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TITLE

"DIFFERENTIAL ADAPTIVE PROPERTIES OF MESOLIMBIC AND MESOCORTICAL DOPAMINE TRANSMISSION TO TASTE STIMULI, NEUROINFLAMMATORY EFFECTS AND BEHAVIORAL CORRELATES AFTER REPEATED EXPOSURE TO THE SYNTHETIC CANNABINOID JWH-018"

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Contents

Abbreviations

Δ^9-THC	Δ^9- TetraHydroCannabinol
2-AG	2-Arachidonoyl Glycerol
5-HT	5-HydroxyTryptamine (serotonin)
6-OHDA	6-HydroxyDopamine
AC	Anterior Cingulate
AEA	Anandamide
Ach	Acetylholine
ACSF	Artificial Cerebrospinal Fluid
AdC	Adenylyl Cyclase
Amb	Ambiguous nucleus
AMPA	α-Amino-3-hydroxy-5-Methyl-4-isoxazolePropionic Acid receptor
AOB	Accessory Olfactory Bulbs
BBB	Blood Brain Barrier
BDNF	Brain Brain-Derived Neurotrophic Factor
BLA	Basolateral Amygdala
BNST	Bed Nucleus of Stria Terminalis
CB	CannaBinoid
CBR	CannaBinoid Receptor
CCK	CholeCystoKinin
CeA	Central Amygdala
Cg	anterior Cingulate cortex
COX-2	CycloOXygenase-2
CNS	Central Nervous System

CPA	Conditioned Place Aversion
CPP	Conditioned Place Preference
CPu	Caudate Putamen
CSF	CerebroSpinal Fluid
DA	Dopamine
DAGL	DiacylGlycerol-Lipase
DAT	Dopamine Transporter
DB	Diagonal Band
DCAA	Aromatic L-Amino Acid Decarboxylase
DRN	Dorsal Raphe Nucleus
EAAT-2	Excitatory Amino Acid Transporter-2
eCB	endoCannaBinoid
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
FAAH	Fatty Acid Amide Hydrolase
FC	Frontal Cortex
GABA	Gamma-AminoButyric Acid
GFAP	Glial Fibrillary Acidic Protein
GLAST	Glutamate Aspartate Transporter
GLT-1	Glutamate Transporter-1
Glu	Glutamate
GP	Globus Pallidus
GPCR	G - Protein Coupled Receptor
Hipp	Hippocampus
HPLC	High Performance Liquid Chromatography
Hypo	Hypothalamus

io	intraoral
ip	intraperitoneal
iv	intravenous
ID	Inside Diameter
IL	InfraLimbic cortex
IL-1α	InterLeukin-1α
IL-6	InterLeukin-6
IL-10	InterLeukin-10
IFNγ	InterFeron γ
iNOS	inducible Nitric Oxide Synthase
IVSA	Intravenous Self-Administration
LC	Locus Coeruleus
LHb	Lateral Habenular nucleus;
LTD	Long term Depression
LTP	Long term Potentiation
MAGL	MonoAcylGlycerol Lipase
MD	MedioDorsal thalamic nucleus
mGlutR1	metabotropic Glutamate Receptors
mHb,	Medial Habenular nucleus;
MOB /OB	Olfactory Bulbs
mPFC	medial PreFrontal Cortex
MSNs	Medium Spiny Neurons
NA	Noradrenaline
NAc	Nucleus Accumbens
NAPE-PLD	N-Arachidonyl-PhosphatidylEthanolamine PhosphoLipase- D

NAT	Arylamine N-Acetyltransferase
NMDA	N-Methyl-D-Aspartate
NPS	New Psychoactive Substance
OCF	OrbitoFrontal Cortex,
OD	Outside Diameter
PAG	PeriAqueductal Gray
PBS	Phosphate Buffer Solution
PEA	PalmitoylEthanolAmide
PET	Positron Emission Tomography
PFC	PreFrontal Cortex
PL	PreLimbic cortex
PPAR	Peroxisome Proliferator Activated Receptor
SCRA	Synthetic Cannabinoid Receptor Agonist
sEPSC	spontaneous Excitatory PostSynaptic Currents
sIPSC	spontaneous Inhibitory PostSynaptic Currents
SN	Substantia Nigra
SNc	Substantia Nigra pars Compacta
SNr	Substantia Nigra pars reticulate
Sol	nucleus of the Solitary tract
SOM	Somatostatin
SP	Substance P
STh	SubThalamic nucleus (ventral thalamus)
TGF-β	Transforming Growth Factor- β
TH	Tyrosine Hydroxylase
TNFα	Tumor Necrosis Factor α

TRPV1	Transient Receptor Potential Vanilloid 1
Tu	olfactory Tubercle
VTA	Ventral Tegmental Area

A-Introduction

1 Dopamine and Dopaminergic systems

Dopamine (DA), a biogenic amine that derives from the aminoacid tyrosine (Fig. 1), is a neurotransmitter that acts via G-protein-coupled receptors (GPCRs) in a typical neuromodulatory fashion (Missale *et al.*, 1998) characterized by large temporal and spatial dimensions (Greengard, 2001; Di Chiara *et al.*, 2004). In particular, DA acts through different GPCRs named from D1 to D5 according to their ability to stimulate or inhibit adenylyl cyclase (AdC): the D1-like family receptors (which includes D1 and D5 receptors) stimulate the synthesis of AdC and the D2-like family (D2, D3 and D4 receptors) inhibit it (Fig. 1) (Webster, 2001).

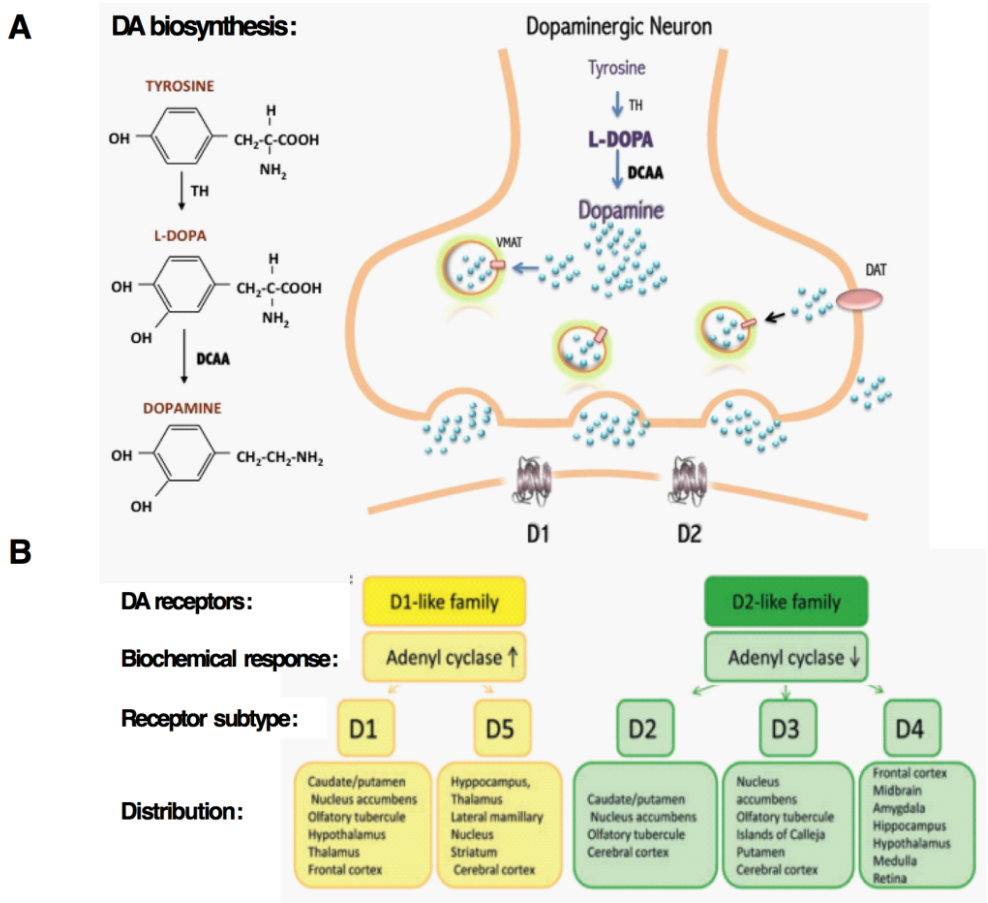

Figure 1 The biosynthetic DA pathway (A) and DA receptors features (B).
Abbreviations: TH, tyrosine hydroxylase; DCAA, aromatic L-amino acid decarboxylase. Adapted from Allabaran Bravo et al. (2014) and Ares-Santos et al. (2013)

All DA receptors are slow (response time exceeding 100ms) metabotropic receptors with the function to modulate other receptor systems and/or ion channels. Therefore, DA is a "pure" neuromodulator that exerts slow control over fast neurotransmission, in contrast to many other neurotransmitter systems that have both fast-acting ionotropic and slower acting GPCR-mediated actions (Covey *et al.*, 2017). Consequently, DA cannot be classified as either an excitatory or an inhibitory neurotransmitter, which may explain why DA does not yield identical effects under all experimental conditions.

Based on the anatomical and functional differences of the subgroups of DA midbrain neurons it is possible to identify at least three major DAergic systems of the mammalian brain (Carlsson *et al.*, 1962; Dahlstroem & Fuxe, 1964) (Fig. 2). The *nigrostriatal or mesostriatal pathway* rises from the cell bodies of the neurons of the substantia nigra pars compacta (SNc, lateral A9 group) which project primary to the dorsal striatum. This pathway plays an important role in the control of voluntary movement and is also related to the initiation and execution of habitual behavior. In particular, selective degeneration of this pathway is responsible for the motor symptoms observed in patients with Parkinson's disease. On the other hand, the DA pathways that rise from cell bodies located in the ventral tegmental area (VTA, medial A10 group), are those implicated in reward processes. In particular, the VTA DA neurons project to either the Nucleus Accumbens (NAc) and related limbic regions, establishing *mesolimbic DA pathways*, or to the prefrontal cortex (PFC), establishing *mesocortical DA pathways*. Besides reward, these pathways are importantly involved in the regulation of cognition, emotions and have been implicated in pathological conditions such as depression and schizophrenia, as well as drug addiction. Although the A9 cells preferentially innervate the striatum, and the A10 cells mainly target the limbic and cortical regions, the projections of the midbrain DA cells are not restricted to these regions. For instance, many of the SN DA neurons give off collateral branches and ramify within more than one region. Therefore, the projection fields of the A9 and A10 cell groups are collectively known as the *mesotelencephalic DA system*(Elsworth & Roth, 2009) (Fig. 2).

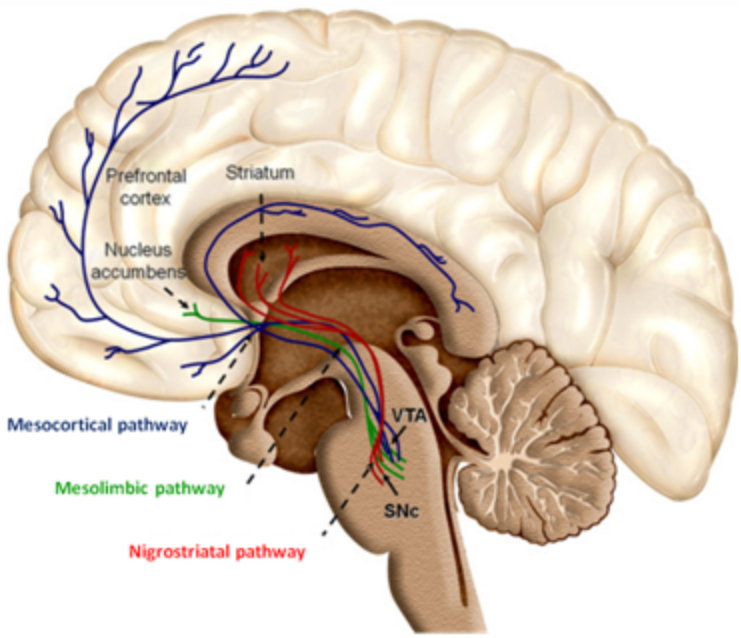

Figure 2 Schematic representation of main DA pathways: the nigrostriatal (red), mesolimbic (green) and mesocortical pathway (blue).
Abbreviations: VTA, ventral tegmental area; SNc, substantia nigra pars compacta. Modified from (Arias-Carrión *et al.*, 2010).

Regarding the activity of DA neurons, the DA cells fire in two independent but interactive releasing modes with distinct temporal properties, which have a marked impact on the release of DA: a phasic release (fast and transient) mediated primarily by bursting events (>15-20 Hz) at the level of the cell body that leads to a much larger DA release, which is necessary to stimulate D1 receptors (D1R, low-affinity receptors) in the NAc (Grace, 1991; Phillips *et al.*, 2003; Dreyer *et al.*, 2010). The second release mode is named tonic release (constant), mediated by irregular single-spike events (1-5 Hz) (Grace, 1991; Dreyer *et al.*, 2010). In this mode, DA is released slowly with prolonged onset and the whole process takes long periods of time, from minutes to hours. Although DA tonic release results in lower DA increases than from phasic firing, this DA increase is sufficient to stimulate D2 receptors (D2R, high-affinity receptors) signaling. These different firing DA neurons lead to a different DA increases regulated primarily by GABAergic and glutamatergic afferents (either locally or through projecting neurons) that synapse with the DA neurons.

Moreover, since DA cells are able to switch between these modes, the transition in activity is a mechanism for altering the impact of DA neurotransmission on receptive cells, a condition observed

in several neuropsychiatric diseases such as in addiction. Indeed, the bursting activity of DA neurons is thought to represent a key component of reward circuitry, as in reward-related learning processes (Grace, 1991; Gonon, 1997; Grace, 2000; Dreher & Burnod, 2002), while, alterations in tonic levels of DA efflux are implicated on a variety of motor, cognitive, and motivational processes (Elsworth & Roth, 2009). Nevertheless, as discussed in the next sections, it is difficult to directly correlate stimulus-bound DA activity (burst firing) and its functional activity in the receptive elements, due to the temporal and spatial dimensions of DA transmission (Gonon, 1997; Di Chiara, 2002; Di Chiara *et al.*, 2004; Di Chiara & Bassareo, 2007).

1.1 The Nucleus Accumbens

Nucleus Accumbens (NAc) is a small nucleus ventral at the head of the caudate nucleus (Fig. 2 and 3), and together with the ventromedial part of the caudate-putamen (CPu) and the olfactory tubercles comprise the anatomical complex often referred as "ventral striatum" (Nakano, 2000. NAc is described as an "integral, but specialized part of the striatal complex" {Heimer, 1991 #149) and consists mainly (90-95%) of medium spiny neurons (MSNs), which receive afferent fibers from interneurons, the cortex and other areas (Somogyi *et al.*, 1979; Freund *et al.*, 1984; Smith & Bolam, 1990).

In the middle of the 1980s, the studies of Groenewegen & Russchen (1984) and Zaborszky et al. (1985) demonstrated significant differences between the projections of the lateral and the medial NAc, introducing the division of the NAc into two histochemically distinct compartments: the *core* and the *shell*. Further anatomical (Heimer *et al.*, 1991; Zahm & Brog, 1992; Zahm & Heimer, 1993), histochemical(Jongen-Relo *et al.*, 1993; Jongen-Relo *et al.*, 1994), behavioral (Deutch & Cameron, 1992; Prinssen *et al.*, 1994; Pierce & Kalivas, 1995; Corbit & Balleine, 2011) and neurochemical (Pontieri *et al.*, 1995; Pontieri *et al.*, 1996; Tanda *et al.*, 1997; Bassareo *et al.*, 2002) studies established scientifically the division of NAc in these two compartments, the ventro-medial

"shell" and dorsolateral "*core"*, which have distinct structures, properties and physiological roles (see section 2.1).

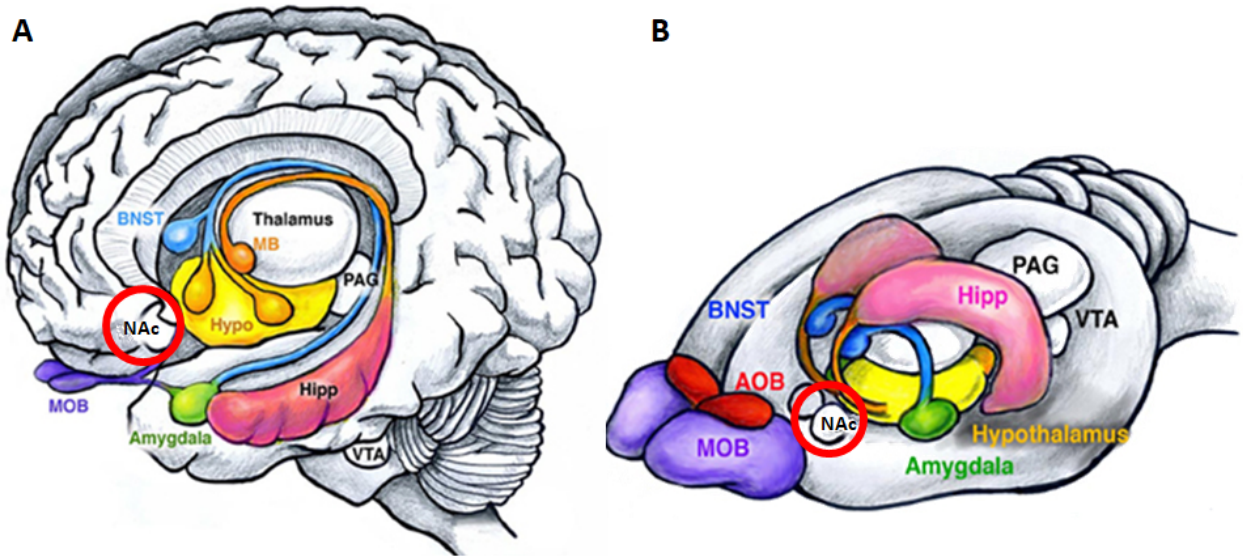

Figure 3 Main structures of the human (A) and rodent (B) limbic system.
The red circle indicate the position of the Nucleus Accumbens (NAc) in the human (A) and in the rat brain (B). Abbreviations: BNST, bed nucleus of stria terminalis; Hypo, hypothalamus; Hipp, hippocampus; MOB, olfactory bulbs; VTA, ventral tegmental area; PAG, periacqueductal gray; AOB, accessory olfactory bulbs. Adapted from Sokolowski and Corbin (2012).

As mentioned above, NAc is the main target of the mesolimbic DA system, since dense DA innervation from the VTA project into this area. Moreover, it receives inputs from a lot of cortical and non-cortical areas, including the hippocampal formation, the medial prefrontal cortex (mPFC), the insular cortex, the amygdala and the thalamus, and is characterized as a "portal" of prefrontal inputs to the striatum (Nakano, 2000). At the same time, prominent projections of the NAc reach the pallidum, the SN and the VTA (Swanson & Cwan, 1975; Williams *et al.*, 1977; Nauta *et al.*, 1978). As concerns the two distinct compartments of the nucleus, they receive afferents from and project to different areas of the brain. Tract-tracing methods have shown that both *shell* and *core* areas project to pallidal, hypothalamic and mesencephalic areas, even though *core* projections arrive more prominently to the SN while those of the *shell* to the VTA. In addition, *shell* projects also to the extended amygdala unlike *core* (Heimer *et al.*, 1991), that explains the significant role of the *shell* rather than *core* division in emotional salience processing (Tan *et al.*, 2014). In parallel, besides DA, several neuroactive substances such as glutamate (Glu), GABA, endocannabinoids

(eCBs) (AEA and 2-AG), acetylcholine (Ach), serotonin (5-HT), noradrenaline (NA), somatostatin (SOM), substance P (SP), neurotensin, Leu-enkephalin, Met-enkephalin, cholecystokinin (CCK), melatonin, histamine, galanine and neuropeptide Y, which might modulate the activity of NAc have been identified (Voorn *et al.*, 1989; Selden *et al.*, 1994; Parent *et al.*, 1995).

As explained below (see section 2.1), the NAc is considered to be an interface between limbic and motor functions (Mogenson *et al.*, 1980; Mogenson & Yang, 1991) and to be involved, primarily through DA activity, in several aspects of behavior such as motivation, reward, water and food intake, sexual behavior, emotions and cognitive functions, playing a major role in drug dependence (Wise, 1987; Alexander & Crutcher, 1990; Smith & Bolam, 1990; Di Chiara, 2002; Di Chiara *et al.*, 2004; Di Chiara & Bassareo, 2007; Anselme, 2009; Bimpisidis *et al.*, 2013; De Luca, 2014). In particular, it has been suggested that the *shell* of the NAc has more limbic functions (a more specific role in emotion and motivation) while the *core* compartment is involved mainly in the motor expression of motivated behaviors (Zahm & Brog, 1992; Bassareo & Di Chiara, 1999; Brauer *et al.*, 2000; Bimpisidis *et al.*, 2013; De Luca, 2014).

1.2 Prefrontal Cortex and medial Prefrontal Cortex

The prefrontal cortex (PFC) represents part of the frontal cortex located in its anterior part. Although the PFC shows considerable variation across species, Jersey Rose and Clinton Woolsey in the late 1950s, nominated the area of the frontal lobes of mammals of all species that has strong reciprocal projections with the mediodorsal thalamic nucleus (MD) as “prefrontal cortex” (Groenewegen *et al.*, 1990; Steketee, 2003; 2005).

In humans, PFC is divided into three main regions: the dorsolateral PFC (Broadmann areas 9 & 46), the inferior ventral PFC (areas 11, 12, 13 & 14 – also known as orbitofrontal cortex, OCF) and the medial prefrontal cortex (mPFC) which is considered part of the anterior cingulate (AC) area (Kolb & Whishaw, 2003) (Fig. 4).

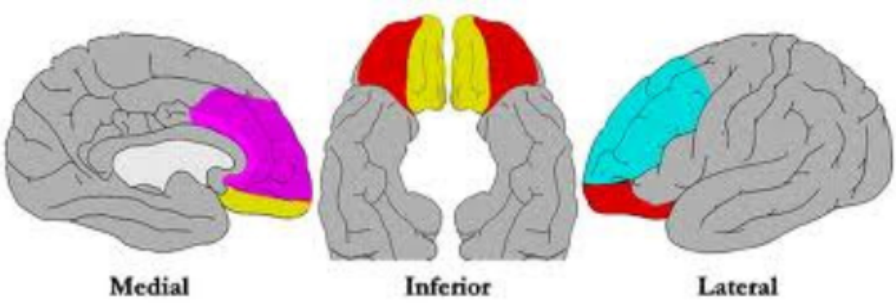

Figure 4 Schematic representation of the prefrontal cortex (PFC) and its subdivisions in the human brain.
Image from www.focus.psychiatryonline.org

Focusing on mPFC, it is located along the medial wall of the brain hemispheres anterior and dorsal to the genu of the corpus callosum (Heidbreder & Groenewegen, 2003), and in rats can be divided into infralimbic (IL), prelimbic (PL) (also called Cg3 of the anterior cingulate), dorsal and ventral anterior cingulate (Cg1 & Cg2) (Krettek & Price, 1977; Van Eden & Uylings, 1985; Groenewegen *et al.*, 1990; Ray & Price, 1992; Van Eden *et al.*, 1992; Steketee, 2003; 2005) (Fig. 5).

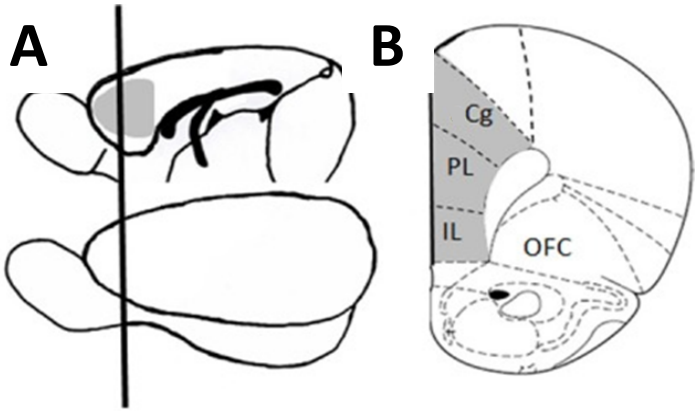

Figure 5 Schematic representation of mPFC and its subdivision in the rat brain.
The black line in panel (A) shows the approximate plane of the section for the coronal view that is shown in panel (B), which illustrates the subdivisions of mPFC (in grey). Abbreviations: Cg, anterior cingulate cortex; PL, prelimbic cortex; IL, infralimbic cortex; OFC, orbitofrontal cortex. Adapted from Bizon et al., 2012.

The mPFC receives the most prominent cortical afferents from the contralateral mPFC and the most prominent subcortical afferents from the MD, as mentioned above. Both of these afferent connections are glutamatergic, along with the inputs that arrive to mPFC from the hippocampus and

the amygdala (Conde *et al.*, 1990; Groenewegen *et al.*, 1990; Ray & Price, 1992; Kuroda *et al.*, 1993; Conde *et al.*, 1995). Furthermore, the mPFC receives NA projections from the locus coeruleus (LC) and, as already mentioned, DA projections from the VTA (Thierry *et al.*, 1973; Bjorklund *et al.*, 1978; Foote *et al.*, 1983). Besides the most prominent connections of mPFC with the MD (with a direct feedback loop) (Kuroda *et al.*, 1993), heavy innervation to subcortical areas like the NAc and the VTA has been described (Thierry *et al.*, 1983; Christie *et al.*, 1985a; Sesack *et al.*, 1989; Sesack & Pickel, 1992). Each subarea of the mPFC appears to have distinct afferent and efferent connections, which might also suggest different functional roles. For instance, regarding their efferent connections, these mPFC subareas show topographical organization: dorsal parts (Cg1-2 and dorsal PL cortices) project to the striatum and dorsal parts of the NAc (*core*), while the ventral mPFC subareas (ventral PL and IL cortices) send fibers to ventral aspects of the NAc (*shell*) (Sesack *et al.*, 1989; Berendse *et al.*, 1992). More specifically, Cg1 and Cg2 project mainly to the dorsal striatum, the caudal MD and to other neocortical and brainstem areas; PL projects to the NAc core (as mentioned above), rostral MD and basolateral amygdala among other forebrain structures, while the IL cortex efferents principally target the NAc shell but also the rostral MD, the hypothalamus, and the amygdala; in addition, pyramidal neurons send glutamatergic efferents to the VTA (Taber *et al.*, 1995). As concerns the mPFC afferents, the IL cortex receives inputs from the hippocampus and the VTA but not from the MD, while the PL cortex receives inputs from the MD**,** the hippocampus and the VTA. Notably, the PL cortex receives the densest DAergic input among the other mPFC subareas and it also has the strongest projection to the VTA. The anterior cingulate subareas (Cg1 and Cg2) receive inputs from the caudal MD thalamus, from other neocortical areas and from the VTA (Thierry *et al.*, 1973; Lindvall *et al.*, 1978; Beckstead, 1979; Sesack *et al.*, 1989; Groenewegen *et al.*, 1990) (Hurley *et al.*, 1991; Berendse *et al.*, 1992) (Tzschentke, 2001)

Overall, the DAergic innervation of the mPFC is less dense than that of the NAc or the striatum and the basal extracellular and tissue levels of DA are much lower in this area (Garris *et al.*, 1993; Ihalainen *et al.*, 1999; Tzschentke, 2001). The distribution of DA per se and of the other elements of

the DAergic system (i.e DAT) also differs along the ventrodorsal axis of the mPFC. The DAT is more densely distributed in the AC than in the PL cortex (Sesack *et al.*, 1998), while DA terminals are denser in the PL than the other subregions of the mPFC (Thierry *et al.*, 1973; Van Eden & Uylings, 1985), which innervate from VTA the deep layers of mPFC pyramidal cells (Berger *et al.*, 1991; Dreher & Burnod, 2002) and synapse also with GABAergic interneurons, modulating their function (Penit-Soria *et al.*, 1987; Pirot *et al.*, 1992).

Besides DA, Glu and NA, other neuroactive substances like GABA (mostly from interneurons), endogenous opioids and endocannabinoids (eCB), 5-HT and neurotensin are also present in the mPFC (Mansour *et al.*, 1994; Moldrich & Wenger, 2000; Tzschentke, 2001).

DA in the mPFC serves as an inhibitory neuromodulator either directly on the pyramidal neurons or indirectly by modulating the GABAergic interneuron activity, which synapse with the pyramidal neurons (Ferron *et al.*, 1984; Godbout *et al.*, 1991; Pirot *et al.*, 1992; Sesack *et al.*, 1995). Indeed, it has been shown that, either DA agonists administered locally in the VTA or chemical/electrical stimulation of the VTA induces a release of DA in the mPFC that leads to an inhibition of the mPFC pyramidal cells (Bernardi *et al.*, 1982; Ferron *et al.*, 1984; Thierry *et al.*, 1986; Peterson *et al.*, 1987; Mantz *et al.*, 1988; Yang & Mogenson, 1990; Karreman & Moghaddam, 1996). Despite this clear evidence of an inhibitory role of DA in the mPFC, the data in the literature suggest a more complex role of DA in this area and, overall in the PFC. Indeed, being a heterogeneous area, PFC has been reported to be involved in a great variety of functions-behavior, such as planning of voluntary movement, adjustment of behavior in relation to space, arousal and attention, temporal sequencing of actions, planning of forthcoming behavior based on previously acquired information, response selection and response inhibition, stress responses, mood, spatial and associative learning, memory retrieval and working memory (Tzschentke, 2001; Curtis & D'Esposito, 2003; Ramnani & Owen, 2004; Surmeier, 2007). Disturbances in the structure and function of mPFC have been reported to exist in several neuropsychiatric disorders, such as schizophrenia, depression, bipolar disorder and also addiction. It has been suggested that decreased mPFC DA activity is responsible

for the negative symptoms that occur in schizophrenia while in depression its reduced activity is correlated with disturbances in memory and attention (Blumberg *et al.*, 1999; Webster, 2001). Regarding substance abuse disorders (see next sections), it has been suggested that long-term abuse of substances might induce changes in important cognitive/executive functions such as decision making and correct judgment. Therefore, the mPFC seems to be implicated in many functions, in particular in "higher order cognitive functions" such as attentional processes, working memory and behavioral flexibility (Heidbreder & Groenewegen, 2003).

2 Dopamine and reward

2.1 The Role of NAc DA

The role of DA in the mesolimbic and mesocortical circuitries has been discussed for almost 40 years. It is generally accepted that DA in these areas plays a critical role in the response of the mesocorticolimbic system to conventional and drug reward stimuli, but the exact nature of this role is still unclear.

Ungerstedt (1971) was one of the first that linked DA to motivation by conventional rewards, showing that lesions of the nigrostriatal DA system by 6-hydroxydopamine (6-OHDA) induced severe aphagia and adipsia in rats. Afterwards, based on experiments by DA receptor antagonists, the *"anhedonia hypothesis"* was theorized, where DA is considered the substrate of the hedonic/pleasant effects of all rewards, from natural stimuli, such as sex and food, to drugs of abuse (Yokel & Wise, 1975; Wise, 1980; Wise, 1982). For a long time, this theory has remained influential among scientists, including Koob and Le Moal (1997, 2001), who speculated that drug exposure changes the "reward set point" of the organism, that leads to reduced DA transmission during withdrawal in addicted individuals and in a state of anhedonia. However, the *"anhedonia hypothesis"* was not able to explain the evidence that pharmacological blockade of DA receptors does not suppress hedonic responses estimated by the taste reactivity paradigm (Pecina *et al.*, 1997; Berridge & Robinson, 1998; Di Chiara *et al.*, 2004).

Indeed, it has been demonstrated that food reward is independent of DA transmission (Salamone *et al.*, 1997; Berridge & Robinson, 1998) and DA is also released by aversive events and stress (McCullough & Salamone, 1992; Salamone, 1994; Kalivas & Duffy, 1995). In line with this evidence, as discussed in the next section, microdialysis and *in vivo* voltammetry studies have shown that DA transmission can be activated whether in anticipation of the consumption of drug or food rewards or by presentation of reward-conditioned stimuli (Blackburn *et al.*, 1989; Phillips *et al.*, 1993; Bassareo *et al.*, 2007). Thus, Berridge and colleagues proposed another theory, in which

the DA play a pivotal role, attributing incentive salience to conditioned stimuli, rather than mediating the hedonic properties of rewards. In this theory, they distinguish two components of rewarding stimuli: *"liking"*, "the unconscious hedonic evaluation of a stimulus", and *"wanting",* "the underlying core process that instigates goal-directed behavior, attraction to the stimulus, and consumption of the goal object" (Berridge & Robinson, 1998). Consistent with this theory, DA participates in *"wanting"* and not in *"liking"* (Berridge *et al.*, 1989; Robinson & Berridge, 1993; Berridge, 1996; Berridge & Robinson, 1998). Moreover, according to Salamone and colleagues *"wanting"* can be distinguished into two separate components: *"directional"* and *"activational"*, where directional aspects can be appetitive in nature, while activational aspects include "the initiation and sustaining of instrumental actions or the tendency to work for food"; and "the tendency to work for motivational stimulus, and overcome response constraints" (Salamone & Correa, 2002). Consistent with these views, DA in the NAc is involved in instrumental action and in tasks with a high degree of "work-related response costs" (Salamone *et al.*, 2001; Salamone & Correa, 2002).

However, it has been discussed by Di Chiara and colleagues (2002) that DA, acting during the preparatory phase of feeding behavior, is the substrate of the appetitive properties of food-conditioned incentive stimuli, even though it is not the substrate of the hedonia associated to food consumption. This kind of hedonia, called *"incentive arousal"* (Di Chiara, 2002), characterized by euphoria and behavioral arousal, is also mimicked by psychostimulants like cocaine and amphetamine and it might be related to stimulation of DA transmission in the NAc shell (Di Chiara, 2002). On this basis, two kinds of hedonia have been distinguished: a *preparatory* or *state-hedonia DA-dependent*, associated with the incentive arousal state of food search and approach, and a *consummatory* or *stimulus-bound hedonia DA-independent*, such as that associated with food taste. These two kinds of hedonia correspond to distinct phases of motivated behavior, *appetitive/preparatory* and *consummatory,* due to distinct stimuli, distal (visual, acoustic, olfactory) and proximal (tactile, proprioceptive, taste) respectively. Moreover, they are associated with distinct

behavioral response patterns of search; exploration and approach independent of the specific reward (*appetitive behavior*), while reward specific motor responses is associated with *consummatory* responses (Di Chiara, 2002).

Another popular theory of the role of DA is the *"reward-prediction error hypothesis"* of Schultz and colleagues, based on evidence obtained from extracellular recording of VTA DA neurons in the monkey. These studies showed that when a monkey receives a reward (apple), DA neurons increase their firing rate but not when the light that signals the reward is turned on. After training, DA neurons increase their activity when the animal sees the light (conditioning stimulus) and not when it receives the actual reward (apple). However, if after the light the reward is not presented, DA neurons decrease their firing activity. Thus, these studies indicate that DA neurons are sensitive to both like (reward) and dislike (absence of an ''expected'' reward). The increase in firing observed after learning upon turning on the light suggests that the ''reward value'' is now attributed (by the animal) to the light that signals the forthcoming ''real'' reward. Therefore, according to the *"reward-prediction error hypothesis"*, DA codes for the occurrence of an unpredicted reward and for the omission of a predicted one (Schultz *et al.*, 1993; Schultz *et al.*, 1997; Schultz, 2007), and DA released as a result of an error in the prediction of reward, might act as a teaching signal in associative and learning functions.

Nevertheless, this hypothesis does not necessarily apply to the action of DA on its receptive neurons. In fact, although this theory can account for the phasic activity of DA neurons in response to rewards and their conditioned stimuli, the temporal and spatial properties of DA mediated signals are quite different from those of a fast transmitter acting via ionotropic receptors (Di Chiara & Bassareo, 2007). Thus, from the time DA is released to the time it reaches and activates its receptors, the phasic response of DA neurons is transformed into a tonic influence on a widely distributed range of DA sensitive neural elements. Therefore, the computational properties that are attributed to DA by the Schultz theory do not fit with those of a neuromodulator like DA but with those of a fast, ionotropic excitatory transmitter acting like glutamate instead. As a matter of fact,

recent evidence has shown that glutamate is a co-transmitter of DA in mesolimbic neurons (Lavin *et al.*, 2005).

Finally, an *"anticipatory dynamics model"* (and its *"uncertainty processing theory of motivation"* revised form) for the role of DA in motivation and reward has recently been proposed. According to this theory, phasic DA release is induced by events that comprise "uncertainty with respect to the stimulus arrival". DA in limbic and cortical areas facilitates anticipation, which is necessary for the organism to deal with uncertainty. The NAc (the core compartment in particular) is the region where signals from other corticolimbic areas regarding anticipation converge. Due to its connections with motor areas, it can fast reduce uncertainty by adopting appropriate motor responses to environmental challenges. This theory also includes another important role of DA: the facilitation of task related attention which serves for motivational specificity (towards a particular reward) to take place (Anselme, 2009; 2010).

2.2 The role of mPFC DA

Based on a nonspecific function, DA in the mPFC might play the same role in all the processes mentioned already for the NAc (Anselme, 2009). Today it is widely accepted that DA in the mPFC has a modulatory role and changes of DA transmission in this area do not produce a clear behavioral output. Indeed, although much evidence shows an inhibitory role of DA in the mPFC, the literature suggest a more complex role of DA in this area.

Besides the fact that the mesocortical DA system shows a higher responsiveness to stress, both acute and chronic, compared to the other DA pathways (Thierry *et al.*, 1976; Blanc *et al.*, 1980; Deutch *et al.*, 1985; Jedema & Moghaddam, 1994; Cuadra *et al.*, 1999), DA in the mPFC is also increased by other salient stimuli, such as novelty, food and conditioned to food stimuli (Feenstra & Botterblom, 1996; Bassareo & Di Chiara, 1997; Bassareo *et al.*, 2002; 2007). Similarly to DA in the NAc, mPFC DA seems to play a role in reward processes, even though its role is not yet clear. Indeed, it has been demonstrated that an increase of DA in the mPFC doesn't seem to be induced by the consumption of a reward *per se*, but seems to be associated with "novelty, excitement, expectancy, operant and classical conditioned responses" and "to code for generic motivational value" (Bassareo & Di Chiara, 1997; Tzschentke, 2001; Bassareo *et al.*, 2002). As suggested by Di Chiara and colleagues (1997) , differently from DA in the NAc (shell in particular), DA in the mPFC is not (mainly) related to the ability of some drugs to induce abusive behaviors. This view is supported by evidence that drugs that increase DA (or NA) preferentially in the mPFC are not abused by humans or they have low abuse potential (Tanda *et al.*, 1994; Bassareo *et al.*, 1996).

As in the case of NAc DA in reward processes, several theories have tried to explain the exact role and function of mesocortical DAergic projections. Briefly, Cohen and collegues (Servan-Schreiber *et al.*, 1990; Cohen & Servan-Schreiber, 1992; Braver *et al.*, 1999)proposed a model of mPFC DA function, in which DA serves " to increase signal-to-noise ratio to facilitate inputs, both excitatory and inhibitory". Thus, the role of DA is to regulate access of information to active memory (i.e. to

provide the updating of useful, task-relevant information), but also as a protection mechanism from irrelevant information interference. These effects occur in the synapses through "transient potentiation of both excitatory afferent and local inhibitory input". Consistent with this theory, cognitive deficits in disorders characterized by a cortical *hypodopaminergic state*, as with schizophrenia, result from a failure in "regulating" incoming information, from an increase in "noise". This "noise" results in "disturbances in updating and maintaining important and context relevant information in the active working memory, characteristics of vital importance for cognitive control" (Braver *et al.*, 1999).

In order to correlate reward processes and higher order cognitive functions like working memory and decision making, Durstewitz and colleagues proposed a different model (Durstewitz *et al.*, 1999; Durstewitz & Seamans, 2002) where mPFC DA serves to increase "the robustness of representations encoding goal-related information" by reducing the impact of intervening stimuli. Nevertheless, limited behavioral data exist regarding the role of DA in each subarea of the mPFC. One of these studies (Hitchcott *et al.*, 2007), showed that DA in the ventral parts of the mPFC (i.e. the IL cortex) is necessary for adjustment of behavior in relation to outcome expectancy. The authors discussed the possibility that DA in the IL part of the mPFC played an important role in attentional processes, and this in turn had major impact in the behavioral manifestations observed during their experiments (Hitchcott *et al.*, 2007). In another recent study, Naneix and colleagues (2009) made specific DA lesions in IL and PL cortices of adult rats to understand their specific role. Using behavioral paradigms they showed that DA dennervation within each area is not necessary for the acquisition of an instrumental response to receive a reward. However, animals with DA lesions in the PL cortex showed deficits to adapt their instrumental behavior to contingency changes between behavior expression and reward delivery. Thus, DA in the mPFC might also be related to the role of DA in behavioral flexibility (Naneix *et al.*, 2009). Further studies are necessary to clarify the role of DA in each subregion of the mPFC on several aspects of behavior.

In conclusion, although these data support the hypothesis of a non-specific role of mPFC DA, it is suggested that DA can serve in all three major functions where it is supposed to be involved (motor, reward, cognitive) to help the organism to "learn about, predict and respond appropriately to events that lead to reward" (Braver *et al.*, 1999).

2.3 Food-reward vs. drug-reward: the differential role of DA

Regarding the role of DA in reward processes and its meaning, it is important to underlie the different pattern of DA activation in response to natural rewarding stimuli, such as food and sex, and to drug of abuse. Indeed, in these years, several studies have tried to understand, other than the role of DA transmission in these processes, how the drugs of abuse influence the DA systems and how these effects on mesocorticolimbic DA transmission are implicated in the ability of drugs to elicit addiction (Bassareo & Di Chiara, 1999; Bassareo *et al.*, 2002; Di Chiara *et al.*, 2004; Kalivas & Volkow, 2005; Goldstein & Volkow, 2011; De Luca, 2014).

2.3.1 DA response to food reward

Considering the DA response to natural rewarding stimuli, such as taste stimuli, Di Chiara and colleagues (Bassareo & Di Chiara, 1997; 1999; Bassareo *et al.*, 2002), through *in vivo* brain microdialysis experiments, showed that exposure to unfamiliar taste stimuli increases DA in the NAc core and in the mPFC without delay and independent of positive (appetitive) or negative (aversive) valence. Differently, aversive and appetitive stimuli differentially affect DA of the NAc shell. Indeed, whether NAc shell DA rapidly increases in response to an appetitive unfamiliar taste (i.e. sweet chocolate, Fonzies®) is unaffected by long-lasting (10 min application of aversive tastes (quinine; saturated NaCl solutions) (Bassareo *et al.*, 2002). On the other hand, a shorter-lasting (5 min, 1 ml) application of aversive stimuli, either gustatory (quinine) or olfactory (red fox urine), elicits a delayed and transitory activation of DA transmission in the NAc shell (Bassareo *et al.*, 2002). Thus, this observation suggests that, although necessary "positive valence is not sufficient for short-latency activation of DA transmission in the NAc shell by motivational stimuli" (Bassareo *et al.*, 2002). Hence, this might explain why intraoral 20% sucrose solution, despite being no less effective in eliciting hedonic taste reactions than sweet chocolate, fails to stimulate DA transmission in the NAc shell (Bassareo & Di Chiara, 1997; 1999; Bassareo *et al.*, 2002. Therefore, besides the

positive valence of stimuli, relative novelty is also necessary for stimulation of NAc shell DA transmission. This role of novelty in the NAc shell DA response has been supported by the evidence that unfamiliar palatable food stimulates DA release in the NAc shell, NAc core, and in the mPFC but only in the NAc shell does this effect undergo habituation following repeated exposure (Table 1) {Bassareo, 1997 #268; De Luca, 2014). Moreover, this habituation of DA response in the NAc shell to natural rewards is taste specific, and it is reversed by food deprivation and modified by the presentation of cues associated with the stimulus (Bassareo & Di Chiara, 1997). Therefore, this property has been related to a role of DA in associative learning mediated by food reward. According to this view, DA release in the NAc shell by food taste would be necessary to consolidate the memory trace of taste stimulus, in turn, allowing association with its post-ingestive consequences, as observed in a taste aversion paradigm(Fenu *et al.*, 2001; Bassareo *et al.*, 2007). With novelty being a prerequisite of stimulation of DA release in the NAc shell but not of behavioral hedonic reactions, release of DA in the NAc shell is likely to be a consequence rather than the cause of the appetitive properties of taste stimuli, according to the idea that taste-hedonia does not depend on DA (Berridge & Robinson, 1998).

This and other evidence supports the *"incentive learning"* hypothesis (Di Chiara, 1998), in which DA, specifically in the NAc shell, "is involved in the acquisition of incentive-motivational properties by stimuli conditioned to rewards" (Di Chiara *et al.*, 2004; Di Chiara & Bassareo, 2007). Moreover, the influence of NAc shell DA on incentive learning is retrograde, affecting the association of stimuli that precede or are coincident with reward. On the other hand, the properties of DA transmission in the NAc core and in the mPFC are more consistent with a role in the expression of motivation, in agreement with the notion of the NAc as an interface between motivation and action (Mogenson & Yang, 1991).

Thus, this evidence shows a different role, as well as meaning, of DA in the mPFC and in the NAc in the salience attribution processes of rewarding stimuli. In order to better understand this reciprocal relationship between mPFC and NAc DA responsiveness to rewading stimuli, De Luca

and colleagues studied the DA response to unfamiliar appetitive stimuli under different pharmacological and anatomical manipulation (Table 1) (De Luca *et al.*, 2011; Bimpisidis *et al.*, 2013; De Luca, 2014). In one of these studies, following the paradigm used by Bassareo et al. (2002), it was demonstrated that opiate sensitization (morphine) induces differential adaptive changes to the response of DA transmission with repeated exposure to natural rewarding stimuli. In particular, morphine sensitization was able to induce an increased and delayed DA response in the NAc core to the first chocolate exposure, while an immediate increase of NAc core DA was observed in pre-exposed rats. Moreover, this study showed an opposite change of DA transmission in the NAc shell and mPFC to repeated chocolate exposure: indeed, the unexpected appearance of habituation in mPFC DA responsiveness to taste stimuli was accompanied by a loss of habituation in the NAc shell of morphine sensitized animals (De Luca *et al.*, 2011). Meanwhile, no changes in behavioral taste reactivity have been observed, although sensitization to morphine is associated with long-term changes in mesocorticolimbic DA responsiveness to taste stimuli. The latter evidence supports the hypothesis that taste-hedonia does not depend on DA (Berridge & Robinson, 1998). Thus, these long term changes in DA response of different terminal areas were proposed to have implications in motivational processes and probably to result in increased incentive arousal and learning (De Luca *et al.*, 2011). Indeed, since the habituation of mPFC DA response to chocolate releases NAc shell DA from inhibition, abolishing the single-trial habituation of DA might facilitate repeated approaches toward a motivational stimulus (De Luca *et al.*, 2011). Moreover, this lack of NAc DA habituation may be considered per se as a marker of drug dependence and its liability (De Luca, 2014).

According to this hypothesis, several studies showed an inhibitory role of mPFC DA on DA subcortical areas in reward and motivational processes. Indeed, it has been demonstrated that increasing DA levels in the mPFC corresponds to a decrease of DA in the NAc, while DA antagonists have the opposite effect (Louilot *et al.*, 1989; Jaskiw *et al.*, 1991; Vezina *et al.*, 1991; Kolachana *et al.*, 1995; Lacroix *et al.*, 2000). This top-down control of mPFC on subcortical areas

has been confirmed by results obtained studying the effect of mPFC 6-OHDA lesions on DA responsiveness to repeated chocolate exposure (Bimpisidis *et al.*, 2013). It has been shown that 6-OHDA bilateral infusions in the mPFC were able to change the responsiveness of NAc DA to gustatory stimuli administered by an intraoral catheter (Table 1). In particular, while sham and mPFC lesioned rats showed a similar DA increase in the NAc shell after the first exposure to chocolate, lesions of mPFC DA terminals abolished the habituation of NAc shell DA response to sweet taste. Similar to morphine sensitization, lesions of mPFC DA terminals produced an elevated, delayed, and prolonged increase of DA in the NAc core in response to the first exposure to appetitive taste stimulus, while differently, did not affect NAc core DA responsiveness of pre-exposure animals to chocolate. Meanwhile, DA terminal lesions, were ineffective neither on hedonic taste score nor on motor activity (Bimpisidis *et al.*, 2013). These observations might suggest that mPFC DA inhibitory control of DA responsiveness of subcortical striatal areas is different depending on the ventral striatum sub-region studied. In fact, as described in the previous section (section 1.2) different sub-regions within the mPFC (i.e PL, IL) have different projections to different compartments of the NAc, leading to a different role and DA response to discrete stimuli and conditions (Di Chiara *et al.*, 2004; Di Chiara & Bassareo, 2007; Aragona *et al.*, 2009; Corbit & Balleine, 2011; Cacciapaglia *et al.*, 2012).

Altogether these studies, in agreement with the literature (described above), confirm the crucial role of mesocorticolimbic DA pathways in the evaluation of motivational value of natural rewarding stimuli, and showed the inhibitory role of mPFC DA on subcortical areas involved in these processes. Moreover, the results described above, might help to explain, the reason why mesocorticolimbic DA dysregulations have been identified as critical features in different neuropsychiatric disorders, such as schizophrenia and in drug addiction (Kalivas & Volkow, 2005; Jensen *et al.*, 2008; Goldstein & Volkow, 2011; Deserno *et al.*, 2013; De Luca, 2014).

stimulus	Nac shell DA	Nac core DA	mPFC DA	references
CHOCOLATE 1st exposure	↑	↑	↑	Bassareo et al., 2002
CHOCOLATE 2nd exposure	HABITUATION	↑	↑	
CHOCOLATE 1st exposure after MORPHINE SENSITIZATION	↑	↑ (Higher and delayed)	↑	De Luca et al., 2011
CHOCOLATE 2nd exposure after MORPHINE SENSITIZATION	↑	↑ (Higher)	HABITUATION	
CHOCOLATE 1st exposure in mPFC 6-OHDA LESIONED RATS	↑	↑ (Higher and delayed)	✖	Bimpisidis et al., 2013
CHOCOLATE 2nd exposure in mPFC 6-OHDA LESIONED RATS	↑	↑	✖	

Table 1 Schematic representation of results of DA response to unfamiliar appetitive taste stimulus

2.3.2 DA response to drug of abuse: involvement in addiction

All the drugs of abuse, independent of their pharmacological mechanisms, increase DA preferentially in the NAc shell (Imperato & Di Chiara, 1986; Di Chiara & Imperato, 1988a; b; Carboni *et al.*, 1989). Moreover, differently from what has been observed with natural rewarding stimuli, habituation of NAc shell DA response is not present after repeated exposure to drugs of abuse (i.e., nicotine, opiates, psychostimulants, cannabinoids) (Pontieri *et al.*, 1995; Pontieri *et al.*, 1996; Tanda *et al.*, 1997).

Consistent with this property of addictive drugs on NAc shell DA, as well with the role of NAc DA in motivational processes, is the idea that drug addiction is a condition of disturbed motivation related to drug-induced stimulation of NAc DA (Di Chiara, 1998). Based on this idea, Di Chiara proposed the theory of an *"abnormal Pavlovian incentive learning hypothesis of drug addiction"* to correlate the disturbance of motivation to the DA stimulant action in the NAc shell of addictive drugs (Di Chiara, 1998; 2002). In this view, the motivational abnormality of drug addiction is considered as "the result of the excessive strengthening of Pavlovian stimulus-drug associations due to repeated drug-induced stimulation of DA transmission in the NAc". These abnormal associations would lead to the lack of adaptive proprieties of DA responsiveness observed with natural rewarding stimuli (i.e food), such as single-trial habituation and inhibition by exposure to conditioned stimuli (Bassareo & Di Chiara, 1997; 1999; Bassareo *et al.*, 2002; 2007; Bassareo *et al.*, 2011). As a consequence, the excessive activation of these processes by drugs of abuse (and by their associated stimuli), that induce a dysadaptive stimulation of DA transmission in the NAc shell, might lead to compulsively focused motivation on drugs and drug-related stimuli typical of drug addiction (Volkow *et al.*, 2003; Di Chiara *et al.*, 2004; Volkow & Morales, 2015).

In this subcortical dysfunction, which may occur in different stages of drug addiction, the mPFC seems to play a crucial role. As matter of fact, several studies showed the direct involvement of mPFC, in particular mPFC DA in drug addiction, drug seeking, craving and relapse, which are

related to drugs taken either by humans or animals (Schenk *et al.*, 1991; Weissenborn *et al.*, 1997; Bolla *et al.*, 2003; Kalivas & Volkow, 2005). In accordance with the evidence, as described above, the habituation usually present in the NAc shell is ruled by intact DA transmission within the mPFC (Bimpisidis *et al.*, 2013; De Luca, 2014). In this respect, the appearance of habituation in the mPFC observed in sensitized to morphine animals (De Luca *et al.*, 2011) could be considered as a marker of mPFC dysfunction in its ability to inhibit crucial subcortical functions. Thus, mPFC dysfunction may lead to excessive motivation for inappropriate actions originating from a clear loss of impulse control (De Luca, 2014).

Consistent with dysregulation of the DA system in drug addiction, it has been demonstrated that repeated exposure to different drugs of abuse is associated with down-regulation of D2Rs in the striatum (Thanos *et al.*, 2001; Volkow *et al.*, 2001; Nader *et al.*, 2006). In particular, studies in rodents and non-human primates have shown reduced levels of D2Rs in the striatum, including in the NAc, upon chronic drug exposures (Everitt *et al.*, 2008). In rodents, low levels of D2Rs in this brain area are associated with impulsivity and compulsive administration of cocaine (Everitt *et al.*, 2008) since low levels of D2Rs in the striatum result in reduced DA inhibition of the indirect pathway. Indeed, D2Rs have a high affinity for DA, so they are stimulated by the relatively low DA levels achieved through tonic DA cell firing. Thus, a reduction of D2R-mediated DA inhibition of the indirect pathway may lead to reduced thalamo-cortical stimulation and consequently reduced activity in PFC brain regions (Volkow & Morales, 2015). Clinical studies have confirmed that striatal D2R reduction (dorsal and ventral) in drug abusers is associated with decreased activity in the PFC, which is necessary for self-control and for processing salience attribution, and its disruption is associated with impulsive and compulsive behaviors (Volkow & Fowler, 2000).

Glutamatergic pathways seem to be involved in this reduction of PFC activity, as well in DA dysregulation observed in drug addiction. Since the main sources of glutamatergic inputs to the VTA arise from the PFC (Thierry *et al.*, 1983; Christie *et al.*, 1985b; Sesack & Pickel, 1992; Taber *et al.*, 1995; Smith *et al.*, 1996; Carr & Sesack, 2000), glutamate is thought to regulate the

spontaneous activity of VTA DA cells in vivo (Grenhoff *et al.*, 1988; Svensson & Tung, 1989; Charlety *et al.*, 1991) through activation of ionotropic (Chergui *et al.*, 1993; Wang & French, 1993a; b; 1995) and metabotropic receptors (Mercuri *et al.*, 1993; Shen & Johnson, 1997). Growing evidence suggests that glutamatergic transmission in the VTA plays an important role in the actions of many drugs of abuse and addiction (Kalivas & Stewart, 1991; Pulvirenti & Diana, 2001; Kauer, 2004). Besides glutamatergic control, VTA DA cells possess an additional self-regulatory mechanism that involves the endocannabinoid (eCB) system, through retrograde messengers (Piomelli, 2003). In fact, VTA DA cells release eCBs in an activity-dependent manner, which depresses glutamatergic afferents on mesolimbic DA cells (Melis *et al.*, 2004b) and ultimately their own firing activity and pattern (Melis *et al.*, 2004a).

Both in the human condition as well as in animal models of drug addiction, mesolimbic DA transmission appears to be drastically reduced in its activity, in particular after chronic drug intake and withdrawal, partly due to the reduction of cortical functions, as well as D2R down-regulation in the striatum (Melis *et al.*, 2005). Indeed, as imaging studies have shown, if acute drug administration increases DA neurotransmission, chronic exposure to the drug results in a marked decrease in DA activity that persist months after detoxification and which is associated with the dysregulation of frontal brain regions (Volkow *et al.*, 2003)(Volkow et al., 2002, 2003). In particular, VTA DA neurons seem to be able to adapt to the presence of drugs of abuse without necessarily changing their own responsiveness to the drug itself. However, as observed in DA terminal areas, VTA DA neurons undergo adaptive changes that might be unmasked during withdrawal from addictive drugs (Melis *et al.*, 2005). For instance, morphine withdrawal causes a profound reduction of firing rate and bursting activity of VTA DA cells (Diana *et al.*, 1995), which persists long after the behavioral signs of withdrawal have ceased (Diana *et al.*, 1999). Similarly effects on VTA DA neuronal activity have been observed during ethanol(Diana *et al.*, 1993; Diana *et al.*, 1996; Pulvirenti & Diana, 2001) and Δ9-THC withdrawal (Diana *et al.*, 1998). Interestingly, regarding cannabinoid (CB) withdrawal, a reduction in VTA DA neuronal function is also observed

when somatic signs of withdrawal are not detectable (Diana *et al.*, 1998). Meanwhile, changes in VTA DA cell morphology after repeated addictive drugs exposure have been also reported. In fact, it has been shown that morphine-dependent animals undergoing acute withdrawal experience a dramatic reduction (about 25%) in the size of VTA DA cells (Sklair-Tavron *et al.*, 1996). Similarly, by using confocal microscopy, it has been demonstrated that during acute withdrawal from chronic morphine the morphological features (e.g., circularity, area, and perimeter) of VTA DA neurons are profoundly reduced (Spiga *et al.*, 2003). VTA DA cells are reduced in their size also during acute withdrawal from chronic ethanol (Diana *et al.*, 2003), and from Δ^9-THC (Spiga *et al.*, 2003; Spiga *et al.*, 2010). This reduction in area, perimeter, and circularity, might represent an additional plastic change leading to the neurons being more excitable, to overcome a *hypodopaminergic state*. Thus, these changes in the structural and functional properties of neurons, drug-induced within the mesolimbic DA system, might be implicated in long-term behavioral changes observed during drug addiction (Nestler, 1996; 2001; 2004). Collectively, such evidence supports the concept of a *"hypodopaminergic state"* at both a presynaptic and postsynaptic level, which might lead to an impoverishment of already defective DA transmission (Melis *et al.*, 2005).

Considering this and other evidence from imaging studies, Volkow and colleagues (2003) proposed a model (Fig. 6) in which, "the saliency value of the drug of abuse and its associated cues in addicted subjects is enhanced in the reward and motivation/drive circuits, while that of other reinforcers is markedly decreased". According to this theory, during addiction, the enhanced value of the drug in reward, motivation, and memory circuits overcomes the inhibitory control exerted by the PFC, thereby favoring a positive-feedback loop initiated by the consumption of the drug and protracted by the enhanced activation of motivation/drive and memory circuits (Fig.6). This enhanced saliency value of addictive compounds is initiated partly by the higher intrinsic DA rewarding properties of drugs of abuse rather than those of natural reinforcers (Wise, 2002; Di Chiara *et al.*, 2004). As mentioned above, the lack of habituation of DA response to drugs of abuse might represent another important cause of enhanced saliency (Bassareo & Di Chiara, 1997; 1999;

Bassareo *et al.*, 2002). Thus, it has been postulated that the high reward value of drugs leads to a resetting of reward thresholds, which then results in decreased sensitivity to the reinforcing properties of naturally occurring stimuli (Koob & Le Moal, 2001).

In conclusion, although addiction is a disorder that involves complex interactions between different biological and environmental variables (Leshner, 1997), altogether the evidence confirms the predominance of the mesocorticolimbic DA system and its disregulation in all phases of drug addiction, even though many aspects of this neuropsychatric disorder are still unknown.

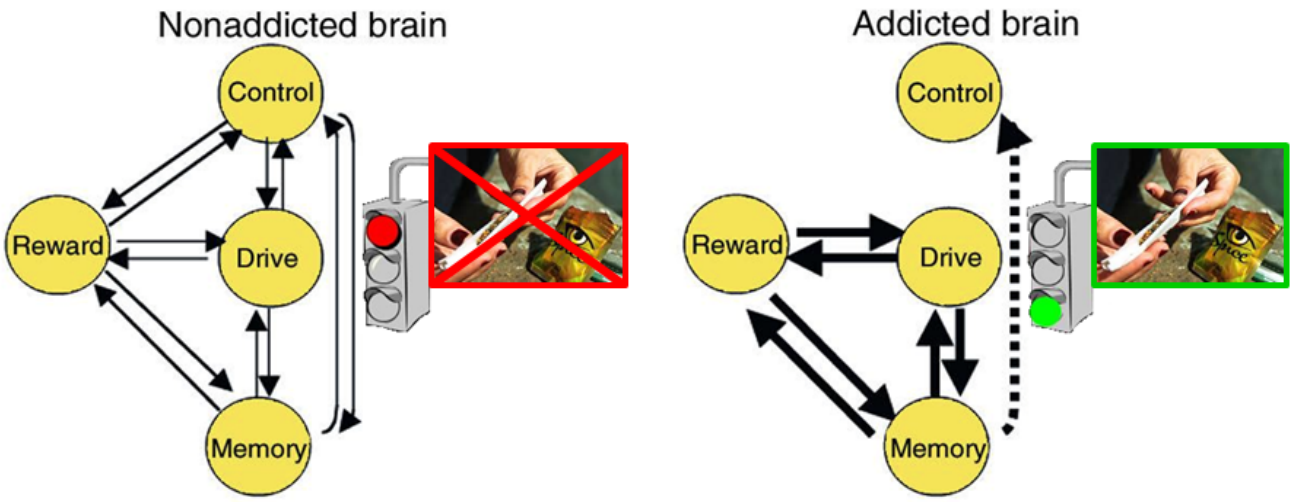

Figure 6 Model proposing a network of four circuits involved with addiction: reward, motivation/drive, memory, and control.
Adapted from Volkow et al. (2003).

3 The endocannabinoid system

The endocannabinoid (eCB) is a receptor-based signaling system composed of three core elements: cannabinoid receptors (CBRs), endogenous ligands, and several proteins responsible for their synthesis and degradation (Maldonado *et al.*, 2006; Lu & Mackie, 2016) (Fig. 7). Two subtypes of CBRs, CB1 and CB2, have been well characterized and cloned, even though it has recently been shown that eCBs also act on other receptors, such as on transient receptor potential vanilloid 1 (TRPV1) and peroxisome proliferator activated receptors (PPARs). CB1Rs represent one of the most abundant G-protein-coupled receptors in the CNS (Fig. 8), particularly in the cortex, basal ganglia, hippocampus, and cerebellum (Mackie, 2005), and mostly on axon terminals and preterminal axon segments (Nyiri *et al.*, 2005). Although CB1Rs have been detected on many neurons, CB1R expression in glial elements (Rodriguez *et al.*, 2001; Molina-Holgado *et al.*, 2002; Han *et al.*, 2012), as well as in perhipheral tissues (Herkenham *et al.*, 1991) has also been reported. The CB2Rs are mainly located in peripheral and brain immune cells(Galiegue *et al.*, 1995; Nunez *et al.*, 2004), but recently have also been identified in neurons (brainstem, cortex and cerebellum neurons) (Van Sickle *et al.*, 2005) and in glial cells (see section 4) (Gong *et al.*, 2006; Xi *et al.*, 2011), although they are less widely expressed in the CNS compared to CB1Rs, as well as showing much lower levels of expression (Atwood & Mackie, 2010).

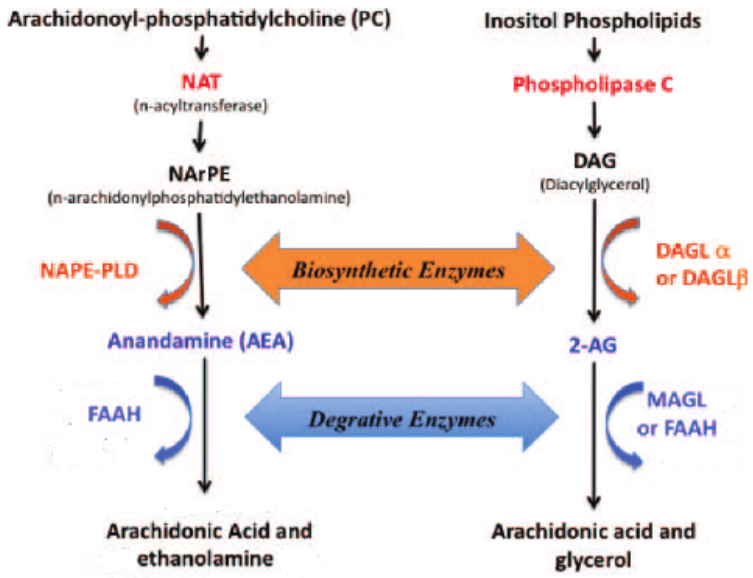

Figure 7 Simplified representation of eCBs synthesis and degradation pathway.
Abbreviations:NAT, arylamine N-acetyltransferase; NAPE-PLD, N-arachidonyl-phosphatidylethanolamine phospholipase- D; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; 2-AG, 2-arachidonoyl glycerol; DAGL, diacylglycerol-lipase. Adapted from Scotchie et al. (2014)

Regarding the endogenous ligands, anandamide (AEA) (a partial agonist of CB1Rs) and 2-arachidonoylglycerol (2-AG) (a full agonist of both CB1Rs and CB2Rs) have been isolated from brain tissue and characterized (Devane *et al.*, 1992; Mechoulam *et al.*, 1995). Although other eCBs have been identified, AEA and 2-AG remain the most well-studied and characterized. These endogenous ligands are synthesized by neurons "on-demand" and, acting as retrograde messengers in the CNS (Wilson & Nicoll, 2002) they behave as neuromodulators in many physiological processes. Accordingly, eCBs released from postsynaptic neurons upon depolarization activate presynaptic CB1Rs, resulting in inhibition of the release of both excitatory and inhibitory neurotransmitters. Following release, AEA and 2-AG signaling is quickly terminated through cellular reuptake and hydrolysis by the fatty acid amide hydrolase (FAAH) enzyme and monoacylglycerol lipase (MAGL), respectively (FAAH can also hydrolyze 2-AG) (Fig.7).

Several studies have demonstrated that eCBs exert retrograde control after synaptic activation of group I metabotropic glutamate receptors (mGlutR1) (Jung *et al.*, 2005) and D2R, supporting the view that the eCB system represents a new candidate for the control of drug rewarding properties (Maldonado *et al.*, 2006) (see next section). Indeed, CB1Rs, as described in Fig. 8, are widely

expressed in the brain reward circuitries and participate in the addictive properties induced by different drugs of abuse. As discussed above, the DA neurons of the mesocorticolimbic pathway are controlled by excitatory and inhibitory inputs that are modulated by CB1Rs. Thus, eCBs can be released in the NAc, following depolarization (Robbe *et al.*, 2002), and from DA neurons in the VTA (Melis *et al.*, 2004b; Riegel & Lupica, 2004), modulating glutamatergic and GABAergic afferents by acting as retrograde messengers on CB1Rs. The presence of CB1Rs in other structures related to motivation and reward, such as the basolateral amygdala and the hippocampus, also contributes to this function of the eCB system (Katona *et al.*, 2001), which also participate in synaptic plasticity in the mesolimbic system. In fact, the long-term depression (LTD) of NAc glutamatergic synapses due to stimulation of prelimbic (PL) cortex afferents is mediated by eCBs release and presynaptic CB1Rs (Gerdeman *et al.*, 2002; Robbe *et al.*, 2002). Endocannabinoids also produce LTD of inhibitory synaptic transmission and are implicated in long-term potentiation (LTP) of synaptic transmission (Chevaleyre & Castillo, 2004), which contributes to the plasticity mechanisms reported in the learning processes related to addictive behavior.

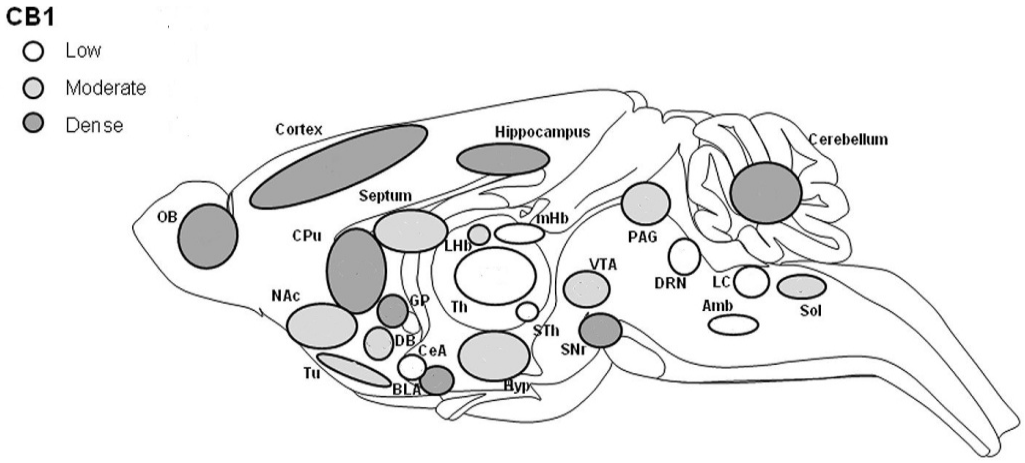

Figure 8 Schematic representation of the distribution (indicated by circle shapes) and expression (indicated by grayscale) of CB1R in the rat brain.

Abbreviations: Amb, ambiguous nucleus; BLA, basolateral amygdala; CeA, central amygdala; CPu, caudate putamen; DB, diagonal band; DRN, dorsal raphe nucleus; GP, globus pallidus; Hyp, hypothalamus; LC, locus coeruleus; LHb, lateral habenular nucleus; mHb, medial habenular nucleus; NAc, nucleus accumbens; OB, olfactory bulb; PAG, periaqueductal gray; SNr, substantia nigra pars reticulate; Sol, nucleus of the solitary tract; STh, subthalamic nucleus (ventral thalamus); Th, dorsal thalamus; Tu, olfactory tubercle; VTA, ventral tegmental area. Adapted from Beford, 2015.

3.1 The endocannabinoid system and reward: involvement in addiction

The eCB system is certainly the primary site of action for the rewarding and pharmacological responses induced by CBs (Ledent *et al.*, 1999; Lichtman & Martin, 2005). However, this system plays an overall modulatory effect on the reward circuitry and also participates in the rewarding and addictive properties of all drugs of abuse (Gardner, 2005; Maldonado *et al.*, 2006).

As mentioned above (Fig. 9), CB1Rs are present in the different regions of the brain reward circuitry, including the VTA, the NAc, and in their projecting areas, such as the PFC, the central amygdala and the hippocampus (Gardner, 2005). The eCBs, acting as a retrograde messenger, modulate the glutamatergic excitatory and GABAergic inhibitory synaptic inputs into the VTA and the glutamate transmission in the NAc (Maldonado *et al.*, 2006). Thus, the activation of CB1Rs present on axon terminals of GABAergic neurons in the VTA would inhibit GABA transmission, removing this inhibitory input on DA neurons (Riegel & Lupica, 2004; Lupica & Riegel, 2005). Similarly, the activation of CB1Rs, is able to modulate glutamate synaptic transmission from neurons of the PFC in the VTA and NAc (Robbe *et al.*, 2001; Melis *et al.*, 2004b). The resulting effect on the modulation of VTA DA activity by eCBs would depend on the functional balance between these inhibitory GABAergic and excitatory glutamatergic inputs, which are both inhibited by eCBs under different physiological conditions. Therefore, the modulatory role of the eCB system on the primary rewarding effects of drugs of abuse might depend on eCBs release in the VTA (Lupica & Riegel, 2005).

The eCB system seems to be involved in the rewarding effects of CBs, opioids, nicotine and alcohol, since these drugs increase DA neuron firing rates through the release of eCBs in the VTA. However, chronic treatment with Δ^9-THC, nicotine or alcohol increases eCBs levels in the limbic areas, while chronic cocaine reduces 2-AG content in these brain structures, indicating that different classes of drugs of abuse could regulate eCB transmission differently (Gonzalez *et al.*, 2002). The CB1Rs are widely present in the PFC, a brain area which represents a nexus for sensory integration,

emotional processing and hedonic experience. As widely discussed in the previous sections, this brain area is an important component in the addictive phenomenon because it processes the reward to become a "hedonic experience" (Kringelbach, 2005). Thus, eCBs could be involved in the motivation to obtain the drug by linking the reward to a "hedonic experience" in the PFC. In particular, the mechanisms underlying the role of the eCB system in relapse to drug-seeking behaviour (produced by drug-related stimuli and drug re-exposure) seem related to the modulation of reward-related memory processes. Indeed, since eCBs mediate LTP and/or LTD of synaptic transmission in several addiction and memory-related brain areas, including the NAc, PFC, amygdala and hippocampus (De Vries & Schoffelmeer, 2005), this eCB-mediated synaptic plasticity might consolidate the reward-driven behaviour required to establish the addictive processes.

In line with evidence that supports the important contribution of CB1Rs in the mPFC activity, a recent study (den Boon *et al.*, 2015), besides confirming the presence of functional presynaptic CB1Rs and their ability to modulate both excitatory and inhibitory inputs to layer II/III pyramidal neurons of the PL area of the mPFC, demonstrated in vitro that exposure to CB agonists (WIN 55,212-2 and CP-55940) modulates the balance between excitation and inhibition (E/I balance) of these pyramidal neurons. In particular, this treatment caused a significant shift of the E/I balance towards excitation, from 18/82 % to 25/75 % (WIN) and from 17/83% to 30/70 % (CP). Importantly, this result was confirmed in vivo, since rats injected with these synthetic cannabinoids receptor agonists (SCRA), showed a shift of the E/I balance (measured in vitro) towards excitation 1 h after WIN (24/76 %) or after CP injection (30/70 %) compared to vehicle-injected rats (18/82 %) (den Boon *et al.*, 2015).

The recent identification of CB2Rs in the CNS presents other sites of action for eCBs (Van Sickle *et al.*, 2005), which might be involved in CB-induced central effects, that have previously been attributed to CB1Rs. In fact, several groups have produced data demonstrating the presence of CB2Rs in different neural tissues and have shown that CB2R knock-out (KO) animals displayed not

only altered electrophysiological responses (Garcia-Gutierrez *et al.*, 2013) but also behavioral memory deficits (Li & Kim, 2016). In the CNS, CB2R expression is detected mostly in microglia cells, even though it is lower than in peripheral immune cells and tissues (Galiegue *et al.*, 1995). However, as discussed in the paragraph 4, several studies have provided strong evidence that CB2Rs are up-regulated primarily on microglial cells upon activation in response to various insults and stimuli (Cabral & Griffin-Thomas, 2009). Furthermore, microglial cells have been shown to produce eCBs at higher levels than neurons in vitro (Walter *et al.*, 2003), suggesting that eCB production by activated microglial cells could play a pivotal role during neuroinflammation processes. These processes, as recently highlighted (Cutando *et al.*, 2013; Lacagnina *et al.*, 2017; Melis *et al.*, 2017) could be implicated also in the effects of drug abuse.

Therefore, all of this evidence supports the hypothesis that the eCB system represents a key component in the common neurobiological substrate of drug addiction, even though further studies are necessary to understand its exact role (Maldonado *et al.*, 2006; Wenzel & Cheer, 2018).

4 Glial cells

Glial cells, also called neuroglia or simply glia, are non-neuronal cells (astrocytes, microglia and oligodendrocytes) identified in the CNS. In 1864, Virchow was the first to propose the existence of non-neuronal tissue in the CNS. He named it 'neuroglia' (neural putty), because it appeared to stick the neurons together. Afterwards, Deiters (1865) and Golgi (1885) identified glial cells and distinguished them from neurons. Nowadays, glial cells can be divided into several categories depending on their anatomical position, origin and role. Considering the CNS, there are roughly twice as many glial cells as there are neurons (Hammond *et al.*, 2015), occupying the space between neurons, neuronal processes and separate neurons from blood vessels (Fig. 9). Once viewed as passive support elements for neurons, it is now clear that both microglia and astrocytes can actively regulate many aspects of neuronal function, including neurotransmitter release, gene regulation, electrophysiology, dendritic morphology, synaptic connectivity, and cell viability (Eroglu & Barres, 2010; Kettenmann *et al.*, 2013; Araque *et al.*, 2014; Salter & Beggs, 2014). Moreover, as immunocompetent sentinels of the CNS, they also show to have a critical role in the development and protection of the CNS, including neural development, cell migration, programmed cell death, and regulation of synapse maturation and elimination (Hanisch & Kettenmann, 2007; Bilbo & Schwarz, 2012; Schafer & Stevens, 2013; Chung *et al.*, 2015).

Interestingly, as described in the section 4.3, recent studies have demonstrated that glial cells (microglia and astrocytes), are also influenced by exposure to abused drugs, and their responses are likely to contribute to the behavioral outcomes associated with substance abuse (Miguel-Hidalgo, 2009; Coller & Hutchinson, 2012; Lacagnina *et al.*, 2017; Melis *et al.*, 2017).

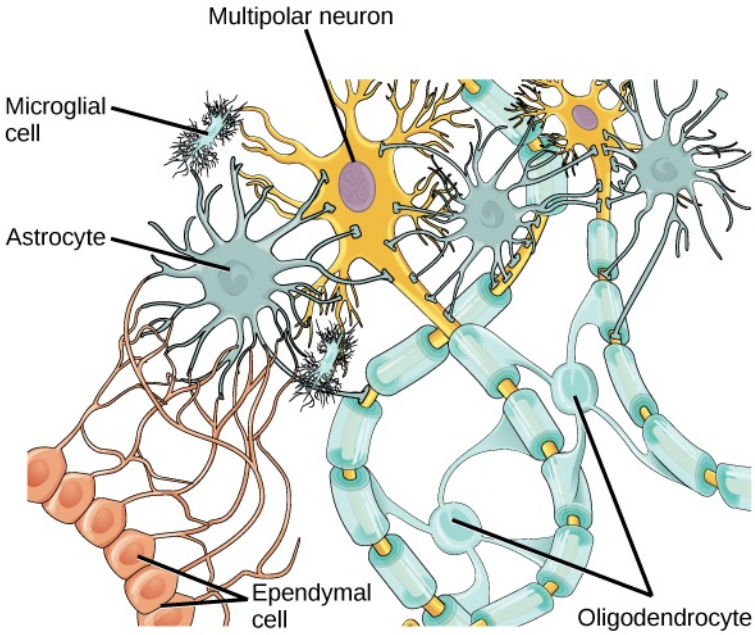

Figure 9 Schematic representation of glial cells in the CNS.
Adapted from: https://opentextbc.ca/biology/chapter/16-1-neurons-and-glial-cells/

4.1 Astrocytes

Astrocytes have traditionally been characterized as supportive cells in the brain for their roles in maintaining neuron homeostasis and survival. They represent the preponderant glial component of the CNS and occupy 20 to 50% of entire brain volume. Astrocytes can be identified histologically since they express the intermediate filament glial fibrillary acidic protein (GFAP).

Astrocytes are essential to the mammalian nervous system; since genetic elimination of astrocyte precursors is lethal to pups within 2 weeks of birth, which coincides with the period of exuberant synaptic outgrowth in the rodent cerebral cortex (Li *et al.*, 2012). Similar to microglia, in the healthy brain, astrocytes extend a network of cellular processes. In fact, a single astrocyte can make contact with a large number of synapses emerging from a single neuron as well as across a population of neurons, ranging from hundreds of synaptic contacts per astrocyte in the cortex to tens of thousands of synapses in the hippocampus (Bushong *et al.*, 2002; Halassa *et al.*, 2007). Thus, astrocytes are well-positioned to integrate signals from a great number of synapses, which may have implications for higher-order information processing. At the synaptic cleft the role of astrocytes is

multiple and involves reuptake of neurotransmitters, buffering of extracellular ion concentrations, and release of many diffusible signaling molecules (Araque *et al.*, 2014; Fields *et al.*, 2015). Thus, from numerous studies the concept of the 'tripartite synapse', proposing that astrocytes are functional components of synapses (Hammond *et al.*, 2015), has emerged.

Indeed, astrocytes are required for the synthesis of Glu and GABA. In particular, they have been shown to be essential for Glu regulation between neurons, since in vitro, inhibition of astrocyte proliferation leads to profound sensitivity to glutamate toxicity (Rosenberg & Aizenman, 1989), while in vivo disruption of astrocyte potassium channel activity results in impaired synaptic Glu clearance with devastating effects to survival (Djukic *et al.*, 2007). Indeed, Glu is primarily uptaken by astrocytes through the glutamate transporters: the Glutamate Aspartate Transporter (GLAST), the Glutamate Transporter-1.(GLT-1), and the Excitatory Amino Acid Transporter-2 (EAAT-2), which are highly expressed in these cells and are critical for clearing synaptic Glu (Fig 10). This was confirmed by intracerebroventricular delivery of antisense oligonucleotides against GLAST or GLT-1, which results in excessive Glu release, striatal neurotoxicity, and progressive motor deficits (Rothstein *et al.*, 2005).

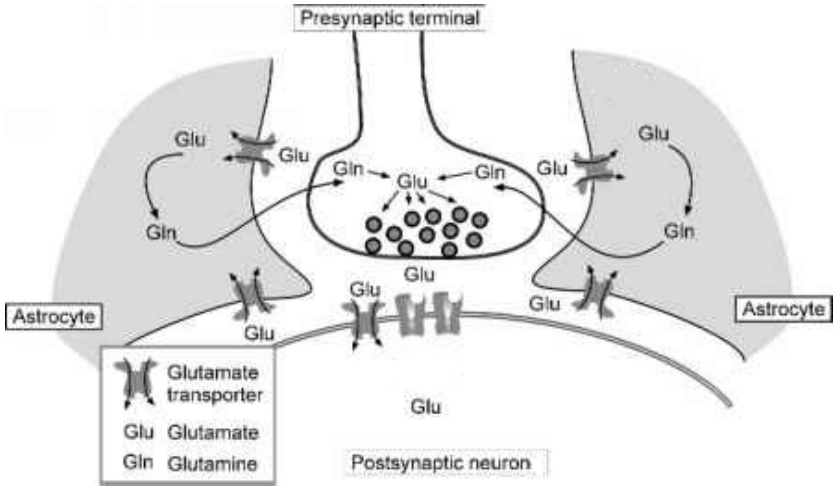

Figure 10 Schematic representation of glutamate uptake by astrocytes. Adapted from www.guwsmedical.info/schwann-cells/regulation-of-extracellular-glutamate-concentration.html

Although the immunoregulatory properties of microglia are more appreciated than those of astrocytes, they can also participate in inflammatory signaling within the CNS as well as regulating

immune cell trafficking at the blood vessels (Tian *et al.*, 2012). As a matter of fact, in response to injury or other insults astrocytes can secrete a variety of neuroimmune signaling molecules, including Interleukin-1α (IL-1α), Interleukin-6 (IL-6), Tumor Necrosis Factor α (TNFα), and Interferon γ (IFNγ) (Lau & Yu, 2001). The release of immune-related molecules from astrocytes can regulate glial cell function as well as modulate synaptic function (Lacagnina *et al.*, 2017).

Much evidence strongly associates a range of changes in astrocyte structure and function (i.e changes in the expression of GFAP) with neuropsychiatric diseases including major depressive disorder, schizophrenia, and addiction (Kim *et al.*, 2018). These changes observed both in human and in animal models can range from astrocytopathy (i.e. degeneration and loss of function) to astrogliosis, which can be either adaptive or maladaptive. In particular, in response to different drugs of abuse, either astrocytopathy or astrogliosis has been observed varying across drug classes, route of administration and length of withdrawal (Kim *et al.*, 2018). Specifically, astrogliosis as well as an atrophy of astrocytes were reported in post-mortem human material from different types of addiction (Suarez *et al.*, 2000; Miguel-Hidalgo, 2009). Preclinical studies have shown that treatment of animals with several drugs of abuse, including cocaine (Fattore *et al.*, 2002; Bowers & Kalivas, 2003), methamphetamine (Friend & Keefe, 2013; Castelli *et al.*, 2014) and morphine (Song & Zhao, 2001), resulted in up-regulation of GFAP expression and reactive astrogliosis.

4.2 Microglia

Microglia have long been considered the resident immune cells of the brain and spinal cord, sharing many functional characteristics with macrophages of myeloid origin (del Río-Hortega, 1932; Thomas, 1992). Microglia cells are distributed in all brain regions with different densities, showing the highest concentration in the SN, basal ganglia, and hippocampus (Lawson *et al.*, 1990). Each microglia cell has numerous ramified processes that extend throughout the surrounding neuropil, without overlapping with those of a neighboring microglial cell (Kierdorf & Prinz, 2013).

Similar to astrocytes, this spatial organization in the CNS demonstrates how microglia are active surveyors of their brain area (microenvironment), sensing the location of other microglia and receiving regulatory signals from surrounding neurons that influence their location (Fig. 9). Microglia cells, exceptional sensors of their microenvironment, respond to different insults by undergoing remarkable changes in morphology and gene expression (Aguzzi *et al.*, 2013). During pathological insults, activated microglial cells thicken and retract their processes, extend filopodia, proliferate and migrate. Microglia cells express a great diversity of receptors for stimulus-specific responses against a variety of potential threats to tissue homeostasis (Hanisch & Kettenmann, 2007; Lucin & Wyss-Coray, 2009; Ransohoff & Perry, 2009; Saijo & Glass, 2011). This includes an extensive number of exogenous factors, such as infections, microbes, toxins, and other foreign substances (i.e. xenobiotics), as well as noxious endogenous compounds released from dead or dying cells due to traumatic brain injury, ischemia, or neurodegeneration (Xanthos & Sandkuhler, 2014). In turn, microglia responses with several actions, such as phagocytosis and lysosomal degradation; and secretion of cytokines, chemokines, or growth factors that can affect neuronal function, recruit additional immune cells, to induce either tissue repair or apoptosis (Kierdorf & Prinz, 2013).

Considering their morphology, it was traditionally assumed that microglia, under healthy conditions, remained in a "*resting or quiescent state*" before rapid activation, that represented a

stimulus-specific response to various signals of injury or danger (Kreutzberg, 1996). Microglia activation lead to alterations in cell morphology, in which resting microglia retracted their highly ramified processes and adopted an *"amoeboid"* shape distinguished by high motility (Stence *et al.*, 2001). However, the notion of microglia *"resting"* in the healthy CNS and *"amoeboid"* after insults was upended by different studies (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005), which revealed that, in physiological conditions, the soma of cortical microglia remain relatively stationary, but in contrast, their ramified processes are highly mobile, retracting and extending protrusions throughout the parenchyma and surveying the entire brain every few hours (Nimmerjahn *et al.*, 2005). Therefore, this surveillance allows the microglia to dynamically respond to signs of injury, which rapidly extend their processes toward sites injured (Davalos *et al.*, 2005). These results indicate that microglia are not simply "reactive" immune cells that mobilize following infection or injury but are active sentinels that are constantly surveying the healthy CNS. Based on these observations, several groups have hypothesized that microglia might have an essential role in monitoring synaptic activity (Graeber, 2010; Bilbo & Schwarz, 2012; Kettenmann *et al.*, 2013; Schafer & Stevens, 2013). This hypothesis has been supported by ultrastructural analysis in mice that revealed that microglial processes make direct contact not only with axon terminals and dendritic spines but also astrocytes and their perisynaptic processes (Tremblay *et al.*, 2010). Similarly, simultaneous in vivo two-photon imaging of fluorescently labeled neurons and microglia confirmed that microglial processes make frequent but brief contact with dendritic spines (Wake *et al.*, 2009). Interestingly, the frequency of this contact is regulated by neuronal activity. Indeed, it is decreased by sensorial deprivation, pharmacological block of action potential, as well as a decrease in body temperature. Thus, microglial cells can sense and react to neuronal activity. In turn, they can locally influence neuronal excitability by releasing several factors such as cytokines, neurotrophic factors, and neurotransmitters (Lacagnina *et al.*, 2017).

4.3 Glial cells and the eCB system: interplay and involvement in drug addiction

The majority of the preclinical and clinical research on addiction have focused on understanding neuronal adaptations due to exposure to drugs of abuse, considerably improving our knowledge about this neuropsychiatric disorder. However, the underlying mechanisms of addiction behaviors, such as drug-seeking, drug-taking, and relapse behavior following periods of abstinence, remain unsolved.

Although drug-induced alterations in neuronal activity play a pivotal role in drug addiction (Koob & Volkow, 2010), accumulating evidence suggests that neurons are not the only cells impacted by drugs of abuse. Indeed, glial cells are markedly influenced by exposure to substances of abuse, including opioids, alcohol, psychostimulants, and cannabinoids (Lacagnina *et al.*, 2017; Melis *et al.*, 2017; Kim *et al.*, 2018). In addition, much evidence strongly supports the hypothesis that drug-induced alterations in glial cells within brain regions critically involved in addiction mechanisms, such as the PFC, NAc, VTA, amygdala and hippocampus, might contribute to the vulnerability and persistence of addictive behaviors (Scofield & Kalivas, 2014; Scofield *et al.*, 2016; Lacagnina *et al.*, 2017).

Indeed, the ability of microglia and astrocytes to monitor, regulate, create and eliminate synapses, allow the hypothesis that glia-mediated synaptic remodeling may occur under the conditions of drug exposure and abuse and could contribute to the persistent behavioral effects characteristic of drug addiction (Miguel-Hidalgo, 2009; Coller & Hutchinson, 2012). In particular, drugs of abuse could influence glia, either directly through interactions at surface receptors, or indirectly through their effects on neighboring neurons surveyed by microglia or astrocytes. These effects include changes in neurotransmitter release or synthesis, protein trafficking, patterns of action potentials, and release of cytokines, chemokines, or other immune signaling molecules (Lacagnina *et al.*, 2017). Meanwhile, glial responses to drugs of abuse could influence neuronal function directly through physical remodeling of structural components (Corty & Freeman, 2013; Brown & Neher, 2014), or

indirectly through a variety of released compounds (Block *et al.*, 2007), which can be both anti-inflammatory and neuroprotective [i.e., Iinterleukin-10 (IL-10), Transforming Growth Factor- β (TGF-β), and Brain Brain-Derived Neurotrophic Factor (BDNF)] or pro- inflammatory and neurotoxic (i.e. IL-1β, and TNFα). In particular, considering drug-induced glial alterations and their role in drug addiction, important involvement of the eCB system has been suggested. In this respect, it has been observed that microglial cells in resting homeostatic condition express low levels of both CB1Rs and CB2Rs. CB2Rs expression rises following microglia cell activation (Carlisle *et al.*, 2002; Walter *et al.*, 2003; Stella, 2010), suggesting that the eCB system could be sensitive to alterations in glia cell physiology. In particular, several studies reported strong evidence that CB2Rs are up-regulated primarily on microglial cells upon activation in response to various insults and stimuli (Cabral & Griffin-Thomas, 2009). In addition, an in vitro study showed that microglial cells produce eCBs at higher levels than neurons (Walter *et al.*, 2003), suggesting that eCBs production by activated microglial cells could play a pivotal role during neuroinflammation processes. Consistently eCBs like AEA, 2-AG and Palmitoylethanolamide (PEA) affect immune function mostly through CB2Rs (Cabral *et al.*, 2015). Considering astrocytes, several in vitro and in vivo studies have showed that astrocytes functionally express CB1Rs (Navarrete & Araque, 2008; Han *et al.*, 2012; Bosier *et al.*, 2013). Similar to microglia, astrocytes can produce eCBs (Stella, 2010) and recent data indicate that astroglial CBRs might control eCB turnover in the brain (Belluomo *et al.*, 2015).

Therefore, the crucial role of the eCB system on glial cell function has been suggested, since it is able to modulate both inflammation and synaptic transmission (Rossi *et al.*, 2015). Thereby, it has been proposed as a key modulator of the synaptic effects of inflammation. In fact, increasing evidence suggests the involvement of the eCB system in cytokine modulation of synaptic transmission and recently bidirectional interplay between inflammation (i.e. pro-inflammatory cytokines) and the eCB system has been demonstrated. Indeed, it has been reported that IL-1β abrogates the sensitivity of CB1Rs, regulating both glutamate and GABA transmission through

different mechanisms. For instance, the depressant effect of CB1R agonists on sEPSC and sIPSC frequency was completely abolished in the presence of IL-1β (Rossi *et al.*, 2012; De Chiara *et al.*, 2013). Moreover, the interaction between IL-1β and CB1Rs has been suggested as a plausible synaptic mechanism at the basis of anxiety associated with an inflammatory response, such as in depression disorders and schizophrenia (Rossi *et al.*, 2008; Rossi *et al.*, 2012). Thus, the eCB system is a crucial modulator of cytokine-dependent synaptic alterations, reciprocally, inflammation can directly alter the activity of eCB system on synaptic transmission. Consistently, growing evidence suggests that inflammatory cytokines are involved in molecular mechanisms of brain plasticity and in complex affective, motivational and cognitive processes, which are altered in drug addiction disorder (Beattie *et al.*, 2002; Ross *et al.*, 2003; Yirmiya & Goshen, 2011; Rossi *et al.*, 2012; Marin & Kipnis, 2013).

Based on this evidence, besides neurons, chronic CB administration, both natural and synthetic, might affect glial cells, likely inducing an abnormal interaction between these two cell populations, which in turn could be implicated in CB-induced behavioral effects (Melis *et al.*, 2017). However, there is a lack of information on the possible contribution of glial cells in effects induced by repeated CB exposure, especially in adulthood, while there are a limited number of studies regarding the effects of adolescent Δ^9-THC exposure on glial cells (Zamberletti *et al.*, 2014; Zamberletti *et al.*, 2015; Zamberletti *et al.*, 2016).

In this respect, Cutando and colleagues (2013) reported initial evidence for the contribution of microglial cells in the effect of sub-chronic Δ^9-THC administration in adulthood. In particular, they demonstrated the involvement of microglial cells in cerebellar conditioned learning (evaluated in the delayed eye-blink conditioning test) and motor coordination deficits induced by sub-chronic Δ^9-THC administration in adult male mice. Δ^9-THC-induced impairments in the learning paradigm was associated with alterations in microglial morphology, mainly in the molecular layer of the cerebellum, and with enhanced mRNA expression of specific pro-inflammatory molecules, such as IL-1β. Moreover, expression of CB2R and IL1β mRNA was increased under neuroinflammatory

conditions in activated CD11b-positive microglial cells. Notably, microglia activation was also associated with cerebellar CB1R down-regulation, showing a similar neuroinflammatory phenotype that was observed in the cerebellum of CB1R KO mice. Furthermore, all these alterations were region-specific since no changes were present in the hippocampus, striatum or PFC (Cutando *et al.*, 2013). Interestingly, all of these deficits, observed both in sub-chronically Δ^9-THC-treated mice and in CB1R-KO mice, were prevented by pharmacological blockade of microglial activation or IL-1 receptor signaling, thus providing a functional association between THC-induced microglia activation and cerebellar-dependent associative learning, as well as motor impairments. These results reveal the critical role of microglia-mediated signaling in cerebellar dysfunctions triggered by CB1R down-regulation (Cutando *et al.*, 2013). In line with Cutando's results, Zamberletti et al. (2015), demonstrated that adolescent Δ^9-THC treatment is associated with long-term microglia activation specifically within the PFC of adult female rats showing cognitive impairment and depressive-like behaviors. The neuroinflammatory phenotype state in the PFC was characterized by altered microglia morphology, increased expression of the pro-inflammatory cytokines such as TNF-α, inducible Nitric Oxide Synthase (iNOS) and Cyclooxygenase-2 (COX-2), and a reduction of the anti- inflammatory cytokine, IL-10. As reported by Cutando (2013), THC-induced microglia activation was region-specific since no alterations were detected in the NAc, hippocampus and amygdala. Moreover, the neuroinflammatory phenotype induced by adolescent Δ^9-THC treatment was associated with a down-regulation of CB1Rs on neuronal cells and a concomitant up-regulation of CB2Rs on microglial cells within the PFC. Interestingly, pharmacological inhibition of glia activation during Δ^9-THC exposure attenuated short-term memory impairment present in adult rats, and prevented increases in TNF-α , iNOS, and COX-2 levels, as well as the up-regulation of CB2Rs on microglial cells (Zamberletti *et al.*, 2015).

Similar to microglial cells, only few papers have reported the consequences of prolonged CB exposure on astrocytes. One of these studies, showed that adolescent Δ^9-THC treatment induces an

increase in the levels of GFAP in the hilus of the dentate gyrus of both male and female rats (Lopez-Rodriguez *et al.*, 2014). More recently, Zamberletti and colleagues (2016), using the same treatment protocol applied to females (Zamberletti *et al.*, 2015), demonstrated that the behavioral effects induced by Δ^9-THC treatment in adolescent male rats overlaps only partially with those observed in females. In fact, although male rats showed poorer memory performance and psychotic-like behaviors, differently from female, in male rats no alterations in the emotional component were observed. Thus, these studies demonstrated sex-differences in terms of the brain region affected and the profile of pro-neuroinflammatory biomarkers. In line with previous studies, an alteration in astrocyte reactivity has also been reported, since an increase of GFAP levels in the hippocampus after adolescent Δ^9-THC treatment has been observed. Moreover, astrocyte activation was associated with increased protein expression of TNF-α and iNOS, together with a concomitant reduction of the anti-inflammatory cytokine IL-10. Parallel to these alterations, a significant increase in the expression of the NMDA and AMPA receptor sub-units, as well as the pre-synaptic marker Synaptophysin and the post-synaptic marker PSD95 (Zamberletti *et al.*, 2016) has been observed.

The coexistence of synapse and astrocyte alterations in the same brain region is very interesting, supporting the idea that astrocytes are crucially involved in the control of surrounding synapses. Indeed, as already discussed, since astrocytes sense neuronal and synaptic activity, the evidence suggests that activated astrocytes, by promoting a pro-inflammatory phenotype, might contribute to the alterations in glutamatergic synapses induced by adolescent Δ^9-THC exposure (Zamberletti *et al.*, 2015; Zamberletti *et al.*, 2016). However, since the effects of CB exposure-induced on glial cells appears sex- , region-dependent, as well as age/period of drug-exposure dependent (Cutando *et al.*, 2013; Zamberletti *et al.*, 2015; Zamberletti *et al.*, 2016), understanding the exact contribution of glial cells still appears hard.

Collectively, available data on the effects of chronic Δ^9-THC exposure on microglia and astrocytes suggest an important role played by these cells in response to chronic activation of CBRs, supporting the hypothesis that both cell populations might have an essential role in monitoring synaptic activity (Graeber, 2010; Bilbo & Schwarz, 2012; Kettenmann *et al.*, 2013; Schafer & Stevens, 2013). In particular, these data allow speculation that excess glutamate in the synaptic cleft resulting from reduced inhibitory control exerted by CB1Rs at glutamatergic terminals might activate microglia and/or astrocytes (brain region- and sex-dependent) that in turn trigger an inflammatory response (Melis *et al.*, 2017). Meanwhile, it has also been suggested that CB1Rs activity on GABAergic terminals might regulate the homeostatic balance between pro-and anti-inflammatory processes, since an increase in neuroinflammatory markers has been found in CB1R deficient mice and is dependent on CB1Rs in GABAergic neurons (Albayram *et al.*, 2011). Moreover, several mediators of this response (i.e., IL-1β, TNF-α, iNOS) might affect neuronal functionality, leading to the learning deficits observed after repeated Δ^9-THC exposure (Cutando *et al.*, 2013; Zamberletti *et al.*, 2015).

Altogether, these data suggest an important contribution of glial cells in the effects observed after chronic CBs exposure, most likely due to the interaction between the eCB system and inflammation processes. Unfortunately, in the literature there is no evidence regarding the effects of SCRA exposure on astrocytes and microglia. Therefore, specific studies are needed in order to understand how a possible alteration of eCB signaling and glial cell activity could contribute to neuroinflammation processes associated with chronic CBs exposure.

5 Synthetic cannabinoid receptor agonist (SCRA) and the "Spice drugs phenomenon"

Synthetic cannabinoid receptor agonists (SCRAs), such as JWH-018, represent a wide class of potent and efficacious full agonists at CB1R and CB2R. As matter of fact, SCRAs have been developed for pharmaceutical purposes either to understand the pharmacology of the eCB systems or as therapeutic agents for treatment of different human pathological conditions (i.e. epilepsy, spasticity, inflammation, eating disorders, certain types of pain, and cancer) (De Luca & Fattore, 2018). JWH-018 belongs to the so-called "first generation" SCRAs since it was synthesized in 1994 by John W. Huffman (Clemson University) as a compound with structure-activity relationships and good affinity for the CB2R but weak affinity for the CB1R. Although *in vitro* studies demonstrated that JWH-018 shows nanomolar affinity for both receptors (Ki: CB1R=approximately 9 nM, CB2R approximately 3 nM) (Huffman & Padgett, 2005), *in vivo* this compound appeared no selective for CB2Rs, since it didn't show a preponderant action on CB2R (De Luca & Fattore, 2018). Thus, despite it being synthetized for scientific purposes, during the last decade, JWH-018 has appeared on the global drug market in herbal preparations proposed as marijuana-like mixtures (Fattore & Fratta, 2011; Pintori *et al.*, 2017; De Luca & Fattore, 2018); in the meantime globally, underground chemists have also used the scientific literature to create and market new illicit SCRAs referred to as the "second and third" generation SCRAs (Rosenbaum *et al.*, 2012; Seely *et al.*, 2012b; Castaneto *et al.*, 2014; Miliano *et al.*, 2016; De Luca & Fattore, 2018).

From 2004, Spice drugs (known also as "K2 drugs" in the USA) appeared on websites and in specialized shops (i.e. smart shops) as novel recreational drugs (Dresen *et al.*, 2010) and were sold as mild hallucinogens with prominent cannabis-like effects. They soon became popular in Central European countries, consequently attracting the attention of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) and the Early Warning System on new drugs (EMCDDA, 2009). However, only at the end of 2008 were two SCRAs identified for the first-time as the main

active, although not declared, ingredients of a herbal blend called "Spice": CP-47,497-C8, the C8 homolog of the non-classical cannabinoid CP-47,497, and JWH-018 a cannabimimetic aminoalkylindole (Auwarter *et al.*, 2009; Uchiyama *et al.*, 2011). Differently from Δ^9-THC, the SCRAs contained in Spice drugs are able to induce more adverse reactions, long-term effects and psychiatric consequences (Papanti *et al.*, 2014; Schifano *et al.*, 2015), several withdrawal symptoms, and present more prevalence of dependence after their chronic use (see next sections). Most likely these effects are due to their higher potency/efficacy and affinity for CBRs (full agonist) compared with Δ^9-THC (partial agonist) (Pintori *et al.*, 2017). Safety data information and active pharmacological agents are not usually described in the packages (EMCDDA, 2009), whereas composition and dosages can vary considerably among different products (Table 2), posing a significant health risk to users (Brents & Prather, 2014; Castaneto *et al.*, 2014).

	Synthetic cannabinoids												
Brands	JWH-018	JWH-073	JWH-081	JHW-250	CP-47,497	CP-55,940	5F-AK848	5F-PB22	AM-2201	APICA	APINACA	AB-APINACA	AB-FUBINACA
K2 blonde	x	x											
K2 standard	x	x											
K2 blue	x												
K2 latte	x	x		x									
K2 mint	x	x											
K2 silver	x			x									
Spike gold	x	x											
Spike max	x			x									
Space	x												
K3 mango	x					x							
K3 original	x					x							
K3 legal			x	x									
Earthquake		x		x	x								
Ocean blue		x		x	x								
Woodstock		x		x	x								
Atomic bomb[a]							x	x					
Black mamba[a]							x	x					
Haze[a]							x	x					
Juicy leaf[a]							x	x					
Spike 99[a]							x	x					
Bonzai summer									x				
Boost													
Fragrance										x	x	x	x
Powder													

[a]Primarily compound contained.
x, drug presence.
Reference: *http://www.uatests.com/drug-test-by-drug/spice-drug-test.php*; NMS Labs online seminar 16 September 2010 (*http://www.slideshare.net/nmslabs/k2-and-synthetic-cannabinoids-pharmacology-effectschemical-analysis*); *https://twitter.com/hashtag/mexedrone*; Uchiyama *et al.* (2012, 2013); NPS Update and National Action Plan, National Early warning System, Department for Antidrug Policies, presidency of the Council of Ministers, September 2013.

Table 2 Spice drug street names and related SCRAs. Adapted from Pintori et al. (2017)

5.1 SCRA classification

Considering their chemical structures, SCRAs have been classified into four different groups: the "JWH group" synthesized in the 80s by Huffman, including JWH-018; the "CP group" (cycloexylphenols) synthesized in the 70s by Pfizer, including CP-47,497; the "HU group" or "classical cannabinoid class", synthesized in the 60s by Mechoulam with the parent compound HU-210; and finally, the "benzoyl-indole group", which includes AM-694 and RCS-4.

Although these SCRAs have been developed as pharmaceutical/scientific tools, they can also be classified based on their appearance in the market as "Spice drugs". Indeed, as already mentioned above, new SCRAs have been synthesized introducing slight modifications to the parent compound structures to avoid legal consequences, resulting in the emergence of at least three consecutive generations of SCRAs (Fig. 11): the first one includes JWH-018, CP-47,497 and HU-210; the second one includes alkyl derivatives (AM-2201, MAM-2201, AM-694), N-methylpiperidines (AM-2233 and AM-1220) and benzoylindoles (AM679, RCS-4 and derivatives); the third includes molecules where the indole ring is replaced with an indazole or a benzimidazole group (AKB-48, 5F-AKB-48, FUBIMINA) or molecules where the carbonyl group is replaced with the carboxylic or the carboxy-amide functional group (APICA, SDB005) and quinolones [PB-22 (QUPIC), 5F- PB-22, BB-22 (QUCHIC)] with secondary cyclic structures (ABDICA, AB-PINACA, 5F-AB-PINACA) and new nitrogen groups (AB-FUBINACA, AB-FUBICA) (Fattore & Fratta, 2011; Miliano *et al.*, 2016; Pintori *et al.*, 2017). The continuous evolution of SCRAs in their chemical composition, apart from attempting to avoid legal issues, leads to higher potency and abuse potential of these compounds generation after generation.

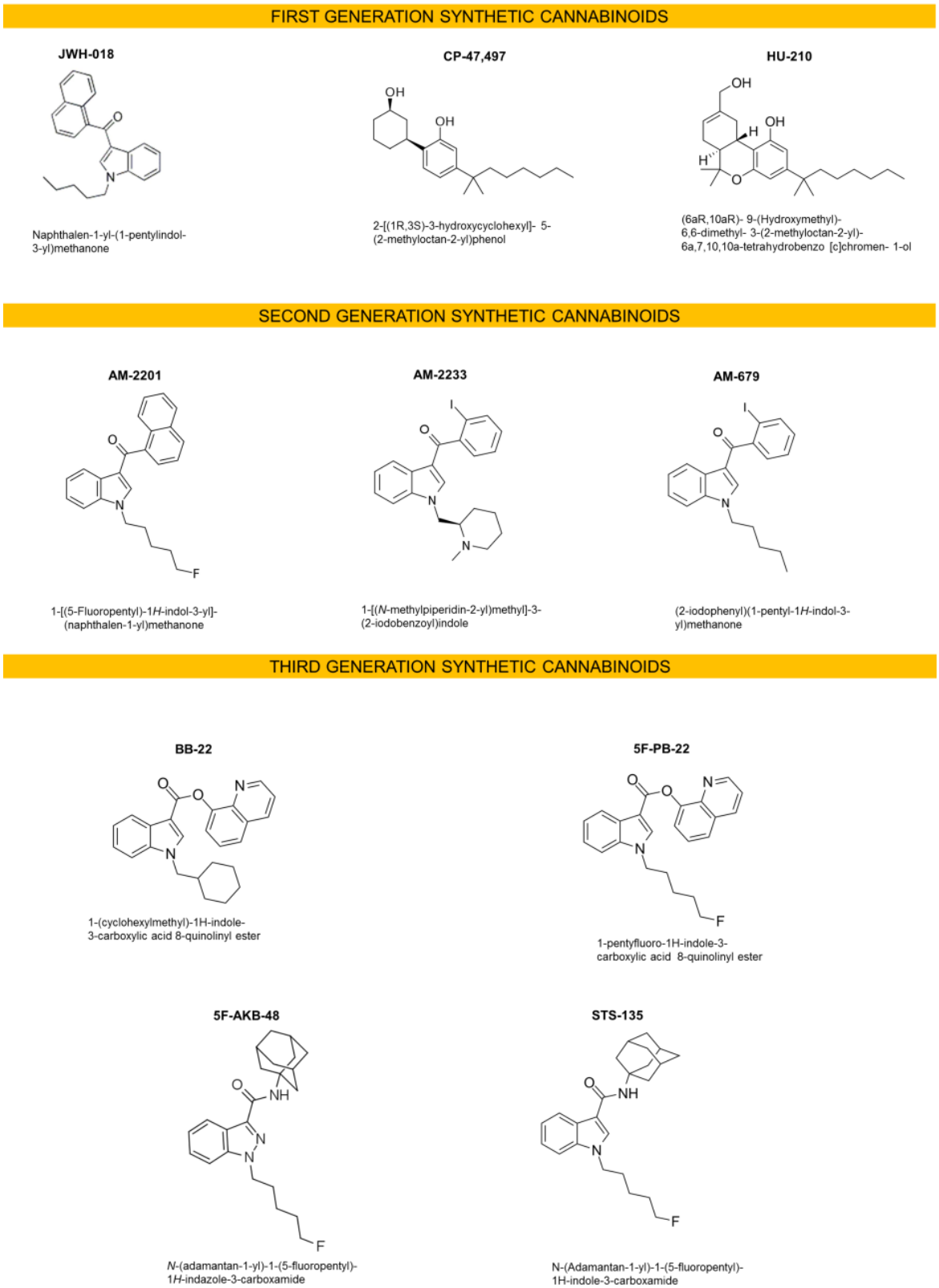

Figure 11 Classification of SCRAs contained in Spice drugs. Adapted from Pintori et al., 2017.

5.2 The widespread growing Spice phenomenon

The increasing success of Spice drugs is due to several reasons. Besides their great psychoactive effects, their marketing via the internet, with several dedicated websites, in a totally anonymous way represents an important factor for the increase in their popularity. Moreover, these Spice drugs have an affordable price (27–36 EUR/sachet, 9–12 EUR/g of mixture) compared to "classical" drugs. Another important reason is that most of the SCRAs sprayed on the Spice drugs are very difficult to detect with commonly used drug-screening procedures (immunoassays of body fluids). This has important consequences, because it encourages not only cannabis users but also curious people with no previous experience of illicit drugs, to use these cannabis-like drugs without legal prosecution. Furthermore, wherever drug screening is routinely performed (i.e. hospital or institutions, forensic psychiatric centers), people may be motivated to substitute cannabis with Spice products (Fattore & Fratta, 2011). Moreover, every effort made to improve toxicological screening procedures, becomes useless due to the continuous evolution of the chemical (i.e. structure) composition of these compounds. Indeed, the Spice drugs market, as well as for all classes of NPS, is "resilient": in fact, when one of these compounds become illegal, the "drug dealers" change its structure or substitute it with a new and legal compound, avoiding detection and legal problems. Finally, another factor implicated in the Spice phenomenon is the poor perception of the risk associated with their use, most likely due to a lack of safety information and commercial advertisements that describe Spice as "natural herbs" or "harmless incense blends". For all these reasons, since 2008 SCRAs have represented the widest class of NPS monitored by the EMCDDA. In fact, 160 different SCRAs have been detected in different types of products so far. In 2014, SCRAs represented 60% of the total NPS-related seizures detected in Europe, while in 2015, 98 new substances were reported to the Early Warning System of the EU for the first time, with 24% of these being classified as SCRAs. It has been shown that SCRAs currently available on the market are able to induce severe peripheral and central effects, including drug dependence and psychosis.

5.3 SCRA vs Δ^9-THC

5.3.1 Pharmacodynamics

Although SCRAs have been designed to "mimic" the pharmacological actions of Δ^9-THC, this class of compounds includes full agonists with a higher affinity at the CB1Rs and the CB2Rs and higher dose-dependent efficacy compared to the partial agonist Δ^9-THC (Fattore & Fratta, 2011; Miliano *et al.*, 2016), leading to them being considered as "super CBR agonists" (Miliano *et al.*, 2016; Pintori *et al.*, 2017).

In fact, if the Ki of Δ^9-THC has been reported to range from 3.87 (Hess, 2016) to 41 nM (Showalter *et al.*, 1996) depending on the specific assay conditions; by contrast, SCRAs contained in seized K2/Spice products exhibit CBR1 affinity in a nanomolar range of concentration such as JWH-018 (Brents *et al.*, 2011), AB-PINACA (Wiley *et al.*, 2015), JWH- 250 (Wiley *et al.*, 2012), STS-135 (De Luca *et al.*, 2016), but also subnanomolar CB1R affinity [i.e., 5F-PB-22, AK-B48 (De Luca *et al.*, 2016), AB- FUBINACA, ADB-FUBINACA (Banister *et al.*, 2015), JWH-210 (Huffman & Padgett, 2005), JWH-122 (Huffman *et al.*, 2003)]. In particular, in vitro JWH-018 ($K_i = 9$ nM), CP-47,497 ($K_i = 2.2$ nM) and HU-210 ($Ki = 0.06$ nM) showed respectively 4.5-fold, 8.6-fold and 55-fold higher affinity at CB1R compared to Δ^9-THC(Ki= 40 nM) (Brents *et al.*, 2011; Fattore & Fratta, 2011; Spaderna *et al.*, 2013; Schifano *et al.*, 2015).

Moreover, SCRAs modulate intracellular signaling pathways via CBRs with high potency and full efficacy when compared with Δ^9-THC. In fact, most studies quantified the intrinsic activity of SCRAs by measuring the potency (i.e. EC50/IC50) and efficacy (i.e. Emax/Imax) of these compounds to activate G proteins (i.e. GTPgS assay), and/or to inhibit AdC activity in brain homogenates or in whole cells expressing native or transfected CB1R, respectively. Activation of CB1Rs by Δ^9-THC results in activation of G proteins [EC50 values from 81 nM (Breivogel *et al.*, 2001) to 167 nM (Brents *et al.*, 2011)], but only as a partial agonist. On the contrary, examined SCRAs act as full CB1Rs [ie, JWH-018 (Brents *et al.*, 2011), 5F-PB-22, STS-135 (De Luca *et al.*,

2016), MAM-2201 (Costain *et al.*, 2016), JWH- 250 (Wiley *et al.*, 2012), XLR-11 (Wiley *et al.*, 2013)]. Moreover, Δ^9-THC also potently inhibits AdC activity via CB1Rs [i.e., IC50 values ranging from 5.0 (Bonhaus *et al.*, 1998) to 44 nM (Canazza *et al.*, 2016)], but exhibits reduced efficacy indicative of a partial agonist. In marked contrast, almost all SCRAs, acting as full agonists, inhibit AdC activity with high potency (nM range) and high efficacy [ie, 5F-PB-22, PB-22, AK-B48, STS-135 (De Luca *et al.*, 2016), AB-PINACA, EAM-2201, MAM-2201 (Costain *et al.*, 2016), JWH-250, and XLR-11(Wiley *et al.*, 2012; Wiley *et al.*, 2013)]. Similar full agonist activity for SCRAs at CB1Rs has been demonstrated by fluorometric assay to measure membrane potential (Banister *et al.*, 2015) and in primary hippocampal neurons by quantifying calcium transients (Costain *et al.*, 2016).

Consistently, these SCRAs can stimulate *in vivo* DA transmission in the NAc shell at doses consistent with their *in vitro* affinity at the CB1Rs (De Luca *et al.*, 2016). For instance, JWH-018 increases DA transmission in the NAc shell at doses 4-fold lower than Δ^9-THC (De Luca *et al.*, 2015); while SCRAs of the third generation, like BB-22 do so at doses 10-fold lower than JHW-018 (De Luca *et al.*, 2016). Altogether, these data demonstrate the higher efficacy and potency of SCRAs that, besides having greater acute effects and toxicity than Δ^9-THC, might also enhance chronic effects, thereby leading to tolerance and dependence, as well as harmful effects.

5.3.2 Behavioral effects: In vivo Cannabinoid efficacy

Over the years, the behavioral effects induced by SCRAs have been extensively investigated in animal models by performing tests used in preclinical characterization of CB1 ligands, (Wiley *et al.*, 1998; Fantegrossi *et al.*, 2014; Canazza *et al.*, 2016; Ossato *et al.*, 2016). In particular, these studies have been focused on a characteristic cluster of effects, such as tetrad effects, neurological alterations and sensorimotor changes induced by acute exposure to SCRAs, that could be

considered a consequence, as well as a reliable indicator/marker of cannabinoid *in vitro* efficacy (Table 3).

Tetrad effect

In vivo animal studies showed that SCRAs elicit the typical "cannabinoid tetrad" observed with Δ^9-THC, which includes hypothermia, analgesia, hypolocomotion, and catalepsy (Table 3) (Wiley *et al.*, 1998; Fantegrossi *et al.*, 2014).

SCRA-induced hypothermia has been observed throughout all the generations of SCRAs (Wiley *et al.*, 2012; Rajasekaran *et al.*, 2013; Vigolo *et al.*, 2015; Canazza *et al.*, 2016; Ossato *et al.*, 2016). In this respect, administrations of JWH-250 and JWH-073 (0.01-15 mg/kg, ip) are effective in reducing both rectal and surface temperatures in mice similar to Δ^9-THC but with shorter duration compared with JWH-018 (0.01-6 mg/kg, ip). However, the extent of the decreased rectal temperature in mice produced by the following generations of SCRAs such as AKB-48 and 5F-AKB-48 (3 and 6 mg/kg, ip) is greater than that induced by Δ^9-THC and similar to that produced by JWH-018 (Canazza *et al.*, 2016). In addition, 5F-AKB-48 and JWH-018 (3 and 6 mg/kg, ip) seem to be more powerful than AKB-48 in decreasing surface temperature.

Moreover, it has been shown that SCRAs induce analgesia, since mechanical and thermal pain thresholds are increased after their administration. In particular, JWH-250 and JWH-073 (0.01-15 mg/kg, ip) elicit a less powerful mechanical and thermal nociception in mice compared with JWH-018 in the tail-pinch and tail-withdrawal tests, respectively (Vigolo *et al.*, 2015; Ossato *et al.*, 2016). In addition, AKB-48 (6 mg/kg, ip) and 5F-AKB-48 (0.01-6 mg/kg, ip) induce an increase in the acute mechanical pain threshold in mice. Interestingly, this experiment showed that 5F-AKB-48 is more potent than AKB-48, since even the lowest doses produce significant analgesia (Canazza *et al.*, 2016). However, AKB-48 and 5F-AKB-48 (0.01–6 mg/kg, ip) elicit an increased threshold to an acute thermal pain stimulus in mice (tail-withdrawal test), with the latter drug producing a more persistent elevation (≤5 h) at the highest dose tested (6 mg/kg, ip) compared with that produced by

AKB-48 (145 min) at the same dose. Overall, the highest doses tested induce an analgesic effect similar to that induced by the same dose of Δ^9-THC.

Moreover, considering locomotor activity, JWH-250 and JWH-073 (0.01-15 mg/kg, ip) induce motor impairment in mice similar to that induced by Δ^9-THC but less powerful than that elicited by JWH-018 (Ossato *et al.*, 2016). In addition, 5F-AKB-48 (3 and 6 mg/kg, ip) induces a more persistent and significant impairment in stimulated locomotion compared with AKB-48 (6 mg/kg, ip) and both are less effective than JWH-018 (6 mg/kg, ip). However, it has been shown in mice that AKB-48 and 5F- AKB-48 can reduce the total distance travelled (6 mg/ kg, ip) and increase immobility time also at the dose of 1 mg/kg. Notably, only AKB-48 induces a transient facilitation of locomotor activity at low doses (1 mg/kg, ip) similar to Δ^9-THC (Canazza *et al.*, 2016).

Finally, SCRAs induce catalepsy, as shown by an augmentation of catalepsy in the bar test (Canazza *et al.*, 2016; Ossato *et al.*, 2016). In particular, JWH-250 and JWH-073 induces altered motor activity at the higher dose tested (6 and 15 mg/kg, ip), showing a stronger effect than that observed with Δ^9-THC and less potent than that elicited by JWH-018. In addition, 5F-AKB-48 (3 and 6 mg/kg, ip) is more powerful in catalepsy induction compared with AKB-48, JWH-018 and Δ^9-THC at the same doses (Canazza *et al.*, 2016). All Cannabinoid-tetrad effects are prevented by pretreatment with AM-251 (6 mg/kg, ip), which does not affect body temperature, pain threshold, locomotor activity and does not induce akinesia and catalepsy when administered alone (Vigolo *et al.*, 2015; Canazza *et al.*, 2016; Ossato *et al.*, 2016).

Neurological alteration

Besides typical tetrad effects, SCRAs also induce neurological alterations, especially at high doses, such as myoclonia, tail elevation, seizures and hyper-reflexia, that have been reported immediately after their administration (Table 4) (Vigolo *et al.*, 2015; Canazza *et al.*, 2016; Ossato *et al.*, 2016). Indeed, high doses of JWH-250 and JWH-073 (6 and 15 mg/kg, ip) induce convulsions, hyperreflexia, myoclonias and aggressive responses in mice, which are not usually observed after

the administration of Δ^9-THC (Ossato *et al.*, 2016). In addition, 5F-AKB-48 (6 mg/kg, ip) induces myoclonias with longer latency and duration than those produced by AKB-48 and JWH-018 (6 mg/kg, ip). Moreover, 5F-AKB-48 (6 mg/kg, ip) induces tail elevation with higher latency, duration and frequency compared with AKB-48 and JWH-018 (3 mg/kg, ip), and seizures with longer duration, but the same latency as observed after administration of AKB-48 and JWH-018 at 6 mg/kg, ip. However, regarding hyper-reflexia, AKB-48, 5F-AKB-48 and JWH-018 (6 mg/kg, ip) induce it showing the same latency and longer duration (Canazza *et al.*, 2016). In addition, consistent with clinical studies, SCRAs cause significant alterations in visual, acoustic and tactile sensorimotor reflexes and induce aggressive behavior in mice (Canazza *et al.*, 2016; Ossato *et al.*, 2016). In this respect, JWH-250 (0.01-15 mg/kg, ip) and JWH-073 (0.01-15 mg/kg, ip) inhibit the visual object and acoustic response in a less potent way compared with JWH-018 and Δ^9-THC. Moreover, 5F-AKB-48 is more potent than AKB-48 in inhibiting the responses of mice to tactile and acoustic stimuli, with tactile activity being similar to that induced by JWH-018 at the same doses (3 and 6 mg/kg, ip). However, AKB-48 and 5F-AKB-48 inhibit the visual placing response, with the latter drug being more effective than the former, acting similarly to Δ^9-THC (Canazza *et al.*, 2016). In terms of the aggressive behavioral aspect, JWH-250 and JWH-073 (15 mg/kg, ip) promote aggressive behaviour in mice (Ossato *et al.*, 2016). Moreover, 5F-AKB-48 (6 mg/kg, ip) is more effective than AKB-48 in inducing aggressiveness, showing a comparable effect and duration compared to JWH-018. As observed for tetrad effects, all neurological changes are prevented by pretreatment with AM-251 (6 mg/kg, ip), which does not produce any alterations when administered alone (Canazza *et al.*, 2016; Ossato *et al.*, 2016).

Overall, these behavioral studies, along with *in vitro* receptor binding and *in vivo* neurochemical studies, confirm the higher potency of SCRAs as compared to Δ^9-THC, and suggest that SCRA effects are mostly mediated by CB1Rs since they can be prevented by pretreatment with the CB1R antagonist/inverse agonist AM-251. Altogether the evidence obtained from animal studies confirms

the harmful effects induced by the human consumption of SCRAs, even though the knowledge of their effects are not still enough, especially as concerns the consequences of their chronic use.

	Substances									
Effects	Δ^9-THC	WIN-55, 212-2	JWH-018	JWH-073	JWH-250	AKB-48	5F-AKB-48	Species	Dose regimen	References
Hypothermia	xx							Male Sprague-Dawley rats	Δ^9-THC (0.3–3 mg/kg, i.p.)	Wiley *et al.* (1998)
Analgesia	xx									
Hyperlocomotion	xx									
Catalepsy	xx									
Hypothermia	x		xx					Male CD-1 mice	Δ^9-THC (0.01–100 mg/kg, i.p.) JWH-018 (0.01–6 mg/kg, i.p.)	Vigolo *et al.* (2015)
Analgesia	x		xx							
Hypolocomotion	x		xx							
Catalepsy	x		xx							
Impaired sensorimotor responses (visual, acoustic, and tactile)	x		xx							
Seizures	x		xx							
Aggressiveness	x		xx							
Hypothermia						x	xx	Male CD-1 mice	Δ^9-THC (0.01–100 mg/kg, i.p.) JWH-018 (0.01–6 mg/kg, i.p.) AKB-48 (0.01–6 mg/kg, i.p.) 5FAKB48 (0.01–6 mg/kg, i.p.)	Canazza *et al.* (2016)
Analgesia						x	xx			
Hypolocomotion						x	xx			
Catalepsy						x	xx			
Impaired sensorimotor responses (visual, acoustic, and tactile)						x	xx			
Seizures						x	xx			
Aggressiveness						x	xx			
Hypothermia				x	x			Male CD-1 mice	Δ^9-THC (0.01–100 mg/kg, i.p.) JWH-018 (0.01–6 mg/kg, i.p.) JWH-073 (0.01–15 mg/kg, i.p.) JWH-250 (0.01–15 mg/kg, i.p.)	Ossato *et al.* (2016)
Analgesia				x	x					
Hypolocomotion				x	x					
Catalepsy				x	x					
Impaired sensorimotor responses (visual, acoustic, and tactile)				x	x					
Seizures				x	x					
Aggressiveness				x	x					

i.p., intraperitoneal; x, behavioural effect observed; xx, major behavioural effect observed than x; Δ^9-THC, Δ^9-tetrahydrocannabinol.

Table 3 Studies related to the behavioral effects of SCRAs. Adapted from Pintori et al. (2017)

5.3.3 Pharmacokinetics and metabolism

Another important tool in order to understand the differences between Δ^9-THC and SCRA and thereby their effects on the brain/body, is to study their pharmacokinetics. Focusing on JWH-018 (the reference compound used in our study), the studies on its pharmacokinetics are limited and reported with equal conditions are small, therefore, the comparison of the different studies is difficult. Another point to be made is that different routes of administration, for instance, inhalation might have a faster pharmacokinetic effect than intraperitoneal injection. Moreover, since much clinical evidence indicates that SCRAs can cause severe and persistent, as well as adverse responses in users (Table 4), consistent with preclinical studies, it seems reasonable to hypothesize that this longer duration and severity compared to Δ^9-THC effects, may be due to differences in blood-brain

permeability or the intracerebral distribution of these compounds (Dhawan *et al.*, 2006; Wiebelhaus *et al.*, 2012).

As a matter of fact, Wiebelhaus and collegues (2012) studying the pharmacokinetics of Δ^9-THC and JWH-018 inhalation and their correlation with tetrad effects, reported that 50 mg Buzz (unknown material containing 2.7 mg JWH-018) and 200 mg marijuana (containing 14.8 mg Δ^9-THC) produce antinociception and hypothermia that are similar in magnitude and duration. However, concentrations of JWH-018 (83 ± 42.8 ng/g) in the brain after inhalation of 50 mg of incinerated Buzz were lower than brain concentrations of Δ^9-THC (433 ± 66 ng/g) following 200 mg of incinerated marijuana inhalation (Wilson *et al.*, 2006) indicating that Buzz, and specifically JWH-018, is a potent intoxicant (Wiebelhaus *et al.*, 2012), consistent with its higher potency and efficacy as compared to Δ^9-THC (Atwood *et al.*, 2010; Brents *et al.*, 2011). Moreover, evaluating the tissue concentrations of JWH-018 in the blood and different organs (brain, heart, kidney, liver, lung, and spleen) a dose-dependent relationship between JWH-018 tissue concentration and the quantity of Buzz burned was observed. Although lower concentrations of JWH-018 were found in the blood compared to all organs, JWH-018 was widely distributed throughout all tissues assessed, which is likely due to the lipophilic nature of the compound. Interestingly, the highest concentrations of JWH-018 were found in the tissues associated with absorption (lung), metabolism (liver), and elimination (kidney) (Wiebelhaus *et al.*, 2012).

As matter of fact, Δ^9-THC metabolism has been well studied in the last decades. On the contrary, data about SCRA metabolism and its potential involvement in the pharmacological and toxicological effects of these novel compounds are still lacking. Δ^9-THC is extensively metabolized by cytochrome P450 enzymes CYP2C9 and CYP3A4, but only to a single major active metabolite (11-OH- Δ^9-THC), which shows equivalent CB1Rs affinity (Compton *et al.*, 1993) and slightly higher potency in antinociceptive assays (Ford *et al.*, 1977) as compared to Δ^9-THC. Afterwards, 11-OH- Δ^9-THC is subsequently oxidized to an inactive intermediate 11-nor-9-carboxy- Δ^9-THC that is conjugated to form the O-ester glucuronide, the major metabolite detected in urine (Mazur *et*

al., 2009). In marked contrast, it has been reported that several hydroxylated metabolites of the SCRAs JWH-073, JWH-018, and AM-2201 bind to CB1Rs and CB2Rs, showing a similar affinity compared to the parent compound, as well as biological activity in both in vitro and in vivo assays (Brents *et al.*, 2012; Chimalakonda *et al.*, 2012; Rajasekaran *et al.*, 2013). Moreover, several of the hydroxylated compounds detected following administration of different SCRAs, such as JWH-018, AM-2201, JWH-122, JWH-210, PB-22, MAM-2201, EAM-2201, and 5F-PB-22 represent the major Phase I metabolites formed (Chimalakonda *et al.*, 2012; Rajasekaran *et al.*, 2013; Cannaert *et al.*, 2016), and importantly retain higher in vitro affinity and activity than Δ^9-THC. Focusing on JWH-018, used in our study as a reference SCRA compound, although a major glucuronide conjugate of this compound [JWH-018-N-(5-hydroxypentyl) β-D-glucuronide (also known as 018-gluc)] exhibits reduced affinity for CB1Rs, this metabolite still binds to CB1Rs (in high nM range) and acts as a competitive CB1R antagonist (Seely *et al.*, 2012a). Therefore, these active CB1R metabolites (i.e. hydroxylated JWH-018 metabolites) may contribute to increased half-life, efficacy, and toxicity of SCRAs compared to Δ^9-THC (Weinstein *et al.*, 2017), while competitive CB1R antagonist metabolites (i.e., 018-gluc) could lead to increased SCRA consumption in an attempt to overcome blunted psychoactive effects (Seely *et al.*, 2012a; Ford *et al.*, 2017). Considering JWH metabolites, a recent study evaluating the pharmacokinetics of JWH-018 in mice, reported the presence of at least 3 metabolites in the serum after acute JWH-018 (2.5 mg/kg ip) administration (Malyshevskaya *et al.*, 2017). Besides JWH-018, JWH-018 N-(4-hydroxypentyl), [JWH-018 N-(4-OH)], JWH-018 N-pentanoic acid metabolites [JWH-018 N-COOH], and JWH-018 5-hydroxyindole [JWH- 018 5-OH indole] metabolites were detected. Regarding their pharmacokinetics, serum JWH-018 concentration reached 45.5 ng/ml 5 min after administration, with a peak serum concentration of 87.8 ng/ml at 15 min post-injection (Malyshevskaya *et al.*, 2017), consistent with the effects observed *in vivo* after acute JWH-018 administration (De Luca *et al.*, 2015). After 1 h the serum concentration still remained at 55.2 ng/ml and after 3 h the level of JWH-018 persisted at 35.7 ng/ml, which was still >40% of the peak concentration. Interestingly,

JWH-018 N-(4-OH) metabolite increased in the serum concentration with a time profile similar to JWH-018, reaching its peak of 9.1 ng/ml at 15 min and slowly decaying to 6.4 ng/ml after 1 h and at 2.5 ng/ml at 3 h. On the contrary, the JWH-018-N-pentanoic acid metabolite (JWH-018 N-COOH) displayed a much slower increase in concentration, with the highest measured concentration of 4.9 ng/ ml detected at 3 h after JWH-018 administration. JWH-018 5-OH indole metabolite increased in the serum at a much lower range of concentrations, with a peak at 0.8 ng/ml 15 min after administration (Malyshevskaya *et al.*, 2017). Although these JWH-018 metabolites, as well as the overall SCRA metabolites, might be important since they could exert synergistic effects and reinforce SCRA toxicity, their activity is still unknown. Among JWH-018 metabolites, the JWH-018 N-(4-OH) metabolite exhibited the highest concentration and mimicked the time/concentration curve of JWH-018 (Malyshevskaya *et al.*, 2017). Notably, if in humans the JWH-018-N-pentanoic acid metabolite usually shows the highest concentration among metabolites (Toennes et al., 2017), in mice Malyshevskaya and collegues (2017) observed that its concentration slowly begins to rise after 15 min. Therefore, this discrepancy may reflect a species difference for JWH-018 metabolism and thereby different toxicity. Moreover, as discussed above, different routes of administration represent another important factor that complicates the comparison between different results.

In conclusion, since many SCRA metabolites (but not Δ^9-THC) retain biological activity, defining the metabolic processing of SCRAs is required to fully understand their pharmacokinetic and pharmacodynamic properties, as well the effect/consequences induced by chronic SCRA exposure.

5.3.4 Abuse liability

In these years, the DA rewarding properties of JHW-018 have been studied widely in rodents. In vivo microdialysis, studies in rats showed an increase in DA release in the NAc shell at 0.25 mg/kg (i.p.) (De Luca *et al.*, 2015). Interestingly, lower (0.125 mg/kg, i.p.) and higher doses (0.5 mg/kg, i.p.) were ineffective on DA release, suggesting a dose-dependent effect of JHW-018 with a very

narrow dose range (De Luca *et al.*, 2015). A similar effect was experimentally observed with the compound BB-22, confirming that this peculiar characteristic is also preserved in the subsequent generations of SCRAs. Overall, all generations of SCRAs that share common properties with Δ^9-THC (Tanda *et al.*, 1997) and similarly to other drugs of abuse, are able to stimulate extracellular release of DA selectively in the NAc shell compared with the NAc core (Di Chiara *et al.*, 2004).

Moreover, to evaluate drug abuse liability and reinforcing properties in experimental animals the intravenous self-administration (IVSA) procedure has been considered one of the best validated and most reliable behavioral models for the study of drug dependence (Mansbach *et al.*, 1994; Fattore *et al.*, 2001). Although Δ^9-THC has not been reported to maintain reliable self-administration behavior in rodents, the SCRA WIN 55,212 and JWH-018 have been reported to maintain intravenous self-administration behavior in mice and rats (Martellotta *et al.*, 1998; Fattore *et al.*, 2001; De Luca *et al.*, 2015), suggesting that other high efficacy SCRAs, such as those present in Spice products, might also display reinforcing effects in self-administration procedures. In particular, these studies showed that WIN-55,212-2 (0.5 and 0.1 mg/ kg/injection through the lateral tail vein in mice and 6.25 to 50 μg/kg/injection through the jugular vein in rats) is dose dependently self-administered in rodents according to a concentration-dependent bell-shaped curve, producing either positive and aversive effects depending on the dose administered. In addition, under proper experimental conditions, adult rats and mice intravenously self-administer JWH-018 (30 and 20 μg/kg/infusion, respectively, through the jugular vein) (De Luca *et al.*, 2015). The reinforcing effects of these SCRAs are significantly reversed by the administration of the CB1R antagonist/inverse agonist Rimonabant (Martellotta *et al.*, 1998; Fattore *et al.*, 2001; De Luca *et al.*, 2015), suggesting an involvement of CB1R in SCRA dependence.

These observations are consistent with results obtained using the conditioned place preference (CPP) paradigm, which is widely used to evaluate the motivational effects of drugs in animals (Tzschentke, 2007). Studies carried out in rodents treated with the JWH-073, JWH-081, JWH-210 and HU-210 show that low doses of JWH-073, JWH-081 and JWH-210 (from 0.005 to 0.5mg/kg,

i.p.) elicit CPP whereas higher doses (0.5-1mg/ kg, i.p.) produce conditioned place aversion (CPA) in mice (Brents & Prather, 2014; Cha *et al.*, 2014). However, the potent CBR agonist HU-210 produces CPA in rats starting from low doses (20 and 60 μg/kg, i.p). Notably, CPA produced by SCRAs is consistent with the aversive motivational states induced by these drugs (Cheer *et al.*, 2000). Finally, as described below, repeated CB exposure, both natural and synthetic, induces tolerance to some behavioral effects and withdrawal symptoms, which represent two other important features of drug addiction (Volkow & Morales, 2015).

5.3.5 Tolerance

A well-characterized consequence of chronic marijuana exposure is tolerance to its behavioral effects (Maldonado & Rodriguez de Fonseca, 2002). Indeed, repeated exposure to cannabinoid agonists, including SCRAs, promotes rapid tolerance to the neurophysiological effects of these drugs, both in animals (Abood & Martin, 1992; Maldonado & Rodriguez de Fonseca, 2002; Tai *et al.*, 2015; Elmore & Baumann, 2018) and in humans (Benowitz & Jones, 1975; Hunt & Jones, 1980; Jones *et al.*, 1981; Hollister, 1986; Ramaekers *et al.*, 2011; Gorelick *et al.*, 2013). Considering the natural cannabinoid, rats exposed to Δ^9-THC for 14 consecutive days exhibit less Δ^9-THC-induced hypothermia, catalepsy, and hypolocomotion, and this behavioral desensitization is associated with a reduced ability of Δ^9-THC to increase DA cell firing rate (Wu & French, 2000). As is well known, all drugs of abuse induce an alteration of eCB-dependent mesolimbic plasticity, which could support some behavioral aspects that support addiction (Childress *et al.*, 1993; Hyman *et al.*, 2006). In this respect, similarly to cocaine, sub-chronic (7 day) Δ^9-THC exposure (10mg/kg) can block the eCB-LTD of glutamatergic synapses in the NAc (Huffman *et al.*, 2003). However, unlike cocaine, which did not alter CB1 receptor function, loss of Δ^9-THC-induced eCB-LTD was associated with CB1R desensitization, that has been observed also with a lower dose (3mg/kg) (Mato *et al.*, 2005).

As a matter of fact, it has been demonstrated that long-term Δ^9-THC administration produces region-dependent CB1R desensitization and down-regulation, contributing to the development of CB tolerance (Oviedo *et al.*, 1993; Rodriguez de Fonseca *et al.*, 1994; Ameri, 1999). Moreover, since the CB1R is critically involved in normal NAc signaling, such as in the mediation of food and drug reward (Oleson & Cheer, 2012), its functional disruption by Δ^9-THC exposure is likely to have widespread behavioral implications beyond an altered sensitivity to Δ^9-THC. Clinical studies confirm this evidence, since in the striatum and midbrain of regular marijuana users, CB1R binding capacity was reduced compared with non-users in postmortem human brains (Villares, 2007). As suggested for DA transmission, it is possible that the internalization of CB1Rs is one of the early pharmacodynamic adaptive mechanisms that lead to reduced responsiveness and tolerance in the case of prolonged activation (Martin *et al.*, 2004).

Considering SCRA-induced behavioral tolerance, Sim-Selley and colleague (2002) reported that chronic administration of considerably higher doses (from 3 to 48 mg/kg, sc, twice daily for 15 days) of WIN55,212-2 in mice produces tolerance to cannabinoid-mediated hypoactivity. Moreover, a recent study showed that rats exposed daily for 7 days to JWH-018 (1 mg/kg sc) develop profound tolerance to its hypothermic and cataleptic effects (Elmore & Baumann, 2018). However, if different studies showed a similar tolerance to hypothermic effects of Δ^9-THC, CP-55,940 and WIN 55,212-2 following a Δ^9-THC pretreatment (Pertwee *et al.*, 1993), others showed that these SCRAs partially surmount Δ^9-THC-induced tolerance to locomotor suppression, hypothermia, and antinociception (Fan *et al.*, 1994). Therefore, further studies are required to better understand this phenomenon.

Moreover, as observed after repeated Δ^9-THC exposure, animal studies have demonstrated widespread desensitization and down-regulation of CB1Rs after long-term, but not acute, administration of SCRAs, such as WIN55,212-2 and CP55,940 (Sim-Selley & Martin, 2002; Sim-Selley *et al.*, 2006), while a reduction of CB1R mRNA levels have also been reported in the striatum of animals treated with CP55,940 (Rubino *et al.*, 1994). Consistently, repeated JWH-018

exposure in mice (3mg/kg ip, daily for 4 days) induces region-specific down regulation and desensitization of CB1Rs, together with long-lasting tolerance to some in vivo effects (Tai *et al.*, 2015). Moreover, in vitro JWH-018 potently induces rapid and robust CB1R internalization (Atwood *et al.*, 2010).

Consistently, in parallel with long-lasting alterations in mesostriatal DA function, Perdikaris and colleague (2017) reported a reduction of CB1R binding and mRNA levels in the SN and striatum after chronic WIN55,212-2 administration, providing evidence for a possible regulatory role of the eCB system on DA function.

5.3.6 Withdrawal

Although several animal studies have reported the absence of somatic signs of spontaneous withdrawal after chronic Δ^9-THC treatment in different animal models, even after the administration of extremely high doses of Δ^9-THC (Diana *et al.*, 1998; Maldonado & Rodriguez de Fonseca, 2002), a recent study (Trexler *et al.*, 2018) reported (for the first time) spontaneous Δ^9-THC withdrawal signs after repeated exposure at high doses (10 or 50 mg/kg sc. twice daily for 6 days) in both male and female mice. However, these behaviors were generally of a lower magnitude than those observed when withdrawal was precipitated with CB1R antagonists/inverse agonist Rimonabant (Trexler *et al.*, 2018). In fact, CB1R antagonists are commonly used in animal models of drug dependence to unmask somatic withdrawal signs CB-induced (Diana *et al.*, 1998; Aceto *et al.*, 2001). In particular, following clinical studies (Jones *et al.*, 1976; Budney *et al.*, 1999; Haney *et al.*, 1999) "cannabis-withdrawal syndrome", which is composed of several countable signs, such as scratching, wet dog shakes, facial rubbing, and licking, has been well characterized also in rodents (Diana *et al.*, 1998; Aceto *et al.*, 2001). Moreover, in chronic cannabis users, withdrawal induces stress and anxiety (Haney *et al.*, 1999), effects observed also in rodents (Harte-Hargrove & Dow-Edwards, 2012; Trexler *et al.*, 2018). As a matter of fact, it has been reported that Δ^9-THC

withdrawal, in particular after precipitation, affects stress-related behaviors (i.e. suppression of marble burying), increases plasma corticosterone levels, in addition to well-characterized somatic signs of withdrawal (Trexler *et al.*, 2018). Moreover, marked differences in the EPM have been reported between adolescent male and female rats during spontaneous Δ^9-THC withdrawal (Harte-Hargrove & Dow-Edwards, 2012). Altogether, these data revealed that, in addition to somatic withdrawal signs, emotional circuits results altered during Δ^9-THC withdrawal (Trexler *et al.*, 2018).

Considering SCRAs, differently from Δ^9-THC, different studies reported SCRA-withdrawal signs without precipitation, as well as abnormalities of emotional states in rodents (Aceto *et al.*, 2001; Oliva *et al.*, 2003; 2004; Aracil-Fernandez *et al.*, 2013; Trexler *et al.*, 2018). Spontaneous withdrawal of moderate intensity was observed for the first time after repeated administration of a high dose regiment of WIN 55212-2 (4, 8, 16 and 16 mg/kg/day, ip. on days 1–4, respectively) (Aceto *et al.*, 2001). Moreover, it has been reported that repeated CP-55,940 administration induces somatic withdrawal signs and increases plasma corticosterone (Oliva *et al.*, 2003; 2004), and also reduces time in the dark side of the light/dark box in male mice (Aracil-Fernandez *et al.*, 2013). Recently, Trexler and colleagues (2018) reported spontaneous withdrawal signs after repeated JWH-018 exposure (1mg/kg, sc, twice daily for 6 days) in mice. Notably, as reported during precipitated Δ^9THC withdrawal, together with somatic signs alteration of stress-related behaviors (Trexler *et al.*, 2018) were observed, confirming an alteration of emotional state also during SCRA withdrawal. In human users, robust withdrawal signs are reported after SCRA discontinuation (Nacca *et al.*, 2013). Unfortunately, clinical reviews of SCRA-withdrawal symptoms (Zimmermann *et al.*, 2009; Nacca *et al.*, 2013; Macfarlane & Christie, 2015; Sampson *et al.*, 2015; Cooper, 2016) do not report specific SCRA compounds used, most likely because the users didn't know the composition of the Spice drugs used.

5.3.7 DA dysregulation and cognitive impairment after chronic cannabinoid exposure

In the last years, several research groups have focused their studies on the addictive and rewarding properties of natural cannabinoids (i.e. Δ^9-THC), as well as the cognitive consequences of chronic exposure to them, while there is a lack of data in the literature concerning those cognitive consequences induced by repeated SCRA exposure.

DA dysregulation

Considering the effects on the DA system, while acute Δ^9-THC administration to naive subjects elevates VTA dopamine cell firing and NAc DA release (French *et al.*, 1997; Gessa *et al.*, 1998), repeated exposure depresses DA neuron function. For instance, as discussed above (section 3.2), rats repeatedly (6.5 day) treated with high dose (15mg/kg) of Δ^9-THC exhibit decreased baseline rates of VTA DA cell firing and suppressed bursting activity (Diana *et al.*, 1998). This is consistent with PET measurements of chronic marijuana abusers that suggest a blunted DA signal in the ventral striatum (i.e. NAc) (Volkow *et al.*, 2014). This decreased DA signal correlates with the duration and severity of marijuana abuse and is also associated with greater measures of negative emotional states. In fact, in heavy marijuana users, negative affect "is accompanied by a greater propensity to take non-cannabinoid drugs, and the alleviation or avoidance of a negative emotional state is the primary motivation for marijuana use in this population" (Hyman & Sinha, 2009). Consistently, repeated administration of Δ^9-THC (10 mg/kg twice daily for 14 days) decreases DA PFC turnover in rats, which persist for up to 2 weeks after Δ^9-THC discontinuation (Jentsch *et al.*, 1998; Verrico *et al.*, 2003).

Considering SCRAs, similarly to Δ^9-THC, if acute SCRA administration (i.e. WIN 55,212-2 and HU-210) elevates VTA dopamine cell firing and NAc DA release (Cheer *et al.*, 2003; Cheer *et al.*, 2004), repeated exposure depresses DA neuron function. In fact, it has been reported that animals treated with WIN 55,212-2 for 3 days develop robust tolerance to WIN-induced increases in VTA

DA cell firing (Pistis *et al.*, 2004). Although, only few studies investigated the effects induced by chronic SCRA exposure on the DA system, a recent study (Perdikaris *et al.*, 2018) showed that chronic WIN55,212-2 administration induces long-lasting alterations in mesostriatal DA function and CB1R dysregulation. In particular**,** this study indicates that chronic (20 days), but also acute WIN55,212-2 exposure, at a relatively moderate dose (1 mg/kg, ip) decreased dopamine transporter (DAT) binding and mRNA levels, as well as tyrosine hydroxylase (TH) mRNA expression in the substantia nigra (SN) and VTA. Interestingly, in the striatum, chronic but no acute WIN55,212-2 administration led to decreased protein expression of DAT and TH, suggesting a diminished DA uptake and synthesis after chronic treatment, which might be explained as a compensatory mechanism against excessive DA transmission (Rabinovic *et al.*, 2000). Interestingly, either 7 or 20 days after WIN55,212-2 cessation, a rebound increase in mRNA , binding and total protein levels of DAT and TH in the VTA, the SN and striatum, as well as in CB1R binding levels has been observed, suggesting the presence of a biphasic expression pattern (Perdikaris *et al.*, 2018). Consistently, Fanarioti et al. (2014) previously reported a reduction of DAT mRNA and binding levels in the SNpc and VTA after chronic WIN55,212-2 treatment (20 days) at a similar dose (1 mg/kg, ip), as well as at lower drug doses (0.1-0.3 mg/kg, ip). Moreover, as observed after repeated Δ^9-THC administration, Verrico and colleagues (2003) demonstrated that repeated administration of WIN55212-2 (10 mg/kg, ip, twice daily for 14 days) also induces a persistent (up to week after drug discontinuation) decrease DA turnover in the PFC, an effect reversed by an amphetamine challenge (Verrico *et al.*, 2003). Interestingly, this alteration of DA activity, different from Δ^9-THC, was observed also after 7 days (twice daily) of WIN 55212-2 (10 mg/kg, ip) administration (Verrico *et al.*, 2003), and might be due to the higher potency of SCRAs as compared to Δ^9-THC. However, no evidence of DA dysregulation induced by repeated JWH-018 exposure has been reported.

Cognitive impairments

Considering natural CB-induced cognitive impairments, both acute and chronic marijuana exposure are associated with dose-related cognitive impairments, especially in attentional processes, working memory, and memory functions in animals (Castellano *et al.*, 2003; Zanettini *et al.*, 2011) and in humans (Pope *et al.*, 2001; Bolla *et al.*, 2002; Solowij & Battisti, 2008). However, besides reduced learning, heavy cannabis use is also associated with a decreased mental flexibility, increased perseveration and reduced ability to sustain attention (Lundqvist, 2005). Hence, long-term heavy cannabis users show impairments in memory and attention that, in some cases are persistent and get worse with increasing years of regular cannabis use (Solowij *et al.*, 2002). Moreover, marijuana use has been associated with low academic achievement, legal problems, unemployment and risk for the development of psychotic disorders (Friedman *et al.*, 2001; Ferdinand *et al.*, 2005; Henquet *et al.*, 2005; Hall & Degenhardt, 2009). Importantly, chronic cannabis use in humans is associated behaviorally with amotivational syndrome and cognitive impairments (Lane *et al.*, 2005; Hester *et al.*, 2009), while neurochemically with hypofrontality (Hester *et al.*, 2009; Martin-Santos *et al.*, 2010). Hypofrontality is defined as "a reduction in neuronal activity/ function of the frontal cortex observed by brain imaging glucose metabolism and fMRI studies" (Egerton *et al.*, 2006; Goldstein & Volkow, 2011), while, at the neurochemical level, hypofrontality "is associated with an imbalanced cortical–subcortical network that involves dysregulation of DA, but also of other neurotransmitter systems such as glutamate" (Prohovnik *et al.*, 1997; Del Arco & Mora, 2008; El Khoury *et al.*, 2012). Alterations of cognitive functions following Δ^9-THC exposure have been reported also in animal models. For instance, attentional deficits have been observed following sub-chronic Δ^9-THC administration (10 mg/kg twice daily for 14 days) in the rat (Verrico *et al.*, 2004). Interestingly, attentional deficits after repeated CB exposure seem similar to those observed with lesions of the medial PFC or striatum (Burk & Mair, 2001; Christakou *et al.*, 2001; Egerton *et al.*, 2006).

Considering SCRA-induced cognitive impairments, few studies have investigated the impact of chronic SCRA exposure on cognitive functions and whether these impairments persist after periods of drug abstinence (Egerton *et al.*, 2006). However, as discussed above, similarly to Δ^9-THC, repeated administration of WIN55212-2 (10 mg/kg, ip, twice daily for 14 days) induces a persistent (up to a week after drug discontinuation) decrease in DA turnover in the PFC, an effect reversed by an amphetamine challenge (Verrico *et al.*, 2003). Therefore, it seems reasonable to hypothesize that, as observed after chronic Δ^9-THC exposure, this DA hypoactivity may lead to some cognitive impairments (Verrico *et al.*, 2003; Verrico *et al.*, 2004). In line with this hypothesis, it has recently been demonstrated both in vitro and in vivo, that acute exposure to WIN 55,212-2 and CP-55,940 caused a significant shift of E/I balance inputs, toward excitation, in layer II/III pyramidal neurons of the PL area of the mPFC (den Boon *et al.*, 2015). Therefore, this modulation of the E/I balance by SCRAs may be fundamental in the regulation of local PL cortical network activity and might represent a mechanism through which chronic SCRA exposure might affect cognitive functions (den Boon *et al.*, 2015).

Therefore, further studies are required to understand the possible alteration of the DA system, as well as cognitive impairments induced by chronic SCRA exposure.

5.3.8 Toxicity in humans

As discussed above, although advertised as "safe and legal" alternatives to marijuana on the Internet, SCRAs have proved to be dangerous novel chemicals, inducing several and severe adverse effects that are distinct from those produced by marijuana (Ford *et al.*, 2017) (Table 4). In particular, clinical case studies have documented markedly greater toxicity following acute use of K2/Spice compared to marijuana, across a broad number of organ/tissue systems, including gastrointestinal (Gurney *et al.*, 2014; Ukaigwe *et al.*, 2014; Zawilska & Wojcieszak, 2014; Abrams & Guzman, 2015; Besli *et al.*, 2015), neurological (Muller *et al.*, 2010; Vardakou *et al.*, 2010; Benford & Caplan, 2011; de Havenon *et al.*, 2011; Schneir *et al.*, 2011; Peglow *et al.*, 2012; Vandrey *et al.*, 2012; Young *et al.*, 2012; Hermanns-Clausen *et al.*, 2013; Yeakel & Logan, 2013; Haro *et al.*, 2014; Meijer *et al.*, 2014; Sheikh *et al.*, 2014; Takematsu *et al.*, 2014; Zawilska & Wojcieszak, 2014; Abrams & Guzman, 2015; Chase *et al.*, 2016), cardiovascular (Jones, 2002; Schneir *et al.*, 2011; Schneir & Baumbacher, 2012; Young *et al.*, 2012; Hermanns-Clausen *et al.*, 2013; Clark *et al.*, 2015; McIlroy *et al.*, 2016), and renal (2013; Bhanushali *et al.*, 2013; Kazory & Aiyer, 2013) systems. In addition, it has been reported that SCRAs induce seizures and convulsions (Lapoint *et al.*, 2011; Schneir & Baumbacher, 2012; McQuade *et al.*, 2013; Schep *et al.*, 2015; Katz *et al.*, 2016) although in these years a growing amount of interest in medical cannabis as a treatment for epilepsy and other seizure disorders (Reddy & Golub, 2016) has been observed. This latter difference between SCRAs and Δ^9-THC has been confirmed by animal studies, supporting the notion that high-efficacy SCBs exhibit unexpected proconvulsant effects (Vigolo *et al.*, 2015; Canazza *et al.*, 2016; Ossato *et al.*, 2016).

Furthermore, although development of dependence to marijuana is rare, chronic use of SCRAs can lead to tolerance, dependence, and withdrawal (Wikler, 1976; Zimmermann *et al.*, 2009; Every-Palmer, 2011). Moreover, the most alarming reports have shown fatalities after SCRA intoxication

(Trecki *et al.*, 2015). Taken altogether, the clinical cases reported in the literature clearly indicate that SCRAs cannot be considered as a "safe alternative" to marijuana (Ford *et al.*, 2017).

Adverse effects and toxicities	Observed with K2/Spice products (SCBs)	Observed with marijuana (THC)
Gastrointestinal		
• Nausea	Common	Rare
• Vomiting	Common	Rare
• Hyperemesis syndrome	Common	Rare
Neurological		
• Euphoria	Common	Common
• Appetite stimulation	Common	Common
• Nystagmus	Reported	Reported
• Slurred speech	Reported	Reported
• Ataxia/lethargy	Reported	Reported
• Psychosis in susceptible individuals	Extreme	Mild
• Hypothermia	Reported	None reported
• Hallucinations	Common	Rare
• Delusions	Common	Rare
• Confusion	Common	Rare
• Anxiety	Common	Rare
• Panic attacks	Common	Rare
• Agitation	Common	Rare
• Irritability	Common	Rare
• Confusion	Common	Rare
• Memory disturbances	Reported	Common
• Self-mutilation	Reported	None reported
• Seizures	Reported	None reported
• Catatonia	Reported	Very rare
• Acute cerebral ischemia	Reported	None reported
Cardiovascular		
• Tachycardia	Reported (can lead to tachyarrhythmia)	Reported (devoid of tachyarrhythmia)
• Hypertension	Reported	None reported
• Hypotension	None reported	Reported
• Chest pain	Reported	None reported
• Cardiotoxicity (i.e., myocardial toxicity)	Reported	None reported
Renal		
• Acute tubular necrosis	Reported	None reported
• Acute interstitial nephritis	Reported	None reported
• Acute kidney failure	Reported	None reported
Effects of chronic use		
• Tolerance	Common	Common
• Marked withdrawal	Reported	Mild
• Dependence	Reported	Rare
Deaths (between 2011 and 2014)	Over 20 deaths reported	None reported

Table 4. SCRA toxicity in Humans: a comparison with marijuana. Modified from Ford et al. (2017)

B-Aim of the study

JWH-018 has been detected in more than 140 specimens of cannabinoid-containing herbal blends, broadly known as Spice and K2 drugs, which started being used recreationally as "legal, natural, and safe" alternatives to marijuana (Carroll *et al.*, 2012), and have been sold worldwide in smart shops and online to circumvent legal issues since 2004 (Fattore & Fratta, 2011; Gurney *et al.*, 2014) http://www.deadiversion.usdoj.gov/). JWH-018 is a synthetic cannabinoid receptor agonist (SCRA), highly potent and efficacious at CB1 and CB2 receptors (CB1R and CB2R)(Huffman & Padgett, 2005; Wiley *et al.*, 2012). It is considered the prototypical compound of the so-called 'first generation' class of SCRA since newer generations arose from slight modifications to the chemical structure of JWH-018 (De Luca *et al.*, 2016; Miliano *et al.*, 2016; De Luca & Fattore, 2018). These modifications not only generated legal drugs, at least for a certain period of time, but also led to SCRA with higher potency and efficacy at CBR, and with increased abuse potential generation after generation (De Luca *et al.*, 2016; De Luca & Fattore, 2018). In humans, differently from Δ^9-THC, SCRAs are able to induce more adverse reactions, long-term effects and psychiatric consequences, several withdrawal symptoms, and more prevalence of dependence after their chronic use (Papanti *et al.*, 2014; Schifano *et al.*, 2015). Most likely, these effects are due to their higher potency and affinity for CB1R (full agonist) compared with Δ^9-THC (partial agonist) (De Luca *et al.*, 2016; Miliano *et al.*, 2016; Pintori *et al.*, 2017). Previous studies by our group demonstrated that JWH-018 shares with Δ^9-THC CB1 receptor-dependent reinforcing properties and dopamine (DA) stimulant actions, displaying a preferential effect on the nucleus accumbens (NAc) shell at the dose of 0.25 mg/kg (ip), thus confirming the higher potency of this compound when compared to Δ^9-THC, which is able to increase NAc shell DA at the dose of 1 mg/kg ip (De Luca *et al.*, 2015). It has been demonstrated that the ability to stimulate DA transmission in the NAc shell is a marker of abuse liability of the drugs, independent of their pharmacological mechanisms of action (Di Chiara *et al.*, 2004). The role of DA transmission in processing emotionally salient information and in

reward processes is well known. As matter of fact, the mesolimbic and the mesocortical DA pathways play a pivotal role in the evaluation of the motivational value of rewarding stimuli. In particular, both drugs of abuse and natural rewarding stimuli, as highly appetitive taste stimuli, increase DA transmission in the NAc shell (Di Chiara, 1990a; Bassareo *et al.*, 2002; Volkow *et al.*, 2003; Fillenz, 2005). However, for taste stimuli this effect undergoes adaptive regulation (termed "habituation") after a single pre-exposure to the same taste stimuli in the NAc shell but not in mPFC, supporting the hypothesis that NAc shell DA is activated by unfamiliar appetitive stimuli while DA in the mPFC codes for generic motivational value (Di Chiara, 1990a; Bassareo & Di Chiara, 1997; 1999; Bassareo *et al.*, 2002; Di Chiara *et al.*, 2004). Moreover, previous studies by our group showed that habituation of DA responsiveness in NAc shell to gustatory stimuli is dependent upon an intact mPFC DA function (Bimpisidis *et al.*, 2013; De Luca, 2014). In fact, bilateral lesions of mPFC by 6-OHDA did not affect NAc shell DA responsiveness to a first chocolate exposure (naive rats), but abolished habituation to the second chocolate exposure. These data, consistent with the results observed in morphine sensitized rats (De Luca *et al.*, 2011), suggest top-down control of mPFC DA on NAc DA and a possible role in the loss of control of the motivational value of stimuli, confirming a reciprocal relationship between these two DAergic areas. It is well established that endocannabinoid (eCB) signalling plays a crucial role in the processing of emotionally salient information and its relationship with DA transmission (Hernandez & Cheer, 2012; Tan *et al.*, 2014; Covey *et al.*, 2015; Covey *et al.*, 2017). Indeed, considerable evidence has shown an important interaction between eCB system and DA transmission in reward processes (Maldonado *et al.*, 2006; Volkow *et al.*, 2017). In particular, it has been demonstrated that dysfunction of eCB signaling, may lead to a dysregulation of mesocorticolimbic DA transmission in the processing of evaluation of salience information related to several neuropsychiatric disorders and addiction (Tan *et al.*, 2014). This evidence is consistent with the effects of eCB signalling dysregulation induced by the heavy use of Δ^9THC (Volkow *et al.*, 2003; Volkow & Morales, 2015; Volkow *et al.*, 2017). In fact, chronic use of marijuana in humans increases impulsivity (Cousijn *et*

al., 2013; Dougherty *et al.*, 2013), induces psychomotor and cognitive deficits, reduces impulse control and increases risk-taking behaviours (Hall, 1998; Iversen, 2003), which are probably associated with dysfunctions of the cortico-limbic-striatal DA circuit (Lane *et al.*, 2005; Hester *et al.*, 2009; Martin-Santos *et al.*, 2010). In addition, accumulating evidence strongly supports an important contribution of glial cells in brain plasticity and in affective, motivational and cognitive processes, which are altered in drug addiction disorder, and might contribute to the vulnerability and persistence of addictive behaviors (Beattie *et al.*, 2002; Ross *et al.*, 2003; Yirmiya & Goshen, 2011; Rossi *et al.*, 2012; Marin & Kipnis, 2013). Consistently, important alterations of the inflammatory system, as well as of glial cells, produced by heavy use of CBR agonists have been observed (Melis *et al.*, 2017). Moreover, it has been suggested that there is important involvement of the eCB system in drug-induced glial alterations and their role in drug addiction (Scofield & Kalivas, 2014; Scofield *et al.*, 2016; Lacagnina *et al.*, 2017).

Despite the growing use of Spice/K2 drugs, the effects induced by repeated exposure to SCRA on DA transmission and their putative relation with the neurobiological basis of drug dependence and withdrawal, as well as their neuroinflammatory effects, have not been yet extensively characterized.

Based on these premises, the aim of this study is to evaluate, in adult male Sprague-Dawley rats, neurochemical, electrophysiological, behavioral, and neuroinflammatory effects of repeated JWH-018 exposure, at a dose able to selectively increase DAergic transmission in the NAc shell (0.25 mg/kg, ip, qd, 14 days). In particular, we focused our research on:

1) Dysregulation of DA system that plays a crucial role in drug addiction, in particular either on basal neuronal activity or on responsiveness of mesocortical and mesolimbic DA transmission to repeated exposure to appetitive (i.e. motivational) taste stimuli; 2) spontaneous withdrawal signs; 3) behavioral effects (i.e. anxiety, compulsive-like behavior, sensorimotor-gating); 4) neuroinflammatory effects (i.e. astrocytes and microglia cells alterations) on DA brain areas that might contribute to the differential effects induced by repeated JWH-018 exposure.

C-Materials and Methods

1 Animals

Male Sprague-Dawley rats (Harlan, Italy) weighing 275-350 g were used for all these studies. Animals were housed in groups of six in standard plastic cages with wood chip bedding, at temperature of 22 ± 2 °C and 60% humidity and under a 12 h light/dark cycle (lights on from 7.00 a.m.). Tap water and standard laboratory rodent chow (Mucedola, Settimo Milanese, Italy) were provided *ad libitum* in the home cage. All animal experiments were carried out in accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research according to Italian (D.L. 116/92 and 152/06) and European Council directives (609/86 and 63/2010) and in compliance with the approved animal policies by the Ethical Committee for Animal Experiments (CESA, University of Cagliari) and the Italian Ministry of Health *(Aut. N.* 162/2016-PR). We made all efforts to minimize pain and suffering, and to reduce the number of animals used.

2 Experimental design timeline

Rats were injected daily with either vehicle or JWH-018 (0.25 mg/kg) intraperitoneally (ip) for 14 consecutive days. Afterward, animals were assigned randomly to different experimental groups, as described below and in the timeline of experiment (Fig.12):

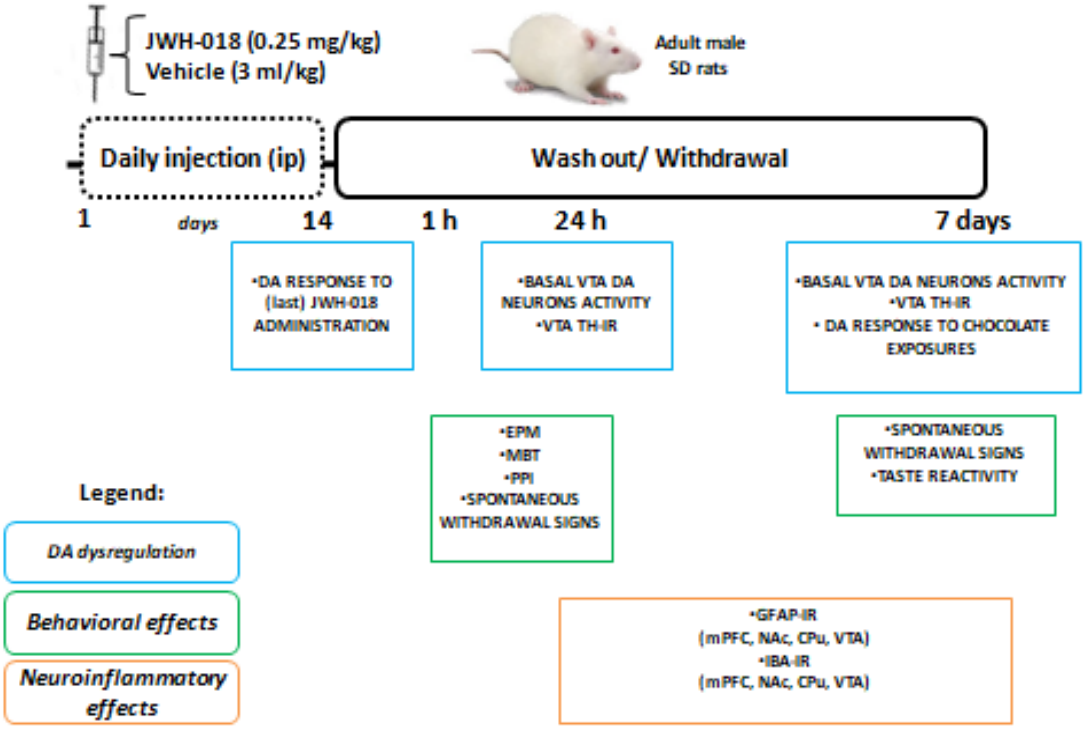

Fig.12 The timeline of the experiments

1- DA dysregulation: to disclose whether repeated JWH-018 administration induced alteration on DA systems we performed, at different time point, the following experiments: 1) evaluation of DA response to JWH-018 during the last day of administration; 2) evaluation of basal VTA DA neurons activity, as well TH-immunoreactivity 24 hours and 3) seven days after repeated JWH-018 exposure; 4) evaluation of DA response to repeated chocolate exposure 7 days after JWH-018 discontinuation.

2- Behavioral effects: to disclose whether repeated JWH-018 administration induced behavioral effects and spontaneous signs of withdrawal, we performed at different time

point the following behavioral tests: 1) marble burying (MB), elevated place maze (EPM), prepulse inhibition (PPI) 1 hour and 2) 24 hours after last JWH-018 injection; 3) behavioral observation of spontaneous withdrawal signs JWH-018- induced 24 hours and 4) seven days after JWH-018 discontinuation; 5) taste reactivity test during chocolate exposures (seven days after repeated JWH-018 exposure).

3- Neuroinflammatory effects: to disclose whether repeated JWH-018 administration induced astrocytes and microglia cells alterations on DA brain areas, we performed at different time point the following immunohistochemical assays: 1) evaluation of Glial Fibrillary Acidic Protein.(GFAP) and Ionized calcium-Binding Adapter molecule 1 (IBA-1) Immunoreactivity (IR) on selected DA brain areas 24 hours and 2) seven days after repeated JWH-018 administration.

A detailed description of different techniques used in the present study is provided in the following paragraphs.

3 Drugs and solutions

JWH-018 was purchased from Tocris (Bristol, UK), and solubilized in 0.5% EtOH, 0.5% Tween 80 and 99% saline. A chocolate solution, containing chocolate syrup (Nesquik Squeeze©, Nestle, S.A., Vevey, Switzerland) and tap water (1:1), has been used as gustatory taste stimulus. The chocolate syrup contained sucrose, water, cacao, corn syrup, citric acid, salt, potassium sorbate and artificial flavour.

4 Probe and oral catheter preparation

Vertical microdialysis probes, with an active dialysing portion of 1.5 mm for NAc and 3 mm for mPFC, were prepared with AN69 fibers (Hospal Dasco, Bologna, Italy), as previously described (De Luca *et al.*, 2011) according to the method of Di Chiara et al. (1993) as modified by Tanda et al. (1996). The oral catheters were made of a 22-G stainless steel needle and polyethylene (PE)

tubing (Portex Ltd, Hythe, Kent, England) (ID 0.58 mm, OD 0.96 mm). The needle was cut at one side (total length of 2 cm from the tip), the cut part was blunted and inserted in the PE tubing, which ending at a perforated circular disc.

5 Surgery

In order to evaluate the neurotransmitters extracellular levels by in vivo brain microdialysis, and to infuse the taste stimulus (i.e chocolate solution), rats were anaesthetized with Equithesin (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) and were implanted with microdialysis probe and oral catheters. The microdialysis probe (1.5 or 3 mm dialyzing portion for NAc or mPFC, respectively) was implanted in the NAc shell (A+2.2, L+1.0 from bregma, V-7.8 from dura) or core (A+1.4, L+1.6 from bregma, V-7.6 from dura) or in the mPFC (A+3.7, L+0.8 from bregma, V-5.0 from dura) according to Paxinos & Watson (2007). In the same session, an oral catheter was inserted at the level of the first molar, passed along the space between the temporalis muscle and the skull by the tip of the 22G needle, and fixed on the top of the head of the rat with a small plastic tip filled with cyanoacrylate glue (Fig. 3).

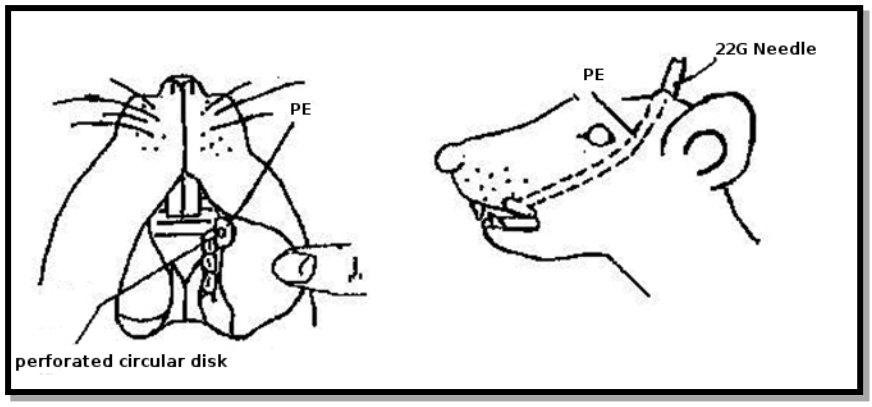

Fig. 13 Schematic representation of the position of implantation of the intraoral catheter in the rat

6 Neurochemical and neurofunctional assays

6.1 In vivo brain microdialysis: DA extracellular levels

In vivo brain microdialysis is a method that permits the collections of samples from the extracellular space of any desired brain region while the animal is freely moving. The method of in vivo brain microdialysis is based on the diffusion of substances between the extracellular space in the brain and an artificial cerebrospinal fluid (ACSF – Ringer's solution) through a microdialysis probe equipped with a semipermeable membrane (Hernandez *et al.*, 1986; Di Chiara, 1990b). In the present study, within the two days following the surgery, *in vivo* microdialysis experiments were performed to measure DA extracellular levels in the NAc shell, NAc core and mPFC according to the procedure previously described (Bimpisidis *et al.*, 2013).

The day of experiment, probes were perfused with Ringer's solution (147 mM NaCl, 4 mM KCl, 2.2 mM $CaCl_2$) at a constant rate of 1 µl/min. Dialysate samples (10 µl) were injected into an HPLC equipped with a reverse phase column (C8 3.5 um, Waters, USA) and a coulometric detector (ESA, Coulochem II) to quantify DA. The first electrode of the detector was set at +130 mV (oxidation) and the second at -175 mV (reduction). The composition of the mobile phase was: 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.

After the evaluation of DA basal levels, estimated as the mean of three consecutive samples which values did not differ more than ±10%, animals either were injected with the last JWH-018 dose (day 14) or seven days after the last injection were intraorally infused with a chocolate solution (1 mL/5 min) (see Fig.12) and the DA extracellular levels were monitored up to 2 hours after JWH-018 injection/chocolate infusion. At the end of microdialysis experiments, animals were sacrificed, brains were removed and stored in formalin (8%) for histological examination to verify the correct placement of the microdialysis probe.

6.2 In vivo electrophysiology: VTA DA neurons single unit recording

24 hours or seven days after JWH-018 discontinuation, rats were anaesthetized with urethane (1.3 g/kg, ip) and placed in the stereotaxic apparatus (Kopf, Tujunga, CA, USA) with their body temperature maintained at 37±1°C by a heating pad. The scalp was retracted, and one small hole was drilled above the parabrachial pigmented nucleus (PBP) of the posterior VTA according to the Atlas of Rat Brain (Paxinos and Watson, 2007). Extracellular single-unit activity of dopamine neurons located in the VTA (A: -5.8 to -6.2 from bregma, L: +0.4 to +0.6 from midline, V: -7.0 to -8.0 from the cortical surface) was recorded with glass micropipettes filled with 2% pontamine sky blue (PSB) dissolved in 0.5 M sodium acetate (impedance 2.5–5 MΩ). The population spontaneous activity of VTA dopamine cells was determined descending the electrode within the area in 6-9 predetermined tracks, separated by 200 μm each other.

Putative VTA DA neurons were selected when all criteria for identification were fulfilled: firing rate <10 Hz and duration of action potential >2.5 ms as measured from start to end (Grace & Bunney, 1983). Bursts were defined as the occurrence of two spikes at interspike interval <80 ms, and terminated when the interspike interval exceeded 160 ms (Grace & Bunney, 1984). The electrical activity for each neuron was recorded for 2-3 minutes. Single-unit activity was filtered (bandpass 0.1–10000 Hz) and individual action potentials were isolated and amplified (Neurolog System, Digitimer, Hertfordshire, UK), displayed on a digital storage oscilloscope (TDS 3012, Tektronics, Marlow, UK). Experiments were sampled on line and off line with Spike2 software (Cambridge Electronic Design, Cambridge, UK) by a computer connected to CED 1401 interface (Cambridge Electronic Design, Cambridge, UK). At the end of recording sessions, DC current (15 mA for 15 min) was passed through the recording micropipette in order to eject PSB for marking the recording site. Brains were then rapidly removed and were frozen in isopentane cooled to -40 °C. The position of the electrodes was microscopically identified on serial 60 μm sections stained with Neutral Red.

7 Behavioural assays

7.1 Marble Burying test

The Marble Burying test (MB) has been proposed as animal model of compulsive activity/ repetitive like- behaviours (Albelda & Joel, 2012). In this study, MB was performed 1 hour, and 24 hours after the last injection in order to evaluate the presence of compulsive activity in rats induce by repeated treatment with JWH-018 and during acute JWH-018 withdrawal period. Before starting the experiment, rats were placed for a 2 h period of acclimation into the experimental room. In the test performed 1 hour after the last injection, animals were pre-treated with either JWH-018 (0.25 mg/kg ip, 14 consecutive days) or vehicle (3ml/kg) 1 hour before starting MB. The MB was conducted into an open transparent plastic cage (54×34.5×20cm) with 5 cm of fresh hardwood chip bedding as previously described (Satta *et al.*, 2016). Twenty-four standard glass marbles (1.5 cm in diameter, arranged in six rows of four marbles each) were placed uniformly over the surface. Individual rats were placed in the test cage and the activity was monitored for 30 min by a video camera placed above the cage. At the end of the session, animals were gently removed from the cages, and the number of marbles partially (≥67%) and totally (>95%) buried was counted (Anymaze Software, Stoelting, Illinois, USA) as previously characterized (Satta *et al.*, 2016; Zanda *et al.*, 2017). New bedding was used for each animal, and marbles were cleaned with soap and tap water.

7.2 Elevated Plus Maze

The elevated plus maze (EPM) has been described as a simple method for assessing anxiety responses of rodents (Dawson *et al.*, 1994; Rodgers *et al.*, 1997). In the present study, the EPM paradigm was used to evaluate possible anxiogenic effects in rats induced by repeated JWH-018 administration and due to acute JWH-018 withdrawal syndrome. Rats' performance in the EPM was measured 1 hour and 24 hours after the last injection in order to evaluate respectively anxiogenic

effects during and after repeated treatment with JWH-018 The day of experiment, rats were placed for a 2 h period of acclimation into the experimental room. In the test performed the 1 hours after the last inection, rats were pre-treated with either JWH-018 (0.25 mg/kg ip) or vehicle (3ml/kg) 1 hour before starting the test. The EPM was made of white PVC and consisted of two opposite open arms (length 50 cm, width 10 cm) and two opposite closed arms (length 50 cm, width 10 cm), the latter enclosed by 40 cm high walls along their length. The four arms converged to a central square (10×10 cm), thus reproducing the shape of a plus sign. The apparatus was elevated 50 cm from the floor. Rats having no prior experience of the EPM were placed in the central square, and were left free to explore the whole apparatus for a single 5 min test session. The experiments were performed under an illumination of 40 lux, which was uniform in both the open and closed arms of the apparatus. Rats' performance was videotaped, and percentages of arm entries as well as of time spent in open and closed arms were calculated with respect to the total number of entries and to the total amount of time spent in the arms, respectively. A rat was considered inside a specific arm when it had all the four paws inside the arm.

7.3 Prepulse Inhibiton

The prepulse inhibition (PPI) was performed 1 hour, 24 hours after the last injection in order to evaluate attentional deficits induced by repeated JWH-018 exposure and due to acute JWH-018 withdrawal syndrome. The day of experiment, rats were placed for a 2 h period of acclimation into the experimental room. In the test performed the 1 hour after the last administration, rats were pre-treated with either JWH-018 (0.25 mg/kg ip) or vehicle (3ml/kg) 1 hour before starting the test. The startle reflex system consisted of 4 standard cages each placed inside a sound-attenuated and ventilated chamber (Med Associated, USA). Startle cages were non-restrictive Plexiglas cylinders (diameter 9 cm) mounted on a piezoelectric accelerometer platform connected to an analogue-digital converter. Background noise and acoustic bursts were conveyed through two speakers placed in proximity to the startle cage so as to produce a variation in sound intensity within 1 dB. On test

day, each rat was placed in the experimental cage for a 5 min acclimatization period with a 70 dB white noise background; this was continued for the remainder of the session. Animals were then tested on 3 consecutive trial blocks. The first and the third blocks consisted of 5 pulse-alone trials of 40 ms at 115 dB, while the second block (test block) was a pseudorandom sequence of 50 trials including 12 pulse-alone trials, 30 pulse trials preceded by 73, 76 or 82 dB prepulses (10 for each level of prepulse loudness), and 8 nostimulus trials (where the only background noise was delivered). The percent (%) PPI was calculated based only on the values relative to the second block, and using the following formula: 100- [(mean startle amplitude for prepulse + pulse trials/mean startle amplitude for pulse-alone trials) × 100].

7.4 Spontaneous behavioural signs of withdrawal

24 hours and seven days after repeated JWH-018 administration, we evaluated behavioural signs of withdrawal JWH-018 induced. Before electrophysiological recordings, individual rats were placed in plastic cages (30x25x45 cm) with standard rat bedding on the floor. Cages were located in a sound-proof room for behavioural observation. Point-scoring was performed by an observer blind to treatment (placed behind a one-way window). The spontaneous cannabinoid withdrawal signs were scored (Diana *et al.*, 1998) with counted signs (total number of events over a 30 min period of time) such as scratching, wet dog shakes, facial rubbing, and licking.

7.5 Taste reactivity

The taste reactivity test has been utilized as an operational estimate of hedonic valence (positive or negative) and hedonic impact of tastes (Grill & Norgren, 1978; Berridge, 2000). In the present study, behaviour was recorded during the microdialysis experiments; the effect on taste reactivity of the chocolate solutions through the oral catheter has been recorded, as previously reported (De Luca *et al.*, 2011; Bimpisidis *et al.*, 2013). The oral catheter was connected to an infusion pump and the chocolate solution was pumped at a constant rate of 0.2 mL/min, to a total of 1 mL/5 min. During

the taste reactivity test, animals were monitored and two classes of taste reactivity patterns were scored – positive hedonic (appetitive) and negative hedonic (aversive). Positive hedonic reactions were characterized by paw licks, lateral tongue protrusions, and rhythmic tongue protrusion; aversive reactions were characterized by face washing, forelimb flails, gapes, chin rubs, paw tread, and locomotion. Each reaction was assigned with one point if the duration was between 1 and 5 s and two points if the duration was more than 5 s.

8 Immunohistochemical assays

8.1 Brain tissue preparation and GFAP, IBA-1 and TH immunofluorescent staining

24 hours or seven days after the last injection, rats were deeply anaesthetized with Equithesin (0.97 g pentobarbital, 2.1 g magnesium sulphate, 4.25 g chloral hydrate, 42.8 mL propylene glycol, 11.5 mL ethanol 90%, 5 mL kg ^{1}, ip) and then transcardially perfused with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4). Brains were rapidly removed and post-fixed in the same fixative for 6 h. After repeated washing in 0.1 M PBS, brains were cryoprotected in 30% sucrose in PBS for 48 h. Immunostaining was performed on free-floating coronal sections (thickness: 40 mm) which were obtained using a vibratome at levels comprising the brain areas selected for this study. To facilitate the identification of the selected brain areas, adjacent sections were also collected and stained with Neutral Red.

Pre-blocking of tissue sections was performed using normal goat serum (NGS, 10%), bovine serum albumin (BSA, 1%) and Triton X-100 (0.2%) in PBS for 1 h at room temperature. As concerns GFAP-immunofluorescence, tissue sections were incubated at 4 C for 24 h with a mouse monoclonal anti-GFAP antibody (1:5000; Millipore Temecula, CA, USA) in PBS containing 0.2% Triton X-100, 0.1% BSA, and 1% NGS. Then, sections were washed in PBS containing 0.2% Triton X-100 and incubated them with Alexa Fluor 594-labeled goat anti-mouse IgG (1:500; Molecular Probes, Eugene, OR, USA) for 1 h in the dark at room temperature.

As concerns IBA-1 immunofluorescence, we incubated tissue sections at 4°C for 24 h with a rabbit monoclonal anti-IBA-1 antibody (1:2000; Wako Pure Chemical Industries, Chuo-Ku, Osaka, Japan) in PBS containing 0.2% Triton X-100, 0.1% BSA, and 1% NGS. Then, sections were washed in PBS containing 0.2% Triton X-100 and incubated them with Alexa Fluor 488-labeled goat anti-rabbit IgG (1:500; Molecular Probes, Eugene, OR, USA) for 1 h in the dark at room temperature. For TH-immunofluorescence, tissue sections were incubated at 4 °C for 24 h with a mouse monoclonal anti-TH antibody (1:1000; Millipore Temecula, CA, USA) in PBS containing 0.2% Triton X-100, 0.1% BSA, and 1% NGS. Then, sections were washed in PBS containing 0.2% Triton X-100 and incubated them with Alexa Fluor 488-labeled goat anti-mouse IgG (1:500; Molecular Probes, Eugene, OR, USA) for 1 h in the dark at room temperature. Finally, all sections were rinsed and mounted on slides using VectaShield anti-fade mounting media (Vector Inc.). Moreover, standard control experiments by omitting of either the primary or secondary antibody have been performed; no cellular labeling was yielded.

8.2 Imaging and quantitative analysis of GFAP, IBA-1 and TH immunofluorescent staining

An Olympus IX 61 microscope, furnished with 2.5, 4, 10, 20 and 60X planapochromatic oil immersion objectives, was used for observations. An Olympus 12-bit cooled F View II camera (Hamburg, Germany) was used for capturing the images. Excitation light was attenuated with a 6% transmittance neutral density filter.

For each animal, analysis of GFAP and IBA-1 immunoreacitivity (IR) was performed on one tissue section out of every 3 successive sections, for a total of 8, 6, 16, and 12 sections containing the Prefrontal Cortex (PFC), the Nucleus Accumbens (NAc shell and core), the Caudate Putamen (CPu) and Ventral Tegmental Area (VTA), respectively. TH-IR analysis was performed only in the VTA sections.

In order to include almost the whole area (either PFC, CPu, NAc, VTA), according to the extension of the region under analysis, we have chosen the total size of the examined area in which GFAP-IR, TH- and IBA-1-IR cells were counted. The selected coronal levels of these sections corresponded to the levels of plates 7-9 for the PFC (AP: +3.70 to +2.70), 13-15 for NAc shell and core (AP: +1.20 to +0.70), 11–19 for the CPu (AP: +1.70 to -0.30), and 37-43 for VTA (AP: -4.80 to -6.30) according to the Atlas of Rat Brain (Paxinos and Watson, 2007).

Semi-quantitative analysis of GFAP was carried out using the 20X objective on 3 non-overlapping regions of interest (ROIs, roughly 140000 mm^2) from one out of either every 3 slices of the PFC, NAc and CPu region or every 2 slices of the VTA region. The focus depth was extended by summing the maximum intensity of several images taken at focus steps of 0.25 mm depth intervals to a total of 2 mm thickness using the Z-stack module (Olympus Soft Imaging Solution, GNHB, Munster, Germany). After capture, images were analyzed using the Cell P Analysis software module. Density thresholding to the single channel grey scale images was applied to detect positively stained fibers. Subsequently, for each image we estimated the proportion (%) of area occupied by fibers, and for each animal we calculated average values from images of all tissue sections.

The number of both IBA-1 and TH positive cells was counted bilaterally in 8 (PFC), 6 (NAc), 16 (CPu) and 12 (VTA) sections per animal. In these sections, non-overlapping randomly selected ROIs of 0.15 mm^2, 6 and 4 respectively for IBA-1 and TH, were examined with a 20X objective by two trained observers blind to drug treatment. Limits of the ROI were defined based on structural details within the tissue sections to ensure the ROIs did not overlap. The distance among the 6/4 ROIs was superior to 40 μm to avoid overlapping. IBA-1 and TH positive cells touching the inferior or the right sides of the ROI were excluded from counting. The number of IBA-1 and TH cells was expressed as mean/mm^2 $\pm$ SEM.

9 Statistical analysis

Means ± S.E.M. were calculated for the following parameters: i) basal values of DA ii) percentages of change in DA extracellular levels; iii) numbers of spontaneously active VTA DA neurons, frequency of DA neuron firing rate, and percentages of spikes in bursts iv) number of taste reactions in the taste reactivity test; v) percentages of time spent in open arms in the EPM; vi) number of marbles totally buried in the MB; vii) of startle amplitude and percentage of PPI at prepulse intensities of 74, 78, and 82 dB; viii) numbers of withdrawal signs, and ix) densities of GFAP-IR, and numbers of TH- and IBA-1 positive cells. Data were analyzed with one factor (treatment), two factors (treatment × time, treatment x chocolate exposure, or treatment x prepulse), or three factors (treatment x time x chocolate exposure) analysis of variance (ANOVA) (DA response to JWH-018 and to chocolate, PPI, GFAP and IBA-1 immunoreactivity) or with Student's *t*-test (basal DA extracellular levels, electrophysiological data, EPM, MB, somatic withdrawal signs and taste reactivity test data, acoustic startle response amplitude, TH immunoreactivity). ANOVA was followed by Tukey or Bonferroni post-hoc test, when appropriate. Statistical analysis was performed with Statistica (StatSoft, Tulsa, OK, USA), Prism (GraphPad, La Jolla, CA, USA). Significance was set at $p < 0.05$ for each analysis.

D-Results

1-Dysregulation of DA systems induced by repeated JWH-018 administration

1.1 Effect of JWH-018 (0.25 mg/kg ip, daily for 14 days) on DA transmission in the NAc shell and mPFC during the last day of administration

In attempt to evaluate the effect of a single administration of JWH-018 (0.25 mg/kg ip) within the experimental protocol described in this study, in this experiment we evaluated the basal DA extracellular levels in the NAc shell and in the mPFC and the effect of the last administration of JWH-018 in rats administered for 13 days with Veh or JWH-018 (0.25 mg/kg ip).

Rat basal values of DA, expressed as fmoles/10 µl sample, were analyzed separately for brain area using unpaired Student's t-test. As shown in Fig. 14A, no significant differences on basal DA levels between JWH-018 and Veh-treated group were observed [NAc shell: $t_{(5)}$= 0.08, p= 0.93; JWH-018: n=4; Veh: n=3; mPFC: $t_{(18)}$= 1.42; p= 0.17; JWH-018: n=12; Veh: n=8].

Afterwards, we studied the effect of repeated administration of JWH-018 (0.25 mg/kg ip) on DA transmission in the NAc shell and in the mPFC during the last day of administration (i.e. 14th day). As shown in Fig. 14B, rats pre-treated with vehicle (n=7) showed a selective significant increase of extracellular DA in the NAc shell in response to JWH-018 (0.25 mg/kg ip) administration, while rats pre-treated with JWH-018 (n=6) failed to increase DA in the same area. Two- way ANOVA showed a main effect of treatment [$F_{(1,11)}$=10.43; p< 0.01], time [$F_{(12,132)}$=5.03; p< 0.0001], and a significant treatment x time interaction [$F_{(12,132)}$= 2.28; p< 0.05]. Tukey's post hoc tests showed an increase of dialysate DA in the NAc shell at 20, 30, and 40 min after JWH-018 administration in Veh pre-treated group as compared to basal values and at 20 min with respect to JWH-018 pre-treated group. Moreover, as shown in Fig. 14C, no significant effects were observed on DA response in the mPFC after JWH-018 (0.25 mg/kg ip) administration between JWH-018 (n=8) and Veh-treated group (n=4). Two- way ANOVA analysis showed no significant main effect of

treatment [$F_{(1,120)}$=0.14; p= 0.71], time [$F_{(12,120)}$=1.48; p= 0.13] and no significant treatment x time interaction [$F_{(12,120)}$=1.63; p= 0.09].

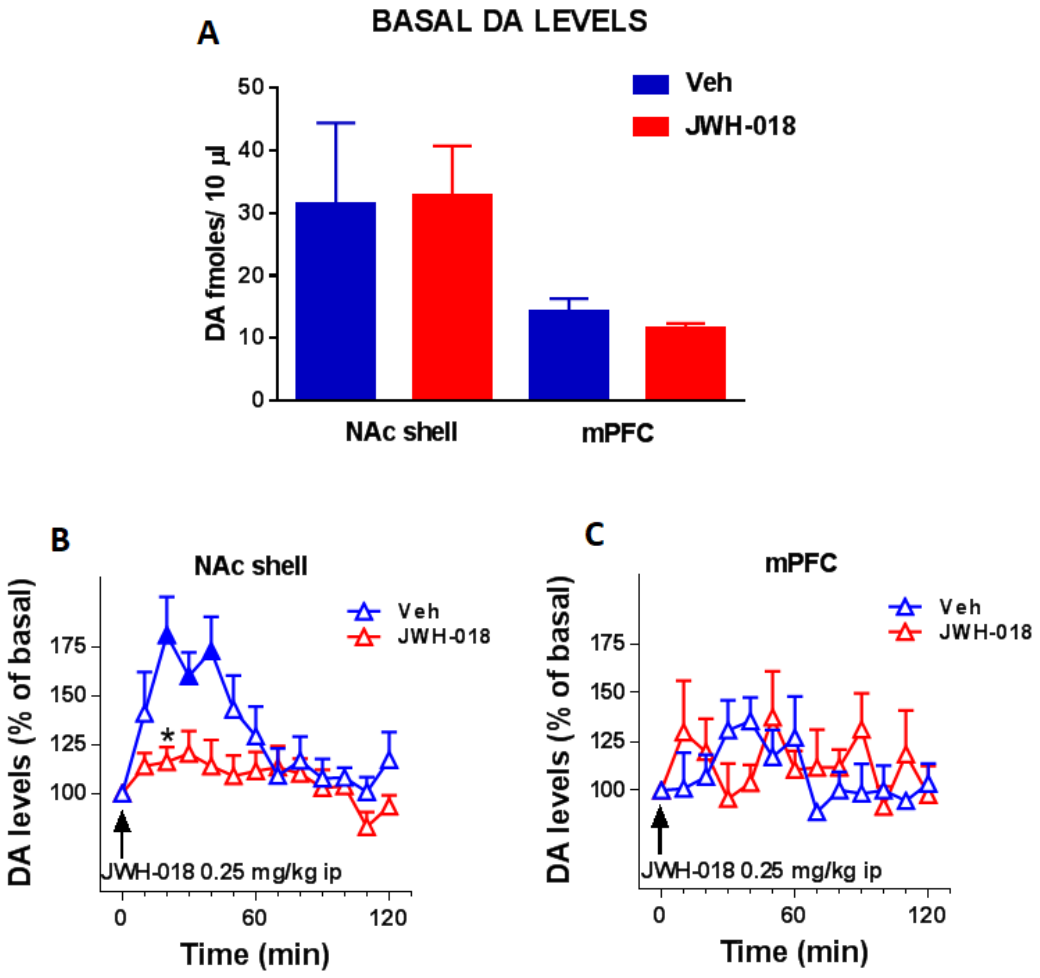

Figure 14 Effect of JWH-018 (0.25 mg/kg ip, daily for 14 days) administration on DA transmission in the NAc shell and in the mPFC during the last day of treatment. Results are expressed as mean ± SEM of basal values of DA expressed as fmoles/10 µl sample (unpaired Student's t-test, JWH-018 NAc shell: n=4; Veh NAc shell: n=3; JWH-018 mPFC: n= 12; Veh mPFC: n=8; panel A), and of change in DA extracellular levels expressed as the percentage of basal values (Two-way ANOVA, Tukey's HSD post hoc, JWH-018 NAc shell: n=6; Veh NAc shell: n=7, panel B; JWH-018 mPFC: n=8; Veh mPFC: n=4; panel C). The arrow indicates the start of JWH-018 ip injection. Solid symbol: $p < 0.05$ with respect to basal values; *$p < 0.05$ JWH-018 pre-treated group vs vehicle (Veh) pre-treated group

1.2 Spontaneous VTA DA neurons activity and TH-immunoreactivity 24h after JWH-018 (0.25 mg/kg ip,daily for 14 days) discontinuation

Electrophysiological recording of DA neurons located in the parabrachial pigmented nuclei of posterior VTA were performed 24 hours after repeated JWH-018 (0.25 mg/kg ip,daily for 14 days) administration. The subregion has been selected for the larger density of DA cells projecting to NAc as compared to the more medial levels of VTA (Yamaguchi *et al.*, 2011; Lammel *et al.*, 2015). As shown in Fig. 15A, analysis by two-tailed unpaired Student's t-tests showed a decrease of the number of spontaneously active DA cells (during spontaneous withdrawal) in JWH-018 treated group as compared to Veh treated group [JWH-018: n=11; Veh: n=10; $t_{(19)}$=2.21, p<0.05]. However, analysis of VTA DA bursting activity (Fig 15C) showed an increased in percentage of spikes in burst [JWH-018: n=132; Veh: n=144, $t_{(274)}$=1.87, p< 0.05] in JHW-018 treated rats as compared to Veh-treated rats. Notably/Moreover, as shown in Fig. 15B,no significant difference of average firing rate was not changed between groups [JWH-018: n=132, Veh: n=144; $t_{(274)}$=0.67. p= 0.25].

Meantime, we evaluated also TH-immunoreactivity of VTA neurons (Fig. 16). As shown in Fig. 15D, JWH-018 treatment significantly decreased the number of TH positive cells in the VTA [JWH-018: n= 5; Veh: n=4; $t_{(7)}$ =2.49, p< 0.05].

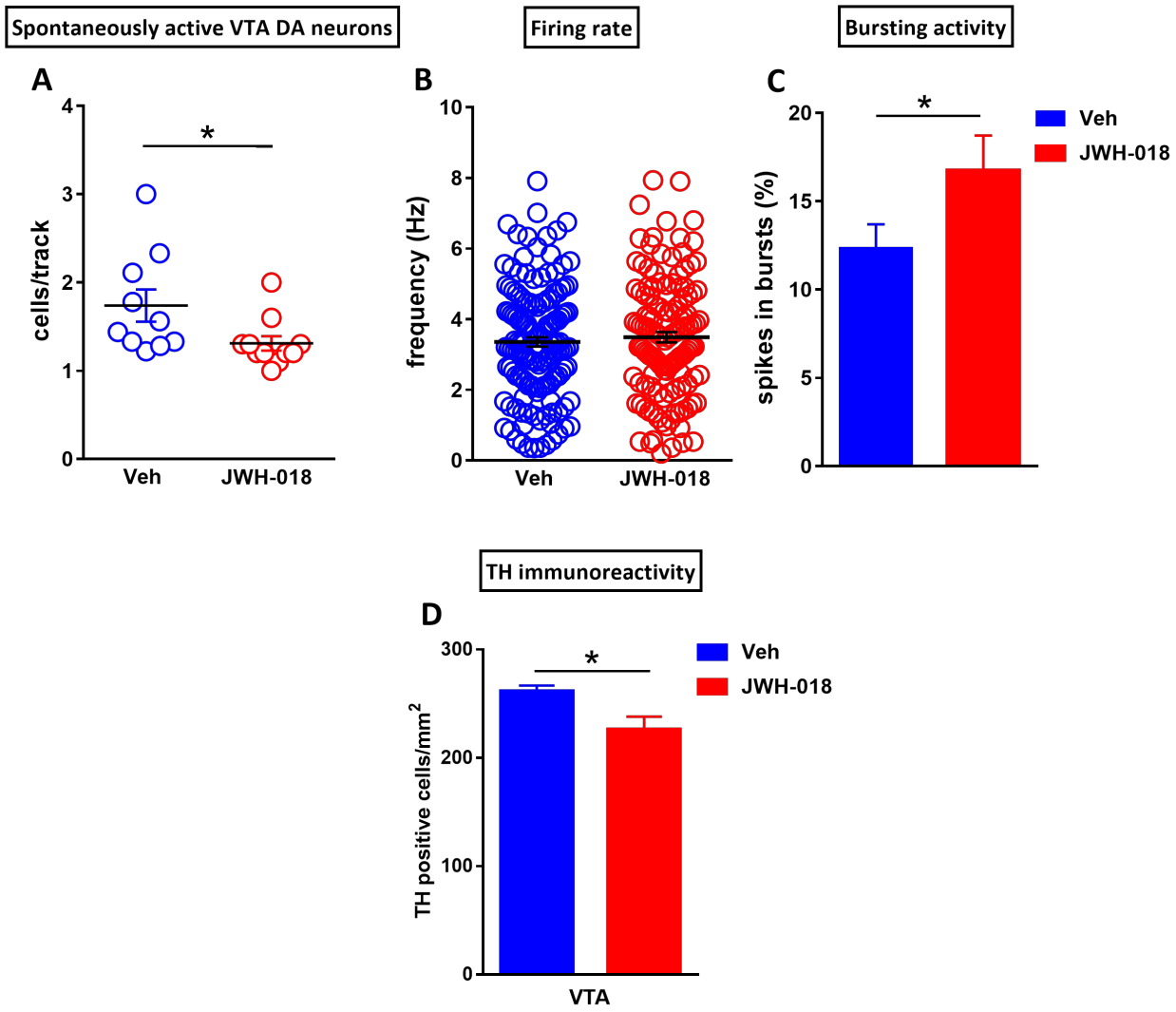

Figure 15 Spontaneous VTA DA neurons activity and TH-IR 24h after JWH-018 (0.25 mg/kg ip, daily for 14 days) discontinuation. The scatter plot in (A) shows the number of spontaneously active VTA DA neurons (JWH-018: n=11; Veh: n=10), while in (B) displays individual DA neuron firing rate in JWH-018 and Veh-treated group (JWH-018: n=132; Veh: n=144). The horizontal lines represent average values that are significantly different between the 2 groups. The Bars in (C) represent the percentage of spikes in bursts (JWH-018: n=132; Veh: n=144), while in (D) shows the number of TH positive cells in the VTA (JWH-018: n=5; Veh: n=4), expressed per mm^2. Data are expressed as percentage or mean ± SEM. *p< 0.05 JWH-018 treated group vs vehicle (Veh) treated group (unpaired Student's t-tests)

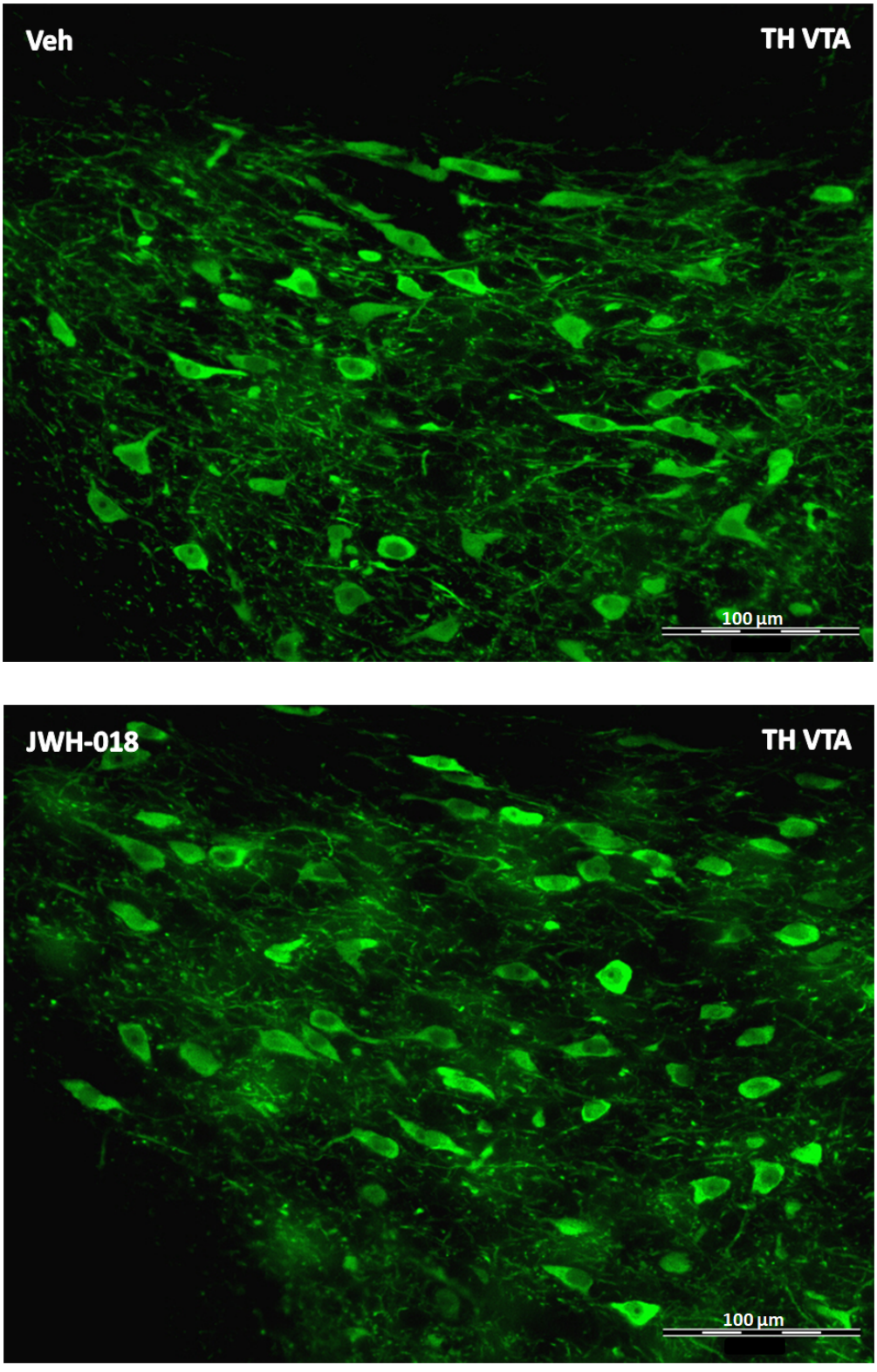

Figure 16. TH- Immunoreactivity of VTA neurons 24h after JWH-018 (0.25 mg/kg ip, daily for 14 days) discontinuation

1.3 Spontaneous VTA DA neurons activity and TH-Immunoreactivity seven days after JWH-018 (0.25 mg/kg ip, daily for 14 days) discontinuation

Following a washout period of seven days, we evaluated the spontaneous activity of putative VTA DA neurons in JWH-018 and Veh-treated rats. As shown in Fig. 17A, analysis by two- tailed unpaired Student's t-tests of electrophysiological recordings revealed a reduced number of spontaneously active DA cells in JWH-018 treated rats as compared to Veh treated rats [JWH-018: Veh: n=8 for both groups; $t_{(14)}$=1.85, $p< 0.05$]. Moreover, as shown in Fig. 17B, we found that average firing rate was lower in JWH-018 treated rats [JWH-018: n=96; Veh: n=128; $t_{(222)}$=1.94, $p< 0.05$], whereas no differences between groups in the percentage of spikes in burst was observed (Fig.17C) [JWH-018: n=96; Veh: n=128; p= 0.31].

Finally, as showed in Fig. 17D, JWH-018 treatment significantly decreased the number of TH positive cells in the VTA [JWH-018: n=5; Veh: n=4; $t_{(7)}$ =2.48, $p< 0.05$] seven days after JWH-018 discontinuation.

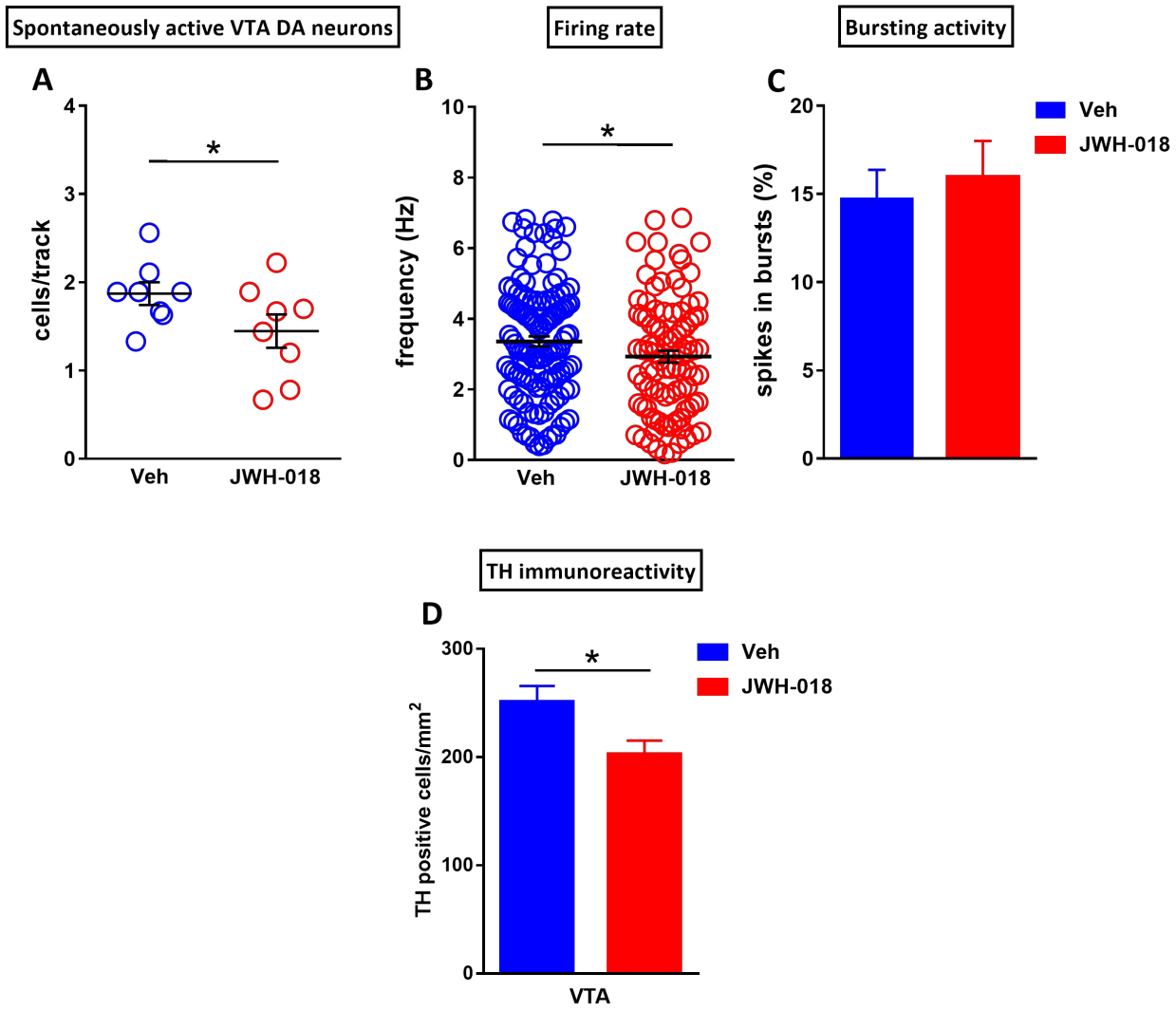

Figure 17 Spontaneous VTA DA neurons activity and TH-IR seven days afterJWH-018 (0.25 mg/kg ip, daily for 14 days) discontinuation. The scatter plot in (A) shows the number of spontaneously active VTA DA neurons (n=8 for group), while in (B) displays individual DA neuron firing rate in JWH-018 and Veh-treated group (JWH-018: n=96; Veh: n=128). The horizontal lines represent average values that are significantly different between the 2 groups. The Bars in (C) represent the percentage of spikes in bursts (JWH-018: n=96; Veh: n=128), while in (D) shows the number of TH positive cells in the VTA (JWH-018: n=5; Veh: n=4), expressed per mm^2. Data are expressed as percentage or mean ± SEM. *p< 0.05 JWH-018 treated group vs vehicle (Veh) treated group (unpaired Student's t-tests)

1.4 Effect of repeated chocolate exposure on DA transmission seven days after JWH-018 (0.25 mg/kg ip,daily for 14 days) discontinuation

Seven days after JWH-018 discontinuation, we evaluated the responsiveness of DA transmission to repeated chocolate exposure. Before each chocolate exposure, we evaluated basal DA extracellular levels in the NAc shell, in the NAc core and in the mPFC. Rat basal values of DA, expressed as fmoles/ 10 µl sample, were analyzed separately for brain areas and chocolate exposures using two-tailed unpaired Student's t-test. As shown in Fig. 18A, no significant differences of basal DA levels between JWH-018 and Veh-treated group were observed [1st chocolate exposure; NAc shell: JWH-018 n=6, Veh n=4, $t_{(8)}$=0.80; p= 0.44; NAc core: JWH-018 n=5, Veh n=3, $t_{(6)}$=0.45; p=0.66; mPFC: JWH-018 n=6, Veh n=8, $t_{(12)}$=1.39; p= 0.18; 2nd chocolate exposure: NAc shell: JWH-018, n=7, Veh: n=2; $t_{(7)}$=0.30; p= 0.77; NAc core: JWH-018: n=4, Veh: n=2, $t_{(4)}$=0.47; p= 0.66; mPFC: JWH-018: n=9; Veh: n=7, $t_{(14)}$=1.32; p= 0.20].

Afterwards, we studied the effect of repeated chocolate exposure (1ml/ 5 min io) on DA transmission in the same brain areas. As showed in Fig. 18 (panel B-G), repeated JWH-018 exposure induces changes on responsiveness of mesocorticolimbic DA transmission to repeated chocolate exposure.

Analysis of NAc shell DA response by three- way ANOVA showed a significant main effect of treatment [$F_{(1,16)}$=9.46; p< 0.01], time [$F_{(12,192)}$=5.55; p< 0.00001], treatment x chocolate exposure interaction [$F_{(1,16)}$=18.04; p< 0.001], and treatment x chocolate exposure x time interaction [$F_{(12,192)}$=1.80; p< 0.05] while no significant main effect of chocolate exposure [$F_{(1,16)}$=2.79; p= 0.11], time x chocolate exposure interaction [$F_{(12,192)}$=0.82; p= 0.62], and time x treatment interaction [$F_{(12,192)}$=0.97; p= 0.47] were observed. Tukey's post hoc tests showed an increase of dialysate DA in the NAc shell at 30 and 40 min after 2nd chocolate exposure in JWH-018 treated group as compared either to basal values, or to JWH-018 treated group response to the 1st chocolate exposure.

To better understand the effects of repeated JWH-018 administration on NAc shell DA response to each chocolate exposure, data were analyzed separately using two way-ANOVA. Analysis of NAc shell DA response to 1st chocolate exposure (n= 6 for group) showed a significant main effect of time [$F_{(12,120)}$=6.38; p< 0.0001], while no significant main effect of treatment [$F_{(1,10)}$=3.46; p= 0.92] nor time x treatment interaction [$F_{(12,120)}$=1.41; p= 0.16] were observed. As showed in Fig. 18B, Tukey's post hoc tests revealed an increase of dialysate DA in the NAc shell at 20 and 30 min after chocolate exposure in Veh pre-treated group as compared to basal values, and to treated JWH-018 group at 30 min after the 1st chocolate exposure. Analysis of NAc shell DA response to 2nd chocolate exposure (n= 5 for group) by two-way ANOVA showed a significant main effect of treatment [$F_{(1,6)}$=9.74; p< 0.05], while no significant main effect of time [$F_{(12,72)}$=1.78; p= 0.06] nor time x treatment interaction [$F_{(12,72)}$=0.95; p= 0.49] were observed. As showed in fig.18C, Tukey's post hoc tests confirmed a significant increase of dialysate DA in the NAc shell at 30 and 40 min after chocolate exposure in JWH-018-treated group as compared to basal values.

Regarding DA response in NAc core (Fig. 18D-E), three-way ANOVA analysis showed a significant main effect of treatment [$F_{(1,18)}$=12.03; p< 0.01], chocolate exposure [$F_{(1,18)}$=8.23; p< 0.05], treatment x chocolate exposure interaction [$F_{(1,18)}$=13.39; p< 0.005], time x chocolate exposure interaction [$F_{(12,216)}$=2.04; p< 0.05], time x treatment interaction [$F_{(12,216)}$=2.43; p< 0.05] treatment x chocolate exposures x time interaction [$F_{(12,216)}$=3.19; p< 0.05], while no significant main effect of time [$F_{(12,216)}$=1.22; p= 0.26] was observed. Tukey's post hoc tests showed an increase of dialysate DA in the NAc core at 80, 90, 110 and 120 min after 2nd chocolate exposure in JWH-018 treated group as compared either to basal values, or to JWH-018 treated group response to the 1st chocolate exposure, or to Veh-treated group response to the 2nd chocolate exposure, two-way ANOVA analysis of NAc core DA response to 1st chocolate exposure (n= 6 for group) showed no significant differences between JWH-018- and Veh-treated group (Fig. 18D), while two-way ANOVA analysis of NAc core DA response to 2nd chocolate exposure (n=5 for group) showed a main effect of treatment [$F_{(1,8)}$=11.77; p< 0.01], time x treatment interaction [$F_{(12,96)}$=2.88; p<

0.005], while no significant main effect of time [$F_{(12,96)}$=1.61; p= 0.10] was observed. As showed in Fig. 18E, Tukey's post hoc tests revealed an increase of dialysate DA in the NAc core at 80, 90, 110, and 120 min after 2nd chocolate exposure in JWH-018-treated group as compared to basal values, and to Veh-treated group at 90 min after chocolate exposure.

Finally, analysis of mPFC DA response (Fig. 18F-G) by three- way ANOVA showed a significant main effect of time [$F_{(12,192)}$=8.83; p< 0.00001], treatment x chocolate exposure interaction [$F_{(1,16)}$=8.05; p< 0.05], and treatment x chocolate exposures x time interaction [$F_{(12,192)}$=5.31; p< 0.00001], while no significant main effect of chocolate exposure [$F_{(1,16)}$=0.48; p= 0.49], treatment [$F_{(1,16)}$=1.73; p= 0.20], time x chocolate exposure interaction [$F_{(12,192)}$=1.62; p= 0.08], and time x treatment interaction [$F_{(12,192)}$=1.74; p= 0.05] were observed. Tukey's post hoc tests showed an increase of dialysate DA in the mPFC at 20 min after 1st chocolate exposure in JWH-018 treated group as compared to basal values, and to JWH-018 treated group at 20 and 40 min after 2nd chocolate exposure. Moreover, Tukey's post hoc revealed an increase of dialysate DA in mPFC at 10 and 20 min after 2nd chocolate exposure in Veh-treated group as compared to basal values.

To better understand the effects of repeated JWH-018 administration on mPFC DA response to each chocolate exposure, data were analyzed separately using two way-ANOVA. Analysis of mPFC DA response to 1st chocolate exposure (n=4 for group) showed a main effect of time [$F_{(12,72)}$=4.36; p< 0.0001], and time x treatment interaction $F_{(12,72)}$=3.06; p< 0.005], while no significant main effect of treatment [$F_{(1,6)}$=0.52; p=0.49] was observed. Tukey's post hoc tests showed a significant increase of dialysate DA in the mPFC at 20 min after chocolate exposure in JWH-018- treated group as compared to basal values (Fig. 18F). Analysis of mPFC DA response to 2nd chocolate exposure (n=6 for group) by two- way ANOVA showed a main effect of time [$F_{(1,10)}$=21.77; p< 0.001], time [$F_{(12,120)}$=4.51; p< 0.0001],and a significant time x treatment [$F_{(12,120)}$=2.65; p< 0.005]. As showed in Fig. 18G, Tukey's post hoc tests revealed a significant increase of dialysate DA in the mPFC at 10, 20 and 30 min after chocolate exposure in Veh- treated group as compared to basal values, and to JWH-018-treated group at 20 and 30 min after 2nd chocolate exposure.

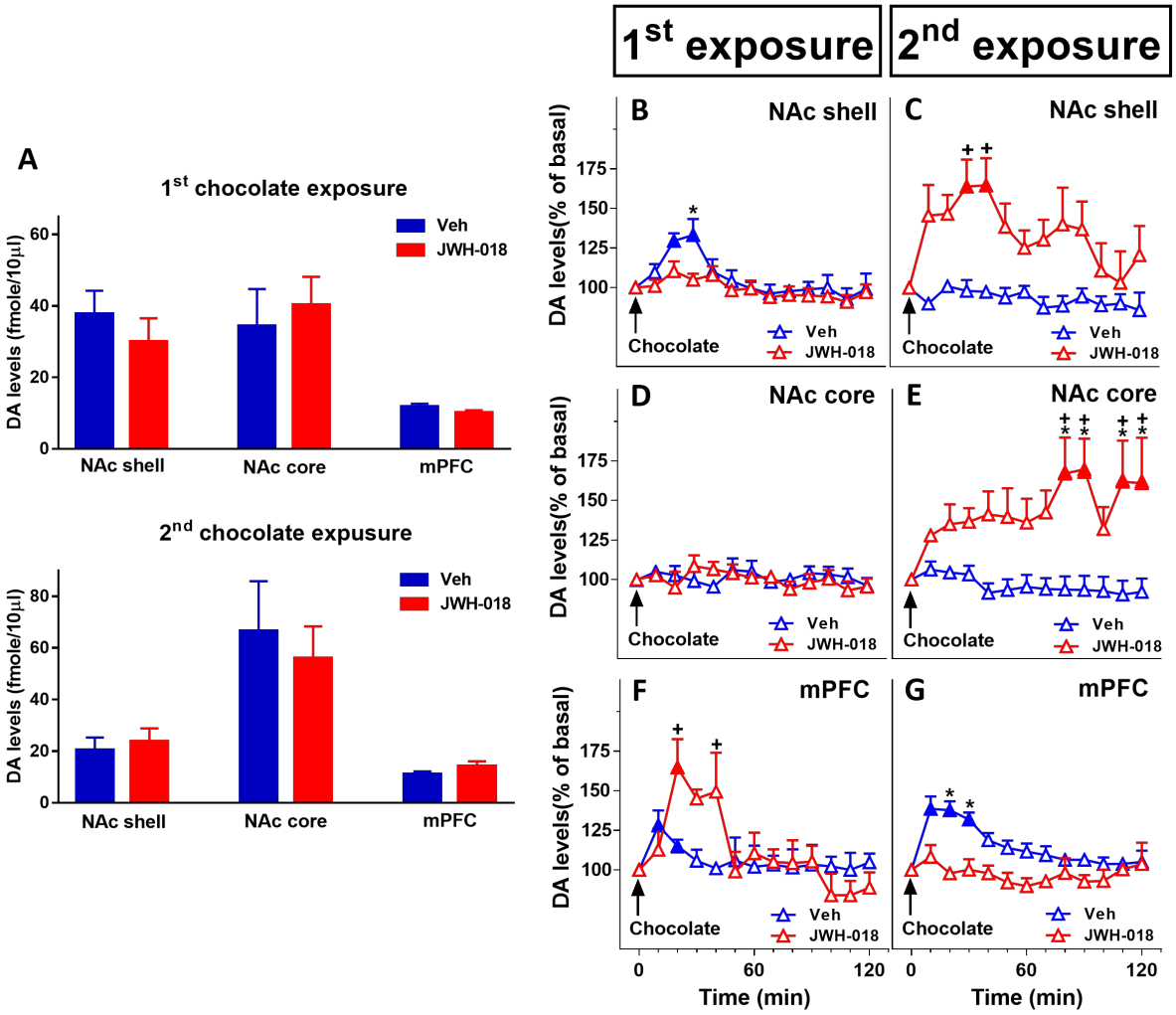

Figure 18 Effect of repeated chocolate exposure on DA transmission seven days after JWH-018 (0.25 mg/kg ip, daily for 14 days) discontinuation. The bars in (A) represent the basal DA levels in the NAc shell (1st chocolate exposure: JWH-018 n=6, Veh n=4; 2nd chocolate exposure: JWH-018: n=7; Veh: n=2), in the NAc core (1st chocolate exposure: JWH-018 n=5, Veh n=3; 2nd chocolate exposure: JWH-018: n=4; Veh: n=2) and in the mPFC (1st chocolate exposure: JWH-018 n=6, Veh n=8; 2nd chocolate exposure: JWH-018: n=9; Veh: n=7) before repeated chocolate exposure. Data expressed as mean ± SEM of basal values of DA expressed as fmoles /10 µl sample (unpaired Student's t-tests). Effect of repeated chocolate exposure on DA transmission in the NAc shell (1st chocolate exposure:n=6 for both group, panel B; 2nd chocolate exposure:n=5 for both group, panel C), in the NAc core (1st chocolate exposure:n=6 for both group, panel D; 2nd chocolate exposure:n=5 for both group, panel E) and in the mPFC (1st chocolate exposure:n=4 for both group, panel F; 2nd chocolate exposure: n=6 for both group, panel G)**.** 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 chocolate intraoral infusion. Solid symbol: $p < 0.05$ with respect to basal values. * $p < 0.05$ Veh vs JWH within 1st or 2nd exposure group; + $p<0.05$ between 1st or 2nd exposure in JWH treated group. (Three-way ANOVA, Tukey's post hoc).

2-Behavioral effects of repeated JWH-018 administration

2.1 Evaluation of compulsive-like behavior, anxiety, and sensorimotor-gating 1 hour after repeated JWH-018 (0.25 mg/kg ip, daily for 14 days) administration

One hour after the last injection, we evaluated compulsive-like behavioral activity (MB), anxiety state (EPM), and attentional deficits (PPI) induced by repeated JWH-018 (0.25 mg/kg ip, 14 days) administration. As shown in Fig. 19A, the total number of marbles buried (partially + fully covered) by JWH-018 treated rats was significantly higher than those buried by Veh-treated rats [n=6 for group; $t_{(10)}$=2.87, p< 0.05]. As showed in Fig. 19B, the time spent in open arms of EPM by JWH-018 treated rats was significant reduced than Veh-treated group. [n= 8 for group: $t_{(14)}$=2.72, p< 0.05]. Finally, considering attentional deficits, no significant differences between Veh- and JWH-018 treated group in acoustic-startle response amplitude were observed [JWH-018: n=8; Veh: n=9; $t_{(15)}$=1.16, p=0.26;Fig. 19C]. However, JWH-018 treated rats showed a significant reduction of the inhibition (PPI) of startle response 1h after repeated JWH-018 administration (Fig. 19D). Two-way ANOVA analysis of PPI 1 hour after repeated JWH-018 administration showed a significant main effect of treatment [$F_{(1,15)}$=8.27; p< 0.05], while no significant main effect of prepulse [$F_{(2,30)}$=0.79; p= 0.45], and treatment x prepulse interaction [$F_{(2,30)}$=0.81; p= 0.45] were observed. Bonferroni's post hoc showed a decreased of PPI at 74 and 78 dB in JWH-018 treated rats as compared to Veh-treated rats.

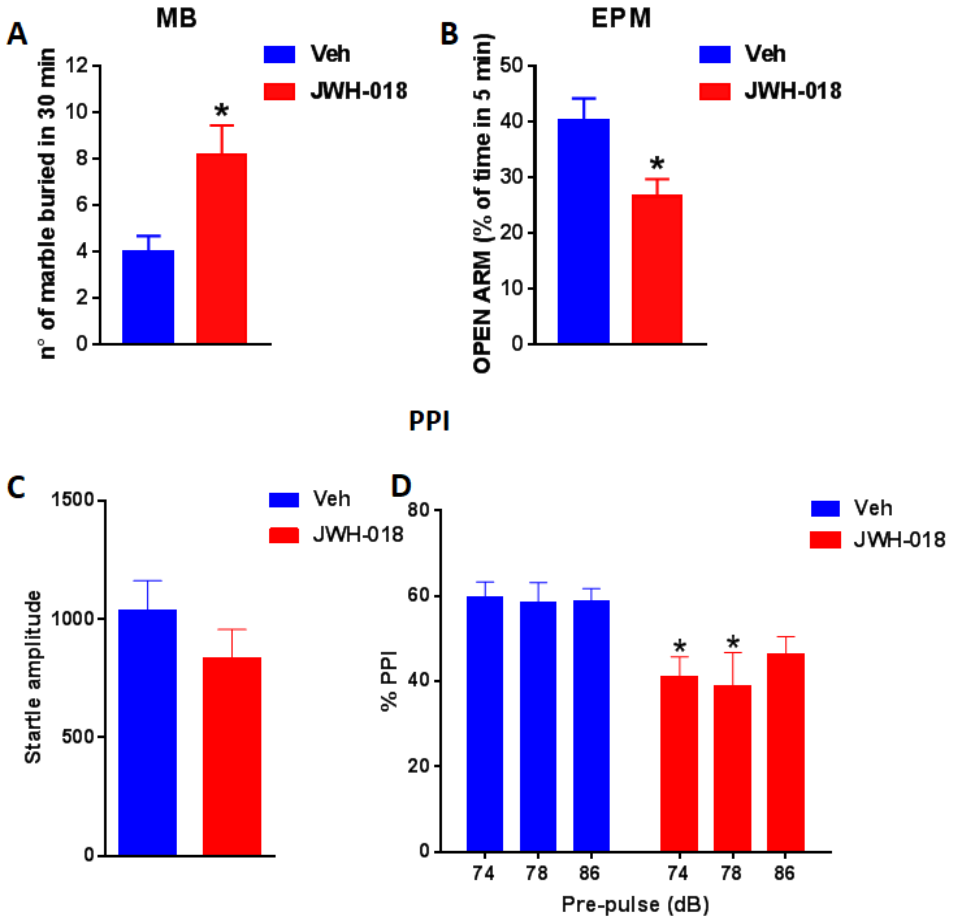

Figure 19 Evaluation of compulsive-like behavior (MB) , anxiety (EPM), and sensorimotor-gating (PPI) 1 hour after repeated JWH-018 (0.25 mg/kg ip, daily for 14 days) exposure. Data are shown as means ±SEM of the total number of marbles covered with bedding during the 30 min of marble burying test (n= 6 for group, panel A), of percentages of time spent in open arms in EPM (n=8 for group; panel B), of startle amplitude (panel C) (unpaired Student's t-tests) and percentage of PPI at prepulse intensities of 74, 78, and 82 dB (JWH-018: n=8; Veh: n=9; panel D) (Two-way ANOVA, Bonferroni's post hoc) of Veh- and JWH-018-treated rats. *p< 0.05 vs vehicle (Veh) treated group.

2.2 Evaluation of compulsive-like behavior, anxiety, and sensorimotor-gating 24 hours after JWH-018 (0.25 mg/kg ip,daily for 14 days) discontinuation

24 hours after the last injection, we evaluated compulsive activity (MB), anxiety state (EPM), and attentional deficits (PPI) during acute JWH-018 withdrawal. Considering compulsive activity, as shown in Fig. 20A, the total number of marbles buried (partially + fully covered) by JWH-018 treated rats was significantly higher than in Veh-treated group [JWH-018: n=8; Veh: n=7; $t_{(13)}$=2.2, $p< 0.05$]. Moreover, as showed in Fig. 20B, the time spent in open arms of EPM by JWH-018 treated rats was significant reduced than Veh-treated group. [n= 8 for group; $t_{(14)}$=2.15, $p< 0.05$]. Finally, considering attentional deficits, no significant differences between Veh- and JWH-018-treated rats were observed both in acoustic-startle response amplitude[JWH-018: n=10; Veh: n=9; $t_{(17)}$=0.10, p=0.91; Fig. 20C] and on PPI 24 hours after JWH-018 (0.25 mg/kg ip) discontinuation [two- way ANOVA: treatment $F_{(1,16)}$=1.40; p=0.53; prepulse $F_{(2,32)}$=6.01; $p< 0.01$]; treatment x prepulse interaction $F_{(2,32)}$=1.01; p= 0.37] (Fig. 20D).

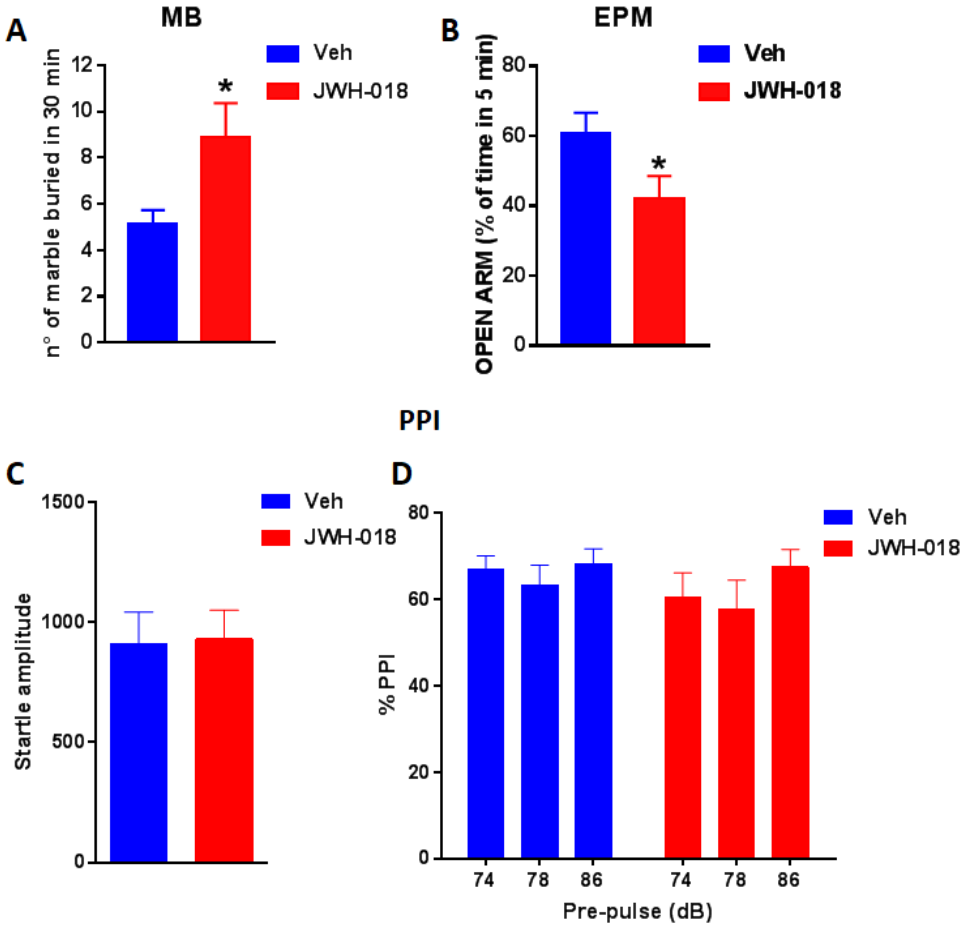

Figure 20 Evaluation of compulsive-like behavior (MB), anxiety (EPM), and sensorimotor-gating (PPI) 24 hours after JWH-018 (0.25 mg/kg ip,daily for 14 days) discontinuation. Data are shown as means ±SEM of the total number of marbles covered with bedding during the 30 min of marble burying test (JWH-018: n=8; Veh: n=7; panel A), of percentages of time spent in open arms in EPM (n=8 for group; panel B), of startle amplitude (C) (unpaired Student's t-tests) and percentage of PPI at prepulse intensities of 74, 78, and 82 dB (JWH-018: n=10; Veh: n=9; panel D) (Two-way ANOVA, Bonferroni's post hoc) of Veh- and JWH-018-treated rats. *p< 0.05 vs vehicle (Veh) treated group.

2.3 Spontaneous JWH-018 withdrawal signs 24 h after JWH-018 (0.25 mg/kg ip,daily for 14 days) discontinuation

24 hour after JWH-018 discontinuation, we performed behavioral observation of withdrawal signs. As shown in Fig. 21, JWH-018 treated rats exhibited a significant increase in several commons signs, such as licking (U:44, $p< 0.05$), headshakes (U:42, $p< 0.05$) and tongue rolling (U:29.5, $p< 0.01$) as well as biting (U:33.5, $p< 0.01$) and chewing (U:27.5, $p< 0.01$), as compared to Veh-treated rats (JWH-018: n=13; Veh: n=11).

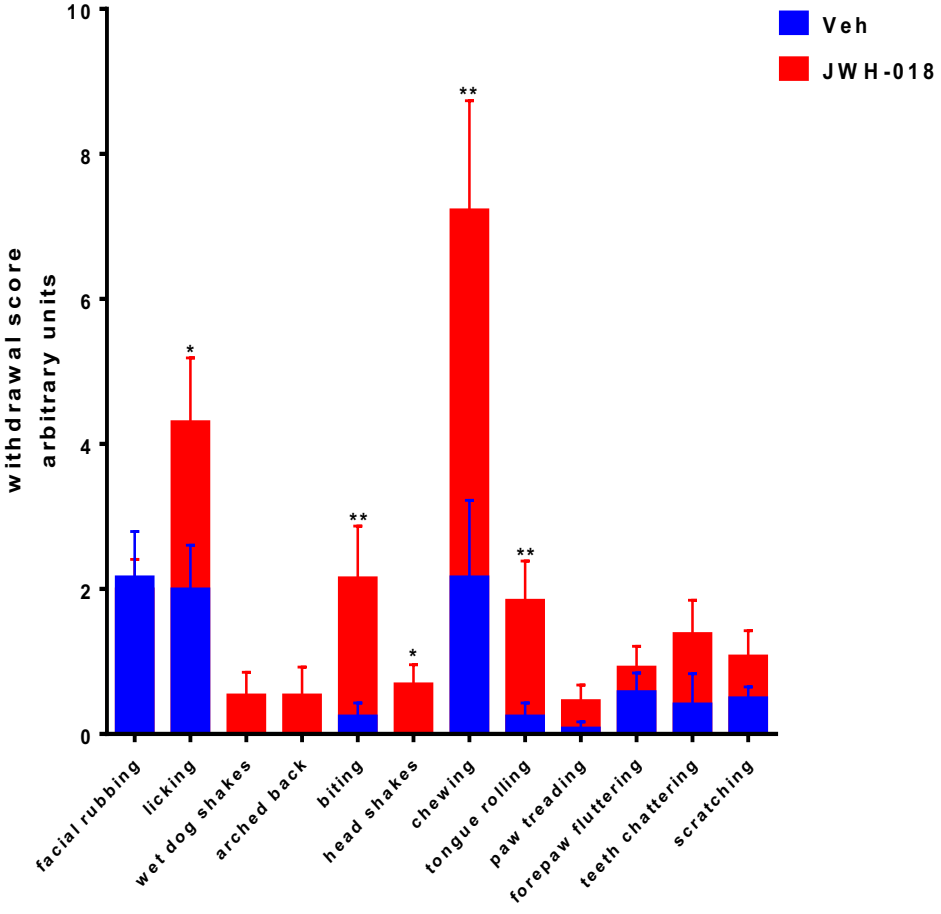

Figure 21 Spontaneous JWH-018 withdrawal signs 24 h afterJWH-018 (0.25 mg/kg ip,daily for 14 days) discontinuation. Results are expressed as mean ± SEM of summed behavioral cannabinoid-withdrawal scores observed for a total of 30-min observation period before electrophysiological experiments (JWH-018: n=13; Veh: n=11). *p < 0.05 JWH-018 treated group vs vehicle (Veh) treated group (Mann–Whitney U test)

2.4 Spontaneous JWH-018 withdrawal signs seven days after JWH-018 (0.25 mg/kg ip,daily for 14 days) discontinuation

Seven days after JWH-018 discontinuation, we performed behavioral observation to evaluate the persistence of JWH-018 withdrawal signs. As shown in Fig. 22, an increased in biting has been found in JWH-018 treated group as compared to Veh treated group (n=9 for group; U:18, $p < 0.05$), while no significant differences have been observed in all other signs considered.

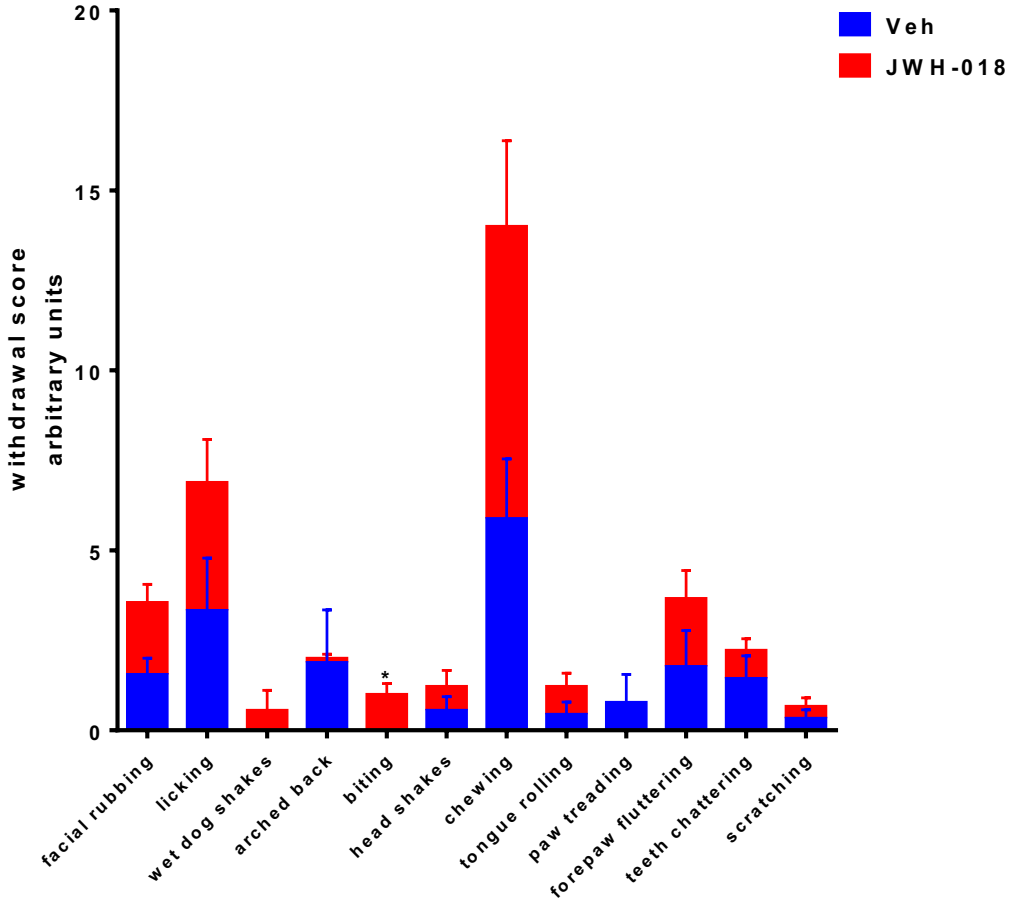

Figure 22 Spontaneous JWH-018 withdrawal signs seven days after JWH-018 (0.25 mg/kg ip, daily for 14 days) discontinuation. Results are expressed as mean ± SEM of summed behavioral cannabinoid-withdrawal scores observed for a total of 30-min observation period before electrophysiological experiments (n=9 for group). *p < 0.05 JWH-018 treated group vs vehicle (Veh) treated group (Mann–Whitney U test).

2.5 Taste reactions to chocolate seven days after JWH-018 (0.25 mg/kg ip,daily for 14 days) discontinuation

Seven days after JWH-018 discontinuation, we evaluated either behavioral appetitive or aversive reactions to chocolate in the taste reactivity test. As shown in Fig. 23A, no significant differences in appetitive reactions to both chocolate exposure were observed [1st chocolate exposure: n=12 for group; $t_{(22)}$= 0.55, p= 0.58; 2nd chocolate exposure: JWH-018: n=11; Veh: n=6; $t_{(15)}$= 1.31; p= 0.20]. However, as shown in Fig. 23B, JWH-018 treated rats showed a significant increase of aversive reactions to the 1st chocolate exposure than Veh-treated group [JWH-018: n=12; Veh: n=11; $t_{(21)}$ =2.11, p< 0.05], while no significant differences in aversive reactions to the 2nd chocolate exposure were observed [JWH-018: n=10; Veh: n=7; $t_{(15)}$ =0.15, p= 0.88].

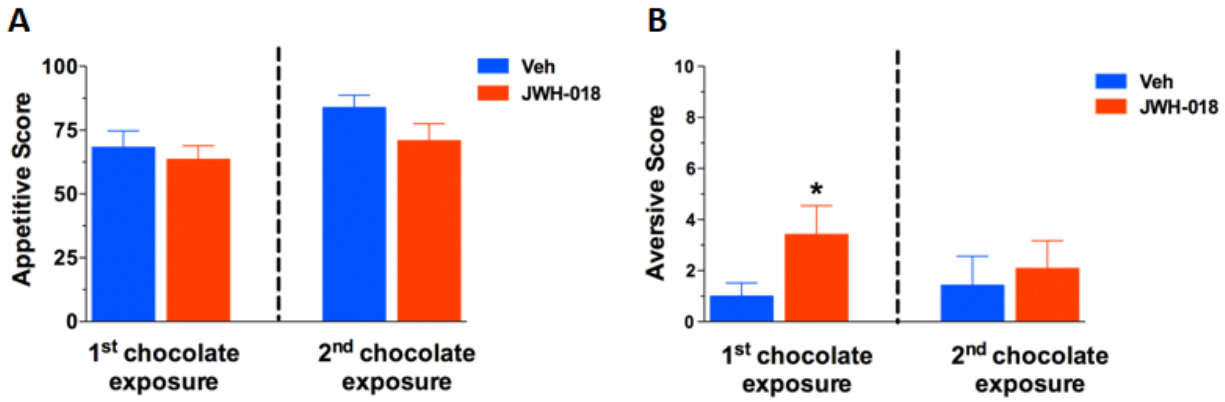

Figure 23 Taste reactions to chocolate in taste reactivity test seven days after JWH-018 (0.25 mg/kg ip,daily for 14 days) discontinuation. Data are shown as means ±SEM of behavioral appetitive (1st chocolate exposure: n=12 for group; 2nd chocolate exposure: JWH-018: n=11; Veh: n=6; panel A) and aversive (1st chocolate exposure: JWH-018 n=12; Veh: n=11; 2nd chocolate exposure: JWH-018: n=10; Veh: n=7; panel panel B) score to chocolate of Veh- and JWH-018-treated rats. *p< 0.05 vs vehicle (Veh) treated group (unpaired Student's t-tests)

3 Neuroinflammatory effects of repeated JWH-018 administration

3.1 GFAP- and IBA-1-IR in DA brain areas 24 hours after JWH-018 (0.25 mg/kg ip, daily for 14 days) discontinuation

The effect of repeated JWH-018 exposure on GFAP- and IBA-1-IR in selected DA brain areas was evaluated 24 hours after JWH-018 discontinuation. Two-way ANOVA analysis of GFAP-IR, showed a main effect of treatment [$F_{(1,37)}$= 71.96, p< 0.00001] and brain area [F(4,37)= 15.49, p< 0.00001], while no effect of area x treatment interaction was observed [$F_{(4,37)}$= 0.29, p= 0.88]. Bonferroni's post hoc showed a higher expression of GFAP in JWH-018- than in Veh-treated rats and a higher GFAP-IR in the mPFC in comparison to the NAc shell, the NAc core and the CPu, as well as in the VTA compared to the NAc shell, the NAc core and the CPu .

Then, to better evaluate in each brain area the effect of JWH-018 treatment on GFAP- IR, data were analyzed separately using two- tailed unpaired Student's t-tests. As shown in Fig.24A, JWH-018 administration significantly increased the GFAP-IR levels in different brain areas, as compared to Veh-treated group. Specifically, rats treated with JWH-018 showed a significant increase of GFAP-IR in the mPFC [n=5 for group; $t_{(8)}$ =4.82, p< 0.01 Fig.25], NAc shell [n=4 for group; $t_{(6)}$ =3.78, p <0.01], NAc core [JWH-018: n=5; Veh: n=4; $t_{(6)}$ =5.97, p<0.001], CPu [n=6 for group $t_{(10)}$ =2.73, p<0.05], and VTA [JWH-018: n=5; Veh: n=4; $t_{(7)}$ =4.10, p< 0.01].

As regard IBA-1 expression, two-way ANOVA showed a main effect of brain area [$F_{(4,36)}$= 12.37, p< 0.00001] and treatment [$F_{(1,36)}$= 34.26, p< 0.00001], while no effect of area x treatment interaction was observed [$F_{(4,36)}$= 1.21, p= 0.32]. Bonferroni's post hoc revealed an higher number of IBA-1 positive cells in JWH-018 treated group, as compared to Veh treated group. Additionally, the IBA-1-IR was significantly higher in the mPFC, in the NAc shell and core as compared to the CPu and to the VTA.

Then, to better evaluate in each brain area the effect of JWH-018 treatment on IBA-1-IR, data were analyzed separately using two- tailed unpaired Student's t-tests. As shown Fig. 24B, JWH-018

administration significantly increased the number of IBA-1-positive cells in different brain areas, as compared to Veh-treated group. In particular, rats treated with JWH-018 showed a significant higher number of IBA-1 positive cells in the NAc shell [n=4 for group; $t_{(6)}$ =2.79, p< 0.05], in the NAc core [n=4 for group; $t_{(6)}$ =4.73, p< 0.01, Fig. 26], and in the CPu [n=6 for group; $t_{(10)}$ =2.36, p< 0.05].

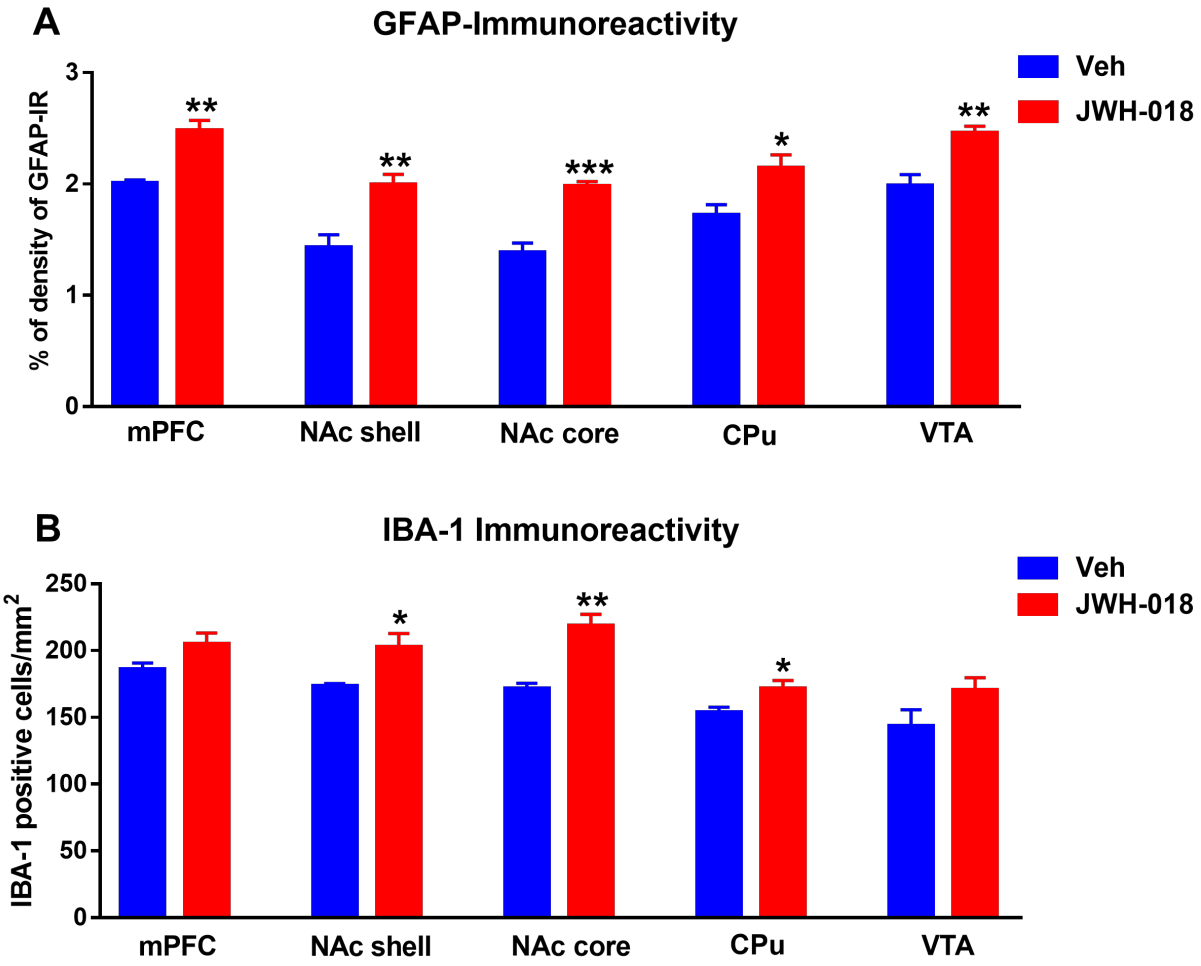

Figure 24 GFAP- and IBA-1-immnunoreactivity in DA brain areas 24 hours after JWH-018 (0.25 mg/kg ip, daily for 14 days) discontinuation. Values represent means ± SEM of density reading expressed as a percentage of the area covered by GFAP immunoreactivity (IR) (mPFC: n=5 for group; NAc shell: n=4 for group; JWH-018 NAc core and VTA: n=5, Veh NAc core and VTA: n=4; CPu: n=6 for group; panel A); and of number of IBA-1 positive cells, expressed per mm^2 (mPFC: n=5 for group; NAc shell, core and VTA: n=4 for group; CPu: n=6 for group; panel B). * p< 0.05, ** p< 0.005, ***p<0.001 vs Vehicle (Veh) group (unpaired Student's t-tests).

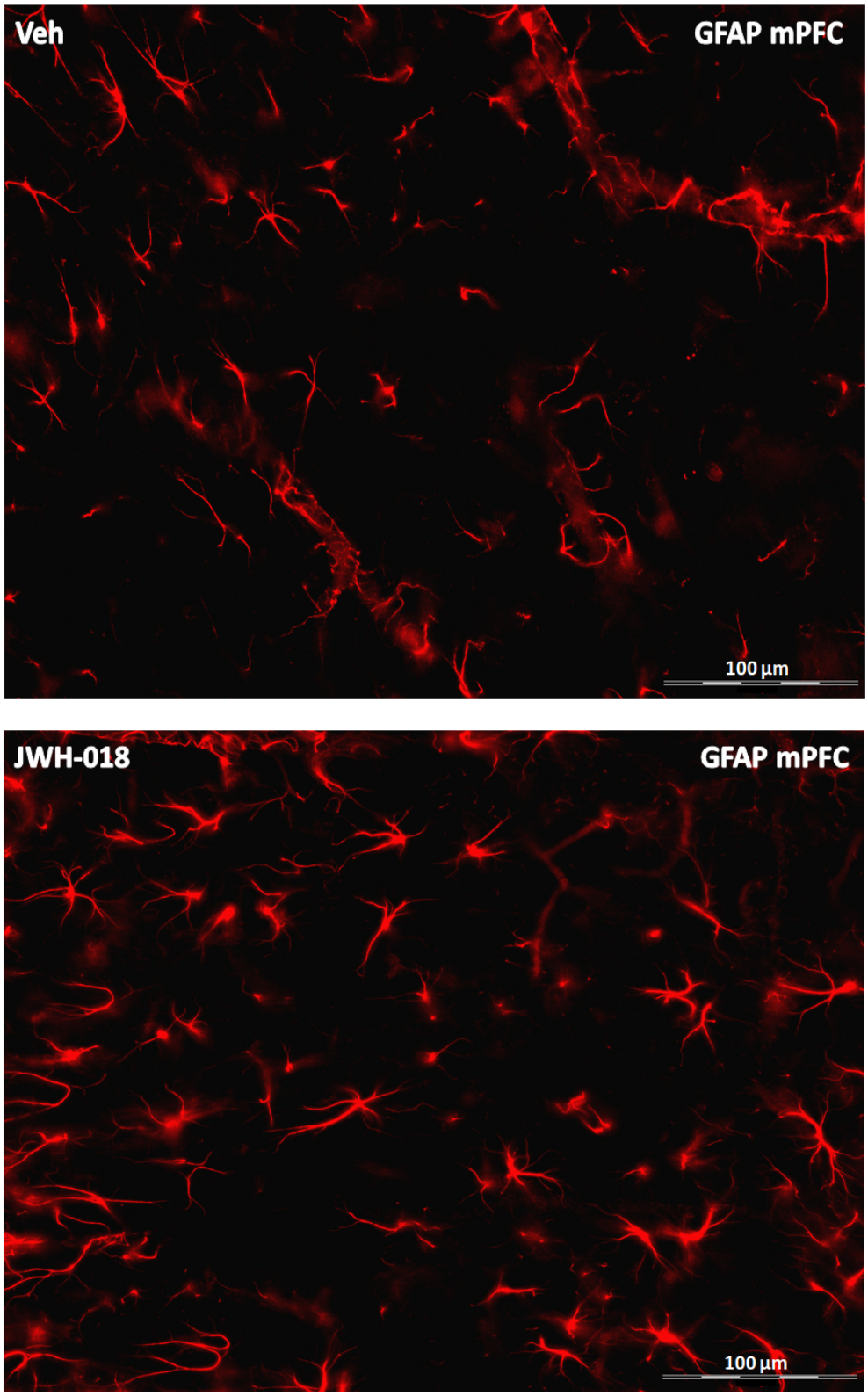

Figure 25 GFAP- Immnunoreactivity in the mPFC 24 hours after JWH-018 (0.25 mg/kg ip, daily for 14 days) discontinuation.

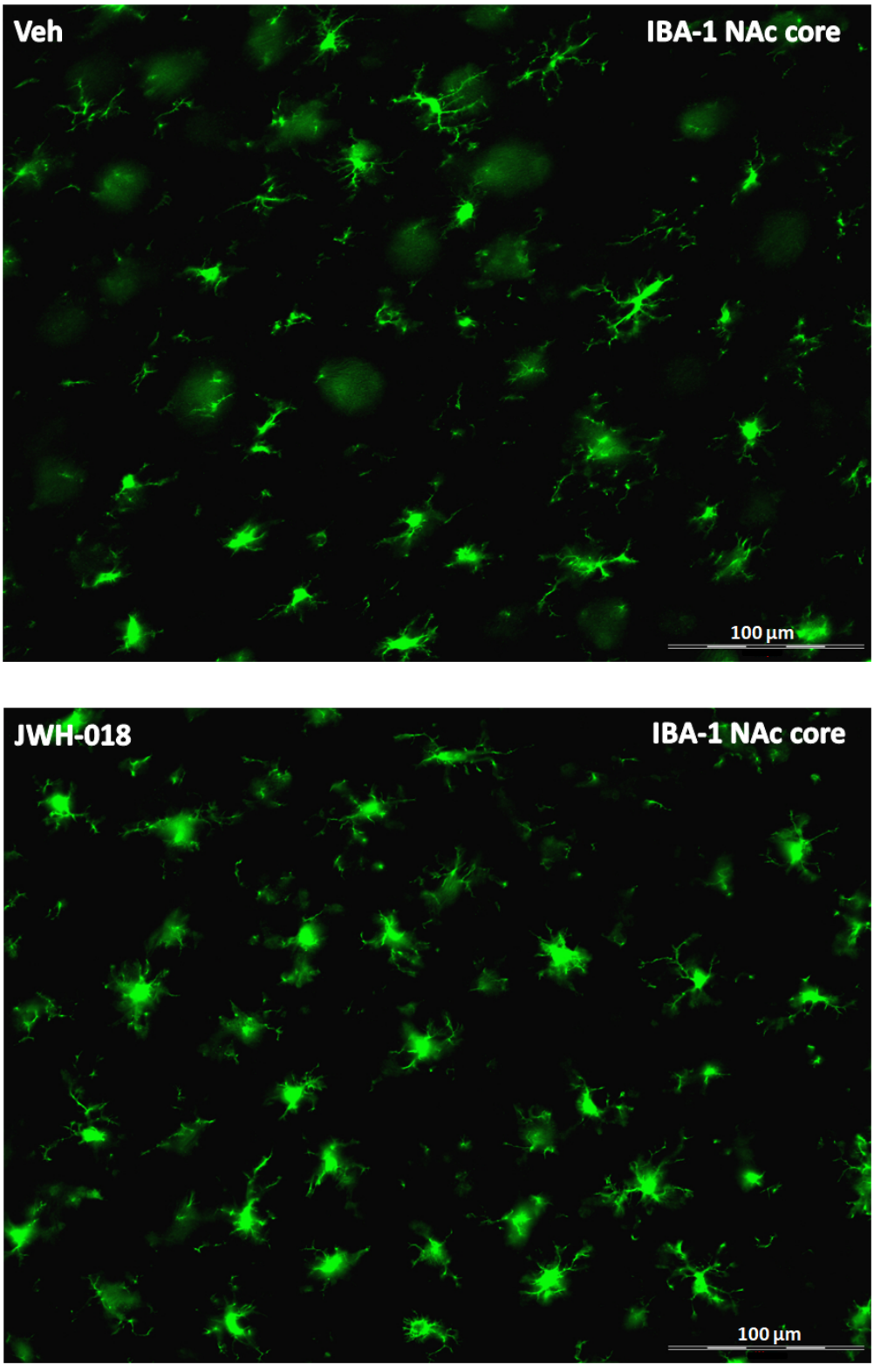

Figure 26 IBA-1-immnunoreactivity in the NAc core 24 hours after JWH-018 (0.25 mg/kg ip, daily for 14 days) discontinuation.

3.2 GFAP- and IBA-1-IR in DA brain areas seven days after JWH-018 (0.25 mg/kg ip, daily for 14 days) discontinuation

The effects of repeated JWH-018 exposure on GFAP-, IBA-1- IR was also investigated seven days after JWH-018 discontinuation. Two-way ANOVA analysis of GFAP-IR, showed a main effect of area [$F_{(4,33)}$=4.18, p< 0.01] and treatment [$F_{(1,33)}$=31.35, p< 0.00001], while no effect of area x treatment interaction was observed [$F_{(4,33)}$= 0.38, p= 0.81]. Bonferroni's post hoc revealed that JWH-018 treated group, as compared to Veh treated group, displayed a significant increase of GFAP levels, and a higher GFAP-IR in the mPFC than in the VTA. To better evaluate in each brain area the effect of JWH-018 treatment on GFAP-IR, data were analyzed separately using two- tailed unpaired Student's t-tests. As showed in Fig. 27A, JWH-018-treated rats, as compared to Veh-treated group, showed a significant increase of GFAP- IR in the mPFC [JWH-018: n=5, Veh: n=4; $t_{(7)}$ =2.63, p< 0.05], in the NAc shell [JWH-018: n=5, Veh: n=4; $t_{(7)}$ =2.61, p< 0.05], in the NAc core [JWH-018: n=5, Veh: n=4; $t_{(7)}$ =2.91, p< 0.05], in the CPu [n= 4 for group; $t_{(6)}$ =2.47, p< 0.05] and in the VTA [JWH-018: n=5, Veh: n=3; $t_{(6)}$ =4.91, p< 0.01].

Regarding IBA-1 IR, two-way ANOVA, showed a main effect of area [$F_{(4,33)}$=9.15, p< 0.0001] and treatment [$F_{(1,33)}$=17.59, p< 0.001] while no effect of area x treatment interaction was observed [$F_{(4,33)}$= 1.37, p=0.26]. Bonferroni's post hoc revealed JWH-018 treated group, as compared to Veh treated group, displayed a significant increase of IBA-1 levels. Moreover, the expression of IBA-1 positive cells was higher in the mPFC than in the NAc shell, in the NAc core core, in the CPu, and in the VTA. To better evaluate in each brain area the effect of JWH-018 treatment on IBA-1 IR, data were analyzed separately using two- tailed unpaired Student's t-tests. As shown in Fig. 27B a significant increase of IBA-1 positive cells in the NAc shell [$t_{(7)}$ =2.76, p< 0.05], in the NAc core [$t_{(7)}$ =2.65, p< 0.05] was detected in JWH-018-treated group as compared to Veh-treated group.

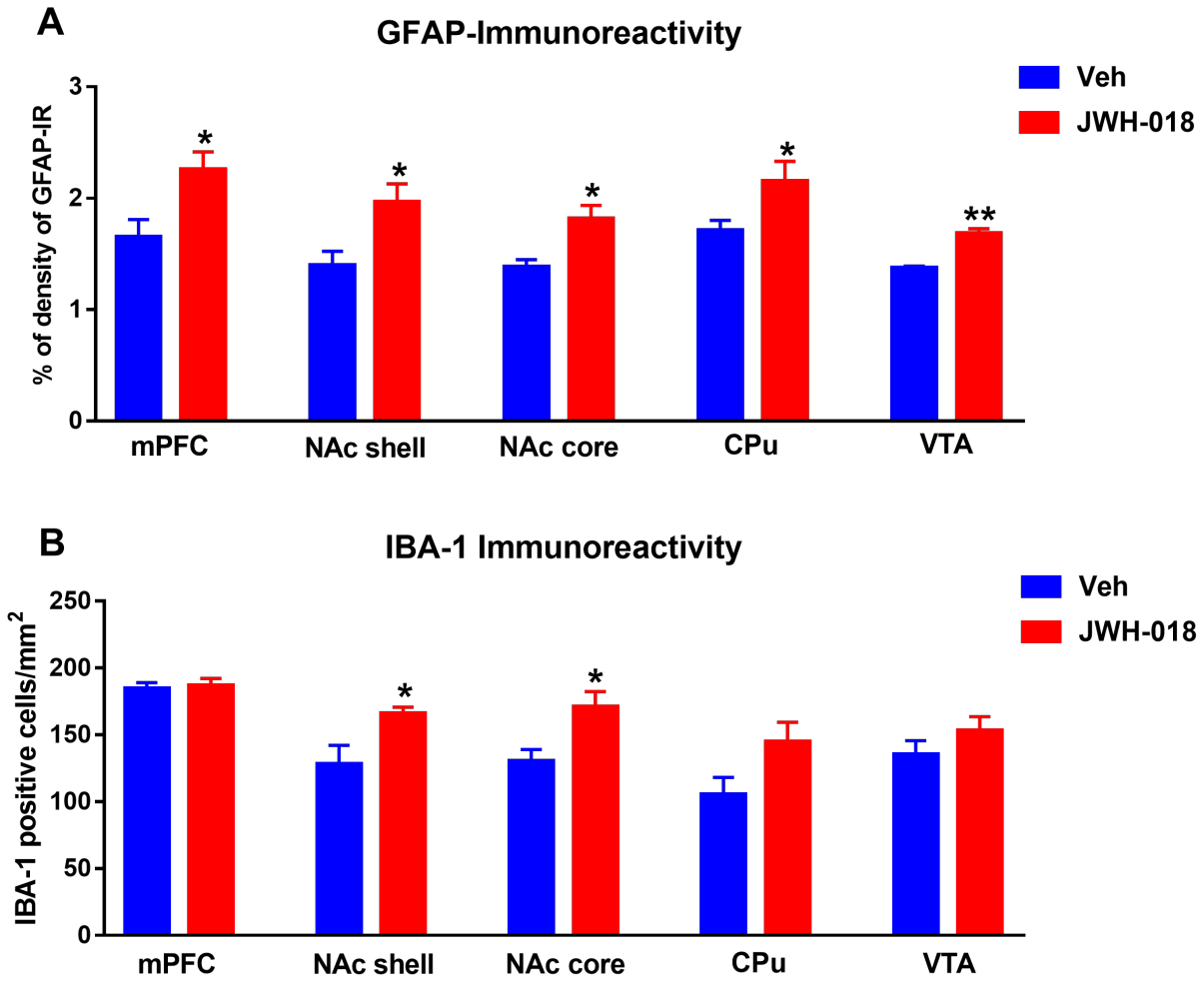

Figure 27 GFAP- and IBA-1-immunoreactivity in DA brain areas seven days after JWH-018 (0.25 mg/kg ip, daily for 14 days) discontinuation. Values represent means ± SEM of density reading expressed as a percentage of the area covered by GFAP immunoreactivity (IR) (JWH-018 mPFC, NAc shell and core: n=5; Veh mPFC, NAc shell and core: n=4; CPu: n=6 for group; VTA: JWH-018 n=5, Veh n=3; panel A); and of number of IBA-1 positive cells, expressed per mm^2 (JWH-018 mPFC, NAc shell and core: n=5; Veh mPFC, NAc shell and core: n=4; CPu: n=6 for group; VTA JWH-018 n=5, Veh n=3; panel B). * $p< 0.05$, ** $p< 0.005$ vs Vehicle (Veh) group (unpaired Student's t-tests).

E-Discussion

The present study evaluated the possible alterations of the DA system in relationship to the behavioral and neuroinflammatory effects elicited by repeated exposure to JWH-018 in rats.

1 Dysregulation of DA transmission induced by repeated JWH-018 administration

Regarding the effect of JWH-018 on the DA system, our results showed that repeated treatment with JWH-018 (0.25 mg/kg, ip, qd, 14 days) induces differential responsiveness on mesolimbic DA transmission in a challenge with JWH-018. While no changes in basal DA levels in the mPFC and in the NAc shell between JWH-018 and the vehicle-treated group (Fig.14A) have been observed, only control animals showed a significant increase of NAc shell DA. JWH-018-treated rats (Fig.14B), showed no significant DA increase in the same area. Moreover, no significant differences were observed in DA response in the mPFC after JWH-018 administration (Fig. 14C). This reduced DA response of the NAc shell displayed by JWH-018 treated rats is consistent with previous studies that reported reduced responsiveness of NAc shell DA to Δ^9-THC challenge in animals pre-exposed to a sensitizing regimen of the natural cannabinoid Δ^9-THC (Cadoni *et al.*, 2001; Cadoni *et al.*, 2008). Therefore, our result showed a decreased NAc shell DA response, that might reflect a tolerance phenomenon, most likely due to a fixed dose regiment used in our experimental protocol. Consistently, repeated exposure to CB agonists, including SCRAs, induces rapid tolerance to the neurophysiological effects of these drugs, both in animals (Abood & Martin, 1992; Maldonado & Rodriguez de Fonseca, 2002; Tai *et al.*, 2015; Elmore & Baumann, 2018) and in humans (Benowitz & Jones, 1975; Jones *et al.*, 1976; Hunt & Jones, 1980; Jones *et al.*, 1981; Hollister, 1986; Ramaekers *et al.*, 2011; Gorelick *et al.*, 2013). Moreover, it has been suggested that the internalization of CB1Rs and their down-regulation is one of the early pharmacodynamic adaptive mechanisms that leads to reduced responsiveness and tolerance in the case of prolonged

activation (Ameri, 1999; Martin *et al.*, 2004). Therefore, although we did not pharmacologically and molecularly evaluate this aspect, the data from the literature allows us to speculate that dysregulation of eCB signaling (i.e. CB1R down-regulation and desensitization, alteration eCB expression), like those observed after both repeated natural and synthetic cannabinoid administration (Fan *et al.*, 1994; Rubino *et al.*, 1994; Romero *et al.*, 1995; Romero *et al.*, 1997; Sim-Selley & Martin, 2002; Sim-Selley *et al.*, 2006; Volkow *et al.*, 2014; Volkow *et al.*, 2017), might alter eCB-mediated synaptic plasticity, leading to alterations of the DA rewarding system (i.e. changes in responsiveness, tolerance, and desensitization). However, discrepancies between this study and the previous reports might be due to differences in drugs, dose regiments, as well as in time of evaluation of DA responsiveness.

Afterwards, focusing on DA activity during an early withdrawal phase from repeated JWH-018 administration, electrophysiological and immunohistochemical experiments showed that repeated JWH-018 exposure is able to induce changes in basal VTA neuron activity. In particular, 24 hours after the last JWH-018 administration, we observed a decrease of the number of spontaneously active VTA DA cells (Fig. 15A), consistent with a decrease of TH-positive cells observed in the same brain area (Fig.15D). However, JWH-018 treated rats showed an increase in percentage of spikes in burst activity (Fig. 15C), while, as shown in Fig. 15B, no significant difference of the average firing rate was observed between JWH-018- and Veh-treated rats. These results are partially consistent with the literature about VTA DA neuron activity during acute withdrawal from addictive drugs; since repeated JWH-018 exposure induces a decrease of TH-positive neurons, similar to that observed after chronic exposure to morphine, ethanol, and Δ^9-THC (Sklair-Tavron *et al.*, 1996; Diana *et al.*, 2003; Spiga *et al.*, 2003; Spiga *et al.*, 2010). On the contrary, we observed a decrease of the number of spontaneously active VTA DA cells; no differences in their firing rate but an increase in their burst activity (increase of percentage of spikes in bursts) (Diana *et al.*, 1995; Diana *et al.*, 1998). Notably, in our experiments we used a lower dose (0.25 mg/kg ip) of JWH-018 than the dose regiment used in these studies (i.e. Δ^{9-}THC 15 mg/kg ip). These results, in particular

with the increase of burst activity, could be considered a compensatory mechanism in response to either this change observed in the DA terminal area (reduced DA NAc shell response), or to a parallel decrease of spontaneously active VTA DA cells.

Moreover, our results on DA neuronal activity seven days after JWH-018 discontinuation (Fig. 17) pointed out that this compensatory mechanism seems to be a transitory response/reaction of DA neurons, since we observed no difference in the burst activity of VTA DA neurons. Instead we observed a reduction of their firing rate, together with a reduction of spontaneous active VTA DA cells, as well as a decrease of VTA TH-positive neurons. This reduction of basal VTA DA neuron activity is consistent with the effects observed with other drugs of abuse (Sklair-Tavron *et al.*, 1996; Diana *et al.*, 2003; Spiga *et al.*, 2003; Spiga *et al.*, 2010), indicating an *hypodopaminergic state* unmasked during withdrawal from addicting drugs, which might lead to an impoverishment of already defective DA transmission (Melis *et al.*, 2005).

Therefore, our results demonstrated that repeated JWH-018 exposure induces an alteration/decreased VTA DA neuronal activity, which persist at least a week after JWH-018 discontinuation. Although only few studies investigated the effects of repeated SCRA exposure on DA activity, our data are consistent with alterations in mesostriatal DA function observed after chronic WIN55,212-2 administration (Fanarioti *et al.*, 2014; Perdikaris *et al.*, 2018). Interestingly, Perdikaris and colleagues (2018) reported a parallel reduction of CB1R binding in DA areas, consistent with widespread desensitization and down-regulation of CB1Rs after long-term, but not acute, administration of Δ^9-THC, WIN55,212-2 or CP55,940 (Rubino *et al.*, 1994; Romero *et al.*, 1995; Romero *et al.*, 1997; Sim-Selley & Martin, 2002; Sim-Selley *et al.*, 2006), supporting the idea of the important involvement of eCB signaling in the DA dysregulation observed.

A crucial result of the present study's focus on dysregulation of DA transmission induced by repeated JWH-018 administration is the changes observed on responsiveness of mesocorticolimbic DA transmission during repeated exposure to appetitive taste stimulus (chocolate solution) seven days after JWH-018 discontinuation.

As shown in Fig.18B, different from the NAc shell DA response shown by Veh- treated rats (an increase DA extracellular levels), the first chocolate exposure did not affect DA response in the NAc shell of JWH-treated rats, while no differences in the NAc core and the mPFC DA response to taste in naïve rats were observed (Fig.18D,F). Thus, we reported, for the first time using this experimental paradigm, that repeated JWH-018 exposure, different from morphine sensitization and mPFC DA lesions, induces decreased sensitivity for natural reward in the NAc shell. This lack of response of NAc shell DA to the first chocolate exposure might represent decreased sensitivity to the reinforcing properties of natural rewarding stimuli, consistent with the idea that repeated exposure to high reward value, such as addictive drugs leads to a resetting of reward thresholds, resulting in a decrease of reinforcing properties of non-drug stimuli (Koob & Le Moal, 2001).

As a matter of fact, although no difference in mPFC DA response in naive rats was observed, JWH-018 treated rats showed opposite changes in the DA response of the mPFC and the NAc shell to the second[d] chocolate exposure. As shown in Fig.18G, the unexpected appearance of habituation in mPFC DA responsiveness to taste stimuli was accompanied by a loss of habituation in the NAc shell (Fig.18C). Interestingly, the same changes to DA responsiveness were observed in morphine sensitized chocolate pre-exposed rats (De Luca *et al.*, 2011), suggesting that different classes of addictive drugs might affect an evaluation of the motivational value of rewarding stimuli.

Therefore, it seems reasonable to hypothesize that these long-term changes in the DA response of different terminal areas observed either after repeated JWH-018 exposure or morphine sensitization are implicated in motivational processes and probably result in the dysregulation of incentive arousal and learning. (De Luca *et al.*, 2011), as well as that of motivational evaluation of rewarding stimuli. Indeed, since the habituation of the mPFC DA response to chocolate disinhibits NAc shell DA, abolishing the single-trial habituation of DA might facilitate repeated approaches toward a motivational stimulus (De Luca *et al.*, 2011; De Luca, 2014). This top-down control of the mPFC on DA subcortical areas has been confirmed using the same experimental paradigm used in our study by Bimpisidis and colleagues (2013), since they demonstrated that lesions of mPFC DA

terminals abolished the habituation of the NAc shell DA response to repeated chocolate exposure. Therefore, our observation in accordance with both morphine sensitization and mPFC DA lesion results, confirm top-down control of NAc DA by the mPFC and its putative role in the loss of control of the motivational value of natural stimuli (De Luca, 2014). Moreover, as suggested for morphine sensitization, this lack of NAc DA habituation may be considered per se as a marker of drug dependence and its liability(De Luca, 2014).

In this experiment we also observed changes in NAc core DA response, which were completely different than those observed in morphine sensitization and mPFC lesioned rats (De Luca *et al.*, 2011; Bimpisidis *et al.*, 2013; De Luca, 2014). In particular, we observed no difference in the NAc core response to the first chocolate exposure between JWH-018 and Veh treated-rats(Fig.18D), on the contrary, morphine sensitization and lesions of mPFC DA terminals produced an elevated, delayed, and prolonged increase of DA in the NAc core. Moreover, JWH-018 treatment potentiated, delayed and prolonged a DA response to the second chocolate exposure(Fig.18E). Diversely, morphine sensitization was able to induce an immediate increase of NAc core DA, while lesions of mPFC DA terminals did not affect NAc core DA responsiveness of pre-exposed rats to chocolate (De Luca *et al.*, 2011; Bimpisidis *et al.*, 2013; De Luca, 2014). Therefore, although our results confirm an alteration of both NAc subdivision DA responsiveness to repeated chocolate exposure after repeated JWH-018 administration, understanding their meaning is still hard. In this respect, is important to remark that the mPFC DA inhibitory control of DA responsiveness in subcortical areas is different depending on the ventral striatum sub-region studied (i.e. the PL, and the IL cortex), since they have different projections to different compartments of the NAc., consistent with the different responsiveness of NAc shell and core DA to discrete stimuli and conditions (Di Chiara *et al.*, 2004; Di Chiara & Bassareo, 2007; Aragona *et al.*, 2009; Corbit & Balleine, 2011; Cacciapaglia *et al.*, 2012). Moreover, discrepancies between our results and the previous reports might be due to differences in drugs and dose regiment, as well as experimental conditions.

Unfortunately, no evidence about the effects on DA responsiveness to taste stimuli induced by either repeated Δ^9-THC or other SCRA exposure has been reported. However, using the same experimental paradigm used in our study, De Luca and colleagues (2012) demonstrated that acute Δ^9-THC (1mg/kg ip) administration through activation of CB1Rs, enables oral sucrose (sucrose solution 20%, io) to increase DA in the NAc shell, but not in the NAc core, and this effect shows the same adaptive property of a highly palatable food, since it undergoes habituation after a single exposure to the food taste (De Luca *et al.*, 2012).Thus, this evidence suggests different effects between acute and repeated cannabinoid exposure, as well as between natural and synthetic CB on DA responsiveness to taste stimuli.

In conclusion, our study demonstrated that repeated JWH-018 exposure induces a profound dysregulation of the DA system, since we observed changes either in the basal activity of VTA DA neurons (reduction) or in responsiveness to mesocorticolimbic DA transmission (either to JWH-018 or to taste stimuli). Moreover, our data confirm the crucial role of the mesocorticolimbic DA pathway in the evaluation of the motivational value of rewarding stimuli, and the pivotal inhibitory role of mPFC DA on subcortical DA areas involved in these processes. In this context, this JWH-018 induced mesocorticolimbic DA dysregulation might be associated with a loss of control of motivational evaluation of rewarding stimuli, thereby leading to compulsively focused motivation on drugs and drug-related stimuli typical of drug addiction (Volkow *et al.*, 2003; Di Chiara *et al.*, 2004; Volkow & Morales, 2015).

2 Behavioral effects induced by repeated JWH-018 administration

In this study we performed multiple behavioral tests, at different time points, to understand the effects of repeated JWH-018 exposure, as well as JWH-018 withdrawal on behavioral tasks. Aspects of emotional state, cortical processing, attentional filtering, sensorimotor-gating, and pre-attentional processes were evaluated, in addition to somatic signs of withdrawal and taste reaction to natural rewarding stimulus (chocolate solution).

Repeated JWH-018 exposure influences the emotional state of rats, as revealed by the reduction of time spent on the open arms of the EPM; increased compulsive activity, as revealed by the higher number of marbles buried in the MB; and induces impairments of sensorimotor-gating in PPI (Fig.19).

The influence of JWH-018 on EPM performance suggests that this SCRA influences the emotional state of rats, in particular indicating an anxiogenic effect of JWH-018 (Pellow et al., 1985; Kliethermes, 2005). Moreover, JWH-018 did not influence the total number of arm entries, suggesting that reduction of the time spent on the open arms observed in rats exposed to JWH-018 is not a epiphenomenon due to a non-specific action of this SCRA on rat behavior, but rather stems from a genuine effect of JWH-018 on the emotional state. This JWH-018-induced alteration of emotional state ,is supported by MB results, since we observed an increase of the total number of marbles buried by JWH-018 treated rats (Fig19A). The marble burying test is generally used to evaluate compulsive activity/repetitive like behaviors (Albelda & Joel, 2012; Zanda *et al.*, 2017) but also to screen anxiolytic drugs (Jimenez-Gomez *et al.*, 2011; Kinsey *et al.*, 2011). Thus, one limitation of the MB test is related to the question of whether marble burying represents a compulsive-like behavior or anxiety-like responses in mice, as revealed by several studies (Thomas *et al.*, 2009; Angoa-Perez *et al.*, 2013; Kedia & Chattarji, 2014; Murphy *et al.*, 2017). However, the increase of marble burying displayed by JWH-018 treated rats, together with the reduction of the time spent on the open arms of the EPM, might represent different aspects of anxiogenic effects, as

well as an alteration of emotional state, induced by repeated JWH-018 exposure. This result is consistent with the anxiogenic effects induced by sub-chronic JWH-018/ WIN in mice, even though no difference in the time spent on the open arm of the EPM was observed (Macri *et al.*, 2013). This discrepancy might be due to difference in time and dose regiment, as well as species. Therefore, although we did not pharmacologically and molecularly evaluate this aspect. Data in the literature support the view that dysregulation of the eCB system, as those observed after repeated CB administration (Fan *et al.*, 1994; Sim-Selley & Martin, 2002; Sim-Selley *et al.*, 2006; Volkow *et al.*, 2014; Volkow *et al.*, 2017) might induce abnormalities in the modulation of anxiety-related response, since the eCB system is involved in the regulation of emotional behaviors, particularly in the PFC and amygdala, likely via CB1R activation (Rubino *et al.*, 2008; Parolaro *et al.*, 2010). In line with this view, regular marijuana use is associated with an increased risk of anxiety and depression (Patton *et al.*, 2002), and an increased sensitivity to anxiety symptoms (Buckner, et al., 2009).

However, the increase of marble burying observed in JWH-018 treated rats could represent a compulsive response in rats, likely a consequence of cortical alteration, like those observed in the present study (see in Results *DA dysregulation* and *Neuroinflammatory effects induced by repeated JWH-018 administration*). In line with this speculation, we observed that repeated JWH-018 administration induces attentional deficits as revealed by impairments of sensorimotor-gating in the PPI (Fig19D). Few clinical studies have investigated the impact of cannabis consumption on PPI (Quednow *et al.*, 2004; Kedzior & Martin-Iverson, 2007; Mathias *et al.*, 2011), producing mixed results due to different dose and time regiments. To the best of our knowledge, only two studies have investigated the effects of chronic Δ^9-THC exposure on PPI in rodents (Long *et al.*, 2010; Tournier & Ginovart, 2014). In one of these studies in particular, no effects on PPI was reported at either 7 days or 18 days of Δ^9-THC treatment (Long *et al.*, 2010), while in the other study, the authors reported that chronic Δ^9-THC treatment (1 mg/kg/day for 21 days) produced significant reductions in PPI 30 min after the last Δ^9-THC THC injection (21st day) (Tournier & Ginovart,

2014). Therefore, the reduction of PPI that we observed 1 hour after the last JWH-018 injection (14th day) is consistent with this latter evidence (Tournier & Ginovart, 2014).

Altogether, our results, which are consistent with the literature, demonstrated that repeated JWH-018 administration induces behavioral abnormalities (changes in emotional state and attentional deficits) that might be correlated with an alteration of mesocorticolimbic DA transmission, as well as with eCB signaling.

Afterwards, we repeated these behavioral tasks 24 hours after JWH-018 discontinuation, to evaluate possible behavioral effects during acute JWH-018 withdrawal. In addition, we evaluated classical somatic signs of spontaneous CB withdrawal (Diana *et al.*, 1998; Aceto *et al.*, 2001). As shown in Fig.20, similarly to results obtained 1 hour after the last JWH-018 injection, we observed an alteration of emotional state in JWH-018 treated rats, as revealed by the reduction of time spent on open arms of the EPM and by the higher number of total marbles buried in the MB test; on the contrary, no impairment in the PPI was observed during acute withdrawal from repeated JWH-018 exposure.

Therefore, these results confirmed that behavioral abnormalities induced by repeated JWH exposure persist also during the acute withdrawal phase (24 hours after JWH-018 discontinuation). Moreover, as shown in Fig.21, JWH-018 treated rats exhibited a significant increase in several common signs of spontaneous CB withdrawal, such as licking, headshakes and tongue rolling, as well as biting and chewing, as compared to Veh-treated rats. Our results are consistent with previous studies that reported spontaneous withdrawal signs after WIN 55,212-2 and CP 55,940 administration, together with other behavioral effects related to emotional circuits (Aceto *et al.*, 2001; Oliva *et al.*, 2003; 2004; Aracil-Fernandez *et al.*, 2013). Recently, spontaneous withdrawal signs induced by high doses of Δ^9-THC (10 or 50 mg/kg/day per 6 days, sc) and JWH-018 (1 mg/kg/day per 6 days, sc) (Trexler *et al.*, 2018) have been reported in mice. Importantly, the signs reported in these studies (Aceto *et al.*, 2001; Oliva *et al.*, 2003; 2004; Trexler *et al.*, 2018), like in our results, were generally of a lower magnitude than rimonabant-precipitated withdrawal, suggesting that spontaneous

withdrawal is less intense than precipitated withdrawal. Interestingly, similarly to what has been observed in rodents (Oliva *et al.*, 2003; 2004; Aracil-Fernandez *et al.*, 2013), in chronic cannabis users, withdrawal induces stress and anxiety (Haney *et al.*, 1999), indicating that, in addition to somatic withdrawal signs, emotional circuits result as being altered/activated during CB withdrawal (Trexler *et al.*, 2018).

Therefore altogether our results, consistent with the literature demonstrated, show/indicate that acute JWH-018 withdrawal induces an alteration of emotional state, in addition to somatic signs of withdrawal, that might be correlated to the dysregulation of DA activity, as well neuroinflammation, observed during acute withdrawal.

In the present study we also evaluated possible behavioral abnormalities seven days after JWH-018 discontinuation. In particular we focused on possible somatic signs of withdrawal, and parallel to the evaluation of DA response to repeated chocolate exposure, we quantified both hedonic and aversive taste reactions to chocolate through the taste reactivity test. As shown in Fig.22, different from the somatic signs of spontaneous withdrawal observed 24 hours after the last JWH-018 injection, only an increase in biting was found in the JWH-018 treated group. Therefore, as we expected, no spontaneous withdrawal signs were detected seven days after JWH-018 discontinuation. In fact, no evidence of spontaneous CB withdrawal signs have been reported both in humans and in rodents after seven days, from either natural or synthetic CB discontinuation. Ongoing studies are investigating the persistence of emotional state abnormalities, since apart from being short-lasting, in humans, somatic withdrawal signs are not the primary symptoms of cannabinoid withdrawal, compared to altered stress, emotional states, or impaired social or occupational functioning (American Psychiatric Association, 2013).

Considering taste reaction to chocolate, as shown in Fig.23, no significant differences in appetitive taste reactions to each chocolate exposure were observed, while JWH-018 treated rats showed an increase in aversive taste reaction after the first but not the second chocolate exposure. Thus, although repeated JWH-018 administration induces changes in DA responsiveness to repeated

chocolate exposure (see in Results *DA dysregulation induced by repeated JWH-018 administration*), consistent with the results observed in morphine sensitizated and mPFC lesioned rats (De Luca *et al.*, 2011; Bimpisidis *et al.*, 2013; De Luca, 2014), changes in behavioral taste reactivity are lacking, supporting the hypothesis that taste-hedonia does not depend on DA (Berridge & Robinson, 1998). Moreover, like with morphine sensitization, the increase of NAc shell DA response to the second chocolate exposure might be due to the increase of motivational, and not hedonic properties, of the taste (Bassareo & Di Chiara, 1999; Bassareo *et al.*, 2002; De Luca *et al.*, 2011; Bimpisidis *et al.*, 2013; De Luca, 2014).

On the contrary, unlike these studies (De Luca *et al.*, 2011; Bimpisidis *et al.*, 2013; De Luca, 2014) we observed that JWH-018 treated rats showed an increase of aversive taste reactions after the first chocolate exposure, while no differences in rats pre-exposed to chocolate were observed. Although this increase of aversive reactions is concomitant to a decreased sensitivity of NAc shell DA to the first chocolate exposure, that could allow one to hypothesize a correlation between DA and taste reaction. As already discussed, several studies have reported no correlation between DA response and taste reactions to taste stimuli (i.e. chocolate solution) (Bassareo & Di Chiara, 1999; Bassareo *et al.*, 2002; De Luca *et al.*, 2011; Bimpisidis *et al.*, 2013; De Luca, 2014) . Therefore, it seems reasonable to hypothesize that this increase of aversive reactions to novel taste stimulus might represent either a "*neophobia*" phenomenon, or an "*anhedonia-state*" maybe due to abnormalities of emotional state, as with those observed during acute JWH-018 withdrawal. In addition, dysregulation of eCB signaling, such as CB1 down regulation/desensitization reported after repeated CB administration, might be involved in this increase of aversive taste reactions, since the eCB system has a prominent influence on the hedonic effects of natural rewards, such as food (Silvestri & Di Marzo, 2013) (Parsons & Hurd, 2015). In line with this hypothesis, different from repeated JWH-018 administration, acute exposure to THC (0.5- 1.0 mg/kg ip) increases hedonic taste reactions to a sucrose solution, effects mediated by CB1Rs (Jarrett *et al.*, 2005; De Luca *et al.*,

2012). However, since these reactions were of a lower magnitude than hedonic taste reactions (at least ten-fold lower), an interpretation of the biological relevance of this finding is precluded.

In conclusion, altogether our results showed that repeated JWH-018 administration induces an alteration of emotional state, compulsive like-behaviors, which also persist during an acute withdrawal period, in addition to classical somatic signs of cannabinoid withdrawal. Moreover, JWH-018 exposure induces attentional deficits and increases aversive taste reaction to chocolate seven days after JWH-018 discontinuation, that might be correlated with abnormalities of emotional states and might represent a JWH-018 induced "*anhedonia-state*".

Thus, our data in accordance with the literature allows us to speculate that repeated exposure to JWH-018 might induce perturbation of eCB signaling, that in turn might be implicated in behavioral abnormalities observed in our study. In this view, the parallel DA neuronal and glial cell alteration might represent two other crucial factors implicated in the behavioral effects induced by repeated JWH-019 administration.

3 Neuroinflammatory effects induced by repeated JWH-018 administration

In this study, in parallel with an evaluation of the DA system and behavioral effects, we evaluated possible neuroinflammatory effects induced by repeated JWH-018 exposure (0.25 mg/kg ip, daily for 14 days). In particular, we focused on astrocyte and microglia expression in different DA brain areas, using specific markers for these two types of glial cells (GFAP and IBA-1, respectively).

As shown in Fig 24, 24 hours after the last injection, we observed that repeated JWH-018 exposure induces astrogliosis (an increase in GFAP expression) in all DA brain areas examined (the mPFC, the NAc shell and core, the CPu, and VTA), together with an increase of IBA-1 positive cells in the NAc shell, in the Nac core, and in the CPu.

Considering GFAP expression, our result shows for the first time that repeated exposure to the SCRA JWH-018, induces changes in astrocytes in mesocorticolimbic DA areas, which are involved in reward and addiction mechanisms. In fact, despite there being an abundance of evidence about alteration of astrocytes after repeated exposure to different classes of addictive drugs, such as opioids, alcohol and cocaine (Lacagnina *et al.*, 2017; Kim *et al.*, 2018) as well as Δ^9-THC (Cutando *et al.*, 2013; Zamberletti *et al.*, 2016; Melis *et al.*, 2017), so far no evidence about SCRA-induced astrocyte changes has been reported.

Furthermore, considering cannabinoid exposure, to the best of our knowledge, the limited number of studies has not investigated the effects of SCRA treatment/exposure at adulthood but has been focused on the effects of natural cannabinoid, such as Δ^9-THC, exposure in prenates (Suarez *et al.*, 2000) or adolescents (Lopez-Rodriguez *et al.*, 2014; Zamberletti *et al.*, 2016). The research reveals either an increase (astrogliosis) or a decrease (astrocytopathy) of astrocytes. Interestingly, opposite astrocytes alteration/adaptation has been observed also in other neuropsychiatric disorders, such as in schizophrenia (Trepanier *et al.*, 2016). However, by analyzing the available literature a preponderance of evidence for astrocytopathy associated with depression, with generally inflammatory effects (astrogliosis) with drugs of abuse (also schizophrenia) that may normalize or

revert to astrocytopathy over a period of withdrawal (Kim *et al.*, 2018) emerge. In the case of drugs of abuse, it is important to consider the time course, route of administration, drug classes, dose, length of withdrawal, and other methodological factors when considering their effects on properties and the function of astrocytes, since either astrogliosis or astrocytophaty have been reported (Song & Zhao, 2001; Fattore *et al.*, 2002; Bowers & Kalivas, 2003; Evrard *et al.*, 2006; Dalcik *et al.*, 2009; Miguel-Hidalgo, 2009; Friend & Keefe, 2013; Castelli *et al.*, 2014; Schwalm *et al.*, 2014; Scofield *et al.*, 2016).

Of note, considering GFAP expression during abstinence, we observed an astrogliosis (increase of GFAP expression) in different DA areas during acute JWH-018 withdrawal (24 hours after the last injection), which is consistent with the increase of GFAP expression observed during acute cocaine, methamphetamine, and morphine withdrawal (Fattore *et al.*, 2002; Granado *et al.*, 2011; Berta *et al.*, 2012; Harada *et al.*, 2013; O'Callaghan *et al.*, 2014). Interestingly, in parallel with alteration of astrocytes, several studies reported changes in astrocyte glutamate transport-function (i.e. GLT-1 reduction) during withdrawal from cocaine, ethanol, and morphine (Wu *et al.*, 2008; Fischer-Smith *et al.*, 2012; Abulseoud *et al.*, 2014; Das *et al.*, 2016; Kim *et al.*, 2018), supporting the role of astrocytes in glutamate homeostasis at neuronal synapses (Danbolt, 2001; Bjornsen *et al.*, 2014; Takahashi *et al.*, 2015) and in line with the concept of the "tripartite synapse" (Hammond *et al.*, 2015). In this view, eCB signaling seems to play a pivotal role, since several studies point out a strong relationship between glial cells (i.e. astrocytes and microglia) and the eCB systems and disruptions in glial cell function and/or maladaptive astrocyte response may be implicated in drug abuse liability and addictive behavior (Navarrete & Araque, 2008; Stella, 2010; Han *et al.*, 2012; Bosier *et al.*, 2013; Navarrete *et al.*, 2014; Belluomo *et al.*, 2015).

Therefore, the increase of reactive astrocytes in DA areas that we observed during acute JWH-018 withdrawal might be considered either a neuroinflammatory effect of repeated exposure to JWH-018 (likely due to alterations of eCB signaling) or, one of the reasons/causes of neurochemical (DA

dyregulation) and behavioral abnormalities (emotional state alteration) observed in our study. In fact, a reciprocal relationship between glial cells and drug of abuse (also drug-induced effects) has been demonstrated (Block *et al.*, 2007; Corty & Freeman, 2013; Brown & Neher, 2014) (see *Introduction*).

In our study we also observed an increase of microglia cells, as revealed by increased IBA-1-IR in the NAc shell, core, and in the CPu of JWH-018 treated rats. Thus, besides astrogliosis, our results pointed out an increase of microglia cells specific to DA limbic areas, supporting the idea of the important involvement of glial cells in drug addiction (Lacagnina *et al.*, 2017; Melis *et al.*, 2017). Consistent with our results, the implication of microglial cells (as well as astrocytes) in the effects of repeated Δ^9-THC exposure (Cutando *et al.*, 2013; Zamberletti *et al.*, 2015) has been demonstrated, supporting the involvement of the eCB system and its dysregulation in glial cell physiology, as well as in neuroinflammatory processes. These studies reported that cognitive impairments and depressive-like behaviors induced by repeated Δ^9-THC exposure were associated with alterations in microglial morphology (increased CD11b-positive cells), neuronal CB1R down regulation, microglial CB2R up regulation, increased expression of the pro-inflammatory cytokines, and reduction of the anti-inflammatory cytokines (Cutando *et al.*, 2013; Zamberletti *et al.*, 2015). Collectively these data from the literature allows us to speculate that the observed perturbation of glial cells (increased GFAP and IBA-1 expression) induced by repeated JWH-018 exposure, might play an important role in the alteration of DA activity (reduced basal DA neuronal activity and decreased NAc shell DA sensitivity to rewarding stimuli) and behavioral abnormalities (increased anxiety and compulsive-like behavior) observed during acute JWH-018 withdrawal. Further studies to further elucidate possible cytokine perturbation, as well eCB signaling (CBR expression) alteration induced by repeated JWH-018 administration are in progress.

Moreover, our study demonstrated that this neuroinflammatory state of mesocorticolimbic DA areas endures also seven days after JWH-018 discontinuation. In fact, as shown in Fig. 27, a persistent astrogliosis (increased GFAP-IR) in all examined DA areas (in the mPFC, NAc shell, NAc core,

CPu and VTA) or an increase of microglia cells (increased IBA-1 IR) in the NAc shell and core is present in JWH-018 treated rats as compared to Veh-treated rats. Similar to the changes observed after acute JWH-018 withdrawal, we demonstrated an increase of GFAP expression in all analyzed DA areas. These results are consistent with the increase of GFAP expression observed after "prolonged withdrawal" (at least 6 days) from cocaine (Bowers & Kalivas, 2003) and amphetamine (Granado *et al.*, 2011; Occhieppo *et al.*, 2017), even though, as observed during acute withdrawal, other studies showed contradictory results (Bowers & Kalivas, 2003; Harada *et al.*, 2013; Bull *et al.*, 2015; Scofield *et al.*, 2016).

Indeed, our immunohistochemical results are associated with the parallel changes in either basal DA neuronal activity (reduction) or in responsiveness of mesocorticolimbic DA transmission observed seven days after JWH-018 discontinuation (see results "DA dysregulation induced by repeated JWH-018 administration"), confirming the possible involvement of astrocytes in DA dysregulation.

Considering microglia, our study revealed an increase of IBA-1 positive cells in the NAc shell and core also seven days after JWH-018 discontinuation, while no difference in the CPu was observed. Thus, our data evidenced that changes in microglia cells persist only in the NAc (shell and core), showing that alteration of JWH-018-induced microglia cells is brain region-specific as compared to astrocyte alteration. Thus, it is reasonable to hypothesize the specific involvement of activated microglia cells in mesolimbic DA pathway alteration, that might reflect either an important involvement of CB2R (i.e. CB2R upregulation, see Introduction) in the observed mesolimbic DA dysregulation, or a stronger neuroinflammation of the NAc (i.e. astroglyosis and activation of microglia), thereby a stronger impact of glial cells in JWH-018-induced DA alterations in the NAc.

In conclusion, altogether our results show that, besides neurons, repeated JWH-018 administration affect glial cells, likely inducing an abnormal interaction between these two cell populations, which in turn could be implicated in JWH-018-induced neurochemical and behavioral abnormalities.

Further studies to better understand either the underlining mechanisms or the relationship between JWH-induced neuroinflammation, DA dysregulation and behavioral abnormalities are required. However, considering our results and those of the literature, it is plausible to hypothesize that repeated JWH-018 exposure may induce changes in eCB signaling (i.e. down-regulation CB1R and up-regulation CB2R), which either directly might induce a perturbation of glutamate signaling, as well as of glutamate-GABA balance, or indirectly through changes in glial cell expression (i.e. astrogliosis and increased microglia) and activity (i.e. decreased GLT-1 and increased pro-inflammatory citokines) that in turn might lead to a dysregulation of the mesocorticolimbic DA system and behavioral abnormalities.

F-Conclusion

Initially developed for therapeutic proposed, the SCRAs, such as JWH-018, have been detected in herbal blend called Spice drugs (known also as "K2 drugs" in the USA) sold on websites as cannabis-like mixtures since 2004(Dresen *et al.*, 2010). Despite the long history and the initial proposed, the SCRAs, in particular those of the last generations appear to not possess any of the therapeutic effects of Δ^9-THC (De Luca & Fattore, 2018). Hence, clinical reports showed that SCRAs induce more adverse reactions, long-term effects and psychiatric consequences (Papanti *et al.*, 2014; Schifano *et al.*, 2015), several withdrawal symptoms, and more prevalence of dependence after their chronic use (Miliano *et al.*, 2016; Pintori *et al.*, 2017).

In the present study we demonstrated for the first time, that repeated exposure to JWH-018 (0.25 mg/kg ip, daily for 14 days) during the adulthood induces dysregulation of mesocorticolimbic DA system, either on basal DA neuronal activity or on DA responsiveness to drug and non-drug stimuli, which persist at least seven days after JWH-018 discontinuation and might be associated with loss of control of motivational evaluation of rewarding stimuli. In parallel with DA dysregulation, JWH-018 treatment induces alteration of glial cells (neuroinflammatory phenotype) in the same DA areas, and behavioral abnormalities in particular during acute JWH-018 withdrawal. Therefore, our data, besides confirming the high abuse liability of the SCRA JWH-018 and the possible severe health risk lead to their chronic use, support the evidence that identified mesocorticolimbic DA dysregulation as critical features in drug addiction (Kalivas & Volkow, 2005; Jensen *et al.*, 2008; Goldstein & Volkow, 2011; Deserno *et al.*, 2013; De Luca, 2014). Moreover, this study supported by the literature, pointed out the possible relationship between neuroinflammation and dysregulation of DA system in the drug addiction (Lacagnina *et al.*, 2017; Melis *et al.*, 2017; Kim *et al.*, 2018).

Taken together, these results suggest that JWH-018 repeated treatment may reflect a model of addiction, and this study could be useful to understand if dysfunctions of cortical-limbic-striatal DA circuit, as well as glial cells alterations, can lead or are related to specific detrimental effects of recurring use of Spice/K2 drugs.

G-References

Abood, M.E. & Martin, B.R. (1992) Neurobiology of marijuana abuse. *Trends in pharmacological sciences*, **13**, 201-206.

Abrams, D.I. & Guzman, M. (2015) Cannabis in cancer care. *Clinical pharmacology and therapeutics*, **97**, 575-586.

Abulseoud, O.A., Camsari, U.M., Ruby, C.L., Kasasbeh, A., Choi, S. & Choi, D.S. (2014) Attenuation of ethanol withdrawal by ceftriaxone-induced upregulation of glutamate transporter EAAT2. *Neuropsychopharmacology*, **39**, 1674-1684.

Aceto, M.D., Scates, S.M. & Martin, B.B. (2001) Spontaneous and precipitated withdrawal with a synthetic cannabinoid, WIN 55212-2. *European journal of pharmacology*, **416**, 75-81.

Acute kidney injury associated with synthetic cannabinoid use--multiple states, (2012). *MMWR. Morbidity and mortality weekly report*, **62**, 93-98.

Aguzzi, A., Barres, B.A. & Bennett, M.L. (2013) Microglia: scapegoat, saboteur, or something else? *Science (New York, N.Y.)*, **339**, 156-161.

Albayram, O., Alferink, J., Pitsch, J., Piyanova, A., Neitzert, K., Poppensieker, K., Mauer, D., Michel, K., Legler, A., Becker, A., Monory, K., Lutz, B., Zimmer, A. & Bilkei-Gorzo, A. (2011) Role of CB1 cannabinoid receptors on GABAergic neurons in brain aging. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 11256-11261.

Albelda, N. & Joel, D. (2012) Animal models of obsessive-compulsive disorder: exploring pharmacology and neural substrates. *Neurosci Biobehav Rev*, **36**, 47-63.

Alexander, G.E. & Crutcher, M.D. (1990) Functional architecture of basal ganglia circuits: neural substrates of parallel processing. *Trends in neurosciences*, **13**, 266-271.

Ameri, A. (1999) The effects of cannabinoids on the brain. *Progress in neurobiology*, **58**, 315-348.

Angoa-Perez, M., Kane, M.J., Briggs, D.I., Francescutti, D.M. & Kuhn, D.M. (2013) Marble burying and nestlet shredding as tests of repetitive, compulsive-like behaviors in mice. *Journal of visualized experiments : JoVE*, 50978.

Anselme, P. (2009) The effect of exposure to drugs on the processing of natural rewards. *Neurosci Biobehav Rev*, **33**, 314-335.

Anselme, P. (2010) The uncertainty processing theory of motivation. *Behav Brain Res*, **208**, 291-310.

Aracil-Fernandez, A., Almela, P. & Manzanares, J. (2013) Pregabalin and topiramate regulate behavioural and brain gene transcription changes induced by spontaneous cannabinoid withdrawal in mice. *Addict Biol*, **18**, 252-262.

Aragona, B.J., Day, J.J., Roitman, M.F., Cleaveland, N.A., Wightman, R.M. & Carelli, R.M. (2009) Regional specificity in the real-time development of phasic dopamine transmission patterns during acquisition of a cue-cocaine association in rats. *Eur J Neurosci*, **30**, 1889-1899.

Araque, A., Carmignoto, G., Haydon, P.G., Oliet, S.H., Robitaille, R. & Volterra, A. (2014) Gliotransmitters travel in time and space. *Neuron*, **81**, 728-739.

Arias-Carrión, O., Stamelou, M., Murillo-Rodriguez, E., Menéndez González, M. & Pöppel, E. (2010) *Dopaminergic reward system: A short integrative review*.

Atwood, B.K., Huffman, J., Straiker, A. & Mackie, K. (2010) JWH018, a common constituent of 'Spice' herbal blends, is a potent and efficacious cannabinoid CB receptor agonist. *Br J Pharmacol*, **160**, 585-593.

Atwood, B.K. & Mackie, K. (2010) CB2: a cannabinoid receptor with an identity crisis. *Br J Pharmacol*, **160**, 467-479.

Auwarter, V., Dresen, S., Weinmann, W., Muller, M., Putz, M. & Ferreiros, N. (2009) 'Spice' and other herbal blends: harmless incense or cannabinoid designer drugs? *Journal of mass spectrometry : JMS*, **44**, 832-837.

Banister, S.D., Stuart, J., Kevin, R.C., Edington, A., Longworth, M., Wilkinson, S.M., Beinat, C., Buchanan, A.S., Hibbs, D.E., Glass, M., Connor, M., McGregor, I.S. & Kassiou, M. (2015) Effects of bioisosteric fluorine in synthetic cannabinoid designer drugs JWH-018, AM-2201, UR-144, XLR-11, PB-22, 5F-PB-22, APICA, and STS-135. *ACS chemical neuroscience*, **6**, 1445-1458.

Bassareo, V., De Luca, M.A. & Di Chiara, G. (2002) Differential Expression of Motivational Stimulus Properties by Dopamine in Nucleus Accumbens Shell versus Core and Prefrontal Cortex. *J Neurosci*, **22**, 4709-4719.

Bassareo, V., De Luca, M.A. & Di Chiara, G. (2007) Differential impact of pavlovian drug conditioned stimuli on in vivo dopamine transmission in the rat accumbens shell and core and in the prefrontal cortex. *Psychopharmacology (Berl)*, **191**, 689-703.

Bassareo, V. & Di Chiara, G. (1997) Differential influence of associative and nonassociative learning mechanisms on the responsiveness of prefrontal and accumbal dopamine transmission to food stimuli in rats fed ad libitum. *J Neurosci*, **17**, 851-861.

Bassareo, V. & Di Chiara, G. (1999) Differential responsiveness of dopamine transmission to food-stimuli in nucleus accumbens shell/core compartments. *Neuroscience*, **89**, 637-641.

Bassareo, V., Musio, P. & Di Chiara, G. (2011) Reciprocal responsiveness of nucleus accumbens shell and core dopamine to food- and drug-conditioned stimuli. *Psychopharmacology (Berl)*, **214**, 687-697.

Bassareo, V., Tanda, G., Petromilli, P., Giua, C. & Di Chiara, G. (1996) Non-psychostimulant drugs of abuse and anxiogenic drugs activate with differential selectivity dopamine transmission in the nucleus accumbens and in the medial prefrontal cortex of the rat. *Psychopharmacology (Berl)*, **124**, 293-299.

Beattie, E.C., Stellwagen, D., Morishita, W., Bresnahan, J.C., Ha, B.K., Von Zastrow, M., Beattie, M.S. & Malenka, R.C. (2002) Control of synaptic strength by glial TNFalpha. *Science (New York, N.Y.)*, **295**, 2282-2285.

Beckstead, R.M. (1979) An autoradiographic examination of corticocortical and subcortical projections of the mediodorsal-projection (prefrontal) cortex in the rat. *The Journal of comparative neurology*, **184**, 43-62.

Belluomo, I., Matias, I., Pernegre, C., Marsicano, G. & Chaouloff, F. (2015) Opposite control of frontocortical 2-arachidonoylglycerol turnover rate by cannabinoid type-1 receptors located on glutamatergic neurons and on astrocytes. *J Neurochem*, **133**, 26-37.

Benford, D.M. & Caplan, J.P. (2011) Psychiatric sequelae of Spice, K2, and synthetic cannabinoid receptor agonists. *Psychosomatics*, **52**, 295.

Benowitz, N.L. & Jones, R.T. (1975) Cardiovascular effects of prolonged delta-9-tetrahydrocannabinol ingestion. *Clinical pharmacology and therapeutics*, **18**, 287-297.

Berendse, H.W., Galis-de Graaf, Y. & Groenewegen, H.J. (1992) Topographical organization and relationship with ventral striatal compartments of prefrontal corticostriatal projections in the rat. *The Journal of comparative neurology*, **316**, 314-347.

Berger, B., Gaspar, P. & Verney, C. (1991) Dopaminergic innervation of the cerebral cortex: unexpected differences between rodents and primates. *Trends in neurosciences*, **14**, 21-27.

Bernardi, G., Cherubini, E., Marciani, M.G., Mercuri, N. & Stanzione, P. (1982) Responses of intracellularly recorded cortical neurons to the iontophoretic application of dopamine. *Brain research*, **245**, 267-274.

Berridge, K.C. (1996) Food reward: brain substrates of wanting and liking. *Neurosci Biobehav Rev*, **20**, 1-25.

Berridge, K.C. (2000) Measuring hedonic impact in animals and infants: microstructure of affective taste reactivity patterns. *Neurosci Biobehav Rev*, **24**, 173-198.

Berridge, K.C. & Robinson, T.E. (1998) What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience? *Brain research. Brain research reviews*, **28**, 309-369.

Berridge, K.C., Venier, I.L. & Robinson, T.E. (1989) Taste reactivity analysis of 6-hydroxydopamine-induced aphagia: implications for arousal and anhedonia hypotheses of dopamine function. *Behavioral neuroscience*, **103**, 36-45.

Berta, T., Liu, T., Liu, Y.C., Xu, Z.Z. & Ji, R.R. (2012) Acute morphine activates satellite glial cells and up-regulates IL-1beta in dorsal root ganglia in mice via matrix metalloprotease-9. *Molecular pain*, **8**, 18.

Besli, G.E., Ikiz, M.A., Yildirim, S. & Saltik, S. (2015) Synthetic Cannabinoid Abuse in Adolescents: A Case Series. *The Journal of emergency medicine*, **49**, 644-650.

Bhanushali, G.K., Jain, G., Fatima, H., Leisch, L.J. & Thornley-Brown, D. (2013) AKI associated with synthetic cannabinoids: a case series. *Clinical journal of the American Society of Nephrology : CJASN*, **8**, 523-526.

Bilbo, S.D. & Schwarz, J.M. (2012) The immune system and developmental programming of brain and behavior. *Frontiers in neuroendocrinology*, **33**, 267-286.

Bimpisidis, Z., De Luca, M.A., Pisanu, A. & Di Chiara, G. (2013) Lesion of medial prefrontal dopamine terminals abolishes habituation of accumbens shell dopamine responsiveness to taste stimuli. *Eur J Neurosci*, **37**, 613-622.

Bjorklund, A., Divac, I. & Lindvall, O. (1978) Regional distribution of catecholamines in monkey cerebral cortex, evidence for a dopaminergic innervation of the primate prefrontal cortex. *Neuroscience letters*, **7**, 115-119.

Bjornsen, L.P., Hadera, M.G., Zhou, Y., Danbolt, N.C. & Sonnewald, U. (2014) The GLT-1 (EAAT2; slc1a2) glutamate transporter is essential for glutamate homeostasis in the neocortex of the mouse. *J Neurochem*, **128**, 641-649.

Blackburn, J.R., Phillips, A.G., Jakubovic, A. & Fibiger, H.C. (1989) Dopamine and preparatory behavior: II. A neurochemical analysis. *Behavioral neuroscience*, **103**, 15-23.

Blanc, G., Herve, D., Simon, H., Lisoprawski, A., Glowinski, J. & Tassin, J.P. (1980) Response to stress of mesocortico-frontal dopaminergic neurones in rats after long-term isolation. *Nature*, **284**, 265-267.

Block, M.L., Zecca, L. & Hong, J.S. (2007) Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nature reviews. Neuroscience*, **8**, 57-69.

Blumberg, H.P., Stern, E., Ricketts, S., Martinez, D., de Asis, J., White, T., Epstein, J., Isenberg, N., McBride, P.A., Kemperman, I., Emmerich, S., Dhawan, V., Eidelberg, D., Kocsis, J.H. & Silbersweig, D.A. (1999) Rostral and orbital prefrontal cortex dysfunction in the manic state of bipolar disorder. *The American journal of psychiatry*, **156**, 1986-1988.

Bolla, K.I., Brown, K., Eldreth, D., Tate, K. & Cadet, J.L. (2002) Dose-related neurocognitive effects of marijuana use. *Neurology*, **59**, 1337-1343.

Bolla, K.I., Eldreth, D.A., London, E.D., Kiehl, K.A., Mouratidis, M., Contoreggi, C., Matochik, J.A., Kurian, V., Cadet, J.L., Kimes, A.S., Funderburk, F.R. & Ernst, M. (2003) Orbitofrontal cortex dysfunction in abstinent cocaine abusers performing a decision-making task. *NeuroImage*, **19**, 1085-1094.

Bonhaus, D.W., Chang, L.K., Kwan, J. & Martin, G.R. (1998) Dual activation and inhibition of adenylyl cyclase by cannabinoid receptor agonists: evidence for agonist-specific trafficking of intracellular responses. *The Journal of pharmacology and experimental therapeutics*, **287**, 884-888.

Bosier, B., Bellocchio, L., Metna-Laurent, M., Soria-Gomez, E., Matias, I., Hebert-Chatelain, E., Cannich, A., Maitre, M., Leste-Lasserre, T., Cardinal, P., Mendizabal-Zubiaga, J., Canduela, M.J., Reguero, L., Hermans, E., Grandes, P., Cota, D. & Marsicano, G. (2013) Astroglial CB1 cannabinoid receptors regulate leptin signaling in mouse brain astrocytes. *Molecular metabolism*, **2**, 393-404.

Bowers, M.S. & Kalivas, P.W. (2003) Forebrain astroglial plasticity is induced following withdrawal from repeated cocaine administration. *Eur J Neurosci*, **17**, 1273-1278.

Brauer, K., Hausser, M., Hartig, W. & Arendt, T. (2000) The core-shell dichotomy of nucleus accumbens in the rhesus monkey as revealed by double-immunofluorescence and morphology of cholinergic interneurons. *Brain research*, **858**, 151-162.

Braver, T.S., Barch, D.M. & Cohen, J.D. (1999) Cognition and control in schizophrenia: a computational model of dopamine and prefrontal function. *Biol Psychiatry*, **46**, 312-328.

Breivogel, C.S., Griffin, G., Di Marzo, V. & Martin, B.R. (2001) Evidence for a new G protein-coupled cannabinoid receptor in mouse brain. *Mol Pharmacol*, **60**, 155-163.

Brents, L.K., Gallus-Zawada, A., Radominska-Pandya, A., Vasiljevik, T., Prisinzano, T.E., Fantegrossi, W.E., Moran, J.H. & Prather, P.L. (2012) Monohydroxylated metabolites of the K2 synthetic cannabinoid JWH-073 retain intermediate to high cannabinoid 1 receptor (CB1R) affinity and exhibit neutral antagonist to partial agonist activity. *Biochemical pharmacology*, **83**, 952-961.

Brents, L.K. & Prather, P.L. (2014) The K2/Spice phenomenon: emergence, identification, legislation and metabolic characterization of synthetic cannabinoids in herbal incense products. *Drug metabolism reviews*, **46**, 72-85.

Brents, L.K., Reichard, E.E., Zimmerman, S.M., Moran, J.H., Fantegrossi, W.E. & Prather, P.L. (2011) Phase I hydroxylated metabolites of the K2 synthetic cannabinoid JWH-018 retain in vitro and in vivo cannabinoid 1 receptor affinity and activity. *PLoS One*, **6**, e21917.

Brown, G.C. & Neher, J.J. (2014) Microglial phagocytosis of live neurons. *Nature reviews. Neuroscience*, **15**, 209-216.

Budney, A.J., Novy, P.L. & Hughes, J.R. (1999) Marijuana withdrawal among adults seeking treatment for marijuana dependence. *Addiction (Abingdon, England)*, **94**, 1311-1322.

Bull, C., Syed, W.A., Minter, S.C. & Bowers, M.S. (2015) Differential response of glial fibrillary acidic protein-positive astrocytes in the rat prefrontal cortex following ethanol self-administration. *Alcoholism, clinical and experimental research*, **39**, 650-658.

Burk, J.A. & Mair, R.G. (2001) Effects of intralaminar thalamic lesions on sensory attention and motor intention in the rat: a comparison with lesions involving frontal cortex and hippocampus. *Behav Brain Res*, **123**, 49-63.

Bushong, E.A., Martone, M.E., Jones, Y.Z. & Ellisman, M.H. (2002) Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains. *J Neurosci*, **22**, 183-192.

Cabral, G.A. & Griffin-Thomas, L. (2009) Emerging role of the cannabinoid receptor CB2 in immune regulation: therapeutic prospects for neuroinflammation. *Expert reviews in molecular medicine*, **11**, e3.

Cabral, G.A., Rogers, T.J. & Lichtman, A.H. (2015) Turning Over a New Leaf: Cannabinoid and Endocannabinoid Modulation of Immune Function. *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology*, **10**, 193-203.

Cacciapaglia, F., Saddoris, M.P., Wightman, R.M. & Carelli, R.M. (2012) Differential dopamine release dynamics in the nucleus accumbens core and shell track distinct aspects of goal-directed behavior for sucrose. *Neuropharmacology*, **62**, 2050-2056.

Cadoni, C., Pisanu, A., Solinas, M., Acquas, E. & Di Chiara, G. (2001) Behavioural sensitization after repeated exposure to Delta 9-tetrahydrocannabinol and cross-sensitization with morphine. *Psychopharmacology (Berl)*, **158**, 259-266.

Cadoni, C., Valentini, V. & Di Chiara, G. (2008) Behavioral sensitization to delta 9-tetrahydrocannabinol and cross-sensitization with morphine: differential changes in accumbal shell and core dopamine transmission. *J Neurochem*, **106**, 1586-1593.

Canazza, I., Ossato, A., Trapella, C., Fantinati, A., De Luca, M.A., Margiani, G., Vincenzi, F., Rimondo, C., Di Rosa, F., Gregori, A., Varani, K., Borea, P.A., Serpelloni, G. & Marti, M. (2016) Effect of the novel synthetic cannabinoids AKB48 and 5F-AKB48 on "tetrad", sensorimotor, neurological and

neurochemical responses in mice. In vitro and in vivo pharmacological studies. *Psychopharmacology (Berl)*, **233**, 3685-3709.

Cannaert, A., Storme, J., Franz, F., Auwarter, V. & Stove, C.P. (2016) Detection and Activity Profiling of Synthetic Cannabinoids and Their Metabolites with a Newly Developed Bioassay. *Analytical chemistry*, **88**, 11476-11485.

Carboni, E., Imperato, A., Perezzani, L. & Di Chiara, G. (1989) Amphetamine, cocaine, phencyclidine and nomifensine increase extracellular dopamine concentrations preferentially in the nucleus accumbens of freely moving rats. *Neuroscience*, **28**, 653-661.

Carlisle, S.J., Marciano-Cabral, F., Staab, A., Ludwick, C. & Cabral, G.A. (2002) Differential expression of the CB2 cannabinoid receptor by rodent macrophages and macrophage-like cells in relation to cell activation. *International immunopharmacology*, **2**, 69-82.

Carlsson, A., Falck, B. & Hillarp, N.A. (1962) Cellular localization of brain monoamines. *Acta physiologica Scandinavica. Supplementum*, **56**, 1-28.

Carr, D.B. & Sesack, S.R. (2000) Projections from the rat prefrontal cortex to the ventral tegmental area: target specificity in the synaptic associations with mesoaccumbens and mesocortical neurons. *J Neurosci*, **20**, 3864-3873.

Carroll, F.I., Lewin, A.H., Mascarella, S.W., Seltzman, H.H. & Reddy, P.A. (2012) Designer drugs: a medicinal chemistry perspective. *Annals of the New York Academy of Sciences*, **1248**, 18-38.

Castaneto, M.S., Gorelick, D.A., Desrosiers, N.A., Hartman, R.L., Pirard, S. & Huestis, M.A. (2014) Synthetic cannabinoids: epidemiology, pharmacodynamics, and clinical implications. *Drug and alcohol dependence*, **144**, 12-41.

Castellano, C., Rossi-Arnaud, C., Cestari, V. & Costanzi, M. (2003) Cannabinoids and memory: animal studies. *Current drug targets. CNS and neurological disorders*, **2**, 389-402.

Castelli, M.P., Madeddu, C., Casti, A., Casu, A., Casti, P., Scherma, M., Fattore, L., Fadda, P. & Ennas, M.G. (2014) Delta9-tetrahydrocannabinol prevents methamphetamine-induced neurotoxicity. *PLoS One*, **9**, e98079.

Cha, H.J., Lee, K.W., Song, M.J., Hyeon, Y.J., Hwang, J.Y., Jang, C.G., Ahn, J.I., Jeon, S.H., Kim, H.U., Kim, Y.H., Seong, W.K., Kang, H., Yoo, H.S. & Jeong, H.S. (2014) Dependence Potential of the Synthetic Cannabinoids JWH-073, JWH-081, and JWH-210: In Vivo and In Vitro Approaches. *Biomolecules & therapeutics*, **22**, 363-369.

Charlety, P.J., Grenhoff, J., Chergui, K., De la Chapelle, B., Buda, M., Svensson, T.H. & Chouvet, G. (1991) Burst firing of mesencephalic dopamine neurons is inhibited by somatodendritic application of kynurenate. *Acta physiologica Scandinavica*, **142**, 105-112.

Chase, P.B., Hawkins, J., Mosier, J., Jimenez, E., Boesen, K., Logan, B.K. & Walter, F.G. (2016) Differential physiological and behavioral cues observed in individuals smoking botanical marijuana versus synthetic cannabinoid drugs. *Clinical toxicology (Philadelphia, Pa.)*, **54**, 14-19.

Cheer, J.F., Kendall, D.A. & Marsden, C.A. (2000) Cannabinoid receptors and reward in the rat: a conditioned place preference study. *Psychopharmacology (Berl)*, **151**, 25-30.

Cheer, J.F., Kendall, D.A., Mason, R. & Marsden, C.A. (2003) Differential cannabinoid-induced electrophysiological effects in rat ventral tegmentum. *Neuropharmacology*, **44**, 633-641.

Cheer, J.F., Wassum, K.M., Heien, M.L.A.V., Phillips, P.E.M. & Wightman, R.M. (2004) Cannabinoids Enhance Subsecond Dopamine Release in the Nucleus Accumbens of Awake Rats. *The Journal of Neuroscience*, **24**, 4393-4400.

Chergui, K., Charlety, P.J., Akaoka, H., Saunier, C.F., Brunet, J.L., Buda, M., Svensson, T.H. & Chouvet, G. (1993) Tonic activation of NMDA receptors causes spontaneous burst discharge of rat midbrain dopamine neurons in vivo. *Eur J Neurosci*, **5**, 137-144.

Chevaleyre, V. & Castillo, P.E. (2004) Endocannabinoid-mediated metaplasticity in the hippocampus. *Neuron*, **43**, 871-881.

Childress, A.R., Hole, A.V., Ehrman, R.N., Robbins, S.J., McLellan, A.T. & O'Brien, C.P. (1993) Cue reactivity and cue reactivity interventions in drug dependence. *NIDA research monograph*, **137**, 73-95.

Chimalakonda, K.C., Seely, K.A., Bratton, S.M., Brents, L.K., Moran, C.L., Endres, G.W., James, L.P., Hollenberg, P.F., Prather, P.L., Radominska-Pandya, A. & Moran, J.H. (2012) Cytochrome P450-mediated oxidative metabolism of abused synthetic cannabinoids found in K2/Spice: identification of novel cannabinoid receptor ligands. *Drug metabolism and disposition: the biological fate of chemicals*, **40**, 2174-2184.

Christakou, A., Robbins, T.W. & Everitt, B.J. (2001) Functional disconnection of a prefrontal cortical-dorsal striatal system disrupts choice reaction time performance: implications for attentional function. *Behavioral neuroscience*, **115**, 812-825.

Christie, M.J., Bridge, S., James, L.B. & Beart, P.M. (1985a) Excitotoxin lesions suggest an aspartatergic projection from rat medial prefrontal cortex to ventral tegmental area. *Brain research*, **333**, 169-172.

Christie, M.J., James, L.B. & Beart, P.M. (1985b) An excitant amino acid projection from the medial prefrontal cortex to the anterior part of nucleus accumbens in the rat. *J Neurochem*, **45**, 477-482.

Chung, W.S., Allen, N.J. & Eroglu, C. (2015) Astrocytes Control Synapse Formation, Function, and Elimination. *Cold Spring Harbor perspectives in biology*, **7**, a020370.

Clark, B.C., Georgekutty, J. & Berul, C.I. (2015) Myocardial Ischemia Secondary to Synthetic Cannabinoid (K2) Use in Pediatric Patients. *The Journal of pediatrics*, **167**, 757-761.e751.

Cohen, J.D. & Servan-Schreiber, D. (1992) Context, cortex, and dopamine: a connectionist approach to behavior and biology in schizophrenia. *Psychological review*, **99**, 45-77.

Coller, J.K. & Hutchinson, M.R. (2012) Implications of central immune signaling caused by drugs of abuse: mechanisms, mediators and new therapeutic approaches for prediction and treatment of drug dependence. *Pharmacology & therapeutics*, **134**, 219-245.

Compton, D.R., Rice, K.C., De Costa, B.R., Razdan, R.K., Melvin, L.S., Johnson, M.R. & Martin, B.R. (1993) Cannabinoid structure-activity relationships: correlation of receptor binding and in vivo activities. *The Journal of pharmacology and experimental therapeutics*, **265**, 218-226.

Conde, F., Audinat, E., Maire-Lepoivre, E. & Crepel, F. (1990) Afferent connections of the medial frontal cortex of the rat. A study using retrograde transport of fluorescent dyes. I. Thalamic afferents. *Brain research bulletin*, **24**, 341-354.

Conde, F., Maire-Lepoivre, E., Audinat, E. & Crepel, F. (1995) Afferent connections of the medial frontal cortex of the rat. II. Cortical and subcortical afferents. *The Journal of comparative neurology*, **352**, 567-593.

Cooper, Z.D. (2016) Adverse Effects of Synthetic Cannabinoids: Management of Acute Toxicity and Withdrawal. *Curr Psychiatry Rep*, **18**, 52.

Corbit, L.H. & Balleine, B.W. (2011) The general and outcome-specific forms of Pavlovian-instrumental transfer are differentially mediated by the nucleus accumbens core and shell. *J Neurosci*, **31**, 11786-11794.

Corty, M.M. & Freeman, M.R. (2013) Cell biology in neuroscience: Architects in neural circuit design: glia control neuron numbers and connectivity. *The Journal of cell biology*, **203**, 395-405.

Costain, W.J., Tauskela, J.S., Rasquinha, I., Comas, T., Hewitt, M., Marleau, V. & Soo, E.C. (2016) Pharmacological characterization of emerging synthetic cannabinoids in HEK293T cells and hippocampal neurons. *European journal of pharmacology*, **786**, 234-245.

Cousijn, J., Watson, P., Koenders, L., Vingerhoets, W.A., Goudriaan, A.E. & Wiers, R.W. (2013) Cannabis dependence, cognitive control and attentional bias for cannabis words. *Addictive behaviors*, **38**, 2825-2832.

Covey, D.P., Mateo, Y., Sulzer, D., Cheer, J.F. & Lovinger, D.M. (2017) Endocannabinoid modulation of dopamine neurotransmission. *Neuropharmacology*, **124**, 52-61.

Covey, D.P., Wenzel, J.M. & Cheer, J.F. (2015) Cannabinoid modulation of drug reward and the implications of marijuana legalization. *Brain research*, **1628**, 233-243.

Cuadra, G., Zurita, A., Lacerra, C. & Molina, V. (1999) Chronic stress sensitizes frontal cortex dopamine release in response to a subsequent novel stressor: reversal by naloxone. *Brain research bulletin*, **48**, 303-308.

Curtis, C.E. & D'Esposito, M. (2003) Persistent activity in the prefrontal cortex during working memory. *Trends in cognitive sciences*, **7**, 415-423.

Cutando, L., Busquets-Garcia, A., Puighermanal, E., Gomis-Gonzalez, M., Delgado-Garcia, J.M., Gruart, A., Maldonado, R. & Ozaita, A. (2013) Microglial activation underlies cerebellar deficits produced by repeated cannabis exposure. *The Journal of clinical investigation*, **123**, 2816-2831.

Dahlstroem, A. & Fuxe, K. (1964) EVIDENCE FOR THE EXISTENCE OF MONOAMINE-CONTAINING NEURONS IN THE CENTRAL NERVOUS SYSTEM. I. DEMONSTRATION OF MONOAMINES IN THE CELL BODIES OF BRAIN STEM NEURONS. *Acta physiologica Scandinavica. Supplementum*, Suppl 232:231-255.

Dalcik, H., Yardimoglu, M., Filiz, S., Gonca, S., Dalcik, C. & Erden, B.F. (2009) Chronic ethanol-induced glial fibrillary acidic protein (GFAP) immunoreactivity: an immunocytochemical observation in various regions of adult rat brain. *The International journal of neuroscience*, **119**, 1303-1318.

Danbolt, N.C. (2001) Glutamate uptake. *Progress in neurobiology*, **65**, 1-105.

Das, S.C., Althobaiti, Y.S., Alshehri, F.S. & Sari, Y. (2016) Binge ethanol withdrawal: Effects on post-withdrawal ethanol intake, glutamate-glutamine cycle and monoamine tissue content in P rat model. *Behav Brain Res*, **303**, 120-125.

Davalos, D., Grutzendler, J., Yang, G., Kim, J.V., Zuo, Y., Jung, S., Littman, D.R., Dustin, M.L. & Gan, W.B. (2005) ATP mediates rapid microglial response to local brain injury in vivo. *Nat Neurosci*, **8**, 752-758.

Dawson, G.R., Crawford, S.P., Stanhope, K.J., Iversen, S.D. & Tricklebank, M.D. (1994) One-trial tolerance to the effects of chlordiazepoxide on the elevated plus maze may be due to locomotor habituation, not repeated drug exposure. *Psychopharmacology (Berl)*, **113**, 570-572.

De Chiara, V., Motta, C., Rossi, S., Studer, V., Barbieri, F., Lauro, D., Bernardi, G. & Centonze, D. (2013) Interleukin-1beta alters the sensitivity of cannabinoid CB1 receptors controlling glutamate transmission in the striatum. *Neuroscience*, **250**, 232-239.

de Havenon, A., Chin, B., Thomas, K.C. & Afra, P. (2011) The secret "spice": an undetectable toxic cause of seizure. *The Neurohospitalist*, **1**, 182-186.

De Luca, M.A. (2014) Habituation of the responsiveness of mesolimbic and mesocortical dopamine transmission to taste stimuli. *Frontiers in integrative neuroscience*, **8**, 21.

De Luca, M.A., Bimpisidis, Z., Bassareo, V. & Di Chiara, G. (2011) Influence of morphine sensitization on the responsiveness of mesolimbic and mesocortical dopamine transmission to appetitive and aversive gustatory stimuli. *Psychopharmacology (Berl)*, **216**, 345-353.

De Luca, M.A., Bimpisidis, Z., Melis, M., Marti, M., Caboni, P., Valentini, V., Margiani, G., Pintori, N., Polis, I., Marsicano, G., Parsons, L.H. & Di Chiara, G. (2015) Stimulation of in vivo dopamine transmission and intravenous self-administration in rats and mice by JWH-018, a Spice cannabinoid. *Neuropharmacology*, **99**, 705-714.

De Luca, M.A., Castelli, M.P., Loi, B., Porcu, A., Martorelli, M., Miliano, C., Kellett, K., Davidson, C., Stair, J.L., Schifano, F. & Di Chiara, G. (2016) Native CB1 receptor affinity, intrinsic activity and accumbens shell dopamine stimulant properties of third generation SPICE/K2 cannabinoids: BB-22, 5F-PB-22, 5F-AKB-48 and STS-135. *Neuropharmacology*, **105**, 630-638.

De Luca, M.A. & Fattore, L. (2018) Therapeutic Use of Synthetic Cannabinoids: Still an Open Issue? *Clinical therapeutics*.

De Luca, M.A., Solinas, M., Bimpisidis, Z., Goldberg, S.R. & Di Chiara, G. (2012) Cannabinoid facilitation of behavioral and biochemical hedonic taste responses. *Neuropharmacology*, **63**, 161-168.

De Vries, T.J. & Schoffelmeer, A.N. (2005) Cannabinoid CB1 receptors control conditioned drug seeking. *Trends in pharmacological sciences*, **26**, 420-426.

Del Arco, A. & Mora, F. (2008) Prefrontal cortex-nucleus accumbens interaction: in vivo modulation by dopamine and glutamate in the prefrontal cortex. *Pharmacology, biochemistry, and behavior*, **90**, 226-235.

den Boon, F.S., Werkman, T.R., Schaafsma-Zhao, Q., Houthuijs, K., Vitalis, T., Kruse, C.G., Wadman, W.J. & Chameau, P. (2015) Activation of type-1 cannabinoid receptor shifts the balance between excitation and inhibition towards excitation in layer II/III pyramidal neurons of the rat prelimbic cortex. *Pflugers Archiv : European journal of physiology*, **467**, 1551-1564.

Deserno, L., Boehme, R., Heinz, A. & Schlagenhauf, F. (2013) Reinforcement learning and dopamine in schizophrenia: dimensions of symptoms or specific features of a disease group? *Front Psychiatry*, **4**, 172.

Deutch, A.Y. & Cameron, D.S. (1992) Pharmacological characterization of dopamine systems in the nucleus accumbens core and shell. *Neuroscience*, **46**, 49-56.

Deutch, A.Y., Tam, S.Y. & Roth, R.H. (1985) Footshock and conditioned stress increase 3,4-dihydroxyphenylacetic acid (DOPAC) in the ventral tegmental area but not substantia nigra. *Brain research*, **333**, 143-146.

Devane, W.A., Hanus, L., Breuer, A., Pertwee, R.G., Stevenson, L.A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A. & Mechoulam, R. (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science (New York, N.Y.)*, **258**, 1946-1949.

Dhawan, J., Deng, H., Gatley, S.J., Makriyannis, A., Akinfeleye, T., Bruneus, M., Dimaio, A.A. & Gifford, A.N. (2006) Evaluation of the in vivo receptor occupancy for the behavioral effects of cannabinoids using a radiolabeled cannabinoid receptor agonist, R-[125/131I]AM2233. *Synapse (New York, N.Y.)*, **60**, 93-101.

Di Chiara, G. (1990a) Brain dialysis of neurotransmitters: a commentary. *Journal of neuroscience methods*, **34**, 29-34.

Di Chiara, G. (1990b) In-vivo brain dialysis of neurotransmitters. *Trends in pharmacological sciences*, **11**, 116-121.

Di Chiara, G. (1998) A motivational learning hypothesis of the role of mesolimbic dopamine in compulsive drug use. *Journal of psychopharmacology (Oxford, England)*, **12**, 54-67.

Di Chiara, G. (2002) Nucleus accumbens shell and core dopamine: differential role in behavior and addiction. *Behav Brain Res*, **137**, 75-114.

Di Chiara, G. & Bassareo, V. (2007) Reward system and addiction: what dopamine does and doesn't do. *Current opinion in pharmacology*, **7**, 69-76.

Di Chiara, G., Bassareo, V., Fenu, S., De Luca, M.A., Spina, L., Cadoni, C., Acquas, E., Carboni, E., Valentini, V. & Lecca, D. (2004) Dopamine and drug addiction: the nucleus accumbens shell connection. *Neuropharmacology*, **47 Suppl 1**, 227-241.

Di Chiara, G. & Imperato, A. (1988a) Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proceedings of the National Academy of Sciences of the United States of America*, **85**, 5274-5278.

Di Chiara, G. & Imperato, A. (1988b) Opposite effects of mu and kappa opiate agonists on dopamine release in the nucleus accumbens and in the dorsal caudate of freely moving rats. *The Journal of pharmacology and experimental therapeutics*, **244**, 1067-1080.

Diana, M., Brodie, M., Muntoni, A., Puddu, M.C., Pillolla, G., Steffensen, S., Spiga, S. & Little, H.J. (2003) Enduring effects of chronic ethanol in the CNS: basis for alcoholism. *Alcoholism, clinical and experimental research*, **27**, 354-361.

Diana, M., Melis, M., Muntoni, A.L. & Gessa, G.L. (1998) Mesolimbic dopaminergic decline after cannabinoid withdrawal. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 10269-10273.

Diana, M., Muntoni, A.L., Pistis, M., Melis, M. & Gessa, G.L. (1999) Lasting reduction in mesolimbic dopamine neuronal activity after morphine withdrawal. *Eur J Neurosci*, **11**, 1037-1041.

Diana, M., Pistis, M., Carboni, S., Gessa, G.L. & Rossetti, Z.L. (1993) Profound decrement of mesolimbic dopaminergic neuronal activity during ethanol withdrawal syndrome in rats: electrophysiological and biochemical evidence. *Proceedings of the National Academy of Sciences of the United States of America*, **90**, 7966-7969.

Diana, M., Pistis, M., Muntoni, A. & Gessa, G. (1996) Mesolimbic dopaminergic reduction outlasts ethanol withdrawal syndrome: evidence of protracted abstinence. *Neuroscience*, **71**, 411-415.

Diana, M., Pistis, M., Muntoni, A.L. & Gessa, G.L. (1995) Ethanol withdrawal does not induce a reduction in the number of spontaneously active dopaminergic neurons in the mesolimbic system. *Brain research*, **682**, 29-34.

Djukic, B., Casper, K.B., Philpot, B.D., Chin, L.S. & McCarthy, K.D. (2007) Conditional knock-out of Kir4.1 leads to glial membrane depolarization, inhibition of potassium and glutamate uptake, and enhanced short-term synaptic potentiation. *J Neurosci*, **27**, 11354-11365.

Dougherty, D.M., Mathias, C.W., Dawes, M.A., Furr, R.M., Charles, N.E., Liguori, A., Shannon, E.E. & Acheson, A. (2013) Impulsivity, attention, memory, and decision-making among adolescent marijuana users. *Psychopharmacology (Berl)*, **226**, 307-319.

Dreher, J.C. & Burnod, Y. (2002) An integrative theory of the phasic and tonic modes of dopamine modulation in the prefrontal cortex. *Neural networks : the official journal of the International Neural Network Society*, **15**, 583-602.

Dresen, S., Ferreiros, N., Putz, M., Westphal, F., Zimmermann, R. & Auwarter, V. (2010) Monitoring of herbal mixtures potentially containing synthetic cannabinoids as psychoactive compounds. *Journal of mass spectrometry : JMS*, **45**, 1186-1194.

Dreyer, J.K., Herrik, K.F., Berg, R.W. & Hounsgaard, J.D. (2010) Influence of phasic and tonic dopamine release on receptor activation. *J Neurosci*, **30**, 14273-14283.

Durstewitz, D., Kelc, M. & Gunturkun, O. (1999) A neurocomputational theory of the dopaminergic modulation of working memory functions. *J Neurosci*, **19**, 2807-2822.

Durstewitz, D. & Seamans, J.K. (2002) The computational role of dopamine D1 receptors in working memory. *Neural networks : the official journal of the International Neural Network Society*, **15**, 561-572.

Egerton, A., Allison, C., Brett, R.R. & Pratt, J.A. (2006) Cannabinoids and prefrontal cortical function: insights from preclinical studies. *Neurosci Biobehav Rev*, **30**, 680-695.

El Khoury, M.A., Gorgievski, V., Moutsimilli, L., Giros, B. & Tzavara, E.T. (2012) Interactions between the cannabinoid and dopaminergic systems: evidence from animal studies. *Prog Neuropsychopharmacol Biol Psychiatry*, **38**, 36-50.

Elmore, J.S. & Baumann, M.H. (2018) Repeated Exposure to the "Spice" Cannabinoid JWH-018 Induces Tolerance and Enhances Responsiveness to 5-HT1A Receptor Stimulation in Male Rats. *Front Psychiatry*, **9**, 55.

Elsworth, J.D. & Roth, R.H. (2009) Dopamine. In Squire, L.R. (ed) *Encyclopedia of Neuroscience*. Academic Press, Oxford, pp. 539-547.

Eroglu, C. & Barres, B.A. (2010) Regulation of synaptic connectivity by glia. *Nature*, **468**, 223-231.

Everitt, B.J., Belin, D., Economidou, D., Pelloux, Y., Dalley, J.W. & Robbins, T.W. (2008) Review. Neural mechanisms underlying the vulnerability to develop compulsive drug-seeking habits and addiction. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, **363**, 3125-3135.

Every-Palmer, S. (2011) Synthetic cannabinoid JWH-018 and psychosis: an explorative study. *Drug and alcohol dependence*, **117**, 152-157.

Evrard, S.G., Duhalde-Vega, M., Tagliaferro, P., Mirochnic, S., Caltana, L.R. & Brusco, A. (2006) A low chronic ethanol exposure induces morphological changes in the adolescent rat brain that are not fully recovered even after a long abstinence: an immunohistochemical study. *Exp Neurol*, **200**, 438-459.

Fan, F., Compton, D.R., Ward, S., Melvin, L. & Martin, B.R. (1994) Development of cross-tolerance between delta 9-tetrahydrocannabinol, CP 55,940 and WIN 55,212. *The Journal of pharmacology and experimental therapeutics*, **271**, 1383-1390.

Fanarioti, E., Mavrikaki, M., Panagis, G., Mitsacos, A., Nomikos, G.G. & Giompres, P. (2014) Behavioral and neurochemical changes in mesostriatal dopaminergic regions of the rat after chronic administration of the cannabinoid receptor agonist WIN55,212-2. *Int J Neuropsychopharmacol*, **18**.

Fantegrossi, W.E., Moran, J.H., Radominska-Pandya, A. & Prather, P.L. (2014) Distinct pharmacology and metabolism of K2 synthetic cannabinoids compared to Delta(9)-THC: mechanism underlying greater toxicity? *Life sciences*, **97**, 45-54.

Fattore, L., Cossu, G., Martellotta, C.M. & Fratta, W. (2001) Intravenous self-administration of the cannabinoid CB1 receptor agonist WIN 55,212-2 in rats. *Psychopharmacology (Berl)*, **156**, 410-416.

Fattore, L. & Fratta, W. (2011) Beyond THC: The New Generation of Cannabinoid Designer Drugs. *Front Behav Neurosci*, **5**, 60.

Fattore, L., Puddu, M.C., Picciau, S., Cappai, A., Fratta, W., Serra, G.P. & Spiga, S. (2002) Astroglial in vivo response to cocaine in mouse dentate gyrus: a quantitative and qualitative analysis by confocal microscopy. *Neuroscience*, **110**, 1-6.

Feenstra, M.G. & Botterblom, M.H. (1996) Rapid sampling of extracellular dopamine in the rat prefrontal cortex during food consumption, handling and exposure to novelty. *Brain research*, **742**, 17-24.

Fenu, S., Bassareo, V. & Di Chiara, G. (2001) A role for dopamine D1 receptors of the nucleus accumbens shell in conditioned taste aversion learning. *J Neurosci*, **21**, 6897-6904.

Ferdinand, R.F., Sondeijker, F., van der Ende, J., Selten, J.P., Huizink, A. & Verhulst, F.C. (2005) Cannabis use predicts future psychotic symptoms, and vice versa. *Addiction (Abingdon, England)*, **100**, 612-618.

Ferron, A., Thierry, A.M., Le Douarin, C. & Glowinski, J. (1984) Inhibitory influence of the mesocortical dopaminergic system on spontaneous activity or excitatory response induced from the thalamic mediodorsal nucleus in the rat medial prefrontal cortex. *Brain research*, **302**, 257-265.

Fields, R.D., Woo, D.H. & Basser, P.J. (2015) Glial Regulation of the Neuronal Connectome through Local and Long-Distant Communication. *Neuron*, **86**, 374-386.

Fillenz, M. (2005) In vivo neurochemical monitoring and the study of behaviour. *Neurosci Biobehav Rev*, **29**, 949-962.

Fischer-Smith, K.D., Houston, A.C. & Rebec, G.V. (2012) Differential effects of cocaine access and withdrawal on glutamate type 1 transporter expression in rat nucleus accumbens core and shell. *Neuroscience*, **210**, 333-339.

Foote, S.L., Bloom, F.E. & Aston-Jones, G. (1983) Nucleus locus ceruleus: new evidence of anatomical and physiological specificity. *Physiological reviews*, **63**, 844-914.

Ford, B.M., Tai, S., Fantegrossi, W.E. & Prather, P.L. (2017) Synthetic Pot: Not Your Grandfather's Marijuana. *Trends in pharmacological sciences*, **38**, 257-276.

Ford, R.D., Balster, R.L., Dewey, W.L. & Beckner, J.S. (1977) delta 9-THC and 11-OH-delta 9-THC: behavioral effects and relationship to plasma and brain levels. *Life sciences*, **20**, 1993-2003.

French, E.D., Dillon, K. & Wu, X. (1997) Cannabinoids excite dopamine neurons in the ventral tegmentum and substantia nigra. *Neuroreport*, **8**, 649-652.

Freund, T.F., Powell, J.F. & Smith, A.D. (1984) Tyrosine hydroxylase-immunoreactive boutons in synaptic contact with identified striatonigral neurons, with particular reference to dendritic spines. *Neuroscience*, **13**, 1189-1215.

Friedman, A.S., Glassman, K. & Terras, B.A. (2001) Violent behavior as related to use of marijuana and other drugs. *Journal of addictive diseases*, **20**, 49-72.

Friend, D.M. & Keefe, K.A. (2013) Glial reactivity in resistance to methamphetamine-induced neurotoxicity. *J Neurochem*, **125**, 566-574.

Galiegue, S., Mary, S., Marchand, J., Dussossoy, D., Carriere, D., Carayon, P., Bouaboula, M., Shire, D., Le Fur, G. & Casellas, P. (1995) Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *European journal of biochemistry*, **232**, 54-61.

Garcia-Gutierrez, M.S., Ortega-Alvaro, A., Busquets-Garcia, A., Perez-Ortiz, J.M., Caltana, L., Ricatti, M.J., Brusco, A., Maldonado, R. & Manzanares, J. (2013) Synaptic plasticity alterations associated with memory impairment induced by deletion of CB2 cannabinoid receptors. *Neuropharmacology*, **73**, 388-396.

Gardner, E.L. (2005) Endocannabinoid signaling system and brain reward: emphasis on dopamine. *Pharmacology, biochemistry, and behavior*, **81**, 263-284.

Garris, P.A., Collins, L.B., Jones, S.R. & Wightman, R.M. (1993) Evoked extracellular dopamine in vivo in the medial prefrontal cortex. *J Neurochem*, **61**, 637-647.

Gerdeman, G.L., Ronesi, J. & Lovinger, D.M. (2002) Postsynaptic endocannabinoid release is critical to long-term depression in the striatum. *Nat Neurosci*, **5**, 446-451.

Gessa, G., Melis, M., Muntoni, A. & Diana, M. (1998) Cannabinoids activate mesolimbic dopamine neurons by an action on cannabinoid CB1 receptors. *European journal of pharmacology*, **341**, 39-44.

Godbout, R., Mantz, J., Pirot, S., Glowinski, J. & Thierry, A.M. (1991) Inhibitory influence of the mesocortical dopaminergic neurons on their target cells: electrophysiological and pharmacological characterization. *The Journal of pharmacology and experimental therapeutics*, **258**, 728-738.

Goldstein, R.Z. & Volkow, N.D. (2011) Dysfunction of the prefrontal cortex in addiction: neuroimaging findings and clinical implications. *Nature reviews. Neuroscience*, **12**, 652-669.

Gong, J.P., Onaivi, E.S., Ishiguro, H., Liu, Q.R., Tagliaferro, P.A., Brusco, A. & Uhl, G.R. (2006) Cannabinoid CB2 receptors: immunohistochemical localization in rat brain. *Brain research*, **1071**, 10-23.

Gonon, F. (1997) Prolonged and extrasynaptic excitatory action of dopamine mediated by D1 receptors in the rat striatum in vivo. *J Neurosci*, **17**, 5972-5978.

Gonzalez, S., Fernandez-Ruiz, J., Sparpaglione, V., Parolaro, D. & Ramos, J.A. (2002) Chronic exposure to morphine, cocaine or ethanol in rats produced different effects in brain cannabinoid CB(1) receptor binding and mRNA levels. *Drug and alcohol dependence*, **66**, 77-84.

Gorelick, D.A., Goodwin, R.S., Schwilke, E., Schwope, D.M., Darwin, W.D., Kelly, D.L., McMahon, R.P., Liu, F., Ortemann-Renon, C., Bonnet, D. & Huestis, M.A. (2013) Tolerance to effects of high-dose oral delta9-tetrahydrocannabinol and plasma cannabinoid concentrations in male daily cannabis smokers. *Journal of analytical toxicology*, **37**, 11-16.

Grace, A.A. (1991) Phasic versus tonic dopamine release and the modulation of dopamine system responsivity: a hypothesis for the etiology of schizophrenia. *Neuroscience*, **41**, 1-24.

Grace, A.A. (2000) The tonic/phasic model of dopamine system regulation and its implications for understanding alcohol and psychostimulant craving. *Addiction (Abingdon, England)*, **95 Suppl 2**, S119-128.

Grace, A.A. & Bunney, B.S. (1983) Intracellular and extracellular electrophysiology of nigral dopaminergic neurons--1. Identification and characterization. *Neuroscience*, **10**, 301-315.

Grace, A.A. & Bunney, B.S. (1984) The control of firing pattern in nigral dopamine neurons: burst firing. *J Neurosci*, **4**, 2877-2890.

Graeber, M.B. (2010) Changing face of microglia. *Science (New York, N.Y.)*, **330**, 783-788.

Granado, N., Ares-Santos, S., Oliva, I., O'Shea, E., Martin, E.D., Colado, M.I. & Moratalla, R. (2011) Dopamine D2-receptor knockout mice are protected against dopaminergic neurotoxicity induced by methamphetamine or MDMA. *Neurobiol Dis*, **42**, 391-403.

Greengard, P. (2001) The neurobiology of dopamine signaling. *Bioscience reports*, **21**, 247-269.

Grenhoff, J., Tung, C.S. & Svensson, T.H. (1988) The excitatory amino acid antagonist kynurenate induces pacemaker-like firing of dopamine neurons in rat ventral tegmental area in vivo. *Acta physiologica Scandinavica*, **134**, 567-568.

Grill, H.J. & Norgren, R. (1978) The taste reactivity test. I. Mimetic responses to gustatory stimuli in neurologically normal rats. *Brain research*, **143**, 263-279.

Groenewegen, H.J., Berendse, H.W., Wolters, J.G. & Lohman, A.H. (1990) The anatomical relationship of the prefrontal cortex with the striatopallidal system, the thalamus and the amygdala: evidence for a parallel organization. *Progress in brain research*, **85**, 95-116; discussion 116-118.

Gurney, S.M., Scott, K.S., Kacinko, S.L., Presley, B.C. & Logan, B.K. (2014) Pharmacology, Toxicology, and Adverse Effects of Synthetic Cannabinoid Drugs. *Forensic science review*, **26**, 53-78.

Halassa, M.M., Fellin, T., Takano, H., Dong, J.H. & Haydon, P.G. (2007) Synaptic islands defined by the territory of a single astrocyte. *J Neurosci*, **27**, 6473-6477.

Hall, W. (1998) Cannabis use and psychosis. *Drug and alcohol review*, **17**, 433-444.

Hall, W. & Degenhardt, L. (2009) Adverse health effects of non-medical cannabis use. *Lancet (London, England)*, **374**, 1383-1391.

Hammond, C., Cayre, M., Panatier, A. & Avignone, E. (2015) Chapter 2 - Neuron–glial cell cooperation. In Hammond, C. (ed) *Cellular and Molecular Neurophysiology (Fourth Edition)*. Academic Press, Boston, pp. 25-37.

Han, J., Kesner, P., Metna-Laurent, M., Duan, T., Xu, L., Georges, F., Koehl, M., Abrous, D.N., Mendizabal-Zubiaga, J., Grandes, P., Liu, Q., Bai, G., Wang, W., Xiong, L., Ren, W., Marsicano, G. & Zhang, X. (2012) Acute cannabinoids impair working memory through astroglial CB1 receptor modulation of hippocampal LTD. *Cell*, **148**, 1039-1050.

Haney, M., Ward, A.S., Comer, S.D., Foltin, R.W. & Fischman, M.W. (1999) Abstinence symptoms following smoked marijuana in humans. *Psychopharmacology (Berl)*, **141**, 395-404.

Hanisch, U.K. & Kettenmann, H. (2007) Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci*, **10**, 1387-1394.

Harada, S., Nakamoto, K. & Tokuyama, S. (2013) The involvement of midbrain astrocyte in the development of morphine tolerance. *Life sciences*, **93**, 573-578.

Haro, G., Ripoll, C., Ibanez, M., Orengo, T., Liano, V.M., Meneu, E., Hernandez, F. & Traver, F. (2014) Could spice drugs induce psychosis with abnormal movements similar to catatonia? *Psychiatry*, **77**, 206-208.

Harte-Hargrove, L.C. & Dow-Edwards, D.L. (2012) Withdrawal from THC during adolescence: sex differences in locomotor activity and anxiety. *Behav Brain Res*, **231**, 48-59.

Heidbreder, C.A. & Groenewegen, H.J. (2003) The medial prefrontal cortex in the rat: evidence for a dorso-ventral distinction based upon functional and anatomical characteristics. *Neurosci Biobehav Rev*, **27**, 555-579.

Heimer, L., Zahm, D.S., Churchill, L., Kalivas, P.W. & Wohltmann, C. (1991) Specificity in the projection patterns of accumbal core and shell in the rat. *Neuroscience*, **41**, 89-125.

Henquet, C., Krabbendam, L., Spauwen, J., Kaplan, C., Lieb, R., Wittchen, H.U. & van Os, J. (2005) Prospective cohort study of cannabis use, predisposition for psychosis, and psychotic symptoms in young people. *BMJ (Clinical research ed.)*, **330**, 11.

Herkenham, M., Lynn, A.B., Johnson, M.R., Melvin, L.S., de Costa, B.R. & Rice, K.C. (1991) Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. *J Neurosci*, **11**, 563-583.

Hermanns-Clausen, M., Kneisel, S., Szabo, B. & Auwarter, V. (2013) Acute toxicity due to the confirmed consumption of synthetic cannabinoids: clinical and laboratory findings. *Addiction (Abingdon, England)*, **108**, 534-544.

Hernandez, G. & Cheer, J.F. (2012) Effect of CB1 receptor blockade on food-reinforced responding and associated nucleus accumbens neuronal activity in rats. *J Neurosci*, **32**, 11467-11477.

Hernandez, L., Stanley, B.G. & Hoebel, B.G. (1986) A small, removable microdialysis probe. *Life sciences*, **39**, 2629-2637.

Hester, R., Nestor, L. & Garavan, H. (2009) Impaired error awareness and anterior cingulate cortex hypoactivity in chronic cannabis users. *Neuropsychopharmacology*, **34**, 2450-2458.

Hitchcott, P.K., Quinn, J.J. & Taylor, J.R. (2007) Bidirectional modulation of goal-directed actions by prefrontal cortical dopamine. *Cereb Cortex*, **17**, 2820-2827.

Hollister, L.E. (1986) Health aspects of cannabis. *Pharmacological reviews*, **38**, 1-20.

Huffman, J.W., Mabon, R., Wu, M.J., Lu, J., Hart, R., Hurst, D.P., Reggio, P.H., Wiley, J.L. & Martin, B.R. (2003) 3-Indolyl-1-naphthylmethanes: new cannabimimetic indoles provide evidence for aromatic stacking interactions with the CB(1) cannabinoid receptor. *Bioorganic & medicinal chemistry*, **11**, 539-549.

Huffman, J.W. & Padgett, L.W. (2005) Recent developments in the medicinal chemistry of cannabimimetic indoles, pyrroles and indenes. *Current medicinal chemistry*, **12**, 1395-1411.

Hunt, C.A. & Jones, R.T. (1980) Tolerance and disposition of tetrahydrocannabinol in man. *The Journal of pharmacology and experimental therapeutics*, **215**, 35-44.

Hurley, K.M., Herbert, H., Moga, M.M. & Saper, C.B. (1991) Efferent projections of the infralimbic cortex of the rat. *The Journal of comparative neurology*, **308**, 249-276.

Hyman, S.E., Malenka, R.C. & Nestler, E.J. (2006) Neural mechanisms of addiction: the role of reward-related learning and memory. *Annual review of neuroscience*, **29**, 565-598.

Hyman, S.M. & Sinha, R. (2009) Stress-related factors in cannabis use and misuse: implications for prevention and treatment. *Journal of substance abuse treatment*, **36**, 400-413.

Ihalainen, J.A., Riekkinen, P., Jr. & Feenstra, M.G. (1999) Comparison of dopamine and noradrenaline release in mouse prefrontal cortex, striatum and hippocampus using microdialysis. *Neuroscience letters*, **277**, 71-74.

Imperato, A. & Di Chiara, G. (1986) Preferential stimulation of dopamine release in the nucleus accumbens of freely moving rats by ethanol. *The Journal of pharmacology and experimental therapeutics*, **239**, 219-228.

Iversen, L. (2003) Cannabis and the brain. *Brain*, **126**, 1252-1270.

Jarrett, M.M., Limebeer, C.L. & Parker, L.A. (2005) Effect of Delta9-tetrahydrocannabinol on sucrose palatability as measured by the taste reactivity test. *Physiol Behav*, **86**, 475-479.

Jaskiw, G.E., Weinberger, D.R. & Crawley, J.N. (1991) Microinjection of apomorphine into the prefrontal cortex of the rat reduces dopamine metabolite concentrations in microdialysate from the caudate nucleus. *Biol Psychiatry*, **29**, 703-706.

Jedema, H.P. & Moghaddam, B. (1994) Glutamatergic control of dopamine release during stress in the rat prefrontal cortex. *J Neurochem*, **63**, 785-788.

Jensen, J., Willeit, M., Zipursky, R.B., Savina, I., Smith, A.J., Menon, M., Crawley, A.P. & Kapur, S. (2008) The formation of abnormal associations in schizophrenia: neural and behavioral evidence. *Neuropsychopharmacology*, **33**, 473-479.

Jentsch, J.D., Verrico, C.D., Le, D. & Roth, R.H. (1998) Repeated exposure to delta 9-tetrahydrocannabinol reduces prefrontal cortical dopamine metabolism in the rat. *Neuroscience letters*, **246**, 169-172.

Jimenez-Gomez, C., Osentoski, A. & Woods, J.H. (2011) Pharmacological evaluation of the adequacy of marble burying as an animal model of compulsion and/or anxiety. *Behavioural pharmacology*, **22**, 711-713.

Jones, R.T. (2002) Cardiovascular system effects of marijuana. *Journal of clinical pharmacology*, **42**, 58s-63s.

Jones, R.T., Benowitz, N. & Bachman, J. (1976) Clinical studies of cannabis tolerance and dependence. *Annals of the New York Academy of Sciences*, **282**, 221-239.

Jones, R.T., Benowitz, N.L. & Herning, R.I. (1981) Clinical relevance of cannabis tolerance and dependence. *Journal of clinical pharmacology*, **21**, 143s-152s.

Jongen-Relo, A.L., Groenewegen, H.J. & Voorn, P. (1993) Evidence for a multi-compartmental histochemical organization of the nucleus accumbens in the rat. *The Journal of comparative neurology*, **337**, 267-276.

Jongen-Relo, A.L., Voorn, P. & Groenewegen, H.J. (1994) Immunohistochemical characterization of the shell and core territories of the nucleus accumbens in the rat. *Eur J Neurosci*, **6**, 1255-1264.

Jung, K.M., Mangieri, R., Stapleton, C., Kim, J., Fegley, D., Wallace, M., Mackie, K. & Piomelli, D. (2005) Stimulation of endocannabinoid formation in brain slice cultures through activation of group I metabotropic glutamate receptors. *Mol Pharmacol*, **68**, 1196-1202.

Kalivas, P.W. & Duffy, P. (1995) Selective activation of dopamine transmission in the shell of the nucleus accumbens by stress. *Brain research*, **675**, 325-328.

Kalivas, P.W. & Stewart, J. (1991) Dopamine transmission in the initiation and expression of drug- and stress-induced sensitization of motor activity. *Brain research. Brain research reviews*, **16**, 223-244.

Kalivas, P.W. & Volkow, N.D. (2005) The neural basis of addiction: a pathology of motivation and choice. *The American journal of psychiatry*, **162**, 1403-1413.

Karreman, M. & Moghaddam, B. (1996) The prefrontal cortex regulates the basal release of dopamine in the limbic striatum: an effect mediated by ventral tegmental area. *J Neurochem*, **66**, 589-598.

Katona, I., Rancz, E.A., Acsady, L., Ledent, C., Mackie, K., Hajos, N. & Freund, T.F. (2001) Distribution of CB1 cannabinoid receptors in the amygdala and their role in the control of GABAergic transmission. *J Neurosci*, **21**, 9506-9518.

Katz, K.D., Leonetti, A.L., Bailey, B.C., Surmaitis, R.M., Eustice, E.R., Kacinko, S. & Wheatley, S.M. (2016) Case Series of Synthetic Cannabinoid Intoxication from One Toxicology Center. *The western journal of emergency medicine*, **17**, 290-294.

Kauer, J.A. (2004) Learning mechanisms in addiction: synaptic plasticity in the ventral tegmental area as a result of exposure to drugs of abuse. *Annual review of physiology*, **66**, 447-475.

Kazory, A. & Aiyer, R. (2013) Synthetic marijuana and acute kidney injury: an unforeseen association. *Clinical kidney journal*, **6**, 330-333.

Kedia, S. & Chattarji, S. (2014) Marble burying as a test of the delayed anxiogenic effects of acute immobilisation stress in mice. *Journal of neuroscience methods*, **233**, 150-154.

Kedzior, K.K. & Martin-Iverson, M.T. (2007) Attention-dependent reduction in prepulse inhibition of the startle reflex in cannabis users and schizophrenia patients--a pilot study. *European journal of pharmacology*, **560**, 176-182.

Kettenmann, H., Kirchhoff, F. & Verkhratsky, A. (2013) Microglia: new roles for the synaptic stripper. *Neuron*, **77**, 10-18.

Kierdorf, K. & Prinz, M. (2013) Factors regulating microglia activation. *Front Cell Neurosci*, **7**, 44.

Kim, R., Healey, K.L., Sepulveda-Orengo, M.T. & Reissner, K.J. (2018) Astroglial correlates of neuropsychiatric disease: From astrocytopathy to astrogliosis. *Prog Neuropsychopharmacol Biol Psychiatry*, **87**, 126-146.

Kinsey, S.G., O'Neal, S.T., Long, J.Z., Cravatt, B.F. & Lichtman, A.H. (2011) Inhibition of endocannabinoid catabolic enzymes elicits anxiolytic-like effects in the marble burying assay. *Pharmacology, biochemistry, and behavior*, **98**, 21-27.

Kolachana, B.S., Saunders, R.C. & Weinberger, D.R. (1995) Augmentation of prefrontal cortical monoaminergic activity inhibits dopamine release in the caudate nucleus: an in vivo neurochemical assessment in the rhesus monkey. *Neuroscience*, **69**, 859-868.

Koob, G.F. & Le Moal, M. (2001) Drug addiction, dysregulation of reward, and allostasis. *Neuropsychopharmacology*, **24**, 97-129.

Koob, G.F. & Volkow, N.D. (2010) Neurocircuitry of addiction. *Neuropsychopharmacology*, **35**, 217-238.

Krettek, J.E. & Price, J.L. (1977) Projections from the amygdaloid complex to the cerebral cortex and thalamus in the rat and cat. *The Journal of comparative neurology*, **172**, 687-722.

Kreutzberg, G.W. (1996) Microglia: a sensor for pathological events in the CNS. *Trends in neurosciences*, **19**, 312-318.

Kringelbach, M.L. (2005) The human orbitofrontal cortex: linking reward to hedonic experience. *Nature reviews. Neuroscience*, **6**, 691-702.

Kuroda, M., Murakami, K., Oda, S., Shinkai, M. & Kishi, K. (1993) Direct synaptic connections between thalamocortical axon terminals from the mediodorsal thalamic nucleus (MD) and corticothalamic neurons to MD in the prefrontal cortex. *Brain research*, **612**, 339-344.

Lacagnina, M.J., Rivera, P.D. & Bilbo, S.D. (2017) Glial and Neuroimmune Mechanisms as Critical Modulators of Drug Use and Abuse. *Neuropsychopharmacology*, **42**, 156-177.

Lacroix, L., Broersen, L.M., Feldon, J. & Weiner, I. (2000) Effects of local infusions of dopaminergic drugs into the medial prefrontal cortex of rats on latent inhibition, prepulse inhibition and amphetamine induced activity. *Behav Brain Res*, **107**, 111-121.

Lammel, S., Steinberg, E.E., Foldy, C., Wall, N.R., Beier, K., Luo, L. & Malenka, R.C. (2015) Diversity of transgenic mouse models for selective targeting of midbrain dopamine neurons. *Neuron*, **85**, 429-438.

Lane, S.D., Cherek, D.R., Lieving, L.M. & Tcheremissine, O.V. (2005) Marijuana effects on human forgetting functions. *Journal of the experimental analysis of behavior*, **83**, 67-83.

Lapoint, J., James, L.P., Moran, C.L., Nelson, L.S., Hoffman, R.S. & Moran, J.H. (2011) Severe toxicity following synthetic cannabinoid ingestion. *Clinical toxicology (Philadelphia, Pa.)*, **49**, 760-764.

Lau, L.T. & Yu, A.C. (2001) Astrocytes produce and release interleukin-1, interleukin-6, tumor necrosis factor alpha and interferon-gamma following traumatic and metabolic injury. *Journal of neurotrauma*, **18**, 351-359.

Lavin, A., Nogueira, L., Lapish, C.C., Wightman, R.M., Phillips, P.E. & Seamans, J.K. (2005) Mesocortical dopamine neurons operate in distinct temporal domains using multimodal signaling. *J Neurosci*, **25**, 5013-5023.

Lawson, L.J., Perry, V.H., Dri, P. & Gordon, S. (1990) Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. *Neuroscience*, **39**, 151-170.

Ledent, C., Valverde, O., Cossu, G., Petitet, F., Aubert, J.F., Beslot, F., Bohme, G.A., Imperato, A., Pedrazzini, T., Roques, B.P., Vassart, G., Fratta, W. & Parmentier, M. (1999) Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. *Science (New York, N.Y.)*, **283**, 401-404.

Leshner, A.I. (1997) Addiction is a brain disease, and it matters. *Science (New York, N.Y.)*, **278**, 45-47.

Li, X., Newbern, J.M., Wu, Y., Morgan-Smith, M., Zhong, J., Charron, J. & Snider, W.D. (2012) MEK Is a Key Regulator of Gliogenesis in the Developing Brain. *Neuron*, **75**, 1035-1050.

Li, Y. & Kim, J. (2016) CB2 Cannabinoid Receptor Knockout in Mice Impairs Contextual Long-Term Memory and Enhances Spatial Working Memory. *Neural plasticity*, **2016**, 9817089.

Lichtman, A.H. & Martin, B.R. (2005) Cannabinoid tolerance and dependence. *Handbook of experimental pharmacology*, 691-717.

Lindvall, O., Bjorklund, A. & Divac, I. (1978) Organization of catecholamine neurons projecting to the frontal cortex in the rat. *Brain research*, **142**, 1-24.

Long, L.E., Chesworth, R., Huang, X.F., McGregor, I.S., Arnold, J.C. & Karl, T. (2010) A behavioural comparison of acute and chronic Delta9-tetrahydrocannabinol and cannabidiol in C57BL/6JArc mice. *Int J Neuropsychopharmacol*, **13**, 861-876.

Lopez-Rodriguez, A.B., Llorente-Berzal, A., Garcia-Segura, L.M. & Viveros, M.P. (2014) Sex-dependent long-term effects of adolescent exposure to THC and/or MDMA on neuroinflammation and serotoninergic and cannabinoid systems in rats. *Br J Pharmacol*, **171**, 1435-1447.

Louilot, A., Le Moal, M. & Simon, H. (1989) Opposite influences of dopaminergic pathways to the prefrontal cortex or the septum on the dopaminergic transmission in the nucleus accumbens. An in vivo voltammetric study. *Neuroscience*, **29**, 45-56.

Lu, H.C. & Mackie, K. (2016) An Introduction to the Endogenous Cannabinoid System. *Biol Psychiatry*, **79**, 516-525.

Lucin, K.M. & Wyss-Coray, T. (2009) Immune activation in brain aging and neurodegeneration: too much or too little? *Neuron*, **64**, 110-122.

Lundqvist, T. (2005) Cognitive consequences of cannabis use: comparison with abuse of stimulants and heroin with regard to attention, memory and executive functions. *Pharmacology, biochemistry, and behavior*, **81**, 319-330.

Lupica, C.R. & Riegel, A.C. (2005) Endocannabinoid release from midbrain dopamine neurons: a potential substrate for cannabinoid receptor antagonist treatment of addiction. *Neuropharmacology*, **48**, 1105-1116.

Macfarlane, V. & Christie, G. (2015) Synthetic cannabinoid withdrawal: a new demand on detoxification services. *Drug and alcohol review*, **34**, 147-153.

Mackie, K. (2005) Distribution of cannabinoid receptors in the central and peripheral nervous system. *Handbook of experimental pharmacology*, 299-325.

Macri, S., Lanuzza, L., Merola, G., Ceci, C., Gentili, S., Valli, A., Macchia, T. & Laviola, G. (2013) Behavioral responses to acute and sub-chronic administration of the synthetic cannabinoid JWH-018 in adult mice prenatally exposed to corticosterone. *Neurotoxicity research*, **24**, 15-28.

Maldonado, R. & Rodriguez de Fonseca, F. (2002) Cannabinoid addiction: behavioral models and neural correlates. *J Neurosci*, **22**, 3326-3331.

Maldonado, R., Valverde, O. & Berrendero, F. (2006) Involvement of the endocannabinoid system in drug addiction. *Trends in neurosciences*, **29**, 225-232.

Malyshevskaya, O., Aritake, K., Kaushik, M.K., Uchiyama, N., Cherasse, Y., Kikura-Hanajiri, R. & Urade, Y. (2017) Natural ((9)-THC) and synthetic (JWH-018) cannabinoids induce seizures by acting through the cannabinoid CB1 receptor. *Scientific reports*, **7**, 10516.

Mansbach, R.S., Nicholson, K.L., Martin, B.R. & Balster, R.L. (1994) Failure of Delta(9)-tetrahydrocannabinol and CP 55,940 to maintain intravenous self-administration under a fixed-interval schedule in rhesus monkeys. *Behavioural pharmacology*, **5**, 219-225.

Mansour, A., Fox, C.A., Burke, S., Meng, F., Thompson, R.C., Akil, H. & Watson, S.J. (1994) Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: an in situ hybridization study. *The Journal of comparative neurology*, **350**, 412-438.

Mantz, J., Milla, C., Glowinski, J. & Thierry, A.M. (1988) Differential effects of ascending neurons containing dopamine and noradrenaline in the control of spontaneous activity and of evoked responses in the rat prefrontal cortex. *Neuroscience*, **27**, 517-526.

Marin, I. & Kipnis, J. (2013) Learning and memory ... and the immune system. *Learn Mem*, **20**, 601-606.

Martellotta, M.C., Cossu, G., Fattore, L., Gessa, G.L. & Fratta, W. (1998) Self-administration of the cannabinoid receptor agonist WIN 55,212-2 in drug-naive mice. *Neuroscience*, **85**, 327-330.

Martin-Santos, R., Fagundo, A.B., Crippa, J.A., Atakan, Z., Bhattacharyya, S., Allen, P., Fusar-Poli, P., Borgwardt, S., Seal, M., Busatto, G.F. & McGuire, P. (2010) Neuroimaging in cannabis use: a systematic review of the literature. *Psychological medicine*, **40**, 383-398.

Martin, B.R., Sim-Selley, L.J. & Selley, D.E. (2004) Signaling pathways involved in the development of cannabinoid tolerance. *Trends in pharmacological sciences*, **25**, 325-330.

Mathias, C.W., Blumenthal, T.D., Dawes, M.A., Liguori, A., Richard, D.M., Bray, B., Tong, W. & Dougherty, D.M. (2011) Failure to sustain prepulse inhibition in adolescent marijuana users. *Drug and alcohol dependence*, **116**, 110-116.

Mato, S., Robbe, D., Puente, N., Grandes, P. & Manzoni, O.J. (2005) Presynaptic homeostatic plasticity rescues long-term depression after chronic Delta 9-tetrahydrocannabinol exposure. *J Neurosci*, **25**, 11619-11627.

Mazur, A., Lichti, C.F., Prather, P.L., Zielinska, A.K., Bratton, S.M., Gallus-Zawada, A., Finel, M., Miller, G.P., Radominska-Pandya, A. & Moran, J.H. (2009) Characterization of human hepatic and extrahepatic UDP-glucuronosyltransferase enzymes involved in the metabolism of classic cannabinoids. *Drug metabolism and disposition: the biological fate of chemicals*, **37**, 1496-1504.

McCullough, L.D. & Salamone, J.D. (1992) Anxiogenic drugs beta-CCE and FG 7142 increase extracellular dopamine levels in nucleus accumbens. *Psychopharmacology (Berl)*, **109**, 379-382.

McIlroy, G., Ford, L. & Khan, J.M. (2016) Acute myocardial infarction, associated with the use of a synthetic adamantyl-cannabinoid: a case report. *BMC pharmacology & toxicology*, **17**, 2.

McQuade, D., Hudson, S., Dargan, P.I. & Wood, D.M. (2013) First European case of convulsions related to analytically confirmed use of the synthetic cannabinoid receptor agonist AM-2201. *European journal of clinical pharmacology*, **69**, 373-376.

Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N.E., Schatz, A.R., Gopher, A., Almog, S., Martin, B.R., Compton, D.R. & et al. (1995) Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochemical pharmacology*, **50**, 83-90.

Meijer, K.A., Russo, R.R. & Adhvaryu, D.V. (2014) Smoking synthetic marijuana leads to self-mutilation requiring bilateral amputations. *Orthopedics*, **37**, e391-394.

Melis, M., Frau, R., Kalivas, P.W., Spencer, S., Chioma, V., Zamberletti, E., Rubino, T. & Parolaro, D. (2017) New vistas on cannabis use disorder. *Neuropharmacology*, **124**, 62-72.

Melis, M., Perra, S., Muntoni, A.L., Pillolla, G., Lutz, B., Marsicano, G., Di Marzo, V., Gessa, G.L. & Pistis, M. (2004a) Prefrontal cortex stimulation induces 2-arachidonoyl-glycerol-mediated suppression of excitation in dopamine neurons. *J Neurosci*, **24**, 10707-10715.

Melis, M., Pistis, M., Perra, S., Muntoni, A.L., Pillolla, G. & Gessa, G.L. (2004b) Endocannabinoids mediate presynaptic inhibition of glutamatergic transmission in rat ventral tegmental area dopamine neurons through activation of CB1 receptors. *J Neurosci*, **24**, 53-62.

Melis, M., Spiga, S. & Diana, M. (2005) The dopamine hypothesis of drug addiction: hypodopaminergic state. *International review of neurobiology*, **63**, 101-154.

Mercuri, N.B., Stratta, F., Calabresi, P., Bonci, A. & Bernardi, G. (1993) Activation of metabotropic glutamate receptors induces an inward current in rat dopamine mesencephalic neurons. *Neuroscience*, **56**, 399-407.

Miguel-Hidalgo, J.J. (2009) The role of glial cells in drug abuse. *Current drug abuse reviews*, **2**, 72-82.

Miliano, C., Serpelloni, G., Rimondo, C., Mereu, M., Marti, M. & De Luca, M.A. (2016) Neuropharmacology of New Psychoactive Substances (NPS): Focus on the Rewarding and Reinforcing Properties of Cannabimimetics and Amphetamine-Like Stimulants. *Front Neurosci*, **10**, 153.

Missale, C., Nash, S.R., Robinson, S.W., Jaber, M. & Caron, M.G. (1998) Dopamine receptors: from structure to function. *Physiological reviews*, **78**, 189-225.

Mogenson, G.J., Jones, D.L. & Yim, C.Y. (1980) From motivation to action: functional interface between the limbic system and the motor system. *Progress in neurobiology*, **14**, 69-97.

Mogenson, G.J. & Yang, C.R. (1991) The contribution of basal forebrain to limbic-motor integration and the mediation of motivation to action. *Advances in experimental medicine and biology*, **295**, 267-290.

Moldrich, G. & Wenger, T. (2000) Localization of the CB1 cannabinoid receptor in the rat brain. An immunohistochemical study. *Peptides*, **21**, 1735-1742.

Molina-Holgado, F., Molina-Holgado, E., Guaza, C. & Rothwell, N.J. (2002) Role of CB1 and CB2 receptors in the inhibitory effects of cannabinoids on lipopolysaccharide-induced nitric oxide release in astrocyte cultures. *Journal of neuroscience research*, **67**, 829-836.

Muller, H., Huttner, H.B., Kohrmann, M., Wielopolski, J.E., Kornhuber, J. & Sperling, W. (2010) Panic attack after spice abuse in a patient with ADHD. *Pharmacopsychiatry*, **43**, 152-153.

Murphy, M., Mills, S., Winstone, J., Leishman, E., Wager-Miller, J., Bradshaw, H. & Mackie, K. (2017) Chronic Adolescent Delta(9)-Tetrahydrocannabinol Treatment of Male Mice Leads to Long-Term Cognitive and Behavioral Dysfunction, Which Are Prevented by Concurrent Cannabidiol Treatment. *Cannabis and cannabinoid research*, **2**, 235-246.

Nacca, N., Vatti, D., Sullivan, R., Sud, P., Su, M. & Marraffa, J. (2013) The synthetic cannabinoid withdrawal syndrome. *Journal of addiction medicine*, **7**, 296-298.

Nader, M.A., Morgan, D., Gage, H.D., Nader, S.H., Calhoun, T.L., Buchheimer, N., Ehrenkaufer, R. & Mach, R.H. (2006) PET imaging of dopamine D2 receptors during chronic cocaine self-administration in monkeys. *Nat Neurosci*, **9**, 1050-1056.

Nakano, K. (2000) Neural circuits and topographic organization of the basal ganglia and related regions. *Brain & development*, **22 Suppl 1**, S5-16.

Naneix, F., Marchand, A.R., Di Scala, G., Pape, J.R. & Coutureau, E. (2009) A role for medial prefrontal dopaminergic innervation in instrumental conditioning. *J Neurosci*, **29**, 6599-6606.

Nauta, W.J., Smith, G.P., Faull, R.L. & Domesick, V.B. (1978) Efferent connections and nigral afferents of the nucleus accumbens septi in the rat. *Neuroscience*, **3**, 385-401.

Navarrete, M. & Araque, A. (2008) Endocannabinoids mediate neuron-astrocyte communication. *Neuron*, **57**, 883-893.

Navarrete, M., Diez, A. & Araque, A. (2014) Astrocytes in endocannabinoid signalling. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, **369**, 20130599.

Nestler, E.J. (1996) Under siege: The brain on opiates. *Neuron*, **16**, 897-900.

Nestler, E.J. (2001) Molecular basis of long-term plasticity underlying addiction. *Nature reviews. Neuroscience*, **2**, 119-128.

Nestler, E.J. (2004) Historical review: Molecular and cellular mechanisms of opiate and cocaine addiction. *Trends in pharmacological sciences*, **25**, 210-218.

Nimmerjahn, A., Kirchhoff, F. & Helmchen, F. (2005) Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science (New York, N.Y.)*, **308**, 1314-1318.

Nunez, E., Benito, C., Pazos, M.R., Barbachano, A., Fajardo, O., Gonzalez, S., Tolon, R.M. & Romero, J. (2004) Cannabinoid CB2 receptors are expressed by perivascular microglial cells in the human brain: an immunohistochemical study. *Synapse (New York, N.Y.)*, **53**, 208-213.

Nyiri, G., Cserep, C., Szabadits, E., Mackie, K. & Freund, T.F. (2005) CB1 cannabinoid receptors are enriched in the perisynaptic annulus and on preterminal segments of hippocampal GABAergic axons. *Neuroscience*, **136**, 811-822.

O'Callaghan, J.P., Kelly, K.A., VanGilder, R.L., Sofroniew, M.V. & Miller, D.B. (2014) Early activation of STAT3 regulates reactive astrogliosis induced by diverse forms of neurotoxicity. *PLoS One*, **9**, e102003.

Occhieppo, V.B., Marchese, N.A., Rodriguez, I.D., Basmadjian, O.M., Baiardi, G. & Bregonzio, C. (2017) Neurovascular unit alteration in somatosensory cortex and enhancement of thermal nociception induced by amphetamine involves central AT1 receptor activation. *Eur J Neurosci*, **45**, 1586-1593.

Oleson, E.B. & Cheer, J.F. (2012) A brain on cannabinoids: the role of dopamine release in reward seeking. *Cold Spring Harb Perspect Med*, **2**.

Oliva, J.M., Ortiz, S., Palomo, T. & Manzanares, J. (2003) Behavioural and gene transcription alterations induced by spontaneous cannabinoid withdrawal in mice. *J Neurochem*, **85**, 94-104.

Oliva, J.M., Ortiz, S., Palomo, T. & Manzanares, J. (2004) Spontaneous cannabinoid withdrawal produces a differential time-related responsiveness in cannabinoid CB1 receptor gene expression in the mouse brain. *Journal of psychopharmacology (Oxford, England)*, **18**, 59-65.

Ossato, A., Canazza, I., Trapella, C., Vincenzi, F., De Luca, M.A., Rimondo, C., Varani, K., Borea, P.A., Serpelloni, G. & Marti, M. (2016) Effect of JWH-250, JWH-073 and their interaction on "tetrad", sensorimotor, neurological and neurochemical responses in mice. *Prog Neuropsychopharmacol Biol Psychiatry*, **67**, 31-50.

Oviedo, A., Glowa, J. & Herkenham, M. (1993) Chronic cannabinoid administration alters cannabinoid receptor binding in rat brain: a quantitative autoradiographic study. *Brain research*, **616**, 293-302.

Papanti, D., Orsolini, L., Francesconi, G. & Schifano, F. (2014) *Noids in a nutshell: Everything you (don't) want to know about synthetic cannabimimetics*.

Parent, A., Cote, P.Y. & Lavoie, B. (1995) Chemical anatomy of primate basal ganglia. *Progress in neurobiology*, **46**, 131-197.

Parolaro, D., Realini, N., Vigano, D., Guidali, C. & Rubino, T. (2010) The endocannabinoid system and psychiatric disorders. *Experimental Neurology*, **224**, 3-14.

Parsons, L.H. & Hurd, Y.L. (2015) Endocannabinoid signalling in reward and addiction. *Nature reviews. Neuroscience*, **16**, 579-594.

Patton, G.C., Coffey, C., Carlin, J.B., Degenhardt, L., Lynskey, M. & Hall, W. (2002) Cannabis use and mental health in young people: cohort study. *BMJ (Clinical research ed.)*, **325**, 1195-1198.

Pecina, S., Berridge, K.C. & Parker, L.A. (1997) Pimozide does not shift palatability: separation of anhedonia from sensorimotor suppression by taste reactivity. *Pharmacology, biochemistry, and behavior*, **58**, 801-811.

Peglow, S., Buchner, J. & Briscoe, G. (2012) Synthetic cannabinoid induced psychosis in a previously nonpsychotic patient. *The American journal on addictions*, **21**, 287-288.

Penit-Soria, J., Audinat, E. & Crepel, F. (1987) Excitation of rat prefrontal cortical neurons by dopamine: an in vitro electrophysiological study. *Brain research*, **425**, 263-274.

Perdikaris, P., Tsarouchi, M., Fanarioti, E., Natsaridis, E., Mitsacos, A. & Giompres, P. (2018) Long lasting effects of chronic WIN55,212-2 treatment on mesostriatal dopaminergic and cannabinoid systems in the rat brain. *Neuropharmacology*, **129**, 1-15.

Pertwee, R.G., Stevenson, L.A. & Griffin, G. (1993) Cross-tolerance between delta-9-tetrahydrocannabinol and the cannabimimetic agents, CP 55,940, WIN 55,212-2 and anandamide. *Br J Pharmacol*, **110**, 1483-1490.

Peterson, S.L., St Mary, J.S. & Harding, N.R. (1987) cis-Flupentixol antagonism of the rat prefrontal cortex neuronal response to apomorphine and ventral tegmental area input. *Brain research bulletin*, **18**, 723-729.

Phillips, A.G., Atkinson, L.J., Blackburn, J.R. & Blaha, C.D. (1993) Increased extracellular dopamine in the nucleus accumbens of the rat elicited by a conditional stimulus for food: an electrochemical study. *Canadian journal of physiology and pharmacology*, **71**, 387-393.

Phillips, P.E., Stuber, G.D., Heien, M.L., Wightman, R.M. & Carelli, R.M. (2003) Subsecond dopamine release promotes cocaine seeking. *Nature*, **422**, 614-618.

Pierce, R.C. & Kalivas, P.W. (1995) Amphetamine produces sensitized increases in locomotion and extracellular dopamine preferentially in the nucleus accumbens shell of rats administered repeated cocaine. *The Journal of pharmacology and experimental therapeutics*, **275**, 1019-1029.

Pintori, N., Loi, B. & Mereu, M. (2017) Synthetic cannabinoids: the hidden side of Spice drugs. *Behavioural pharmacology*, **28**, 409-419.

Piomelli, D. (2003) The molecular logic of endocannabinoid signalling. *Nature reviews. Neuroscience*, **4**, 873-884.

Pirot, S., Godbout, R., Mantz, J., Tassin, J.P., Glowinski, J. & Thierry, A.M. (1992) Inhibitory effects of ventral tegmental area stimulation on the activity of prefrontal cortical neurons: evidence for the involvement of both dopaminergic and GABAergic components. *Neuroscience*, **49**, 857-865.

Pistis, M., Perra, S., Pillolla, G., Melis, M., Muntoni, A.L. & Gessa, G.L. (2004) Adolescent exposure to cannabinoids induces long-lasting changes in the response to drugs of abuse of rat midbrain dopamine neurons. *Biol Psychiatry*, **56**, 86-94.

Pontieri, F.E., Tanda, G. & Di Chiara, G. (1995) Intravenous cocaine, morphine, and amphetamine preferentially increase extracellular dopamine in the "shell" as compared with the "core" of the rat nucleus accumbens. *Proceedings of the National Academy of Sciences of the United States of America*, **92**, 12304-12308.

Pontieri, F.E., Tanda, G., Orzi, F. & Di Chiara, G. (1996) Effects of nicotine on the nucleus accumbens and similarity to those of addictive drugs. *Nature*, **382**, 255-257.

Pope, H.G., Jr., Gruber, A.J., Hudson, J.I., Huestis, M.A. & Yurgelun-Todd, D. (2001) Neuropsychological performance in long-term cannabis users. *Archives of general psychiatry*, **58**, 909-915.

Prinssen, E.P., Balestra, W., Bemelmans, F.F. & Cools, A.R. (1994) Evidence for a role of the shell of the nucleus accumbens in oral behavior of freely moving rats. *J Neurosci*, **14**, 1555-1562.

Prohovnik, I., Arnold, S.E., Smith, G. & Lucas, L.R. (1997) Physostigmine reversal of scopolamine-induced hypofrontality. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*, **17**, 220-228.

Pulvirenti, L. & Diana, M. (2001) Drug dependence as a disorder of neural plasticity: focus on dopamine and glutamate. *Reviews in the neurosciences*, **12**, 141-158.

Quednow, B.B., Kuhn, K.U., Hoenig, K., Maier, W. & Wagner, M. (2004) Prepulse inhibition and habituation of acoustic startle response in male MDMA ('ecstasy') users, cannabis users, and healthy controls. *Neuropsychopharmacology*, **29**, 982-990.

Rabinovic, A.D., Lewis, D.A. & Hastings, T.G. (2000) Role of oxidative changes in the degeneration of dopamine terminals after injection of neurotoxic levels of dopamine. *Neuroscience*, **101**, 67-76.

Rajasekaran, M., Brents, L.K., Franks, L.N., Moran, J.H. & Prather, P.L. (2013) Human metabolites of synthetic cannabinoids JWH-018 and JWH-073 bind with high affinity and act as potent agonists at cannabinoid type-2 receptors. *Toxicology and applied pharmacology*, **269**, 100-108.

Ramaekers, J.G., Theunissen, E.L., de Brouwer, M., Toennes, S.W., Moeller, M.R. & Kauert, G. (2011) Tolerance and cross-tolerance to neurocognitive effects of THC and alcohol in heavy cannabis users. *Psychopharmacology (Berl)*, **214**, 391-401.

Ramnani, N. & Owen, A.M. (2004) Anterior prefrontal cortex: insights into function from anatomy and neuroimaging. *Nature reviews. Neuroscience*, **5**, 184-194.

Ransohoff, R.M. & Perry, V.H. (2009) Microglial physiology: unique stimuli, specialized responses. *Annual review of immunology*, **27**, 119-145.

Ray, J.P. & Price, J.L. (1992) The organization of the thalamocortical connections of the mediodorsal thalamic nucleus in the rat, related to the ventral forebrain-prefrontal cortex topography. *The Journal of comparative neurology*, **323**, 167-197.

Reddy, D.S. & Golub, V.M. (2016) The Pharmacological Basis of Cannabis Therapy for Epilepsy. *The Journal of pharmacology and experimental therapeutics*, **357**, 45-55.

Riegel, A.C. & Lupica, C.R. (2004) Independent presynaptic and postsynaptic mechanisms regulate endocannabinoid signaling at multiple synapses in the ventral tegmental area. *J Neurosci*, **24**, 11070-11078.

Robbe, D., Alonso, G., Duchamp, F., Bockaert, J. & Manzoni, O.J. (2001) Localization and mechanisms of action of cannabinoid receptors at the glutamatergic synapses of the mouse nucleus accumbens. *J Neurosci*, **21**, 109-116.

Robbe, D., Kopf, M., Remaury, A., Bockaert, J. & Manzoni, O.J. (2002) Endogenous cannabinoids mediate long-term synaptic depression in the nucleus accumbens. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 8384-8388.

Robinson, T.E. & Berridge, K.C. (1993) The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain research. Brain research reviews*, **18**, 247-291.

Rodgers, R.J., Cao, B.J., Dalvi, A. & Holmes, A. (1997) Animal models of anxiety: an ethological perspective. *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas*, **30**, 289-304.

Rodriguez de Fonseca, F., Gorriti, M.A., Fernandez-Ruiz, J.J., Palomo, T. & Ramos, J.A. (1994) Downregulation of rat brain cannabinoid binding sites after chronic delta 9-tetrahydrocannabinol treatment. *Pharmacology, biochemistry, and behavior*, **47**, 33-40.

Rodriguez, J.J., Mackie, K. & Pickel, V.M. (2001) Ultrastructural localization of the CB1 cannabinoid receptor in mu-opioid receptor patches of the rat Caudate putamen nucleus. *J Neurosci*, **21**, 823-833.

Romero, J., Garcia-Palomero, E., Castro, J.G., Garcia-Gil, L., Ramos, J.A. & Fernandez-Ruiz, J.J. (1997) Effects of chronic exposure to delta9-tetrahydrocannabinol on cannabinoid receptor binding and mRNA levels in several rat brain regions. *Brain research. Molecular brain research*, **46**, 100-108.

Romero, J., Garcia, L., Fernandez-Ruiz, J.J., Cebeira, M. & Ramos, J.A. (1995) Changes in rat brain cannabinoid binding sites after acute or chronic exposure to their endogenous agonist, anandamide, or to delta 9-tetrahydrocannabinol. *Pharmacology, biochemistry, and behavior*, **51**, 731-737.

Rosenbaum, C.D., Carreiro, S.P. & Babu, K.M. (2012) Here today, gone tomorrow...and back again? A review of herbal marijuana alternatives (K2, Spice), synthetic cathinones (bath salts), kratom, Salvia divinorum, methoxetamine, and piperazines. *Journal of medical toxicology : official journal of the American College of Medical Toxicology*, **8**, 15-32.

Rosenberg, P.A. & Aizenman, E. (1989) Hundred-fold increase in neuronal vulnerability to glutamate toxicity in astrocyte-poor cultures of rat cerebral cortex. *Neuroscience letters*, **103**, 162-168.

Ross, F.M., Allan, S.M., Rothwell, N.J. & Verkhratsky, A. (2003) A dual role for interleukin-1 in LTP in mouse hippocampal slices. *Journal of neuroimmunology*, **144**, 61-67.

Rossi, S., De Chiara, V., Musella, A., Kusayanagi, H., Mataluni, G., Bernardi, G., Usiello, A. & Centonze, D. (2008) Chronic psychoemotional stress impairs cannabinoid-receptor-mediated control of GABA transmission in the striatum. *J Neurosci*, **28**, 7284-7292.

Rossi, S., Motta, C., Musella, A. & Centonze, D. (2015) The interplay between inflammatory cytokines and the endocannabinoid system in the regulation of synaptic transmission. *Neuropharmacology*, **96**, 105-112.

Rossi, S., Sacchetti, L., Napolitano, F., De Chiara, V., Motta, C., Studer, V., Musella, A., Barbieri, F., Bari, M., Bernardi, G., Maccarrone, M., Usiello, A. & Centonze, D. (2012) Interleukin-1beta causes anxiety by interacting with the endocannabinoid system. *J Neurosci*, **32**, 13896-13905.

Rothstein, J.D., Patel, S., Regan, M.R., Haenggeli, C., Huang, Y.H., Bergles, D.E., Jin, L., Dykes Hoberg, M., Vidensky, S., Chung, D.S., Toan, S.V., Bruijn, L.I., Su, Z.Z., Gupta, P. & Fisher, P.B. (2005) Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. *Nature*, **433**, 73-77.

Rubino, T., Massi, P., Patrini, G., Venier, I., Giagnoni, G. & Parolaro, D. (1994) Chronic CP-55,940 alters cannabinoid receptor mRNA in the rat brain: an in situ hybridization study. *Neuroreport*, **5**, 2493-2496.

Rubino, T., Realini, N., Castiglioni, C., Guidali, C., Vigano, D., Marras, E., Petrosino, S., Perletti, G., Maccarrone, M., Di Marzo, V. & Parolaro, D. (2008) Role in anxiety behavior of the endocannabinoid system in the prefrontal cortex. *Cereb Cortex*, **18**, 1292-1301.

Saijo, K. & Glass, C.K. (2011) Microglial cell origin and phenotypes in health and disease. *Nature reviews. Immunology*, **11**, 775-787.

Salamone, J.D. (1994) The involvement of nucleus accumbens dopamine in appetitive and aversive motivation. *Behav Brain Res*, **61**, 117-133.

Salamone, J.D. & Correa, M. (2002) Motivational views of reinforcement: implications for understanding the behavioral functions of nucleus accumbens dopamine. *Behav Brain Res*, **137**, 3-25.

Salamone, J.D., Cousins, M.S. & Snyder, B.J. (1997) Behavioral functions of nucleus accumbens dopamine: empirical and conceptual problems with the anhedonia hypothesis. *Neurosci Biobehav Rev*, **21**, 341-359.

Salamone, J.D., Wisniecki, A., Carlson, B.B. & Correa, M. (2001) Nucleus accumbens dopamine depletions make animals highly sensitive to high fixed ratio requirements but do not impair primary food reinforcement. *Neuroscience*, **105**, 863-870.

Salter, M.W. & Beggs, S. (2014) Sublime microglia: expanding roles for the guardians of the CNS. *Cell*, **158**, 15-24.

Sampson, C.S., Bedy, S.M. & Carlisle, T. (2015) Withdrawal seizures seen in the setting of synthetic cannabinoid abuse. *The American journal of emergency medicine*, **33**, 1712.e1713.

Satta, V., Scherma, M., Giunti, E., Collu, R., Fattore, L., Fratta, W. & Fadda, P. (2016) Emotional profile of female rats showing binge eating behavior. *Physiol Behav*, **163**, 136-143.

Schafer, D.P. & Stevens, B. (2013) Phagocytic glial cells: sculpting synaptic circuits in the developing nervous system. *Current opinion in neurobiology*, **23**, 1034-1040.

Schenk, S., Horger, B.A., Peltier, R. & Shelton, K. (1991) Supersensitivity to the reinforcing effects of cocaine following 6-hydroxydopamine lesions to the medial prefrontal cortex in rats. *Brain research*, **543**, 227-235.

Schep, L.J., Slaughter, R.J., Hudson, S., Place, R. & Watts, M. (2015) Delayed seizure-like activity following analytically confirmed use of previously unreported synthetic cannabinoid analogues. *Human & experimental toxicology*, **34**, 557-560.

Schifano, F., Orsolini, L., Duccio Papanti, G. & Corkery, J.M. (2015) Novel psychoactive substances of interest for psychiatry. *World psychiatry : official journal of the World Psychiatric Association (WPA)*, **14**, 15-26.

Schneir, A.B. & Baumbacher, T. (2012) Convulsions associated with the use of a synthetic cannabinoid product. *Journal of medical toxicology : official journal of the American College of Medical Toxicology*, **8**, 62-64.

Schneir, A.B., Cullen, J. & Ly, B.T. (2011) "Spice" girls: synthetic cannabinoid intoxication. *The Journal of emergency medicine*, **40**, 296-299.

Schultz, W. (2007) Behavioral dopamine signals. *Trends in neurosciences*, **30**, 203-210.

Schultz, W., Apicella, P. & Ljungberg, T. (1993) Responses of monkey dopamine neurons to reward and conditioned stimuli during successive steps of learning a delayed response task. *J Neurosci*, **13**, 900-913.

Schultz, W., Dayan, P. & Montague, P.R. (1997) A neural substrate of prediction and reward. *Science (New York, N.Y.)*, **275**, 1593-1599.

Schwalm, M.T., Pasquali, M., Miguel, S.P., Dos Santos, J.P., Vuolo, F., Comim, C.M., Petronilho, F., Quevedo, J., Gelain, D.P., Moreira, J.C., Ritter, C. & Dal-Pizzol, F. (2014) Acute brain inflammation and oxidative damage are related to long-term cognitive deficits and markers of neurodegeneration in sepsis-survivor rats. *Mol Neurobiol*, **49**, 380-385.

Scofield, M.D. & Kalivas, P.W. (2014) Astrocytic dysfunction and addiction: consequences of impaired glutamate homeostasis. *Neuroscientist*, **20**, 610-622.

Scofield, M.D., Li, H., Siemsen, B.M., Healey, K.L., Tran, P.K., Woronoff, N., Boger, H.A., Kalivas, P.W. & Reissner, K.J. (2016) Cocaine Self-Administration and Extinction Leads to Reduced Glial Fibrillary Acidic Protein Expression and Morphometric Features of Astrocytes in the Nucleus Accumbens Core. *Biol Psychiatry*, **80**, 207-215.

Seely, K.A., Brents, L.K., Radominska-Pandya, A., Endres, G.W., Keyes, G.S., Moran, J.H. & Prather, P.L. (2012a) A major glucuronidated metabolite of JWH-018 is a neutral antagonist at CB1 receptors. *Chemical research in toxicology*, **25**, 825-827.

Seely, K.A., Lapoint, J., Moran, J.H. & Fattore, L. (2012b) Spice drugs are more than harmless herbal blends: a review of the pharmacology and toxicology of synthetic cannabinoids. *Prog Neuropsychopharmacol Biol Psychiatry*, **39**, 234-243.

Selden, N., Geula, C., Hersh, L. & Mesulam, M.M. (1994) Human striatum: chemoarchitecture of the caudate nucleus, putamen and ventral striatum in health and Alzheimer's disease. *Neuroscience*, **60**, 621-636.

Servan-Schreiber, D., Printz, H. & Cohen, J.D. (1990) A network model of catecholamine effects: gain, signal-to-noise ratio, and behavior. *Science (New York, N.Y.)*, **249**, 892-895.

Sesack, S.R., Deutch, A.Y., Roth, R.H. & Bunney, B.S. (1989) Topographical organization of the efferent projections of the medial prefrontal cortex in the rat: an anterograde tract-tracing study with Phaseolus vulgaris leucoagglutinin. *The Journal of comparative neurology*, **290**, 213-242.

Sesack, S.R., Hawrylak, V.A., Matus, C., Guido, M.A. & Levey, A.I. (1998) Dopamine axon varicosities in the prelimbic division of the rat prefrontal cortex exhibit sparse immunoreactivity for the dopamine transporter. *J Neurosci*, **18**, 2697-2708.

Sesack, S.R. & Pickel, V.M. (1992) Prefrontal cortical efferents in the rat synapse on unlabeled neuronal targets of catecholamine terminals in the nucleus accumbens septi and on dopamine neurons in the ventral tegmental area. *The Journal of comparative neurology*, **320**, 145-160.

Sesack, S.R., Snyder, C.L. & Lewis, D.A. (1995) Axon terminals immunolabeled for dopamine or tyrosine hydroxylase synapse on GABA-immunoreactive dendrites in rat and monkey cortex. *The Journal of comparative neurology*, **363**, 264-280.

Sheikh, I.A., Luksic, M., Ferstenberg, R. & Culpepper-Morgan, J.A. (2014) Spice/K2 synthetic marijuana-induced toxic hepatitis treated with N-acetylcysteine. *The American journal of case reports*, **15**, 584-588.

Shen, K.Z. & Johnson, S.W. (1997) A slow excitatory postsynaptic current mediated by G-protein-coupled metabotropic glutamate receptors in rat ventral tegmental dopamine neurons. *Eur J Neurosci*, **9**, 48-54.

Showalter, V.M., Compton, D.R., Martin, B.R. & Abood, M.E. (1996) Evaluation of binding in a transfected cell line expressing a peripheral cannabinoid receptor (CB2): identification of cannabinoid receptor subtype selective ligands. *The Journal of pharmacology and experimental therapeutics*, **278**, 989-999.

Silvestri, C. & Di Marzo, V. (2013) The endocannabinoid system in energy homeostasis and the etiopathology of metabolic disorders. *Cell metabolism*, **17**, 475-490.

Sim-Selley, L.J. & Martin, B.R. (2002) Effect of chronic administration of R-(+)-[2,3-Dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxaz inyl]-(1-naphthalenyl)methanone mesylate (WIN55,212-2) or delta(9)-tetrahydrocannabinol on cannabinoid receptor adaptation in mice. *The Journal of pharmacology and experimental therapeutics*, **303**, 36-44.

Sim-Selley, L.J., Schechter, N.S., Rorrer, W.K., Dalton, G.D., Hernandez, J., Martin, B.R. & Selley, D.E. (2006) Prolonged recovery rate of CB1 receptor adaptation after cessation of long-term cannabinoid administration. *Mol Pharmacol*, **70**, 986-996.

Sklair-Tavron, L., Shi, W.X., Lane, S.B., Harris, H.W., Bunney, B.S. & Nestler, E.J. (1996) Chronic morphine induces visible changes in the morphology of mesolimbic dopamine neurons. *Proceedings of the National Academy of Sciences of the United States of America*, **93**, 11202-11207.

Smith, A.D. & Bolam, J.P. (1990) The neural network of the basal ganglia as revealed by the study of synaptic connections of identified neurones. *Trends in neurosciences*, **13**, 259-265.

Smith, Y., Charara, A. & Parent, A. (1996) Synaptic innervation of midbrain dopaminergic neurons by glutamate-enriched terminals in the squirrel monkey. *The Journal of comparative neurology*, **364**, 231-253.

Solowij, N. & Battisti, R. (2008) The chronic effects of cannabis on memory in humans: a review. *Current drug abuse reviews*, **1**, 81-98.

Solowij, N., Stephens, R., Roffman, R.A. & Babor, T. (2002) Does marijuana use cause long-term cognitive deficits? *Jama*, **287**, 2653-2654; author reply 2654.

Somogyi, P., Hodgson, A.J. & Smith, A.D. (1979) An approach to tracing neuron networks in the cerebral cortex and basal ganglia. Combination of Golgi staining, retrograde transport of horseradish peroxidase and anterograde degeneration of synaptic boutons in the same material. *Neuroscience*, **4**, 1805-1852.

Song, P. & Zhao, Z.Q. (2001) The involvement of glial cells in the development of morphine tolerance. *Neuroscience research*, **39**, 281-286.

Spaderna, M., Addy, P.H. & D'Souza, D.C. (2013) Spicing things up: synthetic cannabinoids. *Psychopharmacology (Berl)*, **228**, 525-540.

Spiga, S., Lintas, A., Migliore, M. & Diana, M. (2010) Altered architecture and functional consequences of the mesolimbic dopamine system in cannabis dependence. *Addict Biol*, **15**, 266-276.

Spiga, S., Serra, G.P., Puddu, M.C., Foddai, M. & Diana, M. (2003) Morphine withdrawal-induced abnormalities in the VTA: confocal laser scanning microscopy. *Eur J Neurosci*, **17**, 605-612.

Steketee, J.D. (2003) Neurotransmitter systems of the medial prefrontal cortex: potential role in sensitization to psychostimulants. *Brain research. Brain research reviews*, **41**, 203-228.

Steketee, J.D. (2005) Cortical mechanisms of cocaine sensitization. *Critical reviews in neurobiology*, **17**, 69-86.

Stella, N. (2010) Cannabinoid and cannabinoid-like receptors in microglia, astrocytes, and astrocytomas. *Glia*, **58**, 1017-1030.

Stence, N., Waite, M. & Dailey, M.E. (2001) Dynamics of microglial activation: a confocal time-lapse analysis in hippocampal slices. *Glia*, **33**, 256-266.

Suarez, I., Bodega, G., Ramos, J.A., Fernandez-Ruiz, J.J. & Fernandez, B. (2000) Neuronal and astroglial response to pre- and perinatal exposure to delta-9-tetra- hydrocannabinol in the rat substantia nigra. *Developmental neuroscience*, **22**, 253-263.

Surmeier, D.J. (2007) Dopamine and working memory mechanisms in prefrontal cortex. *The Journal of physiology*, **581**, 885.

Svensson, T.H. & Tung, C.S. (1989) Local cooling of pre-frontal cortex induces pacemaker-like firing of dopamine neurons in rat ventral tegmental area in vivo. *Acta physiologica Scandinavica*, **136**, 135-136.

Swanson, L.W. & Cwan, W.M. (1975) A note on the connections and development of the nucleus accumbens. *Brain research*, **92**, 324-330.

Taber, M.T., Das, S. & Fibiger, H.C. (1995) Cortical regulation of subcortical dopamine release: mediation via the ventral tegmental area. *J Neurochem*, **65**, 1407-1410.

Tai, S., Hyatt, W.S., Gu, C., Franks, L.N., Vasiljevik, T., Brents, L.K., Prather, P.L. & Fantegrossi, W.E. (2015) Repeated administration of phytocannabinoid Delta(9)-THC or synthetic cannabinoids JWH-018 and JWH-073 induces tolerance to hypothermia but not locomotor suppression in mice, and reduces CB1 receptor expression and function in a brain region-specific manner. *Pharmacol Res*, **102**, 22-32.

Takahashi, K., Foster, J.B. & Lin, C.L. (2015) Glutamate transporter EAAT2: regulation, function, and potential as a therapeutic target for neurological and psychiatric disease. *Cellular and molecular life sciences : CMLS*, **72**, 3489-3506.

Takematsu, M., Hoffman, R.S., Nelson, L.S., Schechter, J.M., Moran, J.H. & Wiener, S.W. (2014) A case of acute cerebral ischemia following inhalation of a synthetic cannabinoid. *Clinical toxicology (Philadelphia, Pa.)*, **52**, 973-975.

Tan, H., Ahmad, T., Loureiro, M., Zunder, J. & Laviolette, S.R. (2014) The role of cannabinoid transmission in emotional memory formation: implications for addiction and schizophrenia. *Front Psychiatry*, **5**, 73.

Tanda, G., Carboni, E., Frau, R. & Di Chiara, G. (1994) Increase of extracellular dopamine in the prefrontal cortex: a trait of drugs with antidepressant potential? *Psychopharmacology (Berl)*, **115**, 285-288.

Tanda, G., Pontieri, F.E. & Di Chiara, G. (1997) Cannabinoid and heroin activation of mesolimbic dopamine transmission by a common mu1 opioid receptor mechanism. *Science (New York, N.Y.)*, **276**, 2048-2050.

Thanos, P.K., Volkow, N.D., Freimuth, P., Umegaki, H., Ikari, H., Roth, G., Ingram, D.K. & Hitzemann, R. (2001) Overexpression of dopamine D2 receptors reduces alcohol self-administration. *J Neurochem*, **78**, 1094-1103.

Thierry, A.M., Blanc, G., Sobel, A., Stinus, L. & Glowinski, J. (1973) Dopaminergic terminals in the rat cortex. *Science (New York, N.Y.)*, **182**, 499-501.

Thierry, A.M., Chevalier, G., Ferron, A. & Glowinski, J. (1983) Diencephalic and mesencephalic efferents of the medial prefrontal cortex in the rat: electrophysiological evidence for the existence of branched axons. *Experimental brain research*, **50**, 275-282.

Thierry, A.M., Le Douarin, C., Penit, J., Ferron, A. & Glowinski, J. (1986) Variation in the ability of neuroleptics to block the inhibitory influence of dopaminergic neurons on the activity of cells in the rat prefrontal cortex. *Brain research bulletin*, **16**, 155-160.

Thierry, A.M., Tassin, J.P., Blanc, G. & Glowinski, J. (1976) Selective activation of mesocortical DA system by stress. *Nature*, **263**, 242-244.

Thomas, A., Burant, A., Bui, N., Graham, D., Yuva-Paylor, L.A. & Paylor, R. (2009) Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. *Psychopharmacology (Berl)*, **204**, 361-373.

Tian, L., Ma, L., Kaarela, T. & Li, Z. (2012) Neuroimmune crosstalk in the central nervous system and its significance for neurological diseases. *Journal of neuroinflammation*, **9**, 155.

Tournier, B.B. & Ginovart, N. (2014) Repeated but not acute treatment with (9)-tetrahydrocannabinol disrupts prepulse inhibition of the acoustic startle: reversal by the dopamine D(2)/(3) receptor antagonist haloperidol. *Eur Neuropsychopharmacol*, **24**, 1415-1423.

Trecki, J., Gerona, R.R. & Schwartz, M.D. (2015) Synthetic Cannabinoid-Related Illnesses and Deaths. *The New England journal of medicine*, **373**, 103-107.

Tremblay, M.E., Lowery, R.L. & Majewska, A.K. (2010) Microglial interactions with synapses are modulated by visual experience. *PLoS biology*, **8**, e1000527.

Trepanier, M.O., Hopperton, K.E., Mizrahi, R., Mechawar, N. & Bazinet, R.P. (2016) Postmortem evidence of cerebral inflammation in schizophrenia: a systematic review. *Molecular psychiatry*, **21**, 1009-1026.

Trexler, K.R., Nass, S.R., Crowe, M.S., Gross, J.D., Jones, M.S., McKitrick, A.W., Siderovski, D.P. & Kinsey, S.G. (2018) Novel behavioral assays of spontaneous and precipitated THC withdrawal in mice. *Drug and alcohol dependence*, **191**, 14-24.

Tzschentke, T.M. (2001) Pharmacology and behavioral pharmacology of the mesocortical dopamine system. *Progress in neurobiology*, **63**, 241-320.

Tzschentke, T.M. (2007) Measuring reward with the conditioned place preference (CPP) paradigm: update of the last decade. *Addict Biol*, **12**, 227-462.

Uchiyama, N., Kikura-Hanajiri, R. & Goda, Y. (2011) Identification of a novel cannabimimetic phenylacetylindole, cannabipiperidiethanone, as a designer drug in a herbal product and its affinity for cannabinoid CB(1) and CB(2) receptors. *Chemical & pharmaceutical bulletin*, **59**, 1203-1205.

Ukaigwe, A., Karmacharya, P. & Donato, A. (2014) A Gut Gone to Pot: A Case of Cannabinoid Hyperemesis Syndrome due to K2, a Synthetic Cannabinoid. *Case reports in emergency medicine*, **2014**, 167098.

Van Eden, C.G., Lamme, V.A. & Uylings, H.B. (1992) Heterotopic Cortical Afferents to the Medial Prefrontal Cortex in the Rat. A Combined Retrograde and Anterograde Tracer Study. *Eur J Neurosci*, **4**, 77-97.

Van Eden, C.G. & Uylings, H.B. (1985) Cytoarchitectonic development of the prefrontal cortex in the rat. *The Journal of comparative neurology*, **241**, 253-267.

Van Sickle, M.D., Duncan, M., Kingsley, P.J., Mouihate, A., Urbani, P., Mackie, K., Stella, N., Makriyannis, A., Piomelli, D., Davison, J.S., Marnett, L.J., Di Marzo, V., Pittman, Q.J., Patel, K.D. & Sharkey, K.A. (2005) Identification and functional characterization of brainstem cannabinoid CB2 receptors. *Science (New York, N.Y.)*, **310**, 329-332.

Vandrey, R., Dunn, K.E., Fry, J.A. & Girling, E.R. (2012) A survey study to characterize use of Spice products (synthetic cannabinoids). *Drug and alcohol dependence*, **120**, 238-241.

Vardakou, I., Pistos, C. & Spiliopoulou, C. (2010) Spice drugs as a new trend: mode of action, identification and legislation. *Toxicology letters*, **197**, 157-162.

Verrico, C.D., Jentsch, J.D. & Roth, R.H. (2003) Persistent and anatomically selective reduction in prefrontal cortical dopamine metabolism after repeated, intermittent cannabinoid administration to rats. *Synapse (New York, N.Y.)*, **49**, 61-66.

Verrico, C.D., Jentsch, J.D., Roth, R.H. & Taylor, J.R. (2004) Repeated, intermittent delta(9)-tetrahydrocannabinol administration to rats impairs acquisition and performance of a test of visuospatial divided attention. *Neuropsychopharmacology*, **29**, 522-529.

Vezina, P., Blanc, G., Glowinski, J. & Tassin, J.P. (1991) Opposed Behavioural Outputs of Increased Dopamine Transmission in Prefrontocortical and Subcortical Areas: A Role for the Cortical D-1 Dopamine Receptor. *Eur J Neurosci*, **3**, 1001-1007.

Vigolo, A., Ossato, A., Trapella, C., Vincenzi, F., Rimondo, C., Seri, C., Varani, K., Serpelloni, G. & Marti, M. (2015) Novel halogenated derivates of JWH-018: Behavioral and binding studies in mice. *Neuropharmacology*, **95**, 68-82.

Villares, J. (2007) Chronic use of marijuana decreases cannabinoid receptor binding and mRNA expression in the human brain. *Neuroscience*, **145**, 323-334.

Volkow, N.D., Chang, L., Wang, G.J., Fowler, J.S., Ding, Y.S., Sedler, M., Logan, J., Franceschi, D., Gatley, J., Hitzemann, R., Gifford, A., Wong, C. & Pappas, N. (2001) Low level of brain dopamine D2 receptors in methamphetamine abusers: association with metabolism in the orbitofrontal cortex. *The American journal of psychiatry*, **158**, 2015-2021.

Volkow, N.D. & Fowler, J.S. (2000) Addiction, a disease of compulsion and drive: involvement of the orbitofrontal cortex. *Cereb Cortex*, **10**, 318-325.

Volkow, N.D., Fowler, J.S. & Wang, G.J. (2003) The addicted human brain: insights from imaging studies. *The Journal of clinical investigation*, **111**, 1444-1451.

Volkow, N.D., Hampson, A.J. & Baler, R.D. (2017) Don't Worry, Be Happy: Endocannabinoids and Cannabis at the Intersection of Stress and Reward. *Annual review of pharmacology and toxicology*, **57**, 285-308.

Volkow, N.D. & Morales, M. (2015) The Brain on Drugs: From Reward to Addiction. *Cell*, **162**, 712-725.

Volkow, N.D., Wang, G.J., Telang, F., Fowler, J.S., Alexoff, D., Logan, J., Jayne, M., Wong, C. & Tomasi, D. (2014) Decreased dopamine brain reactivity in marijuana abusers is associated with negative emotionality and addiction severity. *Proceedings of the National Academy of Sciences of the United States of America*, **111**, E3149-3156.

Voorn, P., Gerfen, C.R. & Groenewegen, H.J. (1989) Compartmental organization of the ventral striatum of the rat: immunohistochemical distribution of enkephalin, substance P, dopamine, and calcium-binding protein. *The Journal of comparative neurology*, **289**, 189-201.

Wake, H., Moorhouse, A.J., Jinno, S., Kohsaka, S. & Nabekura, J. (2009) Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. *J Neurosci*, **29**, 3974-3980.

Walter, L., Franklin, A., Witting, A., Wade, C., Xie, Y., Kunos, G., Mackie, K. & Stella, N. (2003) Nonpsychotropic cannabinoid receptors regulate microglial cell migration. *J Neurosci*, **23**, 1398-1405.

Wang, T. & French, E.D. (1993a) Electrophysiological evidence for the existence of NMDA and non-NMDA receptors on rat ventral tegmental dopamine neurons. *Synapse (New York, N.Y.)*, **13**, 270-277.

Wang, T. & French, E.D. (1993b) L-glutamate excitation of A10 dopamine neurons is preferentially mediated by activation of NMDA receptors: extra- and intracellular electrophysiological studies in brain slices. *Brain research*, **627**, 299-306.

Wang, T. & French, E.D. (1995) NMDA, kainate, and AMPA depolarize nondopamine neurons in the rat ventral tegmentum. *Brain research bulletin*, **36**, 39-43.

Webster (2001) Dopamine (DA) *Neurotransmitters, Drugs and Brain Function*.

Weinstein, A.M., Rosca, P., Fattore, L. & London, E.D. (2017) Synthetic Cathinone and Cannabinoid Designer Drugs Pose a Major Risk for Public Health. *Front Psychiatry*, **8**, 156.

Weissenborn, R., Robbins, T.W. & Everitt, B.J. (1997) Effects of medial prefrontal or anterior cingulate cortex lesions on responding for cocaine under fixed-ratio and second-order schedules of reinforcement in rats. *Psychopharmacology (Berl)*, **134**, 242-257.

Wenzel, J.M. & Cheer, J.F. (2018) Endocannabinoid Regulation of Reward and Reinforcement through Interaction with Dopamine and Endogenous Opioid Signaling. *Neuropsychopharmacology*, **43**, 103-115.

Wiebelhaus, J.M., Poklis, J.L., Poklis, A., Vann, R.E., Lichtman, A.H. & Wise, L.E. (2012) Inhalation exposure to smoke from synthetic "marijuana" produces potent cannabimimetic effects in mice. *Drug and alcohol dependence*, **126**, 316-323.

Wikler, A. (1976) Aspects of tolerance to and dependence on cannabis. *Annals of the New York Academy of Sciences*, **282**, 126-147.

Wiley, J.L., Compton, D.R., Dai, D., Lainton, J.A., Phillips, M., Huffman, J.W. & Martin, B.R. (1998) Structure-activity relationships of indole- and pyrrole-derived cannabinoids. *The Journal of pharmacology and experimental therapeutics*, **285**, 995-1004.

Wiley, J.L., Marusich, J.A., Lefever, T.W., Antonazzo, K.R., Wallgren, M.T., Cortes, R.A., Patel, P.R., Grabenauer, M., Moore, K.N. & Thomas, B.F. (2015) AB-CHMINACA, AB-PINACA, and FUBIMINA: Affinity and Potency of Novel Synthetic Cannabinoids in Producing Delta9-Tetrahydrocannabinol-Like Effects in Mice. *The Journal of pharmacology and experimental therapeutics*, **354**, 328-339.

Wiley, J.L., Marusich, J.A., Lefever, T.W., Grabenauer, M., Moore, K.N. & Thomas, B.F. (2013) Cannabinoids in disguise: Delta9-tetrahydrocannabinol-like effects of tetramethylcyclopropyl ketone indoles. *Neuropharmacology*, **75**, 145-154.

Wiley, J.L., Marusich, J.A., Martin, B.R. & Huffman, J.W. (2012) 1-Pentyl-3-phenylacetylindoles and JWH-018 share in vivo cannabinoid profiles in mice. *Drug and alcohol dependence*, **123**, 148-153.

Williams, D.J., Crossman, A.R. & Slater, P. (1977) The efferent projections of the nucleus accumbens in the rat. *Brain research*, **130**, 217-227.

Wilson, D.M., Varvel, S.A., Harloe, J.P., Martin, B.R. & Lichtman, A.H. (2006) SR 141716 (Rimonabant) precipitates withdrawal in marijuana-dependent mice. *Pharmacology, biochemistry, and behavior*, **85**, 105-113.

Wilson, R.I. & Nicoll, R.A. (2002) Endocannabinoid signaling in the brain. *Science (New York, N.Y.)*, **296**, 678-682.

Wise, R. (1982) *Neuroleptics and operant behavior: The anhedonia hypothesis*.

Wise, R.A. (1980) The dopamine synapse and the notion of 'pleasure centers' in the brain. *Trends in neurosciences*, **3**, 91-95.

Wise, R.A. (1987) The role of reward pathways in the development of drug dependence. *Pharmacology & therapeutics*, **35**, 227-263.

Wise, R.A. (2002) Brain reward circuitry: insights from unsensed incentives. *Neuron*, **36**, 229-240.

Wu, N., Lu, X.Q., Yan, H.T., Su, R.B., Wang, J.F., Liu, Y., Hu, G. & Li, J. (2008) Aquaporin 4 deficiency modulates morphine pharmacological actions. *Neuroscience letters*, **448**, 221-225.

Wu, X. & French, E.D. (2000) Effects of chronic delta9-tetrahydrocannabinol on rat midbrain dopamine neurons: an electrophysiological assessment. *Neuropharmacology*, **39**, 391-398.

Xanthos, D.N. & Sandkuhler, J. (2014) Neurogenic neuroinflammation: inflammatory CNS reactions in response to neuronal activity. *Nature reviews. Neuroscience*, **15**, 43-53.

Xi, Z.X., Peng, X.Q., Li, X., Song, R., Zhang, H.Y., Liu, Q.R., Yang, H.J., Bi, G.H., Li, J. & Gardner, E.L. (2011) Brain cannabinoid CB(2) receptors modulate cocaine's actions in mice. *Nat Neurosci*, **14**, 1160-1166.

Yamaguchi, T., Wang, H.L., Li, X., Ng, T.H. & Morales, M. (2011) Mesocorticolimbic glutamatergic pathway. *J Neurosci*, **31**, 8476-8490.

Yang, C.R. & Mogenson, G.J. (1990) Dopaminergic modulation of cholinergic responses in rat medial prefrontal cortex: an electrophysiological study. *Brain research*, **524**, 271-281.

Yeakel, J.K. & Logan, B.K. (2013) Blood synthetic cannabinoid concentrations in cases of suspected impaired driving. *Journal of analytical toxicology*, **37**, 547-551.

Yirmiya, R. & Goshen, I. (2011) Immune modulation of learning, memory, neural plasticity and neurogenesis. *Brain, behavior, and immunity*, **25**, 181-213.

Yokel, R.A. & Wise, R.A. (1975) Increased lever pressing for amphetamine after pimozide in rats: implications for a dopamine theory of reward. *Science (New York, N.Y.)*, **187**, 547-549.

Young, A.C., Schwarz, E., Medina, G., Obafemi, A., Feng, S.Y., Kane, C. & Kleinschmidt, K. (2012) Cardiotoxicity associated with the synthetic cannabinoid, K9, with laboratory confirmation. *The American journal of emergency medicine*, **30**, 1320.e1325-1327.

Zahm, D.S. & Brog, J.S. (1992) On the significance of subterritories in the "accumbens" part of the rat ventral striatum. *Neuroscience*, **50**, 751-767.

Zahm, D.S. & Heimer, L. (1993) Specificity in the efferent projections of the nucleus accumbens in the rat: comparison of the rostral pole projection patterns with those of the core and shell. *The Journal of comparative neurology*, **327**, 220-232.

Zamberletti, E., Beggiato, S., Steardo, L., Jr., Prini, P., Antonelli, T., Ferraro, L., Rubino, T. & Parolaro, D. (2014) Alterations of prefrontal cortex GABAergic transmission in the complex psychotic-like phenotype induced by adolescent delta-9-tetrahydrocannabinol exposure in rats. *Neurobiol Dis*, **63**, 35-47.

Zamberletti, E., Gabaglio, M., Grilli, M., Prini, P., Catanese, A., Pittaluga, A., Marchi, M., Rubino, T. & Parolaro, D. (2016) Long-term hippocampal glutamate synapse and astrocyte dysfunctions underlying the altered phenotype induced by adolescent THC treatment in male rats. *Pharmacol Res*, **111**, 459-470.

Zamberletti, E., Gabaglio, M., Prini, P., Rubino, T. & Parolaro, D. (2015) Cortical neuroinflammation contributes to long-term cognitive dysfunctions following adolescent delta-9-tetrahydrocannabinol treatment in female rats. *Eur Neuropsychopharmacol*, **25**, 2404-2415.

Zanda, M.T., Fadda, P., Antinori, S., Di Chio, M., Fratta, W., Chiamulera, C. & Fattore, L. (2017) Methoxetamine affects brain processing involved in emotional response in rats. *Br J Pharmacol*, **174**, 3333-3345.

Zanettini, C., Panlilio, L.V., Alicki, M., Goldberg, S.R., Haller, J. & Yasar, S. (2011) Effects of endocannabinoid system modulation on cognitive and emotional behavior. *Front Behav Neurosci*, **5**, 57.

Zawilska, J.B. & Wojcieszak, J. (2014) Spice/K2 drugs--more than innocent substitutes for marijuana. *Int J Neuropsychopharmacol*, **17**, 509-525.

Zimmermann, U.S., Winkelmann, P.R., Pilhatsch, M., Nees, J.A., Spanagel, R. & Schulz, K. (2009) Withdrawal phenomena and dependence syndrome after the consumption of "spice gold". *Deutsches Arzteblatt international*, **106**, 464-467.