated the effect of two doses of 25I-NBOMe (0.3 and 1.0 mg/kg, i.p.) on extracellular DA levels in NAc shell and core and the mPFC (**Figures 2D–F**). The dopamine transmission is affected by the administration of the drug in the NAc shell and lightly in the mPFC, but not in the NAc core. Three-way ANOVA showed a main effect of treatment ($F_{2,83} = 10.33$, $^*p < 0.0001$), time ($F_{6,498} = 6.63$, $^*p < 0.0001$), time × area interaction ($F_{12,498} = 2.55$, $^*p < 0.005$), and time × treatment interaction ($F_{12,498} = 3.08$, $^*p < 0.0005$). In animals implanted in NAc shell, two-way ANOVA showed a main effect of treatment ($F_{2,31} = 3.65$, $^*p < 0.05$), and time × treatment interaction ($F_{12,186} = 2.47$, $^*p < 0.01$). Tukey's *post hoc* tests showed a larger increase of dialysate DA in the NAc 16 shell after 25I-NBOMe 0.3 mg/kg, i.p., revealing differences at the at the 40-, 60-, and 120-min samples with respect to basal values (**Figure 2D**). In animals implanted in NAc core, two-way ANOVA showed no effects (**Figure 2E**). In animals implanted in mPFC, two-way ANOVA showed a main effect of treatment ($F_{1,124} = 4.17$, $^*p < 0.05$) and time ($F_{6,144} = 5.49$, $^*p < 0.0001$). Tukey's *post hoc* test showed a larger increase of dialysate DA in the mPFC after 25I-NBOMe 0.3 mg/kg, i.p., revealing differences at the 40-min samples with respect to basal values (**Figure 2F**).

In an attempt to compare the effects observed in male and female rats, a four-way ANOVA has been performed. No statistically significant differences between sexes have been shown.

Effect of 25I-NBOMe Administration on 5-HT Transmission in the NAc Shell and Core and in the mPFC

Males

In this experiment, we evaluated the effect of two doses of 25I-NBOMe (0.3 and 1.0 mg/kg, i.p.) on extracellular 5-HT levels in NAc shell and core and in the mPFC. As shown in **Figures 2G–I**, the compound did not affect the serotonergic transmission in all the areas studied. Three-way ANOVA showed a significant time × treatment interaction ($F_{12,156} = 1.89$, $^*p < 0.05$). Two-way ANOVA analysis did not highlight significative differences between vehicle-treated animals and 25I-NBOMe-treated animals either for the three areas.

Females

In this experiment, we evaluated the effect of 25I-NBOMe (0.3 and 1.0 mg/kg, i.p.) on extracellular 5-HT levels in NAc shell, NAc core, and mPFC. As shown in **Figures 2J–L**, the compound affects the serotonergic transmission to a small extent only in NAc shell. Three-way ANOVA showed a main effect of area ($F_{2,75} = 14.28$, $^*p < 0.05$), treatment ($F_{2,75} = 4.58$, $^*p < 0.05$), and area × treatment interaction ($F_{42,75} = 4.9$, $^*p < 0.005$). Tukey's *post hoc* tests showed no differences. In animals implanted in NAc shell (**Figure 2J**), two-way ANOVA showed a main effect of treatment ($F_{2,35} = 15.24$, $^*p < 0.0001$) and time ($F_{6,210} = 3.12$, $^*p < 0.01$), but no significant differences were revealed by Tukey's *post hoc* test. Two-way ANOVA analysis did not highlight significant differences between vehicle-treated animals and 25I-NBOMe-treated animals for the NAc core (**Figure 2K**). In animals implanted in the mPFC (**Figure 2L**), two-way ANOVA showed a main effect of treatment ($F_{2,23} = 3.48$, $^*p < 0,05$), without any significant results in the Tukey's *post hoc* test. In an attempt to compare the effects observed in male and female rats, a four-way ANOVA has been performed. No statistically significant differences between sexes have been shown.

Effects of 25I-NBOMe on Behavioral Tests

Sensorimotor Studies

Evaluation of the Visual Object Response

Visual object response did not change in both vehicle-treated male and female rats over 60 minutes Of observation (**Figures 3A**, **B**). Systemic administration of 25I-NBOMe (0.1–1 mg/kg, i.p.) reduced

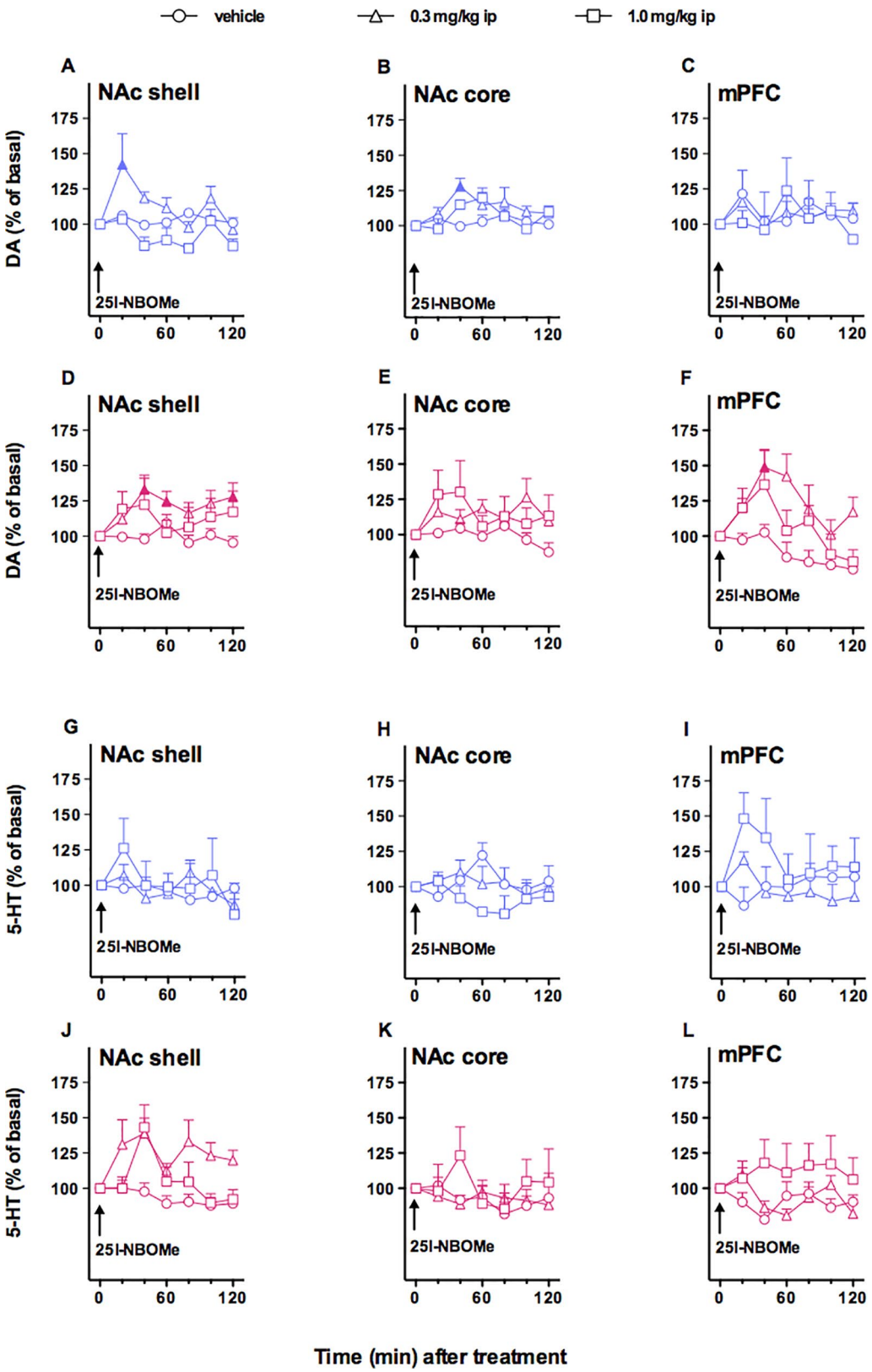

FIGURE 2 | Continued

FIGURE 2 | Effect of 4-iodo-2,5-dimethoxy-*N*-(2-methoxybenzyl)phenethylamine (25I-NBOMe) administration (0.3 and 1.0 mg/kg, i.p) on dopamine (DA) **(A–F)** and serotonin (5-HT) **(G–L)** transmissions in the nucleus accumbens (NAc) shell, NAc core, and medial prefrontal cortex (mPFC) in male (*blue symbols*) and female (*magenta symbols*) rats. Results are expressed as the mean ± SEM of change in DA/5-HT extracellular levels expressed as the percentage of basal values. The *arrow* indicates the start of i.p. injection at of vehicle (*circles*) or 25I-NBOMe 0.3 mg/kg (*triangles*) or 25I-NBOMe 1.0 mg/kg (*squares*) in NAc shell, NAc core, and mPFC. Statistical analysis was performed by three-way or two-way ANOVA followed by the Tukey's HSD *post hoc* test for multiple comparisons. *Solid symbol*: $p < 0.05$ with respect to basal values (DA males: NAc shell, *N* = 15; NAc core, *N* = 10; mPFC, *N* = 14; DA females: NAc shell, *N* = 34; NAc core, *N* = 31; mPFC, *N* = 27; 5-HT males: NAc shell, *N* = 11; NAc core, *N* = 10; mPFC, *N* = 11; 5-HT females: NAc shell, *N* = 38; NAc core, *N* = 20; mPFC, *N* = 26).

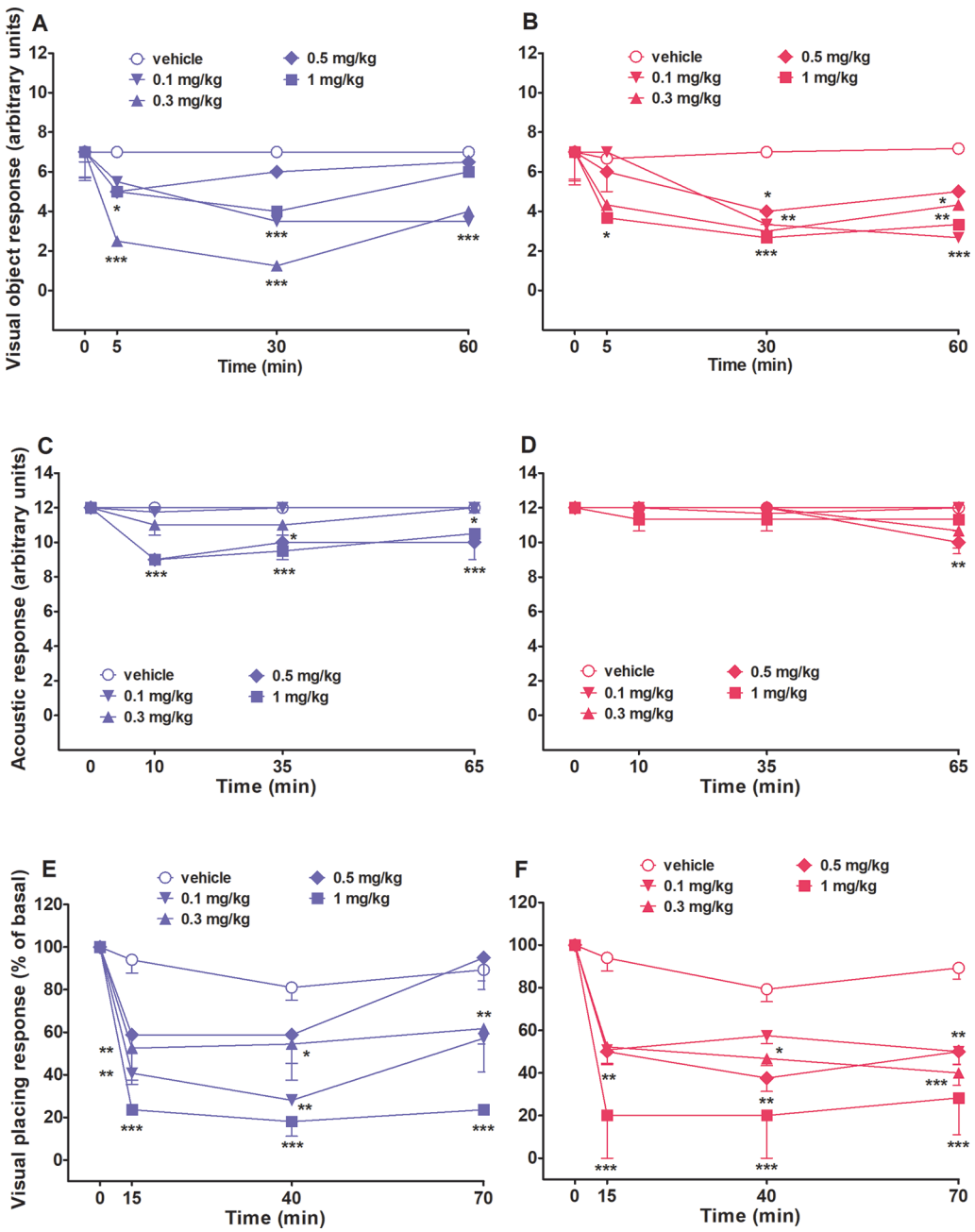

FIGURE 3 | Intraperitoneal injection (0.1–1 mg/kg) of 25I-NBOMe in male and female rats on the visual object test **(A** and **B)**, on the acoustic response **(C** and **D)**, and on the visual placing test **(E** and **F)**. Data are expressed as arbitrary units **(A**, **B**, **C**, and **D)** or percentage of basal **(E** and **F)** and represent the mean ± SEM of six determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparisons for the dose–response curve at different times. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 versus vehicle.

the visual object response in both sex rats and the effect persisted up to 60 min (**Figures 3A**, **B**). Two-way ANOVA followed by the Bonferroni's test for multiple comparisons in male rats showed a significant effect of treatment ($F_{4,140}$ = 22.24, p < 0.0001), time ($F_{3,140}$ = 22.47, p < 0.0001), and time × treatment interaction ($F_{12,140}$ = 4.478, p < 0.0001). The same statistical analysis for female rats showed a significant effect of treatment ($F_{4,140}$ = 8.207, p < 0.0001), time ($F_{3,140}$ = 15.79, p < 0.0001), and time × treatment interaction ($F_{12,140}$ = 2.149, p < 0.05).

Evaluation of the Acoustic Response

Acoustic response did not change in both vehicle-treated male and female rats over 60 min of observation (**Figures 3C**, **D**). Systemic administration of 25I-NBOMe impairs the acoustic response only in male rats at the two highest doses tested, 0.5 and 1 mg/kg, and this effect is persistent up to 60 min after the treatment (**Figure 3C**). Two-way ANOVA for male rats showed a significant effect of treatment ($F_{4,140}$ = 14.54, p < 0.0001), time ($F_{3,140}$ = 9.144, p < 0.0001), and time × treatment interaction ($F_{12,140}$ = 2.061, p < 0.05). The acoustic response was not inhibited in female rats by 25I-NBOMe. Two-way analysis showed a significant effect of time ($F_{3,140}$ = 3.694, p < 0.05), and Bonferroni's test for multiple comparisons showed a tardive little effect displayed by the dose of 0.5 mg/kg, i.p., at 60 min (**Figure 3C**, **D**).

Evaluation of the Visual Placing Response

Visual placing response slightly decreased in both vehicle-treated male and female rats over 70 min of observation (~17% of reduction at 70 min; **Figures 3E**, **F**). Systemic administration of 25I-NBOMe reduced the visual placing response in both rat sexes at all the doses tested (0.1–1 mg/kg, i.p.) and the effect persisted up to 70 min, as shown in **Figure 3E**. Two-way analysis showed a significant effect of treatment ($F_{4,140}$ = 17.25, p < 0.0001), time ($F_{3,140}$ = 31.63, p < 0.0001), and time × treatment interaction ($F_{12,140}$ = 2.582, p < 0.005) for male rats. For female rat, as shown in **Figure 3F** statistical analysis showed a significant effect of treatment ($F_{4,140}$ = 16.23, p < 0.0001), time ($F_{3,140}$ = 39.89, p < 0.0001), and time × treatment interaction ($F_{12,140}$ = 2.135, p < 0.05).

Evaluation of the Tactile Response

Overall tactile responses did not change in both vehicle-treated male and female rats over 65 min of observation (**Figures 4A, B**). As shown in **Figure 4A**, intraperitoneal injection (0.1–1 mg/kg) of 25I-NBOMe affected male tactile responses with a significant effect of treatment ($F_{4,140}$ = 8.942, p < 0.0001) and time ($F_{3,140}$ = 4.916, p < 0.05). No effects on females' tactile responses were observed (**Figure 4B**).

Evaluation of Core and Surface Body Temperature

Core body temperature did not change in both vehicle-treated male and female rats over 65 min of observation (**Figures 4C**, **D**). Systemic administration of 25I-NBOMe (0.1–1 mg/kg, i.p.) did not affect core (**Figure 4C**) body temperatures in male rats. Two-way ANOVA showed a significant effect of treatment ($F_{4,105}$ = 8.880, p < 0.0001). The dose of 0.5 mg/kg, i.p., affected significantly the core temperature in female rats (**Figure 4D**), with a significant effect of treatment ($F_{4,105}$ = 12.07, p < 0.0001). 25I-NBOMe did not affect the surface temperature in male rats; neither in females (data not shown).

Evaluation of Pain Induced by a Mechanical Stimulus

The threshold to acute mechanical pain stimulus did not change in both vehicle-treated male and female rats over 70 min of observation (**Figures 4E**, **F**). Systemic administration of the highest dose of 25I-NBOMe (1 mg/kg, i.p.) heavily increased the threshold to acute mechanical pain stimulus in male rats in the tail pinch test [significant effect of treatment ($F_{4,105}$ = 9.822, p < 0.001) and time ($F_{2,105}$ = 3.110, p < 0.05] (**Figure 4E**), whereas in female rats there is a lower effect with the same dose (**Figure 4F**). Statistical analysis showed a significant effect of treatment ($F_{4,105}$ = 4.988, p < 0.001).

Startle/Prepulse Inhibition Studies

Vehicle injection did not change startle/PPI response in male and female rats, and the effect was similar in naive untreated animals (data not shown). Administration of 25I-NBOMe (0.1–1 mg/kg, i.p.) inhibited the PPI in male rats at 68 dB ($F_{4,30}$ = 6.072, p < 0.001) and 75 dB ($F_{4,30}$ = 7.266, p < 0.001) of prepulse intensity (**Figure 5A**), while it inhibited the PPI in female rats at 68 dB ($F_{4,30}$ = 7.046, p < 0.001), 75 dB ($F_{4,30}$ = 6.635, p < 0.001), and 85 dB ($F_{4,30}$ = 3.501, p < 0.05) of prepulse intensity (**Figure 5B**). Moreover, 25I-NBOMe impaired the startle amplitude in male (about ~50% inhibition; $F_{4,30}$ = 5.98, p < 0.05) and female (about ~50% inhibition; $F_{4,30}$ = 13.07, p < 0.0001) rats at 1 mg/kg at 30 min after drug administration (**Figure 5C**).

DISCUSSION

The psychedelic compound 25I-NBOMe belongs to the phenethylamines that are a class of NPS spread among youth, with greater diffusion in girls than in boys (Wu et al., 2010; UNODC, 2016). 25I-NBOMe is a 5-HT2A receptor agonist used as a legal substitute of LSD and to mimic the effect of methamphetamine as well (Le Roux et al., 2015; Palamar et al., 2016). In this preclinical study, we evaluated the dopamine (DA) and serotonin (5-HT) releasing properties and the behavioral effects of 25I-NBOMe. Our results showed that 25I-NBOMe affects the DA transmission in the shell of the NAc in both sexes, while the higher variability on the serotonergic transmission's response, compared to DArgic response, leads to a lack of significative effect when analyzed by three- or two-way ANOVA. However, behavioral data showed that this compound causes visual alterations in both sexes, whereas core temperature is heavily affected in females, and the highest dose tested exerts an analgesic effect particularly prominent in male rats. Moreover, it impairs the startle amplitude and inhibits the PPI in both sexes.

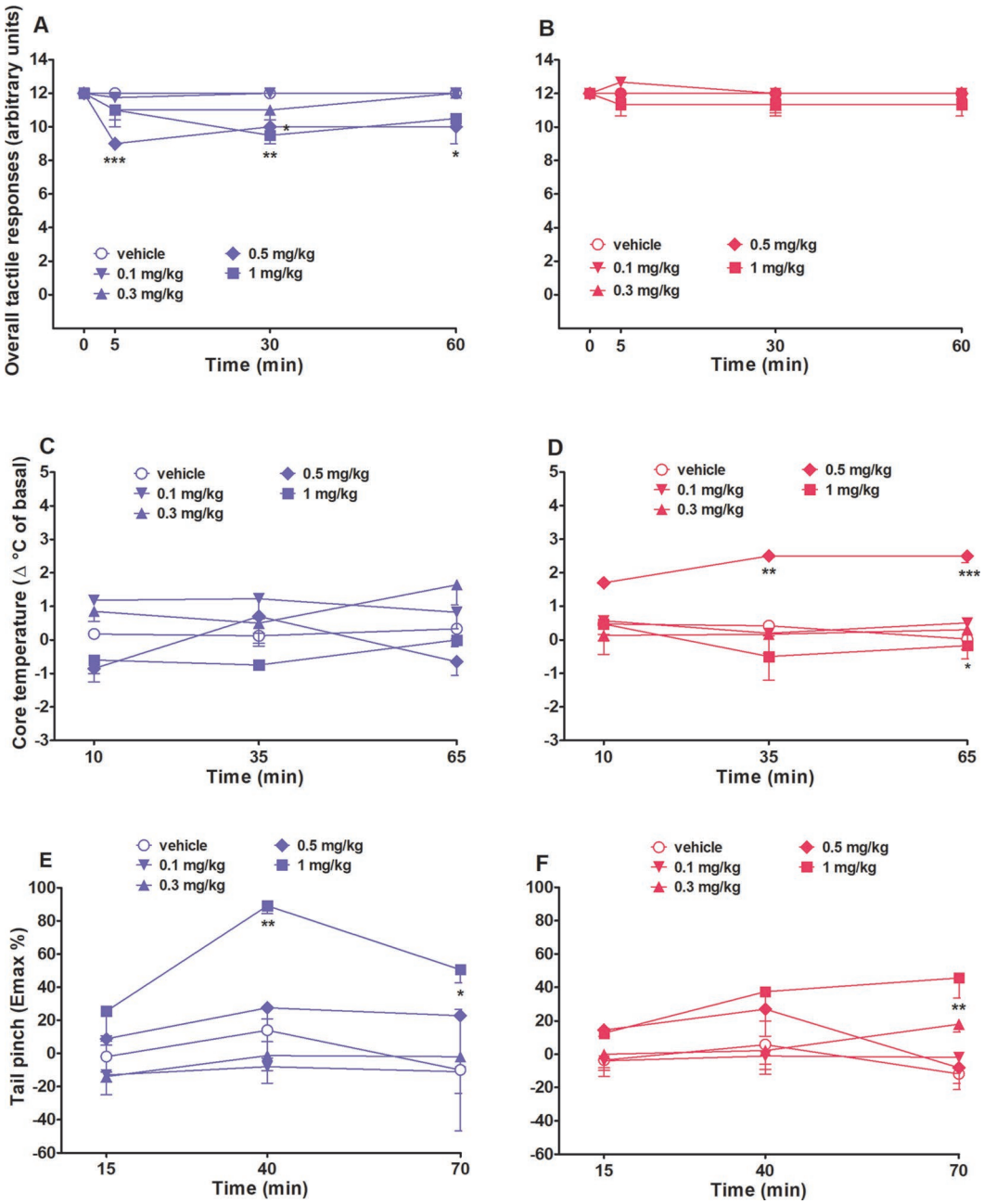

FIGURE 4 | Intraperitoneal injection (0.1–1 mg/kg) of 25I-NBOMe in male and female rats on the overall tactile responses **(A** and **B)** and on core temperature **(C** and **D)** and tail pinch test **(E** and **F)**. Data are expressed as arbitrary units **(A** and **B)**, as difference between control temperature (before injection) and temperature following drug administration (Δ°C; see *Material and Methods*) **(B** and **C)**, or as percentage of maximum effect ($E_{max\%}$; (see *Material and Methods*) **(E** and **F)** and represent the mean ± SEM of six animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparisons for the dose–response curve of each compound at different times. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus vehicle.

25I-NBOMe Affects the Dopaminergic Transmission in the NAc Shell of Both Sexes

It is well established that variability of the basal output may affect the extent of increasing or decreasing in neurotransmitters in brain areas (Di Chiara, (1990); De Luca et al., 2018). Therefore, we started comparing DA and 5-HT basal outputs in male and female rats, and we did not observe any differences in any of the brain areas studied (i.e., NAc shell and core and the mPFC), in agreement with previous microdialysis studies aimed at CPu of male and female groups (Xiao and Becker, 1994). Oppositely, Lazenka et al. (2017) observed a lower baseline NAc DA levels in female compared to male rats. Results obtained by monitoring estrous cycle in female rats suggested that, although the treatment affected significantly the DA transmission in all

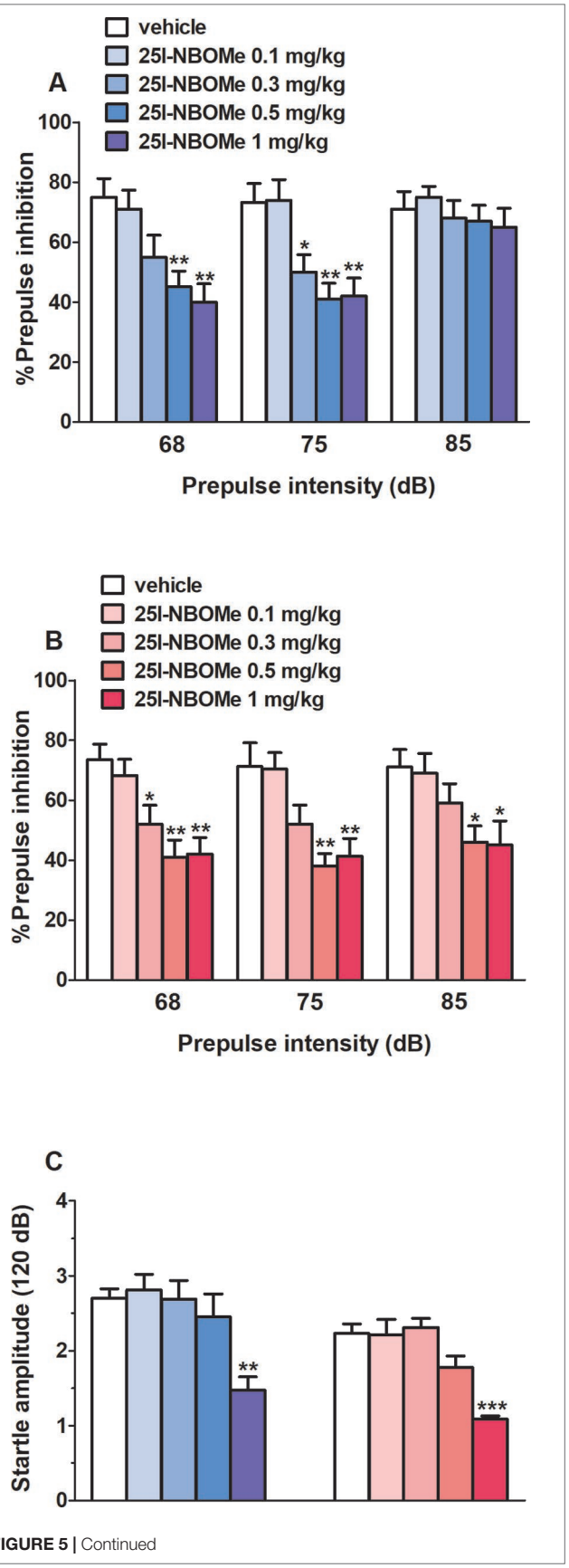

FIGURE 5 | Continued

FIGURE 5 | Effect of the systemic administration of 25I-NBOMe (0.1–1 mg/kg, i.p.) on prepulse inhibition (PPI) in male **(A)** and female **(B)** rats and on startle amplitude for both sexes **(C)**. Effects on PPI are shown for the three prepulse intensities (68, 75, and 85 dB) 30 min after treatment **(A** and **B)**. Data are expressed (see *Material and Methods*) as percentage decrease in the amplitude of the startle reactivity caused by presentation of the prepulse (% PPI) **(A** and **B)** and absolute values (in dB) **(C)**. Values represent the mean ± SEM of seven animals for each treatment. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's test for multiple comparisons. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus vehicle.

the areas studied and the 5-HT transmission in the NAc shell, estrous cycle phases are not effective in changing the effects of 25I-NBOMe, at least in the present set of experiments. Therefore, we combined the female data across the estrous cycle. Our *in vivo* microdialysis studies showed that the lowest dose of 25I-NBOMe tested (0.3 mg/kg, i.p.) affects the DA transmission in male rats in the NAc shell, with a maximal peak of 36% over basal value, 20 min after the injection, in the NAc core, with an extent of 27% at the 40-min sample, whereas it has no effect on the mPFC DA transmission (both doses). No effect has been observed in the 5-HT transmission in all the three areas tested, with both doses in male rats. The dose of 0.3 mg/kg, i.p., was active in female rats as well, increasing both DA and 5-HT dialysates in the NAc shell, with a maximal peak of 30% over basal value 40 min after the administration, and this effect lasted more than 2 h after the drug administration, whereas in the mPFC only DA extracellular levels are increased with an extent of 45%, and the highest dose of 1.0 mg/kg, i.p., did not show any effect in female rats. It is well known that all the drugs of abuse exert their rewarding effect increasing the DA transmission preferentially in the shell of NAc (Di Chiara et al., 2004; De Luca et al., 2015). Therefore, these results suggest that this compound has a common and alarming feature with them. These results are in line with previous studies showing an increased *ex vivo* striatal DA levels (Jeon et al., 2019) as well as an increased DA transmission in cortical areas of male rats (Herian et al., 2019). Moreover, 25I-NBOMe seems to act differently on DA and 5-HT levels in male and female rats, highlighting sex differences that might influence the frequency of ingestion, as well as the psychoactive effects and the long-term effects. Notably, the higher dose tested was not effective in increasing the DA level; this effect can be due to the formation of active metabolites acting as 5-HT2A antagonists, or it can be the result of off-target effects on other receptors, as previously suggested for other NPS classes (e.g., synthetic cannabinoids) (De Luca et al., 2016). Sex differences have been reported in the initiation of drug use, affecting the continuation of drug use as well as the phases of abstinence and relapse (Becker and Hu, 2008; Fattore et al., 2010), but also in the codification of reinforcement and related cues (Zlebnik, 2019). The greater increase of DA extracellular levels in females compared to males is consistent with previous studies reporting that amphetamine (Virdee et al., 2014), cocaine (Holly et al., 2012), and MDMA (Lazenka et al., 2017) are more effective in increasing DA release in the NAc of female rats. The reason for these neurochemical sex discrepancies has been historically ascribed to deep biological differences, such as

sex dimorphisms in the anatomy of DArgic systems in areas like SN and VTA (Walker et al., 2012), as well as ovarian hormone fluctuations (Becker and Hu, 2008). Furthermore, other factors, as pharmacokinetic (Fonsart et al., 2009), pharmacodynamic, and sociocultural differences, have been proposed to take part in the propensity to addiction (Franconi et al., 2013). Additionally, it has been widely demonstrated that female rats exhibit greater sensitivity to psychostimulants compared to males (Walker et al., 2012), with several experimental paradigms such as self-administration and conditioned place preference (Savageau and Beatty, 1981; Becker et al., 1982; Lynch and Carroll, 1999; Walker et al., 2000; Russo et al., 2003; Roth and Carroll, 2004; Harrod et al., 2005; Kantak et al., 2007). Even if we did not observe differences in each stage of the estrous cycle, further investigations are necessary to examine in depth the role of hormones in mediating the effects of this compound. These differences among males and females in responding to these synthetic compounds could explain recent surveys reporting that adolescent girls are more likely, compared to boys, to be ecstasy and/or other hallucinogen users (Wu et al., 2010). In addition, it has also been reported that a given dose of MDMA tends to produce more intense negative psychoactive effects in women than in men (Liechti et al., 2001) and that girls may generally be more vulnerable than boys to developing symptoms of hallucinogen dependence (Wu et al., 2009).

25I-NBOMe Causes Visual Alterations in Both Sexes

Data obtained showed that this compound decreases visual responses, causing dangerous visual alterations in both sexes. Sensorimotor alterations, especially visual ones, may be due to the pro-hallucinogenic action of 25I-NBOMe (Halberstadt and Geyer, 2014) and as typically reported for other 5-HT2A agonists (Canal and Morgan, 2012; Halberstadt and Geyer, 2013). All such hallucinogenic compounds exhibit high affinity for 5-HT2A receptors (Roth et al., 1998; González-Maeso and Sealfon, 2009). In fact, genetic or pharmacological inactivation of 5-HT2A receptor signaling blocks the behavioral effects of hallucinogenic compounds in a variety of species, including mice, rats, and humans (Fiorella et al., 1995; Vollenweider et al., 1998; González-Maeso et al., 2003; González-Maeso et al., 2007). Taken together, these findings indicate that 25I-NBOMe, by activating 5-HT2A receptor in cortico-visual circuits, could impair sensorimotor responses by promoting a hallucinogenic state.

25I-NBOMe Effects on the Acoustic and Tactile Responses

The decrease in the acoustic response is consistent with previous studies demonstrating that the administration of the analogue (±)-2,5-Dimethoxy-4- iodoamphetamine (DOI), in the dose range of 0.25–1.0 mg/kg, disrupted the startle response in Sprague–Dawley rats (Swerdlow et al., 2006). Recently, it has been demonstrated the role of 5-HT in modulating auditory brainstem responses in mice, starting from the cochlear nucleus (Papesh and Hurley, 2016); indeed, in the dorsal region of this nucleus, the activation of 5-HT2 receptors acts increasing the electrical activity of neurons, leading to a final suppression of auditory process (Felix et al., 2017; Tang and Trussell, 2017). Moreover, it has been recently shown that MDMA reduces acoustic and tactile responses as well (Marti et al., 2019), and this is a 5-HT2 receptor-mediated effect (Geyer and Tapson, 1988).

25I-NBOMe Effects on Body Temperature

Hyperthermia, which is one of the symptoms of the serotonin syndrome, was observed only in females with the dose of 0.5 mg/kg. This difference can be related to a distinct pharmacokinetic and pharmacodynamic compared to males, as previously described for other substances, which included amphetamine (Brady and Randall, 1999; Becker et al., 2001; Lynch et al., 2002; Carroll et al., 2004). Importantly, these results could explain why MDMA and hallucinogens seem to be more effective in women compared to men (Liechti et al., 2001; Wu et al., 2010).

25I-NBOMe Has a Greater Analgesic in Males

The highest dose tested (1 mg/kg, i.p.) exerts an analgesic effect prominent in male rats and minor in female rats, increasing the threshold to acute mechanical pain stimulus. This effect in male rats is higher than the effect obtained with compounds acting by the cannabinoid pathway (De Luca et al., 2015; Vigolo et al., 2015). This compound has a great affinity for rat 5-HT2A receptors (Ki = 0.087 nM) (Braden et al., 2006), but it has lower affinity also for μ-opioid receptors (Ki = 82 nM) and Ki greater than 500 nM for 5-HT1A receptors (Nichols et al., 2008). Therefore, it is possible to assume that the highest dose tested binds 5-HT2A receptors first, and further with other receptors such as 5-HT1A and μ-receptors, producing the analgesic effect. It is well known that serotonergic pathways running from the brainstem to the spinal cord are considered to be essential to the mechanisms of descending pain controls (Mayer et al., 1971; Zemlan et al., 1980; Clatworthy et al., 1988; Fields et al., 1991), and 5-HT2A receptors appear to play a critical role on nociceptive responses (Bardin et al., 2000; Sasaki et al., 2001; Kjørsvik et al., 2001). In particular, the administration of 5-HT2A receptor agonist DOI mediates antinociceptive effects in the craniofacial nociception (Okamoto et al., 2007). Different nociceptive responses have been observed before (Gamaro et al., 2014), and they are probably due to sex dimorphism in the localization of serotonergic receptors (Araldi et al., 2017), as well as different microglia activation within the periaqueductal gray (Doyle et al., 2017). Indeed, the activation of 5-HT2A receptors has been demonstrated to stimulate the secretion of various hormones (Van de Kar et al., 2001), among these the estradiol in both animals and humans (Moses et al., 2000; Kugaya et al., 2003; Frokjaer et al., 2010; Moses-Kolko et al., 2011). It is well known that estradiol is able to increase mechanical pain threshold in both sexes (Lu et al., 2012) while estriol fails it. The reason for this could be the ability of females to metabolize estradiol in estriol more quickly than males.

25I-NBOMe Impairs the Acoustic Startle Reflex in Male and Female Rats

Notably, 25I-NBOMe in our experiments showed to impair the acoustic startle reflex with the same extent (about 50% inhibition) in both sexes at the highest dose of 1 mg/kg, i.p., and to disrupt the sensorimotor gating significantly in both male and females compared to the vehicle-treated group (dose, 0.3–1 mg/kg, i.p.), with a tendency to an increased impairment at the prepulse intensity of 85 dB in females compared to males. It has been shown before that LSD exerts the same effects on PPI by activating 5-HT2A receptors in rats (Halberstadt and Geyer, 2010), and MDMA as well in both rats and mice (Marti et al., 2019). These results are alarming since the PPI has been widely defined as a marker of vulnerability to develop a neuropsychiatric disorder (Marti et al., 2019).

CONCLUSIONS

In conclusion, we have shown that the synthetic hallucinogen 25I-NBOMe affects the DArgic transmission in the NAc shell in a sex-independent manner, while mPFC DA seems to be more responsive in females compared to males. However, behavioral data proved that the severity of side effects occurring after 25I-NBOMe ingestion, probably mediated by serotonin pathways, can be different in male and female rats, as suggested also by a tendency, although not statistically significant, to a preferential increase of extracellular 5-HT in the NAc shell and a long-lasting stimulation in the mPFC of females compared to males. Regrettably, the experimental conditions of the present study have not been adequate to correlate either basal or 25I-NBOMe-stimulated brain levels of DA and 5-HT with hormonal fluctuations. In order to expand our knowledge on sex differences in the response to NPS, further experiments, most likely based on the direct evaluation of blood levels of hormones instead of indirect estimation by vaginal smears, would be needed. On the other hand, the observation of a higher core temperature in female rats and a marked analgesic effect in male rats after the administration of 25I-NBOMe may account for a gender-specific toxicity, thus highlighting possible distinct pharmacokinetics and pharmacodynamics as well as impact of enzyme genotype among sexes. These features suggest that the habit of consuming the psychedelic agent 25I-NBOMe and its analogues (Gee et al., 2016) pose a high risk of developing hyperthermia (i.e., serotonin syndrome) and has altered nociceptive responses. Moreover, the decreased acoustic reflex and the impaired visual responses observed in this preclinical study, coupled with the unawareness of what is going to be ingested in humans, pose a significant issue for public health and safety. Notably, in 2016, over 10 million people have been reported to drive under the influence of illicit drugs (DUID) (Substance Abuse and Mental Health Services Administration, 2017), and the impairment of visual and acoustic reflexes may clearly lead to fatal DUID, as reported after the ingestion of NBOMes (Rajotte et al., 2017). Although the findings of the present research give us important preclinical information, further investigations are necessary to clarify sex differences in toxicological responses to different drugs. Moreover, studies including pharmacological, toxicological, and forensic evidence at both preclinical and clinical levels are needed in order to more widely profile NPS effects and intoxication.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

All animal experiments were carried out in accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research according to Italian (D.L. 116/92 and 152/06) and European Council directives (609/86 and 63/2010) and in compliance with the approved animal policies by the Ethical Committee for Animal Experiments (CESA, University of Cagliari) and the Italian Ministry of Health (Aut. N. 162/2016-PR; Aut. N.352/2015-PR).

AUTHOR CONTRIBUTIONS

CM designed the experiment, and wrote the first draft of the paper. CM and NP performed the microdialysis experiments and the data analysis. MM, MT and RA contributed with all the behavioral experiments and figures. MM aided in interpreting the results and worked on the manuscript. MPC provided useful contribution to the content and substantially revised the manuscript. MDL conceived the topic, supervised and coordinated the work and wrote the final version of the manuscript. All the coauthors contributed to the present piece of work before approving it for final submission.

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Virtual Reality (VR) in Assessment and Treatment of Addictive Disorders: A Systematic Review

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Background: Substance Use Disorder (SUD) and behavioral addictions are common and require a multidisciplinary approach. New technologies like Virtual Reality could have the potential to improve assessment and treatment of these disorders.

Objective: In the present paper, we therefore present an overview of Virtual Reality (Head Mounted Devices) in the field of addiction medicine for craving assessment and treatment.

Method: We conducted a systematic review by querying PubMed database for the titles of articles published up to March 2019 with the terms [virtual] AND [addictive] OR [addiction] OR [substance] OR [alcohol] OR [cocaine] OR [cannabis] OR [opioid] OR [tobacco] OR [nicotine] OR [methamphetamine] OR [gaming] OR [gambling].

Results: We screened 319 abstracts and analyzed 37 articles, dividing them into two categories, the first for assessment of cue reactivity (craving, psychophysiological response and attention to cue) and the second for intervention, each drug (nicotine, cocaine, alcohol, cannabis, gambling) being detailed within each category.

Conclusions: This overview suggest that VR provide benefits in the assessment and treatment of substance use disorders and behavior addictions and achieve high levels of ecological validity. While, craving provocation in VR is effective across addiction disorders, treatments based exclusively on virtual exposure to drug related cues as shown heterogenous results.

Keywords: virtual reality, addictive behavior, alcohol, nicotine, cocain

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INTRODUCTION

Substance Use Disorder (SUD) and behavioral addictions are prevalent in many countries (Degenhardt et al., 2017) and require a multidisciplinary approach. Unfortunately, only a minority (7.1%) of patients, even in high income countries, receives adequate treatment. Individuals diagnosed with SUD experience several relapses after interventions and a lower quality of life because of the chronic nature of these disorders (Degenhardt et al., 2017). Therefore, there is an urgent need to conduct more research to expand assessment and treatment approaches. Virtual reality (VR) is emerging as one of the technological keys, it is increasingly accessible, and easy-to-use, and has recently attracted attention because of its potential utility for individuals with SUD (Carter and Tiffany, 1999).

VR is a computer-generated simulation that is a set of images and sounds that represents a real place or situation, which can be interacted with, in a seemingly real or physical way by a person using special electronic equipment. It can transmit visual, auditory, and various sensations to users through a headset to make them feel as if they are in a virtual or imagined environment.

Cue reactivity, which is based on classical and instrumental conditioning theory, involves conditioned response such as craving, psychophysiological response (heart rate, skin conductance…), attention bias and drug seeking behaviors triggered by stimuli previously associated with drug use (Ferreri et al., 2018). Traditional cue reactivity studies (scripts, photographs, videos and objects related to drug use), have allowed for a better understanding of situations leading to continued use and factors triggering relapse (Conklin and Tiffany, 2002). However, many limitations exist: lack of standardization, control and generalization, lack of contextual, and complex cues variety resulting in limited ecological validity and decreasing extinction probability through cue-exposure therapy. Therefore, because VR is immersive, both the environment and the perceptual stimuli can be modulated to trigger and assess pathological behaviors or sensations (e.g., craving), as well as to evaluate behavioral responses to a given situation that can elicit distress. Patients cans learn how to cope with their problems better. Beyond the use for addictive psychopathologies it has been shown to be useful for other psychiatric disorders by Park et al. (2019).

Moreover, VR has the potential to ameliorate many issues like craving assessment; active treatment of craving using cue exposure therapy (CET) or cognitive behavioral therapy (CBT)-driven techniques applied in real time. VR may also facilitate the link between clinician and patients and improve our understanding of addictive behavior.

Until recently, VR was limited by its cost and by the quality of the multimedia content. There has been a recent democratization of these systems (Playsatation4 VR, Oculus 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 an ecological assessment of cognition, emotions, and behavior in real-time.

The purpose of this systematic review is to evaluate the usefulness and efficacy of immersive VR in cue reactivity assessment and craving management for patients undergoing SUD or behavioral addictions. To this end, the proposed systematic review will answer the following questions:

- When compared to VR neutral stimuli, are VR cues able to elicit cue reactivity in adult patients suffering from addictive disorders? Is there an advantage of using VR cue reactivity over traditional cue reactivity methods?
- Can VR be used as an effective tool for craving reduction compared to standard therapy in this aforementioned population?

METHOD

Eligibility Criteria

To identify appropriate resources and search for relevant evidence. We used a PICOS framework, to form a well-focused question and facilitate the literature search (Schardt et al., 2007).

Population	Adolescent or adult humans with SUD or behavioral addiction
Intervention	Immersive VR (using Head-Mounted Display) simulating drug-related cues for assessment or treatment
Comparators	- Assessment: virtual neutral stimuli por traditional exposition (photos, videos, imagination), *in vivo* - Treatment: traditional therapy (CET, CBT, replacement therapy)
Outcomes	- Assessment: variation of cue reactivity (level of craving, physiological responses, attention to cue) - Treatment: level of craving, level of dependence, maintenance of abstinence
Study designs	Randomized controlled trial (RCT), controlled trial (CT), trial (T), case series
Timing	Studies running up to March 2019
Language	English or French

We conduct a systematic review by querying PubMed and Embase using the MeSH terms and keywords [virtual] AND [addictive] OR [addiction] OR [substance] OR [alcohol] OR [cocaine] OR [cannabis] OR [opioid] OR [tobacco] OR [nicotine] OR [methamphetamine] OR [gaming] OR [gambling]. TS and TB screened 471 articles, and 37 were then analyzed and divided into two categories, the first for assessment of cue reactivity (craving, psychophysiological response and attention to cue) and the second for treatment, each drug being detailed within each category.

See PRISMA diagram (**Figure 1**).

RESULTS

Conceptual Overview

A brief description of the concepts underlying virtual reality (VR) and cue features in the field of addiction is summarized in **Table 1**.

Assessment

Studies and results described below are summarized in **Table 2**.

Nicotine

Seventeen studies ($n = 465$) assessed cue reactivity after virtual nicotine-related cues in adult smokers. Nicotine addiction criteria were reported in 13 studies, using DSM 4 (Guze, 1995) and/or Fagerström Test for Nicotine Dependence criteria (Heatherton et al., 1991). Dependence severity was disparate across 8 studies, ranging from low to high (Lee et al., 2003, 2005; Traylor et al., 2008, 2009, 2011; Acker and MacKillop, 2013; Gamito et al., 2014; Thompson-Lake et al., 2015). Virtual reality exposure was controlled by a randomized healthy control group in 2 studies (Traylor et al., 2011; Gamito et al., 2014)

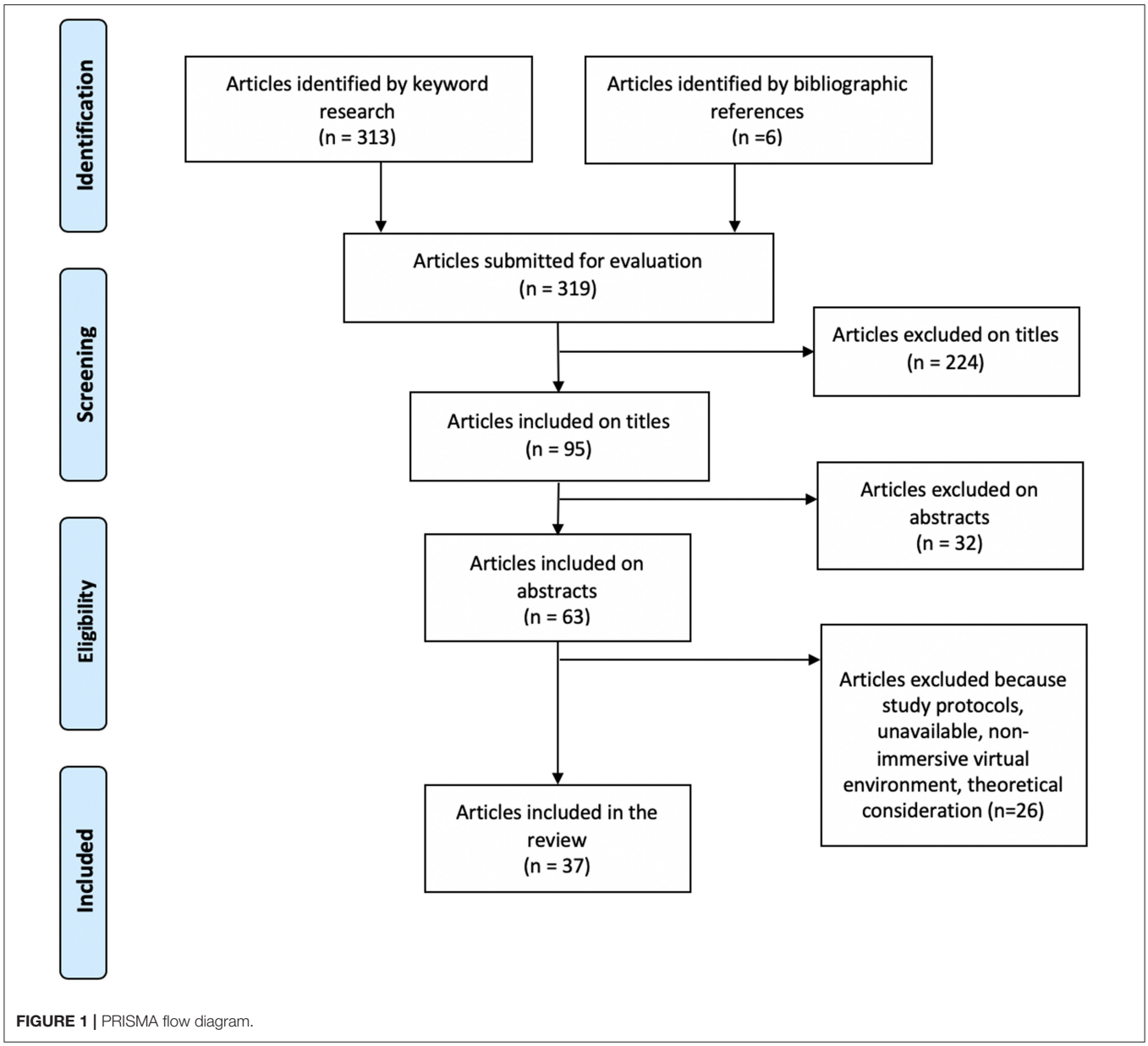

FIGURE 1 | PRISMA flow diagram.

and active 2D image comparators in 2 others (Lee et al., 2003, 2005). In most of the remaining studies, virtual nicotine cues were controlled by virtual neutral environments with random exposure protocols. Virtual environments included multisensory exposure with visual, auditory and olfactory stimuli in 8 studies (Carter et al., 2008; Traylor et al., 2008, 2009, 2011; Kaganoff et al., 2012; Acker and MacKillop, 2013; García-Rodríguez et al., 2013; Thompson-Lake et al., 2015) and visual and auditory environment for others (Ferrer-García et al., 2010; Paris et al., 2011; Pericot-Valverde et al., 2011; Acker and MacKillop, 2013; García-Rodríguez et al., 2013; Thompson-Lake et al., 2015).

Craving

All studies investigating craving ($n = 445$) in VR showed craving induction in adult smokers. Various cues (proximal: lighter, ashtray, pack of cigarettes, contextual: convenience store, party, complexes: smokers at a party or at a pub, having lunch at home) were associated with craving induction.

In most cases (17–24, 26, 27, 29, 32, 33), complex cues were evaluated and often compared to other types of cues (Bordnick et al., 2005; Traylor et al., 2008; Paris et al., 2011; Pericot-Valverde et al., 2011; Kaganoff et al., 2012; Gamito et al., 2014; Thompson-Lake et al., 2015). Some results suggested greater craving induction following complex cues compared to proximal (Thompson-Lake et al., 2015) or contextual cues (Gamito et al., 2014). Pericot-Valverde et al. (2011) suggested that social pressure in the form of virtual avatar could induce a faster craving increase in a complex-cue environment, although no difference in overall craving intensity was found. Conversely, García-Rodríguez et al. (2013) showed that smoking a virtual

TABLE 1 | Summary of concepts underlying Virtual Reality and type of cue.

Concept	Description	References
Virtual reality	An advanced human-computer interface that simulates a realistic environment and allows participants to interact with it. Its purpose is to allow a person sensory-motor and cognitive activity in an artificial world, created numerically, which can be imaginary, symbolic or a simulation of certain aspects of the real world. Each VR application is characterized by two key criteria: presence and autonomy.	Ouramdane et al., 2009
Virtual environment	The place suggested by the VR, represented by a 3D model of real or imaginary data that can be visualized and with which participants can interact in real time.	Ouramdane et al., 2009
Immersion	The objective description of what a VR display can provide in terms of technologies. It includes the extent to which the display is extensive (number of sensory systems involved), surrounding (information can arrive from any direction), inclusive (all information from the real world is shut out), vivid (richness and quality of the sensory information generated) and matching (a match is needed between information generated and participant's proprioceptive feedback). It also requires a self-presentation in the virtual environment i.e., a virtual body.	Slater et al., 1999
Presence	The subjective, psychological feeling of "being there", in the place depicted by the virtual environment. Immersion, control over environments, naturalness and realness of interactions all together contribute to the sense of presence.	Witmer and Singer, 1998
Head-Mounted Display (HMD)	The most immersive device: it displays separated images for each eye allowing stereo vision with stereo earphones and head tracking continually capturing the position and orientation of the participant's head. Rather than being a passive, external observer of video images, it allows participants to see a surrounding 3D stereo scene that can change dynamically.	Anderson et al., 2001; Slater, 2009
Cybersickness	A constellation of motion sickness-like symptoms occurring during and upon VR exposure. It includes symptoms such as disorientation, dizziness and nausea. and may be considered as a potential threat to the ultimate usability of virtual reality.	Stanney et al., 1997
Craving	Defined by the subjective preoccupation or strong desire to use a drug, craving has become a major diagnostic criterion of addictive disorders and is considered a central feature of addiction.	Sayette, 2016
Psycho-physiological response	These responses, controlled by the autonomic nervous system, are considered objective markers of cue-reactivity. Heart rate, skin conductance and temperature are the most studied psychological responses.	Conklin and Tiffany, 2002
Attention to cue	Referring to attention bias, attention to cue is the motivational trend to focus on drug cues while neglecting or ignoring others type of stimuli.	Field et al., 2014
Proximal cues	This is the most frequent type of cue used in traditional cue-reactivity studies. Proximal cues are ubiquitous across drug use. They are more often visual cues such as cigarette, ashtray, lighters, bottle of alcohol but can also be olfactory, auditory and tactile.	Conklin et al., 2008
Contextual (or distal) cues	They refer to the environment or context, with or without social interaction, in which substance use occurs such as bar or party. As well as proximal cues and despite being less reliable, they can elicit conditioned responses by being previously paired with drug use.	Conklin et al., 2008
Complex cues	A combination of proximal and contextual cues. They represent a more complete picture of real-world stimuli (people drinking alcohol in a party or smokers gathering outside a bar).	Conklin et al., 2008

cigarette could act as a stronger proximal cue on craving induction compared to complex situations (García-Rodríguez et al., 2013). Altogether, 3 out of 7 studies failed to find differences in craving induction between types of cues and there are several possible explanations for these results. First of all, the limited use of random exposure protocol could have exposed craving measurements to a report effect, which therefore could limit cues comparability; only one study truly controlled this bias (Thompson-Lake et al., 2015). Secondly, most studies performed craving assessment using single-item visual analog scales, reducing craving to a single dimension, and being then more sensitive to the ceiling effect. Conversely, multidimensional scales can highlight interesting craving differences by assessing it on various motivational dimensions using the Questionnaire of Smoking Urges (QSU) (Thompson-Lake et al., 2015) or through a neuro-economic assessment of craving as proposed by Acker and MacKillop (2013) which found subjects more likely to spend money on cigarettes, continue to smoke even at higher cost and are less sensitive to cigarette prices. Finally, Traylor et al. showed no difference in nicotine craving between subjects who are nicotine and alcohol dependent vs. nicotine deprendent only subjects after exposure to virtual complex environments (Traylor et al., 2011). The relationship between craving induction and dependence levels in VR were mixed and understudied with only one positive result showing predictive value of dependence and withdrawal scores on craving intensity (Thompson-Lake et al., 2015), while no correlation between craving and cigarette consumption was found in Ferrer-García et al. (2010), this possibly reflects a lower nicotine dependence in the smoker group. Surprisingly, no studies evaluated the effect of sensorial

TABLE 2 | Clinical trial on Virtual Reality (VR) assessment of craving in addiction.

Study	Population	VR procedure	Assessment	Outcome
NICOTINE				
Lee et al. (2003)	- 22 TS, moderate ND[a]	- C: complex/CC: smoking-related pictures - Randomized, 5 min exposure	- VAS	- ↑ Craving
Lee et al. (2005)	- 8 TS, moderate ND[a]	- C: complex/CC: smoking-related pictures or VR neutral - Randomized, 5 min exposure	- VAS - fMRI	- ↑ Craving Activation of PFC
Bordnick et al. (2004)	- 13 NTS, ND	- C: proximal or complex cues/CC: VR neutral - Randomized, 3 min exposure each	- VAS	- ↑ Craving
Bordnick et al. (2005)	- 10 NTS, ND		- VAS - SCR	- ↑ Craving and SCR
Carter et al. (2008)	- 22 NTS, ND[b]	- C: complex/CC: VR neutral - 3 min exposure	- MDS, brief QSU items	- ↑ Craving
Traylor et al. (2008)	- 20 NTS, moderate ND[a,b]	- C: proximal or complex cues/CC: VR neutral - Randomized, 3 min exposure	- VAS,	- ↑ Craving
Traylor et al. (2009)	- 20 NTS, moderate ND[a,b]	- C: proximal or complex/CC: VR neutral - Randomized, 3 min exposure	- ACVAS, SoSQ	- ↑ Attention to cues - ↑ Attention to sight in proximal vs. complex environment
Ferrer-García et al. (2010)	- 25 NTS, smokers (>10 cig/d)	- C: 7 complex/CC: VR neutral - Randomized, 6 min exposure to each scene	- VAS, cig/day - PQ	- ↑ Craving - Presence significantly correlated with craving
Traylor et al. (2011)	- 14 NTS, moderate ND[a,b]/intermediate AD[b,c] - Control: 7 NTS, moderate ND[a,b]/daily drinkers	- C: 2 complex/CC: VR neutral - 3 min exposure	- VAS	- ↑ Craving
Pericot-Valverde et al. (2011)	- 46 NTS, ND[b]	- C: 2 complex (+/– social pressure) - Randomized, 6 min exposure	- VAS	- ↑ Craving
Kaganoff et al. (2012)	- 46 TS (NRT +/– CBT), ND[b]	- C: proximal or complex/CC: VR neutral - Randomized exposure	- Intake, week 4 and week 10 of treatment: VAS, SAS	- ↑ Craving - ↑ Attention to cue
Paris et al. (2011)	- 24 NTS, smokers (>10 cig/day)	- C: complex or contextual/CC: 2 VR neutral - 3 min exposure	- VAS	- ↑ Craving - ↑ Craving in complex vs. contextual environment - ↑ Craving between last and first neutral environment
García-Rodríguez et al. (2012)	- 46 NTS, smokers (>10 cig/day) - 44, no smokers	- C: 7 complex/CC: VR neutral - Randomized, 6 min exposure	- VAS - HR, SR, T	- ↑ Craving in smoker group. - ↓ HR: in five environments in smoker group - ↑ T in two environments in smoker group - No difference in SR
García-Rodríguez et al. (2013)	- 45 NTS, smokers (>10 cig/day)	- C: Smoking a virtual cigarette in complex environment/CC: virtual darts in complex environment or complex environment alone - Randomized, 6 min exposure	- VAS - HR	- ↑ Craving - ↑ HR
Acker and MacKillop (2013)	- 47 NTS, low to moderate ND[1] (>10 cig/day)	- C: proximal/CC: VR neutral - 3 min exposure	- VAS - Cigarette Purchase Task and self-administration paradigm	- ↑ Craving - ↑ Motivational value - Craving and motivational indices correlated to number of cigarettes purchased
Gamito et al. (2014)	- 21 NTS, low ND[a] - 24, no smokers	- C: complex / CC: contextual - 5 min exposure	- QSU-Brief - Eye tracking in complex cue exposure - PQ, SSQ	- ↑ In smoker group in complex environment - ↑ Eye fixations on cues in smoker group
Thompson-Lake et al. (2015)	- 36 NTS, high ND[a,b] (one overnight nicotine deprived)	- C: proximal or complex. CC: VR neutral—Randomized, 3 min exposure	- QSU-Brief, FTND, Withdrawal Scale (12 h Deprived) - ACVAS - HR	- ↑ Craving - ↑ Craving in complex vs. proximal environment - Craving correlated with dependence and withdrawal in complex environment - ↑ HR in three of four smoking cues - ↑ Attention to cues

(Continued)

TABLE 2 | Continued

Study	Population	VR procedure	Assessment	Outcome
ALCOHOL				
Lee et al. (2008)	- 14 TS (abstinent 3 weeks), substantial AD [b,c] - Control: 14 NTS, social drinkers	- C: complex +/– social pressure. CC: VR neutral +/– social pressure - Randomized exposure	- VAS	- ↑ Craving in alcoholic group vs. social drinkers in complex environment - ↑ Craving after social pressure craving in all groups and environments except for alcoholic group in complex environment
Bordnick et al. (2008)	- 40 NTS, AD[d]	- C: 4 complex (2 with social pressure) / CC: VR neutral - Randomized, 3 min exposure to each scene	- VAS - AAS	- ↑ Craving - ↑ Attention to cues - ↓ Craving and Thought about Drinking in 1 complex environment ("argument" room)
Ryan et al. (2010)	- 15 NTS, binge drinkers - Control: 8, non-binge drinkers	- C: 4 complex (2 with social pressure) / CC: VR neutral - 3 min exposure to each scene	- VAS - AAS	- ↑ Craving in binge vs. non-binge drinkers in two complex environments (1 with social pressure) - ↑ Attention to cues in both groups - ↑ In Though About Drinking score in the two environments with social pressure in binge drinkers group
Traylor et al. (2011)	- 14 NTS, moderate ND[a,b] / intermediate AD [b,c] - Control: 7 NTS, moderate ND[a,b]/daily drinkers	- C: 2 complex smoking cues / CC: VR neutral - 3 min exposure	- VAS	- ↑ OH craving in OH group vs. non-OH group in one complex environment - ↑ Nicotine craving in both groups.
Kim and Lee (2015)	- 18, HSD[e] - Control: 18, LSD [f]	- C: 4 complex / CC: 4 VR neutral - Randomized exposure	- VAAT (Duration of push or pull response) - AUDIT, BDI	- ↑ Attention to cue in HSD - ↑ Depression score in HSD vs. LSD
COCAINE				
Saladin et al. (2006)	- 11 NTS, CoD[b]	- C: 7 contextual or complex /CC: VR neutral - Randomized, 3 min exposure	- VAS - HR, SCR	- ↑ Craving - ↑ HR in four cocaine-related environments - No difference in SCR
CANNABIS				
Bordnick et al. (2009)	- 20 NTS, CaD[b]	- C: 1 proximal and 1 complex / CC: VR neutral - Randomized, 6 min exposure	- VAS - CAS	- ↑ Craving - ↑ Attention to cues
GAMBLING DISORDER				
Bouchard et al. (2017)	- 28 NTS, frequent gamblers [g] - Control: 36 NTS, occasional gamblers [g]	- C: 2 complex / CC: real VLT and real board game - Randomized, 7 min exposure	- GCS - SOGS	- ↑ Craving (anticipation and desire scores) vs. real board game in frequent gamblers group - Craving correlated with dependence
INTERNET GAMING DISORDER				
Shin et al. (2018)	- –34 NTS, IGD[h,i] - Control: 30, healthy subjects (adolescents and young adults)	- C: 4 complex - Randomized exposure	- VAS - Modified-IAT - PQ, SSQ	- ↑ Craving in IGD group vs. control group - Craving correlated with dependence in one environment in IGD group - ↑ SSQ in IGD group vs. control group

[a] *FTND.*
[b] *DSM-IV.*
[c] *ADS (Alcohol dependence scale).*
[d] *DSM-IV-TR.*
[e] *AUDIT > 8.*
[f] *AUDIT < 8.*
[g] *SOGS.*
[h] *DSM-V.*
[i] *occasional or frequent problems because of internet at IAT.*

AAS, Alcohol Attention Scale; ACVAS, Attention to Cue Visual Analog Scale; AD, Alcohol dependent; AUDIT, Alcohol Use Disorder Identification Test; BDI, Beck Depression Inventory; C, Cue; CaD, Cannabis dependent; CAS, Cannabis, Attention Scale; CC, Control Cue; CoD, Cocaine dependent; CT, Controlled Trial; fMRI, functional Magnetic Resonance Imaging; FTND, Fagerström Test for Nicotine Dependence; GCS, Gambling Craving Scale; HR, Heart Rate; HSD, Heavy, Social Drinkers; IAT, Young Internet Addiction Test; IG, Internet Gaming; LSD, Light Social Drinkers; MDS, Multi-Dimensional Scale; ND, Nicotine dependent; NTS, Non Treatment Seeking; OH, alcohol; PFC, Pre-Frontal Cortex; PQ, Presence Questionnaire; QSU, Questionnaire of Smoking Urge; RCT, Randomized Controlled Trial; SAS, Smoking Attention Scale; SCR, Skin Conductance Response; SOGS, South Oak Gambling Scale; SoSQ, attitude toward Sense of Smell Questionnaire; SR, Skin Resistance; T, Temperature; TS, Treatment Seeking; VAAT, Virtual Approach Avoidance Task; VAS, Visual Analog Scale; VLT, Video Lottery Terminal; VR, Virtual Reality.

diversity on craving, which was supposed to increase immersion, especially since presence and craving levels were correlated in one study (Ferrer-García et al., 2010).

Physiological response

The physiological response to virtual nicotine-related cues was assessed in 5 studies ($n = 145$ participants) (Bordnick et al., 2005; Lee et al., 2005; García-Rodríguez et al., 2012, 2013; Thompson-Lake et al., 2015). Results are inconsistant. On the one hand, proximal and complex cues triggered increased skin conductance (Bordnick et al., 2005) and heart rate (García-Rodríguez et al., 2013; Thompson-Lake et al., 2015) compared to neutral environments in smokers. On the other hand, complex cues were associated with decreased heart rate and no change in skin resistance in smokers compared to non-smokers (García-Rodríguez et al., 2012). Ultimately, these contradictory findings may reflect a low nicotine dependence in the smoker group. Temperature was assessed in one study only (García-Rodríguez et al., 2012) and they found that its unfrequent increase in only 2 out of the 7 environments. Finally, one functional MRI study showed a pre-frontal cortex activation in response to the VR nicotine stimuli compared to neutral stimuli (Lee et al., 2005).

Attention to cue

3 studies evaluated the impact of virtual nicotine-related cues on attentional bias by using the self-administered questionnaires (ACVAS) or the Smoking Attention Scale (SAS) (Traylor et al., 2009; Kaganoff et al., 2012; Thompson-Lake et al., 2015) ($n = 102$). All studies reported a significant increase of attentional bias compared to virtual neutral environments. Adding olfactory nicotine cues were surprisingly not associated with greater attention to cue in Traylor et al. (2009) study. One study showed the feasibility of using implicit measurement of attentional bias in VR through eye-tracking and found greater ocular fixations on nicotine cues in smokers compared to non-smokers (Gamito et al., 2014).

Alcohol

Five studies ($n = 101$) assessed cue reactivity after presentation of virtual alcohol-related cues in adults suffering from alcohol use disorder (Bordnick et al., 2008; Lee et al., 2008; Ryan et al., 2010; Traylor et al., 2011; Kim and Lee, 2015). Diagnosis criteria were reported in 4 studies (Bordnick et al., 2008; Lee et al., 2008; Traylor et al., 2011; Kim and Lee, 2015) using DSM 4 (Guze, 1995) or Alcohol Use Disorder Identification Scale [AUDIT (Saunders et al., 1993)]. Dependence level was disparate and ranged from intermediate to severe dependence [AUDIT or Alcohol Dependence Scale (ADS) (Kivlahan et al., 1989)] in 3 studies (Lee et al., 2008; Traylor et al., 2011; Kim and Lee, 2015). Virtual alcohol-related cues were controlled with virtual neutral environment in each study and randomly exposed in 3 studies. Four studies included a control group (Lee et al., 2008; Ryan et al., 2010; Traylor et al., 2011; Kim and Lee, 2015) of which one was randomized (Traylor et al., 2011). Virtual environments included a multisensory exposure with visual, auditory and olfactory stimuli in 3 studies (Bordnick et al., 2008; Ryan et al., 2010; Traylor et al., 2011) and visual and auditory stimuli in 2 studies (Lee et al., 2008; Kim and Lee, 2015).

Craving

All studies investigating craving ($n = 93$) showed its induction after VR cue exposure (Bordnick et al., 2008; Lee et al., 2008; Ryan et al., 2010; Traylor et al., 2011). Cues usually associated with alcohol consumption (proximal: bottle of alcohol, contextual: bar, complex: party with alcohol) were those inducing craving. All studies investigated craving through single-item VAS. Three studies (Bordnick et al., 2008; Lee et al., 2008; Ryan et al., 2010) studied social interaction impact on craving. Bordnick et al. (2008) found that social pressure (such as offering a cigarette in a party) in addition to complex cues showed no difference in craving level whereas negative social experiences were associated to craving reduction in subjects with alcohol use disorders. Lee et al. (2008) found similar results regarding social pressure in alcohol dependent subjects; unlike with social drinkers for which craving was increased, suggesting a greater sensitivity to the social context in this population (Ryan et al., 2010). Finally, Traylor et al. assessed cross-cue reactivity and didn't find differences in alcohol craving after nicotine-related cue exposure among nicotine and alcohol dependent subjects compared to nicotine only dependent subjects (Traylor et al., 2011). These results may however have been limited by the duration of alcohol abstinence (12 h) which could have result in a ceiling effect, reflected by a greater baseline craving.

Attention to cue

Two studies assessed attentional bias upon virtual alcohol-related cue exposure using the self-report questionnaire Alcohol Attention Scale (AAS) (Bordnick et al., 2008; Ryan et al., 2010). Attentional bias increased every virtual complex cue compared to virtual neutral environments among alcohol dependent subjects (Bordnick et al., 2008). Ryan et al. (2010) found that the subscore "Thinking about alcohol" was higher after social pressure for the binge drinker group compared to healthy controls. Through implicit measurement (with a Virtual Approach-Avoidance Task), Kim and Lee (2015) highlighted attentional bias in heavy social drinkers (HSD) that showed increased difficulty avoiding virtual alcohol-related situations compared to light social drinkers. High depression score may however have affected response time in the HSD group, thus limiting this result.

Cocaine

One study (Saladin et al., 2006) concerning adults ($n = 11$) suffering from cocaine use disorder (DSM-IV) (Guze, 1995) assessed cue reactivity after virtual cocaine-related cues compared to virtual neutral environment, randomly exposed. Virtual environments included visual and auditory cues.

Craving

Craving induction through virtual cues has been demonstrated with contextual and complex environments compared to neutral virtual environments in cocaine-dependent subjects. Craving assessment was performed using single-item VAS. Situations triggering highest craving are those related to cocaine use and interactions with a dealer.

Physiological response

Physiological reactivity was also measured. Heart rate increased in a manner comparable to craving in scenes of cocaine use and interaction with a dealer compared to neutral scenes. Besides, there was no change in skin conductance measurements between virtual neutral exposure or virtual cocaine-related exposure.

Cannabis

One study (Bordnick et al., 2009) concerning adults ($n = 20$) suffering from cannabis use disorder (DSM-IV) (Guze, 1995) assessed cue reactivity after virtual cannabis-related cues were introduced. The study was controlled with a virtual neutral environment that was randomly exposed. Virtual environments consisted of multisensory exposure with visual, auditory and olfactory cues.

Craving

Bordnick et al. (2009) showed craving induction after virtual proximal and complex cues compared to virtual neutral stimuli in cannabis dependent subjects. Craving assessment was performed using single-item VAS.

Attention to cue

In this study (Bordnick et al., 2009), sub-scores of the 3 items of the Cannabis Attention Scale (CAS) self-questionnaire was increased after cannabis cues in VR compared to neutral stimuli in VR.

Gambling Disorder

One study (Bouchard et al., 2017) assessed cue reactivity after virtual gambling-related cues in 28 male frequent gamblers [SOGS (Lesieur and Blume, 1987)]. Virtual gambling cue exposure was controlled with 1 real complex environment (video lottery terminal) and 1 real neutral environment (board game), randomly exposed. Virtual environments consisted of multisensory exposure with visual and auditory cues.

Craving

Craving (both anticipation and desire to play dimensions) were induced after exposure to a virtual and real slot machine environments in Bouchard et al. (2017). Craving assessment was performed using multi-dimensional scale CGS. Increase in desire was higher compared to "casual gamers." Moreover, craving measured by the total score of the GCS was significantly correlated to gambling dependence.

Internet Gaming Disorder

One study (Shin et al., 2018) concerning adolescents and young adults ($n = 34$) suffering from internet gaming disorder [DSM-5 (Petry and O'Brien, 2013)] controlled with a group of 30 healthy subjects assessed cue reactivity after virtual internet gaming-related cues. Virtual environments consisted of multisensory exposure with visual and auditory cues.

Craving

Shin et al. (2018) found induced craving after randomized exposure to 4 virtual complex internet gaming-related cues (cyber-cafes). Craving assessment was performed using a single-item VAS. Craving was higher in the 2 active environments (coffee entrance and gaming invitation task) compared to a passive observation of a gaming conversation. In the environment associated with the task of refusal skills practice, the use of coping with gaming invitation strategies reduced craving compared to other environments in the IGD group. In addition, craving level and severity of dependence (modified-Internet Addiction Test) were correlated in the "coffee entrance" situation within the IGD group and a trend was found in the observation and gaming invitation situation. Although cybersickness score was higher in the IGD group compared to the control group, scores reported in both groups were low by SSQ (Simulator Sickness Questionnaire) standards.

Treatment

Studies and results described below are summarized in **Table 3**.

Nicotine

Virtual Exposure Therapy (VET)

5 VET trials (Lee et al., 2004; Park et al., 2014; Pericot-Valverde et al., 2014, 2015, 2019) assessed craving and nicotine addiction reduction in nicotine dependent adults ($n = 241$). One VET trial was effective on craving and cigarette consumption reduction (controlled on exhaled CO concentration) after 5 weekly sessions on 48 subjects with low to moderate nicotine dependence in association with a tobacco education group (Pericot-Valverde et al., 2014). In a second trial, the team showed an association between craving reduction and younger age, higher cigarette consumption, impulsivity and depressive symptoms with the same exposure protocol (Pericot-Valverde et al., 2015). However, 1 CT and 1 RCT (Park et al., 2014; Pericot-Valverde et al., 2019) found mixed results (Pericot-Valverde et al., 2019). In the first study, similar changes were observed in both VET and CBT groups on cigarette consumption reduction, exhaled CO concentration and dependence score post-treatment and at follow-up in moderate dependent subjects (Park et al., 2014). However, craving decrease wasn't significant in either group and higher baseline Fagerström score in the CBT group may have limited comparability. The second study, a RCT combination of VET plus CBT for smoking cessation, showed no benefit on retention and cessation rate compared with CBT alone after randomization in 102 nicotine moderate dependent subjects (Pericot-Valverde et al., 2019). While VET was associated with a reduction in nicotine craving, the 1-year relapse rate was higher in the VET + CBT group. Explanation concerning this last surprising result could be that cue induced craving responses extinguished after CET protocol re-emerged, a phenomenon known as spontaneous recovery. Otherwise, individuals assigned to CBT only protocol may have over time developed and rehearsed strategies for coping with both craving and withdrawal symptoms, reducing their susceptibility to relapse.

Virtual Cognitive Behavioral Therapy (VCBT)

Two randomized controlled trials (n = 137) studied VCBT in nicotine addiction reduction (Girard et al., 2009; Bordnick et al., 2012).

TABLE 3 | Clinical trial on Virtual Reality (VR) treatment in addiction.

Study	Design	Population	Control conditions	VR intervention	A	Assessment	Outcomes
NICOTINE							
Lee et al. (2004)	- Trial	- 15 adolescent males, low to moderate ND[1]		- VET (20 min, 1 session) 20 min sessions	N	- Baseline, end of treatment: VAS, morning and daily smoking count, planning (min), FTND, SSQ, PQ	- No change in craving and others variables
Pericot-Valverde et al. (2014)	- Trial	- 48 TS, low to moderate ND[a,b]		- VET: progressive individualized exposure (30 min, 5 sessions, 1/w)	Y	- Baseline, end of treatment: VAS, cig/d, air expired CO	- ↓ Craving - ↓ Cig/day and air expired CO
Pericot-Valverde et al. (2015)	- Trial	- 41 TS, low to moderate ND[a,b]		- VET: degressive individualized exposure (30 min, 5 sessions, 1/w)	N	- Baseline: gender, age, years of education, marital status, duration of daily smoking, FTND, NDSS, MNWS, STAI, BDI-II, DD - Week 1 and 5 post-treatment: VAS	- ↓ Craving; correlated with younger age, higher Cig/day, DD, BDI-II
Park et al. (2014)	- CT	- 30 TS males, moderate ND[a,c]	- TTT: CBT (4 sessions, 1/w)	- VET: 2 complex and 2 neutral (25 min, 4 sessions, 1/w)	N	- Baseline, end of treatment, week 12: QSU, cig/d, air expired CO, FTND, MNWS (per protocole analysis)	- No change in craving-No difference on craving - ↓ Dependence (daily smoking count, expiratory CO levels, FTND)
Pericot-Valverde et al. (2019)	- RCT	- 102 TS, moderate ND[a,c]	- TTT: CBT (60 min, 6 sessions, 1/w)	- VET (+CBT): progressive individualized exposure (30 min, 5 sessions, 1/w)	N	- Baseline and month 1, 6, 12: VAS, abstinence, relapse rate, treatment retention (ITT analysis)	- ↓ Craving - ↑ Relapse at 12 months
Girard et al. (2009)	- RCT	- 91 outpatients, moderate to high ND[a]	- TTT: Grasp up to 60 virtual balls (30 min, 4 sessions, 1/w)	- VBT: find and crush up to 60 virtual cigarettes (30 min, 4 sessions, 1/w)	N	- Baseline, end of treatment, week 12: cig/d, air expired CO, FTND, PQ, SSQ (ITT analysis)	- ↓ Dependence - ↑ Abstinence at week 12 - ↓ Treatment drop out - ↑ Presence and ↓ cybersickness at end of treatment
Bordnick et al. (2012)	- RCT	- 46 TS, moderate to high ND[a,d]	- TTT: NRT	- VCBT: progressive individualized exposure and coping skill training (1 h, 10 sessions, 1/w)	Y	- Baseline, end of treatment: QSU-Brief, cig/d, SASE - 1 month post treatment: SCQ (No ITT analysis)	- ↓ Craving - ↓ cig/d - ↑ Self-efficacy and SCQ
ALCOHOL							
Lee et al. (2009)	- CT	- 38 inpatient males, AD[d] (abstinent from a week)	- TTT: CBT + education (45 min, 10 sessions, 2/w) - Group: VET in 15 healthy males	- VET: relaxation, exposure, aversive situation (25 min, 10 sessions, 2/w)	Y	- Baseline, end of treatment: VAS, EEG	- ↓ Craving - ↑ Right Frontal EEG alpha power
Son et al. (2015)	- Trial	- 12 inpatient, AD[d,e] (abstinent from a week)	- Group: 15 healthy subjects	- VET: relaxation, exposure, aversive situation (25 min, 10 sessions, 2/w)	Y	- Baseline, end of treatment: VAS, TEP-FDG	- ↓ Craving - No correlation between change in craving and brain metabolism

(Continued)

TABLE 3 | Continued

Study	Design	Population	Control conditions	VR intervention	A	Assessment	Outcomes
Choi and Lee (2015)	- Trial	- 20 male, HSD[e]	- Group: 20 male LD[f]	- VET: 2 social aversive situations (20 min, 1 session)	N	- Baseline, end of treatment: AUQ, alcohol-IAT, eye-tracking test, alcohol-Stroop test.	- ↓ Craving - ↓ Alcohol-IAT and reaction times for alcohol-related stimuli
GAMBLING DISORDER							
Giroux et al. (2013)	- Trial	- 10 outpatient, gamblers		- VET: progressive exposure (20 min, 1 session)	Y	- Baseline, post treatment: VAS, self-efficacy	- No change in craving and self-efficacy
Bouchard et al. (2017)	- CT	- Study 1: 28, frequent gamblers[g]	- TTT: *in vivo* exposure or neutral exposure (1 session) - Group: 36 occasional gamblers [g]	- VET: 2 complex (7 min each, 1 session)	Y	- Baseline, post treatment: GCS, SOGS	- ↑ Craving in VR gambling and real VLT; correlated to baseline SOGS; correlated to baseline SOGS
		- Study 2: 34 inpatient, pathological gamblers[b] undergoing CBT	- TTT: imaginal exposure (2 sessions)	- VET: 2 complex (20 min, 2 session)	Y	- Baseline, post treatment: GCS, SSQ	- ↓ Craving
		- Study 3: 25, pathological gamblers[b] undergoing CBT	- TTT: imaginal exposure (4 sessions)	- VET: 2 complex scenes (20 min, 4 session)	Y	- Baseline, post treatment: My treatment, questionnaire, effectiveness (CPGI, DIG, GRCS)	- ↓ Craving - ↑ Effectiveness
INTERNET GAMING DISORDER							
Park et al. (2016)	- RCT	- 24, IGD[h]	- TTT: CBT (2 h, 8 sessions, 2/w)	- VET: relaxation, exposure, aversive situation (2 h, 8 sessions, 2/w)	Y	- Baseline, post treatment: YIAS, fMRI	- ↓ YIAS in both conditions - ↑ Connectivity from the PCC seed to the left middle frontal and bilateral temporal after VET

[a] *FTND.*
[b] *DSM-IV-TR.*
[c] *DSM-V.*
[d] *DSM-IV.*
[e] *AUDIT>8.*
[f] *AUDIT<8.*
[g] *SOGS.*
[h] *IYAS>50.*

A, Assisted by a therapist; AUDIT, Alcohol Use Disorder Identification Test; AUQ, Alcohol Urge Questionnaire; CBT, Cognitive-Behavioral Therapy; CO, Carbone monOxyde; CPGI, Canadian Problem Gambling Index; CT, Controlled Trial; DD, Delay Discounting; DIG, Diagnostic Interview for Gambling; EEG, Electroencephalography; FDG-PET, Fluoro-Deoxy-Glucose Positron Emission Tomography; FTND, Fagerström Test for Nicotine Dependence; GCS, Gambling Craving Scale; GRCS, Gambling Related Cognition Scale; HSD, Heavy social drinkers, IAT, Implicit Association Test; ITT, Intention To Treat; LD, Light Drinkers; MNWS, Minnesota Nicotine Withdrawal Scale; NDSS, Nicotine Dependence Syndrome Scale; NTS, Non-Treatment Seeking; PCC, Posterior Cingulate Cortex; PQ, Presence Questionnaire; QSU, Questionnaire of Smoking Urge; RCT, Randomized Controlled Trial; SASE, Smoking Abstinence self-efficacy; SCQ, Smoking Confidence Questionnaire; SSQ, Simulator Sickness Questionnaire; STAI, State-Trait Anxiety Inventory; TS, Treatment Seeking; VAS, Visual analog Scale; VBT, Virtual Behavioral Therapy; VCBT, Virtual cognitive-Behavioral Therapy; VET, Virtual Exposure Therapy; VLT, Video Lottery Terminal; YIAS, Young Internet Addiction Scale; YIAS, Young Internet Addiction Scale.

The first VBT program consisted of finding and crushing 60 cigarettes in a virtual environment without therapist assistance. Implemented 4 weekly in conjunction with a psychosocial support program, the VBT was effective on nicotine addiction reduction and abstinence rate in 91 moderate to high dependent smokers after treatment and during follow-up compared to a virtual task control (Girard et al., 2009).

The second VCBT consisted of 10 weekly sessions including a gradual and personalized exposure to craving with training in coping skills guided by a therapist. Implemented in combination with a nicotine replacement therapy, the VCBT was effective on cigarette consumption and craving reduction compared to nicotine replacement therapy alone in 46 moderates to high dependent smokers (Bordnick et al., 2012). In addition, the retention rate and self-confidence at the end of the intervention and coping skills during follow-up were better in this group. However, these results should be interpreted with caution because of low overall adherence to treatment.

Alcohol

Three controlled studies ($n = 70$) in Korea evaluated virtual exposure therapy, including aversive exposure situations on alcohol craving reduction (Lee et al., 2009; Choi and Lee, 2015; Son et al., 2015).

VET was more effective than a standardized CBT on reducing alcohol craving after 10 biweekly sessions in 38 males who were abstinent for a week. The virtual exposure included relaxation time, craving and aversive exposure guided by a therapist (Lee et al., 2009). Using the same protocol, this team found no correlation between craving reduction and brain metabolism in FDG-PET in 12 patients abstinent for 1 week (Son et al., 2015). On the other hand, VET was effective on craving reduction in heavy social drinkers after one session compared to light consumers (Choi and Lee, 2015). The exposure consisted of two aversive social situations including visual and auditory stimuli not supervised by a therapist. Moreover, HSD's ranking of alcohol markers in positive categories decreased (alcohol-IAT behavioral task) after treatment and VET was associated with decreased ocular fixation times for alcohol-related markers and improved alcohol-Stroop test scores for both groups.

Despite these encouraging results, evidence of VET effectiveness on alcohol dependence reduction had still not been studied.

Gambling Disorder

One study found no reduction on urge to gamble after a single VET on 10 lottery players (Giroux et al., 2013). More recently, pilot data from Bouchard et al. showed promising results (Bouchard et al., 2017): after demonstrating the effectiveness of a VET protocol on reducing desire to gamble similarly to a real-life exposure in 28 frequent players, they found no difference in craving reduction between randomized VET and imagined exposure on 34 pathological gamblers, both group receiving standardized CBT. Finally, 4 VET sessions were associated with stronger sense of control over gambling-related disorders after the third exposure compared to an exposure in imagination after randomization on 25 pathological players.

Internet Gaming Disorder

One VET study showed dependence reduction after 8 biweekly sessions compared to standard CBT on 24 randomized subjects (Park et al., 2016). The VET consisted of relaxation time followed by personalized craving and aversive cue exposure assisted by a therapist, with positive reinforcement. However, no craving measure was performed.

DISCUSSION

In the present review, we discuss major results related to VR that may provide solutions in the field of addiction especially for assessment and treatment.

Several results suggest that immersing subjects in virtual environments related to an addiction can combat many issues regarding craving assessment (Hone-Blanchet et al., 2014; Pericot-Valverde et al., 2016). VR have proved to be effective in triggering craving in both substance use disorder and behavioral addiction. Specifically designed to be immersive, the VR multisensorial cue exposition is able to drive cue attentional bias in order to report cognitive distortion related to addiction and trigger interoceptive reaction such as heart rate variation.

Although lack of statistical power makes it difficult to expose differences in craving response between types of cues, some results from nicotine craving studies (cf table) suggest that greater craving can be triggered by complex environments. Moreover, recent interest in proximal stimuli has emerged (virtual cigarettes) and new studies on other substance consumption behavior in virtual reality could further enrich the exposition realism.

Social interaction with avatars can efficiently induce cravings for multiple substances, mainly assessed in cigarette smoking and alcohol studies. However, difficulties persist in separating social craving from context craving. Indeed, several questions remain regarding stimuli specification assessment in VR craving induction. A significant number of studies were exposed to carry over effect while a better understanding of craving stimuli characteristics could be obtained by using a two by two comparison protocols or by an effective carry over effect control (for example, by controlling a return to baseline of craving level between each active stimulus exposure).

The lack of stimuli comparability resulting from the craving ceiling effect may have been increased by VAS widespread use by limiting the measurement to its intensity level, while a dimensional analysis of scales such as QSU could have revealed interesting qualitative differences (Rosenberg, 2009). More ecological assessments (eye tracking or virtual approach-avoidance task) could be proposed in order to provide an objective craving assessment by the use of real time behavioral measures (Cox et al., 2014). Its implementation in future research would help craving assessment but also offer insight and motivation to treatment via a feedback use. Feeling of presence and cybersickness should be assessed more rigorously to improve the level of ecological validity while increasing the acceptability of VR, especially since these two components are negatively correlated (Weech et al., 2019).

Traditional Cue Exposure Therapy studies' designs are mainly on substance-related cues detached from social environmental or *in vivo* scenarios. However, metanalyses report disappointing results (Mellentin et al., 2017; Ferreri et al., 2018), highlighting methodological biases preventing clear conclusions on its effectiveness in addictive disorders. VR has the potential to exceed those limits by providing a virtual immersion in environments closely related to everyday life and typical drug administration scenarios in order to better trigger craving in an individualized and progressive level. Indeed, Virtual Exposure Therapy (VET) has shown to be effective on craving reduction in nicotine and gambling disorders. Korean studies Lee et al. (2009), Son et al. (2015), and Choi and Lee (2015) find interesting results by adding aversive expositions to VET in alcohol use disorder. The combination of positive and negative affective states could lead to a restructuring of the conditioned stimulus response relation in addiction and should eventually result in avoidance of the administered drug.

In the future, attention to potential limits should be considered:

- Cost-effective studies are needed.
- Access to VR and therapists training need to be evaluated.
- Ethical considerations remain regarding the use of aversive exposures.
- Cybersickness could represent a threat to virtual reality usability and effectiveness and would also require integration in future studies (Gallagher and Ferrè, 2018).
- Patient's adherence should be accurately assessed before implementing aversion therapy in routine practice.

Both positive and negative studies show numerous methodological biases: confusion bias due to the absence of control groups, selection bias due to the absence of randomization, attrition bias due to little intent to treat analysis, altogether indicating low internal validity and limiting the possibility of drawing clear conclusions.

Future studies appear necessary and should evaluate dependence and abstinence maintenance as primary endpoint, offer more long-term follow-up, and include more control treatment groups to limit confusion bias.

Efficacy of treatment based exclusively on virtual exposure to drug related cues appears to be limited, but interesting results point the great potential of implementing VR in CBT. Pericot-Valverde et al. (2019) shows how VR could be disruptive by pointing out how an unsupervised behavior task in VR, such as finding and crushing cigarettes, can achieve positive results by making it enjoyable. Besides, it suggests that integrating several concepts such as patient empowerment to increase self-efficacy and self-regulation with more advances models on addiction associating action cue-exposure to positive mood state could achieve a full embodied experience for addiction behavior change in VR (Riva et al., 2017).

Despite these limitations, VR has the potential to offer new opportunities for treatment through its ability to provide a more ecological environment with more control and safety over exposition. The ability to fine-tune environments composition according to patient's preferences and most relevant data makes it a future tool for personalized medicine.

Because VR has the potential to be disruptive [for review (Freeman et al., 2017)] effort should be made to ensure good acceptability among therapist by providing reliable information, developing interest and allowing them to contribute to the development of these new technologies (Bourla et al., 2018). Furthermore, there is an urgent need to specifically assess the user experience. Virtual reality tends to develop in psychiatric practice (Freeman et al., 2017). Although its acceptability seems good in patients with mental disorders (Navarro-Haro et al., 2017), it has yet to be evaluated among health professionals. Its diffusion can challenge our professional heritage, the younger generations being potentially more sensitized to it. Efforts should be made through greater theoretical and practical information, especially since its application for health professionals could allow them to improve their skills in the assessment and treatment of addictive disorders (Fleming et al., 2009).

Besides its acceptability, its accessibility also raises questions. It could be limited due to the lack of financial support. The populations suffering from substance disorders being frequently in situation of social precariousness. The question of the cost of VR will also have to be evaluated in order to consider its wide implementation in health centers.

CONCLUSION

The studies presented in this review suggest that VR provide benefits in the assessment and treatment of substance use disorders, behavior addictions and achieve high levels of ecological validity. While, craving provocation in VR is effective across addiction disorders, treatments based exclusively on virtual exposure to drug related cues as shown heterogenous result. The addition of learning coping strategies in VRCBT studies are promising, however more rigorous methodological studies are warranted.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

TS and TB screening and abstract analysis, both authors contributed equally to the writing. AB, SM, and FF conceptual framework, supervised development of work, helped in data interpretation and manuscript evaluation. C-SP and J-VB helped to evaluate and edit the manuscript.

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Mephedrone and Alcohol Interactions in Humans

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Mephedrone (4-MMC, mephedrone) is a synthetic cathinone derivative included in the class of new psychoactive substances. It is commonly used simultaneously with alcohol (ethanol). The aim of the present study was to evaluate the interactions on subjective, cardiovascular and hormone effects and pharmacokinetics between mephedrone and alcohol in humans. Eleven male volunteers participated as outpatients in four experimental sessions in a double-blind, randomized, cross-over, and placebo-controlled clinical trial. Participants received a single oral dose of 200 mg of mephedrone plus 0.8 g/kg of alcohol (combination condition); 200 mg of mephedrone plus placebo alcohol (mephedrone condition); placebo mephedrone plus 0.8 g/kg of ethanol (alcohol condition); and placebo mephedrone plus placebo alcohol (placebo condition). Outcome variables included physiological (blood pressure, heart rate, temperature, and pupil diameter), psychomotor (Maddox wing), subjective (visual analogue scales, Addiction Research Center Inventory 49 item short form, and Valoración de los Efectos Subjetivos de Sustancias con Potencial de Abuso questionnaire), and pharmacokinetic parameters (mephedrone and ethanol concentrations). The study was registered in ClinicalTrials.gov, number NCT02294266. The mephedrone and alcohol combination produced an increase in the cardiovascular effects of mephedrone and induced a more intense feeling of euphoria and well-being in comparison to the two drugs alone. Mephedrone reduced the sedative effects produced by alcohol. These results are similar to those obtained when other psychostimulants such as amphetamines and 3,4-methylenedioxymethamphetamine are combined simultaneously with alcohol. The abuse liability of mephedrone combined with alcohol is greater than that induced by mephedrone alone.

Keywords: 4-methylmethcathinone (mephedrone), new psychoactive substance, alcohol (ethanol), interaction, pharmacologic effects, pharmacokinetics

INTRODUCTION

During the previous decade, numerous non-conventionally listed psychoactive compounds, or new psychoactive substances (NPS), have emerged on the illicit drug market to replace controlled ones (EMCDDA, 2016). Ease of availability combined with relatively low prices, and high purity compared to classical street drugs, plus popularization *via* Internet-driven social media, have contributed notably to their increasing presence on the drug scene. Of the 670 NPS detected by the European Union's early warning system, synthetic cathinones make up the second largest group (ACMD, 2010; Papaseit et al., 2014; EMCDDA, 2019).

Mephedrone (4-methylmethcathinone, 4MMC, drone, M-CAT, White Magic, meow meow), also known as "bath salt," "plant feeder," and/or "legal high," has emerged as a prototypical synthetic cathinone surpassing the popularity of other NPS (Vardakou et al., 2011; Bretteville-Jensen et al., 2013). It is a beta-keto amphetamine analogue, structurally and pharmacological related to 3,4-methylenedioxymethamphetamine (MDMA) which it was become its legal alternative (Green et al., 2014; Liechti, 2015). In vitro pharmacological assays have characterized mephedrone as a non-selective releaser and inhibitor of their uptake at the monoamine transporter (Baumann et al., 2012; Simmler et al., 2013; Baumann et al., 2013; Rickli et al., 2015; Saha et al., 2015). Recently, initial data regarding the human pharmacology of mephedrone have confirmed its psychostimulant-like effects which were first reported by recreational users in forums, surveys, and naturalistic and observational studies (Dargan et al., 2010; Winstock et al., 2011; Freeman et al., 2012; Papaseit et al., 2016; Homman et al., 2018). Following controlled oral administration, mephedrone induces cardiovascular-stimulant and euphoric effects with a high abuse liability characterized by earlier onset and shorter duration in comparison to MDMA and other amphetamine derivatives (d-amphetamine, methamphetamine and methylphenidate) (Mayo and de Wit, 2015; Papaseit et al., 2016; Dolder et al., 2017; Dolder et al., 2018).

In spite of its illicit status, recreational use of mephedrone continues to be present on the drug scene (Winstock et al., 2011; Deluca et al., 2012; González et al., 2013; Kelly et al., 2013; Mixmag's Drug Survey: The Results, 2014; CSEW, 2018). In 2015, data from the European Drug Report estimated a previous year prevalence of 3% among club-goers (EMCDDA, 2015). Based on results from the Crime Survey for England and Wales, previous year use of mephedrone among 16- to 34-year-olds was estimated at 0.2%; down from 0.5% in 2015/2016, and 1.1% in 2014/15 (CSEW, 2018; EMCCDA, 2018). In the United Kingdom, there have also been reports of "slamming"—the intravenous injection of mephedrone and other drugs, such as methamphetamine and gamma hydroxybutyrate, immediately before/during sex in groups of men who have sex with men at "chemsex" parties (Melendez-Torres et al., 2018). Mephedrone injecting has been reported as occurring mainly among individuals who have previously injected other drugs (e.g. heroin users), those who have switched from snorting mephedrone, and among younger users (UNODC, 2017).

In addition, mephedrone is linked to an intensive and repetitive administration pattern in which other drugs are concomitantly consumed (Schifano et al., 2011; Deluca et al., 2012). In these social scenes (e.g., nightclubs, music festivals, rave parties), the use of mephedrone and alcohol is the most common two-drug combination reported among NPS recreational users (EMCDDA, 2010; Carhart-Harris et al., 2011; Winstock et al., 2011). Under these conditions, users often report combining mephedrone with alcohol to either heighten its effects or ameliorate the come-down, a particularly unpleasant experience following mephedrone consumption (Newcombe, 2009; Carhart-Harris et al., 2011; O'Neill and McElrath, 2012).

With respect to acute mephedrone intoxication cases, it is notable that in 18.2% of cases, alcohol was also present (EMCDDA, 2016). Indeed, this concomitant use of mephedrone and alcohol in humans has led to several acute toxicities (McGaw and Kankam, 2010) and fatalities (Maskell et al., 2011; Regan et al., 2011; Cosbey et al., 2013; Elliott and Evans, 2014; Loi et al., 2015; Corkery et al., 2017; Papaseit et al., 2017). The latest results for acute drug toxicity presentations from the European Drug Emergencies Network (Euro-DEN) indicate mephedrone as the most common NPS involved (n = 88). The main acute effects include agitation, anxiety, palpitations and chest pain. Recently, in Poland an increased number of acute intoxications with mephedrone (binge episodes) in combination primarily with alcohol and also other substances have been detected (Ordak et al., 2018).

Despite the potentially added risks of mephedrone combined with alcohol, experimental data in humans about interactions between mephedrone and alcohol are very limited. Experimental studies in animal models concerning the effects induced after single and repeated mephedrone and alcohol administration, demonstrated that alcohol increases stimulant and rewarding effects of mephedrone (Ciudad-Roberts et al., 2015), and can also potentiate the neurotoxic properties of mephedrone in adolescent mice (Ciudad-Roberts et al., 2016). A recent study has concluded that mephedrone in combination with alcohol enhances the psychostimulant effect of mephedrone measured as locomotor activity. Given that both serotonin (5-HT) and dopamine are also related with reward and impulsivity, the observed effects point to an increased risk of abuse liability when combining mephedrone with alcohol compared with the sole administration of these drugs (López-Arnau et al., 2018). A part of the present study, including the neurocognitive performance effects of mephedrone and alcohol, have been formerly published (de Sousa Fernandes Perna et al., 2016). The results showed that whilst alcohol intoxication generally impaired performance, mephedrone improved psychomotor performance, impaired spatial memory but it did not affect divided attention performance. Nevertheless, the stimulatory effects of mephedrone were not enough to compensate for the impairing effects of alcohol on most performance parameters.

The present study was designed to assess the subjective, cardiovascular and hormone effects and pharmacokinetics following the interaction between mephedrone and alcohol

under controlled co-administration in humans. The findings presented in this paper are part of the previously mentioned study focusing on the physiological and subjective effects and pharmacokinetics of both substances.

MATERIALS AND METHODS

Subjects

Twelve healthy male subjects were recruited by word of mouth. Eligibility criteria required the recreational use of amphetamines, ecstasy, mephedrone, or cathinones with a lifetime minimum of six times, and on at least two occasions during the year prior to participation, without any serious adverse reaction; recreational use of alcohol (less than four units of alcohol per day) and previous experience of acute alcohol intoxication; and no history of abuse or drug dependence according to the Diagnosis and Statistical Criteria for Mental Disorders IV-R for any other substances except nicotine (in smokers).

All participants completed the study except for one who dropped out due to personal circumstances after participating in three experimental sessions (final n = 11). The participants had a mean age of 28 years (range 22–39 years), mean weight 71.8 kg (range 56.8–83.3 kg), mean height 173.5 cm (range 164.0–180.0 cm) and body mass index 23.8 kg/m^2 (range 19.0–26.8 kg/m^2). They had experience with mephedrone (36.4%), MDMA (90.9%), and cocaine (100%). The participants drank an average of 2.4 units of alcohol per day (range 1.0–4.0). All but five were smokers (mean five cigarettes/day, range 2–15).

Prior to their inclusion the participants underwent a general medical examination, including blood laboratory tests, urinalysis, 12-lead electrocardiogram (ECG), and a Psychiatric Research Interview for Substance and Mental Disorders. Participants completed a training session to familiarize themselves with testing procedures and questionnaires. In addition, in order to reduce variability in pharmacokinetics of mephedrone and to avoid the possibility that subject carriers of allelic variants leading to the PM phenotype for CYP2D6 might be at increased risk of acute toxicity, only subjects who were phenotypically CYP2D6 extensive metabolizers were included (de la Torre et al., 2005). The protocol was approved by the local Research Ethics Committee (CEIC-Parc de Salut Mar, Barcelona, Spain). The study was conducted in accordance with the Declaration of Helsinki and Spanish laws concerning clinical trials and registered in ClinicalTrials.gov (number NCT02294266). The volunteers were financially compensated.

Safeguard measures taken to ensure participant welfare while they were participating in the study included health controls before each session (a phone call one day before each session to ensure health status), during sessions (medical examinations at baseline, clinical monitoring with continuous ECG during 10 h and a psychiatric evaluation) and after sessions (medical examination 24 h after each session), a final control including medical examination and blood and urine chemistry were done 3–7 days after the last session, and a final phone call after 3–4 weeks.

Drugs

Mephedrone was supplied by the Spanish Ministry of *Justice and Ministry of Health*. Mephedrone and placebo capsules were prepared as opaque, white, soft gelatin capsules under the supervision of the Pharmacy Unit of the Hospital del Mar. Acute alcohol intoxication was induced by the ingestion of a beverage containing vodka (Absolut®, Ahus, Sweden) diluted in lemon-flavored water (Fontvella®) to mask the placebo drink. The alcohol-placebo drink was lemon-flavored water (Fontvella®).

Study Design

The study was double-blind, double-dummy, randomized, cross-over, and placebo-controlled. The four drug conditions consisted of a single oral dose: 200 mg of mephedrone plus 0.8 g/kg of alcohol (combination condition); 200 mg of mephedrone plus placebo alcohol (mephedrone condition); placebo mephedrone plus 0.8 g/kg of alcohol (alcohol condition); and placebo mephedrone plus placebo alcohol (placebo condition). The dose of mephedrone was selected based on previous results of the first study evaluating pharmacological effects of mephedrone in humans under controlled and experimental administration. This dose was selected after a series of pilot studies that included single oral doses of 50, 100, 150, and 200 mg of mephedrone, and 100 mg MDMA, being the dose of 200 mg well tolerated and produced similar effects to MDMA (Papaseit et al., 2016). The dose of alcohol and the combination were selected based in previous psychostimulant drug and alcohol interaction studies (Farré et al., 1993; Hernández-López et al., 2002).

Experimental Sessions

Subjects were admitted to the Clinical Research Unit facilities at 07:45 a.m. after an overnight fast. Upon arrival, they were questioned about any drug consumption or event that could affect their participation. They had been requested to refrain from using any psychoactive drug for a minimum of seven days prior to the study and throughout it, and from consuming caffeinated products for 24 h and alcohol for 48 h. A urine sample was collected for drug testing (Instant-View®, Multipanel 10 Test Drug Screen, Alfa Scientific Designs Inc., Poway, CA-USA). They remained in a calm and comfortable laboratory environment during the entire session. Tobacco smoking was not permitted in the sessions. Last cigarette was allowed 2 h before admission.

At the beginning of each experimental session baseline measures were performed. Mephedrone or matched placebo (one capsule) was administered at 8:30 a.m. in a fasting state with 100 milliliters (ml) of bottled water (Fontvella®). Alcohol or matched placebo was administered at 9:00 a.m., 30 min after mephedrone or matched placebo administration. The total volume of the beverage (350 ml) was consumed in 15 min (one-third volume every 5 min).

Four, 6, and 10 h after administration a light breakfast, a meal, and a snack were provided to the participants/subjects, respectively. A psychiatric evaluation was performed 8 h after

administration and adverse effects were assessed during each experimental session and the following day.

Physiological Measures

Non-invasive systolic blood pressure (SBP), diastolic blood pressure (DBP), heart rate (HR), and oral temperature (T) were repeatedly recorded at: -45 min, 0 h (baseline, immediately prior to capsule administration), 0.25, 0.5 (immediately prior to beverage administration), 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, and 24 h following initial drug administration. All assessments were carried out with a DinamapTM 8100-T vital signs monitor (Critikon, Tampa, Fla., US). Pupil diameter (PD) and the Maddox-wing device were recorded at: 0 h (baseline, immediately prior to capsule administration), 0.25, 0.5 (immediately prior to beverage administration), 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, and 24 h following initial drug administration. Pupillary diameter was recorded with a Haab pupil gauge and Maddox wing measures were expressed in diopters along the device's horizontal scale. The Maddox-wing device measures the balance of extraocular muscles and quantifies exophoria as an indicator of extraocular muscle relaxation, and esophoria as an indicator of extraocular muscle tension. For safety reasons ECG was continuously monitored along 10 h using a DinamapTM Plus vital signs monitor (Critikon, Tampa, Fla., US).

Subjective Effects

Subjective effects were measured using a set of 23 visual analogue scales (VAS), the 49-item Addiction Research Center Inventory short form (ARCI), the Evaluation of Subjective Effects of Substances with Abuse Potential questionnaire (VESSPA-SEE), and a pharmacological class identification questionnaire.

VAS (100 mm) were labeled at opposite ends with different adjectives ranging from "not at all" to "extremely" (Farré et al., 2004; Peiró et al., 2013; Farré et al., 2015; Papaseit et al., 2016). Subjects were asked to rate effects from among "high," "drunkenness," "stimulated," "any effect," "good effects," "bad effects," "liking," "content," "drowsiness," "dizziness," "confusion," "fear," "depression or sadness," "changes in distances," "changes in colors," "changes in shapes," "changes in lights," "hallucinations-seeing of lights or spots," "changes in hearing," "hallucinations-hearing sounds or voices," "hallucinations-seeing animals, things, insects or people," "different or changed unreal body feeling," and "different or unreal surroundings".

The Spanish validated version of the short-form ARCI (Lamas et al., 1994), which consists of a true/false 49-item questionnaire, it is a validated instrument for determining subjective drug effects including five subscales: PCAG (pentobarbital-chlorpromazine-alcohol group, a measure of sedation); MBG (morphine-benzedrine group, a measure of euphoria); LSD (lysergic acid diethylamide group, a measure of dysphoria and somatic symptoms); BG (benzedrine group, a stimulant subscale relating to intellectual efficiency and energy); and A (amphetamine, a measure of d-amphetamine effects) (Farré et al., 2015).

The VESSPA-SEE is a questionnaire that measures changes in subjective effects caused by different drugs including MDMA. It includes six subscales: sedation (S), psychosomatic anxiety (ANX), changes in perception (CP), pleasure and sociability (SOC), activity and energy (ACT), and psychotic symptoms (PS) (Poudevida et al., 2003; Papaseit et al., 2016).

The pharmacological class identification questionnaire asks about the class of drugs the participants believed they had been given at each administration (Rush et al., 1995). The options included placebo, benzodiazepine (e.g., valium, diazepam, tranxilium, rophipnol), alcohol, stimulant (amphetamine), designer drugs (ecstasy), cocaine, hallucinogen (e.g., LSD, mescaline), cannabinoids (e.g., marijuana, hashish), ketamine (special K), and gamma hydroxybutyrate (liquid ecstasy).

The VAS were administered at: -45 min (baseline), 0.25, 0.5 (immediately prior to beverage administration), 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, and 24 h following initial drug administration. ARCI and VESSPA-SEE were administered at: -45 min, 0.25, 0.5 1.5, 2, 3, 4, 6, 8, and 10 h following initial drug administration. The pharmacological class identification questionnaire was given at 8 h following initial drug administration.

Pharmacokinetics

Blood samples for the determination of mephedrone were collected during each experimental session at -5 min (0 h, baseline), 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, and 24 h following drug administration. Urine was collected until 24 h (data not shown). Mephedrone plasma concentrations were quantified by gas chromatography-mass spectrometry (GC-MS). A liquid–liquid extraction was performed with tert-butyl methyl etherm and the silylation reagent [N-methyl-N-(trimethylsilyl) trifluoroacetamide] was used for the derivatization of mephedrone (Papaseit et al., 2016; Olesti et al., 2017).

Blood samples for the determination of alcohol were collected during each experimental session at -5 min (0 h, baseline), 0.75, 1, 1.5, 2, 3, 4, 6, 8, and 10 h after drug administration. Urine was collected until 24 h (data not shown). Alcohol plasma concentrations were determined with an enzymatic test (DRI Ethyl Alcohol Assay, Thermo Scientific) in an autoanalyzer (Indiko Plus, Thermo Fisher Scientific).

Blood samples for the determination of cortisol were collected during each experimental session at -5 min (0 h, baseline), 1, 2, 4, 6, and 8 h following drug administration. They were centrifuged at 3,000 rpm for 10 min at 4°C, plasma and serum were removed and frozen at -220°C until analysis. Cortisol plasma concentrations were determined by fluorescence polarization immunoassay (Abbott Laboratories, Chicago, IL) according to the manufacturer's instructions (Kobayashi et al., 1979).

Statistical Analysis

Statistical analysis was performed for eleven participants.

Values from physiological and subjective effects were transformed to differences from baseline. The peak effects in the first 6 h after first drug administration (maximum absolute change from baseline values, E_{max}), and the 6-h area under the curve (AUC) of effects versus time were calculated by the trapezoidal rule for each variable.

Peak concentration (Cmax) and time to reach peak concentrations (Tmax) from mephedrone, alcohol and cortisol plasma concentrations were determined using PKSolver, a freely available add-in program for Microsoft Excel (Joel Usansky, Atul Desai, and Diane Tang-Liu, Department of Pharmacokinetics and Drug Metabolism, Allergan, Irvine, CA, USA). Area under the concentration-time curve from mephedrone, alcohol and cortisol (AUC0-6), mephedrone (AUC0-24), and alcohol (AUC0-10) were calculated by the linear trapezoidal rule.

Firstly, these transformations were analyzed using a two-way analysis of variance (ANOVA) test to study the influence of some participant factors as age, body mass index, weight, smoking and alcohol use in the different parameters calculated. Because the results showed only marginal statistically significant results for factors/interactions, the analysis was rejected (11 variables showed significant results for a total number of 200 comparison, percentage 5.5%). Subsequently, the statistical analysis presented was performed without considering those factors.

Then, these transformations were analyzed by means of one-way repeated-measures analysis of variance (ANOVA) with drug condition as factor. In case of significant differences among treatment conditions in ANOVA, post-hoc multiple comparisons were performed using the Tukey test. Time course (T-C) of effects was analyzed employing two-way repeated-measures ANOVA with Treatment condition and Time (0–6 h) as factors. When Treatment condition, or the Treatment condition × Time interaction, were statistically significant, multiple Tukey post-hoc comparisons were performed at each time point. The difference in time to reach peak effects (T_{max}) values among conditions was assessed with the nonparametric Friedman test. When significant results among conditions were detected post-hoc multiple comparison was performed applying the Wilcoxon signed rank test adjusting the p value to six comparisons ($p < 0.008$).

All statistical tests were performed at each time point using the PASW Statistics 18.0 (SPSS Inc., Chicago, IL, USA). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Global Results

Table 1 shows the summary of physiological and subjective effects where at least one statistical difference (peak, AUC) was found in the ANOVA and multiple comparison post-hoc test analyses. Furthermore, it includes T-C points that presented significant differences in ANOVA and the multiple-comparison post-hoc tests. **Table 2** shows the Peak effect values of variables where at least one statistical difference was found in the ANOVA.

The T-C (differences from baseline) for the most relevant physiological, psychomotor, and subjective effects are shown in **Figures 1** and **2**, respectively. Concentrations over time and pharmacokinetic parameters of mephedrone and alcohol in plasma are presented in **Figure 3** and **Table 3**, respectively. The effects of all drug/experimental conditions on plasma cortisol levels are depicted in **Tables 1** and **2**.

No serious adverse events were observed. No hallucinations, psychotic episodes, or any other psychiatric symptoms were experienced during the sessions. None of the participants required specific therapy or special care during the study. All 11 subjects completed the study.

Physiological Effects

Regarding physiological effects, the two conditions including mephedrone (combination condition and mephedrone condition) produced an increase in SBP, DBP, HR, and PD as compared with the placebo condition (when considering both peak effects and AUC). The more relevant differences for HR increase appeared between the combination and mephedrone conditions. The former produced an increase of 40 ± 17 beats per min (bpm) at 1 h following the first drug administration whilst for the latter the peak effects was 32 ± 20 bpm at 0.75 h. In statistical terms, only significant differences were detected for $AUC_{0\text{-}6}$ (142.33 $_{mm}$ × h for the combination condition, and 70.91 mm × h for the mephedrone one) and T-C points (1.5–6 h) for both. For the remaining physiological variables (SBP, DBP, T, and PD), only significant differences in several T-C points were observed between the combination and mephedrone conditions (no AUC or peak effects).

The alcohol condition produced very slight effects on SBP, DBP, HR, and T and none on DP compared with the placebo condition (non-statistically significant). Comparing the combination and alcohol condition, the mephedrone-alcohol effects on SBP, DBP, HR and DP were significantly higher than those produced by alcohol alone (when considering both peak effects and AUC) with significant differences at several T-C points.

In the Maddox-wing device, the combination and mephedrone conditions led to an increase in the degree of esophoria compared with placebo (when considering peak effects and/or AUC). Esophoria induced by the combination condition was approximately two to three-fold lower (-1.41 ± 2.91 diopters) in comparison with the mephedrone one (-3.45 ± 2.73 diopters) with only significant differences in several T-C points (1–1.5 h). Alcohol slightly increased exophoria (+1.41 ± 1.30 diopters), relaxation of ocular musculature, but only significant differences in several T-C points were obtained when compared with the placebo. The combination condition scored approximately midway between the mephedrone and alcohol conditions.

Subjective Effects

The two conditions containing mephedrone caused an increase in subjective effects (VAS, ARCI, and VESSPA-SEE) compared with placebo (**Tables 1** and **2**).

The combination and mephedrone conditions led to significant changes in the ratings of "high," "stimulated," "any effect," "good effects," "liking," "content" and also in "change in distances and different, changed or unreal body feeling" in comparison with placebo. In general terms, the combination condition produced higher and more prolonged scores with statistically significant

TABLE 1 | Summary of statistically significant results of the physiological parameters and subjective effects (n = 11) observed after administration of mephedrone plus alcohol (mephedrone–alcohol), mephedrone plus placebo alcohol (mephedrone), placebo mephedrone plus alcohol (alcohol), and placebo mephedrone plus placebo alcohol (placebo condition).

Variable				Tukey's Multiple Comparison Test (*p < 0.05; **p < 0.01)					
	ANOVA			Placebo			Mephedrone		Alcohol
	Parameter	F/X^2	p Value	Alcohol	Mephedrone	Mephedrone–alcohol	Alcohol	Mephedrone–alcohol	Mephedrone–alcohol
Physiological effects									
SBP	AUC (df = 3,30)	22.107	<0.001	NS	**	**	**	NS	**
	Peak (df = 3,30)	38.667	<0.001	NS	**	**	**	NS	**
	T_{max}	11.97	0.007	NS	NS	*	NS	NS	NS
	T-C (df = 3,270)	13.924	<0.001	0.75*,4*	0.5**,0.75**,1**, 1.5**,2**,3**	0.5**,0.75**,1**, 1.5**,2**,3**	0.5**,0.75**,1**, 1.5**,2**,3**,4**	2*	0.5**,0.75**,1**,1.5**,2**,3**,4**
DBP	AUC (df = 3,30)	17.087	<0.001	NS	**	**	**	NS	**
	Peak (df = 3,30)	20.138	<0.001	NS	**	**	**	NS	**
	T_{max}	9.54	0.023	NS	NS	NS	NS	NS	NS
	T-C (df = 3,270)	5.526	<0.001	6*	0.5*,0.75**,1**,1.5**,2**	0.5**,0.75**,1**, 1.5**,2**	0.5**,0.75**,1**, 1.5**,2**,3*,4**,6**		0.5**,0.75**,1**,1.5**, 2**,3*,4**
HR	AUC (df = 3,30)	25.960	<0.001	NS	**	**	NS	**	**
	Peak (df = 3,30)	23.570	<0.001	NS	**	**	**	NS	**
	T_{max}	9.38	0.025	NS	*	NS	NS	NS	NS
	T-C (df = 3,270)	10.750	<0.001	0.75**,1**,3*, 4**,6*	0.5**,0.75**,1**, 1.5**,2**,3**,4**, 6*	0.5**,0.75**,1**, 1.5*,2**,3**,4**,6**	0.5**,0.75**,1**, 1.5**,2**,3**	1.5**,2**,3**,4**,6**	0.5*,0.75**,1**, 1.5**, 2**,3**,4**, 6**
T	AUC (df = 3,30)	6.475	0.002	NS	**	NS	*	NS	NS
	Peak (df = 3,30)	1.420	0.252						
	T_{max}	2.93	0.402						
	T-C (df = 3,270)	4.598	<0.001	0.75**	1.5**,2**,3**,4**, 6**	0.75**,1**,2**,4**,6**	0.75**,1.5**,2**,3**,4**,6**	0.75**,1**,1.5**	0.75**,1**,1.5*,2*,3**,4**
PD	AUC (df = 3,30)	20.973	<0.001	NS	**	**	**	NS	**
	Peak (df = 3,30)	25.333	<0.001	NS	**	**	**	NS	**
	T_{max}	14.24	0.003	NS	*	NS	NS	NS	NS
	T-C (df = 3,270)	8.972	<0.001		0.5*,0.75**,1**, 1.5**,2*	0.5**,0.75**,1**,1.5**, 2**, 3**	0.5*,0.75**,1**,1.5**,2*	2*,3**	0.5**,0.75**,1**,1.5**,2**,3**
Maddox-wing	AUC (df = 3,30)	7.674	0.001	NS	NS	NS	**	NS	*
	Peak (df = 3,30)	13.1320	<0.001	NS	**	NS	**	NS	**
	T_{max}	6.21	0.102						
	T-C (df = 3,270)	4.585	<0.001	1**,1.5*,4*	0.75**,1**,1.5**,2*	0.75**,1*	0.5**,0.75**,1**,1.5**,2**,3**,4**	1**, 1.5*	0.75**,1**,1.5**,2**,4**
Visual Analogue Scales									
High	AUC (df = 3,30)	14.998	<0.001	NS	NS	**	NS	**	**
	Peak (df = 3,30)	27.105	<0.001	NS	**	**	**	NS	**
	T_{max}	28.39	<0.001	NS	*	*	NS	*	*
	T-C (df = 3,270)	10.564	<0.001		0.5**,0.75**, 1**,1.5**,2*	0.75**, 1**, 1.5**, 2**,3**, 4**	0.5**, 0.75**, 1**, 1.5**, 2*	0.5*, 0.75**, 1.5**,2**,3**,4*	0.75**, 1**,1.5**,2**,3**,4**
Drunkenness	AUC (df = 3,30)	20.076	<0.001	**	NS	**	**	**	NS
	Peak (df = 3,30)	30.600	<0.001	**	NS	**	**	**	NS
	T_{max}	30.12	<0.001	*	NS	*	*	*	NS
	T-C (df = 3,270)	8.780	<0.001	0.75**,1**,1.5**,2**,3**		0.75**,1**,1.5**,2**,3*	0.75**,1**,1.5**, 2**,3**	0.75**,1**,1.5**,2**,3*	0.75**,1**,1.5**
Stimulated	AUC (df = 3,30)	9.434	<0.001	NS	NS	**	NS	*	*
	Peak (df = 3,30)	17.026	<0.001	*	**	**	NS	NS	**
	T_{max}	23.69	<0.001	*	*	*	NS	NS	NS
	T-C (df = 3,270)	5.568	<0.001	0.75**, 1**,1.5**	0.5**,0.75**,1**,1.5**	0.5**,0.75**,1**,1.5**,2**,3**	0.5**,0.75**	1.5**,2**,3**	0.5**,0.75**,1**,1.5**, 2**
Any effect	AUC (df = 3,30)	13.702	<0.001	**	NS	**	NS	**	NS
	Peak (df = 3,30)	21.813	<0.001	**	**	**	NS	NS	NS
	T_{max}	24.38	<0.001	*	*	*	NS	NS	NS
	T-C (df = 3,270)	6.411	<0.001	0.75**,1**,1.5**,2**	0.5**,0.75**,1**,1.5**	0.5**,0.75**,1**, 1.5**,2**,3**	0.5**	1*,1.5**,2**,3**	0.5**,1.5**,2*

(Continued)

TABLE 1 | Continued

Variable				Tukey's Multiple Comparison Test (*p < 0.05; **p < 0.01)					
	ANOVA			Placebo			Mephedrone		Alcohol
	Parameter	F/X^2	p Value	Alcohol	Mephedrone	Mephedrone–alcohol	Alcohol	Mephedrone–alcohol	Mephedrone–alcohol
Good effects	AUC (df = 3,30)	14.115	<0.001	NS	NS	**	NS	**	**
	Peak (df = 3,30)	22.754	<0.001	NS	**	**	*	NS	**
	T_{max}	19.21	<0.001	*	*	*	NS	NS	NS
	T-C (df = 3,270)	6.173	<0.001	1**.1.5*	0.5**,0.75**,1**, 1.5**,2**	0.25**,0.5**,0.75**, 1**,1.5**,2**,3**	0.75**,1*,1.5*	0.25**,0.5**,3**	0.25**,0.5**,0.75**, 1**,1.5**,2*,3**
Bad effects	AUC (df = 3,30)	3.426	0.030	*					
	Peak (df = 3,30)	2.384	0.089						
	T_{max}	8.29	0.040	NS	NS	NS	NS	NS	NS
	T-C (df = 3,270)	1.164	0.268						
Liking	AUC (df = 3,30)	12.629	<0.001	NS	NS	**	NS	*	**
	Peak (df = 3,30)	15.248	<0.001	NS	**	**	*	NS	**
	T_{max}	17.67	0.001	NS	*	*	NS	NS	NS
	T-C (df = 3,270)	5.756	<0.001		0.5*,0.75**,1**, 1.5**	0.5**,0.75**,1**, 1.5**,2**,3**	0.5*,0.75**,1**	1.5**,2**,3**	0.5**,0.75**,1**, 1.5**,2**,3**
Content	AUC (df = 3,30)	10.094	<0.001	NS	NS	**	NS	**	**
	Peak (df = 3,30)	16.287	<0.001	**	**	**	NS	NS	*
	T_{max}	22.4	<0.001	*	*	*	NS	NS	NS
	T-C (df = 3,270)	4.726	<0.001	1**,1.5**,2*	0.75**,1**,1.5**, 2*	0.5**,0.75**,1**, 1.5**,2**,3**	0.75**	1.5**,2**,3**	0.5**,0.75**,1**,1.5**, 2**,3**
Drowsiness	AUC (df = 3,30)	6.629	0.001	**	NS	NS	**	NS	*
	Peak (df = 3,30)	5.383	0.004	**	NS	NS	*	NS	NS
	T_{max}	14.73	0.002	NS	NS	NS	NS	*	NS
	T-C (df = 3,270)	2.117	<0.001	2*,3**,4**		6*	2**,4**	6*	2**,3**,4*
Dizziness	AUC (df = 3,30)	3.375	0.031	*	NS	NS	NS	NS	NS
	Peak (df = 3,30)	3.055	0.043	*	NS	NS	NS	NS	NS
	T_{max}	13.13	0.004	NS	NS	NS	NS	NS	NS
	T-C (df = 3,270)	1.521	0.052						
Confusion	AUC (df = 3,30)	2.347	0.093						
	Peak (df = 3,30)	2.395	0.088						
	T_{max}	8.10	0.044	NS	NS	NS	NS	NS	NS
	T-C (df = 3,270)	1.679	0.022	0.5*,0.75*,2**		0.5**,0.75**,1**, 1.5**,2*	2**		1.5*
Changes in	AUC (df = 3,30)	2.071	0.125						
Distances	Peak (df = 3,30)	1.530	0.226						
	T_{max}	9.87	0.020	NS	NS	NS	NS	NS	NS
	T-C (df = 3,270)	1.513	0.054						
Changes in Colors	AUC (df = 3,30)	3.184	0.038	NS	NS	*	NS	NS	NS
	Peak (df = 3,30)	2.310	0.096						
	T_{max}	6.38	0.095						
	T-C (df = 3,270)	1.352	0.120						
Changes in lights	AUC (df = 3,30)	2.880	0.052						
	Peak (df = 3,30)	2.652	0.067						
	T_{max}	7.03	0.071						
	T-C (df = 3,270)	1.661	0.024		1*	0.5**,0.75**,1**, 1.5**,2**		0.5**,0.75**,1.5*	0.5**,0.75**,2*
Changes in hearing	AUC (df = 3,30)	1.102	0.364						
	Peak (df = 3,30)	1.574	0.216						
	T_{max}	7.32	0.062						
	T-C (df = 3,270)	1.587	0.037		0.5**,0.75**,1*		0.5**,0.75**,1*	0.5*	
Different body	AUC (df = 3,30)	4.905	0.007	NS	NS	**	NS	NS	NS
feeling	Peak (df = 3,30)	7.766	0.001	NS	**	**	NS	NS	NS
	T_{max}	14.43	0.002	NS	NS	*	NS	NS	NS
	T-C (df = 3,270)	3.535	<0.001		0.5**,0.75**,1**	0.5**,0.75**,1**, 1.5**,2**,3**	0.5**,0.75**,1**	1.5*,3**	0.5**,0.75**,1**,1.5**,3*

(Continued)

TABLE 1 | Continued

Variable		ANOVA		Tukey's Multiple Comparison Test (*p < 0.05; **p < 0.01)					
				Placebo			Mephedrone		Alcohol
	Parameter	F/X^2	p Value	Alcohol	Mephedrone	Mephedrone–alcohol	Alcohol	Mephedrone–alcohol	Mephedrone–alcohol
Different surroundings	AUC (df = 3,30)	1.426	0.255						
	Peak (df = 3,30)	2.147	0.115						
	T_{max}	9.04	0.029	NS	NS	NS	NS	NS	NS
	T-C (df = 3,270)	1.243	0.195						
ARCI questionnaire subscales									
ARCI-PCAG	AUC(df = 3,30)	12.704	<0.001	**	NS	NS	**	NS	**
	Peak (df = 3,30)	6.992	0.001	**	NS	NS	*	NS	**
	T_{max}	11.64	0.009	*	NS	NS	NS	NS	NS
	T-C (df = 3,150)	6.992	<0.001	1.5*,2**,3**,4**,6**		1.5**,2*,6**	1.5**,2**,3**,4**,6**	2**,3**,6**	1.5**,2**,3**,4**
ARCI-MBG	AUC (df = 3,30)	20.441	<0.001	NS	*	**	NS	**	**
	Peak (df = 3,30)	23.217	<0.001	NS	**	**	*	*	**
	T_{max}	18.88	<0.001	*	*	*	NS	NS	NS
	T-C (df = 3,150)	11.162	<0.001		1.5**,2**	1.5**,2**,3**,4*	1.5**,2**	1.5**,2**,3**	1.5**,2**,3**
ARCI-LSD	AUC (df = 3,30)	3.787	0.020	NS	NS	*	NS	NS	NS
	Peak (df = 3,30)	3.290	0.034	NS	NS	NS	NS	NS	NS
	T_{max}	15.43	0.001	*	NS	*	NS	NS	NS
	T-C (df = 3,150)	2.357	0.005/ 0.025	1.5**,2**,3**	1.5**	1.5**,2**,3*,4**	3*	4**	
ARCI-BG	AUC (df = 3,30)	13.389	<0.001	NS	NS	*	**	NS	**
	Peak (df = 3,30)	12.759	<0.001	NS	NS	**	**	NS	**
	T_{max}	14.90	0.001	*	NS	*	NS	NS	NS
	T-C (df = 3,150)	5.124	<0.001	3**	1.5**	1.5**,2**,3**	1.5**,2**,3**,4*	2*,3**	1.5**,2**,3**,4**
ARCI-A	AUC (df = 3,30)	17.750	<0.001	NS	**	**	*	NS	**
	Peak (df = 3,30)	24.674	<0.001	NS	**	**	*	NS	**
	T_{max}	17.60	0.001	NS	*	*	NS	NS	NS
	T-C (df = 3,150)	9.197	<0.001	1.5*	1.5**,2**,3*	1.5**,2**,3**,4**	1.5**,2**	2**,3**	1.5**,2**,3**,4**
VESSPA questionnaire subscales									
VESSPA-S	AUC (df = 3,30)	7.671	0.001	**	NS	NS	**	NS	NS
	Peak (df = 3,30)	9.517	<0.001	**	NS	*	*	NS	NS
	T_{max}	21.03	<0.001	*	*	*	NS	NS	NS
	T-C (df = 3,150)	2.989	<0.001	1.5**, 2**,3**,4**,6**		6**	1.5**, 2**,3**,4*,6**	6**	1.5**,2**,3**
VESSPA-ANX	AUC (df = 3,30)	28.198	<0.001	NS	**	**	*	**	**
	Peak (df = 3,30)	31.959	<0.001	NS	**	**	**	NS	**
	T_{max}	21.51	<0.001	*	*	*	NS	NS	NS
	Time Course (df = 3,150)	20.031	<0.001		1.5**,2**,3**	1.5**,2**,3**,4**,6**	1.5**,2**,3*	2**,3**,4**,6**	1.5**,2**,3**,4**,6**
VESSPA-CP	AUC (df = 3,30)	3.445	0.029	NS	NS	NS	NS	NS	NS
	Peak (df = 3,30)	3.604	0.025	NS	NS	NS	NS	NS	NS
	T_{max}	8.88	0.031	NS	NS	NS	NS	NS	NS
	T-C (df = 3,150)	2.159	0.010	1.5**,2**,3*		1.5**,2**,3**	1.5**,2*,3*	1.5**,2**,3*	
VESSPA-SOC	AUC (df = 3,30)	11.817	<0.001	NS	**	**	NS	NS	**
	Peak (df = 3,30)	13.779	<0.001	NS	**	**	NS	NS	**
	T_{max}	16.53	0.001	NS	NS	NS	NS	NS	NS
	T-C (df = 3,150)	8.619	<0.001	1.5**,2*	1.5**,2**	1.5**,2**,3**,4*	1.5**	1.5**,2**,3**	1.5**,2**,3**
VESSPA-ACT	AUC (df = 3,30)	15.151	<0.001	NS	NS	**	NS	**	**
	Peak (df = 3,30)	20.330	<0.001	NS	**	**	NS	NS	**
	T_{max}	15.03	0.002	*	NS	NS	NS	NS	NS
	T-C (df = 3,150)	10.297	<0.001	1.5**	1.5**,2**	1.5**,2**,3**,4*	1.5**	1.5**,2**,3**	1.5**,2**,3**

(Continued)

TABLE 1 | Continued

Variable		ANOVA		Tukey's Multiple Comparison Test (*p < 0.05; **p < 0.01)					
				Placebo			Mephedrone		Alcohol
	Parameter	F/X^2	p Value	Alcohol	Mephedrone	Mephedrone–alcohol	Alcohol	Mephedrone–alcohol	Mephedrone–alcohol
VESSPA-PS	AUC (df = 3,30)	3.463	0.028	NS	NS	*	NS	NS	NS
	Peak (df = 3,30)	5.497	0.004	NS	NS	**	NS	NS	*
	T_{max}	13.53	0.004	NS	NS	NS	NS	NS	NS
	T-C (df = 3, 150)	4.025	<0.001		1.5**	1.5**,2**,3*,4*		2**,4*	1.5**,2**
Cortisol	AUC (df = 3,30)	19.266	<0.001	NS	**	**	**	NS	**
	Peak (df = 3,30)	17.240	<0.001	NS	**	**	**	NS	**
	T_{max}	17.340	0.001	NS	NS	NS	NS	NS	NS
	T-C (df = 3, 120)	8.404	<0.001	NS	1**,2**	1**,2**	1**,2**	NS	1**,2**

Peak = peak effects from 0 to 6 hours measured by mmHg (systolic blood pressure [SBP]; diastolic blood pressure [DBP]), bpm (heart rate [HR]),
°C (temperature (T)), mm (pupil diameter (PD); mm visual analog scale (VAS0)), and score (Addiction Research Center Inventory (ARCI), score Evaluation of Subjective Effects of Substances with Abuse Potential questionnaire (VESSPA-SEE)] and expressed as mean.
AUC = area under the curve from 0 to 6 h measured by units x hours and expressed as mean.
Tmax = time to reach peak effects measured by hours and expressed as median.
df = degree of freedom.
*For Peak, AUC and T-C an ANOVA and multiple Tukey post hoc comparisons were used. Statistical differences among conditions are presented as **p < 0.01 and *p < 0.05.*
*For Tmax a Friedman test and multiple Wilcoxon post-hoc comparisons were used. Differences among conditions are presented as *p < 0.008; **p < 0.002.*

differences in AUC and several T-C points compared with mephedrone alone. In addition, for "stimulated," "any effect," "good effects," "liking," and "content" scores these differences were statistically significant for peak effects and, in the case of "high," also for T_{max}. As expected, statistically significant differences were detected in drunkenness rating scores between conditions (AUC, peak, T_{max}, and T-C, **Tables 1** and **2**).

The alcohol condition produced an increase in the ratings of "drunkenness," "content," "stimulated," "any effect," "good effects," and "liking" compared with placebo (**Table 2**).

Regarding "drunkenness," the most characteristic effect for alcohol, a no significant lower score was observed for the combination condition in comparison to the alcohol condition (peak effect 34.18 ± 21.53 vs 49.36 ± 25.70 mm, respectively; **Table 2**), but significant differences, however, were only found at several T-C points (0.75–1.5 h). In contrast, significant differences were detected for "content" effects in AUC, peak effects, and T-C points (0.5–3 h) with higher scores in the combination condition compared with the alcohol one (**Tables 1** and **2**).

In the ARCI questionnaire, both conditions including mephedrone produced an increase in the scores of MBG (euphoria), BG (intellectual efficiency and energy) (**Tables 1** and **2**), A (amphetamine-like effects), and LSD (dysphoria) subscales in comparison to placebo (when considering AUC, peak effects, and both). The MBG, BG, and A peak scores were 10.36 ± 3.91, 3.82 ± 3.52, and 6.55 ± 2.73 points for the combination condition and 6.45 ± 5.80, 2.64 ± 2.94, and 4.82 ± 2.79 points for the mephedrone condition, respectively. For MBG, significant differences were detected in AUC, peak effects, and several T-C points (1.5–3h) between conditions. For the other subscales only significant differences were detected at several T-C points. Conversely, for the LSD subscale no significant differences were detected between both conditions.

Alcohol produced a statistically significant increase in the PCAG-sedation subscale in comparison to placebo. Compared with the alcohol condition, the combination with mephedrone reduced the sedation induced by alcohol (peak effect 6.18 ± 3.60 vs 5.26 ± 1.58) with statistical differences in peak effects and AUC that remained statistically significant during 2.5 h in the T-C analysis (1.5–4 h). In contrast, MBG peak difference scores were lower for alcohol compared to the combination condition (2.27 ± 1.62 vs 10.36 ± 3.91, respectively) with statistical differences in peak effects, AUC, and T-C analysis (1.5–3 h) (**Tables 1** and **2**).

Regarding the VESSPA-SEE questionnaire, the combination and mephedrone conditions increased all the subscales compared with placebo. The combination condition, in comparison to the mephedrone one, presented statistical differences in peak effects for ACT (activity and energy) and ANX (psychosomatic anxiety) subscales and in several T-C points for all the subscales (**Tables 1** and **2**). With respect to the alcohol condition, statistical differences in peak effects, AUC, and T-C points for ANX, SOC, (pleasure and sociability) and ACT subscales and only in peak effects for CP subscale were detected compared to the combination one.

TABLE 2 | Summary of Peak effects (maximal effect, mean value and standard error) for physiological, subjective and cortisol concentrations (Cmax) (n = 11).

	Placebo	Alcohol	Mephedrone	Mephedrone + alcohol
Physiological effects				
SBP	-5.82 ± 2.96	-5.00 ± 4.52	35.45 ± 4.28	40.36 ± 3.57
DBP	0.27 ± 3.10	-1.73 ± 3.86	19.64 ± 2.08	22.45 ± 1.83
HR	-7.18 ± 2.28	9.91 ± 4.35	32.27 ± 6.03	40.09 ± 5.25
T	-0.94 ± 0.12	-0.47 ± 0.14	-0.56 ± 0.21	-0.56 ± 0.16
PD	0.00 ± 0.00	0.00 ± 0.00	1.64 ± 0.35	1.91 ± 0.33
Maddox-wing	0.00 ± 0.27	1.41 ± 0.39	-3.45 ± 0.82	-1.41 ± 0.88
Visual Analogue Scales				
High	0.00 ± 0.00	1.55 ± 1.55	47.36 ± 9.31	55.09 ± 7.90
Drunkenness	0.00 ± 0.00	49.36 ± 7.75	0.00 ± 0.00	34.18 ± 6.49
Stimulated	0.00 ± 0.00	23.00 ± 5.77	42.73 ± 8.56	53.18 ± 8.17
Any effect	0.00 ± 0.00	49.91 ± 7.40	51.18 ± 9.27	56.09 ± 7.68
Good effects	0.00 ± 0.00	23.18 ± 5.20	51.09 ± 8.59	63.36 ± 8.49
Bad effects	0.00 ± 0.00	14.18 ± 7.26	3.45 ± 2.06	4.55 ± 2.05
Liking	0.00 ± 0.00	17.18 ± 7.19	44.09 ± 8.59	50.27 ± 8.31
Content	0.00 ± 0.00	30.91 ± 6.73	43.64 ± 8.24	56.09 ± 8.16
Drowsiness	8.45 ± 2.62	29.09 ± 8.14	12.36 ± 4.81	17.00 ± 3.95
Dizziness	0.00 ± 0.00	20.27 ± 8.07	6.18 ± 5.70	11.36 ± 8.00
Confusion	0.00 ± 0.00	20.64 ± 7.67	11.00 ± 5.85	15.73 ± 9.29
Changes in Distances	0.00 ± 0.00	11.55 ± 6.14	11.64 ± 6.53	13.09 ± 6.78
Changes in Colors	0.00 ± 0.00	4.73 ± 2.46	6.27 ± 3.45	8.55 ± 3.84
Changes in lights	0.00 ± 0.00	6.36 ± 2.76	6.64 ± 4.11	11.64 ± 5.73
Changes in hearing	0.00 ± 0.00	3.18 ± 1.58	14.18 ± 9.29	6.27 ± 4.13
Different body feeling	0.00 ± 0.00	13.27 ± 5.21	30.91 ± 9.01	33.18 ± 9.67
Different surroundings	0.00 ± 0.00	9.91 ± 5.39	10.73 ± 3.61	15.36 ± 7.80
ARCI questionnaire subscales				
ARCI-PCAG	1.00 ± 0.65	6.18 ± 1.09	2.82 ± 1.19	1.73 ± 1.58
ARCI-MBG	0.09 ± 0.09	2.27 ± 0.49	6.45 ± 1.75	10.36 ± 1.18
ARCI-LSD	-0.45 ± 0.25	1.55 ± 0.85	0.73 ± 0.74	1.55 ± 0.96
ARCI-BG	0.00 ± 0.23	-2.27 ± 0.54	2.64 ± 0.89	3.82 ± 1.06
ARCI-A	0.27 ± 0.14	2.00 ± 0.47	4.82 ± 0.84	6.55 ± 0.82
VESSPA questionnaire subscales				
VESSPA-S	1.09 ± 1.09	9.36 ± 2.49	4.27 ± 1.47	6.09 ± 1.40
VESSPA-ANX	0.00 ± 0.00	1.55 ± 0.41	9.00 ± 1.72	12.36 ± 1.46
VESSPA-CP	0.00 ± 0.00	1.64 ± 0.65	0.45 ± 0.25	1.91 ± 0.91
VESSPA-SOC	0.09 ± 0.09	4.09 ± 1.46	7.36 ± 2.22	13.00 ± 1.73
VESSPA-ACT	0.27 ± 0.19	4.36 ± 0.96	8.18 ± 2.14	12.55 ± 1.52
VESSPA-PS	0.00 ± 0.00	0.64 ± 0.39	1.27 ± 0.45	2.45 ± 0.87
Cortisol	14.93 ± 4.50	16.20 ± 4.88	22.23 ± 6.70	23.85 ± 7.19

SBP: systolic blood pressure, mm; DBP: diastolic blood pressure, mmHg; HR: heart rate, beats per minute; T: temperature, °C; PD: pupil diameter, mm; visual analogue scale, mm, ARCI: Addiction Research Center Inventory [ARCI], score; VESSPA: Evaluation of Subjective Effects of Substances with Abuse Potential questionnaire, score.
*Only statistically significant results are showed (see **Table 1** for comparisons). Conditions are mephedrone plus alcohol (mephedrone–alcohol), mephedrone plus placebo alcohol (mephedrone), placebo mephedrone plus alcohol (alcohol), and placebo mephedrone plus placebo alcohol (placebo).*

In the pharmacologic drug class identification questionnaire, the combination condition (mephedrone plus alcohol) was correctly identified by all subjects (100% designer drug and 100% alcohol). For the mephedrone condition (mephedrone plus placebo), mephedrone was identified by eight subjects as a designer drug (72.7%), as a stimulant (18.2%) by two, and as placebo (9.1%) by one, whilst placebo was identified correctly by all the subjects (100%). With respect to the alcohol/placebo alcohol conditions, the placebo was identified as such by nine subjects (81.8%), and as a designer drug (9.1%) and cannabinoids (9.1%) by one subject each, whilst alcohol was identified correctly by all subjects (100%).

All the active conditions (mephedrone, alcohol, and the combination) were well tolerated and no mania, hallucinations or psychotic reactions were observed or reported during the study.

Pharmacokinetic Measures

Mephedrone and Alcohol Concentrations

The pharmacokinetic parameters for mephedrone and alcohol are summarized in **Table 3** and **Figure 3**.

When the two conditions containing mephedrone were compared, no significant differences were found in the pharmacokinetic parameters (**Table 3**). In the combination condition, mephedrone concentrations peaked at 1.5 h. In the mephedrone condition, mephedrone concentrations peaked at 1.5 h. In both conditions, at 10 h following first drug administration, mephedrone concentrations declined to mean values of 15.73 and 12.76 ng/ml until undetectable levels at 24 h, respectively (**Figure 3**).

Regarding alcohol, significant differences in pharmacokinetics were detected. Alcohol concentrations peaked at 2 h (in the combination condition and at 1.5 h in the alcohol one.

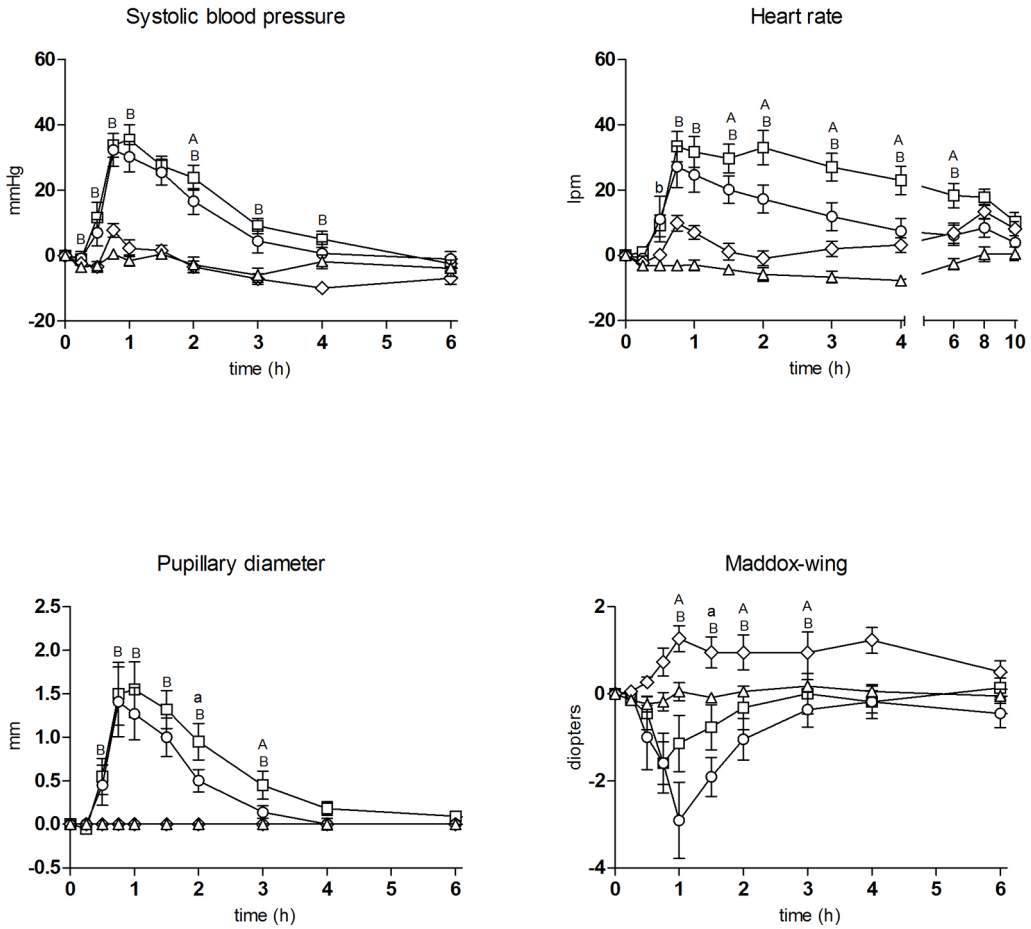

FIGURE 1 | Time course of drug effects (n = 11, mean, standard error) on physiological and psychomotor performance (differences from baseline). □ mephedrone + alcohol; ○ mephedrone; ◊ alcohol; Δ placebo; Significant differences between mephedrone vs mephedrone + alcohol (a: $p < 0.05$/A: $p < 0.01$); alcohol vs mephedrone + alcohol (b: $p < 0.05$/B: $p < 0.01$).

Significant differences in AUC were detected between alcohol and combination conditions.

Cortisol Concentrations

Kinetic parameters for cortisol are summarized in **Tables 1** and **2**. Plasma cortisol concentrations were significantly higher (peak and AUC) after the administration of the combination and mephedrone conditions as compared with placebo.

Cortisol concentrations peaked at 2 h with a mean peak of 23.85 ± 1.31 μg/dl after the combination condition and 22.23 ± 1.23 μg/dl after mephedrone administration. The combination condition also showed significant differences in comparison to alcohol, cortisol concentrations peaked at 2 h with a mean peak of 16.20 ± 0.82 μg/dl. AUC for combination, mephedrone and alcohol conditions were 96.05, 87.29 and 62.31 ng/ml·h^{1}, respectively.

Significant differences in peak, AUC and several T-C points (1 and 2 h) were observed between the combination and mephedrone conditions in comparison to placebo, and also between alcohol and the combination conditions in comparison to mephedrone.

DISCUSSION

To the best of our knowledge, this study provides the first data in humans about the pharmacodynamics and pharmacokinetics of mephedrone and alcohol interactions and completes previous results on neurocognitive performance effects (de Sousa Fernandes Perna et al., 2016). Our findings demonstrate the increased pharmacological effects of the co-administration of mephedrone and alcohol compared to single drug administration.

The 200 mg oral administration of mephedrone reproduced pharmacological effects which concurred with the sole experimental study performed to date in humans (Papaseit et al, 2016). Our results demonstrate that mephedrone produced a significant increase in BP, HR, and PD. It also

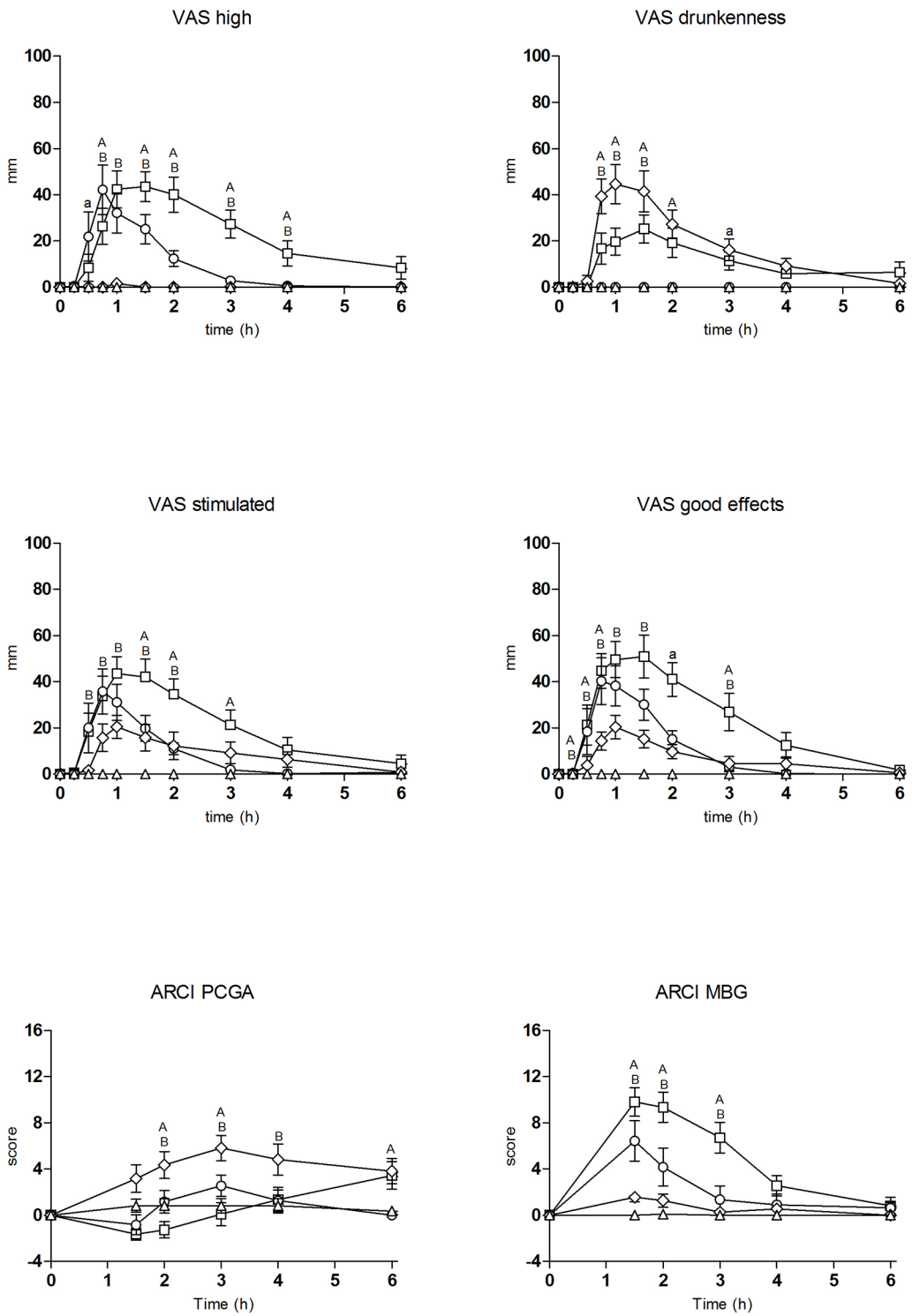

FIGURE 2 | Time course of drug effects (n = 11, mean, standard error) on subjective effects (differences from baseline). □ mephedrone + alcohol; ○ mephedrone; ◊ alcohol; Δ placebo; Significant differences between mephedrone vs mephedrone + alcohol (a: $p < 0.05$/A: $p < 0.01$); alcohol vs mephedrone + alcohol (b: $p < 0.05$/B: $p < 0.01$).

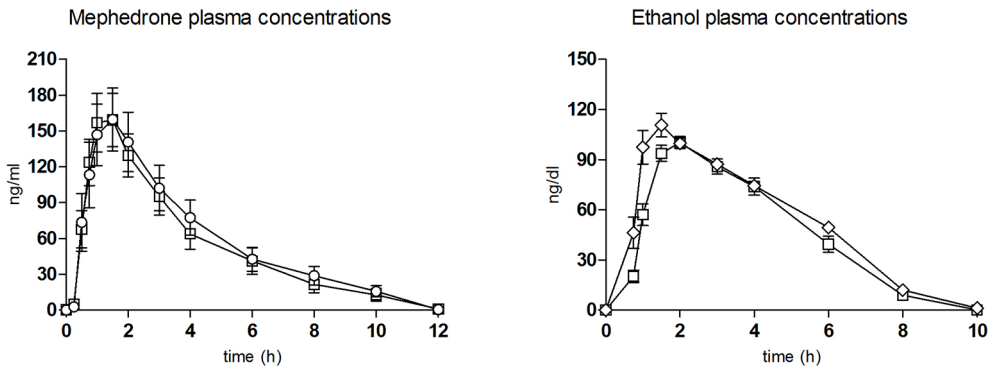

FIGURE 3 | Plasma concentration over time curves of mephedrone (left) and ethanol (right) (n = 11, mean, standard error). ○ mephedrone; ◊ alcohol; □ mephedrone + alcohol.

TABLE 3 | Pharmacokinetics parameters of mephedrone and alcohol in plasma (n = 11).

Pharmacokinetic parameters	Cmax (ng/ml)	AUC_{0-6} (ng/ml h^{-1})	AUC_{0-24} (ng/ml h^{-1})	Tmax (h)	Ke (h^{-1})	t1/2 (h)
Mephedrone						
Mephedrone and ethanol	175.7 ± 71.1	516.8 ± 264.6	709.8 ± 477.1	1.5 (0.75–2)	0.35 ± 0.14	2.32 ± 1.01
Mephedrone	172.6 ± 82.9	549.0 ± 315.0	778.4 ± 512.9	1.5 (0.5–2)	0.29 ± 0.09	2.68 ± 0.92
p-value	NS	NS	NS	NS	NS	NS
	Cmax (ng/ml)	**AUC_{0-6} (ng/ml h^{-1})**	**AUC_{0-10} (ng/ml h^{-1})**	**Tmax (h)**		
Ethanol						
Mephedrone and ethanol	103.8 ± 14.1	389.8 ± 73.3	447.0 ± 96.8	2.0 (1.5–2)		
Ethanol	121.1 ± 14.9	438.4 ± 36.8	512.9 ± 45.2	1.5 (1–2)		
p-value	0.030	0.048	0.020	0.014		

AUC: area under the curve. SD: standard deviation. T_{max} is shown as median (range) values. NS: not statistically significant. A value of $p < 0.05$ was considered statistically significant.

induced stimulant-like effects (euphoria, well-being, feelings of pleasure) and mild changes in perceptions. All these physiological and subjective effects were of rapid onset and short duration. The increase in VAS scores (stimulated, high, good effects, liking) and ARCI (subscales MBG, BG, and A) caused by mephedrone were within the range of previous studies with psychostimulant drugs with a well-known abuse potential (Farré et al., 1993; Hernández-López et al., 2002). Moreover, its faster and shorter duration confirmed results obtained in the previous human investigation (Papaseit et al., 2016). The administration of an oral dose of 0.8 g/kg of alcohol replicated the typical effects of acute alcohol intoxication characterized by an increase in the ratings of drunkenness, sedation, and mild effects on physiological responses compared with placebo.

The co-administration of mephedrone and alcohol amplified cardiovascular effects, producing a more marked increase in HR in comparison with mephedrone alone. In turn, these results are consistent with a previous description of cardiovascular toxicity associated with mephedrone and alcohol co-ingestion (McGaw and Kankam, 2010). Similar findings have also been observed after the concomitant administration of other psychostimulants drugs (MDMA, methamphetamine, cocaine) in combination with alcohol in healthy volunteers (Pérez-Reyes and Jeffcoat, 1992; Farré et al., 1993; Higgins et al., 1993; McCance-Katz et al., 1993; Mendelson et al., 1995; Hernández-López et al., 2002; Dumont et al., 2010).

In addition, the combination of mephedrone and alcohol produced mydriasis and esophoria, an indicator of extraocular muscle tension, two specific acute psychostimulant-like effects, although slighter in comparison to mephedrone alone. These results are in line with those observed after other psychostimulant drugs and alcohol co-administration (Mas et al., 1999; de la Torre et al., 2000; Farré et al., 2015). Alcohol, as expected from the extrapolation of results obtained from other psychostimulant-alcohol interaction studies, attenuated the rise in PD and extraocular musculature contraction induced by mephedrone. Furthermore, the addition of alcohol to mephedrone increased the maximal psychostimulant effects, maintaining higher measures for euphoria and well-being for a longer period of time in comparison to mephedrone alone, which maximal effects are faster (Papaseit et al., 2016). Among conditions, the most remarkable difference was in the mephedrone-alcohol combination. It produced during 4 h relevant increases in subjective scores which were intense during the three first hours compared with 1–2 h following mephedrone alone.

Alcohol administration, equivalent four to six alcoholic beverages, in combination with mephedrone resulted in

decreased drunkenness and reduced sedative effects producing mixed scores (ARCI-PCAG and VAS drowsiness) between the mephedrone and alcohol alone conditions. Mephedrone moderated the effects induced by alcohol in a similar manner to that observed after MDMA experimental administration (Hernández-López et al., 2002).

Overall, the combination of mephedrone and alcohol slightly delayed peak effects and increased maximal effects which remained high with no changes in their total duration. Cardiovascular and subjective effects after mephedrone-alcohol co-administration started at 0.25–5 h, peaked at approximately 0.75–1.5 h after administration, and returned to pre-dose values at 4–8 h after administration with approximately 3–4 h of marked effects. In general terms, mephedrone induced lower effects than the combination and almost overlapped with our own observations in a previous study in which the same dose of mephedrone was administered (Papaseit et al., 2016). Mephedrone effects were observed between 0.5-1 h and most returned to baseline 2–3 h after drug administration, but some last more than 3 h (HR, temperature, stimulated or any effect).

With respect to the pharmacokinetics of the mephedrone-alcohol combination, the most relevant finding was that alcohol did not modify the plasma levels of mephedrone. The maximal concentrations of mephedrone after the combination administration were within the range of those obtained after mephedrone alone and concurred with previously published data (Papaseit et al., 2016). This finding suggests that the pharmacokinetics of mephedrone is not altered when alcohol is concurrently administered.

With reference to alcohol pharmacokinetics, statistically significant differences were detected in C_{max}, AUC, and T_{max}. In this respect, other psychostimulant-alcohol studies have also reported slightly lower alcohol C_{max} in combination conditions related to changes in alcohol metabolism and absorption rate (Farré et al., 1993; McCance-Katz et al., 1993). In the case of mephedrone, initial results suggest a similar kinetic scenario.

An adequate pharmacological effect in relation to pharmacokinetics was thus observed despite the biological variability among subjects. Our results suggest that the higher abuse liability exhibited for mephedrone when concomitantly consumed with alcohol can be attributed to the early onset and maintenance of the subjective/pleasant effects in comparison to mephedrone alone. It should be emphasized that the short mephedrone half-life and T_{max} following its co-administration with alcohol could partially explain the mephedrone-alcohol binge pattern among regular users.

Higher increases in cortisol plasma concentrations were found after the mephedrone-alcohol co-administration and mephedrone alone in comparison to the rest of conditions. This pattern of response has already been observed after cocaine and alcohol (Farré et al., 1997) and MDMA and alcohol administration (Hernández-López et al., 2002). Interestingly, both combinations with alcohol, and also MDMA and cocaine alone, produced significant cortisol increases (Farré et al., 1997; Harris et al., 2002; Hernández-López et al., 2002; Kuypers et al., 2013; Kuypers et al., 2015). As far as we know, the precise mechanism of this effect is poorly understood. Although the role of cortisol in the acute effects of mephedrone has as yet to be described, it could be extrapolated to MDMA and related-amphetamines (Mas et al., 1999). The serotonergic effects of mephedrone might stimulate the hypothalamo-pituitary-adrenal axis, leading to an increase in cortisol plasma concentrations as previously described by other psychostimulants as MDMA (Seibert et al., 2014; Papaseit et al., 2016; Strajhar et al., 2019).

Integrating the results of the present study and the previous one on psychomotor performance (de Sousa Fernandes Perna et al., 2016), it seems that mephedrone reduced some of the subjective feelings of sedation induced by alcohol, but its stimulatory effects were not enough to compensate for the impairing effects of alcohol on most performance parameters. This dissociation between subjective and objective sedation measures is of interest. Subjects may feel less sedated by alcohol and psychomotor abilities remain impaired or unchanged. The potential impact of this dissociation in terms of driving safety is unknown, but it may be plausible that subjects would consider they are driving better when actual performance continues to be impaired by the effect of alcohol. Similar dissociation has been reported when other psychostimulants (e.g. cocaine, MDMA) have been administered simultaneously with alcohol (Farré et al., 1993; Farré et al., 1997; Hernández-López et al., 2002).

The present study has several limitations which are mainly associated with its experimental design. Firstly, the moderate sample size. Secondly, a non-representative sample that not allows to generalize the results across gender (absence of women due to unknown and potential seriously fetal risk). Thirdly, the evaluation of only one dose level of mephedrone and alcohol (users usually are subjected to repeated drug consumption in a single session). Fourthly, the alcohol blind was potentially insufficient. Nonetheless, we conducted a placebo controlled study, randomized, within-subjects, various control conditions (combination condition, mephedrone condition, alcohol condition, and placebo condition). The results provide novel data on pharmacodynamics and pharmacokinetics of mephedrone-alcohol combination.

In summary, the concomitant administration of mephedrone and alcohol produced a significant increase in cardiovascular effects and induced more intense and prolonged feelings of euphoria and well-being, in comparison to mephedrone alone. Mephedrone reduced the drunkenness and sedation produced by alcohol. These effects could encourage the consumption of larger amounts of mephedrone and alcohol, placing the recreational user at a heightened risk for potentially toxic effects. The results presented are like those obtained with the combination of alcohol and other psychostimulants, suggesting that the abuse liability of the simultaneous consumption is greater than that induced by mephedrone alone.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the senior author (Magi Farré, magi.farre@uab.cat).

ETHICS STATEMENT

The protocol was approved by the local Research Ethics Committee (CEIC-Parc de Salut Mar, Barcelona, Spain). The study was conducted in accordance with the Declaration of Helsinki and Spanish laws concerning clinical trials and registered in ClinicalTrials.gov (number NCT02294266).

AUTHOR CONTRIBUTIONS

MF, EP, CP-M, ES, RT, KK, ET, and JR conceptualized the study design. EP, CP-M, JM, ES, FF, MT, and MF collected the data. RT and EO analyzed mephedrone and alcohol concentrations. EP, CP-M, EO, and MF analyzed the data. EP, MF, CP-M, MT, FF, RT, ES, KK, ET, and JR wrote and reviewed the manuscript.

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Hazard Characterization of Synthetic Cathinones Using Viability, Monoamine Reuptake, and Neuronal Activity Assays

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Synthetic cathinones are the second largest class of new psychoactive substances (NPS) on the drug market. Despite the large number of different cathinones and their abundant use, hazard characterization is mainly limited to their potential to inhibit monoamine transporters. To expand the current hazard characterization, we first investigated the acute effects of several synthetic cathinones [4-methylethcathinone (4-MEC), 3-methylmethcathinone (3-MMC), 4-MMC, methylone, pentedrone, α-pyrrolidinovalerophenone (α-PVP), and 3,4-methylenedioxypyrovalerone (MDPV)] on human dopamine, norepinephrine, and serotonin reuptake transporters (hDAT, hNET, and hSERT), which were stably transfected in human embryonic kidney (HEK) 293 cells. Next, we examined effects on spontaneous neuronal activity in rat primary cortical cultures grown on microelectrode arrays (MEAs) as an integrated endpoint for neurotoxicity. Changes in neuronal activity were assessed after acute (30 min) and prolonged (4.5 h) exposure. Moreover, we investigated whether neuronal activity recovered after washout of the exposure (24 h after the start of the 5 h exposure). Low micromolar concentrations of synthetic cathinones inhibited monoamine uptake via hDAT and hNET, while higher cathinone concentrations were needed to inhibit uptake via hSERT. Comparable high concentrations were needed to inhibit spontaneous neuronal activity during acute (30 min) and prolonged (4.5 h) exposure. Notably, while the inhibition of neuronal activity was reversible at low concentrations, only partial recovery was seen following high, but non-cytotoxic, concentrations of synthetic cathinones. Synthetic cathinones with either a pyrrolidine moiety or long alkyl-tail carbon chain more potently inhibit monoamine uptake via hDAT and neuronal activity. Monoamine uptake via hNET was most potently inhibited by synthetic cathinones with a pyrrolidine moiety. The combination of integrated measurements (MEA recordings of neuronal activity) with single target assays (monoamine reuptake transporter inhibition) indicates inhibition of hDAT and hNET as the primary mode of action of these synthetic cathinones. Changes in neuronal activity, indicative for additional mechanisms, were observed at higher concentrations.

Keywords: bath salts, designer drugs, hazard characterization, synthetic cathinones, *in vitro* neurotoxicity assays

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INTRODUCTION

Over the last decade, new psychoactive substances (NPS) have acquired a steady interest and place on the drug market. After the synthetic cannabinoids, synthetic cathinones are the most popular and abundant class of NPS on the drug market (United Nations Office on Drugs and Crime [UNODC], 2017). At the end of 2017, 148 synthetic cathinones were monitored by the United Nations Office on Drugs and Crime (United Nations Office on Drugs and Crime [UNODC], 2018).

Synthetic cathinones are derivatives from the phenylalkylamine cathinone, which is the naturally occurring stimulant in the khat plant (*Catha edulis*). Structurally, synthetic cathinones resemble amphetamine and 3,4-methylenedioxymethamphetamine (MDMA) with the addition of a ketone group (=0). The first synthetic cathinone derivative [i.e., methcathinone (MCAT)] was produced in the late 1920s (Glennon, 2014). Due to its abuse, MCAT was categorized as a Schedule 1 drug in 1971 by the United Nations' Convention on Psychotropic Substances. Decades later, novel synthetic cathinones were introduced, starting with mephedrone (4-MMC) in 2006. In the following year, when mephedrone popularity peaked, novel cathinones methylone, 3,4-methylenedioxypyrovalerone (MDPV), α-pyrrolidinovalerophenone (α-PVP), pentedrone, 3-methylmethcathinone (3-MMC), and 4-methylethcathinone (4-MEC) were introduced to the drug market (Hondebrink et al., 2018; Majchrzak et al., 2018). The cathinone market peaked in 2014 when 30 novel synthetic cathinones were notified to the EU Early Warning System (European Monitoring Centre for Drugs and Drug Addiction [EMCDDA], 2018).

Desired and adverse effects of cathinones overlap with cocaine, amphetamine, and MDMA, and include mild to severe sympathomimetic toxicity and an altered mental status. Consequently, the use of cathinones has resulted in many emergency department visits, poisonings, and fatalities (Wiegand et al., 2012; Gunderson et al., 2013; Hondebrink et al., 2018). Anecdotal user reports suggest variation in effect profile between cathinones (Gunderson et al., 2013). Effects could also differ between drug batches, as the online advertised purity of >95% is not always reached (Gunderson et al., 2013).

Comparable to well-known illicit drugs, cathinones inhibit the reuptake of monoamines via the dopamine (DA), norepinephrine (NE), and/or serotonin (5-HT) reuptake transporters (DAT, NET, SERT) (Simmler et al., 2014; Hondebrink et al., 2018). The pharmacological and toxicological profile of cathinones is not fully elucidated, as hazard characterization is usually based solely on the effects of cathinones on these main targets. However, other targets like receptors and ion channels could also be affected by cathinones. Testing various (secondary) targets would increase knowledge on additional mechanisms of action, but would be time- and money consuming when using single target assays. In addition, toxicity may be over- or underestimated, as effects on other targets could mitigate or exacerbate effects. Therefore, applying an integrated method to measure the effects of cathinones on a diverse range of neuronal targets in a single assay could aid hazard characterization.

In previous research, the applicability of neuronal cultures grown on microelectrode arrays (MEAs) as an efficient screening tool to determine the neurotoxicity of pharmaceuticals, toxins, illicit drugs, and NPS was shown (Dingemans et al., 2016; Vassallo et al., 2017; Strickland et al., 2018; Zwartsen et al., 2018, 2019). MEA measurements allow for determining effects of synthetic cathinones on spontaneous neuronal network activity, thereby including a range of neuronal targets in a single assay. Combining results from single target assays, like inhibition of monoamine reuptake transporters, with integrated methods, like inhibition of neuronal network activity, would strengthen hazard characterization of synthetic cathinones. In the present study, we therefore investigated the potencies of several synthetic cathinones to affect both the uptake via monoamine transporters and neuronal activity (for chemical structures see **Figure 1**).

MATERIALS AND METHODS

Chemicals

3-Methylmethcathinone (2-(methylamino)-1-(3-methylphenyl) propan-1-one), 4-MMC (2-(methylamino)-1-(4-methylphenyl) propan-1-one), 4-MEC (2-(ethylamino)-1-(4-methylphenyl) propan-1-one), methylone (1-(1,3-benzodioxol-5-yl)-2-(methy lamino)propan-1-one), pentedrone (2-(methylamino)-1-phenyl pentan-1-one), and MDPV (1-(1,3-benzodioxol-5-yl)-2-pyrro lidin-1-ylpentan-1-one) hydrochloride salts (purity > 98.5%) were obtained from Lipomed (Weil am Rhein, Germany). Data on α-PVP were published previously (Zwartsen et al., 2017, 2019). 3-MMC and 4-MEC were also purchased from a commercial website for "research chemicals" and are described in this article as $3\text{-MMC}_{\text{internet}}$ and $4\text{-MEC}_{\text{internet}}$. Gas chromatography–mass spectrometry (GC–MS) analysis showed a purity of >99.5% of the online purchased drugs (see **Supplementary Materials** for methods and **Supplementary Figure 1**). All other chemicals were purchased from Life Technologies (Bleiswijk, Netherlands) unless otherwise stated. Stock solutions and dilutions were freshly prepared in HBSS (1×) at the day of usage.

Effects on Monoamine Reuptake Transporters in Transfected HEK 293 Cells

Culture of Transfected HEK 293 Cells

Human embryonic kidney (HEK) 293 cells stably expressing human DAT, NET, or SERT (kindly provided by Dr. Hoener from F. Hoffmann-La Roche Ltd., Basel, Switzerland) were cultured as described in Zwartsen et al. (2017).

Monoamine Uptake Assay

Uptake activity of hNET, hDAT, and hSERT was measured using the Neurotransmitter Transporter Uptake Assay Kit from MDS Analytical Technologies (Sunnyvale, CA, United States) as described in detail previously (Zwartsen et al., 2017). The kit contained a mixture consisting of a fluorescent substrate, which resembles the biogenic amine neurotransmitters, and a masking

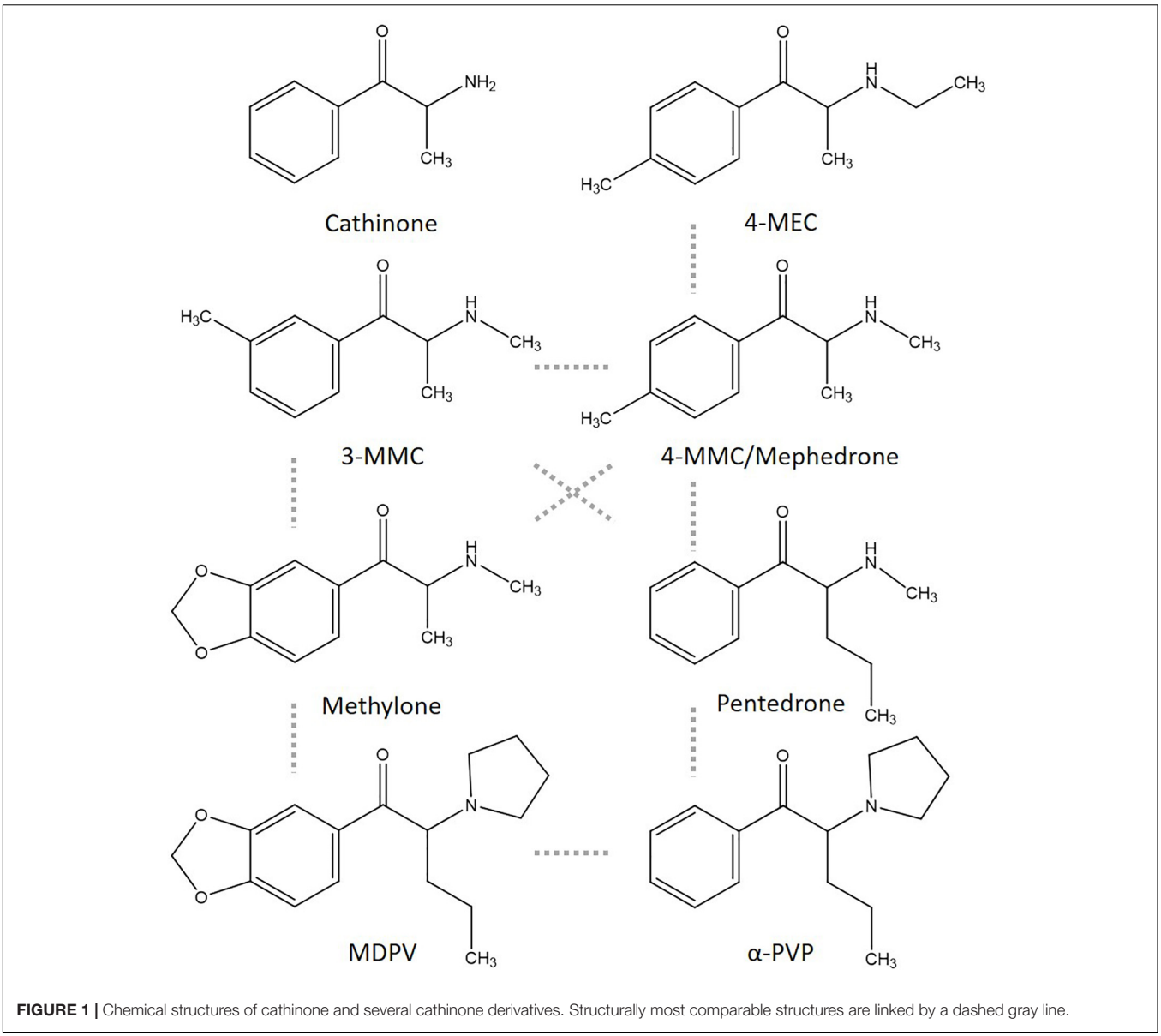

FIGURE 1 | Chemical structures of cathinone and several cathinone derivatives. Structurally most comparable structures are linked by a dashed gray line.

dye that extinguishes extracellular fluorescence. Uptake of the fluorescent substrate, a measure of neurotransmitter transporter function, increases intracellular fluorescence, while extracellular fluorescence is blocked by the masking dye (Jorgensen et al., 2008). The mixture was dissolved in Hank's Balanced Salt Solution (1×) (HBSS) with 20 mM HEPES, and kept up to 1 week at −20°C.

On day 0, HEK 293 cells were seeded (60,000 cells/well) in clear-bottom black-walled 96-well plates coated with poly-L-lysine (PLL, 50 mg/L). On day 1, cells were pre-incubated with the fluorescent substrate mixture (95 μL/well, comprising of the fluorescent substrate and the extracellular masking dye, dissolved in HBSS) for 12 min (t = −12 to t = 0). During incubation, intracellular fluorescence was measured every 3 min. After 12 min (at t = 0), 100 μL/well HBSS without (control) or with drug was added and uptake was continuously measured for 48 min to determine drug-induced inhibition of the monoamine transporters. Stock solutions and dilutions were freshly prepared in HBSS (1×) at the day of usage. Effects of 3-MMC, 4-MMC, 4-MEC, methylone, pentedrone, MDPV, 3-$MMC_{internet}$, and 4-$MEC_{internet}$ were measured at final concentrations of 0.03–300/1000 μM. Fluorescence was measured with a microplate reader (Tecan Infinite M200 microplate; Tecan Trading Männedorf, Switzerland) at 37°C at 430/515 nm excitation/emission wavelength. For more experimental detail see Zwartsen et al. (2017).

Uptake Analysis and Statistics

Data from the monoamine uptake assay were analyzed as described by Zwartsen et al. (2017), with minor modifications. Data for α-PVP were reanalyzed from Zwartsen et al. (2017). In short, the fluorescence of each well was background

corrected and uptake was determined per well by calculating the change in fluorescence (ΔFU) at 12 min after drug exposure (t = 12) compared to the fluorescence at the start of exposure (t = 0), as a percentage of the fluorescence at the start of exposure (%ΔFU = ((FU t = 12 − FU t = 0)/FU t = 0) ∗ 100%; see Zwartsen et al., 2017). Plate-matched control values for each compound (e.g., methylone) above or below two times the standard deviation of the average normalized control value were considered outliers and were removed from analysis (4.8%). Thereafter, uptake in drug-exposed wells was expressed as a percentage of control wells on the same plate and all uptake values were scaled between 0 and 100%. Outliers (>mean ± 2×SD) in exposed groups were removed (2.7%) and uptake (% compared to control) was expressed as mean ± SEM of n_{wells} from N_{plates}.

Cell Viability of HEK 293 Cells

To exclude that effects of cathinones on transporter inhibition were due to a reduction in cell viability, cytotoxicity was investigated using a Neutral Red assay. At day 0, 100 μL of a cell suspension of 600,000 cells/mL was added to each well of a transparent 96-well plate (Greiner Bio-one, Solingen, Germany), coated with PLL. At day 1, medium was removed, and cells were exposed to 3-MMC, 4-MMC, 4-MEC, methylone, pentedrone, MDPV, 3-$MMC_{internet}$, or 4-$MEC_{internet}$ [final concentrations 10–1000 μM in HBSS (1×)]. After 48 min, the exposure medium was changed into DMEM culture medium before the plates were stored at 37°C, 5% CO_2/95% air atmosphere until cell viability was tested 24 h after the start of exposure. At least 20 min before testing cell viability, several non-exposed wells were lysed to obtain background values.

Cell viability was determined as described in Zwartsen et al. (2019). In short, medium and lysis buffer were removed from all wells after which 100 μL NR solution (Invitrogen, Breda, Netherlands; 12 μM in phenol-red free NB-A medium w/o supplements) was added to the cells. Following 1 h incubation in the dark at 37°C, the solution was removed, and the cells were lysed using 100 μL NR lysis buffer. The plate, covered in aluminum foil, was placed on a plate shaker for ∼30 min before fluorescence was measured at 530/645 nm using the Tecan Infinite M200 microplate (Tecan Trading Männedorf, Switzerland). Cell viability was calculated according to Zwartsen et al. (2019), and expressed as mean ± SEM of n_{wells} from N_{plates}. In total, 4.0% of the values were considered outliers (>mean ± 2×SD).

Effects on Spontaneous Neuronal Network Activity Using Rat Cortical Cultures

Culture of Neuronal Networks Derived From Rat Cortices

Rat pups born of timed-pregnant Wistar rats (Envigo, Horst, Netherlands) were sacrificed on postnatal day 0–1 to prepare cortical cultures grown on MEA plates as described previously (Zwartsen et al., 2018, 2019). Briefly, a 50 μL drop of cell suspension was added to each well (1×10^5 cells/well) of a 48-well MEA plate (Axion BioSystems Inc., Atlanta, GA, United States, M768-GL1-30Pt200) coated with 0.1% polyethyleneimine (PEI). After 2 h, 450 μL dissection medium was added to each well. The day after the isolation (day *in vitro* 1; DIV1), 450 μL/well dissection medium was replaced with 450 μL/well glutamate medium. At DIV4, 450 μL/well glutamate medium was replaced with 450 μL/well FBS medium (for medium supplements see Zwartsen et al., 2018, 2019). Cultures were kept in FBS medium at 37°C, 5% CO_2/95% air atmosphere until use at DIV9-10.

MEA Recordings of Spontaneous Neuronal Network Activity

Microelectrode array recordings were performed as described in Zwartsen et al. (2019). In short, neuronal activity was measured using a Maestro 768-channel amplifier (Axion BioSystems Inc., Atlanta, GA, United States). Baseline spontaneous neuronal activity was recorded for 30 min at 37°C, after which wells were exposed to selected synthetic cathinones under sterile conditions. Next, neuronal activity was determined during a 30 min "acute exposure" recording. As the half-life of most illicit drugs and NPS *in vivo* ranges from 0.5 to 5 h in plasma [see Zwartsen et al. (2019) for references], the plate was subsequently incubated for an additional 4 h of exposure at 37°C, after which activity was measured during a 30 min "prolonged exposure" recording. Next, exposure medium was replaced with fresh FBS medium and the plate was incubated for 19 h at 37°C, until the 30 min "washout" recording, i.e., 24 h after the start of the exposure.

Effects of 3-MMC, 4-MMC, 4-MEC, pentedrone, 3-$MMC_{internet}$, and 4-$MEC_{internet}$ were tested at 1–1000 μM (final concentration). At the day of exposure, stocks and dilutions were freshly made in NB-A FBS medium (for medium supplements see Zwartsen et al., 2019). Data on methylone, α-PVP, and MDPV exposure have been published previously in Zwartsen et al. (2019). For each experimental condition, primary cultures from two to three different isolations were used and tested in four to five plates (N_{plates}). The number of wells (n_{wells}) represents the number of replicates per condition.

MEA Analysis

Microelectrode array data were analyzed as described in Zwartsen et al. (2019). In short, parameters of interest after acute exposure were expressed as a percentage of the parameters prior to exposure to obtain a treatment ratio for each well (paired comparison; $parameter_{exposure}$/$parameter_{baseline}$ as % of control wells). The parameters after prolonged exposure and washout were also expressed as a percentage of the baseline parameters. Next, treatment ratios were grouped per parameter, condition, drug [e.g., mean spike rate (MSR) 10 μM 3-MMC] and exposure scenario (acute, prolonged, or washout).

Outliers (>mean ± 2×SD) for MSR (5.0%) were used to exclude wells on all parameters. Outliers for mean burst rate (MBR; 2.6%) and mean network burst rate (MNBR; 1.4%) were used to exclude wells on specific parameters (burst, network burst, and synchronicity parameters, or network burst and synchronicity parameters, respectively). Finally, the acute, prolonged, and washout control treatment ratios were set to 100% and treatment ratios of exposed wells were normalized

to the average treatment ratio of medium control wells of the corresponding parameter and exposure scenario. Treatment ratios of exposed wells were averaged per parameter (e.g., MSR), condition (e.g., 100 μM), drug (e.g., methylone), and exposure scenario (e.g., acute exposure) and used for further statistical analyses [see Zwartsen et al. (2019) for more details on criteria, parameters, and exposure scenarios]. Neuronal activity (as % of control) is expressed as mean ± SEM of n_{wells} from N_{plates}. As the spike, burst, and network burst rates were among the most sensitive parameters, these parameters are presented in the manuscript. Additional parameters are presented in **Supplementary Figure 2**.

Cell Viability of Neuronal Cultures

To exclude that effects of cathinones on neuronal activity were due to cytotoxicity, cell viability was investigated as described in the section "Cell Viability of HEK 293 Cells," with minor modifications. Briefly, 100 μL of a cell suspension of rat cortical cells (3.0×10^4 cells/well) was added to each well of a transparent 96-well plate (Greiner Bio-one, Solingen, Germany). Medium was changed at DIV1 and DIV4 as described for the 48-wells MEA plates, only at smaller volumes (100 μL/well). In addition, the glutamate to FBS medium change on DIV4 was done using phenol-red free FBS medium (medium supplements described in Zwartsen et al., 2019). At DIV9-10, cells (three to six plates from two to three different primary cultures) were exposed for 4.5 h to 3-MMC, 4-MMC, 4-MEC, pentedrone, 3-$MMC_{internet}$, and 4-$MEC_{internet}$ (final concentrations 1–1000 μM in phenol-red free NB-A FBS medium). Thereafter, the exposure medium was replaced with phenol-red free FBS medium before the plates were stored at 37°C, 5% CO_2/95% air atmosphere until the cell viability was tested 19.5 h later, 24 h after the start of exposure. Neutral Red cell viability assay was performed and analyzed as described in Zwartsen et al. (2019). Following the exclusion of outliers (>mean ± 2×SD; 5.4% in the normalized control values and 4.8% in the experimental conditions), cell viability was expressed as mean ± SEM of n_{wells} from N_{plates}.

Statistical Analysis

Concentration–response curves were made for transporter, MEA, and cell viability assays. To calculate IC_{50} values, a four-parameter logistic curve with a variable slope was used ($Y = \text{Bottom} + (\text{Top}-\text{Bottom})/(1 + 10\wedge((\text{LogIC}_{50} - \text{X}) * \text{HillSlope}))$) (GraphPad Prism, version 7.04). When applicable, significance between concentrations and controls (for MEA) and different IC_{50} values [between different transporters (DAT/NET/SERT), MEA parameters (MSR/MBR/MNBR), exposure settings (acute/prolonged/recovery), and drug grade (pharmaceutical/internet)] were determined using unpaired *t*-tests (two values) or one-way ANOVA's followed by a *post hoc* Dunnet's test (>2 values). All statistical tests were performed using GraphPad Prism. For recovery of neuronal activity, IC_{50} values and 95% confidence intervals could not always be determined and are reported as higher than the highest concentration tested (e.g., >1000 μM). In these cases, statistical analysis comparing activity following recovery to activity during acute or prolonged exposure could not be performed. Effects on monoamine reuptake, neuronal activity, and cell viability were considered relevant when the effect was statistically significant ($p < 0.05$) and above the biological variation of the control values (≥15, ≥30, and ≥10%, respectively).

Estimated Brain Concentrations

To correlate reported effect concentrations to expected concentrations in the human brain during recreational use a brain partitioning factor (BPF; serum/brain ratio) was applied, as actual human brain concentrations following recreational drug use are unknown. Expected concentrations in the human brain were estimated by multiplying recreational human blood/serum/plasma concentrations with the BPF. Human recreational blood, serum, or plasma levels were obtained from literature (driving under the influence or accidental non-fatal intoxications). The BPF was determined for each drug by dividing the brain concentration by the blood/serum/plasma concentration found in human post mortem reports, or animal studies (rat/mice) when human data were insufficient.

RESULTS

Effects on Monoamine Reuptake

All cathinones inhibited monoamine uptake via hDAT, hNET, and hSERT (**Figure 2**; data for α-PVP re-analyzed from Zwartsen et al., 2017). Uptake via hSERT was less potently inhibited compared to uptake via hDAT and hNET. 3-MMC, 4-MEC, and pentedrone inhibited hDAT at lower concentrations compared to hNET, while 4-MMC, α-PVP, and MDPV more potently inhibited hNET ($p < 0.05$; **Table 1**). Methylone inhibited hDAT and hNET with comparable potency. MDPV was the most potent hDAT and hNET inhibitor with IC_{50} values of 0.07 and 0.03 μM, respectively (**Table 1**). For hDAT inhibition the rank order was MDPV < α-PVP/pentedrone < 4-MMC/4-MEC < methylone/3-MMC. hNET was inhibited with the following rank order: MDPV < α-PVP < 4-MMC < pentedrone/methylone < 3-MMC/4-MEC. Uptake via hSERT was inhibited by MDPV and pentedrone with IC_{50} values of 4.5 and 16 μM, respectively, while all other cathinones inhibited hSERT with IC_{50} values ≥100 μM (**Table 1**).

While 4-$MEC_{internet}$ inhibited hDAT with comparable potency compared to pharmaceutical grade 4-MEC, less than twofold differences in potencies between the pharmaceutical grade and internet-bought drugs for 4-MEC at hNET and 3-MMC at hDAT and hNET were seen ($p < 0.05$; **Figures 2B,E** and **Table 1**). Inhibition of uptake via hSERT did not significantly differ between the pharmaceutical grade cathinones and the cathinones bought via the internet.

Effects on Neuronal Activity

Effects of methylone, α-PVP, and MDPV were published previously in Zwartsen et al. (2019) (**Supplementary Figure 2**). Here, we extend on these results by measuring the effects of 3-MMC, 4-MMC, 4-MEC, and pentedrone on neuronal activity. All investigated synthetic cathinones inhibited the MSR, MBR, and MNBR after acute exposure (**Figure 3**; for

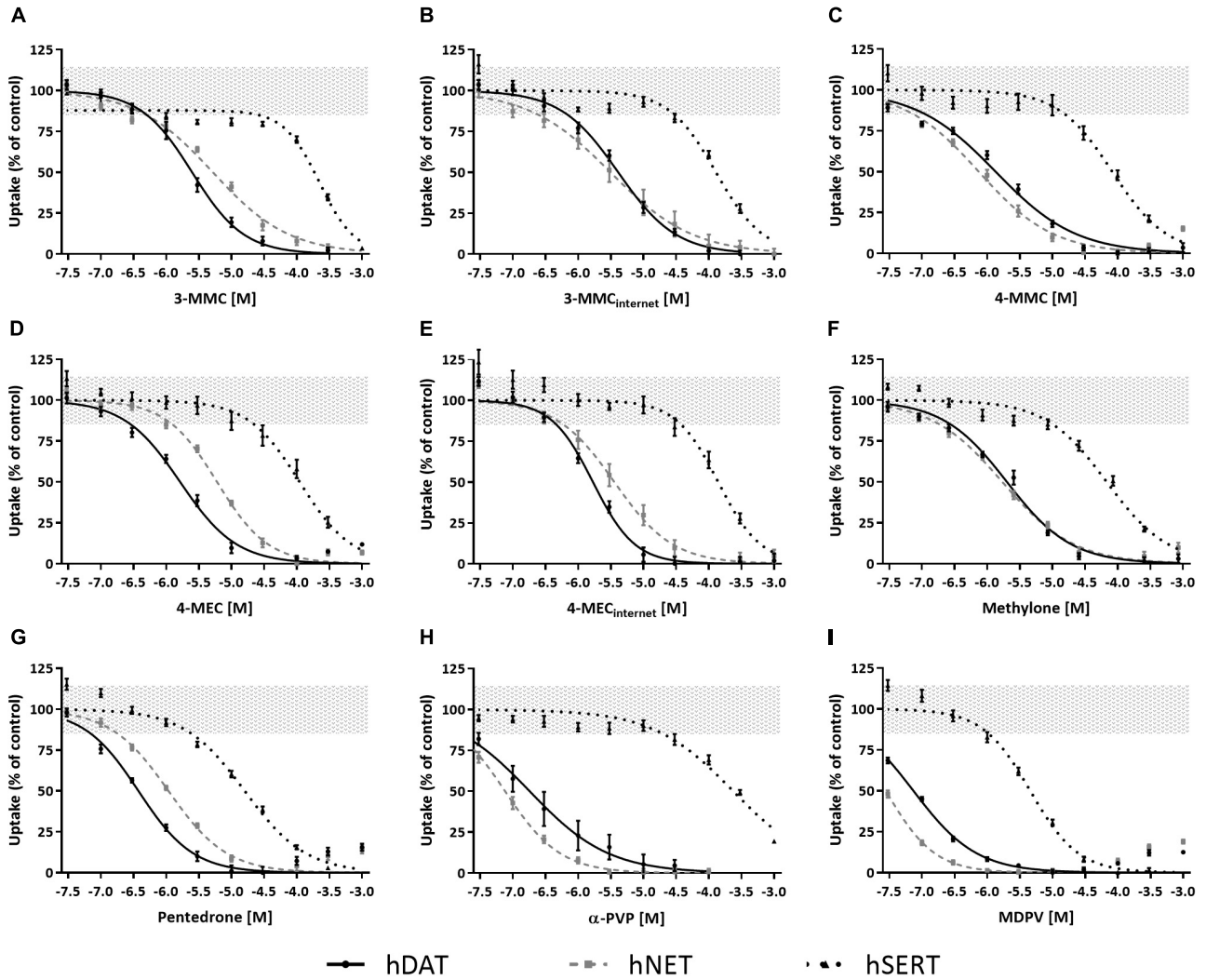

FIGURE 2 | Concentration–response curves of synthetic cathinones for the inhibition of uptake of fluorescent substrate via monoamine transporters. The inhibition of uptake via hDAT (solid line), hNET (dashed gray line), and hSERT (dotted line) was tested for 3-MMC **(A)**, 3-MMC$_{internet}$ **(B)**, 4-MMC **(C)**, 4-MEC **(D)**, 4-MEC$_{internet}$ **(E)**, methylone **(F)**, pentedrone **(G)**, α-PVP **(H)**, and MDPV **(I)**. Uptake is shown as mean ± SEM percentage compared to control (n_{wells} = 13–41, N_{plates} = 4–5). Effects ≤ 15% (i.e., the variation of medium control) are considered not to be of (toxicological) relevance, which is depicted by the gray area. The corresponding IC_{50} values can be found in **Table 1**. α-PVP was reanalyzed from previously published data (Zwartsen et al., 2017).

other parameters and heatmaps see **Supplementary Figure 2**). Based on IC_{50} values, no significant difference in sensitivity was seen between MSR, MBR, and MNBR (**Table 1**). Of the four synthetic cathinones, pentedrone most potently inhibited neuronal activity during acute exposure (MSR IC_{50} value of 23 μM), followed by 3-MMC, 4-MEC, and 4-MMC (MSR IC_{50} values of 65, 73, and 101 μM, respectively, **Table 1**). For most synthetic cathinones, neuronal activity was slightly less inhibited following prolonged exposure compared to acute exposure. Following washout, neuronal networks partially or fully recovered from exposure. However, neuronal networks exposed to the highest concentration of pentedrone remained fully inhibited.

During acute exposure, 3-MMC$_{internet}$ inhibited the MSR and MBR, yet not the MNBR, significantly more potently compared to pharmaceutical grade 3-MMC (**Figures 3D–F** and **Table 1**). During prolonged exposure, no significant differences were seen between the pharmaceutical grade 3-MMC and the 3-MMC$_{internet}$. The potency of 4-MEC$_{internet}$ did not significantly differ from the potency of pharmaceutical grade 4-MEC to inhibit MSR, MBR, or MNBR at any exposure scenario (except for the IC_{50} for the MSR during prolonged exposure) (**Figures 3M–O**).

Cell Viability

In general, no cytotoxicity was seen in HEK 293 cells stably transfected with hDAT, hNET, and hSERT or rat primary cortical cultures after exposure to ≤1000 μM pharmaceutical grade cathinones or cathinones bought via the internet (**Supplementary Figures 3**, **4**).

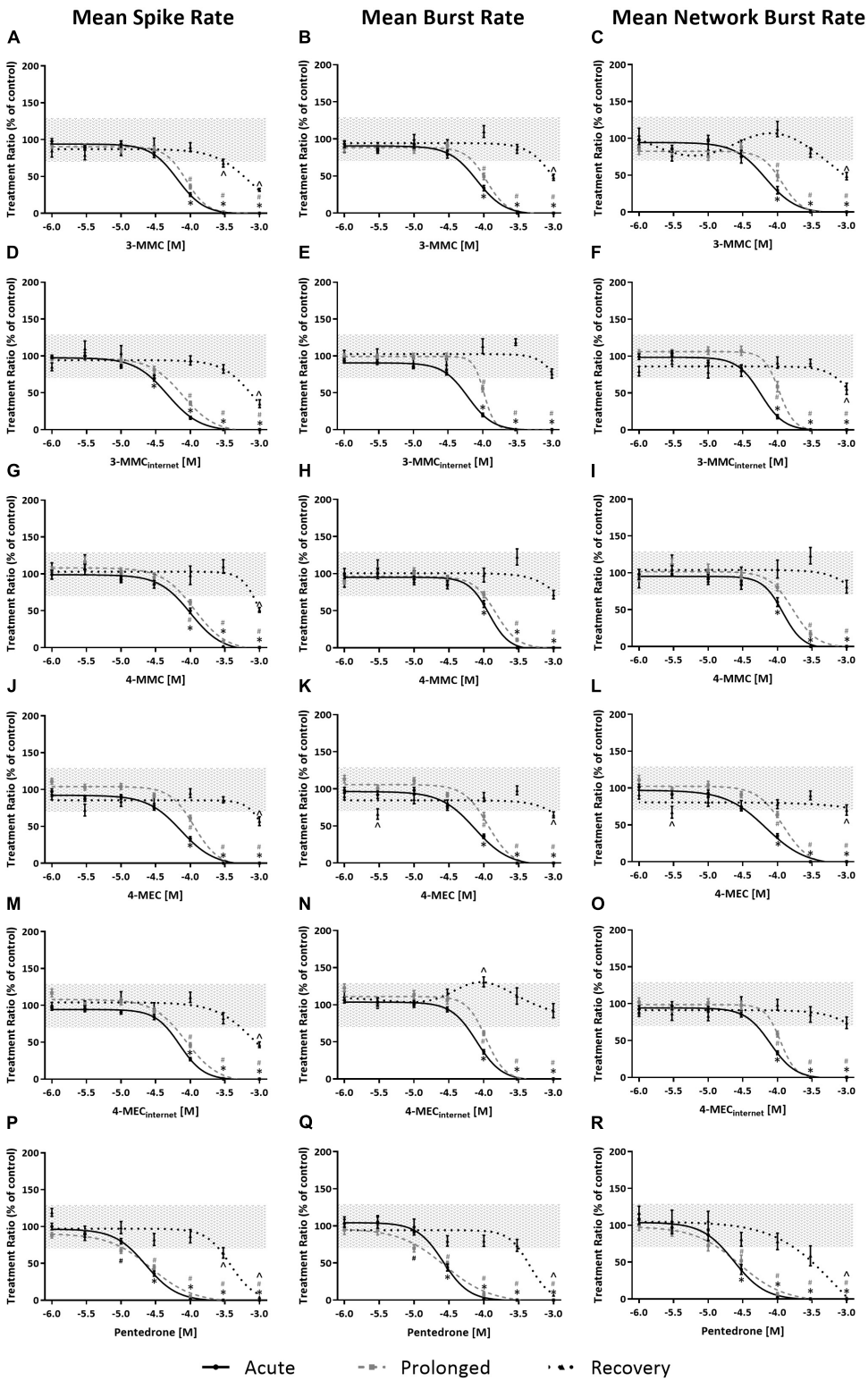

FIGURE 3 | Concentration–response curves of synthetic cathinones for neuronal activity. The mean spike rate (MSR), mean burst rate (MBR), and mean network burst rate (MNBR) after acute exposure (solid line, 30 min), prolonged exposure (dashed gray line, 4.5 h) and 19 h washout (dotted line, 24 h from the start of exposure) are shown for 3-MMC **(A–C)**, 3-MMC$_{internet}$ **(D–F)**, 4-MMC **(G–I)**, 4-MEC **(J–L)**, 4-MEC$_{internet}$ **(M–O)**, and pentedrone **(P–R)** (n_{wells} = 8–31, N_{plates} = 4–5). Neuronal activity is depicted as the mean treatment ratio ± SEM (parameter$_{exposure}$/parameter$_{baseline}$ as % of control wells). Effects ≤ 30% (i.e., the variation of medium control) are considered not to be of (toxicological) relevance, which is depicted by the gray area. Relevant effects that are statistically different from control ($p < 0.05$) are indicated with * for acute exposure, # for prolonged exposure and ∧ for washout.

TABLE 1 | IC_{50} values for the inhibition of neuronal activity (MSR, MBR, and MNBR) and monoamine reuptake transport (hDAT, hNET, and hSERT) of cathinones.

Cathinones	Neuronal activity IC_{50} values (μM [CI])									Transporter inhibition IC_{50} values (μM [CI])		
	Mean spike rate (MSR)			Mean burst rate (MBR)			Mean network burst rate (MNBR)					
	Acute	Prolonged	Recovery	Acute	Prolonged	Recovery	Acute	Prolonged	Recovery	hDAT	hNET	hSERT
3-MMC	65 [56–75]	87* [75–98]	710*# [490–1051]	79 [65–94]	109* [96–126]	>1000	67 [51–87]	116* [98–139]	>1000	2.5 [2.1–2.9]	5.2* [4.5–6.0]	134*# [108–165]
3-$MMC_{internet}$	47^ [40–54]	77* [65–91]	776* [532–1224]	61^ [54–69]	104* [99–121]	>1000	57 [50–66]	106* [96–117]	>1000	4.1^ [3.5–4.8]	3.1*^ [2.2–4.5]	129*# [110–151]
4-MMC	101 [80–125]	114 [94–140]	>1000	118 [101–145]	147 [121–184]	>1000	120 [101–149]	167 [122–222]	>1000	1.4 [1.2–1.6]	0.7* [0.6–0.9]	83*# [66–104]
4-MEC	73 [63–85]	114* [101–129]	>1000	74 [62–89]	115* [100–132]	>1000	68 [56–84]	122* [105–141]	>1000	1.6 [1.4–1.9]	6.0* [5.4–6.7]	111*# [87–139]
4-$MEC_{internet}$	70 [62–79]	83*^ [73–101]	850* [618–1271]	79 [68–90]	108* [96–125]	>1000	79 [67–91]	111* [100–123]	>1000	1.7 [1.4–2.0]	3.6*^ [2.8–4.6]	139*# [110–175]
Methylone[1]	244 [210–275]	283 [240–313]	>1000	266 [211–366]	322 [266–385]	>1000	323 [267–1075]	362 [316–431]	>1000	2.0 [1.7–2.3]	1.7 [1.5–2.0]	68*# [58–80]
Pentedrone	23 [20–27]	27 [22–33]	379*# [296–501]	26 [22–31]	28 [20–37]	451*# [351–615]	23 [18–30]	28 [18–41]	671*# [466–982]	0.4 [0.3–0.4]	1.1* [0.9–1.2]	16*# [14–18]
α-PVP[1,2]	26 [21–32]	39* [31–51]	>300	33 [28–39]	58* [39–131]	>300	33 [28–42]	69* [43–111]	>300	0.2 [0.1–0.3]	0.08* [0.07–0.09]	237*# [196–291]
MDPV[1]	30 [25–38]	33* [27–40]	358*# [250–558]	40 [30–75]	61 [45–76]	309*# [229–403]	35 [30–87]	74* [40–87]	281*# [139–362]	0.07 [0.07–0.08]	0.03* [0.02–0.03]	4.5*# [4.0–5.2]

*Neuronal activity: Following recovery, several IC_{50} values and 95% confidence intervals could not be determined and were depicted as higher than highest concentration tested (e.g., >1000 μM). * depicts IC_{50} values of prolonged exposure or following washout that differ significantly from acute exposure ($p < 0.05$). # depicts IC_{50} values following washout that differ significantly from prolonged exposure ($p < 0.05$). IC_{50} values for MBR and MNBR significantly different from MSR at the same exposure scenario (acute, prolonged, or recovery) are underlined ($p < 0.05$; on ">" values statistics could not be performed). IC_{50} values for MNBR and MBR at the same exposure scenario (acute, prolonged, or recovery) did not significantly differ ($p < 0.05$). Transporter inhibition: * depicts hNET or hSERT IC_{50} values significantly different from hDAT IC_{50} values ($p < 0.05$). # depicts hSERT values significantly different from hNET IC_{50} values ($p < 0.05$). Internet vs. pharmaceutical grade cathinones: ^ depicts IC_{50} values from cathinones bought via the internet different from pharmaceutical grade IC_{50} values ($p < 0.05$). [1]Neuronal activity data from Zwartsen et al. (2019). [2]Transporter inhibition data reanalyzed from Zwartsen et al. (2017).*

DISCUSSION

Toxicological profiles used for hazard characterization of synthetic cathinones are usually based on single target assays (i.e., specific receptors, ion channels, or transporters). As such, the current study aimed at strengthening the hazard characterization of synthetic cathinones (**Figure 1**) by combining effects obtained with a single target assay (monoamine reuptake; **Figure 2**) and those obtained with an integrated assay (neuronal activity; **Figure 3**). To enlarge the data set, monoamine transporter data for α-PVP and MEA data for methylone, α-PVP, and MDPV from previous publications have been included (**Table 1**).

Our results confirm previous reports on the potent inhibitory effects of cathinones on monoamine transporters hDAT and hNET, and the less potent inhibition of hSERT [**Table 1**; for review see Hondebrink et al. (2018); also see Baumann et al., 2013; Eshleman et al., 2013; Simmler et al., 2013; Kolanos et al., 2015; Saha et al., 2015; McLaughlin et al., 2017]. The magnitude of the hSERT IC_{50} values for the cathinones without pyrrolidine structure or lengthened alkyl chain (3-MMC, 4-MMC, 4-MEC, and methylone) showed some deviations compared to literature. These differences are probably due to the use of different assays. Instead of using the traditional assays that rely on radioactively labeled monoamines, we used a fluorescent-based assay that relies on a fluorescent substrate, which resembles the biogenic amine neurotransmitters. While this is a relatively new assay, it has advantages over the traditional assays, primarily related to allowing for kinetic measurements at more physiological settings. For example, we measured effects at a physiological temperature (37°C), whereas experiments using radioactively labeled monoamines are mostly performed at room temperature. This can affect the results since the uptake of and binding to hSERT is temperature dependent (Elfving et al., 2001; Saldana and Barker, 2004; Oz et al., 2010; Tsuruda et al., 2010). For example, decreasing the temperature from 37°C to room temperature increases the potency of MDMA to inhibit hSERT threefold (Zwartsen et al., 2017). Possibly, a lower temperature also increases the potency of synthetic cathinones, including 3-MMC, 4-MMC, 4-MEC, and methylone for which we obtained higher IC_{50} values on hSERT compared to literature.

Furthermore, we determined monoamine uptake using a monolayer of interconnected cells, while in radioactive assays cells in suspension are often used. The process of obtaining cells in suspension can cause changes in cell morphology and damage to membrane proteins, resulting in cellular dysfunction and stress responses (Huang et al., 2010). This may increase the sensitivity of cells to the effect of toxicants, as was reported by Simmler and Liechti (2017), who stated that drugs were less potent releasers when attached cells were used compared to cells in suspension.

Notably, only one study investigated effects of recreational drugs on hSERT using radioactively labeled monoamines at 37°C while using attached cells. In line with our data, this study also reported lower potencies for amphetamine, 4-fluoroamphetamine, and MDMA to inhibit hSERT

(Rosenauer et al., 2013). Similar to our data, for other compounds no differences in potencies were observed between both methods when experimental conditions like temperature and cell attachment were comparable (Jorgensen et al., 2008; Tsuruda et al., 2010).

Moreover, the fluorescent-based assay can detect potent hSERT inhibition, since we observed an IC_{50} value of 0.1 μM for fluoxetine. The fact that the fluorescent-based assay effectively detects SERT inhibition by this selective serotonin reuptake inhibitor (SSRI, Zwartsen et al., 2017) highlights the usability of the fluorescent-based assay. Which assay (radioactive of fluorescent-based) approximates the effects actually occurring in humans best remains to be determined.

Although the dataset is too narrow to perform an extensive structure–activity analysis, the presence of a pyrrolidine moiety, as present in MDPV and α-PVP, increases the potency to inhibit uptake via hNET and hDAT ($p < 0.05$), in line with other studies (Kolanos et al., 2013; Marusich et al., 2014). In addition, the lengthened alkyl-tail carbon chain (α-carbon side chain) also appears to increase the potency to inhibit uptake via hDAT, as seen by the IC_{50} values of pentedrone, α-PVP, and MDPV compared to 4-MMC, 4-MEC, and methylone. This is in line with research published by others (Marusich et al., 2014; Eshleman et al., 2017). Interestingly, Eshleman et al. (2017) reported a 100-fold increased affinity for hDAT when the alkyl-tail carbon chain changed from one to five carbons. This is possibly due to the increase of lipophilicity and molecular size of the lengthened drugs (Kolanos et al., 2015).

As this study is the first to describe the effects of cathinones on spontaneous neuronal activity, comparisons to literature could only be based on non-cathinone substances. Cathinones without a lengthened chain or methylenedioxy group (3-MMC, 4-MMC, and 4-MEC) showed comparable potency to inhibit neuronal activity following acute and prolonged exposure to and after washout from amphetamine-type stimulants MDMA, PMMA, and methamphetamine (Zwartsen et al., 2019). While the chemical structure of methylone and MDMA only differs in the addition of a keton group for methylone, this reduces the potency to inhibit neuronal activity threefold (Zwartsen et al., 2019). Cathinones with a lengthened alkyl chain (as present in pentedrone, α-PVP, and MDPV) show comparable potencies to inhibit neuronal activity to 2C-B, a hallucinogenic phenethylamine of the 2C family (Zwartsen et al., 2019).

In line with the monoamine reuptake transporters pentedrone, α-PVP, and MDPV had a two to fourfold increased potency to inhibit the neuronal activity (MSR, MBR, and MNBR) following both acute and prolonged exposure, compared to cathinones lacking the lengthened alkyl chain. In addition, increased neurotoxicity was observed for these pentedrone, α-PVP, and MDPV, as illustrated by the lack of (full) recovery of neuronal activity (**Table 1**; Zwartsen et al., 2019). As cortical cultures have a low expression of monoamine transporters (Dahlin et al., 2007), this lack of recovery of neuronal activity could suggest the involvement of additional neurotoxic mechanisms of cathinones.

We also investigated the effects of two cathinones bought via the internet (3-$MMC_{internet}$ and 4-$MEC_{internet}$) on neuronal activity and monoamine reuptake transporters. Effects on hSERT and neuronal activity did not differ between the pharmaceutical grade and internet-bought drugs. Between 4-MEC and 4-$MEC_{internet}$, small differences were observed on hDAT and between 3-MMC and 3-$MMC_{internet}$ small differences were observed for both hDAT and hNET. These differences all concerned a less than twofold change in potency likely due to biological or technical variation. Contaminations of the cathinones bought online is unlikely as GC–MS analysis showed 0≤0.5% contamination by structurally related compounds (>99.5% purity; see **Supplementary Figure 1**).

To identify the relevance of effects and potentially harmful drugs, effect concentrations (IC_{50} values) were related to the

TABLE 2 | Estimated human brain concentrations of cathinones compared to the monoamine reuptake transporter and neuronal activity inhibition potencies.

Cathinones	Human (serum/blood/plasma) (μM)	Brain partitioning factor (BPF)	Estimated human (brain) (μM)	IC_{50} value at or close to estimated human (brain)
3-MMC	0.005–9.0[A,b,**c**]	x	0.005–9.0	hDAT > hNET
4-MMC	0.05–6.0[**a**,d,E,f]	0.7–6.2[1,**2**,**3**]	0.04–37	hNET > hDAT
4-MEC	0.1–4.7[**a**,d]	1.5–6.2[4]	0.2–29	hDAT > hNET >> MSR_a, MBR_a, $MNBR_a$
Methylone	0.01–18[a,d,g,h]	1.4–4.5[5,**6**,**7**]	0.01–81	hNET, hDAT >> hSERT
Pentedrone	0.04–1.9[**a**,d,I]	1.6[8]	0.06–3.0	hDAT > hNET
α-PVP	0.01–2.8[b,j,K]	0.1–1.8[4,8,9,10]	0.001–5.0	hNET > hDAT
MDPV	0.02–6.9[d,L,M,n]	0.8–4.4[4,11,12]	0.02–30	hNET > hDAT >> hSERT >> MSR_a, MSR_p, MBR_a, $MNBR_a$

Estimated brain concentrations were calculated using human blood, serum (caps), or plasma (bold) concentrations and brain partition factors (BPF) found in literature. All human concentrations were obtained from reports on recreational use (voluntary intake, driving under the influence or accidental non-fatal intoxications). BPFs were based on blood/serum (underlined)/plasma (strikethrough) and brain concentrations of human or rat (bold) data. Estimated brain concentrations for 3-MMC were based on the observation that most BPFs are >1. Small to large differences in effect concentrations for different endpoints are depicted as > or >>, respectively. hDAT, human dopamine reuptake transporter; hNET, human norepinephrine reuptake transporter; hSERT, human serotonin reuptake transporter; MSR_a, mean spike rate during acute exposure; MBR_a, mean burst rate during acute exposure; $MBNR_a$, mean network burst rate during acute exposure; MSR_p, mean spike rate during prolonged exposure. References for serum concentrations: [a]Turcant et al. (2017), [b]Adamowicz et al. (2016b), [c]Backberg et al. (2015), [d]Elliott and Evans (2014), [e]Wood et al. (2010), [f]Cosbey et al. (2013), [g]deRoux and Dunn (2017), [h]Cawrse et al. (2012), [i]World Health Organisation [WHO] (2016), [j]Adamowicz et al. (2016a), [k]Beck et al. (2016), [l]Kriikku et al. (2011), [m]Thornton et al. (2012), [n]Marinetti and Antonides (2013). References for BPF calculation: [1]Gerace et al. (2014), [2]Hadlock et al. (2011), [3]Martinez-Clemente et al. (2013), [4]Lehmann et al. (2018), [5]deRoux and Dunn (2017), [6]Lõpez-Arnau et al. (2013), [7]Štefková et al. (2017), [8]Sykutera et al. (2015), [9]Hasegawa et al. (2014), [10]Potocka-Banas et al. (2017), [11]Wyman et al. (2013), [12]Marinetti and Antonides (2013).

estimated human brain concentration during recreational use [(brain), **Table 2**]. By comparing these concentrations, the most relevant neurotoxic mechanisms of cathinones can be identified. All synthetic cathinones inhibited uptake via hDAT and hNET within the estimated human brain concentrations, while methylone and MDPV also inhibited hSERT at concentrations relevant for human exposure (**Table 2**). Only 4-MEC and MDPV inhibited neuronal activity during acute exposure at concentrations within (MDPV) or close to (4-MEC) the estimated brain concentration. Although the effect concentrations of MDPV and 4-MEC for neuronal activity are far above those of the transporters, this suggests that additional neurotoxic mechanisms could be at play during recreational drug use.

As neuronal activity is inhibited at concentrations several magnitudes above inhibition of uptake via hDAT and hNET, the monoamine transporters are a more sensitive endpoint for the cathinones tested. While this is true for cathinones, we have previously shown that neuronal activity is a more sensitive endpoint for other drugs like methoxetamine (MXE) and hallucinogenic phenethylamines 2C-B and multiple NBOMes (Hondebrink et al., 2017; Zwartsen et al., 2017, 2018). As neuronal activity reflects many neuropharmacological targets of interest, in contrast to single targets like monoamine transporters, these measurements can be very valuable in screening for neuropharmacological effects of NPS, especially when the mechanism of action is *a priori* unknown.

To conclude, by combining results from different methods to investigate the neurotoxicity of a (group of) drug(s), the most sensitive drug target can be determined. Inhibition of monoamine reuptake transporters is the most sensitive target for this set of synthetic cathinones, although the inhibition of neuronal activity at concentrations relevant for recreational exposure indicated the possible relevance of additional targets for some cathinones. Therefore, hazard characterization of emerging NPS can be optimized by applying several *in vitro* screening methods directed to different specific or integrated targets.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Animal experiments were performed in agreement with Dutch law, the European Community directives regulating animal research (2010/63/EU) and reviewed and approved by the Ethical Committee for Animal Experiments of Utrecht University. All efforts were made to minimize the number of animals used and their suffering.

AUTHOR CONTRIBUTIONS

AZ, RW, and LH designed the study. AZ and MO performed the experiments. AZ performed the statistical analysis. AZ wrote the first draft of the manuscript. All authors contributed to the manuscript revision, and read and approved the submitted version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.00009/full#supplementary-material

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Childhood Trauma, Cognitive Emotion Regulation and Motivation for Behavior Change Among Clients of Opioid Substitution Treatment With and Without Past Year Synthetic Cathinone Use During Therapy

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Background: With a decrease in heroin's purity and availability in the European drug market, Hungarian opioid dependent patients started to substitute heroin with novel psychoactive substances (NPS) and especially with synthetic cathinones.

Goal: This study aims to assess whether clients of opioid substitution treatment (OST) with and without a history of synthetic cathinone use during therapy differ in (1) the rate and type of experienced childhood trauma, (2) the way they cope with negative life events, (3) their motivation to change substance use behavior, (4) the rate of treatment retention.

Methods: A total of 198 clients of an outpatient centers (Nyírõ Gyula National Institute of Psychiatry and Addictions, Budapest) OST were asked to provide information about their general substance use experiences, including the consumption of NPS during treatment, their childhood traumatic experiences (Childhood Trauma Questionnaire), cognitive emotion regulation strategies (Cognitive Emotion Regulation Questionnaire), their motivation to change substance use behavior (University of Rhode Island Change Assessment Scale) and current psychiatric symptoms (Brief Symptom Inventory). Baseline data was collected in the summer of 2015, while 4 years follow-up data on treatment retention was obtained in the summer of 2019.

Results: The majority of the clients were male (N = 141, 71.2%), receiving methadone as a substitute for opioids (N = 178, 89.9%), while mean age of the full sample was 39.7 (SD = 6.8). Based on a logistic regression model, the odds for past year synthetic cathinone use was higher among clients with more severe psychiatric symptoms (B = 0.8, OR = 2.2, $p < 0.01$) and among clients who were in treatment for a shorter period of time (B = 0.1, OR = 0.9, $p < 0.05$). Synthetic cathinone use during treatment was further associated with less adaptive strategies to cope with negative life events.

Synthetic cathinone use was also a risk factor for reduced treatment retention ($B = -0.8$, OR = 0.4, $p < 0.05$) and was associated with lower odds of being member of a latent class with less severe psychopathological profile ($B = -0.9$, OR = 0.4, $p < 0.05$).

Conclusion: Synthetic cathinone use during treatment is associated with poorer treatment outcomes and might be characterized by more severe psychiatric symptoms and amotivation to change substance use among opioid dependent clients.

Keywords: NPS, synthetic cathinone, opioid substitution therapy, trauma, emotion regulation

INTRODUCTION

Considering the ever-changing world of contemporary drug scene, the past two decades were characterized by the emergence of NPS, started with the appearance of SC, as first reported in the early 2000s (Urquhart, 2004). Cathinone-derivatives, such as mephedrone, 4-MEC, methylone, pentedrone or MDPV became popular alternates of formerly scheduled psychostimulants and even depressants (e.g., opioids). Regular SC users usually attributed their consumptive choices to easy availability, perception of safety, low prices and pleasurable drug effects (e.g., Van Hout and Brennan, 2012). Although most countries have implemented some forms of control legislations, varying in their type or depth (Corazza and Roman-Urrestarazu, 2018), online trafficking that provides relative anonymity for NPS users (EMCDDA, 2016) and the fact that NPS consumption is now an integral part of the drug scene still support SC popularity among members of certain subpopulations (such as club-goers, prisoners, psychiatric patients, and injecting drug users). Based on the results of the European Syringe Collection and Analysis Project Enterprise (ESCAPE) 2017 campaign (EMCDDA, 2019), traces of SC were found in a high proportion of the 1521 syringes collected from six sentinel European cities (Amsterdam, Budapest, Glasgow, Helsinki, Lausanne, and Paris), indicating that SC use among people who inject drugs is a continuous phenomenon.

Injecting SC use was reported as a low-level and localized incident in many European countries, except for Hungary and Romania where a more substantial level of SC injecting was observed. Injection of SC is of decisive relevance among those who are injectors of other psychoactive substances, including opioids and amphetamines (EMCDDA, 2015). Certain reports denoted that long-term abstinent ex-opiate users shifted to SC injecting (e.g., Van Hout and Bingham, 2012; Péterfi et al., 2014; Rácz et al., 2015) throughout a time period of sustained heroin shortage in the drug market. As part of this shift, clients of OST started to abuse SC during their maintenance treatment (Heikman et al., 2017; Kapitány-Fövény et al., 2017), explained by either treatment deficiencies (inadequate dose of OST medicine), easy SC availability or specific individual factors (e.g., severity of psychiatric symptoms).

From a clinical perspective it is of high relevance to understand the potential reasons of substance use during treatment among OST clients, and more specifically to explore whether or not these reasons differ by the substance being consumed. The local phenomenon of opioid dependent patients' SC use enables the study of SC-specific consumption determinants in a clinical sample. Of the many possible factors underlying substance use during OST – e.g., shorter treatment duration (Li et al., 2012), a medium 60–100 mg/day medication dose (Baumeister et al., 2014), low treatment attendance, drug using friends, family conflicts (Sullivan et al., 2014), male gender (Vigna-Taglianti et al., 2016), younger age (Amiri et al., 2018), or the quality and supportiveness of the social network (Shen et al., 2018) – this paper focuses on the presumed impact of unresolved childhood trauma, with special emphasis placed on individual patterns of cognitive emotion regulation as related to stressful live events.

Posttraumatic stress disorder frequently co-occurs with SUD. An estimated prevalence of PTSD in SUD patients may range from 25 to 49%, a three times higher prevalence than that in the general population (Gielen et al., 2012). PTSD and SUD may result in shared pathological features, such as heightened stress sensitivity, reward deficiency or impulsive behavior with overlapping impaired neurocircuitry (Enman et al., 2014). Furthermore, as both pathologies can be interpreted as the outcomes of hyperactivity to reminding cues, some argues that PTSD and SUD are in fact disorders of memory (Gisquet-Verrier and Le Dorze, 2019). Exposure to either stress or psychoactive substances can induce epigenetic alterations resulting in similar neurobiological, molecular and behavioral changes (e.g., glucocorticoids can induce excitation patterns by activating dopamine neurons) that facilitate drug-seeking behavior (Pizzimenti and Lattal, 2015). However, experiencing traumatic events does not necessarily lead to PTSD. Emotion regulation – connected to cognitive processes – helps the individual in recognizing, evaluating and influencing the course and expression of emotions (Domaradzka and Fajkowska, 2018). Posttraumatic cognitive emotion regulation plays an important mediatory role in the relationship between trauma and either PTSD or PTG. For instance, intrusive rumination might be associated with PTSD, while deliberate rumination can be linked to PTG (Zhang et al., 2018), indicating different cognitive

Abbreviations: 4-MEC, 4-methylethcathinone; BSI, Brief Symptom Inventory; CERQ, Cognitive Emotion Regulation Questionnaire; CTQ-SF, Childhood Trauma Questionnaire; GSI, Global Severity Index; MDPV, 3,4-methylenedioxypyrovalerone; mephedrone, 4-methylmethcathinone; methylone, 3,4-methylenedioxy-*N*-methylcathinone; NPS, novel psychoactive substance(s); OST, opioid substitution treatment; pentedrone, 2-(methylamino)-1-phenylpentan-1-one; PTG, posttraumatic growth; PTSD, posttraumatic stress disorder; SC, synthetic cathinone(s); SES, socioeconomic status; SUD, substance use disorder(s); TTM, Transtheoretical Model; URICA, University of Rhode Island Change Assessment Scale.

processes in case of negative or positive trauma outcomes, even if PTSD and PTG may also coexist on an individual level. Similarly, while some forms of cognitive processes are thought to be unproductive (e.g., overgeneralization, avoidance), there are many constructive ways (e.g., decentering, accommodation of corrective information) of trauma processing (Hayes et al., 2017).

Regarding the subpopulation of OST clients, early childhood trauma has been linked to poorer treatment retention in OST programs (Kumar et al., 2016), childhood sexual abuse was found to be a predictor of drug use during OST (Schiff et al., 2010), while NPS use among treatment seeking opioid dependent patients was evaluated as an attempt of self-medication for earlier psychological trauma (Gittins et al., 2018). Additionally, OST patients with a history of childhood trauma show impaired ability to associate a stimulus with its outcome when the stimuli is presented in a drug-related context (Weiss et al., 2019), hence clients' cognitive efficacy might be reduced by drug-related stimuli. Cues that are associated with either stress or drugs also increase opioid craving and anxiety (Hyman et al., 2007) and pose negative impact on OST prognosis (Jaremko et al., 2015).

As regards SC use among OST clients, the subgroup of patients who consume any SC during treatment is characterized by younger age, shorter treatment duration, more severe psychiatric symptoms and higher stress reactivity to interpersonal conflicts (Kapitány-Fövény et al., 2017). Based on these previous findings, the current study aimed to assess whether OST clients with and without a history of SC use during treatment differ in experienced childhood trauma, cognitive emotion regulation strategies (i.e., how they cope with stressful life events), the severity of their psychiatric symptoms, their motivation for behavioral change and the rate of treatment retention.

MATERIALS AND METHODS

Procedure

A total of 206 clients of Hungary's biggest drug outpatient centers (Nyírõ Gyula National Institute of Psychiatry and Addictions, Budapest) OST were involved in the study. Data collection was implemented by face-to-face interviews conducted by trained psychologists. Inclusion criteria were: (1) being a client of either methadone or Suboxone (a combination formulation of buprenorphine and naloxone) maintenance therapy for at least 1 year, (2) 18 < years of age, (3) signing the informed consent form; while exclusion criteria were: (1) acute drug effects, (2) agitation or violent behavior. After eight patients were excluded from the study, the final sample consisted of 198 participants. In order to prevent respondents from being influenced by the acute sedative effects of OST medication, data was collected just before the administration of either methadone or buprenorphine-naloxone.

Baseline data was collected in the summer of 2015, while follow-up data on treatment retention was obtained in the summer of 2019 (4 years follow up).

Ethical approval was provided by the hospital's Research Ethics Committee.

Measures

Demographics and Treatment Characteristics

Demographic items were comprised of questions regarding gender, age, educational background, marital status, perceived SES and occupation. Perceived SES was measured by a 7-point Likert scale (from 1 = the lowest possible SES to 7 = the highest possible SES). Treatment indices included the length of treatment, the type of OST medication (methadone vs. buprenorphine-naloxone) and medication dose. The following rule-breaking behaviors were also explored: buying street methadone/buprenorphine-naloxone, intravenous administration of methadone/buprenorphine-naloxone.

Synthetic Cathinone Use

Participants provided information about their past year and past month SC use experiences during OST. Mephedrone, pentedrone and MDPV as the most prevalent SC in Hungary were listed in the questionnaire.

Childhood Trauma

Childhood traumatic experiences (CTQ) were assessed by the short form of the CTQ-SF. CTQ-SF is a retrospective recall based measure (Bernstein et al., 2003), containing 28 items of which three are validity items. The remaining 25 clinical items load on five factors: (1) Emotional abuse (e.g., "*Family said hurtful things*"), (2) Physical abuse (e.g., "*Punished with hard objects*"), (3) Sexual abuse (e.g., "*Was sexually abused*"), (4) Emotional neglect (e.g., the reversed item of "*Made to feel important,*" (5) Physical neglect (e.g., "*Not enough to eat*"). Respondents evaluate each statement by using a 5-point Likert scale (from 0 = never to 4 = very often).

Good reliability was found regarding the five factors, with the following Cronbach's α scores: Emotional abuse = 0.89, Physical abuse = 0.92, Sexual abuse = 0.94, Emotional neglect = 0.95, Physical neglect = 0.87.

Cognitive Emotion Regulation

The short 18-item version of the CERQ (Garnefski and Kraaij, 2006; Miklósi et al., 2011) was applied to measure subjects' stress-related cognitive-affective processing style. The short form of the CERQ comprises of nine conceptual scales: (1) Self-blame (e.g., "*I feel that I am the one to blame for it*"), (2) Other-blame (e.g., "*I feel that others are responsible for what has happened*"), (3) Rumination (e.g., "*I dwell upon the feelings the situation has evoked in me*"), (4) Catastrophizing (e.g., "*I continually think how horrible the situation has been*"), (5) Positive refocusing (e.g., "*I think about pleasant experiences*"), (6) Refocus on planning (e.g., "*I think about how to change the situation*"), (7) Positive reappraisal (e.g., "*I think I can learn something from the situation*"), (8) Putting into perspective (e.g., "*I think that it all could have been much worse*") and (9) Acceptance (e.g., "*I think that I have to accept the situation*"), with two items per scale. The questionnaire's items are evaluated on a 5-point Likert scale (from 1 = almost never to 5 = almost always). The higher the subscale score, the more inherent the cognitive strategy is. CERQ's conceptual scales are additionally interpreted as either adaptive (Positive refocusing, Planning, Positive reappraisal,

Putting into perspective and Acceptance) or non-adaptive (Self-blame, Other-blame, Rumination, Catastrophizing) strategies.

With regard to reliability testing, the following good, acceptable or low Cronbach's α scores were found for the nine scales: Self-blame = 0.63, Other-blame = 0.72, Rumination = 0.83, Catastrophizing = 0.82, Positive refocusing = 0.68, Refocus on planning = 0.63, Positive reappraisal = 0.62, Putting into perspective = 0.53, Acceptance = 0.67.

Motivation for Behavior Change

Respondents' current motivational state regarding a potential decision to change their substance use habits (e.g., stop using any psychoactive substances during treatment or reducing their OST medication dose) was measured by the 32-item version of the URICA (DiClemente and Hughes, 1990). URICA is based on the TTM of behavioral change (Prochaska and DiClemente, 1983) and as such theoretically driven measure, contains the following subscales: (1) Precontemplation (e.g., "*I'm not the problem one. It doesn't make much sense for me to consider changing*"), (2) Contemplation (e.g., "*I've been thinking that I might want to change something about myself*"), (3) Action (e.g., "*I am really working hard to change*") and (4) Maintenance (e.g., "*I'm struggling to prevent myself from having a relapse of my problem*"). Readiness to change as a second-order factor is yielded by using the formula: Contemplation + Action + Maintenance - Precontemplation.

Responses are given on a 5-point Likert scale (from 1 = strongly disagree to 5 = strongly agree).

In the current study, the following low or good Cronbach's α scores were observed: Precontemplation = 0.53, Contemplation = 0.49, Action = 0.79, Maintenance = 0.73.

Psychiatric Symptoms

Current (last week's) psychiatric symptoms were assessed by the BSI (Derogatis, 1975; Urbán et al., 2014). The 53 items of the BSI are evaluated by using a 5-point Likert scale (from 0 = not at all to 4 = extremely). BSI comprises nine symptom scales: (1) Somatization (e.g., "*Pain on heart or chess*"), (2) Obsession-compulsion (e.g., "*Feeling blocked in getting things done*"), (3) Interpersonal sensitivity (e.g., "*Your feeling being easily hurt*"), (4) Depression (e.g., "*Feeling lonely*"), (5) Anxiety (e.g., "*Feeling tense or keyed up*"), (6) Hostility (e.g., "*Getting into frequent arguments*"), (7) Phobic anxiety (e.g., "*Feeling afraid to travel on buses, subways or trains*"), (8) Paranoid ideation (e.g., "*Feeling that you are watched or talked about by others*") and Psychoticism (e.g., "*The idea that something is wrong with your mind*"). A GSI was computed as the mean of the 53 items.

Good or acceptable reliability results were found: Cronbach's α scores regarding the nine scales were: Somatization = 0.87, Obsession-compulsion = 0.8, Interpersonal sensitivity = 0.73, Depression = 0.86, Anxiety = 0.85, Hostility = 0.82, Phobic anxiety = 0.78, Paranoid ideation = 0.62, and Psychoticism = 0.66.

Urine Samples

As part of the OST protocol, clients were randomly screened for the following substances by applying standard rapid urine tests: opiate, cocaine, amphetamine, MDMA, THC, benzodiazepine. Randomization was done by the hospital's medical software as based on clients' Treatment Demand Indicator (TDI) codes. Past year's positive or negative test results were entered in our database retrospectively. Since the outpatient centers urine tests could not be used to detect SC, past year SC use was based on respondents' report only (see section "Synthetic Cathinone Use"). Questionnaire data and urine test results were matched by clients' TDI codes, however, in the final database only unique identifiers were recorded in order to protect personality rights.

Statistical Analysis

Data were analyzed by SPSS 17 (SPSS Inc, 2008). Demographic characteristics and treatment indices were explored by descriptive statistics, potential differences between SC and non-SC-using clients were analyzed by non-parametric Mann Whitney *U* test and chi-square statistics, the association between childhood trauma types and non-adaptive cognitive emotion regulation strategies was tested by linear regression model, while significant predictors of past year SC use and treatment retention were identified by logistic regression models. Participants were classified by their highest standardized *z* score achieved on any of the BSI factors, referred to as "*prominent psychopathological dimension*," based on a very similar approach presented by Maremmani et al. (2016). Psychopathological symptom profiles were explored by using latent class analysis in Mplus v5 (Muthén and Muthén, 1998–2007). Finally, predictors of latent class memberships were examined by logistic regression models.

RESULTS

Sample Characteristics

Table 1 summarizes detailed sample characteristics in terms of demographics and treatment indices.

Clients with and without a history of past year SC use were compared regarding demographic and treatment-related variables. SC using clients were younger [SC-using: M = 37.1, SD = 6.6, non-SC-using: M = 40.5, SD = 6.6, $t(196) = 2.9$, $p < 0.01$], being in treatment for a shorter period of time [SC-using: M = 5.1 years, SD = 6.1, non-SC-using: M = 7.4 years, SD = 5.5, $t(196) = 2.9$, $p < 0.01$], showed lower rates (41.9 vs. 59.7%) of methadone/buprenorphine-naloxone street-buying [$\chi 2$ (1, $N = 177$) = 4.2, $p < 0.05$] and lower rates (37.2 vs. 61.9%) of methadone/buprenorphine-naloxone injecting [$\chi 2$ (1, $N = 177$) = 8.1, $p < 0.01$]. Lower rates (22.7%) of treatment retention was observed among SC using OST clients [$\chi 2$ (1, $N = 198$) = 5.2, $p < 0.05$] as compared to those without a history of past year SC consumption (41.6%) at 4-years follow-up. Clients receiving either methadone or buprenorphine-naloxone were similarly compared in terms of their age, gender, years spent in treatment, highest academic degree, SES, occupation, taking other medication (e.g., benzodiazepine), rate of successful dose reduction during treatment, IV use of OST medication, SC use during treatment. Those receiving buprenorphine-naloxone have spent less years in treatment [methadone clients: M = 7.2, SD = 5.7, buprenorphine-naloxone clients: M = 3.6, SD = 4.5,

TABLE 1 | Demographics and treatment characteristics.

Demographics		
Age Mean (SD)		39.7 (6.8)
Gender distribution	Male *N* (%)	141 (71.2)
	Female *N* (%)	57 (28.8)
Educational background	Elementary *N* (%)	52 (26.3)
	Vocational/technical school *N* (%)	91 (46)
	High school *N* (%)	28 (14.1)
	Incomplete higher education *N* (%)	8 (4)
	Completed higher education *N* (%)	19 (9.6)
Marital status	Single *N* (%)	120 (60.6)
	In a relationship *N* (%)	33 (16.7)
	Married *N* (%)	24 (12.1)
	Divorced *N* (%)	19 (9.6)
	Widowed *N* (%)	2 (1)
Perceived socioeconomic status Mean (SD)		3.9 (1.1)
Occupation	Unemployed *N* (%)	42 (21.2)
	Temporary job *N* (%)	23 (11.6)
	Permanent job *N* (%)	116 (58.6)
	Maternity leave *N* (%)	2 (1)
	Disability pension *N* (%)	14 (7.1)
	Student *N* (%)	1 (0.5)
Treatment indices		
Length of treatment Mean (SD)		6.9 (5.7)
Type of OST medication *N* (%)	Methadone *N* (%)	178 (89.9)
	Buprenorphine-naloxone *N* (%)	20 (10.1)
OST medication dose (mg)	Methadone Mean (SD)	77.9 (33.8)
	Buprenorphine-naloxone Mean (SD)	8.6 (5.5)
Buying street methadone/buprenorphine-naloxone *N* (%)		98 (49.5)
Intravenous administration of methadone/ buprenorphine-naloxone *N* (%)		99 (50)
4 year treatment retention rate *N* (%)		74 (37.4)

TABLE 2 | Self-reported past year and past month SC use.

	Past year	Past month
Mephedrone *N* (% of full sample)	21 (10.7)	3 (1.5)
Pentedrone *N* (% of full sample)	36 (18.2)	10 (5.1)
MDPV *N* (% of full sample)	16 (8.1)	3 (1.5)
SC use in general *N* (% of the full sample)	44 (22)	12 (6.1)

$t(198) = 3.4$, $p < 0.01$], but did not differ from methadone clients in any other measures.

Substance Use Profiles

Past month and past year SC use frequencies are presented in **Table 2**.

In order to increase the sample size of SC using clients' subgroup, we decided to merge distinct past year SC use categories (mephedrone, pentedrone, MDPV) into one SC category (N = 44) for further analyses. The overall sample size of the merged subgroup was lower than the sum of the three categories as some of the clients used more than one SC derivative. Nevertheless, the most frequently used SC was pentedrone among our OST clients. Intravenous SC use was identified in a high proportion of past year SC users, with altogether 28 participants (63.6%) reported SC injecting.

Last year use of opiate, cocaine, amphetamine, MDMA, cannabis/hashish and the misuse of benzodiazepines during OST was identified by urine test results. THC was found in 92 samples (46.5%), opiates in 86 (43.4%), benzodiazepine in 31 (15.7%), amphetamine in 19 (9.6%), MDMA in 8 (4%), and cocaine in 6 (3%).

The current study also aimed to explore past year concurrent substance use patterns during treatment. As self-reports were based on a 1-year recall period, only urine samples were able to provide objective measures regarding co-occurring substance use. But since our study focuses on SC using clients, **Figure 1** presents the association between self-reported past year SC use and urine sample-based concomitant substance use profiles as well.

Past year SC use was mainly associated with opiate (n = 20) and THC (n = 19) consumption in the same year, but not necessarily in the same time period. Regarding concomitant substance use patterns, opiates were most commonly co-ingested with cannabis/hashish (53 clients concurrently used these substances), while among those clients with a history of past year SC use, the consumption of any other stimulants was uncharacteristic. The co-occurrence of more than one stimulant in the urine samples was also rare, indicating that OST clients don't often mix substances from the same drug class. There were no significant differences in the rate of positive urine samples between SC and non-SC-using clients.

Childhood Traumatic Experiences

Synthetic cathinone using and non-SC using OST clients were compared regarding their CTQ. **Table 3** summarizes the results of comparative statistics.

Potential gender differences in the frequency of CTQ were also analyzed, however, there were no significant differences between males and females.

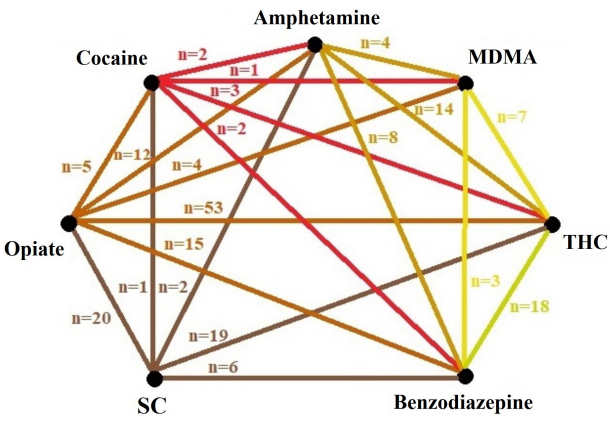

FIGURE 1 | Profile of past year's concurrent substance use based on urine test results and self-reports (in case of SC). SC, synthetic cannabinoids; MDMA, methylenedioxymethamphetamine; THC, tetrahydrocannabinol.

TABLE 3 | Childhood traumatic experiences among OST clients with and without a history of past year SC use.

	SC use reported *N* = 44	SC use not reported *N* = 154	Mann Whitney *U* test (*U*)	Effect size (*r*)
Emotional neglect Mean (SD)	4.1 (5.9)	5.6 (6.7)	2384.5	0.12
Emotional abuse Mean (SD)	2.7 (4.3)	3.8 (5.2)	2454	0.11
Physical abuse Mean (SD)	2.2 (3.9)	2.6 (4.6)	2832	0.05
Physical neglect Mean (SD)	0.7 (1.3)	2.5 (4.5)	2302.5*	0.26
Sexual abuse Mean (SD)	0.2 (0.7)	0.9 (3.2)	2820.5	0.15

*$p < 0.05$.

TABLE 4 | Cognitive emotion regulation related to stressful live events in SC and non-SC using subgroups.

	SC use reported *N* = 43	SC use not reported *N* = 133	Mann Whitney *U* test (*U*)	Effect size (*r*)
Self-blame Mean (SD)	5.6 (1.9)	6.3 (2.3)	2381.5	0.17
Other-blame Mean (SD)	3.5 (1.4)	3.5 (1.5)	2747	0.00
Rumination Mean (SD)	5.6 (2)	5.9 (2.4)	2646.5	0.07
Catastrophizing Mean (SD)	4.2 (2.2)	4.5 (2.3)	2582	0.07
Positive refocusing Mean (SD)	4.1 (2.3)	4.3 (2.1)	2632	0.05
Refocus on planning Mean (SD)	5.9 (1.9)	6.9 (2)	2076.5**	0.25
Positive reappraisal Mean (SD)	6.6 (1.8)	7 (2.1)	2549	0.10
Putting into perspective Mean (SD)	5.9 (1.7)	6.1 (1.9)	2766.5	0.06
Acceptance Mean (SD)	5.8 (1.9)	6.1 (2.3)	2618	0.07
Adaptive strategies Mean (SD)	5.7 (1.2)	6.1 (1.4)	2343	0.15
Non-adaptive strategies Mean (SD)	4.7 (1.2)	5.1 (1.4)	2492	0.15

**$p < 0.01$.

Cognitive Emotion Regulation Strategies

As for cognitive emotion regulation strategies in the SC and non-SC using subgroups, only one scale (Refocus on planning) showed significant difference between these subgroups (**Table 4**).

Those clients without a past year history of SC use were more prone to use planning as an adaptive strategy to cope with stressful live situations. Refocus on planning as assessed by the CERQ refers to thinking about potential ways to handle a frustrating event.

The association between the type and frequency of endured CTQ and non-adaptive cognitive emotion regulation strategies were analyzed by regression models and compared by past year SC use as a grouping variable. **Figure 2** presents the results of the two regression models.

In the non-SC-using subgroup, childhood physical neglect showed significant association with non-adaptive cognitive emotion regulation strategies, although only a low proportion of the outcome variable's variance was explained by the models ($R^2 = 0.15$ and 0.19).

Motivation to Change

In terms of motivation for behavior change, SC-using clients were more likely to show higher scores on the precontemplation domain of the URICA (**Table 5**).

This result indicates that SC-using clients might be less motivated to change their substance use habits. This might also be associated with lower treatment retention rates among SC-using participants.

Psychiatric Symptom Profiles

Psychiatric symptoms were also measured and compared between the two subgroups. **Table 6** presents the results.

Synthetic cathinone-using OST clients showed higher scores on almost every psychiatric symptom scales with the exception of Somatization and Hostility. Furthermore, applying a 63 or above GSI T (raw) score as cut-off for identifying cases of clinical severity, a higher rate of screened psychiatric patients were found among SC (N = 22, 53.7%) than non-SC-using (N = 39, 25.7%) respondents [$\chi 2$ (1, N = 193) = 11.7, $p < 0.01$]. As a next step, participants were classified on the basis of their prominent psychopathological dimension, according to the highest standardized z score achieved on the BSI factors. The three most dominant dimensions were depression (n = 51, 25.8%), somatization (n = 15.7%) and hostility (n = 19, 9.6%). **Figure 3** presents the distribution of the dominant psychopathological dimensions within the subgroups of SC-using and non-SC-using respondents.

When compared, SC- and non-SC-using using subgroups did not differ significantly in the rate of any prominent psychopathological dimensions: Somatization [$\chi 2$ (1, N = 171) = 2.9, $p > 0.05$], Obsession-compulsion [$\chi 2$ (1, N = 171) = 0.3, $p > 0.05$], Interpersonal sensitivity [$\chi 2$ (1, N = 171) = 1.8, $p > 0.05$], Depression [$\chi 2$ (1, N = 171) = 0.9, $p > 0.05$], Anxiety [$\chi 2$ (1, N = 171) = 0.1, $p > 0.05$], Hostility [$\chi 2$ (1, N = 171) = 1.4, $p > 0.05$], Phobic anxiety [$\chi 2$ (1, N = 171) = 0.2, $p > 0.05$], Paranoid ideation [$\chi 2$ (1, N = 171) = 0.6, $p > 0.05$], Psychoticism [$\chi 2$ (1, N = 171) = 3.8, $p > 0.05$]. Furthermore, none of the dominant psychopathological dimensions showed significant association with treatment retention rates, indicating that psychiatric subtypes among OST clients might not differ in their treatment outcomes.

A person-centered approach was also applied in order to examine potential latent classes of users regarding their psychopathological profile based on achieved BSI factor scores. Latent profile analysis is a form of latent variable analyses (e.g., Collins and Lanza, 2010) with categorical latent variable and continuous manifest indicators (in this case the BSI factor scores). The Bayesian information criteria parsimony index (BIC), entropy and the interpretability of clusters were used during the process of determining the number of latent classes. Lower BIC value and higher value of entropy are preferable in model selection. The determination of the number of classes is usually supported by the results of the likelihood-ratio difference test [Lo–Mendell–Rubin adjusted likelihood-ratio test (LRT)], which compares the estimated model with a model of one less class (k - 1). In this case low p value ($p < 0.05$) indicates that the model with one less class is rejected in favor of the estimated model, however, none of our tested models yielded significant LRT test, therefore final model determination was based on BIC and entropy values. One- (BIC = 121420.8), two- (BIC = 123256.6), three- (121481.3), and four-class (BIC = 121534.1) solutions were

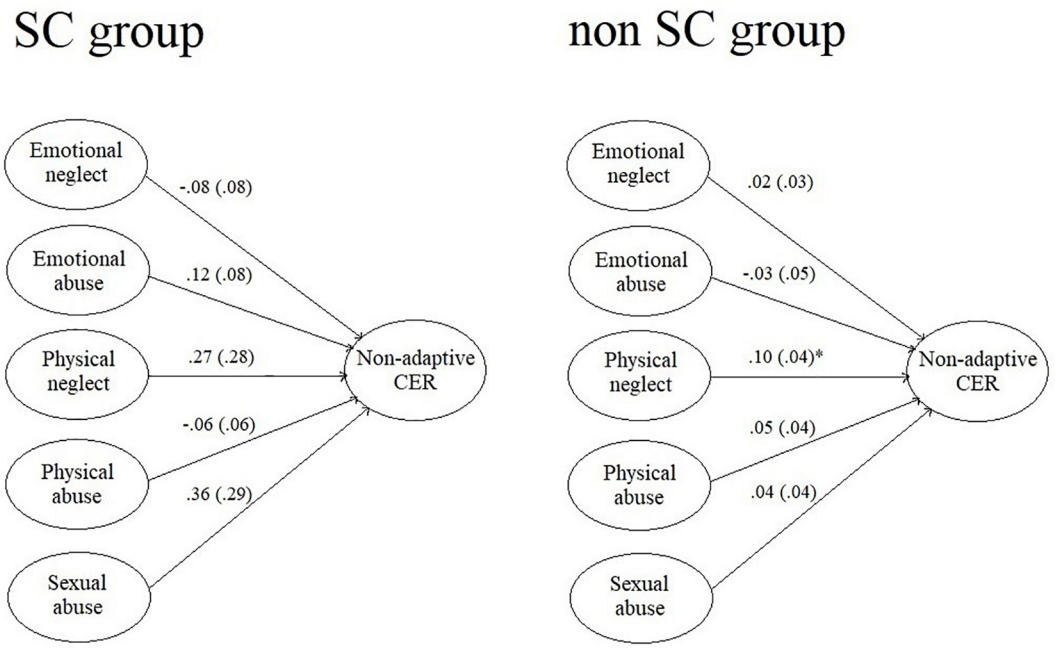

FIGURE 2 | The variability of non-adaptive cognitive emotion regulation strategies explained by childhood traumatic experiences among SC and non-SC-using clients. *$p < 0.05$; unstandardized *B* coefficients are presented with standard errors (in brackets). SC, synthetic cannabinoids; CER, cognitive emotion regulation.

estimated throughout the analysis. Entropy increased at two-class (0.887), reached its peak at three-class (0.947) and decreased at four-class solution (0.483). Finally, a three-class solution was accepted. **Figure 4** presents the psychiatric symptom profiles of the three classes.

Based on their most likely latent class membership, 111 participants belonged to the first class (less severe symptomatology), 57 participants to the second class (moderate symptom severity), while 25 respondents belonged to the third class (most severe symptomatology). These symptom profiles differ in their global severity but not in the pattern of dominant psychopathologies, indicating that OST clients might be similar in terms of psychiatric symptom patterns. SC using clients were more likely to belong to either the second ($n = 16$, 39%) or the third class ($n = 8$, 19.5%) than non-SC-using participants (second class: $n = 41$, 27%, third class: $n = 17$, 11.2%) [$\chi 2$ (1, $N = 193$) = 5.5, $p < 0.05$]. Clients receiving methadone vs. buprenorphine-naloxone did not differ in the rate of their class memberships [$\chi 2$ (1, $N = 193$) = 1.4, $p > 0.05$]. Additionally, there were no differences in treatment retention rates between the members of the three latent classes (the retention rates for the first, second and third class were 38.7, 42.1, and 24%, respectively) [$\chi 2$ (2, $N = 193$) = 2.5, $p > 0.05$].

Finally, class membership was predicted by using logistic regression analysis, entering treatment indices and SC use during treatment as covariates in the model. **Table 7** summarizes the result of these analyses. The only significant predictor was SC use during treatment regarding first latent class membership,

TABLE 5 | Stages of motivation for behavior change in SC and non-SC using OST clients.

	SC use reported *N* = 42	SC use not reported *N* = 132	Mann Whitney *U* test (*U*)	Effect size (*r*)
Precontemplation Mean (SD)	18.6 (5.9)	16.6 (4.9)	2200.5*	0.18
Contemplation Mean (SD)	30.4 (5.1)	30.7 (7.5)	2845	0.02
Action Mean (SD)	30.1 (6.9)	30.7 (5.9)	2764.5	0.05
Maintenance Mean (SD)	27.6 (6.3)	28.6 (6.9)	2558	0.08
Readiness to change Mean (SD)	17.4 (4.3)	18.3 (4.2)	2416	0.11

*$p < 0.05$.

TABLE 6 | Psychiatric symptoms of SC and non-SC using subgroups.

	SC use reported *N* = 43	SC use not reported *N* = 154	Mann Whitney *U* test(*U*)	Effect size (*r*)
Somatization Mean (SD)	1.3 (1.1)	1.1 (0.9)	2990	0.09
Obsession-Compulsion Mean (SD)	1.4 (0.9)	0.9 (0.8)	2250**	0.28
Interpersonal Sensitivity Mean (SD)	1.4 (1.1)	0.8 (0.8)	2299**	0.29
Depression Mean (SD)	1.8 (1.3)	1.2 (0.9)	2492*	0.26
Anxiety Mean (SD)	1.5 (0.9)	0.9 (0.9)	2357.5**	0.32
Hostility Mean (SD)	1.1 (1.1)	0.8 (0.9)	2594.5	0.15
Phobic Anxiety Mean (SD)	1.2 (1.1)	0.7 (0.8)	2599.5*	0.25
Paranoid Ideation Mean (SD)	1.3 (0.8)	0.9 (0.7)	2271.5**	0.26
Psychoticism Mean (SD)	0.9 (0.9)	0.5 (0.6)	2467***	0.25
Global Severity Index Mean (SD)	1.3 (0.9)	0.9 (0.7)	2220**	0.24

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

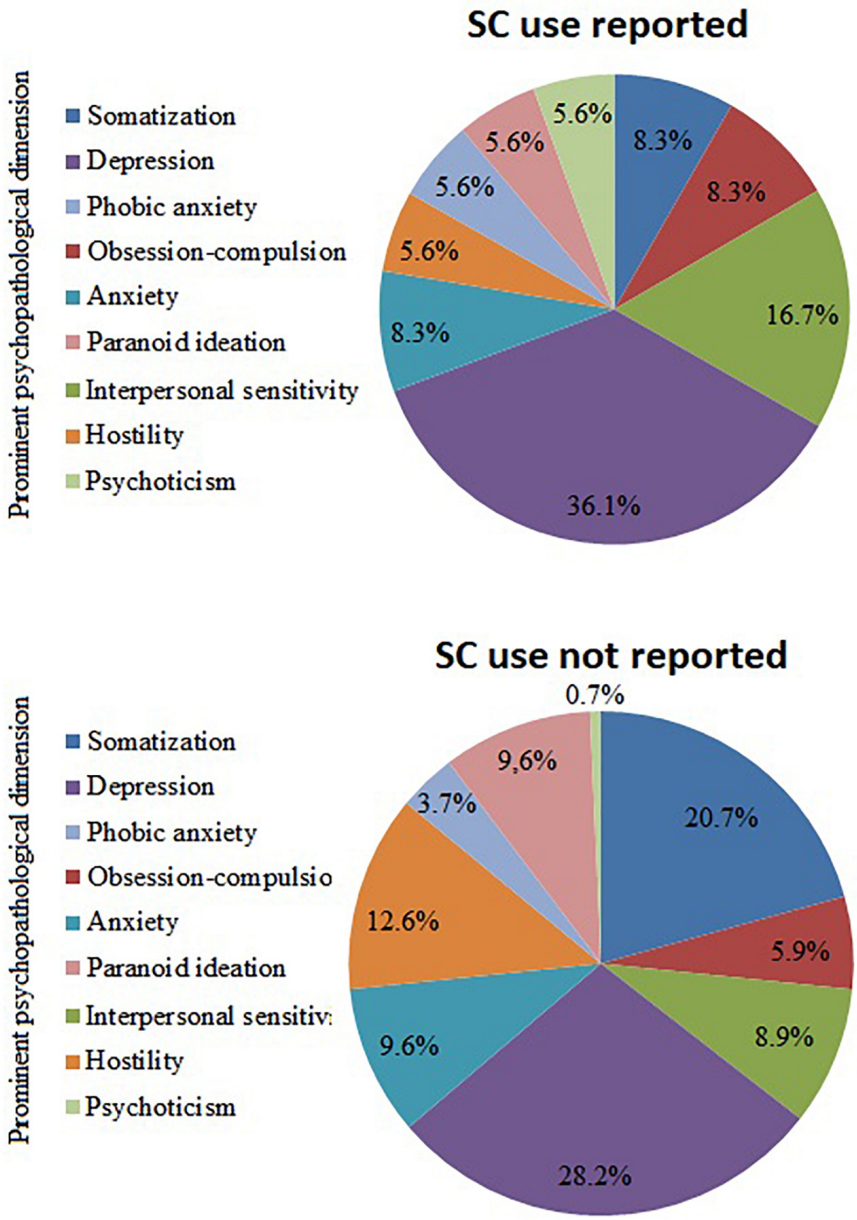

FIGURE 3 | Distribution of prominent psychopathological dimensions with and without reported SC use. SC, synthetic cannabinoids.

indicating that treatment indices (such as OST medication type, dose, years in treatment or receiving other medication such as benzodiazepines) do not show significant association with the severity of psychiatric symptom profiles. Those clients, however, who used SC during treatment had lower odds of belonging to the less severe psychiatric symptom class.

Predictors of SC Use

In order to examine the association between past year SC use and those variables that resulted in significant differences between SC-using and non-SC-using subgroups, a logistic regression model was tested (**Table 8**).

Clients with more severe psychiatric symptoms showed elevated risk for past year SC use (OR = 2.03), while those who spent longer time in treatment had lower odds of SC consumption (OR = 0.91).

Predictors of Treatment Retention

Finally, a logistic regression model was applied to test potential explanatory variables of treatment retention. Besides SC use, explanatory variables (age, gender, years in treatment, OST medication dose) were selected as commonly reported factors associated with treatment retention. **Table 9** summarizes the results.

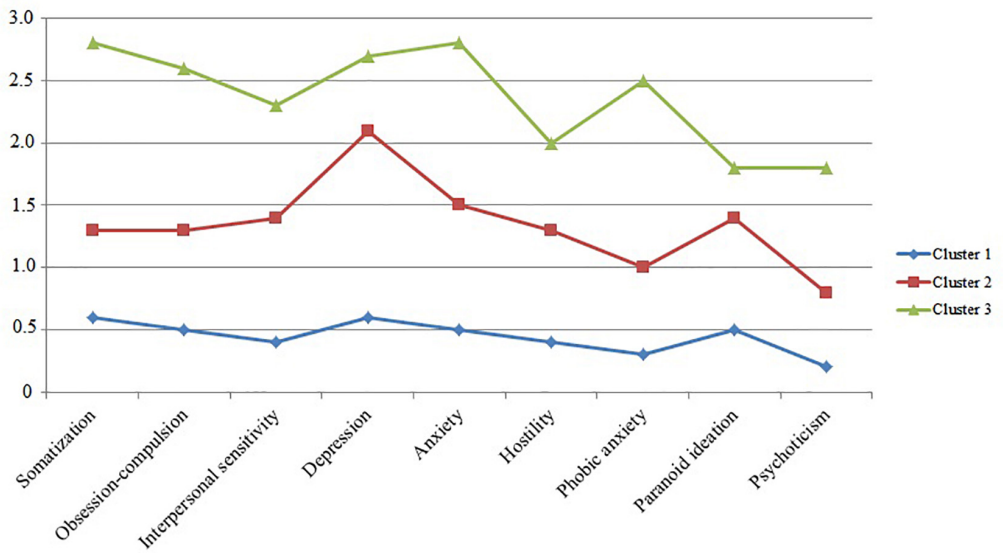

FIGURE 4 | Latent profiles of psychiatric symptom severities.

Past year SC use was the only significant predictor in the model, associated with decreased treatment retention odds (OR = 0.44).

DISCUSSION

Our study indicated differences between OST clients with and without a history of past year SC use in terms of treatment indices, childhood trauma experiences, cognitive emotion regulation strategies, motivation for behavior change and psychiatric symptom severity.

As regards treatment indices, the result that SC-using clients were less likely to buy street methadone/buprenorphine-naloxone as well as to inject their OST medication can primarily be explained by the assumption that instead of misusing/overusing opioid medication they are more prone to use SC in order to experience euphoria during treatment. Clients receiving buprenorphine-naloxone have spent less years in treatment than those who were taking methadone. This finding might be attributed to the fact that Suboxone was only introduced in 2007 in Hungary (Demetrovics et al., 2009), and further years have passed until clinicians started to prescribe this medication instead of methadone as a first-line OST medication. The buprenorphine-naloxone clients participating in this study were most likely among the first new clients who started their OST treatment with buprenorphine-naloxone and not methadone. Within the scope of the current study clients receiving methadone or buprenorphine-naloxone did not differ in any further measures. 4-year retention rate among non-SC-using clients (41.6%) was relatively high as compared to previous findings that identified a 18 months retention rate of 32.3% (Lin et al., 2013), a 1-year retention rate of 34.4% (Sheikh Fathollahi et al., 2016) or a 3-year retention rate of 20% (Yang et al., 2013) among OST clients, although some reported much higher, 87% 1-year retention rate (Jiang et al., 2014) or a 57% retention rate at 4-years follow-up (Wei et al., 2013). Past year SC use was associated with higher proportion of treatment drop-outs. Given the finding that almost every second client was screened positive for past year cannabis or opiate use during treatment and considering that there were no significant differences in the rate of positive urine test results between SC and non-SC-using clients, it might be assumed that SC use *per se* is a significant risk factor for drop-out. This assumption was further supported by the results of the second logistic regression model, namely that past year's SC use was a significant predictor of reduced treatment retention. SC using OST clients had approximately two times higher risk of drop out. In contrast to previous findings, age, gender, years spent in treatment or the dose of methadone or buprenorphine-naloxone did not show significant association with treatment retention. SC users were more likely to be in the precontemplation stage of behavior change, showing limited or no motivation to stop using psychoactive substances. Therefore frequent screening of clients' current motivation for behavior change should be an important routine in addiction care and especially in low-threshold services such as OST programs, accompanied by motivational interviewing.

Childhood physical neglect was related to non-adaptive cognitive emotion regulation strategies among non-SC-using clients. Previous results indicate that childhood neglect may predict deficits in the recognition of positive emotions (Young and Widom, 2014), that might explain difficulties in applying adaptive strategies, such as refocus on planning, positive reappraisal or putting a stressful life event into a more acceptable perspective. On a neurobiological level, others found that right amygdale volume mediates the association between neglect and anxiety (Roth et al., 2018) or that early exposure to either

TABLE 7 | Treatment indices and SC use during treatment as predictors of latent class memberships.

	B (SE)	*p*	Odds ratio	95% CI
Class 1 membership	**R^2 = 0.052 (Cox and Snell), 0.070 (Nagelkerke), *N* = 173**			
OST medication dose	−0.01-(0.01)	0.671	0.99	0.99, 1.01
Years in treatment	−0.04-(0.03)	0.154	0.96	0.90, 1.02
OST medication: methadone vs. buprenorphine-naloxone	−0.97-(0.64)	0.129	0.38	0.11, 1.33
Receiving other medication (e.g., benzodiazepines)	−0.23-(0.32)	0.478	0.79	0.42, 1.51
SC use during treatment	−0.94-(0.39)	0.018	**0.39**	0.18, 0.85
Class 2 membership	**R^2 = 0.036 (Cox and Snell), 0.051 (Nagelkerke), *N* = 173**			
OST medication dose	−0.00-(0.01)	0.916	0.99	0.99, 1.01
Years in treatment	0.02-(0.03)	0.472	1.02	0.96, 1.09
OST medication: methadone vs. buprenorphine-naloxone	0.84-(0.65)	0.196	2.32	0.65, 8.29
Receiving other medication (e.g., benzodiazepines)	0.43-(0.35)	0.220	1.54	0.77, 3.08
SC use during treatment	0.67-(0.41)	0.100	1.94	0.88, 4.29
Class 3 membership	**R^2 = 0.023 (Cox and Snell), 0.045 (Nagelkerke), *N* = 173**			
OST medication dose	0.01-(0.01)	0.470	1.01	0.99, 1.02
Years in treatment	0.05-(0.05)	0.269	1.05	0.96, 1.15
OST medication: methadone vs. buprenorphine-naloxone	0.27-(0.99)	0.783	1.31	0.19, 9.05
Receiving other medication (e.g., benzodiazepines)	−0.32-(0.48)	0.513	0.73	0.28, 1.88
SC use during treatment	0.71-(0.54)	0.189	2.04	0.70, 5.91

Significant explanatory variables and related values are boldfaced. CI, confidence interval.

TABLE 8 | Logistic regression model to test the retrospective odds for past year SC use.

	B (SE)	*p*	Odds ratio	95% CI
R^2 = 0.179 (Cox and Snell), 0.271 (Nagelkerke), *N* = 168				
Age	−0.05-(0.03)	0.142	0.95	0.89, 1.02
Years in treatment	−0.09-(0.04)	0.034	**0.91**	0.84, 0.99
Childhood physical neglect (CTQ)	−0.15-(0.09)	0.090	0.86	0.72, 1.03
Refocus on planning (CERQ)	−0.16-(0.10)	0.104	0.85	0.69, 1.03
Precontemplation (URICA)	0.06-(0.04)	0.133	1.06	0.98, 1.14
Global Severity Index (BSI)	0.71-(0.29)	0.014	**2.03**	1.16, 3.56

Significant explanatory variables and related values are boldfaced. CI, confidence interval.

emotional or physical neglect is related to amygdala hypertrophy and mood disorders (Herzog and Schmahl, 2018). Increased risk for anxiety and mood disorders thus further reduce the capability to focus on positive outcomes in stressful live situations. Non-SC-using clients reported a more frequent exposure to physical neglect in their childhood, denoting that SC use is probably not a maladaptive self-medication attempt in terms of dealing with early trauma experiences.

TABLE 9 | A logistic regression model to predict treatment retention.

	B (SE)	*p*	Odds ratio	95% CI
R^2 = 0.056 (Cox and Snell), 0.057 (Nagelkerke), *N* = 198				
Age	−0.02-(0.03)	0.465	0.98	0.94, 1.03
Gender	−0.25-(0.35)	0.477	0.78	0.39, 1.54
Years in treatment	−0.05-(0.03)	0.093	1.05	0.99, 1.11
OST medication dose	−0.01-(0.01)	0.253	1.01	0.99, 1.01
SC use during treatment	−0.82-(0.42)	0.049	**0.44**	0.19, 0.99

Significant explanatory variables and related values are boldfaced. CI, confidence interval.

Approximately half of SC-using clients were characterized by psychiatric symptoms of clinical severity, and global severity of these symptoms was a significant explanatory variable of SC use during treatment. Considering the identified latent profiles of psychiatric symptom severities, none of the treatment indices could predict class memberships, however, SC use during treatment was significantly associated with lower odds to belong to the less severe class of psychiatric symptoms. Prominent psychopathological dimensions were additionally examined. Our result that the most dominant psychopathologies of opioid-dependent clients are depression, somatization and hostility is in line with former findings, including the self-medication hypothesis of Khantzian (1985) that highlights the powerful muting action of opiates on the threatening affect of aggression. OST clients might also use or misuse their medication as a form of maladaptive self-medication in order to ease their depressive symptoms and violent urges. These findings emphasize the importance of psychiatric screening among OST clients in order to identify patients with potential unrecognized dual diagnoses. On the other hand, unlike Maremmani et al. (2016) we could not identify any significant associations between treatment outcomes and prominent psychopathological subtypes. Nevertheless, as other authors have already pointed out (e.g., Schulte et al., 2010), unidentified comorbid disorders may increase the risk of drop-out from addiction treatment, yet psychiatric comorbidity often remains a latent factor. Another related anomaly also needs to be addressed: according to the results of McGovern et al. (2014), only a minority of addiction treatment programs is capable of providing integrated services. Hence, public access to integrated care should be increased. A possible solution for enhancing the efficacy of dual diagnosis recognition would be the synthesis of addiction/psychiatry and primary care services, stressing the role of GPs and community services in early identification of both addiction problems (among other things, anamnestic information about SC or NPS use in general) and co-occurring mental disorders, including PTSD as well.

Limitations

The current study is not without limitations. While past year's opiate, cocaine, amphetamine, MDMA, THC or benzodiazepine misuse were described by positive urine test results as objective measures, SC use was based on clients' self-reports. Furthermore, past year's SC use as a retrospective and self-reported measure

was predicted by variables that were subsequently assessed (e.g., GSI). In these cases, a relative persistence was assumed regarding the values of explanatory variables, however, this is not necessarily realistic as the severity of psychiatric symptoms, the motivation for behavior change or the current cognitive emotion regulation strategies may markedly change over a time-span of 1 year. As regards our statistical analyses, only small – or in some cases medium – effect sizes were found, as based on Cohen's rule of thumb. Childhood traumatic experiences were also assessed by clients' retrospective self-reports, therefore these results might have been impacted by potential recall biases. Finally, OST clients' psychiatric profiles assessed by BSI could not be supported/confirmed by their clinical diagnoses as all clients of the drug outpatient center received the exact same comorbid diagnoses of opioid dependence (F11.20) and mixed anxiety and depressive disorder (F41.2), most likely due to a rather simplifying diagnostic procedure that mainly justifies the applied pharmacotherapy but does not necessarily aim to provide an elaborate psychopathological profile of the clients.

Future Directions

This study did not assess PTSD as a potential outcome of childhood trauma, nor the mediatory role of psychiatric symptom severity between trauma exposure and non-adaptive cognitive emotion regulation strategies. These associations were partly tested by others (e.g., Garnefski et al., 2017; McLaughlin and Lambert, 2017; Karatzias et al., 2018), but not in a sample of OST clients or in light of concurrent SC consumption. Differences in emotion regulation and psychopathology between SC-using clients and those who consume other stimulant drugs (e.g., cocaine, amphetamine, MDMA) would highlight further specificities of SC regarding the consecutive neuropsychological traits of its use.

CONCLUSION

Synthetic cathinone use is a risk factor of poorer treatment outcomes, characterized by more severe psychiatric symptoms as well as a lack of motivation to change substance use behavior among opioid dependent clients. The availability of rapid urine tests able to detect NPS like SC need to be increased in order to more efficiently screen OST clients' SC consumption during therapy.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Research Ethics Committee of the Nyírõ Gyula National Institute of Psychiatry and Addictions, Budapest, Hungary. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MK-F, AK, KK, and JF conceived the presented idea and developed the questionnaire for the study. PP and JH were involved in data collection procedure. All authors discussed the results and contributed to the manuscript. ZD helped in supervising the project. MK-F, AK, KK, JF, and ZD took part in writing the manuscript. MK-F was responsible for statistical analyses.

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REVIEW
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NBOMes–Highly Potent and Toxic Alternatives of LSD

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Recently, a new class of psychedelic compounds named NBOMe (or 25X-NBOMe) has appeared on the illegal drug market. NBOMes are analogs of the 2C family of phenethylamine drugs, originally synthesized by Alexander Shulgin, that contain a *N*-(2-methoxy)benzyl substituent. The most frequently reported drugs from this group are 25I-NBOMe, 25B-NBOMe, and 25C-NBOMe. NBOMe compounds are ultrapotent and highly efficacious agonists of serotonin 5-HT_{2A} and 5-HT_{2C} receptors (Ki values in low nanomolar range) with more than 1000-fold selectivity for 5-HT_{2A} compared with 5-HT_{1A}. They display higher affinity for 5-HT_{2A} receptors than their 2C counterparts and have markedly lower affinity, potency, and efficacy at the 5-HT_{2B} receptor compared to 5-HT_{2A} or 5-HT_{2C}. The drugs are sold as blotter papers, or in powder, liquid, or tablet form, and they are administered sublingually/buccally, intravenously, via nasal insufflations, or by smoking. Since their introduction in the early 2010s, numerous reports have been published on clinical intoxications and fatalities resulting from the consumption of NBOMe compounds. Commonly observed adverse effects include visual and auditory hallucinations, confusion, anxiety, panic and fear, agitation, uncontrollable violent behavior, seizures, excited delirium, and sympathomimetic signs such mydriasis, tachycardia, hypertension, hyperthermia, and diaphoresis. Rhabdomyolysis, disseminated intravascular coagulation, hypoglycemia, metabolic acidosis, and multiorgan failure were also reported. This survey provides an updated overview of the pharmacological properties, pattern of use, metabolism, and desired effects associated with NBOMe use. Special emphasis is given to cases of non-fatal and lethal intoxication involving these compounds. As the analysis of NBOMes in biological materials can be challenging even for laboratories applying modern sensitive techniques, this paper also presents the analytical methods most commonly used for detection and identification of NBOMes and their metabolites.

Keywords: new psychoactive substances, NBOMe, phenethylamines, psychedelics, toxicity, metabolism, analytical methods

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INTRODUCTTION

The last decade witnessed the emergence of new psychoactive substances (NPSs), followed by a rapid increase in their prevalence and the constant introduction of new compounds into the clandestine market in order to circumvent the existing laws. From 2009 to 2018, 899 different NPSs were reported worldwide (United Nations Office on Drugs and Crime [UNODC], 2019).

Over the course of 2018, a total of 687 NPSs were notified to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). In 2018, one new NPS was reported to EMCDDA every week (EMCDDA, 2019). The five main classes of NPSs are synthetic cannabinomimetics, stimulants (dominated by derivatives of cathinone), opioids, psychedelics, and non-pharmaceutical benzodiazepines. By analogy to other NPSs, psychedelic compounds, which produce marked alterations of perception, mood, and cognition, are widely used for recreational purposes. Psychedelics (also called classical or serotoninergic hallucinogens) are divided into two main groups based on their chemical structure: indoleamines (termed also indolealkylamines; e.g., ergolines, including LSD and its analogs, and simple tryptamines, such as *N*,*N*-dimethyltryptamine and 5-methoxy-*N*,*N*-dimethyltryptamine) and phenylalkylamines. Phenylalkylamines are highly selective for serotonin 5-HT_2 receptors, while indoleamines are relatively non-selective for 5-HT receptors, displaying moderate to high affinity for 5-HT_1 and 5-HT_2 receptor subtypes. The phenylalkylamines can be further divided into two subgroups, one group being the phenylisopropylamines (analogs of amphetamine), e.g., 2,5-dimethoxy-4-bromoamphetamine (DOB) and 2,5-dimethoxy-4-methylamphetamine (DOM), and the other being the phenethylamines, including mescaline, 2C-X compounds and their derivatives (**Figure 1**) (reviewed by Halberstadt, 2017). The name "2C" refers to an acronym created by the 'godfather' of psychedelic drugs Alexander Shulgin to describe their chemical structure, where two carbon atoms separate the amine group from the phenyl ring (Shulgin and Shulgin, 1991). The prototype of the 2C series, 2C-B, was synthesized by Shulgin in 1974. Since 2010, a new group of 2C compounds containing an *N*-(2-methoxy)benzyl (*N*-benzoylmethoxy) substituent, known as *N*-(2-methoxybenzyl)phenethylamines (aka 25X-NBOMes or simply NBOMes), has emerged in the illicit drug market. Structure-activity studies indicate that this substituent significantly increases the affinity of the drug toward the 5-HT_{2A} receptor and its pharmacological activity (Hansen et al., 2014). It is important to note that stimulation of the 5-HT_{2A} receptors is required for the psychedelic effects of compounds such as LSD, mescaline, and psilocybin (Glennon et al., 1984; Titeler et al., 1988; Sadzot et al., 1989; Vollenweider et al., 1998; Rickli et al., 2016). The first NBOMes were originally synthesized by Ralf Heim at the Free University of Berlin in a search for pharmacological tools to study the 5-HT_{2A} receptor (Heim, 2003). Since then, [^{11}C]25I-NBOMe and [^{11}C]25C-NBOMe have been used to map the distribution of 5-HT_{2A} receptors in the brain by positron emission tomography (PET) imaging (Ettrup et al., 2010, 2014; for an excellent review see Poulie et al., 2019).

The first recreationally used drug from this group was 25I-NBOMe (2-(4-iodo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine), identified in seven green blotters seized by the Swedish police in May 2012 (EMCDDA, 2014). It is likely that 25I-NBOMe was the first NBOMe to be used recreationally in the United States (Palamar and Le, 2019). Following this, several potent NBOMes were synthesized and introduced into the drug market. In these, the iodine atom was exchanged for other halogens: e.g., bromine (25B-NBOMe) or chlorine (25C-NBOMe), a hydrogen atom (25H-NBOMe), a nitro group (25N-NBOMe) or an organic functional group, - methyl (25D-NBOMe), -ethyl (25E-NBOMe), or –isopropyl (25iP-NBOMe) (Wood et al., 2015; Poulie et al., 2019). Three compounds from this group, namely 25I-NBOMe, 25B-NBOMe, and 25C-NBOMe, accounted for 0.03% of the total quantity of hallucinogens (other than ketamine) seized globally between 2011 and 2017 (United Nations, 2019). In the United States, 2,129 reports for 25I-NBOMe, 1,273 reports for 25C-NBOMe, and 924 reports for 25B-NBOMe were collected by the System to Retrieve Information from Drug Evidence and the National Forensic Laboratory Information System between January 2014 and April 2018 (Drug Enforcement Administration, 2018).

METHODS

This literature review was based on an exhaustive search of PubMed (U.S. National Library of Medicine) that used "NBOMe" and each of the compound names listed in **Table 1** as keywords. Only papers written in English and with full texts available by November 2019 were included. Additionally, official reports published by the United Nations Office on Drugs and Crime (UNODOC), EMCDDA, and the World Health Organization (WHO) were studied. Furthermore, in each article and report obtained, references were checked carefully in order to identify possible additional publications missed during the initial search.

PHARMACOLOGY OF NBOMes

In vitro studies indicated that NBOMe compounds are ultrapotent and highly efficacious agonists of 5-HT_{2A} and 5-HT_{2C} receptors (Ki values in low nanomolar range), with more than 1000-fold selectivity for 5-HT_{2A} compared with 5-HT_{1A}. The compounds display higher affinity for 5-HT_{2A} receptors than their 2C counterparts and have markedly lower affinity, potency, and efficacy at the 5-HT_{2B} receptor than at 5-HT_{2A} or 5-HT_{2C} (Juncosa et al., 2013; Nichols et al., 2015; Rickli et al., 2015; Elmore et al., 2018; Eshleman et al, 2018). In addition, NBOMes have a significant affinity (Ki $<$ 300 nM) for adrenergic α_1 receptors but not so H_1-histamine, dopamine D_1, D_2, and D_3 receptors or the monoamine transporters DAT, NET, or SERT (Nichols et al., 2015; Elmore et al., 2018; Eshleman et al, 2018). Molecular modeling and molecular dynamics simulation studies performed on a human 5-HT_{2A} receptor model identified several amino acid residues as putative binding sites of NBOMes. It is suggested that the binding pocket, localized among transmembranes (TM) III, V, VI, and VII, includes Trp-151(TMIII), Ile-152(TMIII), Asp-155(TMIII), Ser-159(TMIII), Ser-239(TMV), Phe-339 (TMVI), Phe-340(TMVI), Val-336(TMVII), and Tyr-370(TMVII) (Braden et al., 2006; Silva et al., 2011; Ísberg et al., 2011). Among them, a highly conserved Asp-155 forms a salt bridge with the amine nitrogen, Ser-159 and Ser-239 form H-bonds with the 2-methoxy and 5-methoxy group, respectively, and Phe-340 forms a van der Waals interaction with the benzene ring. It should be emphasized

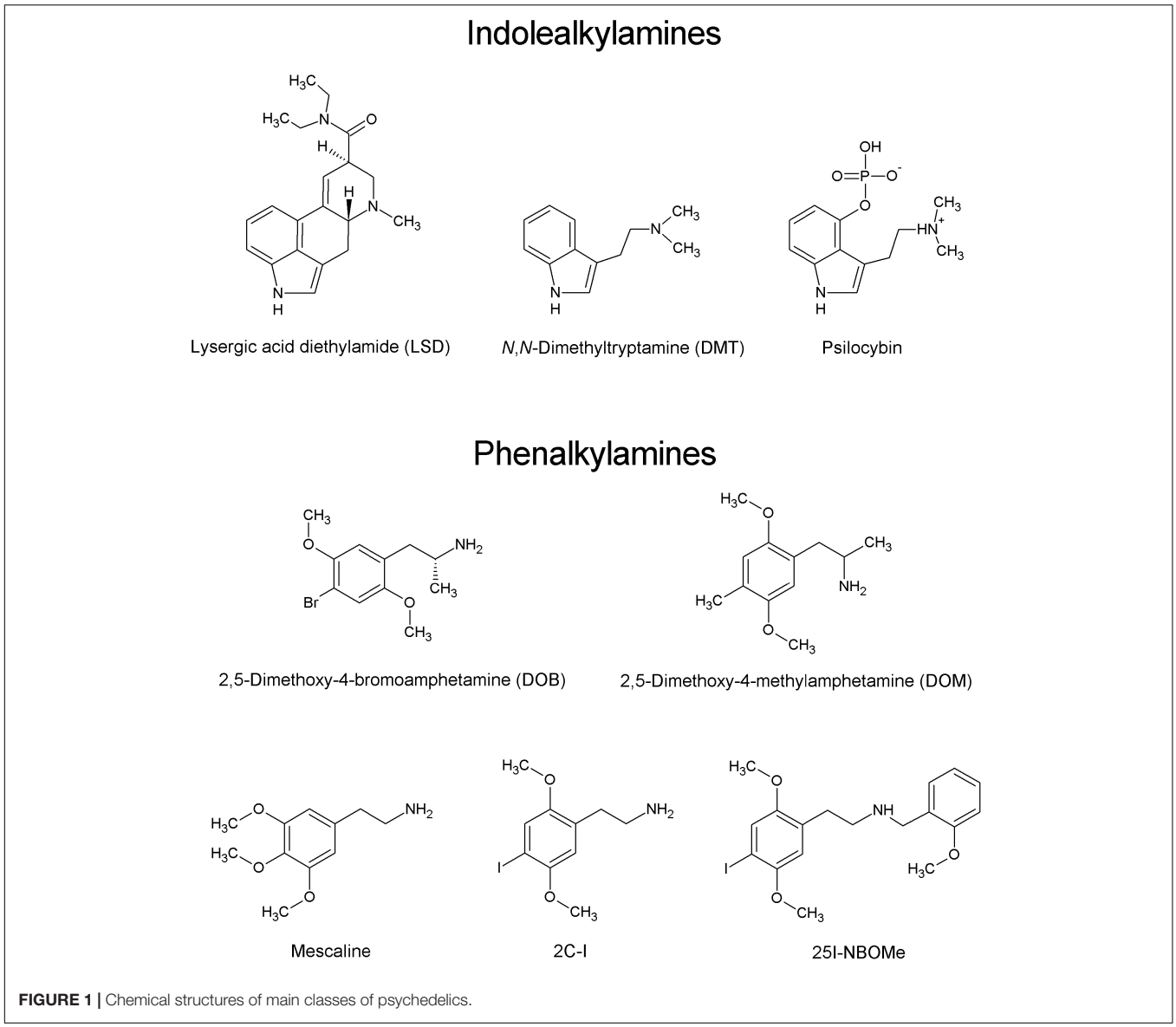

FIGURE 1 | Chemical structures of main classes of psychedelics.

TABLE 1 | Doses and duration of action of NBOMe compounds (TripSit a, b, c, d, e).

Compound (route of administration)	Dose (μg)				Action		
	Threshold	Light	Common	Strong	Onset	Duration	After effects
25B-NBOMe							
Sublingual	100	100–300	350–500	500–700+	20–40 min	8–12 h	2–6 h
Insufflation	50	50–200	200–350	350–500+	2–5 min	8–12 h	2–6 h
25C-NBOMe							
Sublingual	50–250	250–500	500–750	750–1250+	45–90 min	4–10 h	1–24 h
25E-NBOMe							
Sublingual	50–100	50–200	200–400	400–800	15–60 min	5–10 h	2–10 h
25I-NBOMe							
Sublingual	50–250	200–500	500–750	700–1000+	45–90 min	5–10 h	6–24 h
25N-NBOMe							
Buccal/sublingual		100–300	300–800	800–1300+	45–75 min	5–10 h	1–12 h

that Asp-155, Ser-159, Ser-239, Phe-340 are also important for binding and efficacy of different agonists and partial agonists at 5-HT_{2A} receptor (Silva et al., 2011). On the other hand, the van der Waals interaction between Phe-339 and *N*-benzyl ring of NBOMes and the hydrogen bond formed by Tyr-370 with the 2-position oxygen on this ring are considered to play a key role in the high potency and affinity of these compounds binding to 5-HT_{2A} receptor (Silva et al., 2011; Ísberg et al., 2011).

The activation of cortical 5-HT_{2A} receptors induces the head twitch response (HTR) in mice and rats, also referred to as wet dog shakes (Willins and Meltzer, 1997; Abiero et al., 2019). The HTR is widely used as a behavioral marker for hallucinogen effects in humans (Halberstadt and Geyer, 2018). 25C-NBOMe, 25I-NBOMe, and 25B-NBOMe induced an HTR response in rodents with a potency several-fold higher than their 2C counterparts, 2C-C and 2C-I (Halberstadt and Geyer, 2014; Elmore et al., 2018; Custodio et al., 2019; Herian et al., 2019). Two lines of evidence support a notion that this behavioral effect is mediated by cortical 5-HT_{2A} receptors. Thus, ketanserin, a 5-HT_{2A} antagonist, blocked the 25B-NBOMe-evoked HTR and normalized 5-HT_{2A} mRNA levels in the mouse prefrontal cortex upregulated by a prolonged administration of the drug (Custodio et al., 2019).

Gatch et al. (2017) tested 25B-NBOMe, 25C-NBOMe, and 25I-NBOMe for discriminative stimulus effects similar to a prototypical psychedelic/hallucinogen DOM and to an empathogen, 3,4-methylenedioxymethamphetamine (MDMA). In DOM-trained rats 25B-NBOMe and 25C-NBOMe, but not 25I-NBOMe, fully substituted for this drug. 25B-NBOMe also fully substituted for MDMA. In both tests, the dose-effect curves for 25B-NBOMe had an inverted U-shape. It is suggested that 25B-NBOMe and 25C-NBOMe are most likely used as recreational psychedelics, although 25B-NBOMe may also be used as an empathogenic compound (Gatch et al., 2017). However, the latter assumption should be taken with caution, as some compounds (e.g., fenfluramine) that substitute for MDMA in rats do not produce MDMA-like empathogenic effects in humans (Schechter, 1988).

Using a battery of tests, behavioral effects of 25I-NBOMe (0.5 and 1 mg/kg) were examined in male and female Sprague-Dawley rats (Miliano et al., 2019). In both sexes, the systemic administration of the drug reduced visual object and placing responses–an effect likely related to its pro-hallucinogenic action–and decreased acoustic and tactile responses. Furthermore, by analogy to LSD and MDMA (Halberstadt and Geyer, 2010; Marti et al., 2019), 25I-NBOMe impaired the acoustic startle response [prepulse inhibition, a preclinical behavioral marker of vulnerability to develop a neuropsychiatric disorder (Marti et al., 2019)]. The drug increased body temperature only in females. On the other hand, it exerted an analgesic affect in males. It is suggested that the observed differences could be related to a sex-dependent pharmacodynamic profile of 25I-NBOMe (Miliano et al., 2019).

Psychedelic drugs interact with various neurotransmitter systems, namely serotonergic, glutamatergic, dopaminergic, cholinergic, and GABA-ergic. Among them, the glutamatergic system appears to play a prominent role in the action of these drugs (Aghajanian and Marek, 1999). In vivo microdialysis after systemic administration to rats of DOI, 5-methoxy-*N*,*N*-diisopropyltryptamine (MeO-DIPT) or LSD revealed markedly elevated extracellular levels of glutamate in the cortex (Scruggs et al., 2003; Muschamp et al., 2004; Noworyta-Sokołowska et al., 2016, 2019). DOI also increased the dopamine level in the cortex and ventral tegmental area (VTA) (Bortolozzi et al., 2005; Pehek et al., 2006). Recently, Herian et al. (2019), using microdialysis in freely moving male Wistar-Han rats, demonstrated increased extracellular levels of glutamate, dopamine, and 5-HT in the frontal cortex after administration of 25I-NBOMe. The drug also increased the tissue content of 5-HT and its metabolite hydroxyindoleacetic acid (5-HIAA) but did not affect the tissue content of dopamine and its metabolites: 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanilic acid (HVA). Subsequent studies performed by Miliano et al. (2019) on Sprague-Dawley rats, both males and females, showed that 25I-NBOMe increased extracellular dopamine levels in the nucleus accumbens (NAc) shell (but not core) in a sex-independent way. The drug markedly elevated dopamine levels in the medial prefrontal cortex (mPFC) of females but not males. No statistically significant changes in extracellular levels of 5-HT in the three analyzed brain structures were found in both sexes (Miliano et al., 2019). These results suggest that in rats effects of 25I-NBOMe on dopaminergic and serotoninergic transmission depend not only on the brain structure but also strain and sex.

An important question that remains to be fully resolved is whether NBOMe compounds are endowed with an abuse potential. Two behavioral tests, conditioned place preference (CPP) and self-administration (SA), are widely used in studies examining the abuse potential of drugs by analyzing their rewarding and reinforcing effects. 25I-NBOMe (0.3 mg/kg), 25B-NBOMe (1 mg/kg), and 25N-NBOMe (3 mg/kg) produced CPP in mice with a magnitude comparable to 1 mg/kg of methamphetamine (Custodio et al., 2019; Jeon et al., 2019; Seo J.Y. et al., 2019). The 25B-NBOME-elicited CPP was blocked by antagonists of D_1- and D_2-dopamine receptors, SCH 23390 and haloperidol, respectively, but was not affected by ketanserin, an observation indicating an important role of dopaminergic transmission in this phenomenon (Custodio et al., 2019). In the SA test performed on mice, 25B-NBOMe used at doses of 0.03, 0.1 and 0.3 mg/kg/infusion significantly increased both a number of infusions/session and an active lever pressing/session, albeit with a weaker potency than methamphetamine (Custodio et al., 2019). On the contrary, 25N-NBOMe (0.01 mg/kg/infusion) weakly increased the number of infusions/session, but not the active lever pressing/session in mice (Seo J.Y. et al., 2019), whereas 25I-NBOMe (0.03 mg/kg/infusion) did not significantly affect these two SA parameters in rats (Jeon et al., 2019). These findings suggest that 25I-NBOMe, 25B-NBOMe, and 25N-NBOMe might have some dependence liability.

As activation of the mesolimbic dopaminergic pathway plays a critical role in drug abuse and addiction, effects of 25N-NBOMe and 25B-NBOMe on the expression of D_1- and D_2-dopamine receptors, dopamine transporter (DAT), and tyrosine hydroxylase (TH) at the protein level were examined in two

studies. One was performed on mice that, in the course of the CPP test, received in total four injections of 25N-NBOMe (3 mg/kg/injection) and were sacrificed two days after the last injection (Seo J.Y. et al., 2019). In the second study, mice were repeatedly treated with 25B-NBOMe (1 mg/kg) for 7 days; they were sacrificed 30 min after the last injection (Custodio et al., 2019). Results of these studies are, however, not uniform. The level of D_1 receptor protein was increased in the NAc of mice pretreated with 25B-NBOMe but was not affected in NAc and dorsal striatum (DSt) of 25N-NBOMe mice. Markedly lower levels of D_2 receptors were found in the ventral tegmental area (VTA) after administration of 25B-NBOMe, in NAc and DSt (25N-NBOMe and 25B-NBOMe). 25N-NBOME decreased expression of DAT and TH in the NAc but not in DSt. 25B-NBOMe-induced a decrease of DAT and did not change TH protein levels in the VTA. Among several factors that might contribute to the above discrepancies, different dosing protocols appears to play an important role.

TOXICITY *IN VITRO*

Recent studies demonstrated that NBOMes exhibit neurotoxic and cardiotoxic activity. 25C-NBOMe was cytotoxic against neuronal cell lines SH-SY5Y, PC12, and SN4741 with respective calculated IC_{50} values of 89, 78, and 62 μM. The compound was 56, 25, and 64 times more potent than methamphetamine at reducing the viability of SH-SY5Y, PC12, and SN4741 cells, respectively. The neurotoxic action of 25C-NBOMe involves activation of the MAP/ERK cascade and inhibition of the Akt pathway (Xu et al., 2019). Acute (30 min) and prolonged (5 h) exposure of primary rat cortical cultures to 25B-NBOMe decreased spontaneous neuronal activity, measured as firing rate and burst rate (Zwartsen et al., 2018, 2019). The compound was 10-fold more potent than its precursor, 2C-B. Importantly, neuronal activity did not recover after 19 h of washout following prolonged exposure to 10 and 30 μM of 25B-NBOMe (Zwartsen et al., 2019).

25D-NBOMe and 25C-NBOMe reduced viability of H9c2 cells (cardiomyocytes). Both compounds used at doses of 0.75 and 2 mg/kg downregulated expression levels of p21 (CDC42/RAC)-activated kinase 1 (PAK1), an enzyme with documented cardiac protective effects, and prolonged QT intervals in rat ECG. 25D-NBOMe inhibited the hERG potassium channel, a phenomenon that might play a role in QT interval prolongation (Yoon et al., 2019).

AVAILABLE FORMS OF PRODUCTS AND PATTERN OF USE

The three most popular compounds from the 25X-NBOMe series are 25I-NBOMe (2-(4-iodo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine), 25B-NBOMe (2-(4-bromo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine), and 25C-NBOMe (2-(4-chloro-2,5-dimethoxy phenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine) (**Figure 1**; Al-Iman and AbdulMajeed, 2017; Halberstadt, 2017; Palamar and Le, 2019). 25I-NBOM is known under street names "Solaris", "25I", "Dots", "legal acid", "N-Bomb", "NE-BOME", "Smiles", "INBMeO", "BOM-Cl", "Hoffman", "N-boom", and "Holand Film"; 25C-NBOMe as "C-Boom", "Cimbi-82", "Pandora", and "Dime"; and 25B-NBOMe as "Nova", "legal acid", "NBomb", "NE-BOME", "New Nexus", "NBOMe-2-B", and "BOM 2-CB" (Zuba et al., 2013; EMCDDA, 2014; World Health Organization, 2014a,b; Al-Iman and AbdulMajeed, 2017).

NBOMes are typically available in the form of preloaded paper doses (blotters) with images and logos from popular cartoons and music/movie posters and, less frequently, in powder or liquid form (Zuba et al., 2013; EMCDDA, 2014; World Health Organization, 2014a,b; Andrabi et al., 2015; Halberstadt, 2017). As NBOMes undergo extensive first-pass metabolism (see section BIOTRANSFORMATION), preferred and common patterns of their use include sublingual, buccal, and nasal. Thus, 25C-NBOMe and 25I-NBOMe are usually taken by holding the blotter in the mouth (sublingually or buccally), insufflated as powder, or in solution as a nose spray (Zuba et al., 2013; EMCDDA, 2014; Lawn et al., 2014; World Health Organization, 2014a,b; Nikolaou et al., 2015; Suzuki et al., 2015; Halberstadt, 2017; Marchi et al., 2019). In order to improve buccal absorption, 25I-NBOMe may be complexed with cyclodextrin. Other less common routes of administration include oral, rectal, vaginal, intravenous or intramuscular injection, and smoking (EMCDDA, 2014; World Health Organization, 2014a,b). Doses and duration of action depend on the route of administration (see **Table 1**).

According to the limited information available from user websites and clinical case reports, NBOMes have been sold as a 'legal' alternative to LSD ("legal LSD") or as LSD due to the very potent psychedelic activity (Bersani et al., 2014; EMCDDA, 2014; World Health Organization, 2014a,b; UNODC, 2017). 25I-NBOMe was found in blotters seized in China sold as LSD (Zhang et al., 2018). A recent report from Columbia documents identification of 25I-NBOMe in 21 out of 70 blotters marked as LSD; the drug was combined in the same blotter with MDMA, 25I-NBOMe amine, and/or 25H-NBOMe (Mendoza-Valencia et al., 2019). NBOMes (25I-, 25C-, 25B-, and 25H-) were also found in tablets sold as Ecstasy (Chia et al., 2019). Thus, users may accidentally ingest NBOMe as counterfeited LSD or MDMA. According to information given on drug fora, users may find that LSD has a slight metallic taste or no taste at all, while 25I-NBOMe will have a bitter taste. The two substances can also be tested using a black light/UV source: LSD will glow whereas 25I-NBOMe will not (Zamnesia Blog, 2016).

Similar to other NPSs, users may combine NBOMes with various psychoactive substances: psychedelics (e.g., 2C compounds, mescaline, psilocybin and LSD), empathogens, psychostimulants, and/or depressants, including alcohol and marijuana, and medicines, both intentionally and unintentionally (Halberstadt, 2017; Madsen et al., 2017; Marchi et al., 2019). Importantly, the use of serotoninergic drugs, e.g., selective serotonin re-uptake inhibitors (SSRIs) or MAO-A inhibitors and/or substances known to increase

extracellular serotonin levels may increase the risk of developing serotonergic toxicity, the symptoms of which include tachycardia, hypertension, hyperthermia, muscle rigidity, and convulsions (Volpi-Abadie et al., 2013).

EFFECTS RELATED TO USE OF NBOMes

NBOMes are used for recreational purposes and psychedelic/hallucinogenic experiences. Subjective 'positive' effects reported by users resemble those of other psychedysleptics and include euphoria, mild stimulation, mood lift, feelings of love and empathy, change in perception, ego softening, insight, brightened and vibrant colors, enhanced appreciation of music, strong closed/open eye visuals, enhanced tactile sensation, mental/physical stimulation, increase in associative and creative thinking, erotic, sexual thoughts and sensations, and life-changing spiritual experiences (Zuba et al., 2013; EMCDDA, 2014; World Health Organization, 2014a,b; Erowid.org). "*Wow! Visuals are crazy, and the music is intense–waves of 3D objects have taken over my living room and everything looks beautiful! [...] Amazing party drug! I don't feel very stimulated even though this is a psychedelic stimulant? But euphoria I feel quite allot, and this is a really social drug, although on high doses it's a bit hard to have a real conversation.*" "*I literally FEEL the beauty of the universe in its infinite complexity. My perception of myself is erased. There is no longer a 'me'*". "*I am most certainly in a profound psychedelic headspace*" *(i.e., I intuitively understand the universe, society, etc. It's much more of a pure psychedelic than its non-benzyl substituted cousin 2C-I while still retaining some of the entactogen qualities.*" (Erowid.org).

NBOMes produce an array of adverse effects (Hill et al., 2013; Rose et al., 2013; Bersani et al., 2014; EMCDDA, 2014; Forrester, 2014; Grautoff and Kähler, 2014; Lawn et al., 2014; Stellpflug et al., 2014; Suzuki et al., 2014; Tang et al., 2014; World Health Organization, 2014a,b; Hieger et al., 2015; Nikolaou et al., 2015; Poklis et al., 2015b; Srisuma et al., 2015; Wood et al., 2015; Gee et al., 2016; Kristofic et al., 2016; Hermanns-Clausen et al., 2017; Humston et al., 2017; Madsen et al., 2017; Rajotte et al., 2017; Schetz et al., 2017; Wiergowski et al., 2017; Zygowiec et al., 2017; Marchi et al., 2019; Erowid.org); for comprehensive reviews see Suzuki et al., 2015; Halberstadt, 2017).

Psychoactive

Severe agitation, agitated delirium, intensive unpleasant hallucinations, aggression that sometimes progresses to violent and self-destructive behavior, paranoia, suicidal attempts, psychosis with delusions, dysphoria, irritability, fear, and panic attacks.

Neurological

Hyperthermia, convulsions, clonus, motor incoordination, mouth numbing and impaired speech, insomnia, blurred vision, and leucoencephalopathy.

Cognitive

Loss of location and time, confusion, short-term memory deficits, cognitive impairment, mental fatigue, altered mental state, loosening of association, and disorganized thoughts.

Cardiovascular

Tachycardia, hypertension, cardiac arrest, and vasoconstriction leading to ischemia.

Miscellaneous

Nausea, vomiting, sweating/chills, diaphoresis, tachypnea, respiratory and metabolic acidosis, leukocytosis, hyperglycemia, hyperkalemia, muscle rigidity, and compartment syndrome.

In severe cases, the use of NBOMes can led to comas, disseminated intravascular coagulation, liver failure, heart failure, pulmonary edema, cardiopulmonary arrest, rhabdomyolysis [a case of massive rhabdomyolysis with serum kinase creatinine concentration over 500,000 U/I was reported after ingestion of a 25I-NBOMe containing party pill named "Alice in Wonderland" (Schetz et al., 2017)], acute kidney failure, and multiorgan failure.

Srisuma et al. (2015) analyzed 148 cases of intoxication with NBOMe drugs and 193 with 2C compounds reported to the National Poison Data System in the United States from 1st September 2012 to 30th September 2014. They reported higher numbers of hallucinations/delusions, single-episode seizures, and benzodiazepine administration in NBOMe exposures (40.5, 8.8, and 50.0%, respectively) than those of 2C exposures (25.4, 3.1, and 32.6%, respectively).

In general, the features of NBOMe toxicity are also induced by other psychedelics. The main difference is an intensity and frequency of severe intoxication symptoms. The incidence of seizures is higher with NBOMes compared with other psychedysleptics, whereas muscle spasms, hyperreflexia, and tremors are rarely noted in cases of intoxication with NBOMes. The progression from rhabdomyolysis to metabolic acidosis, anuria, and acute renal failure is a common complication of severe NBOMe toxicity, but this is reported less frequently in cases of intoxication with other drugs.

By analogy to other NPSs, except for opioids and benzodiazepines, at present there are no specific antidotes for NBOMes, and all treatments used are symptomatic. Clinical management of acute toxicity resulting from the use of NBOMe compounds consists of monitoring, including fluids, electrolytes, acid-base balance, and supportive treatment: mechanical ventilation and intravenous administration of fluids; benzodiazepines (e.g., midazolam and lorazepam) given intravenously are used for sedation, to treat aggression, tremors, and convulsions; an infusion of catecholamines (noradrenaline, dopamine) to overcome sinus bradycardia; antiarrhythmic drugs (e.g., cardioselective β-blockers, amiodarone) to treat supraventricular tachyarrhythmia; and antipyretics/mechanical cooling in cases of hyperthermia. Gross hematuria and anuria require continuous venovenus hemodialysis (CVVHD), while oliguria demands CVVHD with citrate calcium. Patients with hematological disturbances require transfusion(s) of

blood preparations (frozen plasma, frozen erythrocytes, or platelet concentrate). Severely aggressive patients may require antipsychotic drugs in addition to benzodiazepines (Hill et al., 2013; Rose et al., 2013; Forrester, 2014; Stellpflug et al., 2014; Hieger et al., 2015; Gee et al., 2016; Humston et al., 2017; Schetz et al., 2017; Wiergowski et al., 2017). Some emergency interventions are specifically intended to treat rhabdomyolysis, which may lead to severe complications, particularly acute kidney injury/failure and metabolic acidosis. They include discontinuation of further skeletal muscle damage by infusion of muscle relaxants (midazolam, rocuronium), early and aggressive fluid administration with a goal of maintaining an urinary flow of 200–300 mL/h, as well as urine alkalization to prevent myoglobin precipitation in a renal tract and management of hyperkalemia and hypocalcemia (Tang et al., 2014; Cervellin et al., 2017; Schetz et al., 2017)

Table 2 presents clinical fatality cases due to intoxication with NBOMes.

BIOTRANSFORMATION

In recent years, biotransformation studies have been carried out for many NBOMe compounds. The metabolites have mainly been identified via *in vitro* study with microsomes and polled human hepatocytes or by the analysis of mouse or rat urine or authentic human samples of blood and urine collected from drug users. An accumulating body of data clearly indicates that NBOMes undergo extensive biotransformation that results in the production of numerous metabolites. For example, Caspar et al. (2015, 2017, 2018c,d) list more than 60 metabolites for each of the analogs, 25B-NBOMe, 25C-NBOMe, and 25I-NBOMe, as well as 36 phase I and 33 phase II metabolites for 4-EA-NBOMe, 17 phase I and 21 phase II metabolites for 3,4-DMA-NBOMe, and 19 phase I and 14 phase II metabolites for 4-MMA-NBOMe. The calculated intrinsic clearance values for 25I-NBOMe and 25I-NBOH were found to be 70.1 and 118.7 mL/min/kg, respectively (Nielsen et al., 2017).

The reported biotransformation steps include oxidative deamination, oxidative *N*-dealkylation also in combination with hydroxylation, oxidative *O*-demethylation possibly combined with hydroxylation, oxidation of secondary alcohols, mono- and dihydroxylation, oxidation of primary alcohols, and carboxylation of primary alcohols (**Figure 2**). In the case of 25N-NBOMe, reduction of the aromatic nitro group and *N*-acetylation of the primary aromatic amine have also been reported. The dominant phase I biotransformation was *O*-demethylation, followed by *O*-di-demethylation and hydroxylation; accordingly, the most abundant metabolites were the *O*-demethylated and hydroxylated forms. The major cytochrome P450 isoenzymes involved in the metabolism of NBOMes were identified as CYP1A2, CYP3A4, CYP2B6, CYP2C9, CYP2C19, and CYP2D6 (Caspar et al., 2015, 2018b,c,d; Nielsen et al., 2017; Richter et al., 2019). Phase I metabolites subsequently undergo glucuronidation and sulfation (Caspar et al., 2015; Leth-Petersen et al., 2016; Temporal et al., 2017; Wohlfarth et al., 2017; Richter et al., 2019; Seo H. et al., 2019).

Forensic casework samples have also identified demethyl metabolites of 25C-NBOMe (Soh and Elliott, 2014; Andreasen et al., 2015). Poklis et al. (2015a) analyzed urine samples from two patients intoxicated with 25I-NBOMe. One sample contained 25I-NBOMe together with 15 metabolites, while the other contained no parent 25I-NBOMe; it was found to contain three *O*-demethyl metabolites. Seven 25I-NBOMe metabolites were detected in the urine of a severely intoxicated man: two demethyl-25I-NBOMe, one demethyl-hydroxy-25I-NBOMe, one hydroxy-25I-NBOMe, one di-demethyl-25I-NBOMe, one demethyl-25I-NBOMe glucuronide, and one hydroxy-25I-NBOMe glucuronide (Richeval et al., 2017). The presence of 25H-NBOMe in biological samples of people who used both 25B-NBOMe and 25I-NBOMe (Soh and Elliott, 2014; Stellpflug et al., 2014) suggests an alternative route of NBOMes biotransformation, i.e., removal of the halogen atom. However, it is also possible that 25H-NBOMe is not formed during metabolic processes in the body but, more likely, is already present in the consumed product as a contaminant. Assuming that the halogenation step performed during synthesis in a clandestine laboratory was incomplete and the unreacted material was not adequately removed by purification, it appears likely that 25H-NBOMe could be present in the final drug product.

The fact that NBOMe compounds undergo extensive first-pass metabolism by the liver (Leth-Petersen et al., 2016; Halberstadt, 2017) fits well with data demonstrating very fast clearance of parent compounds from plasma. This makes the signals of the parent compounds approximately 100-fold lower than those of the most abundant metabolites (Stellpflug et al., 2014; Leth-Petersen et al., 2016). The significantly greater intensity of glucuronated metabolites when compared to the parent compounds in plasma make them prime candidates to be used as markers for NBOMe intoxication (Leth-Petersen et al., 2016).

An important issue worth pointing out is the fact that metabolites can also be responsible for the toxic effects of NBOMes (Leth-Petersen et al., 2016). Two other groups of active formed compounds, which are also sold on the drug market, include 2C phenethylamines or NBOH derivatives (Pasin et al., 2015; Nisbet et al., 2019). For all investigated NBOMes, the corresponding 2,5-dimethoxyphenethylamine (2C-X) metabolite formed during *N*-demethoxybenzylation was detected; however, they were mostly seen at low levels (Temporal et al., 2017; Grafinger et al., 2018).

DETECTION AND IDENTIFICATION OF NBOMes AND THEIR METABOLITES IN BIOLOGICAL MATERIALS

Due to the high receptor affinities of NBOMes and functional activities as full agonists, only very low doses, often in the range of 50–1000 μg, are needed to induce psychoactive effects, and, as a consequence, the resulting biological fluid concentrations tend to be very low, ranging from about 0.1 ng/mL to several ng/mL in blood and up to several dozen ng/mL in urine. Hence, only sensitive and specific analytical methods can be used for the detection, identification, and determination of NBOMes

TABLE 2 | Fatal cases related to use of NBOMe compounds.

Gender/ Age	Case data	Toxicological findings	References
25I-NBOMe			
M/21	At a rave party, the decedent took two 'hits of acid' and smoked marijuana. On the way home with his friend, he was driving a car. He started hallucinating, damaged the interior of the car and caused a car accident. The man died before the arrival of the emergency services. Autopsy: Ecchymoses of the forehead and face, erythema around earlobes, hemorrhages in the conjunctivae.	25I-NBOMe and Δ^9-THC metabolite were detected in heart blood and urine.	Walterscheid et al., 2014
F/15	A woman ingested a clear, unknown liquid at the party. On the way home she became irresponsible and was taken to a hospital. On arrival she had asystole and 39.9 °C temperature (rectal). She died approx. 18 min after arrival to the hospital. Autopsy: Hemorrhages of the conjunctivae. Endotracheal tube and oropharyn were filled by a white foam. Many abrasions and contusions over the shoulders, left hip and right buttock.	25I-NBOMe and Δ^9-THC metabolite detected in heart blood and urine.	Walterscheid et al., 2014
F/23	Following insufflation of a white powder she believed to be "synthetic LSD", a woman began acting strangely and uttering random words; she became aggressive, then vomited, had convulsions, collapsed and died. Autopsy: Multiple bruises and abrasions on most of the body. Some dried apparent vomitus was presented on her face. Congested lungs and mild pulmonary edema.	Aortic blood: 25I-NBOMe, 28 μg/L; 25H-NBOMe, 1 μg/L; 25C-NBOMe, 0.7 μg/L; Methamphetamine, 0.39 μg/L; Δ^9-THC 3.4 μg/L	Kueppers and Cooke, 2015
M/19	Following ingestion of a blotter paper with "acid", a man began behaving strangely and appeared paranoid. He went for a walk with his friends, but abruptly walked away from them. The decedent started to hallucinate, had delusions and finally jumped/fell from his balcony. Autopsy: Multiple blunt impact, injuries of the heart, aorta, liver and spleen. The skull displayed a large number of fractures. No non-traumatic abnormalities or lesions were identified.	25I-NBOMe (direct analysis): peripheral blood, 405 pg/mL; heart blood, 410 pg/mL; urine, 2860 pg/mL; vitreous humor, 99 pg/mL; bile,12.1 ng/g; brain, 2.78 ng/g; liver, 5.64 ng/g.	Poklis et al., 2014b
M/16	The decedent was at the party with his friends where he used a drug that was spotted on a blotter paper. In the morning his friend found a body and a number of pieces of glass in the floor. Autopsy: Abrasions on the chest, arms, neck, knees and shins. Presence of many red contusions. Mild cerebral edema, severe and diffuse pulmonary edema present within all five pulmonary lobes (right > left). The bronchial tree contained frothy edematous fluid.	25I-NBOMe: heart blood, 19.8 ng/mL; urine, qualitative identification.	Shanks et al., 2015
M/15	A teenager was at a party where he ingested 25I-NBOMe and mushrooms. Also he was drinking an unknown fluid. About an hour later, he started to vomit and convulse and finally lost consciousness. Three days later he died at the hospital from multi-system organ failure following unsuccessful cardiopulmonary resuscitation.	Serum: Δ^9-THC, 4.1 ng/mL; 11-nor-9-carboxy-THC, 83 ng/mL. Blood: 25I-NBOMe, 0.76 ng/mL. Urine: 25C-NBOMe, 25H-NBOMe, 25I-NBOMe and psilocin, qualitative identification.	Lowe et al., 2015
F/17	A girl presented in status epilepticus shortly after ingesting an unknown substance on a blotter paper at a music concert. She then acutely developed hyperthermia, metabolic acidosis, rhabdomyolysis, elevated transaminases, acute kidney injury, hypokalemia and hypocalcemia. Later on she developed irreversible cerebral edema and died after 7 days.	Whole blood: 25I-NBOMe, 0.25 ng/mL; lithium 0.34 mmol/L.	Umemura et al., 1988

(Continued)

TABLE 2 | Continued

Gender/ Age	Case data	Toxicological findings	References
25B-NBOMe			
M/18	After ingesting 'NBOMe' in the form of a blotter paper, the decedent started to behave destructively, collapsed and became unresponsive. The man was transported to the local hospital where he died. Autopsy: Trauma like lacerations, abrasions and contusions. severe and diffuse pulmonary edema present within all five pulmonary lobes, gastric content in the bronchi.	Heart blood: 25B-NBOMe, 1.59 ng/mL; Δ^9-THC, 2.4 ng/mL; 11-nor-9-carboxy-THC, 17.1 ng/mL; caffeine, qualitative identification. Urine: 25B-NBOMe, qualitative identification; 11-nor-9-carboxy-THC, 361 ng/mL.	Shanks et al., 2015
M/20	After ingestion of a recreational drug called 'Blue Magic Master', the decedent became agitated and experienced convulsions. He developed serotonin syndrome the next day and died two days later.	Plasma: 25B-NBOME, 3.15 ng/mL after admission; 0.45 ng/mL and 0.16 ng/mL, at six and twelve h post-admission, respectively.	Shintani-Ishida et al., 2018
M/teenager	The decedent was at the party where alcoholic beverages were present, as well as various drugs: marijuana, hashish, cocaine and 'hallucinogenic blotters'. On the way back home, he reportedly reiterated "I want to kill myself" before jumping into the waterway. Autopsy: Cause of death: drowning.	Heart blood: 25H-NBOMe, 0.13 ng/mL; 25C-NBOMe, 1.43 ng/mL. Central blood: Δ^9-THC, 9.9 ng/mL; 11-nor-9-carboxy-THC, 8.5 ng/mL.	Morini et al., 2017
M/23-24	Three men had a party in a flat on the fifth floor. One of them jumped out of a window and was found dead.	Blood: 25B-NBOMe, 661 ng/mL; 4-CMC, 0.8 ng/mL	Wiergowski et al., 2017
	The other two were aggressive and very agitated. They were shouting at each other, speaking illogically, screaming incomprehensibly and moving anxiously in the flat.		
	One of them experienced strong convulsions, heavy breathing and salivation; he died at the hospital. The second man survived. He had a bag with him with the description: "25B NBOMe 1 GR" and "4 CMC 1 GR".	Blood: 25B-NBOMe, 66.5 ng/mL; 4-CMC, 2.14 ng/mL	
M/19	After consumption of LSD, a man with no known past medical history, started to behave oddly and uncontrollably. He was sent to rest in a bedroom, where was found unresponsive one h later. The deceased died in a hospital after unsuccessful cardiopulmonary resuscitation. Cause of death: 25B-NBOMe toxicity.	25B-NBOMe: blood 10 ng/mL; bile and stomach content - present	Chan et al., 2019
25C-NBOMe			
M/22	The deceased had a short history of alcohol and drug use. After sniffing 25C-NBOMe, he started running and pulling down the curtains in his house, experiencing hallucinations, clenched jaw and convulsions. At the hospital, the following symptoms were observed: hyperthermia with a core temperature of 40 °C, pulse of 140 bpm, diffuse bleeding from all mucosa, respiratory and	25C-NBOMe: peripheral blood 0.6 μg/kg, urine 2.93 μg/kg, liver 0.82 μg/kg, vitreous humor 0.33 μg/kg, gastric content 0.32 μg total. Amphetamine: peripheral blood 470 μg/kg.	Andreasen et al., 2015
	metabolic acidosis, rhabdomyolysis, high lactic acid, anuria and hyperkalemia. He died approximately 12 h after ingestion due to the multiorgan failure. Autopsy: Free fluid in thorax and abdomen, mucosal hemorrhage. Bruises and abrasions.	Δ^9-THC: peripheral blood 1.5 μg/kg	

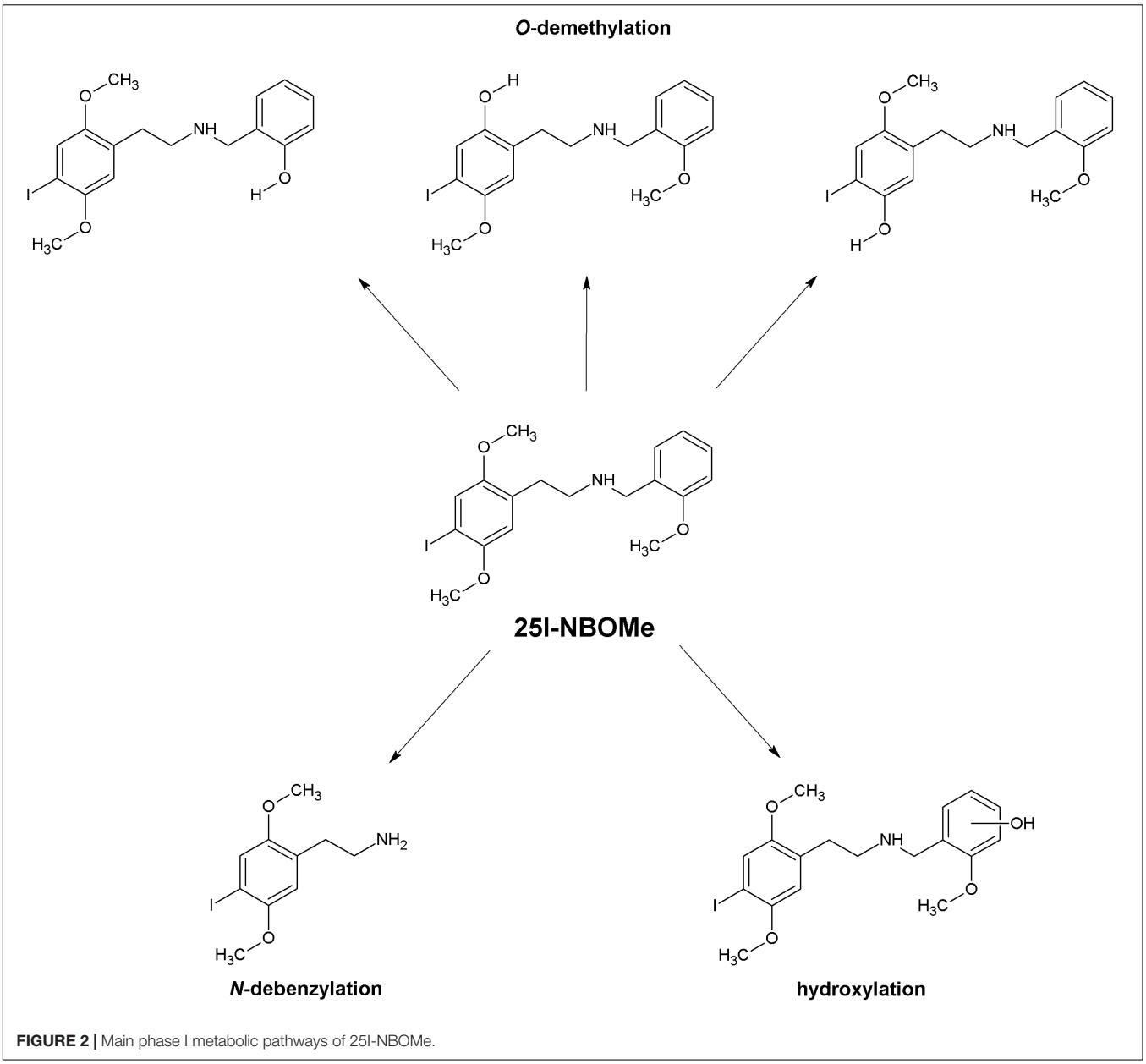

FIGURE 2 | Main phase I metabolic pathways of 25I-NBOMe.

in biological materials (Kyriakou et al., 2015). Even in post-mortem cases, the reported blood concentrations of NBOMe compounds also tend to be low and are often below 0.5 ng/mL (Anilanmert et al., 2018). Although the compounds are generally present at higher concentrations in urine than in blood, detection methods should be targeted to the metabolites rather than the parent compounds.

The analysis of NBOMes can be a challenging task, even for laboratories equipped with sensitive modern methods, and popular immunochemical tests are not effective. Common analytical methods used in laboratories, such as gas chromatography coupled with mass spectrometry (GC-MS) or high-performance liquid chromatography with diode array detection (HPLC-DAD) without derivatization of the sample, are also inadequate for identifying NBOMe compounds due to insufficient sensitivity. Analytical methods must have low limits of detection (LOD); therefore, the most common techniques of detection of NBOMes in biological fluids are those implementing tandem mass spectrometry (MS-MS). Both high-performance liquid chromatography (HPLC or LC) and ultra-performance liquid chromatography (UPLC) coupled to either MS-MS or high resolution time-of-flight spectrometry (TOF-MS) are preferred. This latter technique allows for accurate determination of molecular and fragmentation ions, which in turn makes it possible to elucidate the chemical structure of compounds and consequently unambiguously identify not only the parent substance but also many metabolites. For the isolation of NBOMes from blood (as well as serum or plasma) and urine

both liquid-liquid extraction (LLE) and solid phase extraction (SPE) can be used. Sometimes, a simple precipitation or just a dilution of a sample is sufficient.

Although many methods have been developed for the detection of NBOMe analogs in biological materials, both in metabolic studies and authentic forensic sample analyses, only a few screening methods covering more than one or two NBOMe compounds have been published. Caspar et al. (2018a) developed a method for the identification and determination of 21 low-dosed psychedelics and opioids, including 25B-NBOMe, 25C-NBOMe, 25E-NBOMe, 25I-NBOMe, and 25H-NBOMe, in blood plasma. A diethyl ether:ethyl acetate mixture was applied for a two-step extraction. Analyses were carried out using LC high resolution (HR) MS (orbitrap analyzer) with alternating HR full scan (HRFS) MS and "All-ions fragmentation" (AIF) MS. The approach allowed the detection of these analytes down to concentrations of 0.1 ng/mL.

Pasin et al. (2015) developed and validated an analytical method for the detection and quantification of 37 new designer drugs, including 25B-NBOMe, 25C-NBOMe, 25H-NBOMe, and 25I-NBOMe in the whole blood. Salting-out-assisted LLE with acetonitrile was performed to isolate compounds, followed by LC with an analysis combined with a quadrupole time-of-flight mass spectrometer (Q-TOF-MS). The method required only 100 μL of blood, but the limits of detection for NBOMe compounds was relatively high at 5 ng/mL. Temporal et al. (2017) describe the analysis of the same set of NBOMes with their major metabolites in blood and urine samples by UPLC-Q-TOF system; authentic samples underwent LLE before analysis using an n-butyl chloride:ethyl acetate mixture.

Poklis et al. (2014a) described the use of the LC-MS-MS method for the identification and quantification of nine NBOMe derivatives (25H-NBOMe, 2CC-NBOMe, 25I-NBF, 25D-NBOMe, 25B-NBOMe, 2CT-NBOMe, 25I-NBMD, 25G-NBOMe, and 25I-NBOMe) in human urine. The method used dilution of urine samples and extraction by Clean Screen FASt^{TM} SPE columns to reduce the amount of matrix. A Q-Trap apparatus was used in multiple reaction monitoring (MRM) acquisition mode, which allowed the compounds to be detected at a level of 0.1 ng/mL. SPE was also used to identify and quantify five different 25-NBOMes (25B-NBOMe, 25C-NBOMe, 25D-NBOMe, 25H-NBOMe, and 25I-NBOMe) in blood and urine. The applied LC–MS-MS (Q-Trap) system allowed LOD to be obtained at a level of 0.05 ng/mL (Morini et al., 2017).

The UPLC-MS-MS (Q-Trap) system was used for the simultaneous quantification of six NBOMe analogs (25B-NBOMe, 25C-NBOMe, 25D-NBOMe, 25H-NBOMe, 25I-NBOMe, and 25T2-NBOMe) in the whole blood, plasma, and urine. The SPE was performed with the use of UCT Clean Screen DAU mixed mode columns. The method, characterized by LODs as low as 0.005–0.01 ng/mL, offered sufficient sensitivity to detect any of these compounds following use (Johnson et al., 2014).

Caspar et al. (2017) analyzed 25B-NBOMe and 25C-NBOMe along with their metabolites in human and rat urine. Depending on the experiment, urine was either incubated with a mixture of glucuronidase/arylsulfatase and then extracted with the use an HCX SPE columns or just precipitated with acetonitrile. The obtained samples were analyzed on the LC-HR-MS/MS system. In subsequent studies, nanoLC-HRMS/MS and UHPLC-HRMS/MS systems were applied for the detection and identification of metabolites of 3,4-DMA-NBOMe and 4-MMA-NBOMe (Caspar et al., 2018c). Urine samples were analyzed directly after dilution. Mass spectrometers were operated in positive ionization mode using full scan (FS) data and a subsequent data-dependent acquisition (DDA) mode. Both applied systems were comparable, but nanoLC allowed much lower eluent consumption: flow rate of 0.7 μL/min for nanoLC compared to 500–800 μL/min for UHPLC. Wohlfarth et al. (2017) also employed the simple dilution of urine to analyze samples with or without a prior hydrolysis step (with ß-glucuronidase/arylsulfatase mixture). 25C-NBOMe and 25I-NBOMe, and their metabolites, were detected and identified by LC-QTOF-MS in DDA mode (Wohlfarth et al., 2017).

Yu et al. (2019) proposed a new method of NBOMes identification. The fragmentation patterns of nine NBOMe derivatives (25H-NBOMe, 25B-NBOMe, 25E-NBOMe, 25N-NBOMe, 25C-NBOH, 25I-NBOH, 25B-NBF, 25C-NBF, and 25I-NBF) were analyzed using LC-QTOF-MS and an approach known as molecular networking, one that organizes MS-MS data by mining the MS-MS fragmentation similarity. The resulting MS-MS spectral data was used to establish a molecular networking map for different NBOMes, as these compounds generally showed similar product ion spectral patterns. The map was applied to spiked urine samples, confirming that it can be used for the rapid detection and identification of unknown NBOMes.

In addition to body fluids, other biological materials have also been analyzed for NBOMe compounds, including post-mortem tissues. Tissue homogenates were subjected to SPE using UCT mixed mode silica-based columns. LC–MS-MS analyses were performed on a Q-Trap apparatus (Kristofic et al., 2016). A validated method for the detection of 32 NPS, including 25C-NBOMe, 25B-NBOMe, and 25T4-NBOMe, in oral fluid has also been presented in which samples were prepared using a simple protein precipitation in acetonitrile and analyzed using the UHPLC-MS-MS system. All analytes were found to have a LOD at 1 ng/mL (Williams et al., 2017). Ameline et al. (2017) presented an analysis of hair samples (9.5 cm) collected in an acute poisoning case. The hairs were collected 6.5 months after a drug consumption and were analyzed by UPLC-MS-MS working in MRM mode. As a result of the analyses, the presence of 25I-NBOMe was demonstrated in two of five 2 cm hair segments at concentrations of 1.0 pg/mg and 4.9 pg/mg.

GC-MS is used less frequently but it can be a great tool in screening analyses when used in combination with a derivatization step. A validated GC-MS method for the quantification of 23 NPSs, including 25B-NBOME, 25C-NBOME, 25D-NBOMe, 25E-NBOME, 25H-NBOME, 25I-NBOME, Mescaline-NBOME, and 25P-NBOME in blood and urine samples have been presented. Sample preparation

was carried out using SPE followed by derivatization with pentafluoropropionic anhydride (PFPA). The LODs for NBOMes were in the range of 0.2–0.3 ng/mL in urine and 0.3–0.4 ng/mL in blood. It should be emphasized that the proposed method can be used for detection of NBOMes in acute fatalities by laboratories that do not have access to an LC–MS-MS (Nisbet et al., 2019).

CONCLUDING REMARKS

In recent years, NBOMe derivatives, a specific set of psychedelic phenylalkylamines, have been encountered on the drugs of abuse market. These compounds are used in very low doses (in the range between 50 and 1000 μg) due to their high pharmacological activity. NBOMe drugs are highly toxic and their intake has been associated with severe adverse reactions including deaths. The analysis of NBOMes and their metabolites is a challenging task; only sensitive and specific analytical methods can be used for their detection, identification, and determination in biological materials.

AUTHOR CONTRIBUTIONS

JZ: literature search, writing the sections "Abstract,Introduction, Methods, Pharmacology of NBOMes, Toxicity in vitro, Available forms of products, Pattern of use, Effects related to use of NBOMes, and Concluding remarks." MK: preparation of tables and co-writing the section "Effects related to use of NBOMes." PA: writing the sections "Detection and identification of NBOMes and their metabolites in biological materials, Biotransformation"; preparation of figures, and literature search.

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Acute Effects of 2C-E in Humans: An Observational Study

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2,5-Dimethoxy-4-ethylphenethylamine (2C-E) is psychedelic phenylethylamine, with a chemical structure similar to mescaline, used as new psychoactive substance (NPS). It inhibits norepinephrine and serotonin uptake and, more relevant, acts as a partial agonist of the serotonin 2A (5-HT_{2A}), 2B (5-HT_{2B}), and (5-HT_{2C}) receptors. Consumers have reported that 2C-E induces mild-moderate psychedelic effects, but its pharmacology in humans, including pharmacological effects and pharmacokinetics, have not yet studied. To assess the acute effects of 2C-E on physiological and subjective effects and evaluate its pharmacokinetics, an observational study was carried-out. Ten recreational users of psychedelics self-administered a single oral dose of 2C-E (6.5, 8, 10, 15, or 25 mg). Blood pressure and heart rate were evaluated at baseline, 2, 4, and 6 h post-administration. Three rating scales were administered to evaluate subjective effects: a set of Visual Analog Scales (VAS), the 49-item short form version of the Addiction Research Centre Inventory (ARCI), and the Evaluation of the Subjective Effects of Substances with Abuse Potential (VESSPA-SSE) at baseline, 2, 4, and 6 h after self-administration. To assess 2C-E concentrations oral fluid (saliva) was collected during 6 h. 2C-E induced primarily alterations in perceptions, hallucinations, and euphoric-mood. Saliva maximal concentrations were achieved 2 h after self-administration. Administration of oral 2C-E at recreational doses produces a group of psychedelic-like effects such to 2C-B and other serotonin-acting drugs.

Keywords: 2C-E (2,5-Dimethoxy-4-ethylphenethylamine), novel psychoactive substances (NPS), psychedelic, phenylethylamines, psychostimulants

INTRODUCTION

Classical psychedelics (serotonergic psychedelics) have traditionally been defined as a class of psychoactive substances that induce in humans a wide range of complex physiological, behavioral and psychological effects through serotonin 5-HT2$_A$ receptors stimulation (Nichols, 2016). In the past few years, however, phenethylamine psychedelics have emerged as a class of new psychoactive substances (NPS) able to induce similar effects to those of controlled psychedelic substances (Vollenweider, 2001; Aarde and Taffe, 2017). 2C-compounds (2C-s) are ring-substituted phenylethylamines derived from the modification of the mescaline structure with two methoxy groups on the benzene ring (2nd and 5th positions) (Tracy et al., 2017). Although they are widely considered a family of substances with hallucinogenic/psychedelic and psychostimulant properties, information available on their pharmacology and toxicology in humans is very limited.

2,5-Dimethoxy-4-ethylphenethylamine [2C-E, or 2-(4-ethyl-2,5-dimethoxyphenyl) ethanamine] is colloquially known as "Aquarust," "Eternity," "Europe," and "Hummingbird" (Sutherland et al., 2016). Synthesized in 1977 by Alexander Shulgin it is one of the most potent 2C-compounds (Shulgin and Shulgin, 1990). 2C-E is structurally very closely related to other 2C-s and to other well-studied phenethylamine substitutes such as mescaline and MDMA (ecstasy). It first came out the club scene in the mid-1980s as a quick replacement for MDMA which had been banned in the United States. 2C-E then remerged on the psychedelic scene and lately has been present as part of the NPS phenomenon. In fact, 2C-E has been documented as being contained in pills sold as ecstasy in America and Europe (United Nations Office on Drugs and Crime [UNODC], 2014), and more recently in Colombia and other Latin American countries, where it is considered an NPS due to its new presence on the drug market (Observatorio de Drogas de Colombia [ODC], 2017).

Pharmacologically, 2C-E, in a similar manner to other 2C-compounds, inhibits the uptake of serotonin and norepinephrine by membrane transporters (SERT and NET, respectively), although with very low activity in relation to amphetamine (Nagai et al., 2007; Van Vrancken et al., 2013; Eshleman et al., 2014). 2C-E mainly acts as a partial agonist at the 5-HT2$_A$, 5-HT2$_B$, and 5HT2$_C$ receptors (related to its psychedelic effects) (Rickli et al., 2015). Also it binds mostly at the adrenergic α-2 receptor (Rickli et al., 2015).

Relatively little information is available regarding human 2C-E metabolism. Nevertheless, research has suggested that it follows similar metabolic pathways to 2C-Bwhich are carried out by O-demethylation and N-acetylation (Theobald et al., 2007).

With respect to epidemiological data on 2C consumption, the information available from web-based questionnaires and population-based surveys is particularly infrequent. In a self selected sample from the 2013 Global Drug Survey[1], including 2,282 participants in the United States, reporting attendance to nightclubs in the previous year, 46.4% described lifetime use of at least one of the 58 NPS assessed (age range 16–60 years). Among the psychedelic phenethylamines, consumption of 2C-compounds was the most commonly reported (21.7%), and 8.55% admitted taking 2C-E (n = 195) (Palamar et al., 2016). In the latest Global Drug Survey there are no specific data regarding the prevalence of 2C-E (Global Drug Survey [GDS], 2018).

In Australia, national cross-sectional surveys among regular ecstasy users (n = 693, year 2010) and regular psychostimulant users (n = 1260, years 2012/2013) reported a 2 and 3% prevalence of 2C-E use in the previous 6 months, respectively (Bruno et al., 2012; Matthews et al., 2016). In 2014, a sample of Australian NPS users (n = 800) described a 5.9% use in the previous 6 months (Sutherland et al., 2017).

In a survey done in Spain among 230 research chemical users a 25.7% had taken 2C-E in the previous year. It was the fifth most frequent substance consumed, and rarely used in combination with other psychostimulants or psychedelics (2C-E + MDMA 1.8%, 2C-E + amphetamine 0.9%, 2C-E + mephedrone 0.9%, and 2C-E + psilocybin 0.4%) (González et al., 2013). In a recent study in the United States, including 356,413 respondents to the 2008–2016 National Survey on Drug Use and Health, 0.12% reported lifetime novel psychedelic use. Of these, 30.1, 14.8, and 23.9%, reported lifetime use of 2C-B (2,5-dimethoxy-4-bromophenethylamine), 2C-E and 2C-I (2,5-dimethoxy-4-iodophenethylamine), respectively (Sexton et al., 2019).

The first description of 2C-E effects was published in *PiHKAL: A Chemical Love Story*, which considered the drug to be one of the "magical half-dozen" or more intense psychedelic phenethylamines (Shulgin and Shulgin, 1990). In recent years, 2C-E recreational users have reported its effects as being a combination of hallucinogenic and stimulating ones, like those of ecstasy and LSD. Like other psychedelics drugs and 2C compounds, 2C-E at low doses usually produces stimulant effects and increased auditory, visual and tactile sensations. At moderate doses it leads to mild hallucinations, and at high ones can cause the user to experience unpleasant hallucinations and sympathomimetic effects. In general, effects from 2C-E are reportedly more intense in comparison to 2C-B (Dean et al., 2013).

An average dose of 2C-E ranges from 10 to 20 mg (medium dose 15–25 mg, high dose 25–40 mg) although exceptionally elevated doses up to 100 mg have been reported (Dean et al., 2013)[2]. Recommendations for an initial dose are between 6 and 20 mg depending on the user's previous experience with similar drugs, whilst 3 mg is considerate a "microdose" which produces intense effects on cognitive processes and well-being without the typical ones on consciousness (Polito and Stevenson, 2019). As with most psychedelics, the effects of 2C-E are long-acting, lasting typically for 6–12 h, depending on the dose and individual.

To date, a dozen cases of acute intoxication (tachycardia, hypertension, agitation, delirium, and hallucinations) have been reported (Van Vrancken et al., 2013; Iwersen-Bergmann et al., 2019) and, although very rare, some deaths have been linked to 2C-E (Topeff et al., 2011; Sacks et al., 2012). Alarmingly, no human research has been conducted with 2C-E in spite of the relatively long history of its recreational use and the recent

[1]https://www.globaldrugsurvey.com/

[2]https://www.erowid.org/

resurgence of interest in psychedelic drugs. The aim of our study was to evaluate the pharmacological effects and pharmacokinetics of 2C-E in recreational users.

MATERIALS AND METHODS

Participants

Ten healthy subjects were selected (4 females and 6 males). Volunteers were recreative drug users who had experienced a 2C-series compound at least once in a lifetime. Exclusion criteria were a history of any serious medical or psychopathological disorder including substance use disorder (except nicotine), a previous serious adverse reaction with 2C-series, and chronic medicines use.

Participants were recruited by word-of-mouth and snowball sampling through the harm reduction, non-governmental organization, Energy Control (ABD). The study protocol was submitted and approved by the Clinical Research Ethics Committee (CEIC Parc de Salut Mar, Barcelona, Spain, ref. 2016/6700/I). It was conducted according to the Declaration of Helsinki recommendations. All the participants were correctly and fully informed, both orally and in writing, of the purpose, methods and means of the study. All of them indicated their agreement to participate and signed an informed consent prior inclusion. Participants received monetary compensation for their participation.

Design and Treatments

The design was a non-controlled prospective observational study with minimal intervention in subjects who self-administrated 2C-E orally. Most evaluations and procedures were similar to a previous naturalistic observational study evaluating acute effects of 2C-B (Papaseit et al., 2018). Each participant participated in one session. Treatment consisted of oral self-administration of one 2C-E capsule, that they brought to the testing site themselves, which they had obtained from an unknown source. Although no information was available about the synthesis of the drug, similar capsules tested by Energy Control, a harm reduction organization that provides a Drug Checking Service for users, showed that the capsules contained 2C-E at 95% purity with no toxic adulterants. The 2C-B pill content was previously analyzed by means of gas chromatography associated with mass spectrometry (GC/MS). The method used permits to check for most common drugs of abuse including most of the NPSs and to know the exact purity of 2C-E in the powder to prepare dosing by a precision scale (Papaseit et al., 2018). The dose of 2C-E self-administrated was selected by the participants based presumably on their previous experience. The mean 2C-E dose was 11.95 $\pm$ 5.30 mg [1 female ingested 6.5 mg, 1 female 8 mg, 5 males 10 mg, 2 subjects (1 male and 1 female) 15 mg, and 1 female 25 mg]. In order to standardize dosing for statistical analysis and to evaluate dose-response relationship, we grouped doses in two intervals: 6.5–10 and 15–25 mg (taken by 7 and 3 subjects, respectively). All the selected doses were well tolerated.

Procedures

Prior to study session, the participants were submitted to a general medical examination and a psychiatric diagnostic examination. They received training with respect to questionnaires and procedures employed in the study. Upon arrival, they were questioned about any event that could affect their participation. They were asked to refrain from any drug use 2 days prior to the session. Participants were not allowed to consume alcohol or beverages containing caffeine the previous 24 h. Sessions took place on two different days (5 participants each day and administration were separated by various minutes among participants) at a private club with ambient music and participants could talk, read, or play table games during the session and interact in exception to the evaluation times. Also, they were instructed not to talk about the effects of the substance during the session. Assessments were performed by at baseline (pre-dose) and 2, 4, and 6 h after 2C-E self-administration. The experiment was conducted from 15:00 to 22:00 h. Urine spot samples were collected prior administration to exclude prior substance drug use (benzodiazepines, barbiturates, morphine, cocaine, amphetamines, methamphetamine, MDMA, marijuana, phencyclidine) with Instant-View, Multipanel 10 Test Drug Screen Alfa Scientific Designs Inc., Poway, CA, United States. Self-administration of 2C-E took place around 16.00 h. The sequence of procedures at each time point of the session was: physiological measures, oral fluid collection, and subjective effects questionnaires. A psychiatry was present during the entire session. Adverse effects were assessed during study session.

Physiological Effects

Non-invasive systolic and diastolic blood pressure (SBP and DBP), and heart rate (HR) were determined with an Omron® monitor at baseline and 2, 4, and 6 h after administration. Oral temperature was measured simultaneously.

Subjective Effects

Subjective effects of 2C-E were reported at baseline and at 2, 4, and 6 h after self-administration. They were measured using a set of Visual Analog Scales (VAS), the 49-item Addiction Research Centre Inventory (ARCI) short form, and the Evaluation of the Subjective Effects of Substances with Abuse Potential (VESSPA-SSE) questionnaires. VAS (100 mm, from "not at all" to "extremely") were used to rate intensity; stimulated; high; good effects; liking; content; changes in colors; changes in shapes; changes in lights; hallucinations-seeing of lights or spots; hallucinations-seeing animals, things, insects or people; changes in hearing; hallucinations-hearings of sounds or voices; different body feeling; unreal body feeling; changes in distances; different surroundings; unreal surroundings; confusion; fear; depression or sadness; drowsiness; dizziness; bad effects; headache; nausea; vertigo; breathing difficulty and face flushing (González et al., 2015; Papaseit et al., 2016, 2018).

The ARCI 49-item short form is a validated instrument that includes five subscales related to drug sedation (pentobarbital-chlorpromazine-alcohol group, PCAG), euphoria (morphine-benzedrine group, MBG), dysphoria and somatic symptoms (lysergic acid diethylamide group, LSD), intellectual efficiency and energy (benzedrine group, BG) and d-amphetamine-like effects (A) (Lamas et al., 1994; Papaseit et al., 2016; Martínez-Riera et al., 2019).

The VESSPA-SE is a questionnaire that measures changes in subjective effects caused by different drugs including stimulants and psychedelics and includes six subscales: sedation (S), psychosomatic anxiety (ANX), changes in perception (CP), pleasure and sociability (SOC), activity and energy (ACT), and psychotic symptoms (PS) (González et al., 2015; Papaseit et al., 2016).

Oral Fluid Concentrations of 2C-E

To assess 2C-E concentrations in oral fluid (saliva), it was collected with Salivette® tubes at baseline, 2, 4, and 6 h after self-administration. After collection samples were centrifuged and frozen at -20°C until analysis. 2C-E concentrations were analyzed by a modified and validated liquid chromatography–mass spectrometry method LC-MS/MS) (Papaseit et al., 2018).

Statistical Analysis

For physiological (SBP, DBP, HR, and T) and subjective effects (VAS, ARCI, and VESSPA), differences with respect to baseline were calculated. Maximum effects (E_{max}) were determined and the area under the curve of the effects ($AUC_{0-6\,h}$) were calculated using the trapezoidal rule.

For 2C-E oral fluid concentrations, the maximum concentration (C_{max}), the time needed to reach the maximum concentration (T_{max}) and the $AUC_{0-6\ h}$ were determined using the Pharmacokinetic Functions for Microsoft Excel (Joel Usansky, Atul Desai, and Diane Tang-Liu, Department of Pharmacokinetics and Drug Metabolism, Allergan, Irvine, CA, United States).

Although it is remarkably that the participant that selected the lowest dose (6.5 mg) presented higher acute effects and oral fluid concentrations in comparison to others, this subject was included in all the analysis.

A one-way analysis of variance (ANOVA) test including all doses as a factor was used for E_{max} and AUC_{0-6}. When the dose factor was statistically significant, a *post hoc* analysis for the two defined groups were done using a Student *T*-test (lower dose group: 6.5–10 mg, $n = 7$; higher dose group: 10–25 mg, $n = 3$).

To evaluate the effects along time and to study the effects of the substance in comparison to baseline, a one-way repeated measures ANOVA, with time as factor (baseline, 2, 4, and 6 h), was done to evaluate the time-course of effects (for all doses). When the time condition was statistically significant, a Dunnett multiple comparison *post hoc* test was conducted to compare the different time points with baseline (0–2 h, 0–4 h, 0–6 h).

All statistical tests were conducted using PAWS Statistics version 18 (SPSS Inc., Chicago, IL, United States). A $p < 0.05$ value was considered statistically significant.

RESULTS

Participants

All ten selected subjects participated in the study (4 females and 6 males). Demographics were a mean age of 27 ± 4 years (range 24–37), mean weight of 64.60 ± 8.77 kg (range 58–78), and mean body mass index (BMI) of 20.26 ± 2.55 kg/m^2 (range 16–24). The mean weight-adjusted dose of 2C-E was 0.19 ± 0.09 mg/kg (range 0.13–0.43). All subjects had previous recreative experience with 2Cs, psychedelics/hallucinogens, cocaine, MDMA, amphetamines, and cannabis. Seven of them were current tobacco smokers (range 0.5–7 cigarettes/day) and all consumed alcohol daily (mean 1.4 units/day). All drugs of abuse urine tests were negative at baseline. As explained in the statistical analysis for dose-response analysis we grouped doses in two groups (6.5, 8–10, and 15–25 mg), **Figures 1–3** are showed as the two doses groups. **Supplementary Figures S1–S3** presented individual data in order to show the elevated variability of the acute effects and concentrations.

Physiological Effects

Effects of 2C-E on physiological signs are summarized in **Table 1** and **Figure 1**, and **Supplementary Figure S1** (individual data). 2C-E produced a non-significant increase in SBP, DBP, HR and T. For HR significant differences were detected in the comparison of baseline and 4 hand 6 h after administration. Regarding T, only statistically significant differences were detected at 2 and 4 h. No dose-response relationship was observed.

Subjective Effects

The subjective effects induced by 2C-E are presented in **Table 2** and **Figure 2**, and **Supplementary Figure S2** (individual data). In summary, 2C-E significantly increased scores for most of the outcomes measured with VAS. Some effects were related to dose, as higher doses produced more intense effects. The substance produced more intensity of effects in comparison to baseline for most variables.

For VAS scales related to euphoria-stimulation the highest scores were observed for "intensity," "stimulated," "high," "good effects," "liking," and "content." When compared to baseline, significant differences were detected at 2 and 4 h, except for "stimulated" (4 h) and "liking" (2, 4, and 6 h). No dose-response was observed when comparing both groups of doses.

For VAS scales measuring changes in perceptions, statistically significant differences in E_{max} and AUC_{0-6} were detected for all VAS except in "different body feelings." When compared to baseline, significant differences were found in VAS for "changes in colors" (2 h), "changes in lights" (4 h), "different body feeling" (h, 4 h), and "different surroundings" at 4 h and 6 h. A dose-response was observed in all VAS except for "changes in hearing," "changes in distances," and "different body feeling."

With respect to scales measuring hallucinations, the highest scores were found for "hallucination-seeing of lights/spots" (E_{max} 21.00 mm) whilst modest and low scores were observed for "hallucination-seeing animals, things, insects or people" (E_{max} 6.20 mm, no significant) and "hallucination-hearing of sounds or voices" (E_{max} 2.20 mm, significant). Significant effects, baseline differences and dose-response were observed for "hallucinations-seeing of light and spots" (6 h) and "hallucination-hearing of sounds or voices."

In addition, 2C-E induced "confusion," "drowsiness," and "breathing difficulty." Differences from baseline were observed for "drowsiness," "dizziness," "bad effects," and "nausea." No dose-response was observed except for "breathing difficulty."

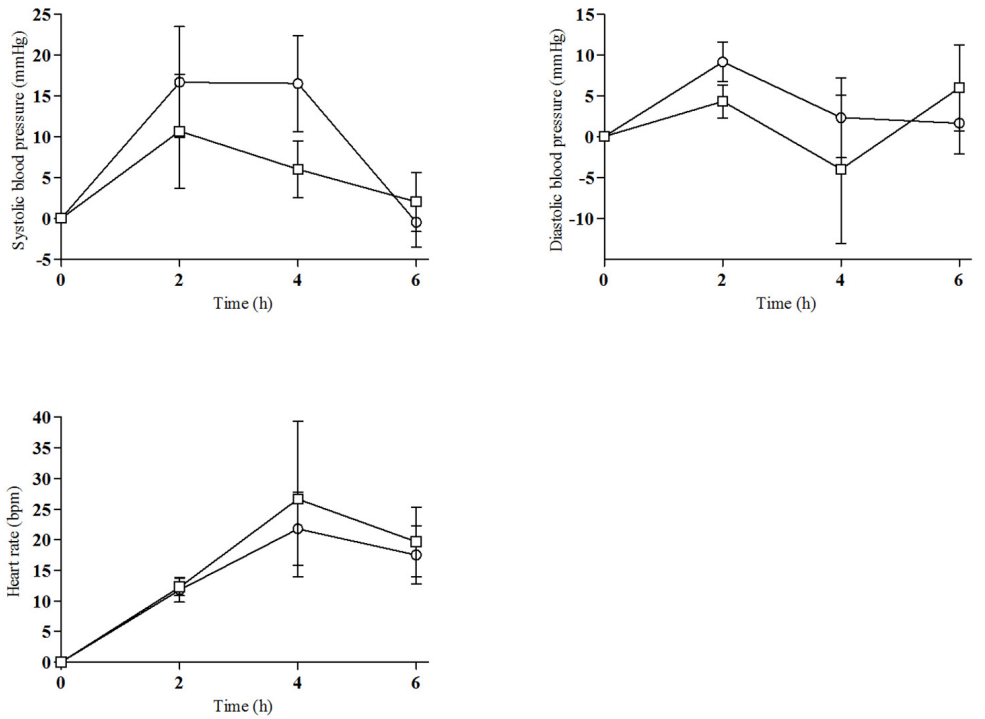

FIGURE 1 | Time course of changes from baseline for physiological effects [○, 6.5–10 mg of 2C-E (*n* = 7), □, 15–25 mg of 2C-E (*n* = 3); mean, standard error].

In relation to ARCI questionnaire, significant increases in the scores of all subscales were detected, however, differences in dose were not statistically significant. Similarly, differences from baseline were observed for all subscales at different times. No dose-response was observed.

With respect to the VESSPA, significant changes were shown in Sedation, Change in perception and Psychotic symptoms, with significant differences from baseline in all except Psychotic symptoms. Dose-response relationship were detected for Changes in Perception and Psychotic symptoms.

Most of the effects dissipated after 6 h, and all subjects returned to their usual routine. Two of them presented residual mild visual hallucinations (lights) at 6 h which disappeared 1–2 h later.

Oral Fluid Concentrations

The oral fluid concentration-time curve for 2C-E are shown in **Figure 3**, and **Supplementary Figure S3** (individual data). Concentrations of 2C-E increased rapidly, reaching a peak 2 h after ingestion. Concentrations rapidly decrease from 2 to 6 h after ingestion. Mean maximum concentration (C_{max}) values of 5.8 ± 6.4 ng/mL (range 0.93–21.54) were obtained at a T_{max} of 2 h following drug administration. The AUC_{0-6} was 18 ± 18 ng·h/mL (range 3.69–57.70). Plasma concentrations varied considerably among doses and subjects. No significant differences between the two grouped doses were found for C_{max} or AUC_{0-6} (**Table 2**). All ten subjects presented positive concentrations of 2C-E at 4 h; only 5, however, had 2C-E concentrations in saliva at 6 h.

DISCUSSION

To the best of our knowledge, this is the first study to assess the acute behavioral (subjective) and physiological effects and oral fluid concentrations of 2C-E after the administration of known doses (6.5–25 mg) in humans. The main finding is that 2C-E induced primarily a group of psychedelic-like effects, a profile consistent with prior data from surveys and poisonings symptoms (Matthews et al., 2016). Moreover, our study provides unique results about concentrations of 2C-E in oral fluid.

In our non-controlled setting, 2C-E only partially mimicked the prototypical sympathomimetic-like effects of other psychedelic and psychostimulant drugs (Schmid et al., 2015; Dolder et al., 2017) and 2C-B (Papaseit et al., 2018). The physiological actions induced by 2C-E included a mild-moderate increase of HR, without changes in blood pressure. The effects were lower than those produced by 2C-B (Papaseit et al., 2018) and by MDMA, mephedrone or other amphetamines administered in dose-controlled conditions (Farré et al., 2015; Papaseit et al., 2016). It is possible that the wide range of doses in the present study (from 6.5 to 25 mg) did not permit differences to be observed in blood pressure when compared to 2C-B (in a narrow range from 10 to 20 mg) (Papaseit et al., 2018). For 2C-E the maximal cardiac effect was observed at the 2 h assessment, maintained over 2–4 h, and returned to baseline at 6 h post-administration.

In this study, 2C-E produced mixed euphoria, pleasure and well-being feelings, and alterations in mental functions like psychedelics such as 2C-B (González et al., 2015;

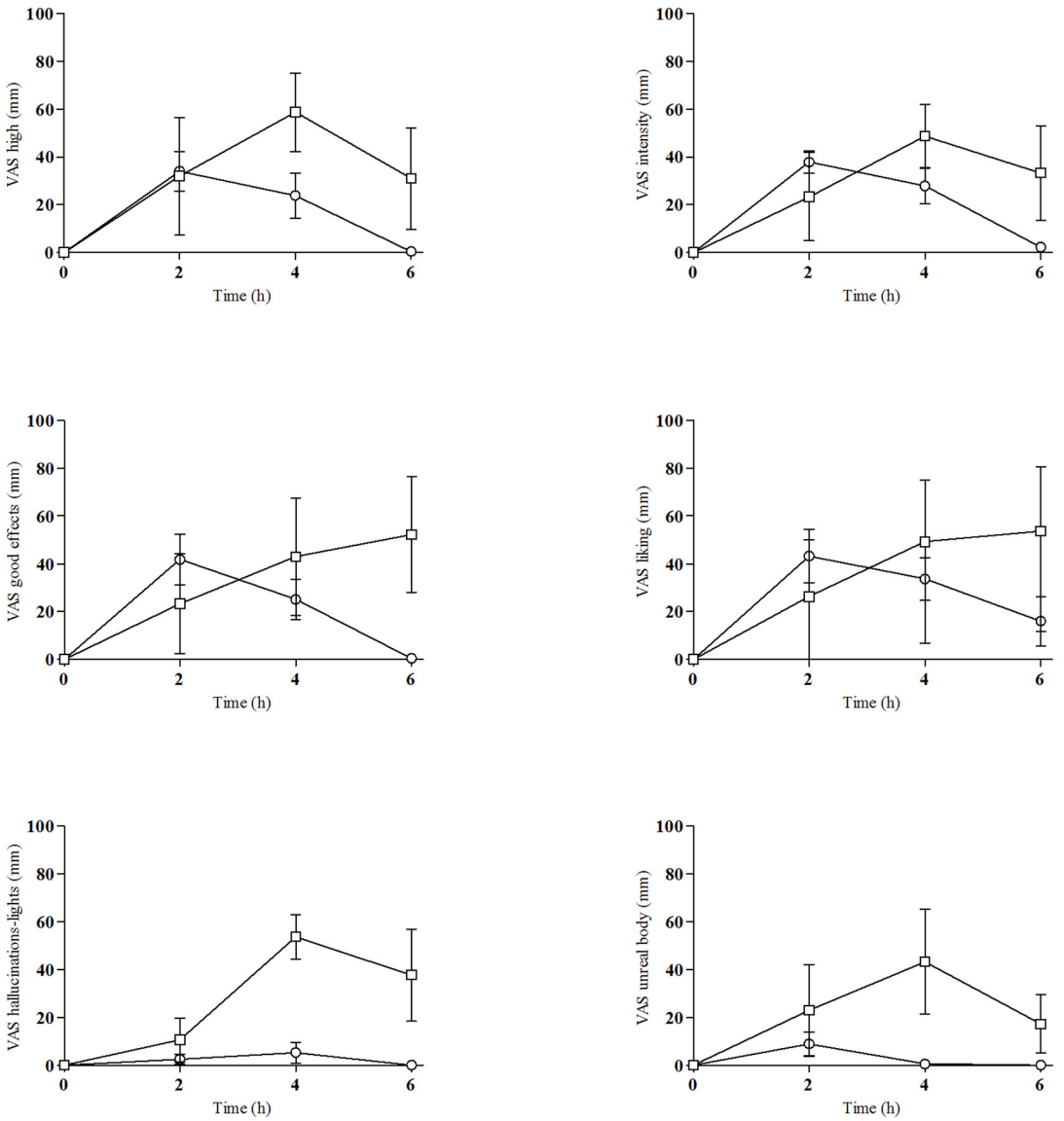

FIGURE 2 | Time course of changes from baseline for subjective effects [○, 6.5–10 mg of 2C-E (*n* = 7); □, 15–25 mg of 2C-E (*n* = 3); mean, standard error].

Papaseit et al., 2018), psilocybin (Griffiths et al., 2006), salvinorin A (Johnson et al., 2011) and ayahuasca (Riba et al., 2001, 2004) and psychostimulants such as MDMA (Papaseit et al., 2016), amphetamine (Cami et al., 2000), and mephedrone (Papaseit et al., 2016). Under 2C-E influence participants reported euphoria, stimulation, and altered state of consciousness due to the psychedelic experience. Changes in mood were more pronounced than perceptual ones. As an example, the mean VAS ratings of "high," "good effects," and "liking" reached up to 50% of the maximum possible VAS scores, but they were still lower than those observed in experimental dose-controlled conditions for 2C-B, MDMA, and other stimulants as mephedrone (Mas et al., 1999; Farré et al., 2015; Papaseit et al., 2016, 2018). It is possible that euphoria could be an important issue of the psychedelic experience after 2C-B or 2C-E use, as previously postulated for other psychedelics (Bouso et al., 2016). It is noteworthy that 2C-E increased some somatic VAS scales (drowsiness, dizziness, and confusion) in a similar manner to 2C-B.

Moreover, alteration in perception varied from changes in perceptions to hallucinations, that were experienced by 5 volunteers (3 only visual and 2 visual and auditory hallucinations). Of these, 5 subjects reported visual (seeing of lights or spots, 14–72 mm), 1 subject visual (seeing things/people, 50 mm) and 2 participants auditory (hearing sounds/voices, 8–14 mm score), effects. Results differ in intensity from other psychedelics probably because in this study subjects self-administered low to moderate doses of the substance. Additionally, 2C-E produced higher increases in sociability

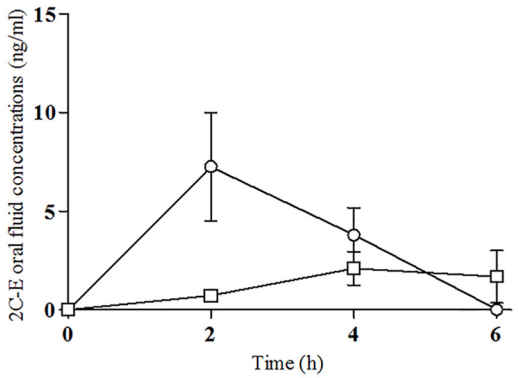

FIGURE 3 | Time course of 2C-E concentrations in oral fluid [Δ, 6.5 mg of 2C-E (n = 1); ∘, 8–10 mg of 2C-E (n = 6), □, 15–25 mg of 2C-E (n = 3); mean, standard error].

(VESPA SOC subscale) and augmented ratings on change perceptions, effects widely related to MDMA and LSD (Papaseit et al., 2016; Dolder et al., 2017; Puxty et al., 2017). Overall, the subjective effects induced by 2C-E appear to be closely related to psychedelic drugs indicating that it produces mind-altering and hallucinogenic effects which could be primarily mediated by the $5HT_{2A}$ receptor.

In a similar manner to 2C-B, the sole 2C-compound with previous observational data in humans after dose-controlled administration, 2C-E induced modest sympathomimetic effects, similar feelings of well-being, euphoria, and changes in perception although with more profound hallucinations (Caudevilla-Gálligo et al., 2012; González et al., 2015; Papaseit et al., 2018).

As expected, in our study 2C-E produced the prototypical effects of psychedelic substances that include visual hallucinations, perceptual changes, somatic symptoms, and activation of euphoria. Although it also induced headache, confusion, and breathing difficulty, no severe adverse reactions were observed. Our results show that in a recreational setting, self-administration of low-moderate doses of 2C-E by healthy experienced users is well tolerated and relatively safe. The results are consistent with a relatively low number of severe acute toxicity cases associated to 2C-E use (Iwersen-Bergmann et al., 2019).

The pharmacokinetics of 2C-E in humans has not yet been fully known. Our results on oral fluid concentrations of 2C-E are the first data in humans to be reported. 2C-E concentrations ranged from 0.93 to 21.54 ng/mL, with an average peak concentration of 5.8 ± 6.4 ng/mL observed at 2 h after administration. Oral fluid 2C-E showed a similar time course with effect outcomes. Nevertheless, because the study included

TABLE 1 | Summary of result on the physiological effects observed after self-administration of 2C-E.

Effects	Parameter	ANOVA			Comparison to baseline	T-Student			
		Doses (6.5–25 mg) (n = 10)			Doses (6.5–25 mg) (n = 10)	6.5–10 mg (n = 7)	15–25 mg (n = 3)	T-value	p-value
		Mean ± SD	F	p-value	Dunnett's test	Mean ± SD	Mean ± SD		
Physiological effects									
Systolic blood pressure	E_{max}	15 ± 23	0.047	0.995		15 ± 28	15 ± 5.8	ND	ND
	AUC_{0-6}	41 ± 74	0.050	0.994		43 ± 89	35 ± 22	ND	ND
	T-C				NS				
Diastolic blood pressure	E_{max}	1.6 ± 20	0.840	0.554		2 ± 22	0.7 ± 20	ND	ND
	AUC_{0-6}	−2.1 ± 63	0.873	0.539		−5.9 ± 74	6.7 ± 39	ND	ND
	T-C				NS				
Heart rate	E_{max}	18 ± 19	2.883	0.138		12 ± 17	33 ± 19	ND	ND
	AUC_{0-6}	58 ± 56	4.799	0.058		41 ± 57	98 ± 34	ND	ND
	T-C				b, c				
Temperature	E_{max}	0.5 ± 0.2	2.366	0.185		0.1 ± 0.2	0.3 ± 0.2	ND	ND
	AUC_{0-6}	0.3 ± 0.5	1.122	0.440		0.2 ± 0.5	0.6 ± 0.6	ND	ND
	T-C				b				

*Emax = peak effects 0–6 h (differences from baseline). AUC_{0-6} = area under the curve from 0 to 6 h. Units: mmHg (systolic blood pressure and diastolic blood pressure), beats per minute (heart rate), °C (temperature). For E_{max} and AUC_{0-6} a one-way ANOVA was used to examine the effect of all doses. A $p < 0.05$ was considered statistically significant. Only if a statistical difference were detected an unpaired T-Student was used to examine differences between the grouped doses (6.5–10 mg vs. 15–25 mg). A $p < 0.05$ was considered statistically significant. ND, not done. For T-C a one-way ANOVA and a post hoc Dunnett's test for multiple comparisons was used. Statistical differences between are presented as "a" $p < 0.05$, "**a**" $p < 0.01$ (times 0–2 h), "b" $p < 0.05$, "**b**" $p < 0.01$ (times 0–4 h), "c" $p < 0.05$, "**c**" $p < 0.01$ (times 0– 6 h).*

TABLE 2 | Summary of result on the subjective effects and saliva concentrations observed after self-administration of 2C-E.

Effects	Parameter	ANOVA			Comparison to baseline	T-Student			
		Doses (6.5–25 mg) (n = 10)			Doses (6.5–25 mg) (n = 10)	6.5–10 mg (n = 7)	15–25 mg (n = 3)	T-value	p-value
		Mean ± SD	F	p-value	Dunnett's test	Mean ± SD	Mean ± SD		
Visual analog scale (VAS)									
Intensity	E_{max}	46 ± 17	1.045	0.468		43 ± 11	55 ± 27	ND	ND
	AUC_{0-6}	147 ± 68	5.464	0.045		134 ± 52	177 ± 104	−0.916	0.387
	T-C				**a**, **b**				
Stimulated	E_{max}	37 ± 28	1.423	0.349		29 ± 25	55 ± 31	ND	ND
	AUC_{0-6}	114 ± 104	3.666	0.093		86 ± 87	179 ± 130	ND	ND
	T-C				**b**				
High	E_{max}	48 ± 23	1.924	0.245		48 ± 21	54 ± 44	ND	ND
	AUC_{0-6}	145 ± 99	6.003	0.038		134 ± 74	185 ± 189	ND	ND
	T-C				**a**, **b**				
Good effects	E_{max}	50 ± 27	0.839	0.555		72 ± 86	62 ± 30	ND	ND
	AUC_{0-6}	150 ± 110	3.875	0.085		116 ± 74	212 ± 133	ND	ND
	T-C				**a,** b				
Liking	E_{max}	51 ± 30	0.751	0.598		49 ± 24	55 ± 48	ND	ND
	AUC_{0-6}	181 ± 134	1.691	0.287		170 ± 113	205 ± 203	ND	ND
	T-C				**a**, **b**, **c**				
Content	E_{max}	47 ± 30	1.048	0.467		44 ± 25	53 ± 47	ND	ND
	AUC_{0-6}	145 ± 110	1.784	0.269		130 ± 92	180 ± 161	ND	ND
	T-C				a, b				
Changes in colors	E_{max}	32 ± 21	6.786	0.030		23 ± 7.9	52 ± 32	−2.426	0.041
	AUC_{0-6}	102 ± 111	51.871	< 0.001		55 ± 16	209 ± 173	−2.545	0.034
	T-C				**a**, b				
Changes in shapes	E_{max}	27 ± 27	3.717	0.091		15 ± 16	53 ± 32	ND	ND
	AUC_{0-6}	73 ± 91	14.974	0.005		34 ± 35	165 ± 128	−2.665	0.029
	T-C				NS				
Changes in lights	E_{max}	35 ± 28	9.468	0.015		23 ± 18	64 ± 32	−2.665	0.029
	AUC_{0-6}	99 ± 90	34.980	0.001		59 ± 39	193 ± 114	−2.930	0.019
	T-C				**c**				
Hallucinations-seeing of lights or spots	E_{max}	21 ± 26	8.564	0.018		6.6 ± 12	55 ± 16	−5.388	0.001
	AUC_{0-6}	61 ± 88	13.026	0.007		16 ± 28	166 ± 92	−4.220	0.003
	T-C				c				
Hallucinations-seeing animals, things, insects, or people	E_{max}	6.2 ± 16	1.002	0.485		1.4 ± 3.8	17 ± 28	ND	ND
	AUC_{0-6}	11 ± 26	0.987	0.491		2.9 ± 7.6	29 ± 46	ND	ND
	T-C				NS				
Changes in hearing	E_{max}	4.1 ± 7.4	15.425	0.005		4.0 ± 8.5	4.3 ± 5.1	−0.062	0.952
	AUC_{0-6}	12 ± 23	19.891	0.003		12 ± 27	11 ± 14	0.080	0.938
	T-C				NS				
Hallucinations-hearings of sounds or voices	E_{max}	2.2 ± 4.9	13.444	0.007		0.0 ± 0.0	7.3 ± 7.0	−3.026	0.016
	AUC_{0-6}	4.9 ± 11	29.642	0.001		0.0 ± 0.0	16 ± 15	−3.189	0.013
	T-C				NS				

(Continued)

TABLE 2 | Continued

Effects	Parameter	ANOVA			Comparison to baseline	T-Student			
		Doses (6.5–25 mg) (n = 10)			Doses (6.5–25 mg) (n = 10)	6.5–10 mg (n = 7)	15–25 mg (n = 3)	T-value	p-value
		Mean ± SD	F	p-value	Dunnett's test	Mean ± SD	Mean ± SD		
Different body feeling	E_{max}	46 ± 23	1.559	0.315		46 ± 20	46 ± 33	ND	ND
	AUC_{0-6}	135 ± 78	3.792	0.088		120 ± 46	169 ± 133	ND	ND
	T-C				**a**, **b**				
Unreal body feeling	E_{max}	20 ± 26	6.413	0.033		9.4 ± 13	43 ± 38	−2.231	0.056
	AUC_{0-6}	58 ± 101	26.999	0.001		19 ± 26	150 ± 161	−2.273	0.053
	T-C				NS				
Changes in distances	E_{max}	22 ± 30	1.286	0.387		13 ± 25	44 ± 34	ND	ND
	AUC_{0-6}	60 ± 98	5.499	0.045		26 ± 50	139 ± 149	−1.899	0.094
	T-C				NS				
Different surroundings	E_{max}	29 ± 29	2.311	0.191		17 ± 18	56 ± 32	ND	ND
	AUC_{0-6}	82 ± 100	8.625	0.018		37 ± 38	187 ± 129	−3.001	0.017
	T-C				b, c				
Unreal surroundings	E_{max}	13 ± 27	14.432	0.006		0.0 ± 0.0	43 ± 36	−3.428	0.009
	AUC_{0-6}	45 ± 102	29.938	0.001		0.0 ± 0.0	150 ± 153	−2.843	0.022
	T-C				NS				
Confusion	E_{max}	15 ± 22	1.891	0.250		0.0 ± 0.0	2.3 ± 2.08	ND	ND
	AUC_{0-6}	35 ± 49	6.297	0.034		9 ± 12	30 ± 37	−1.461	0.182
	T-C				NS				
Fear	E_{max}	3.1 ± 5.2	0.802	0.573		1.1 ± 3.0	7.7 ± 7.1	ND	ND
	AUC_{0-6}	6.7 ± 12	0.785	0.581		2.3 ± 6.1	17 ± 16	ND	ND
	T-C				NS				
Depression or sadness	E_{max}	3.0 ± 5.3	3.774	0.089		1.3 ± 3.0	7.0 ± 8.2	ND	ND
	AUC_{0-6}	7.0 ± 12	2.437	0.178		2.6 ± 6.0	17 ± 16	ND	ND
	T-C				NS				
Drowsiness	E_{max}	22 ± 28	10.050	0.013		15 ± 18	38 ± 44	−1.221	0.257
	AUC_{0-6}	66 ± 89	17.533	0.004		48 ± 64	106 ± 140	−0.933	0.378
	T-C				**a**				
Dizziness	E_{max}	15 ± 21	1.916	0.246		9.9 ± 16	27 ± 30	ND	ND
	AUC_{0-6}	44 ± 71	4.783	0.058		22 ± 36	97 ± 114	ND	ND
	T-C				a				
Bad effects	E_{max}	8.4 ± 10	2.761	0.147		9.3 ± 12	8.7 ± 4.5	ND	ND
	AUC_{0-6}	23 ± 29	1.938	0.243		22 ± 33	26 ± 20	ND	ND
	T-C				a				
Headache	E_{max}	14 ± 17	1.509	0.327		8.3 ± 12	26 ± 22	ND	ND
	AUC_{0-6}	28 ± 33	3.647	0.094		25 ± 39	32 ± 22	ND	ND
	T-C				NS				
Nausea	E_{max}	11 ± 10	0.262	0.891		11 ± 11	12 ± 7.3	ND	ND
	AUC_{0-6}	32 ± 30	0.761	0.593		28 ± 31	40 ± 30	ND	ND
	T-C				a				
Vertigo	E_{max}	12 ± 20	0.316	0.857		8.7 ± 18	19 ± 26	ND	ND
	AUC_{0-6}	20 ± 32	0.143	0.959		17 ± 37	25 ± 23	ND	ND
	T-C				NS				

(Continued)

TABLE 2 | Continued

Effects	Parameter	ANOVA			Comparison to baseline	T-Student			
		Doses (6.5–25 mg) (*n* = 10)			Doses (6.5–25 mg) (*n* = 10)	6.5–10 mg (*n* = 7)	15–25 mg (*n* = 3)	*T*-value	*p*-value
		Mean ± SD	*F*	*p*-value	Dunnett's test	Mean ± SD	Mean ± SD		
Breathing difficulty	E_{max}	2.7 ± 6.5	90.601	< 0.001		0.3 ± 0.8	8.3 ± 11	−2.103	0.069
	AUC_{0-6}	10 ± 27	319.150	< 0.001		0.6 ± 1.6	32 ± 47	−1.910	0.093
	T-C				NS				
Face flushing	E_{max}	13 ± 20	0.374	0.819		16 ± 17	27 ± 29	ND	ND
	AUC_{0-6}	20 ± 20	0.883	0.535		53 ± 59	72 ± 90	ND	ND
	T-C				NS				
Addiction research center inventory (ARCI)									
PCAG (sedation)	E_{max}	3.1 ± 4.6	0.443	0.775		3.1 ± 4.3	3.0 ± 6.1	ND	ND
	AUC_{0-6}	14 ± 13	1.101	0.447		12 ± 13	18 ± 14	ND	ND
	T-C				**a**				
MBG (euphoria)	E_{max}	4.4 ± 4.4	0.904	0.526		3.1 ± 3.5	7.3 ± 5.7	ND	ND
	AUC_{0-6}	16 ± 19	1.549	0.318		11 ± 14	28 ± 28	ND	ND
	T-C				b, **c**				
LSD (dysphoria and somatic symptoms)	E_{max}	4.5 ± 2.7	1.469	0.337		3.6 ± 1.0	6.7 ± 2.5	ND	ND
	AUC_{0-6}	12 ± 9.8	3.802	0.088		7.4 ± 6.3	23 ± 7.55	ND	ND
	T-C				**a**, **b**				
BG (intellectual efficieny and energy)	E_{max}	1.5 ± 2.2	0.330	0.847		1.1 ± 2.0	2.3 ± 3.1	ND	ND
	AUC_{0-6}	4.1 ± 6.6	0.419	0.790		4.0 ± 5.6	4.3 ± 10	ND	ND
	T-C				b				
A (amphetamine-like effects)	E_{max}	4.2 ± 1.9	0.755	0.596		3.7 ± 1.2	5.3 ± 2.9	ND	ND
	AUC_{0-6}	14 ± 8.1	0.658	0.647		13 ± 5.9	19 ± 12	ND	ND
	T-C				**a**, **b**, c				
Evaluation of subjective effects of substances with abuse potential (VESSPA-SEE)									
S (sedation)	E_{max}	6.7 ± 3.3	9.231	0.016		5.8 ± 3.5	8.7 ± 2.08	−1.275	0.238
	AUC_{0-6}	19 ± 11	3.051	0.126		16 ± 11	24 ± 12	ND	ND
	T-C				a				
ANX (psychosomatic anxiety)	E_{max}	4.0 ± 2.9	1.996	0.234		3.3 ± 3.1	5.7 ± 1.5	ND	ND
	AUC_{0-6}	13 ± 10	3.178	0.118		11 ± 10	19 ± 8.7	ND	ND
	T-C				a, **b**				
CP (changes in perception)	E_{max}	4.2 ± 4.7	8.452	0.019		1.7 ± 1.2	10 ± 4.6	−4.736	0.001
	AUC_{0-6}	13 ± 17	17.663	0.004		4.3 ± 3.9	33 ± 18	−4.311	0.003
	T-C				**b**				
SOC (pleasure and sociability)	E_{max}	8.2 ± 7.7	2.389	0.183		5.9 ± 5.2	13 ± 11	ND	ND
	AUC_{0-6}	26 ± 29	3.212	0.116		18 ± 20	47 ± 40	ND	ND
	T-C				b				
ACT (activity and energy)	E_{max}	6.0 ± 6.3	1.205	0.412		3.9 ± 4.4	11 ± 7.9	ND	ND
	AUC_{0-6}	18 ± 20	1.362	0.365		11 ± 12	35 ± 27	ND	ND
	T-C				**b**				

(Continued)

TABLE 2 | Continued

Effects	Parameter	ANOVA			Comparison to baseline	T-Student			
		Doses (6.5–25 mg) (*n* = 10)			Doses (6.5–25 mg) (*n* = 10)	6.5–10 mg (*n* = 7)	15–25 mg (*n* = 3)	*T*-value	*p*-value
		Mean ± SD	*F*	*p*-value	Dunnett's test	Mean ± SD	Mean ± SD		
PS (psychotic symptoms)	E_{max}	3.1 ± 4.1	3.753	0.090		1.2 ± 1.1	7.3 ± 5.7	−2.919	0.019
	AUC_{0-6}	11 ± 18	15.680	0.005		3.1 ± 3.0	28 ± 17	−2.418	0.042
	T-C				NS				
Oral fluid concentrations									
2C-E	C_{max}	5.8 ± 6.4	0.491	0.745		7.3 ± 7.2	2.4 ± 1.7	ND	ND
	AUC_{0-6}	18 ± 18	0.532	0.720		22 ± 21	7.3 ± 4.7	ND	ND
	T-C				**a**				

*E_{max} = peak effects 0–6 h. Emax = peak effects 0–6 h (differences from baseline). AUC_{0-6} = area under the curve from 0 to 6 h. Units: mm [visual analog scale (VAS)], and score [Addiction Research Center Inventory (ARCI), Evaluation of Subjective Effects of Substances with Abuse Potential questionnaire (VESSPA-SEE)] and expressed as mean. C_{max} = maximal concentrations 0–6 h (differences from baseline) measured by ng/mL. For Emax and AUC_{0-6} a one-way ANOVA was used to examine the effect of all doses. A $p < 0.05$ was considered statistically significant. Only if a statistical difference were detected an unpaired T-Student was used to examine differences between the grouped doses (6.5–10 mg vs. 15–25 mg). A $p < 0.05$ was considered statistically significant. ND, not done. For T-C a one-way ANOVA and a post hoc Dunnett's test for multiple comparisons was used. Statistical differences between are presented as "a" $p < 0.05$, "**a**" $p < 0.01$ (times 0–2 h), "b" $p < 0.05$, "**b**" $p < 0.01$ (times 0–4 h), "c" $p < 0.05$, "**c**" $p < 0.01$ (times 0–6 h).*

five different 2C-E doses in a limited number of subjects, a dose-concentration relationship was not observed. We do not have an explanation for the high variability observed, with higher concentrations after lower doses. Problems in the collection of the samples or an erratic distribution of 2C-E in saliva could be possible causes. Concentrations in oral fluid were present in all subjects until 4 h, and5 of them were positive at 6 h post-administration. Oral fluid, in contrast to plasma, is a suitable, non-invasive, and easy biological matrix to collect in a non-controlled setting. Nevertheless, the interpretation of oral fluid 2C-E concentrations without data from plasma is extremely difficult (not obtained in this study or any other).

Our study has several limitations mainly associated with its design as naturalistic-observational. An expectancy bias could appear due to the non-placebo-controlled design. Because participants selected the dose according to their preferences, it resulted in low-moderate doses (ranging from 6.5 to 25 mg), and some doses were only used by one participant. A limited number of subjects could be responsible for a lack of power in some measures. Our findings may not refer to other 2C-E routes of administration. Moreover, the recreational setting could have influenced the effects reported by participants. The limited number of time-point measures did not permit to know the real peak effect/concentration times that will need more intensive evaluations. However, it should be noted that there are a number of strengths: the participation of female subjects, the dose selection by the subjects according to their preferences (6.5–25 mg representing real-life quantities), effects previously experienced with the same or similar psychedelic substances, the recreational scenario, and the use of validated rating scales, questionnaires, and analytic techniques. We cannot discard that a more controlled dose-response study using defined drug doses equal for all subjects would produce a different picture. Future studies should be carried out in controlled conditions and with a larger sample. In addition, it should be noted that 2C-E profiles may vary considerably due to the dose administered and the interindividual differences in pharmacodynamic-pharmacokinetics.

CONCLUSION

The results of this non-controlled, observational study in a real-life setting of recreational use provide useful preliminary data of the acute pharmacodynamic effects and pharmacokinetics in oral fluid of 2C-E. Taken together, the current findings suggest that self-administered oral 2C-E induced a constellation of alterations in perceptions, hallucinations, and euphoric-mood which displayed marked similarities to psychedelic experience. Even at low-moderate doses, notable perceptual changes and hallucinations were the most prominent 2C-E effects. High interindividual variability among doses was observed. Participants with self-administered higher doses were more susceptible to experiencing the most intense subjective effects. Based on these preliminary data, oral fluid can be an appropriate, non-invasive, biologic matrix to detect acute 2C-E use.

It can be concluded that further research in humans is needed to compare the effects of 2C-E with other classical and new psychedelic substances.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the senior author (MF, magi.farre@uab.cat).

ETHICS STATEMENT

The protocol was approved by the local Research Ethics Committee (CEIC-Parc de Salut Mar, Barcelona, Spain). The study was conducted in accordance with the Declaration of Helsinki and Spanish laws concerning clinical research. The participants provided their written informed consent previous to participate in this study.

AUTHOR CONTRIBUTIONS

MF, RT, MV, MG, MT, ES, JR, and EO conceptualized the study design. MF, EO, MG, ES, and MV collected the data. EO and OP analyzed the oral fluid. MV analyzed the 2C-E contents. EP and CP-M analyzed the data. EP, EO, CP-M, MT, MG, MV, OP, ES, JR, RT, and MF wrote, revised, and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.00233/full#supplementary-material

FIGURE S1 | Time course of individual changes from baseline for selected physiological effects (n = 10; mean, standard error).

FIGURE S2 | Time course of individual changes from baseline for selected subjective effects (n = 10; mean, standard error).

FIGURE S3 | Time course of individual 2C-E concentrations in oral fluid (n = 10; mean, standard error).

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Novel Opioids: Systematic Web Crawling Within the e-Psychonauts' Scenario

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Background: A wide range of novel psychoactive substances (NPSs) are regularly searched and discussed online by e-psychonauts. Among NPSs, the range of prescription/non-prescription opioids (fentanyl and non-fentanyl analogs) and herbal derivatives currently represents a challenge for governments and clinicians.

Methods: Using a web crawler (i.e., NPS.Finder®), the present study aimed at assessing psychonaut fora/platforms to better understand the online situation regarding opioids.

Results: The open-web crawling/navigating software identified some 426 opioids, including 234 fentanyl analogs. Of these, 176 substances (162 were very potent fentanyls, including two ohmefentanyl and seven carfentanyl analogs) were not listed in either international or European NPS databases.

Conclusion: A web crawling approach helped in identifying a large number, indeed higher than that listed by European/international agencies, of unknown opioids likely to possess a significant misuse potential. Most of these novel/emerging substances are still relatively unknown. This is a reason of concern; each of these analogs potentially presents with different toxicodynamic profiles, and there is a lack of docking, preclinical, and clinical observations. Strengthening multidisciplinary collaboration between clinicians and bioinformatics may prove useful in better assessing public health risks associated with opioids.

Keywords: psychonauts, novel psychoactive substance, novel synthetic opioids, fentanyl analogs, web crawling, drug misuse, prescribed drug misuse

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INTRODUCTION

Novel psychoactive substances (NPSs) are substances that are not controlled by the United Nations (UN) 1961 Single Convention on Narcotic Drugs or by Psychotropic Substances Conventions (United Nations Office on Drugs and Crime [UNODC], 2019a). By definition, "novel" does not necessarily imply that a drug has been recently developed (Hassan et al., 2017); 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 concern (Schifano et al., 2015). NPSs are mainly of synthetic origin and comprise different drug classes (Schifano et al., 2019b). Among them, over the recent years, there has been an increase in the appearance of novel synthetic opioids (NSOs) on the recreational drug market (Zawilska, 2017). NSOs are frequently used with other illegal or prescribed drugs (Pichini et al., 2018; Pérez-Mañá et al., 2018). Owing to a range of reasons, the non-medical use of opioids such as fentanyl analogs and a range of remaining prescription/non-prescription substances is spreading worldwide (Prekupec et al., 2017; Lovrecic et al., 2019) and is affecting the entire life span, from youngsters to the elderly (Huhn et al., 2018; Kelley-Quon et al., 2019). Opioids are among the most powerful analgesic drugs, but they are burdened by unwanted adverse effects, in particular the abuse liability and the respiratory depression, with the last being the primary cause of death from overdose (Valentino and Volkow, 2018; Algera et al., 2019; Varga et al., 2020). Further, the evidence suggests that opioids' consumption impacts the *in utero* neuronal development and induces in humans long-lasting transgenerational changes in subsequent generations owing to epigenetic alterations (Gilardi et al., 2018). For these reasons, worldwide intensive endeavors are directed from academics, clinicians, and industries toward expanding, intensifying, and coordinating fundamental, translational, and clinical research with respect to opioid abuse. To this respect, there is strong focus at present on the study and development of abuse-deterrent opioid formulations (Pergolizzi et al., 2018). Despite the efforts of law enforcement and other agencies (European Monitoring Centre for Drugs and Drug Addiction [EMCDDA], 2019; United Nations Office on Drugs and Crime [UNODC], 2019c), an unknown number of substances are continuously manufactured, (illicitly) offered for sale, and inappropriately consumed (Davey et al., 2012; Orsolini et al., 2017). Some concerns are related as well to the lack of regulation relating to so-called home-brew opiates (Galanie et al., 2015; Oye et al., 2015).

Number of Opioids Identified

By April 2019, the EMCDDA European Database on New Drugs (European Database on New Drugs [EDND], 2019) contained 751 entries, with 51 of them being classified as opioids. By February 2019, the UNODC listed a total of 892 substances in their NPS database (Unodc Early Warning Advisory on New Psychoactive Substances [UNODC EWA NPS], 2019), with 61 being NSOs. It could, however, be argued that the NPS scenario is much larger than that outlined by those substances that have been seized and formally identified by the European Union (EU) and the UN databases. Because the online NPS scenario typically predicts the real-life NPS market availability (Corazza et al., 2013; Schifano et al., 2015), identifying what is being discussed online by web-based NPS enthusiasts (e.g., the "e-psychonauts") may well be of interest (Orsolini et al., 2015; Corkery et al., 2017).

Although data about the clinical, pharmacological, and toxicological characteristics of a number of opioid drugs have already been made available (Suzuki and El-Haddad, 2017; Armenian et al., 2018; Baumann et al., 2018; Beardsley and Zhang, 2018; Tabarra et al., 2019), there is a clear lack of knowledge relating to the number of novel substances offered to online/real-life customers (Kacinko and Papsun, 2019), also owing to the difficulties in detecting them in both interdiction and clinical settings (Abdulrahim and Bowden-Jones, 2018).

Aims

The aim of the current research was to (a) identify and categorize the number of opioids collected by the NPS.Finder® web crawler from a range of psychonaut, NPS-related, online sources; and (b) compare the NPS.Finder® opioid list with related findings from the UNODC and the EMCDDA.

MATERIALS AND METHODS

Identification of Substances

To facilitate the process of early recognition of the increasing dissemination of new substances online and the variability of information sources, a crawling/navigating software (i.e., "NPS.Finder®") was designed to automatically scan the open/surface web for new/novel/emerging NPSs (for a thorough description of web crawling and data cleaning activities, see Schifano et al., 2019b). This software was designed to map on a 24/7 basis the large variety of psychoactive substances mentioned/discussed within a range of popular online psychonauts websites/fora. NPS.Finder® was designed *de novo* (e.g., it was not designed by the authors; neither was it adapted from another program) by Damicom, an IT enterprise based in Rome (Italy), to extract a range of information regarding NPSs, including chemical and street names; chemical formula; three-dimensional images; and anecdotally reported clinical/psychoactive effects. These data were then automatically stored in an online, restricted-access/password-controlled database located within firewall protected, highly secure, and consistently performing servers. First, a number of proper piloting searches were carried out (see also Schifano et al., 2019b), and any new website of interest was added to the list, whose final version is attached as **Table 1**. Although the language most typically used in these websites was English, further languages analyzed by NPS.Finder® included the following: Dutch, French, Turkish, Swedish, Spanish, German, Russian, and Italian. Afterward, a range of specific web scraper/crawler activities, to extract all accessible posts/entries from November 26, 2017 to May 31, 2019, were carried out. With the help an *ad hoc* check control panel, all data were manually and carefully analyzed by four medically/psychiatrically trained professionals (e.g., FN, DA, CZ, and LG). In this way, a full assessment and editing of each NPS.Finder® data entry were carried out, and the range of unique opioids here commented was identified. Finally, using chemical structure identification and published related data, researchers assigned each molecule to its NPS drug class (Schifano et al., 2019b).

Classification of Opioid Drugs

The web crawler-identified substances' denominations were first searched in Medline/PubMed (PubMed, 2019) and in Google®/Google® Scholar (Google, 2019; Google Scholar, 2019).

TABLE 1 | NPS.Finder® websites' list; search activities carried out on the surface web only.

N	Website name
1	*Avalonmagicplants.com*
2	*Azarius.net*
3	*Bluelight.org*
4	*Bluemorphotours.com*
5	*Cannabis.net*
6	*Chemeurope.com*
7	*Committedpsychonaut.tumblr.com*
8	*Consolidated Index of Controlled Substances*
9	*Daath.hu/psychonauts*
10	*Dancesafe.org*
11	*Deviantart.com/psychonaut-a*
12	*Druglibrary.org*
13	*Drugs.tripsit.me*
14	*Drugs-forum.com*
15	*Drugs-plaza.com*
16	*Dutch-headshop.eu*
17	*Ecstasydata.org*
18	*Elephantos.com*
19	*Energycontrol.org*
20	*http://entheogen-network.com/forums/*
21	*Erowid.org*
22	*Eusynth.org*
23	*https://everything2.com/title/Psychonaut*
24	*Fungifun.org*
25	*Hedweb.com*
26	*Hipforums.com/forum*
27	*Isomerdesign.com*
28	*Knehnav.home.xs4all.nl*
29	*Kratomshop.com*
30	*Legal-high-inhaltsstoffe.de*
31	*Mindstates.org*
32	*Mycotopia.net*
33	*Natmedtalk.com*
34	*Npsproject.eu*
35	*Peyote.com/peyolink.html*
36	*Psychedelic-library.org*
37	*Psychonaut.ca*
38	*Psychonaut.fr*
39	*Psychonautdocs.com*
40	*Psychonautwiki.org*
41	*Psyconauts.tripod.com*
42	*Reddit.com and drug-related subreddits (e.g., Reddit.com/r/Psychonaut/; Reddit.com/r/shroomers/)*
43	*Shayanashop.com*
44	*Sjamaan.com*
45	*Tripzine.com*
46	*Tryptamind.com*
47	*Urban75.net*
48	*Wikipedia List of designer drugs*
49	*Zamnesia.com*

The used terms were "opiates/opioids," "opioids," "novel synthetic opioids," "fentanyl analogs," "opioid receptors," "mu-opioid receptor," "delta-opioid receptor," "kappa-opioid receptor," "receptor binding affinity," and "herbal compounds." An initial screening was performed to classify the substances according to their main description (e.g., fentanyl vs. non-fentanyl analogs). For this purpose, both the PuCchem (PubChem, 2019) and ChEMBL (Davies et al., 2015; Gaulton et al., 2017; EMBL-EBI, 2019) were searched; and whenever possible, the International Union of Pure and Applied Chemistry (IUPAC) name was also used. For fentanyl analogs, to make sure that the index molecule was indeed a fentanyl, the IUPAC name proposed by "isomer design" (PiHKAL, 2019) was here considered. For a handful of substances, when there were errors in published IUPAC names, the ChemDraw approach, able to generate chemical names from structures and/or vice versa, was used (ChemDraw, 2019).

A further screening was performed to compare the NPS.Finder® results with those reported by both the EMCDDA and the UNODC. More precisely, the search was performed on the following UN databases: International Narcotics Control Board (INCB) "Yellow List" (International Narcotics Control Board [INCB], 2019a); the Unodc Early Warning Advisory on New Psychoactive Substances [UNODC EWA NPS] (2019); and the fentanyl-related substances INCB report (International Narcotics Control Board [INCB], 2019a). For European data, the European Database on New Drugs [EDND] (2019) was accessed. To understand if an index opioid was currently being used as a prescribed medication, further screenings were carried out in the United Kingdom and United States lists of controlled substances (GOV.UK, 2017; DEA, 2019), and in the Anatomical Therapeutic Chemical/defined daily dose (ATC/DDD) list (Chen et al., 2012; WHO Collaborating Centre [WHOCC], 2016; WHO Collaborating Centre [WHOCC], 2019). The ATC substances were listed according to the ATC classification by WHO and manually searched in the ATC/DDD Index (WHO Collaborating Centre [WHOCC], 2019). In the ATC/DDD Index, most opioids are grouped with the code N02A (WHO Collaborating Centre [WHOCC], 2016).

RESULTS

Identification and Classification of Opioid Drugs

After about 18 months of operation, the number of substances identified by the web crawler activities was 5,922. By the time of writing, some 4,204 unique NPSs were included in the database, and 1,718/5,922 (29.01%) remaining substances were found to be false positives or duplicates. The most common NPS mentioned in psychonaut fora included the following: psychedelic phenethylamines (30.1%; CI 95%: 28.7–31.5%); synthetic cannabimimetics (29.8%; CI 95%: 28.4–31.2%); and opioids (10.1%; CI 95%: 9.2–11.0%).

The opioids (n = 426) were then divided into two groups: *fentanyl analogs* (n = 234; 54.9%; CI 95%: 50.1–59.5%; **Supplementary Table S1**) and *miscellaneous opioids* (n = 192; 45.0%; CI 95%: 40.0–49.8%; **Supplementary Tables S2A–C**). The miscellaneous opioids group included the following: (a) all the prescribing opioids classified in the ATC/DDD Index

(n = 48; 11.2% of 426; CI 95%: 8.6–14.6%); (b) the non-fentanyl analog opioids (n = 136; 31.9%; CI 95%: 27.6–36.5%); and (c) the herbal derivatives (n = 8; 1.8%; CI 95%: 0.9–3.6%) (**Supplementary Tables S2A–C**).

Comparison With Novel Psychoactive Substance-/Opioid-Focused European and United Nations Databases

Current NPS.Finder® results were compared with those pertaining to opioids listed in the EMCDDA and UN databases (**Supplementary Tables 1**, **2A–C**). Overall, the NPS.Finder® detected a larger number of opioids than was in the remaining databases. The opioids identified by *NPS.Finder® only* were 176 chemically different substances. In particular, out of the 234 *fentanyl analogs*, 162 (69.2% of 234) were listed only in the NPS.Finder®; 7 (2.99%; e.g., 4-fluoroisobutyrfentanyl, acetylfentanyl, acrylfentanyl, butyrylfentanyl, furanylfentanyl, ocfentanil, and tetrahydrofuranylfentanyl) were listed in all databases, and the remaining 65 (27.78%) substances were reported in one or more of the EU or UN databases (i.e., INCB Yellow List; INCB "Fentanyl-related substances with no known legitimate use list"; UNODC EWA NPS; and EDND). Out of the 136 substances in the non-fentanyl analog opioid list, 14 (10.29%) were listed only in NPS.Finder®, and not in any of the following: INCB Yellow list (updated March 2019), EDND (April 2019), and UNODC EWA NPS (July 2019). The remaining 122 substances were reported both in the NPS.Finder® and in at least one of the following: INCB Yellow list (updated March 2019), EDND (April 2019), or UNODC EWA NPS (July 2019).

DISCUSSION

To the best of our knowledge, an unprecedented list of opioid drugs with a possible recreational/misuse potential was generated by these open web-only crawling software activities, which were focusing on a range of psychonaut forum entries. For all these fentanyl/non-fentanyl/miscellaneous drugs, only limited levels of preclinical/clinical data are typically available (Deluca et al., 2012; Armenian et al., 2018; Frisoni et al., 2018; Gerace et al., 2018a; Ventura et al., 2018; European Database on New Drugs [EDND], 2019; Tabarra et al., 2019; United Nations Office on Drugs and Crime [UNODC], 2019b). Apart from the vast range of previously undescribed fentanyl analogs, NPS.Finder® identified a further number of "novel" and potent/very potent, chemically diverse miscellaneous substances. This may suggest that psychonauts are attracted to a variety of drugs, which range from research chemicals and their derivatives to more "traditional" substances, including failed pharmaceuticals or old patents that have been "rediscovered" and marketed for their potential use as "recreational" substances (Corkery et al., 2018). At present, we cannot establish with certainty whether the opioids here identified are all, or in part, currently circulating in the community and are available for consumption. Indeed, the current paper focused on the e-psychonauts' discussions only; these web-based drug enthusiasts, however, have been suggested to somehow represent the drug scenarios' "trend setters" (Schifano et al., 2015, 2019b). Hence, a focus on web-based drug discussion may well be of interest to better assess, and possibly predict (Corazza et al., 2013), the international drug misuse concerns. Ongoing studies from our group will hopefully better identify the following: a) which of the e-psychonauts' substances, including opioids, will make an entry into the future markets; and b) which is the time gap, for an index drug, between the start of the e-psychonauts' interest and their actual identification on the international drug scenarios.

Different from both the UNODC and the EMCDDA, which report in their NPS databases only those substances that have been both seized from the community and chemically analyzed, it was unclear from here if the mentioned opioids have already been synthesized or not. For each mentioned opioid, however, the unique IUPAC name was here identified and reported, and those further sources here are considered (e.g., PubChem, ChEMBL, ChemDraw, and Isomer Design) confirmed that the substance was properly chemically characterized. Hence, one could argue that the rogue producers' synthesis of any of the opioids here commented is indeed a real and distinct possibility (Kata et al., 2018; Financial Crimes Enforcement Network [FinCHEN], 2019; Pardo et al., 2019; Whitehouse, 2019).

Present results, highlighting a strong interest by psychonauts toward opioid drugs, are consistent with previous studies, which have analyzed the opioids' debate both in social media settings (Kalyanam et al., 2017; Kim et al., 2017; Pandrekar et al., 2018; Li et al., 2019) and on the darknet (Mackey et al., 2018; Tzanetakis, 2018; Cunliffe et al., 2019).

Fentanyl Analogs

Most (e.g., 55%) opioids identified here were fentanyl analogs. Although present findings do not necessarily confirm in any possible way these substances' levels of use, they can still reflect the attention given by psychonauts to these drugs and help in explaining aspects of the current "opioid epidemic" (Kakko et al., 2019; Zhao, 2019). A few fentanyl analogs have been mentioned in the literature (Suzuki and El-Haddad, 2017; Zawilska, 2017; Misailidi et al., 2018; Lipiński et al., 2019), especially in terms of the acute clinical toxicity issues relating to their intake (Abdulrahim and Bowden-Jones, 2018). Vulnerable subjects can access online a large number of these substances (Suzuki and El-Haddad, 2017), without even being aware of what they are taking exactly (Ciccarone et al., 2017; Bardwell et al., 2019; McLean et al., 2019; Stein et al., 2019). Indeed, levels of related clinical toxicological information are sometimes available only postmortem (Giorgetti et al., 2017; Concheiro et al., 2018; D'Errico, 2018; Kraemer et al., 2019).

Apart from a range of some well-known fentanyl-related substances, two ohmefentanyl and seven carfentanyl analogs were here identified. It is of interest that the analgesic activity of ohmefentanyl in mice is 6,300 times more potent than that of morphine (Xu et al., 1985) and that carfentanyl has been reported in association with a number of fatalities (Wilcoxon et al., 2018).

Given the wide range of common and street names available for each molecule, even small modifications occurring in the fentanyl family structure can lead to misidentification of the index drug (Akhondi et al., 2015) and potentially provoke clinically unexpected effects. It is also possible that some substances here commented by psychonauts included fentanyl precursors or metabolites (Wilde et al., 2019). Hence, it is particularly relevant, for the vast range of fentanyl analogs, to use unique codes such as those of IUPAC (Gaulton et al., 2017; Hähnke et al., 2018; PiHKAL, 2019).

Miscellaneous: Non-fentanyl Compounds, Prescribing Opioids, Herbals, and Derivatives

The 136 non-fentanyl analog opioids identified in the NPS.Finder® database belong to a range of pharmacological classes, and some 10.2% were not reported by the EU and UN databases. Several of these substances have been used in the past for research purposes and/or were never marketed. The present findings are consistent with the current "top" and "rising" posts on social networks like Reddit (Reddit, 2019d). Some of these substances, for example, BDPC/bromadol have already been described as being some 500 times more potent than morphine (Reddit, 2019a; Sharma et al., 2019; TripSit Factsheets, 2019a), whereas others, for example, embutramide, have been associated with suicidal intent (Lajtai et al., 2016). A range of remaining substances identified here have received little/no attention in the literature and include acetoxyketobemidone (e.g., an alternative to ketobemidone, a prescription analgesic that is scheduled in different countries; United Nations Office on Drugs and Crime [UNODC], 1954; Drugs-forum, 2007; Bluelight, 2019a; Reddit, 2019c); 6-methylenedihydrodesoxymorphine/6-MDDM (e.g., a semi-synthetic derivative of hydromorphone about 80 times more potent than morphine and associated with euphoriant effects; Bluelight, 2019b; TripSit Factsheets, 2019b; Reddit, 2019e); isopropyl-U-47700 (identified for the first time in 2018 and belonging to N-substituted benzamides and acetamide opioid analgesics, colloquially known as "U-compounds" or "U-drugs;" Krotulski and Logan, 2018; Sharma et al., 2019; Yin, 2019); and piperidylthiambutene (developed in the 1950s and equipotent to morphine; Adamson and Green, 1950).

NPS.Finder® identified here virtually all the prescription opioids listed in the ATC/DDD Index. The psychonauts' interest in commenting about these substances may be a reason of concern, because prescription opioid misuse is a challenging issue worldwide, especially so in both North America and Europe (van Amsterdam and van den Brink, 2015; Helmerhorst et al., 2017).

NPS.Finder® identified as well a range of well-known opioid herbal compounds, including *Papaver somniferum* and some crude opiate extracts (e.g., granulate, tincture, and poppy seed tea); *Mitragyna speciosa*/kratom (but not mitragynine and 7-hydroxymitragynine; Fluyau and Revadigar, 2017; Graziano et al., 2017; Coe et al., 2019; Corkery et al., 2019); and *Salvia divinorum*. Although herkinorin (Ventura et al., 2018) was not identified in the current database, salvinorin B ethoxymethyl ether/"Symmetry" (e.g., an unusually potent synthetic salvinorin compound, potently binding to kappa opioid receptors; Peet and Baker, 2011; Erowid, 2015; Reddit, 2019b), salvinorin B methoxymethyl (a potent semi-synthetic derivative of salvinorin A; Baker et al., 2009; Peet and Baker, 2011; Reddit, 2019f; Zjawiony et al., 2019), and salvinorin A received here the attention of psychonauts. This may be a cause for concern, because salvinorin products' psychoactive effects include perceptual disturbances, psychosis, irritability, and anxiety (Ventura et al., 2018).

Finally, it is interesting to note that a range of both endogenous (e.g., amphibian opioid peptides such as dermorphins and deltorphins) and food-derived (e.g., derivatives from milk and soya such as soymorphines and β-casomorphins) opioid peptides, possessing low levels of potency (Teschemacher et al., 1997; Negri et al., 2000; Ohinata et al., 2007; Liu and Udenigwe, 2018), were not mentioned in psychonaut fora.

Comparison With European Union and United Nations Novel Psychoactive Substance-Related Databases

A total of 426 opioids (e.g., 234 fentanyl analogs and 192 non-fentanyl analogs) were identified. The number of NPS.Finder® opioids is indeed higher than that listed by international agencies like the UNODC and the EMCDDA. By May 2019, the INCB identified 115 opioids (International Narcotics Control Board [INCB], 2019a); up to 2019, the UNODC EWA NPS listed 61 different opioids; by June 2018, the INCB listed 93 fentanyl-related substances with no known legitimate use (International Narcotics Control Board [INCB], 2019b); and by April 2019, the EMCDDA reported 51 opioids out a total of 749 EDND substances (European Database on New Drugs [EDND], 2019). There might be different reasons behind these inconsistencies. First, both the UN and EU agencies collect in their databases only those substances that are detected/seized and properly analyzed and most importantly reported, respectively, worldwide or in the European region. Furthermore, the NPS.Finder® carried out a range of open web crawling identification activities focusing on a large range of psychonaut-based, specialized, multilingual, sources with a specific focus on new/old psychoactive substances of likely recreational interest. From this point of view, one could also argue that discussing a molecule on the web is not, *per se*, an indication that the index molecule is being/will be ingested by interested individuals. This can explain why the opioids common to all lists (e.g., the fentanyl analogs 4-fluoroisobutyrfentanyl, acetylfentanyl, acrylfentanyl, butyrylfentanyl, furanylfentanyl, ocfentanil, tetrahydrofuranylfentanyl, and the non-fentanyl analogs AH-7921 and U-47700) are only a small proportion of the total.

Pharmacological and Clinical Considerations

Fentanyl, fentanyl analogs, and the remaining opioids here commented present as partial/full agonists, and with different affinity levels, at the mu, delta, and kappa opioid receptors.

This may well suggest the existence of a possible vast range of ill-health consequences associated with these substances (Stein, 2016). However, a clear understanding of the clinical toxicity of each compound is at present problematic. In fact, although the *in vitro* pKi values/binding affinities, potency, and efficacy levels for a number of opioids are already available (World Health Organization [WHO], 2016; Baumann et al., 2018; World Health Organization [WHO], 2018), these may not provide enough information about the relative *in vivo* potency (Baumann et al., 2018). In fact, there might be variable effects on G-protein-coupled receptors, which could potentially give rise to a great diversity of intracellular consequences following the administration of different analogs with apparently similar pharmacodynamics (Smith et al., 2018). Furthermore, as highlighted using a molecular docking model, some substances are too structurally similar for the scoring function to distinguish between different analogs (Ellis et al., 2018, 2019). For some opioids, larger dosages of naloxone may be required to reverse the opioid toxidrome than needed in case of a typical heroin overdose (Armenian et al., 2018; Lovrecic et al., 2019). The greatest levels of concerns remain related to fentanyl analogs, because of their harmful potential (Schifano et al., 2019a), the continuous high incidence of emerging analogs on the markets over the last years (Schueler, 2017), and the difficulties in identifying them with analytical chemistry techniques (Gerace et al., 2018b; Morrow et al., 2019). Conversely, those substances here identified possessing a full/partial kappa opioid receptor agonist activities (including salvinorin A and its derivatives; tifluadom; and pethidine) are likely to be particularly attractive for opioid and other research chemical consumers prone to a recreational experimentation associated with complex psychoactive effects (Coffeen and Pellicer, 2019).

Limitations

NPS.Finder® crawled only on the open web during the present phase of its development. Future studies by our group will be focused on expanding drug searches on less accessible areas of the web such as the deep web and the darknet (Orsolini et al., 2017). A qualitative/netnographic approach (Loi et al., 2017; Wang, 2019) will be needed as well to better assess the levels of online information relating to the possible psychonauts' preference between analogs and their motivation for use, that is, recreational or self-medication purposes. As previous studies have highlighted their importance in NPS-based studies (Deluca et al., 2012), next NPS.Finder®-based projects will need to focus as well on further languages, for example, Chinese, Japanese, and Arabic. Some consideration needs to be given as well to some opioids not having been reported by NPS.Finder®. The present findings, however, relate only to the psychonauts' interest, who are typically debating/discussing/mentioning only those substances that are considered as "trendy." Furthermore, it is possible that owing to fentanyl/non-fentanyl nomenclature issues, there may be discrepancies between the denomination of substances resulting from international/EU interdiction data and those mentioned by psychonauts.

CONCLUSION

The web crawler activities may well possess the potential to identify a wide range of novel/previously undescribed NPSs, including opioids. The literature base regarding these substances is limited in terms of acute and long-term effects, adverse effects, abuse potential, and manufacturing/distribution in both the virtual and real markets. It is difficult for health professionals to keep up to date with the growing number of opioids being made available. Clinicians are not always aware of the risks relating to novel psychoactives' intake, and, at the same time, they are not typically able to identify a potential NPS user (Simonato et al., 2013). This may be a reason for concern, especially for emergency professionals confronting acute, and at times dramatic, clinical situations that are suspected of being drug related but in which the standard urine specimen turns out to be negative (Guirguis et al., 2017). Hence, clinicians should be informed about the range of opioids, their idiosyncratic drug–drug combinations, and their medical risks (Orsolini et al., 2015). The availability of new digital technologies, and the current systematic web crawling activities, provided here better levels of knowledge about the emerging number of opioid derivatives. This may help in designing and developing a range of efficient opioid on−site screening and detection techniques (Guirguis et al., 2017) and in drafting potent opioids' specific treatment and management guidelines.

Raising awareness and education among users, the general public, and frontline staff and the development of harm reduction techniques are of paramount importance to tackle the flood of opioids. The epidemic of opioid overdose is a complex problem that can only be addressed by concerted and multidisciplinary efforts. Indeed, *in silico*, *in vitro*, and *in vivo* studies could provide important findings; furthermore, it is deemed here essential to monitor the real-life scenarios through drug checking in interdiction, drug outpatient clinics, and critical care settings. The provided lists can be useful in prospective and forward-looking terms. More studies should aim at providing better levels of misusing drugs' clinical pharmacological-related knowledge, so that properly tailored prevention strategies can be drawn up and made available.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

FS and AV have conceived the idea of the manuscript and have coordinated the whole project. FN, CZ, DA, and LG have actually carried out the process of both data collection and systematization. DA performed the literature searches and the analysis of data and drafted the manuscript. FS and JC supervised the writing of the manuscript and contributed to the final version of the manuscript. FS and EA approved the final content of the manuscript. JC provided data from the EMCDDA and UNODC

databases for the purposes of this research. FS, JC, and AG have provided relevant epidemiological data and have contributed as well to the drafting of the manuscript itself.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.00149/full#supplementary-material

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frontiers in Psychiatry

ORIGINAL RESEARCH
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Personality Traits and Psychotic Proneness Among Chronic Synthetic Cannabinoid Users

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Objective: Chronic use of synthetic cannabinoids (SCs) has been associated with a wide range of negative consequences for health including psychotic and affective disturbances. Accumulating evidence indicates that cannabinoids use may be a risk factor for schizophrenia, and chronic natural cannabis users score higher than non-users on measures of schizotypal personality traits. However, little is known regarding the personality characteristics of SC users, especially in comparison with recreational cannabis users and healthy individuals. This study aimed to examine the differences in personality characteristics and schizotypy between SC users, regular cannabis users, and non-users and to compare these measures between groups.

Methods: Forty-two chronic SC users, 39 natural cannabis users, and 47 non-using control participants, without history of mental disorder, or current substance use diagnosis (mean age 26± 4.47 years; 23 females, 105 males), completed the Big-Five Factor Inventory (BFI), the Schizotypal Personality Questionnaire-Brief (SPQ-B), substance use history, rating scales of depression and anxiety, and a demographic questionnaire.

Results: On the BFI, SC users scored higher than natural cannabis users and non-users on neuroticism, but lower on agreeableness and extraversion, and endorsed greater schizotypal symptoms on the SPQ-B. In addition, SC users had lower scores on conscientiousness than non-users, and natural cannabis users were more extroverted than non-users. Higher openness and lower conscientiousness predicted schizotypy for both SC and natural cannabis users. Finally, greater neuroticism predicted schizotypy for natural cannabis users, and introversion predicted schizotypy for non-users.

Conclusions: These results show that chronic SC users differ from natural cannabis users and non-users on dimensions of specific personality traits and schizotypy that may indicate psychotic proneness.

Keywords: synthetic cannabinoids, personality, psychosis, cannabis, addiction

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INTRODUCTION

Epidemiology

Cannabis is the most popular recreational psychoactive substance following tobacco and alcohol (1). Around 4% of the global adult population has used cannabis in their life. In the United States of America (USA) alone, at least 36 million people used cannabis at least once in their lifetime (1, 2). Since several countries have conducted a decriminalization policy regarding the possession of cannabis for recreational use and possession of cannabis in small amounts (1, 3), it seems likely that the consumption of cannabis will increase in the coming years (4). Recently, a new type of cannabinoid-based drugs has started to be consumed recreationally among drug users across the globe (5, 6). These new cannabinoid-based drugs classified as novel psychoactive substances (NPS) and are composed a high concentration of SCs (7–10). Drug brands such as "Spice" and/or K2 are generally used to describe the diverse types of herbal blends that encompass synthetic cannabinoids (SCs), same as other NPS, individuals who consume SCs are typically attracted by these substances due to their intense psychoactive effects and likely lack of detection in routine drug screenings (10–12). Lifetime prevalence of SC use in the general population is similar to other NPS and ranges between 0.2 to 4% (13). Contrary to other types of NPS, SC use has not been associated with low educational levels or low incomes (14) and SC users are mostly young males, high school graduates using other recreational drugs (15). Since the beginning the current decade, the existence of more than a hundred different types of SCs were documented by the European Union Early Warning System. These drugs are mainly sold online as a "legal" alternative to controlled and regulated psychoactive substances. They appear to have a life cycle of about few years before being replaced by a next generation of products. Regulation controlling these NPS has been introduced in several states in order to limit the spread of existing drugs and control potential new analogs (16).

Neurobiology

Synthetic cannabinoids compared with natural cannabis have higher affinity with endogenous cannabinoid receptor type-1 (CB_1) and/or endogenous cannabinoid receptor type-2 (CB_2) with a high affinity/potency and they are full receptor agonists [Δ9-tetrahydrocannabinol (THC) is a partial agonist]. Unlike natural cannabis, in SCs there is no cannabidiol (CBD) (which may protect against psychosis) and they have longer half-life active metabolites. Their; effects are more intense and longer lasting, bringing greater health risks, more powerful, and unpredictable effects, with higher toxicity and overdose potential than THC. SCs are mainly consumed by smoking; solely or with cannabis, when absorbed, SC induce a wide range of adverse effects, some of them are similar to the psychotropic effects of cannabis (7–9). However, the acute effects are more intense, in terms of duration and severity induce both somatic and psychiatric adverse effects (8, 9, 12, 15, 17, 18). Although the chronic toxicity of SC is still not well known, recent studies have found neuronal alterations, cognitive impairments, and mental distress in chronic SC users (19–22). Interestingly, recent data has indicated that SC hold a greater risk for psychosis compared to regular (non-synthetic) cannabis (23, 24). Previous studies have linked chronic use of cannabis to personality dimensions associated with increased psychosis-proneness, or schizotypy (25–27). However, there is limited information regarding the personality characteristics of SC users and the relations of personality and schizotypy to this population. The identification of personality traits specific for SC users could be useful for the development of effective screening instruments and future prevention and intervention strategies of psychosis-proneness in this population (28–30). The main object of the current study was to explore the personality characteristics of SC users compared with those of cannabis users and non-user subjects, and to examine the relationships between personality factors underline schizotypy in SC users compared with regular cannabis users and non-users.

Synthetic Cannabinoids and Related Adverse Effects

Similar to regular cannabis, the primary psychoactive constituents of SC drugs interact with CB_1 and CB_2 receptors (5, 7, 9, 31, 32). There is an agreement that the activation of CB_1 receptors following consumption of an exogenous cannabinoid-agonist may underline the psychoactive effect of cannabinoid-based drugs (33–36). In contrast to regular cannabis, SCs contain extremely potent CB_1-receptor full-agonist as well as additional psychoactive ligands, and are missing anti-psychotic CB_1-receptor-antagonist ligands such as CBD (5, 7, 9, 31, 32, 36). Furthermore, SC drugs are composed of variable concentrations of a wide range of other ingredients, have a longer half-life active metabolite, and induce long-lasting and unpredictable adverse effects bringing greater health risks with higher toxicity and overdose potential than regular cannabis (23, 32, 36). These features hold by SC drugs may indicate their great harmful-potential (37, 38).

Although the acute and chronic toxicity related to SC use is still not well known, the negative consequences associated with SCs include a variety of psychoactive effects, such as: mood alterations, anxiety, paranoia, cognitive impairment, dissociation, excitability and agitation, sedation, and psychosis (17, 24, 37, 39–42). The use of SC may trigger the occurrence of severe psychosis in psychosis-prone users or the exacerbation of a prodromal psychotic syndrome in healthy individuals, due the rigid psychopathological issues associated with SC intoxication it is sometimes referred to as "spiceophrenia" (18). In addition, physical effects included nausea, vomiting, diarrhea, tremors, hypertension, tachycardia, and symptoms of dependency (24, 37, 39–42) **Table 1** describes clinical side-effects of synthetic cannabinoids.

Some symptoms such as cardiovascular events, seizures, agitation, hypertension, emesis, and hypokalemia are features of SC intoxication and are not present even after consuming high doses of regular cannabis (19, 24). Severe toxicity due to SCs has been required medical intervention mostly of neuropsychiatric and cardiovascular clinical manifestations (43–45). Since the use

TABLE 1 | Summary of clinical side-effects of synthetic cannabinoids.

Acute psychopathology	Agitation, manic episode, anxiety, irritability, disorganized behavior, violent behavior, aggression, altered visual/auditory perception or hallucinations, delusion, confusion, altered attention and concentration, amnesia and memory impairment, paranoia, mood alterations, suicidal ideation, sedation, catatonia, (6, 7, 12, 17, 24, 37–50). Chronic use may increase the risk for developing psychotic disorders (18, 32, 36, 46).
Other acute toxicity	Tachycardia, drowsiness/lethargy, hypertension, headache, nausea/vomiting, tremor, dizziness/vertigo, ataxia, dysarthria, angina, palpitations, dyspnea, mydriasis, bradycardia, hypotension, rhabdomyolysis, seizures, stroke, arrhythmias, myocardial infarction, emboli, encephalopathy, acute kidney injury, coma, including agitation, mydriasis, diaphoresis, tremor, clonus, hyperreflexia, hyperthermia (38, 42–45).
Possible long-term adverse effect	Adverse effect on cognitive functions including; memory alteration, attention difficulties, thinking problems, and slow responses, alterations in brain's structure and functions (20–22, 51, 52). Chronic use may increase the risk for developing psychopathology including mood and psychotic disorders (38, 42–45).

of SC is rapidly growing together with it increasing health related events recent works have suggested that actions such as; prompt reliable information available for health professionals, more specific analytic techniques, designed preventive strategies for at-risks categories, and for law enforcement strategy in the commune are all required to face the SC phenomena (53).

Recently, several cohort studies have shown evidence for cognitive deficits and affective alterations in chronic SC users (20–22, 46). Complementary neurobiological studies demonstrated in chronic SC users alterations in brain regions which are involved in cognitive and emotional function (22, 51, 52). The evidence of neuronal damage associated with chronic use of SC is alarming, since it may indicate possible neurotoxic effects of SC drugs (22, 51, 52). Moreover, SC use is common among teenagers and young adults, who are more vulnerable to the negative impact of cannabinoids on the central nervous system (CNS) (19, 54–56).

Cannabinoids and Personality Factors

Beside early age, other factors such as personality predisposition are associated with cannabis use and are linked with greater vulnerability to the adverse effect of cannabinoid use (25–27, 57). Personality characteristics such as sensation seeking, anxiety, and emotional distress are associated with early onset of drug use (29, 58), and greater levels of emotional imbalance and extraversion are associated with increased risk for developing psychosis following chronic regular cannabis use (25). A large-number of studies describes the association between drug-abuse and personality characteristics. Although several studies examined the motives and demographic characteristic of SC users, yet, there is no available information on the personality dimensions for this population (11, 47, 59, 60). On the other hand, there are few studies that have characterized the personalities of chronic regular cannabis users. Flory et al. (61) have found that symptoms of cannabis dependency were negatively correlated with agreeableness and conscientiousness and were associated with openness. However, after controlling additional factors such as alcohol consumption, antisocial personality disorder, and internalizing disorders, cannabis dependency was positively correlated with openness and negatively associated to extraversion (61). Later on, Terracciano et al. have conducted an epidemiological study and found that current cannabis users were lower in agreeableness and conscientiousness, but higher in openness, relative to healthy non-users control subjects. However, in their study additional confounding factors were not controlled (62). Allen and Holder have found that regular cannabis use has been associated with lower agreeableness and lower conscientiousness (63). Similar result was found by Tartagila et al. who showed an association between cannabis use and low levels of agreeableness and conscientiousness, and higher levels of openness in a sample of university students (64). In an epidemiological study conducted by Hengartnet et al. cannabis consumption has been found to be associated with higher scores on extraversion and openness, and lower scores on conscientiousness (28). Altogether, these studies provide strong evidence on the association between personality traits and regular cannabis use, yet the pattern of the result is inconsistent, possibly due to the heterogeneity in the studied populations and confounding factors. Interestingly, Friedberg and colleagues have shown schizotypal features that were common among cannabis users and had been associated with certain personality characteristics. In their study, they have compared a group of cannabis users with a group of healthy drug-naïve control subjects and they have found higher scores of openness, and lower levels of agreeableness and conscientiousness in regular cannabis users compared with control subjects. Moreover, higher levels of neuroticism predicted schizotypy in all participants and extraversion predicated negative schizotypal symptoms (25). Greater levels on schizotypal measures were reported in earlier several studies in regular heavy cannabis users (25–27, 65–67). However, it is unclear whether the association between schizotypal symptoms and regular cannabis use is a result of repeated cannabis use, inherited predisposition, or additional confounding factors (25, 67).

Rational and Aims of the Current Study

The main aim of the present study was to investigate the personality characteristics of SC users compared with natural cannabis users and non-users on measures of the Big-Five Factors (BFI) (68, 69) and schizotypy (70), and to examine the relations among those measures within each group. To our knowledge, this is the first study to investigate the personality profile of SC users compared with natural cannabis users. We hypothesized that SC users would show higher levels of schizotypy compared with natural cannabis users and non-users, suggesting greater psychosis-proneness. In addition, we have predicted that SC users would present greater levels of neuroticism and introversion and lower conscientiousness, than the two control groups. Finally, we plan to investigate the contribution of depression, anxiety, and personality traits to the variance of schizotypal scores in all groups.

METHODS

Participants

One hundred and twenty-eight participants were recruited for the study, including 105 males and 23 females. The mean age was 26.21 (SD=4.46) years. The total sample was divided to three groups based on their self-reported substance use history: a) SC users, b) regular cannabis users, and c) non-users. SC users were recruited from the Israeli Ministry of Health drug addiction treatment programs. Both regular cannabis users and non-users were recruited by convenient sampling *via* friends, relatives, or social networks.

Synthetic Cannabinoid Users

The SC users group comprised of 42 subjects, 32 males, and 7 females, who have frequently consumed SC drugs during the last 2 years. We have defined the inclusion criteria for SC users as a regular use on a monthly basis with a minimal usage of at least 10 times in the last year and without binge consumption defined as more than 4 usages of SC during the last month. The mean age was 27.1 (SD=5) years. Participants were cannabinoid-free for at least 1 week prior the study, were evaluated by a senior psychiatrist and diagnosed as not suffering from current psychosis or comorbid psychiatric or neurological disorders or a past or current substance use disorder other than cannabinoids.

Natural Cannabis Users

The group of natural cannabis users included 32 males and 7 females. Altogether, there were 39 subjects that used cannabis on a monthly basis with minimal usage of at least 10 times in the last year and without binge consumption defined as more than four usages of cannabis during the last month and they were cannabinoid-free for at least 1 week. The mean age in the natural cannabis user group was 25.25 (SD=3.51) years. Exclusion criteria for natural cannabis participants were history of neurological or psychiatric disorders and history or current or past substance use disorder other than cannabis.

Non-Users

The group of non-users included 40 males and 7 females, altogether 47 healthy individuals, who have reported that they did not consume cannabinoid-based drugs during the last 2 years. The participants' mean age was 26.2 (SD=4.5) years. Exclusion criteria for healthy control participants were history of neurological or psychiatric disorders and history or current substance use disorder.

Ethical Approvals

The Ariel University Review Board and the Israeli Ministry of Health have approved the study. All participants volunteered to participate in the study and did not get any reward for their participation. All the participants were above the age of 18 years, and signed an informed consent prior to participation.

Materials and Design

Sample Characteristic and Substance Use History

The demographic questionnaires included items on education level, age, gender, and information regarding current or past neurological or psychiatric disorders. The questionnaires also contained items regarding the use of psychoactive substances, focusing on cannabis and SCs, as well as tobacco and alcohol. Data on the age of first use, the frequency of usages past month, and past year of cannabis and SC use were recorded.

Depression and Anxiety Levels

Depression and anxiety symptoms levels were recorded as well using the Beck Depression Inventory (BDI) (Cronbach internal reliability of $\alpha = 0.91$) (71, 72), and the Spielberger State-Trait Anxiety Inventory (STAI-S, STAI-T) (Cronbach's $\alpha = 0.91$ and 0.85; respectively) (73).

Big-Five Factors Inventory

The BFI questionnaire was used to asses personality traits (68, 69). The BFI consists of 44 self-rated items on a five severity scores from 1 =strongly disagree to 5 =strongly agree. Each item represents one of the core traits that define each big five domains; extraversion, neuroticism, agreeableness, conscientiousness, and openness to experience. Total mean scores for each of the personality factors were recorded for each participant. The Hebrew version of the BFI was translated and validated previously, Cronbach's α reliability of the Hebrew version domains ranged from 0.63 to 0.83 (74). In this study, the BFI Cronbach's α reliability score ranged from 0.86 to 0.34.

Schizotypal Personality Questionnaire

The Schizotypal Personality Questionnaire-Brief (SPQ-B) was used to measure psychotic proneness (70). The SPQ-B is a 22-item (true/false) self-report for the assessment of schizotypal personality disorder or dimensional schizotypy. The SPQ-B consists of three subscales: a) cognitive-perceptual deficits, b) interpersonal problems, and c) disorganized symptoms. Each "true" response counts as one-point, total scores ranging from 0 to 22. The internal consistency indices of the SPQ-B ranged from 0.75 to 0.83 (from 0.58 to 0.83 for the subscales) and the test–retest reliability from 0.82 to 0.90 (75). In this study, the SPQ-B had a Cronbach internal reliability of $\alpha = 0.87$.

Statistical Analysis

The analysis of the results was performed on Statistical Package for Social Science (SPSS) for windows v.21 (IBM Corp. Armonk, NY). Differences between groups in terms of gender were tested using chi-square test and a multivariate analysis of variance (MANOVA) was used to calculate the effect of group on BFI domains, further one-way ANOVAs indicated the sources of significant group main effects. One-way ANOVA was conducted to examine group main effects on SPQ-B overall and sub-scale scores; Student's t-tests followed by Bonferroni *post hoc* corrections were used for group comparisons. In additional analyses, anxiety rates and depression were added as covariate factors to the initial models in order to explore the possibility of confounding variables. Finally, hierarchal regression models were computed separately for each group in order to explore relationships between SPQ-B and BFI factors, depression and anxiety.

RESULTS

Sample Characteristics and Substance Use History

Participant's drug use history and demographic data are described in **Table 2**. Groups did not differ by gender, age, education level, or by rates of alcohol use history. SC users have consumed more tobacco cigarettes per day than either non-users and natural cannabis users SC users had used cannabinoid-based drugs at an early age than natural cannabis users In addition, SC users have scored higher on the BDI than non-users and natural cannabis users but there were no differences in BDI scores between natural cannabis and non-user groups SC users had higher scores on STAI Trait and State scales compared to natural cannabis users and non-users. There were no differences in STAI State and Trait scores between natural cannabis users and non-users.

The Big-Five Factors

The mean scores on BFI factors by group are presented in **Table 3**. Initial analysis showed a significant effect of the groups on BFI [Wilks' lambda=0.61, F (10, 240) = 6.91, $p < 0.001$]. Further one-way ANOVAs have indicated a main effect of group on neuroticism, extraversion, conscientiousness, and agreeableness. SC users had higher neurotic scores, lower ratings of agreeableness, and lower ratings of extraversion, than natural cannabis users and non-users. Furthermore, SC users had lower conscientiousness scores than non-users yet, there were no differences in conscientiousness between SC and natural cannabis users and between natural cannabis users and non-users. The groups did not differ on scores of openness (**Figure 1**). Finally, when anxiety or depression were entered to the MANOVA as covariate factors, the effects of group on neuroticism [$F(2,121)= 1.31$, $p=0.27$; $F(2,121)= 4.07$, $p < 0.05$, respectively], extraversion [$F(2, 121)= 4.49$, $p < 0.05$; $F(2,121)= 5.48$, $p < 0.01$, respectively], conscientiousness [$F(2, 121)= 1.27$, $p=0.28$; $F(2,121)= 1.51$, $p=0.22$, respectively], and agreeableness [$F(2, 121)= 3.05$, $p=0.05$; $F(2,121)= 2.7$, $p=0.07$, respectively] were reduced.

Schizotypal Personality Questionnaire

Table 4 shows Schizotypy questionnaire dimensions by all groups of participants.

Analysis revealed the main effect of group on SPQ-B scores. SC users had greater score on the SPQ-B compared with natural cannabis users [t (79) = 6.44, $p < 0.01$] and non-users [t (87) = 6.84, $p < 0.01$]. There were no differences on the SPQ-B between natural cannabis and non-users [t (84) = 0.47, $p=1.00$]. There were main effects of groups on SPQ-B's sub-scales: cognitive-perceptual, interpersonal, and disorganization. SC users have scored higher than natural cannabis users [$t(79)= 2.80$, $p < 0.001$ and $t(79)=3.76$, $p < 0.001$; $t(79)= 4.65$, $p < 0.001$, respectively]

TABLE 2 | Demographic and questionnaires' ratings in all participants.

	Synthetic	Cannabis	None	Comparison	Significance
N, frequencies (male: female)	42 (32:7)	39 (32:7)	47 (40:7)	[a] 0.63	$p > 0.05$
Age, mean (SD)	27.1 (5)	25.25 (3.51)	26.2 (4.5)	[b]1.63	$p = 0.2$
Education level (SD)	12.3 (2)	12.1 (0.9)	12.8 (2.17)	[b]1.5	$p = 0.21$
Alcohol consumption (SD)	2.83 (2.61)	3.74 (1.72)	3.54 (2.55)	[b]1.99	$p = 0.14$
Tabaco consumption (SD)	16.85 (10)	4.82 (6.6)	2.7 (4.66)	[b]43.78	$p < 0.001$
Age of first use for cannabinoids	15 (6.56)	18.41 (4.72)	–	[c]2.66	$p < 0.01$
Frequency of cannabinoids use during the last month	21.53 (93.7)	19.5 (32.82)	–	[c]0.13	$p = 0.89$
Frequency of cannabinoids use during the last year	208 (146.31)	185 (134)	–	[c]0.75	$p = 0.45$
BDI, mean (SD)	39.19 (8.7)	27 (7.73)	24.9 (4.37)	[b]49.4	[d]$p < 0.001$
STAI Trait, mean (SD)	49.64 (6.58)	34.35 (7.47)	34.84 (8)	[b]51.54	[d]$p < 0.001$
STAI State, mean (SD)	48.38 (7.4)	32.97 (9.91)	32.84 (9.54)	[b]41.13	[d]$p < 0.001$

Age and education level reported in years; alcohol consumption habits drink defined as glass of wine or 250 ml of beer or one shot of alcoholic beverages; tobacco consumption, cigarettes per day; frequencies of cannabinoids use defined as event of consummation of cannabinoid-based drugs; BDI, Beck Depression Inventory scores; STAI, Silberberg Trait or State Anxiety inventory scores; SPQ-B, Schizotypal Personality Questionnaire Brief; significant level of difference between drug groups within the total sample; [a]χ^2; [b]$F(2,125)$; [c]$t(78)$, differences observed for SC vs. cannabis; [d]$t(78)$, differences between SC vs. cannabis users, and $t(84)$ SC vs. non-users.

TABLE 3 | Mean scores of groups for each of the Big-Five Factor Inventory (BFI) sub-scales.

	Group			Comparison	
	SC	Cannabis	None	$F(2,125)$	P-value
Extraversion	3.12 (0.51)	3.76 (0.74)	3.45 (0.55)	12.26	[a] [b]$p < 0.001$
Neuroticism	3.25 (0.63)	2.27 (0.69)	2.4 (0.76)	24.10	[a]$p < 0.001$
Agreeableness	3.31 (0.51)	3.72 (0.61)	3.81 (0.55)	8.45	[a]$p < 0.001$
Conscientiousness	3.47 (0.52)	3.83 (0.44)	3.83 (0.44)	5	[c]$p < 0.01$
Openness	3.35 (0.65)	3.53 (0.47)	3.36 (0.47)	0.9	0.42

Values are mean (SD).[a]Difference observed for SC vs. cannabis and for SC vs. non-users; [b]differences observed for non-users vs. cannabis; [c]difference observed for SC vs. non-users.

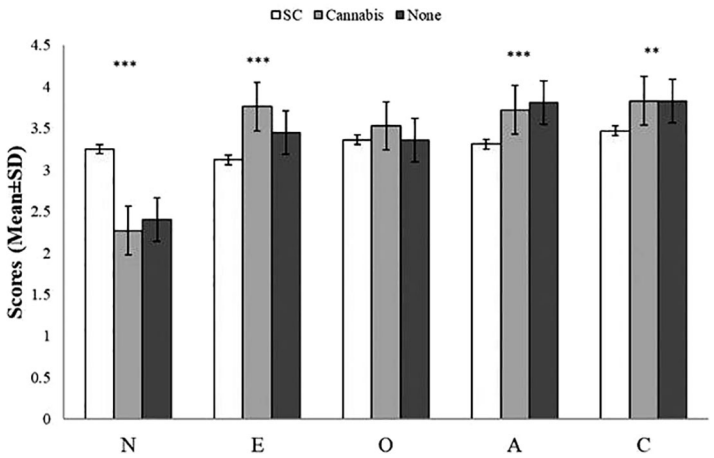

FIGURE 1 | Scores (mean ± SD) of the Big-Five Factor Inventory (BFI) sub-scales by group. There was a main effect of group on neuroticism (SC>Cannabis, SC>Non-users, Cannabis=Non-users), extraversion (SC<Cannabis, SC<Non-users, Cannabis>Non-users), aggreableness (SC<Non-users, SC<Cannabis, Cannabis=Non-users), and conscientiousness (SC<Non-users, SC=Cannabis, Cannabis=Non-users); there were no differences among group in openness; ***p < 0.0001, **p < 0.01; A, agreeableness; C, conscientiousness; E, extraversion; O, openness; N, neuroticism.

TABLE 4 | Schizotypy questionnaire dimensions by groups.

	Synthetic	Cannabis	None	Comparison	
				F(2,122)	*p*-value[a]
SPQ-B total score	11.64 (5)	5.35 (3.58)	4.22 (3.19)	41.98	*p < 0.001*
SPQ-B cognitive-perceptual	4.57 (2.27)	1.76 (1.5)	1.43 (1.46)	38.73	*p < 0.001*
SPQ-B interpersonal	3.88 (2.26)	2.10 (1.97)	1.84 (1.52)	13.84	*p < 0.001*
SPQ-B disorganized	3.19 (1.75)	1.48 (1.51)	0.95 (1.18)	25.77	*p < 0.001*

[a]*Difference observed for SC vs. cannabis and for SC vs. non-users.*

and non-users [$t(87)= 6.95$, $p < 0.001$, $t(87)= 4.20$, $p < 0.001$ and $t(87)= 5.53$, $p < 0.001$, respectively]. There were no differences between natural cannabis users and non-users in either cognitive-perceptual [t (84) = 0.70, p=1.00], interpersonal [t (84) =0.04, p=1.00], or disorganization [t (84) = 0.75, p=1.00] sub-scales. The effect of group on SPQ-B score remained significant when anxiety [$F(2,121)=17.87$, $p < 0.001$], and depression [$F(2,121)=16.37$, $p < 0.001$] were used as covariates, a similar pattern was observed for SPQ-B's sub-scales; cognitive-perceptual [$F(2,121)= 20.54$, $p < 0.001$; $F(2,121)= 18.04$, $p < 0.001$, respectively], interpersonal [$F(2,121)= 3.26$, $p= < 0.05$; $F(2,121)=3.12$, $p < 0.05$, respectively], and disorganization [$F(2,121)= 12.61$, $p < 0.001$; $F(2,121)= 11.68$, $p < 0.001$, respectively].

Association Between Schizotypal and Personality

In order to explore the relationships between schizotypal trait and personality factors a serial of hierarchical multiple regression analyses was conducted; first for the whole sample and then for each group separately with the schizotypal scores as a dependent variable and BFI domains as predictors. In order to account for group differences, depression and anxiety variables were entered in the first step of the model and scores of all BFI factors were entered in the second step. In the first repression model, personality traits significantly contributed to the variance of schizotypy after controlling for depression and trait and state anxiety scores. Beside depression, higher scores of openness and neuroticism and lower scores of extraversion predicted schizotypy. Specific analysis of each group showed that high scores of openness and lower scores of conscientiousness predicted schizotypy for SC and natural cannabis users. Finally, greater neuroticism predicted schizotypy for natural cannabis users and introversion predicted schizotypy for non-users. **Table 5** shows hierarchical multiple regression analysis predicting schizotypal scores by the scores of depression, anxiety, and personality traits for the three groups.

DISCUSSION

The purpose of the present study was to explore the personality characteristics of SC users and compare them to those of natural cannabis users and non-users. Our results showed that chronic SC users differ from both natural cannabis users and non-users on the BFI personality traits and schizotypy measures. On the BFI, SC users had higher scores of neuroticism and lower scores of agreeableness and extraversion compared with natural

TABLE 5 | Hierarchical multiple regression analysis predicting schizotypal scores by the scores of depression, anxiety, and personality traits for the three groups.

	Factors	B	β	Total R^2	*F* Value	*p*-Value
Total sample	First step			0.31	26.57	$p < 0.0001$
	Depression	0.22	0.41$^{**}_{\|\|}$			
	Anxiety	0.08	0.17			
	Second step			0.46	14.06	$p < 0.0001$
	Neuroticism	2.15	0.34**			
	Extraversion	–1.67	–0.22**			
	Openness	1.47	0.19*			
	Conscientiousness	–1.4	–0.15$^{¥}$			
	Agreeableness	–0.1	–0.01			
Non-users	First step			0.16	3.84	0.03
	Depression	0.22	0.3			
	Anxiety	0.05	0.11			
	Second step			0.43	3.92	0.003
	Neuroticism	1.31	0.28			
	Extraversion	–2.6	–0.46**			
	Openness	–0.4	–0.06			
	Conscientiousness	0.93	0.12			
	Agreeableness	0.65	0.12			
Cannabis	First step			0.09	1.72	0.19
	Depression	0.12	0.23			
	Anxiety	0.04	0.1			
	Second step			0.47	3.78	0.005
	Neuroticism	2.74	0.52*			
	Extraversion	–0.92	–0.20			
	Openness	2.55	0.36*			
	Conscientiousness	–1.79	–0.34*			
	Agreeableness	–0.52	–0.9			
Synthetic	First step			0.12	2.53	0.09
	Depression	0.13	0.25			
	Anxiety $^{\|\|}$	–0.31	–0.41*			
	Second step			0.43	3.53	0.006
	Neuroticism	1	0.13			
	Extraversion	–1.31	–0.45			
	Openness	2.02	0.39*			
	Conscientiousness	–4.16	–0.46 *			
	Agreeableness	0.57	0.08			

Depression and anxiety scores were entered simultaneously in step 1; rhe scores of depression (Beck Depression Inventory), anxiety (mean score of both Spielberger state and trait anxiety inventory), and Big-Five Factor Inventory (BFI) personality traits were entered simultaneously in step 2;$p < 0.05$, **$p < 0.01$, $^{||}p < 0.01$ in step 2, $^{¥}p = 0.05$.*

cannabis users and non-users. In addition, SC users have presented lower levels of conscientiousness relative to non-users, and similar scores of openness compared to both control groups. These results are consistent with previous studies that showed an association between drug use disorders including cannabis and higher neuroticism, lower conscientiousness and agreeableness, and scores on the extroversion-introversion scale (29, 62, 76–82).

Synthetic Cannabinoids and Neuroticism

Neurotic individuals usually experience high levels of negative affect, suffer from anxiety and depression, and have a low activation threshold in the face of external or internal stressors (83). SC users in this study, as in previous studies, also showed elevated symptoms of depression and anxiety (21, 22, 84). According to recent studies on the role of neuroticism in the etiology of addictive disorders, high levels of neuroticism predispose individuals to both personality and substance use disorders. Thus, neurotic individuals are prone to use psychoactive agents which accord their excessive physiological arousal (85). Interestingly, neuroticism was found to be associated with cocaine, opioids, and amphetamine use (69, 79, 86). However, the association between neuroticism and cannabis is mixed, as it seems to be influenced by additional factors such as: extensive cannabis use, mood, anxiety, and psychiatric conditions (25, 64, 87). Chowdhury et al. (2015) have found an association between cannabis and neuroticism among regular cannabis users in a community-based study. Yet, most of the participants in their research have been met the criteria of depressive disorder, generalized anxiety disorder, or alcohol abuse (87). In addition, the sample contained a mixture of both regular and occasional cannabis users. Later-on, a series of studies have failed to show an association between neuroticism and cannabis use, these investigations composed samples of cannabis user who did not suffer from psychiatric symptoms as well as current substance-abuse (25, 63, 64). Furthermore, similar to the present study, the samples of cannabis users in these studies (25, 63, 64) were composed from recreational cannabis users. Thus, it is reasonable to assume that regular cannabis users show high neuroticism whereas recreational users show low neuroticism. The low neuroticism score in recreational cannabis users in our study is therefore since our subjects were not regular users and did not show the psychological distress that may affect the association between cannabis use and neuroticism (25, 63, 64).

Synthetic Cannabinoids and Conscientiousness

SC had lower scores on conscientiousness compared to non-users as well as lower scores of agreeableness than both control groups. Recent reports had indicated that SC users were prone to manifest antisocial behaviors and tend to be aggressive, manipulate, impulsive, and hostile toward others (47, 84, 88, 89). Low levels of conscientiousness are often associated with impulsivity, mental distress, risk taking behaviors (including health risks), and maladaptive coping strategies (69). Low scores of conscientiousness not only enhance the chance of health risk taking behavior, but also affect the mechanisms which regulate the maintenance of drug abuse (90). Low scores of agreeableness are associated with emotional detachment from others, suspiciousness as well antagonism, and dishonesty (91). Together, low agreeableness and conscientiousness characterize the personality profile of chronic drug users (92, 93). Accordingly, low levels of these traits may predispose individuals to abuse substances or may account for problems in interpersonal relationships which are commonly associated with drug use disorders (94). The low levels of agreeableness and conscientiousness of SC users in the current study may be associated with difficulties with authority and health risk taking behaviors in SC users (29, 94, 95).

Synthetic Cannabinoids and Extraversion

SC users also showed lower scores on extraversion (i.e., *introverts*), while natural cannabis users had higher scores on extraversion (i.e., *extroverts*) compared with non-users. Introverts are less interested in the external world, they are imaginative, tend to live within themselves, and they avoid referring themselves to social supports in order to minimize confrontation with stressful situations (96). Higher scores on this scale are associated with greater risk for suicidal attempt (97). Previous studies have linked SC use with suicidal ideation and relatively high incidence of suicide attempts (98–100). On the other hand, extroverts tend to be sociable and sensation seeking, they often exhibit lack of behavioral constrain and fail to conform to conventional norms (100). Consistent with the former, Bozkurt and colleagues (2014) have found that SC users preferred to use SC alone rather than with a companion (59), and a recent report indicated that users attempted to consume SC secretly (60). Additionally, drug users prefer to communicate with others *via* social media rather than direct interpersonal communication (92). Contrary to SC users, natural cannabis use is common in social settings (101), and extroverts may appreciate and pursue the social ritual and support associated with cannabis use (102). The former indicates that while SCs are commonly used individually and secretly, regular cannabis is mostly consumed in a group setting, as a part of social activity. This observation is consistent with the differences between SC and natural cannabis users in extraversion levels obtained in our study. However, the association between cannabis use and extroversion is inconsistent. Flory et al. (61) have reported that introversion has been associated with cannabis dependence (61). On the other hand, Hengartnet et al. (2016) showed that extraversion is associated with cannabis use (28). Later on, several studies have reported no association between introversion or extraversion and natural cannabis users (25, 62–64). Importantly, while acute intoxication of cannabis has been associated with increased levels of sociability and empathy toward others (103), chronic natural cannabis use often induces "amotivational syndrome," a psychological condition in which social withdrawal is considered to be a prominent expression (104, 105). It is possible that the high introversion scores in SC users may reflect this syndrome, as a result of chronic consumption of potent SCs. The natural cannabis users were young adults who smoked relatively small amounts of low-potency cannabis and therefore may not show the "amotivational syndrome" and this may explain the differences between SC and regular cannabis users, at least with regard to introversion. Finally, the current study suggests that neuroticism, low agreeableness, high introversion, and low conscientiousness are the personality characteristics of SC users. Although there is insufficient evidence on an exclusive personality profile for drug users (106), our results are in accord with a well-designed meta-analysis study which showed that a personality profile of high neuroticism, low agreeableness, introversion, and low conscientiousness is associated with a wide range of psychiatric disorders (107). Accordingly, a common pattern of personality characteristics is observed in drug dependent individuals regardless of the specific drug being used (107). In light of the current results, it is reasonable to assume that individuals with increased risk for developing drug use disorder tend to use SC drugs. Moreover, it may further imply that SC users and natural cannabis users represent a different type of population.

In summary, the personality characteristics that were identified in the current study for SC users are: a) consistent with previous studies described behavioral and psychological symptoms in chronic SC users, b) may underlie part of the psychological mechanisms of SC addiction, and c) may indicate that SCs attract individuals with a unique, problematic personality characteristics, which are different from natural cannabis users.

Synthetic Cannabinoids and Psychosis

We have found that SC users have shown greater scores of schizotypy traits compared to natural cannabis users and non-users. This finding accords previous indications for the association between psychotic proneness and chronic cannabis use disorder (25–27, 65–67, 108). The association between cannabinoids and psychosis is well documented and recognized (1, 19, 33, 35, 54, 109–112). Converging data suggests that cannabis use has the potential for inducing psychosis (1, 19, 33, 35, 54, 109–112). The evidence may explain the relatively high incidence of severe psychosis that have been observed in chronic SC users. Vallersnes and colleagues (2016) have reported that SCs were the drugs most frequently involved in presentation of psychosis to an emergency department in Europe (48). In England, 28% of the SC users who were referred to health professionals due to SC intoxication had presented a severe psychotic episode (45). Recent reports in Europe suggest that 15% of SC users who report to emergency departments present psychotic symptoms (48). These figures are far greater compared to those using other types of psychoactive substance (48). In addition, compared with natural cannabis, psychotic symptoms that are associated with SC are more aggressive and rigid, accompanying with elevated and prolonged mental distress (49, 84, 89). In an Israeli retrospective cohort study, Shalit et al. retrieved data from a period of 7 years in order to examine demographic and clinical characteristic of SC users admitted to a mental-health center in comparison to regular cannabis users. Patients admitted following use of SC had higher severity of psychotic symptoms, were more likely to be admitted by criminal court order, and required longer hospitalization periods in comparison to regular cannabis users (84). However, SC users have not shown higher rates of depression, anxiety, or physiological symptoms compared with natural cannabis users. Recently, Mensen have investigated mental health consequences associated with SC use in a non-clinical sample (47). The authors have shown that compared to natural cannabis use, SC use is more strongly associated with a broad range of self-reported mental health problems such as: sleep problems, manic ideation, somatization,

obsessive-compulsive behaviors, hyper interpersonal sensitivity, depression, anxiety, hostility, phobic anxiety, paranoid ideation, and psychoticism. They have suggested that there are more severe problems related to SC use compared to natural cannabis use (47). Consistently, recent studies have indicated that SC use is associated with greater psychoticism, and a broad range of psychological symptoms. There are differences between the population in Shalit's study of patients that were admitted to hospital due to psychotic episodes and the population in our study who were not admitted due to psychosis and the general population reported by Mensen (47, 84). These differences may account for the variations in the adverse effects of SC drugs, including anxiety and depression.

Possible Mechanisms for the Association Between Synthetic Cannabinoids and Psychosis

A possible explanation for SC induced psychosis is that SC products contain compounds which act as highly potent CB_1 and CB_2 full agonists, and in contrast to natural cannabis, contain no CBD (5, 7, 9, 31, 32, 36). Due to the psychoactive features of SC drug ingredients it is not surprising that there are numerous reports on healthy and vulnerable individuals who suffer from recurrent psychosis after an acute or repeated consumption of SC drugs (37, 38). Converging evidence suggests that the adverse effects of cannabinoids are dose-dependent, thus, as the concentration of the CB_1 agonist increases, the adverse effects of cannabinoid-based drugs increase (34, 109, 110, 113). Accordingly, greater cannabinoid psychoactive effect is associated with greater risk for developing psychosis (10, 18, 19, 31, 33, 35, 54, 107), and individuals with schizotypal personality are more sensitive to the psychoactive effect of cannabinoids (111, 114–116). Moreover, several studies have shown that some cognitive and emotional deficits observed in chronic cannabis users are associated with schizotypal symptoms, suggesting that greater schizotypy may reflect a risk factor for the long-term adverse effects of cannabinoids (111, 115, 116). It is reasonable to assume that the psychoactive features of SC drugs along with the schizotypy characteristics of SC users, which were demonstrated in the current study, may underlie the severe adverse effects that have been associated with SCs, especially the high rates of prolonged psychotic episodes. The relationship between cannabinoid use and psychosis is well documented. Yet, the nature of the relationships between cannabinoid consumption and psychotic proneness is not fully understood (34, 86, 117). However, the phenomenon could be explained by three possible mechanisms: a) direct pharmacological effects of cannabinoids lead to schizotypal traits; b) schizotypal traits lead to cannabinoids use; or c) further factors influences both tendency toward psychosis or schizotypal traits and cannabinoids use (27). Recent data from clinical and pre-clinical studies show that acute consumption of cannabinoid agonists tend to induce brief psychotic symptoms in both vulnerable and healthy individuals (112, 117). Accordingly, when absorbed, cannabinoid agonists stimulate brain's CB_1 receptors which in turn modulate the firing rates of dopaminergic neurons in the ventral tegmental area—mesolimbic circuitry (34, 35). This pharmaco-dynamic mechanism may explain the short-term psychotic-like effects induced following cannabinoid-based drugs, yet there is a limited evidence for this relation in term of a long-term effect (33, 35). An alternative explanation is that individuals with schizotypal traits may use cannabinoids in order to "self-medicate" their schizotypal symptoms. Accordingly, individuals with schizotypal personality could attempt to reduce their negative symptoms by consuming cannabinoids, in order to return their control over their mental distress (27). Interestingly, earlier studies have indicated that SC users reported that despite the adverse effects, SC drugs induce pleasurable experiences such as: good mood, relaxation, and clear thought (12, 15, 88). Accordingly, beside drug-related prosecution issues, SC users most commonly consume these drugs in order to obtain positive effects, although the acute effect of SCs is unpredictable (6, 11, 14, 15, 50, 59). These results, together with the high scores of neuroticisms may support the view that SC users may use SC in an attempt to acquire a mental relief and to reduce their mental distress.

The Relationship Between Personality Factors and Schizotypy

For both natural cannabis users and SC users, openness to experiences, and lower conscientiousness predicted schizotypy. This observation may indicate a partial common mechanism that underlies schizotypy features in these two groups. The correlation between openness to experience and schizotypy in the general population was recognized in previous studies and reflect idiosyncratic cognitive processes, unconventional ideas and elevated risk for developing schizophrenia (118, 119). Low levels of conscientiousness were also associated previously with schizotypal symptoms in non-clinical and clinical populations (119). A positive correlation between neuroticism and schizotypy for natural cannabis users is unsurprising given the association between negative affect and greater risk for developing psychosis (25). Yet, it is possible that this relationship was not observed for SC users due to their elevated levels of anxiety and depression that reduce the effect of neuroticism on schizotypy in the present study.

Notably, natural cannabis users did not differ from non-users in schizotypal measures. Although elevated schizotypal measures were previously observed among chronic cannabis users, recent studies have shown inconsistent findings. In few studies there were lower scores of negative symptoms of schizotypy in natural cannabis users compared to healthy control subjects (120, 121). Yet, earlier observations have indicated greater scores on either positive or negative schizotypal symptoms in natural cannabis users (25, 65, 108, 110). An alternative explanation to this inconsistency is that in most of these studies, additional factors which are associated with schizotypal traits such as: alcohol consumption, depression, and anxiety symptoms or current use of additional substances were not recorded or controlled (25). Finally, recent studies showed no differences in schizotypy measures between natural cannabis users and healthy control participants (122), an observation that may suggest the involvement of other moderators in this association. The

presented result support this view, since after controlling for anxiety and depression levels, different traits predicted schizotypy for natural cannabis users and control participants, indicating further evidence for the complex relationships between these factors.

Limitations of the Current Study

Studying drugs use, which is a prohibited behavior, by self-reported measures may be biased by subjective factors such as: social desirability, poor insight, and impression management (123). However, there is a consensus that self-report methods for assessing substance users have validity and reliability similar to that of biomarkers of drug consumption (63). Moreover, although the anonymity of the participants was kept in the current study, which may help to reduce socially desirable responses, we were unable to control over subjective biases, and objective measures of cannabinoids use as well as the possible use of additional psychoactive compounds. Future studies may use additional measures, such as biological assays of drugs in order to improve the reliability of the data. Secondly, the association between SCs and BFI factors was diminished when anxiety or depression were entered to the model as covariates, this result is not surprising as there is a large agreement that psychological distress, anxiety, and depression are correlated with personality dimensions such as; low conscientiousness, neuroticism, and introversion, yet, there is still a debate regarding the nature of this association in terms of causality and the involvement of additional factors in this phenomena (124). Thirdly, the current study showed an association between schizotypy and SC use, but it does not provide evidence for the direction of the relationship as the data are correlational, and therefore it is impossible to conclude whether prolonged SC use leads to schizotypy or the opposite. Future studies may consider conducting longitudinal study designs in order to better address these issues. Finally, the sample size of the current study is not large and the cross-sectional design does not allow for causal inferences. Chronic SC users are a very unique and rare cohort and difficult to recruit, therefore unfortunately our sample size was limited. Future studies may consider replicate our study using larger samples in order to confirm or disprove the current results.

CONCLUSIONS

In conclusion, the current study provides initial evidence for the association between specific personality characteristics, schizotypal traits, and chronic SC use. On the BFI, SC users showed higher scores of neuroticism than natural cannabis users and non-users. SC users had lower agreeableness and introversion scores than both control groups, while natural cannabis users had higher extroversion scores than non-users. In addition, SC users had lower scores on conscientiousness than non-users. These effects were diminished when anxiety and depression scores were used as covariates. On the SPQ-B, SC users presented more schizotypal symptoms than both control groups. Finally, there were no differences between non-users and natural cannabis users in other personality variables. In addition, elevation of depressive and anxiety levels was observed in SC users. For SC and natural cannabis users, high measures of openness and low measures of conscientiousness have predicted schizotypy. To the best of our knowledge this is the first study presenting the complex relationships between specific personality characteristics, schizotypal traits, and SC use. The present results add initial information of the personality factors associated with SC use and their association with psychosis proneness. Yet, further studies are needed to replicate and expand the current observations.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ministry of Health, Jerusalem, Israel. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All the authors contributed substantially to the conception and design of the study. KC and SR have collected the data. KC and AvW were responsible for the analysis of the results. All the authors contributed to further drafts of the manuscript.

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The Role of Dopamine in the Stimulant Characteristics of Novel Psychoactive Substances (NPS)—Neurobiological and Computational Assessment Using the Case of Desoxypipradrol (2-DPMP)

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Stimulant drugs, including novel psychoactive substances (NPS, formerly "legal highs") have addictive potential which their users may not realize. Stimulants increase extracellular dopamine levels in the brain, including the reward and addiction pathways, through interacting with dopamine transporter (DAT). This work aimed to assess the molecular and atomistic mechanisms of stimulant NPS actions at DAT, which translate into biological outcomes such as dopamine release in the brain's reward pathway. We applied combined *in vitro*, *in vivo*, and *in silico* methods and selected 2-diphenylmethylpiperidine (2-DPMP) as an example of stimulant NPS for this study. We measured *in vitro* binding of 2-DPMP to rat striatum and accumbens DAT by means of quantitative autoradiography with a selective DAT-radioligand [^{125}I]RTI-121. We evaluated the effects of intravenously administered 2-DPMP on extracellular dopamine in the accumbens-shell and striatum using *in vivo* microdialysis in freely moving rats. We used dynamic modeling to investigate the interactions of 2-DPMP within DAT, in comparison with cocaine and amphetamine. 2-DPMP potently displaced the radioligand in the accumbens and striatum showing dose-dependence from 0.3 to 30 μM. IC_{50} values were: 5.65×10^{-7}M for accumbens shell and 6.21×10^{-7}M for dorsal striatum. Dose-dependent responses were also observed in accumbens-shell and striatum *in vivo,* with significant increases in extracellular dopamine levels. Molecular dynamics simulations identified contrasting conformational changes of DAT for inhibitors (cocaine) and releasers (amphetamine). 2-DPMP led to molecular rearrangements toward an outward-facing DAT conformation that suggested a cocaine-type effect. The present combination of molecular modeling with experimental neurobiological procedures allows for extensive

characterization of the mechanisms of drug actions at DAT as the main molecular target of stimulants, and provides an insight into the role of dopamine in the molecular and neurobiological mechanisms of brain responses to stimulant NPS that have addictive potential. Such knowledge reveals the risk of addiction related to NPS use. The research presented here can be adapted for other psychostimulants that act at their membrane protein targets.

Keywords: addiction, autoradiography, brain, dopamine transporter, microdialysis, molecular modelling, cocaine, amphetamine

INTRODUCTION

Typical drugs of addiction, stimulants such as cocaine or amphetamine, have been known to share among them the ability to activate the brain's reward system and increase extracellular levels of dopamine (DA) in the mesolimbic pathway, and preferentially in the nucleus accumbens (NAc) (Di Chiara and Imperato, 1988; Volkow et al., 2007). Elevated DA availability in the NAc shell, which associates with the perception of pleasure and reward, plays a role in the complex biological phenomenon of drug dependence (Di Chiara et al., 2004). Extracellular DA concentrations can be increased by stimulant-related inhibition or reversal of the monoamine reuptake transporters, mainly the presynaptic dopamine transporter (DAT) which is addressed below.

Stimulants can share similar structural moieties, such as phenylethylamine which is a common structural feature found embedded in many stimulants like amphetamine and methylamphetamine and is also found in the naturally occurring neurotransmitter dopamine (**Figure 1**). 2-DPMP, pipradrol and methylphenidate are also structurally similar with a diphenylmethane moiety (**Figure 1**). However, despite structural similarities, stimulants can modulate DAT structure and function through different mechanisms. Cocaine (**Figure 1**) acts as an inhibitor (or blocker) of DAT by directly binding DAT and preventing the reuptake of DA (Kuhar et al., 1991; Jones et al., 1995), while amphetamine (AMPH) (**Figure 1**) competes and displaces newly synthesized DA, thus inducing reversal of DAT in a calcium-independent manner i.e. irrespective of action potential, triggering the reverse transport (efflux) of DA from the cell interior to the synapse (Butcher et al., 1988). In either case, extracellular DA concentrations increase, and the acute effect is thought to associate with the user's perception of a "high" following a stimulant dose. The faster the onset, the more pronounced the perceived "high" and the greater the addictive potential of the stimulant drug (Volkow et al., 2007).

Stimulants can be found among novel/new psychoactive substances (NPS) (Miliano et al., 2016); it is important to investigate their possible addictive properties, which, together with the pharmacological peripheral effects, result in an increasing mortality and in emergency admissions for overdoses, as reported by several poison centers (UNODC, 2017). We and others have previously reported prodopaminergic, thus presumed stimulant effects among synthetic cathinones (Opacka-Juffry et al., 2014) and benzofurans (Dawson et al., 2014; Sahai et al., 2017) as well as dissociative diarylethylamines. Of the latter group diphenidine engages in effective interactions with the DAT, unlike some of its analogs, e.g. methoxyphenidine (Wallach et al., 2016; Luethi et al., 2018; Sahai et al., 2018). We have characterized those stimulants by means of neurobiological *in vitro* methods, and *in silico,* using molecular modeling of interactions between the drugs and DAT (Sahai et al., 2017; Sahai et al., 2018). We have also predicted their respective prodopaminergic properties without *in vivo* evidence of dopamine release caused by those stimulants.

In the present paper, we chose desoxypipradrol, also known as 2-diphenylmethylpiperidine (2-DPMP) (**Figure 1**) as an example of a stimulant NPS with high abuse potential (Davidson and Ramsey, 2012; Simmler et al., 2014). In the *in vitro* assays in human embryonic kidney 293 cells (HEK 293) that express DAT, NET and SERT, 2-DPMP was most potent at DAT: the IC_{50} values were 0.07 μM for DAT, 0.14 μM for NET and >10 for SERT (Simmler et al., 2014). 2-DPMP is able to stimulate evoked DA efflux in NAc brain slices to a greater extent than cocaine (Davidson and Ramsey, 2012). Importantly, 2-DPMP is a pure dopamine transporter inhibitor without release ability (Davidson and Ramsey, 2012).

In the present study, we assessed the stimulant profile of 2-DPMP by means of *in vitro* as well as *in vivo* approaches, the latter testing the effects on DA release in the brain's reward pathway in freely moving rats. We hypothesized that those *in vitro* and *in vivo* findings can be interpreted at the atomistic level, by means of *in silico* methods of computational biophysics with molecular modeling and simulations, and based on the consensus that there are distinct differences in the mode of action between dopamine releasers/substrates and dopamine reuptake inhibitors. Regardless of whether the binding and transport of these compounds are driven by entropy (substrates and releasers) or enthalpy (inhibitors) (Ferris and Tang, 1979) they induce a conformational change in their target protein (Shan et al., 2011; Khelashvili et al., 2015b; Khelashvili et al., 2015a; Cheng and Bahar, 2019).

As the pharmacology of 2-DPMP has been reasonably described (Davidson and Ramsey, 2012; Simmler et al.,

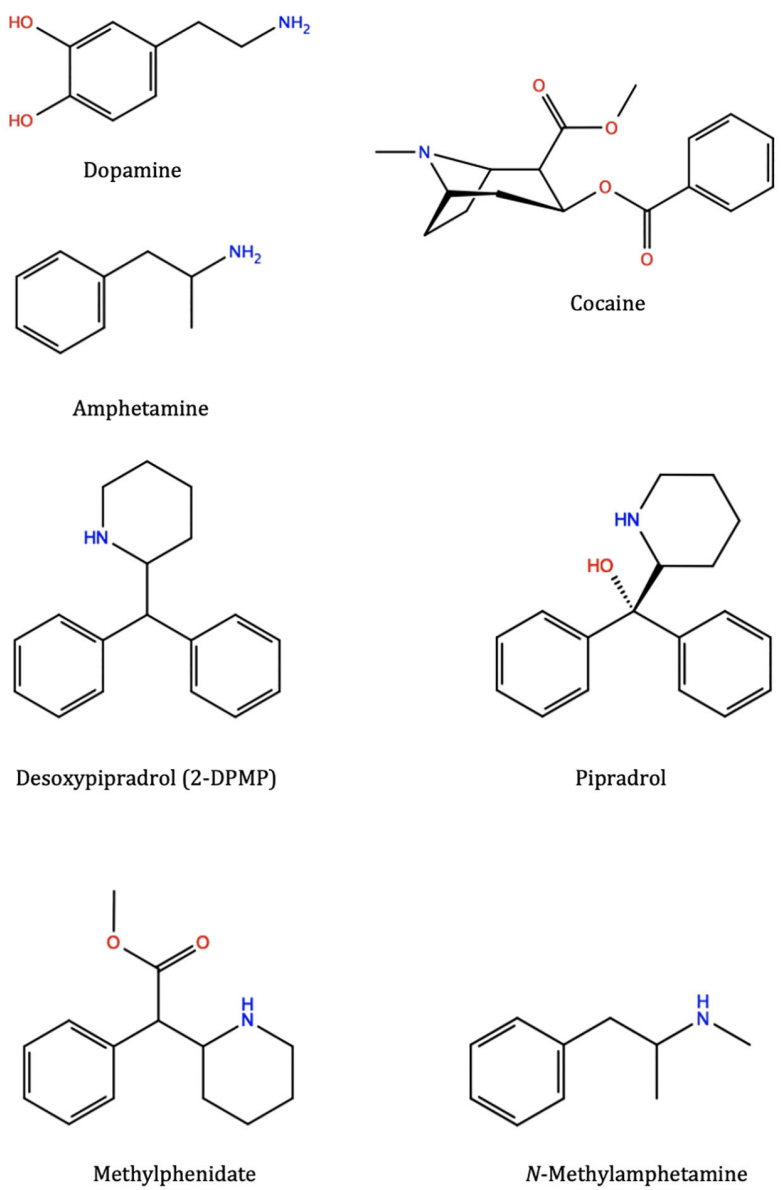

FIGURE 1 | Molecular structures of various substrates and stimulants: dopamine, amphetamine, cocaine, 2-diphenylmethylpiperidine (2-DPMP), pipradrol, methylphenidate, and methylamphetamine (which structurally belongs to the substituted amphetamine class of compounds). These different compounds, although occupying the same central binding site in the dopamine transporter, trigger different downstream effects and prefer different conformational states of the protein. Desoxypipradrol (2-DPMP), shares similar structural and pharmacological characteristics with pipradrol and methylphenidate. They have a hydrophobic diphenylmethyl group attached to the α-carbon atom of a cyclic amine (Dargan and Wood, 2013). Figures were produced with Maestro 2D Sketcher (Schrödinger Release 2019-1: Maestro, Schrödinger, LLC, New York, NY)

2014), that together with the evidence of its harm to users (Banks et al., 2014), makes it a suitable case of NPS stimulant for the present study. By employing a novel multi-method approach, we aimed to investigate the mechanisms of the addictive potential of NPS that continue to be misused with no awareness of harm, including the risk of addiction. At the level of scientific inquiry, it is important to understand the molecular mechanisms of the stimulant effects of NPS, which determine their distinct interactions with DAT and translate into biological outcomes, including their addictive potential.

METHODS

Animals

For the *in vitro* autoradiography study, 8-week-old male Wistar rats (Charles River, UK) were used. They were kept six per cage

on a 12/12-h light/dark cycle (lights on at 7 AM) with food and water freely available. Temperature and humidity were 18° C–22°C and 55% ± 15% respectively. Rats were treated in accordance with the U.K. Animals (Scientific Procedures) Act 1986 and sacrificed by cervical dislocation.

For the *in vivo* microdialysis study, adult male Wistar rats (body weight: 275–300 g; Envigo, Harlan Laboratories, Italy) were housed in groups of four per cage under an inverted 12:12-h light/dark cycle and at a constant temperature of 22°C ± 2°C and humidity of about 60%. Tap water and standard food were available ad libitum in the home cage. The *in vivo* animal experiments were conducted in the University of Cagliari (Italy) and carried out in accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research according to Italian (D.L. 116/92 and 152/06) and European Council directives (609/86 and 63/2010) and in compliance with the approved animal policies by the Ethical Committee for Animal Experiments (CESA, University of Cagliari) and the Italian Ministry of Health (Aut. N.162/2016-PR). Every effort was made to avoid pain and suffering, and to reduce the number of animals used.

Chemicals

All chemicals, including desoxypipradrol hydrochloride solution D-082, were purchased from Sigma Chemicals (Poole, UK). The radioligand for the dopamine transporter, [^{125}I]RTI-121 (specific activity 81.4TBq/mmol) was purchased from Perkin Elmer.

2-DPMP was purchased from Sigma-Aldrich (Ref. Nr. D-082) as 1 ml/ml solution in methanol. For microdialysis studies, the methanol was evaporated by N2 stream to dryness and the resulting residue was dissolved in a vehicle containing 2% ethanol, 2% Tween 80 and saline. The drug was administered intravenously at the volume of 1 ml/kg. Control rats received the vehicle alone.

Radioligand Binding

Brains were rapidly removed and frozen at −40°C, then stored at −80°C. Frozen brains were cut into 20-µm coronal sections to harvest the ventral and dorsal striatum areas at +1.7mm to −0.3mm against bregma (Paxinos and Watson, 2007). Serial sections were collected onto polysine-coated slides and stored at −80°C prior to autoradiography. The autoradiography procedure was based on Strazielle et al. (1998) and conducted according to Dawson et al. (2014). After preincubation in 0.05 M sodium phosphate buffer (NaPB) pH 7.4, sections were treated with 20 pM [^{125}I]RTI-121 in the NaPB with increasing concentrations of 2-DPMP (0–30 µM) for 60 min at room temperature. Nonspecific binding was assessed in the presence of 200 µM nomifensine (control – "block"). Kodak BioMax MR films were applied over the rinsed and air-dried slides for three days. Autoradiograms were analyzed using MCID™, Version 7.0, Imaging Research Inc. (Interfocus Ltd, U.K.). Flat-field correction was applied. Each drug concentration was tested in six brains. The dorsal striatal (caudate-putamen, CPu) and accumbens (NAc) regions of interest (ROIs) were sampled in duplicates for relative optical density, left and right ROI values were averaged and their means were calculated to assess the specific binding.

In Vivo Microdialysis Procedures

Rats were anesthetised with isoflurane gas (4%–5%), and maintained under anesthesia using a breathing tube under a scavenging system while placed in a stereotaxic apparatus, and implanted with a catheter consisting of a polyethylene tubing (Dow Corning Corporation, Michigan, USA) in the right jugular vein and stable fixed in the mid-scapular region of the back. During the same surgical session, rats were implanted with vertical dialysis probes aimed at the NAc shell and CPu. The following coordinates were used according to Paxinos and Watson (1998): NAc shell: A/P+2.2, L ±1.1 from bregma, and V -7.8 from dura; CPu: A/P +1.2, L ±3.0 from bregma, and V -5.5 from dura. On the day after surgery, rats with the microdialysis probes implanted in either the NAc shell or CPu were treated with vehicle or 2-DPMP i.v. at the following doses (mg/kg): 0.01; 0.1; 0.3; 0.5; and 1.0. Dialysate samples were collected at 1 µl/min every 20 min. The composition of Ringer's solution as artificial CSF was 147 mM NaCl, 4 mM KCl, 2.2 mM $CaCl_2$ (Tanda et al., 2015). Samples were analyzed by means of HPLC with a coulometric detector (ESA; Coulochem II, Bedford, MA). Briefly, the mobile phase consisted of 50 mM NaH_2PO4, 0.1 mM Na_2-EDTA, 0.5 mM n-octyl sodium sulfate, 15% (v/v) methanol, pH 5; the detector settings were +125 mV (first electrode/oxidation) and −175 mV (second electrode/reduction). Extracellular dopamine was monitored for a total period of 180 min.

At the end of the experiment, all animals were sacrificed under the anesthesia, the probes were gently removed, and the brains harvested and cut in coronal sections with a vibratome to check the correctness of a microdialysis probe location in every brain. Fiber placement was determined as consistent with the coordinates by Paxinos and Watson (1998). Each treatment group contributed data of n = 3–4 each with the microdialysis probe position confirmed *post mortem*. High reproducibility of the procedure allowed reducing the group size with the intention of limiting the animal use to minimum.

Statistical Analysis

Autoradiography data were analyzed using two-way ANOVAs followed by post-hoc Tukey's test. Data were expressed as mean percentage ± standard error of mean (SEM) against the control value. Binding of [^{125} I]RTI-121 was analyzed in the presence of increasing concentrations of the drugs in both CPu and NAc shell.

Microdialysis DA data were analyzed by ANOVA for repeated measures followed by the post-hoc Tukey's test; data were presented as mean ± SEM. Statistica for Windows (Version 10) software was used throughout; significance was set at $p < 0.05$.

Construction of the Simulated Systems

The Schrödinger Release 2019-1 (Schrödinger Release 2019-1: Maestro, Schrödinger, LLC, New York, NY) with the OPLS3e force field was used to prepare the compounds and dock them into a homology model of rDAT, the construction and validation

of which has been previously described (Sahai et al., 2017; Sahai et al., 2018). Three addictive substances known to exhibit different pharmacology and mechanisms of action at DAT were docked into the binding site of rDAT before equilibration: amphetamine (monoamine releaser), cocaine (reuptake inhibitor) and 2-DPMP (presumed reuptake inhibitor). While the LigPrep module was used to build 2-DPMP, the structures of cocaine and amphetamine were retrieved from the crystal structures of these compounds bound to dDAT; PDB IDs: 4XP4 and 4XP9, respectively (Wang et al., 2015). The Epik module assigned a net positive charge to each of these compounds before the rDAT homology model was prepared for the Induced Fit Docking (IFD) protocol in the Schrödinger suite. A docking grid box was defined no more than 7Å from the central binding residues of Phe76, Asp79, Ser149, Val152, Tyr156, Asn157, Phe326, Val328, and Ser422, previously identified as important for binding stimulants of comparable size. Dockings were then performed using a standard protocol whereby conformations of the ligand were screened for clashes with the protein and subsequently refined by allowing flexibility of the side-chains in the binding. The CHARMM36 force field parameters for the compounds were obtained from the Acellera small molecule parameterization tool implemented in the HTMD 1.15.2 suite using the quantum mechanical calculations protocol at the Hartree-Fock level of theory and the 6–31G(d) basis set (Doerr et al., 2016).

A standard protocol was used to study these docked homology models of rat DAT (rDAT) based on the crystal structure of the *Drosophila Melanogaster* dopamine transporter (dDAT) with all-atom MD simulations in explicit models of the hydrated lipid membrane environment (Hamilton et al., 2013; Hansen et al., 2014; Khelashvili et al., 2015a; Khelashvili et al., 2015b; Sahai et al., 2017; Sahai et al., 2018). To summarize, a multistep equilibration protocol was performed with the NAMD software, version 2.13 (Phillips et al., 2005), to remove the close contacts in the structure, the backbones were initially fixed and then harmonically constrained, and water was restrained by small forces from penetrating the protein-lipid interface. The constraints on the protein were released gradually in three steps of 300 ps each, changing the force constants from 1 to 0.5 and 0.1 kcal/(mol Å2) respectively, with a time step of 1 fs. This was then followed by a short (100 ns) unbiased MD simulation performed with a 2 fs integration time step and under constant temperature (310 K) maintained with Langevin dynamics, and 1 atm constant pressure achieved by using the hybrid Nosé-Hoover Langevin piston method on a flexible periodic cell to capture long-range effects. The simulated system, including the transporter embedded in a membrane patch and water layers on each side containing Na+ and Cl− ions (corresponding to a concentration of 150 mM NaCl), was composed of approximately 149,664 atoms in a box with the final dimensions of 121 × 121 × 139 Å. After this equilibration phase, 3 each of unbiased production MD simulations were carried out using GPUS and the ACEMD software (Harvey et al., 2009) with an established protocol for a further 400 ns (Khelashvili et al., 2015b; Sahai et al., 2017; Sahai et al., 2018).

Unbiased atomistic molecular dynamics (MD) simulation trajectories (totaling 4.5 μs) were analyzed and images were created with VMD (Humphrey et al., 1996).

RESULTS

DAT Autoradiography

The displacement of [^{125}I]RTI-121 was examined in NAc and CPu. **Figure 2** shows the autoradiograms of the relevant brain sections labeled with [^{125}I]RTI-121 in the presence of varying concentrations of 2-DPMP, ranging from 0 (total binding) to 30 μM. 2-DPMP caused a marked, concentration-dependent reduction in the radioligand signal in the brain tissue, indicating potent competition between the drug and the radioligand in NAc shell and CPu. IC_{50} values were: 5.65×10^{-7}M for NAc shell and 6.21×10^{-7}M for CPu.

In detail, two-way ANOVA revealed a significant effect of the drug concentrations ($F_{5,60} = 705.4$; $P < 0.0001$), a significant effect of the area ($F_{1,60} = 23.49$; $P < 0.0001$), and a significant concentration x area interaction ($F_{5,60} = 3.18$; $P < 0.05$) (see **Figure 2**). Additionally, *post hoc* Tukey's test showed a highly significant effect of all 2-DPMP concentrations in both CPu ($^{++++}P < 0.0001$) and NAc shell ($^{****}P < 0.0001$) against control values where no drug was present. It is worth noting that 2-DPMP significantly displaced the radioligand in both CPu and NAc, even at the lowest concentrations tested (0.3 μM), unlike several other potent stimulants that we have analyzed using this very methodology (Dawson et al., 2014; Opacka-Juffry et al., 2014; Sahai et al., 2017; Sahai et al., 2018).

In Vivo Microdialysis

The acute i.v. administration of 2-DPMP elicited a dose-dependent increase in extracellular DA in the NAc shell and in the CPu of freely moving animals, with an onset of action within the first hour after the treatment (**Figure 3**). In the NAc shell, the maximal peak effect (300% over basal levels) was observed in the first sample collected within 20 min of the highest dose injection (1 mg/kg) with the dopamine levels decreasing at 60 min and achieving a plateau around the baseline over the rest of the period of sampling. The injection of 2-DPMP at 0.3 mg/kg elicited a less pronounced increase (about 200% over basal levels) occurring between 20 and 40 min after treatment, while lower doses were ineffective. Similar responses were observed in the CPu with the maximal peak effect in the second sample collected within 40 min of the highest dose injection (1 mg/kg). The higher doses of 2-DPMP (0.5 and 1.0 mg/kg) seemed to produce stimulant-dependent behavioral effects such as piloerection, facial stereotypy with gnawing, licking, biting, grooming and sniffing the walls of the home cage, as well as rearing and scratching (quantitative data are not presented).

Three-way ANOVA revealed a significant effect of the brain area [$F(1,22)=8.78$; $p < 0.001$], dose [$F(3,22)=13.10$; $p < 0.0001$], time [$F(9,198)=46.24$; $p < 0.000001$] and a significant brain area × time [$F(9,198)=1.95$; $p < 0.05$], dose × time [$F(27,198)=10.57$; $p < 0.000001$], and brain area × dose × time

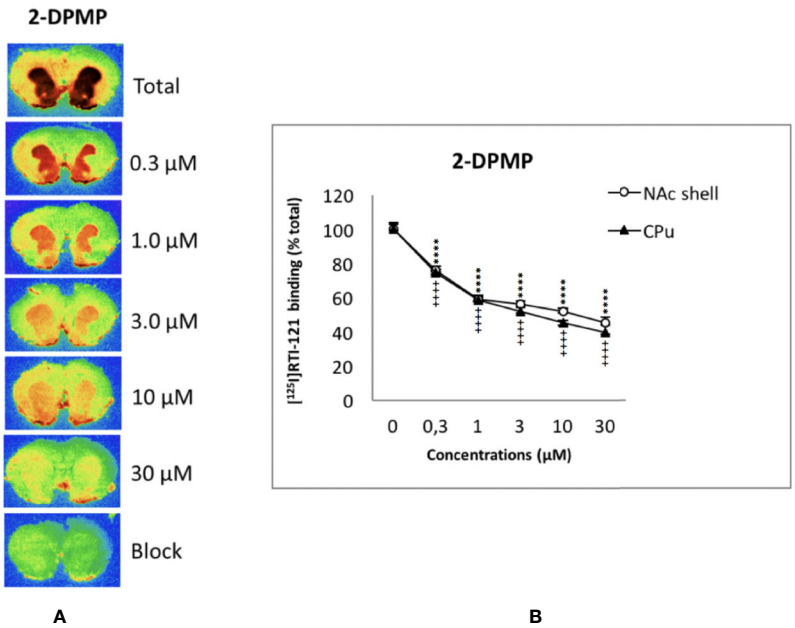

FIGURE 2 | Concentration-dependent displacement of [^{125}I]RTI-121 by 2-diphenylmethylpiperidine (2-DPMP) in rat nucleus accumbens (NAc) and caudate-putamen (CPu) sections. **(A)** Representative computer-enhanced images of brain sections incubated with the selective dopamine transporter ligand [125I]RTI121 and exposed to increasing concentrations of 2-DPMP. **(B)** [^{125}I]RTI21 displacement by 2-DPMP in the caudate-putamen and nucleus accumbens. Values are means ± SEM. Two-way ANOVA analysis (****P < 0.0001 versus control (0 µM) in the NAc, n = 6 per drug concentration; $^{++++}$P < 0.0001 versus control (0 µM) in the CPu, n = 6 per drug concentration, post-hoc Tukey's test).

interaction [F(27,198)=1.69; p < 0.05]. In the NAc shell, *post hoc* Tukey's test showed a significant difference of DA with respect to basal levels at 20 and 40 min after injection of 2-DPMP (0.3 and 1 mg/kg) and significant differences at 20- and 40-min samples between 2-DPMP (0.3 and 1.0 mg/kg, i.v.) and vehicle treated animals, and between the highest dose tested (1 mg/kg) and both 0.3 and 0.1 mg/kg. In the CPu, *post hoc* Tukey's test showed a significant difference of DA with respect to basal levels at 20 min after injection of the intermediate dose (0.3 mg/kg) and at 20 and 40 min after the higher dose tested (1 mg/kg), and significant differences at 20- and 40-min samples between 2-DPMP (1.0 mg/kg, i.v.) and vehicle treated animals, at 20-min samples between 2-DPMP (0.3 mg/kg, i.v.) and vehicle treated animals, and at 20 min between the highest dose tested (1 mg/kg) and both 0.3 and 0.1 mg/kg.

In Silico Findings

The *in silico* part of the study aimed to investigate the molecular changes when 2-DPMP is bound to DAT and compare it to the DA releaser, amphetamine that induces an inward-facing conformation of DAT and the DAT inhibitor, cocaine that locks DAT in an outward-facing conformation.

Docking studies were performed by the Induced Fit Protocol in Schrödinger to identify the potential binding sites for AMPH, cocaine and 2-DPMP. The protocol performed in the presence of the internal ions $2Na^+$ and Cl^- obtained positions for all three drugs near a common high affinity binding site that is near the primary substrate-binding S1 site (**Figure 5**) (Quick et al., 2009). Since many NPS have molecular structures comparable to illicit drugs and also induce comparable intended effects, their mechanisms of action likely overlap. Thus, this observation is not surprising and is comparable to previous findings (Sahai et al., 2017; Sahai et al., 2018).

The binding poses of AMPH and cocaine were consistent with previous studies (Beuming et al., 2008; Bisgaard et al., 2011; Sahai et al., 2017) and the crystal structures PDB IDs: 4XP4 and 4XP9, respectively (Wang et al., 2015). All three drugs are positively charged and take part in an attractive electrostatic interaction with the amino acid Asp 79 (D79) found in the S1 site. Unsurprisingly, the initial docking position shows that the amine group of 2-DPMP also interacts with D79, which highlights the critical role of this residue in stabilizing the binding of drugs in the S1 site (**Figure 4**).

MD Simulations Reveal Different Mechanisms of Transport

In our study, unbiased MD simulations show a preferential substrate translocation for AMPH, whereby the DAT adopts an inward-facing conformation indicated by the structural dynamics of the salt-bridge forming pairs of the intracellular (IC) vestibule (R60-D436, K66-D345 and E428–R445) (**Figures 5A, B**) as well as Na2 destabilization from the primary binding site (not shown). This has previously been observed for the substrate dopamine in microsecond simulations (Khelashvili et al., 2015b), the NPS 5-MAPB (Sahai et al., 2017) and diarylethylamine derivatives (Sahai et al., 2018). There is no indication in these time scales that the extracellular gates (R85-

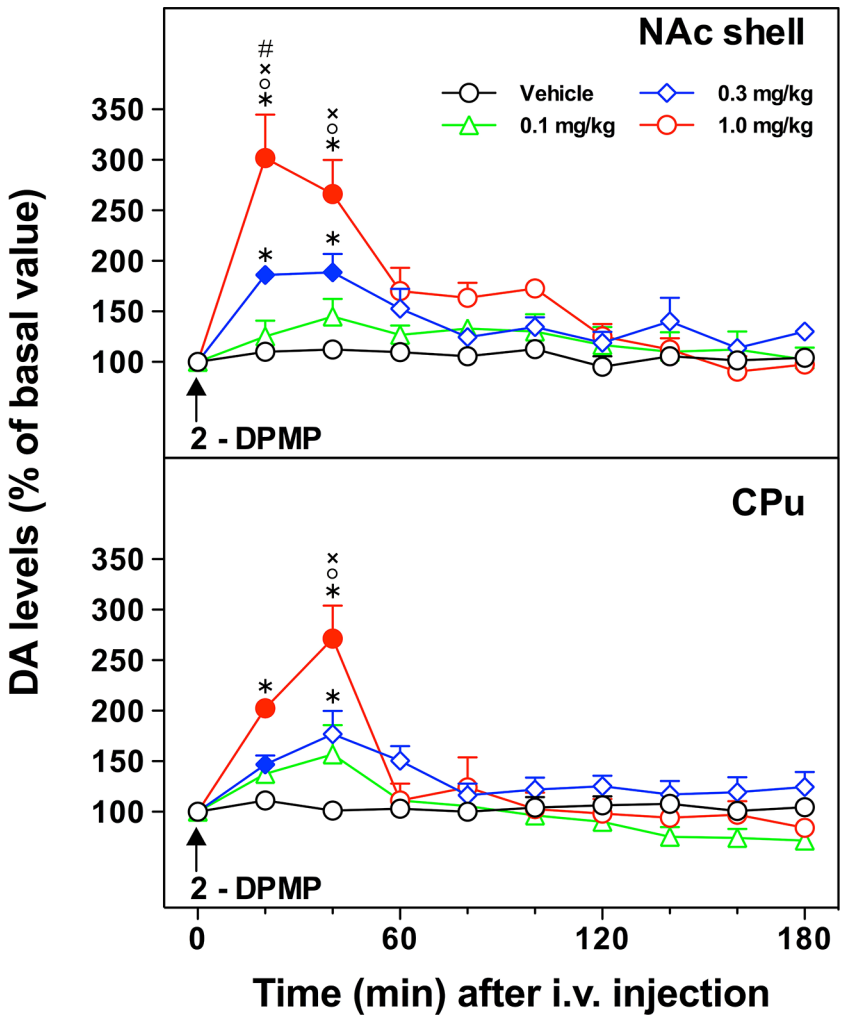

FIGURE 3 | *In vivo* effect of i.v. 2-diphenylmethylpiperidine (2-DPMP) on dopamine (DA) extracellular levels in dialysates from the nucleus accumbens (NAc) shell and the caudate-putamen (CPu) of freely moving rats. Data are presented as mean ± SEM of the amount of DA expressed as the percent of basal values; n per dose/brain region. The arrow indicates the i.v. injection of either vehicle (black circles, n = 4) or 2-DPMP at the following doses: 0.1 mg/kg (green triangles, n = 4); 0.3 mg/kg (blue diamonds, n = 4); 1.0 mg/kg (red circles, n = 3) in NAc shell or CPu. Solid symbols indicate a significant difference vs the baseline, $^{*}p < 0.05$ vs. Veh group, $^{\circ}p < 0.05$ vs. 0.1, $^{\times}p < 0.05$ vs. 0.3, $^{\#}p < 0.05$ vs. CPu group (Three-way ANOVA, post-hoc Tukey's test).

D476, Y156-F320) show any opening to the extracellular (EC) vestibule (**Figure 5B**).

Cocaine, on the other hand, adopts a position in the S1 site where there is minor destabilization of the EC gate Y156–F320 bond at these time scales. As the extracellular salt-bridge R85-D476 remains closed, we interpret it as showing that the DAT is maintained in the original outward-facing conformation (**Figures 5C, D**). This feature prevents DAT from transforming into an inward-facing conformation later in the simulations. The IC gates remains closed as there is negligible dynamics for the salt-bridge pairs (R60-D436, K66-D345 and E428–R445) in the IC vestibule (**Figure 5D**).

With the understanding that 2-DPMP binds in the S1 site, our simulations provide evidence whereby the structural arrangement leaves DAT in the outward-facing conformation, similarly to cocaine (**Figures 5E, F**). Since there are negligible changes in the distances of the EC gates Y156–F320 and R85-D476 (**Figure 5F**), 2-DPMP appears to arrest in this particular conformation and is also prevented from being transported *via* the transporter. This is supported by the IC gates also remaining closed at these time scales for the salt-bridge pairs (R60-D436, K66-D345 and E428–R445) in the IC vestibule (**Figure 5F**).

To sum up, our *in silico* findings confirmed the following: (1) the primary binding site S1 is the same for all three investigated drugs, (2) the mechanisms of interaction of DAT with amphetamine, cocaine and 2-DPMP highlight the critical role of the intracellular and extracellular salt-bridges, R60-D436 and R85-D476 respectively' and negatively charged aspartate

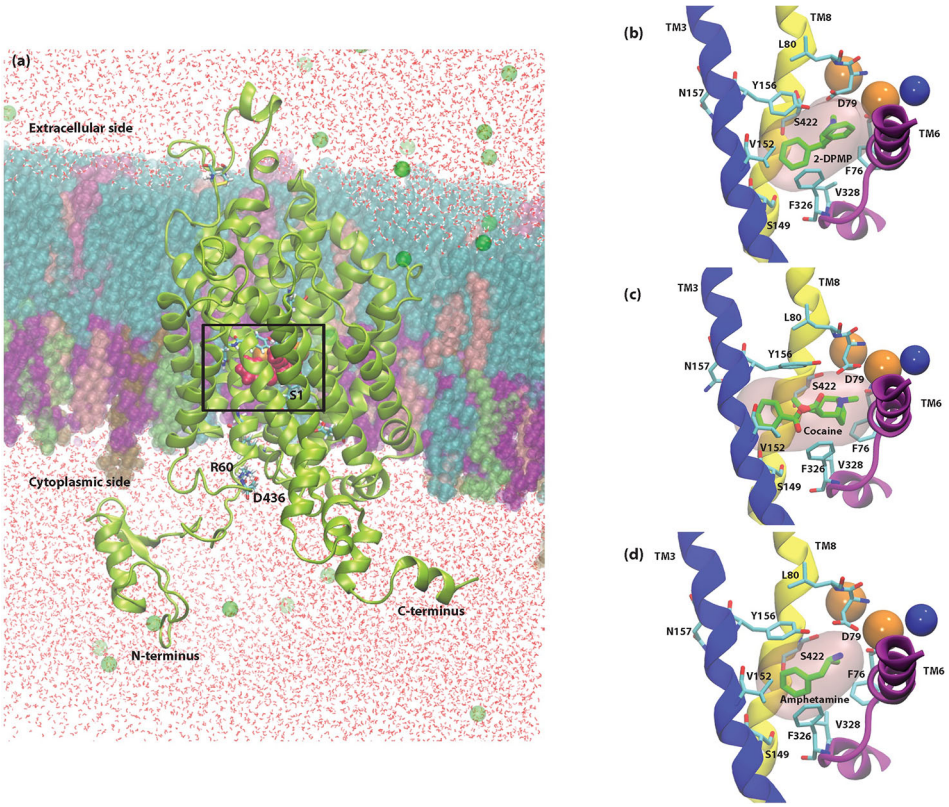

FIGURE 4 | Cross-sectional illustration of the dopamine transporter (DAT) and molecular models of DAT/ligand complexes. **(A)** Cartoon ribbons (in green) imbedded in the physiological membrane used in this study. The water box and ionizable salts (green spheres) are also included in this representation. The extracellular and intracellular sides as well as the N- and C- termini are clearly illustrated. The substrate binding pocket, S1 as well as the location of the internal ions is highlighted with a box. **(B–D)** Molecular models of DAT/ligand complexes. These illustrations indicate the equilibrated poses of 2-DPMP **(B)**, cocaine **(C)**, and amphetamine **(D)** shown in green in the S1 binding site (pink filled surface) as well as the S1 binding residues that are named explicitly. Sodium (orange spheres) and chloride (blue sphere) ions around the S1 site are represented as well as the Transmembrane domains (TM) 3, 6, and 8, which are shown in blue, purple and yellow, respectively; the other transmembrane domains and intra- and extracellular loops have been removed for clarity.

residue (D79) in stabilizing the drug in the S1 binding site, and (3) MD simulations show contrasting conformational changes of DAT for inhibitors and releasers.

DISCUSSION

We employed *in vitro*, *in vivo*, and *in silico* approaches to assess the dopaminergic effects of 2-DPMP as the chosen NPS stimulant, selected on the basis of the existing literature (Davidson and Ramsey, 2012; Simmler et al., 2014). Our *in vitro* and *in vivo* findings are consistent with the characteristics of a potent stimulant that directly interacts with the brain's rewards system. Our *in silico* study uniquely extends the analysis into the atomistic level of interactions between a prodopaminergic NPS ligand and DAT as its biological target.

Radioligand Binding at DAT

As hypothesized, 2-DPMP behaved like a highly potent DAT ligand. The effects of 2-DPMP were more pronounced than those of cocaine, which, when tested under the same conditions, was able to significantly displace [^{125}I]RTI-121, starting at higher concentrations than 2-DPMP in the rat NAc (Opacka-Juffry et al., 2014). The present findings are consistent with the previously published *in vitro* study using fast scan cyclic voltammetry (Davidson and Ramsey, 2012) that demonstrated the ability of 2-DPMP to stimulate evoked dopamine efflux in NAc brain sections to a greater extent than cocaine.

In Vivo Microdialysis Findings

In vivo brain microdialysis enables the monitoring of neurotransmitters in the extracellular brain compartment and provides information on the relationship between pharmacological manipulations, neurotransmitters release and behavior (Di Chiara, 1990; De Luca et al., 2018). Monitoring extracellular DA in the accumbens and striatum in animals is a useful preclinical method to identify new drugs of abuse through revealing their rewarding properties (De Luca et al., 2015).

The present profile of the "on-and-off" of extracellular DA in response to i.v. 2-DPMP, with a decisive descending phase,

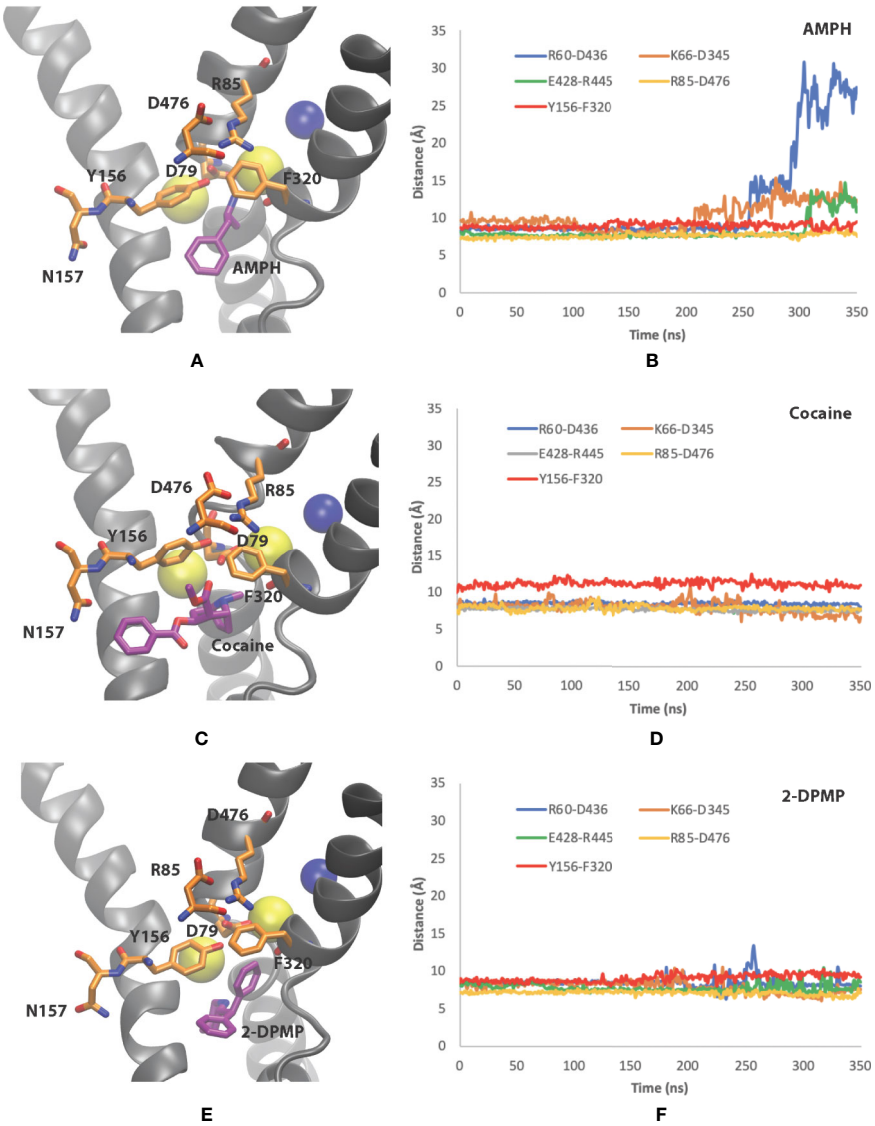

FIGURE 5 | Conformational changes of dopamine transporter (DAT) when bound to the three different compounds. **(A, B)** amphetamine, **(C, D)** cocaine and **(E, F)** 2-DPMP in the last 350 ns in one representative simulation for each compound. Panels **(A**, **C**, **E)** show the poses of the compounds at the end of the simulation, while panels **(B, D**, **F)** show the time dependency changes in salt-bridge-forming pairs at the EC vestibule (Asp476– Arg85) and IC vestibule (Asp436–Arg60, Asp345–Lys66 and Glu428–Arg445).

resembles that of DA responses to cocaine as observed in a comparable experimental design applied previously in the same research lab of Di Chiara (Pontieri et al., 1995). Amphetamine-induced extracellular DA response in the NAc shell has a slow descending phase that does not return to the basal levels within the same time scale (Pontieri et al., 1995). Thus, the present *in vivo* findings not only prove that 2-DPMP causes a direct dose-dependent increase in extracellular DA in the brain's reward pathway, but also suggest that the mode of its action is more similar to that of cocaine. The DA response to 2-DPMP observed here may account for the high abuse potential of this drug, higher than that of cocaine (Banks et al., 2014; Negus and Miller, 2014).

The present findings of the potent direct effects of 2-DPMP on DAT and DA availability in the reward pathway help explain why, according to users' reports, physical and psychoactive effects of 2-DPMP, such as pleasure, euphoria, and increased energy and sociability may start as early as after 15 min of the dose (and last for hours), depending on the dose and route of administration (ACMD, 2010). The most often routes of 2-DPMP administration are oral, insufflation, injection or rectal with dosages ranging from 1 to 10 mg (Corkery et al., 2012). Untoward effects include hypertension, tachycardia, sweating, tremors, insomnia, aggression and psychosis with hallucination, paranoia, and often long-lasting agitation for up to five days

(Corkery et al., 2012). Evidence of harm supported by numerous acute toxicology reports and several fatalities linked to 2-DPMP use (Corkery et al., 2012) led to the decision to classify desoxypipradrol as a Class B substance in the UK under the Misuse of Drugs Act, 1971 (Home Office, 2012).

In Silico Evidence

In the present study, we elaborated the molecular features that may determine the mode of drug binding at DAT by modeling two structurally distinct inhibitors as well as the releaser amphetamine at the binding site of DAT. MD simulations provide a suitable computational method to reveal structural changes within DAT in response to stimulant effects. Amphetamine, transported *via* DAT like a substrate, stimulates the efflux of intracellular DA; MD simulations have shown structural rearrangements of the transporter toward an inward-facing conformation, while cocaine binding inhibits DA transport through forcing DAT to remain in the outward-facing conformation (Cheng and Bahar, 2019).

To date, there is no structural information available about the binding of 2-DPMP to DAT. We know from the docking study that 2-DPMP can bind to the S1 site, which also supports the autoradiography findings where 2-DPMP competed with the radioligand. Previously mentioned *in vitro* evidence from fast cyclic voltammetry suggests that 2-DPMP is more cocaine-like than amphetamine-like (Davidson and Ramsey, 2012), which is also consistent with Simmler et al. (2014).

Overall, amphetamine and cocaine behaved structurally as we expected at the atomistic level, with structural rearrangements of DAT toward the inward-facing conformation and outward-facing conformation respectively. Several residues in both the transmembrane regions TM1 and TM6, including D79 directly interacts with the cationic amine of typical drugs of addiction, including cocaine-like molecules that occupy the primary binding site (Vaughan et al., 2007; Parnas et al., 2008). The importance of transmembrane regions, TM1 and TM6, in stabilizing the S1 binding site has been previously studied. The binding of cocaine makes the cysteine residue (C90), located on the extracellular side of TM1, more reactive toward impermeable sulfhydryl-reducing reagents, suggesting that cocaine binding in DAT changes the conformation of TM1 (Reith et al., 2001). Additionally, site-directed mutagenesis of TM1 D79 to glutamate (D79E) decreases the binding affinity of cocaine and its analogs, while mutation of D79 to alanine (D79A) or leucine (D79L) prevents binding completely (Ukairo et al., 2005).

2-DPMP in the simulated time-frame shows characteristics toward an outward-facing conformation and 2-DPMP binding to DAT is a plausible reason for an increase in extracellular dopamine observed *in vivo* in the NAc and striatum of free-moving rats. The atomistic and molecular properties of 2-DPMP, which is highly lipophilic with a longer duration of action when compared to most psychostimulants of the same class, can explain its behavioral effects reported in humans. The absence of polar functional groups that are usually targeted by metabolic enzymes accounts for its persistent biological activity after use and a long elimination half-life (Coppola and Mondola, 2012).

Although DAT features as the main molecular target for typical stimulants, responsible for their DA-enhancing effects, and dopamine is involved in the acute effects of stimulants, the complex and long-term process of addiction involves as well other neurotransmitter systems in addition to that of dopamine. Thus, the noradrenaline/norepinephrine transporter (NET) and serotonin transporter (SERT) contribute to the stimulant characteristics to a varying degree across the range of stimulant drugs (Gibbons, 2012; Iversen et al., 2013; Vaughan and Foster, 2013; Iversen et al., 2014; Simmler et al., 2014). Both noradrenaline and serotonin transporters play a role in the case of 2-DPMP and other stimulant NPS (Gibbons, 2012; Iversen et al., 2013; Vaughan and Foster, 2013; Iversen et al., 2014; Simmler et al., 2014), and not only DAT but also NET interactions positively correlate with the clinical potency of stimulant NPS, while SERT inhibition potency inversely correlates with human doses (Luethi et al., 2018). The dopamine theory of addiction that informs the present work has been unchallenged for typical stimulants and supported even for opioids such as morphine (Pontieri et al., 1995) and heroin (Corre et al., 2018), despite some contradicting evidence that opioids do not seem to cause substantial dopaminergic responses in humans (Daglish et al., 2008) and mice (Castañé et al., 2008). However, it is a valid argument that addiction is a highly complex phenomenon and reducing it to just one neurotransmitter is limiting (Nutt et al., 2015). Future computational studies will address the monoaminergic mechanisms of stimulant effects beyond those of dopamine.

The present study is not free from limitations. Thus, quantitative behavioral observations of rat mobility, freezing, piloerection, grooming, sniffing, gnawing, rearing and scratching during the *in vivo* microdialysis experiments would have provided information on the level of behavioral stimulation caused by the drug. Additionally, the time scales for the simulations in this study may not be enough to sample the conformational space accessed by these compounds in DAT. Further studies can be expected to expand on these time scales, with an emphasis on comparing the signature dynamics of various drugs of addiction and stimulations at DAT. These observations will include structural changes of important interhelical distances of transmembrane TM1b–TM10 and TM6a–TM10 and TM1a–TM6b, representative of EC vestibule openings and IC vestibule openings, respectively.

One outcome of this research is the development of a framework for studying stimulant abuse by utilizing both *in silico* and *in vitro* methods. The research presented here and in our previous studies (Sahai et al., 2017; Sahai et al., 2018) can be adapted for other psychostimulants that act at their membrane protein targets. This framework also fulfills the 3Rs, offering alternative methods (replacement, reduction and refinement) to animal experimentation *via* computational approaches. As structural information increasingly becomes available, our understanding at the molecular level improves. Realistic molecular models can be combined with docking and MD to help us study the dynamic nature of various stimulants when bound to their membrane protein targets. Not only can we can

study the influence of the surrounding environment but we can also observe how the stimulants bind to the protein. This permits us to characterize the transport mechanisms at the atomistic level, and thus complement experimental evidence.

To conclude: our combination of the *in vivo* and *in vitro* pharmacological approaches with *in silico* methods—novel in research on NPS—allows for the extensive characterization of a new psychoactive compound and its effects on the mammalian brain, with its target regions and molecular and atomistic mechanisms. The present findings prove that molecular modeling has an important place in future 3Rs methodology used in research on the mechanisms of addiction; thus, the present study demonstrates the new methodological directions in research on drug dependence. Beyond methodological considerations, our present work provides an insight into not only the properties of the drug and the mechanisms of its action but more broadly into the role of dopamine in the molecular and neurobiological mechanisms of brain responses to stimulant drugs which have addictive potential.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by: All animal experiments were carried out in accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research according to Italian (D.L. 116/92 and 152/06) and European Council directives (609/86 and 63/2010) and in compliance with the approved animal policies by the Ethical Committee for Animal Experiments (CESA, University of Cagliari) and the Italian Ministry of Health (Aut. N.162/2016-PR).

AUTHOR CONTRIBUTIONS

BL, MS, and JO-J were responsible for the study concept and design. BL and JO-J conducted the ligand binding experiments and analyzed the data. ML facilitated and designed the microdialysis study. BL collected and interpreted the microdialysis data with ML's input. HS parameterized the ligands and performed the docking studies. MS performed the molecular simulations and interpreted the findings. JO-J, BL, and MS drafted the manuscript. All authors critically reviewed and edited the content and approved the final version.

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Chronic Use of Synthetic Cannabinoids Is Associated With Impairment in Working Memory and Mental Flexibility

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Background: We have recently shown that chronic use of Synthetic Cannabinoids (SCs) has been associated with mood disorders and impairments in executive functions. There is also evidence indicating that chronic SC users have higher rates of comorbidity with depression and psychotic symptoms. Here, we investigate performance on executive function and emotional processing tasks in regular SC users and a measure of schizotypal traits.

Method: Thirty chronic SC users, 32 recreational cannabis users, and 32 non-using control participants, without history of mental disorder, or current substance abuse diagnosis (mean age 26 ± 4.27 years; 85 males, 9 females), were tested in addiction treatment centers in Israel. Computerized neurocognitive function tests; the N-back task, Go/No-Go task, Wisconsin Sorting Card-like Task (WSCT), and emotional face recognition task and questionnaires of depression, anxiety and schizotypal traits and symptoms were used.

Results: SC users have performed worse than recreational cannabis users and non-cannabis users on the N-back working-memory task (lower accuracy) and the WSCT cognitive flexibility task. SC users showed greater schizotypal traits and symptoms compared with recreational cannabis users and non-user control participants. A positive association was found in cannabinoid-user groups between schizotypal traits and symptoms and cognitive and emotional processing measures. Finally, SC users have scored higher on depression and state-trait anxiety measures than recreational cannabis users or healthy control participants.

Conclusions: Repeated use of SCs is associated with impairment in executive functions and emotional processing. These alterations are associated with depression and schizotypal traits and symptoms. This adds to existing evidence on the long-term consequences of SC drugs and their risks for mental health.

Keywords: synthetic cannabinoids, response inhibition, mental flexibility, emotional processing, cannabis

INTRODUCTION

There is a growing use of novel psychoactive substances (NPSs) which contain various psychoactive agents (1, 2). Some of these NPSs contain Synthetic Cannabinoid (SC) compounds which are marketed as a natural herbal mixture under different brands names (3–5). These drugs are composed of multiple types of extremely potent synthetic cannabinoid-agonists as well as additional psychoactive constituents, of which some are unknown (5, 6). The intoxicating effects of SC drugs are similar to the effects of cannabis, commonly with; SC drugs induce reactions such as relaxation, euphoria, perceptual disturbances, and alterations in cognitive abilities (7–9). Importantly, the adverse effects in terms of duration and severity of SCs are more intense than natural cannabis. SC use has been linked with a range of undesired physiological effects ranging in intensity, from nausea to more severe symptoms such as psychomotor agitation, diaphoresis, and palpitations (10, 11). Furthermore, converging evidence has shown an association between SC use and severe affective alterations and cognitive deficits (3, 12). Although SC drugs are gaining popularity, the information regarding their adverse effect and long-term impact on health is limited as well as the general awareness regarding the damaging potential of these drugs (13, 14).

Similar to herbal cannabis, SCs induce their effect through the activation of cannabinoid receptors (CB_1 and CB_2) within the Central Nerves System (CNS) (15). In contrast to the psychoactive and non-psychoactive compounds in herbal cannabis such as Δ-9-tetrahydro-cannabinol (THC) and Cannabidiol (CBD) (3–5), SC drugs contain a mixture of psychoactive ingredients, which are more potent and efficacious at the CB_1 and CB_2 receptors (16–18). Therefore, although SC drugs are designed to mimic the effect of cannabis, their effects even in low doses are more severe, persistent and unpredictable (8, 19, 20).

There is an agreement that the central psychoactive effect of cannabinoid-based drugs is exerted through direct stimulation of CB_1 receptors (21, 22). These receptors are observed in high densities in brain regions including the prefrontal cortex, hippocampus, basal ganglia, anterior cingulate, and cerebellum (23). An activation of CB_1 receptors induces alterations in the release of neurotransmitters and amino-acids in a wide range of neural networks in sub-cortical and cortical brain regions (20). As CB_1 receptors interact with additional neurotransmitter systems, cannabinoids exert their effects on a variety of cognitive functions, emotional processing, sensory perception and regulation of incoming sensory information (24).

When administered acutely, CB_1 agonist agents such as THC, the main psychoactive compound of cannabis and different types of SCs, can impair cognitive function as well as emotional processing (20, 25–27). Such effects were observed in animal and human studies (12, 28). Several studies have shown that acute administration of cannabinoid-agonists alters the ability to recognize emotions in others and may induce anxiety symptoms (25, 26, 29). Furthermore, D'Souza and colleagues have reported dose-related adverse effects which were induced following acute consumption of THC in healthy participants; THC has produced a broad range of transient symptoms, including anxiety symptoms and cognitive deficits in healthy individuals that resemble some aspects of psychosis (26). Bedi and colleagues have conducted a well-designed, double-blind, randomized clinical study, and they have reported a dose-related cognitive decline that has been observed in current cannabis users following a treatment with Nabilone (30). More recently, Theunissen and colleagues showed that acute consumption of SC JWH-018 has induced poor coordination, memory deficits, and perception alteration in current cannabis users (20).

The long-term effects of cannabinoid-agonists on cognitive and emotional functions in humans is both mixed and limited, and it is mostly focused on herbal cannabis (27, 31, 32). Neuro-imaging studies have shown that repeated use of cannabis was associated with structural and functional alterations in the CNS. Interestingly, alterations were observed in pre-frontal and limbic regions that are involved in cognitive and emotional processing functions (33). However, while behavioral manifestations of these neuronal alterations in humans are modest (27, 31–33), pre-clinical studies have shown that chronic treatment with cannabinoid-agonists such as SCs has caused severe and persistent cognitive impairment as well as an affective imbalance (34–37). These studies have indicated an association between repeated treatment with cannabinoid-agonists and cognitive deficits in a wide range of domains including; attention, working memory and cognitive flexibility (12). Moreover, treatment with cannabinoid-agonists has induced depression-like and anxiety-like states, and chronic treatment with CB_1-receptor agonists are considered as applied animal models for affective disorders (38–40). Interestingly, the disruptive effects of cannabinoid-agonists were associated with exposure at an early age, genetic predisposition, and with a higher dosage (34, 41–43).

Clinical reports have indicated a similar phenomenon, in which severe affective disturbances and cognitive deficits were observed among chronic SC users. Cengel and colleagues have shown generalized cognitive impairments in SC users (44). Still, most of the reports regarding this association are based on self-report measurements and surveys (8, 45, 46). Castellanos and Thornton have reported that young SC users have experienced alterations in short-term memory with their main symptom; a severe psychotic episode (47). Additional studies have reported similar clinical symptoms including severe deficits in cognitive functions and psychosis (19, 48, 49). The association between cannabinoids and psychosis is well established (50, 51). There is accumulating evidence of an association between cannabinoids use and psychotic proneness, yet a causal relationship between these two factors is missing (52). Some authors have suggested that psychotic proneness may underline an individual's genetic predisposition, moderates the adverse effects of cannabis (53, 54). Accordingly, several studies have shown a correlation between cannabinoids use and schizotypal traits which appear to represent individual psychotic proneness among students (55–57). Moreover, schizotypal traits have been associated with greater psychotic experiences and worse undesired effects of cannabis intoxication (58, 59).

In our previous study, we have shown initial evidence for impairments of Working Memory (WM), response inhibition and long-term memory among SC users, compared with non-synthetic cannabis users and healthy control participants (60). SC users displayed lower accuracy and longer reaction time on performance of cognitive tasks compared with non-users and cannabis users (60). In a further study, we have shown a WM impairment in SC users which was associated with structural and functional deficits in several brain regions including the middle frontal gyrus, frontal orbital gyrus, inferior frontal gyrus, insula, anterior cingulate cortex, and the precuneus. Surprisingly, response inhibition in SC users was preserved compared with healthy participants (61). In both studies, SCs have shown depression alongside these cognitive impairments, yet we were unable to control possible confounding factors such as educational levels (60, 61). Altogether, these studies have indicated that SC consumption is associated with severe cognitive impairments, while, there is disagreement regarding the specific cognitive distortion associated with repeated SC use. Moreover, although there is strong evidence for emotional disturbances associated with repeated SC use, there are no studies on emotional processing in SC users. Finally, no study has shown any association between cognitive and emotional function and psychosis proneness in SC users. The purpose of the current study was to expand existing knowledge regarding the effects of SCs on executive function and emotional processing. In addition, we aimed to explore the possible association of these functions with schizotypal traits. We have hypothesized that chronic use of SC would be associated with poorer performance on WM and on response inhibition and cognitive flexibility tasks compared to recreational cannabis users and non-users. Furthermore, we have expected SC users to show worse performance on the emotional processing task compared with performance of both control groups. Finally, we have hypothesized that executive function and emotional processing impairments would be associated with schizotypal proneness among cannabinoid users and not among healthy control participants.

METHODS

Ethical Approvals

The Institutional Review Board of Ariel University and the Israeli Ministry of Health Office have approved the study. All participants have volunteered to participate in the study and they did not get any incentives for their participation. All the participants have signed an informed consent prior to participation.

Participants

Ninety-four participants were recruited for the study, including 85 males and 9 females. The mean age was 26.01 (SD = 4.26) years. The total sample was divided into three groups based on their self-reported substance use history: (a) SC users (b) recreational cannabis users and (c) non-users. Both regular cannabis users and non-users were recruited by using convenient snowball sampling *via* friends, relatives or advertisements in social networks. SC users were recruited from three drug addiction treatment inpatient units supervised by the Israeli Ministry of Health located in Ashdod, Eilabun, and Malcishua in Israel. All the participants were administered a screening interview that covered the following areas: medical history, illicit drug use, current psychiatric status, personal psychiatric history and native language. The screening interview, the explanation of the procedure, and the data collection were conducted by a licensed Psychologist (KC).

Synthetic Cannabinoids-Users

The SC users' group was initially comprised of 38 participants, 36 males and 2 females, who have frequently consumed SC drugs over the last 2 years. We have defined the inclusion criteria for SC users as regular SC use on a monthly basis, with minimal usage of at least 10 times in the last year and without binge consumption defined as more than 4 usages of SC during the last month. Eight male participants from this group have not completed the experiment and were excluded following the initial screening interview and their data were excluded, thus, the group was finally composed of 30 participants. The mean age of the remaining 30 participants was 26.97 years (SD = 4.17). All participants were evaluated and diagnosed by a senior Psychiatrist prior to the experiment. They were confirmed as not suffering from current psychosis, having co-morbidity with other psychiatric or neurological disorders or a past or current substance use disorder other than cannabinoids.

Recreational Cannabis-Users

The recreational cannabis users' group has included 32 participants (28 males and 4 females), who consumed cannabis for recreational purpose more than 10 times in the last year and have never consumed SCs. The mean age in the cannabis user group was 26.99 (SD = 4.17) years. Two participants have not finished the emotional processing task, and the missing data was omitted from related analyses. Exclusion criteria for cannabis participants were history of neurological or psychiatric disorder and history or current substance use disorder.

Non-Users

The group of non-users has included 29 males and 3 females, altogether 32 healthy individuals, who have reported that they did not consume cannabinoid-based drugs during the past 2 years and have never consumed SCs. Participants' mean age was 25.41 (SD = 4.53) years. One participant has not finished the emotional processing task, the missing data was omitted from related analyses. Exclusion criteria for healthy control participants were history of neurological or psychiatric disorder and history or current substance use disorder.

Materials, Stimuli, and Design

Demographic and Self-Reported Questionnaires

The demographic questionnaire included items on education level, age, and gender. The questionnaire also contained items regarding the use of psychoactive substances, focusing on

cannabinoid-based drugs, and including additional psychoactive substances, tobacco, and alcohol. The date of the last use, frequency of past week, past month and frequency of past year drug use were also assessed. In addition, for measuring psychotic proneness, participants have completed self-reported measures of Schizotypal Personality Questionnaire (SPQ-B) (62, 63). The internal consistency of the SPQ-B ranged from 0.75 to 0.83. In this study, the SPQ-B had a Cronbach internal reliability of α = 0.86. Furthermore, participants have answered the Beck Depression Inventory (BDI) (64) (Cronbach α = 0.86), and the Spielberger state (Cronbach α = 0.86)-trait (Cronbach α = 0.86) anxiety inventory (STAI-S, STAI-T) (65).

Executive Function Measures (EF)

For assessing EF, we used computer versions of three tasks which measure; (a) response inhibition (b) WM, and (c) cognitive flexibility (66). (a) The Go/No-Go task was used for assessing response inhibition and sustained attention. In this task participants are required to tap on a corresponding key when "Go" stimuli (blue rectangles) are presented, and to inhibit responses when "No-Go" stimuli (black rectangles) are presented (67, 68). The task has included 150 trials, and the probabilities of occurrence of "Go" and "No-Go" stimuli were equal and randomized (68). The task's measures RTs, and two types of errors; (1) commission errors (percentages of non-responses for "go" stimuli) (2) omission errors (percentages of responses for "no-go" stimuli). Increased commission or omission error rates in the task have indicated greater impulsivity or sustained attention impairments (69).

(b) The n-back task is considered a "gold-standard" measure for WM function and it consists of alternating conditions with two WM load levels: 1-back and 2-back (70). In the 1-back condition, participants are required to decide if a stimulus on the screen is identical to the previous stimulus. In the 2-back condition, participants are required to decide if a stimulus on the screen is identical to the stimulus presented two steps beck. Accuracy percentages of the two conditions are recorded (71). The two conditions of the n-back represent measures of WM at low and high load (72), in our previous work we have demonstrated WM deficits in SC users, in both 1-back and 2-back conditions (60).

(c) A modified short version of the Wisconsin Card Sorting-like Task (WCST) (73) was used for measuring cognitive flexibility. The short version of the WCST includes 64 response cards and 4 stimulus cards. The stimulus cards are presented in a standard left-to-right order, while response cards are presented one by one according to a specific criterion (color, shape, or number). In the sorting task, the response card should correspond to a feature of the target card. After a sequence of 10 correct responses, the sorting criterion changes and a new sorting criterion must be discovered. The task includes 64 trials. The following indices were recorded; (a) number of completing sets, (b) number of maintaining set failures (c) number of perseveration errors (set-shifting failures), and (d) number of non-perseveration errors. These indices are associated with chronic consumption of cannabis and were observed in schizophrenic patients (74, 75).

Emotional Processing

The static facial affect recognition task was used to assess emotion recognition (76). During this task, participants are required to recognize different types of facial expressions of five emotions: happiness, sadness, anger, disgust, fearfulness, and neutral facial expressions of 4 different faces (2 males and 2 females). We have calculated participants' proportion of accuracy, false alarms, sensitivity (Pr) and response bias (Br) in each emotion (76, 77).

RESULTS

Statistical Analysis

The analysis of the results was performed on a Statistical Package for Social Science (SPSS) for windows v.21 (IBM Corp. Armonk, NY, USA). There were three cases of missing data, all missing values were excluded from the analysis. Differences among groups in terms of gender were tested using chi-square test. The group effects on cognitive and emotional processing measures were analyzed with univariate Analysis of Variance (ANOVAs); Bonferroni corrections for t-test were used for *post hoc* group comparisons. In a further analysis, demographic variables and depression, anxiety and tobacco consumption were added as covariates to the ANOVA, in order to investigate the possibility of confounding variables. In order to examine the relationships between age of first cannabinoid use and cognitive performances further Pearson correlations were computed separately for SC and cannabis user groups. Pearson correlations were computed separately for each group in order to explore correlation between SPQ-B, cognitive performance and emotional processing factors.

Sample Characteristic and Substance Use History

Participants' drug use history and demographic data are described in **Table 1**. The groups did not significantly differ by gender (χ^2 = 6.11, $p > 0.05$), age [F (2, 91) = 1.46, p = 0.32], education level [F (2, 91) = 1.49, p = 0.53] or by alcohol use history [F (2, 90) = 1.17, p = 0.31]. While there were no differences in tobacco consumption between cannabis and non-users [t (61) = 0.69, p = 0.77], SC users have consumed more tobacco than non-users [t (60) = 12.16, $p < 0.01$] and recreational cannabis users [t (59) = 11.49, $p < 0.01$]. SC users have used cannabinoid-based drugs earlier in life than recreational cannabis users [t (60) = 2.19, $p < 0.05$]. However, there were no differences between the groups in cannabinoid-consumption frequencies during the last year [t (59) = 0.66, p = 0.13].

There was a main effect of group on depression, anxiety and schizotypal trait measurers. SC users had greater scores on the SPQ-B than non-users [t (60) = 6.26, $p < 0.01$] and recreational cannabis users [t (59) = 5.63, $p < 0.01$], No differences was found in SPQ-B between non-users and recreational cannabis users [t (62) = 0.63, p = 0.8].

SC users have scored higher on the BDI than non-users [t (60) = 7.77; $p < 0.01$] and recreational cannabis users

TABLE 1 | Demographic and participants characteristics for each group.

	Synthetic	Cannabis	None	Significance
N, frequencies (male: female)	30 (28:2)	32 (28:4)	32 (29:3)	$p = 0.73$
Age, mean (SD)	25.93 (4.27)	27.71 (3.15)	25.4 (4.53)	$p = 0.32$
Education level (sd)	11.96 (1.29)	12.21 (0.69)	12.12 (0.55)	$p = 0.53$
Alcohol consumption (SD)	3.17 (2.72)	4.25 (3.12)	4.15 (3.14)	$p = 0.31$
Tabaco consumption (SD)	19 (8.23)	2.37 (4.75)	1.4 (2.83)	$p < 0.001$
Age of first use for cannabinoids	17.3 (4.61)	19.17 (2.87)	–	*p < 0.05*
Age of first use for SC	22.9 (5.7)	–	–	–
Age of first use for cannabis	17.34 (4.1)	19.17 (2.87)	–	*p = 0.05*
Frequency of cannabinoids use during the last year	202.68 (145)	186.87 (135.46)	–	$p = 0.13$
BDI, mean (SD)	40.17 (9.18)	24.93 (5)	25.90 (6.88)	$p < 0.001$
STAI trait, mean (SD)	49.44 (7.98)	34.04 (7.30)	35.21 (9.7)	$p < 0.001$
STAI state, mean (SD)	49.39 (9.75)	31.53 (9.11)	32.03 (10.03)	$p < 0.001$
SPQ-B, mean (SD)	11.66 (4.37)	5.5 (4.38)	4.81 (4.26)	*p < 0.001*

Age and education level reported in years; Alcohol consumption habits drink defined as glass of wine or 250 ml of beer or one shot of alcoholic beverages; Tabaco consumption, cigarettes per day; BDI, Back depression inventory scores; STAI, Silberberg Trait or State anxiety inventory scores; SPQ-B, Schizotypal Personality Questionnaire Brief; significant level of difference between drug groups within the total sample; n.s., non-significant difference.

[t (59) = 8.31, $p < 0.01$] but there were no differences on BDI score between non-users and recreational cannabis users [t (62) = 0.54; p =1]. SC users had higher scores on the STAI Trait and State compared with non-users [t (60) = 6.89, $p < 0.01$; t (60) = 6.6, $p < 0.01$] and recreational cannabis users [t (59) =7.01, $p < 0.01$; $p < 0.01$; t (59) = 7.15, $p < 0.01$]. There were no differences in STAI State and Trait scores between non-users and recreational cannabis users [t (62) = 6.6, $p = 1$; t (62) = 0.21, $p = 0.83$].

Cognitive Performance

The Go/No-Go Task

Reaction Time

A one-way ANOVA was conducted to explore the effect of group (SC, recreational cannabis users, non-users) on RTs in each condition. Results reveal a main group effect [F (2, 90) = 10.95, $p < 0.001$]. SC users were significantly slower in their responses than non-users [t (60) = 3.43 $p < 0.001$] and cannabis users [t (59) = 4.34, $p < 0.001$; t (59) = 3.9, $p < 0.001$]. There were no differences in reaction times of non-cannabis users and recreational cannabis users [t (61) = 0.38, $p = 0.75$] (**Table 2**). This effect remained significant when anxiety [F (2, 88) = 5.63, $p < 0.01$], depression [F (2, 88) = 6.7, $p < 0.01$], and schizotypal trait [F (2, 88) = 6.4, $p < 0.01$] were used as covariates. However, this effect was diminished when consumption of cigarettes with tobacco [F (2, 88) = 1.1, $p = 0.33$] was entered as a covariate.

Commission and Omission Errors

Analysis has revealed a main group effect on rate of omissions in the Go/No-go task [F(2,90) = 3.5, $p < 0.05$]. SC participants have made more omission errors than non-users [t(60) = 2.98, $p < 0.05$]. There were no differences in omission errors between recreational cannabis users and SC users [t(59) = 1.04, $p = 0.3$] and non-users [t(61) = 1.5, $p = 0.13$]. This effect remained significant when anxiety [F(2,88) = 3.77, $p < 0.05$], depression [F(2,88) = 1.42, $p < 0.05$] and schizotypal trait [F(2,88) = 3.16, $p < 0.05$] were used as covariates, yet, it was no longer significant when consumption of cigarettes with tobacco [F(2,88) = 1.41, $p = 0.24$] was used as a covariate. Further analysis has failed to show differences between the groups in the rate of commission errors [F(2,90) = 0.43, $p = 0.64$] (**Table 2**).

TABLE 2 | Means (standard deviations) of performance on the Go/No Go task in SC, cannabis users and non-user group.

Go/No Go	Group			Comparison	
	SC	Cannabis	Non-users	*F* (2,90)	*P*-value
Reaction time	508 (147.08)	399.78 (59.52)	406.34 (76.54)	10.95	$p < 0.001$
Omission	1.04 (1.47)	0.64 (1.51)	0.19 (0.64)	3.50	$p < 0.05$
Commission	1.11 (1.84)	0.9 (1.56)	0.75 (1.1)	0.43	$p < 0.65$

Values express mean (SD), reaction time are in milliseconds, errors reported as percentages.

The N-Back Task

For the analysis of WM performances, accuracy data were analyzed using a repeated measures ANOVA with group (SC, recreational cannabis, and non-users) as the between-subject factor and memory load (1-back, 2-back) as the within-subject factors. Results have revealed a significant main effect for memory load, [F (1, 90) = 82.75, $p < 0.001$]. The accuracy scores in the 1-back condition were significantly higher than the accuracy scores of the 2-back condition [t (92) = 8.91, $p < 0.001$]. Additionally, a main group effect was observed, [F (2, 90) = 18.52; $p < 0.001$]. Post hoc analyses with Bonferroni corrections has revealed that SC users were significantly less accurate than both non-users [t (60) = 5.67, $p < 0.001$] and recreational cannabis users [t (61) = 4.80, $p < 0.01$]. There was no difference in accuracy between recreational cannabis users and non-users [t (62) = 0.83, $p = 1$] (**Table 3**). The effect on accuracy remained significant when tobacco [F (2,88) = 5.53, $p < 0.01$], schizotypal trait [F (2,88) = 5.58, $p < 0.01$], anxiety [F (2,88) = 7.23, $p < 0.01$], and depression [F (2,88) = 3.7, $p < 0.05$] were used as covariates.

The Wisconsin Sorting Card-Task

Analysis of Number of Completing Sets

There was a main group effect on the number of completing sets [F (2, 91) = 35.84, $p < 0.001$], SC had completed less sets (M = 1.66, SD = 1) than non-users (M = 3.96, SD = 4.03)

TABLE 3 | Accuracy of N-back performance by group.

	Group			Comparison	
	SC	Cannabis	None	*F*(2,90)	*P*-value
1-back	86.09 (7.4)	91.15 (4)	92.39 (2.68)	18.52	$p < 0.001$
2-back	79.05 (9.25)	87.211 (6.09)	87.87 (4.8)		

Accuracy values are expressed as mean correct percentage (SD).

[t (60) = 8.17, $p < 0.001$] and recreational cannabis users (M = 3.75, SD = 1.29) [t (60) = 6.82, $p < 0.001$]. No differences in number of completing sets between non-users and recreational cannabis users [t (62) = 0.72, $p = 1$]. The main effect remained significant in further ANCOVAs when tobacco-cigarette consumption [F (2, 89) = 14.24, $p < 0.01$], schizotypal trait [F (2, 88) = 14.75, $p < 0.01$], anxiety [F (2, 88) = 16.15, $p < 0.001$], and depression [F (2, 89) = 10.7, $p < 0.01$] were used as covariates.

Analysis of Maintaining Set Failures

There was a main group effect on maintaining set failures [F (2, 91) = 3.43, $p < 0.05$], SC had performed more failures in maintaining sets (M = 1, SD = 1.20) than non-users (M = 0.34, SD = 0.75) [t (60) = 2.6, $p < 0.05$]. There was no difference between SC users and recreational cannabis user (M = 0.56, SD = 0.75) [t (60) = 1.72, $p = 0.19$] and non-users vs. recreational cannabis users [t (62) = 1.63, $p = 1$]. This main effect remained significant in further ANCOVAs when tobacco cigarette consumption [F (2, 89) = 14.24, $p < 0.01$] and anxiety [F (2, 88) = 16.15, $p < 0.001$], were used as covariates. However, this effect was reduced to a trend when depression was used as a covariate [F (2, 89) = 2.5, $p = 0.08$] and it was diminished when schizotypal scores were entered as a covariate [F (2, 88) = 2.1, $p = 0.12$].

Analysis of Non-Perseverative and Perseverative Errors

A one-way ANOVA has indicated a main group effect on non-perseverative errors [F (2, 91) = 43.58, $p < 0.01$] and perseverative errors [F (2, 91) = 19.98, $p < 0.01$]. SC users had performed more non-perseverative errors (M = 12.86, SD = 5.07) and perseverative errors (M = 11.53, SD = 3.76) than non-users (M = 5.65, SD = 2.75; M = 6.09, SD = 2.58) [t (60) = 8.16, $p < 0.001$; t(60) = 5.83, p < 0.001] and recreational cannabis users (M = 5.71, SD = 2.6; M = 7, SD = 4.28) [t (59) = 8.80, $p < 0.01$; t(59) = 4.76, $p < 0.01$]. There were no differences in these measurers between non-users and recreational cannabis users [t (61) = 0.07, $p = 1$; t (61) = 1.09, $p = 1$]. The group effect on error rates remained significant in further ANCOVAs when consumption of cigarettes with tobacco [F (2, 89) = 13.24, $p < 0.001$], schizotypal trait [F (2, 88) = 19.56, $p < 0.001$] anxiety [F (2, 88) = 23.05, $p < 0.001$] and depression [F (2, 89) = 13.5, $p < 0.01$], were used as covariates.

Emotional Processing Task

A repeated measures ANOVA was conducted on hit rates, false alarms, Pr, and Br with six emotions (anger, sadness, disgust, happiness, fear, and neutrality) as within-subject factors and group (non-users, cannabis users, SC users) as the between-subject factor.

Hit Rates

There was a main group effect on hit rates [F (2, 88) = 4.6, $p < 0.05$]. Post-hoc tests has indicated that while there was no difference between non-users (M = 0.73, SD = 0.07) and recreational cannabis users (M = 0.71, SD = 0.12) [t (61) = 0.6, $p = 1$], SC users had less hits (M = 0.62, SD = 0.14) compared with non-users [t (58) = 4.23, $p < 0.01$] and marginally less than accurate then recreational cannabis users [t (59) = 4.98, $p = 0.08$]. There was not interaction between group and emotion [F (10, 440) = 0.38, $p = 0.25$]. The group effect on hit rates remained significant in further ANCOVAs when consumption of cigarettes with tobacco [F (2, 86) = 4.61, $p < 0.05$] and anxiety [F (2, 85) = 3.83, $p < 0.05$] were used as covariates. This was no longer significant when depression [F (2, 86) = 1.03, $p = 0.35$] or schizotypal score were used as covariates [F (2, 88) = 1.12, $p = 0.32$] (**Figure 1A**).

False Alarms

There was a main group effect on false alarms [F (2, 88) = 338.74, $p < 0.001$] (**Figure 1B**). Post-hoc tests has indicated that SC-users had more false alarms (M = 0.09, SD = 0.06) than non-users (M = 0.06, SD = 0.016) [t (58) = 2.74, $p < 0.05$] and marginally more false alarms then recreational cannabis users (M = 0.06, SD =

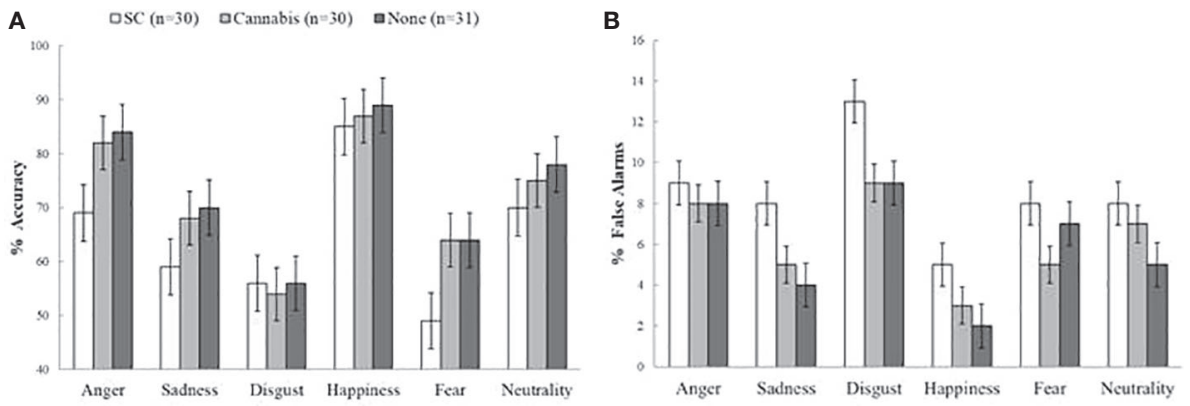

FIGURE 1 | Accuracy and false alarm rates on the emotion recognition task. **(A)** Main effect of group on accuracy (mean percentage of correct responses). Synthetic cannabinoids (SC) users were less accurate compared to non-users and marginally less accurate than natural cannabis users. **(B)** Main group effect on false alarms (mean percentage of false alarms). SC users had more false alarms than non-users, and marginally more false alarms than natural cannabis users. The error bars represent standard error of the mean.

0.02) [t (59) = 2.3, p = 0.07]. There was no difference in false alarms between recreational cannabis users and non-users [t (61) = 0.7, p = 1]. Finally, there was not interaction effect of group and emotion [F (10, 440) = 0.65, p = 0.72]. The group effect on false alarms rates remained significant in further ANCOVAs when consumption of cigarettes with tobacco [F (2, 86) = 5.74, p < 0.01], and anxiety [F (2, 85) = 3.21, p < 0.05] were used as covariates. This was no longer significant when depression [F (2, 86) = 1.13, p = 0.36] or schizotypal [F (2, 88) = 1.12, p = 0.33] were used as covariates.

Sensitivity

There was a main group effect on Pr rates [F (2, 88) = 4.67, p < 0.05] (**Figure 2A**). Post-hoc tests indicated that SC-users showed less sensitivity (M = 0.55, SD = 0.18) than non-users (M = 0.66, SD = 0.09) [t (58) = 2.88, p < 0.05] and marginally lower scores then recreational cannabis users (M = 0.64, SD = 0.14) [t (59) = 2.27, p = 0.07]. There were no differences between recreational cannabis users and non-users [t (61) = 0.6, p = 1]. Finally, there was no interaction effect of group and emotion [F (10, 440) = 1.42, p = 0.33]. The effect of group on Pr rates remained significant in further ANCOVAs when tobacco [F (2, 86) = 5.03, p < 0.001], and anxiety [F (2, 85) = 4.23, p < 0.01] were used as covariate factors. Yet, the effect was diminished when depression [F (2, 89) = 1.17, p = 0.31] or schizotypal [F (2, 88) = 1.87, p=0.31] were used as covariates.

Response Bias

There was a main effect of emotion type on Br score [F (5, 440) = 11.34, p < 0.01] but analysis of group effects on response bias has shown no significant differences between groups [F (10, 440) = 2, p = 0.17] (**Figure 2B**).

Analyses Between Age of First Cannabinoid Use and Tasks Performance

We have investigated the association between age of first cannabinoids use and task performance using simple Pearson's correlation for SC and cannabis groups. For SC users, there were no significant correlations between age of first cannabis and SC consumption with RTs (r = 0.78, p = 0.68; r = 0.02, p = 0.89), omission errors (r = −0.09, p = 0.622; r = 0.03, p = 0.85) on the Go-No/Go task, WM performance on the N-back task (r = 0.16, p = 0.39; r = −0.23, p = 0.21), number of completing sets (r = 0.24, p = 0.19; r = 0.11, p = 0.53), maintaining sets failures (r = −0.22, p = 0.23; r = −0.12, p = 0.52) and error rates (r = −0.09, p = 0.60; r = 0.19, p = 0.31) on the WCST, and accuracy (r = 0.20, p = 0.27; r = −0.43, p = 0.82), false-alarm (r = −0.11, p = 0.53; r = −0.03, p = 0.84), sensitivity (r = 0.19, p = 0.30; r = −0.04, p = 0.81) or response bias (r = −0.17, p = 0.35; r = −0.06, p = 0.72) on the emotional processing task. Similarly, for cannabis users there was no significant correlation between age of first cannabis use and WM performance (r = 0.11, p = 0.54), RTs (r = −0.25, p = 0.17) and omission errors (r = −0.15, p = 0.41) on the Go-No/Go task, number of completing sets (r = −0.04, p = 0.80), maintaining set failures (r = 0.05, p = 0.75) and error rates (r = 0.04, p = 0.81) on the WCST, and accuracy (r = −0.31, p = 0.09), false-alarm (r = 0.22, p = 0.23), sensitivity (r = −0.31, p = 0.09) or response bias (r = −0.08, p = 0.66) on the emotional processing task.

Exploratory Analyses Between Schizotypal and Tasks Performances

We have investigated the association between SPQ-B scores and task performance using simple Pearson's correlation within each group separately. For SC users, there was a negative correlation between schizotypal traits and WM performance (r = −0.45, p < 0.01). A similar pattern was observed for recreational cannabis users; greater scores on the schizotypal trait scale were positively associated with less accuracy on the WM task (r = −0.36, p < 0.05). Moreover, for SC users, greater scores on the schizotypal trait scale were associated with poorer accuracy (r = 0.45, p < 0.05) and less sensitivity (r = 0.44, p < 0.01) on the emotional processing task. Among non-users there were no significant associations between SPQ-B scores and WM performance (r = −0.05, p = 0.37), emotional processing accuracy (r = −0.13, p = 0.23) or sensitivity (r = −0.11, p = 0.28) measurers.

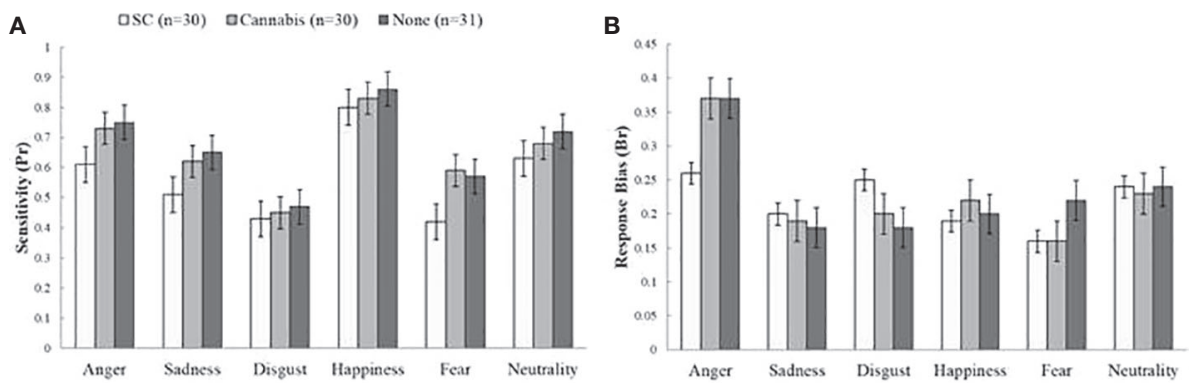

FIGURE 2 | Sensitivity (Pr) and response bias (Br) values on the emotional processing task. **(A)** Main effect of group on sensitivity (Pr). Synthetic cannabinoids (SC) users showed less sensitivity than non-users and marginally lower score than natural cannabis users. **(B)** No differences between groups in response bias index (Br). The bars represent mean scores ± standard error of the mean.

DISCUSSION

The current study has shown impairments in mental flexibility of SC users. These cognitive deficits cannot be explained by demographic variables such as age, gender, alcohol consumption or educational levels. Previous studies have indicated a generalized impairment in high-order cognitive function of SC users, impairments which were accompanied with neuronal alterations and depression.

The main findings of this study indicate executive function deficits of chronic SC users. These impairments demonstrate poor accuracy on the n-back task, indicating an impairment of WM. Performance on the WCST task has also shown an impairment of mental flexibility indicated by more errors, less completed categories and more failures to maintain sets. These deficits were not observed in recreational cannabis users or healthy control participants. These results are consistent with our previous findings on WM impairment (60, 61) and with additional human and pre-clinical studies examining the effects cannabinoid-agonists on cognitive function. Cengel and colleagues reported impairments in several cognitive functions such as attention, memory, executive, and visual-spatial functions of SC users that were more severe than individuals with cannabis use disorder and healthy control group (44). Furthermore, SC users made more omission errors on the Go/No-go task, indicating impairment in response inhibition. Further analysis of covariance has indicated that adding depression, anxiety, schizotypal trait, and tobacco consumption as covariates has reduced this effect.

In contrast, negative results were reported by Altintas and colleagues who have examined several cognitive domains in SC users who have experienced psychotic episodes and compared their performance with hospitalized schizophrenic patients. Interestingly, there were no differences between the schizophrenic patients and SC users in cognitive function (78).

Recently, Livny and colleagues have reported WM impairment in SC users that were tested on the n-back test and these impairments were associated with structural and functional deficits in several brain regions including the middle frontal gyrus, frontal orbital gyrus, inferior frontal gyrus, insula, anterior cingulate cortex and the precuneus (61). Yet, the response inhibition ability in SC using the same task as ours was preserved compared with control participants. Our results, together with Livny and colleagues suggest that unlike WM impairments, there is no strong evidence for response inhibition impairment in SC users as this variable was confounded by other variables such as tobacco smoking and depression.

The Pharmacological approach may provide an appropriate explanation for the association between the consumption of cannabinoid-agonists and impairment of cognitive functions (35, 37). Accordingly, a consumption of exogenous CB_1 receptor agonists may alter CB_1 modulation of additional neurotransmitters such as dopamine, serotonin and noradrenaline (20, 22, 35). In a pre-clinical study, chronic consumption of THC has led to dopamine receptors down-regulation as well as WM deficits (79). Additional rodent studies have indicated that administration of CB_1 receptor agonists has induced a decrease in prefrontal serotonin levels in a way which alters cognitive function in general and learning abilities specific (80). Finally, activation of CB_1 receptors produces an inhibitory effect on GABAergic neurons, an effect which alters the neuronal activity of prefrontal brain regions (81). Furthermore, studies show the inhibitory effects of cannabinoid-agonists on GABA activity in the rat's frontal cortex, amygdala, hippocampus and cerebellum (82, 83). This inhibition has produced a down-regulation in GABAergic transmission in the prefrontal cortex that is associated with cognitive impairments (83). Altogether, these may explain the wide range of cognitive dysfunction which was observed among SC users.

Impaired emotional processing was observed among SC users compared with regular cannabis users and non-users. The impairment was demonstrated by lower accuracy, more false alarms and lower sensitivity. However, when depression ratings were added as a covariate the effect was diminished. This finding implies that depression has a strong effect on emotional processing and it can explain why SC users have made errors in recognizing facial emotional expressions in other people. It is well established that depressed patients have difficulties in processing facial emotional expressions (84) and we now demonstrate the association between emotional processing and depression in SC users. Our results support previous human and pre-clinical studies which have shown the adverse effect of long-term SC and cannabis consumption on affective states and emotional function (8, 29, 45, 46, 85). On the other hand, in contrast to recent studies (76, 77), in the present study no differences between recreational cannabis users and non-users in emotional processing were found. Several explanations are proposed for this inconsistency. First, this lack of effect may be due to inherent differences in the task designs, for example in contrast to Hindocha and colleagues we did not use emotional faces in different intensity (76), nor dynamic emotion expression faces as Platt and colleagues (77). Second, in the current sample we were able to control for alcohol consumption. This is important since long-term use of alcohol affects emotional processing abilities among cannabis users in previous studies (76).

Finally, we have found a negative correlation between schizotypal traits and WM performance in SC and recreational cannabis user groups. Moreover, for SC users greater schizotypal traits were associated with poorer performance on the emotional processing task, and may have confounded the effect of SC on emotional processing. These associations stand in line with current research which showed that the adverse effects of cannabinoids are partially associated with psychotic proneness (68, 77). The present data may support the last notion and supports the evidence of the involvement of endo-cannabinoid system in the psychopathology of schizophrenia, yet, the current study could not examine the moderation effect of psychosis proneness on the association between SC use and emotional processing.

Limitations of the Current Study

While interpreting the results of the current study, some potential limitations should be taken into account. First, objective measures of participants' cannabinoids use as well as other psychoactive compounds were not taken. These assessments may be important since there is a relationship

between blood concentrations of those psychoactive ingredients and cognitive function as well as emotional processing (20, 26). However, it is important to take into account that some of these SC drugs are composed of psychoactive ingredients that may not be detected by urine or blood test (17). Furthermore, SC users have consumed regular cannabis as well and the current study could not assess whether the cognitive and emotional deficits presented by SC users are induced due to excessive use of SC rather than the interaction of SC chronic use combined with regular cannabis. Moreover, the current research could not assess whether the observed effects are dose-related or perhaps an expression of a genetic predisposition. In addition, we have reported an elevation of depressive and anxiety symptoms as well as schizotypal traits in SC users. Anxiety and depression have been previously associated with schizotypal symptoms, as well as chronic drug use. SC users were administered a screening interview and psychiatric evaluation in order to exclude participants with a history of neurological or psychiatric disorder. However, the current study could not assess whether the elevation of schizotypal measure in SC users is due to the influence of anxiety or depression or a result of prolonged drug abuse. Prospective studies with more objective measurements and details regarding patients' substance use and clinical presentations are therefore needed to address these limitations. Furthermore, we have found that depression and consumption of cigarettes with nicotine has reduced the observed effect of SC on response inhibition and emotional processing. These findings indicate that they are confounding variables affecting the association between the use of SCs on cognitive and emotional processing. Finally, the sample size of the current study was relatively small since chronic SC users are a very unique and rare cohort and difficult to recruit. Due to the relatively small sample size we were unable to conduct additional analyses of further potential confounding variables or to infer causality. Future studies may consider using larger samples in order to investigate cognitive, emotional and psychotic proneness among SC users.

CONCLUSIONS

The current study provides further evidence of impaired cognitive and emotional function in chronic SC users. SC users have presented deficits in; WM, mental flexibility and response inhibition. In addition, elevation of depressive and anxiety symptoms as well as schizotypal traits were observed. Some of those cognitive and emotional processing dysfunctions were associated with schizotypal traits in the cannabinoid users' groups. It is plausible that these deficits are a result of the toxic effects of extremely potent cannabinoids may have on the human's brain. Yet, further studies are needed to replicate and expand the last conclusions.

DATA AVAILABILITYSTATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ministry of Health Jerusalem Israel. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All individuals included as authors of the paper have contributed substantially to the scientific process leading up to the writing of the paper. The authors have contributed to the conception and design of the project, performance of the experiments, analysis and interpretation of the results and preparing the manuscript for publication.

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Complications Related to Sexualized Drug Use: What Can We Learn From Literature?

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Chemsex is described as the use of specific psychoactive substances (PS) during sexual activity to sustain, enhance, disinhibit or facilitate the sexual experience. It preferentially concerns men who have sex with men (MSM). They use new synthetic substances like cathinones, methamphetamines, gamma-butyrolactone/gamma-hydroxybutyrate (GBL/GHB), ketamine, and cocaine. The prevalence of chemsex varies from 3 to 31% during lifetime. The Internet has participated significantly in the evolution of sexual behaviors, both in terms of sexual dating and the availability of new synthetic substances. The advent of geolocation applications contributed to the development of chemsex. The literature describes many complications linked to these sexual practices; the main clinical effects related to cathinones consumption were psychiatric symptoms; agitation, hallucinations, anxiety, suicidal ideation, paranoia, and confusion. Regular GBL/GHB consumption alter cognitive functions, particularly memory and emotion management. Use of these drugs in party and play is dramatically associated with high-risk sexual behaviors. The prevalence of hepatitis B, hepatitis C syphilis, and HIV is higher in men who use methamphetamine and Viagra and/or who declared they practiced slamming, chemsex, and fisting. Other sexually transmitted infections (STIs) such as gonorrhea have increased with methamphetamine and GHB/GBL use. Actually, the care of individuals who practice Chemsex in a problematic way is currently not codified, but the use of integrative and specific interventions is necessary.

Keywords: psychoactive substances, sexual behaviors, cathinones, sexualized drug use, GBL/GHB

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INTRODUCTION

Drug use in a sexual context has been described since antiquity in order to improve sexual performance or to promote desinhibition. In this review we specifically studied the recent phenomenon of "chemsex" secondary to geolocation applications and new designer substances, which has increased exponentially since the 2000s. Numerous substances are linked to sex

Abbreviations: PS, psychoactive substances; BBV, bloodborne viruses; STI, sexually transmitted infections; MSM, men who have sex with men; EDDs, erectile disorder drugs.

use and they are associated with different populations and sexual behaviors. Alcohol, cannabis and MDMA (methylenedioxyméthamphétamine) are more commonly used in heterosexual practices (Lawn et al., 2019), whereas men who have sex with men (MSM) use cathinones, methamphetamines, gamma-butyrolactone/gamma-hydroxybutyrate (GBL/GHB), ketamine, and cocaine. This behavior is referred to "chemsex" (formerly "sexualized drug use"). Chemsex is described as the use of specific psychoactive substances (PS) during sexual activity to sustain, enhance, disinhibit, or facilitate the sexual experience. (Giorgetti et al., 2017). The Internet has participated significantly in the evolution of sexual behaviors, both in terms of sexual dating and the availability of new synthetic substances. Chemsex is associated with a high risk of contraction of sexually transmitted infection (STI) and bloodborne viruses (BBV). The care of individuals who practice Chemsex in a problematic way is currently not codified. The objective of this review was to describe complications related to drug use in a sexual context in order to adapt specific care.

METHODS

A review of the literature was conducted using PubMed. The search was carried out per theme using the keywords reported in **Table 1**.

These keywords were used alone or in association with the keyword "chemsex." The **Table 1** shows the number of articles identified for each keyword and in association from 2000 to 2020. This technique has allowed to find many articles with keyword alone and/or association with the main keyword "chemsex." We have selected those that seemed more relevant for this review about complications related to drug use (**Figure 1**). Original animal articles have been excluded.

PREVALENCE

Currently, the prevalence of chemsex is difficult to estimate because its definition varies between countries (Schmidt et al., 2016). It varies from 3 to 31% during lifetime and from 0.4 to 16.3% the last month. The frequency of chemsex depending on the city of residence and countries studied, and the taking into account or not of HIV-positive individuals (Elliot et al., 2017), the use of dating applications and the type of PS used (Maxwell et al., 2019).

Slamming is the term used for intravenous injection of these PS during party and play. In Australia, the prevalence of slamming is 10% in MSM, and in England it was found to be 16% in an MSM population diagnosed with *Shigella flexneri* 3a infection (Gilbart et al., 2015). Slamming is more common in MSM and women who have sex with women (WSW) than in libertine heterosexuals practicing chemsex (Heinsbroek et al., 2018). These differences in prevalence show how interesting it would be to define the practice of chemsex in an international way: target population, drugs used, and exclusion criteria.

TABLE 1 | Keywords (from 2000 to 2020).

Themes	Keywords	Alone/Association
Sexual behavior	Chemsex	228
	Slamming	52/9
Drugs	Cathinones	766/17
	Mephedrone	830/77
	Methamphetamine	335/123
	GHB	1704/34
	GBL	456/23
	GBL/GHB	10/3
	Ketamine	13 711/76
	Erectile designer drugs (Sildenafil, Tadalafil, and Vardenafil)	2859/14
Applications	Sex meeting application	122/22

SEX MEETING APPLICATIONS

The practice of combining sex and PS has increased steadily with the development of mobile applications. The advent of geolocation applications in 1990 transformed the way gay and bisexual men meet (Grov et al., 2014) and contributed to the development of chemsex. These new applications allow to meet one or more partners very quickly in their surroundings and to express their sexual desires, and their preferences in the practice of chemsex. Sex meeting applications made it possible for "clubbers" and "sexers" to meet. These 2 populations did not have the same sexual practices. "Sexers" had harder practices and used drugs, which have since become widespread amongst MSM.

At least 40% of MSM and 68% of the youngest individuals (Garofalo et al., 2007) find their partners using the Internet. Finding a sex partner on Internet or using a mobile application increases condomless anal intercourse and the risk of HIV transmission (Lewnard and Berrang-Ford, 2014; Whitfield et al., 2017). Sociologists are worried about the disappearance of non-virtual meeting places associated with the development of phone applications and describe the increase of solitude felt by homosexuals (Renninger, 2018).

DESIGNER SUBSTANCES AND ERECTILE DISORDER DRUGS (EDDs)

New designer substances appeared in the early 2000s and participated in the development of chemsex. New synthetic substances are detailed in the **Figure 2** and in the **Table 2**. The main complications are described in **Table 3**. Most of these studies come from emergency or intensive care.

Synthetic Cathinones

The most used PS are synthetic cathinones, the leader of which being mephedrone or 4 MMC (4-methylmetcathinone). Their development was inspired by the Khat plant (*Catha edulis*) that is considered as a natural amphetamine and is used in certain countries of East Africa and the Arabian Peninsula (Kalix, 1990). These new designer substances are sold as bath salts "not for human consumption" to circumvent

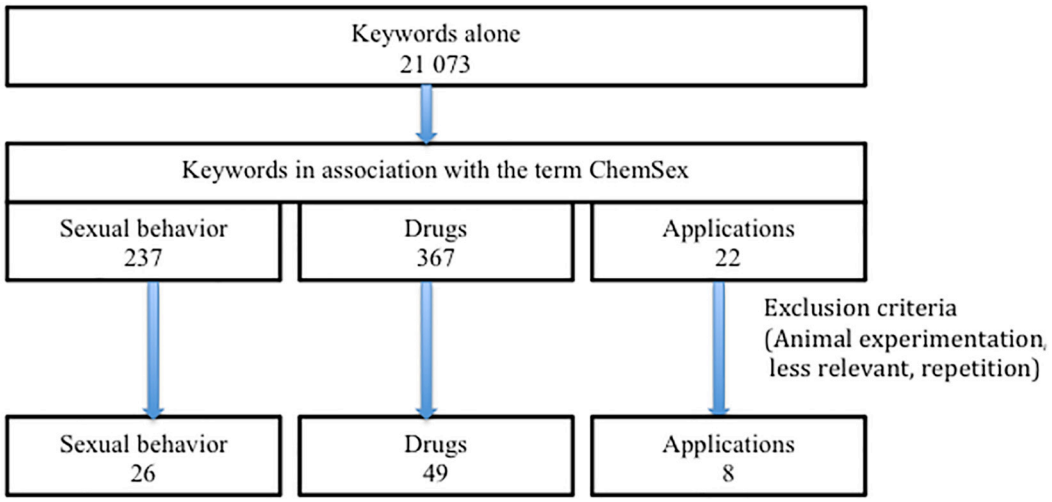

FIGURE 1 | Flow chart.

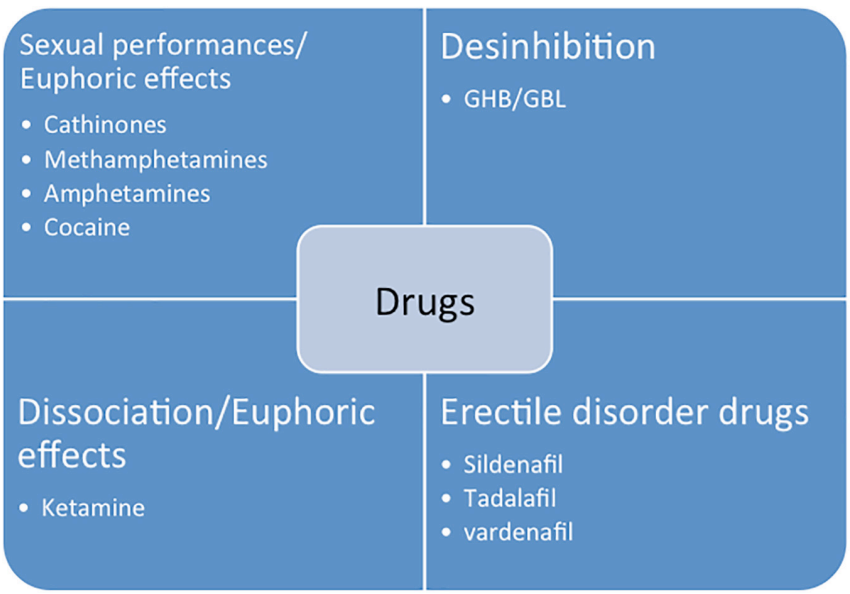

FIGURE 2 | New designer drugs and their expected effects.

the legislation (Coppola and Mondola, 2012). They belong to the phenylethylamine family (Petit et al., 2013) and are psychostimulants with an amphetamine- or cocaine-like effect. They generate agitation, euphoria, and empathy in users and increase libido and sexual performance.

These PS have a strong addictogenic effect with dependence levels of 30% according to DSM-IV criteria (Dargan et al., 2011; Winstock et al., 2011). The main acute complications are cardiac, psychiatric, and neurological, and they can be fatal (Busardo et al., 2015; Ezaki et al., 2016; Sande, 2016; Kronstrand et al., 2018; Riley et al., 2019; **Table 3**).

Blood levels in case of lethal intoxication are higher than in cases of non-lethal poisoning, and lethal situations arise from the combining of several drugs (Papaseit et al., 2017). These substances have been classified as narcotics in Europe since 2010 (Forsyth, 2012) and it is currently illegal to purchase or possess them, but they are usually obtained on the Internet or directly during party and play. Cathinones take the form of a white powder or small crystals. They are taken orally, by snorting, intrarectally (booty bump), or by injection (slamming).

In the study of Spiller et al. (2011) the main clinical effects (>10%) related to cathinones consumption were agitation (82%), combative violent behavior (57%), tachycardia (56%), hallucinations (40%), paranoia (36%), confusion (34%), myoclonus (19%), hypertension (17%), chest pain (17%), and mydriasis (13%).

TABLE 2 | New designer drugs and their expected effects.

Drugs name	Common names	Expected effects	Way of administration
Cathinones	MDPV	Psychostimulants amphetamine-like effect	Orally
	4-MMC	Euphoria	Sniff
	Mephedrone	Empathy	Intrarectally
	4-MEC	Increase libido and sexual performance	(booty bump)
	M-Cath		Injection
Methamphetamine	Meth	Powerful psychostimulant	Orally
	Crystal	Euphoria	Sniff
	Ice	Empathy	Smoke
			Injection
GBL/GHB	"G"	Relaxation disinhibiting Increases desire facilitates penetration	Orally
Ketamine	"Ke"	Psychostimulant effect	Sniff
		Euphoria	Orally

In another serie, main complications of cathinones related to chemsex were psychotic symptoms, agitation, anxiety, suicidal ideation, or suicide attempt (Batisse et al., 2014). More recently, severe psychiatric symptoms have been observed with ephylone, a recent available synthetic cathinones, in a context of chemsex (Serre et al., 2019).

Excited delirium has been reported with the use of synthetic cathinones, with a challenging combination of paranoia, confusion, severe agitation, and violent behavior (Diestelmann et al., 2018; Schmoll et al., 2018). The presentation of these patients has been frequently complicated by evidence of skeletal muscle damage, dehydration, renal dysfunction, and hyperthermia that may lead to multiorgan failure and death. The precise pathophysiology underlying the syndrome of excited delirium is incompletely understood. However, the role of the central dopamine dysregulation, inducing a thermoregulation dysfunction has been suggested (Penders et al., 2012).

Cathinones are often combined with other illicit PS such as methamphetamine (Maxwell et al., 2019), GBL/GHB (Bourne et al., 2015; Edmundson et al., 2018), ketamine, and cocaine (Melendez-Torres et al., 2018b).

Methamphetamine

Methamphetamine (crystal meth), like cocaine and amphetamines, inhibits the reuptake of monoamine transporters and stimulate the release of dopamine, noradrenaline, adrenaline, and serotonin (Liechti, 2015). Crystal is characterized by its stronger psychostimulant effects and its immense addictogenic potential. Use of this dangerous substance is mainly described in the United States and the United Kingdom (Degenhardt et al., 2010) with prevalence ranging from 27% (lifetime use) to 7% (recent use) (Benotsch et al., 2012). Methamphetamine can be swallowed, smoked injected or intrarectally administrated. Methamphetamine has been linked to a lot of cardiac, psychiatric, neuropsychological and dental effects (**Table 3**).

TABLE 3 | Drugs and their main complications (without STI and BBV).

Drugs	Main risks	Treatment	Studies	Number of subjects
Cathinones	Psychiatric: panic attack, feeling of persecution, depression, confusion, hallucinations, suicide attempt or suicidal ideation, severe agitation, or violence	Psychiatric hospitalization	Batisse et al., 2014	21 subjects
		Symptomatic treatment	Spiller et al., 2011	236 subjects
			Sande, 2016	249 subjects
			Schmoll et al., 2018	96 subjects
			Diestelmann et al., 2018	50 subjects
	Cardiac: hypertension, tachycardia, and chest pain	Symptomatic treatment	Ezaki et al., 2016	61 autopsy cases
	Cardiac ischemia		Spiller et al., 2011	236 subjects
	Dependence		Winstock et al., 2011	100 subjects
	Sympathomimetic syndrome and rhabdomyolysis	Intensive care	O'Connor et al., 2015	102 subjects
		Benzodiazepines	Froberg et al., 2015	23 subjects
		Psychiatric care	Umebachi et al., 2016	8 subjects
Methamphetamine	Cardiac: methamphetamine-associated cardiomyopathy, heart rhythm disturbance, acute coronary syndromes, pulmonary arterial hypertension, and hypertension	β-blockers	Sevak et al., 2011	6 subjects
		Blockage of the renin–angiotensin system	Neeki et al., 2018	449 subjects
		Aripiprazole		
	Psychiatric: depression, confusion, acute psychosis and psychotic symptoms, and anxiety disorder	Antipsychotic medications	McKetin et al., 2013	278 subjects
		Benzodiazepines		
	Neuropsychological effects: alteration of executive functions, episodic memory, psychomotor functions, and complex information processing speed.		Scott et al., 2007	Meta-analysis (18 studies)
	Dependence	*N*-acetyl-cysteine	McKetin et al., 2019	180 subjects
	Dental and periodontal disease		Shetty et al., 2015	571 subjects
			Rommel et al., 2016	100 subjects
GBL/GHB	Coma, presentation to a emergency department, and neurological complications	Intensive care	Wood et al., 2013	158 subjects
			Raposo Pereira et al., 2020	27 subjects
			Raposo Pereira et al., 2018	27 subjects
	Dependence	Baclofen	Bell and Collins, 2011	19 subjects
		Diazepam		

Gamma-hydroxybutyrate/Gamma-butyrolactone

Gammahydroxybutyrate is a central nervous system depressant that has a double stimulant and sedative effect (Raposo Pereira et al., 2019b). GBL, which is cheaper, is taken orally in liquid form and transformed into GHB (Busardo et al., 2018).

Gamma-butyrolactone/gamma-hydroxybutyrate is most often mixed with another drink. The sought-after effects are relaxation, disinhibition, increased desire and sensuality, and easer penetration. The main acute risk is overdose with significant sleepiness and hypothermia that can lead to coma and death.

An increase in the number of deaths in London related to GHB use was observed between 2011 and 2015 with 61 reported deaths, while a 119% increase in deaths between 2014 and 2015 was observed versus 25% for cocaine (Hockenhull et al., 2017). Regular GBL/GHB consumption and repeated comas alter cognitive functions, particularly memory (Raposo Pereira et al., 2018) and emotion management (Raposo Pereira et al., 2019a; **Table 3**). GBL/GHB dependence can be established using physical and psychological criteria with consumption outside of sexual intercourse.

Ketamine, Cocaine, and Speed

Ketamine, an anesthetic used in human or veterinary medicine, is a phencyclidine (PCP) derivative that blocks non-competitively the glutamate *N*-methyl-*D*-aspartate (NMDA) receptor (Corazza et al., 2013). It is used as a psychoactive substance in sexual sessions for its euphoric effects. It can cause hallucinations at higher doses. Urologic complications are described with ketamine. Cases of bladder dysfunction have been reported in the literature, mainly ulcerative cystitis. Cases of hydronephrosis have also been reported. Symptoms described by users are frequency and urgency of urination, dysuria, urge incontinence, and occasionally painful hematuria (Morgan and Curran, 2012).

Cocaine and speed are still used in a sexual context but less frequently since the advent of the new designer drugs (Busardo et al., 2019).

Erectile Dysfunction Agents

Non-psychoactive substances such as erectile dysfunction agents (sildenafil, tadalafil, and vardenafil) are used to facilitate or enhance sexual performances. These drugs are diverted from their medical use to counterbalance the negative effects of psychoactive drugs and to prolong the duration of sexual intercourse (Giorgetti et al., 2017). Combining all these drugs is associated with high cardiac toxicity (Bracchi et al., 2015).

STI AND BBV

In addition to the complications already described, the major risks are related to the sexual behaviors of the users, and the injection of drugs that increases the risk of spreading BBV and STI.

Use of illicit and licit drugs in party and play is dramatically associated with high-risk sexual behaviors. Use of methamphetamine is strongly associated with condomless anal intercourses (Fisher et al., 2011), multiple sex partners (Melendez-Torres et al., 2018a), sex marathons, and sex with HIV-positive MSM (Benotsch et al., 2012; Bui et al., 2018). The prevalence of hepatitis B, syphilis (Rana et al., 2019), and HIV is higher in men who use methamphetamine and Viagra which is associated with serodiscordant unprotected sexual intercourse (Spindler et al., 2007). Hepatitis C seroprevalence is higher in HIV-positive MSM who declared they practiced slamming, chemsex, and fisting (Vaux et al., 2019). Other STIs such as gonorrhea have increased with methamphetamine and GHB/GBL use (Kohli et al., 2019) and there has been a worldwide increase in all STIs among MSM that is linked to chemsex and Internet, which facilitates high-risk sexual behaviors (Soriano and Romero, 2018).

PREVENTION AND INTERVENTION

Phone applications would be ideal to promote messages in terms of sexual health, STI and prevention of addictions. Some applications have started to emit HIV prevention messages (Chan et al., 2016). Medina et al. (2019) explained that future HIV prevention approach should pass through dating applications. Moreover another study showed that interventions that facilitate condom negotiation could exist in future applications (Tang et al., 2016).

Providing care to individuals suffering from the problematic practice of chemsex is complex; there are no explicit recommendations on specific drugs that could be used for withdrawal or regulation and the care must simultaneously take into account pharmacological, addictological, and psychological and sexual aspects. The use of integrative and specific interventions is necessary. Different types of therapy are tested as cognitive behavior therapy, contingency management, and gay-specific cognitive behavior therapy (GCBT). These therapies are used with or without medications for withdrawal or maintaining abstinence. GCBT integrated elements from standard cognitive behavior therapy with cultural and social elements of chemsex users. Reback has proved the efficiency of this therapy in methamphetamines users in the United States (Reback and Shoptaw, 2014). CBT and motivation interview improve adherence to HIV medication in gay and bisexual men (Parson et al., 2018).

For GBL/GHB withdrawal, the most used drugs are benzodiazepines, neuroleptics, and sometimes barbiturates (Cappetta and Murnion, 2019). Serious complications may occur with these, including hallucinations, delusions, and epileptic fits (Liao et al., 2018; Neu, 2019). Baclofen has been tested for the long-term maintenance of GBL/GHB abstinence, but randomized studies are required before specific recommendations can be issued (Beurmanjer et al., 2018).

Drugs such as atypical antidepressants (mirtazapine) or naltrexone have been tested to reduce sexual risk-taking and metamphetamine use (Knight et al., 2019).

Fighting against the transmission of infections requires preventive interventions and harm reduction including condom

distribution and needle exchange programs. These practices need to be developed.

Special attention should be paid to MSM with problematic chemsex behavior so they may have access to the best possible post-exposure prophylaxis (PEP) and pre-exposure prophylaxis (PrEP) (Hammoud et al., 2018; Sewell et al., 2019).

Actions focusing on prevention, addiction management and sexual health need to be increased and new community spaces such as sexual health centers need to be opened to break down barriers and help alleviate the shame of chemsex drug users (Giorgetti et al., 2017; Sewell et al., 2018).

CONCLUSION

Chemsex is a complex issue. The behavior is at the crossroads of sociology, infectious medicine, addiction, and sexology. Its users are confronted with medical, psychiatric, and sexual risks. Some users engage in chemsex seeking sexual disinhibition, others in order to embrace their sexual preferences, and others still for the effects of the drugs.

The phenomenon can be a claim of part of the MSM population that wants to have unbridled sex with protection against HIV. There is still enormous stigma and shame associated with HIV infection and being homosexual (Dubov et al., 2018). With chemsex, caregivers are confronted with a continuum between normal and pathological sexual and drug use behaviors. The care to be provided to chemsex users must be validated by large sample studies. CGBT and LGBT-specific-e-therapy (Lucassen et al., 2018) are interesting ways to facilitate prevention and access to care for problematic chemsex users.

AUTHOR CONTRIBUTIONS

HD-R and HP reviewed the literature and wrote the manuscript. LK and AB revised the manuscript. All authors contributed equally to this manuscript.

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