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Neuropharmacology of new psychoactive substances (NPS): focus on the rewarding properties of cannabimimetics and phenethylamines

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Presentata da:	Dott.ssa Cristina Miliano
Coordinatore Dottorato :	Prof. Antonio Argiolas
Tutors	Prof. Gaetano Di Chiara Dott.ssa Maria Antonietta De Luca

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Abbreviations

5F-AKB-48N-(adamantan-1-yl)-1-(5-fluoropentyl)-1H-indazole-3-carboxamide

5F-PB-22 1-pentyfluoro-1*H*-indole-3-carboxylic acid 8-quinolinyl ester

AKB48 N-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide

AM 251 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-(1-piperidyl)pyrazole-3-carboxamide

BB-221-(cyclohexylmethyl)-1*H*-indole-3-carboxylic acid 8-quinolinyl ester

CPPconditioned place preference

DA dopamine

mPFCmedial Prefrontal Cortex

NAcNucleus Accumbens

NPSNew Psychoactive Substances

JWH-0181-pentyl-3-(1-naphthoyl)indole

STS-135*N*-(Adamantan-1-yl)-1-(5-fluoropentyl)-1*H*-indole-3-carboxamide

SCssynthetic cannabinoids

THC delta-9-tetrahydrocannabinol

VTA ventral tegmental area

WIN-55,212-2 (R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone

Abstract

In the last decade the trend of drug consumption has completely changed and the "classical" drugs of abuse, such as opiates, cocaine, cannabis, amphetamines, and lysergic acid diethylamide (LSD), have been replaced by several synthetic compounds. These molecules, namely New Psychoactive Substances (NPS), recently appeared in the drug market becoming very popular worldwide. NPS are designed to mimic the effects of illicit drugs, and consequently to be sold as legal alternative to them, mainly via the Internet. Scientific literature and clinical knowledge on NPS is minimal. Moreover, users are usually unaware of what they are ingesting. These factors often lead to severe cases of intoxications, difficult to understand and treat, considering that the forensic identification of these substances is complicated, also because NPS's market adapts very quickly to changes introduced by legal controls. Besides peripheral toxicological effects, many NPS seem to have addictive properties.

In order to fill the gap of scientific knowledge, the primary aim of this study was to evaluate the pharmacological effects and the abuse potential of selected NPS; in addition, this study aimed at disseminating information on the alarming consequences of using them, in order to prevent their use. Among the different classes of NPS, we chose synthetic cannabinoids (SC) and phenethylamines, that are the two most used classes, according to UNODC, Early Warning Advisory, 2014. In particular, we studied the pharmacological profile of third generation SC (AK-B48, BB-22, 5F-PB-22, 5F-AKB48, STS-135); we evaluated their *in vitro* affinity and agonist properties for rat and mice CB1 receptors, and their *in vivo* stimulant properties on dopamine transmission in the rat nucleus accumbens (NAc) shell, NAc core, and medial prefrontal cortex (mPFC). Among phenethylamines, we chose 25I-NBOMe, that is one of the most used among young people as alternative to LSD. *In vivo* microdialysis studies were performed to evaluate the effect of this compound on dopamine (DA) and serotonine (5-HT) transmissions, both in male and female rats, moreover, behavioral tests, such as sensorimotor studies, body temperature evaluation and nociception test, were performed.

The main results of this work were that third generation cannabinoids, BB-22, 5F-PB-22, 5F-AKB-48, and STS-135 are full agonists of CB1 receptors and they are more potent compared to AKB-48, which belongs to the same generation but appeared earlier in the market, as well as compared to JWH-018, belonging to the first generation of SC.

They all affect DA transmission selectively in the NAc shell, displaying a putative abuse liability; furthermore, we demonstrated that the phenethylamine 25I-NBOMe is more active in females, compared to males, in increasing DA transmission in NAc shell and in the mPFC; behavioral data showed that this compound caused visual alterations in both sexes, whereas core temperature in females is heavily affected, compared to males; indeed, the highest dose tested exerts an analgesic effect prominent in male rats, compared to female rats. Finally, we disseminated the toxicological effects related to the consumption of NPS by organizing conferences in some high schools, and sharing this information on Facebook and on the blog http://infonuovedroghe.blogspot.it/.

Considering the growing evidence of the widespread use of NPS, this work helps us to understand the new trends in the field of drug reward and drug addiction by revealing the rewarding properties of NPS, and will be helpful to gather reliable data regarding the abuse potential of these compounds.

Further investigations in the future might be useful to assess if these properties can explain the high acute toxicity and the addiction liability of these compounds, as well as the cases of death reported after their ingestion.

1. INTRODUCTION

1.1 Drug Addiction

According to the DSM-IV (Diagnostic and Statistical Manual of Mental Disorders, 4th edition; American Psychiatric Association, 1994), drug addiction is a chronically relapsing disorder characterized by compulsion to seek and take the drug, impaired control in limiting intake, emergence of a withdrawal state and negative emotional state (e.g., dysphoria, anxiety, irritability) when addicted individuals remain drug free for an extended period, and development of tolerance. The emergence of typical physical symptoms associated with thedrug abstinenceusually leads to negative emotional feelings and, consequently, social withdrawal (Koob and Le Moal, 1997; Wise and Koob, 2014) which make protracted abstinence difficult to sustain (O'Brien, 2005); all these events, in addition to the craving (i.e. the strong, often uncontrollable desire to use the drug) causethe relapse, that is the return to drug use in abstinent individuals.

The transition from occasional, controlled drug use and the loss of behavioral control over drug seeking and drug taking is due toneuropharmacological and neuroadaptive mechanisms that occur in specific neurocircuits (Koob, 2009).

As shown in Figure 1, the addiction cycle is typically composed by three stages -'binge/intoxication', 'withdrawal/negative affect', and 'preoccupation/anticipation' (craving)- and different neurocircuits are involved in each stage (Wise and Koob, 2014). Key elements of the binge/ intoxication stage are the basal ganglia, including the nucleus accumbens (NAc), dorsal striatum (DS), globus pallidum (GP) and thalamus (Thal); the extended amygdala, including the central nucleus of the amygdala (AMG), bed nucleus of the stria terminals (BNST), and a transition area in the shell of the nucleus accumbens (NAc) is responsible for the withdrawal/negative affect stage; the frontal cortex and allocortex, including the prefrontal cortex (PFC), orbitofrontal cortex (OFC), hippocampus (Hippo), and insula (Insula) playkey role in the preoccupation/anticipation stage (Koob and Volkow, 2010; Wise and Koob, 2014). Molecular, synaptic, and neurocircuitry neuroadaptations of all these pathway, combined with other factors, such as individual vulnerability, stress and environmental stimuli, underlie drug addiction.

All these modifications influence addicted people lives leading to adverse social and health consequences (Hyman et al., 2006) , as well as, cognitive impairments (Bechara, 2005; Jentsch and Taylor, 1999; Kalivas and Volkow, 2005; Spiga et al., 2008).

Individual vulnerability to drugs of abuse is related to several factors such as genetic factors (Cadoni, 2016), social relationships, environmental stimuli, as well as stressful events, that can influence the drug intake; different types of drug users also exist. Sensation-seeking and novelty- seeking are personality characteristic that affect the propensity to use drugs (Piazza and Le Moal, 1998). Indeed, it has been shown that the hypothalamic-pituitary-adrenal axis and the brain stress system, are dysregulated by chronic administration of drugs of abuse, and that corticotropin-releasing factor (CRF) levels are increased during acute withdrawal in the extended amygdala(Kreek and Koob, 1998). Therefore, the dysregulation of the HPA axis may facilitate both the positive reinforcing effects of drugs via modulation of the mesolimbic dopamine system and the negative reinforcing effects of drugs by activating the extended amygdala (George and Koob, 2010).

At the social psychology level, the failure of self-regulation, deeply affects several brain function, such as stress, anxiety, reward, pain, habits, and decision-making (George and Koob, 2010) resulting in a loss of control, typical of an addicted individual, that is attributed to a dysfunction of the frontal cortex or hypofrontality (Pribram and Mishkin, 1956; Mishkin, 1964; Bechara, 2005) and subsequent dysregulation of subcortical cognitive systems controlled by the prefrontal cortex.

Also environment stimuli constitute an important factor, in fact increasing evidence indicates that exposure to environmental enrichment (EE) during early stages of life decreases the vulnerability to develop addiction and reduces the effects of drugs of abuse (Carroll et al, 2009; Laviola et al, 2008; Solinas et al, 2010; Stairs and Bardo, 2009).

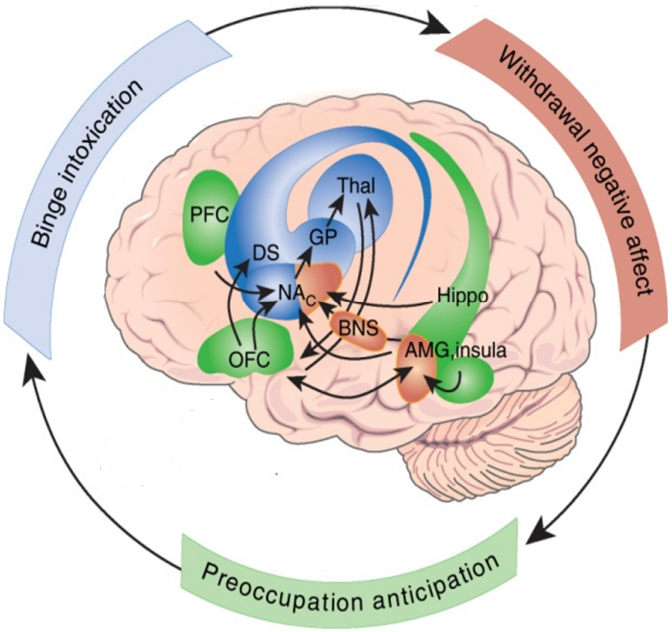

Figure 1. The addiction cycle : 'binge/intoxication' (blue), 'withdrawal/negative affect' (red), and 'preoccupation/anticipation' (craving) (green).Adapted from Wise and Koob, 2014.

1.2 Reward and mesocorticolimbic system

The term "reward" refers to a pleasure or hedonic impact of a stimulus, that has positive effects on behavior, attitude, relationships and reinforces behavior (Ikemoto and Bonci, 2014).The midbrain and forebrain are involved in motivated behavior through connections of the medial forebrain bundle, composed of ascending and descending pathways, including most of the brain monoamine systems (Koob, 1992); even if the anhedonia hypothesis suggests that mainly brain dopamine systems mediate the pleasure produced by food and other unconditioned incentives such as sex or drugs of abuse (Berridge and Robinson, 1998).

Midbrain structures, basal ganglia and cerebral cortex are anatomically and functionally connected by dopamine (DA) pathways that cooperate modulating different functions in the Central Nervous System. The mesolimbic system arises from cell bodies of DArgic neurons located in the ventral tegmental area (medial VTA- A10) projecting to nucleus accumbens (NAc), central nucleus of amygdala, and hippocampus (Dahlstrom and Fuxe, 1964); it plays a key role in mediating rewarding effects of drugs of abuse (Bowers et al., 2010; Fibiger and Phillips, 1986; Koob, 1992; Robbins and Everitt, 1996; Wise and Bozarth, 1987).

The VTA has also a GABAergic neurons population able to inhibit dopamine cells and affect other structures, such as the pedunculopontine tegmental nucleus and glutamatergic neurons (Dobi et al., 2010). Ventral tegmental excitatory afferents are glutamatergic and cholinergic arriving from the ventromedial prefrontal cortex (ventral prelimbic, infralimbic, dorsal peduncular cortices), ventral subiculum, subthalamic nucleus, parabrachial nucleus, pedunculopontine tegmental nucleus, and laterodorsal tegmental nucleus (Kalivas, 1993); also the nucleus accumbens shell and the ventromedial ventral pallidum project to the VTA (Oades and Halliday, 1987). Many reports have also demonstrated a role for the extended amygdala , composed of the bed nucleus of the stria terminalis (BNST), the central nucleus of the amygdala (CeA), basolateral amigdala (BLA) and a transition zone in the shell of the nucleus accumbens (Koob and Le Moal, 2001; Koob, 2009), that represents the specific brain areas that interface classical limbic (emotional) structures with the extrapyramidal motor system (Alheid et al., 1995).

Dysregulation of the extended amygdala has been hypothesized to play a key role in disorders related to stress and negative emotional states, such as posttraumatic stress disorder, general anxiety disorder, and affective disorders (Shin and Liberzon, 2010). Neuroadaptive changes in this extended amygdala circuit may also lead to the aversive effects and dysregulated reward system hypothesized to be the motivation for the transition to drug addiction (Koob and Le Moal, 2008).

A second DArgic neuronal subpopulation of the A8 VTA, projects to the prefrontal cortex, orbito-frontal cortex, and anterior cingulated (Lindval et al., 1974; Gardner and Ashby, 2000); this circuit, known as mesocortical, is likely to be involved in the conscious experience of drug intoxication, drug incentive salience, drug expectation/craving, and compulsive drug administration (Goldestein and Volkow, 2002). In addition, DA terminals from VTA modulate prefrontal cortex function, synapsing with GABAergic interneurons (Penit-Soria et al., 1987; Pirot et al., 1992); whereas, mPFC projects glutamatergic efferents to the NAc and the VTA(Taber et al., 1995). When this circuit is compromised, loss of control and cognitive impairments occur.

1.2.1Drugs of abuse and the rewarding circuits

Similar to natural rewards (Bassareo and Di Chiara, 1997), addictive drugs, with several action mechanisms, increase synaptic concentrations of dopamine in ventral striatum, namely nucleus accumbens (NAc) in rats,in a greater and prolonged way compared to natural rewards(Hernandez and Hoebel, 1988). It is well established that mostaddictive drugs increase extracellular DA preferentially in the rat NAc, as compared to the dorsal caudate-putamen; it was shown by in vivo microdialysis studies in rats (Imperato and Di Chiara, 1986; Imperato et al., 1986; Di Chiara and Imperato, 1988; Carboni et al., 1989; Di Chiara et al., 2004), but also in non-human primate after cocaine self-administration with microdialysis (Bradberry et al., 2000) and after amphetamine administration with brain imaging (Drevets et al. 1999), and in human ventral-striatumwith brain imaging (Drevets et al., 2001; Leyton et al., 2002; Boileau et al., 2003).

The NAc can be divided in two regions, the medial shell and the lateral core; the shell portion of the accumbens appears to be more important than the core for drug reward (Ikemoto, 2007); drugs of abuse preferentially increase dialysate DA in the NAc shell as compared to the core in rats (Pontieri et al., 1995;Tanda et al., 1997); indeed, rats learn to self-administer stimulants such as amphetamine or cocaine or dopamine receptor agonists into the accumbens shell, but not in the core (Carlezon et al., 1995; Ikemoto et al., 1997a; Rodd-Henricks et al., 2002; Ikemoto, 2003; Ikemoto et al., 2005). In addition, microinjections of dopaminergic antagonists into the shell, but not in the core, disrupt conditioned place preference induced by systemic nicotine or morphine (Fenu et al., 2006; Spina et al., 2006). These results confirm functional differences between the two accumbens compartments confirming anatomical observations that afferents and efferents differ significantly between shell and core (Zahm and Brog, 1992).

The acute reinforcing effects of drugs of abuse, that occur during the binge/intoxication stage, are mediatedeither by direct actions in the basal forebrain (notably the nucleus accumbens and central nucleus of the amygdala) or by indirect actions in the ventral tegmental area (Koob and Le Moal, 2001; Nestler, 2005; Koob, 2006; Koob, 2009); during such acute withdrawal, decreased activity of the mesocorticolimbic dopamine system occurs, as well as decreased functional activity in opioid peptide, GABA, and glutamate systems in the nucleus accumbens and extended amygdala leading to the negative reinforcement

mechanisms associated with abstinence and protracted abstinence of the withdrawal/negative affect stage of the addiction cycle.

Repeated exposure to drugs of abuse causes pharmacological effects such as tolerance and withdrawal and provokes neuroadaptive mechanisms that mediate the transition from occasional, controlled drug use and the loss of behavioral control over drug-seeking and drug-taking that defines chronic addiction changes in brain circuits. Some drugs, such as cocaine and amphetamine, act inhibiting the reuptake of DA in the synaptic cleft, but multiple drugs of abuse persistently enhance neurotransmission at excitatory synapses on dopamine cells in the VTA (Borgland et al., 2004; Faleiro et al., 2004; Thomas et al., 2003; Ungless et al., 2001), while opioids and cocaine both persistently depress inhibitory synapses on dopamine cells (Nugent et al., 2007). These drugs appear to promote or block forms of plasticity that are candidate mechanisms of learning and memory in other brain regions, and therefore have the potential to influence long-term storage of reward-related memories that may lead to addiction (Kauer and Malenka, 2007; Hyman et al., 2006). Long-term potentiation or depression (LTP or LTD) is a long-lasting increase or decrease, respectively, in synaptic transmission. These cellular mechanisms are hypothesized to underlie information storage in the brain as they are rapidly established, maintained for long periods of time and strengthened by repetition (Niehaus et al., 2009).A hypodopaminergic state (Melis et al., 2005), a reduced activity of the nucleus accumbens (Kalivas and Hu, 2006; Spiga et al., 2010), and a general malfunctioning of the prefrontal cortex (Nogueira et al., 2006), have been proposed for an “addicted brain”.

1.4 New trend in addiction: New Psychoactive Substances (NPS)

Drug use is a worldwide problem that challenges public health causing hundreds of drug-related deaths every year (WDR,2016). In the last decade, the “classical” drugs of abuse, such as opiates, cocaine, cannabis, amphetamines, andlysergic acid diethylamide (LSD) were replaced by several synthetic compounds, changing completely the trend of drug consumption.

These molecules, namely New Psychoactive Substances (NPS), recently appeared in the drug market becoming very popular worldwide, as shown by the alarming number of 644 NPS reported between 2008 and 2015 by 102 countries and territories (UNODC, 2016). NPSwere defined as “substances of abuse, either in a pure form or a preparation, that are not controlled by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances, but which may pose a public health threat”(UNODC, Global Smart Update, 2013).All these substances, known also as ‘designer drugs’, and ‘legal highs’ are synthetic compounds designed to mimic the effects of the established illicit drugs, and consequently to be sold as legal alternative to them; besides the term ‘new’ does not necessarily allude to new inventions, because some NPS were synthesized many years ago – often for research purpose – but refers to substances that have recently emerged(UNODC, Global Smart Update, 2013; Schifano et al., 2015). Key to the success of these new drugs is a combination of factors that makes them very attractive for users of all ages such as legal status, availability, cost, as well as the desire to avoid detection, and user preferences for particular pharmacological properties (Helander et al., 2013; González et al., 2013; Helander et al., 2014; EMCDDA, New psychoactive substances in Europe, 2015; EMCDDA, European Drug Report, 2015; Miliano et al., 2016).Unfortunately, users are often unaware of what they are ingesting because seizure reports indicate that NPS are sold like mixtures of several compounds (more than a NPS for each sample) but also including controlled drugs, pharmaceutical products, and adulterants, added both intentionally or not (UNODC, 2016). They are mainly produced in Eastern Europe, Central Asia and China in clandestine, unsanitary laboratories that are improperly equipped, and then shipped and sold to Europe and the USA (UNODC, 2015).The lack of knowledge on NPS available to professionals performing the analytical analysis makes the forensic identification very difficult, and many laboratories have no appropriate equipment for their recognition (Drug Policy Department, Italian Presidency of the Council of Ministers, National Action Plan on New

Psychoactive Substances, 2013). Indeed, the high potency of these substances further complicates their detection, as they will be present only at very low concentrations in the blood, and this has implications for law enforcement, as even small quantities of these drugs can be converted into multiple doses (EMCDDA, 2014). All these features challenge Policy, national governments, and all the international institutions that are trying to control this global phenomenon that, despite the tons of synthetic NPS seized over the past few years, it still represents a public health concern, considering that the number of NPS increases every year (EMCDDA, 2014; UNODC, 2014b;Miliano et al., 2016; UNODC, 2016), (Figure 2).

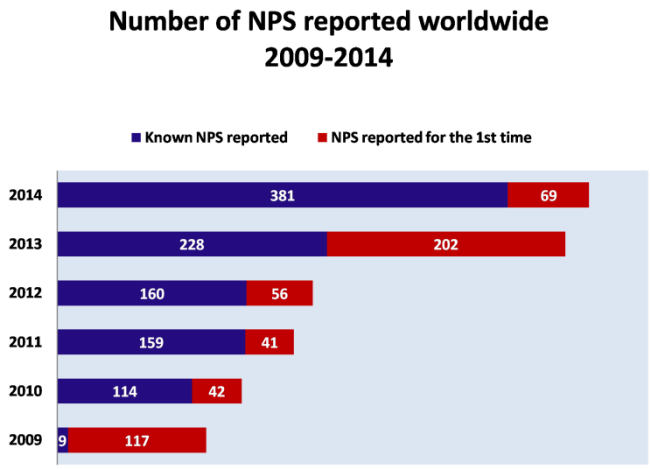

Figure 2. Number of NPS reported worldwide (2009-2014); from Miliano et al., 2016

NPS reported for the first time
Known NPS reported

Effective risk communication is also essential to prevent and control NPS spread. Prevention awareness programmes could significantly raise the knowledge on the harmful consumption of NPS; using also the Internet to disseminate information, making accessible to everyone published literature on toxicology, pharmacology and use of NPS.

1.4.1 Sales channels

In a world where by now the communication is based on internet and social networks, of course there is also the other side of the coin; in fact, online sites operate on both the surface and the deep web (Deluca et al., 2012;Drug Policy Department, 2013;Burns et al., 2014;Corazza et al.,2014; Miliano et al., 2016) selling NPS labeled as 'not for human consumption', and sold as plant fertilizers, incenses,bath salts, as well as other aliases in order to avoid legislative controls (Smith et al., 2015).Therefore, the "dark net" plays a key role in this "super safe drug dealing", whereas buyers and sellers can access anonymously and provide drugs, paying with a virtual wallet (UNODC, 2016); essentially, few clicks are enough to supply highly psychoactive substances, cheaply and in a low risk way (Fattore and Fratta, 2011; Schmidt et al., 2011), even through smartphone apps (Ramo et al., 2015; Bierut et al., 2016). Therefore, NPS can be sold to everyone, also to very young people, in complete anonymity and easily avoiding law enforcement (Drug Policy Department, 2013; UNODC, 2014; EMCDDA, 2015). Because not everyone have the finances or the technical skills to create or manage an Internet site, Facebook is used as an alternative site for sales and for "advertising" the use of this kind of products (Drug Policy Department, NPS Update and National Action Plan, 2013), as well as trend forums, where these compounds are discussed and promoted (e.g. www.drugs-forum.com, www.erowid.org, www.alkemico.com) (Deluca et al., 2012). The changing policy on marijuana use in some States of North America, seems to lead to an increase rate of cannabis use both in young and adult people, even if it has not been demonstrated the causal effect of the legalization (Cerdá et al., 2012; Harper et al., 2012); on the other hand, young people do not perceive the risk of marijuana consumption, if the law allows for using it for medical purpose, and this could represents a "gateway of curiosity" (D'Amico et al., 2015). Together, the growth of online and virtual drug markets strongly contributes to the uncontrolled widespread of these substances, to increase health risks for consumers and to pose major challenges to drug control policies.

1.4.1.1 Deep web and surface web: the market resilience

Recently, online drug dealing is replacing the old way to supply drugs of abuse. Both surfing in the Surface and in the Deep web, it is possible to find out and buy traditional illicit drugs but also “temporary legal” new psychoactive substances (Miliano et al., 2016).

The Deep web, also known as dark net, is a cryptomarket where, accessing through The Onion Router (TOR), administrators, sellers, and customers can have an anonymous identity (AlQahtani and El-Alfy, 2015; Martin, 2014a; Christin, 2012). Developed in 2010 by U.S. military, in order to make possible anounymous communications, this software can encrypt the IP address (Van Hout and Bingham, 2014), making all the operations untraceable; the payment of all illicit goods, obviously, occurs by means of cryptocurrencies – mainly bitcoins – virtual coins not controlled by government (Rhumorbarbe et al., 2016). In this dark world, the most famous platform is the Silk Road hub; born in 2011, and shutdown by the Federal Bureau of Investigation (FBI) in October 2013, it impressively reappered after a month under the name of Silk Road 2.0 in order to supply to demanding customers(Dolliver, 2015). Although it was closed in November 2014, it got back on track in May 2016, and it is now available as Silk Road 3.0. (http://silkroaddrugs.org/guide-on-how-to-access-the-silk-road-3-0/).

The Deep web remains anyway not accessible to everyone and for this reason the research of novel substances occurs also into the web surface, where several websites sell NPS using links advertising products such as incenses, bath salts, fresheners etc.,. Indeed, writing on Google key words like “legal highs” or “herbal highs”, many websites offer drugs still considered “legal”, considering the time lag from the appearance of a new substance into the market and the introduction of it in the list of substances controlled by the law (Schmidt et al., 2011). In few of these websites (http://www.herbal-smoke-shop.com, http://www.legalhighlabs.com, http://legalhighsshop.de/legal-highs/), NPS are sold explicitly. These websites are designed to attract the attention of younger consumers, with gaudy pictures , reduced price for the first purchase, advertising on new equipments (such as vaporizers and smoking pipes), “gift ideas” and “holidays sales”. Everyone who is looking for a new substance is encourage to make the purchase with guaranteed secure payment and fast shipment.

1.4.1.1.1 Sharing the information: drugs forum and Youtube

Drug forums (such as www.drugs-forum.com, www.erowid.org, www.alkemico.com) are very popular among consumers of NPS; they usually use them to report their experienceson positive and negative effects of substances, giving advices on doses, routes of administration , and how to obtain them easily (Deluca et al., 2012), frequently sharing their favourite substance, and using a pharmacological language .

In addition to this kind of promotion, it is very common to find "trip reports" on Youtube platform, web channel widely used by teenagers and beyond. Previously used to report Marijuana, tobacco, and alcohol experiences (Krauss et al., 2015), a few videos of various NPS are available on Youtube; consumers tell in first person all proven effects including negative aspects of their experiences; sometimes live shooting after the ingestion of the drug are posted. It is well established thatlimbic regions, associated with reward, develop before cortical regions(Galvan et al., 2006; Casey et al., 2008), and this imbalance, lead to a greater novelty-seeking in young people, that might result in a greater vulnerability to this on line strong promotion of these substances.

1.4.1.2 Social networks and smartphones Apps

Currently, the way to surf the Internet has radically changed and social networks are the new leaders of this trend, with a big percentage of use by teenagers (EMCDDA, 2015). Sellers, obviously, try to be up to date with these changes; for this reason it is possible to find information and direct links to proceed with the purchase of several NPS, simply looking on Facebook ("https://www.facebook.com/legalhighs.de/?fref=ts;" "https://www.facebook.com/Legal-Highs-553983508039987/?fref=ts"; "https://www.facebook.com/Legal-High-Labs-222645141258818/"; "https://www.facebook.com/herbalheadshoponline/"). Even the social network Instagram, despite the different use compared to the most famous Facebook, is used to look for new possible customers (Cavazos-Rehg et al., 2016);profiles such as "Newvisionsheadshop", "Outtadaboxspice", and "Buylegalhighs" are used to post pictures of their product with hashtags as #cannabiseeds, #headshop, #herbalicense, #over18sonly.

Also on Twitter, typing #legalhighs, it is possible to buy, paying with bitcoins, "the blue stuff" of the famous "Breaking Bad" series, otherwise known as methamphetamine.

Finally, in a technological world where peopleuse constantly a smartphone, and Apps to play any online business or simplify it, also drug dealers create simple Apps that make easier buying psychoactive substances.

In North America, the number of Smartphone Apps Cannabis-related is remarkable.. In 2014, the number of apps searched under terms like “Cannabis” and “Marijuana” were respectively 124 and 218 in Apple’s Store, and 250 for both on Google Play (Ramo et al., 2015). These Apps have several content codes, whereas information on different Cannabis strains and synthetic cannabinoids mixture (e.g.“K2-Spice”), advises for growing Cannabis, recipes for cooking “special meals”; therefore, several apps create a connection with medical marijuana doctors to obtain a prescription and others like “Eaze”, “Nugg”, “Meadow”, and “WeedMaps”, trace medical dispensaries of Marijuana, giving to users the closest spot based on their location (Ramo et al., 2015; Bierut et al., 2016). Additionally, using the app “High There”, it is possible to match people to smoke together; “MassRoots”, very similar to Instagram, is used for posting photos, videos or texts related to Marijuana; noteworthy, Apps like “Disposable Number” or “Burner”, are becoming very popular to make untraceable calls to contact drug dealers.

1.4.2NPS users

The target of this aggressive marketing advertising online are obviously adolescents and young adults, vulnerable to attractive names, colorful packaging and free sample to test; theseproducts seem to be “safe” and “enjoyable”, free from law problems and drug screenings, making young people unconscious of all the risks hidden behind consuming NPS(Bersani et al., 2014; Corazza et al., 2014; Martinotti et al., 2015;Santacroce et al., 2015). However, it has been reported that adults up to60 years old also smoke herbal mixtures(UNODC 2016).The overview of the situation is worsened by poly-drug users who usually ingest more than one drug and drink alcohol and /or energy drink at the same time, exacerbating health consequences due to the increased toxicity, overdose and death (UNODC,2014).

Adolescence represents a critical period commonly associated with an increase in drug abuse, because limbic regions, associated with reward, develop before cortical regions, that are responsible for the decision making (Galvan et al., 2006; Casey et al.,2008) leading to novelty-seekingand a consequent vulnerability to the effects of the new psychoactive drugs (Spear,2000; Johnston et al., 2013). Moreover, most of the brain

receptor systems have been shown to mature slowly, reaching maximal levels around age 20 (Paus, 2005). Indeed, the use of these drugs might influence neurodevelopment inducing psychiatric disorders or other mental deficits (Sussman et al., 2008), after all further evidence support a correlation between using synthetic cannabinoids and the onset of acute/chronic psychotic episodes (Papanti et al., 2013; Schifano et al., 2015; Fattore, 2016).

Surveys on NPS use have shown that consumers are school students, party-goers, psychonauts, prisoners, and injecting drug users(EMCDDA, 2015;Miliano et al., 2016); recently, in some European countries (Belgium, France, Germany, UK,etc.), drug users who used to inject heroin and amphetamines have switched to injecting NPS, such as synthetic cathinones,with high risk of acquiring and transmitting HIV and other blood-borne diseases (UNODC,2016).

It is well established that there are gender differences in drug addiction (EMCDDA, 2005; UNODC, 2013; Fattore et al., 2014) because the hormonal status and estradiol levels affect drug related behavior, as well as pharmacokinetic, pharmacodynamic, and sociocultural differences could influenced the response to the exposition to drugs of abuse (Fattore et al., 2008; Franconi et al., 2012). According to recent surveys, adolescent girls prefer ingesting ecstasy(Wu et al., 2010), and boys tend to use more smokable herbal blend (UNODC, 2016); indeed, 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).These sex differences have been widely demonstrated also in laboratory rodents, such as a more rapid acquisition of females in cocaine-drug taking behavior ,increased by high levels of estradiol (Jackson et al., 2006; Becker and Hu, 2008; Zhao and Becker, 2010); besides, Cummings and collegues in 2014 showed that estradiol affect the DA transmission in dorsolateral striatum, shifting female rats behavior from recreational to compulsive drug use.

1.4.3Legal status

The NPS market adapts very quickly to changes introduced by legal controls. A good example of NPS market resilience involves synthetic cannabinoids; this chemical class evolves continuously to keep those substances in an ambiguous legal status. For instance, the emergence of the naphthoylindoles to which JWH-018 belongs, was quickly followed by the emergence of indazole carboxamides (e.g. AKB-48).Currently, not all NPS are under international control. Many countries worldwide have established permanent control measures for some substances or issued temporary bans [EMCDDA (New psychoactive substances in Europe), 2015b; UNODC, 2015]. Only a few NPS have been reviewed by the mechanisms established under the international drug conventions. Existing laws covering issues unrelated to controlled drugs, such as consumer safety legislation, have been used in some countries such as Poland and UK; in others (Hungary, Finland, Italy, France, Denmark, etc.) existing drug laws or processes have been extended or adapted; additionally, in Ireland, Austria, Portugal, Romania, and Sweden new legislation has been designed [EMCDDA (New psychoactive substances in Europe), 2015b; UNODC, 2015].Unfortunately, putting a potentially harmful substance under legal control may be a lengthy process that often requires evidence-gathering, a scientific review of harms and consultations. NPS manufacturers take advantage of the delay that occurs between the appearance of a new drug in the market, and the introduction of legal control on it; during this time, in fact, they create new “legal compounds”, manipulating existing NPS formulas(Zuba et al., 2012; Commission on Narcotic Drugs, 2016).In Italy, in the last 2 years many substances such as synthetic cannabinoids and phenethylamines (see Table 1) were included in the table 1 of illegal psychoactive drugs (DL 36/2014) (Ministry of Health, updated on the 1st August 2016).

Chemical class	Substances	
Synthetic cannabinoids	JWH-018	5F-APP-PINACA
	JWH-073	5F-PB22
	JWH-122	AB-FUBINACA
	JWH-250	APP-FUBINACA
	CP 47,497	BB-22
	AM-694	
Phenethylamines	2C-B	
	25B-NBOMe	
	25C-NBOMe	
	25I-NBOMe	

Table 1. NPS defined as illicit psychoactive substances in the last years in Italy.

1.4.4 Classification and pharmacological effects

NPS can be divided into six chemical classes (Schifano et al., 2015; Martinotti et al., 2015): *phenethylamines, piperazines, tryptamines, synthetic cathinones, alkylindoles (synthetic cannabinoids) and arylcyclohexylamines*(see Table 2). Alternatively, a different classification is based on pharmacological and clinical effects: stimulants, entactogens, hallucinogens, and cannabis-like compounds.

Phenethylamines, piperazines, tryptamines and synthetic catinones exhibit stimulant and hallucinogenic effects, making up the distinct class of 'entactogens', which are described as psychoactive substances that enhance feelings of empathy, love, and emotional closeness to others (Schifano et al., 2007). Entactogens can be chemically divided into phenethylamines, amphetamines, synthetic cathinones, piperazines, pipradrols/piperidines, aminoindanes, benzofurans, and tryptamines. Stimulant drugs usually inhibit monoamine reuptake, increasing the quantity of noradrenaline, dopamine and serotonin in the synaptic cleft leading to sympathomimetic effects (Schifano,2013). *Phenethylamines* are synthetic compounds

commercially known as ‘party pills’(e.g. tablets of different colors/shapes, capsules, powder/crystal). They act on serotoninergic receptors leading to psychedelic effects and some of them inhibit the monoamine reuptake as well (Nelson et al., 2014); 3,4-methylenedioxy-methamphetamine (MDMA), widely known as‘ecstasy’, is one of the most popular drugs among young people because of its stimulant effects. But, recently a growing use of new dangerous molecules on the recreational drug scene, such as 2C and its derivatives (e.g. ‘N-Bomb’, ‘B-Fly’ and ‘Dr. Death’), 2-D series drugs, 3C-bromo-Dragonfly, 4-MTA, 6-APB, 4,4’-DMAR and MPA, that are novel derivatives of classic psychedelic phenethylamines/MDMA-like drugs (Nelson et al., 2014) has been reported; several cases of intoxications have been reported with symptoms such as hypertension, vomiting, hyperthermia, convulsions, dissociation, hallucinations, respiratory deficits, liver, and kidney failure and death in case of overdose (Winstock and Schifano, 2009; Schifano et al.,2010; Corazza et al.,2011; Dean et al., 2013; Bersani et al., 2014; Maas et al., 2015; Le Roux et al., 2015).The lead compound in *piperazines*, N-Benzylpiperazin (BZP), has a typical central nervous system stimulant structure so it triggers the release of dopamine and norepinephrine and inhibits the uptake of dopamine, norepinephrine and serotonin (Smith et al., 2015). Although BZP is structurally similar to amphetamine, it is reported to have only one-tenth the potency (Wikström et al., 2004). However, at higher dosages, hallucinations can be reported as well (Kersten and McLaughlin, 2015). Before legal restrictions were placed on it, BZP was used as a safe alternative to amphetamines such MDMA (Monteiro et al., 2013). *Tryptamines* (the most common is the lysergic acid diethylamide-LSD) are a group of monoamine alkaloids, very similar to the endogenous neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) (Tittarelli et al., 2015), so they act both as 5HT2A receptor agonist and serotonin reuptake inhibitor (Lessin et al., 1965; Nichols, 2004; Fantegrossi et al., 2008; Cozzi et al., 2009; Fontanilla et al., 2010) provoking visual hallucinations, alterations in sensoryperception, and depersonalization (Sogawa et al., 2007); novel tryptamines, as 5-MeO-AMT or 5-MeO-DMT, continue to appear on the online drug market and on the ‘dark net’ (Schifano et al., 2015; Araújo et al., 2015). *Syntheticcathinones* (mephedrone, methylone,butylone, MDPV, and α-PVP) are structural analogs of cathinones (a molecule present in the psychoactive plant Khat) and are available in tablets, capsules, powder/crystal and generally labeled as ‘bath salts’ or ‘plant fertilizers’ (Fass et al., 2012;Valente et al., 2014; German et al., 2014; Karila et al., 2015). Clinical effects most commonly reported with cathinones include anxiety, impaired concentration and

memory, irritation of the nasal mucosa, headache, tachycardia, and hypertension. The typical clinical symptoms are indistinguishable from the acute effects of MDMA or cocaine (Prosser and Nelson, 2012; Baumann et al., 2013; Valente et al., 2014); among their psychoactive effects, agitation, restlessness, vertigo, abdominal pain, paranoia, rhabdomyolysis, convulsions, and death are included (Schifano et al., 2012; Corkery et al.,2012;Corkery et al.; 2014; Loi et al.,2015).

Synthetic cannabinoids belong to the *alkylindole* and *cyclohexylphenos* classes which show high affinity for CB1 and CB2 cannabinoid receptors and act like Δ^9-THC but with prolonged psychoactive effects and more side effects (Fattore and Fratta, 2011; Brents and Prather, 2014). They are generally consumed by inhalation through the consumption of cigarettes containing herbal substances as well as these synthetic molecules to obtain euphoria, anxiolytic and antidepressant-like effects. However, reports presented by the EMCDDA (2009) and by the Italian Early Warning System – N.E.W.S. (Anti-drug Policies Department) have shown effects like paranoia, tachycardia, panic, convulsions, psychosis, visual/auditory hallucinations, vomiting and seizures (Hermanns-Clausen et al., 2013;Winstock and Barratt, 2013).

Finally, *arylcyclohexylamine* (ketamine, phencyclidine- PCP and methoxetamine) are dissociative anesthetics that distort perceptions of sight and sound and produce feelings of detachment (or dissociation) from the environment and self without hallucinations (Nishimura and Sato, 1999; ACMD,2013).

CHEMICAL CLASS	PHARMACOLOGICAL EFFECTS	References
Phenethylamines	Serotoninergic receptor agonists that cause psychedelic effects and inhibit monoamine reuptake Effects: hypetension, vomiting,hyperthermia, convulsions, dissociation, hallucinations, respiratory deficits, liver and kidney failure and death in case of overdose	*Nelson et al., 2014* *Schifano et al.,2010* *Winstockand Schifano,2009* *Corazza et al.,2011* *Bersani et al., 2014*
Piperazines	Stimulants that promote the release of dopamine and norepinephrine and inhibits the uptake of monoamines Effects: hyperthermia, convulsions and kidney failure. Hallucinations and death have been reported at high doses	*Smith et al., 2015* *Kersten and McLaughlin, 2015*
Tryptamines	5HT2A receptor agonists and serotonin reuptake inhibitors Effects:visual hallucinations, alterations in sensory perception, depersonalization	*Lessin et al., 1965* *Cozzi et al., 2009* *Fantegrossi et al., 2008* *Nichols, 2004* *Fontanilla et al., 2010* *Sogawa et al., 2007*
Synthetic cathinones	Sympathomimetic drugs that act on serotonin, dopamine and noradreline pathways Effects: agitation, restlessness, vertigo, abdominal pain, paranoia, rhabdomyolysis, convulsions and death	*Corkery et al., 2014* *Schifano et al., 2012* *Corkery et al.,2012* *Loi et al.,2015*
Synthetic cannabinoids	CB1 and CB2 receptors agonists displaying higher affinity, efficacy, and potency compared to Δ^9-THC EFFECTS: euphoria, anxiolytic and antidepressant-like effects, paranoia, tachycardia, panic, convulsions, psychosis, visual/auditory hallucinations, vomiting and seizures	*Fattore and Fratta, 2011* *Brents and Prather, 2014* *De Luca et al., 2015a* *De Luca et al., 2015b* *Hermanns-Clausen et al., 2013* *Winstock et al.,2013*
Arylcyclohexylamine	Dissociative anesthetics that act as 5HT2A agonist and NMDA receptor antagonist and show high affinity for opioid receptors Effects: distort perceptions of sight and sound, dissociation from the environment and selfwithout hallucinations	*Nishimura and Sato, 1999* *ACMD,2013* *Schifano et al., 2015*

Table 2. New Psychoactive Substances classification.From Miliano et al., 2016

1.4.5 Synthetic cannabinoids (SCs)

Among NPS, synthetic cannabinoids (SCs) are the most popular; indeed, the 39% of NPS reported in 2014 belongs to this class (UNODC, Early Warning Advisory, 2014), (Figure 3). These products are broadly known as "Spice", and have been sold under many different names (Spice Gold, nJoy, K2, etc.) marketed as a safe, legal alternative to Cannabis, composed by shredded plant material laced with a variety of SC compounds (NIDA, 2012; De Luca et al., 2015). Recently, Drugs-fora showed a new trend in Spice consumption; in fact, to enhance psychoactive effects consumers prefer to vaporize pure powder or buying solutions suitable for electronic cigarettes(www.drugs-forum.com).

According to their chemical structures they can be divided into naphtoylindoles (e.g. JWH- 018, JWH-073, JWH-210, WIN-55212), phenylacetylindoles (e.g. JWH-250 e JWH-251), benzoylindoles (e.g. WIN-48,098, AM-694, RSC-4), cyclohexylphenols (e.g. CP-47497, CP-55940, CP-55244) (Smith et al., 2015). These cannabimimetic agents are "smokable" since they are small (typically 20–26 carbon atoms) and highly lipophilic molecules;they have pharmacological properties similar todelta-9-tetrahydrocannabinol (Δ9- THC) assessed by in vitro and in vivo animal studies such as binding studies and functional assays (Compton et al., 1992; EMCDDA, 2009b).They are considered to be CB1 "super agonist" because of their high affinity for cannabinoid receptors, with a dose-response efficacy significantly higher than Δ9-THC itself (Brents et al., 2011; Fattore and Fratta, 2011; Schifano et al., 2015). Indeed, while THC is a partial CB1 agonist, in vitro studies have clearly shown that these compounds are full agonists with higher potency and efficacy as compared to Δ9-THC (Atwood et al., 2010, 2011; Marshell et al., 2014). In 2009-2010 variousEuropean countries (Austria, Germany, France, Luxembourg, Poland, Lithuania, Sweden, and Estonia, and UK) and US States (Alabama, Arkansas, Georgia, Kansas, Kentucky, and Missouri) regulated the sale and use of cannabimimetic ingredients of Spice(ACMD, 2009;US Department of Justice Drug Enforcement Administration Drugs and Chemicals of Concern, 2010). The increasing demand to synthesize new compounds, in order to avoid controls, led to a drastic reduction of the presence of 1st generation SC in and their substitution with 2nd generation SC (ACMD, 2012). These compounds included haloalkyl derivatives of JWH-018, AM-2201 and its methyl derivative MAM-2201 and the fluoro alkyl, iodobenzyl derivative AM-694, the n-methylpiperidinyl AM-2233 and AM-1220, the benzoyl indoles AM-679, RCS-4 and derivatives, and ada- mantoindoles AM-1248 and AB-001. The 3rd generation include compounds with an indazole or

benzimidazole core replacing the indole (e.g. AKB-48, 5F-AKB-48, FUBIMINA), replacement of the carbonyl link of JWH-018 with carboxamide or carboxylate groups (e.g. APICA, SDB005), quinolinyl (PB-22 “QUPIC”, 5F-PB-22, BB-22 “QUCHIC”) or non-cyclic (ABDICA, AB PINACA, 5F-AB-PINACA) secondary structures and novel nitrogenized tails (AB-FUBINACA, AB-FUBICA) (Uchiyama et al., 2012, 2013a,b; ACMD, 2014; De Luca et al., 2016). According to the literature, SC displayed locomotor depressant effects and a characteristic tetrad profile in rats and mice at lower doses compared to Δ9- THC (Chaperon and Thiébot, 1999; Wiley et al., 2012, 2014; Gatch and Forster, 2014, 2015; Vigolo et al., 2015). In addition, JWH-018 and its congeners are metabolized in other cannabimimetic compounds (Seely et al., 2012). That, together with the presence of several different SC in herbal mixture and the unknown range doses (Kronstrand et al., 2014), might explain their acute severe toxicity and even lethal medical complications in humans (Papanti et al., 2013; Brents and Prather, 2014; Brewer and Collins, 2014; Santacroce et al., 2015), leading to severe withdrawal syndrome and dependence as well in some cases (Zimmermann et al., 2009; Gunderson et al., 2012; Macfarlane and Christie, 2015). In addition, clinical evidence indicates that JWH-018 (Every-Palmer, 2011) but also other synthetic cannabinoids abuse can generate/cause psychosis episodes in vulnerable individuals(Papanti et al., 2013; Schifano et al., 2015; Fattore, 2016).Notably, an higher incidence of anxiety, agitation/panic attacks, paranoid ideation, suicidal ideation, and hallucinations episodes misuse has been associated with the misuse of SC (Fattore and Fratta, 2011; Wells and Ott, 2011; Thomas et al., 2012; Besli et al., 2015) in comparison to those seen with Δ9-THC use (Papanti et al., 2013; Spaderna et al., 2013; Van Amsterdamet al., 2015).All these alarming effects associated with a low life satisfaction can lead to the typical “amotivational syndrome" that has been described for cannabis users as a general apathy and an inability to progress through life successfully (McGlothlin and West, 1968). Binding CB1 receptors , cannabimimetics act in brain regions where they are heavily expressed, such as the amygdala, prefrontal cortex (PFC), ventral pallidum, caudate putamen, nucleus accumbens (NAc), ventral tegmental area (VTA), CeA, BNST and lateral hypothalamus (Glass et al., 1997; Wang et al., 2003). It is well established that all these areas are involved in reward, addiction and cognitive functions (Koob and Volkow, 2010), and obviouslythe integration of excitatory and inhibitory inputs, coming from these structures, modulate reward processing (Sidhpura and Parsons, 2011; Panagis et al., 2014). Several studies in mice and rats showed that these compounds affect the mesolimbic

dopaminergic transmission and influence conditioned behaviors in paradigms such as self-administration, conditioned place preference, etc., (see Table 3).

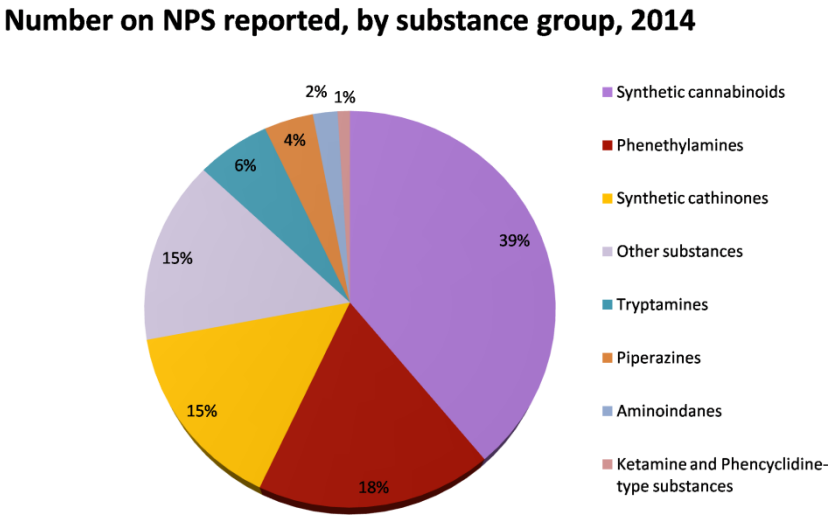

Figure 3. Source: Miliano et al., 2016

Studies related to the rewarding properties of cannabimimetics

Substance	Dosage Regimen	Studies	Reference
WIN 55212-2	Intravenous self-administration model in drug-naive mice of WIN 55212-2 (0.5 and 0.1 mg/kg per injection).	WIN 55,212-2 was intravenously self-administered by mice in a concentration-dependent manner according to a bell-shaped curve.	*Martellotta et al., 1998*
HU210	Conditioned place preference (CPP) in male rats: HU210 (20, 60 and 100 µg/kg), and delta9-THC (1.5 mg/kg)	HU210 and delta9-THC produced aversion as expressed by time spent in the drug-paired compartment of the CPP apparatus	*Cheer et al., 2000*
WIN 55212-2	Intravenous SA in rats WIN 55,212-2 at doses ranging from 6.25 to 50 µg/kg per injection, under a fixed-ratio 1 (FR1) schedule of reinforcement and nose-pokes as the operant responses.	Response rate depended on the drug dose available, with maximum rates occurring at 12.5 microg/kg per injection.	*Fattore et al., 2001*
WIN 55212-2	Fast-scan cyclic voltammetry: systemic administration at a dose of 125 µg/kg	WIN55,212–2 enhances dopamine transients but depresses electrically evoked release	*Cheer et al., 2004*
WIN 55212-2	After Intracranial self-stimulation (ICSS) of	With the exception of the highest dose of all	*Vlachou et al., 2005*

CP 55940 **HU-210**	the medial forebrain bundle, rats received intraperitoneal injections of WIN 55,212-2 (graded doses 0.1, 0.3, 1 and 3 mg/kg), CP 55,940 (graded doses 10, 30, 56 and 100 µg/kg), or HU-210 (graded doses 10, 30, 100 µg/kg).	cannabinoid agonists tested, which significantly increased the threshold frequency required for ICSS into the medial forebrain bundle, all other doses of the tested drugs did not affect ICSS thresholds. The CB1 receptor antagonist SR141716A reversed the actions of WIN 55,212-2 and CP 55,940, but not HU-210.	
WIN 55212-2	Intravenous self-administration (SA). Rats, trained for 3 weeks to self-administer WIN 55,212-2 (12.5 µg/kg) in single daily 1-h sessions under a fixed ratio 1 (FR 1) schedule, then switched to FR 2 for a further week. During SA sessions, microdialysis assays were performed every 3rd day, and then daily starting from the 13th session. Dialysate DA from the NAc shell and core was monitored before, during, and for 30 min after SA.	Response-contingent WIN 55,212-2 SA preferentially increases the NAc shell DA output as compared to that of the core independently from the duration of the WIN 55,212-2 exposure. Increase in NAc DA is strictly related to WIN 55,212-2 actions because it is not observed during extinction despite active responding.	*Lecca et al.,2006*
WIN 55212-2	Rats received intraperitoneal injections of WIN55,212-2 (0.1, 0.3 or 1mg/kg) for 20 subsequent days. Thresholds for ICSS were measured before and after each injection.	WIN55,212-2 (1mg/kg) significantly increased ICSS thresholds from the first day of administration, an effect that remained stable across the subsequent days of administration. These findings indicate that repeated WIN55,212-2 administration elicited a sustained increase in ICSS.	*Mavrikaki et al., 2010*
JWH-018 **JWH-073** **JWH-210**	Adult male rats trained to discriminate 3mg/kg Δ(9)-THC or 0.3mg/kg JWH-018 from vehicle.	JWH-018, JWH-073, and JWH-210 fully substituted in Δ(9)-THC-trained rats and Δ(9)-THC substituted in JWH-018-trained rats.	*Wiley et al., 2014*
JWH-018 **JWH-073** **JWH-250** **JWH-200** **JWH-203** **AM-2201** **CP 47,497-C8-homolog**	These compounds were then tested for substitution in rats trained to discriminate Δ-THC (3 mg/kg, intraperitoneally).	Each of the compounds fully substituted for the discriminative stimulus effects of Δ-THC, mostly at doses that produced only marginal amounts of rate suppression. JWH-250 and CP 47,497-C8-homolog suppressed response rates at doses that fully substituted for Δ-THC.	*Gatch and Foster, 2014*
CP 55940	Acute and repeated administration (7 days) of CP55,940 (0.12-0.18)mg/kg).on operant responding for electrical brain stimulation of the medial forebrain bundle in C57BL/6J mice.	CP55,940 attenuated ICSS in a dose-related manner. This effect was blocked by the CB1 receptor antagonist rimonabant.	*Grim et al., 2015*
JWH-018	Microdialysis studies in rats: 0.125 mg/kg ip 0. 25 mg/kg ip 0. 5 mg/kg ip Rats self-administered JWH-018 (20 µg/kg/infusion) in single daily 1 h FR3 sessions. C57BL/6 mice self-administered JWH-018 (30 µg/kg/infusion) in single daily 2 h FR1 sessions.	JWH-018 0.25 mg/kg ip increases dopamine transmission in Nac shell, but not in NAc core nor in mPFC. The lower and the higher doses do not stimulate DA transmission so the dose-response curve of this compound has an inverted U-shape. Both rats and mice readily acquired two different operant behaviors: nose-poking into an optical switch (rats) and lever-pressing (mice).	*De Luca et al., 2015a*

Table 3. From Miliano et al., 2016

1.4.6 Phenethylamines

Phenethylamines are a large family of compounds thatare molecular variants of the core compounds, i.e., amphetamines, MDMA, etc. (Le Roux et al., 2015). They are recently abused for their psychedelic and entactogenic effects mainly by people who attend electronic dance music (EDM), parties at nightclubs, and festivals (Palamar et al., 2016).The N-benzylmethoxy derivatives of the 2C hallucinogens (i.e., 2C-I, 2C-B, and 2C-C), commonly called NBOMes, are probably the most famous; marketed as a legal lysergic acid, with names such as “Smiles,”“N-bombs”, they act as full agonist of 5-HT2A receptor with high affinity (Braden et al., 2006; Halberstadt and Geyer, 2014). As a consequence, low doses of the order of 50 μg are able to produce psychoactive effects (Suzuki et al., 2015). For example, 25I-NBOMe is 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 500-800μg (Erowid, 2013; Halberstadt and Geyer, 2014). The duration of action of 25I-NBOMe depends on the route of administration, ranging from 4–6 h (insufflation) to 6–10 h (sublingual). Several intoxication cases and some fatalities have been reported after the recreational use of 25I-NBOMe (Walterscheid et al., 2014; Suzuki et al.,2015). Overdoses of “N-Bomb” can cause several toxicological effects such as tachycardia, hypertension, seizures, and agitation persisting for up to three days (Kelly et al., 2012; Rose et al., 2012, 2013; Hill et al., 2013; Spellpflug et al., 2013). Indeed, given that NBOMes are potent 5-HT2A agonists,the use of these substances may contribute to develop the serotonine syndrome; this is a consequence of excess serotonergic agonism that results in clinical manifestations, such as tremor , diarrhea (in mild cases), and delirium, neuromuscular rigidity and hyperthermia in life-threatening cases (Boyer and Shannon, 2005).

Central 5-HT2A receptors are heavily expressed in cortical and forebrain areas, various brainstem nuclei, and the hippocampus (Cornea-Hébert et al., 2002).They are localizedon the dendrites (Miner et al., 2003) of cortical pyramidal glutamatergic projection neurons(Amargos-Bosch, 2004), local GABAeric interneurons(Burnet et al., 1995)and on cholinergic neurons(Morilak and Ciaranello, 1993).

5-HT2A receptors seem to be presynaptic on monoamine axons, and postsynaptic in the prefrontal cortex (Miner et al., 2003).

This class of receptors in the central nervous system modulate GABAergic and glutamergic neurotransmission (Leysen,2004). Activation of 5-HT2Areceptors stimulates the secretion of various hormones (Van de Kar et al., 2001). 5-HT2A receptors play a physiological role in working memory,(Williams et al., 2002) the regulation of cognitive states, and associative learning (Harvey, 2003). Moreover, 5-HT2Areceptors influence neuronal plasticity through processes in which brain-derived neurotrophic factor (BDNF) is involved (Vaidya et al., 1997).

Despite the widespread use of these compounds, and toxicological effects reported, there is a lack of knowledge about their behavioral or toxicological effects (see Table 4).

Substance	Methods	Studies	Reference
25I-NBOMe	Binding affinity on human and rat 5HT2A receptor	Affinity of 25I-NBOMe (Ki= 0.044 nM) on human 5-HT2A (Ki= 0.087 nM) on rat 5-HT2A	*Braden et al., 2006*
25I-NBOMe	Head twitches response in C57BL/6J mice 25I-NBOMe (0.1–1 mg/kg s.c.)	25I-NBOMe induced the HTR with 14-fold higher potency than 2C-I, and this effect is completely blocked by the selective 5-HT2A antagonist M100,907.	*Halberstadt and Geyer, 2014*

Table 4. Pharmacological studies.

2.AIM OF THE STUDY

Recently, classical drugs of abuse were replaced by synthetic compounds, called New Psychoactive Substances (NPS), that became very popular at a global level, as shown by the alarming number of 644 NPS reported between 2008 and 2015 (UNODC, 2016). These substances, also known as "legal highs", were designed in order to mimic the effects of illicit drugs, becoming very attractive for users of all ages because of their legal status and the possibility to avoid detection as well as their availability and low cost(Helander et al., 2013; González et al., 2013; Helander et al., 2014; EMCDDA, 2015; Miliano et al., 2016). Unfortunately, limited information are available on NPS, both in the scientific literature and in clinical knowledge.

Given these premises,in order to fill the gap of scientific knowledge, the aim of this study was to evaluate the pharmacological effects and the abuse potential of selected NPS.

Generally, men are considered to have more opportunities than women to use drugs, but both genders are equally likely to use drugs when they have that opportunity (Van Etten et al., 1999; Van Etten and Anthony, 2001); nevertheless,the 12% of males ≥12 years currently use illegal drugs compared with over 7.3% of same age group females (AMHSA, 2013; UNODC, 2015).

Among the different classes of NPS, we chose syntheticcannabinoids (SC) and phenethylamines, that are the two most used classes,according to UNODC, Early Warning Advisory, 2014.

Indeed, epidemiological data reported that male consumers prefer to use cannabimimetics, while females prefer to take pills and blotters with psychostimulants and psychedelic substances (Wu et al., 2010;UNODC, 2016). In light of this fact, we decided to test synthetic cannabinoids in male rats and a phenethylamine in both males and females to evaluate if there were gender differences in the pharmacological effects caused by this compound.

The main aim of this work was to study the pharmacological profile of selected third generation SC that became very popular because of their greater psychoactive effects compared to Δ^9-THC; besides, their toxicological effects increased hospital emergencies and caused some drug-induced deaths, calling the attention of law enforcement agency; consequently drug designers synthesized new compounds, leading to a quick substitution of 1st generation SC with the more potent 2nd generation SC (ACMD, 2012), and

successively with the third one. All these compounds are metabolized in other cannabimimetic compounds (Seely et al., 2012); indeed, herbal mixtures often contain several SC in unknown range doses (Kronstrand et al., 2014). 5F-AKB-48 and 5-FPB-22 (‘clockwork orange’, ‘exodus’) have been reported as the most identified NPS overall (Wedinos, 2014).

We first studied the *in vitro* affinity to CB1 and CB2 receptors for third generation cannabinoids such as BB-22, 5F-PB-22, 5F-AKB48, and STS135, of which binding properties were unknown; afterwards, we evaluated the effects of AK-B48, BB-22, 5F-PB-22, 5F-AKB48, STS-135on dopamine transmission by *in vivo* microdialysis in male rats.

Among phenethylamines, we chose 25I-NBOMe, that is one of the most used among young people as alternative to LSD, and to mimic the effect of methamphetamine as well (Le Roux et al., 2015; Palamar et al., 2016). In vivo microdialysis studies were performed to evaluate the effect of 25I-NBOMe on dopamine and serotonine transmissions, both in male and female rats; moreover, behavioral tests, such as sensorimotor studies, body temperature evaluation and nociception test, were performed in collaboration with Dr. Marti of the University of Ferrara. These behavioral tests are widely used in studies of "safety-pharmacology" for the preclinical characterization of new molecules in rodents (Irwin, 1968; Mattsson et al., 1996; Porsolt et al., 2002; Redfern et al., 2005; Hamdam et al., 2013; ICH S7A, 2001); in particular, the evaluation of visual and acoustic responses is really important if we consider that 25I-NBOMe is a psychedelic compound that can lead to hallucinations; indeed, these tests might give us some information about possible alterations that can occur in people driving a car after the ingestion.

For all the substances tested, the microdialysis was performed in three terminal areas strongly involved in the motivation to take drug and in the cognitive impairment induced by chronic drug use, NAc shell and core, and mPFC.

3. MATERIALS AND METHODS

3.1 Animals

Male and female Sprague-Dawley rats (Harlan Italy), C57BL/J6 and CB1 knockout (KO)mice (originally bred on C57BL/6J background were kindly donated by Dr Aaron H Lichtman, Department of Pharmacology and Toxicology, Virginia, Commonwealth, Virginia) were used for *in vivo* microdialysis (rats of 275-300 g) and *in vitro* experiments (rats of 200-250g and mice of 17-20 g), respectively. Rats and mice were housed 4 and 10 per cage, respectively, 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 homecage. 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. All animals were handled once daily for 5 minutes for 5 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.

3.2 Substances and doses

5'-O-(3-[^{35}S]thiotriphospate) ([^{35}S]GTPγS) (1250 Ci/mmol), [^{3}H]CP,55940 (131.8 Ci/mmol) ((-)-*cis*-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol) were purchased from Perkin-Elmer Life Sciences, Inc. (Boston, MA, USA). Guanosine5′-diphosphate (GDP), and guanosine5′-*O*-(3-thiotriphosphate) (GTPγS) were obtained from Sigma/RBI (St. Louis, MO, USA). CP55,940, WIN-55,212-2 (WIN) JWH-018 and AM 251 were purchased by Tocris (Bristol, UK). 5F-AKB-48, 5F-PB-22, BB-22, and STS-135 were purchased from an Internet source (www. researchchemist.co.uk). AKB-48 and 25I-NBOMe, were purchased from LGC Standards S.r.l (Milan, Italy). For biochemical experiments, drugs were dissolved in dimethyl sulfoxide (DMSO). DMSO concentration in the different assays never exceeded 0.1% (v/v) and had no effects on [^{3}H]CP-55,940 binding and [^{35}S]GTPγS binding assay. For *in vivo* microdialysis and behavioral tests, drugs were solubilized in 2% EtOH, 2% Tween 80 and

94 % saline and administered intravenously (i.v.; 1 ml/kg) or intraperitoneally (i.p.; 3 ml/kg) at a different doses depending on the group of animals. BB-22: 0.003-0.1mg/kg/iv; 5F-PB-22: 0.01mg/kg/iv; 5F-AKB-48 0.1mg/kg/iv; STS-135: 0.15mg/kg/iv. AM 251: 1mg/kg; AKB-48: 0.125-5 mg/kg/ip; 25I-NBOMe: 0.3mg/kg/ip(microdialysis dose) and 0.1-1 mg/kg/ip (behavioural tests).

3.2.1Chemical Characterization of Cannabinoids Sourced from the Internet

In order to confirm their identity and purity, the four cannabinoids (5F-AKB-48, 5F-PB-22, BB-22, and STS-135)were evaluated using gas chromatography mass spectrometry with electron ionisation (GC-EI-MS), 400 MHz nuclear magnetic resonance spectroscopy (NMR), and high performance liquid chromatography (HPLC). Reference standards of the four cannabinoids were purchased from Chiron (Norway) for comparison. GC-EI-MS was used for the initial identification where the fragmentation pattern of all four Internet products correlated to the cannabinoid on the label claim, when compared to that of the reference standard as well as the SWG Drug library (Version 2.1). The identification was further confirmed using NMR where the number of peaks and splitting patterns were consistent with the cannabinoid chemical structures and in line with spectra produced by SWG Drug. HPLC was then used to evaluate the purity of the cannabinoid products where the purity of 5F-AKB-48, 5F-PB-22, BB-22, and STS-135 were determined to be 93 ± 1%, 95.2 ± 0.8%, 90.6 ± 0.6%, and 91 ± 2%, respectively.

3.3 In Vitro Experiments

3.3.1[^{3}H]CP-55,940 Binding Assay. Rats and mice were sacrificed by decapitation, brains were collected and cerebral cortices were rapidly dissected and placed on an ice-cold plate. After thawing, tissues were homogenated in 20 volumes (w/v) of ice-cold TME buffer (50 mMTris-HCl, 1 mM EDTA, and 3 mM MgCl2, pH 7.4). The homogenates were centrifuged at 1000*g* for 10 min at 4 °C, and the resulting supernatants were centrifuged at 45000*g* for 30 min at 4 °C. Aliquots of membranes were frozen at -80 °C until the day of experiment.The Bradford protein assay was used for protein determination using bovine serum albumin (BSA) as a standard in accordance with the supplier protocol (Bio-Rad, Milan, Italy).[^{3}H]CP-55,940 binding was carried out as previously described (Manera et al.,2006). Briefly, the membranes (40-50 µg of protein) were incubated for 1 h at 30 °C with [^{3}H]CP-55,940 (0.5 nM) in a final volume of 0.5 mL of

TME buffer containing 5 mg/mL BSA. Non specific binding was determined in the presence of 10 μM CP-55,940. Incubation was terminated by rapid filtration through Whatman GF/C filters pretreated with 0.5% (w/v) polyethyleneimine (PEI), using a Brandell 30-sample harvester (Gaithersburg, MD). Filters were washed three times with ice-cold Tris-HCl buffer (pH 7.4) containing 1 mg/ml BSA. Filter-bound radioactivity was counted in a liquid scintillation counter (Packard Tricarb 2810 TR, Packard, Meridien, CT), using 3 mL of scintillation fluid (Ultima Gold Packard, MV, Meridien, CT).[^{3}H]CP-55,940 displacement curves were plotted using serial dilutions ranging from 10^{-11} to 10^{-5} M unlabeled compounds and [^{3}H]CP-55,940 (0.5 nM). Independent experiments were repeated on membrane preparations from at least three different experiments.The calculation of the IC_{50} (the concentration that inhibits 50% of specific radioligand binding) was performed by nonlinear curve fitting of the concentration-effect curves using the GraphPad Prism program, San Diego, CA. The *F*-test was used to determine the best approximation of a nonlinear curve fitting to a one- or two- site model ($P < 0.05$). IC_{50} values were converted to *K*i values by means of the Cheng and Prusoff equation (Cheng and Prussoff, 1973).

3.3.2[^{35}S]GTPγS Binding Assay. Rat and mouse cortical membranes were suspended in 20 volumes of cold centrifugation buffer (50 mMTris-HCl, 3 mM MgCl2, 1 mM EDTA, pH 7.4) and homogenized using a homogenizer system (Glas-Col, Terre Haute, IN). The homogenate was centrifuged at 48000*g* for 10 min at 4 °C. The pellet was then resuspended in the same buffer, homogenized, and centrifuged as previously described. The final pellet was subsequently resuspended in assay buffer (50 mMTris-HCl, 3 mM MgCl2, 0.2 mM EGTA, 100 mMNaCl, pH 7.4), homogenized, and diluted to a concentration of □2 mg/mL with assay buffer. Membrane aliquots were then stored at -80 °C until use. [^{35}S]GTPγS binding was measured as previously described (Manera et al., 2006). Briefly, mouse and rat brain membranes (5-10 μg of protein) were incubated with compounds at 30 °C in assay buffer containing 0.1% BSA in the presence of 0.05 nM [^{35}S]GTPγS and 30 μM GDP in a final volume of 1 ml. After 60 min incubation, samples were filtered using a Packard Unifilter-GF/B, washed twice with 1 ml of ice-cold 50 mMTris-HCl, pH 7.4 buffer, and dried for 1 h at 30 °C. The radioactivity on the filters was counted in a liquid microplate scintillation counter (TopCount NXT, Packard, Meridien, CT) using 30 μl of scintillation fluid (Microscint 20, Packard, Meridien, CT). Concentration-effect curves were determined by incubating membranes with various concentrations of compounds (0.1 nM-10 μM) in the presence of 0.05 nM [^{35}S]GTPγS and 30 μM GDP. Non

specific binding was measured in the presence of 10 μM unlabeled GTPγS. Basal binding was assayed in the absence of agonist and in the presence of GDP. Stimulation by the agonist was defined as a percentage increase above basal levels (i.e., {[dpm(agonist) - dpm(no agonist)]/dpm(no agonist)} × 100). Nonlinear regression analysis of concentration-response data was performed using Prism 6.0 software (GraphPad Prism program, San Diego, CA) to calculate *E*max (maximal stimulation over basal levels) and EC_{50} (concentration of agonist to obtain 50% of the maximal effect) values.

3.4*In vivo*microdialysis

3.4.1 Surgery. Male and female Sprague-Dawley rats (275-300 g; Harlan, Italy) were anaesthetized with Equitesin (3ml/kg ip; chloral hydrate 2.1 g, sodium pentobarbital 0.46 g, $MgSO_4$ 1.06 g, propylene glycol 21.4 ml, ethanol (90%) 5.7 ml, H_2O 3 ml),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 ratbrain atlas of Paxinos and Watson (1998).In order to performintravenous (i.v.) drug administration in some experimental groups,a catheter (Silastic, Dow Corning Corporation, Michigan,USA) was inserted in the right jugular vein according to thetechnique previously described (De Luca et al., 2014).

3.4.2 ***Analytical Procedure.*** On the day following surgery, probes were perfused with Ringer's solution (147 mMNaCl, 4 mMKCl, 2.2 mM $CaCl_2$) at a constant rate of 1 μl/min. Dialysate samples (10 or 20 μl) were injected into an HPLC equipped with a reverse phase column (C8 3.5 um, Waters, USA) and a coulometric detector (ESA, Coulochem II) to quantify DA. The first electrode of the detector was set at +130 mV (oxidation) and the second at -175 mV (reduction). The composition of the mobile phase was: 50 mM NaH_2PO_4, 0.1 mM Na_2-EDTA, 0.5 mM n-octyl sodium sulfate, 15% (v/v) methanol, pH 5.5. The sensitivity of the assay for DA was 5 fmol/sample.

3.4.3 Histology. 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.

3.4.4 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 or T-test. Results from treatments showing significant overall changes were subjected to Tukey's tests or Dunnett's tests for *post hoc* comparisons, with significance for $p < 0.05$.

3.5 Behavioural studies

Thanks to a collaboration with Dr. Marti of the University of Ferrara, the effects of 25I-NBOMe were investigated using a battery of behavioral tests widely used in studies of "safety-pharmacology" for the preclinical characterization of new molecules in rodents (Irwin, 1968; Mattsson et al., 1996; Porsolt et al., 2002; Redfern et al., 2005; Hamdam et al., 2013; ICH S7A, 2001); indeed, evaluation of body temperature and nociception test (Compton et al., 1992; De Luca et al., 2015; Vigolo et al., 2015; Ossato et al., 2015; Ossato et al., 2016) were performed to better understand the effect of this compound because few information were available on scientific literature. To reduce the number of animals used, the behaviour of rats were evaluated in four consecutive experimental sections. Moreover, 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 are trained 2 times per week for 2 weeks before the pharmacological treatment. All experiments were performed between 8:30 AM to 2:00 PM. Experiments were conducted in blind by trained observers working together in pairs (Redfern et al., 2005). The behaviour of rats (sensorimotor responses) was videotaped and analyzed off-line by a different trained operator that gives test scores.

3.5.1. Sensorimotor studies

We studied the voluntary and involuntary sensorimotor responses resulting from different rat reaction to visual, acoustic and tactile stimuli (Koch, 1999; Ossato et al., 2015).

3.5.1.1. Evaluation of the visual response

Visual response was verified by two behavioural tests, which evaluated the ability of the rat to capture visual information even when the animal is moving (the visual placing response) or when it is stationary (the visual object response). 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/sec (modified from Ossato et al., 2015). The downward movement of the rat is videotaped by a camera. The analysis frame by frame allows to evaluate 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 millimetres 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, 5, 30 and 60 min post injection. Visual object response test was used to evaluate the ability of the rat to see an object approaching from the front or the side, than inducing the animal to shift or turn the head or retreat it (modified from Ossato et al., 2015). For the frontal visual response, a white horizontal bar was moved frontally to the rat head and the manoeuvre was repeated 3 times. For the lateral visual response, a small dentist's mirror was moved into the rat's field of view in an horizontal arc, until the stimulus was between the rat's eyes. The procedure was conducted bilaterally and was repeated 3 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.

3.5.1.2. Evaluation of acoustic response

Acoustic response measures the reflex of the rat in replay to an acoustic stimulus produced behind the animal (Koch, 1999). In particular, four acoustic stimuli of different intensity and frequency were tested (see Ossato et al., 2015). Each sound test was repeated 3 times, giving a value of 1 if there was a response, 0 if not present, for a total score of 3 for each sounds. 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 at 0, 5, 30, and 60 min post injection.

3.5.1.3. Evaluation of tactile response

The overall tactile response in the rat was verified through vibrissae, pinna and corneal reflexes (modified from Ossato et al., 2015). 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, 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.

3.5.2. 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 sec; Vigolo et al., 2015; De Luca 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 at 1 cm from the surface of the

abdomen of the rat (Vigolo et al., 2015). Core and surface rat body temperatures were measured at 0, 5, 35 and 60 min.

3.5.2.2. 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 remove the hind paw, 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 3 obtained scores. Acute mechanical nociception was measured at 0, 5, 40 and 60 min min post injection.

3.5.3 Statistical analysis of behavioural 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 {EMax%=[(test - control latency)/(cut off time - control)] X 100}. Data are expressed in absolute values, Δ°C (core and surface temperature), Emax% (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 analysis of the effects of the individual substances in different concentrations over time and that of antagonism studies in histograms were performed by ANOVA analysis followed by Bonferroni's test for multiple comparisons. The statistical analysis was performed with the program Prism software (GraphPad Prism, USA).

4. RESULTS

4.1*In vitro*studies

4.1.1 Agonist-stimulated [^{35}S]GTPγS binding to CB1 receptor

As shown in Figure 3A-B, at 1 μM concentration WIN and JWH-018, our reference compounds, stimulated [^{35}S]GTPγS binding to rat cortex membranes to approximately 150% and 170%, respectively, of the basal activity. BB-22, 5F-PB-22, 5F-AKB-48, and STS-135 produced greater G-protein stimulation than the full CB1 receptor agonist, WIN. Specifically, the stimulation of GTPγS induced by 1 μM of BB-22 and 5F-PB-22 was significantly ($p<0.01$) greater than the amount of stimulation produced by WIN (Figure 3A). WIN and all compounds produced no GTPγS stimulation when co-incubated with AM 251 (0.1 μM), a CB1 receptor antagonist/inverse agonist (Figure 3A-B), suggesting that all four test compounds activate a G protein coupled to CB1 receptor.

[^{35}S]GTPγS binding was stimulated in a concentration-dependent and saturable manner by the prototypic indole-derived synthetic cannabinoid JWH-018 and by all four synthetic cannabinoids 5F-AKB-48, STS-135, BB-22 and 5F-PB-22 (Figure 4, Table 5). All compounds possess nanomolar potency at CB1 receptors, with BB-22 and 5F-PB-22 being approximately 5-7 fold more potent than JWH-018. EC_{50} values for BB-22 and 5F-PB-22 were significantly lower than EC_{50} value for JWH-018 (ANOVA: $F_{(4,14)} = 14.78$, $p< 0.0001$, $p<0.05$, Dunnett's test), while no difference was recorded in the EC_{50} value for STS-135 and 5F-AKB-48(ANOVA: $F_{(4,14)} = 14.78$ $p< 0.001$). These latter compounds display similar potency to JWH-018 for stimulating GTPγS binding-CB1 mediated (Table 5). The maximal efficacy (*E*max) of G-protein activation by JWH-018 and STS-135 was similar, being 163 ± 3.0 %, and 168 ± 9.0 % respectively, while the others compounds (5F-AKB-48, BB-22 and 5F-PB-22) exhibited significant enhanced efficacy compared to JWH-018 (ANOVA $F_{(4,14)} = 11.56$ $P< 0.001$). Rank order of potency and efficacy was BB-22 = 5FP-22 > JWH-018 = 5F-AKB-48= STS-135 and BB-22 = 5FP-22 >5F-AKB-48>STS-135 = JWH-018, respectively (Table 5). Lastly, to confirm the involvement of cannabinoid CB1 receptor in the activation of G protein we performed concentration-effect curves of our compounds in mouse cortex membrane homogenates of CB1-KO and wild-type mice. As shown in Figure 5, all compounds stimulated [^{35}S]GTPγS binding in a concentration-manner in cortex of wild-type mice with EC_{50} and Emax values of 38 ± 5.7 nM and 158 ± 2.4

%, 28 ± 3.2 nM and 167 ± 3.7 %, 15 ± 1.7 nM and 159 ± 1.5 %, 4 ± 0.9 nM and 183 ± 5.5 %, 1.46 ± 0.14 nM and 187 ± 3.6 %, for JWH-018, 5F-AKB-48, STS-135, 5F-PB-22 and BB-22, respectively.Importantly, no activation of G protein was observed in CB1-KO mice.

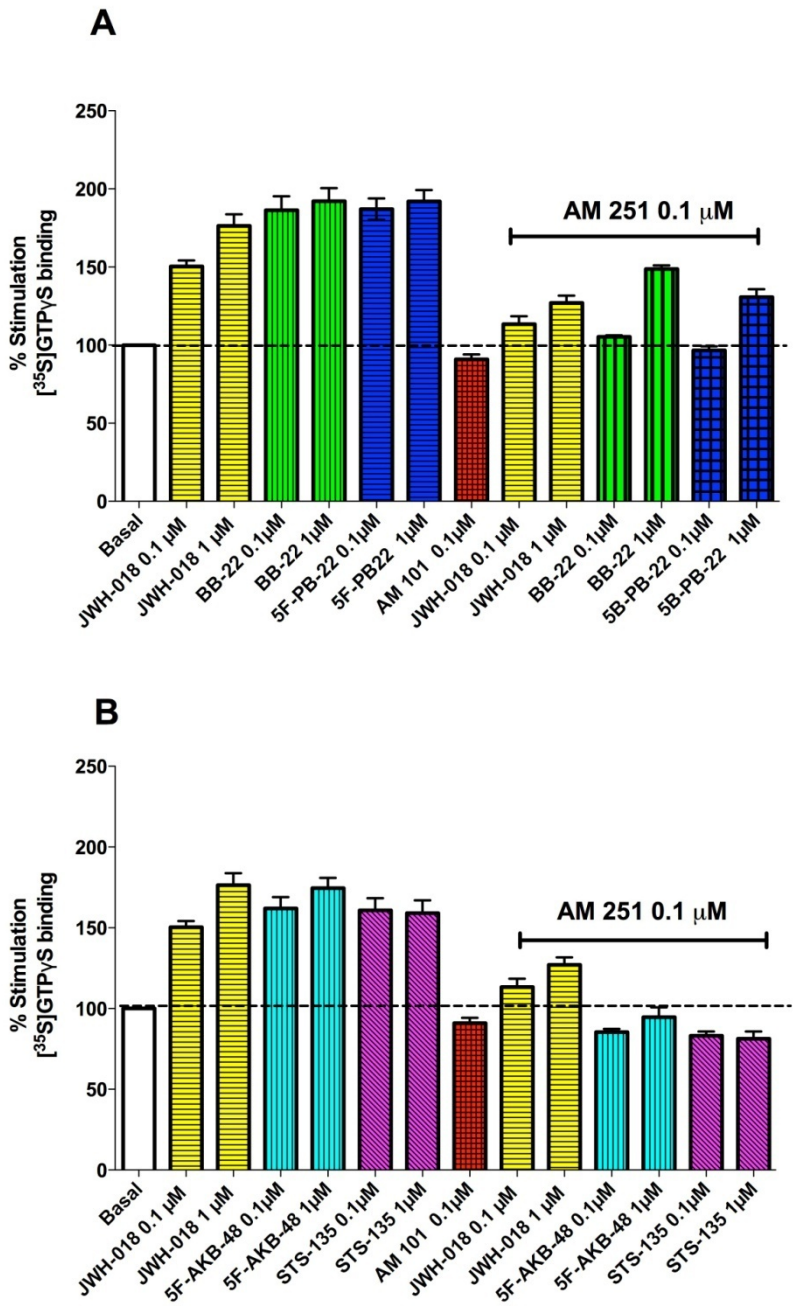

Figure 3.Effect of WIN, JWH-018 and its derivatives on [^{35}S]GTPγS binding in rat cortical membranes. WIN, JWH-018, BB-22, 5F-PB-22 (3A), 5F-AKB-48 and STS-135 (3B) were tested alone or in combination with the CB1 antagonist/inverse agonist, AM 251(0.1 μM). Data, expressed as percentage of basal values, are means ± SEM of at

least three determinations in triplicate. Horizontal dotted line indicates baseline values.One-way ANOVA: 3A, $F_{(9,39)}$=42,45 p<0.0001; 3B: $F_{(9,39)}$= 37,30 p< 0.0001 **p< 0.01 vs JWH-018, Tukey's test. From: De Luca et al., 2016.

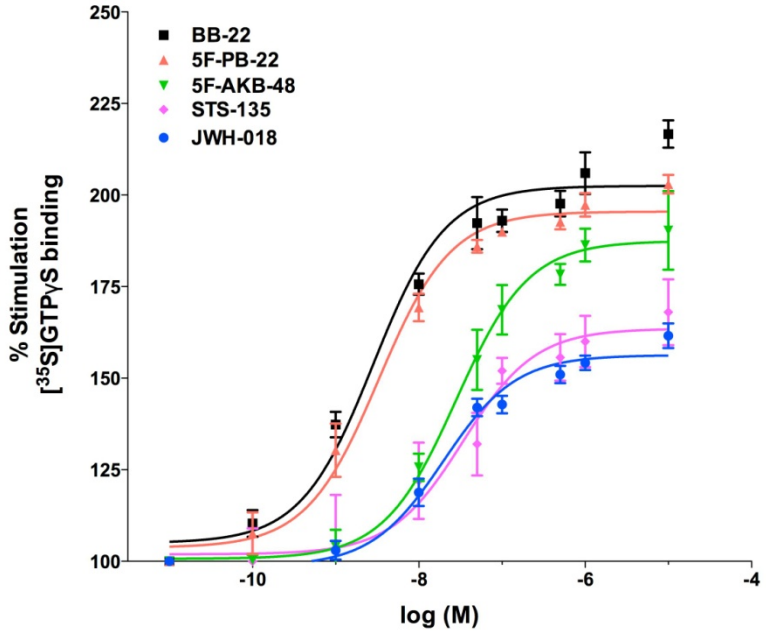

Figure 4. Concentration-response curves of compounds-stimulated [^{35}S]GTPγS binding in rat cortical membranes. Data are expressed as mean percentage of basal values of GTPγS binding ± SEM of at least four independent experiments. Rat cortical membranes were incubated with various concentrations of BB-22 (*black squares*), 5F-PB-22 (*red triangles*),5F-AKB-48 (*green triangles*), STS-135 (*magenta diamonds*), and JWH-018 (*blue circles*),as described in Material and Methods. The parameters describing the different curves are given in Table 5.From: De Luca et al., 2016.

Compounds	CB1	GTPγS binding	
		EC_{50}	Emax
	Ki (nM)	nM	% over basal
BB-22	0.11± 0.03***	2.9 ± 0.6*	217 ± 4**
5F-PB-22	0.13 ± 0.01***	3.7 ± 0.6*	203 ± 2**
5F-AKB48	0.87 ± 0.14***	31.0 ± 7.5	190 ± 11*
STS-135	1.93 ± 0.18*	32.3 ± 2.9	168 ± 9
JWH-018	3.38 ± 0.63	20.2 ± 1.3	163 ± 3

Table 5. Binding affinity, potency and efficacy for stimulation of [^{35}S]GTPγS binding in rat cortical membranes. Data are the means ± SEM of at least four experiments, each performed in triplicate. The calculation of IC_{50} was performed by non-linear curve fitting of the concentration-effect curves using Graphpad Prism Program. IC_{50} values were converted to *K*i values by means of the Cheng and Prusoff equation (Cheng and Pursoff, 1973). Compounds-mediated [^{35}S]GTPγS binding data represent percentage of stimulation over basal values (set as 100%). Emax and EC50 were determined by non linearregressioncurve fit (GraphPad Prism). One way ANOVA: Ki: $F_{(4,14)}$=21.24, P<0.0001; EC_{50}: $F_{(4,14)}$ = 14.78 P<0.0001; Emax: $F_{(4,14)}$ =11.56 p< 0.001*p<0.05, **p<0.01 and ***p<0.001 compared to JWH-018 (Dunnett's test).From: De Luca et al., 2016.

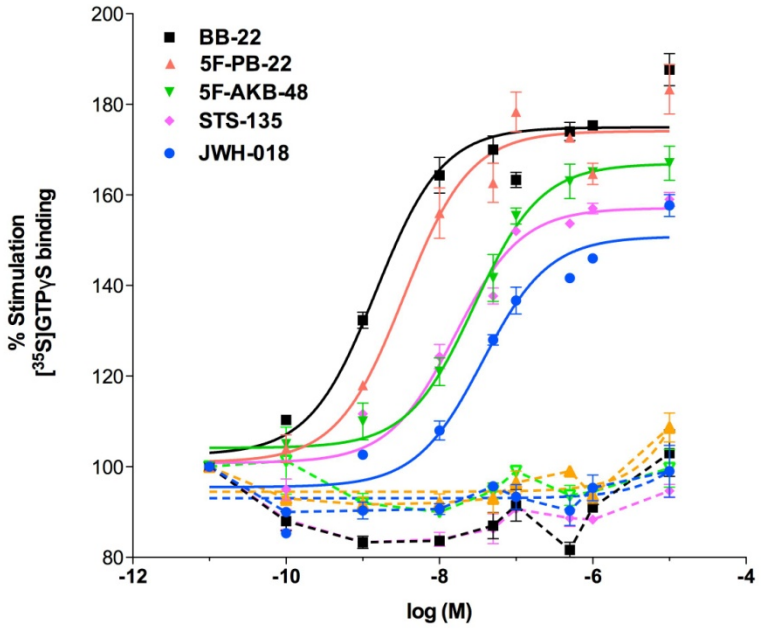

Figure 5.Concentration-response curves of compounds-stimulated [^{35}S]GTPγS binding in mouse cortical membranes of CB1-KO and wild-type mice. Data represent a typical experiment out of three independent experiments. $EC_{50,}$ of Wild-Type mice: BB-22 (*black squares*), 1.7 nM; 5F-PB-22 (*red triangles*), 3.4 nM; 5F-AKB-48 (*green triangles*), 28 nM; STS-135 (*magenta diamonds*),15nM; JWH-018 (*blue circles*): 36 nM. All compounds fail to activate GTPgS binding in CB1-KO mice (*dotted lines*).From: De Luca et al., 2016.

4.1.2 Effects of JWH-018, 5F-AKB48, STS-135, BB-22 and 5F-PB-22 on CB1 receptor binding

To determine the affinity of JWH-018 and the other compounds to the CB1 receptor we used a radiolabelled competition binding assay in rat cortical membranes. Indeed, high levels of CB1 receptors are expressed in the central nervous system, while only negligible CB2 receptors quantities are present (Pertwee, 2005). In good agreement with previous published data (Devane et al., 1988; Thomas et al., 1998) Kd and Bmax obtained by Scatchard analysis of [^{3}H]CP55,940 saturation binding were 2.08 ± 0.16 picomol/mg protein and 0.33 ± 0.06 nM, respectively (n=3, data not shown). As expected, JWH-018 in rat cortical membranes caused complete inhibition of the specific binding of [^{3}H]CP55,940 with a Ki of 3.4 ± 0.6 nM (Figure 6). As shown in Table 5, all four test compounds displaced [^{3}H]CP55,940 binding with varying affinities ranging from 0.11 ± 0.03 for BB-22 to 1.9 ± 0.18. Indeed, Ki values of these compounds were significantly lower

compared to our reference compound JWH-018, being rank order of CB1 receptor affinity BB-22 = 5FPB-22 >5F-AKB-48 > STS-135 > JWH-018 (Table 5).

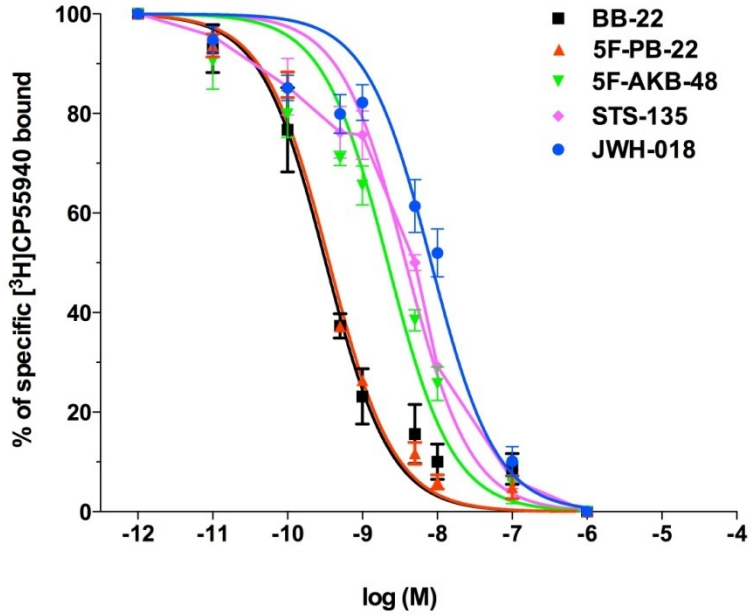

Figure 6. Displacement curves of [^{3}H]CP55,940 in rat cortical membranes by BB-22, 5F-PB-22, 5F-AKB-48, STS-135, and JWH-018. Data are expressed as means ± SEM of at least four independent experiments, each performed in triplicate. The calculation of IC_{50} was performed by non-linear curve fitting of the concentration-effect curves using GraphPad Prism Program. The F-test was used to determine the best approximation of a non-linear curve fitting to one or two site model ($p< 0.005$). IC_{50} values were converted to *K*i values by means of the Cheng and Prusoff equation (Cheng and Pursoff, 1973). From: De Luca et al., 2016.

4.2 *In vivo* microdilaysis studies

Rat basal values of DA, expressed as fmoles/10 µl sample (mean ± SEM), were: NAc shell 52 ± 5 (N=50), NAc core 55 ± 4 (N =25), mPFC 16± 2 (N =21).

4.2.1 Effect of AKB-48 administration on DA transmission in the NAc shell and core, and in the mPFC

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), mPFC14± 4 (N=13). In this experiment we evaluated the effect of three doses of AKB48 (0.125, 0.25, 0.5 mg/kg i.p.) on extracellular DA levels in NAc shell and only two doses (0.125 and 0.25 mg/kg i.p.) on NAc core, and mPFC DA levels. As shown in Figure 7, this synthetic cannabinoid increased DA levels preferentially in the NAc shell (panel A) as compared to the NAc core (panel B) and mPFC (panel C). 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) (Figure 8). In animals implanted in 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 dialysate 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 (Figure7 panel A). In animals implanted in NAc core, two-way ANOVA showed a main effect of time ($F_{18,108}$=3.24; *p<0.0001) and a significant time x treatment interaction ($F_{36,108}$=3.97; *p<0.0001).Tukey's post hoc tests showed a larger increase of dialysate DA in the NAc core after 0.25 mg/kg i.p. of AKB-48 and after 0.125mg/kg i.p. revealing differences with respect to basal values (Figure7 panel B). In animals implanted in mPFC, two-way ANOVA showed no significant effects, (Figure 7 panel C).

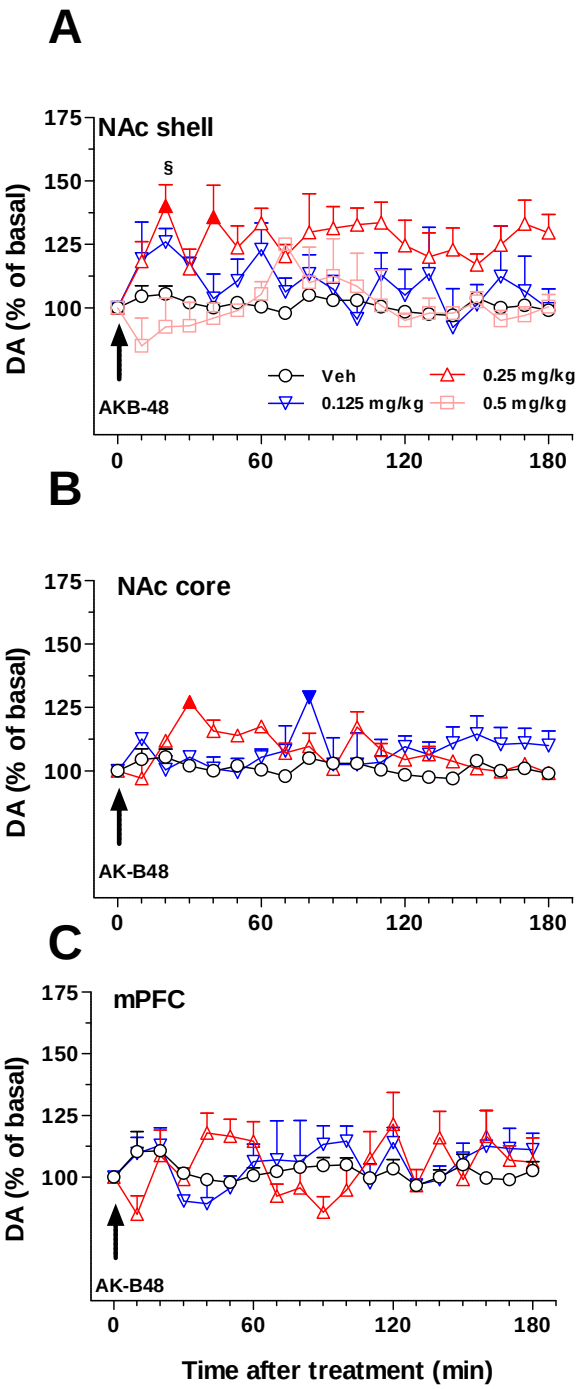

Figure 7. Effect of AKB48 administration on DA transmission in the NAc shell (panel A), NAc core (panel B), and mPFC (panel C). Results are expressed as mean ± SEM of change in DA extracellular levels expressed as the percentage of basal values. The arrow indicates the start of AKB48 i.p. injection at the dose of 0.125 mg/kg (*blue triangles*), 0.25 mg/kg (*red triangles*), 0.5 mg/kg (*pink squares*) or vehicle (*black circles*) in the NAc shell (panel A), NAc core (panel B), and mPFC (panel C). Statistical analysis was performed by Three-way or two-way ANOVAfollowed 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).

4.2.2Effect of BB-22 administration on DA transmission in the NAc shell and core, and in the mPFC

In this first experiment, we studied the effect of four doses of BB-22 (0.003, 0.01, 0.03, 0.1, mg/kg i.v.) on extracellular DA levels in NAc shell and core, and mPFC.As shown in Figure 8, the dose-response curve of the effect of BB-22 on dialysate DA is bell-shaped with the dose of 0.01 mg/kg increasing DA levels preferentially in the NAc shell as compared to the NAc core and mPFC. No significant effects were observed in the NAc core and mPFC. Three-way ANOVA showed a main effect of dose ($F_{3,75}$=4.46; $p < 0.01$), brain area ($F_{2,75}$=7.72; $p <0.001$)and time ($F_{12,900}$=4.24; $p < 0.001$), and a significant dose x brain area interaction ($F_{6,75}$=6.46; $p < 0.0001$). Tukey *post hoc* tests showed a larger increase of dialysate DA in the NAc shell after 0.01 mg/kg of BB-22 revealing differences at the 20-40 and 90-120 min sample with respect to basal value, to vehicle treated animals implanted in NAc shell, and to the same dose (0.01 mg/kg) treated animals implanted in the NAc core (90 min sample) and in the mPFC (30, 90 min sample).

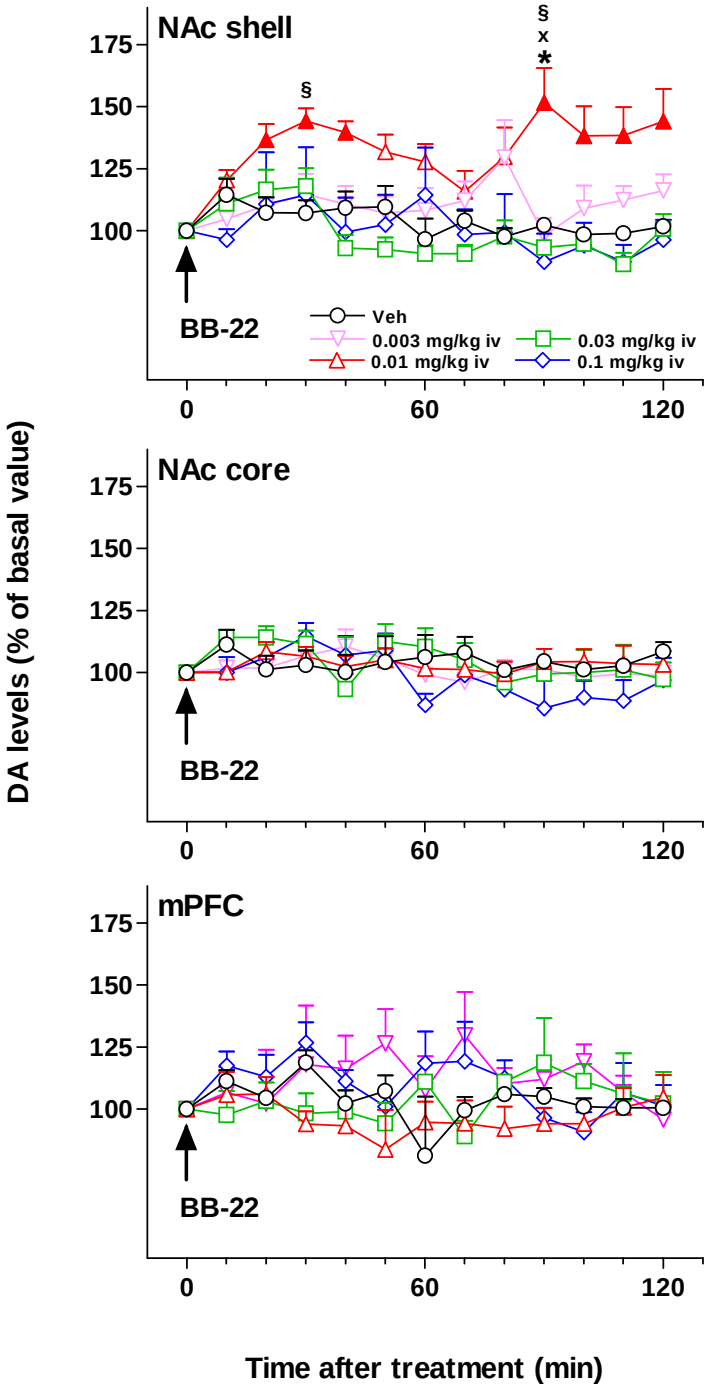

Figure 8. Effect of BB-22 administration on DA transmission in the NAc shell, NAc core, and mPFC. Results are expressed as mean ± SEM of change in DA extracellular levels expressed as the percentage of basal values. The arrow indicates the start of BB-22 i.v.injection at the dose of 0.003 mg/kg (*magenta triangles*), 0.01 mg/kg (*red triangles*), 0.03 mg/kg (*green squares*),0.1 mg/kg (*blue diamonds*), or vehicle (*blackcircles*) in the NAc shell (A), NAc core (B), and mPFC (C). Solid symbol: $p < 0.05$ with respect to basal values; *$p < 0.05$ vsvehNAc shell group; ×$p < 0.01$ vs0.01 NAc core group; § $p < 0.01$ vs 0.01 mPFC group; (NAc shell N= 29; NAc core N= 27; mPFC N= 21) (Three-way ANOVA, Tukey'*spost hoc*).From: De Luca et al., 2016.

4.2.3Role of CB1 receptors on the NAc shell DA stimulation induced by BB-22

In this experiment, we studied the effect of CB1 receptor blockade by inverse agonists/antagonists AM 251 on the NAc shell DA response to BB-22 (0.01 mg/kg i.v.) in rats (Figure9). In AM 251 pre-treated animals, two-way ANOVA showed a main effect of treatment ($F_{1,11}$=12.07; p<0.005), and treatment x time significant interaction ($F_{18,198}$=2.2; p < 0.005). Tukey's*post hoc* tests revealed that pre-treatment with AM 251 reduced significantly dialysate DA in the NAc shell as compared to rats pre-treated with vehicle (90, 140,150,190 min sample).

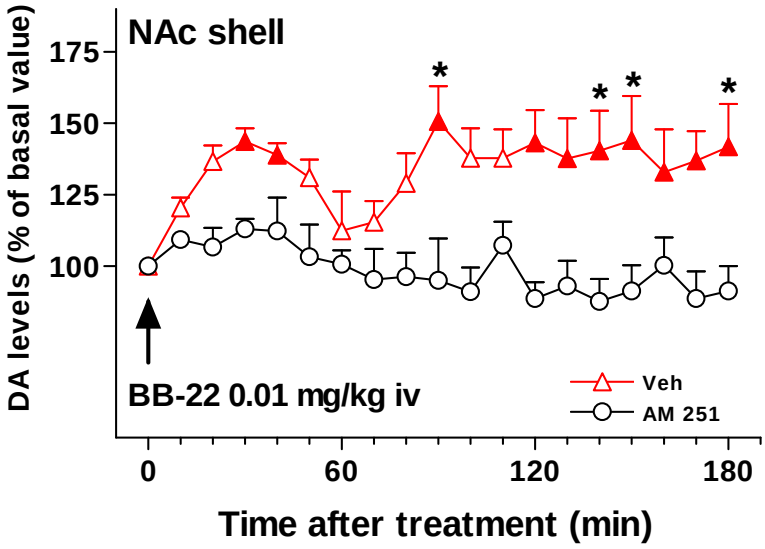

Figure 9. Blockade of BB-22 effect on increase of DA transmission in the NAc shell by AM 251. Results are expressed as mean ± SEM of change in DA extracellular levels expressed as the percentage of basal values. The arrow indicates the start of BB-22 i.v.injection at the dose of 0.01 mg/kg in rats pre-treated with AM 251 (1.0 mg/kg i.p., 30 min before agonist) (*circles*) or vehicle (*triangles*). Solid symbol: $p < 0.05$ with respect to basal values; *$p < 0.05$ vsveh group. (NAc shell veh N=6; NAc shell AM251 N=3) (Two-way ANOVA, Tukey's*post hoc*).From: De Luca et al., 2016.

4.2.4Effect of 5F-PB-22, 5F-AKB-48, and STS-135administration on DA transmission in the NAc shell

In this set of experiments, we studied the effect of 5F-PB-22, 5F-AKB-48, and STS-135 on extracellular DA levels in NAc shell. As shown in Figure10, all the drugs tested stimulated DA transmission in the NAc shell. Two-way ANOVA showed the following main effects:5F-PB-22 treatment ($F_{1,10}$=15.97; p< 0.005); 5F-AKB-48 treatment ($F_{1,11}$=63.39; p< 0.001), 5F-AKB-48 time x treatment ($F_{18,198}$=1.7; p< 0.05); STS-135 time ($F_{18,144}$=2.16; p< 0.05), STS-135 time x treatment ($F_{18,144}$=2.1; p< 0.005). Tukey*post hoc* tests showed a larger increase of dialysate DA in the NAc shell after all the cannabinoids tested revealing differences at the 30, 40 min sample with respect to basal value(5F-PB-22); at the 60, 100, 150 min sample with respect to basal value and at the 60 and 100 min sample compared to vehicle (5F-AKB-48); at the 60 with respect to basal value and to vehicle (STS-135).

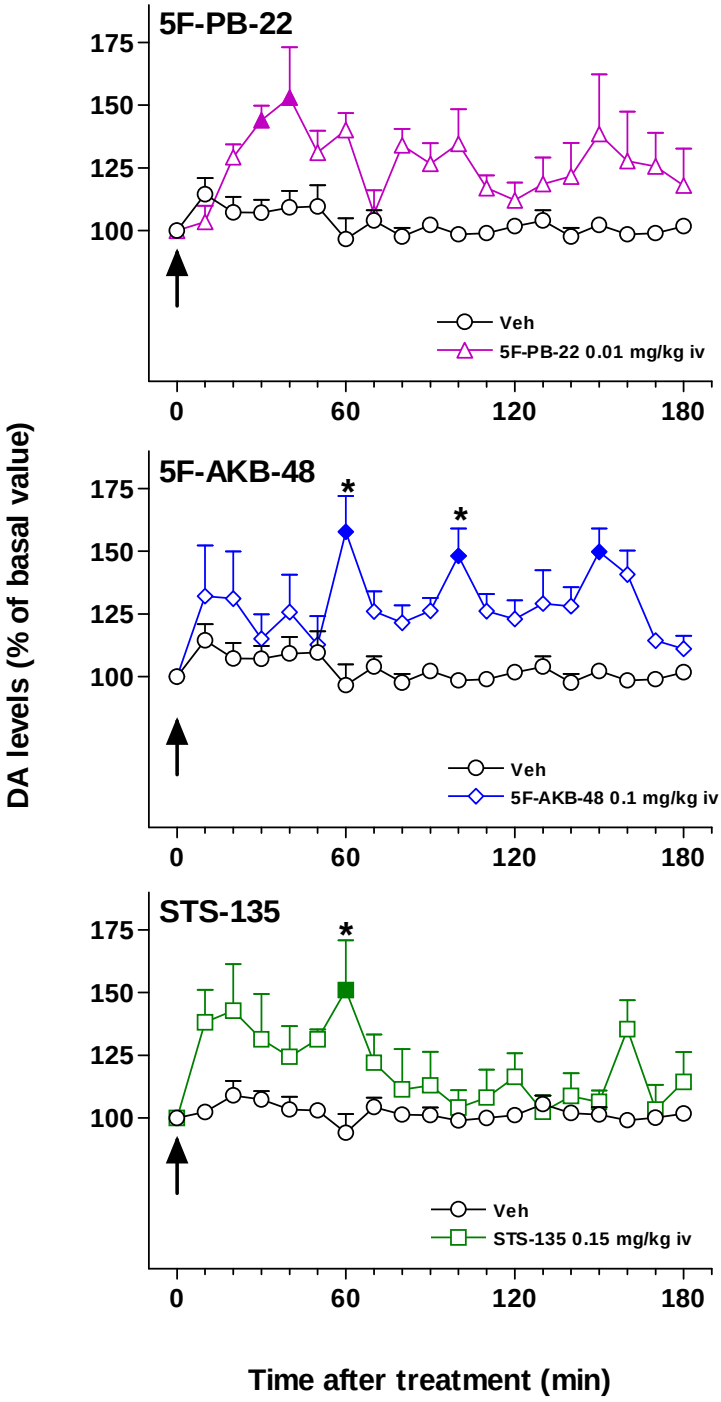

Figure 10. Effect of 5F-PB-22, 5F-AKB-48, STS-135 administration on DA transmission in the NAcshell. Results are expressed as mean ± SEM of change in DA extracellular levels expressed as the percentage of basal values. The arrow indicates the start of cannabinoid i.v.injection: (A) 5F-PB-220.01 mg/kg (*triangles*), (B) 5F-AKB-48 0.1 mg/kg (*diamonds*), and (C) STS-1350.15 mg/kg (*squares*), or vehicle (*circles*) in the NAc shell. Solid symbol: $p < 0.05$ with respect to basal values; *$p < 0.05$ vsVeh group (5F-PB-22, N= 6; 5F-AKB-48N= 7 ;STS-135 N= 5; Veh N=17) (Three-way ANOVA, Tukey's*post hoc*).From: De Luca et al., 2016.

4.2.5 Effect of 25I-NBOMe administration on DA and 5-HT transmissions in male and female rats

4.2.5.1 Effect of 25I-NBOMe administration on DA transmission in the NAc shell and core, and in the mPFC

Males

Rat basal values of DA, expressed as fmoles/20µl sample (mean ± SEM), were NAcshell60 ± 14 (N=10),NAc core55 ± 2(N=7),mPFC18± 3(N=11).In this experiment we evaluated the effect of one dose of 25I-NBOMe (0.3 mg/kg i.p.) on extracellular DA levels in NAc shell and core, and mPFC. As shown in Figure 11, this phenethylamine affectsDA transmission to a small extent only in NAc shelland core, but not in mPFC.Three-way ANOVA showed a main effect of treatment ($F_{1,22}$=4.6; *p<0.05). In animals implanted in NAc shell, two-way ANOVA showed a main effect of time ($F_{6,48}$=2.56; *p<0.05). 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 11, panel A).In animals implanted in NAc core, two-way ANOVA showed a main effect of treatment ($F_{1,5}$=7.54; *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 (Figure11, panel B). In animals implanted in mPFC, two-way ANOVA showed no significant effects (Figure11, panel C).

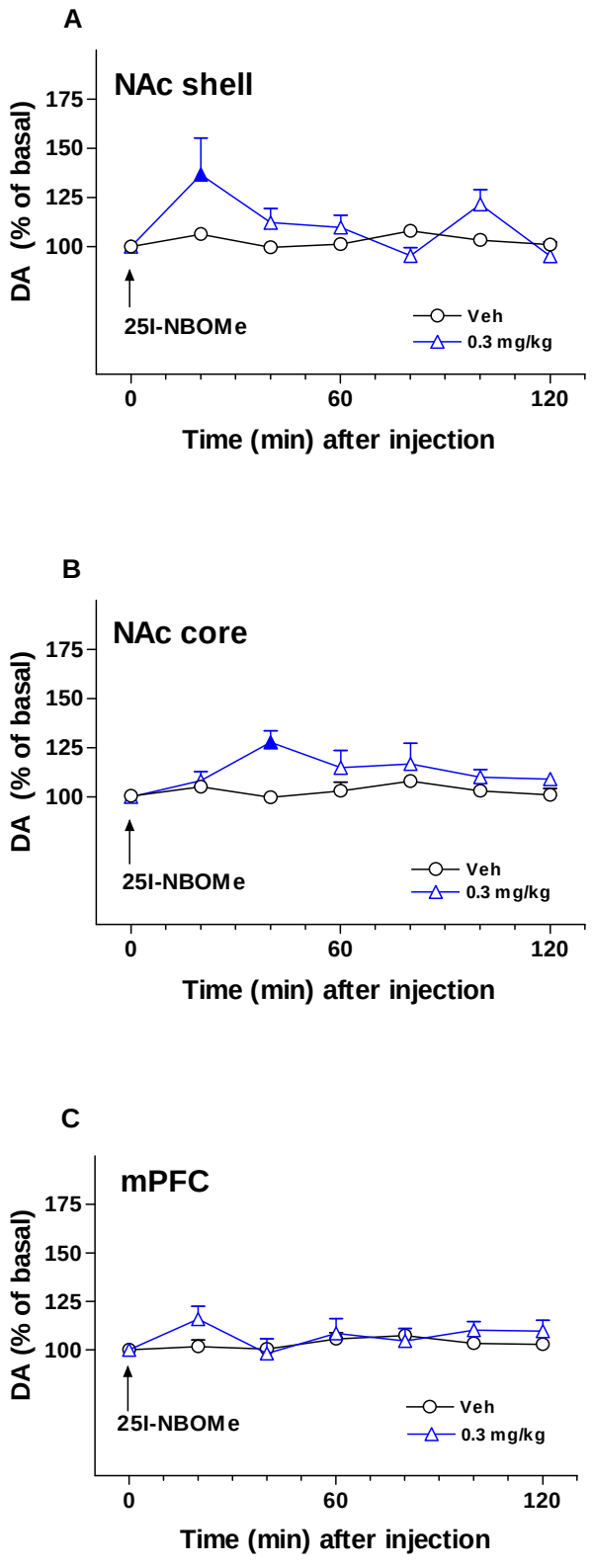

Figure 11. Effect of 25I-NBOMe administration(0.3 mg/kg i.p) on DA transmission in the NAc shell, NAc core, and mPFC in male rats. Results are expressed as mean ± SEM of change in DA extracellular levels expressed as the percentage of basal values. The arrow indicates the start ofi.p. injection at of vehicle (*blackcircles*) or 25I-NBOMe 0.3 mg/kg(*blue triangles*) in NAc shell (panel A), NAc core (panel B),andmPFC (panel 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, (NAc shell N=10;NAc core N=7; mPFC N=11).

Females

Rat basal values of DA, expressed as fmoles/20 µl sample (mean ± SEM), were NAc shell 36 ± 4 (N=28), NAc core 39 ± 6 (N=25), mPFC14± 1 (N=20). In this experiment we evaluated the effect of one dose of 25I-NBOMe (0.3 mg/kg i.p.) on extracellular DA levels in NAc shell and core, and mPFC. As shown in figure 12, 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 ($F_{1,67}$=15.88; *p<0.0005), time ($F_{6,402}$=4.38; *p < 0.0005), time x area interaction ($F_{12,402}$=2.34; *p < 0.01) and time x treatment interaction ($F_{6,402}$=3.0; *p < 0.01). In animals implanted in NAc shell, two-way ANOVA showed a main effect of treatment ($F_{1,26}$=7.65 ; *p< 0.05), time ($F_{6,156}$=3.23; *p < 0.01) and time x treatment interaction ($F_{6,156}$=3.55; *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 at the 40, 60, 100, 120 min samples with respect to basal values and a significant difference at 40 min sample compared to vehicle (Figure12, panel A). In animals implanted in NAc core, two-way ANOVA showed a main effect of treatment ($F_{1,23}$=7.08 ; *p < 0.05); tukey's post hoc tests showed no differences (Figure12, panel B). In animals implanted in mPFC, two-way ANOVA showed a main effect of treatment ($F_{1,18}$=5.75; *p < 0.05) and time ($F_{6,108}$=3.48; *p < 0.01); tukey *post hoc* test showed a larger increase of dialysate DA in the mPFC after 25I-NBOMe 0.3 mg/kg i.p revealing revealing differences at the 40 and 60min samples with respect to basal values (Figure 12, panel C).

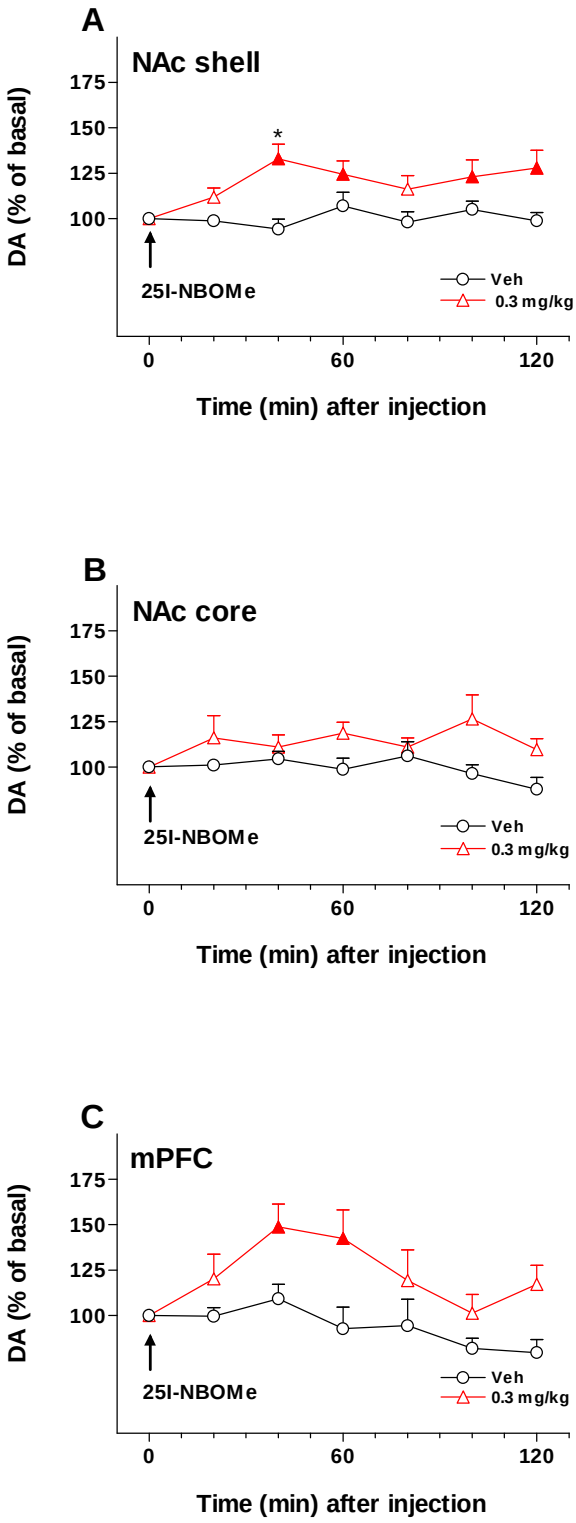

Figure 12. Effect of 25I-NBOMe 0.3 mg/kg i.p administration on DA transmission in the NAc shell, NAc core, and mPFC in female rats. Results are expressed as mean ± SEM of change in DA extracellular levels expressed as the percentage of basal values. The arrow indicates the start ofi.p. injection at the dose of vehicle (*black circles*) or 25I-NBOMe 0.3 mg/kg(*red triangles*) in NAc shell (panel A), NAc core (panel B),andmPFC (panel 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 vehicle (NAc shell N=28; NAc core N=25; mPFC N=20).

4.2.5.2 Effect of 25I-NBOMe administration on 5-HT transmission in the NAc shell and core, and in the mPFC

Males

Rat basal values of 5-HT, expressed as fmoles/20 µl sample (mean ± SEM), wereNAc shell8 ± 1(N=10), NAccore 8 ± 0.5 (N=7),mPFC 7 ± 0.6 (N=8). In this experiment we evaluated the effect of one dose of 25I-NBOMe (0.3 mg/kg i.p.) on extracellular5-HT levels in NAc shell and core, and mPFC. As shown in figure 13, the compound does not affect the serotoninergic transmission in all the areas studied. Three-way ANOVA showed a significant time x treatment interaction ($F_{6,114}$=2.3; *p<0.05). Two-way ANOVA analysis does not highlight significative differences between vehicle treated animals and 25I-NBOMe treated animals neither for the three areas (Figure13, panel A, panel B, panel C).

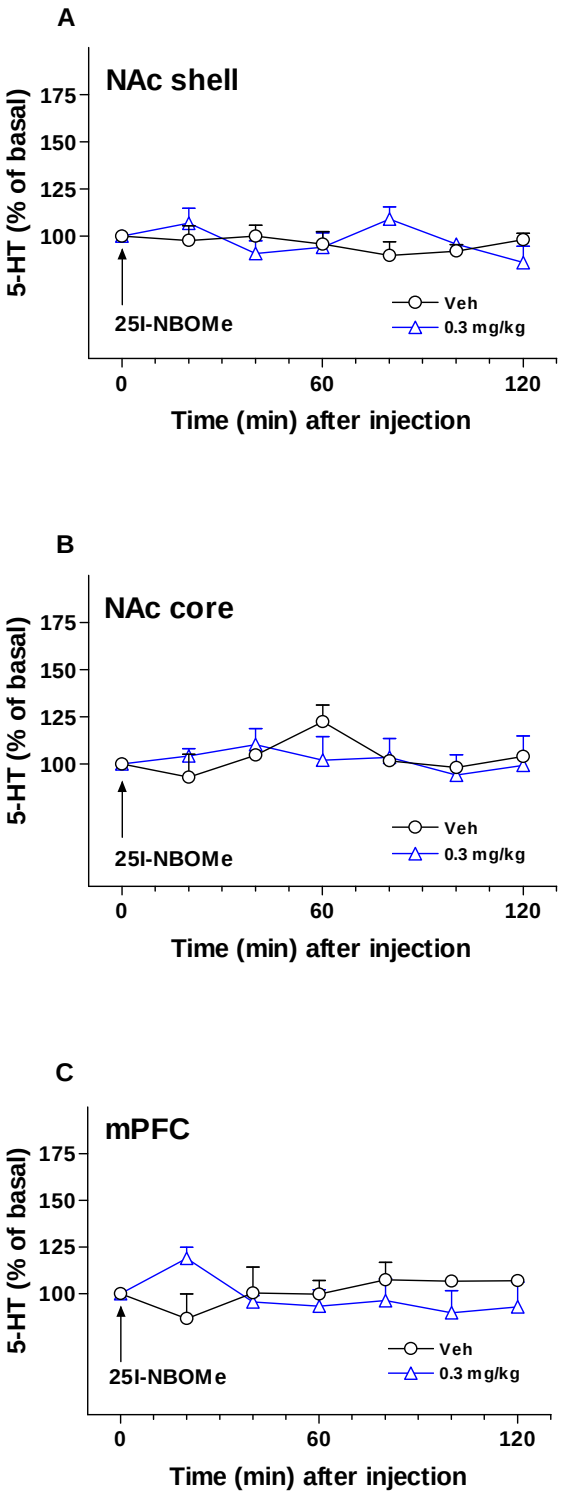

Figure 13. Effect of 25I-NBOMe 0.3 mg/kg i.p administration on 5-HT transmission in the NAc shell, NAc core, and mPFC in male rats. Results are expressed as mean ± SEM of change in 5-HT extracellular levels expressed as the percentage of basal values. The arrow indicates the start ofi.p. injection at the dose of vehicle (*blackcircles*) or 25I-NBOMe 0.3 mg/kg(*blue triangles*) in NAc shell (panel A), NAc core (panel B),andmPFC (panel 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, (NAc shell N=10; NAc core N=7; mPFC N=8).

Females

Rat basal values of 5-HT, expressed as fmoles/20 µl sample (mean ± SEM), wereNAc shell8 ± 0.8 (n=29), NAc core 8 ± 1 (n=14),mPFC 11 ± 0.8 (n=20). In this experiment we evaluated the effect of of25I-NBOMe (0.3 mg/kg i.p.) on extracellular5-HT levels in NAc shell, NAccore, and mPFC. As shown in figure 14, the compound affects the serotoninergic transmission to a small extent only in NAc shell. Three-way ANOVA showed a main effect of area($F_{2,57}$=11.28; *p <0.0001), treatment($F_{1,57}$=9.4; *p <0.005), area x treatment interaction ($F_{2,57}$=8.7; *p <0.001) and time x treatment interaction ($F_{6,342}$=0.96; *p <0.05); tukey's post hoc tests showed a larger increase of dialysate 5-HT in the NAc shell after 25I-NBOMe 0.3 mg/kg i.p revealing differences at the at the 40min sample with respect to basal values and a significant difference at 80 min sample compared to vehicle (Figure 14, panel A).In animals implanted in NAc shell, two-way ANOVA showed a main effect of treatment [$F_{(1,27)}$=33.81;*p < 0.0001], but no significant differences were revealed by tukey's post hoc test. Two-way ANOVA analysis does not highlight significative differences between vehicle treated animals and 25I-NBOMe treated animals for theNAc core (Figure14, panel B). In animals implanted in the mPFC(Figure14, panel C), two-way ANOVA showed a main effect of time($F_{6,108}$=3.84;*p < 0.005) and time x treatment interaction ($F_{6,108}$=3.06;*p < 0.01), without any significant results in the tukey's post hoc test.

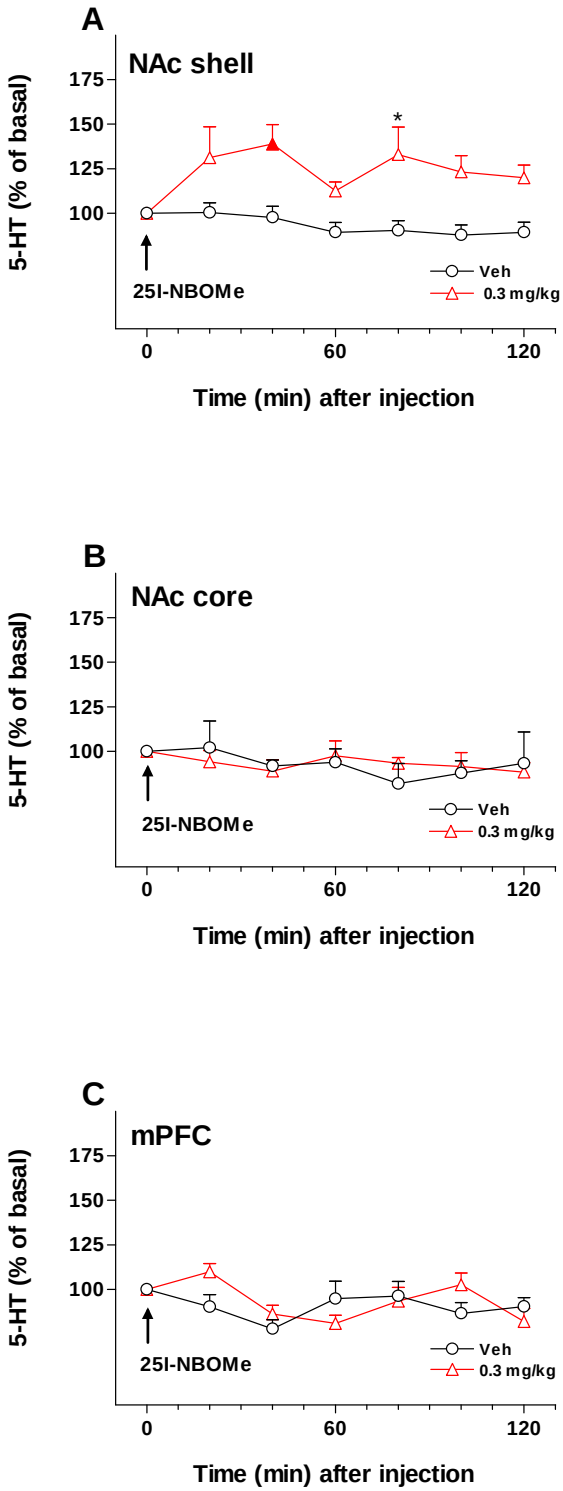

Figure 14. Effect of 25I-NBOMe 0.3 mg/kg i.p administration on 5-HT transmission in the NAc shell, NAc core, and mPFC in female rats. Results are expressed as mean ± SEM of change in 5-HT extracellular levels expressed as the percentage of basal values. The arrow indicates the start ofi.p. injection at the dose of vehicle (*black circles*) or 25I-NBOMe 0.3 mg/kg(*red triangles*) in NAc shell (panel A), NAc core (panel B),andmPFC (panel 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 vehicle (NAc shell N=29; NAc core N=14; mPFC N=20).

4.3 Effects of 25I-NBOMe on behavioural tests

4.3.1Sensorimotor studies

4.3.1.1 Evaluation of the visual response

4.3.1.1.1 Evaluation of the visual object response

Visual object response did not change in both vehicle-treated male and female rats over 60 minutes observation (Figure15panel A and B). Systemic administration of 25I-NBOMe (0.1-1 mg/kg i.p.) reduced the visual object response in both sex rats and the effect persisted up to 60 minutes (Figure15). Two-way ANOVA followed by the Bonferroni's test for multiple comparisons in male ratsshoweda significant effect of treatment($F_{4,140}$=22.24, p<0.0001), time ($F_{3,140}$=22.47, p<0.0001) and time x 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 x treatment interaction ($F_{12,140}$=2.149, p<0.05).

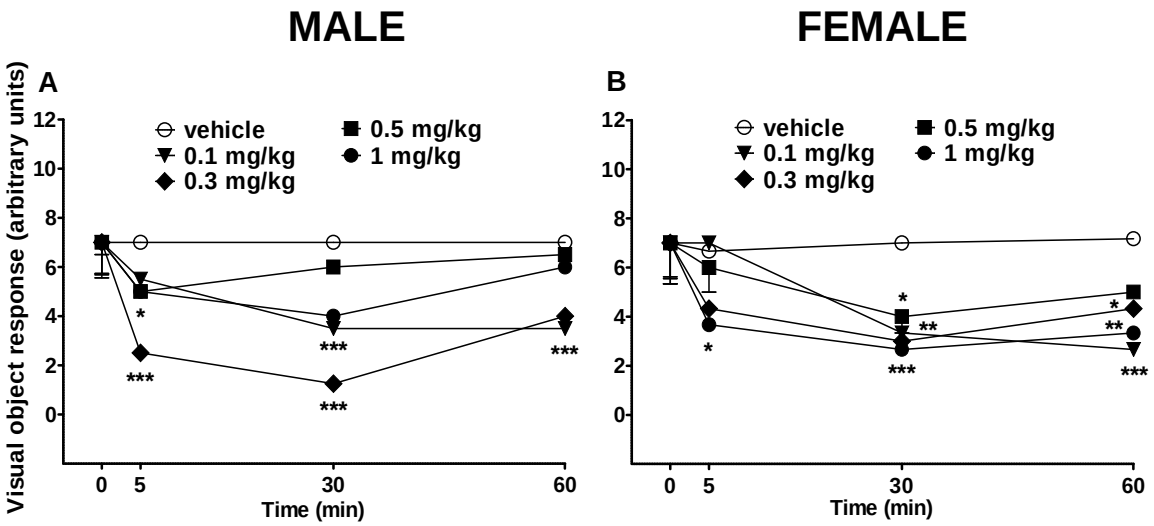

Figure 15. Intraperitoneal injection (0.1-1 mg/kg) of 25I-NBOMe on the visual object test in male (panel A) and female (panel B) rats. Data are expressed as arbitrary units andrepresent the mean ± SEM of 6 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose response curve at different times. *p<0.05, **p<0.01, ***p<0.001 versus vehicle.

4.3.1.1.2 Evaluation of the visual placing response

Visual placing response slightly decreased in both vehicle-treated male and female rats over 60 minutes observation (~17% of reduction at 60 min; Figure16panel A and B). Systemic administration of 25I-NBOMereduced the visual placing response in both sex rats at all the doses tested (0.1-1 mg/kg i.p.) and the effectpersistedup to 60 minutes;as shown in Figure16 (panel A), 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 x treatment interaction ($F_{12,140}$=2.582, p<0.005) for male rats; for female rat, as shown in the panel B, statical 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 x treatment interaction ($F_{12,140}$=2.135, p<0.05).

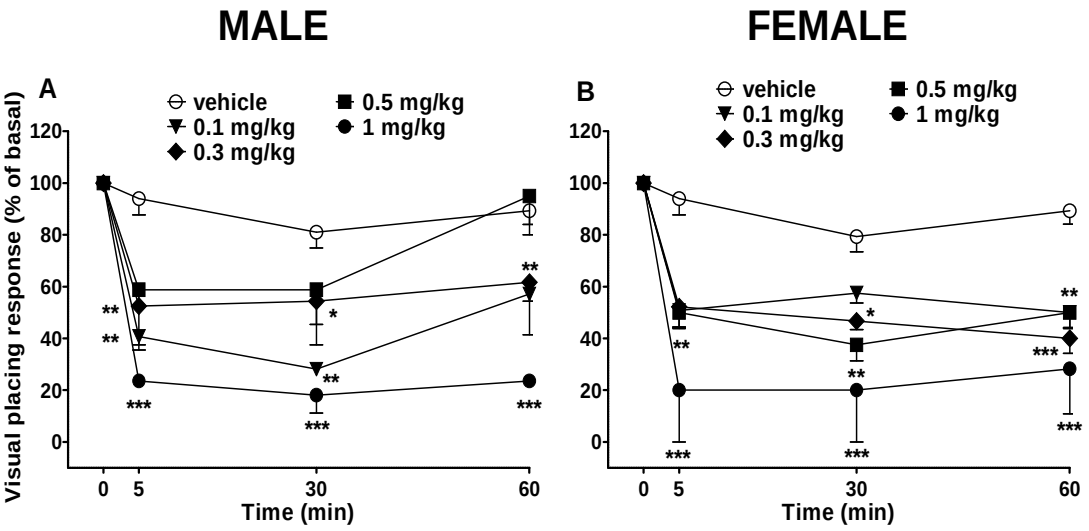

Figure 16. Intraperitoneal injection (0.1-1 mg/kg) of 25I-NBOMe on the visual placing test in male (panel A) and female (panel B) rats. Data are expressed as percentage of basal and represent the mean ± SEM of 6 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose response curve at different times. *p<0.05, **p<0.01, ***p<0.001 versus vehicle.

4.3.1.2 Evaluation of the acoustic response

Acoustic response did not change in both vehicle-treated male and female rats over 60 minutes observation (Figure17panel A and B). Systemic administration of 25I-NBOMe affect the acoustic response only in male rats at the two highest doses tested 0.5 and 1 mg/kg and, reducing it and this effect is persistent up to 60 minutes after the treatment (Figure17panel A); 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 x 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 comparisonsshowed a tardive little effect displayed by the dose of 0.5 mg/kg i.p. at 60 minutes(Figure17, panel B).

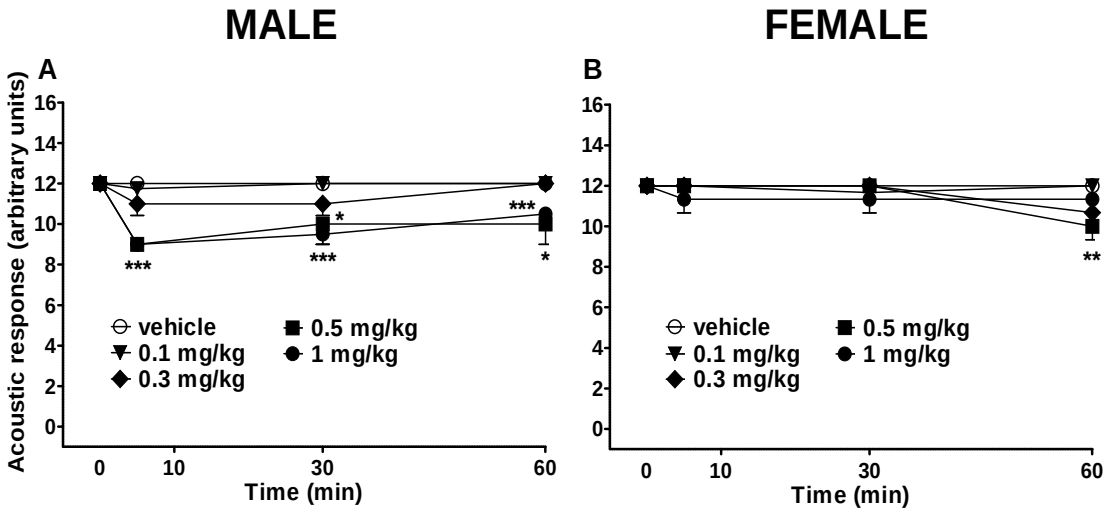

Figure 17. Intraperitoneal injection (0.1-1 mg/kg) of 25I-NBOMe on the acoustic response in male (panel A) and female (panel B) rats. Data are expressed as arbitrary units andrepresent the mean ± SEM of 6 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose response curve at different times. *p<0.05, **p<0.01, ***p<0.001 versus vehicle.

4.3.1.3 Evaluation of the tactile response

Overall tactile responses did not change in both vehicle-treated male and female rats over 60 minutes observation (Figure18, panel A and B).As shown in Figure 18, panel A, intraperitoneal injection (0.1-1 mg/kg) of 25I-NBOMe affected males tactile responses with asignificant 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 (Figure18, panel B).

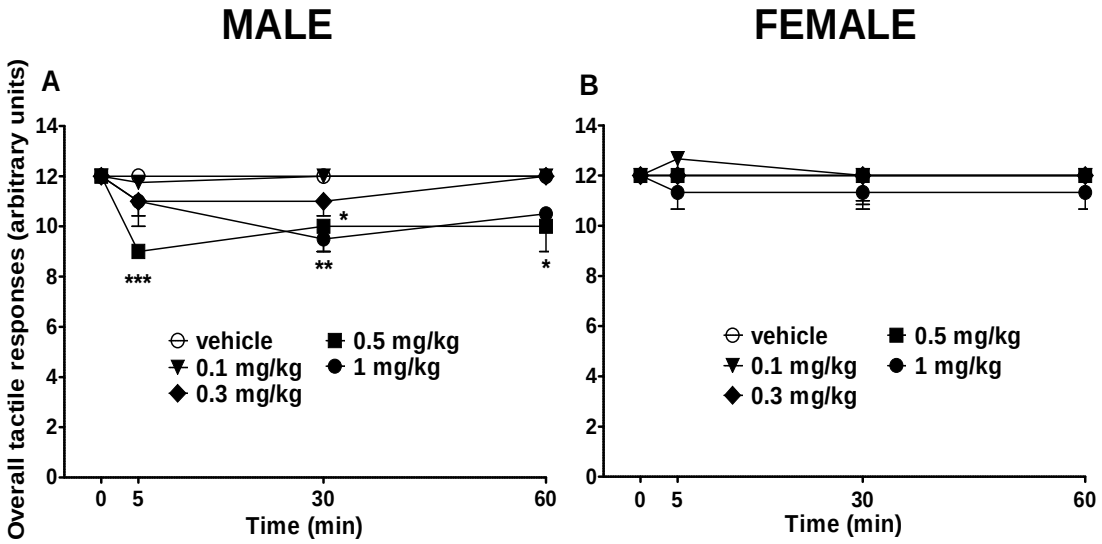

Figure 18. Intraperitoneal injection (0.1-1 mg/kg) of 25I-NBOMe on the overall tactile response in male (panel A) and female (panel B) rats. Data are expressed as arbitrary units andrepresent the mean ± SEM of 6 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose response curve at different times. *p<0.05, **p<0.01, ***p<0.001 versus vehicle.

4.3.2 Evaluation of core and surface body temperature

Core and surface body temperature did not change in both vehicle-treated male and female rats over 60 minutes observation (Figure19, panel A,B, C, D). Systemic administration of 25I-NBOMe(0.1-3 mg/kg i.p.) did not affect core (Figure19, panel A) 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 o.5 mg/kg i.p.affected significantly the core temperature in female rats (Figure19, panel B) with a significant effect of treatment ($F_{4,105}$=12.07, p<0.0001). As shown in the last two panels, C and D, 25I-NBOMe did not affect the surface temperature in male rats, neither in females.

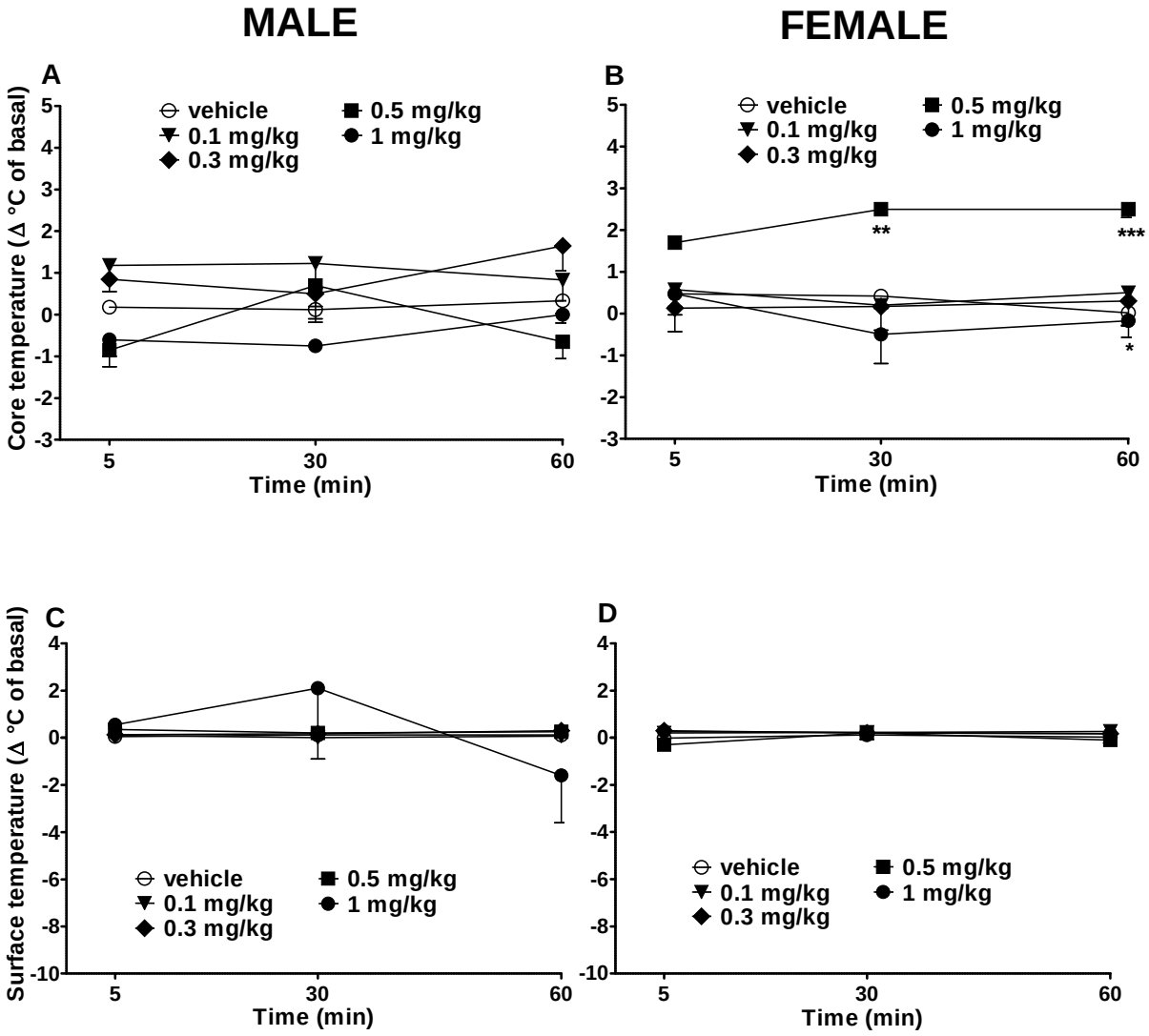

Figure 19. Intraperitoneal injection (0.1-1 mg/kg) of 25I-NBOMe on core temperature in male (panel A) and female (panel B) rats and in surface temperature in male (panel C) and in female (panel D) rats..Data are expressed as the difference between control temperature (before injection) and temperature following drug administration (Δ°C; see material and methods) and represent the mean ± SEM of 6 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 compounds at different times. *p<0.05, **p<0.01, ***p<0.001 versus vehicle.

4.3.3 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 60 minutes observation (Figure 20, panel A and B). Systemic administration of the highest dose of 25I-NBOMe(1 mg/kg i.p.) heavilyincreased the threshold to acute mechanical pain stimulus in male rats in the tail pinch test (Figure20panel A: significant effect of treatment ($F_{4,105}$=9.822, p<0.001), time ($F_{2,105}$=3.110, p<0.05); whereas, in female rats there is a lower effect with the same dose (Figure20, panel B); statistical analysis showed a significant effect of treatment ($F_{4,105}$=4.988, p<0.001).

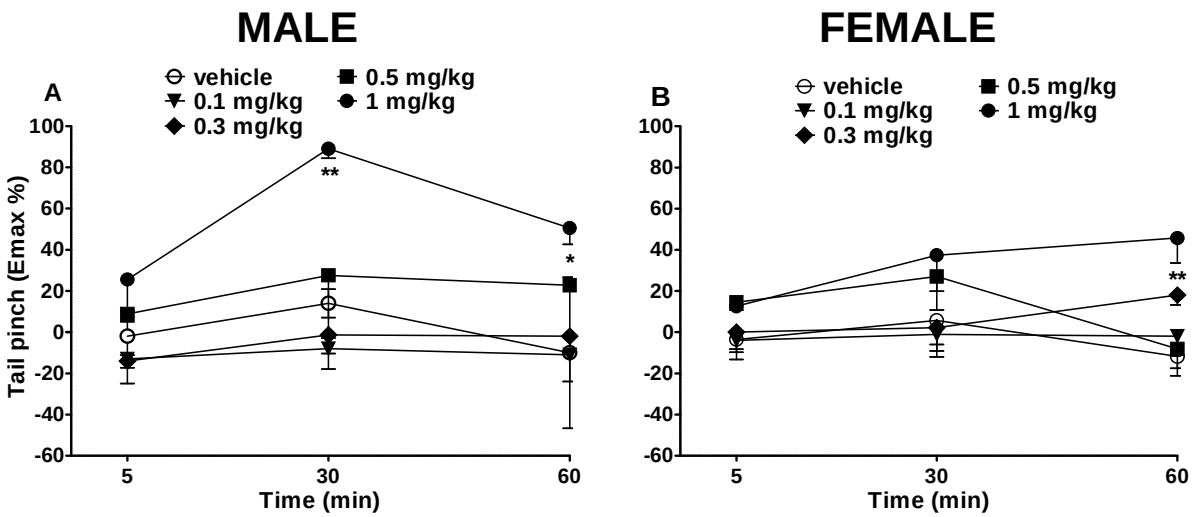

Figure 20. Intraperitoneal injection (0.1-1 mg/kg) of 25I-NBOMe on tail pinch test in male (panel A) and female (panel B) rats.Data are expressed as percentage of maximum effect (see material and methods) and represent the mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose response curve of each compounds at different times. *p<0.05, **p<0.01, ***p<0.001 versus vehicle.

5. DISCUSSION

In the present study, we mainly evaluated the dopamine releasing properties and behavioral effects of different compounds chosen between two of the most popular classes of Novel Psychoactive Substances: synthetic cannabinoids and phenyletylamines. The main results of this work were that selected third generation cannabinoids, namely BB-22, 5F-PB-22, 5F-AKB-48, and STS-135 are full agonists of the CB1 receptors and they are more potent compared to AKB-48, which belongs to the same generation but appeared earlier in the market, as well as compared to JWH-018, a first generation cannabinoid. All the SC studied affect dopamine transmission selectively in the NAc shell, displaying a putative abuse liability. Furthermore, we demonstrated that the phenethylamine 25I-NBOMe is more active on females, compared to male, in increasing DA transmission in NAc shell and in the mPFC; however, behavioral data showed that this compound caused 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.

As to SC, the in vitro results of this study showed that third generation cannabinoids, BB-22, 5F-PB-22, 5F-AKB-48, and STS-135 possess a very high affinity and act as potent full agonists at the native rat and mice brain CB1 receptors; therefore they are more potent compared to AKB-48, the non-fluorinated APICA which belongs to the same generation but appeared before in the market(Uchiyama et al. 2013; Canazza et al., 2016), as well as compared to the JWH-018that r epresents, in this study, the reference compound of the first generation of SC. JWH-018,BB-22, 5F-PB-22, 5F-AKB-48, and STS-135 bind with nanomolar affinity to CB1 receptors in rat cerebral cortex homogenates, and stimulate [^{35}S]GTPγS binding in a concentration-dependent manner; this effect is completely suppressed by the CB1 antagonist/inverse agonist AM 251 and totally absent in CB1-KO mice, leading us to conclude that they activate a G-protein coupled CB1 receptor with high potency and efficacy. Ki values of third generation SCs tested were significantly lower compared to the reference compound, with the following rank order of CB1 receptor affinity: BB-22=5FPB-22 > 5F-AKB-48 > STS-135 > JWH-018 (De Luca et al.,2016). In particular, BB-22 and the fluorinate 5F-PB-22 possess 5 and 7 fold, respectively greater CB1 receptor agonist potency and efficacy and a higher binding affinity (26 and 30 fold, respectively) at CB1 receptors compared to JWH-018. 2005; De Luca et al., 2012; De Luca et al., 2015), it is easy to understand that the consumption of these SC might have critical outcomes for the public health. Among the SC studied, AKB-48 was the first that appeared in the market of “legal

marijuana"; as shown from our microdialysis results, this compound affects the DArgic transmission in the NAc shell only at the dose 0.25 mg/kg/i.p., increasing significantly extracellular DA levels selectively in the NAc shell as compared to NAc core and mPFC. Interestingly, JWH-018 stimulates the dialysate DA in the NAc shell at the same dose (De Luca et al., 2015). However, compared to the present observations, the effect observed after the administration of JWH-018 was a long-lasting effect with a maximal increase of DA in the NAc shell of 65% over basal value; the lower efficacy of AKB-48 could be due to the steric hindrance of the adamantly group that delays the passage through the blood brain barrier or limits a quick bond to CB1 receptors. BB-22, the most potent compound *in vitro*, was tested *in vivo* in a range of doses between 0.003 and 0.1 mg/kg iv; results showed that BB-22 increased dialysate DA in the NAc shell but at the intermediate dose of 0.01 mg/kg iv, and that this effect was prevented by CB1 antagonist/inverse agonist AM251 at a dose that per se did not affect basal dialysate DA, thus confirming that the effect is CB1 receptors-mediated. Notably, the effective dose of BB-22 that affect dialysate DA in the NAc shell is about 10 times lower than the dose of JWH-018 that elicits a quantitatively similar peak in dialysate DA in the NAc shell (about 50% over basal) and these differences correspond to the difference in Ki between the two compounds as ligands of native rat CB1 receptors (BB-22, 0.11 nM; JWH-018, 3.38 nM).Indeed, BB-22 has a bell shaped dose-response curve with loss of the effect at the highest dose, as it was previously shown in our lab for the JWH-018 (De Luca et al., 2015), the reason of this could be the action of active metabolites, produced by phase I metabolism, that can readily cross the blood-brain-barrier and act as partial agonists or antagonists, thus retaining the activity of the parent drug (Dhawan et al., 2006; Wiebelhaus et al., 2012), as previously demonstrated for JWH-018 metabolites (Brents et al., 2011).In addition, BB-22 stimulates NAc shell DA release at the dose of 0.01 mg/kg iv, while THC increases extracellular DA in the same area at dose of 0.15mg/kg iv (Tanda et al., 1997). The other three compounds (i.e. 5F-PB-22, 5F-AKB-48, and STS-135) were tested for their effects on dialysate DA only in the NAc shell at a single dose level, selected on the basis of the ratio between the Ki of JWH-018 and BB-22 for CB1 receptors and the doses of the same compounds that activate in vivo NAc shell DA transmission. Thus, doses of 0.01 mg/kg iv of 5F-PB-22, 0.1 mg/kg of 5F-AKB-48 and 0.15 mg/kg of STS-135 were tested. At these doses, all compounds increased dialysate DA in the NAc shell to a similar extent to BB-22 (max < 50% over basal).In the case of 5F-AKB-48 the increase of dialysate DA was delayed, similarly to the non-fluorinated analogue previously tested (i.e. AKB-48). In

fact, the steric hindrance of the adamantly residue limits the passage through the blood brain barrier and a rapid bond to CB1 and CB2 receptors; nevertheless it results more potent, compared to the non-fluorinated APICA, because the new trend of SC fluorination increases the lipophilicity of SC (Schifano et al., 2015). Interestingly, all the SCs studied elicit their effects on DA dialysate in a tight range of doses, this property seems to be different in natural and endogenous cannabinoids (Tanda et al.,1997;De Luca et al., 2014), as well as in psychostimulants, nicotine and narcotic drugs of abuse (Pontieri et al., 1995, 1996; Di Chiara et al., 2004).All the drugs with abuse potential are able to increase DA transmission preferentially in the NAc shell (Di Chiara et al., 2004; Di Chiara and Bassareo, 2007). Furthermore, all the SC tested share the ability to increase DA levels selectively in the shell of NAc with Δ^9-THC as well as with the synthetic cannabinoid WIN 55,212-2 (Tanda et al., 1997; Lecca et al., 2006), suggesting similar rewarding properties. Notably, the demanding "legal" market of these products encourages NPS manufacturers to manipulate existing psychoactive substances formulas and obtain new compounds that are often more potent compared to the preceding compounds.

The second section of this work focused on the study of the pharmacological and neurochemical properties of 25I-NBOMe in male and female rats. 25I-NBOMe belongs to the phenethylamines that are a class of NPS widely used among young people, more from girls than boys(Wu et al., 2010;UNODC, 2016). 25I-NBOMe is a $5HT_2A$ receptor agonist used as legal substitute of LSD, and to mimic the effect of methamphetamine as well (Le Roux et al., 2015; Palamar et al., 2016). In the current literature, there are no data about the abuse liability of this compound and its pharmacological effects. Our results, obtained by *in vivo* microdialysis studies , showed that 25I-NBOMeaffects the DA transmission in male rats in the NAc shell with a maximal peak of 36 % over basal value , 20 min after the injection and in the NAc core, with an extent of 27% at the 40 min sample whereas, it has no effect on the mPFC DA transmission; no effect has been observed in the 5-HT transmission in all the three areas tested. The same dose (0.3 mg/kg/ip) is more active in female rats, increasing both DA and 5-HT dialysates in the NAc shell with a maximal peak of 30% over basal value, 40 minutes after the administration, whereas in the mPFC only DA extracellular levels are increased with an extent of 45%,andthese effects lasted more than two hours after the drug administration. Taken together these results suggest that 25I-NBOMe affects DA and 5-HT transmissions in male and females in a different way, highlighting gender differences that can influence the frequency of ingestion, as well as the

psychoactive effects, and the long-term effects. It is well established that significant gender differences have been reported in the initiation of drug use and that this may be affect the continuation of drug use as well as the phases of abstinence and relapse (Becker and Hu, 2008; Fattore et al., 2009). These differences are due to biological differences, such as ovarian hormone fluctuations (Becker and Hu, 2008), as well as sex dimorphisms in the anatomy of DArgic systems in areas like SN and VTA (Walker et al., 2012).Furthermore other factors, as pharmacokinetic, pharmacodynamic, and socio-cultural differences have been proposed to take part in the propensity to addiction (Fattore et al., 2008;Franconi et al., 2012), These gender differences were widely demonstrated in female rats that exhibit greater sensitivity to psychostimulants compared to males (Walker et al., 2012) with experimental paradigms such as self-administration and conditioned place preference (Becker et al., 1982; Lynch and Carroll, 1999; Harrod et al., 2005; Kantak et al., 2007; Roth and Carroll, 2004; Russo et al., 2003; Savageau and Beatty, 1981; Walker et al., 2001). Indeed, a recent study of Lazenka and colleagues (2016) showed that MDMA significantly increases NAc dialysate DA, culminating in greater peak in females compared to males, and this is in line with our results. Further investigations are necessary to examine in depth the reason of these gender differences; therefore, the next step will be to perform microdialysis experiments with other doses to obtain a dose-response curve in both intact and ovarectomized female rats, otherwise verifying if there are differences in the four phases of estrous cycle to understand the role of hormones in mediating the effects of this compound. These differences among males and females in responding to this synthetic compounds could explain the fact that according to recent surveys adolescent girls are more likely, compared to boys, to be ecstasy and/or other hallucinogens 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). In order to have more information on the toxicological effects of this 25I-NBOMe, in collaboration with Dr. Marti of the University of Ferrara, we performed behavioural tests in both male and females rats. Data obtained showed that this compound decreases visual responses, causing dangerous visual alterations in both sexes; the acoustic and tactile responses is decreased only in male rats, whereas core temperature in females is heavily affected by the compound, compared to males. The highest dose tested exerts an analgesic effect prominent in male rats and lighter in female rats, increasing the threshold to acute mechanical pain

stimulus; this effect in male rats is higher than the effect of a dose 100 fold greater than Δ9-THC and 6 fold greater than the synthetic cannabinoid JWH-018, both acting by cannabinoid pathway (Vigolo et al.,2015).This compound has a great affinity for rat $5HT_{2A}$ receptors (Ki= 0.087 nM) (Braden et al., 2006) but it has lower affinity also for μ opiate receptors (Ki= 82 nM) and Ki greater than 500nM for $5HT_{1A}$ receptors (Nichols et al., 2008); therefore it is possible to assume that the highest dose tested, bound $5HT_{2A}$ receptors first, and further with other receptors such as $5HT_{1A}$ and μ receptors producing the analgesic effect.

6. CONCLUSION

In conclusion, we have shown that four representatives of 3rd generation Spice/K2 cannabinoids are highly potent and effective agonists of CB1 receptors and that they share with AKB-48 the properties of increasing DA transmission selectively in the NAc shell of male rats.

These results showed that these new compounds are more potent than THC and previous generations of SC in inducing NAc shell DA release, suggesting greater rewarding properties and severe side effects. These results provide pre-clinical evidence for a putative abuse liability of these compounds , although further investigastion on the reinforcing properties of these substances are necessary to confirm the abuse potential, as previous demonstrated in self-administration studies for WIN 55,2012-2(Lecca et al., 2006), and JWH-018 (De Luca et al., 2015).Furthermore, we demonstrated that the phenethylamine 25I-NBOMe is more active on females, compared to male, in increasing DA transmission in NAc shell and in the mPFC, suggesting likely rewarding properties, that need to be proved with further investigations.

Collectively, the present findings are a reason for further clinical concern. Users do not seem to be aware of the serious adverse effects related to SC misuse, since these compounds may be perceived to be somehow equivalent to Marijuana and hence “safe” and “natural” (Santacroce et al., 2015; Schifano et al., 2015). The scenario becomes more worrisome if we consider that polydrug users use to mix synthetic cannabinoids and psychostimulants (Parrott et al., 2007; Schulz, 2011), together with alcohol, risking severe effects and fatal events. On this regard, the project “Prevention and Information about New Psychoactive Substances (NPS) and their toxicity”, gave us the opportunity to share all these information with students, parents and teachers by a series of conferences in local high schools, and by the use of a dedicated

blog(http://infonuovedroghe.blogspot.it/) and in the related page on Facebook “Nuovedroghe e Danni alla salute”. Both these tools are effective to share information about the dangerous effects of New Psychoactive Substances (NPS) in the same channels where they are usually promoted.

Considering the growing evidence of the widespread use of NPS, this work helps us to understand the new trends in the field of drug reward and drug addiction by revealing the rewarding properties of NPS, and will be helpful to gather reliable data regarding the abuse potential of these compounds.

Finally, all these results highlight that the NPS issue should not be underestimated by governments and civil society, and that the scientific community has an important role, since it is fundamental to evaluate the pharmacology and toxicological effects of NPS, and develop effective treatments for NPS intoxication. Additionally, this work intends to be useful to update law enforcement agencies, which need even more information to prevent and fight against trafficking and sale of NPS, in order to protect public health and safety.

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