Università degli Studi di Cagliari

DOTTORATO DI RICERCA IN NEUROSCIENZE, SCUOLA DI DOTTORATO IN NEUROSCIENZE E SCIENZE MORFOLOGICHE

Ciclo XXIII

AN ENDOCANNABINOID-LIKE SYSTEM REGULATES NEURONAL RESPONSES TO NICOTINE IN THE MESOLIMBIC SYSTEM: ROLE OF N-ACYLETHANOLAMINES AND NUCLEAR PPAR-α RECEPTORS.

Settore/i scientifico disciplinari di afferenza

BIO/14-Farmacologia

Presentata da: Antonio Luchicchi

Coordinatore Dottorato Prof.ssa Alessandra Concas

Relatore Prof. Marco Pistis

Esame finale anno accademico 2009 – 2010

The greatest challenge to any thinker is stating the problem

in a way that will allow a solution.

Bertrand Russell

to Barbara, my grandfather and my parents

INDEX

INTRODUCTION

Nicotine addiction is one of the most alarming public health problem of this century. In fact, results shown by several agencies, such as the *World Health Organization* (WHO) and the *Center for Disease Control* (CDC) underline a disconcerting situation, which do not involve only developed countries. It has been estimated that over a billion of people smoke tobacco (WHO, 2006), and despite the large amount of pharmacological and non-pharmacological strategies to quit smoking, at the state of art, *tabagism* is still considered one of the first causes of preventable death worldwide (WHO, 2006).

Drug addiction is a complex and relapsing disorder characterized by several symptoms, such as the compulsive seeking of a drug and drug abuse despite its side effects (Koob and Volkow, 2010). Among this spectrum, nicotine addiction obviously represents a crucial problem in terms of prevalence (higher than other drugs) (Markou, 2008) and negative long-term physical problems. Even though only a certain percentage of people who use drugs of abuse becomes addicted (for nicotine is around 50%), some genetic and environmental factors have been described as triggers of addiction. For what concerns nicotine dependence, various polymorphisms in some genes encoding for specific receptors or receptor subunit of the central nervous system (e.g. dopaminergic receptors, nicotinic acetylcholine receptors) (Ray et al., 2009), associated with stressful life events and other non-biochemical modifications, have been suggested so far. In addition, most of these events pinpoint how progresses in the neuroscience and neuropharmacology –related field can be crucial to better understand this problem.

Recently, studies have been focused on the modulatory effect of certain endogenous system in the response to drugs of abuse in the brain reward circuitry. Among these systems, a wide amount of studies are taking into account the possibility that the endogenous cannabinoid (eCb) system might be a suitable candidate to regulate nicotine-induced reinforcing properties. The eCb system is a family of lipid molecules, enzymes and receptors, also described in the brain reward circuitry

(Melis and Pistis, 2007), where cannabinoid type 1 receptors (CB1-Rs) are also a target for the exogenous cannabinoid Δ^9-tetrahydrocannabinol.

In the last five years several studies suggested that the CB1-Rs antagonist SR141716-A (rimonabant) prevents nicotine-induced effect within the brain reward circuitry (using different paradigms and techniques) (Cohen et al., 2002; Cheer et al., 2007). Additionally, it has been recently proposed the role of other components of the eCb system as putative modulators of nicotine effect.

The present thesis is focused on this second possibility and it summarizes the results obtained carrying out experiments using *in vivo* extracellular electrophysiological single unit recordings in anaesthetized rats to study the effect of pharmacological inhibition of fatty acid amide hydrolase (FAAH). FAAH is the main enzyme which inactivates the eCb anandamide (AEA) and other n-acylethanolamides (NAEs), such as oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), structurally similar to eCbs but devoid of CB1-Rs affinity (Fu et al., 2003).

FAAH activity reduces the levels of AEA, OEA and PEA and pharmacological tools to deactivate this metabolic process have been proposed so far. For example, the carbamic acid derivate URB597, an irreversible inhibitor of FAAH, allows a long-lasting increase of AEA and NAEs levels in the brain and peripheral tissue (Kathuria et al., 2003; Fegley et al., 2005).

For this reason, in the present study it was addressed whether FAAH inactivation by URB597 might act as a negative modulator of nicotine-induced effect in two of the most important groups of neurons of the brain reward circuitry: the ventral tegmental area dopaminergic neurons and the GABAergic medium spiny neurons of the nucleus accumbens shell. Furthermore, it was also tried to identify the mechanism by which URB597 modulates these effects and, finally, whether URB597's action is only related to nicotine effects or it can be extended to other drugs of abuse.

In the first chapter, the abuse liability of nicotine, evaluated through some of the most important parameters such as its biochemistry, epidemiology, pharmacology and neural effects in the brain reward circuitry will be discussed. In the second chapter, the eCb system and the novel family of

eCb-like molecules, devoid of CB-Rs affinity, will be introduced to better clarify the *rationale* of this study. In the third chapter, a brief excursus on methods and materials employed to carry out this project will be presented. In the forth chapter, it will be reported a summary of results obtained through the *in vivo* electrophysiological technique. Finally, in the fifth chapter, a detailed discussion of these results will be shown to connect present data to the literature and to provide some important final remarks about the meaning of our study.

Chapter I

NICOTINE ABUSE AND ADDICTION: A MULTIDIMENSIONAL APPROACH

Introduction

Nicotine is the main psychoactive component of tobacco plant and it contributes to tobacco smoking habit (Stolerman and Jarvis, 1995). This compound is an alkaloid mainly found in the *Solanacee* plant *Nicotiana Tabacum*, where it constitutes the 0.6-3.0% of dry tobacco weight (Siegmund et al., 1999). Nicotine is commonly extracted from four different types of *Nicotiana Tabacum* known as *Bright*, *Burley*, *Maryland* and *Turkish* (Hoffmann and Hoffmann, 1998), although other species of *Nicotiana*, such as *N.Rustica*, are widely used to extract nicotine and manufacture cigarettes and cigars (Hoffmann and Hoffmann, 1998).

Acute administration of nicotine to humans produces mild euphoria and mild cognitive enhancement (Markou, 2008) and its continuative use leads to tolerance and withdrawal (depressed mood, irritability and mild cognitive deficits) when the access to the drug is prevented (Shiffman et al., 2004). Most of these effect are due to the high nicotine-binding affinity for a specific class of receptors named "*nicotinic acetylcholine receptors*" (nAChRs), whose properties and localization within the central and peripheral nervous system will be better explained below.

Even though cigarette consumption is the most common route to administer nicotine, the phenomenon known as *tabagism* involves a wide array of different aspects (from the epidemiology of tobacco use to the neural basis that facilitates nicotine-induced effects in the central nervous system) which will be elucidated in this chapter.

1. Physical and social epidemiology of nicotine consumption

Different agencies have provided a large amount of evidence regarding the widespread tobacco use and nicotine addiction. For example, data revealed by the *World Health Organizat*ion (WHO) and the *Center for Disease Control* (CDC) have shown that 1.3 billion of people in the world smoke cigarette (WHO, 2006). In the United States, the 5th world producer of tobacco manufactures, with approximately 45 millions of smokers (CDC, 2008) (male prevalence: 26.3%; female prevalence: 21.5%) (WHO, 2008), tobacco-related health care cost is evaluated around 76 billions of dollars, almost 20 folds over than China (3.5 billions of dollars) (WHO, 2002), where tobacco use involves more than 300,000,000 of people (WHO, 2002) (male prevalence: 59.5%; female prevalence: 3.7%) (WHO, 2008).

These data obviously report a disconcerting problem, which is almost all confined to tobacco smoking behavior. In fact, according to the *National Institute on Drugs of Abuse* (NIDA) the 98% of tobacco users take nicotine through cigarette smoke (Fratta et al., 2005), minimizing to a scarce 2% the rest of consumption.

Furthermore, inhaling tobacco through cigarette smoke remains the most dangerous route of administration because of its sudden release of toxic compounds, which have been used for manufacturing them. In fact, beyond the well-known abuse liability of nicotine, it is now well-established that tobacco smokers have an increasing probability to develop several physical problems. Among them, severe types of cancer (mouth, bladder, lungs etc…), cardiovascular disease and respiratory problems (U.S. DHHS, 2004) have been reported in chronic smokers. Additionally, it has been estimated that tobacco-related illness lead to die with an higher rate than HIV-related disease, other drugs of abuse and motor vehicle accidents (U.S. DHHS, 2004).

The *American Psychiatric Association* (APA) considers nicotine dependence as the occurrence of

both tolerance and withdrawal symptoms related to nicotine use under different forms and the compulsive consumption despite its side effects (APA, 2000). The abuse liability of nicotine is also responsible for nicotine relapse. In fact, despite the development of different strategies (e.g. nicotine replacement therapy), only 3-5% of people trying to quit smoking benefit from them (Stead et al., 2008).

In addition, beyond the strict correlation between tobacco consumption and physical injuries, a lot of interest has been focused on the link between nicotine addiction and psychiatric problems. In fact, according to the APA, smoking behavior and nicotine dependence have an higher prevalence in psychiatric patients than normal people (APA, 2000). For example, recent evidence points toward a self-medication use of nicotine in patients with schizophrenia-related cognitive impairment (Williams and Gandhi, 2008), although a correlation between nicotine use in schizophrenic patient came out earlier (O'Farrell et al., 1983). In addition, also a link between nAChRs (see below) and depression (Bertrand, 2005) has been recently suggested, involving the role of nicotinic receptors in the pathophysiology of mood disorders. These observations are robustly improved by animal studies showing an α4β2 nicotinic receptor involvement in some measures impaired in psychotic patients (Radek et al., 2010). Moreover, further data have shown an α7 nicotinic receptor subunit contribution in the modulation of P20-40, a paradigm set to evaluate animal models of psychosis (Martin and Freedman, 2007).

Furthermore, a series of studies have also postulated the existence of a genetic vulnerability to nicotine dependence. In fact, studies in twins have reported a sustained degree of heritability of cigarette smoking (>50%) (Lessov-Schlaggar et al., 2008) coupled to an heritability of specific withdrawal symptoms (Xian et al., 2005). These studies also include in the predisposition to nicotine use:

(1) the presence of smoking-related phenotypes accountable to the polymorphism of the cytochrome P450 CYP2A6 (Malaiyandi et al., 2005);

(2) the role of variants in some genes encoding for nAChRs specific subunits, such as the CHNRA4 (encoding for the α4) (Li et al., 2005; Hutchison et al., 2007) and the CHNRA3 and 5 (encoding for the α3 and the α5) (Saccone et al., 2007; Berrettini et al., 2008);

(3) the polimorphysm in some dopaminergic (DA) genes (Lerman et al., 1999; Vandenbergh et al., 2007; Huang et al., 2008) which might improve the rewarding properties of nicotine itself;

Taken together, these considerations remarkably point out the alarming problem of nicotine abuse and addiction, which is quickly growing up in developed, less developed, and developing countries.

2. Brief history of nicotine: from the Pre-Columbian medical use to the XXI century

Early information about manufactured cigars from *Nicotiana* plants derives from the artworks painted on 10th century Maya's vessel (Kingsborough, 1825). Nonetheless, there is evidence of tobacco use also in 16,000 B.C., and during the course of 3,000/5,000 B.C. in Ecuador and Peru native's cultures (Dowieko, 2008).

The biggest source of data comes out from the *Cristoforo Colombo* landing in America. While exploring the *Isle of Guahain* (currently San Salvador) the sailors of *Colombo*'s crew noticed a strange behavior defined as "*drinking smoke*" practiced by the so-called "Indians", and which was reported on the Gonzalo Fernandez de Oviedo's milestone "*Historia General y Natural de las Indias*" (1526) (Penn, 2007). Presumably, the name tobacco belongs to this age and it was translated as a mistake from the cane pipe that indigenes utilized to smoke (called *tobago* by them) (Meyer, 1999).

These ancient populations, from Maya to American natives, used to smoke tobacco for religious purposes and some reports describe that Indians offered their pipes filled with tobacco to *Colombo*'s

crew thinking they were divinity. Moreover, a great array of references comes from the exploration of other sailors in the "*New World*", who frequently noticed the therapeutic use of tobacco plants by indigenes (e.g. Pedro Alvarez Cabral, Amerigo Vespucci etc...) (Dickson, 1954; Brookes, 1937, 1952).

After *Colombo's age* tobacco plants were also imported in Europe where they became very popular in Spain and other countries. According to some studies, the first person who cultivated *Nicotiana Tabacum* in Europe was the French Ambassador to Portugal Jean Nicot, who sent seed of *tabacum* to France and introduced its use at the Royal Court of Paris during the XVI century (Penn, 2007). Henceforth, the belief of smoking tobacco medical properties became so popular to induce Caterina De Medici and King Charles IX to treat their migraine headache taking nicotine (Jeffers and Gordon, 1996). In fact, during the same century other empirical opinions, such as those belonging to the Spanish school of the physician Nicolas Monardes, hypothesized a therapeutic role of *Nicotiana* plants for curing a great array of physical problems (Monardes,1596) up to be considered as a *panacea* (Dickson, 1954). Nevertheless, despite this optimistic approach to tobacco use, some studies reported an early evidence of a negative effect caused by smoking behavior. For example, the Italian scientist Francesco Redi (1671) demonstrated an harmful action of tobacco oil injection in animals (Goodman, 1994), which was confirmed by latest studies occurred when nicotine was isolated. Noteworthy, other source of criticism came from Phylaretes (Phylaretes, 1602), Vaughan (Vaughan, 1612), and James Hart (Hart, 1633) most of them concerning the over inclusive consideration of *tobacco* as a *panacea* and its harmful effect when abused.

The headword nicotine was obviously given in honor of Jean Nicot by Jean Liebault, a botanist, who was the first to cultivate this plant in France. He called this plant "*Herba Nicotiana*" rearranging the nickname of Nicot "*ambassador's nicotiane*" (Charlton, 2004). In the XIX century nicotine was, then, isolated by Wilhelm Posselt and Ludwig Reimann (1828), and it was recognized as the main pharmacological ingredient of tobacco plants (Henningfield and Zeller, 2006) with an

extremely high poisonous property (Goodman, 1994). Nevertheless, as early as 1723, nicotine, like crude extract of tobacco, had been already used and recognized as a powerful insecticide agent (Metcalf, 1948), and sometimes as a quick and effective killer-drug (Wennig, 2009).

These steps constituted something essential to reach the nicotine empirical (Melsens, 1844) and chemical structure (Pinnier, 1891-95) and how to synthesize it (Pictet and Crepieux, 1903), which opened the field of study related to nicotine-induced effects in the human body and the manufacture industry of cigarette and cigars.

3. From leaves to lungs: biochemistry and pharmacology of nicotine

Before introducing how nicotine acts on the central nervous system as an abuse substance, it is worth to quickly describe the biochemistry and the pivotal passages that allow the biosynthesis of this compound, together with its main pharmacological features.

3.1 Biochemistry and biosynthesis

As previously mentioned, nicotine ($C_{10}H_{14}N_2$) is an alkaloid found in a *solanacea* plant, whose biosynthesis takes place in the roots and accumulates in the leaves. It reaches the ~95% of total alkaloids of tobacco plants (Baldwin, 1989; Hashimoto and Yamada, 1994), followed by other compounds such as *nornicotine*, *anatabine* and *anabasine* (Benowitz and Jacob, 1998). Nicotine is a compound whose chemical and physical data are summarized in tab 1.1, and it appears like a pale yellow to dark brown liquid with a slight, fishy odor when warm, strongly alkaline in reaction and with a tendency to from salt with acid (Metcalf, 1948). The chemical structure of nicotine is composed by 2 different rings: *pyrrolidine* and *pyridine* (Katoh et al., 2005), and this explains its

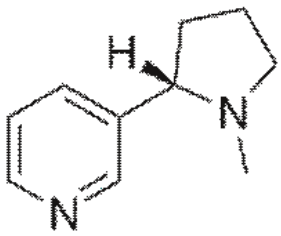
IUPAC name 3-[(2*S*)-1-methylpyrrolidin-2-]pyridine (tab 1.1). The chemical root for the pyrrolidine ring is the ornithine and/or arginine-formed symmetrical diamine putrescine. Putrescine is methylated by the S-adenoshylnmethionine-dependent enzyme putescine N-methyltransferase (Biastoff et al., 2009), a protein isolated from roots of different plants (*Nicotiana Tabacum, Atropa Belladona, Datura Stamonium*) (see for example Walton et al., 1990; 1994), yielding N-methylputrescine (Hashimoto and Yamada, 1994) which is then deaminated by the diamine oxidase. A cyclization product of this oxidation process is the N-methylpirrolinum cation (Katoh et al., 2005). This chemical process is common to almost all known alkaloids (Ziegler and Facchini, 2008).

A

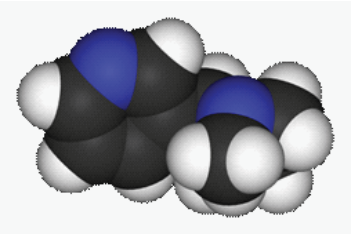
Nicotine/3-[(2*S*)-1-methylpyrrolidin-2-yl]pyridine

Density	1.01 g/cm3
Molecular weight	162.26 g/mol
Boiling point	477°F
Melting point	-277°F
Formula	$C_{10}H_{14}N_2$

B



C



TAB1.1.Nicotine chemical features. (**a**) Panel showing some important chemical parameters of nicotine. (**b**) Chemical structure of nicotine. (**c**). Three dimensional chemical structure of nicotine

The pyridine ring derives from aspartate, which, through the aspartate oxidase, is oxidated in α-amino-succinate (αIS). From αIS, a synthetization process by the quinoliate synthase forms quinoliate and, through quinoliate phospho-ribosyltransferase, it is converted in nicotinic acid mononucleotide (NaNM). Finally, using a nicotinamide adenine dinucleotide (NAD) biosynthesis pathway, NaNM is converted in nicotinate (Dawson et al., 1958; Dawson et al., 1956. Yang et al., 1965) which is an esther of nicotinic acid (niacine or vitamin B3 of β-pyridine carbonic acid).

The condensation between pyrrolinum cation and nicotinic acid creates the 3,6-dihidronicotine which is next dehydrogenated in nicotine by still poorly characterized enzymes (Ziegler and Facchini, 2008). A schematic representation of this anabolic process is presented in fig 1.1. Several studies have demonstrated that nicotine synthesis might have a defensive role against herbivore insects' attack, which can damage and/or kill tobacco plants. In fact, under leaf damage caused by herbivore insect, a specific jasmonic acid-mediated signal is activated leading to a massive increase of the gene encoding for nicotine synthesis in the roots (Shoi et al., 2000; Sinclair et al., 2000). This mechanism leads to protect tobacco plants through the toxic effect of nicotine on the central nervous system of insects.

The translocation from the root to the leaf takes place in the xylem, where nicotine is transported after its synthesis (Shoi et al., 2000; Shoi et al. 2002; Katoh et al.2005). Once in the leaf, nicotine is trapped into the vacuole after being delivered at the mesophyl cells (Hashimoto and Yamada, 2003), forming ion-pairs with organic acids (Katoh, 2005), and here it is accumulated.

Nicotine can be found in 2 different isoforms: the levorotary (S)-nicotine, which is the negative (-) stereoisomer and shows affinity for the nAchRs, and the (R)-nicotine, with scarce or null effect on the above mentioned receptors. Tobacco products contain a larger amount of levorotary nicotine whit just a moderate amount of (R) stereoisomers. In fact, (R)-nicotine reaches the 0.1-0.6% of the total amount of nicotine (Armstrong, 1998), and it is almost all confined to racemization products under combustion (Matta et al., 2007).

These steps are essential to produce tobacco manufactures. In fact, when accumulated in the leafs nicotine is extracted following different methods and different cures, in order to obtain desiccated leafs of tobacco with a variable rate of nicotine inside.

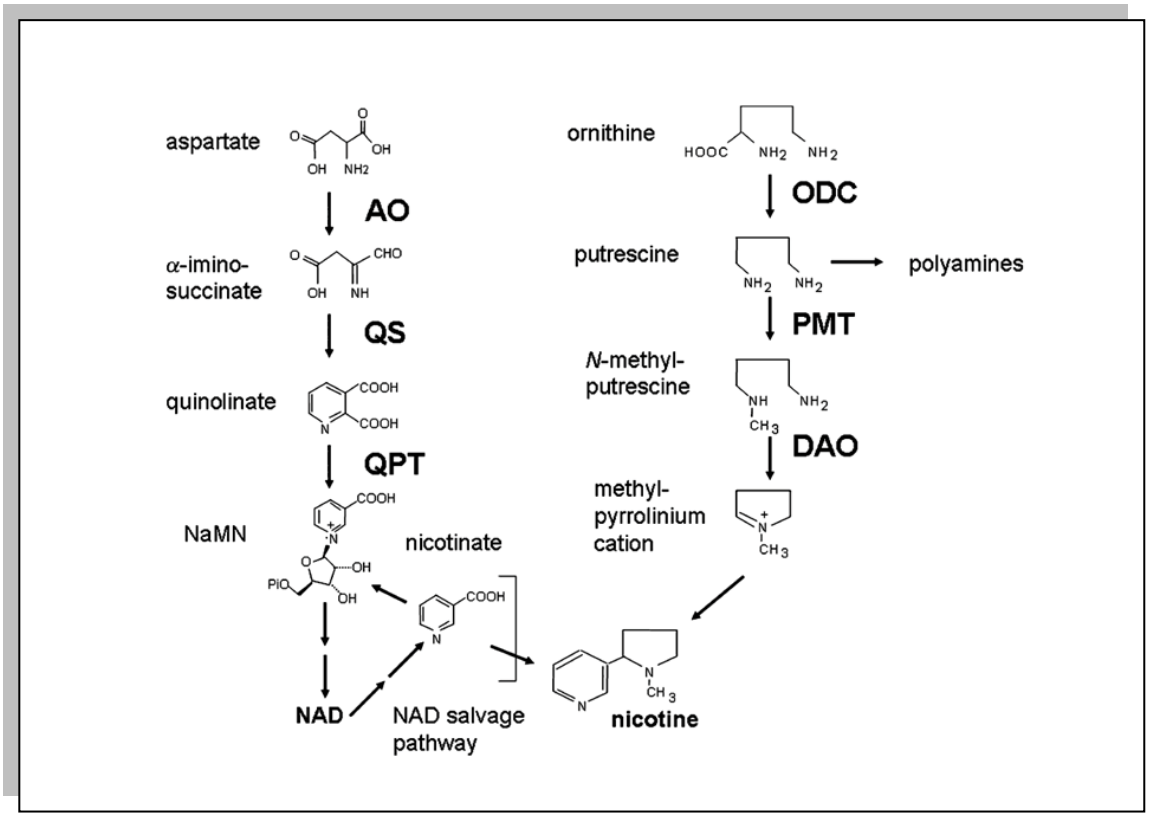


Fig1.1 Nicotine biosynthesis. Schematic representation of nicotine biosynthetic pathway as it happens in the root of *nicotiana tabacum* plants. Once produced, nicotine is unloaded in the leaf vacuoles, where it acts as a protective agent. The covalent binding between the pyridine and pyrrolidine ring derives from two separate processes which involve several enzymes (Katoh et al. 2005). Abbreviations: AO, aspartate oxidase; QS, quinoliate synthase; QPT, quinoliate phosphor-ribolsyltransferase; ODC, ornithine decarboxylase; PMT, putrscine-N-metyltransferase; DAO, diamine oxidase

3.2 Pharmacology and toxicology: how does nicotine act on the mammalian body?

Nicotine, as a drug, once administered to humans and animals, exerts pharmacological and toxicological effects, including addiction. The main pharmacological action of nicotine is due to the high affinity on nAchRs, a family of ion channels-coupled receptors widely expressed in the mammalians central (CNS) and peripheral (PNS) nervous system, and in a great amount of other cell lines and tissues (muscles and cancer cells) (Gotti et al., 2009).

Additionally, depending on different types of tobacco manufactures, nicotine absorption and its effects strongly differ. For example, in a particular type of tobacco, named "*flue-cured*" (very common in cigarettes), whose smoke has an acid pH (5.5-6.0), nicotine, which is a weak base (pKa=8.0), requires to be ionized, and this impairs the buccal absorption of this compound (Benowitz et al., 2009). On the other hand, the *air-cured* tobacco, typical of European cigarettes and pipe/cigar tobacco, producing its smoke more alkaline (pH=6.5), allows an improved nicotine absorption through the mouth (Armitage et al., 1978; Benowitz et al., 2009).

Furthermore, different routes of administration can be an important variable to define the blood concentration and the absorption rate of nicotine through body tissues. In fact, according to a study published on *Clinical Pharmacology and Therapy* by Benowitz and coll. (1988), while tobacco smoking leads to a faster peak than intravenous administration (Benowitz et al., 1988), other pharmacological preparations, such as nicotine gum (2mg/piece) and oral snuff (2.5g) are slower in their effect, with a much more modest peak (Benowitz et al., 1988). A summary of the main pharmacokinetics parameters of nicotine absorption through different route of administration is reported in Tab 1.2 (Benowitz et al., 2009).

Moreover, studies in humans have shown a high variability in blood concentration (10-50 ng/ml in the afternoon of typical smokers) and arterial peak (up to 100 ng/ml) of nicotine (Benowitz, 2009) in chronic smokers. This factor can vary because of individual differences in puff strength,

contextual environmental factors and other parameters, such as the way of smoking to optimize the psychoactive effect of nicotine (U.S. DHHS, 2001). Usually, nicotine blood peak declines after 20 minutes, due to nicotine distribution in other body tissues than bloodstream, with a volume of distribution close to 2.6 times body weight (Benowitz and Jacob, 1984). Only less than 5% of this nicotine binds to plasma proteins, where, depending on the pH (7.4) of blood, it is present under either an ionized (69%) or unionized (31%) form.

Type of nicotine administration	**C_{max}** **ng ml^{-1}**	**T_{max}** **min**	**Bioavailability** **%**
Smoking (one cigarette 5 min, 2 mg/cigarette)	15-30 (V) 20-60 (A)	5-8 (V) 3-5 (A)	80-90 (inhaled nicotine)
Intravenous (5.1 mg)	30	30	100
Nasal spray (1 mg)	5-8 (V) 10-15 (A)	11-18 (V) 4-6 (A)	60-80
Gum (2mg/30 min)	6-9	30	78
Sublingual tablet (2mg/20-30 min)	3.8	60ca.	65
Transdermal patch (15 mg/16 h)	11-14	6-9h	75-100
Transdermal patch (21 mg/24 h)	12-21	9-12	82

Tab 1.2 Nicotine preparations. Panel showing some of the most common route of administration for nicotine coupled with their main pharmacokinetic parameters. Notably, transdermal patch (15 mg/16h) guarantees a wide bioavailability with a very long-lasting T_{max} (adapted from Benowitz et al., 2009).

Several tissues are permeable to nicotine including kidney, liver, spleen, lungs, skeletal muscles, placenta, brain and with a less rate also adipose tissue (Matta et al., 2007). Liver metabolism plays a central role in human and mammalian degradation of nicotine and, despite the major nicotine metabolite known is cotinine (~75%), a wide array of less frequent compounds are products of

nicotine metabolism. Among them, the most found in human urines are nicotine-N-oxide (4-7%), nicotine glucoronide (3-5%), and other cotinine metabolites (Hukkanen et al., 2005) (fig 1.2).

Studies carried out to compare the presence of predominant cytocrome P450 across diverse species have underlined that monkeys, dogs, cats and rabbits degradate nicotine primarily in cotinine, whereas rats and guinea pigs produce more nicotine-N-oxide than cotinine or 3-hydroxycotinine (Matta et al., 2007). In addition, it has been shown that mice metabolize nicotine through the CYP2A5, which is instead inactive in rats (Hammond et al., 1991; Nakayama et al., 1993) supporting the hypothesis of a genetic difference in nicotine deactivation between humans and animals.

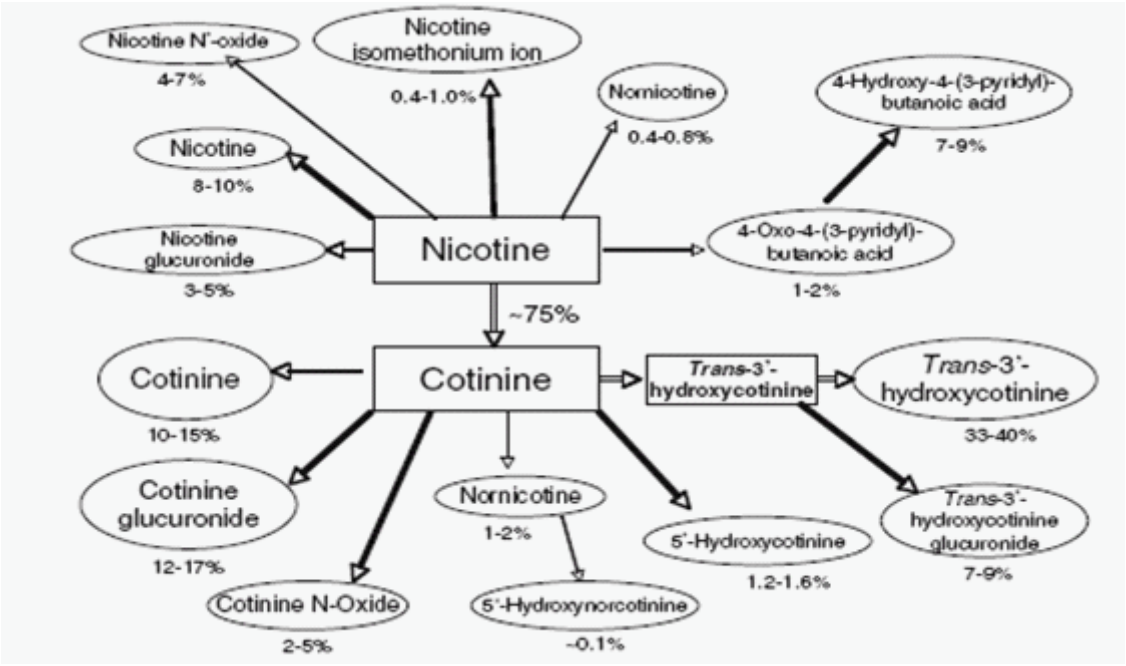


Fig 1.2 Nicotine deactivation pathways. Graphical representation of nicotine metabolites. Although cotinine is the main metabolite obtained by nicotine degradation (75%), it is followed by a wide number of minor compounds (Matta et al., 2007).

It has also been reported that parameters like urine excretion and half-life time ($t_{1/2}$) consistently change among humans. For example, after liver metabolism a small part of nicotine is excreted by kidney through urines, according to their alkaline or acid pH (from 1% to 20% of the total clearance respectively) (Matta et al., 2007). Moreover, although the average plasma half-life elimination for nicotine requires roughly 2h, it has been shown an increase of the *steady state* of plasma nicotine levels over 8h in regular smokers (Matta et al., 2007).

However, studies across species have underlined a remarkably nicotine rate of metabolism difference, with similarity between non-humans and humans primates (Seaton et al., 1991), but with a tendency to observe a faster metabolism in other animals (Gorrod and Jenner, 1975). For example, it has been shown that rodents, like rats and mice, have a shorter nicotine plasma $t_{1/2}$ than primates, which is around 45 minutes and 6-7 minutes respectively (Matta et al.2007).

In addition, other parameters, such as age, sex and ethnic group has been associated to nicotine metabolism variability in humans (Benowitz et al., 2002; Schoedel et al., 2004). Also environmental factors like stress, pathologies like kidney disorders, infective diseases (A Hepatitis), and some drugs like certain anticonvulsivant and oral contraceptives, might influence nicotine metabolism through impairing the activity of CYP2A6 (Matta et al., 2007), contributing to complicate the relation between nicotine consumption and individual differences.

4. Neural basis of nicotine abuse and addiction

The addictive property of nicotine is obviously the main reason which causes a widespread use of tobacco worldwide. The large expression of nAChRs mRNA in the CNS, together with nicotine-induced increase of DA release in the nucleus accumbens (NAc), guarantee a modulation of the brain reward circuitry in response to nicotine administration. Hence, through its effect, nicotine

facilitates short- and long-term synaptic modifications leading to abuse and addiction. Furthermore, nicotine effect in the CNS is not only related to its rewarding properties, additionally contributing to enhance cognitive and locomotor activity.

4.1 Nicotinic acetylcholine receptors: a physiological target for nicotine addiction

Even though this thesis is not primary focused on nAchRs, nothing, from the body/brain effect of nicotine to abuse/addiction liability of this compound, could be really understood unless a paragraph is dedicated to them. As previously mentioned, nAchRs belong to the super-family of Cys-loop ligand-gated ion channels (McGehee and Role, 1995; Role and Berg, 1996; Albuquerque et al., 1997; Wonnacott, 1997; Jones et al., 1999; Dani, 2001; Hogg et al., 2003), selectively activated by the neurotransmitter acetylcholine (Ach) and widely localized within the CNS, PNS and neuromuscular junction (NMJ). Through their activation, nAChRs allow an intracellular influx of positive ions like sodium (Na+), calcium (Ca2+) and potassium (K+) (Taly et al., 2009) inducing a wide array of different physiological modification.

The history related to nAChRs discovery is long and rich of memorable findings, often awarded with prestigious prizes. Some of the earliest hypothesis of a receptor-mediated effect of nicotine in the PNS came out almost ten years before the Ach discovery by Otto Loewi and Henry H.Dale, and is attributed to John Langley (Langley, 1905). Langley's discovery was followed by several decades of enthusiastic research in the field of study of Ach and its binding sites. In fact, during the last century the structure of nAChRs was better studied, taking advantage from both the discovery of nAChRs expression in the electric organ of the *Torpedo Californica*, and the discovery related to nicotinic receptors binding affinity of α-bungarotoxin (Albuquerque et al., 2009).

Both in the CNS and other tissues, nAchRs are assembled from five transmembrane subunits built around a water-filled pore located in the centre (McGehee and Role, 1995; Jones et al., 1999;

Karlin, 2002) with a pseudo-crystalline form primarily identified from the above mentioned *Torpedo* electric organ (Unwin et al., 2002; Unwin, 2005) (Fig 1.4a; 1.4d). However, this similar structure underlines a consistent difference in subunit composition across diverse organs. For example, on NMJ, where nAchRs are mainly located postsynaptically to transduce the motorneuron impulse evoked by Ach release, five different subunits have been isolated (α, β, γ and ε/θ) assembled each other in the same way (Mishina et al., 1986). On the other hand, within the CNS only α and β subunits have been identified, although organized in such a complex way to create over than 20 different types of pentameters, either homomeric or heteromeric (Changeux, 2010). Thus, this variety of subunits composition reflects a substantial difference in pharmacological and kinetic properties of nAChRs (Taly et al., 2009).

In the CNS, nine types of α (α2-10) and three of β (β2-4) isoforms have been described so far, although α8 expression has never been reported in mammals (Gotti and Clementi, 2004; Dani and Bertrand, 2007; Albuquerque et al., 2009). Most of these nicotinic receptors subunits are assembled in a heteromeric fashion (Karlin, 2002), with a less expression of homomeric nAchRs. A putative stoichiometry of heteropentameric nicotinic receptor among different subunit combination has been described as 2α:3β (Deneris et al., 1991; Sargent, 1993). Among these possibilities, α4β2 subunit and α6β2 represent the most common heteromeric receptors detected in the mammalian brain, whereas it is quite ascertain that only α7 and α9 might form homomeric compositions in these vertebrates (Anand et al., 1991; Cooper et al., 1991; Vernallis et al., 1993; Gotti et al., 1994; Le Novere et al., 1996) (Fig 1.4c).

Recent studies carried out through mRNA *in situ* hybridization techniques, have also underlined a marked differences both in the distribution and density of different nicotinic subunits throughout the mammalian brain, with an higher expression of α4, β2 and α7 and low levels of α2, α5, α6, β3 and β4 (Drago et al., 2003; Fowler et al., 2008).

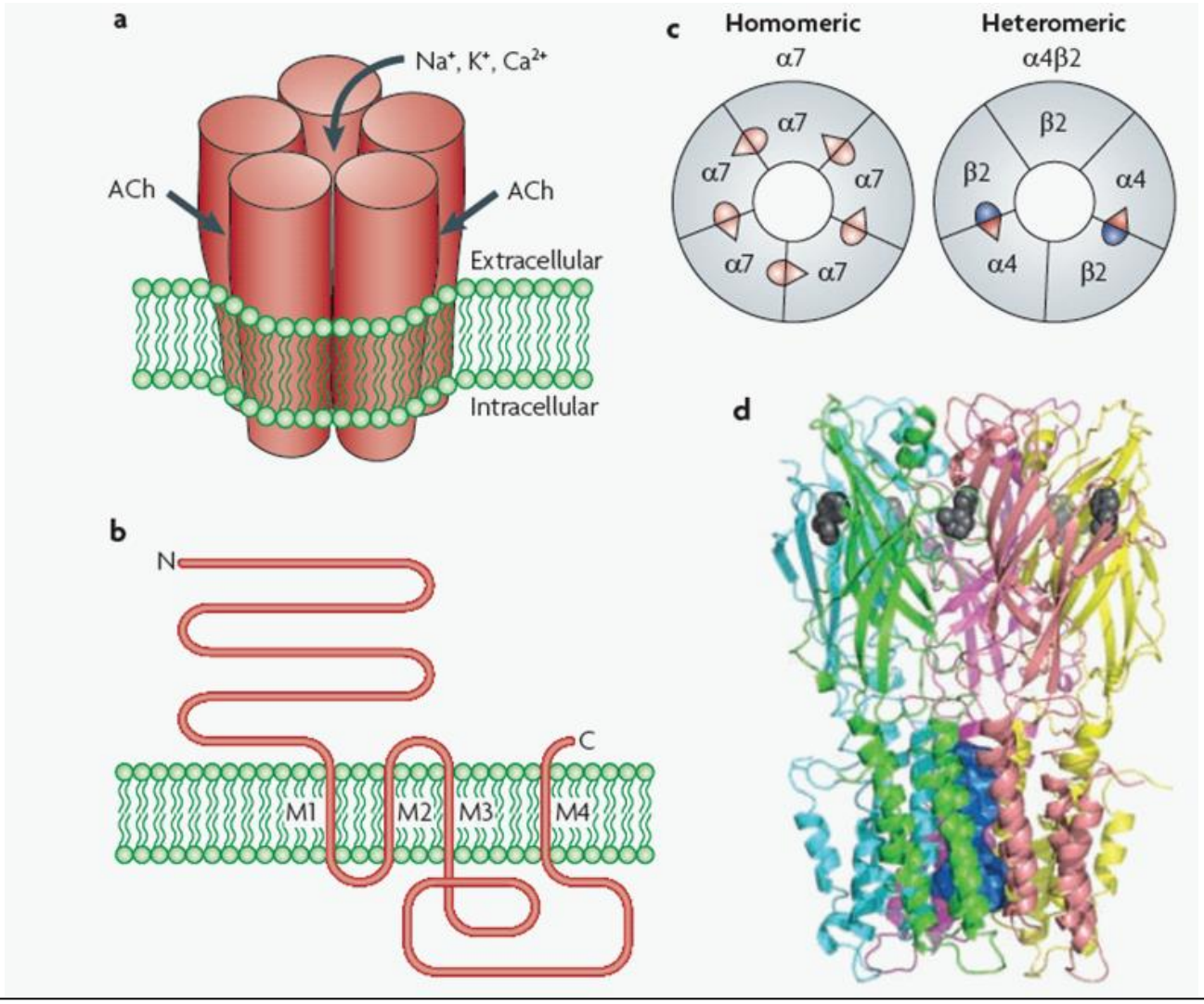


Fig 1.3 Structure of nAChRs. (**a**) nAChR is assembled from five transmembrane subunits with a central pore permeable to positive ions, and lateral binding sites for its agonist acetylcholine (Ach). (**b**) Graphical representation of a typical quaternary structure (M1-M4) of nAChRs. (**c**) Graphical representation of two of the most important nAchRs: the homomeric a7 and the heteromeric a4β2. (**d**) Side view of an a7 pentamer model (Changeux, 2010)

Furthermore, due to the lack of selective agonists and antagonists for specific nicotinic receptors subunits, the precise distribution of different nAChRs on precise neuronal populations was largely unknown, unless the advantage of new tools was available. Nowadays, through techniques like real time-polymerase chain reaction (RT-PCR) combined to *in vitro* electrophysiological recordings and

genetic deletion of specific subunits (see below), different research groups have been investigating this topic.

For example, it is now relatively well-known which are the main subunits expressed on dopaminergic neurons of the ventral tegmental area and on excitatory and inhibitory afferents that modulate their activation or inactivation (Changeux, 2010) (see below). Additionally, specific studies have been carried out on hippocampus cells (Sudweeks and Yakel, 2000), comprising an area strongly influenced by nicotine while exerting its cognitive enhancing properties.

nAChRs subunits have a common structure revealing 4 transmembrane domains (M1-M4) with a long N-terminal chain and a less extended C-terminal, both facing the extracellular space (Fig 1.4b) (Dani and Bertrand, 2007). The M2 transmembrane domain aligns along the pore at the centre of the structure contributing to draw the borders of the ion channel (Dani, 1989; Revah et al., 1991; Bertrand et al., 1993; Karlin, 2002).

Early studies provided evidence of two binding sites for nAChRs agonists, located between certain subunits (Karlin, 2002; Sine, 2002) and later, using crystals obtained from the Ach binding protein (AchBP) secreted by the *Limnaea Stagnalis* glial cells, this data has been confirmed with a <2.6 A resolution in a three-dimensional structure (Smit et al., 2001). Nonetheless, studies on AchBP, whose permeability to agonists is extended to nicotine, Ach, α-bungarotoxin, epibatidine and (+)-tubcurarine (Karlin et al., 2002), have also contributed to understand which subunits are involved in the pharmacological properties of nicotine. For example, in the heterodimer α4β2, Ach binding site is located in a small pocket between the adjacent α4 and β2 subunits, suggesting their equal role in the modulation of nicotine-induced effects (Dani and Bertrand, 2007).

Four functional nAChRs-states have been described so far: the resting state, the open state and the slow and fast-onset desensitized state (Katz and Thesleff, 1957; Sakmann et al., 1980; Neubig et al., 1982; Heidmann et al., 1983; Jackson, 1989; Hess, 1993; Edelstein et al., 1996; Auerbach and Akk,

1998; Prince and Sine, 1999; Reitstetter et al., 1999; Grosman and Auerbach, 2001). Among them, only the open position is functional while the others represent closed states. The presence of a nAChRs agonist, such as nicotine, quickly allows a switch of these receptors from a resting state, which is stable without agonist, to an open state, and then to a slow or fast-onset desensitization (Karlin, 2002).

Moreover, another common feature of nAChRs is that they are allosteric proteins. Basically, nAChRs represent the mainstream model of allosteric protein, since the origin of the name "allosteric" share with these receptors a common history. In fact, this term was coined by Jacques Monod and Francois Jacob in 1961 to qualify an enzymatic nonoverlapping mechanism discovered by a young student named Jean-Pierre Changeux (Changeux, 2009), who will have been, some years later, one of the most eminent researcher on nicotinic receptors. Henceforth through several years of study, mostly carried out by Changeux's group, it has been demonstrated that nAChRs contain multiple binding sites, non-competitive antagonists sites and gates that interact with them modifying the quaternary structure of domains (Karlin, 2002). This allosteric property confers to these receptors the capability to switch from one state to the others, following different possible combinations. This mechanism goes beyond the simple agonist binding and it has been theorized and adapted to nAChRs by the Monod-Wyman-Changeux (MWC) model (Monod et al., 1965; Changeux et al., 1967; Karlin, 1967, , 2002). Furthermore, through this mechanism some compounds might behave as negative or positive effectors on nAChRs in order to modulate its activity. For example, 17-β-estradiol and other neurosteroids act as a positive effector on α4β2 nAChRs (Valera et al., 1992; Paradiso et al., 2001; Curtis et al., 2002), while genistein and src-family kinases (SFKs) act on α7 to dephosphorilate nAChRs in a different way, drastically modifying their Ach-evoked response (Charpantier et al., 2005; Cho et al., 2005).

It is also worth to mention that nAChRs kinetic varies in relation to the pentamer's assembly. For example, while α7 has a relatively low affinity for Ach, with half-effective concentration around

200 μM, α4β2 affinity is higher (low affinity: 62 μM, high affinity: 1.6 μM) (Buisson and Bertrand, 2001) with some modification produced by repeated exposure to agonists (Sallette et al., 2005; Vallejo et al., 2005). As previously mentioned, agonist exposure also produces an inactive state known as desensitization. Due to the less affinity of α7-containing neuronal nAChRs to agonists, it has been demonstrated they desensitize rapidly after a high concentration of them, whereas α4β2 have a slower inactivating kinetic (Dani and Bertrand, 2007). On the other hand, nAChRs also show a slow desensitization process which involves much more α4β2 subunits, when activated by a prolonged lower concentration of agonists, than α7. In this case α7 does not desensitize below 1μM concentration of agonist, while α4β2 does it below 0.1 μM (Dani et al., 2000; Quick and Lester, 2002; Wooltorton et al., 2003). Obviously, the knowledge of this differences in subunit composition and desensitization time is crucial to better understand the multifaceted effect that nicotine exerts in the CNS.

Moreover, the localization of nAChRs in the CNS and NMJ slightly differs. In fact, while in the NMJ they are mainly located on the surface of muscle cell bodies, in the CNS a number of nicotinic receptors are situated on the presynaptic terminal (Sargent, 1993). This surely represents a clever route for Ach to modulate different processes within the brain, and to confer a prominent role to nicotine as a cognitive enhancing drug. Thus, nAChRs play a role in neurotransmitter release (McGehee et al., 1995; McGehee and Role, 1995; Gray et al., 1996; Role and Berg, 1996; Alkondon et al., 1997; Lena and Changeux, 1997; Wonnacott, 1997; Radcliffe and Dani, 1998; Jones et al., 1999; Luetje, 2004; Sher et al., 2004). For example, it has been shown that presynaptic nAChRs on glutamatergic terminals facilitate the release of glutamate when activated, contributing to modulate several brain functions, including synaptic plasticity (Wonnacott, 1997). Therefore, also nicotine application has revealed a high capability to induce the outflow of several neurotransmitters such as choline and acetylcholine (Grady et al., 2001), GABA (Alkondon et al., 1997), serotonin (Kenny et al., 2000), norepinephrine (Singer et al., 2004), endogenous opioid peptides (Pomerleau, 1998),

excitatory aminoacids (Schilstrom et al., 2000), and dopamine (DA) (Di Chiara and Imperato, 1988).

Despite this role, brain nAChRs have been also detected in the postsynaptic space, to allow a fast direct nicotinic transmission, in the preterminal space and onto dendrites, axons and soma (Dani and Bertrand, 2007).

In light of this consideration, nAChRs represent a substrate whose activity is not only related to modulate Ach transmission, being involved in several function within the CNS. Since their distribution is largely extended to the whole brain, it is obvious that these receptors participate in several functions, from learning and memory (Ji et al., 2001; Ge and Dani, 2005) to pathophysiological mechanism involved in Alzheimer's disease, schizophrenia, epilepsy and addiction (Lena and Changeux, 1998; Court et al., 2001; Raggenbass and Bertrand, 2002; Dani and Harris, 2005).

4.2 Neural effects of nicotine

Recently, taking advantage of new tools and strategies in neuroscience-related field, it has been also clarified which nAChR subunits are involved in specific behaviors. For example, the well-known nicotine-induced cognitive and psychomotor enhancing effect and its rewarding properties have been studied with genetically modified mice lacking the gene encoding for some nicotinic subunits (Changeux, 2010). These results, compared to earlier evidence about the main features of nicotine activity in the brain, have enlarged the knowledge about this substance.

Providing a detailed description of nicotine-induced motor and cognitive enhancing properties goes beyond the scope of this thesis, and a pertinent overview of this matter can be found in a number of excellent papers and reviews (see for example Changeux, 2010). Under this circumstance, it is only worth to underline how specific subunits of the nAChR have been selectively implicated in some

aspects of cognitive and motor control. For example, it has been demonstrated that β2 subunit is involved in nicotine-induced memory enhancement related to aversive stimuli (Picciotto et al., 1995; Caldarone et al., 2000; King et al., 2003), social interaction in the resident-intruder test (Granon et al., 2003), and modulation of navigation and exploration in an open field paradigm (Maubourguet et al., 2008). Moreover, α4 and β3 subunits seem to be involved in spontaneous locomotor behavior and acoustic startle response in the prepulse inhibition, respectively (le Novere et al., 1999; Cui et al., 2003). Finally, it has been shown that, while α7 modulates the efficiency in attentional tasks, β3 is employed in the modulation of seizures (Salas et al., 2004; Young et al., 2004).

Despite the role of nAChR subunits in the modulation of locomotor activities and cognitive performance, nicotine also elicits an array of modifications in the brain reward circuitry leading to nicotine abuse and addiction. Nicotine, as an addictive substance, produces rewarding effects acting on the mesolimbic DA circuit, a dense pathway of midbrain projections to forebrain structures and cortical areas, which regulates the response to pleasant and unpleasant stimuli. Through its connection, this circuit, which is one of the first DA-containing pathway discovered (Marsden, 2006), ultimately orientates goal-directed behavior.

Addiction is only the end point of a complex series of brain modification whose features basically are: (1) the compulsive drug seeking and voluntary drug intake, (2) the loss of control related to this behavior, and (3) the drug consumption despite its side effects (Everitt and Robbins, 2005; Koob and Le Moal, 2008). Thus, addiction is a relapsing brain disease which can harmfully interferes with the quality of life and individual wellness, also because of the emerging withdrawal symptoms when the access to drug is prevented (Koob and Le Moal, 2008). Moreover, after a prolonged consumption of an addictive drug, tolerance can be developed, leading the user to increase the rate and dose of drug to perceive its psychotropic effect (APA, 2000).

The activation of the brain reward circuitry by drugs of abuse concerns a wide array of short and

long-term modifications which vary in relation to drug exposure, triggering the neural adaptation to the substance (Koob and Volkow, 2010). Among these changes, it is now well-known how drugs of abuse modulate the synaptic strength and synaptic plasticity in some crucial brain regions, and how up- and down-regulation of specific receptors subunits can guide certain adaptations. In fact, addictive substances modify specific form of synaptic adjustment named long-term potentiation (LTP) and long-term depression (LTD) (Kalivas et al., 2005; Bellone and Luscher, 2006; Kauer and Malenka, 2007). Basically, they are two forms of plasticity that allow synaptic strengthening or weakening to, ultimately, fine modulate neural circuitries and to adapt behavior to a specific environment (Malenka and Bear, 2004). Through a long-lasting impairment of these forms of synaptic plasticity drugs of abuse cause addiction (Kasanetz et al., 2010).

Currently, among different brain areas involved in modulating steps from reward-dependent properties of a drug to the compulsive use (addiction), five main circuitries have been isolated. It is worth to mention that neuroadaptation of these circuitries follows an engagement in succession (Koob and Volkow, 2010), underlining how time-dependent changes are fully implicated in drug dependence. Basically, these circuitries make up the "brain reward circuitry" (fig 1.4) and they are: the mesolimbic DA system, the ventral striatum, ventral striatum/dorsal striatum/thalamus circuits, dorsolateral frontal cortex/inferior frontal cortex/hippocampus circuitries, and amygdala (Koob and Volkow, 2010).

Specifically, the mesolimbic DA system is a pathway of projections which arises from ventral tegmental area (VTA) DA neurons, involving as primary target the NAc, considered as the major component of the ventral striatum (Wise and Bozarth, 1987; Koob and Bloom, 1988). The activation of this pathway triggers the earliest stages of drug intake, and it is crucially implicated in the acute and chronic use of addictive substances. For example, it has been shown that drugs of abuse exposure induces short- and long-term modifications in DA neurons firing rate (Bonci et al., 2003), and that a single *in vivo* exposure to psychostimulants induces AMPA-mediated LTP on DA

neurons (Ungless et al., 2001). The NAc, through its connection to some of the most important cortical and subcortical areas (e.g. the prefrontal cortex) (Groenewegen et al., 1999) plays a crucial role in the modulation of rewarding properties of natural stimuli and drugs of abuse, thus, directing the reward-seeking and goal-directed behaviors (Grace, 2000; Jongen-Relo et al., 2003; Cassaday et al., 2005). In fact, it has been shown that several drugs of abuse, including cocaine, morphine, nicotine and cannabinoids, through their effects on VTA DA neurons, enhance the release of DA in the NAc (Di Chiara and Imperato, 1988), and that DA increase constitutes a biological substrate for natural stimuli rewarding properties (Fibiger et al., 1992; Kelley, 2004). In addition, it has been reported that specific depletion of DA or DA receptor blockade, induce several deficits in appetitive learning, and in approach behavior (Di Ciano et al., 2001; Parkinson et al., 2002; Faure et al., 2005), while lesions of the NAc cause disruption in motivated behavior, operant and emotional learning and behavioral flexibility (Reading and Dunnett, 1991; Cardinal et al., 2001; Cassaday et al., 2005)

Further modifications related to addiction have been described, involving the upregulation of NAc cAMP pathway and cAMP response element binding protein (CREB) in response to chronic administration of opiates and cocaine, whose effect contributes to reduce the rewarding value of these addictive substances (Chao and Nestler, 2004).

It is worth to mention that, despite the apparent simple organization of the VTA-NAc pathway, several and additional subdivision have been proposed so far, which highly complicate the understanding of the regulation of DA release in the NAc. In fact, beyond the comprehensive structural property of the NAc, it is now well-known that this area is divided in two functional subregions: the core (CoNAc) and the shell (ShNAc). These two areas play a well-distinct role, being the latter much more involved in the early steps of drug of abuse response (Goto and Grace, 2008), and strongly connected to other emotion-regulating brain areas (Ito et al., 2004). Nonetheless, also the architecture of VTA has been separated in distinct regions, whose response to drug of abuse administration strongly differs. For example, in an elegant study, Ikemoto et al.

(2006) demonstrated that rats self-administer nicotine in the posterior part of the VTA rather than anterior (Ikemoto et al., 2006), suggesting a distinct functional organization of these two VTA portions.

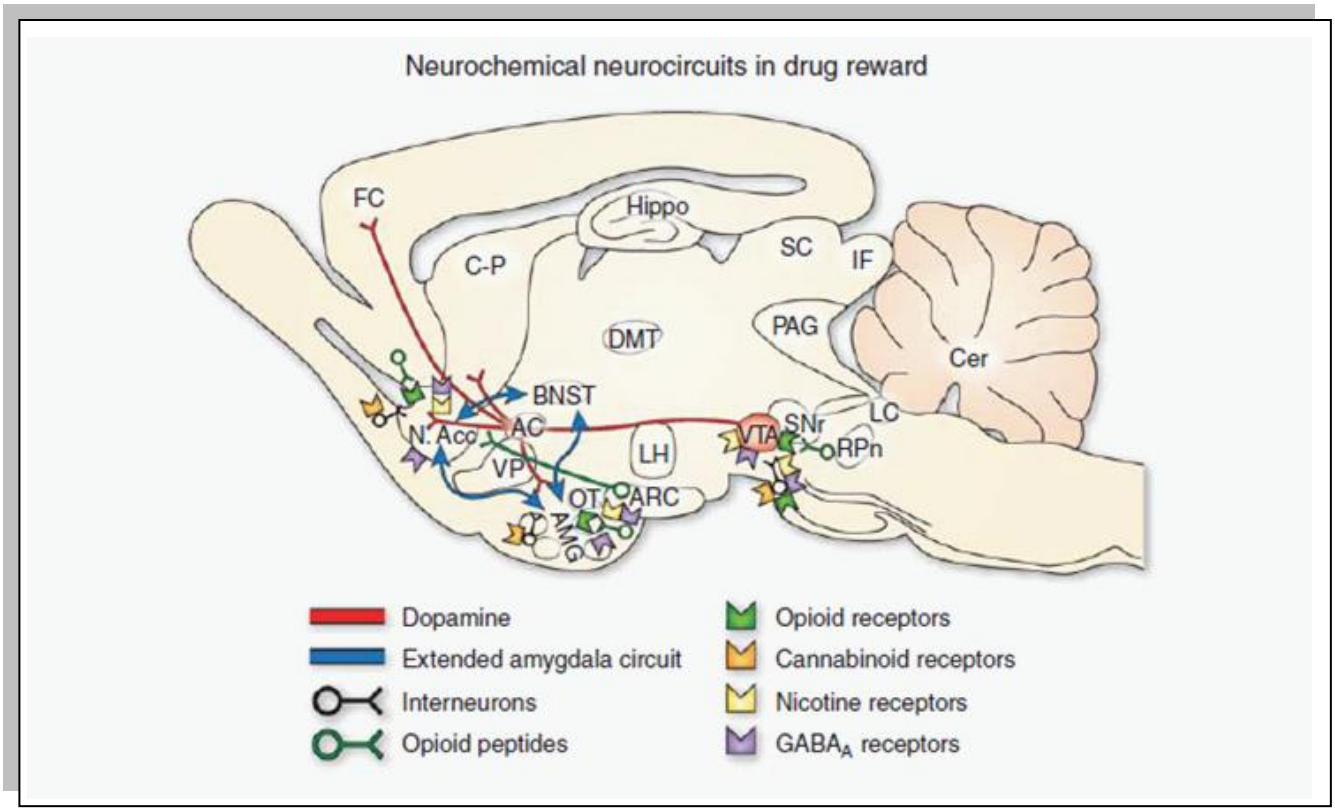


Fig 1.4 Brain reward circuitry and neurociruitry employed in acute response to drugs of abuse. Sagittal view of a rodent brain with a schematic representation of the main areas involved in the acute response to addictive substance. As marked in red, VTA DA neurons work like a *maestro* of this process, sending projections to several cortical, limbic and subcortical areas. Through their connections DA modulates the primary reinforcing properties of drugs of abuse. *Abbreviations*: AC, anterior commissure; AMG, amygdala; ARC, arcuate nucleus; BNST, bad nucleus stria terminalis; Cer, cerebellum; C-P, caudate-putamen; DMT, dorsomedial thalamus; FC, frontal cortex; Hippo, hippocampus; IF, inferior colliculus; LC, locus coeruleus; LH, lateral hipotalamus; NAcc, nucleus accumbens; OT, olfactory tract; PAG, periaqueductal grey matter; RPn, reticular pontine nucleus; SC, superior colliculus; SNr, substantia nigra reticulata; VP, ventral pallidum; VTA, ventral tegmental area (Koob and Volkow, 2010).

In addition, other theories beyond the DA-hypothesis of reward have been proposed, arguing about the exclusive DA enhancement role to modulate reward (Carlezon and Thomas, 2009; Koob and Volkow, 2010). However, the latter hypothesis still remains the best investigated, especially when it concerns the early stages of drug consumption. In fact, under these conditions, the prolonged and unregulated drug-induced DA release contributes to create an habit-learning related to drug-induced rewarding effects (Everitt and Wolf, 2002).

Nicotine, as well as other addictive substances, primarily acts on the mesolimbic/mesoaccumbens pathway to activate the brain reward circuitry, whose earliest modification is the increase of DA release from the VTA to the NAc. In fact, studies carried out with the intracranial self-stimulation (ICSS) in animals have shown that nicotine decreases the ICSS threshold, whereas nicotine withdrawal exerts the opposite effect (Epping-Jordan et al., 1998). Moreover, intravenous self-administration of nicotine is blocked by neurotoxin-specific lesions of mesolimbic DA system (Watkins et al., 2000), although other measures appear to act through a DA-independent mechanism (Laviolette et al., 2002). Additionally, it has been shown that: (1) systemic nicotine-induced increase of DA in the NAc is blocked by intra-VTA infusion of the nAChR antagonist mecamylamine (Nisell et al., 1994), (2) infusion of dihydro-β-erythroidine in the VTA decreases nicotine self-administration (Corrigall et al., 1994), suggesting a preferential effect of nicotine in this area, and (3) specific lesions of the NAc or DA receptors antagonist injections impair nicotine-mediated reinforcing effects (Corrigall and Coen, 1991; Corrigall et al., 1992).

To guide these modifications, nicotine acts through a common fashion to other drugs of abuse, enhancing the firing rate and burst firing activity of VTA DA neurons (Erhardt et al., 2002; Mameli-Engvall et al., 2006). Basically, even under physiological conditions, VTA DA neurons exert a double pattern of firing activity: (1) a single-spike firing or (2) burst rhythms (Grace and Bunney, 1984a, 1984b; Kitai et al., 1999). The latter has been associated to a larger DA release, and to the expression of immediately early genes within the NAc (Chergui et al., 1996; Chergui et al., 1997).

For this reason, a switch from tonic firing to phasic burst firing is a feature of unexpected reward or reward-predicting stimuli (Schultz, 2002). Since VTA DA neurons receive a wide array of glutamatergic (from PFC and peduncule pontine nucleus) and GABAergic projections, it has been shown how the burst firing modulation is due to the balance between excitatory and inhibitory aminoacid release to VTA DA cell bodies, with a crucial involvement of glutamatergic, and also cholinergic, afferents (Floresco et al., 2003; Lodge and Grace, 2006). In addition, closer analysis of VTA DA firing patterns in mice have revealed four different types of firing activity divided in: (1) high firing, high bursts; (2) high firing, low bursts; (3) low firing, low bursts; (4) low firing, high bursts (Mameli-Engvall et al., 2006; Changeux, 2010), and it has been demonstrated that these differences are due to the activation of different types of nAChR subunits. Hence, nAChRs play a crucial role also under physiological condition to modulate the spontaneous activity of mesolimbic neurons.

Taken together, these considerations and findings drew a very *complex* scenario related to nicotine action in the brain reward circuitry, which ultimately directs nicotine addiction. Recent studies with *knock out* and *lentiviral reexpressed* mice have shed some light to better understand which receptor subunits modulate this wide array of both molecular and behavioral modification.

Since nAChRs are widely expressed in the brain reward circuitry, it is obvious that the ultimate effect produced by nicotine depends on the different balance played by distinct subunits involved in this mechanism. A detailed schematic description of these subunits on VTA DA neurons and afferent projections is reported in fig 1.5. Specifically, different studies have investigated which subunit triggers certain physiological behaviors of VTA DA neurons, that represent the crucial start point to modulate the response to rewarding stimuli. A well accepted hypothesis, confirmed by studies in genetically modified mice, involves a central function of β2 and α7-containing nAChRs in the modulation of nicotine reinforcing effects. In particular, β2 subunit is considered as the oligomer mainly involved in the neuron switching from a resting to an excited state, and α7 as a fine

regulator of excited state after β2-containing receptors activation (Mameli-Engvall et al., 2006). In fact, it has been demonstrated that $\beta2^{-/-}$ mice only show a low-frequency, low-bursting activity pattern of VTA DA neurons (Mameli-Engvall et al., 2006). Moreover, VTA DA neurons do not respond to nicotine injection (Picciotto et al., 1998). In addition, lentiviral reexpression of $\beta2^{-/-}$ subunit in the VTA does not fully restore nicotine-induced excitation (Picciotto et al., 1998), being probable that it is necessary a functional activation of β2 also on excitatory projection to the VTA. On the other hand, $\alpha7^{-/-}$ mice have only high frequency, high bursting mode of VTA DA neurons, and nicotine injection leads to a rapid excitation of these neurons followed by a rapid return to baseline activity, also with a tendency to a lower firing activity (Mameli-Engvall et al., 2006).

This evidence underlines that a concomitant activation of both α7 and β2 is necessary to obtain a full expression of events related to nicotine reinforcement (Changeux, 2010). To confirm this involvement, other studies have shown that blockade of α7-containing neurons and N-metyl-d-aspartate receptors (NMDA-Rs) in the VTA diminishes DA release to the NAc (Schilstrom et al., 1998), and post-mortem human studies have observed an over-expression of α4β2 in smokers brain tissue (Benwell et al., 1988; Breese et al., 1997). Moreover, this result on humans has also been reported on living patients through Position Emission Tomography (Wullner et al., 2008). In addition, comparing systemic administration studies in animals with tobacco smoke, it has been reported how the overall excitation of DA neurons and LTP on glutamatergic terminals strongly depend to the balance between desensitization of β2 subunit in DA cell body, and enhanced glutamate release through α7 stimulation (Pidoplichko et al., 1997; Mansvelder and McGehee, 2000; Mansvelder et al., 2002; Pidoplichko et al., 2004; Dani and Harris, 2005). This occurs because the rate of nicotine after smoking is able to desensitize faster β2 than α7 which are less sensitive to nicotine, especially at lower concentration (Wooltorton et al., 2003).

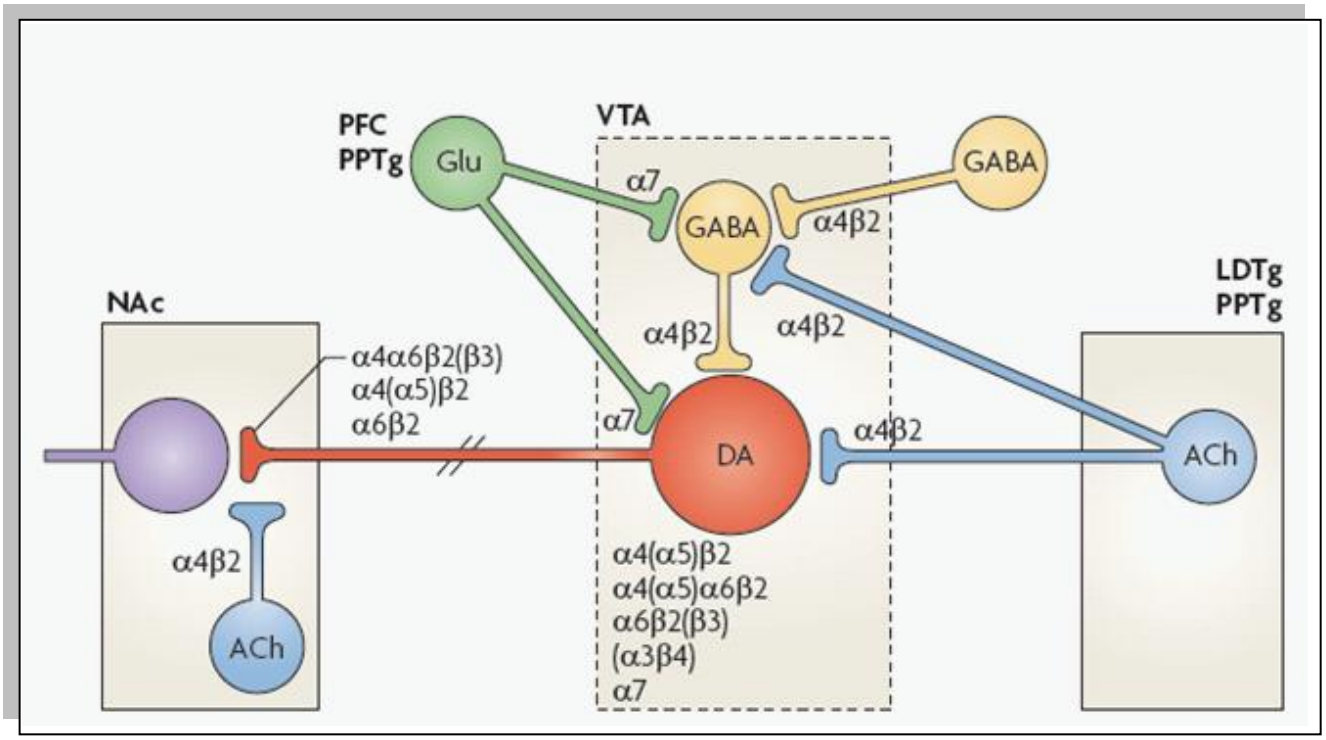


Fig 1.5 Localization of nAchRs on VTA DA neurons and its afferent projections. VTA DA neurons play a crucial role in the early stage of nicotine response. Nicotine acts in these neurons through the widespread localization of different homo- and heteropentameters nAChRs. Notably a4β2 subunits have a wider expression than α7 (Changeux, 2010). Abbreviations: Ach, acetylcholine; DA, dopamine; Glu, glutamate; LDTg, laterodorsal tegmental nucleus; PPTg, pedunculo-pontine tegmental nucleus; VTA, ventral tegmental area; PFC, prefrontal cortex; NAc, nucleus accumbens.

However, not only β2 and α7 have been involved in the physiological modulation of VTA DA neurons and brain reward circuitry. In fact, also α4 and α6 subunits seem to modulate some aspect of nicotine-induced effects in the mesolimbic system. Hence, it has been demonstrated that both α4 and α6 control striatal DA release by Ach or nicotine injection (Faure et al., 2010). In addition, it has been recently observed that α4, but not α6, which is mainly expressed in the terminal regions (Exley et al., 2008), is centrally involved in the switch from tonic to phasic VTA DA firing rate (Faure et al., 2010).

Beside this clear effect of some nAChR subunit in the modulation of VTA DA neuron response,

other studies have evaluated the role of specific nicotinic subunit deletion using behavioral paradigms. For example, it has been demonstrated that mice lacking the β2, α6 or α4 subunits fail to self-administer nicotine, whereas $\alpha7^{-/-}$ mice do not (Pons et al., 2008). Interestingly, nicotine self-administration is restored when a lentiviral injection elicits the reexpression of β2, α4 and α6 (Pons et al., 2008), suggesting a strong involvement of these subunits in the behavioral reinforcing properties of nicotine. Furthermore, using intra-VTA infusion of nicotine, it has been shown that $\beta2^{-/-}$ mice do not self-administer nicotine while they do it when β2 subunit is reexpressed (Maskos et al., 2005). By contrast, $\alpha4^{-/-}$ mice show an initial increase followed by a decrease of intra-VTA nicotine self-administration, while $\alpha6^{-/-}$ do not show any difference to wild type (Maubourguet et al., 2008).

Further studies have also underlined how deletion of α5 or overexpression of β4 enhance nicotine self-administration and that β2 subunit efficiency, rather than α7, plays a pivotal role in nicotine-induced conditioned place preference (Stolerman et al., 2004; Walters et al., 2006). Finally, α4 knock-in mice show a conditioned place preference with doses of nicotine 50-folds lower than wild-type (Tapper et al., 2004) and it has been hypothesized that α6 subunit plays a role in mediating the rewarding properties of nicotine (Jackson et al., 2009).

It is, finally, worth to mention how nAChR functionality may influence the chronic administration of nicotine and withdrawal symptoms. Since nicotine is an abused substance, it is even more interesting to evaluate the role of nAChRs under physiological conditions, which can explain the mechanisms at the downstream of nicotine addiction. As mentioned above, chronic administration of an addictive drug causes a short and a long-term modification within the brain reward circuitry which triggers, together with other events, the switch from abuse to addiction. Nicotine does that through a receptor sensitization to subsequent drug exposure (Caille et al., 2009). The molecular basis of this process depends on a combination of different molecular changes. Among them a possible mechanism might involve an up-regulation, instead of down-regulation typically observed

when other drugs are abused (Gutkin et al., 2006; Kenny and Markou, 2006), of mainly α4β2 nAChRs in the VTA after a long-term nicotine exposure (Nashmi and Lester, 2007; Govind et al., 2009). Moreover, it could be possible that a change in presynaptic cholinergic transmission might happen to modulate this sensitization process. This second mechanism might involve a balance between the decreased involvement of β2 subunits and a compensatory role of the α7 after a chronic nicotine exposure (Besson et al., 2007). Finally, also a long-term nicotine-induced reinforcement of glutamatergic input from cortical regions to VTA DA neuron has been evaluated (Caille et al., 2009). Noteworthy, it has been also reported a VTA and NAc up-regulation of glutamate receptors after chronic exposure to nicotine which would contribute to the long-term rewarding effect of this drug (Ray et al., 2009).

Also nicotine-induced withdrawal symptoms have been studied using knockout mice. To summarize, it seems that nAChRs act on both somatic and affective symptoms after quitting smoking with a prominent role of α2, α5, α7, β4 and β2, α6 respectively (Changeux, 2010).

In conclusion, even though studies on genetically modified mice represent a promising strategy to improve the knowledge on nicotinic subunits involved in specific behaviors, some limitation of this model should be taken into account. In fact, it has been reported that deletions in some genes encoding for certain subunits cause a compensatory over-expression of other proteins, affecting different behavioral measures (Fowler et al., 2008). This occurs especially when the gene inactivation is protracted along the physical development. For example, it has been shown that $\alpha7^{-/-}$ mice exhibit an augmented expression of α3 and α4 subunits, and that $\beta3^{-/-}$ show an increase of α6 (Fowler et al., 2008). Therefore, it is obvious that such kind of unplanned problem may bias against the reliability of measures with these models.

5. Final remarks

In light of all these considerations *tabagism* appears to be a distressing problem, not easy to get fixed and where a wide amount of variables must be taken into account. The large expression of different nicotinic subunits across the brain, combined to several pharmacokinetic and pharmacodynamic parameters related to nicotine action in the brain and whole body, should be correctly evaluated to make up a pharmacotherapy to treat nicotine dependence. Currently, different pharmacological approaches have provided successful results in the treatment of nicotine use disorders. Most of them benefit from previously prescribed compounds which were later approved also for nicotine addiction. Among these molecules we can include the antidepressant buproprion (used as a first-line therapy), the α2 noradrenergic agonist clonidine and the tricyclic antidepressant nortriptiline (both used as second-line therapy) (Ross and Peselow, 2009). In addition, the food and drug administration (FDA) have approved a wide array of nicotine replacement therapies such as gum, transdermal patch, inhaler etc. (Benowitz et al., 2009) although whose efficiency is quite limited. Finally, also the long-acting α4β2 nAChR partial agonist varenicline has been introduced as a drug to quit smoking, and to prevent relapse to nicotine during abstinence (Gonzales et al., 2006; Jorenby et al., 2006; West et al., 2008).

Beside these compounds, some studies, either clinical and pre-clinical, are still evaluating the therapeutic potential of different drugs such as an immune vaccine and the cannabinoid type 1 receptor antagonist rimonabant, clinically tested for obesity but now rejected from approval due to its severe side effects (Ross and Peselow, 2009).

The hypothesis of a modulating role played by the manipulation of the endocannabinoid system in nicotine addiction is an intriguing topic which is captivating several research groups all around the world. In fact, in light of data revealing a co-morbility between tobacco and cannabis use, especially

in adolescents (Viveros et al., 2006), and a common neurobiological substrate where cannabinoids and nicotine act in the brain, a possible modulatory activity of endogenous cannabinoids on nicotine-induced rewarding effects should be carefully taken into account.

Chapter II

THE ENDOCANNABINOID SYSTEM: FROM CLASSICAL CANNABINOIDS TO NUCLEAR RECEPTORS

Introduction

The eCb system is a family of lipid molecules, enzymes and receptors whose discovery is relatively recent (Jonsson et al., 2006; Marsicano and Lutz, 2006). In fact, despite the old interest on *Cannabis Sativa* active principles, the essential steps which allowed the isolation of specific cannabinoid receptors and endogenous compounds occurred within these last 20 years (tab 2.1).

1.Cannabinoid receptors

Once Gaoni and Mechoulam (1964) elucidated the structure of the tricyclic dibenzopyran derivative Δ^9-tetrahydrocannabinol (Δ^9-THC; THC), the main alkaloid of *Cannabis Sativa* (Hollister, 1986), and Howlett's group in 1988 found a specific binding site for THC in the brain (Devane et al., 1988), the intriguing possibility that neurons, and other cell lines, could express specific cannabinoid receptors quickly captivated more than one research group worldwide. This interest became something real when, the so-called cannabinoid type 1 (CB1-R) and cannabinoid type 2 (CB2-R) receptors were cloned, in 1990 (Matsuda et al., 1990) and 1993 (Munro et al., 1993) respectively

YEARS	Discoveries
<1900	1964-isolation of cannabinoids from Cannabis Sativa (Woods)
1900-1950	1940-eluciadtion and synthesis of cannabinol (Todd)
1950-1970	1964-elucidation structure of THC (Gaoni and Mechoulam)
1970-1990	1984-cannabinoids decreases cyclic adenosine monophosphate (Howlett and Flemming) 1988-THC binding site in the brain (Devane) 1990-clonation of CB1-Rs (Matzuda)
>1990	1992-identification of anadamide (Devane, Mechoulam, Petrwee) 1993-identification and clonation of CB2-Rs (Murno) 1994-development of the first CB1-Rs antagonist (Rinaldi-Carmona) 1994-mechanism of cannabinoid biosynthesis (Di Marzo) 1995-identification of 2-AG (Mechoulam, Waku) 1996-clonation of FAAH (Cravatt) 1997-evidence for AEA transport (Beltramo) 1997-Neurobiology of cannabinoid dependence and withdrawal (Rodriguez De Fonseca) 1999-generation of CB1-KO mice (Ledent) 2000-AEA activates TRPV1 (Zygmund) 2001-modulation of synaptic transmission in the brain by eCbs (Kano, Wilson, Nicol) 2001-evidence about other CB-Rs (Hajos, Ledent) 2003-AEA degradation and anxiety (Kathuria) 2003-clonation of eCbs biosynthesizing enzymes (Bisogno)

TAB 2.1 Brief history of cannabinoid research. Major breakthroughs on cannabinoids and endocannabinoids field of study. Notably the biggest piece of evidence about this topic occurred within the last 20 years (>1990). ((Di Marzo, 2006) with some modifications).

Notably, progresses on this topic also stimulated a worthwhile interest regarding the neuropharmacology of cannabinoid receptors which, in 1994, allowed the development of SR 141716-A (SR, rimonabant) the first and well-studied CB1-R antagonist (Rinaldi-Carmona et al., 1994). At the state of art, after sixteen years of research, several CB1- and CB2-R agonist or antagonist are now available as pharmacological tools (Pertwee, 2008) (Tab 2.2).

Ligand	CB1 K_i value (nM)	CB2 K_i value (nM)
CB1 selective agonists		
ACEA	1.4; 5.29	19.5; >2000
R-(+)-methAEA	17.9 to 28.3	815 to 868
Agonist without CB1-CB2 selectivity		
CP55940	0.5 to 5.0	0.17 to 0.52
R-(+)-WIN55212-2	1.89 to 123	0.28 to 16.2
(-)-Δ^9THC	5.05 to 80.3	3.13 to 75.3
AEA	61 to 543	279 to 1940
2-AG	58.3; 472	145; 1400
CB2 selective agonists		
AM1241	280	3.4
JWH-133	677	3.4
CB1 selective antagonists		
SR141716-A	1.8 to 12.3	514 to 13200
AM281	12	4200
AM251	7.49	2290
CB2 selective antagonists		
SR144528	50.3 to >10000	0.28 to 5.6
AM630	5152	31.2

TAB 2.2 K_i values related to the most used CB-R agonists and antagonists. Data shown in this table are related to K_i values of some of the most important CB1 and CB2-Rs agonists and antagonists, whose activity has been studied for the displacement of [^{3}H]-CP59940 and [^{3}H]-HU-243 from CB-R binding sites (Pertwee, 2008)

1.1 Structure and localization of cannabinoid receptors

Both CB1- and CB2-Rs are 7-transmembrane domain G_i-protein-coupled receptors with a widespread localization within the human and animal body. Human studies focused on structure of these receptors have reported a 44% of similarity in aminoacid sequence between CB1 and CB2-Rs with a splice variant of NH2 terminal in CB1 (Kano et al., 2009). In addition, despite evidence

suggesting the likely existence of CB1-Rs as homodimers (Wager-Miller et al., 2002), further studies have reported the presence of a CB1-heteromer dimerized with the dopaminergic receptor type 2 (D2-R) (Kearn et al., 2005; Mackie, 2005). This dimerization allows the formation of a CB1/D2 complex that is activated by CB1-Rs stimulation. Nonetheless, even a dimer with the Orexin type1 receptor has been proposed (Hilairet et al., 2003). Thus, this dimerization might suggest a specific cross-talk between eCb and other systems, whose functional role would be of interest in the pathophysiology of specific brain disorders (Kearn et al., 2005).

Despite the ubiquitous presence of CB-Rs in the mammalian body, there is a substantial difference about the localization of CB1 and CB2-Rs in the CNS and other tissues. In fact, it is now-well known that only a scarce amount of CB2-Rs is located in the CNS (mostly in the cerebellum and brain stem) (Van Sickle et al., 2005; Ashton et al., 2006), with a great abundance at the surface of immune cells (T and B cells, microglial cells), spleen, tonsils and peripheral tissue (Munro et al., 1993; Galiegue et al., 1995; Piomelli, 2003). On the other hand, CB1-Rs expression is higher in the CNS (Howlett et al., 1990; Herkenham et al., 1991) rather than other tissues (muscles, liver, gastrointestinal tract, adipose tissue and pancreas) (Batkai et al., 2001). In the brain, CB1-Rs represent the most abundant G_i-protein-coupled receptor found (Rodriguez de Fonseca et al., 2005) and through their activation, cannabinoids exert their psychotropic effect and eCbs their physiological properties (Ledent et al., 1999; Elphick and Egertova, 2001). Early animal studies with the synthetic cannabinoid radioligand [3H]-CP55,940 reported an high level of CB1-Rs in regions like the innermost layers of the olfactory bulb, CA3 of the hippocampus, lateral part of the striatum, globus pallidus, substantia nigra pars reticulata, and cerebellar molecular layer (Herkenham et al., 1990; Herkenham et al., 1991; Mailleux and Vanderhaeghen, 1992). In contrast, moderate and low levels of these receptors have been found in the cerebral cortex, septum, amygdala, some regions of the hypothalamus, spinal dorsal horn, thalamus, brain stem and spinal ventral horn (Herkenham et al., 1990; Herkenham et al., 1991; Mailleux and Vanderhaeghen, 1992). Further investigations, carried out after the cDNA-CB1-Rs clonation (Matsuda et al., 1990), have

better clarified the cellular localization of these receptors and their mRNA expression through immunoistochemistry and *in situ* hybridization techniques (fig 2.1 a).

Immunoistochemistry has revealed a double pattern of CB1-Rs mRNA-labeling across the CNS, with uniform response in the major neuronal population of the thalamus, striatum (Hohmann and Herkenham, 2000), hypothalamus, and cerebellum. Nonetheless, an un-uniform response was detected in some cell lines of the cerebral cortex (Marsicano and Lutz, 1999), hippocampus (Katona et al., 1999; Katona et al., 2000; Kawamura et al., 2006), and amygdala (Katona et al., 2001; McDonald and Mascagni, 2001). Moreover, a distinct dissociation between mRNA expression and immunoreactivity has been reported to underline a specific target of these receptors in the presynaptic space rather than postsynaptic (Kano et al., 2009), with a much more intense accumulation in the presynaptic portion of the axon (Kawamura et al., 2006). Furthermore, it has been well-demonstrated that CB1-Rs are preferentially distributed on the top of inhibitory synapse depending on specific brain region (Kawamura et al., 2006).

1.2 Functional relevance of cannabinoid receptors and their involvement in signal transduction

Among the main structural and functional characteristics of CB-Rs, it would be taken into account: (1) their critical involvement in the brain development where they control cell differentiation (Rueda et al., 2002), and (2) their full preservation throughout evolution (human, rat and mouse share 97-97% amino acid sequence identity) (Rodriguez de Fonseca et al., 2005). These features suggest that eCbs, through their affinity for CB-Rs, play a central role in cell and system physiology. Moreover, beyond their well-ascertained involvement in some physiological states, other evidence has underlined that blockade, genetic polymorphism and loss of CB-Rs (especially CB1) might be related to certain pathophysiological conditions and that exogenous cannabinoid administration might disrupt certain physiological processes. In fact, studies on genetic variants of *CHNR1* (which encodes for CB1-R) has reported a positive correlation between gene polimorphism and a wide array of different pathological state (e.g. Alzheimer's disease, obesity, attentional

deficits, schizophrenia) (Kano et al., 2009). Additionally, also a loss of striatal CB1-Rs has been recently considered as a key pathogenetic factor for Hungtington's disease(Blazquez et al., 2010) and an agonistic action at the level of CB1-Rs might improve some basal ganglia-related symptoms (Fernandez-Ruiz, 2009).

Basically, cannabinoid receptors activation leads to a signal transduction pathway which inhibits the cyclic adenosinemonophosphate (cAMP) formation, causing consequently a decrease of protein kynase A (PKA)-dependent phosporylation process (Devane et al., 1988; Howlett et al., 1990). Besides the pivotal role of this mechanism, other mechanisms have been proposed to explain the CB-Rs signaling. For example, it has been shown that CB1 and CB2-Rs are also coupled to ion channels through GolfProtein, whose activation inhibits the Ca^{2+} influx at the level of N-P/Q and L Ca^{2+}channels (Mackie and Hille, 1992; Twitchell et al., 1997), additionally causing an increase of inward rectifying potassium conductance and A current (Mackie et al., 1995) (fig 2.1 b). Furthermore, a coupling mechanism between these receptors and different species of intracellular cascades (e.g. mitogen activated kinase cascade and phophatidylinositol-3 kinase) have been suggested by several studies (Bouaboula et al., 1997; Howlett, 2002).

Among the modifications caused by CB-R activation, the most important is the suppression of neurotransmitter release (Schlicker and Kathmann, 2001). Through this process, eCbs exert their specific neuromodulatory effect in the CNS and PNS. To facilitate this mechanism the localization of CB-Rs is, preferentially, presynaptic, and their activation depends on eCbs release from the postsynaptic neuron (see below). For example, it has been shown that CB1-Rs activation suppresses the release of almost all catecholamine (dopamine, norephinephrine, serotonin) (Ishac et al., 1996; Cadogan et al., 1997; Nakazi et al., 2000), acetylcholine (Gifford and Ashby, 1996), excitatory and inhibitory aminoacids (glutamate, GABA and glycine) (Levenes et al., 1998; Szabo et al., 1998; Jennings et al., 2001), and the neuropeptide colecistokinin (Beinfeld and Connolly, 2001), providing evidence about its pivotal role in several brain and peripheral functions.

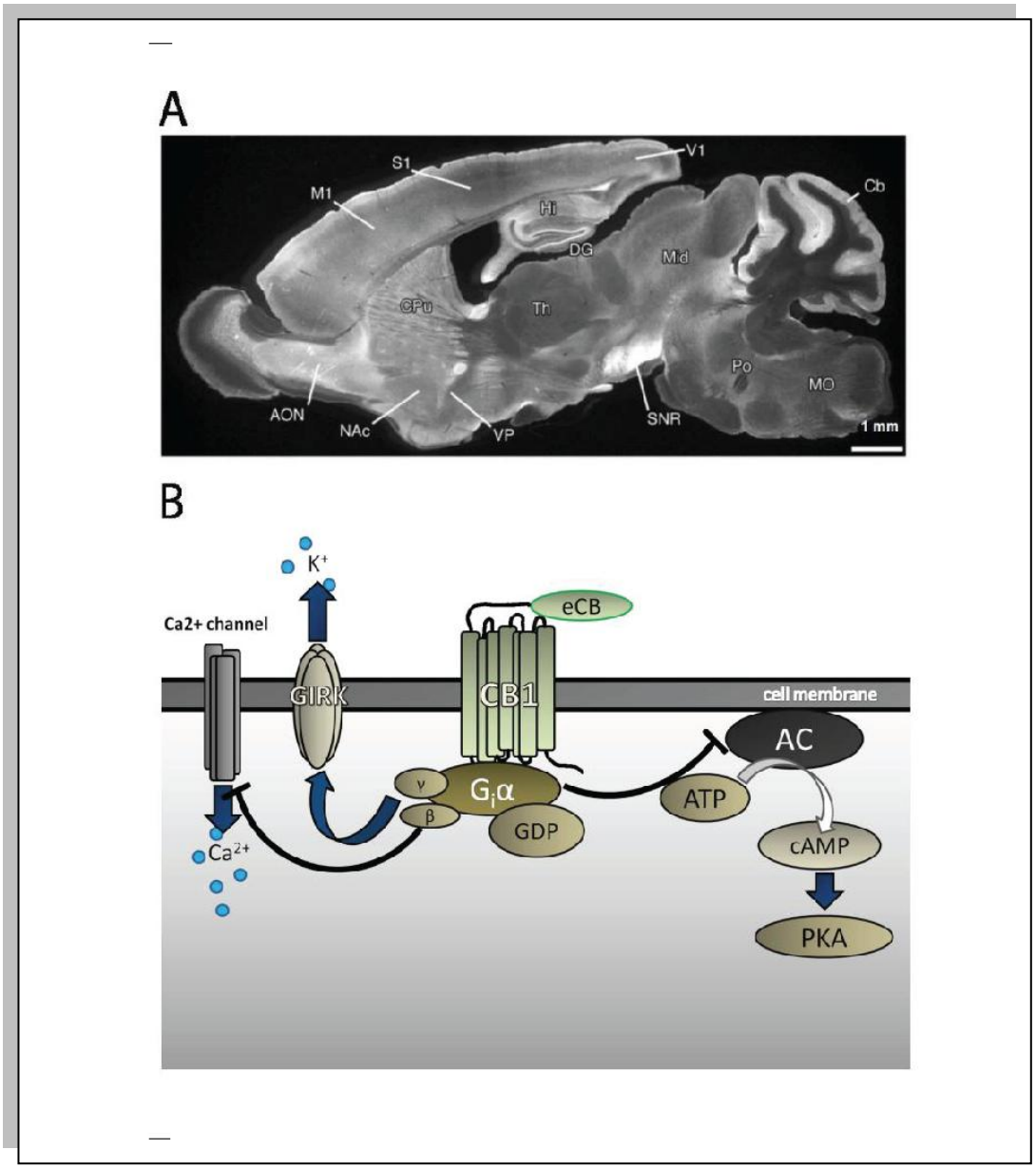


Fig 2.1 CB1 expression and mechanism of action. (a). Medial section of a mouse brain where it is shown the expression of CB1-Rs through *in situ* hybridization techniques (source: Kano et al., 2009). Abbreviations: AON, anterior olfactory nucleus; Cb, cerebellum; CPu, caudate/putamen; DG, dentate gyrus; Hi, hippocampus; M1, primary motor cortex; MO, medulla oblonga; Mid, midbrain; NAc, nucleus accumbens; S1, primary sensory cortex; V1, primary visual cortex; VP, ventral pallidum. (b) Schematic representation of a typical CB1-R activation pathway. When activated, CB1-Rs inhibit the adenilate cyclase (AC) activity, reducing the formation of cAMP. On the other hand, CB1-Rs activation behaves as a positive effector to allow an efflux of potassium (K+) by modulating G-protein-coupled inwardly rectifying potassium channel (GIRK), and as a negative modulator of Ca^{2+} channels.

1.3 Beyond classical cannabinoid receptors: is there anything else?

In light of these considerations, at the first glance it seems that CB-Rs might be necessary and sufficient to regulate the whole eCb-dependent signaling, being largely widespread in some of the most important areas where eCbs act. However, recent and elegant studies have substantially disproved the exclusive involvement of these receptors in the modulation of eCb-mediated effects, hypothesizing the existence of at least other two subfamilies of CB receptors non-CB1 or CB2 (Hajos et al., 2001; Hajos and Freund, 2002). Among the putative CBx receptors, it has been mentioned the so-called CB3-Rs (Hajos and Freund, 2002; Hajos et al., 2001), the transient receptor potential vanilloid type 1 (TRPV1) (Starowicz et al., 2007; Maccarrone et al., 2008), and the orphan G-protein coupled receptor GPR55 (Baker et al., 2006; Brown, 2007; Pertwee, 2007). It is worth to mention that also the peroxisome proliferator activated receptor (PPAR) has been considered as a CBx-R candidate. However, the central role in non-classical cannabinoid transmission of the latter receptor will be displayed below.

2. Endocannabinoids: biosynthesis, release and deactivation

Afterward the amazing discovery of CB1-Rs, a group of Israeli and British researchers leaded by Raphael Mechoulam reported the existence of the first described endocannabinoid in the brain, named anandamide (Devane et al., 1992). The headword anandamide ((5Z,8Z,11Z,14Z)-N-(2-hydroxyethyl)icosa-5,8,11,14-tetraenamide, N-arachidonoylethanolamide, AEA) reminds the Sanskrit word "ananda" which means "bliss, delight", and it has been given in honor of its function. In 1995, was the turn of 2-arachidonyl-glicerol (2-AG), the second eCb found (Mechoulam et al., 1995; Sugiura et al., 1995), and a wide amount of molecules with eCb-like activity has been discovered so far (see below). Through their effect on CB-Rs, and sometimes other receptors, eCbs exert their neuromodulatory function, providing a regulatory mechanism to guarantee the balance between excitatory and inhibitory neurotransmitter action within the nervous system.

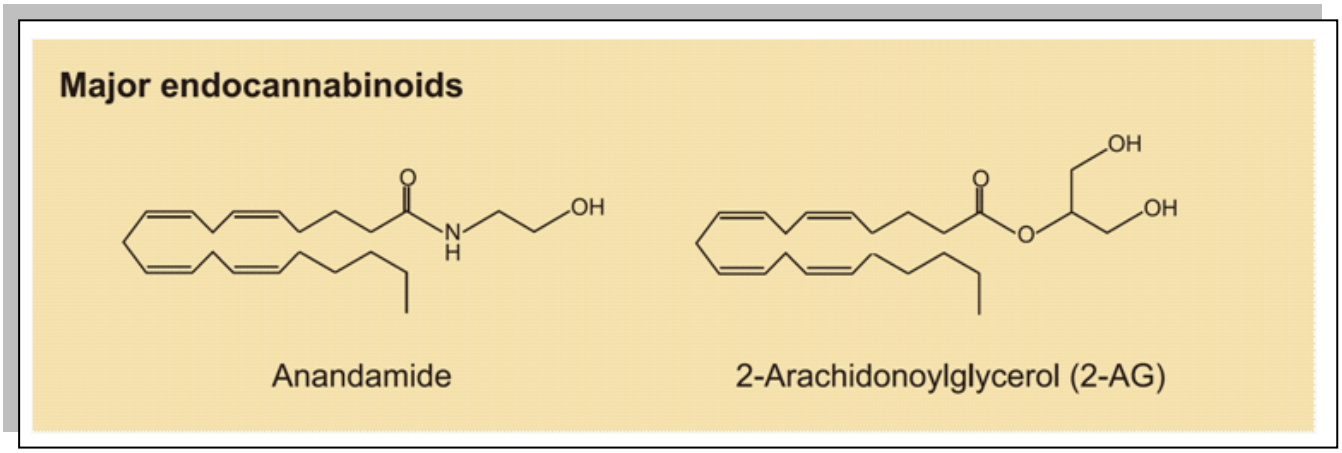


Fig 2.2.Molecular structure of AEA and 2-AG

2.1 Synthesis and release of AEA and 2-AG: the fine modulation of eCbs in the brain

The biosynthetic pathway of AEA and 2-AG is now established, although their involvement in other biochemical mechanisms (e.g. the cascade of arachidonic acid) complicates the *scenario* about their synthesis. Different studies have provided a description of some critical passages to obtain AEA and 2-AG and how they are deactivated by specific enzymes (see Kano, 2009 for a review). Both AEA and 2-AG are derivatives of arachidonic acid coniugated with ethanolamide or glycerol, respectively (Rodriguez de Fonseca et al., 2005). AEA is obtained from the phosphatidylethanolamide, which through the enzyme N-acyltransferase, in presence of Ca^{2+} and cAMP (Cadas et al., 1996; Piomelli, 2003) is transformed in N-arachidonoylphosphatidyl-ethanolamine (NAPE). The cleavage of NAPE in AEA is obtained through a specific pospholipase D (NAPE-PLD), which belongs to the zinc metallhydrolase family of the β-hydrolase fold (Okamoto et al., 2004), and whose activity is regulated by depolarization and/or activation of ionotropic or metabotropic receptors (Giuffrida et al., 1999; Stella and Piomelli, 2001; Piomelli, 2003; Cheer et al., 2007). Interestingly, NAPE-PLD mRNA immunoreactivity has been detected mostly on postsynaptic sites, contributing to clarify how eCbs exert their effects (see below), although also a presynaptic expression of this enzyme has been reported (Cristino et al., 2008; Egertova et al., 2008). An overall evaluation of NAPE-PLD mRNA reactivity in the brain has

underlined high levels of this enzyme in the dentate gyrus of hippocampus, the Ammone's horn, cortex, thalamus, hypothalamus and cerebellum (Cristino et al., 2008; Egertova et al., 2008).

In contrast, 2-AG synthesis does not only involve a linear pathway, but several arrays of it. The main pathway requires a combination of a phospholipase C (PLC) and a diacylglycerol lipase (DAG-L) activity. The first step is the PLC hydrolysis of arachidonic acid-containing membrane phospolipid to obtain arachidonic acid-containing diacylglicerol. From diacylglicerol the action of DAG-L yields 2-AG (Stella et al., 1997; Kondo et al., 1998; Jung et al., 2005). In addition, other studies have proposed the involvement of a phospholipase A1 and a lyso-PI-specific PLC (LPIPLC) (Ueda et al., 1993; Tsutsumi et al., 1994; Sugiura et al., 1995), which transform phospholipids in lysophospholipids (through PLA1) and then to 2-AG (through LPIPLC). Nonetheless, other biosynthesis mechanisms have been suggested (see Kano et al., 2009 for a review).

AEA, whose chemical features resemble that of THC (Ryan et al., 1997; Seltzman et al., 1997), has an higher affinity for CB1-Rs (K_i: 89±10nM) than CB2-Rs (Ki: 371±102nM) (Reggio, 2002), showing also an affinity for TRPV1 (Ross, 2003). Sustained levels of AEA have been found throughout the mammalian brain and peripheral tissues (Felder et al., 1993; 1996), with highest levels in the brain stem and striatum (Bisogno et al., 1999; Yang et al., 1999). On the other hand, 2-AG is able to bind both to CB1-Rs (K_i: 2.4μM) and CB2-Rs (Basavarajappa, 2007a), and its effect mimic that of THC (e.g. immobility, antinociception, immunomodulation) (Mechoulam et al., 1995). Additionally, it has been reported that 2-AG brain levels are roughly 200 folds higher than AEA. However, the two eCbs display a superimposed distribution in the CNS (highest levels: brainstem, medulla, limbic system, striatum) (Sugiura et al., 2002). These higher levels of 2-AG in the brain might reflect its predominant involvement, when compared to other eCbs, in the modulation of certain cerebral physiological processes.

Once synthesized, AEA and 2-AG exert their role in the CNS and PNS through a so-called "*on demand*" process, which is peculiar for this class of neuromodulators.

The "*on demand*" (Di Marzo et al., 1994; Cadas et al., 1996) release of AEA and 2-AG is a feature which involves both a rapid production and a rapid degradation of eCbs, triggering their biosynthesis in response to several physiological and pathopysiological stimuli. The purpose of this mechanism is to allow a sudden eCb release from the postsynaptic neuron to activate CB-Rs located in the presynaptic space and, consequently, to inhibit the neurotransmitter release (Schlicker and Kathmann, 2001; Piomelli, 2003). This represents a clever mechanism to influence and modulate both the short- and long-term form of synaptic plasticity, especially in some brain regions strongly influenced by the role of synaptic re-modeling. For example, in the midbrain, which is crucially involved in reward processes and motor control (e.g. the VTA and substantia nigra), eCbs are released *on demand* after different circumstances and through a Ca^{2+}-dependent fashion (Melis and Pistis, 2007). Some of these events, which trigger the release of eCbs, are: (1) depolarization of DA neurons (Melis et al., 2004a), (2) induction of burst firing (Melis et al., 2004b; Riegel and Lupica, 2004), and (3) stimulation of excitatory afferents (Melis et al., 2004b). Thus, the final step of this process is to suppress GABA and glutamate release from projecting areas to midbrain DA neurons, modulating their activity and also protecting postsynaptic cells. This mechanism of short-term synaptic efficacy modulation has been named differently according to the suppression of GABA, or glutamate release. In fact, in the first case it is named depolarization-induced suppression of inhibition (DSI) (Llano et al., 1991; Pitler and Alger, 1992) and, in the second, depolarization-induced suppression of excitation (DSE) (Kreitzer and Regehr, 2001) . DSI was the first mechanism recognized, and was primarily isolated in the cerebellum (Llano et al., 1991), whereas the specific acronym was given by Pitler and Alger (1992). In fact, they found a particular DSI mechanism in the hippocampus, acting through a Ca^{2+}-dependent mechanism and that would have involved a retrograde messenger, whose primary candidate was glutamate (Pitler and Alger, 1992). Finally, in 2001 eCbs where found to be responsible of GABA release suppression, since DSI was blocked by the CB1-R antagonists SR141716-A, and AM251 or AM281 (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001).

Additionally, in the same year, Wilson and Nicoll demonstrated that eCb release occurs without the presence of a vescicular transport (Wilson and Nicoll, 2001), suggesting a mechanism which differs from the classic neurotransmitter behavior. Among other evidence which strongly suggested that the messenger responsible for DSI was an eCb, it is worth to mention that: (1) CB1-R agonists enhance DSI and selectively reduce the inhibitory post-synaptic currents (IPSCs) in the hippocampus and cerebellum (Katona et al., 1999; Hoffman and Lupica, 2000; Takahashi and Linden, 2000; Ohno-Shosaku et al., 2001; Wilson et al., 2001; Wilson and Nicoll, 2001); (2) DSI is absent in $CB1^{-/-}$ mice (Wilson et al., 2001; Yoshida et al., 2002), and (3) GABA interneurons, involved in DSI, express an high level of CB1 in their axon terminals (Katona et al., 1999).

Afterwards was the hand of DSE discovery, by Kreitzer and Regeher, which includes the same aspect of DSI, but acting on glutamate release instead of GABA (Kreitzer and Regehr, 2001). Also in this case, different studies have reported: (1) an absence of DSE in CB1-KO mice (Ohno-Shosaku et al., 2002), (2) a modulatory effect exerted by CB1-R agonists and antagonists (Kreitzer and Regehr, 2001; Maejima et al., 2001), and (3) a massive suppression of excitatory post-synaptic currents (EPSCs) mediated by eCbs release (Melis et al., 2004b). This mechanism has been studied in some brain areas such as the VTA (Melis et al., 2004b), where it seems that eCb-mediated DSE is triggered by the activation of D2-Rs in the postsynaptic cell (Melis et al., 2004b). A schematic representation of eCb-mediated DSE is reported in fig 2.3.

Despite the central role played by DSI and DSE in the modulation of eCb-mediated synaptic plasticity, also long-term forms of synaptic remodeling have been assigned to eCbs. In fact, different eCb-induced LTDs have been described so far, together with heterosynaptic form of LTP (where eCbs act indirectly). Throughout these mechanisms the eCb system plays a pivotal role in some specific processes which require a long-lasting change in synaptic strengthening, or weakening. For example, in the hippocampus, LTD and LTP are critical for learning/memory consolidation and a common feature of cannabinoids, such as THC, is to impair these cognitive functions.

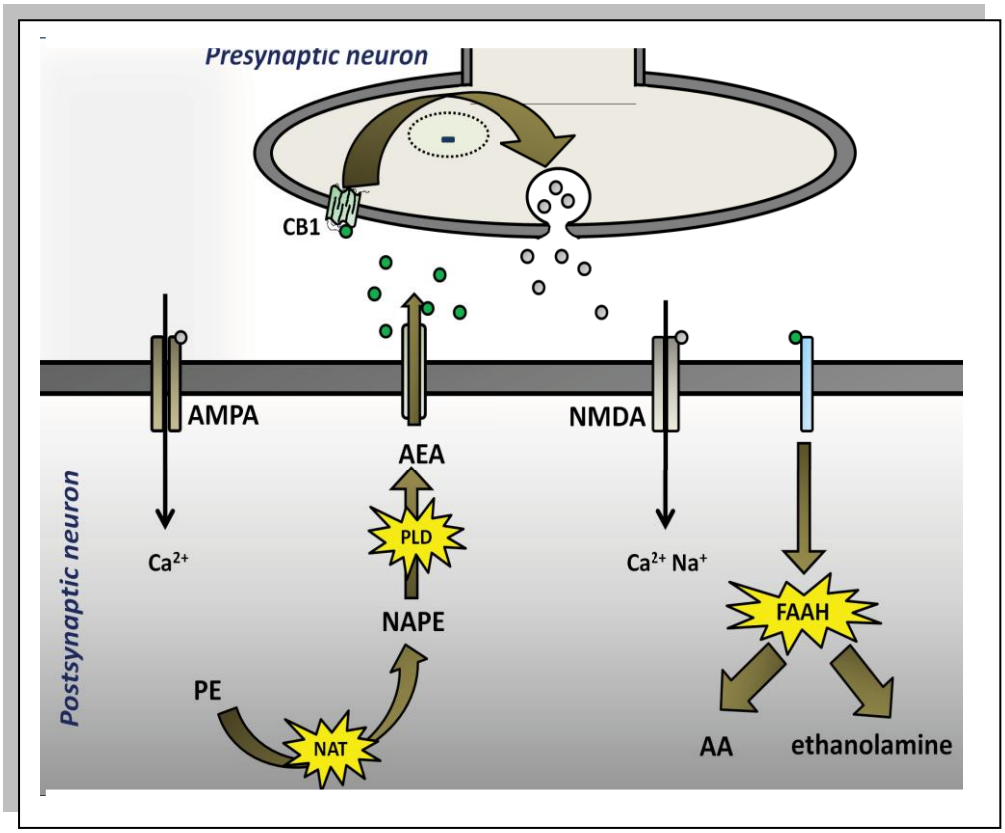


Fig 2.3.eCb signaling in the brain. Graphical representation of a typical AEA-mediated DSE. AEA (green) is produced *on demand* following the influx of Ca^{2+} through the stimulation of AMPA receptors by glutamate (grey). Afterward AEA is released to bind to CB1-Rs located in the axon terminal of presynaptic excitatory afferents. The result is a massive inhibition of glutamate release. Afterward, AEA is reuptaken by a putative carrier protein (sky-blue) and, then, brought to the postsynaptic space where it encounters a FAAH-dependent deactivation process.

These long-term changes affect the synaptic strength, involving modifications whose time length varies from few minutes to several days, and they include a fine regulation of cellular adaptation (e.g.: receptor density; synaptic remodeling) (Piomelli, 2003; Kreitzer, 2005; Chevaleyre et al., 2006; Safo et al., 2006). LTD has been intensively studied and it represents a typical feature of eCb-mediated long-lasting effect. Providing a detailed description of eCb-mediated LTD goes beyond the scope of this thesis, and excellent reviews on this topic are now available (Heifets and Castillo, 2009). The first evidence of this modulatory mechanism of synaptic plasticity emerged in 2002 through studies carried out by Lovinger's group (Gerdeman et al., 2002) that demonstrated an eCb-

mediated LTD at excitatory synapses in the dorsal striatum. Further studied have extended the brain localization of this mechanism to the NAc (Robbe et al., 2002), amygdala (Marsicano et al., 2002; Azad et al., 2004; Chevaleyre et al., 2007), the above mentioned hippocampus (Chevaleyre and Castillo, 2003; Edwards et al., 2008; Lafourcade and Alger, 2008), PFC (Lafourcade et al., 2007), VTA (Pan et al., 2008) and other areas, where synthesis and postsynaptic release of eCbs trigger this specific eCb-mediated plasticity. For example, in the VTA and NAc, whose high relevance for this thesis has been already discussed, it has been shown that moderate *in vitro* stimulations for 5-10 minutes, evoke an eCb-mediated LTD on inhibitory and excitatory afferents, respectively (Robbe et al., 2002; Hoffman et al., 2003; Soler-Llavina and Sabatini, 2006). This effect causes a consequent long-lasting reduction of GABA and glutamate release from presynaptic neurons.

Even though the involvement of the eCb system in drug addiction will be better explained below, it is worth to mention that repeated exposures to drugs of abuse modulate eCb-LTD both in VTA and NAc (Fourgeaud et al., 2004; Mato et al., 2004; Pan et al., 2008), underlining a strict relationship between the eCb system activation and drugs of abuse-induced long-term effect responsible of the switch from abuse to addiction.

In light of these findings, it seems to be quite established that through these fine mechanisms (DSI/DSE, LTP/LTD etc…), the activation of the eCb system guarantees a reliable modulatory assemble to regulate several functions in the CNS, which would also include the information processing and brain protection to injuries (Melis et al., 2006).

2.2 Transport and metabolism of eCbs

Similarly to other neurotransmitter systems, eCbs possess a putative reuptake mechanism and an array of enzymes which inactivate and degrade them. Currently, little is known about AEA and 2-AG membrane transporter. In fact, despite the fact that different models have been proposed, a specific uptake mechanism has not been found yet. Among them, the highest recognized possibility remains the transport of AEA into the postsynaptic cell through a putative carrier protein (Fegley et

al., 2004; Ligresti et al., 2004), but also the hypothesis of simple passive diffusion and endocytosis-induced by a caveolae-related uptake process have been suggested (McFarland and Barker, 2004; McFarland et al., 2004). A very recent study carried out by Vincenzo di Marzo's group has elegantly provided evidence to the first hypothesis, measuring the activity of TRPV1 channels as biosensor of AEA cellular reuptake together with exploiting nanotechnology in TRPV1-overexpressing HEK-293 cells (Ligresti et al., 2010).

For what concerns 2-AG, there are less studies focused on the investigation of uptake mechanism. Mounting evidence suggests the involvement of the same mechanism discussed above for AEA (Beltramo and Piomelli, 2000; Bisogno et al., 2001), or a shared mechanism for AEA and 2-AG (Hermann et al., 2006).

On the other hand, it is quite ascertained that AEA and 2-AG do not share the same metabolizing proteins. In fact, two different enzymes have been involved in AEA and 2-AG degradation, named fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAG-L), respectively. Although the main route to deactivate these eCbs is the hydrolization through the above mentioned enzymes, other mechanisms, such as the oxydation by lipoxigenases and cycloxygenases, have been involved in endocannabinoids degradation (Kano et al., 2009).

FAAH, cloned from the rat liver, but identified also in the brain (Cravatt et al., 1996), is a membrane-bound enzyme belonging to the family of amidase proteins (Basavarajappa, 2007b). FAAH, whose structure comprises a chain formed by 579 aminoacids, is able to recognize an array of fatty acids, although AEA is its preferred substrate, hydrolyzing it in arachidonic acid and ethanolamine (Kano et al., 2009). Recently, it has been demonstrated a role of FAAH in deactivating the n-acylethanolamides (NAEs) oleoylethanolamide (OEA) and palitoylethanolamide (PEA) (see below) (Kathuria et al., 2003), whose levels, together with AEA, are drastically increased after pharmacological blockade of FAAH by URB597 (Fegley et al., 2005). In fact, the development of genetically modified mice lacking the gene encoding for FAAH (FAAH-knock out, FAAH-KO) has shown a more responsiveness to the exogenous administration of AEA in these

animals compared to wild type (WT) (Cravatt et al., 2010). Moreover, also an endogenous level of AEA and other fatty acid ethanolamines higher over ten folds than control mice has been reported in FAAH KO (Cravatt et al., 2001).

Nonetheless, beside FAAH activity, AEA is also metabolized by other enzymes. In fact, also the involvement of cyclooxigenases, such as the COX-2, LOX-2, and the cytochrome P450 has been reported as putative inactivating mechanism for AEA (see Basavarajappa, 2007a for a review).

On the other hand, MAG-L, first cloned from mouse adipocytes cDNA library (Karlsson et al., 1997), plays a pivotal role in the degradation of 2-AG, although some studies have underlined a circumscribed FAAH capability to hydrolyze this eCb (Di Marzo et al., 1998). MAG-L, whose expression has been found in many tissues and cells, works metabolizing 2-AG in arachydonic acid and glycerol (Beltramo and Piomelli, 2000; Maccarrone et al., 2001), and it is formed by 303 aminoacids in several species of animals and in humans (Karlsson et al., 1997; Karlsson et al., 2001). Further information about the critical steps to 2-AG deactivation and metabolism can be found in several excellent reviews (Basavarajappa, 2007b). In addition, it is worth to mention that a substantial difference between site of action of FAAH and MAG-L has been reported. In fact, while FAAH activity is concentrated in the postsynaptic terminal, which requires a transport of eCbs from the presynaptic membrane to the postsynaptic space, MAG-L acts within the presynaptic membrane (Choi and Lovinger, 1997; Dinh et al., 2004). This evidence suggests that MAG-L has a role in terminating retrograde signaling at presynaptic neurons (Kim and Alger, 2004).

3. Not only anandamide: toward the discovery of new endocannabinoids

As already mentioned, the discovery of CB-Rs has not definitely closed the discussion to the beguiling world of receptors activated by eCbs. The hypothesis of the so-called CBx, or CB3-R, and the suggested involvement of TRPV1 as binding site for the eCb AEA, combined with unfitting

studies carried out in knock-out mice for the CB1-Rs, have disproved the exclusive role of classic cannabinoid receptors in eCb-mediated signaling (Wiley and Martin, 2002).

In addition, mounting evidence about the not exclusive affinity of CB1 and CB2-R agonist for their respective binding sites, have underlined the possibility that other systems and oligomers might interact with the classic eCb system to complicate its profile of action in the CNS and PNS. For example, it has been shown that, at concentration of 1 μM, AEA activates TRPV4 and blocks TRPV8, while HU-210 and CP55940 act as agonists of the orphan receptor GPR55 (Pertwee, 2008). In addition, there is evidence about the presence of non-CB1/CB2 or V1-Rs in the brain, which can be activated by methAEA and the synthetic cannabinoid WIN55212-2 (Pertwee, 2008).

Moreover, despite the well-characterized presence of AEA and 2-AG in the brain, other eCb-like molecules have been described in the CNS and PNS. Among them, other eCbs are the 2-arachidonyl-glyceryl ether (nolandin, 2-AGE), O-arachidonyl-ethanolamine (virodhamine), N-arachidonyl-dopamine (NADA), dihomo-γ-linoleonyl ethanolamide, and docostetraenoyl ethanolamide (Hanus et al., 1993; Hanus et al., 2001; Huang et al., 2002; Porter et al., 2002).

In particular, a recent interest has been dedicated to OEA and PEA (fig 2.2), two NAEs, whose pharmacological profile remained elusive until the discovery of their agonistic action on a family of nuclear receptors and transcription factor known as *peroxisome proliferator activated receptors* (PPARs) (Fu et al., 2003).

3.1 n-acylethanolamides, cognate molecules of classic encocannabinoids

Even though the interest for these compounds has grown up in these years, PEA and n-acylethanolamides were already known several decades ago, when it was found that PEA had an antinflammatory activity in guinea pig, and it was present in peanuts, soybean and egg yolk (Hansen, 2010). Additionally, early studies provided the existence of these NAEs as endogenous compounds in the mammalian brain (Bachur et al., 1965).

OEA and PEA share with AEA a common metabolizing substrate, which is the FAAH enzyme, and it has been demonstrated that pharmacological blockade of FAAH by the irreversible inhibitor URB597 increases the levels of both AEA and OEA/PEA (Kathuria et al., 2003) (see above).

OEA and PEA, as well as AEA, belong to the family of NAEs, representing a monounsaturated and saturated form of them, respectively. The most peculiar feature of these endogenous NAEs is that they are devoid of cannabinoid-like activity, being ineffective either on CB1 or CB2-Rs, and sharing affinity for the α-type PPAR (PPAR-α) (O'Sullivan, 2007). Furthermore, even PPAR-α was considered an orphan receptor for a long time unless it has been discovered its affinity for OEA and PEA (Pistis and Melis, 2010).

Recently, a large interest has been dedicated to a putative cross talk between the eCb system and PPAR activation in the modulation of several brain functions.

To corroborate this hypothesis, different studies have demonstrated that CB-R agonists and PPAR ligands play an opposite effect each other in the regulation of certain physiological aspects. For example, weather it is well-known the appetite stimulant properties of the CB1-R agonist THC (Fride et al., 2005), OEA activity is implicated in appetite suppression and weight loss (Fu et al., 2003). Moreover, while THC and CB1-R agonists negatively modulate learning and memory (Sullivan, 2000; Davies et al., 2002), OEA and PEA are involved in different aspects of memory enhancement (Campolongo et al., 2009; Mazzola et al., 2009). Nonetheless, other studies have suggested a co-activation of these two parallel systems in the modulation of specific response both in the CNS and PNS. In fact, it has been recently reported that several CB1-Rs agonists exert their neuroprotective action through activation of PPAR-α (Sun et al., 2006; Sun et al., 2007). Furthermore, NAEs significantly contribute to the anti-nociceptive and anti-inflammatory action of endocannabinoids (LoVerme et al., 2006; Jhaveri et al., 2008; Sagar et al., 2008). In addition, an intriguing synergistic effect between CB1-R activation and PPAR-α have been suggested to explain the cognitive enhancing properties of the FAAH inhibitor URB597 (Mazzola et al., 2009).

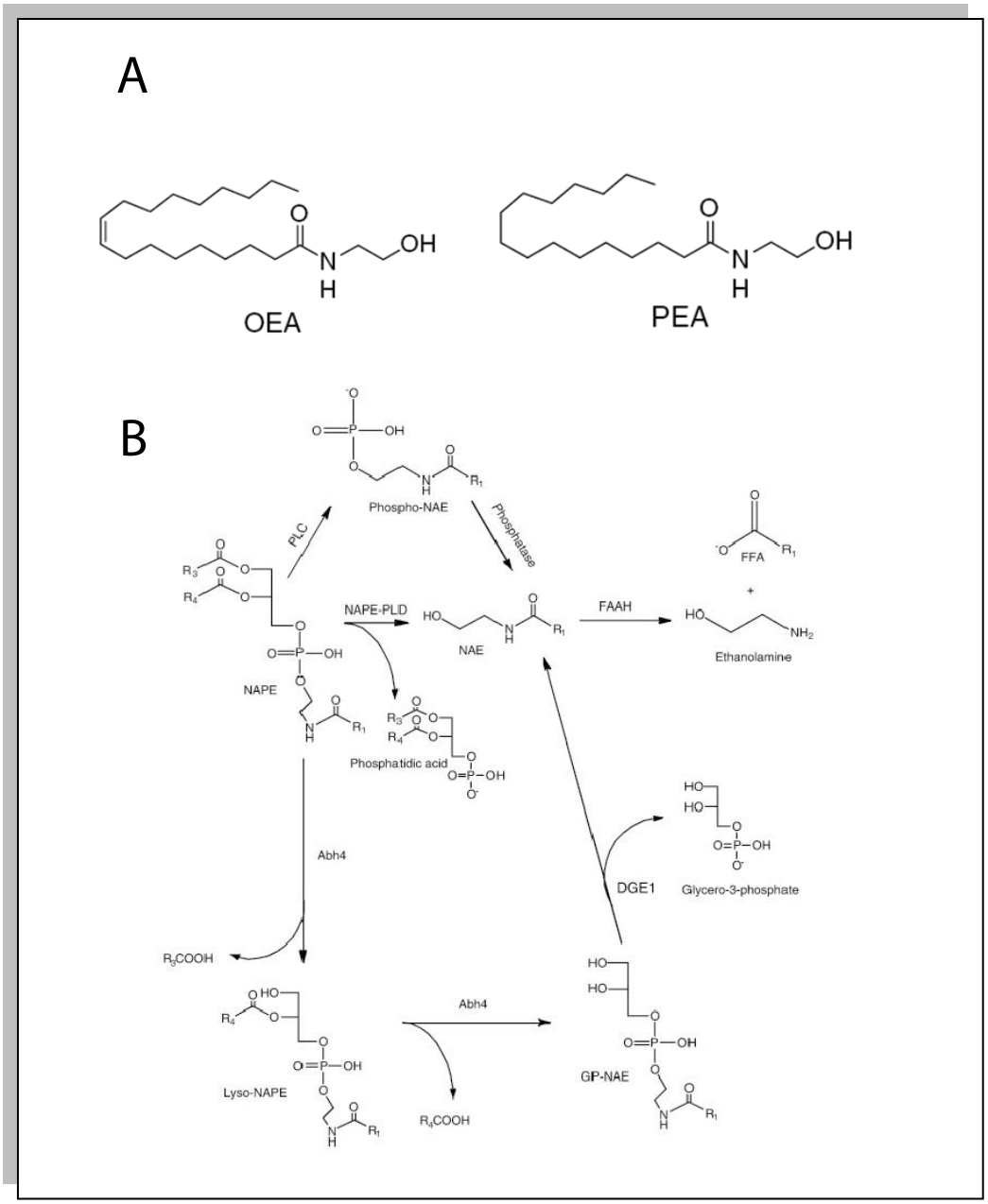


Fig 2.3 Non-classical eCbs and biosynthesis of NAEs. (**a**) Among the large family of eCb-like molecules, a current specific interest has been dedicated to oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), whose chemical structure is represented in this panel. (**b**) Different enzymatic pathways for formation of n-acylethanolamide (NAE) from n-acylphosphatidylethanolamine (NAPE) (Hansen, 2010). Although different pathways are involved in NAE formation, the most studied remains the NAPE-PLD-dependent process. In addition, NAEs can be also syntesized through a phospholipase C (PLC)-, a phosphatase- and a phospholipase B (Abh4)+phosphodiesterase (GDE1)-dependent process. NAEs can be hydrolyzed by a fatty acid amide hydrolase (FAAH), or a NAE-hydrolyzing acid amidase (NAAA).

NAEs, whose presence has been reported in the brain with a concentration in the rat CNS around or above 100 pmol/g each (Hansen, 2010), share with AEA a lipophylic structure with high log P values. The latter parameter gives a reason to their low solubility in water (OEA=6.12±0.64; PEA=5.56±0.58) (Bojesen and Hansen, 2003). NAE synthesis and catabolic process occur independently of classical eCbs, and crucial stimuli at the upstream of their biosynthesis are not fully understood (Pistis and Melis, 2010). Some evidence suggests that, similarly to AEA and 2-AG, these molecules are released *on demand* in response to certain physiological processes concerning injuries and inflammation (Hansen et al., 1997), contributing to modulate physiological response to external threats. The main biosynthesis pathway of OEA and PEA is basically the same observed and discussed for AEA (see above), involving NAPE-PLD (Pistis and Melis, 2010) (fig 2.3).

Interestingly, NAPE-PLD$^{-/-}$ mice show a reduction in Ca^{2+}-dependent conversion of NAPE to NAEs (Hansen and Diep, 2009) with a decrease in OEA and PEA levels, but not AEA (Leung et al., 2006), suggesting an involvement of parallel systems and/or compensatory adaptation for AEA biosynthesis in this mouse strain. In addition, since it is well-known that NAPE-PLD mRNA immunoreactivity (see above) has been highly reported on postsynaptic space, it is possible that NAE synthesis might be functional to allow an autocrine signaling on PPARs expressed in the same cell, although they can also act as modulator of synaptic plasticity once produced in the presynaptic axon terminal (Pistis and Melis, 2010).

Finally, even though it has been already mentioned that FAAH represents the main metabolic substrate for NAEs (see above), at least other 2 enzymes have been considered capable to deactivate these molecules: FAAH-2 and NAE-hydrolyzing acid amidase (NAAA) (Tsuboi et al., 2005; Wei et al., 2006).

3.2 Nuclear receptors: new targets for endocannabinoid-like molecules

As already cited, the affinity of OEA and PEA is basically confined to PPARs, showing no binding properties on CB-Rs. In fact, although it is still under evaluation the possible involvement of other receptors not fully characterized (e.g. GPR55) (Borrelli and Izzo, 2009; Godlewski et al., 2009) in OEA and PEA-mediated effects, PPARs obviously represent the most studied biological target to understand where NAEs act.

The PPAR subfamily is composed in three isoforms: α, γ, and β/δ, whose encoding genes are separated. Belonging to the family of nuclear receptors, PPARs possess a modular structure with two different domains: (1) a DNA-binding domain and (2) a large ligand-binding domain which interacts with a great number of different ligands (e.g. fatty acids, NAEs, eicosanoids, antidiabetic synthetic agonists thiazolidinediones and fibrates) (Laudet et al., 1992; Escriva et al., 1998; Desvergne and Wahli, 1999). Binding studies have confirmed that OEA has an higher half-maximal concentration (EC_{50}) for PPAR β/δ than α (1.1 μM vs. 120 nM), being much more active on the latter and showing no effect on γ subfamily of PPARs (Fu et al., 2003). By contrast, PEA shows affinity only for PPAR α (3 μM), but not for PPAR γ and β/δ (Bouaboula et al., 2005; Lo Verme et al., 2005). Also AEA has been reported as a putative PPAR-α and γ agonist, although studies have been carried out only through *in vitro* techniques and in transfected systems (Sun et al., 2007).

Providing a full description of PPAR activity in the brain, where the localization of these receptors (especially PPAR α) is ubiquitous (Moreno et al., 2004), is beyond the scope of this thesis. However, recent reviews have been focusing on this topic (see Pistis and Melis, 2010 for a review).

In this section it is only worth to mention that PPAR-α is a lipid sensor implicated in the metabolism of fatty acids (Pistis and Melis, 2010). Thus, PPAR-α is expressed in several tissues involved in the regulation of metabolic aspect such as kidney, liver, brown fat, CNS (Braissant et al., 1996; Mandard et al., 2004; Moreno et al., 2004; Galan-Rodriguez et al., 2009), and in other cell lines (e.g. monocytes, vascular endothelium) (Chinetti et al., 1998) where its presence is mainly

involved in the negative modulation of inflammatory response. By contrast, γ-type PPAR participates in the regulation and expression of DA-Rs and DA signaling, neuroprotection and inflammation, while β/δ line is involved in homeostasis control, reproductive capacity in females, tissue repair, and cell proliferation (Wang et al., 2003; Michalik et al., 2006). Taken together, these studies provide an interesting profile of these receptors in the regulation of several physiological functions, mainly connected with metabolism.

Similarly to other members of the nuclear receptor family, they mainly act as gene transcription regulators (Berger and Moller, 2002), with a tendency to develop conformational changes promoting the association of coactivators and corepressor proteins (Pistis and Melis, 2010). Briefly, they take part in gene transcription with an heterodimerization with the retinoid X receptor (RXR) and then binding to specific regions of the DNA called "peroxisome proliferator response element" (PPRE). In addition, beside this conventional genomic effect of PPARs, it has been suggested also a non-genomic effect responsible of short-term modification induced by receptor activation (Gardner et al., 2005; Ropero et al., 2009).

Some studies have provided evidence of this non-genomic mechanism. Among them it has been shown that (1) PPAR activity is still conserved in presence of protein and mRNA synthesis inhibitor (Ropero et al., 2009), (2) they possess a rapid onset (2-5 min) (Ropero et al., 2009) and (3) PPAR activation induces production of cytosolic effectors such as reactive oxygen species (Melis et al., 2008).

4. Endocannabinoids from reward to addiction: do they play a role in the mesoaccumbens pathway?

In light of previous considerations, at a first glance it seems that eCb-mediated fine modulatory action might be an intriguing topic, when it concerns complicated phenomena like drug abuse and addiction. In the first chapter some space was dedicated to draw the hallmarks of the brain reward

circuitry and the main steps that trigger drug dependence. Furthermore, already in the introduction it was highlighted how the manipulation of the eCb system might modulate drug-induced effects in some specific areas related to responses to pleasurable stimuli.

Recently, interest has been dedicated to the putative role of the eCb system in the midbrain DA neuron physiology, where it might play a pivotal function in rewarding processes and addiction. It has been already reported how the activation of the so-called brain reward circuitry is crucial in early stages of reward seeking and goal-directed behavior. In addition, it has also been underlined how a malfunction of this system could trigger drug dependence.

The first evidence of a cannabinoid action in the VTA and in the neighbor *substantia nigra pars compacta* (SNc) arises from the effect of the exogenous CB1-R agonist THC, acting on VTA DA neurons as an abused substance (French et al., 1997; Gessa et al., 1998). This effect was mimicked by the CB1-Rs synthetic agonist WIN 55212-2. Both of them cause an increase of VTA DA neuron firing rate and bust firing, which allows an enhancement in DA release from the VTA to its projecting areas such as the NAc (Tanda et al., 1997; Cheer et al., 2004) and prefrontal cortex (Chen et al., 1990; Pistis et al., 2002b).

Since the levels of CB1-R mRNA in the VTA and SNc are low (Herkenham et al., 1991; Matsuda et al., 1993), it has been suggested that exogenous synthetic and natural agonists act on DA neurons through a CB1-Rs mediated inhibition of GABA and glutamate release from inhibitory and excitatory projections, respectively (Mechoulam et al., 1995; Marsicano and Lutz, 1999). This represents the already mentioned eCb-mediated DSI and DSE, whose functional relevance was already described. To confirm this hypothesis, several electrophysiological studies have indirectly demonstrated that inhibitory and excitatory projections to mesolimbic DA neurons express CB1-Rs (Melis et al., 2004b; Melis et al., 2004a; Riegel and Lupica, 2004), and that perfusion of CB1-Rs agonists depresses excitatory and inhibitory post-synaptic currents on VTA DA neurons (Szabo et al., 2002; Melis et al., 2004a; Marinelli et al., 2007). Moreover, direct evidence of CB1-R

expression on GABAergic terminals was reported by studies carried out through electron microscopic investigation (Katona et al., 1999).

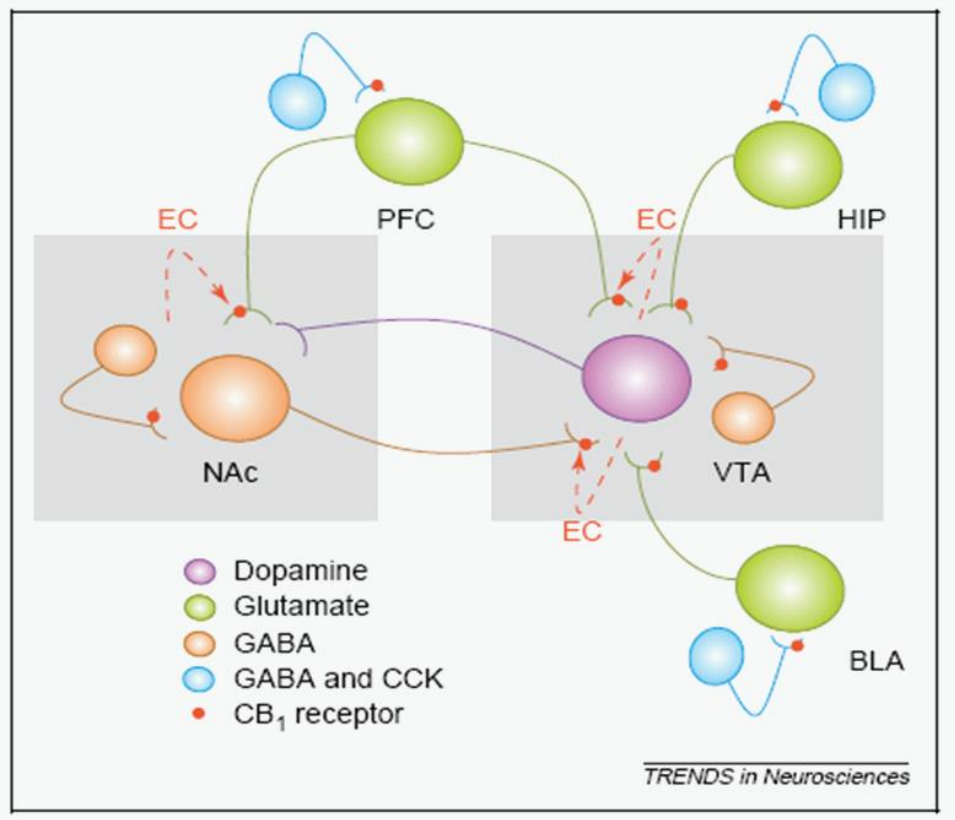


Fig 2.3 localization of CB1-Rs in the brain reward circuitry. The eCb system is considered as a suitable candidate to modulate the brain reward circuitry response to pleasant stimuli, such as those produced by administration of drugs of abuse. In this graphical representation CB-Rs are preferentially located at the top of excitatory and inhibitory presynaptic buttons which project to the mainstream neurons of the mesocorticolimbic system. Abbreviations: BLA, basolateral amygdala; HIP, hippocampus; NAc, nucleus accumbens; VTA, ventral tegmental area; PFC, prefrontal cortex (Maldonado et al., 2006)

In addition, it has been elegantly shown that DA neurons release eCbs after depolarization induced by the enhanced Ca^{2+} postsynaptic influx (Melis et al., 2004b; Riegel and Lupica, 2004) and that, among different eCbs implicated in this mechanism, 2-AG plays a more relevant role than AEA (Melis et al., 2004b).

Moreover, it has been demonstrated that the eCb system exerts a modulatory effect beyond the VTA to regulate the whole activity of the mesoaccumbens pathway. In fact, electrophysiological studies in rats have shown that both THC and synthetic cannabinoids depress BLA and PFC-evoked excitation of GABAergic medium spiny neurons (MSNs) of the ShNAc (Pistis et al., 2002a), which represent a common feature of drug of abuse administration. Nonetheless, it has been clearly elucidated that eCbs are necessary to induce LTD in the NAc and neostriatum, taking part in the critical steps from reward-dependent drug intake to compulsive consumption (Gerdeman et al., 2002; Robbe et al., 2002; Robbe et al., 2003).

Several studies have also demonstrated that the eCb system modulates drug-induced response in the reward pathway, providing evidence regarding its possible role in the pharmacotherapy of addiction (Parolaro and Rubino, 2008). For example, it has been clearly elucidated that the eCb system interacts with the rewarding properties of some, but not all, drugs of abuse. In fact, it has been shown that cannabinoids attenuate both morphine and methadone withdrawal signs while SR 141716-A (SR, rimonabant) precipitates morphine abstinence in addicted rats (Hine et al., 1975; Vela et al., 1995; Yamaguchi et al., 2001; Del Arco et al., 2002; Maldonado, 2002). Moreover, an interplay between opioid and eCb system has been confirmed by studies showing that the opioid μ-antagonist naloxone precipitates abstinence in THC-tolerant rats (Kaymakcalan et al., 1977), and that SR-precipitated withdrawal is attenuated by morphine (Lichtman et al., 2001). Moreover, using self-administration (SA) and conditioned place preference (CPP) paradigms in animals, it has been demonstrated that (1) $CB1^{-/-}$ mice do not show morphine-induced CPP and SA at a dose capable to induce rewarding effects in WT (Ledent et al., 1999; Martin et al., 2000; Cossu et al., 2001), and (2) the same effect was observed treating rats with the CB1-R antagonist SR (Navarro et al., 2001; De Vries et al., 2003; Fattore et al., 2003). Finally, a cannabinoid-dependent mechanism has been reported in the reinstatement of heroin seeking behavior after a long period of abstinence (Fattore et al., 2003). In line with these findings, which tend to be less compelling across different studies as concerns CPP in $CB1^{-/-}$ (Parolaro and Rubino, 2008), also opioid receptor KO mice do not show

rewarding response to THC (Castane et al., 2003). Furthermore, even the involvement of the eCb system in alcohol dependence has been evaluated. In fact, using a genetically selected strain of animals called *Sardinian Preferring* (SP) rats, it has been shown that SR blocks the voluntary alcohol consumption in these animals (Colombo et al., 1998) and that SP rats show a spontaneously reduced eCb-mediated DSI with a general less effect of CB1-Rs agonists on GABA IPSCs as compared with controls (Melis et al., 2009). In addition, electrophysiological studies on BLA glutamatergic neurons have demonstrated that SR prevents ethanol-induced inhibition on these neurons, underlining an active involvement of CB1-Rs on alcohol-mediated effects in this subcortical area (Perra et al., 2008).

On the other hand, these results have not been replicated with cocaine and psychostimulants, leading to a controversial argumentation about the ineffective role of the eCb system in the modulation of cocaine and amphetamine-induced response in the VTA. In fact, although cocaine and amphetamine-induced SA and CPP are not blocked by either SR or genetic deletion of CB1-Rs (Martin et al., 2000; Cossu et al., 2001; Braida et al., 2005; Lesscher et al., 2005) and CB1-Rs blockade does not prevent cocaine-induced increase in DA release in the NAc (Soria et al., 2005), other investigations have reported opposite results. For example, according to some studies the eCb system plays a pivotal role in the acquisition of an operant response to self-administrable cocaine (Soria et al., 2005), SR is able to revert the breakpoint of cocaine-SA in rats under long access sessions (Orio et al., 2009) and SR has a preventive effect on cue and drug induced relapse to cocaine (De Vries et al., 2001; Filip et al., 2006; Wiskerke et al., 2008).

In addition, in recent years the possibility that eCb system manipulation might interfere also with nicotine-induced rewarding effects in animals and humans was also evaluated. This possibility has attracted attention, since both THC and nicotine are usually taken in combination by humans, and that reciprocally can interact with each other in several behavioral paradigms and physiological processes. For example, interactions between THC and nicotine were studied on locomotion, anxiety, nociception and heart rate (Pryor et al., 1978; Valjent et al., 2002) and it was also reported

that nicotine potentiated some THC-induced effects such as hypothermia, bradycardia (Pryor et al., 1978) and anxiolitic-like responses (Valjent et al., 2002). In addition, it was shown that co-administration of THC and nicotine leaded to an higher enhancement of c-Fos immunoreactivity in some of the most important areas of the brain reward circuitry (e.g. ShNAc, BNST, basolateral amygdala) (Valjent et al., 2002).

Furthermore, it was postulated a type of interaction between the eCb system and nicotine addiction. This hypothesis has been strengthened by results showing a rewarding effect of co-infusion of subthreshold doses of THC and nicotine using CPP measures (Valjent et al., 2002) and that THC reduced the incidence of withdrawal signs caused by nicotine (Balerio et al., 2004).

Moreover, in line with results obtained with morphine, it has been demonstrated that CB1-KO mice do not show any CPP for nicotine and that nicotine-induced CPP is blocked by the administration of SR (Le Foll and Goldberg, 2004). Conversely, other high impact studies have ruled out the involvement of CB1-Rs in nicotine SA under fixed-ratio schedule (Cossu et al., 2001; Castane et al., 2002). These findings seem to be in sharp contrast to other evidence about the action of rimonabant on nicotine-induced behavioral effects, and nicotine-induced DA release in the ShNAc (Cohen et al., 2002; Cohen et al., 2005; Cheer et al., 2007).

Finally, very recently it has been shown that pharmacological manipulation of the eCb system, through the inhibition of FAAH by URB597, negatively modulated nicotine-induced behavioral effects such as the CPP, SA and nicotine-induced DA release in the ShNAc (Scherma et al., 2008), thus, opening a new avenue in the study on how eCbs modulate drug addiction.

As mentioned before, one of the most intriguing characteristic of FAAH activity and its pharmacological inhibition is the capability to modulate the endogenous levels of NAEs and eCbs like AEA. Since FAAH blockade not only elevates AEA, but also NAEs levels (e.g. OEA and PEA) it is possible that at least a part of URB597 effect might be ascribed to NAEs rather than AEA, raising an interesting perspective in the pharmacological treatment of tobacco addiction.

5. Final remarks and purpose of our study

At first glance, all these findings underline a possible role of eCbs in the common neurobiological mechanism underlying drug addiction. As a matter of fact, beside the vast literature focused on eCbs, a current interest on the interaction between the eCb system and the effect of addictive substances is growing up. One reason which explains this specific attention might be ascribed to the lack of pharmacological strategies to treat an insidious and relapsing disorder like drug addiction. Hence, the possibility that manipulation of an endogenous system modulates drug-induced response might represent a new avenue for drug development.

As previously mentioned (chapter I), nicotine dependence might be a suitable candidate to test this possible involvement. This is due to different reasons, most of them discussed above, including previously described encouraging results obtained with CB1-R antagonists.

Even though in the present study it was first evaluated the actions of rimonabant on nicotine-induced excitatory effects on VTA DA neurons, we subsequently moved to the pharmacological inhibition of FAAH enzyme, since previous findings had shown a unexpected effect of URB597 on nicotine action (Scherma et al., 2008). Next, we focused on MSNs of the ShNAc, which are the main targets of DA released by VTA neurons, to evaluate whether FAAH inhibition modulates nicotine's action on these cells. To this aim, we carried out *in vivo* single unit extracellular recording from VTA DA neurons and MSNs of the ShNAc in urethane anaesthetized rats. Our results showed that FAAH inhibition modulates nicotine effects both in the VTA and in the NAc. Furthermore, we discovered that NAEs, rather than classical endocannabinoids, are involved, suggesting the possibility of an intriguing cross talk between CB1-R and PPAR-α activation. These results have been sequentially presented under abstract form in several conferences (e.g. Luchicchi et al., 2008; 2009a; 2009b; 2009c; 2010), and in three peer reviewed articles (Melis et al., 2008; Luchicchi et al., 2010; Mascia et al., 2010) (see appendix).

Chapter III

MATERIALS AND METHODS

Experiments were performed in strict accordance with the Guidelines for the Care and Use of Mammalian in Neuroscience and Behavioral Research (National Research Council 2004) and EEC Council Directive of 24 November 1986 (86/609). We made all efforts to minimize pain and suffering, and to reduce the number of animals used. Male Sprague Dawley albino rats (250–350 g) (Harlan) were housed in groups of three to six in standard conditions of temperature and humidity under a 12 h light/dark cycle (with lights on at 7:00 A.M.), with food and water available *ad libitum*.

1. Experiments in the VTA

Animals were anaesthetized with urethane (1300 mg/kg, i.p.), their femoral vein was cannulated for intravenous administration of pharmacological agents, and they were placed in the stereotaxic apparatus (Kopf) with their body temperature maintained at 37±1°C by a heating pad. Thereafter, the scalp was retracted and two burr holes were drilled above the VTA (-6.0 mm anteroposterior from bregma, 0.3-0.6 mm lateral from midline) for the placement of a recording electrode and above the medial part of the ShNAc (+1.5 mm anteroposterior from bregma; 1 mm lateral from midline) for the placement of stimulating electrode. Since only cells identified as projecting to the ShNAc were isolated, we delivered electrical stimuli from the ipsilateral medial ShNAc through a formvar-coated stimulating stainless steel bipolar electrode (250μm tip diameter) (fig 4.1).

For intracerebroventricular drug administration, a guide cannula (23 gauge stainless steel) was placed into the ventricle ipsilateral to the recording side (1.0 mm posterior, 1.4 mm lateral to bregma and 4.0 mm ventral to the cortical surface). Structures were localized according to the

stereotaxic atlas of Paxinos and Watson (1997). Intracerebroventricular injections were made through a prefilled inner cannula (30 gauge stainless steel tubing) connected to a 50 µl Hamilton microsyringe, and extending 1.0 mm below the tip of the guide into the ventricle. Infusion rate was set at 2.5 µl/min by an electrically driven mini-pump.

Single unit activity of neurons located in the VTA (V 7.0–8.0 mm from the cortical surface) was recorded extracellularly with glass micropipettes filled with 2% pontamine sky blue dissolved in 0.5 M sodium acetate (impedance 2–5MΩ). Single unit activity was filtered (bandpass 500–5000 Hz), and individual spikes were isolated by means of a window discriminator (Digitimer), displayed on a digital storage oscilloscope (TDS 3012, Tektronics), and digitally recorded. Experiments were sampled on line and off line with Spike2 software (Cambridge Electronic Design) by a computer connected to CED 1401 interface (Cambridge Electronic Design). Single units were isolated and identified according to already published criteria (Grace and Bunney, 1983, , 1984a; Ungless et al., 2004). To evaluate their selective projection to the ShNAc, we deliver a 1Hz electrical stimulation through the stimulating electrode to observe an antidromic response on VTA DA neurons. Since only one cell was recorded per rat, VTA DA neurons were selected when all criteria for identification were fulfilled: firing rate <10 Hz, duration of action potential >2.5 ms, inhibitory responses to hind paw pinching. Bursts were defined as the occurrence of two spikes at an interspike interval ≤80 ms, and terminated when the interspike interval exceeded 160 ms (Grace and Bunney, 1983). At the end of each recording section, direct current (10µA for 15 min) was passed through the recording electrode to eject Pontamine sky blue, which allowed the identification of the recorded cells. Brains were removed and fixed in 8% formalin solution. The position of the electrodes was microscopically identified on serial sections (60 µm) stained with cresyl violet.

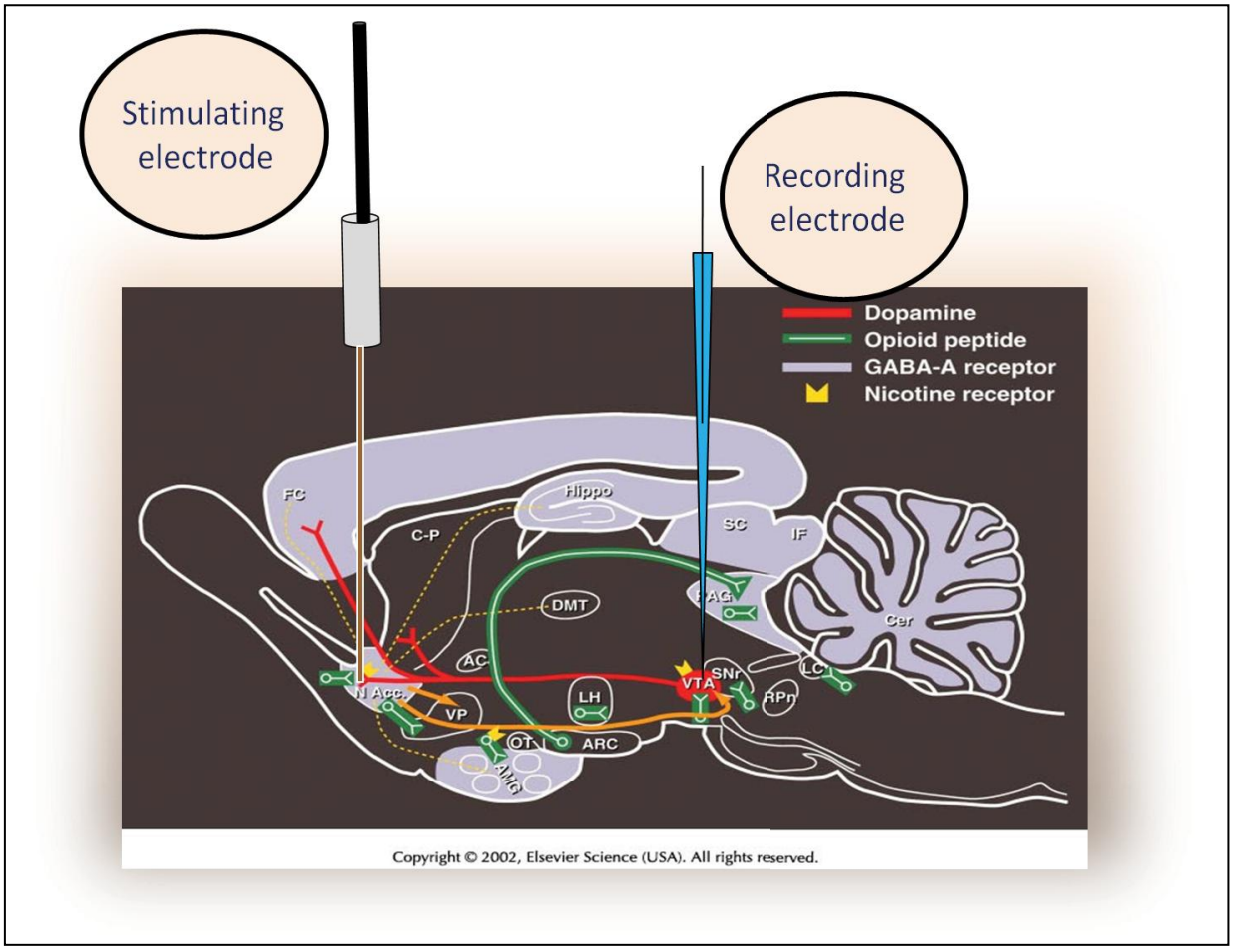


Fig 3.1. Experiments in the VTA. Schematic representation showing the protocol carried out for in vivo electrophysiological recordings from VTA DA neurons. Two burr holes are drilled above the VTA and ShNAc for the placement of a recording and stimulating electrode, respectively. When a neuron is isolated, current from the ShNAc is applied in order to evaluate the antidromic response of VTA DA neuron to NAc stimulation.

2. Experiments in the ShNAc

We recorded extracellularly single-unit activity of neurons located in the medial part of the NAc (shell) (1.5 mm anterior from bregma, 0.8–1.3 mm lateral from the midline, 6.5–7.0 mm ventral from cortical surface) using the same instruments previously described for the VTA experiments. In addition, because MSNs of the ShNAc do not fire spontaneously in anaesthetized animals, we delivered electrical stimuli in the basolateral amygdala (BLA) to evoke spike firing in the NAc cell.

For this reason, we inserted a formvar-coated stimulating stainless steel bipolar electrode with an inclination of 15° anteroposterior on the coronal plane (250 μm tip diameter) in the ipsilateral BLA (3.2 mm posterior from bregma, 5.0 mm lateral from the midline, 7.0 mm ventral from the cortical surface) (fig 4.2), which is a major excitatory projecting area to the NAc. After the glass electrode had been positioned to the dorsal limit of the NAc, we searched cells that responded to the stimulation of the BLA. Stimuli (~0.5 mA) were delivered to the BLA at 1-second intervals, while the microelectrode was lowered incrementally through the NAc. When a cell was detected, we adjusted the position of the microelectrode in order to maximize the spike amplitude relative to background noise. We identified neurons that responded to BLA stimulation by their robust excitatory response (latency range 10–25 ms). We did not include in this study cells whose latencies were longer than 26 ms following BLA stimulation because they could exhibit a polysynaptic response component (Mulder et al., 1998). A graphical representation of BLA-evoked excitation of MSNs is reported in fig 3.2. The experimental protocol was essentially that published by Floresco et al. (2001) (Floresco et al., 2001) with some modifications (Pistis et al., 2002a). When we isolated a cell, we adjusted stimulation currents to approximately half-maximal intensity, such as ~50% of electrical stimuli (1 Hz) in the BLA elicited an action potential in the recorded cell. We calculated evoked spike probability by dividing the number of action potentials observed by the number of stimuli administered in 100-second periods. Once stable levels of evoked spike probability were achieved (< 10% changes over 10-15 minutes), we administered drugs intravenously and assessed spike probability every 100 seconds. Changes in spike probability were an index of changes induced by the studied compounds over the excitation of NAc cells evoked by BLA stimulation. As well as for VTA experiments, we recorded only one cell per rat.

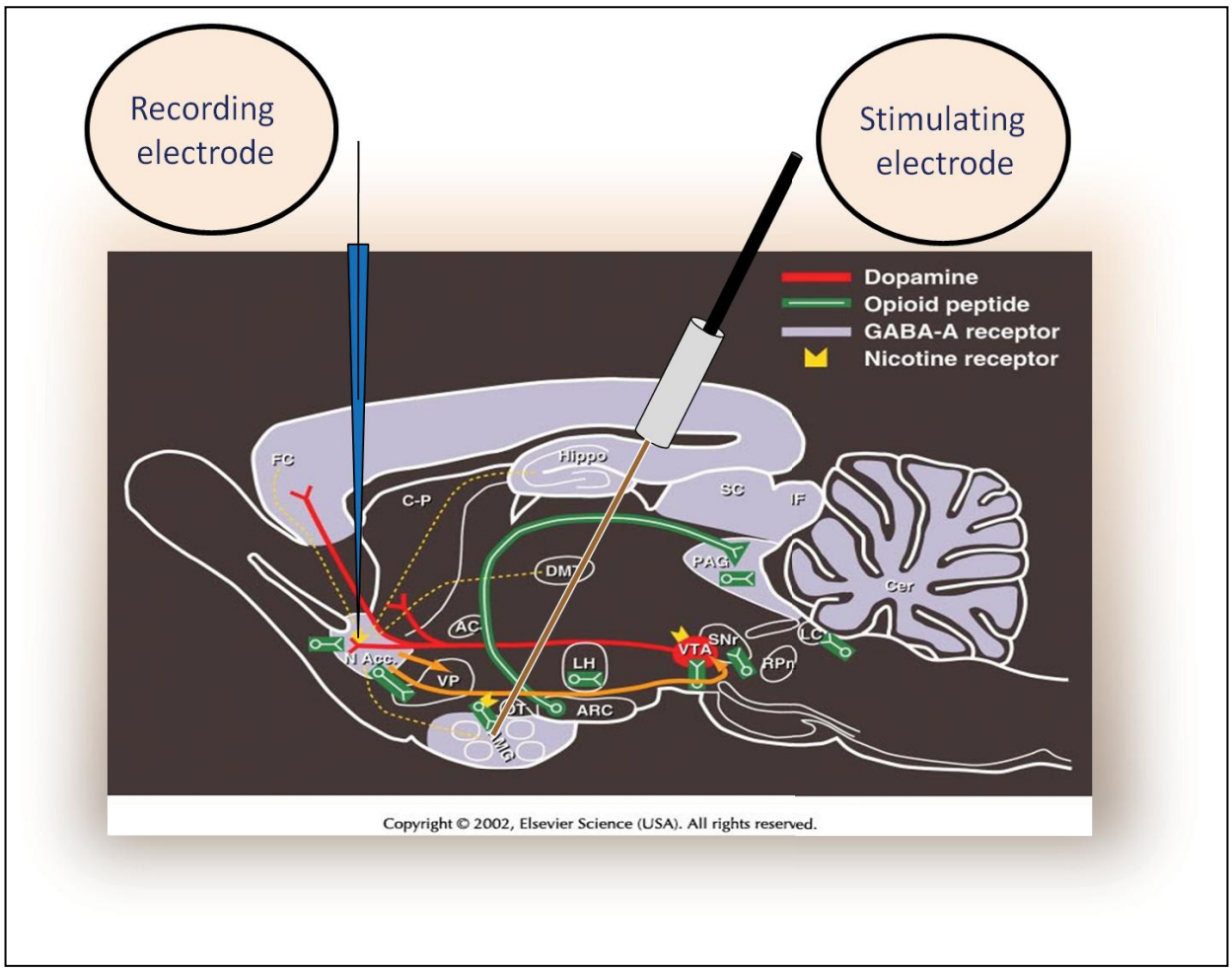


Fig 3.2. Experiments in the Shell of the NAc. Since medium spiny neurons (MSNs) of the ShNAc do not fire spontaneously in anaesthetized animals, a current was applied from the basolateral amygdala (BLA) in order to elicit an evoked spike firing of NAc neurons. For this reason the current was adjusted to obtain a 50% of probability to evoke a MSNs firing rate after BLA stimulation.

3. Statistical analysis

For VTA experiments, we calculated drug-induced changes in firing rate and pattern by averaging the effects after drug administration (2 minutes), and normalizing them to the predrug baseline. For ShNAc experiments, we determined predrug spike probability baseline as the mean of at least three consecutive assessments (100 seconds) over 10 minutes before drug administration. We generated

peristimulus time histograms (1-ms bins, 100 cumulative sweeps) by CED Spike2 software (Cambridge Electronic Design). Following drug administration, we calculated spike probability every 100 seconds, and normalized it to the predrug baseline. All the numerical data are given as mean±standard error of the mean. Data were compared and analyzed by using two-way analysis of variance (ANOVA) for repeated measures (treatment vs. time), or one-way ANOVA, or Student's *t*-test for repeated measures, when appropriate. *Post hoc* multiple comparisons were made using the Dunnett's, or Bonferroni's tests. We performed statistical analysis by means of the NCSS program (Kaysville, UT, USA). The significance level was established at $P < 0.05$.

4. Drugs

Nicotine [(–)-nicotine hydrogen tartrate] was purchased from Sigma (St. Louis, MO). Morphine chloridrate and cocaine chloridrate were purchased from S.a.l.a.r.s (Como, Italy), and Akzo Pharmadivision Diosynth (Oss, the Netherlands). SCH 23390 was purchased from Sigma/RBI, and L-sulpiride was purchased from Ravizza (Latina, Italy). Rimonabant (SR141716-A) was a generous gift of Sanofi-Aventis Recherche (Montpellier, France). URB597 was purchased from Alexis (Lausen, Switzerland). OEA, WY14643, MK886, methAEA, and fenofibrate, and clofibrate were purchased from Tocris (Bristol, UK). MethOEA was a generous gift of Dr. Steven R. Goldberg (NIDA, Baltimore, MD, US). We diluted nicotine, SCH 23390, L-sulpiride, cocaine and morphine in saline. We adjusted nicotine solution to pH = 7 with NaOH. We emulsified rimonabant in 1%Tween80 (Sigma, St Louis, MO, USA), then we diluted in saline and sonicated. We dissolved URB597 in dimethylsulfoxide (DMSO) (100 mg/ml) and diluted to the final concentration in saline. The final concentration of DMSO was 0.1%. We emulsified methOEA, WY14163, MK886, fenofibrate and clofibrate in 10% of Tween80, dissolved in 20% of DMSO and then diluted to the final concentration in distilled water. For i.c.v. administration, either OEA or methAEA were

dissolved in 40% w/v 2-hydroxypropyl-β-ciclodextrin, while methAEA for i.v. injection was dissolved in 2% of Tween80 and 2% of ethanol, and then diluted in saline.

Chapter IV

RESULTS

1. Blockade of CB-1Rs did not affect nicotine-induced effects on VTA DA neurons

We first addressed whether the electrophysiological effect of nicotine on VTA DA neurons is prevented by the CB1-R antagonist SR141716-A (SR, rimonabant). To this aim, we recorded the electrical activity of single VTA DA neurons in anaesthetized animals, and we selected neurons only when they fulfilled all the criteria already reported in literature (see methods). A typical VTA DA neuron waveform, acquired from a digital storage oscilloscope, is presented in fig 4.1. Only one cell was recorded per rat. Moreover we restricted our sample to cells which responded to the stimulation of ShNAc. A total of 183 VTA DA neurons were recorded. We recorded the spontaneous activity of VTA DA neurons for at least 5 minutes, followed by a single administration of vehicle. Then, after 4 minutes of acquisition we administered nicotine (0.2 mg/kg i.v.).

As previously reported in literature (Mereu et al., 1987; Erhardt et al., 2002; Mameli-Engvall et al., 2006), nicotine (0.2 mg/kg i.v.) enhanced the firing rate (144.2±24.2% of baseline firing rate; $F_{(5,71)}$=4.06; n=23; p<0.05; one-way ANOVA for repeated measures and Dunnett's test vs. baseline) and burst firing (+10.6±3.8% of baseline; $F_{(5,71)}$=2.89; n=23; p<0.05; one-way ANOVA for repeated measures and Dunnett's test vs. baseline) of VTA DA neurons identified as projecting to the ShNAc (fig 4.1 c, d), while the administration of vehicle was ineffective *per se* (data not shown). This stimulatory effect, which is common to other drugs of abuse (e.g. opioids, cannabinoids and alcohol) enables the activation of the mesolimbic circuitry and allows the release of DA in the NAc.

The administration of the CB1-R antagonist SR (0.5 mg/kg i.v.), 4 minutes before nicotine administration, did not abolish nicotine-induced activation of VTA DA neurons ($F_{(1;103)}$=1.10, n=11; p>0.05; two-way ANOVA and Bonferroni's test vs. controls) (fig 4.1 c, d). This result is in sharp

contrast to previous findings obtained through other techniques, where it was found that SR blocks neurochemical and behavioral effects of nicotine when tested with paradigms suggestive of addicting properties (Cohen et al., 2005; Cheer et al., 2007).

2. URB597 fully prevented nicotine-induced effects on VTA DA neurons: involvement of CB1-Rs and PPAR-α

Since it has been already reported that FAAH blockade by URB597 fully prevents nicotine-induced self administration, nicotine-induced conditioned place preference and nicotine-induced increase of DA release in the ShNAc (Scherma et al., 2008), our next step was to evaluate whether FAAH inactivation might also modulate the electrophysiological effects of nicotine on the VTA DA neurons. Surprisingly, URB597 pretreatment (0.1 mg/kg i.v., 1-2 hours before recording) abolished nicotine-induced excitation of VTA DA neurons both on firing rate (74.1±6.2% of baseline firing rate; $F_{(1,95)}$=4.95; n=6; p<0.01; two-way ANOVA and Bonferroni's test vs. controls) and burst firing (-16.8±4.1% of baseline burst firing; $F_{(1,107)}$=7.98; n=6; p<0.01; two-way ANOVA and Bonferroni's test vs. controls) (fig 4.1 c, d) causing, on the other hand, a transient inhibition (firing rate: $F_{(3,23)}$=3.73; n=6; p<0.001; one-way ANOVA for repeated measures vs. baseline; burst: $F_{(3,23)}$=9.44; n=6; p<0.01; one-way ANOVA for repeated measures vs. baseline). The administration of URB597, per se, did not induce any change in spontaneous firing activity of VTA DA cells (firing rate: 3.7±0.26 Hz; n=48, in control animals vs. 3.9±0.14; n=14, in URB597 pretreated animals; p>0.05; Student's T test; burst firing: 22.6±3.64% of spikes in burst; n=48, in control animals vs. 23.0±5.15%; n=14, in URB597 pretreated animals; p>0.05; Student's T test) (fig 4.1 e).

Next, we addressed whether URB597-induced effects were due to the activation of CB1-Rs by AEA. To this aim, we pretreated animals with the CB1-R antagonist SR (0.5 mg/kg i.v., 1 minute before URB597). In fact, SR prevented URB597 effect on firing rate of VTA DA neurons (123.7±11.5% of baseline; $F_{(1,23)}$=11.04; n=9; p<0.01; two way ANOVA and Bonferroni's test vs.

UB597) but was ineffective on burst firing (-18.4±8.0% of baseline; $F_{(1,23)}$=2.30; n=9; p>0.05; two way ANOVA and Bonferroni's test for repeated measures vs. URB597) (fig 4.2 a, b). The CB1-R antagonist AM251 (1.0 mg/kg i.v., 1 min before URB597) mimicked the effect of SR (firing: $F_{(1,61)}$=10.96; n=6; p<0.01; two way ANOVA and Bonferroni's test vs.URB597; burst: $F_{(1,75)}$=0.00; n=6; p>0.05; two-way ANOVA vs.URB597) (fig 4.2 a, b). Since URB597 does not only enhance AEA levels, but also OEA and PEA levels, we asked whether the lack of effect of SR and AM251 on burst activity was due to the activation of PPAR-α. In fact, as discussed in chapter II, both OEA and PEA do not show affinity for CB1-Rs, but can be considered endogenous ligands for PPAR-α, a member of nuclear receptor transcription factor family widely expressed in the brain (Moreno et al., 2004). Hence, the pretreatment with the PPAR-α antagonist MK886 (3 mg/kg i.p.,15 min before URB597) fully prevented the effect of URB597 on nicotine-induced burst firing, showing no effect on firing rate (firing: $F_{(1,75)}$=0.00; n=13; p>0.05; two-way ANOVA and Bonferroni's test vs.URB597; burst: 10.5±3.5% of baseline; $F_{(1,72)}$=4.90; n=13; p<0.05; two-way ANOVA and Bonferroni's test vs.URB597) (fig 4.2 a, b). These results draw a more complicated *scenario*, which involves new aspects to take into account when studying the effects of nicotine on VTA DA neurons.

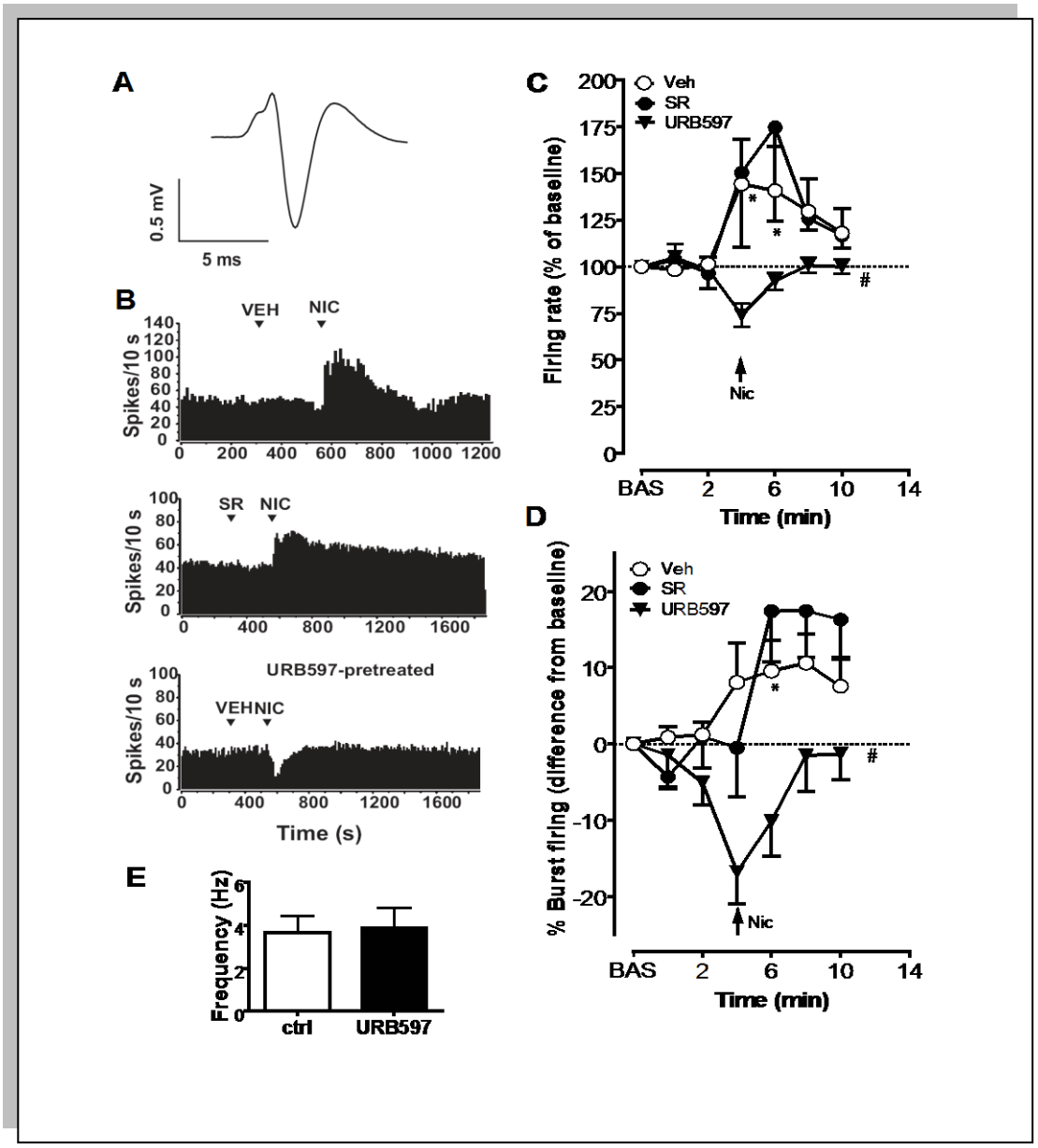


Figure 4.1. Effects of rimonabant and URB597 on activation of VTA dopamine neurons by nicotine. (**a**) Average trace, acquired from a digital storage oscilloscope, showing the typical waveform of a VTA DA neuron recorded from an anaesthetized rat. (**b**) Representative firing rate histograms illustrating effects of intravenous nicotine (NIC, injected at arrowheads) on discharge activity of individual VTA DA neurons recorded from anaesthetized rats. The top panel shows the typical response to 0.2 mg/kg nicotine in control conditions following intravenous injection of vehicle (VEH). The middle panel illustrates the lack of effect of rimonabant (SR, 0.5 mg/kg i.v.) on spontaneous firing rate of dopamine neurons and on the subsequent effects of nicotine. The bottom panel shows

the effect of nicotine in a URB597 pretreated animal, where nicotine induced a transient inhibition of firing activity. (**c; d**) Graphs illustrating the time course of nicotine's actions on firing rate and burst firing of VTA DA neurons. Pretreatment with URB597 (0.1 mg/kg, i.v.), but not rimonabant (0.5 mg/kg, i.v.), prevented the nicotine-induced increases in firing rate (**c**) and burst firing (**d**) of VTA dopamine neurons. (**e**) Histograms showing that URB597 pretreatment does not affect the baseline activity of recorded VTA DA cells ($p>0.05$, Student's t-test). Results are means, with vertical bars representing the SEM of firing rates and burst firing, expressed as a percentage of, or difference from, baseline (BAS) values. Arrows represent time of nicotine injection. *$p<0.05$ versus baseline (one-way ANOVA for repeated measures and Dunnett's test); # $p<0.01$ versus controls (two-way ANOVA and Bonferroni's test)

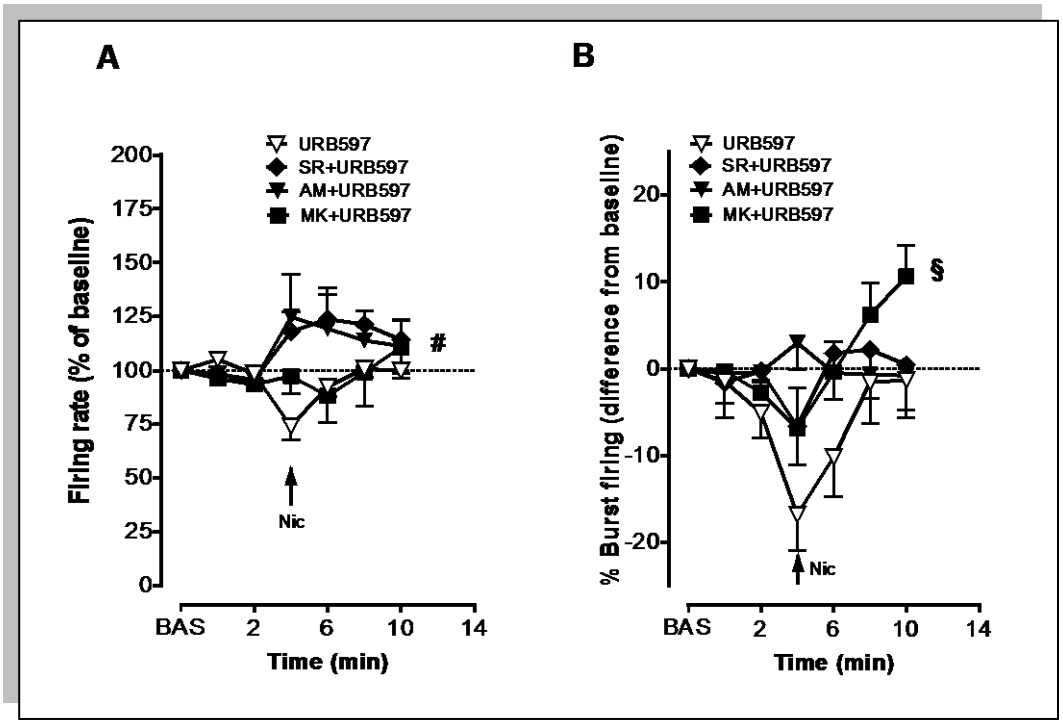


Figure 4.2. Contribution of CB1 receptors and PPAR-a to URB597's reversal of nicotine's effects. (**a**) The CB1 receptor antagonists rimonabant (SR, 0.5 mg/kg i.v.) or AM251(AM, 1.0 mg/kg, i.v.) reversed URB597's blockade of nicotine-induced increase in firing rate of VTA dopaminergic neurons (NIC, 0.2 mg/kg), whereas the PPAR-a antagonist MK886 (MK, 3 mg/kg, i.p.) was ineffective. (**b**) In contrast, MK886 administration reversed URB597's blockade of nicotine-induced increase in bursting activity of VTA dopamine neurons, whereas SR and AM251 were ineffective. Results are means, with vertical bars representing

the SEM of firing rates and burst firing, expressed as a percentage of, or difference from, baseline (BAS) values. Arrows represent time of nicotine injection. # $p<0.05$, SR and AM251 versus controls (two-way ANOVA and Bonferroni's test); § $p<0.05$, MK versus controls (two-way ANOVA and Bonferroni's test)

3. Oleoylethanolamide (OEA), but not methyl-anandamide (methAEA) mimicked URB597 effects

To determine the precise contribution of CB1-R and PPAR-α in the modulation of nicotine effects on VTA DA neurons, we next addressed whether the FAAH resistant analogue of AEA, methAEA prevented the actions of nicotine. To this aim we injected methAEA at doses of 1 and 5 mg/kg i.v. or 5µg/5µl i.c.v. Interestingly, methAEA did not mimic the effects of URB597 (i.e. blockade of the excitatory effect of nicotine) (methAEA 5mg/kg firing: $F_{(3,234)}=0.68$; n=6; $p>0.05$; two-way ANOVA and Bonferroni's test vs. controls) (fig 4.3 b), suggesting that CB1-Rs are scarcely involved in the modulation of nicotine-induced effects on VTA DA neurons.

Furthermore, to test the involvement of PPAR-α, we administered the PPAR-α endogenous agonist OEA. Since OEA is metabolically unstable, we chose to inject it in the lateral ventricle (20 µg/5µl) 4 minutes before nicotine. OEA experiments were compared with controls receiving the same vehicle used to dilute OEA (40% w/v 2-hydroxypropyl-β-cyclodextrin). Interestingly, OEA, without producing any effect *per se*, fully prevented nicotine-induced excitation of VTA DA neurons both on firing rate (92.7±13.5% of baseline; $F_{(1,99)}=5.61$; n=6; $p<0.05$; two-way ANOVA and Bonferroni's test vs. controls), and burst firing ($F_{(1,107)}=4.28$; n=6; $p<0.05$; two-way ANOVA vs. controls). Moreover, the PPAR-α antagonist MK886 (3 mg/kg i.p., 30 minutes before recording) blocked the effects of OEA (firing: 122.8±7.2; $F_{(1,55)}=6.06$; n=8; $p<0.005$; two way ANOVA and Bonferroni's test vs. OEA) (fig 4.3 d), thus restoring the excitatory action of nicotine. These data

strongly highlight the contribution of PPAR-α in the modulation of nicotine effect on mesolimbic DA neurons.

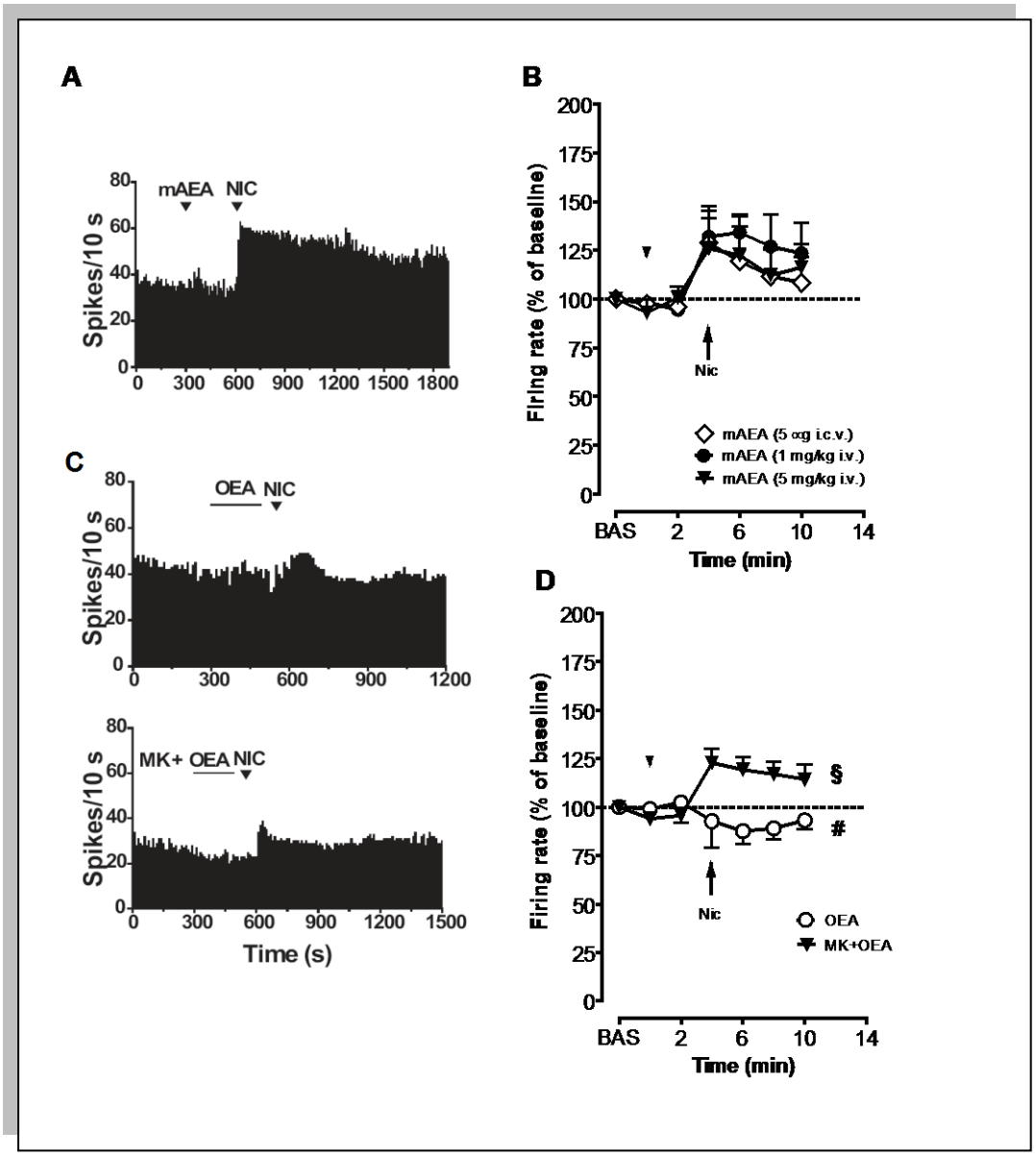


Figure 4.3. OEA, but not methAEA, prevented increases in firing rate of VTA dopaminergic neurons produced by nicotine. (**a**) Representative firing rate histograms showing the effects of nicotine (NIC, 0.2 mg/kg i.v., injected at arrowheads) on the discharge activity of individual VTA dopamine neurons recorded following injection of methAEA(1 mg/kg, i.v.). (**b**) Graph illustrating that nicotine-induced excitation of VTA dopamine neurons was not changed following the administration of methAEA, either intravenously (1 and 5 mg/kg) or intracerebroventricularly (5µg/5µl). (**c**) Representative firing rate histograms showing the effects of nicotine (0.2 mg/kg i.v., injected at arrowheads) on the discharge activity of individual VTA dopamine neurons recorded following injection of OEA (20

μg/5μl, i.c.v.; top). MK886 (MK, 3 mg/kg, i.p.) reversed the OEA-induced blockade of nicotine's effects (bottom). Neither OEA nor vehicle (40% w/v 2-hydroxypropyl-β-cyclodextrin) produced significant changes in spontaneous firing rate or burst firing. (**d**) Graph depicting that nicotine-induced excitation of VTA dopamine neurons was abolished by OEA. MK886 (MK, 3 mg/kg, i.p.) reversed the OEA-induced blockade of nicotine's effects. Results are means, with vertical bars representing SEM of firing rates, expressed as a percentage of baseline (BAS) values. Arrows represent the time of intravenous injections. The horizontal bar represents the time of intracerebroventricular administration. # $p<0.05$, OEA versus controls (two-way ANOVA and Bonferroni's test); § $p<0.05$, MK+OEA versus OEA (two-way ANOVA and Bonferroni's test).

4. The hydrolysis resistant analogue of OEA, methyl-oleoylethanolamide (methOEA) abolished nicotine effects on VTA DA neurons

Our next aim was to test the actions of different PPAR-α agonists to confirm our previous results with the endogenous NAE, OEA. For these studies, in control experiments the administration of nicotine was preceded by a single injection of the vehicle used to dilute different PPAR-α agonists (see methods), which was ineffective *per se*.

Consistent with previous results, the administration of the FAAH resistant analogue of OEA, methOEA (5 and 10 mg/kg i.v.) abolished nicotine-induced effects on VTA DA neurons (Firing rate: $F_{(1,48)}=5.24$; n=7; $p<0.05$; two-way ANOVA vs. controls; burst: $F_{(1,52)}=4.73$; n=7; $p<0.05$ two-way ANOVA and Bonferroni's test vs. controls) (fig 4.4 b, c), without affecting the baseline firing rate, or burst firing *per se* (3.18±0.5 Hz for methOEA 5 mg/kg vs. 3.24±0.2 Hz for controls; $p>0.05$; Student's T test 3.11±0.4 Hz for methOEA 10 mg/kg vs. 3.24±0.2 Hz for controls; $p>0.05$; Student's T test).

5. The synthetic PPAR-α agonist WY14643 (WY) abolished the effects of nicotine in the VTA

Our next step was to pretreat animals with the PPAR-α agonist WY14643 (WY). WY (40 mg/kg i.p., 1-2 hours before recording) blocked nicotine-induced enhancement of firing rate (95.76±5.71% of baseline; $F_{(1,48)}$=20.36; n=7; p<0.01; two-way ANOVA and Bonferroni's test vs. controls), and burst firing ($F_{(1,48)}$=5.98; n=*7;* p<0.05; two-way ANOVA and Bonferroni's test vs. controls) (fig 4.4 e, f). WY pretreatment had any effect neither on firing rate nor burst firing of VTA DA neurons (3.2±0.6 Hz for WY 20 mg/kg vs. 3.24 ±0.2Hz for controls; p>0.05; Student's T test; 3.3±0.6 for WY 40 mg/kg vs. 3.24±0.2 for control; p>0.05; Student's T test). Moreover, pre-administration of the PPAR-α antagonist MK886 (MK, 3 mg/kg i.p., 15 min before WY) abolished the effect of WY on these cells (firing: $F_{(1,60)}$=16.57; n=5; p<0.01; two-way ANOVA and Bonferroni's test vs. WY; burst: $F_{(1,60)}$=18.24; n=5; p<0.05; two-way ANOVA and Bonferroni's test vs. WY) (fig 4.4 e, f), thus confirming that WY acts through a PPAR-α dependent-mechanism. A lower dose of WY (20 mg/kg i.p.) partially, and not significantly, attenuated the effects of nicotine action (data not shown).

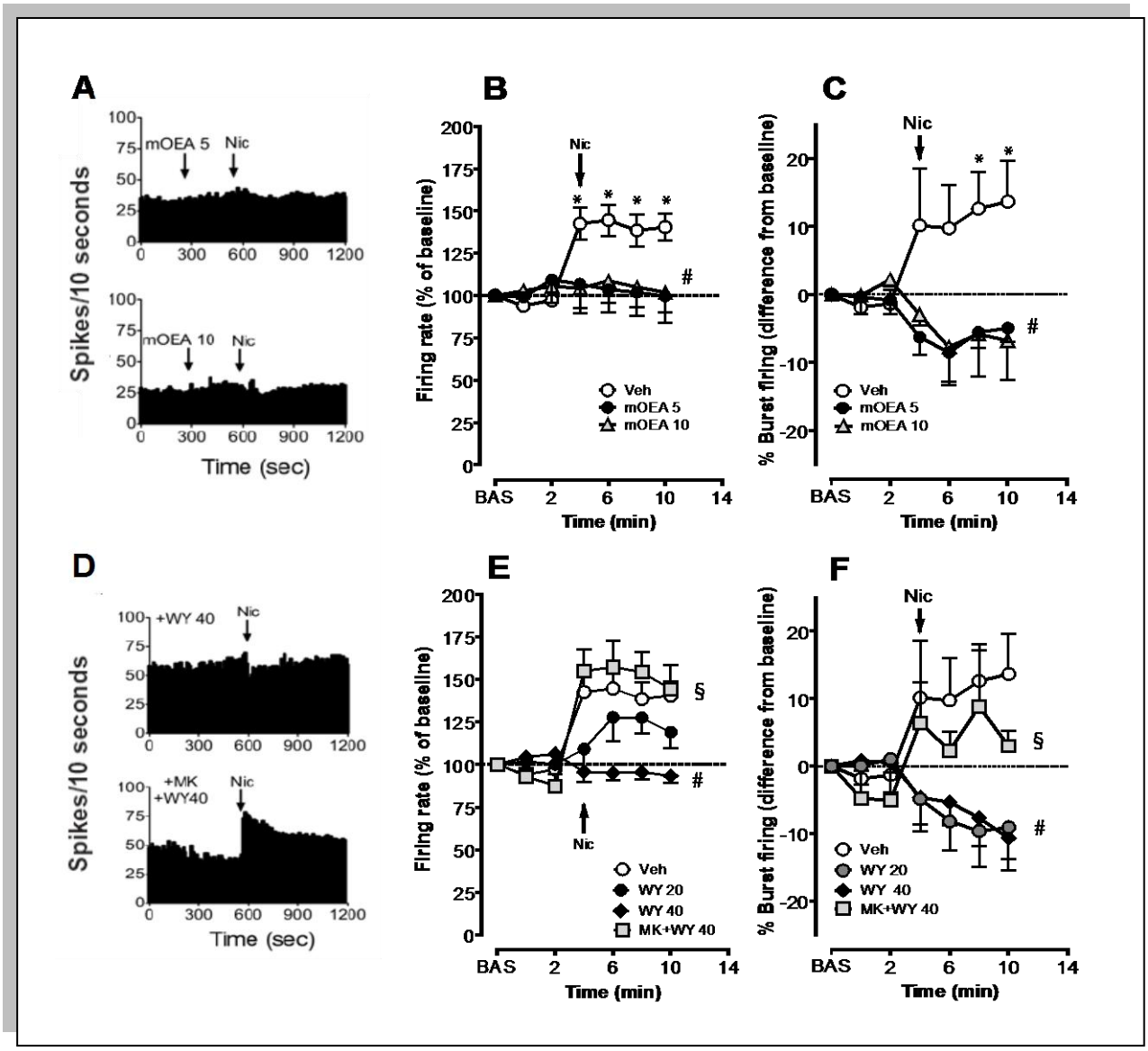


Figure 4.4. The PPAR-a agonists inhibited nicotine-induced activation of VTA dopamine neurons in anaesthetized rats. (**a, d**) Histograms showing the effects of PPAR-a agonists on discharge activity of individual VTA dopamine neurons after nicotine (Nic) administration. **(b, c)** The PPAR-a agonist meth-oleoylethanolamide (methOEA) (5 and 10 mg/kg intravenously injected 4 minutes before nicotine) significantly blocked nicotine-induced increases in firing rate and burst firing. (**e, f**) In line with methOEA experiments, WY1463 (40 mg/kg i.p. > 30 minutes before the start of recordings) blocked nicotine-induced increases in firing rate, and burst firing and the PPAR-a antagonist MK886 (3 mg/kg injected >45 minutes before the start of recordings) significantly abolished the effects produced by WY14643. Results are presented as mean ± SEM of firing rates and burst firing, expressed as percentages of, or differences from, baseline values, respectively. Arrows indicate time of drug injections.* $p<0.01$ versus baseline (one-way ANOVA and Dunnett's test); # $p<0.05$ versus controls (two-way ANOVA and Bonferroni's test); § $p<0.05$ versus WY (two-way ANOVA and Bonferroni's test) Nic, nicotine; MK, MK886; mOEA, methOEA; WY, WY14643.

6. Fenofibrate and clofibrate abolished nicotine effect in the VTA through a PPAR α-dependent mechanism

In addition, we tried to block nicotine action on mesolimbic DA neurons taking advantage of two PPAR-α agonists, fenofibrate and clofibrate, already used in clinical practice for the treatment of lipid disorders. Fenofibrate (200 mg/kg i.p., >1hour before recordings) abolished nicotine effects on VTA DA neurons (firing rate: 83.27±17.21% of baseline; $F_{(1;32)}$=6.59; n=5; p<0.05; two-way ANOVA and Bonferroni's test vs. controls; burst: -6.79±3.45; $F_{(1;36)}$=5.16; n=5; p<0.05; two way ANOVA and Bonferroni's test vs. controls) (fig 4.5 a, b) through a PPAR-α dependent mechanism, since this effect was reverted by the pre-administration of MK (3 mg/kg i.p., 15 minutes before fenofibrate) (firing rate: 149.78±11.14% of baseline; $F_{(1;32)}$=6.59; n=5; p<0.05; two-way ANOVA and Bonferroni's test vs. fenofibrate; burst: 21.62±9.42% of baseline; $F_{(1;32)}$=5.14; n=5; p<0.05; two-way ANOVA and Bonferroni's test vs. fenofibrate) (fig 4.5 a, b). Clofibrate (300 mg/kg i.p., >1 hour before recordings), consistently, prevented nicotine-induced increase in firing rate (97.00±5.04% of baseline; $F_{(4;48)}$=17.59; n=7; p<0.01; two-way ANOVA and Bonferroni's test vs. controls), and burst firing ($F_{(4;44)}$=4.480; p<0.01; two-way ANOVA and Bonferroni's test vs. controls) of VTA DA neurons (fig 4.5 c, d). These data suggest PPAR-α agonists as possible new treatment of tobacco addiction.

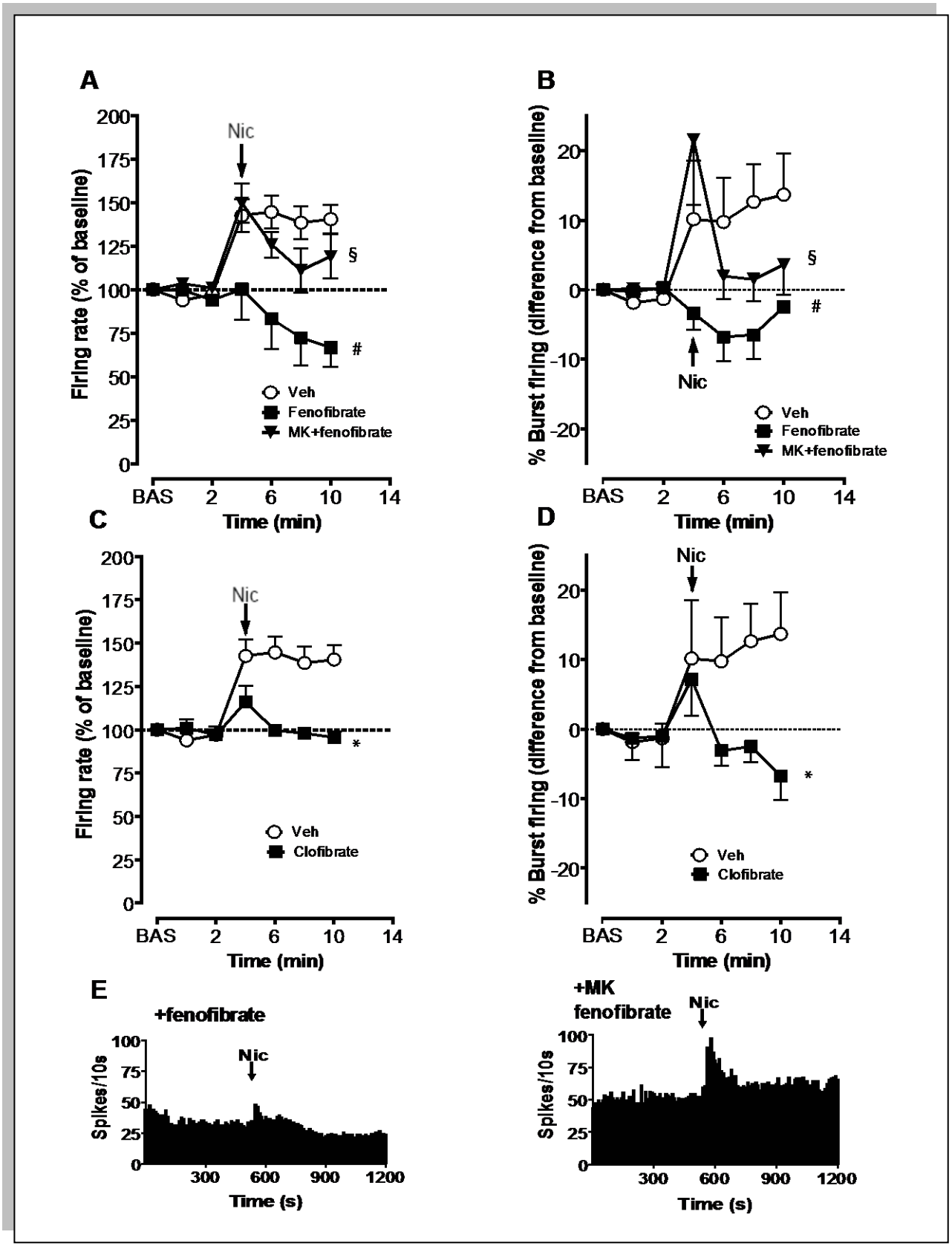


Figure 4.5. Fenofibrate and clofibrate blocked nicotine-induced effects on VTA dopamine neurons. (**a**, **b**) The PPAR-a agonist fenofibrate (200 mg/kg i.p., >1 hour before recordings) blocked nicotine effects on VTA DA neuron firing rate and burst firing. The administration of MK886 (MK; 3 mg/kg i.p., 15 minutes before fenofibrate) abolished the effects produced by fenofibrate. (**c**, **d**) In line with fenofibrate experiments, also clofibrate (300 mg/kg i.p.,

>1 hour before recordings) prevented nicotine-induced effect both on firing rate, and burst firing of VTA DA neurons. (**e**) Representative firing rate histograms showing the action of nicotine in a fenofibrate- (left panel) and a MK+fenofibrate-pretreated rat (right panel). Results are presented as mean ± SEM of firing rates and burst firing, expressed as percentages of or differences from baseline values, respectively. Arrows indicate time of drug injections. # $p<0.05$ versus controls (two-way ANOVA and Bonferroni's test); § $p<0.05$ versus fenofibrate (two-way ANOVA and Bonferroni's test); *$p<0.05$ versus controls (two-way ANOVA and Bonferroni's test).

7. URB597 did not revert cocaine- and morphine-induced effects on VTA DA neurons

Since our previous results have shown that PPAR-α can modulate nicotine-induced effects, our next aim was to better clarify whether this effect was specific for nicotine or can be extended to other drugs of abuse. For this reason, we carried out experiments on VTA DA neurons to evaluate whether URB597 modified their electrophysiological response to the psychostimulant cocaine and the μ-opioid receptor agonist morphine.

Cocaine, in line with previous findings (Einhorn et al., 1988), depressed the firing rate and burst firing of VTA DA neurons in a long-lasting manner (firing: 61.62±9.35% of baseline; $F_{(5;30)}=5.996$; n=6; P<0.001; one-way ANOVA for repeated measures and Dunnett's test vs. baseline; burst: -16.42±6.14 of baseline level; $F_{(5;25)}=4.659$; n=6; P<0.01; one-way ANOVA for repeated measures and Dunnett's test vs. baseline) (fig 4.6 b, c). Using the above reported protocol of pretreatment, URB597 (0.1 mg/kg i.v.) did not prevent cocaine effects on VTA DA neurons (66.5±9.98% of baseline level; $F_{(1;60)}=0.0003$; n=6, P>0.05; two-way ANOVA and Bonferroni's test vs. vehicle+cocaine) (fig 4.6 b, c).

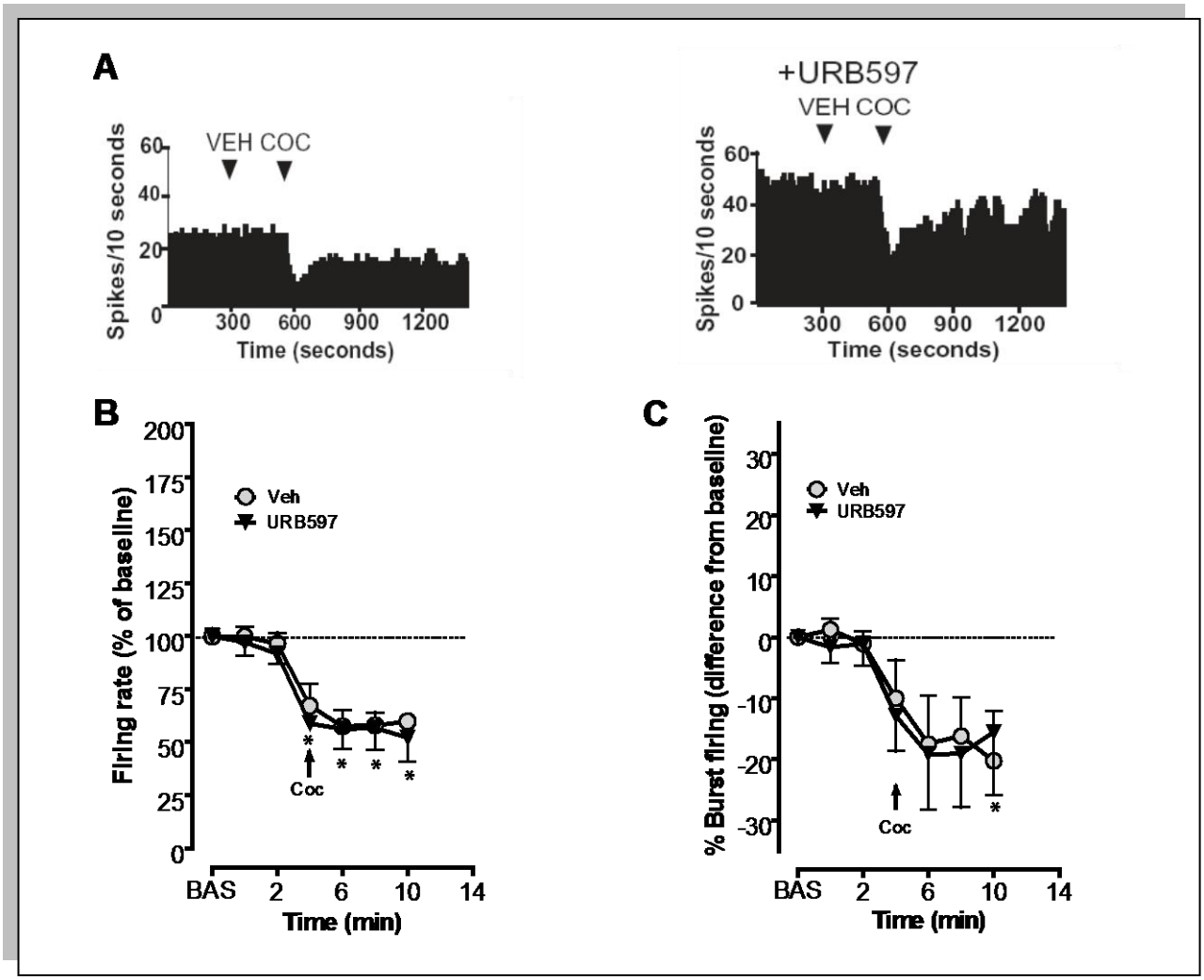


Figure 4.6. Effects of URB597 on the responses of VTA dopamine neurons to cocaine. (**a**) Representative firing rate histograms showing the decrease in firing rate of an individual VTA dopamine neuron produced by intravenous cocaine (COC, 1 mg/kg injected at arrowheads) in control conditions (left panel), and after URB597 (0.1 mg/kg i.v.) pretreatment (right panel). The injection of vehicle (VEH) is ineffective. (**b, c**) Graphs illustrating the time course of cocaine's effects on firing rate and burst firing of VTA DA neurons with and without URB597 pre-treatment. Results are means, with vertical bars representing the standard error of the mean of firing rates and burst firing, expressed as a percentage of, or difference from, the baseline (BAS). *$p < 0.01$ versus baseline (one-way ANOVA and Dunnett's test).

Morphine, consistently with the literature (Matthews and German, 1984), enhanced the firing rate and burst firing of DA neurons in the VTA (139.46±8.17% of baseline level; $F_{(4;20)}=3.299$; n=5; $P<0.05$; one-way ANOVA for repeated measures and Dunnett's test vs. baseline) (fig 4.7 b, c).

Pretreatment with URB597 failed to modify this effect (firing: 159.63±9.06 of baseline level; $F_{(1;40)}$=2.76; n=5, P=0.13 vs. vehicle+morphine; two-way ANOVA and Bonferroni's test; burst: +15.34±5.13 of baseline level; $F_{(1;40)}$=0.12; n=5, P>0.05 vs. vehicle+morphine; two-way ANOVA and Bonferroni's test) (fig 4.7 b, c). These results support the hypothesis that the effects of PPAR-α agonists are specific for nicotine.

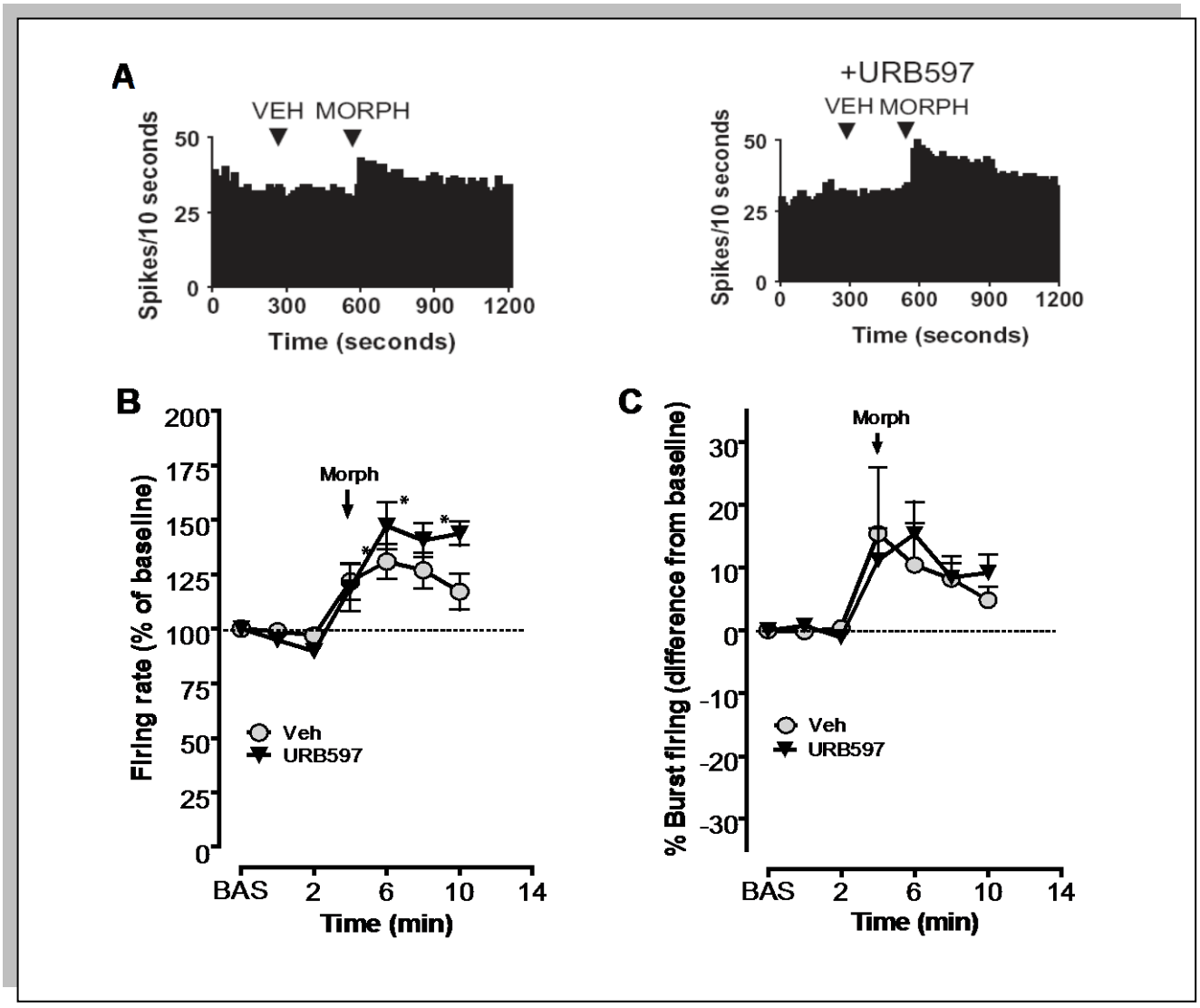


Figure 4.7. Lack of effect of URB597 on morphine-induced increases in firing rate and burst firing of VTA dopamine neurons. (a) Representative firing rate histograms showing that intravenous injection of morphine (MORPH, 4 mg/kg) enhances firing rate of VTA DA neurons in control conditions (left panel) and that this effect is not blocked by the administration of URB597 (0.1 mg/kg, i.v.,1- 2 hours before the recordings). (b, c) Graphical

depiction of the time course of firing rate (b) or burst firing (c) of VTA dopamine neurons following intravenous administration of morphine (MORPH, 4 mg/kg). Pre-treatment with URB597 (0.1 mg/kg, i.v.) did not alter the effects of morphine either on firing rate or burst activity of VTA dopamine neurons. Results are means, with vertical bars representing the standard error of the mean of firing rates and burst firing, expressed as a percentage of, or difference from, the baseline (BAS). *$p < 0.05$ versus baseline (one-way ANOVA and Dunnett's test).

8. URB597 abolished nicotine-induced inhibition of MSNs in the ShNAc

In the third part of our study, we addressed whether the "classical" and "non-classical" eCb systems may affect the actions of nicotine, cocaine, and morphine on medium spiny neurons (MSNs) of the ShNAc. The ShNAc is a crucial area of the reward circuitry, receiving an abundant projection from VTA DA neurons, and modulating the primary reinforcing properties of natural stimuli and drugs of abuse. A total number of 59 MSNs isolated in the medial part of the ShNAc were recorded. These neurons are normally quiescent in anaesthetized animals, and are excited by basolateral amygdala (BLA) stimulation, which is one of the major excitatory afferents. BLA stimulation evoked firing in MSNs of the ShNAc with a mean latency of 18.4±0.7 ms (fig 4.8 a). The average baseline spike probability following BLA stimulation was 46.3±1.5%. We recorded evoked activity of MSNs of the ShNAc for 300 seconds before the administration of nicotine, morphine, or cocaine. We recorded only one neuron per rat.

As previously described in literature (Hakan et al., 1993), nicotine (0.2 mg/kg i.v.) depressed the excitability of MSNs evoked by BLA stimulation (64±12% of baseline level; $F_{(5;40)}=3.44$, n=6, $P<0.01$; one-way ANOVA for repeated measures and Dunnett's test vs. baseline) in a long-lasting manner (fig 4.8 b). This inhibitory effect is due to a combination of activation of DA receptor type-1 and type-2 (D1-Rs, D2-Rs). Hence, the simultaneous administration of the D1R antagonist SCH 23390 (SCH; 1 mg/kg i.v.), and the D2-Rs antagonist L-sulpiride (10 mg/kg i.v.) abolished the

inhibitory response of these neurons after nicotine injection (122.5±10.6% of baseline level; $F_{(1;80)}$=14.09; n=6; P<0.001 vs. control; two-way ANOVA and Bonferroni's test) (fig 4.8 c). By contrast, neither the single injection of SCH nor L-sulpiride was able to revert nicotine effect on MSNs (SCH23390: $F_{(1;70)}$=0.05, n=6, P>0.05; L-sulpiride: $F_{(1;70)}$=0.02, n=6, P>0.05; two-way ANOVA and Bonferroni's test) (fig 4.8 c).

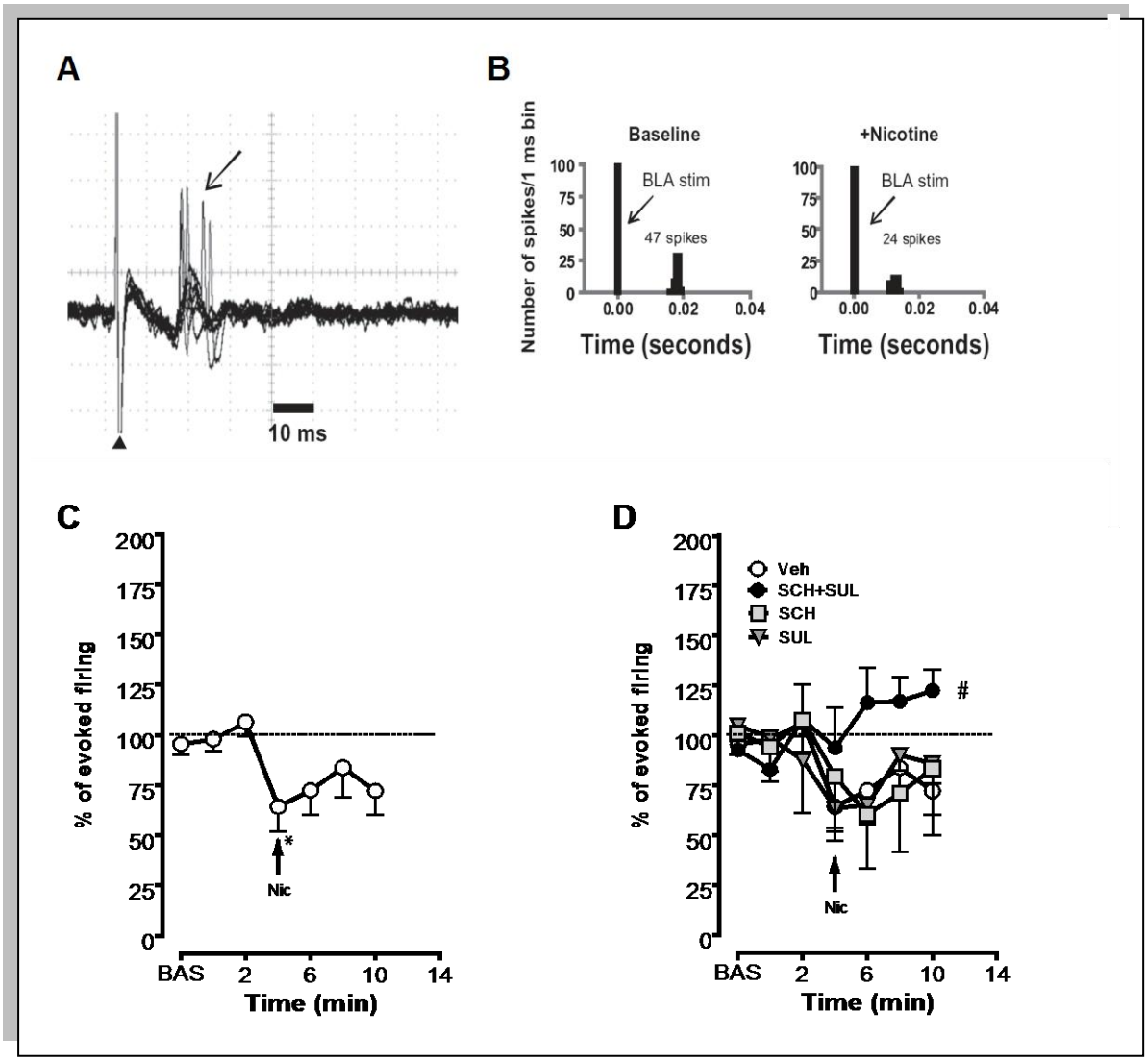


Figure 4.8. Nicotine depresses the excitability of (MSNs) in the shell of the nucleus accumbens (ShNAc). (**a**) Superimposed traces acquired from a digital storage oscilloscope showing a relatively constant latency of the orthodromic responses of a representative MSN after basolateral amygdala (BLA) stimulation. The arrowhead indicates the artifacts produced by BLA stimulation; the arrow shows evoked action

potentials of a MSN. Once a cell was isolated, the current applied to the BLA was adjusted to obtain ~50% of probability to elicit an action potential after a single pulse stimulation. (**b**) Representative peristimulus time histograms displaying the typical inhibitory response of a MSN in the ShNAc after BLA stimulation and injection of nicotine (0.2 mg/kg, i.v.). (**c**) Graph showing the time course of nicotine-induced inhibition of spike firing of MSNs. (**d**) Graphical depiction illustrating that nicotine-induced inhibition was prevented by the combined administration (at arrow), but not by the separate injection, of the D1-R antagonist SCH23390 (SCH, 1 mg/kg, i.v.) and the D2-R antagonist L-sulpiride (L-Sulp, 10 mg/kg, i.v.). Results are means, with vertical bars representing the standard error of the mean of evoked spike firing, expressed as a percentage of the baseline (BAS). *$p < 0.05$ versus baseline (one-way ANOVA and Dunnett's test); #$p < 0.05$ versus controls (two-way ANOVA and Bonferroni's test)

To test whether the eCb system might modulate nicotine-induced inhibitory effect in the ShNAc, we first carried out experiments with the CB1-Rs antagonist SR. In line with VTA experiments, the administration of SR (0.5 mg/kg i.v., 200 seconds before nicotine) failed to prevent nicotine-induced depression of MSNs excitability, suggesting that CB1-Rs are not involved (data not shown).

On the other hand, consistently with the results obtained in the VTA, URB597 pretreatment (0.1 mg/kg i.v., 1-2 hours before recording) abolished nicotine action in the ShNAc (126.6±15.6% of baseline level, $F_{(1;70)}$=9.03, P<0.01 vs. control; two-way ANOVA and Bonferroni's test) (fig 4.9 b). URB597 pretreatment did not show any effect *per se* on spike firing excitability of MSNs (1.52±0.16 mA vs. 1.9±0.4 mA, respectively; n=6; P>0.05; Student's T test), while nicotine administration after URB597 caused an increase in BLA-evoked MSNs excitability ($F_{(8;40)}$=3.32, n=6, P<0.01; one-way ANOVA for repeated measures and Dunnett's test vs. baseline).

These data prove that increasing levels of NAEs and eCbs, rather than CB1-R inactivation, is related to the modulation of nicotine rewarding properties in a crucial pathway of the brain reward circuitry.

9. SR141716-A and MK886 antagonized URB597 effects in the ShNAc

Next, we investigated whether the effects of URB597 depend on activation of CB1-R or PPAR-α. To this aim, we pretreated a group of animals with the CB1-R antagonist SR and another group with the PPAR-α antagonist MK886, both of them injected 15 minutes before URB597 administration.

In line with VTA experiments, both SR (0.5 mg/kg i.v.) and MK (3 mg/kg i.p.), unable to produce any effect *per se* (data not shown), reverted URB597 action on MSNs after nicotine administration (rimonabant+URB597 vs. URB597: 63.5±21.8% of baseline level; $F_{(1;63)}$=10.3, n=5, P<0.05, MK886+URB597 vs. URB597: 56.8,±16% of baseline level; $F_{(1;70)}$= 5.462, n=6, P<0.05, two-way ANOVA and Bonferroni's test) (fig 4.9 b, c). These results might suggest a possible cross-talk between classical eCb system and NAEs (see discussion).

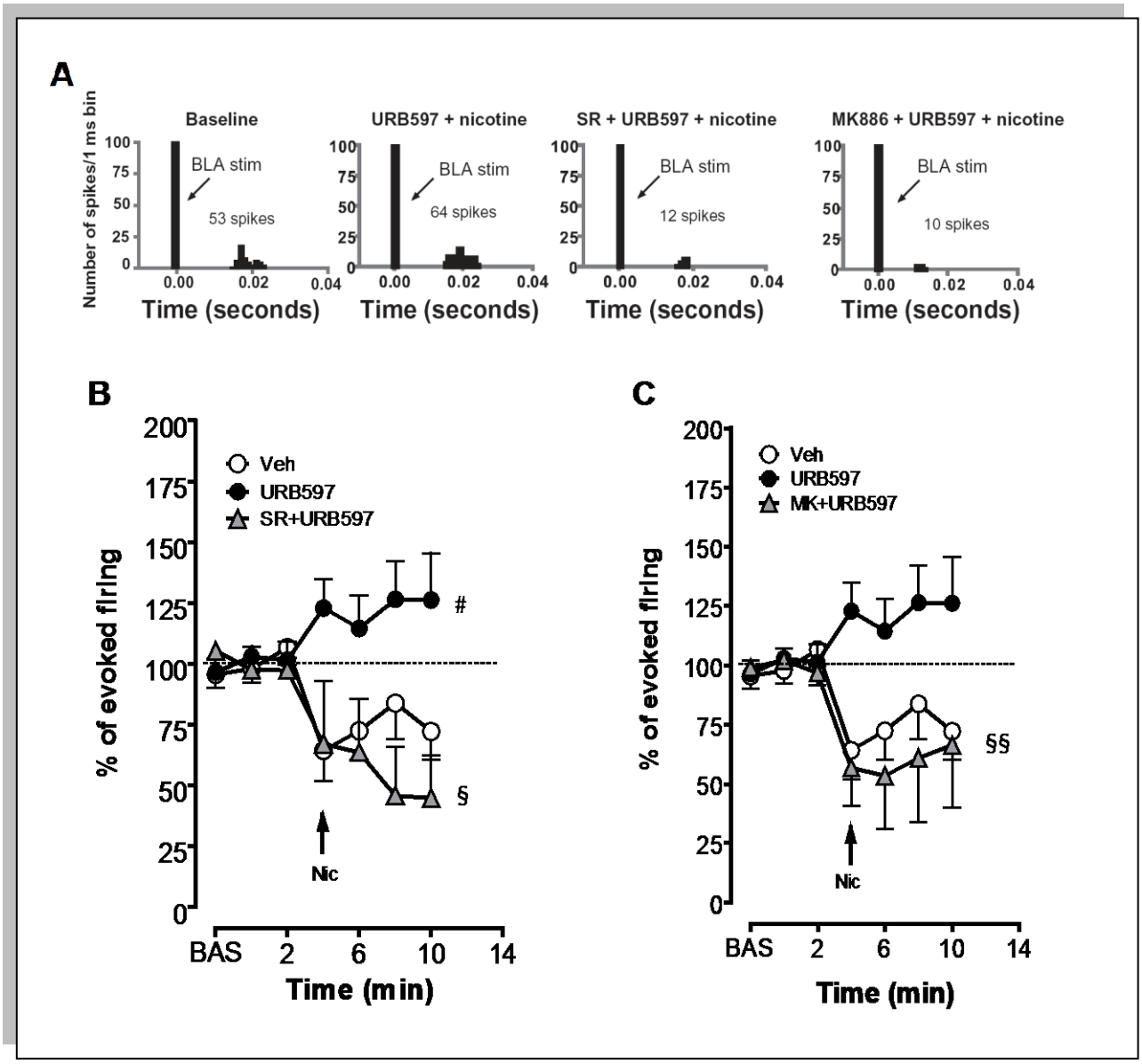


Figure 4.9. URB597 suppresses nicotine's action on MSNs in the ShNAc. (**a**) Exemplificative peristimulus time histograms showing that nicotine-induced decrease of MSN excitability is reversed by URB597, whereas the CB1-R antagonist rimonabant (SR, 0.5 mg/kg) and the peroxisome proliferator-activated nuclear receptor-a antagonist MK886 (3 mg/kg), administered 15 minutes before URB597, prevented the effects of the fatty acid amide hydrolase inhibitor, and restored nicotine-induced inhibition of MSN responses to basolateral amygdala (BLA) stimulation in the ShNAc. (**b,c**) Graphical depiction illustrating that URB597 pre-treatment prevented nicotine-induced inhibition of MSNs, and that this inhibition by nicotine was reversed by rimonabant (SR, 0.5 mg/kg, i.v.) (b), or MK886 (3 mg/kg, i.p.) (c). Results are means, with vertical bars representing the standard error of the mean of evoked spike firing, expressed as difference percentage of the baseline (BAS). #$p < 0.05$ versus controls (two-way ANOVA and Bonferroni's test),§ and §§ $p < 0.001$ versus URB597 (two-way ANOVA and Bonferroni's test).

10. URB597 blocked cocaine effects in the NAc

Since URB597 blocked nicotine effect on MSNs of the NAc, our last step was focused on testing whether the enhanced levels of eCbs and NAEs might also modulate cocaine and morphine action on these neurons.

In line with the literature (Hakan et al., 1987), we did not manage to obtain a stable time course of morphine on GABAergic MSNs. For this reason we carried out our experiments only with cocaine.

Cocaine (1 mg/kg i.v.), as already demonstrated (White et al., 1993), exerted a strong and long-lasting inhibitory effect on BLA-evoked MSN excitability (37.06±27.7% of baseline level; $F_{(6;48)}$=7.28, n=7, P<0.001, one-way ANOVA for repeated measures and Dunnett's test vs. baseline) (fig 4.10 b). Interestingly, URB597 pretreatment (0.1 mg/kg i.v.) abolished cocaine-induced effect on MSNs without affecting the baseline mean current applied to evoke a MSN excitation (95.3±15.1% of baseline level; $F_{(1;77)}$=11.97, n=6, P<0.01 vs. control, two-way ANOVA and Bonferroni's test) (fig 4.10 b).

This result strongly differs from those observed in the VTA experiment, where URB597 was not able to block cocaine, thus pointing to the involvement of different mechanisms by which FAAH blockade may act to prevent cocaine effects.

11. MK886 but not SR141716-A reverted URB597 effect on cocaine in the NAc.

Finally, we tried to elucidate the mechanism by which URB597 acts in the ShNAc to abolish cocaine-induced effects. In contrast, the effects of URB597 depended on the activation of PPAR-α, since the pretreatment with MK (3 mg/kg i.p., 15 minutes before URB597) (58.02±15.59% of baseline level; $F_{(1;70)}$=7.028, n=6, P<0.05 vs. URB597 pretreated animals, two-way ANOVA and Bonferroni's test) (fig 4.10 d), but not with SR (0.5 mg/kg i.v., 15 min before recording) (98.34±18.45% of baseline; $F_{(1;70)}$=0.04, n=6, P>0.05 vs. URB597 pretreated animals, two-way

ANOVA and Bonferroni's test) (fig 4.10 c), reverted URB597 action on cocaine response in the ShNAc. This last result provides evidence about a possible involvement *solo* of PPAR-α activation, which could opens the way for considering these receptors as modulators of reward-seeking behavior.

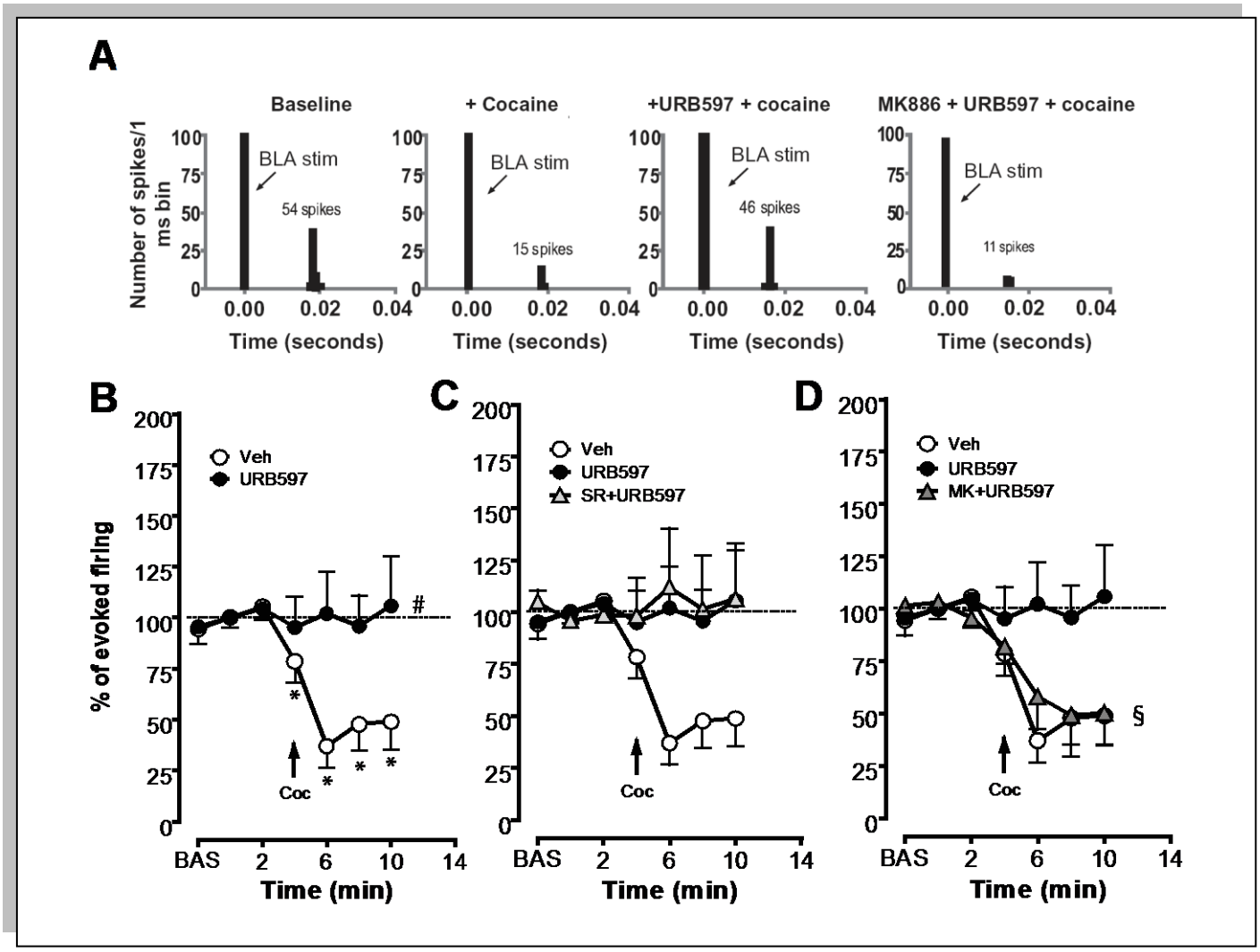


Figure 4.10. URB597 suppresses cocaine's action on MSNs of the shell of the ShNAc. (**a**) Representative peristimulus time histograms showing the response of recorded ShNAc MSNs after BLA stimulation. The probability of evoking MSN responses after BLA stimulation decreased after cocaine administration. Pre-treatment with URB597 reversed cocaine-induced inhibition of MSNs. The peroxisome proliferator-activated nuclear receptor-a antagonist MK886 blocked URB597 effect and restored cocaine-induced inhibition of MSNs. (**b–d**) Graphical depictions of the time course of cocaine's effects on MSN excitability in the ShNAc. Cocaine depresses the excitability of MSNs in a long-lasting manner (b). This effect was blocked by URB597, which fully prevented cocaine-induced inhibition (b). Pre-treatment with the CB1-R antagonist rimonabant (SR; 0.5 mg/kg, i.v.) did not alter URB597's blockade of cocaine's effect (c), whereas MK886 (3 mg/kg, i.p.) (d) completely prevented URB597's blockade of cocaine's actions and restored cocaine-induced inhibition of MSNs. Results are means, with vertical bars representing the standard error of the mean of evoked spike firing, expressed as a percentage of the baseline (BAS).

*$p < 0.05$ versus baseline, one-way ANOVA and Dunnett's test); #$p < 0.05$ versus controls, §$P < 0.05$ versus URB597 (two-way ANOVA and Bonferroni's test).

Chapter V

GENERAL DISCUSSION

In this thesis, it was shown that the manipulation of the eCb system, through inhibition of the enzyme FAAH, blocked nicotine addictive properties by the activation of both the conventional eCb and the "parallel" eCb-like systems, the latter comprising NAEs and their nuclear receptors.

In fact, the inhibition of FAAH by the carbamic acid derivate URB597abolished nicotine-induced enhancement of firing rate and burst firing of VTA DA neurons. The functional consequence of this effect are of importance, since mesolimbic DA neurons play a pivotal role in the acute responses to natural rewarding stimuli and drugs of abuse. Hence, increased DA neuron discharge rate and/or DA release in terminal areas such as the NAc, are among the first steps of the complex and multifaceted neural mechanisms and cellular pathways that lead to addiction (see chapter I).

Previous studies were focused on the involvement of the eCb system in the modulation of nicotine rewarding properties. For instance, early reports showed that the CB1-R antagonist rimonabant decreases nicotine-induced enhancement of DA outflow from the VTA to the NAc (Cohen et al., 2002; Cheer et al., 2007), and conditioned place preference (Le Foll and Goldberg, 2004). However, the electrophysiological data here reported do not support this hypothesis, since URB597 but not rimonabant, abolished the effects of nicotine on VTA DA neurons. This finding suggests that enhanced levels of eCbs, rather than blockade of cannabinoid receptors exert a preventive action on nicotine-induced effects in mesolimbic DA neurons. The reasons of the discrepancy between the lack of effects by rimonabant on neuronal responses to nicotine and its effects on neurochemical and behavioral actions of the drug are currently unknown. However, it might be possible that the suppression by rimonabant of evoked DA release may be independent from the inhibition of firing activity of DA neurons in the VTA, and may be an effect primarily involving their terminal regions or local circuits within the NAc.

Behavioral experiments carried out by Scherma and coll. (2008) support our electrophysiological results, since they demonstrated a significant effect of FAAH inhibition by URB597 in preventing the development of nicotine-induced conditioned place preference (CPP), acquisition of nicotine self-administration, nicotine reinstatement, and nicotine-induced increase of DA release in the ShNAc in rats (Scherma et al., 2008).

It must be pointed out that, despite the role of FAAH in the deactivation of the CB1-R agonist AEA, this enzyme also metabolizes other NAEs like the monounsaturated fatty acid oleoylethanolamide (OEA), and the saturated palmitoylethanolamide (PEA) (Kathuria et al., 2003; Fegley et al., 2005). Interestingly, even though OEA and PEA belong to the family of eCb-like molecules, they are devoid of CB1-R affinity, being ligands at the α-type peroxisome-proliferator activated receptors (PPAR-α), a subfamily of nuclear receptors and transcription factors highly implicated in several metabolic processes (Pistis and Melis, 2010) (see chapter II).

In line with these findings, in our study we observed that the conventional eCb system is not primarily involved in the mechanism of action of URB597. In fact, despite rimonabant prevented the effects mediated by URB597 on VTA DA neuron firing rate after nicotine administration, further experiments showed that the stable analogue of AEA, methAEA failed to mimic the action of the FAAH inhibitor. Conversely, we discovered that OEA blocked nicotine-induced electrophysiological effects on VTA DA cells. This result highlights the role of PPAR-α in the modulation of nicotine rewarding properties. Notably, this finding was also corroborated by patch-clamp experiments *in vitro,* where it was shown that OEA and PEA completely prevented nicotine-induced excitation of VTA DA neurons (Melis et al., 2008).

These results draw a very complex *scenario* which goes beyond our early hypothesis about the modulatory role of the "classic" eCb system in drug-induced effects, and they provide one of the first evidence about a potential, functional role of nuclear receptors in the pathophysiology of CNS diseases. In fact, although it has been recently demonstrated that PPAR-α is ubiquitously expressed in the brain (Moreno et al., 2004), and that OEA and PEA are identified as its endogenous ligands

(Fu et al., 2003), the characterization of its central function and role in brain physiology and pathophysiology remained widely unknown (Hansen, 2010). Similarly, centrally mediated effects of the PPAR-α agonist NAEs have been poorly investigated. Evidence suggests that OEA and PEA might be part of an independent eCb-like system, which exerts modulatory actions by the activation of PPAR-α, rather than CB1-Rs. Moreover, to support this observation, several studies underlined how NAEs are synthesized, released and inactivated independently to conventional eCbs (Hansen et al., 1995; Stella and Piomelli, 2001; Mackie and Stella, 2006).

The findings that the effects of OEA are blocked by the PPAR-α antagonist MK886, and mimicked by the synthetic agonist WY14643 (WY) and by the stable analogue of OEA, methOEA, give further support to the hypothesis that a PPAR-α-mediated mechanism is involved in the modulation of nicotine effects on mesolimbic DA neurons. Notably, also the modulatory effect of fibrates (fenofibrate and clofibrate), already used in the clinical treatment of dyslipidemia, on nicotine-induced actions strongly suggests that these compounds might possess a pharmacological spectrum that goes beyond the lipid sensor ability of PPAR-α (see chapter II).

In line with these results, it has been recently reported that WY and methOEA block the acquisition of nicotine self-administration, and suppress the reinstatement to nicotine seeking behavior in rats and squirrel monkeys through a direct effect on PPAR-α (Mascia et al., 2010).

Noteworthy, different studies by Sun and coll. (Sun et al., 2006; Sun et al., 2007) showed that also AEA displays affinity as an agonist for PPAR-α. However, our results do not support the idea that AEA might act as a PPAR-α agonist on DA neurons since, as already mentioned above, differently from OEA, methAEA did not suppress the effects of nicotine, and further *in vitro* experiments with AEA have confirmed our observation *in vivo* (Melis et al., 2008). In addition, other studies performed in an animal model of analgesia, have disconfirmed that AEA activates PPAR-α, given that the analgesic properties of this eCb were still preserved in PPAR-$\alpha^{-/-}$ mice, whereas the effects of OEA and PEA were abolished (LoVerme et al., 2006). These discrepancies might arise from the fact that the studies of Sun and coll. were carried out in HeLa cells transiently transfected with

PPAR-α, raising the issue that these investigations should be performed under more physiological conditions, and possibly in neurons, to confirm their results.

Besides the activation of PPAR-α, another alternative explanation of our observations might involve a noncompetitive antagonistic action of AEA and NAEs on nAChRs, as it was reported by several studies (Butt et al., 2002; Oz et al., 2003; Barrantes, 2004; Spivak et al., 2007; Butt et al., 2008). However, also in these cases studies were performed in *Xenopus oocytes*, or in mouse thalamic synaptosomes. We tend to exclude this hypothesis since our results show that chemically unrelated PPAR-α agonists are similarly effective, and that their actions are blocked by pharmacological antagonism of PPAR-α.

In this thesis it was also demonstrated that the modulation of both FAAH blockade and PPAR-α activation on VTA DA neurons is specifically associated to nicotine action, since URB597 had no effect on cocaine- and morphine-induced decrease and increase of DA neurons electrical activity, respectively. This finding suggests that PPAR-activation might exclusively target nAChRs (discussed below).

However, we found that the inhibition of FAAH does not specifically target nicotine action in the shell of the NAc, where URB597 blocked both nicotine- and cocaine-induced depression of MSN excitability after BLA stimulation. The ShNAc is crucially involved in the modulation of primary reinforcing properties of natural stimuli and drugs of abuse and reward-seeking behavior (Goto and Grace, 2008). A common electrophysiological feature of GABAergic MSNs is that they are depressed by the administration of the main addictive substances, including nicotine and cocaine (Carlezon and Thomas, 2009). This inhibitory action exerted by drugs of abuse has an important functional role since, by impairing GABAergic transmission to downstream areas encoding for hedonic qualities of stimuli, it disinhibits structures such as the ventral pallidum and VTA, and augment the rewarding value of the incentive (Carlezon and Thomas, 2009).

For this reason, our finding is of particular interest as it shows a modulatory role exerted by increased levels of conventional and unconventional eCbs in drug-induced response of MSNs.

Notably, our study in the ShNAc showed that URB597 action on nicotine-induced effects was prevented by either the administration of SR or MK886, underlining that URB597 effects might require both CB1 and PPAR-α activation. These data extend our results on VTA DA neurons, where both CB1 and PPAR-α played a role in URB597-mediated effect. Thus, this findings sheds some light into the probable cross-talk between these two systems, a possibility already taken into account to explain the effects of URB597 as a cognitive enhancer (Mazzola et al., 2009) .

Further electrophysiological studies have provided additional evidence about a synergistic effect between the eCb system and PPAR-α activation to explain how FAAH inhibition might modulate nicotine-induced effect. In fact, neither methAEA nor methOEA, affect nicotine-induced depression on BLA-evoked MSNs spike firing (Luchicchi, Goldberg and Pistis, work in progress). This lack of effect would suggest that URB597 modulation in the NAc might need the combined activation of both CB1-R and PPAR-α to exert its suppressant action.

Surprisingly, here it was also shown that after URB597 pretreatment, nicotine effect became excitatory rather than inhibitory, in the NAc. This enhancement of BLA-evoked excitability of MSNs might be parsimoniously explained by calling up a combination of different factors. Among them, it is likely that reduction of nicotine-induced DA release from the VTA to the NAc (Scherma et al., 2008), together with activation of CB1-Rs in the ventral striatum by AEA and the consequent depression of nicotine-induced GABA release, may unmask the increase of glutamate release induced by nicotine (Reid et al., 2000), which ultimately excites MSNs.

The finding that URB597 modulates the effects of cocaine in the ShNAc was rather unexpected, since FAAH blockade did not prevent cocaine action on VTA DA neurons. For this reason, this result makes the *scenario* of cocaine-eCb interactions quite complicated. In fact, studies have shown that (1) $CB1^{-/-}$ mice self-administer cocaine (Cossu et al., 2001), (2) rimonabant does not modify the development of cocaine induced CPP (Martin et al., 2000), and (3) URB597 does not alter cocaine self-administration in squirrel monkeys (Justinova et al., 2008). On the contrary, other investigations provided opposing results. For example, Cheer et al. (2007) demonstrated that SR

prevents cocaine-induced increase in DA levels in the NAc, and Orio et al. (2009) that rimonabant increases the breakpoint for cocaine self-administration under a fixed-ratio schedule in rats under long access sessions.

One possible explanation for the discrepancy between our study and that of Justinova and coll. might involve differences in experimental subjects (rats vs. monkeys), and the substantial difference in the experimental protocol used. In fact, while in self-administration studies the injection of cocaine followed 1-h session protocol, and URB597 effect was examined over three consecutive daily sessions, in our study we examined electrical responses of neurons from specific brain areas (ShNAc) to an acute intravenous dose of cocaine. This effect might suggest that URB597 acts by impairing drug-induced electrophysiological effect in discrete manner, whereas it is ineffective on drug-induced associated behavior. Another possibility is that URB597 might prevent the initial acute effects of cocaine. In line with this hypothesis, a recent study has shown that URB597 reduces cocaine reinstatement in abstinent animals, but not the initial cocaine self-administration (Adamczyk et al., 2009).

In the present experiments, some evidence indicating that CB1-Rs are not involved in the modulation exerted by URB597 on cocaine-induced effect in the ShNAc was also provided. In fact, this effect seems to be correlated more to the activation of PPAR-α, since the pretreatment with MK886 abolishes URB597 effects on MSNs. These data indicate that under these circumstances, FAAH inhibition results in the activation of both surface cannabinoid (by AEA) and nuclear receptors (by OEA/PEA).

Taken together, these findings provide evidence of a role for PPAR-α in the CNS, alone or in combination with CB1-Rs, in the modulation of drug-induced effects in the mesoaccumbens pathway, a circuit which mediates the rewarding response to addictive substances and, ultimately, produces some neurobiological changes that leads to abuse and addiction.

Finally, it is worth to mention that, even though the mechanism by which PPAR-α may modulate neuronal response to nicotine and cocaine in the VTA and ShNAc has not been clarify yet, different

hypothesis might be proposed to explain this effect. First of all, the rapid onset of agonist action suggests that PPAR-α may act through a non-genomic mechanism. In fact it is hard to reconcile our result with a classic genomic process of nuclear receptors, which requires a longer timeframe to produce an effect. In addition, studies *in vitro* carried out by our group strongly suggest that PPAR-α activation on DA neurons negatively modulate somatodendritic nAChRs by phosphorylating them (Melis et al., 2008; Melis et al., 2010). In fact, it was previously shown that functional properties of specific nAChRs subunits (such as α7) depend on the status of tyrosine phosphorylation of the receptor (Charpantier et al., 2005), and that phosphorylation/dephosphorylation of tyrosine residues in nAChRs controls the number of functional surface receptors (Cho et al., 2005).

For this reason it is likely that PPAR-α activation may trigger the phosphorylation of specific subunits of nAChRs on DA neurons by a tyrosin kinase-dependent mechanism. This hypothesis is confirmed by findings *in vitro* using the tyrosine kinases inhibitor genistein (Melis et al., 2008). Hence, inhibition of tyrosine kinases restores the excitatory properties of nicotine in the presence of PPAR-α agonists. Although we cannot identify which specific tyrosin kinase is involved in this mechanism, the nAchR subunits primarily involved in the interaction with PPAR-α have been identified. Indeed, in an elegant experimental design utilizing both patch-clamp experiments in rats and lentiviral expression mice, Melis and coll. (2010) demonstrated that PPAR-α decreases both DA neurons activity and VTA net output by negatively modulating the β2 subunit of nAChRs expressed in DA neurons. β2 subunits of nAChRs are critically involved in the switch from regular to activate state of DA neurons (Mameli-Engvall et al., 2006; Melis et al., 2010) and their negative modulation might reduce the responsiveness of DA neurons to external information (Melis et al., 2010). This effect might also explain why URB597 suppresses nicotine-induced depression of MSN in the ShNAc, which is a DA-dependent effect.

How PPAR-α modulated acute neuronal responses to cocaine in the present experiments is not known. Among possible explanations, a conservative hypothesis may involve a negative modulation exerted by PPAR-α agonists, such as OEA and PEA, on cholinergic transmission within

the ShNAc. In fact, cholinergic interneurons of the NAc were shown to modulate the response of MSNs (de Rover et al., 2002). In that study, the authors hypothesized that this effect occurred through an increase of GABAergic interneuron activity within the ventral striatum. These neurons receive inputs from the cholinergic neurons mediated by nAChRs, and their synapses impinge directly to MSNs. Moreover, other studies have demonstrated an increase in acetylcholine release in the NAc after psychostimulant exposure (Guix et al., 1992; Imperato et al., 1992; Bickerdike and Abercrombie, 1997). Since PPAR-α, activated by endogenous agonists OEA and PEA, specifically modulates nAChRs by inducing their inactivation through phosphorylation (Melis et al., 2008), by analogy, it is likely that PPAR-α activation within the NAc might modulate cocaine's response through inactivation of nAChRs in GABAergic interneurons. This should results in an impairment of GABA transmission to the MSNs that could explain the lack of inhibitory effect of cocaine on MSNs after URB597 pretreatment. Interestingly, interactions between OEA and PEA and acetylcholine transmission might be bidirectional, given that their biosynthesis is increased after stimulation of muscarinic receptors (Stella and Piomelli, 2001), which are present in the terminal regions of GABAergic interneurons (de Rover et al., 2002).

A schematic representation of PPAR-α dependent modulation on nicotine and cocaine effects is shown in fig 5.1.

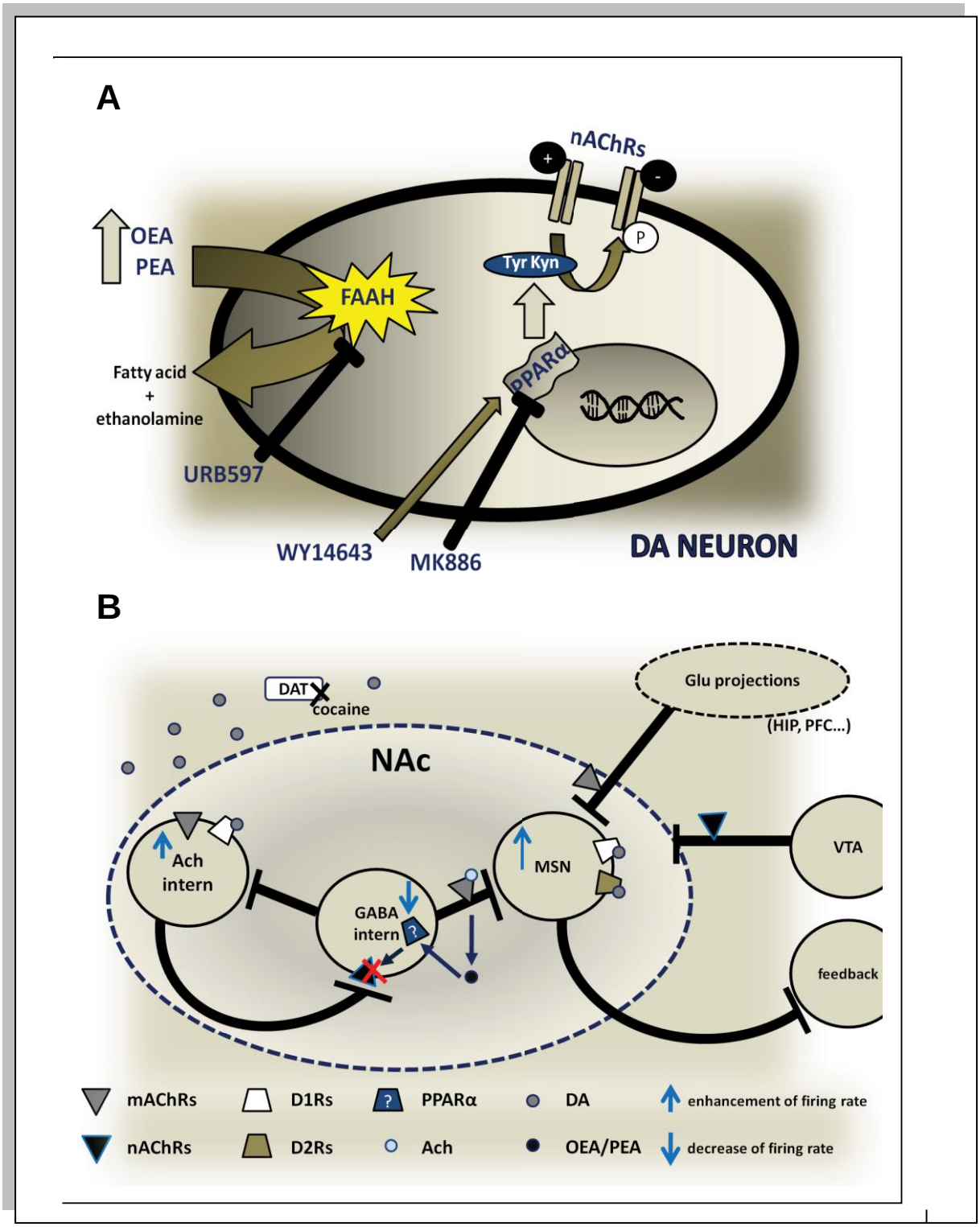


Fig 5.1. A possible mechanism of action by which PPAR-a activation modulates nicotine-induced effects on VTA DA neurons and cocaine-induced depression of MSNs excitability in the ShNAc. (**a**) Schematic diagram illustrating the proposed mechanism of PPAR-a activation, and modulation of responses of DA neurons to nicotine, by the noncannabinoid fatty acid ethanolamides OEA and PEA. Their action is mimicked by the synthetic PPAR-a agonist WY14643, and blocked by the PPAR-a antagonist MK886. URB597 enhances brain levels of OEA and PEA *in vivo* by inhibiting their major catabolizing

enzyme, FAAH. It is proposed that activated PPAR-a stimulates the activity of tyrosine kinases (Tyr Kin) through a nongenomic mechanism. Tyrosine kinases, in turn, induce the phosphorylation (P) of nAChRs, which reduces their responses to the agonists, or promotes rapid internalization. (**b**). Possible mechanisms underlying the modulation by PPAR-a on cocaine-induced effect in the ShNAc. Cocaine, by blocking DA transporters (DAT), increases DA levels in the NAc. In turn, activation of D1-Rs by DA on Ach interneurons (Ach intern) evokes increases of the release of acetylcholine (Ach), which activates GABA interneurons (GABA intern) through the activation of postsynaptic nAChRs (de Rover et al., 2002). Excitation of GABA interneurons depresses the excitability of MSNs. Hence, PPAR-a activation might modulate nAChRs activity by reducing Ach-mediated excitation of GABA interneurons, and modifying the subsequent GABA release to MSNs. This figure also shows that activation of muscarinic receptors (mAChRs) triggers the biosynthesis of OEA and PEA (Stella and Piomelli, 2001), contributing to create a bidirectional interaction between NAEs and acetylcholine transmission.

CONCLUSIONS AND FINAL REMARKS

Altogether, these findings suggest that FAAH inhibition, and the consequent PPAR-α activation by increased levels of FAAH substrates, one can abolish neurochemical, electrophysiological and behavioral effects of nicotine. Importantly our data are also corroborated by previous results obtained through different techniques (Melis et al., 2008; Scherma et al., 2008; Mascia et al., 2010; Melis et al., 2010). Overall, these observations provide a strong preclinical *rationale* and are predictive of a potential use of PPAR-α agonists in the treatment of tobacco addiction in humans. Since fibrates prevent nicotine-induced effects on VTA DA neurons, it seems reasonable to test these drugs, already used in the clinical treatment of metabolic disorders, for detoxification of nicotine addicted patients. Currently, there is no evidence about this possible effect of fibrates in humans, and doubts might arise whether these drugs do effectively reach appropriate concentration in the brain. However, a recent study by Porta and coll. (2009) has demonstrated that fenofibrate possesses anticonvulsant properties in rats (Porta et al., 2009), suggesting that these drugs might cross the blood-brain barrier and directly act in the CNS through a PPAR-α-dependent mechanism. Consistently, neuroprotective effects of PPAR agonists in CNS diseases such as Alzheimer, Parkinson and stroke have been reported (Heneka and Landreth, 2007). Indeed, among fibrates, fenofibric acid (the active metabolite of fenofibrate) does cross the blood-brain barrier though slowly (Deplanque et al., 2003), and its levels in the cerebrospinal fluid might actually be higher than generally expected. Noteworthy, Chikaisa and coll. (2008) report that a two-week feeding of bezafibrate phase advanced sleep-wake rhythm of about 2-3 h, increased the EEG delta-power in non-REM sleep, and attenuated its daily amplitude, thus ultimately supporting the central effects of fibrates (Chikahisa et al., 2008).

Therefore, fibrates might represent a new interesting avenue as a strategy for smoking cessation.

However, studies are needed to ascertain the possibility that patients already treated with fibrates, and with an history of tabagism, show a reduced intake of nicotine after a short- and long- term treatment with these drugs.

Additionally, a very recent study published in the journal *Obesity* has shown an interesting link between a common NAPE-PLD haplotype polymorphism and smoking cessation (Wangensteen et al., 2010), which may provide the first clinical evidence of an interaction between levels of NAEs and vulnerability to nicotine addiction.

Irrespective of whether or not studies on humans would confirm the hypotheses of the feasibility of PPAR-α activation as a pharmacological tool for quitting cigarette smoking, our studies identified new unsuspected players in the complex interplay between neurotransmitter and neuromodulators, which govern neuroadaptations induced by drugs of abuse, leading to addiction.

ACKNOWLEDGEMENT

A special thank goes to all the people who worked in this project. In particular, I would like to thank my supervisor, Prof. Marco Pistis, who has always trusted me, and who made me aware of the fascinating world of "research". Furthermore, I would like to thank Dr. Anna Lisa Muntoni, whose technical and theoretical supervision has been basilar to carry on my experiments, and Dr. Miriam Melis who thought me how to evaluate a "scientific problem" from different points of view. In addition, my thank goes to Dr. Giuliano Pillolla, who helped me to acquire some basilar technical skills and to Dr. Salvatore Lecca and Dr. Stefano Carta for their priceless help.

Furthermore, I would like to show gratitude to young and established researchers that I met during my studies, both in Italy and abroad, who have always provided me interesting suggestions about my project and to Dr. Philippe Faure, who was my supervisor during the internship at the "Pierre et Marie Curie University of Paris, France".

Finally, a special thanks goes to the "University of Cagliari" for the Ph.D. bursary, and to the "Regione Autonoma della Sardegna" for the Young Researchers Bursary. In addition I want to show gratitude to the "International Cannabinoid Research Society" (ICRS) and "European College of Neuropsychopharmacology" (ECNP) for their strong support to young scientists.

REFERENCES

Adamczyk P, McCreary AC, Przegalinski E, Mierzejewski P, Bienkowski P, Filip M (2009) The effects of fatty acid amide hydrolase inhibitors on maintenance of cocaine and food self-administration and on reinstatement of cocaine-seeking and food-taking behavior in rats. J Physiol Pharmacol 60:119-125.

Albuquerque EX, Pereira EF, Alkondon M, Rogers SW (2009) Mammalian nicotinic acetylcholine receptors: from structure to function. Physiol Rev 89:73-120.

Albuquerque EX, Alkondon M, Pereira EF, Castro NG, Schrattenholz A, Barbosa CT, Bonfante-Cabarcas R, Aracava Y, Eisenberg HM, Maelicke A (1997) Properties of neuronal nicotinic acetylcholine receptors: pharmacological characterization and modulation of synaptic function. J Pharmacol Exp Ther 280:1117-1136.

Alkondon M, Pereira EF, Barbosa CT, Albuquerque EX (1997) Neuronal nicotinic acetylcholine receptor activation modulates gamma-aminobutyric acid release from CA1 neurons of rat hippocampal slices. J Pharmacol Exp Ther 283:1396-1411.

American Psychiatric Association (APA) (2000) Diagnostic and Statistic Manual of Mental Disorders, Text Revision, DSM-IV TR, Masson

Anand R, Conroy WG, Schoepfer R, Whiting P, Lindstrom J (1991) Neuronal nicotinic acetylcholine receptors expressed in Xenopus oocytes have a pentameric quaternary structure. J Biol Chem 266:11192-11198.

Armitage A, Dollery C, Houseman T, Kohner E, Lewis PJ, Turner D (1978) Absorption of nicotine from small cigars. Clin Pharmacol Ther 23:143-151.

Armstrong DW, Wang X, and Ercal N (1998) Enantiomeric composition of nicotine in smokeless tobacco, medicinal products and commercial reagents. Chirality 10**:** 587-591.

Ashton JC, Friberg D, Darlington CL, Smith PF (2006) Expression of the cannabinoid CB2 receptor in the rat cerebellum: an immunohistochemical study. Neurosci Lett 396:113-116.

Auerbach A, Akk G (1998) Desensitization of mouse nicotinic acetylcholine receptor channels. A two-gate mechanism. J Gen Physiol 112:181-197.

Azad SC, Monory K, Marsicano G, Cravatt BF, Lutz B, Zieglgansberger W, Rammes G (2004) Circuitry for associative plasticity in the amygdala involves endocannabinoid signaling. J Neurosci 24:9953-9961.

Bachur NR, Masek K, Melmon KL, Udenfriend S (1965) Fatty Acid Amides of Ethanolamine in Mammalian Tissues. J Biol Chem 240:1019-1024.

Baker D, Pryce G, Davies WL, Hiley CR (2006) In silico patent searching reveals a new cannabinoid receptor. Trends Pharmacol Sci 27:1-4.

Baldwin IT (1989) Mechanism of damage-induced alkaloid production in wild tobacco. J Chem Ecol 15(5):1661-80

Balerio GN, Aso E, Berrendero F, Murtra P, Maldonado R (2004) Delta9-tetrahydrocannabinol decreases somatic and motivational manifestations of nicotine withdrawal in mice. Eur J Neurosci 20:2737-2748.

Barrantes FJ (2004) Structural basis for lipid modulation of nicotinic acetylcholine receptor function. Brain Res Brain Res Rev 47:71-95.

Basavarajappa BS (2007a) Neuropharmacology of the endocannabinoid signaling system-molecular mechanisms, biological actions and synaptic plasticity. Curr Neuropharmacol 5:81-97.

Basavarajappa BS (2007b) Critical enzymes involved in endocannabinoid metabolism. Protein Pept Lett 14:237-246.

Batkai S, Jarai Z, Wagner JA, Goparaju SK, Varga K, Liu J, Wang L, Mirshahi F, Khanolkar AD, Makriyannis A, Urbaschek R, Garcia N, Jr., Sanyal AJ, Kunos G (2001) Endocannabinoids acting at vascular CB1 receptors mediate the vasodilated state in advanced liver cirrhosis. Nat Med 7:827-832.

Beinfeld MC, Connolly K (2001) Activation of CB1 cannabinoid receptors in rat hippocampal slices inhibits potassium-evoked cholecystokinin release, a possible mechanism contributing to the spatial memory defects produced by cannabinoids. Neurosci Lett 301:69-71.

Bellone C, Luscher C (2006) Cocaine triggered AMPA receptor redistribution is reversed in vivo by mGluR-dependent long-term depression. Nat Neurosci 9:636-641.

Beltramo M, Piomelli D (2000) Carrier-mediated transport and enzymatic hydrolysis of the endogenous cannabinoid 2-arachidonylglycerol. Neuroreport 11:1231-1235.

Benowitz NL, Jacob P, 3rd (1984) Daily intake of nicotine during cigarette smoking. Clin Pharmacol Ther 35:499-504.

Benowitz NL, Hukkanen J, Jacob P, 3rd (2009) Nicotine chemistry, metabolism, kinetics and biomarkers. Handb Exp Pharmacol:29-60.

Benowitz NL, Porchet H, Sheiner L, Jacob P, 3rd (1988) Nicotine absorption and cardiovascular effects with smokeless tobacco use: comparison with cigarettes and nicotine gum. Clin Pharmacol Ther 44:23-28.

Benowitz NL and Jacob P 3rd (1998) Pharmacokinetics and metabolism of nicotine and related alkaloids, in Neuronal Nicotinic Receptors: Pharmacology and Therapeutic Opportunities (Arneric SP and Brioni JD eds) pp 211-234, Wiley-Liss, Inc., New York.

Benowitz NL, Perez-Stable EJ, Herrera B, Jacob P, 3rd (2002) Slower metabolism and reduced intake of nicotine from cigarette smoking in Chinese-Americans. J Natl Cancer Inst 94:108-115.

Benwell ME, Balfour DJ, Anderson JM (1988) Evidence that tobacco smoking increases the density of (-)-[3H]nicotine binding sites in human brain. J Neurochem 50:1243-1247.

Berger J, Moller DE (2002) The mechanisms of action of PPARs. Annu Rev Med 53:409-435.

Berrettini W, Yuan X, Tozzi F, Song K, Francks C, Chilcoat H, Waterworth D, Muglia P, Mooser V (2008) Alpha-5/alpha-3 nicotinic receptor subunit alleles increase risk for heavy smoking. Mol Psychiatry 13:368-373.

Bertrand D (2005) The possible contribution of neuronal nicotinic acetylcholine receptors in depression. Dialogues Clin Neurosci 7:207-216.

Bertrand D, Galzi JL, Devillers-Thiery A, Bertrand S, Changeux JP (1993) Mutations at two distinct sites within the channel domain M2 alter calcium permeability of neuronal alpha 7 nicotinic receptor. Proc Natl Acad Sci U S A 90:6971-6975.

Besson M, Granon S, Mameli-Engvall M, Cloez-Tayarani I, Maubourguet N, Cormier A, Cazala P, David V, Changeux JP, Faure P (2007) Long-term effects of chronic nicotine exposure on brain nicotinic receptors. Proc Natl Acad Sci U S A 104:8155-8160.

Biastoff S, Brandt W, Drager B (2009) Putrescine N-methyltransferase--the start for alkaloids. Phytochemistry 70:1708-1718.

Bickerdike MJ, Abercrombie ED (1997) Striatal acetylcholine release correlates with behavioral sensitization in rats withdrawn from chronic amphetamine. J Pharmacol Exp Ther 282:818-826.

Bisogno T, Berrendero F, Ambrosino G, Cebeira M, Ramos JA, Fernandez-Ruiz JJ, Di Marzo V (1999) Brain regional distribution of endocannabinoids: implications for their biosynthesis and biological function. Biochem Biophys Res Commun 256:377-380.

Bisogno T, MacCarrone M, De Petrocellis L, Jarrahian A, Finazzi-Agro A, Hillard C, Di Marzo V (2001) The uptake by cells of 2-arachidonoylglycerol, an endogenous agonist of cannabinoid receptors. Eur J Biochem 268:1982-1989.

Blazquez C, Chiarlone A, Sagredo O, Aguado T, Pazos MR, Resel E, Palazuelos J, Julien B, Salazar M, Borner C, Benito C, Carrasco C, Diez-Zaera M, Paoletti P, Diaz-Hernandez M, Ruiz C, Sendtner M, Lucas JJ, de Yebenes JG, Marsicano G, Monory K, Lutz B, Romero J, Alberch J, Gines S, Kraus J, Fernandez-Ruiz J, Galve-Roperh I, Guzman M (2010) Loss of striatal type 1 cannabinoid receptors is a key pathogenic factor in Huntington's disease. Brain.

Bojesen IN, Hansen HS (2003) Binding of anandamide to bovine serum albumin. J Lipid Res 44:1790-1794.

Bonci A, Bernardi G, Grillner P, Mercuri NB (2003) The dopamine-containing neuron: maestro or simple musician in the orchestra of addiction? Trends Pharmacol Sci 24:172-177.

Borrelli F, Izzo AA (2009) Role of acylethanolamides in the gastrointestinal tract with special reference to food intake and energy balance. Best Pract Res Clin Endocrinol Metab 23:33-49.

Bouaboula M, Hilairet S, Marchand J, Fajas L, Le Fur G, Casellas P (2005) Anandamide induced PPARgamma transcriptional activation and 3T3-L1 preadipocyte differentiation. Eur J Pharmacol 517:174-181.

Bouaboula M, Perrachon S, Milligan L, Canat X, Rinaldi-Carmona M, Portier M, Barth F, Calandra B, Pecceu F, Lupker J, Maffrand JP, Le Fur G, Casellas P (1997) A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1. Evidence for a new model of receptor/ligand interactions. J Biol Chem 272:22330-22339.

Braida D, Iosue S, Pegorini S, Sala M (2005) 3,4 Methylenedioxymethamphetamine-induced conditioned place preference (CPP) is mediated by endocannabinoid system. Pharmacol Res 51:177-182.

Braissant O, Foufelle F, Scotto C, Dauca M, Wahli W (1996) Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-alpha, -beta, and -gamma in the adult rat. Endocrinology 137:354-366.

Breese CR, Marks MJ, Logel J, Adams CE, Sullivan B, Collins AC, Leonard S (1997) Effect of smoking history on [3H]nicotine binding in human postmortem brain. J Pharmacol Exp Ther 282:7-13.

Brooks JE (1952) The Mighty Leaf: Tobacco Through the Centuries. Boston: Little Brown

Brooks JE (1937) Tobacco: Its History Illustrated by the Books, Manuscripts and Drawings and

Engravings in the Library of george Arents Jr. Vol.1: 1507-1615. New York: Rosenbach

Brown AJ (2007) Novel cannabinoid receptors. Br J Pharmacol 152:567-575.

Buisson B, Bertrand D (2001) Chronic exposure to nicotine upregulates the human (alpha)4((beta)2 nicotinic acetylcholine receptor function. J Neurosci 21:1819-1829.

Butt C, Alptekin A, Shippenberg T, Oz M (2008) Endogenous cannabinoid anandamide inhibits nicotinic acetylcholine receptor function in mouse thalamic synaptosomes. J Neurochem 105:1235-1243.

Butt CM, Hutton SR, Marks MJ, Collins AC (2002) Bovine serum albumin enhances nicotinic acetylcholine receptor function in mouse thalamic synaptosomes. J Neurochem 83:48-56.

Cadas H, Gaillet S, Beltramo M, Venance L, Piomelli D (1996) Biosynthesis of an endogenous cannabinoid precursor in neurons and its control by calcium and cAMP. J Neurosci 16:3934-3942.

Cadogan AK, Alexander SP, Boyd EA, Kendall DA (1997) Influence of cannabinoids on electrically evoked dopamine release and cyclic AMP generation in the rat striatum. J Neurochem 69:1131-1137.

Caille S, Guillem K, Cador M, Manzoni O, Georges F (2009) Voluntary nicotine consumption triggers in vivo potentiation of cortical excitatory drives to midbrain dopaminergic neurons. J Neurosci 29:10410-10415.

Caldarone BJ, Duman CH, Picciotto MR (2000) Fear conditioning and latent inhibition in mice lacking the high affinity subclass of nicotinic acetylcholine receptors in the brain. Neuropharmacology 39:2779-2784.

Campolongo P, Roozendaal B, Trezza V, Cuomo V, Astarita G, Fu J, McGaugh JL, Piomelli D (2009) Fat-induced satiety factor oleoylethanolamide enhances memory consolidation. Proc Natl Acad Sci U S A 106:8027-8031.

Cardinal RN, Pennicott DR, Sugathapala CL, Robbins TW, Everitt BJ (2001) Impulsive choice induced in rats by lesions of the nucleus accumbens core. Science 292:2499-2501.

Carlezon WA, Jr., Thomas MJ (2009) Biological substrates of reward and aversion: a nucleus accumbens activity hypothesis. Neuropharmacology 56 Suppl 1:122-132.

Cassaday HJ, Horsley RR, Norman C (2005) Electrolytic lesions to nucleus accumbens core and shell have dissociable effects on conditioning to discrete and contextual cues in aversive and appetitive procedures respectively. Behav Brain Res 160:222-235.

Castane A, Robledo P, Matifas A, Kieffer BL, Maldonado R (2003) Cannabinoid withdrawal syndrome is reduced in double mu and delta opioid receptor knockout mice. Eur J Neurosci 17:155-159.

Castane A, Valjent E, Ledent C, Parmentier M, Maldonado R, Valverde O (2002) Lack of CB1 cannabinoid receptors modifies nicotine behavioural responses, but not nicotine abstinence. Neuropharmacology 43:857-867.

Centers for Disease Control (CDC) (2008) Cigarette smoking among adults-United States, 2007. MMWR Morb Mortal Wkly Rep 57(45):1221-1226

Changeux JP (2009) Jean-Pierre Changeux: allostery as neuro-enlightenment. Mol Interv 9:61-67.

Changeux JP (2010) Nicotine addiction and nicotinic receptors: lessons from genetically modified mice. Nat Rev Neurosci 11:389-401.

Changeux JP, Thiery J, Tung Y, Kittel C (1967) On the cooperativity of biological membranes. Proc Natl Acad Sci U S A 57:335-341.

Chao J, Nestler EJ (2004) Molecular neurobiology of drug addiction. Annu Rev Med 55:113-132.

Charlton A (2004) Medicinal uses of tobacco in history. J R Soc Med 97:292-296.

Charpantier E, Wiesner A, Huh KH, Ogier R, Hoda JC, Allaman G, Raggenbass M, Feuerbach D, Bertrand D, Fuhrer C (2005) Alpha7 neuronal nicotinic acetylcholine receptors are negatively regulated by tyrosine phosphorylation and Src-family kinases. J Neurosci 25:9836-9849.

Cheer JF, Wassum KM, Heien ML, Phillips PE, Wightman RM (2004) Cannabinoids enhance subsecond dopamine release in the nucleus accumbens of awake rats. J Neurosci 24:4393-4400.

Cheer JF, Wassum KM, Sombers LA, Heien ML, Ariansen JL, Aragona BJ, Phillips PE, Wightman RM (2007) Phasic dopamine release evoked by abused substances requires cannabinoid receptor activation. J Neurosci 27:791-795.

Chen J, Paredes W, Lowinson JH, Gardner EL (1990) Delta 9-tetrahydrocannabinol enhances presynaptic dopamine efflux in medial prefrontal cortex. Eur J Pharmacol 190:259-262.

Chergui K, Nomikos GG, Mathe JM, Gonon F, Svensson TH (1996) Burst stimulation of the medial forebrain bundle selectively increase Fos-like immunoreactivity in the limbic forebrain of the rat. Neuroscience 72:141-156.

Chergui K, Svenningsson P, Nomikos GG, Gonon F, Fredholm BB, Svennson TH (1997) Increased expression of NGFI-A mRNA in the rat striatum following burst stimulation of the medial forebrain bundle. Eur J Neurosci 9:2370-2382.

Chevaleyre V, Castillo PE (2003) Heterosynaptic LTD of hippocampal GABAergic synapses: a novel role of endocannabinoids in regulating excitability. Neuron 38:461-472.

Chevaleyre V, Takahashi KA, Castillo PE (2006) Endocannabinoid-mediated synaptic plasticity in the CNS. Annu Rev Neurosci 29:37-76.

Chevaleyre V, Heifets BD, Kaeser PS, Sudhof TC, Castillo PE (2007) Endocannabinoid-mediated long-term plasticity requires cAMP/PKA signaling and RIM1alpha. Neuron 54:801-812.

Chikahisa S, Tominaga K, Kawai T, Kitaoka K, Oishi K, Ishida N, Rokutan K, Sei H (2008) Bezafibrate, a peroxisome proliferator-activated receptors agonist, decreases body temperature and enhances electroencephalogram delta-oscillation during sleep in mice. Endocrinology 149:5262-5271.

Chinetti G, Griglio S, Antonucci M, Torra IP, Delerive P, Majd Z, Fruchart JC, Chapman J, Najib J, Staels B (1998) Activation of proliferator-activated receptors alpha and gamma induces apoptosis of human monocyte-derived macrophages. J Biol Chem 273:25573-25580.

Cho CH, Song W, Leitzell K, Teo E, Meleth AD, Quick MW, Lester RA (2005) Rapid upregulation of alpha7 nicotinic acetylcholine receptors by tyrosine dephosphorylation. J Neurosci 25:3712-3723.

Choi S, Lovinger DM (1997) Decreased probability of neurotransmitter release underlies striatal long-term depression and postnatal development of corticostriatal synapses. Proc Natl Acad Sci U S A 94:2665-2670.

Cohen C, Perrault G, Griebel G, Soubrie P (2005) Nicotine-associated cues maintain nicotine-seeking behavior in rats several weeks after nicotine withdrawal: reversal by the cannabinoid (CB1) receptor antagonist, rimonabant (SR141716). Neuropsychopharmacology 30:145-155.

Cohen C, Perrault G, Voltz C, Steinberg R, Soubrie P (2002) SR141716, a central cannabinoid (CB(1)) receptor antagonist, blocks the motivational and dopamine-releasing effects of nicotine in rats. Behav Pharmacol 13:451-463.

Colombo G, Agabio R, Fa M, Guano L, Lobina C, Loche A, Reali R, Gessa GL (1998) Reduction of voluntary ethanol intake in ethanol-preferring sP rats by the cannabinoid antagonist SR-141716. Alcohol Alcohol 33:126-130.

Cooper E, Couturier S, Ballivet M (1991) Pentameric structure and subunit stoichiometry of a neuronal nicotinic acetylcholine receptor. Nature 350:235-238.

Corrigall WA, Coen KM (1991) Selective dopamine antagonists reduce nicotine self-administration. Psychopharmacology (Berl) 104:171-176.

Corrigall WA, Coen KM, Adamson KL (1994) Self-administered nicotine activates the mesolimbic dopamine system through the ventral tegmental area. Brain Res 653:278-284.

Corrigall WA, Franklin KB, Coen KM, Clarke PB (1992) The mesolimbic dopaminergic system is implicated in the reinforcing effects of nicotine. Psychopharmacology (Berl) 107:285-289.

Cossu G, Ledent C, Fattore L, Imperato A, Bohme GA, Parmentier M, Fratta W (2001) Cannabinoid CB1 receptor knockout mice fail to self-administer morphine but not other drugs of abuse. Behav Brain Res 118:61-65.

Court J, Martin-Ruiz C, Piggott M, Spurden D, Griffiths M, Perry E (2001) Nicotinic receptor abnormalities in Alzheimer's disease. Biol Psychiatry 49:175-184.

Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB (1996) Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. Nature 384:83-87.

Cravatt BF, Demarest K, Patricelli MP, Bracey MH, Giang DK, Martin BR, Lichtman AH (2001) Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. Proc Natl Acad Sci U S A 98:9371-9376.

Cristino L, Starowicz K, De Petrocellis L, Morishita J, Ueda N, Guglielmotti V, Di Marzo V (2008) Immunohistochemical localization of anabolic and catabolic enzymes for anandamide and other putative endovanilloids in the hippocampus and cerebellar cortex of the mouse brain. Neuroscience 151:955-968.

Cui C, Booker TK, Allen RS, Grady SR, Whiteaker P, Marks MJ, Salminen O, Tritto T, Butt CM, Allen WR, Stitzel JA, McIntosh JM, Boulter J, Collins AC, Heinemann SF (2003) The beta3 nicotinic receptor subunit: a component of alpha-conotoxin MII-binding nicotinic acetylcholine receptors that modulate dopamine release and related behaviors. J Neurosci 23:11045-11053.

Curtis L, Buisson B, Bertrand S, Bertrand D (2002) Potentiation of human alpha4beta2 neuronal nicotinic acetylcholine receptor by estradiol. Mol Pharmacol 61:127-135.

Dani JA (1989) Site-directed mutagenesis and single-channel currents define the ionic channel of the nicotinic acetylcholine receptor. Trends Neurosci 12:125-128.

Dani JA (2001) Overview of nicotinic receptors and their roles in the central nervous system. Biol Psychiatry 49:166-174.

Dani JA, Harris RA (2005) Nicotine addiction and comorbidity with alcohol abuse and mental illness. Nat Neurosci 8:1465-1470.

Dani JA, Bertrand D (2007) Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. Annu Rev Pharmacol Toxicol 47:699-729.

Dani JA, Radcliffe KA, Pidoplichko VI (2000) Variations in desensitization of nicotinic acetylcholine receptors from hippocampus and midbrain dopamine areas. Eur J Pharmacol 393:31-38.

Davies SN, Pertwee RG, Riedel G (2002) Functions of cannabinoid receptors in the hippocampus. Neuropharmacology 42:993-1007.

Dawson RF, Christman DR, Anderson RC, Solt ML, D'Adamo AF, Weiss U (1956) Biosynthesis of the pyridine ring of nicotine. J Am Chem Soc 78: 2645–2646

Dawson RF, Christman DR, D'Adamo AF, Solt ML, Wolf AP (1958) Pathway of nicotine biogenesis. Chem Ind (London) 1958: 100

de Rover M, Lodder JC, Kits KS, Schoffelmeer AN, Brussaard AB (2002) Cholinergic modulation of nucleus accumbens medium spiny neurons. Eur J Neurosci 16:2279-2290.

De Vries TJ, Homberg JR, Binnekade R, Raaso H, Schoffelmeer AN (2003) Cannabinoid modulation of the reinforcing and motivational properties of heroin and heroin-associated cues in rats. Psychopharmacology (Berl) 168:164-169.

De Vries TJ, Shaham Y, Homberg JR, Crombag H, Schuurman K, Dieben J, Vanderschuren LJ, Schoffelmeer AN (2001) A cannabinoid mechanism in relapse to cocaine seeking. Nat Med 7:1151-1154.

Del Arco I, Navarro M, Bilbao A, Ferrer B, Piomelli D, Rodriguez De Fonseca F (2002) Attenuation of spontaneous opiate withdrawal in mice by the anandamide transport inhibitor AM404. Eur J Pharmacol 454:103-104.

Deneris ES, Connolly J, Rogers SW, Duvoisin R (1991) Pharmacological and functional diversity of neuronal nicotinic acetylcholine receptors. Trends Pharmacol Sci 12:34-40.

Deplanque D, Gele P, Petrault O, Six I, Furman C, Bouly M, Nion S, Dupuis B, Leys D, Fruchart JC, Cecchelli R, Staels B, Duriez P, Bordet R (2003) Peroxisome proliferator-activated receptor-alpha activation as a mechanism of preventive neuroprotection induced by chronic fenofibrate treatment. J Neurosci 23:6264-6271.

Desvergne B, Wahli W (1999) Peroxisome proliferator-activated receptors: nuclear control of metabolism. Endocr Rev 20:649-688.

Devane WA, Dysarz FA, 3rd, Johnson MR, Melvin LS, Howlett AC (1988) Determination and characterization of a cannabinoid receptor in rat brain. Mol Pharmacol 34:605-613.

Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, 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 258:1946-1949.

Dickson SA (1954) Panacea or Precious Bane. Tobacco in 16th Century Literature. New York: New York Public Library

Di Chiara G, Imperato A (1988) Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. Proc Natl Acad Sci U S A 85:5274-5278.

Di Ciano P, Cardinal RN, Cowell RA, Little SJ, Everitt BJ (2001) Differential involvement of NMDA, AMPA/kainate, and dopamine receptors in the nucleus accumbens core in the acquisition and performance of pavlovian approach behavior. J Neurosci 21:9471-9477.

Di Marzo V (2006) A brief history of cannabinoid and endocannabinoid pharmacology as inspired by the work of British scientists. Trends Pharmacol Sci 27:134-140.

Di Marzo V, Bisogno T, Sugiura T, Melck D, De Petrocellis L (1998) The novel endogenous cannabinoid 2-arachidonoylglycerol is inactivated by neuronal- and basophil-like cells: connections with anandamide. Biochem J 331 (Pt 1):15-19.

Di Marzo V, Fontana A, Cadas H, Schinelli S, Cimino G, Schwartz JC, Piomelli D (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons. Nature 372:686-691.

Dinh TP, Kathuria S, Piomelli D (2004) RNA interference suggests a primary role for monoacylglycerol lipase in the degradation of the endocannabinoid 2-arachidonoylglycerol. Mol Pharmacol 66:1260-1264.

Doweiko HE (2008) Concepts of Chemical Dependency. Cengage Learning

Drago J, McColl CD, Horne MK, Finkelstein DI, Ross SA (2003) Neuronal nicotinic receptors: insights gained from gene knockout and knockin mutant mice. Cell Mol Life Sci 60:1267-1280.

Edelstein SJ, Schaad O, Henry E, Bertrand D, Changeux JP (1996) A kinetic mechanism for nicotinic acetylcholine receptors based on multiple allosteric transitions. Biol Cybern 75:361-379.

Edwards DA, Zhang L, Alger BE (2008) Metaplastic control of the endocannabinoid system at inhibitory synapses in hippocampus. Proc Natl Acad Sci U S A 105:8142-8147.

Egertova M, Simon GM, Cravatt BF, Elphick MR (2008) Localization of N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) expression in mouse brain: A new perspective on N-acylethanolamines as neural signaling molecules. J Comp Neurol 506:604-615.

Einhorn LC, Johansen PA, White FJ (1988) Electrophysiological effects of cocaine in the mesoaccumbens dopamine system: studies in the ventral tegmental area. J Neurosci 8:100-112.

Elphick MR, Egertova M (2001) The neurobiology and evolution of cannabinoid signalling. Philos Trans R Soc Lond B Biol Sci 356:381-408.

Epping-Jordan MP, Watkins SS, Koob GF, Markou A (1998) Dramatic decreases in brain reward function during nicotine withdrawal. Nature 393:76-79.

Erhardt S, Schwieler L, Engberg G (2002) Excitatory and inhibitory responses of dopamine neurons in the ventral tegmental area to nicotine. Synapse 43:227-237.

Escriva H, Langlois MC, Mendonca RL, Pierce R, Laudet V (1998) Evolution and diversification of the nuclear receptor superfamily. Ann N Y Acad Sci 839:143-146.

Everitt BJ, Wolf ME (2002) Psychomotor stimulant addiction: a neural systems perspective. J Neurosci 22:3312-3320.

Everitt BJ, Robbins TW (2005) Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. Nat Neurosci 8:1481-1489.

Exley R, Clements MA, Hartung H, McIntosh JM, Cragg SJ (2008) Alpha6-containing nicotinic acetylcholine receptors dominate the nicotine control of dopamine neurotransmission in nucleus accumbens. Neuropsychopharmacology 33:2158-2166.

Fattore L, Spano MS, Cossu G, Deiana S, Fratta W (2003) Cannabinoid mechanism in reinstatement of heroin-seeking after a long period of abstinence in rats. Eur J Neurosci 17:1723-1726.

Faure A, Haberland U, Conde F, El Massioui N (2005) Lesion to the nigrostriatal dopamine system disrupts stimulus-response habit formation. J Neurosci 25:2771-2780.

Faure P et al. (2010) Differential role for alpha4 and alpha6 nicotinic subunits in burst response to nicotine in dopamine neurons. FENS Forum of European Neuroscience (Amsterdam, the Nederlands) P14006

Fegley D, Kathuria S, Mercier R, Li C, Goutopoulos A, Makriyannis A, Piomelli D (2004) Anandamide transport is independent of fatty-acid amide hydrolase activity and is blocked by the hydrolysis-resistant inhibitor AM1172. Proc Natl Acad Sci U S A 101:8756-8761.

Fegley D, Gaetani S, Duranti A, Tontini A, Mor M, Tarzia G, Piomelli D (2005) Characterization of the fatty acid amide hydrolase inhibitor cyclohexyl carbamic acid 3'-carbamoyl-biphenyl-3-yl ester (URB597): effects on anandamide and oleoylethanolamide deactivation. J Pharmacol Exp Ther 313:352-358.

Fernandez-Ruiz J (2009) The endocannabinoid system as a target for the treatment of motor dysfunction. Br J Pharmacol 156:1029-1040.

Fibiger HC, Nomikos GG, Pfaus JG, Damsma G (1992) Sexual behavior, eating and mesolimbic dopamine. Clin Neuropharmacol 15 Suppl 1 Pt A:566A-567A.

Filip M, Golda A, Zaniewska M, McCreary AC, Nowak E, Kolasiewicz W, Przegalinski E (2006) Involvement of cannabinoid CB1 receptors in drug addiction: effects of rimonabant on behavioral responses induced by cocaine. Pharmacol Rep 58:806-819.

Floresco SB, Blaha CD, Yang CR, Phillips AG (2001) Modulation of hippocampal and amygdalar-evoked activity of nucleus accumbens neurons by dopamine: cellular mechanisms of input selection. J Neurosci 21:2851-2860.

Floresco SB, West AR, Ash B, Moore H, Grace AA (2003) Afferent modulation of dopamine neuron firing differentially regulates tonic and phasic dopamine transmission. Nat Neurosci 6:968-973.

Fourgeaud L, Mato S, Bouchet D, Hemar A, Worley PF, Manzoni OJ (2004) A single in vivo exposure to cocaine abolishes endocannabinoid-mediated long-term depression in the nucleus accumbens. J Neurosci 24:6939-6945.

Fowler CD, Arends MA, Kenny PJ (2008) Subtypes of nicotinic acetylcholine receptors in nicotine reward, dependence, and withdrawal: evidence from genetically modified mice. Behav Pharmacol 19:461-484.

Fratta W, Massi M, Nencini P (2005) Tossicodipendenza e Sostanze d'Abuso in Farmacologia: Principi di Base e Applicazioni Terapeutiche, Minerva Edizioni, Torino, Italy

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

Fride E, Bregman T, Kirkham TC (2005) Endocannabinoids and food intake: newborn suckling and appetite regulation in adulthood. Exp Biol Med (Maywood) 230:225-234.

Fu J, Gaetani S, Oveisi F, Lo Verme J, Serrano A, Rodriguez De Fonseca F, Rosengarth A, Luecke Di Giacomo B, Tarzia G, Piomelli D (2003) Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha. Nature 425:90-93.

Galan-Rodriguez B, Suarez J, Gonzalez-Aparicio R, Bermudez-Silva FJ, Maldonado R, Robledo P, Rodriguez de Fonseca F, Fernandez-Espejo E (2009) Oleoylethanolamide exerts partial and dose-dependent neuroprotection of substantia nigra dopamine neurons. Neuropharmacology 56:653-664.

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. Eur J Biochem 232:54-61.

Gardner OS, Dewar BJ, Graves LM (2005) Activation of mitogen-activated protein kinases by peroxisome proliferator-activated receptor ligands: an example of nongenomic signaling. Mol Pharmacol 68:933-941.

Ge S, Dani JA (2005) Nicotinic acetylcholine receptors at glutamate synapses facilitate long-term depression or potentiation. J Neurosci 25:6084-6091.

Gerdeman GL, Ronesi J, Lovinger DM (2002) Postsynaptic endocannabinoid release is critical to long-term depression in the striatum. Nat Neurosci 5:446-451.

Gessa GL, Melis M, Muntoni AL, Diana M (1998) Cannabinoids activate mesolimbic dopamine neurons by an action on cannabinoid CB1 receptors. Eur J Pharmacol 341:39-44.

Gifford AN, Ashby CR, Jr. (1996) Electrically evoked acetylcholine release from hippocampal slices is inhibited by the cannabinoid receptor agonist, WIN 55212-2, and is potentiated by the cannabinoid antagonist, SR 141716A. J Pharmacol Exp Ther 277:1431-1436.

Giuffrida A, Parsons LH, Kerr TM, Rodriguez de Fonseca F, Navarro M, Piomelli D (1999) Dopamine activation of endogenous cannabinoid signaling in dorsal striatum. Nat Neurosci 2:358-363.

Godlewski G, Offertaler L, Wagner JA, Kunos G (2009) Receptors for acylethanolamides-GPR55 and GPR119. Prostaglandins Other Lipid Mediat 89:105-111.

Gonzales D, Rennard SI, Nides M, Oncken C, Azoulay S, Billing CB, Watsky EJ, Gong J, Williams KE, Reeves KR (2006) Varenicline, an alpha4beta2 nicotinic acetylcholine receptor partial agonist, vs sustained-release bupropion and placebo for smoking cessation: a randomized controlled trial. Jama 296:47-55.

Gonzalo Fernandez de Oviedo y Valdéz (1526) Historia General y Natural de las Indias. Madrid, 1959

Goodman J (1994) Tobacco in history: the cultures of dependence. Taylor&Fancis.

Gorrod JW, Jenner P (1975) Metabolic N-oxidation products of aliphatic amines as potential mediators in amine pharmacology. Int J Clin Pharmacol Biopharm 12:180-185.

Goto Y, Grace AA (2008) Limbic and cortical information processing in the nucleus accumbens. Trends Neurosci 31:552-558.

Gotti C, Clementi F (2004) Neuronal nicotinic receptors: from structure to pathology. Prog Neurobiol 74:363-396.

Gotti C, Hanke W, Maury K, Moretti M, Ballivet M, Clementi F, Bertrand D (1994) Pharmacology and biophysical properties of alpha 7 and alpha 7-alpha 8 alpha-bungarotoxin receptor subtypes immunopurified from the chick optic lobe. Eur J Neurosci 6:1281-1291.

Gotti C, Clementi F, Fornari A, Gaimarri A, Guiducci S, Manfredi I, Moretti M, Pedrazzi P, Pucci L, Zoli M (2009) Structural and functional diversity of native brain neuronal nicotinic receptors. Biochem Pharmacol 78:703-711.

Govind AP, Vezina P, Green WN (2009) Nicotine-induced upregulation of nicotinic receptors: underlying mechanisms and relevance to nicotine addiction. Biochem Pharmacol 78:756-765.

Grace AA (2000) Gating of information flow within the limbic system and the pathophysiology of schizophrenia. Brain Res Brain Res Rev 31:330-341.

Grace AA, Bunney BS (1983) Intracellular and extracellular electrophysiology of nigral dopaminergic neurons--1. Identification and characterization. Neuroscience 10:301-315.

Grace AA, Bunney BS (1984a) The control of firing pattern in nigral dopamine neurons: burst firing. J Neurosci 4:2877-2890.

Grace AA, Bunney BS (1984b) The control of firing pattern in nigral dopamine neurons: single spike firing. J Neurosci 4:2866-2876.

Grady SR, Meinerz NM, Cao J, Reynolds AM, Picciotto MR, Changeux JP, McIntosh JM, Marks MJ, Collins AC (2001) Nicotinic agonists stimulate acetylcholine release from mouse interpeduncular nucleus: a function mediated by a different nAChR than dopamine release from striatum. J Neurochem 76:258-268.

Granon S, Faure P, Changeux JP (2003) Executive and social behaviors under nicotinic receptor regulation. Proc Natl Acad Sci U S A 100:9596-9601.

Gray R, Rajan AS, Radcliffe KA, Yakehiro M, Dani JA (1996) Hippocampal synaptic transmission enhanced by low concentrations of nicotine. Nature 383:713-716.

Groenewegen HJ, Wright CI, Beijer AV, Voorn P (1999) Convergence and segregation of ventral striatal inputs and outputs. Ann N Y Acad Sci 877:49-63.

Grosman C, Auerbach A (2001) The dissociation of acetylcholine from open nicotinic receptor channels. Proc Natl Acad Sci U S A 98:14102-14107.

Guix T, Hurd YL, Ungerstedt U (1992) Amphetamine enhances extracellular concentrations of dopamine and acetylcholine in dorsolateral striatum and nucleus accumbens of freely moving rats. Neurosci Lett 138:137-140.

Gutkin BS, Dehaene S, Changeux JP (2006) A neurocomputational hypothesis for nicotine addiction. Proc Natl Acad Sci U S A 103:1106-1111.

Hajos N, Freund TF (2002) Distinct cannabinoid sensitive receptors regulate hippocampal excitation and inhibition. Chem Phys Lipids 121:73-82.

Hajos N, Ledent C, Freund TF (2001) Novel cannabinoid-sensitive receptor mediates inhibition of glutamatergic synaptic transmission in the hippocampus. Neuroscience 106:1-4.

Hakan RL, Hart C, Eyl C (1993) Specific neurophysiological effects of systemic nicotine on neurons in the nucleus accumbens. Synapse 15:191-197.

Hammond DK, Bjercke RJ, Langone JJ, Strobel HW (1991) Metabolism of nicotine by rat liver cytochromes P-450. Assessment utilizing monoclonal antibodies to nicotine and cotinine. Drug Metab Dispos 19:804-808.

Hansen HS (2010) Palmitoylethanolamide and other anandamide congeners. Proposed role in the diseased brain. Exp Neurol 224:48-55.

Hansen HS, Diep TA (2009) N-acylethanolamines, anandamide and food intake. Biochem Pharmacol 78:553-560.

Hansen HS, Lauritzen L, Strand AM, Moesgaard B, Frandsen A (1995) Glutamate stimulates the formation of N-acylphosphatidylethanolamine and N-acylethanolamine in cortical neurons in culture. Biochim Biophys Acta 1258:303-308.

Hansen HS, Lauritzen L, Strand AM, Vinggaard AM, Frandsen A, Schousboe A (1997) Characterization of glutamate-induced formation of N-acylphosphatidylethanolamine and N-acylethanolamine in cultured neocortical neurons. J Neurochem 69:753-761.

Hanus L, Gopher A, Almog S, Mechoulam R (1993) Two new unsaturated fatty acid ethanolamides in brain that bind to the cannabinoid receptor. J Med Chem 36:3032-3034.

Hanus L, Abu-Lafi S, Fride E, Breuer A, Vogel Z, Shalev DE, Kustanovich I, Mechoulam R (2001) 2-arachidonyl glyceryl ether, an endogenous agonist of the cannabinoid CB1 receptor. Proc Natl Acad Sci U S A 98:3662-3665.

Hart J (1633) The Diet of the Diseased. London: John Beale for Thomas Allot.

Hashimoto T, Yamada Y (1994) Alkaloyds biogenesis: Molecular aspects. Ann Rev Plant Physiol Plant Mol Biol 45:257-285

Hashimoto T, Yamada Y (2003) New genes in alkaloid metabolism and transport. Curr Opin Biotechnol 14:163-168.

Heidmann T, Bernhardt J, Neumann E, Changeux JP (1983) Rapid kinetics of agonist binding and permeability response analyzed in parallel on acetylcholine receptor rich membranes from Torpedo marmorata. Biochemistry 22:5452-5459.

Heifets BD, Castillo PE (2009) Endocannabinoid signaling and long-term synaptic plasticity. Annu Rev Physiol 71:283-306.

Heneka MT, Landreth GE (2007) PPARs in the brain. Biochim Biophys Acta 1771:1031-1045.

Henningfield JE, Zeller M (2006) Nicotine psychopharmacology research contributions to United States and global tobacco regulation: a look back and a look forward. Psychopharmacology (Berl) 184:286-291.

Herkenham M, Lynn AB, Johnson MR, Melvin LS, de Costa BR, Rice KC (1991) Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. J Neurosci 11:563-583.

Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, de Costa BR, Rice KC (1990) Cannabinoid receptor localization in brain. Proc Natl Acad Sci U S A 87:1932-1936.

Hermann A, Kaczocha M, Deutsch DG (2006) 2-Arachidonoylglycerol (2-AG) membrane transport: history and outlook. AAPS J 8:E409-412.

Hess GP (1993) Determination of the chemical mechanism of neurotransmitter receptor-mediated reactions by rapid chemical kinetic techniques. Biochemistry 32:989-1000.

Hilairet S, Bouaboula M, Carriere D, Le Fur G, Casellas P (2003) Hypersensitization of the Orexin 1 receptor by the CB1 receptor: evidence for cross-talk blocked by the specific CB1 antagonist, SR141716. J Biol Chem 278:23731-23737.

Hine B, Torrelio M, Gershon S (1975) Attenuation of precipitated abstinence in methadone-dependent rats by delta 9-THC. Psychopharmacol Commun 1:275-283.

Hoffmann D, Hoffmann (1998) Chemistry and Toxicology. Smoking and Tobacco Control

Hoffman AF, Lupica CR (2000) Mechanisms of cannabinoid inhibition of GABA(A) synaptic transmission in the hippocampus. J Neurosci 20:2470-2479.

Hoffman AF, Oz M, Caulder T, Lupica CR (2003) Functional tolerance and blockade of long-term depression at synapses in the nucleus accumbens after chronic cannabinoid exposure. J Neurosci 23:4815-4820.

Hogg RC, Raggenbass M, Bertrand D (2003) Nicotinic acetylcholine receptors: from structure to brain function. Rev Physiol Biochem Pharmacol 147:1-46.

Hohmann AG, Herkenham M (2000) Localization of cannabinoid CB(1) receptor mRNA in neuronal subpopulations of rat striatum: a double-label in situ hybridization study. Synapse 37:71-80.

Hollister LE (1986) Health aspects of cannabis. Pharmacol Rev 38:1-20.

Howlett AC (2002) The cannabinoid receptors. Prostaglandins Other Lipid Mediat 68-69:619-631.

Howlett AC, Bidaut-Russell M, Devane WA, Melvin LS, Johnson MR, Herkenham M (1990) The cannabinoid receptor: biochemical, anatomical and behavioral characterization. Trends Neurosci 13:420-423.

Huang SM, Bisogno T, Trevisani M, Al-Hayani A, De Petrocellis L, Fezza F, Tognetto M, Petros TJ, Krey JF, Chu CJ, Miller JD, Davies SN, Geppetti P, Walker JM, Di Marzo V (2002) An endogenous capsaicin-like substance with high potency at recombinant and native vanilloid VR1 receptors. Proc Natl Acad Sci U S A 99:8400-8405.

Huang W, Payne TJ, Ma JZ, Li MD (2008) A functional polymorphism, rs6280, in DRD3 is significantly associated with nicotine dependence in European-American smokers. Am J Med Genet B Neuropsychiatr Genet 147B:1109-1115.

Hukkanen J, Jacob P, 3rd, Benowitz NL (2005) Metabolism and disposition kinetics of nicotine. Pharmacol Rev 57:79-115.

Hutchison KE, Allen DL, Filbey FM, Jepson C, Lerman C, Benowitz NL, Stitzel J, Bryan A, McGeary J, Haughey HM (2007) CHRNA4 and tobacco dependence: from gene regulation to treatment outcome. Arch Gen Psychiatry 64:1078-1086.

Ikemoto S, Qin M, Liu ZH (2006) Primary reinforcing effects of nicotine are triggered from multiple regions both inside and outside the ventral tegmental area. J Neurosci 26:723-730.

Imperato A, Obinu MC, Demontis MV, Gessa GL (1992) Cocaine releases limbic acetylcholine through endogenous dopamine action on D1 receptors. Eur J Pharmacol 229:265-267.

Ishac EJ, Jiang L, Lake KD, Varga K, Abood ME, Kunos G (1996) Inhibition of exocytotic noradrenaline release by presynaptic cannabinoid CB1 receptors on peripheral sympathetic nerves. Br J Pharmacol 118:2023-2028.

Ito R, Robbins TW, Everitt BJ (2004) Differential control over cocaine-seeking behavior by nucleus accumbens core and shell. Nat Neurosci 7:389-397.

Jackson KJ, McIntosh JM, Brunzell DH, Sanjakdar SS, Damaj MI (2009) The role of alpha6-containing nicotinic acetylcholine receptors in nicotine reward and withdrawal. J Pharmacol Exp Ther 331:547-554.

Jackson MB (1989) Perfection of a synaptic receptor: kinetics and energetics of the acetylcholine receptor. Proc Natl Acad Sci U S A 86:2199-2203.

Jeffers HP, Gordon K (1996) The Good Cigar. New York: Lyons and Burford Publishers.

Jennings EA, Vaughan CW, Christie MJ (2001) Cannabinoid actions on rat superficial medullary dorsal horn neurons in vitro. J Physiol 534:805-812.

Jhaveri MD, Richardson D, Robinson I, Garle MJ, Patel A, Sun Y, Sagar DR, Bennett AJ, Alexander SP, Kendall DA, Barrett DA, Chapman V (2008) Inhibition of fatty acid amide hydrolase and cyclooxygenase-2 increases levels of endocannabinoid related molecules and produces analgesia via peroxisome proliferator-activated receptor-alpha in a model of inflammatory pain. Neuropharmacology 55:85-93.

Ji D, Lape R, Dani JA (2001) Timing and location of nicotinic activity enhances or depresses hippocampal synaptic plasticity. Neuron 31:131-141.

Jones S, Sudweeks S, Yakel JL (1999) Nicotinic receptors in the brain: correlating physiology with function. Trends Neurosci 22:555-561.

Jongen-Relo AL, Kaufmann S, Feldon J (2003) A differential involvement of the shell and core subterritories of the nucleus accumbens of rats in memory processes. Behav Neurosci 117:150-168.

Jonsson KO, Holt S, Fowler CJ (2006) The endocannabinoid system: current pharmacological research and therapeutic possibilities. Basic Clin Pharmacol Toxicol 98:124-134.

Jorenby DE, Hays JT, Rigotti NA, Azoulay S, Watsky EJ, Williams KE, Billing CB, Gong J, Reeves KR (2006) Efficacy of varenicline, an alpha4beta2 nicotinic acetylcholine receptor partial agonist, vs placebo or sustained-release bupropion for smoking cessation: a randomized controlled trial. Jama 296:56-63.

Jung KM, 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.

Justinova Z, Mangieri RA, Bortolato M, Chefer SI, Mukhin AG, Clapper JR, King AR, Redhi GH, Yasar S, Piomelli D, Goldberg SR (2008) Fatty acid amide hydrolase inhibition heightens anandamide signaling without producing reinforcing effects in primates. Biol Psychiatry 64:930-937.

Kalivas PW, Volkow N, Seamans J (2005) Unmanageable motivation in addiction: a pathology in prefrontal-accumbens glutamate transmission. Neuron 45:647-650.

Kano M, Ohno-Shosaku T, Hashimotodani Y, Uchigashima M, Watanabe M (2009) Endocannabinoid-mediated control of synaptic transmission. Physiol Rev 89:309-380.

Karlin A (1967) On the application of "a plausible model" of allosteric proteins to the receptor for acetylcholine. J Theor Biol 16:306-320.

Karlin A (2002) Emerging structure of the nicotinic acetylcholine receptors. Nat Rev Neurosci 3:102-114.

Karlsson M, Contreras JA, Hellman U, Tornqvist H, Holm C (1997) cDNA cloning, tissue distribution, and identification of the catalytic triad of monoglyceride lipase. Evolutionary relationship to esterases, lysophospholipases, and haloperoxidases. J Biol Chem 272:27218-27223.

Karlsson M, Reue K, Xia YR, Lusis AJ, Langin D, Tornqvist H, Holm C (2001) Exon-intron organization and chromosomal localization of the mouse monoglyceride lipase gene. Gene 272:11-18.

Kasanetz F, Deroche-Gamonet V, Berson N, Balado E, Lafourcade M, Manzoni O, Piazza PV (2010) Transition to addiction is associated with a persistent impairment in synaptic plasticity. Science 328:1709-1712.

Kathuria S, Gaetani S, Fegley D, Valino F, Duranti A, Tontini A, Mor M, Tarzia G, La Rana G, Calignano A, Giustino A, Tattoli M, Palmery M, Cuomo V, Piomelli D (2003) Modulation of anxiety through blockade of anandamide hydrolysis. Nat Med 9:76-81.

Katoh A, Hirouki O, Inai K, Hashimoto T (2005) Molecular regulation of nicotine biosynthesis. Plant Biotechnology 22:389-392

Katona I, Sperlagh B, Sik A, Kafalvi A, Vizi ES, Mackie K, Freund TF (1999) Presynaptically located CB1 cannabinoid receptors regulate GABA release from axon terminals of specific hippocampal interneurons. J Neurosci 19:4544-4558.

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

Katona I, Sperlagh B, Magloczky Z, Santha E, Kofalvi A, Czirjak S, Mackie K, Vizi ES, Freund TF (2000) GABAergic interneurons are the targets of cannabinoid actions in the human hippocampus. Neuroscience 100:797-804.

Katz B, Thesleff S (1957) A study of the desensitization produced by acetylcholine at the motor end-plate. J Physiol 138:63-80.

Kauer JA, Malenka RC (2007) Synaptic plasticity and addiction. Nat Rev Neurosci 8:844-858.

Kawamura Y, Fukaya M, Maejima T, Yoshida T, Miura E, Watanabe M, Ohno-Shosaku T, Kano M (2006) The CB1 cannabinoid receptor is the major cannabinoid receptor at excitatory presynaptic sites in the hippocampus and cerebellum. J Neurosci 26:2991-3001.

Kaymakcalan S, Ayhan IH, Tulunay FC (1977) Naloxone-induced or postwithdrawal abstinence signs in delta9-tetrahydrocannabinol-tolerant rats. Psychopharmacology (Berl) 55:243-249.

Kearn CS, Blake-Palmer K, Daniel E, Mackie K, Glass M (2005) Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors enhances heterodimer formation: a mechanism for receptor cross-talk? Mol Pharmacol 67:1697-1704.

Kelley AE (2004) Ventral striatal control of appetitive motivation: role in ingestive behavior and reward-related learning. Neurosci Biobehav Rev 27:765-776.

Kenny PJ, Markou A (2006) Nicotine self-administration acutely activates brain reward systems and induces a long-lasting increase in reward sensitivity. Neuropsychopharmacology 31:1203-1211.

Kenny PJ, File SE, Neal MJ (2000) Evidence for a complex influence of nicotinic acetylcholine receptors on hippocampal serotonin release. J Neurochem 75:2409-2414.

Kim J, Alger BE (2004) Inhibition of cyclooxygenase-2 potentiates retrograde endocannabinoid effects in hippocampus. Nat Neurosci 7:697-698.

King SL, Marks MJ, Grady SR, Caldarone BJ, Koren AO, Mukhin AG, Collins AC, Picciotto MR (2003) Conditional expression in corticothalamic efferents reveals a developmental role for nicotinic acetylcholine receptors in modulation of passive avoidance behavior. J Neurosci 23:3837-3843.

Kingsborough, EK (1825) Antiquities of Mexico. Comparing facsimilies of ancient Mexican paintings. London 1831-1849.

Kitai ST, Shepard PD, Callaway JC, Scroggs R (1999) Afferent modulation of dopamine neuron firing patterns. Curr Opin Neurobiol 9:690-697.

Kondo S, Kondo H, Nakane S, Kodaka T, Tokumura A, Waku K, Sugiura T (1998) 2-Arachidonoylglycerol, an endogenous cannabinoid receptor agonist: identification as one of the major species of monoacylglycerols in various rat tissues, and evidence for its generation through CA2+-dependent and -independent mechanisms. FEBS Lett 429:152-156.

Koob GF, Bloom FE (1988) Cellular and molecular mechanisms of drug dependence. Science 242:715-723.

Koob GF, Le Moal M (2008) Review. Neurobiological mechanisms for opponent motivational processes in addiction. Philos Trans R Soc Lond B Biol Sci 363:3113-3123.

Koob GF, Volkow ND (2010) Neurocircuitry of addiction. Neuropsychopharmacology 35:217-238.

Kreitzer AC (2005) Neurotransmission: emerging roles of endocannabinoids. Curr Biol 15:R549-551.

Kreitzer AC, Regehr WG (2001) Retrograde inhibition of presynaptic calcium influx by endogenous cannabinoids at excitatory synapses onto Purkinje cells. Neuron 29:717-727.

Lafourcade CA, Alger BE (2008) Distinctions among GABAA and GABAB responses revealed by calcium channel antagonists, cannabinoids, opioids, and synaptic plasticity in rat hippocampus. Psychopharmacology (Berl) 198:539-549.

Lafourcade M, Elezgarai I, Mato S, Bakiri Y, Grandes P, Manzoni OJ (2007) Molecular components and functions of the endocannabinoid system in mouse prefrontal cortex. PLoS One 2:e709.

Langley JN (1905) On the reaction of cells and of nerve-endings to certain poisons, chiefly as regards the reaction of striated muscle to nicotine and to curari. J Physiol 33:374-413.

Laudet V, Hanni C, Coll J, Catzeflis F, Stehelin D (1992) Evolution of the nuclear receptor gene superfamily. EMBO J 11:1003-1013.

Laviolette SR, Alexson TO, van der Kooy D (2002) Lesions of the tegmental pedunculopontine nucleus block the rewarding effects and reveal the aversive effects of nicotine in the ventral tegmental area. J Neurosci 22:8653-8660.

Le Foll B, Goldberg SR (2004) Rimonabant, a CB1 antagonist, blocks nicotine-conditioned place preferences. Neuroreport 15:2139-2143.

Le Novere N, Zoli M, Changeux JP (1996) Neuronal nicotinic receptor alpha 6 subunit mRNA is selectively concentrated in catecholaminergic nuclei of the rat brain. Eur J Neurosci 8:2428-2439.

le Novere N, Zoli M, Lena C, Ferrari R, Picciotto MR, Merlo-Pich E, Changeux JP (1999) Involvement of alpha6 nicotinic receptor subunit in nicotine-elicited locomotion, demonstrated by in vivo antisense oligonucleotide infusion. Neuroreport 10:2497-2501.

Ledent C, Valverde O, Cossu G, Petitet F, Aubert JF, Beslot F, Bohme GA, Imperato A, Pedrazzini T, Roques BP, Vassart G, Fratta W, Parmentier M (1999) Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. Science 283:401-404.

Lena C, Changeux JP (1997) Role of Ca2+ ions in nicotinic facilitation of GABA release in mouse thalamus. J Neurosci 17:576-585.

Lena C, Changeux JP (1998) Allosteric nicotinic receptors, human pathologies. J Physiol Paris 92:63-74.

Lerman C, Caporaso NE, Audrain J, Main D, Bowman ED, Lockshin B, Boyd NR, Shields PG (1999) Evidence suggesting the role of specific genetic factors in cigarette smoking. Health Psychol 18:14-20.

Lesscher HM, Hoogveld E, Burbach JP, van Ree JM, Gerrits MA (2005) Endogenous cannabinoids are not involved in cocaine reinforcement and development of cocaine-induced behavioural sensitization. Eur Neuropsychopharmacol 15:31-37.

Lessov-Schlaggar CN, Pergadia ML, Khroyan TV, Swan GE (2008) Genetics of nicotine dependence and pharmacotherapy. Biochem Pharmacol 75:178-195.

Leung D, Saghatelian A, Simon GM, Cravatt BF (2006) Inactivation of N-acyl phosphatidylethanolamine phospholipase D reveals multiple mechanisms for the biosynthesis of endocannabinoids. Biochemistry 45:4720-4726.

Levenes C, Daniel H, Soubrie P, Crepel F (1998) Cannabinoids decrease excitatory synaptic transmission and impair long-term depression in rat cerebellar Purkinje cells. J Physiol 510 (Pt 3):867-879.

Li MD, Beuten J, Ma JZ, Payne TJ, Lou XY, Garcia V, Duenes AS, Crews KM, Elston RC (2005) Ethnic- and gender-specific association of the nicotinic acetylcholine receptor alpha4 subunit gene (CHRNA4) with nicotine dependence. Hum Mol Genet 14:1211-1219.

Lichtman AH, Sheikh SM, Loh HH, Martin BR (2001) Opioid and cannabinoid modulation of precipitated withdrawal in delta(9)-tetrahydrocannabinol and morphine-dependent mice. J Pharmacol Exp Ther 298:1007-1014.

Ligresti A, Morera E, Van Der Stelt M, Monory K, Lutz B, Ortar G, Di Marzo V (2004) Further evidence for the existence of a specific process for the membrane transport of anandamide. Biochem J 380:265-272.

Ligresti A, De Petrocellis L, Hernan Perez de la Ossa D, Aberturas R, Cristino L, Moriello AS, Finizio A, Gil ME, Torres AI, Molpeceres J, Di Marzo V (2010) Exploiting nanotechnologies and TRPV1 channels to investigate the putative anandamide membrane transporter. PLoS One 5:e10239.

Llano I, Leresche N, Marty A (1991) Calcium entry increases the sensitivity of cerebellar Purkinje cells to applied GABA and decreases inhibitory synaptic currents. Neuron 6:565-574.

Lo Verme J, Fu J, Astarita G, La Rana G, Russo R, Calignano A, Piomelli D (2005) The nuclear receptor peroxisome proliferator-activated receptor-alpha mediates the anti-inflammatory actions of palmitoylethanolamide. Mol Pharmacol 67:15-19.

Lodge DJ, Grace AA (2006) The hippocampus modulates dopamine neuron responsivity by regulating the intensity of phasic neuron activation. Neuropsychopharmacology 31:1356-1361.

LoVerme J, Russo R, La Rana G, Fu J, Farthing J, Mattace-Raso G, Meli R, Hohmann A, Calignano A, Piomelli D (2006) Rapid broad-spectrum analgesia through activation of peroxisome proliferator-activated receptor-alpha. J Pharmacol Exp Ther 319:1051-1061.

Luchicchi A, Pillolla G, Lecca S and Pistis M (2009a) Inhibition of anandamide hydrolysis antagonizes nicotine effects on neurons in the nucleus accumbens shell in the rat, ECNP workshop on Neuropsychopharmacology for young scientists in Europe (Nice, France) S86

Luchicchi A, Lecca S, Carta S, Pillola G, Pistis M (2009c) Inhibition of anadamide hydrolisys differentially modulates cocaine-induced effects on rat mesolimbic system. 22nd ECNP congress, European College of Neuropsychopharmacology (Istanbul, Turkey) S662

Luchicchi A, Lecca S, Carta S, Pillola G, Pistis M (2009b) Inhibition of FAAH antagonizes cocaine and nicotine effect on neurons in the nucleus accumbens shell in the rat, 19th symposium of the international cannabinoid research society (St.Charles, IL, USA) P33

Luchicchi A, Pillolla G, Pistis M (2008) Effects of FAAH inhibition on morphine, cocaine and nicotine actions in mesolimbic dopamine system in the rat. 18th symposium of the International cannabinoid research society (Aviemore, Scotland) P82

Luchicchi A, Lecca S, Carta S, Pistis M (2010) Peroxisome proliferator activated receptors type α agonists modulate nicotine effects in the ventral tegmental area in the rat. 23rd ECNP congress, European College of Neuropsychopharmacology (Amsterdam, the Nederlands) S598

Luchicchi A, Lecca S, Carta S, Pillolla G, Muntoni AL, Yasar S, Goldberg SR, Pistis M (2010) Effects of fatty acid amide hydrolase inhibition on neuronal responses to nicotine, cocaine and morphine in the nucleus accumbens shell and ventral tegmental area: involvement of PPAR-alpha nuclear receptors. Addict Biol 15:277-288.

Luetje CW (2004) Getting past the asterisk: the subunit composition of presynaptic nicotinic receptors that modulate striatal dopamine release. Mol Pharmacol 65:1333-1335.

Maccarrone M, Attina M, Bari M, Cartoni A, Ledent C, Finazzi-Agro A (2001) Anandamide degradation and N-acylethanolamines level in wild-type and CB1 cannabinoid receptor knockout mice of different ages. J Neurochem 78:339-348.

Maccarrone M, Rossi S, Bari M, De Chiara V, Fezza F, Musella A, Gasperi V, Prosperetti C, Bernardi G, Finazzi-Agro A, Cravatt BF, Centonze D (2008) Anandamide inhibits metabolism and physiological actions of 2-arachidonoylglycerol in the striatum. Nat Neurosci 11:152-159.

Mackie K (2005) Cannabinoid receptor homo- and heterodimerization. Life Sci 77:1667-1673.

Mackie K, Hille B (1992) Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. Proc Natl Acad Sci U S A 89:3825-3829.

Mackie K, Stella N (2006) Cannabinoid receptors and endocannabinoids: evidence for new players. AAPS J 8:E298-306.

Mackie K, Lai Y, Westenbroek R, Mitchell R (1995) Cannabinoids activate an inwardly rectifying potassium conductance and inhibit Q-type calcium currents in AtT20 cells transfected with rat brain cannabinoid receptor. J Neurosci 15:6552-6561.

Maejima T, Ohno-Shosaku T, Kano M (2001) Endogenous cannabinoid as a retrograde messenger from depolarized postsynaptic neurons to presynaptic terminals. Neurosci Res 40:205-210.

Mailleux P, Vanderhaeghen JJ (1992) Distribution of neuronal cannabinoid receptor in the adult rat brain: a comparative receptor binding radioautography and in situ hybridization histochemistry. Neuroscience 48:655-668.

Malaiyandi V, Sellers EM, Tyndale RF (2005) Implications of CYP2A6 genetic variation for smoking behaviors and nicotine dependence. Clin Pharmacol Ther 77:145-158.

Maldonado R (2002) Study of cannabinoid dependence in animals. Pharmacol Ther 95:153-164.

Malenka RC, Bear MF (2004) LTP and LTD: an embarrassment of riches. Neuron 44:5-21.

Mameli-Engvall M, Evrard A, Pons S, Maskos U, Svensson TH, Changeux JP, Faure P (2006) Hierarchical control of dopamine neuron-firing patterns by nicotinic receptors. Neuron 50:911-921.

Mandard S, Muller M, Kersten S (2004) Peroxisome proliferator-activated receptor alpha target genes. Cell Mol Life Sci 61:393-416.

Mansvelder HD, McGehee DS (2000) Long-term potentiation of excitatory inputs to brain reward areas by nicotine. Neuron 27:349-357.

Mansvelder HD, Keath JR, McGehee DS (2002) Synaptic mechanisms underlie nicotine-induced excitability of brain reward areas. Neuron 33:905-919.

Marinelli S, Di Marzo V, Florenzano F, Fezza F, Viscomi MT, van der Stelt M, Bernardi G, Molinari M, Maccarrone M, Mercuri NB (2007) N-arachidonoyl-dopamine tunes synaptic transmission onto dopaminergic neurons by activating both cannabinoid and vanilloid receptors. Neuropsychopharmacology 32:298-308.

Markou A (2008) Neurobiology of nicotine dependence. *Phil Trans R Soc B* **363**:3159–3168

Marsden CA (2006) Dopamine: the rewarding years. Br J Pharmacol 147 Suppl 1:S136-144.

Marsicano G, Lutz B (1999) Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain. Eur J Neurosci 11:4213-4225.

Marsicano G, Lutz B (2006) Neuromodulatory functions of the endocannabinoid system. J Endocrinol Invest 29:27-46.

Marsicano G, Wotjak CT, Azad SC, Bisogno T, Rammes G, Cascio MG, Hermann H, Tang J, Hofmann C, Zieglgansberger W, Di Marzo V, Lutz B (2002) The endogenous cannabinoid system controls extinction of aversive memories. Nature 418:530-534.

Martin LF, Freedman R (2007) Schizophrenia and the alpha7 nicotinic acetylcholine receptor. Int Rev Neurobiol 78:225-246.

Martin M, Ledent C, Parmentier M, Maldonado R, Valverde O (2000) Cocaine, but not morphine, induces conditioned place preference and sensitization to locomotor responses in CB1 knockout mice. Eur J Neurosci 12:4038-4046.

Mascia P, Pistis M, Justinova Z, Panlilio LV, Luchicchi A, Lecca S, Scherma M, Fratta W, Fadda P, Barnes C, Redhi GH, Yasar S, Le Foll B, Tanda G, Piomelli D, Goldberg SR (2010) Blockade of Nicotine Reward and Reinstatement by Activation of Alpha-Type Peroxisome Proliferator-Activated Receptors. Biol Psychiatry.

Maskos U, Molles BE, Pons S, Besson M, Guiard BP, Guilloux JP, Evrard A, Cazala P, Cormier A, Mameli-Engvall M, Dufour N, Cloez-Tayarani I, Bemelmans AP, Mallet J, Gardier AM, David V, Faure P, Granon S, Changeux JP (2005) Nicotine reinforcement and cognition restored by targeted expression of nicotinic receptors. Nature 436:103-107.

Mato S, Chevaleyre V, Robbe D, Pazos A, Castillo PE, Manzoni OJ (2004) A single in-vivo exposure to delta 9THC blocks endocannabinoid-mediated synaptic plasticity. Nat Neurosci 7:585-586.

Matsuda LA, Bonner TI, Lolait SJ (1993) Localization of cannabinoid receptor mRNA in rat brain. J Comp Neurol 327:535-550.

Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346:561-564.

Matta SG, Balfour DJ, Benowitz NL, Boyd RT, Buccafusco JJ, Caggiula AR, Craig CR, Collins AC, Damaj MI, Donny EC, Gardiner PS, Grady SR, Heberlein U, Leonard SS, Levin ED, Lukas RJ, Markou A, Marks MJ, McCallum SE, Parameswaran N, Perkins KA, Picciotto MR, Quik M, Rose JE, Rothenfluh A, Schafer WR, Stolerman IP, Tyndale RF, Wehner JM, Zirger JM (2007) Guidelines on nicotine dose selection for in vivo research. Psychopharmacology (Berl) 190:269-319.

Matthews RT, German DC (1984) Electrophysiological evidence for excitation of rat ventral tegmental area dopamine neurons by morphine. Neuroscience 11:617-625.

Maubourguet N, Lesne A, Changeux JP, Maskos U, Faure P (2008) Behavioral sequence analysis reveals a novel role for beta2* nicotinic receptors in exploration. PLoS Comput Biol 4:e1000229.

Mazzola C, Medalie J, Scherma M, Panlilio LV, Solinas M, Tanda G, Drago F, Cadet JL, Goldberg SR, Yasar S (2009) Fatty acid amide hydrolase (FAAH) inhibition enhances memory acquisition through activation of PPAR-alpha nuclear receptors. Learn Mem 16:332-337.

McDonald AJ, Mascagni F (2001) Localization of the CB1 type cannabinoid receptor in the rat basolateral amygdala: high concentrations in a subpopulation of cholecystokinin-containing interneurons. Neuroscience 107:641-652.

McFarland MJ, Barker EL (2004) Anandamide transport. Pharmacol Ther 104:117-135.

McFarland MJ, Porter AC, Rakhshan FR, Rawat DS, Gibbs RA, Barker EL (2004) A role for caveolae/lipid rafts in the uptake and recycling of the endogenous cannabinoid anandamide. J Biol Chem 279:41991-41997.

McGehee DS, Role LW (1995) Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. Annu Rev Physiol 57:521-546.

McGehee DS, Heath MJ, Gelber S, Devay P, Role LW (1995) Nicotine enhancement of fast excitatory synaptic transmission in CNS by presynaptic receptors. Science 269:1692-1696.

Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, Gopher A, Almog S, Martin BR, Compton DR, et al. (1995) Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. Biochem Pharmacol 50:83-90.

Melis M, Pistis M (2007) Endocannabinoid signaling in midbrain dopamine neurons: more than physiology? Curr Neuropharmacol 5:268-277.

Melis M, Pistis M, Perra S, Muntoni AL, Pillolla G, Gessa GL (2004a) 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, Pillolla G, Perra S, Colombo G, Muntoni AL, Pistis M (2009) Electrophysiological properties of dopamine neurons in the ventral tegmental area of Sardinian alcohol-preferring rats. Psychopharmacology (Berl) 201:471-481.

Melis M, Pillolla G, Luchicchi A, Muntoni AL, Yasar S, Goldberg SR, Pistis M (2008) Endogenous fatty acid ethanolamides suppress nicotine-induced activation of mesolimbic dopamine neurons through nuclear receptors. J Neurosci 28:13985-13994.

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

Melis M, Carta S, Fattore L, Tolu S, Yasar S, Goldberg SR, Fratta W, Maskos U, Pistis M (2010) Peroxisome proliferator-activated receptors-alpha modulate dopamine cell activity through nicotinic receptors. Biol Psychiatry 68:256-264.

Melis M, Pillolla G, Bisogno T, Minassi A, Petrosino S, Perra S, Muntoni AL, Lutz B, Gessa GL, Marsicano G, Di Marzo V, Pistis M (2006) Protective activation of the endocannabinoid system during ischemia in dopamine neurons. Neurobiol Dis 24:15-27.

Melsens (1844). "Über das Nicotin". Journal für Praktische Chemie 32 (1): 372–377

Mereu G, Yoon KW, Boi V, Gessa GL, Naes L, Westfall TC (1987) Preferential stimulation of ventral tegmental area dopaminergic neurons by nicotine. Eur J Pharmacol 141:395-399.

Metcalf RL (1948) The mode of action of organic insecticides. National Academies.

Meyer FG, Trueblood EE, Heller JL. (1999) The Great Herbal of Leonard Fuchs: De historia stirpium commentaria insignes, 1542, Vol 1: Commentary, Vol 2: Facsimile Chapter 5. The Vienna Codex: Leonard Fuchs's Unpublished Herb Book. Stanford: Stanford University Press

Michalik L, Auwerx J, Berger JP, Chatterjee VK, Glass CK, Gonzalez FJ, Grimaldi PA, Kadowaki T, Lazar MA, O'Rahilly S, Palmer CN, Plutzky J, Reddy JK, Spiegelman BM, Staels B,

Wahli W (2006) International Union of Pharmacology. LXI. Peroxisome proliferator-activated receptors. Pharmacol Rev 58:726-741.

Mishina M, Takai T, Imoto K, Noda M, Takahashi T, Numa S, Methfessel C, Sakmann B (1986) Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. Nature 321:406-411.

Monardes N (1596) Joyfull Newes out of the New Found Word (English Translation by Thomas Hackett). London. (Section on tobacco reprinted in Economic Botany, 1963. 17:83-85)

Monod J, Wyman J, Changeux JP (1965) On the Nature of Allosteric Transitions: A Plausible Model. J Mol Biol 12:88-118.

Moreno S, Farioli-Vecchioli S, Ceru MP (2004) Immunolocalization of peroxisome proliferator-activated receptors and retinoid X receptors in the adult rat CNS. Neuroscience 123:131-145.

Mulder AB, Hodenpijl MG, Lopes da Silva FH (1998) Electrophysiology of the hippocampal and amygdaloid projections to the nucleus accumbens of the rat: convergence, segregation, and interaction of inputs. J Neurosci 18:5095-5102.

Munro S, Thomas KL, Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. Nature 365:61-65.

Nakayama H, Okuda H, Nakashima T, Imaoka S, Funae Y (1993) Nicotine metabolism by rat hepatic cytochrome P450s. Biochem Pharmacol 45:2554-2556.

Nakazi M, Bauer U, Nickel T, Kathmann M, Schlicker E (2000) Inhibition of serotonin release in the mouse brain via presynaptic cannabinoid CB1 receptors. Naunyn Schmiedebergs Arch Pharmacol 361:19-24.

Nashmi R, Lester H (2007) Cell autonomy, receptor autonomy, and thermodynamics in nicotine receptor up-regulation. Biochem Pharmacol 74:1145-1154.

Navarro M, Carrera MR, Fratta W, Valverde O, Cossu G, Fattore L, Chowen JA, Gomez R, del Arco I, Villanua MA, Maldonado R, Koob GF, Rodriguez de Fonseca F (2001) Functional

interaction between opioid and cannabinoid receptors in drug self-administration. J Neurosci 21:5344-5350.

Neubig RR, Boyd ND, Cohen JB (1982) Conformations of Torpedo acetylcholine receptor associated with ion transport and desensitization. Biochemistry 21:3460-3467.

Nisell M, Nomikos GG, Svensson TH (1994) Systemic nicotine-induced dopamine release in the rat nucleus accumbens is regulated by nicotinic receptors in the ventral tegmental area. Synapse 16:36-44.

O'Farrell TJ, Connors GJ, Upper D (1983) Addictive behaviors among hospitalized psychiatric patients. Addict Behav 8:329-333.

O'Sullivan SE (2007) Cannabinoids go nuclear: evidence for activation of peroxisome proliferator-activated receptors. Br J Pharmacol 152:576-582.

Ohno-Shosaku T, Maejima T, Kano M (2001) Endogenous cannabinoids mediate retrograde signals from depolarized postsynaptic neurons to presynaptic terminals. Neuron 29:729-738.

Ohno-Shosaku T, Tsubokawa H, Mizushima I, Yoneda N, Zimmer A, Kano M (2002) Presynaptic cannabinoid sensitivity is a major determinant of depolarization-induced retrograde suppression at hippocampal synapses. J Neurosci 22:3864-3872.

Okamoto Y, Morishita J, Tsuboi K, Tonai T, Ueda N (2004) Molecular characterization of a phospholipase D generating anandamide and its congeners. J Biol Chem 279:5298-5305.

Orio L, Edwards S, George O, Parsons LH, Koob GF (2009) A role for the endocannabinoid system in the increased motivation for cocaine in extended-access conditions. J Neurosci 29:4846-4857.

Oz M, Ravindran A, Diaz-Ruiz O, Zhang L, Morales M (2003) The endogenous cannabinoid anandamide inhibits alpha7 nicotinic acetylcholine receptor-mediated responses in Xenopus oocytes. J Pharmacol Exp Ther 306:1003-1010.

Pan B, Hillard CJ, Liu QS (2008) Endocannabinoid signaling mediates cocaine-induced inhibitory synaptic plasticity in midbrain dopamine neurons. J Neurosci 28:1385-1397.

Paradiso K, Zhang J, Steinbach JH (2001) The C terminus of the human nicotinic alpha4beta2 receptor forms a binding site required for potentiation by an estrogenic steroid. J Neurosci 21:6561-6568.

Parkinson JA, Dalley JW, Cardinal RN, Bamford A, Fehnert B, Lachenal G, Rudarakanchana N, Halkerston KM, Robbins TW, Everitt BJ (2002) Nucleus accumbens dopamine depletion impairs both acquisition and performance of appetitive Pavlovian approach behaviour: implications for mesoaccumbens dopamine function. Behav Brain Res 137:149-163.

Parolaro D, Rubino T (2008) The role of the endogenous cannabinoid system in drug addiction. Drug News Perspect 21:149-157.

Paxinos G, Watson C (1997) The rat brain in stereotaxic coordinates Ed.3rd. San Diego: Academic.

Penn WA (2007) The Soverane Herb: a History of Tobacco. Read Books.

Perra S, Pillolla G, Luchicchi A, Pistis M (2008) Alcohol inhibits spontaneous activity of basolateral amygdala projection neurons in the rat: involvement of the endocannabinoid system. Alcohol Clin Exp Res 32:443-449.

Pertwee RG (2007) GPR55: a new member of the cannabinoid receptor clan? Br J Pharmacol 152:984-986.

Pertwee RG (2008) Ligands that target cannabinoid receptors in the brain: from THC to anandamide and beyond. Addict Biol 13:147-159.

Philaretes (1602) Work for Chimny sweepers or a Warning for Tabacconists. London: Thomas Esat for Thomas Bushell.

Picciotto MR, Zoli M, Rimondini R, Lena C, Marubio LM, Pich EM, Fuxe K, Changeux JP (1998) Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine. Nature 391:173-177.

Picciotto MR, Zoli M, Lena C, Bessis A, Lallemand Y, Le Novere N, Vincent P, Pich EM, Brulet P, Changeux JP (1995) Abnormal avoidance learning in mice lacking functional high-affinity nicotine receptor in the brain. Nature 374:65-67.

Pictet A, Crepieux P (1903) Comptes Rendus. 137:860

Pidoplichko VI, DeBiasi M, Williams JT, Dani JA (1997) Nicotine activates and desensitizes midbrain dopamine neurons. Nature 390:401-404.

Pidoplichko VI, Noguchi J, Areola OO, Liang Y, Peterson J, Zhang T, Dani JA (2004) Nicotinic cholinergic synaptic mechanisms in the ventral tegmental area contribute to nicotine addiction. Learn Mem 11:60-69.

Pinnier A (1891-95) “Ueber Nicotin", in "Berichte", 1891-95, and in "Archiv der Pharmazie",CCXXXI, CCXXXIII

Piomelli D (2003) The molecular logic of endocannabinoid signalling. Nat Rev Neurosci 4:873-884.

Pistis M, Melis M (2010) From surface to nuclear receptors: the endocannabinoid family extends its assets. Curr Med Chem 17:1450-1467.

Pistis M, Muntoni AL, Pillolla G, Gessa GL (2002a) Cannabinoids inhibit excitatory inputs to neurons in the shell of the nucleus accumbens: an in vivo electrophysiological study. Eur J Neurosci 15:1795-1802.

Pistis M, Ferraro L, Pira L, Flore G, Tanganelli S, Gessa GL, Devoto P (2002b) Delta(9)-tetrahydrocannabinol decreases extracellular GABA and increases extracellular glutamate and dopamine levels in the rat prefrontal cortex: an in vivo microdialysis study. Brain Res 948:155-158.

Pitler TA, Alger BE (1992) Postsynaptic spike firing reduces synaptic GABAA responses in hippocampal pyramidal cells. J Neurosci 12:4122-4132.

Pomerleau OF (1998) Endogenous opioids and smoking: a review of progress and problems. Psychoneuroendocrinology 23:115-130.

Pons S, Fattore L, Cossu G, Tolu S, Porcu E, McIntosh JM, Changeux JP, Maskos U, Fratta W (2008) Crucial role of alpha4 and alpha6 nicotinic acetylcholine receptor subunits from ventral tegmental area in systemic nicotine self-administration. J Neurosci 28:12318-12327.

Porta N, Vallee L, Lecointe C, Bouchaert E, Staels B, Bordet R, Auvin S (2009) Fenofibrate, a peroxisome proliferator-activated receptor-alpha agonist, exerts anticonvulsive properties. Epilepsia 50:943-948.

Porter AC, Sauer JM, Knierman MD, Becker GW, Berna MJ, Bao J, Nomikos GG, Carter P, Bymaster FP, Leese AB, Felder CC (2002) Characterization of a novel endocannabinoid, virodhamine, with antagonist activity at the CB1 receptor. J Pharmacol Exp Ther 301:1020-1024.

Posselt W, Reimann L (1828) Chemische Untersuchungen des Tabaks und Darstellung des eigenhumlichen wirksamen Principes dieser Pflanze. Geigers Magazin der Pharmazie. 24:138-161

Prince RJ, Sine SM (1999) Acetylcholine and epibatidine binding to muscle acetylcholine receptors distinguish between concerted and uncoupled models. J Biol Chem 274:19623-19629.

Pryor GT, Larsen FF, Husain S, Braude MC (1978) Interactions of delta9-tetrahydrocannabinol with d-amphetamine, cocaine, and nicotine in rats. Pharmacol Biochem Behav 8:295-318.

Quick MW, Lester RA (2002) Desensitization of neuronal nicotinic receptors. J Neurobiol 53:457-478.

Radcliffe KA, Dani JA (1998) Nicotinic stimulation produces multiple forms of increased glutamatergic synaptic transmission. J Neurosci 18:7075-7083.

Radek RJ, Kohlhaas KL, Rueter LE, Mohler EG (2010) Treating the cognitive deficits of schizophrenia with alpha4beta2 neuronal nicotinic receptor agonists. Curr Pharm Des 16:309-322.

Raggenbass M, Bertrand D (2002) Nicotinic receptors in circuit excitability and epilepsy. J Neurobiol 53:580-589.

Ray R, Schnoll RA, Lerman C (2009) Nicotine dependence: biology, behavior, and treatment. Annu Rev Med 60:247-260.

Reading PJ, Dunnett SB (1991) The effects of excitotoxic lesions of the nucleus accumbens on a matching to position task. Behav Brain Res 46:17-29.

Reggio PH (2002) Endocannabinoid structure-activity relationships for interaction at the cannabinoid receptors. Prostaglandins Leukot Essent Fatty Acids 66:143-160.

Reid MS, Fox L, Ho LB, Berger SP (2000) Nicotine stimulation of extracellular glutamate levels in the nucleus accumbens: neuropharmacological characterization. Synapse 35:129-136.

Reitstetter R, Lukas RJ, Gruener R (1999) Dependence of nicotinic acetylcholine receptor recovery from desensitization on the duration of agonist exposure. J Pharmacol Exp Ther 289:656-660.

Revah F, Bertrand D, Galzi JL, Devillers-Thiery A, Mulle C, Hussy N, Bertrand S, Ballivet M, Changeux JP (1991) Mutations in the channel domain alter desensitization of a neuronal nicotinic receptor. Nature 353:846-849.

Riegel AC, Lupica CR (2004) Independent presynaptic and postsynaptic mechanisms regulate endocannabinoid signaling at multiple synapses in the ventral tegmental area. J Neurosci 24:11070-11078.

Rinaldi-Carmona M, Barth F, Heaulme M, Shire D, Calandra B, Congy C, Martinez S, Maruani J, Neliat G, Caput D, et al. (1994) SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. FEBS Lett 350:240-244.

Robbe D, Alonso G, Manzoni OJ (2003) Exogenous and endogenous cannabinoids control synaptic transmission in mice nucleus accumbens. Ann N Y Acad Sci 1003:212-225.

Robbe D, Kopf M, Remaury A, Bockaert J, Manzoni OJ (2002) Endogenous cannabinoids mediate long-term synaptic depression in the nucleus accumbens. Proc Natl Acad Sci U S A 99:8384-8388.

Rodriguez de Fonseca F, Del Arco I, Bermudez-Silva FJ, Bilbao A, Cippitelli A, Navarro M (2005) The endocannabinoid system: physiology and pharmacology. Alcohol Alcohol 40:2-14.

Role LW, Berg DK (1996) Nicotinic receptors in the development and modulation of CNS synapses. Neuron 16:1077-1085.

Ropero AB, Juan-Pico P, Rafacho A, Fuentes E, Bermudez-Silva FJ, Roche E, Quesada I, de Fonseca FR, Nadal A (2009) Rapid non-genomic regulation of Ca2+ signals and insulin secretion by PPAR alpha ligands in mouse pancreatic islets of Langerhans. J Endocrinol 200:127-138.

Ross RA (2003) Anandamide and vanilloid TRPV1 receptors. Br J Pharmacol 140:790-801.

Ross S, Peselow E (2009) Pharmacotherapy of addictive disorders. Clin Neuropharmacol 32:277-289.

Rueda D, Navarro B, Martinez-Serrano A, Guzman M, Galve-Roperh I (2002) The endocannabinoid anandamide inhibits neuronal progenitor cell differentiation through attenuation of the Rap1/B-Raf/ERK pathway. J Biol Chem 277:46645-46650.

Ryan WJ, Banner WK, Wiley JL, Martin BR, Razdan RK (1997) Potent anandamide analogs: the effect of changing the length and branching of the end pentyl chain. J Med Chem 40:3617-3625.

Saccone SF, Hinrichs AL, Saccone NL, Chase GA, Konvicka K, Madden PA, Breslau N, Johnson EO, Hatsukami D, Pomerleau O, Swan GE, Goate AM, Rutter J, Bertelsen S, Fox L, Fugman D, Martin NG, Montgomery GW, Wang JC, Ballinger DG, Rice JP, Bierut LJ (2007) Cholinergic nicotinic receptor genes implicated in a nicotine dependence association study targeting 348 candidate genes with 3713 SNPs. Hum Mol Genet 16:36-49.

Safo PK, Cravatt BF, Regehr WG (2006) Retrograde endocannabinoid signaling in the cerebellar cortex. Cerebellum 5:134-145.

Sagar DR, Kendall DA, Chapman V (2008) Inhibition of fatty acid amide hydrolase produces PPAR-alpha-mediated analgesia in a rat model of inflammatory pain. Br J Pharmacol 155:1297-1306.

Sakmann B, Patlak J, Neher E (1980) Single acetylcholine-activated channels show burst-kinetics in presence of desensitizing concentrations of agonist. Nature 286:71-73.

Salas R, Cook KD, Bassetto L, De Biasi M (2004) The alpha3 and beta4 nicotinic acetylcholine receptor subunits are necessary for nicotine-induced seizures and hypolocomotion in mice. Neuropharmacology 47:401-407.

Sallette J, Pons S, Devillers-Thiery A, Soudant M, Prado de Carvalho L, Changeux JP, Corringer PJ (2005) Nicotine upregulates its own receptors through enhanced intracellular maturation. Neuron 46:595-607.

Sargent PB (1993) The diversity of neuronal nicotinic acetylcholine receptors. Annu Rev Neurosci 16:403-443.

Scherma M, Panlilio LV, Fadda P, Fattore L, Gamaleddin I, Le Foll B, Justinova Z, Mikics E, Haller J, Medalie J, Stroik J, Barnes C, Yasar S, Tanda G, Piomelli D, Fratta W, Goldberg SR (2008) Inhibition of anandamide hydrolysis by cyclohexyl carbamic acid 3'-carbamoyl-3-yl ester (URB597) reverses abuse-related behavioral and neurochemical effects of nicotine in rats. J Pharmacol Exp Ther 327:482-490.

Schilstrom B, Nomikos GG, Nisell M, Hertel P, Svensson TH (1998) N-methyl-D-aspartate receptor antagonism in the ventral tegmental area diminishes the systemic nicotine-induced dopamine release in the nucleus accumbens. Neuroscience 82:781-789.

Schilstrom B, Fagerquist MV, Zhang X, Hertel P, Panagis G, Nomikos GG, Svensson TH (2000) Putative role of presynaptic alpha7* nicotinic receptors in nicotine stimulated increases of extracellular levels of glutamate and aspartate in the ventral tegmental area. Synapse 38:375-383.

Schlicker E, Kathmann M (2001) Modulation of transmitter release via presynaptic cannabinoid receptors. Trends Pharmacol Sci 22:565-572.

Schoedel KA, Hoffmann EB, Rao Y, Sellers EM, Tyndale RF (2004) Ethnic variation in CYP2A6 and association of genetically slow nicotine metabolism and smoking in adult Caucasians. Pharmacogenetics 14:615-626.

Schultz W (2002) Getting formal with dopamine and reward. Neuron 36:241-263.

Seaton M, Kyerematen GA, Morgan M, Jeszenka EV, Vesell ES (1991) Nicotine metabolism in stumptailed macaques, Macaca arctoides. Drug Metab Dispos 19:946-954.

Seltzman HH, Fleming DN, Thomas BF, Gilliam AF, McCallion DS, Pertwee RG, Compton DR, Martin BR (1997) Synthesis and pharmacological comparison of dimethylheptyl and pentyl analogs of anandamide. J Med Chem 40:3626-3634.

Sher E, Chen Y, Sharples TJ, Broad LM, Benedetti G, Zwart R, McPhie GI, Pearson KH, Baldwinson T, De Filippi G (2004) Physiological roles of neuronal nicotinic receptor subtypes: new insights on the nicotinic modulation of neurotransmitter release, synaptic transmission and plasticity. Curr Top Med Chem 4:283-297.

Shiffman S, West R, Gilbert D (2004) Recommendation for the assessment of tobacco craving and withdrawal in smoking cessation trials. Nicotine Tob Res 6:599-614.

Shoji T, Winz R, Iwasa T, Nakajima K, Yamada Y, Hashimoto T (2002) Expression patterns of two tobacco isoflavone reductase like genes and their possible roles in secondary metabolism in tobacco. Plant Mol Biol 50: 427–440

Shoji T, Yamada Y, Hashimoto T (2000) Jasmonate induction of putrescine N-methyltransferase genes in the root of Nicotiana sylvestris. Plant Cell Physiol 41: 831–839

Siegmund B, Leitner E, Pfannhauser W (1999) Determination of the nicotine content of various edible nightshades (Solanaceae) and their products and estimation of the associated dietary nicotine intake. J Agric Food Chem 47:3113-3120.

Sinclair SJ, Murphy KJ, Birch CD, Hamill D (2000) Molecular characterization of quinolinate phosphoribosyltransferase (QPRTase) in *Nicotiana*. Plant Mol Biol 44: 603–617

Sine SM (2002) The nicotinic receptor ligand binding domain. J Neurobiol 53:431-446.

Singer S, Rossi S, Verzosa S, Hashim A, Lonow R, Cooper T, Sershen H, Lajtha A (2004) Nicotine-induced changes in neurotransmitter levels in brain areas associated with cognitive function. Neurochem Res 29:1779-1792.

Smit AB, Syed NI, Schaap D, van Minnen J, Klumperman J, Kits KS, Lodder H, van der Schors RC, van Elk R, Sorgedrager B, Brejc K, Sixma TK, Geraerts WP (2001) A glia-derived acetylcholine-binding protein that modulates synaptic transmission. Nature 411:261-268.

Soler-Llavina GJ, Sabatini BL (2006) Synapse-specific plasticity and compartmentalized signaling in cerebellar stellate cells. Nat Neurosci 9:798-806.

Soria G, Mendizabal V, Tourino C, Robledo P, Ledent C, Parmentier M, Maldonado R, Valverde O (2005) Lack of CB1 cannabinoid receptor impairs cocaine self-administration. Neuropsychopharmacology 30:1670-1680.

Spivak CE, Lupica CR, Oz M (2007) The endocannabinoid anandamide inhibits the function of alpha4beta2 nicotinic acetylcholine receptors. Mol Pharmacol 72:1024-1032.

Starowicz K, Nigam S, Di Marzo V (2007) Biochemistry and pharmacology of endovanilloids. Pharmacol Ther 114:13-33.

Stead LF, Perera R, Bullen C, Mant D, Lancaster T (2008) Nicotine replacement therapy for smoking cessation. Cochrane Database Syst Rev 1 CD000146.

Stella N, Piomelli D (2001) Receptor-dependent formation of endogenous cannabinoids in cortical neurons. Eur J Pharmacol 425:189-196.

Stella N, Schweitzer P, Piomelli D (1997) A second endogenous cannabinoid that modulates long-term potentiation. Nature 388:773-778.

Stolerman IP, Jarvis MJ (1995) The scientific case that nicotine is addictive. Psychopharmacology (Berl) 117:2-10; discussion 14-20.

Stolerman IP, Chamberlain S, Bizarro L, Fernandes C, Schalkwyk L (2004) The role of nicotinic receptor alpha 7 subunits in nicotine discrimination. Neuropharmacology 46:363-371.

Sudweeks SN, Yakel JL (2000) Functional and molecular characterization of neuronal nicotinic ACh receptors in rat CA1 hippocampal neurons. J Physiol 527 Pt 3:515-528.

Sugiura T, Kobayashi Y, Oka S, Waku K (2002) Biosynthesis and degradation of anandamide and 2-arachidonoylglycerol and their possible physiological significance. Prostaglandins Leukot Essent Fatty Acids 66:173-192.

Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, Yamashita A, Waku K (1995) 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. Biochem Biophys Res Commun 215:89-97.

Sullivan JM (2000) Cellular and molecular mechanisms underlying learning and memory impairments produced by cannabinoids. Learn Mem 7:132-139.

Sun Y, Alexander SP, Kendall DA, Bennett AJ (2006) Cannabinoids and PPARalpha signalling. Biochem Soc Trans 34:1095-1097.

Sun Y, Alexander SP, Garle MJ, Gibson CL, Hewitt K, Murphy SP, Kendall DA, Bennett AJ (2007) Cannabinoid activation of PPAR alpha; a novel neuroprotective mechanism. Br J Pharmacol 152:734-743.

Szabo B, Siemes S, Wallmichrath I (2002) Inhibition of GABAergic neurotransmission in the ventral tegmental area by cannabinoids. Eur J Neurosci 15:2057-2061.

Szabo B, Dorner L, Pfreundtner C, Norenberg W, Starke K (1998) Inhibition of GABAergic inhibitory postsynaptic currents by cannabinoids in rat corpus striatum. Neuroscience 85:395-403.

Takahashi KA, Linden DJ (2000) Cannabinoid receptor modulation of synapses received by cerebellar Purkinje cells. J Neurophysiol 83:1167-1180.

Taly A, Corringer PJ, Guedin D, Lestage P, Changeux JP (2009) Nicotinic receptors: allosteric transitions and therapeutic targets in the nervous system. Nat Rev Drug Discov 8:733-750.

Tanda G, Pontieri FE, Di Chiara G (1997) Cannabinoid and heroin activation of mesolimbic dopamine transmission by a common mu1 opioid receptor mechanism. Science 276:2048-2050.

Tapper AR, McKinney SL, Nashmi R, Schwarz J, Deshpande P, Labarca C, Whiteaker P, Marks MJ, Collins AC, Lester HA (2004) Nicotine activation of alpha4* receptors: sufficient for reward, tolerance, and sensitization. Science 306:1029-1032.

Tsuboi K, Sun YX, Okamoto Y, Araki N, Tonai T, Ueda N (2005) Molecular characterization of N-acylethanolamine-hydrolyzing acid amidase, a novel member of the choloylglycine hydrolase family with structural and functional similarity to acid ceramidase. J Biol Chem 280:11082-11092.

Tsutsumi T, Kobayashi T, Ueda H, Yamauchi E, Watanabe S, Okuyama H (1994) Lysophosphoinositide-specific phospholipase C in rat brain synaptic plasma membranes. Neurochem Res 19:399-406.

Twitchell W, Brown S, Mackie K (1997) Cannabinoids inhibit N- and P/Q-type calcium channels in cultured rat hippocampal neurons. J Neurophysiol 78:43-50.

Ueda H, Kobayashi T, Kishimoto M, Tsutsumi T, Okuyama H (1993) A possible pathway of phosphoinositide metabolism through EDTA-insensitive phospholipase A1 followed by lysophosphoinositide-specific phospholipase C in rat brain. J Neurochem 61:1874-1881.

Ungless MA, Magill PJ, Bolam JP (2004) Uniform inhibition of dopamine neurons in the ventral tegmental area by aversive stimuli. Science 303:2040-2042.

Ungless MA, Whistler JL, Malenka RC, Bonci A (2001) Single cocaine exposure in vivo induces long-term potentiation in dopamine neurons. Nature 411:583-587.

Unwin N (2005) Refined structure of the nicotinic acetylcholine receptor at 4A resolution. J Mol Biol 346:967-989.

Unwin N, Miyazawa A, Li J, Fujiyoshi Y (2002) Activation of the nicotinic acetylcholine receptor involves a switch in conformation of the alpha subunits. J Mol Biol 319:1165-1176.

U.S Department of Health and Human Services (U.S. DHHS). Risks associated with smoking cigarettes with low machine-measured yields of tar and nicotine. Smoking and Tobacco Control Monographs, 2001

U.S Department of Health and Human Services (U.S. DHHS) (2004) The Health Consequences of Smoking: The Surgeon General's Report—Executive Summary. http://www.cdc.gov/tobacco/data_statistics/sgr/sgr_2004/index.htm#full.

Valera S, Ballivet M, Bertrand D (1992) Progesterone modulates a neuronal nicotinic acetylcholine receptor. Proc Natl Acad Sci U S A 89:9949-9953.

Valjent E, Mitchell JM, Besson MJ, Caboche J, Maldonado R (2002) Behavioural and biochemical evidence for interactions between Delta 9-tetrahydrocannabinol and nicotine. Br J Pharmacol 135:564-578.

Vallejo YF, Buisson B, Bertrand D, Green WN (2005) Chronic nicotine exposure upregulates nicotinic receptors by a novel mechanism. J Neurosci 25:5563-5572.

Van Sickle MD, Duncan M, Kingsley PJ, Mouihate A, Urbani P, Mackie K, Stella N, Makriyannis A, Piomelli D, Davison JS, Marnett LJ, Di Marzo V, Pittman QJ, Patel KD, Sharkey KA (2005) Identification and functional characterization of brainstem cannabinoid CB2 receptors. Science 310:329-332.

Vandenbergh DJ, O'Connor RJ, Grant MD, Jefferson AL, Vogler GP, Strasser AA, Kozlowski LT (2007) Dopamine receptor genes (DRD2, DRD3 and DRD4) and gene-gene interactions associated with smoking-related behaviors. Addict Biol 12:106-116.

Vaughan W (1612) Directions for Heath, both Naturall and Artificial: Derived from the best Physitians as well modern and Ancient. London: T Snodham for R Jackson

Vela G, Ruiz-Gayo M, Fuentes JA (1995) Anandamide decreases naloxone-precipitated withdrawal signs in mice chronically treated with morphine. Neuropharmacology 34:665-668.

Vernallis AB, Conroy WG, Berg DK (1993) Neurons assemble acetylcholine receptors with as many as three kinds of subunits while maintaining subunit segregation among receptor subtypes. Neuron 10:451-464.

Viveros MP, Marco EM, File SE (2006) Nicotine and cannabinoids: parallels, contrasts and interactions. Neurosci Biobehav Rev 30:1161-1181.

Wager-Miller J, Westenbroek R, Mackie K (2002) Dimerization of G protein-coupled receptors: CB1 cannabinoid receptors as an example. Chem Phys Lipids 121:83-89.

Walters CL, Brown S, Changeux JP, Martin B, Damaj MI (2006) The beta2 but not alpha7 subunit of the nicotinic acetylcholine receptor is required for nicotine-conditioned place preference in mice. Psychopharmacology (Berl) 184:339-344.

Walton NJ, Robins RJ, Peerless CJA (1990) Enzymes of N-methylputrescine biosynthesis in relation to hyoscyamine formation in transformed root cultures of Datura stramonium and Atropa belladonna, Planta *182*: 136–141.

Wang YX, Lee CH, Tiep S, Yu RT, Ham J, Kang H, Evans RM (2003) Peroxisome-proliferator-activated receptor delta activates fat metabolism to prevent obesity. Cell 113:159-170.

Wangensteen T, Akselsen H, Holmen J, Undlien D, Retterstol L (2010) A Common Haplotype in NAPEPLD Is Associated With Severe Obesity in a Norwegian Population-Based Cohort (the HUNT Study). Obesity (Silver Spring).

Watkins SS, Stinus L, Koob GF, Markou A (2000) Reward and somatic changes during precipitated nicotine withdrawal in rats: centrally and peripherally mediated effects. J Pharmacol Exp Ther 292:1053-1064.

Wei BQ, Mikkelsen TS, McKinney MK, Lander ES, Cravatt BF (2006) A second fatty acid amide hydrolase with variable distribution among placental mammals. J Biol Chem 281:36569-36578.

Wennig R (2009) Back to the roots of modern analytical toxicology: Jean Servais Stas and the Bocarme murder case. Drug Test Anal 1:153-155.

West R, Baker CL, Cappelleri JC, Bushmakin AG (2008) Effect of varenicline and bupropion SR on craving, nicotine withdrawal symptoms, and rewarding effects of smoking during a quit attempt. Psychopharmacology (Berl) 197:371-377.

White FJ, Hu XT, Henry DJ (1993) Electrophysiological effects of cocaine in the rat nucleus accumbens: microiontophoretic studies. J Pharmacol Exp Ther 266:1075-1084.

Wiley JL, Martin BR (2002) Cannabinoid pharmacology: implications for additional cannabinoid receptor subtypes. Chem Phys Lipids 121:57-63.

Williams JM, Gandhi KK (2008) Use of caffeine and nicotine in people with schizophrenia. Curr Drug Abuse Rev 1:155-161.

Wilson RI, Nicoll RA (2001) Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. Nature 410:588-592.

Wilson RI, Kunos G, Nicoll RA (2001) Presynaptic specificity of endocannabinoid signaling in the hippocampus. Neuron 31:453-462.

Wise RA, Bozarth MA (1987) A psychomotor stimulant theory of addiction. Psychol Rev 94:469-492.

Wiskerke J, Pattij T, Schoffelmeer AN, De Vries TJ (2008) The role of CB1 receptors in psychostimulant addiction. Addict Biol 13:225-238.

Wonnacott S (1997) Presynaptic nicotinic ACh receptors. Trends Neurosci 20:92-98.

Wooltorton JR, Pidoplichko VI, Broide RS, Dani JA (2003) Differential desensitization and distribution of nicotinic acetylcholine receptor subtypes in midbrain dopamine areas. J Neurosci 23:3176-3185.

World Health Organization (WHO) (2008) WHO Report on the Global Tobacco Epidemic. 278-287

World Health Organization (WHO) (2006) WHO: The Facts About Smoking and Health. 30 May 2006 (WHO, Geneva).

World Health Organization (WHO) (2002) The Tobacco Atlas, American Cancer Society

Wullner U, Gundisch D, Herzog H, Minnerop M, Joe A, Warnecke M, Jessen F, Schutz C, Reinhardt M, Eschner W, Klockgether T, Schmaljohann J (2008) Smoking upregulates alpha4beta2* nicotinic acetylcholine receptors in the human brain. Neurosci Lett 430:34-37.

Xian H, Scherrer JF, Madden PA, Lyons MJ, Tsuang M, True WR, Eisen SA (2005) Latent class typology of nicotine withdrawal: genetic contributions and association with failed smoking cessation and psychiatric disorders. Psychol Med 35:409-419.

Yamaguchi T, Hagiwara Y, Tanaka H, Sugiura T, Waku K, Shoyama Y, Watanabe S, Yamamoto T (2001) Endogenous cannabinoid, 2-arachidonoylglycerol, attenuates naloxone-precipitated withdrawal signs in morphine-dependent mice. Brain Res 909:121-126.

Yang HY, Karoum F, Felder C, Badger H, Wang TC, Markey SP (1999) GC/MS analysis of anandamide and quantification of N-arachidonoylphosphatidylethanolamides in various brain regions, spinal cord, testis, and spleen of the rat. J Neurochem 72:1959-1968.

Yang KS, Gholson RK, Waller GR (1965) Studies on nicotine biosynthesis. J Am Chem Soc 87:4184–4188

Yoshida T, Hashimoto K, Zimmer A, Maejima T, Araishi K, Kano M (2002) The cannabinoid CB1 receptor mediates retrograde signals for depolarization-induced suppression of inhibition in cerebellar Purkinje cells. J Neurosci 22:1690-1697.

Young JW, Finlayson K, Spratt C, Marston HM, Crawford N, Kelly JS, Sharkey J (2004) Nicotine improves sustained attention in mice: evidence for involvement of the alpha7 nicotinic acetylcholine receptor. Neuropsychopharmacology 29:891-900.

Ziegler, J., Facchini, PJ (2008) Alkaloid biosynthesis: metabolism and trafficking. Annual Review of Plant Biology 59: 735-769

APPENDIX

Cellular/Molecular

Endogenous Fatty Acid Ethanolamides Suppress Nicotine-Induced Activation of Mesolimbic Dopamine Neurons through Nuclear Receptors

Miriam Melis,[1]* Giuliano Pillolla,[1]* Antonio Luchicchi,[1] Anna Lisa Muntoni,[2] Sevil Yasar,[3] Steven R. Goldberg,[4] and Marco Pistis[1]

[1]B. B. Brodie Department of Neuroscience, and [2]Consiglio Nazionale delle Richerche Institute of Neuroscience, University of Cagliari, 09042 Monserrato, Italy, [3]Division of Geriatric Medicine and Gerontology, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21224, and [4]Preclinical Pharmacology Section, Behavioral Neuroscience Research Branch, Intramural Research Program, Department of Health and Human Services, National Institute on Drug Abuse–National Institutes of Health, Baltimore, Maryland 21224

Nicotine stimulates the activity of mesolimbic dopamine neurons, which is believed to mediate the rewarding and addictive properties of tobacco use. Accumulating evidence suggests that the endocannabinoid system might play a major role in neuronal mechanisms underlying the rewarding properties of drugs of abuse, including nicotine. Here, we investigated the modulation of nicotine effects by the endocannabinoid system on dopamine neurons in the ventral tegmental area with electrophysiological techniques *in vivo* and *in vitro*. We discovered that pharmacological inhibition of fatty acid amide hydrolase (FAAH), the enzyme that catabolizes fatty acid ethanolamides, among which the endocannabinoid anandamide (AEA) is the best known, suppressed nicotine-induced excitation of dopamine cells. Importantly, this effect was mimicked by the administration of the FAAH substrates oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), but not methanandamide, the hydrolysis resistant analog of AEA. OEA and PEA are naturally occurring lipid signaling molecules structurally related to AEA, but devoid of affinity for cannabinoid receptors. They blocked the effects of nicotine by activation of the peroxisome proliferator-activated receptor-α (PPAR-α), a nuclear receptor transcription factor involved in several aspects of lipid metabolism and energy balance. Activation of PPAR-α triggered a nongenomic stimulation of tyrosine kinases, which might lead to phosphorylation and negative regulation of neuronal nicotinic acetylcholine receptors. These data indicate for the first time that the anorexic lipids OEA and PEA possess neuromodulatory properties as endogenous ligands of PPAR-α in the brain and provide a potential new target for the treatment of nicotine addiction.

Key words: **dopamine neurons; nicotine; electrophysiology; endocannabinoids; fatty acid amide hydrolase; patch clamp; peroxisome proliferator-activated receptor**

Introduction

Nicotine is the main active component in tobacco smoke, which initiates and sustains tobacco addiction. Hence, nicotine induces drug-seeking behavior in animals and many additional effects commonly seen with addictive drugs (Stolerman and Shoaib, 1991). Among these effects, stimulation of mesolimbic dopamine (DA) transmission is considered to be one of the hallmarks to define the addicting potential of nicotine, being one of the common features of all abused drugs (Di Chiara and Imperato, 1988; Wise, 2004). By acting at neuronal nicotinic acetylcholine receptors (nAChRs), nicotine activates ventral tegmental area (VTA) DA neurons (Mereu et al., 1987; Pidoplichko et al., 1997) and induces DA release in the nucleus accumbens (Di Chiara and Imperato, 1988).

Among medications aimed at achieving smoking cessation, antagonists at the cannabinoid type-1 (CB_1) receptors show promise, based on preclinical data indicating that these compounds, such as rimonabant (SR141716A) or AM251, reduce nicotine self-administration or conditioned place preference (CPP) (Cohen et al., 2002; Le Foll and Goldberg, 2004; Forget et al., 2005; Shoaib, 2008), nicotine-induced DA release in the nucleus accumbens (Cohen et al., 2002; Cheer et al., 2007), or smoking cessation in humans (Cahill and Ussher, 2007). These data strongly point to a facilitatory effect of the endocannabinoid system in the motivational and DA-releasing properties of nicotine. Indeed, endogenous cannabinoids, such as arachidonoylethanolamide (anandamide, AEA) and 2-arachidonoylglycerol, and CB_1 receptors are involved in the neuronal mechanisms underlying the rewarding effects of most drugs of abuse, including nicotine (Castañé et al., 2005; Le Foll and Goldberg, 2005; Le Foll et al., 2008; Solinas et al., 2008).

Received July 9, 2008; revised Nov. 10, 2008; accepted Nov. 12, 2008.

This work was supported by a grant from the Italian Ministry of University (PRIN 2005), Philip Morris USA and Philip Morris International, and by the Intramural Research Program of National Institute on Drug Abuse, National Institutes of Health, and Department of Health and Human Services.

*M. M. and G. P. contributed equally to this work and share first authorship.

Correspondence should be addressed to Marco Pistis, B. B. Brodie Department of Neuroscience, University of Cagliari, Cittadella Universitaria, 09042 Monserrato, Italy. E-mail: mpistis@unica.it.

DOI:10.1523/JNEUROSCI.3221-08.2008

 0270-6474/08/2813985-10$15.00/0

The present study was designed to investigate modulation of nicotine effects by the endocannabinoid system, and to clarify the role of DA neurons in the mediation of the antiaddicting properties of CB_1 antagonists. To this aim, the electrophysiological responses of DA neurons to nicotine administration were studied following either blockade of CB_1 receptors or, conversely, enhancement of brain endocannabinoid levels by inhibiting fatty acid amide hydrolase (FAAH) (Kathuria et al., 2003; Fegley et al., 2005), the major hydrolyzing enzyme for AEA and other endogenous fatty acid ethanolamides (FAEs), such as the N-acylamines oleoylethanolamide (OEA) and palmitoylethanolamide (PEA). Unlike AEA, both OEA and PEA have no affinity for cannabinoid receptors, but bind to the peroxisome proliferator-activated receptor (PPAR), a family of nuclear receptor transcription factors (Fu et al., 2003; Lo Verme et al., 2005). Three subtypes of PPARs (α, β/δ and γ) play important roles in lipid metabolism, insulin sensitivity, glucose homeostasis and inflammation (Berger and Moller, 2002). Through PPAR-α, OEA and PEA are peripherally acting satiety signals that modulate feeding, body weight and lipid metabolism (Rodriguez de Fonseca et al., 2001; Fu et al., 2003) and have antinociceptive effects in visceral and inflammatory pain models (Lo Verme et al., 2005; D'Agostino et al., 2007; Suardiaz et al., 2007).

We discovered that, contrary to our expectations, enhancement of brain FAE levels, rather than blockade of CB_1 receptors, inhibited the responses of DA neurons to nicotine. More importantly, the noncannabinoid FAEs OEA and PEA play a novel and unsuspected role, as PPAR-α agonists, in the negative regulation of neuronal responses to nicotine.

Materials and Methods

Electrophysiology: single unit recordings. We performed the experiments in strict accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2004) and EEC Council Directive of 24 November 1986 (86/609). We made all efforts to minimize pain and suffering and to reduce the number of animals used.

Male Sprague Dawley rats (250–350 g) (Harlan) were housed in groups of three to six in standard conditions of temperature and humidity under a 12 h light/dark cycle (with lights on at 7:00 A.M.) with food and water available *ad libitum*.

Animals were anesthetized with urethane (1300 mg/kg, i.p.), their femoral vein was cannulated for intravenous administration of pharmacological agents, and they were placed in the stereotaxic apparatus (Kopf) with their body temperature maintained at 37 ± 1°C by a heating pad. Thereafter, the scalp was retracted and one burr hole was drilled above the VTA (−6.0 mm anteroposterior from bregma, 0.3–0.6 mm lateral from midline) for the placement of a recording electrode. For intracerebroventricular drug administration, a guide cannula (23 gauge stainless steel) was placed into the ventricle ipsilateral to the recording side (1.0 mm posterior, 1.4 mm lateral to bregma and 4.0 mm ventral to the cortical surface). Structures were localized according to the stereotaxic atlas of Paxinos and Watson (1997). Intracerebroventricular injections were made through a prefilled inner cannula (30 gauge stainless steel tubing) connected to a 50 μl Hamilton microsyringe and extending 1.0 mm below the tip of the guide into the ventricle. Infusion rate was set at 2.5 μl/min by an electrically driven mini-pump.

Single unit activity of neurons located in the VTA (V 7.0–8.0 mm from the cortical surface) was recorded extracellularly with glass micropipettes filled with 2% pontamine sky blue dissolved in 0.5 M sodium acetate (impedance 2–5 MΩ). Single unit activity was filtered (bandpass 500–5000 Hz) and individual spikes were isolated by means of a window discriminator (Digitimer), displayed on a digital storage oscilloscope (TDS 3012, Tektronics) and digitally recorded. Experiments were sampled on line and off line with Spike2 software (Cambridge Electronic Design) by a computer connected to CED 1401 interface (Cambridge Electronic Design).

Single units were isolated and identified according to already published criteria (Grace and Bunney, 1983, 1984; Ungless et al., 2004). Since only one cell was recorded per rat, VTA DA neurons were selected when all criteria for identification were fulfilled: firing rate <10 Hz, duration of action potential >2.5 ms, inhibitory responses to hindpaw pinching. Bursts were defined as the occurrence of two spikes at an interspike interval <80 ms, and terminated when the interspike interval exceeded 160 ms (Grace and Bunney, 1983).

At the end of each recording section, direct current (10 μA for 15 min) was passed through the recording electrode to eject Pontamine sky blue, which allowed the identification of the recorded cells. Brains were removed and fixed in 8% formalin solution. The position of the electrodes was microscopically identified on serial sections (60 μm) stained with cresyl violet.

Electrophysiology: patch-clamp recordings. The preparation of VTA slices was as described previously (Johnson and North, 1992). Briefly, male Sprague Dawley rats (10–28 d) were anesthetized with halothane in a vapor chamber and killed by decapitation. A block of tissue containing the midbrain was rapidly dissected and sliced in the horizontal plane (300 μm) with a vibratome (Leica VT1000S) in ice-cold low-Ca^{2+} solution containing (in mM): 126 NaCl, 1.6 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 0.625 $CaCl_2$, 18 $NaHCO_3$, and 11 glucose. Slices were transferred to a holding chamber with artificial CSF (ACSF, 37°C) saturated with 95% O_2 and 5% CO_2 containing (in mM): 126 NaCl, 1.6 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 18 $NaHCO_3$, and 11 glucose. Slices were allowed to recover for at least 1 h before being placed in the recording chamber and superfused with the ACSF (37°C) saturated with 95% O_2 and 5% CO_2. Cells were visualized with an upright microscope with infrared illumination (Axioskop FS 2 plus, Zeiss), and whole-cell patch-clamp recordings were made by using an Axopatch 200B amplifier (Axon Instruments). Both voltage- and current-clamp experiments were made with electrodes filled with a solution containing the following (in mM): 117 KCl 144, 10 HEPES, BAPTA 3.45, CaCl 1, 2.5 Mg_2ATP, and 0.25 Mg_2GTP (pH 7.2–7.4, 275–285 mOsm). Experiments were begun only after series resistance had stabilized (typically 15–40 MΩ). Series and input resistance were monitored continuously on-line with a 5 mV depolarizing step (25 ms). Data were filtered at 2 kHz, digitized at 10 kHz, and collected on-line with acquisition software (pClamp 8.2, Axon Instruments). DA neurons from the posterior VTA were identified by the presence of a large I_h current (Johnson and North, 1992) that was assayed immediately after break-in, using a series of incremental 10 mV hyperpolarizing steps from a holding potential of −70 mV. Each slice received only a single drug exposure.

Drugs. Nicotine ((−)-nicotine hydrogen tartrate) was purchased from Sigma. OEA, PEA, methanandamide (mAEA), AM281, AM251, capsazepine, WY14643, MK886, genistein, PP2 were purchased from Tocris. Rimonabant (SR141716A) was a generous gift of Sanofi-Aventis Recherche (Montpellier). Nicotine was diluted in saline (pH = 7). For i.c.v. administration, OEA or mAEA were dissolved in 40% w/v 2-hydroxypropyl-β-cyclodextrin. mAEA for i.v. injections was dissolved in 2% Tween 80 and 2% ethanol and then diluted in saline. Rimonabant was emulsified in 1% Tween 80, then diluted in saline solution and sonicated. URB597 and MK886 were dissolved in DMSO (100 μg/μl) and diluted to the final concentration in saline. All drugs for patch-clamp experiments were dissolved in DMSO as stock solutions and then dilute to the final volume in ACSF (final concentration <0.01%).

Statistical analysis. Drug-induced changes in firing rate and pattern were calculated by averaging the effects after drug administration (2 min or 30 s bins for *in vivo* and *in vitro* electrophysiology, respectively) and normalizing to the predrug baseline. All the numerical data are given as mean ± SEM. Data were compared and analyzed by using two-way ANOVA for repeated measures (treatment × time), or one-way ANOVA or Student's *t* test for repeated measures, when appropriate. *Post hoc* multiple comparisons were made using the Dunnett's test. Statistical analysis was performed by means of the NCSS program. The significance level was established at $p < 0.05$.

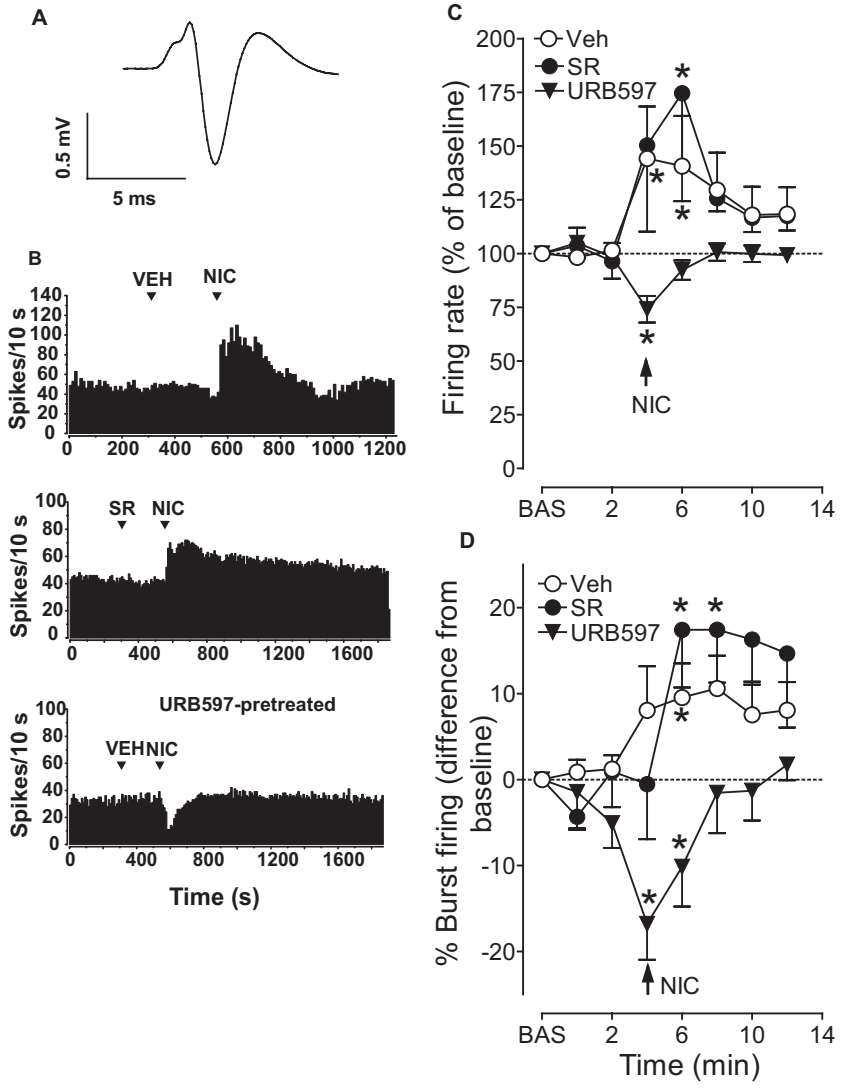


Figure 1. Effects of rimonabant and URB597 on activation of VTA dopamine neurons by nicotine. ***A***, Average trace, acquired from a digital storage oscilloscope, showing the typical, broad, notched waveform of a VTA dopamine neuron recorded from an anesthetized rat. ***B***, Representative firing rate histograms showing effects of intravenous nicotine (NIC, injected at arrowheads) on discharge activity of individual VTA dopamine neurons recorded from anesthetized rats. The top panel shows the typical response to 0.2 mg/kg nicotine in control conditions following intravenous injection of vehicle (VEH). The middle panel shows the lack of effect of rimonabant (SR, 0.5 mg/kg, i.v.) on spontaneous firing rate of dopamine neurons and on the subsequent effects of nicotine. The bottom panel shows the effect of nicotine in a URB597 pretreated animal, where nicotine induced a transient inhibition of firing activity. ***C***, ***D***, Graphs illustrating the time course of nicotine's effects on firing rate and burst firing of VTA dopamine neurons. Pretreatment with URB597 (0.1 mg/kg, i.v.), but not rimonabant (0.5 mg/kg, i.v.), prevented the nicotine-induced increases in firing rate (***C***) and burst firing (***D***) of VTA dopamine neurons. Results are means, with vertical bars representing the SEM of firing rates and burst firing, expressed as a percentage of or difference from baseline (BAS) values. Arrows represent time of nicotine injection. *$p < 0.05$ versus baseline (one-way ANOVA for repeated measures and Dunnett's test).

Results

Effects of rimonabant and URB597 on activation of VTA dopamine neurons by nicotine *in vivo*

We recorded the activity of VTA DA neurons in urethane anesthetized rats. Cells were recorded only when they fulfilled all established criteria for VTA DA neuron identification (see Materials and Methods). A typical DA neuron broad waveform is shown in Figure 1*A*. Consistent with previous *in vivo* studies (Mereu et al., 1987; Erhardt et al., 2002), nicotine (0.2 mg/kg, i.v.) enhanced firing rate of VTA DA neurons to 144.2 ± 24.2% of baseline and burst firing to + 10.6 ± 3.8% ($F_{(5,71)} = 4.06$, $n = 23$, $p < 0.05$; $F_{(5,71)} = 2.89$, $n = 23$, $p < 0.05$, respectively, one-way ANOVA for repeated measure) (Fig. 1*B–D*). As previously reported, nicotine-induced excitation was short lasting, being significant 2 and 4 min following administration (Dunnett's *post hoc*). Injections of all the different vehicles (either intraperitoneal, intravenous, or intracerebroventricular) that were used in all subsequent experiments neither significantly changed the discharge activity of DA neurons nor modulated the effect of the subsequent nicotine administration. Therefore, all control experiments with nicotine were pooled.

We first studied the effect of CB_1 receptor blockade on nicotine-induced excitation of DA neurons. Rimonabant (SR141716A, 0.5 mg/kg, i.v. 4 min before nicotine) did not modify the excitatory response of DA neurons to nicotine compared with vehicle ($F_{(1,103)} = 1.10$, $n = 11$, $p = 0.3$, two-way ANOVA for repeated measures) (Fig. 1*B–D*). Next, we investigated the effect of enhancement of endogenous endocannabinoid tone. Rats were pretreated between 60 and 120 min (average 72.4 min) before electrophysiological recordings with URB597 (0.1 mg/kg, i.v.), an irreversible FAAH inhibitor. The interval between URB597 administration and recordings varied among experiments, nevertheless, this dose of URB597 is within the range of doses shown to enhance persistently (>6 h) brain AEA levels (Kathuria et al., 2003; Fegley et al., 2005), with maximal effects 1–2 h following administration (Fegley et al., 2005). URB597 did not change spontaneous baseline firing rate (3.7 ± 0.26 Hz, $n = 48$, in control animals; 3.9 ± 0.14, $n = 14$, in URB-pretreated animals, $p = 0.36$, Student's t test) or burst firing (22.6 ± 3.64% of spikes in bursts, $n = 48$, in control animals; 23.0 ± 5.15%, $n = 14$; in URB-pretreated animals, $p = 0.96$, Student's t test) of VTA DA neurons assessed before nicotine administration, but, unexpectedly, it completely prevented nicotine's effects on DA neuronal activity (74.1 ± 6.2% and −16.8 ± 4.1% of baseline, for firing rate and burst firing, respectively) (Fig. 1*B*,*C*,*D*). Two-way ANOVA showed a highly significant effect of URB597 treatment on the effects of nicotine on firing rate ($F_{(1,95)} = 4.95$, $n = 6$, $p < 0.01$) and burst firing ($F_{(1,107)} = 7.98$, $n = 6$, $p < 0.01$). Hence, following URB597 pretreatment, nicotine transiently inhibited, rather than excited, firing rate and burst firing of DA neurons ($F_{(3,23)} = 3.73$, $p < 0.001$, $n = 6$; $F_{(3,23)} = 9.44$, $p < 0.01$, one-way ANOVA for repeated measures) (Fig. 1*B*,*C*,*D*).

Contribution of CB_1 receptors and PPAR-α to URB597 antagonism of nicotine effects *in vivo*

URB597 has been shown to increase not only AEA, but also OEA and PEA levels (Kathuria et al., 2003). We first investigated

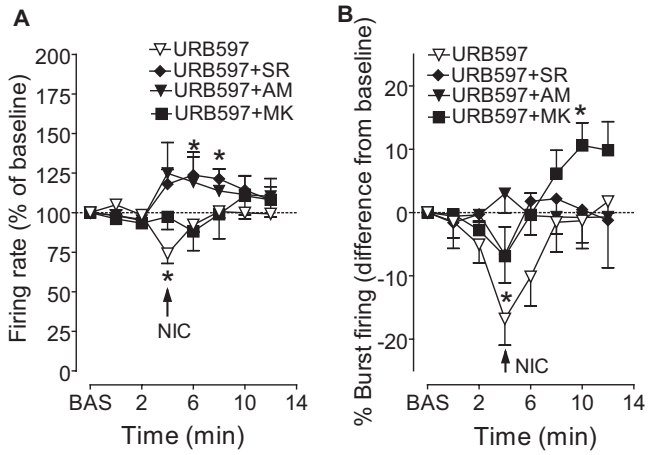


Figure 2. Contribution of CB_1 receptors and PPAR-α to URB597's reversal of nicotine's effects *in vivo*. ***A***, The CB_1 receptor antagonists rimonabant (SR, 0.5 mg/kg, i.v.) or AM251 (AM, 1.0 mg/kg, i.v.) reversed URB597's blockade of nicotine-induced increases in firing rate of VTA dopaminergic neurons produced by nicotine (NIC, 0.2 mg/kg), whereas the PPAR-α antagonist MK886 (MK, 3 mg/kg, i.p.) was ineffective. ***B***, In contrast, MK886 administration reversed URB597's blockade of nicotine-induced increase in bursting activity of VTA dopamine neurons, whereas rimonabant and AM251 were ineffective. Results are means, with vertical bars representing the SEM of firing rates and burst firing, expressed as a percentage of or difference from baseline (BAS) values. Arrows represent time of nicotine injection. *$p < 0.05$ versus baseline (one-way ANOVA for repeated measures and Dunnett's test).

whether AEA mediates URB597's antagonism of nicotine effects by acting at CB_1 receptors. For this purpose, we administered the CB_1 antagonist rimonabant together with URB597. Rimonabant (0.5 mg/kg, i.v. 1 min before URB597) significantly reduced the ability of URB597 to block nicotine-induced stimulation of DA neuron discharge rate (123.7 ± 11.5% of baseline), but not of burst firing (−18.4 ± 8.0% of baseline) ($F_{(1,23)} = 11.04$, $n = 9$, $p < 0.01$; $F_{(1,23)} = 2.30$, $n = 9$, $p = 0.2$, respectively, two-way ANOVA for repeated measures) (Fig. 2*A*,*B*). The CB_1 receptor antagonist AM251 (1.0 mg/kg, i.v. 1 min before URB597, $n = 5$) fully replicated the effects of rimonabant (Fig. 2*A*,*B*) (firing rate: $F_{(1,61)} = 10.96$, $n = 6$, $p < 0.01$; burst firing: $F_{(1,75)} = 0.00$, $n = 6$, $p = 0.9$, two-way ANOVA for repeated measures). This dissociation between URB597 effects on nicotine-induced increases in firing rate and burst firing was further analyzed by assessing the contribution of the noncannabinoid FAEs (OEA and PEA), which are ligands at PPAR-α (Fu et al., 2003). We asked whether the effect of URB597 could be antagonized by the synthetic selective PPAR-α antagonist MK886 (3 mg/kg, i.p.). MK886 significantly prevented URB597 from altering nicotine-induced stimulation of bursting (+10.5 ± 3.5% of baseline) ($F_{(1,72)} = 4.90$, $n = 13$, $p < 0.05$, two-way ANOVA for repeated measures) (Fig. 2*B*), but not firing rate ($F_{(1,75)} = 0.0$, $n = 13$, $p = 0.95$, two-way ANOVA for repeated measures) (Fig. 2*A*). These results suggest that diverse FAEs may modulate nicotine effects on DA neurons through different mechanisms.

Oleoylethanolamide blocks nicotine effects *in vivo* via PPAR-α

To determine the precise contribution of either CB_1 or PPAR-α receptors in the observed effects, we assessed whether mAEA, the metabolically stable analog of AEA, and OEA modulated the response to nicotine of VTA DA neurons. mAEA was administered intravenously at doses of 1 and 5 mg/kg ($n = 6$ each group) (Fig. 3*A*,*B*), or i.c.v. at a dose of 5 μg/5 μl ($n = 6$) (Fig. 3*B*) 4 min before nicotine administration. These doses, which exert CB_1 receptor-mediated behavioral effects *in vivo* (Solinas et al., 2006, 2007), did not affect either baseline firing rate or burst firing of DA neurons or modulate the excitatory response to nicotine administration, compared with vehicle ($F_{(3,234)} = 0.68$, $n = 6$, $p = 0.57$, two-way ANOVA for repeated measures). Due to the poor metabolic stability of OEA, we chose to administer it (20 μg/5 μl), or a corresponding volume of vehicle (40% w/v 2-hydroxypropyl-β-cyclodextrin), into the lateral ventricle 4 min before nicotine. In contrast to mAEA, OEA completely prevented the activation of DA neurons induced by nicotine (92.7 ± 13.5% of baseline at 2 min postnicotine) (Fig. 3*C*,*D*), whereas vehicle injection was inactive ($n = 6$, data not shown). Two-way ANOVA showed a significant effect of OEA treatment on nicotine-induced stimulation of firing rate and burst firing ($F_{(1,99)} = 5.61$, $n = 6$, $p < 0.05$; $F_{(1,107)} = 4.28$, $n = 6$, $p < 0.05$, respectively, two-way ANOVA for repeated measures). Neither OEA nor vehicle produced significant changes in the spontaneous firing rate or burst firing of DA neurons (Fig. 3*C*,*D*). Next, MK886 pretreatment (3 mg/kg, i.p., 30 min before recordings) prevented the blockade by OEA of nicotine's excitatory effects (122.8 ± 7.2% of baseline at 4 min postnicotine), when compared with OEA alone ($F_{(1,55)} = 6.06$, $n = 8$, $p < 0.05$, two-way ANOVA for repeated measures) (Fig. 3*C*,*D*), thus highlighting the role of PPAR-α in the effects of OEA.

Blockade of nicotine-induced excitation of dopamine neurons *in vitro* by noncannabinoid fatty acid ethanolamides

We next asked whether modulation of nicotine effects by PPAR-α could be studied in brain slices containing the mesencephalon by using whole-cell patch-clamp recordings. The effect of nicotine was studied on posterior VTA DA neurons. Figure 4*A* (top) shows a typical action potential of a representative DA neuron, when recorded in the current-clamp mode, with its typical low threshold, broad action potential, and prominent afterhyperpolarization. The second derivative of this action potential originates the waveform (Fig. 4*A*, bottom) that has been used for cell identification of DA neurons in many *in vivo* extracellular studies (Grace and Bunney, 1983, 1984; Ungless et al., 2004), and qualitatively corresponds to the typical action potential recorded *in vivo* and shown in Figure 1*A*. DA neurons recorded under current-clamp mode displayed an average frequency of 1.8 ± 0.1 Hz ($n = 102$) and fired spontaneously in a clock-like, single-spike mode. Consistent with the literature (Pidoplichko et al., 1997), DA neurons responded to bath-applied nicotine (1 μM, 2 min) with a transient excitation of discharge rate (~40%) (Fig. 4*B*–*D*). This excitation peaked (137 ± 12.8% of baseline, $n = 6$), and was statistically significant ($F_{(6,41)} = 8.03$, $p < 0.0001$, one-way ANOVA), during the first minute of application. Under voltage-clamp mode ($V_{holding} = -70$ mV), nicotine caused a transient inward current of 40.3 ± 5.6 pA ($n = 6$) (Fig. 4*F*), due to rapid activation and desensitization of nAChRs (Pidoplichko et al., 1997). We next examined the effects of the three different FAEs (i.e., mAEA, OEA and PEA) on nicotine induced excitation of VTA DA neurons. These drugs were applied for 5 min to assess whether they per se modulated spontaneous firing of DA neurons, and then coapplied with nicotine (1 μM). mAEA was tested at two different concentrations, 30 nM and 1 μM. The lower concentration of mAEA did not significantly affect spontaneous discharge rate of DA neurons or modulate nicotine-evoked excitation (the peak of nicotine with mAEA was 147.7 ± 13.7% of baseline firing rate, $p = 0.58$, *t* test, vs nicotine alone) (Fig. 4*E*,*G*). Since this concentration of mAEA might have been too low, we next tested a concentration of 1 μM, which itself significantly

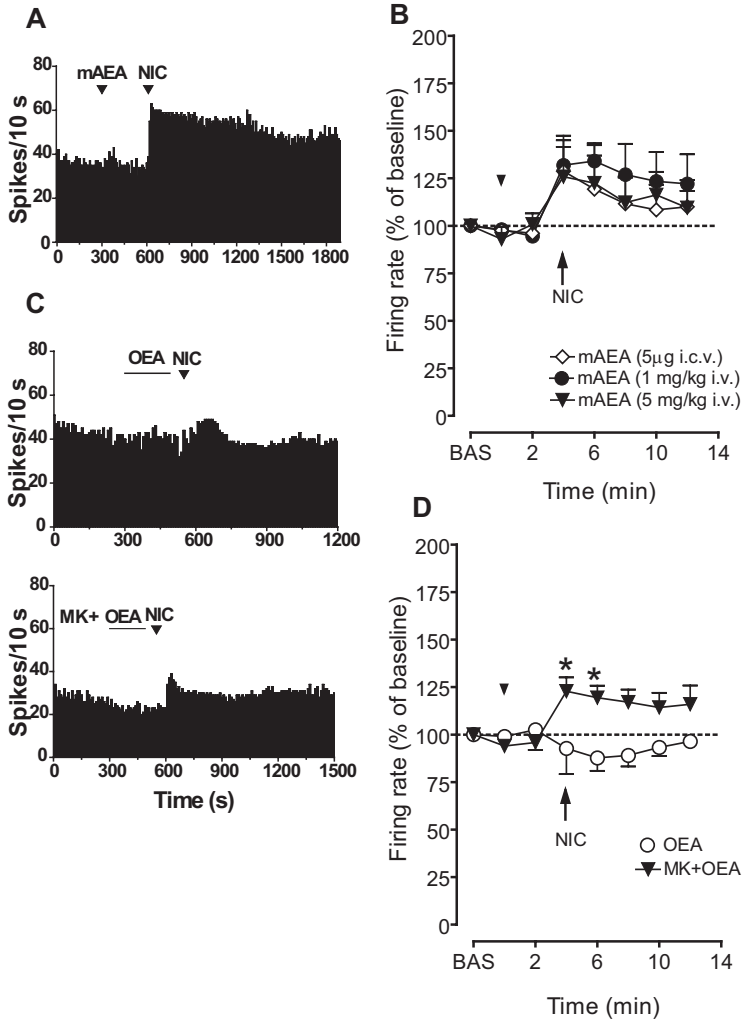


Figure 3. OEA, but not mAEA, prevented increases in firing rate of VTA dopaminergic neurons produced by nicotine. ***A***, Representative firing rate histograms showing the effects of nicotine (NIC, 0.2 mg/kg i.v., injected at arrowheads) on the discharge activity of individual VTA dopamine neurons recorded following injection of mAEA (1 mg/kg, i.v.). ***B***, Graph showing that nicotine-induced excitation of VTA dopamine neurons was not changed following the administration of mAEA, either intravenously (1 and 5 mg/kg) or intracerebroventricularly (5 μg/5 μl). ***C***, Representative firing rate histograms showing the effects of NIC (0.2 mg/kg i.v., injected at arrowheads) on the discharge activity of individual VTA dopamine neurons recorded following injection of OEA (20 μg/5 μl, i.c.v.; top). MK886 (MK, 3 mg/kg, i.p.) reversed the OEA-induced blockade of nicotine's effects (bottom). Neither OEA nor vehicle (40% w/v 2-hydroxypropyl-β-cyclodextrin) produced significant changes in spontaneous firing rate or burst firing. ***D***, Graph showing that nicotine-induced excitation of VTA dopamine neurons was abolished by OEA. MK886 (MK, 3 mg/kg, i.p.) reversed the OEA-induced blockade of nicotine's effects. Results are means, with vertical bars representing SEM of firing rates, expressed as a percentage of baseline (BAS) values. Arrows represent the time of intravenous injections. The horizontal bar represents the time of intracerebroventricular administration. *$p < 0.05$ versus baseline (one-way ANOVA for repeated measures and Dunnett's test).

enhanced firing rate of DA neurons to 370.6 ± 110% of baseline level ($F_{(5,125)} = 21.74$, $n = 6$, $p = 0.0001$, one-way ANOVA for repeated measures; data not shown). We asked whether mAEA-induced stimulation was due to activation of CB_1 and/or TRPV1 receptors. The CB_1 receptor antagonist AM281, at a concentration (500 nM) that fully reverses activation of CB_1 receptors by maximal concentrations of agonists (Melis et al., 2004), had no effect on mAEA-induced stimulation of DA neuron firing rate ($F_{(1,72)} = 0.67$, $n = 5$, $p = 0.4365$, two-way ANOVA; data not shown). However, this stimulation was completely blocked by the TRPV1 receptor antagonist capsazepine (10 μM, $F_{(1,171)} = 8.13$, $n = 5–6$, $p = 0.019$, two-way ANOVA; data not shown). This observation is consistent with other studies showing that TRPV1 agonists stimulate DA neuron activity by enhancing glutamatergic synaptic transmission onto DA neurons (Marinelli et al., 2005). To isolate the agonistic activity of mAEA at CB_1 receptors, or possibly at PPAR-α, and avoid TRPV1-induced excitation of DA cells that could mask or confound the effects of the subsequent application of nicotine, we applied nicotine in the presence of capsazepine. Under these circumstances, excitatory effects of nicotine were unmodified compared with controls (151.4 ± 13.5% of baseline, $t = 0.77$, $n = 6$, $p = 0.4563$, *t* test) (Fig. 4*G*), suggesting that CB_1 receptor stimulation did not affect nicotine-induced excitation of DA neuron firing and that mAEA did not activate PPAR-α (see below).

Next, we tested two different noncannabinoid FAEs, OEA and PEA. Interestingly, OEA (0.3, 1 and 3 μM) dose-dependently prevented nicotine-induced excitation (97.44 ± 5% and 92.01 ± 7% of baseline at 1 and 3 μM, respectively; 1 μM: $n = 5$, $t = 2.65$, $p = 0.01$; 3 μM: $n = 7$, $t = 3.22$, $p = 0.04$) (Fig. 4*H*), without affecting DA neuron spontaneous activity during preapplication (Fig. 4*E*). The effects of OEA were mimicked by PEA (10 μM, 101.9 ± 3% of baseline, $n = 6$, $t = 2.66$, $p = 0.01$) (Fig. 4*E*,*G*). Consistently, under voltage-clamp mode ($V_{holding} = -70$ mV), the nicotine-induced inward current was completely abolished when nicotine was perfused in the presence of OEA (3 μM, 5 min preapplication: −0.3 ± 3.4 pA, $n = 6$, $t = 7.13$, $p = 0.0004$, paired *t* test) (Fig. 4*F*), or PEA (10 μM, 5 min preapplication: −0.6 ± 7.5 pA, N = 5, $t = 4.442$, $p = 0.001$, paired *t* test) (Fig. 4*F*). During preapplication, OEA and PEA did not induce inward or outward currents onto DA neurons (data not shown).

Based on these results, we expected that the PPAR-α antagonist MK886 would block the actions of OEA and PEA on nicotine-induced excitation. As predicted, when OEA or PEA were coapplied with MK886 (300 nM), nicotine's effects on firing rate of DA neurons were restored (155.8 ± 16.6% and 163.8 ± 11.3% of baseline in the presence of OEA and PEA, respectively; OEA+MK886 vs OEA alone: $F_{(2,323)} = 7.59$, $n = 8$, $p = 0.004$, two-way ANOVA; PEA+MK886 versus PEA alone: $F_{(1,228)} = 4.84$, $n = 8$, $p = 0.04$, two-way ANOVA) (Fig. 5*A*,*B*,*C*). MK886 when perfused either alone or in combination with OEA/PEA did not alter spontaneous firing rate of VTA DA neurons (Fig. 5*C*). However, MK886 significantly enhanced nicotine-induced activation of DA neurons (207 ± 27% of baseline, MK886+nicotine vs nicotine: $t = 2.167$, $n = 7$, $p < 0.05$, *t* test) (Fig. 5*B*).

Next, we determined whether the synthetic PPAR-α agonist WY14643 would alter the effects of nicotine on DA cells. WY14643 (300 nM) was per se ineffective on DA neuronal firing rate, but fully prevented nicotine-induced excitation (83.7 ±

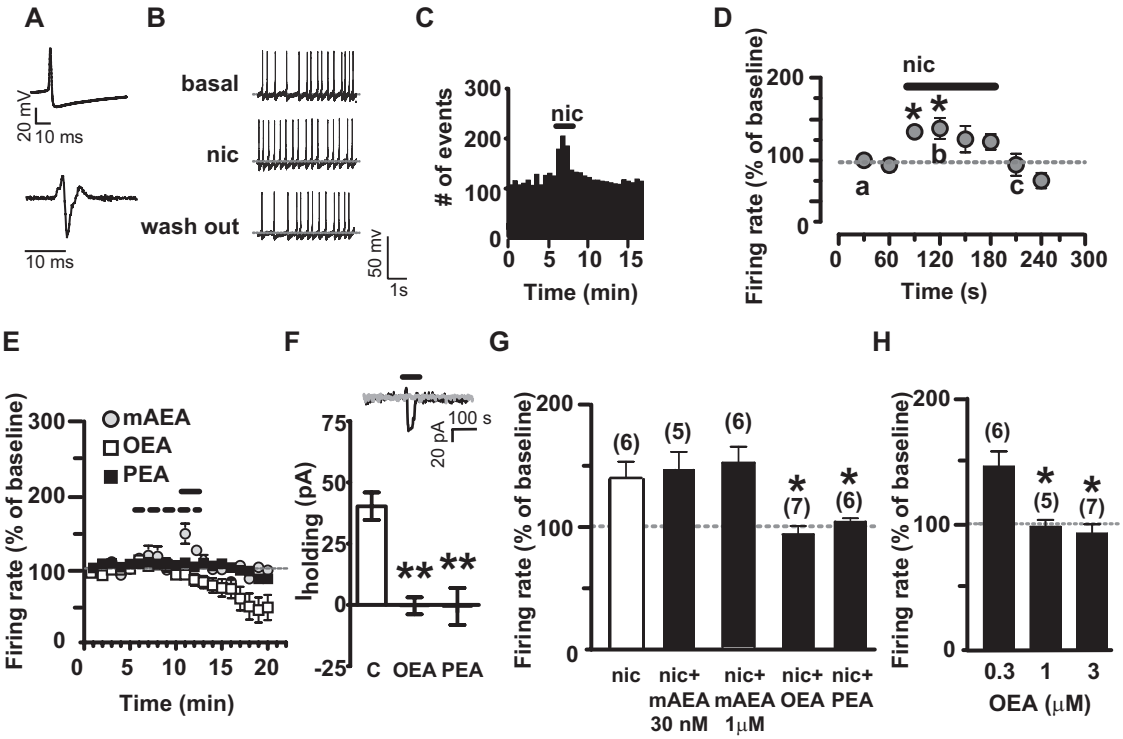


Figure 4. Activation of dopamine neurons by nicotine is prevented by OEA and PEA *in vitro*. ***A***, Typical action potential waveform of a dopamine neuron recorded under current-clamp mode (top) and its second derivative (bottom). ***B***, Representative traces of a dopamine neuron spontaneous activity during baseline level (top), nicotine application (nic, 1 μM for 2 min; middle), and wash out (bottom). ***C***, Rate histogram depicting an example of the effect of nicotine on dopamine neuron firing rate. The horizontal bar represents the time of nicotine application. ***D***, Time course of the excitatory effect of nicotine on dopamine neuron discharge rate (30 s bins). ***E***, OEA (3 μM) and PEA (10 μM), but not mAEA (30 nM), blocked nicotine-induced activation of dopamine neurons. The dashed and the solid bars represent the times of fatty acid ethanolamide (OEA, PEA, or mAEA) or nicotine application, respectively. ***F***, The bar graph shows that OEA (3 μM and PEA 10 μM) also blocked nicotine-induced inward currents ($I_{holding}$) when dopamine neurons were recorded under the voltage-clamp mode ($V_{hold} = -70$ mV). The inset shows that nicotine (black line) caused a 47 pA inward current under voltage-clamp mode, which was completely abolished in the presence of OEA (3 μM; OEA's effect on $I_{holding}$ is superimposed in light gray for comparison). The horizontal bar represents the time of nicotine application. ***G***, Bar graph summarizing the actions of mAEA (30 nM and 1 μM), OEA (3 μM), and PEA (10 μM) on nicotine-induced enhancement of dopamine neuron discharge rate (average of the first minute of nicotine perfusion). mAEA at 1 μM was perfused in the presence of capsazepine (CPZ, 10 μM) to prevent the vanilloid-induced excitation of dopamine neurons. ***H***, Dose-effect relationship of OEA's blockade of nicotine-induced excitation of dopamine neurons. Numbers above bars indicate the *n* values for each group of experiments. Data are expressed as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$.

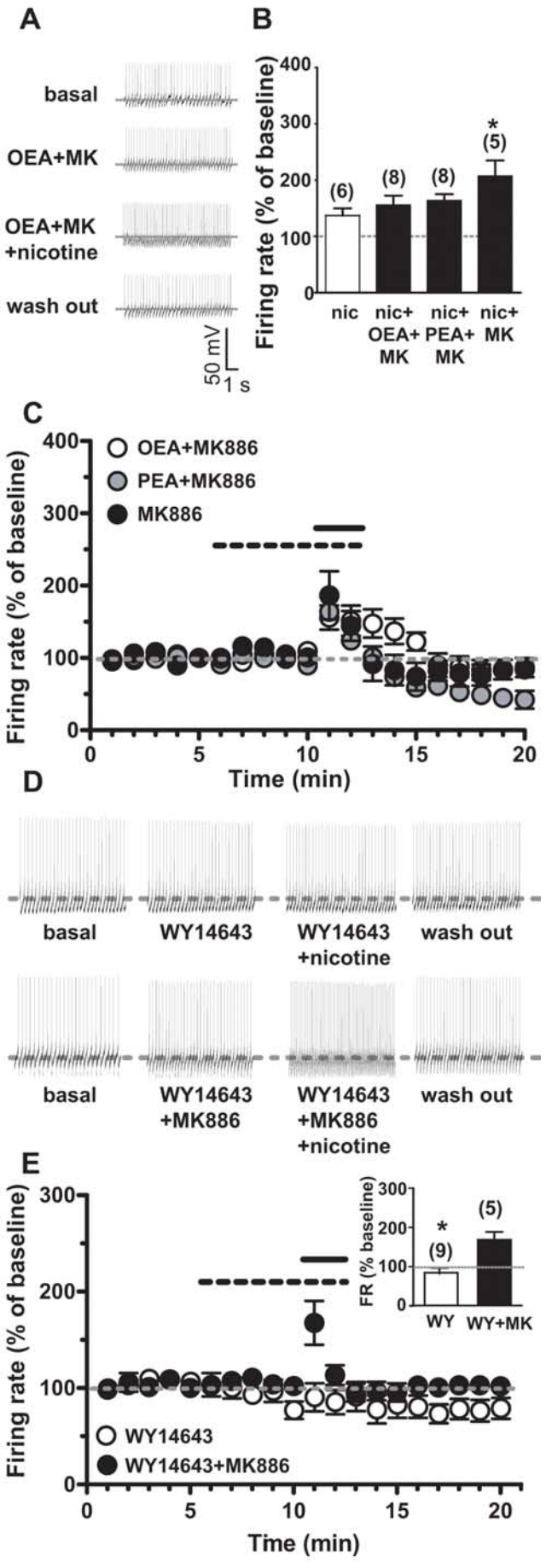
14.7% of baseline, $n = 9$, $t = 2.54$, $p = 0.02$) (Fig. 5*D*,*E*). The effect of WY14643 was also reversed by MK886 (167.7 ± 22.7% of baseline; WY14643+MK886 vs WY14643 alone: $F_{(1,228)} = 5.30$, $n = 5$, $p < 0.05$, two-way ANOVA) (Fig. 5*D*,*E*), confirming the role of PPAR-α in the modulation of DA neuron responses to nicotine.

Mechanisms downstream of PPAR-α activation in the modulation of nicotine effects: involvement of tyrosine kinases

Although it is well established that PPAR-α regulates gene expression (Berger and Moller, 2002), the effects of OEA, PEA and WY14643 observed in the present study were fairly rapid in onset, thus ruling out gene induction as a possible mechanism, and suggesting a more likely nongenomic (Gardner et al., 2005) mechanism occurring in such a short time scale. Among many diverse pathways, we chose to investigate the regulation of tyrosine kinases, because PPAR-α agonists have been shown to activate several tyrosine kinases, such as the Src family kinase (SFK) (Gardner et al., 2005), which phosphorylates and negatively regulates α7 nAChRs (Charpantier et al., 2005).

We hypothesized that phosphorylation of nAChRs could account for PPAR-α mediated inhibition of nicotine effects. To explore this possibility, we incubated slices with the general tyrosine kinase inhibitor genistein (10 μM), which has indirect effects on nAChRs arising from the inhibition of intracellular phosphorylation pathways. Experiments were conducted under voltage-clamp mode on nicotine-induced inward currents, since genistein had aspecific channel blocker properties which led to a complete blockade of action potential generation (data not shown). Genistein was able to prevent OEA blockade of nicotine effects and restored nicotine-evoked inward currents (37.8 ± 4.4 pA, $n = 6$, $t = 6.79$, $p < 0.0001$) (Fig. 6*A*,*B*), demonstrating that inhibition of tyrosine kinases reverses the effect of PPAR-α activation.

To investigate which tyrosine kinase phosphorylates and negatively modulates nAChRs, we focused on SFKs, on the basis of previous reports highlighting the role of SFK in the regulation of α7 nAChRs (Charpantier et al., 2005). We predicted that inhibition of SFK would reverse the effects of OEA. To test this hypothesis, slices were incubated (1 h) and continuously perfused with the SFK inhibitor PP2 (10 μM). This treatment did not change electrophysiological features of recorded DA neurons (data not shown). However, PP2 failed to reverse OEA blockade of nicotine's effects on DA cells under both voltage- and current-clamp modes. Indeed, in the presence of PP2, OEA abolished nicotine-induced inward currents (−1.4 ± 5.7, $n = 5$, $t = 0.16$, $p > 0.5$) (Fig. 6*A*,*B*) as well as the nicotine-induced enhancement of firing rate (100.9 ± 7.1% of baseline, $F_{(1,108)} = 0.06$, $n = 6$, $p = 0.8$, two-way ANOVA) (Fig. 6*C*,*D*), suggesting that SFK is not involved in the negative modulation of nAChRs by PPAR-α agonists.



Discussion

The present study revealed that naturally occurring noncannabinoid FAEs can modulate the responses of VTA DA neurons to nicotine via PPAR-α, possibly by phosphorylation of nAChRs. This is the first evidence of an important functional role of this family of nuclear receptor transcription factors in the brain. It also highlights the role of FAEs, devoid of cannabinoid actions, in the regulation of neuronal functions.

Centrally mediated effects of the noncannabinoid FAEs have been poorly characterized, although OEA and PEA might constitute an independent endocannabinoid-like system. This view is supported by the findings that their synthesis and inactivation occurs independently of "classic" endocannabinoids, although in a similar on demand manner (Hansen et al., 1995; Stella and Piomelli, 2001; Mackie and Stella, 2006). The molecular targets underlying their pharmacological effects have remained elusive until the discovery of their agonistic actions at PPAR-α (Fu et al., 2003). These nuclear receptors are ubiquitously distributed in the CNS (Moreno et al., 2004), but their roles in neuronal physiology, or in pathophysiological mechanisms of brain disorders, are largely unknown.

Indirect enhancement of brain FAEs levels obtained by blockade of their major hydrolyzing enzyme, FAAH, by URB597 (Kathuria et al., 2003) has been reported to produce antidepressant, anxiolytic and analgesic effects in rodents (Kathuria et al., 2003; Gobbi et al., 2005; Piomelli et al., 2006; Russo et al., 2007). All of these effects are prevented by treatment with CB_1 receptor antagonists, and have been ascribed to increased AEA levels, thus suggesting that augmented levels of OEA and PEA do not contribute significantly. However, a PPAR-α antagonist was recently reported to block the peripheral analgesic effects of URB597, suggesting that analgesia may be mediated by FAEs binding at PPAR-α (Jhaveri et al., 2008). In the present experiments, we discovered that inhibition of FAAH, rather than blockade of CB_1 receptors, suppresses nicotine-induced activation of DA neurons. The lack of effect by rimonabant was unexpected in light of recent reports that CB_1 antagonists decrease DA release evoked by nicotine in the nucleus accumbens (Cohen et al., 2002; Cheer et al., 2007). One can argue, however, that the suppression by rimonabant of evoked DA release may be independent from the inhibition of firing activity of DA neurons in the VTA, and may

←

Figure 5. OEA and PEA block nicotine activation of dopamine neurons through a PPAR-α-mediated mechanism. ***A***, Representative traces of the spontaneous activity of a dopamine neuron during baseline (top), OEA (3 μM) plus the PPAR-α antagonist MK886 (0.3 μM) preapplication (5 min, second panel), subsequent nicotine application (1 μM, 2 min, third panel), and wash out (bottom). ***B***, Bar graph illustrating the effect of MK886 on nicotine-induced activation of VTA DA neurons and on OEA- and PEA-mediated inhibition of nicotine excitation (average of the first minute of nicotine perfusion). Note that activation of dopamine neurons by nicotine was fully restored when either OEA or PEA were coapplied with MK886. Notably, MK886 itself significantly potentiated nicotine-induced excitation. ***C***, Time course of the effect of MK886 (0.3 μM), alone or in combination with either OEA or PEA, on nicotine induced excitation. The dashed bar represents the time of fatty acid ethanolamide (OEA, PEA) plus MK886 or MK886 alone application. The solid bar represents the time of nicotine application. ***D***, Representative traces of dopamine neuron firing rate showing that the PPAR-α agonist WY14643 (300 nM) mimicked the actions of OEA and PEA by preventing nicotine-induced excitation (top), which was then restored by the coapplication of MK886 (bottom). ***E***, Time course of the effect of nicotine on dopamine neuron firing rate in the presence of WY14643 (open symbols) or WY14643 plus MK886 (closed symbols). The dashed and the solid bars represent the times of PPAR-α agonist/antagonist or nicotine application, respectively. In the inset, the bar graph summarizes the effects of WY14643 (WY) on nicotine-induced excitation of dopamine neuron firing rate (FR) with or without MK886. Numbers above bars indicate the *n* values for each group of experiments. Data are expressed as mean ± SEM. $^*p < 0.05$.

be an effect primarily involving their terminal regions or local circuits within the nucleus accumbens. Hence, presynaptically located nAChRs potently regulate DA release in the striatum, including the nucleus accumbens (Zhou et al., 2001). The effect of URB597 was even more surprising, since it contradicts the notion that the endocannabinoid system exerts a facilitatory effect on nicotine reward and addiction. Consistent with the present results, recent findings indicate that URB597 prevents the development of nicotine-induced CPP, acquisition of nicotine self-administration and nicotine-induced reinstatement in both CPP and self-administration models of relapse (Scherma et al., 2008).

Here, we found that the effects of URB597 were not entirely dependent on CB_1 receptor stimulation, since nicotine-induced increases in DA neuron bursting were not reversed by rimonabant or AM251, whereas increases in firing rate were. The PPAR-α antagonist MK886 reversed URB597's blockade of nicotine-induced bursting in DA neurons, suggesting that FAEs, other than AEA, play a role in antagonizing the effects of nicotine. More importantly, OEA, but not mAEA, blocked the effects of nicotine on DA neurons *in vivo*. These results were substantiated by the findings that both OEA and PEA, but not mAEA, completely prevented nicotine-induced excitation of DA neurons *in vitro*. Additionally, OEA and PEA actions via PPAR-α were confirmed by the antagonism exerted by MK886, and by the observation that the PPAR-α agonist WY14643 mimicked the actions of noncannabinoid FAEs. Although AEA has been reported to display binding affinity for PPAR-α (Sun et al., 2006, 2007), our results are not consistent with those findings, since mAEA had no effects on nicotine-induced excitation of DA neurons, contrary to OEA and PEA. However, the studies of Sun et al. (2006, 2007) were performed in HeLa cells transiently transfected with PPAR-α, thus other investigations are necessary to confirm the binding properties of AEA at PPAR-α under more physiological conditions and, more importantly, in neurons. Remarkably, the analgesic properties of mAEA are fully preserved in PPAR-α knock-out mice, suggesting a PPAR-α-independent mechanism of action, whereas those of OEA and PEA are abolished (LoVerme et al., 2006).

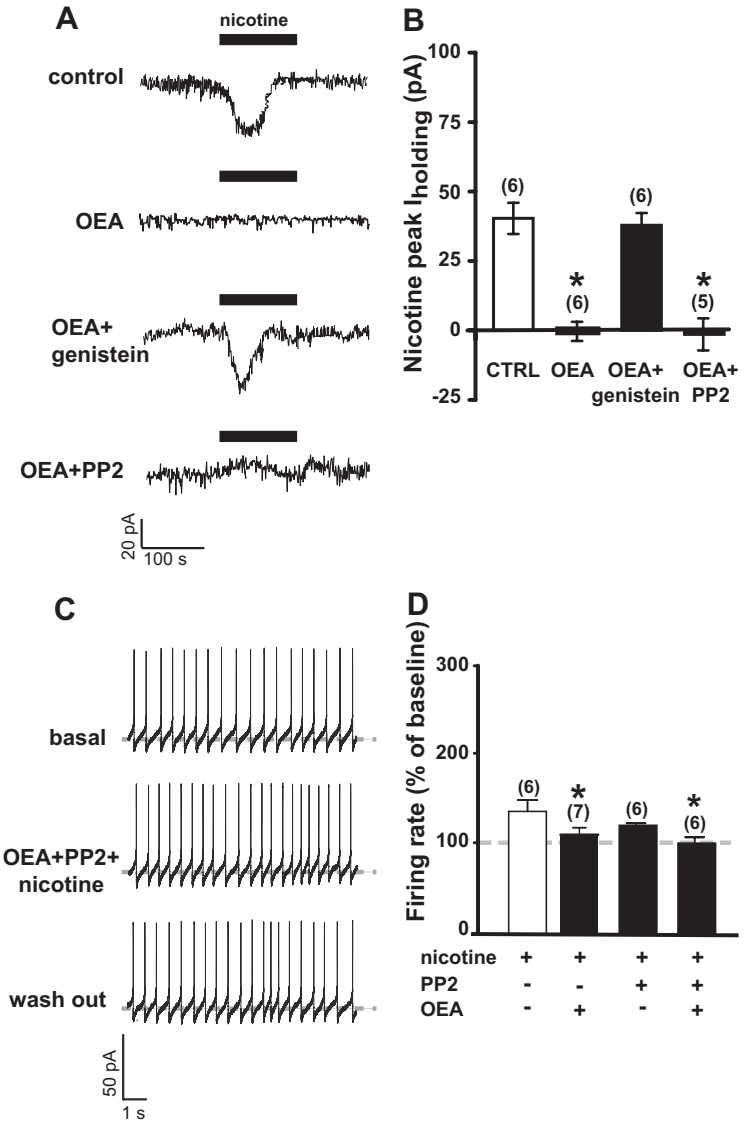


Figure 6. Tyrosine kinases, but not Src kinase, inhibition prevent OEA-induced blockade of nicotine effects. ***A***, In voltage-clamp mode, nicotine (1 μM, 2 min) caused a 43 pA inward current (top) that is blunted by OEA preapplication (3 μM, 5 min; second panel). Pretreatment with the general tyrosine kinase inhibitor genistein (10 μM, 5 min) fully blocked OEA actions by restoring nicotine-induced inward current (third panel). However, pretreatment with the selective Src kinase inhibitor PP2 (10 μM, bottom) failed to prevent OEA actions on nicotine-induced change in holding current ($I_{holding}$). ***B***, Bar graph summarizing the effects of OEA per se and in the presence of either kinase inhibitor on nicotine-induced inward current. ***C***, Representative traces of dopamine neuron firing rate showing that PP2 failed to prevent OEA's action on nicotine-induced excitation (middle). ***D***, Bar graph summarizing the effect of OEA on nicotine-induced enhancement of dopamine neuron discharge rate (average of the first minute of nicotine perfusion) alone or in the presence of PP2. Numbers above bars indicate the *n* values for each group of experiments. Data are expressed as mean ± SEM. $*p < 0.05$.

Studies on recombinant or native nAChRs expressed in *Xenopus* oocytes or in mouse thalamic synaptosomes, respectively, have demonstrated that AEA (Oz et al., 2003; Spivak et al., 2007; Butt et al., 2008) or fatty acids (Butt et al., 2002; Barrantes, 2004) can modulate nAChR function as noncompetitive antagonists. Our results tend to exclude this possibility. In fact, they strongly support the notion that OEA and PEA effects are specifically mediated by PPAR-α, since they are blocked by the selective synthetic antagonist and mimicked by the agonist. Additionally, we found that mAEA did not alter nicotine-induced DA neuron excitation, making its action as nAChR antagonist unlikely.

As mentioned above, rimonabant revealed a significant component mediated by CB_1 receptors in the effects of URB597. This piece of evidence is apparently difficult to reconcile with the results obtained with mAEA. Hence, mAEA does not significantly modulate nicotine's effects either *in vivo* or *in vitro*, arguing against an involvement of CB_1 receptors in the modulation of the excitatory actions of nicotine on VTA DA neurons. There are possible explanations for this discrepancy: first, the effects of URB597 are due to the combination of CB_1- (by AEA) and

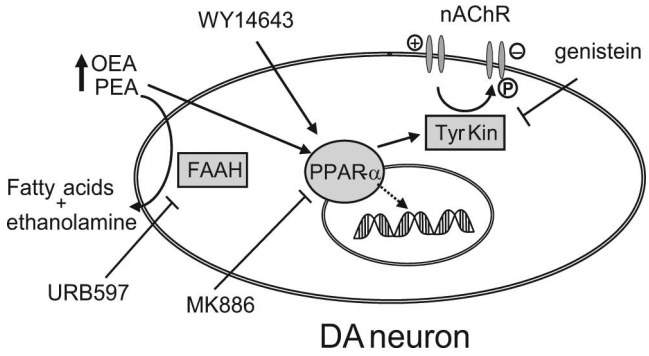


Figure 7. Schematic diagram illustrating the proposed mechanism of PPAR-α activation, and modulation of responses of DA neurons to nicotine, by the noncannabinoid fatty acid ethanolamides OEA and PEA. Their action is mimicked by the synthetic PPAR-α agonist WY14643 and blocked by the PPAR-α antagonist MK886. URB597 enhances brain levels of OEA and PEA *in vivo* by inhibiting their major catabolizing enzyme, FAAH. It is proposed that activated PPAR-α stimulate the activity of tyrosine kinases (Tyr Kin) through a nongenomic mechanism. Tyrosine kinases, in turn, induce the phosphorylation (P) of nAChR, which reduces their responses to the agonists, or promotes rapid internalization. Genistein, a general tyrosine kinase inhibitor, blocks the effects of PPAR-α activation. ↓, activation; ⊥, inhibition.

PPAR-α- (by OEA and PEA) mediated effects. CB_1 receptors and PPAR-α may share opposing or reinforcing intracellular pathways (including modulation of protein kinases) (for review, see Alexander and Kendall, 2007). Second, URB597 enhances brain levels of endogenously released FAEs in a discrete and region-specific manner, and may influence the release of other endocannabinoids as well, including 2-arachidonoylglycerol (Di Marzo and Maccarrone, 2008; Maccarrone et al., 2008). Conversely, exogenously applied mAEA induces the activation of CB_1 receptors throughout the brain. The third possible explanation is a differential involvement of TRPV1 channels, which may be activated by AEA (following URB597 administration) or by mAEA itself. However, our results *in vitro* tend to exclude the possibility that TRPV1 receptors play a significant role in the modulation of nicotine effects, since their activation by mAEA, or blockade by the selective antagonist capsazepine did not change nicotine-induced excitation of DA neurons.

We investigated also the mechanism by which PPAR-α may modulate the effects of nicotine. Due to the rapid onset of agonist actions, we hypothesized that this could be a nongenomic effect. Hence, PPARs exert pleiotropic effects on many different intracellular pathways, including protein kinases (Gardner et al., 2005). It was recently shown that the functional properties of α7 nAChRs depend on the tyrosine phosphorylation status of the receptor, being the result of a balance between SFKs and tyrosine phosphatases (Charpantier et al., 2005), which negatively or positively modulate nAChR-mediated currents, respectively. Additionally, phosphorylation/dephosphorylation of tyrosine residues in nAChRs controls the number of functional surface receptors (Cho et al., 2005).

Interestingly, we found that the effects of the tyrosine kinase inhibitor genistein were consistent with the idea that PPAR-α-mediated nAChR phosphorylation could account for the blockade of neuronal responses to nicotine, although at this stage we cannot identify the specific tyrosine kinase involved. A proposed mechanism is displayed in Figure 7. A constitutive interaction between PPAR-α and tyrosine kinases is also possible, and may tonically control the ratio of phosphorylated/dephosphorylated nAChRs, as indicated by the enhanced effects of nicotine in the presence of the PPAR-α antagonist MK886. It cannot be excluded that OEA and PEA may be endogenous modulators of acetylcholine transmission, since stimulation of muscarinic receptors was shown to stimulate the biosynthesis of OEA and PEA (Stella and Piomelli, 2001), suggesting the intriguing possibility of a reciprocal control between acetylcholine and FAEs.

This novel mechanism of regulation of nAChRs by PPAR-α may represent a new therapeutic avenue for the discovery of medications to support patients during nicotine abstinence. Our data demonstrate that the actions of OEA are not restricted to the periphery and suggest that modulation of neuronal responses to nicotine by OEA may represent an interesting extension of its peripheral anorexic properties. PPAR-α agonists, such as fibrates, are well established medications clinically used in the treatment of lipid metabolism disorders. Their central effects are considered negligible due to their poor ability to cross the blood brain barrier. However, changes in brain lipid metabolism and/or composition, or modifications of the levels of endogenous lipid signaling molecules may exert unsuspected actions on neurotransmitter functions, which might be exploited therapeutically.

References

Alexander SP, Kendall DA (2007) The complications of promiscuity: endocannabinoid action and metabolism. Br J Pharmacol 152:602–623.

Barrantes FJ (2004) Structural basis for lipid modulation of nicotinic acetylcholine receptor function. Brain Res Brain Res Rev 47:71–95.

Berger J, Moller DE (2002) The mechanisms of action of PPARs. Annu Rev Med 53:409–435.

Butt C, Alptekin A, Shippenberg T, Oz M (2008) Endogenous cannabinoid anandamide inhibits nicotinic acetylcholine receptor function in mouse thalamic synaptosomes. J Neurochem 105:1235–1243.

Butt CM, Hutton SR, Marks MJ, Collins AC (2002) Bovine serum albumin enhances nicotinic acetylcholine receptor function in mouse thalamic synaptosomes. J Neurochem 83:48–56.

Cahill K, Ussher M (2007) Cannabinoid type 1 receptor antagonists (rimonabant) for smoking cessation. Cochrane Database Syst Rev:CD005353.

Castañé A, Berrendero F, Maldonado R (2005) The role of the cannabinoid system in nicotine addiction. Pharmacol Biochem Behav 81:381–386.

Charpantier E, Wiesner A, Huh KH, Ogier R, Hoda JC, Allaman G, Raggenbass M, Feuerbach D, Bertrand D, Fuhrer C (2005) Alpha7 neuronal nicotinic acetylcholine receptors are negatively regulated by tyrosine phosphorylation and Src-family kinases. J Neurosci 25:9836–9849.

Cheer JF, Wassum KM, Sombers LA, Heien ML, Ariansen JL, Aragona BJ, Phillips PE, Wightman RM (2007) Phasic dopamine release evoked by abused substances requires cannabinoid receptor activation. J Neurosci 27:791–795.

Cho CH, Song W, Leitzell K, Teo E, Meleth AD, Quick MW, Lester RA (2005) Rapid upregulation of alpha7 nicotinic acetylcholine receptors by tyrosine dephosphorylation. J Neurosci 25:3712–3723.

Cohen C, Perrault G, Voltz C, Steinberg R, Soubrié P (2002) SR141716, a central cannabinoid (CB(1)) receptor antagonist, blocks the motivational and dopamine-releasing effects of nicotine in rats. Behav Pharmacol 13:451–463.

D'Agostino G, La Rana G, Russo R, Sasso O, Iacono A, Esposito E, Raso GM, Cuzzocrea S, Lo Verme J, Piomelli D, Meli R, Calignano A (2007) Acute intracerebroventricular administration of palmitoylethanolamide, an endogenous peroxisome proliferator-activated receptor-alpha agonist, modulates carrageenan-induced paw edema in mice. J Pharmacol Exp Ther 322:1137–1143.

Di Chiara G, Imperato A (1988) Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. Proc Natl Acad Sci U S A 85:5274–5278.

Di Marzo V, Maccarrone M (2008) FAAH and anandamide: is 2-AG really the odd one out? Trends Pharmacol Sci 29:229–233.

Erhardt S, Schwieler L, Engberg G (2002) Excitatory and inhibitory responses of dopamine neurons in the ventral tegmental area to nicotine. Synapse 43:227–237.

Fegley D, Gaetani S, Duranti A, Tontini A, Mor M, Tarzia G, Piomelli D (2005) Characterization of the fatty acid amide hydrolase inhibitor cy-

clohexyl carbamic acid 3′-carbamoyl-biphenyl-3-yl ester (URB597): effects on anandamide and oleoylethanolamide deactivation. J Pharmacol Exp Ther 313:352–358.

Forget B, Hamon M, Thiebot MH (2005) Cannabinoid CB1 receptors are involved in motivational effects of nicotine in rats. Psychopharmacology (Berl) 181:722–734.

Fu J, Gaetani S, Oveisi F, Lo Verme J, Serrano A, Rodríguez De Fonseca F, Rosengarth A, Luecke H, Di Giacomo B, Tarzia G, Piomelli D (2003) Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha. Nature 425:90–93.

Gardner OS, Dewar BJ, Graves LM (2005) Activation of mitogen-activated protein kinases by peroxisome proliferator-activated receptor ligands: an example of nongenomic signaling. Mol Pharmacol 68:933–941.

Gobbi G, Bambico FR, Mangieri R, Bortolato M, Campolongo P, Solinas M, Cassano T, Morgese MG, Debonnel G, Duranti A, Tontini A, Tarzia G, Mor M, Trezza V, Goldberg SR, Cuomo V, Piomelli D (2005) Antidepressant-like activity and modulation of brain monoaminergic transmission by blockade of anandamide hydrolysis. Proc Natl Acad Sci U S A 102:18620–18625.

Grace AA, Bunney BS (1983) Intracellular and extracellular electrophysiology of nigral dopaminergic neurons–1. Identification and characterization. Neuroscience 10:301–315.

Grace AA, Bunney BS (1984) The control of firing pattern in nigral dopamine neurons: burst firing. J Neurosci 4:2877–2890.

Hansen HS, Lauritzen L, Strand AM, Moesgaard B, Frandsen A (1995) Glutamate stimulates the formation of N-acylphosphatidylethanolamine and N-acylethanolamine in cortical neurons in culture. Biochim Biophys Acta 1258:303–308.

Jhaveri MD, Richardson D, Robinson I, Garle MJ, Patel A, Sun Y, Sagar DR, Bennett AJ, Alexander SP, Kendall DA, Barrett DA, Chapman V (2008) Inhibition of fatty acid amide hydrolase and cyclooxygenase-2 increases levels of endocannabinoid related molecules and produces analgesia via peroxisome proliferator-activated receptor-alpha in a model of inflammatory pain. Neuropharmacology 55:85–93.

Johnson SW, North RA (1992) Two types of neurone in the rat ventral tegmental area and their synaptic inputs. J Physiol 450:455–468.

Kathuria S, Gaetani S, Fegley D, Valiño F, Duranti A, Tontini A, Mor M, Tarzia G, La Rana G, Calignano A, Giustino A, Tattoli M, Palmery M, Cuomo V, Piomelli D (2003) Modulation of anxiety through blockade of anandamide hydrolysis. Nat Med 9:76–81.

Le Foll B, Goldberg SR (2004) Rimonabant, a CB1 antagonist, blocks nicotine-conditioned place preferences. Neuroreport 15:2139–2143.

Le Foll B, Goldberg SR (2005) Cannabinoid CB1 receptor antagonists as promising new medications for drug dependence. J Pharmacol Exp Ther 312:875–883.

Le Foll B, Forget B, Aubin HJ, Goldberg SR (2008) Blocking cannabinoid CB1 receptors for the treatment of nicotine dependence: insights from pre-clinical and clinical studies. Addict Biol 13:239–252.

Lo Verme J, Fu J, Astarita G, La Rana G, Russo R, Calignano A, Piomelli D (2005) The nuclear receptor peroxisome proliferator-activated receptor-alpha mediates the anti-inflammatory actions of palmitoylethanolamide. Mol Pharmacol 67:15–19.

LoVerme J, Russo R, La Rana G, Fu J, Farthing J, Mattace-Raso G, Meli R, Hohmann A, Calignano A, Piomelli D (2006) Rapid broad-spectrum analgesia through activation of peroxisome proliferator-activated receptor-alpha. J Pharmacol Exp Ther 319:1051–1061.

Maccarrone M, Rossi S, Bari M, De Chiara V, Fezza F, Musella A, Gasperi V, Prosperetti C, Bernardi G, Finazzi-Agrò A, Cravatt BF, Centonze D (2008) Anandamide inhibits metabolism and physiological actions of 2-arachidonoylglycerol in the striatum. Nat Neurosci 11:152–159.

Mackie K, Stella N (2006) Cannabinoid receptors and endocannabinoids: evidence for new players. Aaps J 8:E298–306.

Marinelli S, Pascucci T, Bernardi G, Puglisi-Allegra S, Mercuri NB (2005) Activation of TRPV1 in the VTA excites dopaminergic neurons and increases chemical- and noxious-induced dopamine release in the nucleus accumbens. Neuropsychopharmacology 30:864–870.

Melis M, Pistis M, Perra S, Muntoni AL, Pillolla G, Gessa GL (2004) Endocannabinoids mediate presynaptic inhibition of glutamatergic transmission in rat ventral tegmental area dopamine neurons through activation of CB1 receptors. J Neurosci 24:53–62.

Mereu G, Yoon KW, Boi V, Gessa GL, Naes L, Westfall TC (1987) Preferential stimulation of ventral tegmental area dopaminergic neurons by nicotine. Eur J Pharmacol 141:395–399.

Moreno S, Farioli-Vecchioli S, Cerù MP (2004) Immunolocalization of peroxisome proliferator-activated receptors and retinoid X receptors in the adult rat CNS. Neuroscience 123:131–145.

Oz M, Ravindran A, Diaz-Ruiz O, Zhang L, Morales M (2003) The endogenous cannabinoid anandamide inhibits alpha7 nicotinic acetylcholine receptor-mediated responses in *Xenopus* oocytes. J Pharmacol Exp Ther 306:1003–1010.

Paxinos G, Watson C (1997) The rat brain in stereotaxic coordinates, Ed 3. San Diego: Academic.

Pidoplichko VI, DeBiasi M, Williams JT, Dani JA (1997) Nicotine activates and desensitizes midbrain dopamine neurons. Nature 390:401–404.

Piomelli D, Tarzia G, Duranti A, Tontini A, Mor M, Compton TR, Dasse O, Monaghan EP, Parrott JA, Putman D (2006) Pharmacological profile of the selective FAAH inhibitor KDS-4103 (URB597). CNS Drug Rev 12:21–38.

Rodríguez de Fonseca F, Navarro M, Gómez R, Escuredo L, Nava F, Fu J, Murillo-Rodríguez E, Giuffrida A, LoVerme J, Gaetani S, Kathuria S, Gall C, Piomelli D (2001) An anorexic lipid mediator regulated by feeding. Nature 414:209–212.

Russo R, Loverme J, La Rana G, Compton TR, Parrott J, Duranti A, Tontini A, Mor M, Tarzia G, Calignano A, Piomelli D (2007) The fatty acid amide hydrolase inhibitor URB597 (cyclohexylcarbamic acid 3′-carbamoylbiphenyl-3-yl ester) reduces neuropathic pain after oral administration in mice. J Pharmacol Exp Ther 322:236–242.

Scherma M, Panlilio LV, Fadda P, Fattore L, Gamaleddin I, Le Foll B, Justinová Z, Mikics E, Haller J, Medalie J, Stroik J, Barnes C, Yasar S, Tanda G, Piomelli D, Fratta W, Goldberg SR (2008) Inhibition of anandamide hydrolysis by URB597 reverses abuse-related behavioral and neurochemical effects of nicotine in rats. J Pharmacol Exp Ther 327:482–490.

Shoaib M (2008) The cannabinoid antagonist AM251 attenuates nicotine self-administration and nicotine-seeking behaviour in rats. Neuropharmacology 54:438–444.

Solinas M, Justinova Z, Goldberg SR, Tanda G (2006) Anandamide administration alone and after inhibition of fatty acid amide hydrolase (FAAH) increases dopamine levels in the nucleus accumbens shell in rats. J Neurochem 98:408–419.

Solinas M, Tanda G, Justinova Z, Wertheim CE, Yasar S, Piomelli D, Vadivel SK, Makriyannis A, Goldberg SR (2007) The endogenous cannabinoid anandamide produces delta-9-tetrahydrocannabinol-like discriminative and neurochemical effects that are enhanced by inhibition of fatty acid amide hydrolase but not by inhibition of anandamide transport. J Pharmacol Exp Ther 321:370–380.

Solinas M, Goldberg SR, Piomelli D (2008) The endocannabinoid system in brain reward processes. Br J Pharmacol 154:369–383.

Spivak CE, Lupica CR, Oz M (2007) The Endocannabinoid Anandamide Inhibits the Function of {alpha}4beta2 Nicotinic Acetylcholine Receptors. Mol Pharmacol 72:1024–1032.

Stella N, Piomelli D (2001) Receptor-dependent formation of endogenous cannabinoids in cortical neurons. Eur J Pharmacol 425:189–196.

Stolerman IP, Shoaib M (1991) The neurobiology of tobacco addiction. Trends Pharmacol Sci 12:467–473.

Suardíaz M, Estivill-Torrús G, Goicoechea C, Bilbao A, Rodríguez de Fonseca F (2007) Analgesic properties of oleoylethanolamide (OEA) in visceral and inflammatory pain. Pain 133:99–110.

Sun Y, Alexander SP, Kendall DA, Bennett AJ (2006) Cannabinoids and PPARalpha signalling. Biochem Soc Trans 34:1095–1097.

Sun Y, Alexander SP, Garle MJ, Gibson CL, Hewitt K, Murphy SP, Kendall DA, Bennett AJ (2007) Cannabinoid activation of PPAR alpha; a novel neuroprotective mechanism. Br J Pharmacol 152:734–743.

Ungless MA, Magill PJ, Bolam JP (2004) Uniform inhibition of dopamine neurons in the ventral tegmental area by aversive stimuli. Science 303:2040–2042.

Wise RA (2004) Dopamine, learning and motivation. Nat Rev Neurosci 5:483–494.

Zhou FM, Liang Y, Dani JA (2001) Endogenous nicotinic cholinergic activity regulates dopamine release in the striatum. Nat Neurosci 4:1224–1229.

ARCHIVAL REPORT

Blockade of Nicotine Reward and Reinstatement by Activation of Alpha-Type Peroxisome Proliferator-Activated Receptors

Paola Mascia, Marco Pistis, Zuzana Justinova, Leigh V. Panlilio, Antonio Luchicchi, Salvatore Lecca, Maria Scherma, Walter Fratta, Paola Fadda, Chanel Barnes, Godfrey H. Redhi, Sevil Yasar, Bernard Le Foll, Gianluigi Tanda, Daniele Piomelli, and Steven R. Goldberg

Background: Recent findings indicate that inhibitors of fatty acid amide hydrolase (FAAH) counteract the rewarding effects of nicotine in rats. Inhibition of FAAH increases levels of several endogenous substances in the brain, including the endocannabinoid anandamide and the noncannabinoid fatty acid ethanolamides oleoylethanolamide (OEA) and palmitoylethanolamide, which are ligands for alpha-type peroxisome proliferator-activated nuclear receptors (PPAR-α). Here, we evaluated whether directly acting PPAR-α agonists can modulate reward-related effects of nicotine.

Methods: We combined behavioral, neurochemical, and electrophysiological approaches to evaluate effects of the PPAR-α agonists [[4-Chloro-6-[(2,3-dimethylphenyl)amino]-2-pyrimidinyl]thio]acetic acid (WY14643) and methyl oleoylethanolamide (methOEA; a long-lasting form of OEA) on 1) nicotine self-administration in rats and squirrel monkeys; 2) reinstatement of nicotine-seeking behavior in rats and monkeys; 3) nicotine discrimination in rats; 4) nicotine-induced electrophysiological activity of ventral tegmental area dopamine neurons in anesthetized rats; and 5) nicotine-induced elevation of dopamine levels in the nucleus accumbens shell of freely moving rats.

Results: The PPAR-α agonists dose-dependently decreased nicotine self-administration and nicotine-induced reinstatement in rats and monkeys but did not alter food- or cocaine-reinforced operant behavior or the interoceptive effects of nicotine. The PPAR-α agonists also dose-dependently decreased nicotine-induced excitation of dopamine neurons in the ventral tegmental area and nicotine-induced elevations of dopamine levels in the nucleus accumbens shell of rats. The ability of WY14643 and methOEA to counteract the behavioral, electrophysiological, and neurochemical effects of nicotine was reversed by the PPAR-α antagonist 1-[(4-Chlorophenyl)methyl]-3-[(1,1-dimethylethyl)thio]-a,a-dimethyl-5-(1-methylethyl)-1H-Indole-2-propanoic acid (MK886).

Conclusions: These findings indicate that PPAR-α might provide a valuable new target for antismoking medications.

Key Words: FAAH, nicotine, OEA, PEA, PPAR-α, reinstatement, reward

It has recently been recognized that peroxisome proliferator-activated nuclear receptors, which are known to be involved in metabolism and other cellular functions in many internal organs, also comprise a cannabinoid-like signaling system in the brain (1). Like the endogenous cannabinoid anandamide, the fatty acid amides oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) are endogenous ligands for the alpha subtype of the peroxisome proliferator-activated receptor (PPAR-α), are synthesized on demand, and are primarily degraded by fatty acid amide hydrolase (FAAH). Drugs that selectively inhibit FAAH prevent degradation and increase brain levels of anandamide, OEA, and PEA (2,3). But, unlike anandamide, OEA and PEA are devoid of action at cannabinoid receptors (4–6).

We recently reported that a FAAH-inhibiting drug can counteract addiction-related effects of nicotine in several animal models (7–9). In rats, FAAH inhibition suppressed the development of nicotine-induced conditioned place preference and nicotine self-administration, widely used animal models of nicotine's habit-forming effects (9). Inhibition of FAAH also suppressed reinstatement of nicotine seeking, an animal model of relapse (7). In addition to these behavioral effects, we found that FAAH inhibition prevented the neurochemical and electrophysiological effects of nicotine on reward circuits of the brain that underlie addictive behavior. That is, FAAH inhibition prevented nicotine-induced elevations of the neurotransmitter dopamine in the nucleus accumbens shell (9), and it attenuated nicotine-induced excitation of dopamine neurons in the ventral tegmental area (VTA) (8). Surprisingly, the latter effect did not appear to be mediated by cannabinoid receptors because it was not mimicked in vivo by intravenous (IV) or intracerebroventricular administration of the cannabinoid methanandamide (a hydrolysis-resistant analog of anandamide) but rather by intracerebroventricular administration of the noncannabinoid FAAH substrate, OEA (8). In addition, in vitro activation of VTA dopamine neurons by nicotine in brain slices was prevented by both OEA and PEA but not by methanandamide (8). OEA and PEA are potent agonists of PPAR-α, which is expressed in many areas of the rat brain (including cortex, VTA, midbrain, medulla, hippocampus, substantia nigra, and olfactory tubercle [10–14]) and might regulate

From the Preclinical Pharmacology Section (PM, ZJ, LVP, CB, GHR, SRG), Behavioral Neuroscience Research Branch, and Psychobiology Section (GT), Medications Discovery Research Branch, Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Department of Health and Human Services; Division of Geriatric Medicine and Gerontology (SY), Johns Hopkins University School of Medicine; and Department of Psychiatry (ZJ), University of Maryland School of Medicine, Baltimore, Maryland; B.B. Brodie Department of Neuroscience (MP, AL, SL, MS, WF, PF), University of Cagliari, Monserrato, Italy; Translational Addiction Research Laboratory (BLF), Centre for Addiction and Mental Health, University of Toronto, Toronto, Canada; and Department of Pharmacology (DP), University of California, Irvine, California.

Authors PM, MP, ZJ, and LVP contributed equally to this work.

Address correspondence to Steven R. Goldberg, Ph.D., National Institute on Drug Abuse, Biomedical Research Center, 251 Bayview Boulevard, Baltimore, MD 21224; E-mail: sgoldber@intra.nida.nih.gov.

Received Feb 20, 2010; revised Jul 8, 2010; accepted Jul 9, 2010.

cholinergic neurotransmission and learning and memory processes (15,16). These findings suggest that FAAH inhibition counteracts the rewarding effects of nicotine by activating PPAR-α.

In the present study, we combined behavioral, neurochemical, and electrophysiological approaches to determine whether directly acting PPAR-α agonists can counteract several reward- and dependence-related effects of nicotine: 1) nicotine self-administration in rats and squirrel monkeys; 2) reinstatement of nicotine seeking precipitated by re-exposure to nicotine in rats and squirrel monkeys after a period of abstinence; 3) the interoceptive effects of nicotine in a drug-discrimination procedure in rats; 4) electrophysiological effects of nicotine on the activity of VTA dopamine neurons in anesthetized rats; and 5) nicotine-induced elevations in dopamine levels in the nucleus accumbens shell of freely moving rats.

Methods and Materials

Animals

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Massachusetts; Harlan-Nossan, Milan, Italy) weighing 300 g to 350 g were housed in temperature- and humidity-controlled rooms on a 12-hour light/dark cycle. Experiments were conducted during the light phase. For self-administration experiments, food intake was limited to 20 g/day. For drug discrimination experiments, food was restricted to maintain weight at ≥85% of the subject's highest recorded weight.

Ten adult male squirrel monkeys (*Saimiri sciureus*) weighing .9 kg to 1.1 kg were housed in individual cages in a temperature- and humidity-controlled room with unrestricted access to water. Monkeys were fed five high-protein biscuits per day (Laboratory Diet 5045, PMI Nutrition International, Richmond, Indiana) and two pieces of Banana Softies (Bio-Serv, Frenchtown, New Jersey). Fresh fruits, vegetables, and environmental enrichment were provided daily. Three monkeys (441, 431, and 577) self-administered nicotine. Three monkeys (70F7, 5045, and 39B) self-administered cocaine. Four monkeys (34A, 27B, 30 A, 1549) were used for food-reinforcement experiments.

Monkeys and rats at the National Institute on Drug Abuse-Intramural Research Program were maintained in facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and all procedures were approved by the National Institute on Drug Abuse Institutional Animal Care and Use Committee and conducted in accordance with the 2003 Guidelines of the National Research Council. Rats in the electrophysiology study were maintained at the University of Cagliari, where all procedures were conducted in accordance with the European Economic Community Council Directive (86/609; DL27/01/92, Number 116).

Drugs

Nicotine (Sigma, St. Louis, Missouri) was dissolved in saline (pH corrected to 7.0) and injected subcutaneously (SC) or IV. Cocaine hydrochloride (RTI International, Research Triangle Park, North Carolina) was injected IV. The PPAR-α agonist methyl oleoylethanolamide [methOEA], (N-[(1R)-2-hydroxy-1-methylethyl-9Z-octadecenamide) (donated by Dr. Daniele Piomelli, University of California, Irvine, California) was dissolved in 2% Tween 80, 2% ethanol, and sterile water. The PPAR-α agonist [[4-Chloro-6-[(2,3-dimethylphenyl)amino]-2-pyrimidinyl]thio]acetic acid (WY14643) (Tocris, Ellisville, MO, USA) and the PPAR-α antagonist 1-[(4-Chlorophenyl) methyl]-3-[(1,1-dimethylethyl)thio]-a,a-dimethyl-5-(1-methylethyl)-1H-Indole-2-propanoic acid (MK886) (Tocris) were dissolved in 2% to 4% Tween 80, 30% dimethyl sulfoxide, and sterile water. Methyl oleoylethanolamide, WY14643, and MK886 were injected intraperitoneally (IP) (1 mL/kg) in rats and intramuscular (IM) (.3 mL/kg) in monkeys, except for electrophysiology, where methOEA was injected IV. Dose selection was based on previous studies using WY14643 (8,16,17), methOEA (18), and MK886 (8,16).

Self-Administration (Rats)

General procedure and apparatus were described previously (9,19). Self-administration sessions (2 hours/day) under a one-response fixed-ratio schedule of IV nicotine injection (10 or 30 μg/kg/injection) began 7 to 10 days after catheterization. The response requirement was increased to a five-response fixed-ratio over 15 to 23 sessions. Responses in the left nose-poke hole produced nicotine and pulsed the house light (5 Hz) for a 20-second time-out. Responses in the other, inactive hole had no scheduled effect. Once a criterion was reached (>9 nicotine injections/session for three consecutive sessions), rats received a pretreatment injection (either drug or vehicle) before each subsequent session, with drugs tested only when the criterion had been met during the preceding three vehicle sessions. Doses of WY14643 (20 or 40 mg/kg IP, 20 minutes before the session, tested in counterbalanced order) or methOEA (10 mg/kg IM, 40 minutes before the session) were each tested for three consecutive sessions. Mean data from the three drug pretreatment sessions for each dose were compared with the mean from the three preceding vehicle pretreatment sessions.

Reinstatement (Rats)

Rats learned to self-administer nicotine (30 μg/kg/injection IV) as described above and then were placed under extinction, during which responding had no programmed consequence. When there were <10 active hole responses per session for three consecutive sessions, reinstatement tests were conducted with a pretreatment injection (vehicle, 20 or 40 mg/kg WY14643, IP, 20 minutes before the session) and a priming injection (vehicle or .2 mg/kg nicotine, SC, 10 minutes before the session). Rats were required to meet the response-cessation requirement before each test. Nine rats were tested at both doses of WY14643, and some (two rats at 20 mg/kg and six rats at 40 mg/kg WY14643) were only tested with one dose of WY14643. During the reinstatement test session, responding in the active hole pulsed the house light for 20 seconds; to increase sensitivity of the reinstatement test, only a single response was required to produce this cue. This reinstatement procedure combines nicotine- and cue-induced reinstatement, which has been used in several previous studies (20–23), has several advantages. First, when the cues are removed during extinction, the response-cessation criterion is met more rapidly (mean ± SEM = 5.2 ± .8 sessions in the present study without the stimulus vs. 11.3 ± 1.5 in a pilot group with the stimulus). Second, reinstatement by the combination of nicotine and cues is more robust than when only nicotine or only the cues are presented. This was important because, consistent with the findings of others (20), we have found that a substantial number of rats fail to show reinstatement when given nicotine alone. Finally, the combination of nicotine and cues may be a more relevant and stringent model of the human relapse situation than cues alone or nicotine alone.

Self Administration (Monkeys)

General procedure and apparatus were described previously (24,25). At the start of the session, the house light was extinguished and a green stimulus light was presented. In the presence of the green light, 10 responses on the lever (10-response fixed-ratio) produced a .2-second, .2-mL, 30 μg/kg injection of nicotine, extin-

guished the green light, and illuminated the amber stimulus light for 2 seconds. Each reinforcement was followed by a 60-second time-out period, during which the chamber was dark and lever presses had no programmed consequences. One-hour sessions were conducted 5 days per week.

After four to five sessions of vehicle pretreatment in which responding was stable (<15% variability), drug pretreatments were given for five consecutive sessions. Data from the five drug pretreatment sessions were compared with the three preceding vehicle pretreatment sessions. The drug pretreatments were WY14643 (10, 20, and 40 mg/kg IM, 20 minutes before the session), MK886 (1 mg/kg IM, 45 minutes before the session), MK886 (1 mg/kg) plus WY14643 (20 mg/kg), and methOEA (10 mg/kg IM, 40 minutes before the session). The WY14643 pretreatments (20 and 40 mg/kg IM, 20 minutes before the session) were also tested in two additional groups of monkeys self-administering cocaine (30 μg/kg/injection) or food pellets under the same fixed-ratio schedule.

Reinstatement (Monkeys)

Monkeys trained to self-administer nicotine (30 μg/kg/injection, IV), as described above, were placed under extinction by substituting vehicle for nicotine but maintaining the response-dependent presentation of the nicotine-paired stimulus. When responding reached a low, stable level (<10 injections per session, with no obvious increasing or decreasing trend), priming injections (vehicle or .1 mg/kg IV nicotine, immediately before the session) were given after pretreatment with WY14643 (20 or 40 mg/kg) or WY14643 (20 or 40 mg/kg) plus MK886 (1 mg/kg). Pretreatments were given in the home cage, and primes were given while the monkey was in the training chamber. Each test was performed for a single session followed by extinction sessions with no pretreatment.

Nicotine Discrimination (Rats)

General procedure and apparatus were described previously (26). Rats were trained under a discrete trial schedule of food pellet delivery (10-response fixed-ratio, 45-second time-out) to respond on one lever after a subcutaneous injection of a training dose of .4 mg/kg nicotine (10 minutes before the session) and on the other lever after an injection of saline. Sessions lasted for 20 reinforcements or 30 minutes. WY14643 (40 mg/kg) was substituted for the training dose of nicotine and was also administered together with various doses of nicotine (.01–.4 mg/kg) to assess possible alterations of the nicotine dose-response curve.

Electrophysiology (Anesthetized Rats)

General procedure was described previously (8). Single-unit activity of VTA neurons was recorded extracellularly with glass micropipettes filled with 2% pontamine sky blue (Sigma-Aldrich, Milan, Italy) dissolved in .5 mol/L sodium acetate (impedance 2–5 MΩ). Single units were isolated and identified according to previously published criteria (27,28). All neurons were antidromically identified as projecting to the nucleus accumbens shell by antidromic spikes elicited by stimulation of the shell of the nucleus accumbens. An antidromic response was defined as the ability of evoked spikes to follow stimulation frequencies of >250 Hz, displaying constant latency and collision with spontaneously occurring spikes (29). Nicotine (.2 mg/kg) was administered IV after 5 to 10 minutes of baseline recording. MethOEA (0, 5, or 10 mg/kg IV) was injected 4 minutes before nicotine. WY14643 (20 or 40 mg/kg IP) was injected ~ 30 minutes before the start of recordings; MK886 (3 mg/kg IP) was injected 15 minutes before WY14643. Only one cell was recorded per rat.

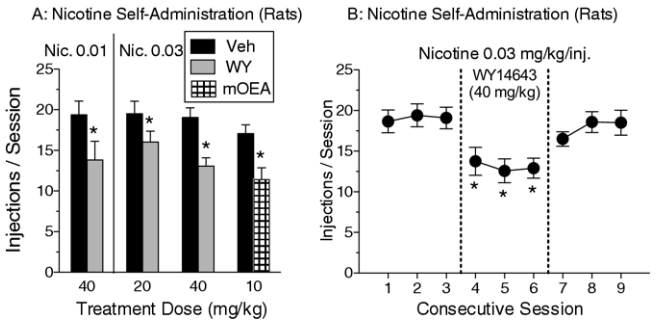


Figure 1. The PPAR-α agonists WY14643 (20 and 40 mg/kg) and methOEA (10 mg/kg) reduced nicotine self-administration in rats. The PPAR-α agonists were given intraperitoneally 20 minutes (WY14643) or 40 minutes (methOEA) before three consecutive sessions in which rats self-administered nicotine (.01 or .03 mg/kg/injection) under a five-response fixed ratio schedule. **(A)** Average rate of injection over three test sessions, compared with average of three sessions of vehicle treatment. **(B)** Rates of nicotine self-administration during individual sessions under baseline conditions (sessions 1–3), after treatment with 40 mg/kg WY14643 (sessions 4–6), and after return to baseline conditions (sessions 7–9). $n = 6$ for rats at the .01 mg/kg/injection nicotine dose; $n = 12$ for rats at the .03 mg/kg/injection nicotine dose, except for methOEA, where $n = 5$. *Significant difference from vehicle treatment. Data are represented as group means ± SEM. inj., injection; mOEA, methOEA; Nic., nicotine; Veh, vehicle; WY, WY14643.

Microdialysis (Freely Moving Rats)

General procedure was described previously (30). Rats were surgically implanted with a concentric dialysis probe aimed at the shell of the nucleus accumbens (anterior +2.0 and lateral 1.1 from bregma, vertical –8.0 from dura) (31). Experiments were performed on freely moving rats 20 to 24 hours after the surgical implant. Ringer's solution (147.0 mmol/L sodium chloride, 2.2 mmol/L calcium chloride, 4.0 mmol/L potassium chloride) was delivered at a constant flow rate of 1.0 μL per minute. Collection of dialysate samples (10 μL) started after 90 minutes, with samples collected every 20 minutes and immediately analyzed by an high-performance liquid chromatography system coupled to electrochemical detection to quantify dopamine. Rats were treated only after stable dopamine values (<10% variability) were obtained for at least three consecutive samples. Probe location in the nucleus accumbens shell was determined histologically after each experiment, and only data from rats with correct probe placement were analyzed. The WY14643 (0, 20, or 40 mg/kg IP) was injected 20 minutes before nicotine (.4 mg/kg SC) or cocaine (3 mg/kg IP), and methOEA (0, 5, or 10 mg/kg IP) was injected 40 minutes before nicotine (.4 mg/kg SC). The MK886 (3 mg/kg IP) was injected 20 minutes before WY14643 or methOEA.

Statistical Analysis

All results are presented as group means (± SEM). For experiments comparing only two conditions, Student *t* test was performed. Self-administration and reinstatement data were analyzed with PROC MIXED (SAS Institute, Cary, North Carolina) with Tukey-Kramer comparisons. For self-administration, the dependent variable was injections per session and the independent variables were pretreatment (dose) and session type (baseline sessions vs. pretreatment sessions), allowing each pretreatment condition to be compared with the most recent baseline. For selected conditions where consecutive sessions are illustrated (Figures 1B, 2B, and 2C), additional analyses were performed using session as a factor. For

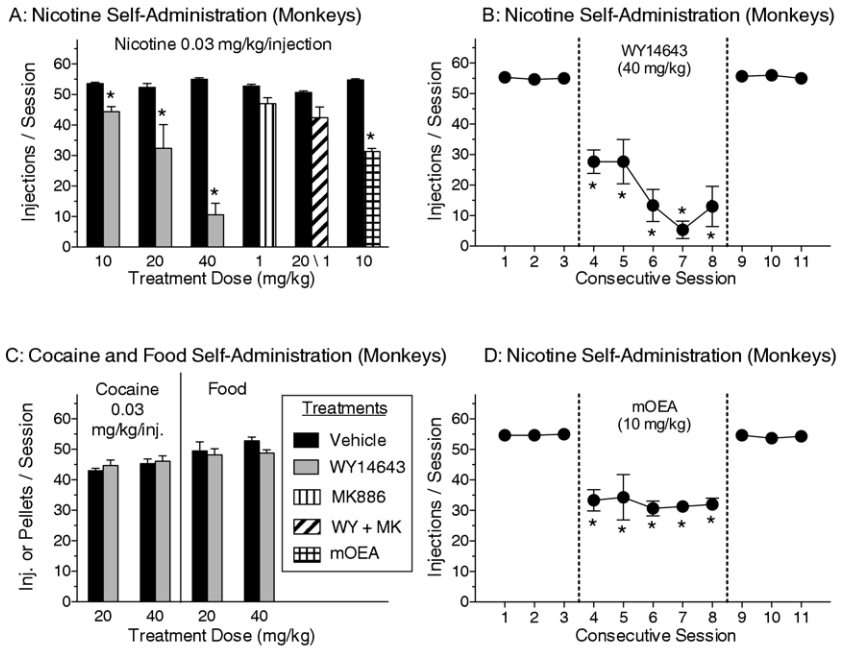


Figure 2. The PPAR-α agonists WY14643 (10, 20 and 40 mg/kg intramuscularly [IM], 20 minutes before session for five consecutive sessions) and methOEA (10 mg/kg IM, 40 minutes before session) significantly reduced the rate of nicotine injections self-administered by squirrel monkeys under a 10-response fixed ratio schedule at a nicotine dose of 30 μg/kg/injection. The effects of WY14643 (20 mg/kg) were reversed by pretreatment with the PPAR-α antagonist MK886 (1 mg/kg IM, 45 minutes before session), which had no significant effect when given alone. **(A)** Average rate of injection over five test sessions, compared with average of five sessions of vehicle treatment. **(B,D)** Rates of nicotine self-administration during individual sessions under baseline conditions (sessions 1–3), after treatment with 40 mg/kg WY14643 (sessions 4–8) or 10 mg/kg methOEA, and after return to baseline conditions (sessions 9–11). **(C)** WY14643 (20 or 40 mg/kg IM, 20 minutes before session) did not alter the number of 30 μg/kg cocaine injections self-administered or the number of food pellets obtained under an identical 10-response fixed ratio schedule in squirrel monkeys. $n = 3$ for monkeys under all conditions except food reinforcement, where $n = 4$. *Significant difference from vehicle treatment. Data are represented as group means ± SEM. inj., injection; MK, MK886; mOEA, methOEA; WY, WY14643.

reinstatement in rats, the factors were nose-poke hole (active vs. inactive), prime (saline vs. nicotine), and pretreatment (dose of WY14643); for monkeys, separate one-way analyses of variance were conducted for the three doses of WY14643. Electrophysiology and microdialysis data were analyzed using analysis of variance with Dunnett or Student-Newman-Keuls comparison procedures, respectively. Experiment-wise significance levels of .05 were maintained in all analyses.

Results

PPAR-α Activation Suppressed Nicotine Self-Administration in Rats and Monkeys

The PPAR-α agonists WY14643 and methOEA significantly decreased ongoing nicotine self-administration in both rats [Figure 1A: main effect of WY14643, $F(1,11) = 5.4$, $p < .05$; main effect of session $F(1,11) = 41.8$, $p < .0001$; for .01 mg/kg nicotine baseline, t test, $t(5) = 5.019$, $p < .05$; methOEA: $t(4) = 5.4$, $p < .006$] and monkeys [WY14643 in Figure 2A: interaction of WY14643 and session $F(2,4) = 8.25$, $p < .05$; methOEA in Figure 2A: $t(2) = 25.8$, $p < .01$]. At the most effective doses, self-administration behavior was decreased significantly throughout the course of PPAR-α agonist treatment [Figure 1B: $F(3,27) = 7.99$, $p < .001$; Figure 2B: $F(5,10) = 11.04$, $p < .001$; Figure 2D: $F(5,10) = 6.23$, $p < .007$] and rapidly returned to higher levels when treatment was discontinued. In rats, response rates in the inactive hole occurred at a fairly constant percentage of response rates in the active hole regardless of pretreatment (mean percentage ± SEM = 26 ± 5 under vehicle treatment, 21 ± 3 under 20 mg/kg WY14643, and 27 ± 9 under 40 mg/kg WY14643). The specificity of WY14643's effects was verified by giving the PPAR-α antagonist MK886 as a pretreatment in monkeys. MK886 reversed the decreases in nicotine self-administration produced by WY14643 but had no effect on nicotine self-administration when given alone. On the final day of treatment with the most effective dose of WY14643 (40 mg/kg), nicotine intake was decreased by 35% in rats and 76% in monkeys.

PPAR-α Activation Suppressed Reinstatement When Abstinent Rats and Monkeys Were Re-exposed to Nicotine

When relapse was modeled using a reinstatement procedure, the nicotine-seeking response (nose poking in rats, lever pressing in monkeys) was reinstated by a noncontingent priming injection of nicotine before the session (Figure 3). WY14643 significantly reduced this reinstatement in rats [Figure 3A: main effect of active vs. inactive nose-poking hole, $F(1,14) = 20.4$, $p < .0005$; interaction of WY14643 dose and nicotine, $F(2,13) = 7.7$, $p < .01$] and monkeys [Figure 3B: 20 mg/kg WY14643: $F(3,6) = 15.4$, $p < .005$; 40 mg/kg WY14643: $F(3,6) = 93.5$, $p < .001$]. WY14643 alone did not reinstate drug seeking. In rats, nicotine also increased responding in the inactive hole, and WY14643 prevented this increase. However, it should be noted that responding in the active hole remained higher than responding in inactive hole under all testing conditions, indicating that the nicotine-induced increases in active-hole responding were due to reward rather than nonspecific increases in locomotor activity. Pretreatment of monkeys with the PPAR-α antagonist MK886 prevented the effects of WY14643 in this model of relapse, demonstrating the receptor specificity of these effects (Figure 3B).

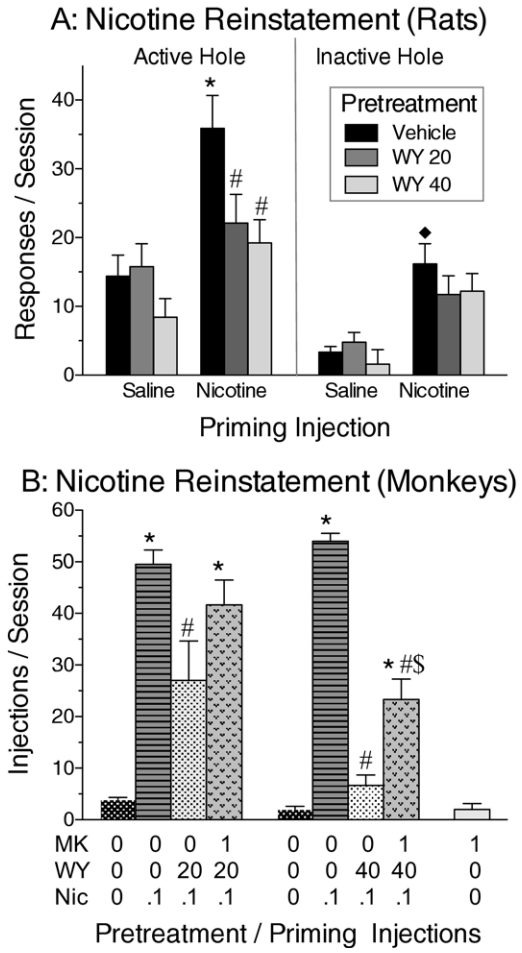


Figure 3. The PPAR-α agonist WY14643 blocked reinstatement of nicotine self-administration after a period of abstinence in rats and monkeys. **(A)** In rats, WY14643 (20 mg/kg intraperitoneally [IP], $n = 11$; and 40 mg/kg IP, $n = 15$) dose-dependently reduced the reinstatement of extinguished nicotine-seeking responses produced by a priming injection of nicotine. **(B)** In squirrel monkeys, WY14643 (20 or 40 mg/kg intramuscularly [IM], 20 minutes before the session) dose-dependently reduced the reinstatement of extinguished nicotine-seeking responses produced by a priming injection of nicotine (.1 mg/kg intravenously) before the session ($n = 3$). This effect of WY14643 was prevented by pretreatment with the PPAR-α antagonist MK886 (1 mg/kg IM, 45 minutes before session). Data are presented as group means ± SEM. *Significant difference from vehicle pretreatment during a saline prime session. # Significant difference from vehicle pretreatment during a nicotine prime session. Diamond represents a significant difference from inactive hole responding during a saline prime session. MK, MK886; Nic, nicotine; WY, WY14643.

PPAR-α Activation Did Not Alter Nicotine's Interoceptive Effects or Produce a General Depression of Operant Behavior

The ability of WY14643 to reduce nicotine self-administration and reinstatement was not due to a nonspecific disruption of operant behavior. WY14643 had no effect on cocaine- or food-reinforced responding in squirrel monkeys under testing conditions identical to those used with nicotine (Figure 2D), and it did not alter food-reinforced responding by rats as measured in the drug discrimination procedure (even when combined with intraperitoneal nicotine injection; Figure 4B) or rats' ability to detect that they had received nicotine (Figure 4A) in a drug-discrimination procedure.

PPAR-α Activation Prevented Nicotine-Induced Changes in Dopamine Cell Firing in the Ventral Tegmental Area of Rats

In single-unit in vivo recording experiments in anesthetized rats, IV injection of .2 mg/kg nicotine enhanced firing rate [Figure 5A: $F(6,7) = 6.99$, $p < .0001$] and burst firing [Figure 5B: $F(6,7) = 2.837$; $p < .05$] of VTA dopamine neurons that were antidromically identified as projecting to the nucleus accumbens. At doses of WY14643 that significantly reduced nicotine self-administration and nicotine-induced reinstatement in the behavioral experiments, WY14643 and methOEA did not alter spontaneous firing rate (Figure 5A,C; basal mean Hz ± SEM; control rats: 3.24 ± .2; WY14643 20 mg/kg: 3.2 ± .6; WY14643 40 mg/kg: 3.3 ± .6; methOEA 5 mg/kg: 3.18 ± .5; MK886 + WY14643 40 mg/kg: 3.6 ± .8; methOEA 10 mg/kg: 3.11 ± .4] or burst firing (Figure 5B,D; basal mean % of spikes/bursts ± SEM; control rats: 12.9 ± 3.72; WY14643 20 mg/kg: 13.6 ± 9.88; WY14643 40 mg/kg: 15.8 ± 6.1; MK886 + WY14643 40 mg/kg: 8.0 ± 6.4; methOEA 5 mg/kg: 8.7 ± 3.57; methOEA 10 mg/kg: 8.9 ± 1.61] of VTA dopamine neurons when given alone. However, when given before nicotine, 20 mg/kg of WY14643 partially blocked and 40 mg/kg of WY14643 completely blocked nicotine-induced excitation of dopamine neurons [Figure 5A,B: effect of WY14643 on firing rate, $F(1,48) = 20.36$, $p < .001$; and burst firing, $F(1,48) = 5.98$, $p < .05$]. Intravenous administration of methOEA (5 and 10 mg/kg) also completely prevented excitation of dopamine

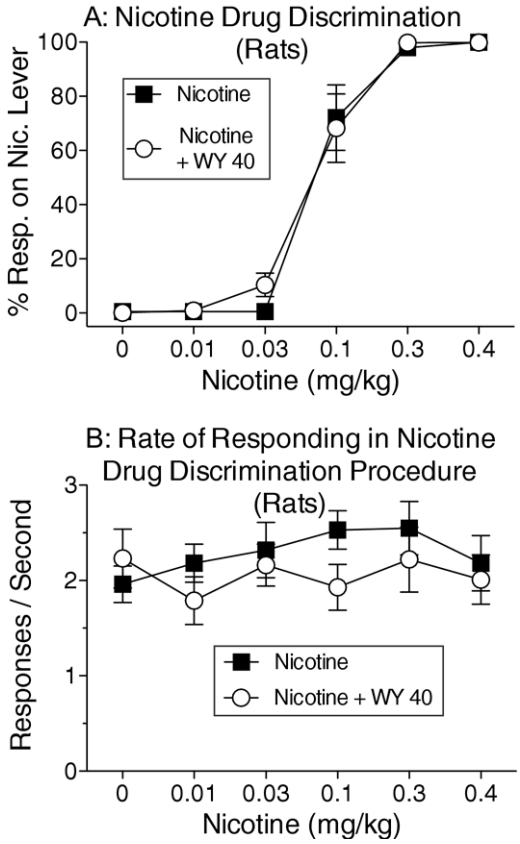


Figure 4. The PPAR-α agonist WY14643 (40 mg/kg intraperitoneal, 20 minutes before session) did not alter the interoceptive effects of nicotine or the rate of food-maintained lever pressing under a nicotine drug discrimination procedure in rats ($n = 12$). When given alone or in combination with any dose of nicotine (.01–.4 mg/kg subcutaneous), WY14643 did not significantly affect the percentage of responses on the nicotine-appropriate lever **(A)** or the rate of lever responding **(B)**. Data are presented as group means ± SEM. Nic., nicotine; Resp., responding; WY, WY14643.

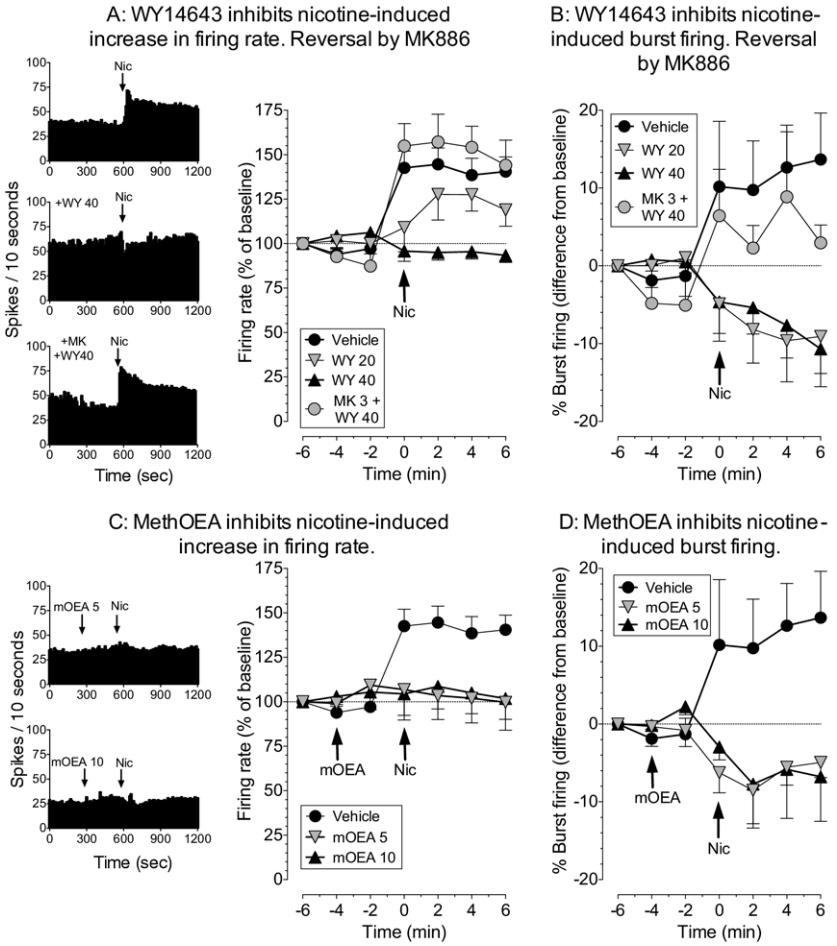


Figure 5. The PPAR-α agonists inhibited nicotine-induced activation of ventral tegmental area dopamine neurons in anesthetized rats. Histograms show the stimulatory effects of nicotine (.2 mg/kg intravenous, $n = 7$) on discharge activity of an individual ventral tegmental area dopamine neuron in a representative rat and the actions of the PPAR-α agonists **(A,C)**. Line graphs show the time course of nicotine's effects. The PPAR-α agonist WY1463 (40 mg/kg intraperitoneally injected ~ 30 minutes before the start of recordings, $n = 7$) significantly blocked nicotine-induced increases in firing rate **(A)** and burst firing **(B)**. The PPAR-α antagonist MK886 (3 mg/kg injected ~45 minutes before the start of recordings, $n = 5$) significantly abolished the effects produced by WY14643 **(A,B)**. Methyl oleoylethanolamide (methOEA) (5 and 10 mg/kg intravenously injected 4 minutes before nicotine, $n = 7$ both) mimicked the effects of WY14643, significantly blocking nicotine-induced increases in firing rate **(C)** and burst firing **(D)**. Results are presented as mean ± SEM of firing rates and burst firing, expressed as percentages of or differences from baseline values, respectively. Note that data for vehicle in **(A)** are repeated in **(C)** and that data for vehicle in **(B)** are repeated in **(D)**. Arrows indicate time of drug injections. The following treatments significantly reduced the effects of nicotine on firing rate ($ps < .05$, Dunnett's post hoc comparisons): WY14643 40 mg/kg, methOEA 5 mg/kg, and methOEA 10 mg/kg. Burst firing was significantly reduced by WY14643 20 and 40 mg/kg and by methOEA 5 and 10 mg/kg. Both firing rate and burst firing differed when WY14643 40 mg/kg was given with versus without MK886 ($ps < .05$). Nic, nicotine; MK, MK886; mOEA, methOEA; WY, WY14643.

neurons by nicotine [Figure 5C,D: effects of two doses of methOEA on firing rate: $F(1,48) = 5.02$, $p < .05$, and $F(1,48) = 5.24$, $p < .05$; and burst firing: $F(1,52) = 6.34$, $p < .05$, and $F(1,52) = 4.73$, $p < .05$]. Pretreatment with the PPAR-α antagonist MK886 completely reversed WY14643's blockade of nicotine-induced increases in firing rate (Figure 5A) and burst firing [Figure 5B: MK886 + WY14643 vs. WY14643, $F(1,60) = 16.57$, $p < .01$; $F(1,60) = 18.24$, $p < .05$, for firing rate and burst firing, respectively].

PPAR-α Activation Prevented Nicotine-Induced Increases in Dopamine Levels in the Nucleus Accumbens Shell of Rats

In vivo microdialysis experiments in freely moving rats showed that systemic injection of .4 mg/kg nicotine increased extracellular dopamine levels in the nucleus accumbens shell by ~100% [Figure 6A: $F(12,48) = 16.23$, $p < .0001$; Figure 6B: $F(13,65) = 58.61$; $p < .0001$]. WY14643 alone did not alter dopamine levels, but it significantly reduced nicotine-induced elevations in dopamine levels in a dose-related manner [Figure 6A: time–treatment interaction, $F(18,108) = 3.01$, $p < .001$; basal level, expressed as mean fmoles/10 μL sample ± SEM for control rats: 31 ± 2.7; WY14643 40 mg/kg: 31.8 ± 5.2]. Administration of methOEA also did not alter dopamine levels by itself (Figure 6D) but markedly reduced nicotine-induced elevations in dopamine levels [Figure 6B: time–treatment interaction, $F(26,169) = 5.95$, $p < .0001$]. The PPAR-α antagonist MK886 had no effect when given alone but completely reversed WY14643's (40 mg/kg) blockade of nicotine-induced elevations in dopamine levels [Figure 6C: time–treatment interaction, $F(26,169) = 4.06$, $p < .05$]. Similarly, MK866 prevented the effects of 10 mg/kg of methOEA [Figure 6D: time–treatment interaction, $F(28,182) = 3.06$, $p < .01$]. In contrast, WY14643 did not alter the effects of cocaine on dopamine levels in the nucleus accumbens shell. Basal levels of dopamine in dialysates, expressed as mean fmoles/10 μL sample ± SEM, did not differ between groups before injections (Figure 5A,C; vehicle + nicotine: 31.2 ± 5.2; WY14643 40 mg/kg +

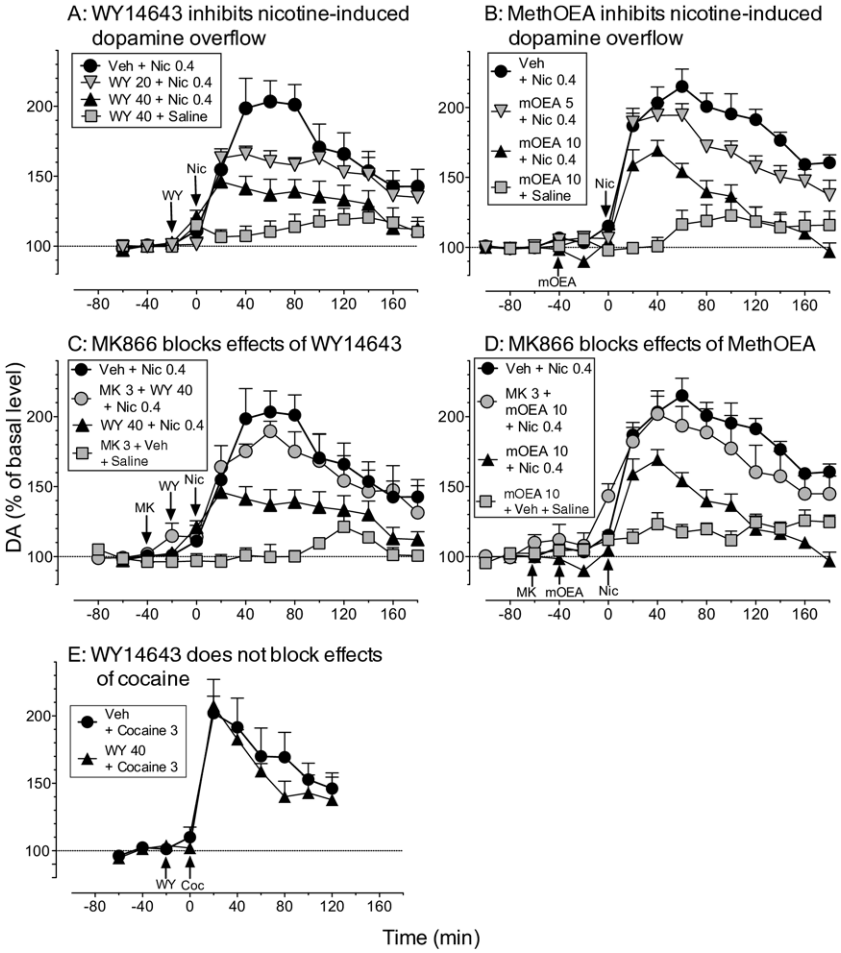


Figure 6. The PPAR-α agonists inhibited nicotine-induced elevations in dopamine levels in the nucleus accumbens shell of freely moving rats. Pretreatment with WY14643 (20 and 40 mg/kg intraperitoneally [IP], $n = 5$ both) or methOEA (10 mg/kg IP, $n = 5$) but not their vehicle ($n = 5$ both), given 20 and 40 minutes, respectively, before nicotine (.4 mg/kg subcutaneous, $n = 6$), significantly reduced the increase in extracellular dopamine levels produced by nicotine **(A,B)**. The PPAR-α antagonist MK886 (3 mg/kg IP) injected 20 minutes before 40 mg/kg WY14643 ($n = 6$) or 10 mg/kg methOEA ($n = 6$) completely reversed the reduction of nicotine-induced elevations in dopamine levels produced by WY14643 (40 mg/kg IP) and methOEA (10 mg/kg IP) **(C,D)**. The following treatments significantly reduced the effects of nicotine (Tukey post hoc comparisons): WY14643 20 mg/kg, ($p < .05$); WY14643 40 mg/kg, ($p < .001$); methOEA 10 mg/kg ($p < .001$). The MK886 (3 mg/kg IP, $n = 4$) had no significant effect when given with the vehicle of WY14643 and saline **(C)**, and methOEA (10 mg/kg IP, $n = 4$) had no significant effect when given with the vehicles of MK886 and saline **(D)**. The following treatments significantly reduced the effects of nicotine (Tukey post hoc comparisons): WY14643 20 mg/kg, ($p < .05$); WY14643 40 mg/kg, ($p < .001$); methOEA 10 mg/kg ($p < .001$). The WY14643 (40 mg/kg IP, $n = 5$) injected 20 minutes before cocaine (3 mg/kg IP; $n = 5$) did not significantly alter the effects of cocaine **(E)**. Note that data for vehicle + nicotine and for WY14643 + nicotine in **(A)** are repeated in **(C)** and that data for vehicle + nicotine and for methOEA + nicotine in **(B)** are repeated in **(D)**. Arrows indicate time of drug or vehicle injection. Results are presented as group means ± SEM, expressed as percent of basal values. DA, dopamine; MK, MK886; mOEA, methOEA; Nic, nicotine; Veh, vehicle; WY, WY14643.

saline: 31.8 ± 5.2; WY14643 20 mg/kg + nicotine: 30.8 ± 4.5; WY14643 40 mg/kg + nicotine: 36.0 ± 5.9; MK886 + vehicle + saline: 41.0 ± 6.0; MK886 + WY14643 40 mg/kg + nicotine: 32.0 ± 4.8; Figure 5B,D; vehicle + nicotine: 30.8 ± 2.9; methOEA 5 mg/kg + nicotine: 31.5 ± 1.3; methOEA 10 mg/kg + nicotine: 33.8 ± 4.2; MK886 + vehicle + saline: 32.5 ± 3.2; MK886 + methOEA 10 mg/kg + nicotine: 30.4 ± 3.3; Figure 5E; vehicle + cocaine: 34.9 ± 5.8; cocaine + WY14643: 36.6 ± 5.4).

Discussion

These findings indicate that activation of PPAR-α can counteract addiction-related effects of nicotine on the brain and behavior. In both rats and monkeys, the PPAR-α agonists WY14643 and methOEA significantly decreased nicotine self-administration and suppressed reinstatement of nicotine seeking, which models relapse, the main obstacle to smoking cessation. At the doses that produced these effects with nicotine self-administration, there was no indication that PPAR-α ligands had any effect on food- or cocaine-maintained behavior. The reduction of nicotine self-administration and reinstatement by PPAR-α agonists was most likely due to these drugs' ability to prevent nicotine-induced excitation of dopaminergic transmission in reward-related areas of the brain. Specifically, PPAR-α agonists prevented nicotine-induced increases in firing rate and burst firing in dopamine neurons in the VTA, and they prevented nicotine-induced (but not cocaine-induced) elevations of dopamine levels in the shell of the nucleus accumbens. These potentially therapeutic behavioral, electrophysiological, and neurochemical effects of PPAR-α agonists were reversed by the PPAR-α antagonist MK886, verifying that they were indeed due to PPAR-α activation.

The fact that the PPAR-α agonist WY14643 did not alter the

interoceptive effects of nicotine in the drug-discrimination procedure is consistent with previous findings that nicotine's reward-related dopaminergic effects are not well captured by this procedure (32). For example, even though the cannabinoid type 1 receptor antagonist rimonabant can block nicotine reward (i.e., self-administration, conditioned place preference) and nicotine-induced increases of dopamine levels in the nucleus accumbens, rimonabant does not alter nicotine discrimination (26,33). Similarly, antagonism of the dopamine D3 receptor blocks nicotine-induced conditioned place preference but does not alter nicotine discrimination (34). The finding that WY14643 blocked nicotine's effects on dopamine but did not alter its discriminative effects is consistent with previous data suggesting that neurobiological substrates between reward-related and interoceptive effects of nicotine are not entirely overlapping (35).

The nicotine-related behavioral, electrophysiological, and neurochemical effects of PPAR-α agonists in the present study are very close to the effects obtained earlier with the FAAH inhibitor URB597 ([3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate) (7,9). The results obtained here with the PPAR-α agonist WY14643 are also consistent with the finding that URB597 does not alter nicotine discrimination (36). All of these findings converge to suggest that the elevation of endogenous PPAR-α ligands OEA and PEA induced by URB597 modulates the rewarding effects of nicotine; further studies are needed to delineate the role of anandamide.

The mechanism by which PPAR-α agonists and FAAH inhibition exert these unanticipated antiaddictive actions is not completely understood. However, the following points are well established. Mesolimbic dopamine plays a pivotal role in nicotine dependence, and the VTA and nucleus accumbens shell are critical brain areas for nicotine's rewarding effects (37–39). Nicotinic receptors in the VTA are located both on cell bodies of dopaminergic neurons and on presynaptic nerve endings of glutamatergic neurons that descend from the medial prefrontal cortex and impinge on these cell bodies (40). Nicotine facilitates dopaminergic neurotransmission and dopamine release in the nucleus accumbens shell by directly stimulating nicotinic receptors on cell bodies of dopaminergic neurons and by indirectly stimulating glutamate release, which in turn stimulates VTA dopaminergic neuron firing and dopamine release in the nucleus accumbens shell. Our data show that activation of PPAR-α, either indirectly through FAAH inhibition or directly by administration of a PPAR-α agonist, prevents nicotine-induced increases in firing rate and burst firing in dopamine neurons in the VTA and as a consequence prevents nicotine-induced elevations of dopamine levels in the shell of the nucleus accumbens. The mechanism underlying these effects was elucidated by our recent in vitro findings showing that activation of PPAR-α produces a nongenomic (rapid) modulation of nicotinic receptors on cell bodies of dopaminergic neurons in the VTA by promoting their phosphorylation by tyrosine kinases (8). Phosphorylated nicotinic receptors show diminished ionic conductance (41) and are rapidly internalized (42), reducing or abolishing the responses of dopamine neurons to nicotine, and we have demonstrated that the general tyrosine kinase inhibitor genistein reverses OEA's ability to block nicotine-induced excitation of VTA dopamine neurons (8). Additionally, we have found that the β2 subunit of nicotinic receptors is critical for PPAR-α effects, because deletion of this subunit abolished the effects of PPAR-α compounds, whereas its selective re-expression in VTA dopamine neurons restores both the behavioral effects of nicotine (motor activity) and PPAR-α actions (43). Taken together, all of these findings suggest a mechanism by which PPAR-α may modulate the reward-related dopaminergic effects of nicotine that provide a basis for nicotine self-administration.

In conclusion, our findings demonstrate that nicotine's ability to have rewarding effects and reinstate drug-seeking behavior after a period of abstinence are suppressed by PPAR-α activation, accomplished directly by PPAR-α agonists or indirectly by FAAH inhibition. These behavioral effects appear to be due to modulation of nicotine's excitatory effects on reward-related mesolimbic dopamine transmission. Notably, PPAR-α agonists and FAAH inhibitors appear to suppress nicotine reward and reinstatement much like the cannabinoid inverse agonist/antagonist rimonabant but do not share the adverse psychoactive effects produced by medications that target cannabinoid type 1 receptors (3,9,19). Thus, PPAR-α is a promising new molecular target for the treatment of the devastating problem of tobacco dependence.

This study was supported in part by the Intramural Research Program of the National Institute on Drug Abuse, National Institutes of Health, Department of Health and Human Services; by the Italian Ministry of University and Scientific Research and the University of Cagliari Center of Excellence on Neurobiology of Dependence; by the Department of Psychiatry, University of Maryland School of Medicine; by the Division of Geriatric Medicine and Gerontology, Johns Hopkins University School of Medicine; and by the Department of Pharmacology, University of California, Irvine.

All authors report no biomedical financial interests or potential conflicts of interest.

1. O'Sullivan SE (2007): Cannabinoids go nuclear: Evidence for activation of peroxisome proliferator-activated receptors. *Br J Pharmacol* 152:576–582.
2. Fegley D, Gaetani S, Duranti A, Tontini A, Mor M, Tarzia G, Piomelli D (2005): Characterization of the fatty acid amide hydrolase inhibitor cyclohexyl carbamic acid, 3′-carbamoyl-biphenyl-3-yl ester (URB597): Effects on anandamide and oleoylethanolamide deactivation. *J Pharmacol Exp Ther* 313: 352–358.
3. Justinova Z, Mangieri RA, Bortolato M, Chefer SI, Mukhin AG, Clapper JR, *et al.* (2008): Fatty acid amide hydrolase inhibition heightens anandamide signaling without producing reinforcing effects in primates. *Biol Psychiatry* 64:930–937.
4. Lambert DM, Vandevoorde S, Jonsson KO, Fowler CJ (2002): The palmitoylethanolamide family: A new class of anti-inflammatory agents? *Curr Med Chem* 9:663–674.
5. Lo Verme J, Gaetani S, Fu J, Oveisi F, Burton K, Piomelli D (2005): Regulation of food intake by oleoylethanolamide. *Cell Mol Life Sci* 62:708–716.
6. Hansen HS (2010): Palmitoylethanolamide and other anandamide congeners. Proposed role in the diseased brain. *Exp Neurol* 224:48–55.
7. Forget B, Coen KM, Le Foll B (2009): Inhibition of fatty acid amide hydrolase reduces reinstatement of nicotine seeking but not break point for nicotine self-administration—comparison with CB(1) receptor blockade. *Psychopharmacology (Berl)* 205:613–624.
8. Melis M, Pillolla G, Luchicchi A, Muntoni AL, Yasar S, Goldberg SR, Pistis M (2008): Endogenous fatty acid ethanolamides suppress nicotine-induced activation of mesolimbic dopamine neurons through nuclear receptors. *J Neurosci* 28:13985–13994.
9. Scherma M, Panlilio LV, Fadda P, Fattore L, Gamaleddin I, Le Foll B, *et al.* (2008): Inhibition of anandamide hydrolysis by cyclohexyl carbamic acid, 3′-carbamoyl-3-yl ester (URB597) reverses abuse-related behavioral and neurochemical effects of nicotine in rats. *J Pharmacol Exp Ther* 327:482–490.
10. Moreno S, Farioli-Vecchioli S, Ceru MP (2004): Immunolocalization of peroxisome proliferator-activated receptors and retinoid X receptors in the adult rat CNS. *Neuroscience* 123:131–145.
11. Cullingford TE, Bhakoo K, Peuchen S, Dolphin CT, Patel R, Clark JB (1998): Distribution of mRNAs encoding the peroxisome proliferator-activated receptor alpha, beta, and gamma and the retinoid X receptor alpha, beta, and gamma in rat central nervous system. *J Neurochem* 70:1366–1375.

12. Deplanque D, Gele P, Petrault O, Six I, Furman C, Bouly M, *et al.* (2003): Peroxisome proliferator-activated receptor-alpha activation as a mechanism of preventive neuroprotection induced by chronic fenofibrate treatment. *J Neurosci* 23:6264–6271.
13. Kainu T, Wikstrom AC, Gustafsson JA, Pelto-Huikko M (1994): Localization of the peroxisome proliferator-activated receptor in the brain. *Neuroreport* 5:2481–2485.
14. Kreisler A, Gele P, Wiart JF, Lhermitte M, Destee A, Bordet R (2007): Lipid-lowering drugs in the MPTP mouse model of Parkinson's disease: Fenofibrate has a neuroprotective effect, whereas bezafibrate and HMG-CoA reductase inhibitors do not. *Brain Res* 1135:77–84.
15. Campolongo P, Roozendaal B, Trezza V, Cuomo V, Astarita G, Fu J, *et al.* (2009): Fat-induced satiety factor oleoylethanolamide enhances memory consolidation. *Proc Natl Acad Sci U S A* 106:8027–8031.
16. Mazzola C, Medalie J, Scherma M, Panlilio LV, Solinas M, Tanda G, *et al.* (2009): Fatty acid amide hydrolase (FAAH) inhibition enhances memory acquisition through activation of PPAR-alpha nuclear receptors. *Learn Mem* 16:332–337.
17. Fu J, Gaetani S, Oveisi F, Lo Verme J, Serrano A, Rodriguez De Fonseca F, *et al.* (2003): Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha. *Nature* 425:90–93.
18. Astarita G, Di Giacomo B, Gaetani S, Oveisi F, Compton TR, Rivara S, *et al.* (2006): Pharmacological characterization of hydrolysis-resistant analogs of oleoylethanolamide with potent anorexiant properties. *J Pharmacol Exp Ther* 318:563–570.
19. Panlilio LV, Solinas M, Matthews SA, Goldberg SR (2007): Previous exposure to THC alters the reinforcing efficacy and anxiety-related effects of cocaine in rats. *Neuropsychopharmacology* 32:646–657.
20. LeSage MG, Burroughs D, Dufek M, Keyler DE, Pentel PR (2004): Reinstatement of nicotine self-administration in rats by presentation of nicotine-paired stimuli, but not nicotine priming. *Pharmacol Biochem Behav* 79:507–513.
21. Wing VC, Shoaib M (2008): Contextual stimuli modulate extinction and reinstatement in rodents self-administering intravenous nicotine. *Psychopharmacology (Berl)* 200:357–365.
22. Shoaib M (2006): Effects of isoarecolone, a nicotinic receptor agonist in rodent models of nicotine dependence. *Psychopharmacology (Berl)* 188: 252–257.
23. Shoaib M (2008): The cannabinoid antagonist AM251 attenuates nicotine self-administration and nicotine-seeking behaviour in rats. *Neuropharmacology* 54:438–444.
24. Goldberg SR (1973): Comparable behavior maintained under fixed-ratio and second-order schedules of food presentation, cocaine injection or d-amphetamine injection in the squirrel monkey. *J Pharmacol Exp Ther* 186:18–30.
25. Justinova Z, Tanda G, Redhi GH, Goldberg SR (2003): Self-administration of delta(9)-tetrahydrocannabinol (THC) by drug naive squirrel monkeys. *Psychopharmacology (Berl)* 169:135–140.
26. Le Foll B, Goldberg SR (2004): Rimonabant, a CB1 antagonist, blocks nicotine-conditioned place preferences. *Neuroreport* 15:2139–2143.
27. Grace AA, Bunney BS (1984): The control of firing pattern in nigral dopamine neurons: Burst firing. *J Neurosci* 4:2877–2890.
28. Ungless MA, Magill PJ, Bolam JP (2004): Uniform inhibition of dopamine neurons in the ventral tegmental area by aversive stimuli. *Science* 303: 2040–2042.
29. Lipski J (1981): Antidromic activation of neurones as an analytic tool in the study of the central nervous system. *J Neurosci Methods* 4:1–32.
30. Solinas M, Scherma M, Fattore L, Stroik J, Wertheim C, Tanda G, *et al.* (2007): Nicotinic alpha 7 receptors as a new target for treatment of cannabis abuse. *J Neurosci* 27:5615–5620.
31. Paxinos G, Watson C (1998): *The Rat Brain in Stereotaxic Coordinates*. San Diego: Academis Publishing Group.
32. Corrigall WA, Coen KM (1994): Dopamine mechanisms play at best a small role in the nicotine discriminative stimulus. *Pharmacol Biochem Behav* 48:817–820.
33. Cohen C, Perrault G, Voltz C, Steinberg R, Soubrie P (2002): SR141716, a central cannabinoid (CB(1)) receptor antagonist, blocks the motivational and dopamine-releasing effects of nicotine in rats. *Behav Pharmacol* 13:451–463.
34. Le Foll B, Sokoloff P, Stark H, Goldberg SR (2005): Dopamine D3 receptor ligands block nicotine-induced conditioned place preferences through a mechanism that does not involve discriminative-stimulus or antidepressant-like effects. *Neuropsychopharmacology* 30:720–730.
35. Smith JW, Stolerman IP (2009): Recognising nicotine: The neurobiological basis of nicotine discrimination. *Handb Exp Pharmacol* 295–333.
36. Zaniewska M, McCreary AC, Przegalinski E, Filip M (2006): Evaluation of the role of nicotinic acetylcholine receptor subtypes and cannabinoid system in the discriminative stimulus effects of nicotine in rats. *Eur J Pharmacol* 540:96–106.
37. Corrigall WA, Coen KM, Adamson KL (1994): Self-administered nicotine activates the mesolimbic dopamine system through the ventral tegmental area. *Brain Res* 653:278–284.
38. Nisell M, Nomikos GG, Svensson TH (1994): Systemic nicotine-induced dopamine release in the rat nucleus accumbens is regulated by nicotinic receptors in the ventral tegmental area. *Synapse* 16:36–44.
39. Pontieri FE, 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.
40. Mansvelder HD, McGehee DS (2002): Cellular and synaptic mechanisms of nicotine addiction. *J Neurobiol* 53:606–617.
41. Charpantier E, Wiesner A, Huh KH, Ogier R, Hoda JC, Allaman G, *et al.* (2005): Alpha7 neuronal nicotinic acetylcholine receptors are negatively regulated by tyrosine phosphorylation and Src-family kinases. *J Neurosci* 25:9836–9849.
42. Cho CH, Song W, Leitzell K, Teo E, Meleth AD, Quick MW, Lester RA (2005): Rapid upregulation of alpha7 nicotinic acetylcholine receptors by tyrosine dephosphorylation. *J Neurosci* 25:3712–3723.
43. Melis M, Carta S, Fattore L, Tolu S, Yasar S, Goldberg SR, *et al.* (2010): Peroxisome proliferator-activated receptors-alpha modulate dopamine neuron activity through nicotinic receptors. *Biol Psychiatry* 68:256–264.

Addiction Biology

PRECLINICAL STUDY: FULL ARTICLE

doi:10.1111/j.1369-1600.2010.00222.x

Effects of fatty acid amide hydrolase inhibition on neuronal responses to nicotine, cocaine and morphine in the nucleus accumbens shell and ventral tegmental area: involvement of PPAR-α nuclear receptors

Antonio Luchicchi[1], Salvatore Lecca[1], Stefano Carta[1], Giuliano Pillolla[1], Anna L. Muntoni[2,3], Sevil Yasar[4], Steven R. Goldberg[5] & Marco Pistis[1,3]

B.B. Brodie Department of Neuroscience[1], CNR Neuroscience Institute-Cagliari[2], Center of Excellence for the Neurobiology of Addiction[3], University of Cagliari, Italy, Division of Geriatric Medicine and Gerontology, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA[4] and Preclinical Pharmacology Section, Behavioral Neuroscience Research Branch, Intramural Research Program, Department of Health and Human Services, National Institute on Drug Abuse—National Institutes of Health, Baltimore, MD, USA[5]

ABSTRACT

The endocannabinoid system regulates neurotransmission in brain regions relevant to neurobiological and behavioral actions of addicting drugs. We recently demonstrated that inhibition by URB597 of fatty acid amide hydrolase (FAAH), the main enzyme that degrades the endogenous cannabinoid N-acylethanolamine (NAE) anandamide and the endogenous non-cannabinoid NAEs oleoylethanolamide and palmitoylethanolamide, blocks nicotine-induced excitation of ventral tegmental area (VTA) dopamine (DA) neurons and DA release in the shell of the nucleus accumbens (ShNAc), as well as nicotine-induced drug self-administration, conditioned place preference and relapse in rats. Here, we studied whether effects of FAAH inhibition on nicotine-induced changes in activity of VTA DA neurons were specific for nicotine or extended to two drugs of abuse acting through different mechanisms, cocaine and morphine. We also evaluated whether FAAH inhibition affects nicotine-, cocaine- or morphine-induced actions in the ShNAc. Experiments involved single-unit electrophysiological recordings from DA neurons in the VTA and medium spiny neurons in the ShNAc in anesthetized rats. We found that URB597 blocked effects of nicotine and cocaine in the ShNAc through activation of both surface cannabinoid CB1-receptors and alpha-type peroxisome proliferator-activated nuclear receptor. URB597 did not alter the effects of either cocaine or morphine on VTA DA neurons. These results show that the blockade of nicotine-induced excitation of VTA DA neurons, which we previously described, is selective for nicotine and indicate novel mechanisms recruited to regulate the effects of addicting drugs within the ShNAc of the brain reward system.

Keywords Cocaine, dopamine neurons, electrophysiology, nicotine, nucleus accumbens, peroxisome proliferator-activated receptor-α.

Correspondence to: Marco Pistis, B.B. Brodie Department of Neuroscience, Center of Excellence for the Neurobiology of Addiction, University of Cagliari, Cittadella Universitaria, 09042 Monserrato (CA), Italy. E-mail: mpistis@unica.it

INTRODUCTION

The endocannabinoids are a family of lipid signaling molecules, which play a pivotal role in the modulation of several physiological and pathophysiological conditions within the central nervous system (CNS) and in the periphery. Although there are a number of endogenous compounds with endocannabinoid-like activity, the best characterized are n-arachidonoylethanolamide (anandamide) (Devane *et al.* 1992) and 2-arachidonoyl glycerol (2-AG) (Sugiura *et al.* 1995). Within the CNS, anandamide and 2-AG are synthesized *on demand* in postsynaptic cell membranes and show affinity for type-1 cannabinoid receptors (CB1), which are mainly located on presynaptic neurons (Kano *et al.* 2009). Once produced, endocannabinoids inhibit neurotransmitter release and then are moved intracellularly by a putative carrier protein (Hillard & Jarrahian 2000), where they

are then deactivated by two main enzymes, fatty acid amide hydrolase (FAAH), which catabolizes anandamide (Cravatt *et al.* 1996), and monoacylglycerol lipase, which catabolizes 2-AG (Dinh *et al.* 2002). Two non-cannabinoid N-acylethanolamines (NAEs), the anorexiant oleoylethanolamide (OEA) and the anti-inflammatory palmitoylethanolamide (PEA), which are structurally similar to anandamide but are endogenous ligands for alpha-type nuclear peroxisome proliferator-activated receptors (PPAR-α), are also endogenous substrates for FAAH (Rodriguez de Fonseca *et al.* 2001; Cravatt & Lichtman 2002). Their centrally mediated effects have been poorly characterized, although OEA and PEA might be involved in modulation of synaptic signaling as endogenous ligands for an independent endocannabinoid-like system. Evidence is accumulating, which suggests a significant contribution of OEA and PEA, and PPAR-α nuclear receptors in effects observed following pharmacological inhibition of FAAH (Mazzola *et al.* 2009).

The endocannabinoid system regulates neurotransmission in brain regions relevant to neurobiological and behavioral actions of addicting drugs or natural rewarding stimuli (Maldonado, Valverde & Berrendero 2006; Solinas, Yasar & Goldberg 2007; Solinas, Goldberg & Piomelli 2008). Several lines of evidence indicate that endocannabinoids are released by midbrain dopamine (DA) neurons (Melis *et al.* 2004; Riegel & Lupica 2004) to regulate their own afferents. As a consequence, pharmacological manipulation of endocannabinoid signaling fine-tunes the effects of different addicting drugs. For example, recent studies have investigated how pharmacological inhibition of FAAH, and the consequent increase in anandamide levels, modulates the effects of nicotine (Merritt *et al.* 2008; Scherma *et al.* 2008; Forget, Coen & Le Foll 2009). In rats, the FAAH inhibitor cyclohexyl carbamic acid 3′-carbamoyl-3-yl ester (URB597) blocked nicotine-induced conditioned place preference, acquisition of nicotine self-administration behavior, nicotine-induced relapse to drug-seeking behavior and nicotine-induced DA increases in the shell of the nucleus accumbens (ShNAc) (Scherma *et al.* 2008). We also found that URB597 completely prevents nicotine-induced increases in firing rate and burst firing of ventral tegmental area (VTA) DA neurons of anesthetized rats (Melis *et al.* 2008), thus inhibiting one of the primary neuronal responses to nicotine administration in the brain reward system (Maskos *et al.* 2005).

In this study, we asked whether inhibition of FAAH might prevent not only the effects of nicotine but also the effects of other addicting drugs, such as cocaine and morphine, on VTA DA neurons. In addition, because DA neurons in the VTA directly project to the ShNAc, we also compared the effects of FAAH inhibition on responses to nicotine, cocaine and morphine of GABAergic medium spiny neurons (MSNs) in the ShNAc. Together with the VTA, the ShNAc plays a crucial role in the primary reinforcing properties of addicting drugs and orients reward-seeking behavior (Carlezon & Thomas 2009). We found that FAAH inhibition by URB597 specifically modulates neuronal responses to different substances in these two distinct areas through actions on both cannabinoid CB1-receptors and PPAR-α nuclear receptors. This suggests that both endogenous cannabinoid (anandamide) and non-cannabinoid (OEA and PEA) fatty acid ethanolamides, which are all substrates for FAAH, participate in the fine-tuning of neurophysiological and behavioral effects of addicting drugs.

MATERIALS AND METHODS

Male Sprague Dawley rats (250–350 g) (Harlan, San Pietro al Natisone, Italy) were used in both the NAc and the VTA experiments. We housed animals in groups of three to six in standard conditions of temperature and humidity under a 12-hour light/dark cycle (with lights on at 7:00 a.m.) with food and water available *ad libitum*. We anesthetized animals with urethane (1300 mg/kg, intraperitoneal [i.p.]), cannulated their femoral vein for intravenous administration of pharmacological agents and placed in the stereotaxic apparatus (Kopf, Tujunga, CA) with their body temperature maintained at 37 ± 1°C by a heating pad.

We performed the experiments in strict accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (Committee on Guidelines for the Use of Animals in Neuroscience and Behavioral Research 2003) and EEC Council Directive of 24 November 1986 (86/609). We made all efforts to minimize pain and suffering, and to reduce the number of animals used.

Experiments in the VTA

The scalp was retracted and one burr hole was drilled above the VTA (6.0 mm posterior from bregma, 0.3–0.6 mm lateral from midline) for the placement of a recording electrode. We localized structures according to the stereotaxic atlas of Paxinos & Watson (1997). Single-unit activity of neurons located in the VTA (V 7.0–8.0 mm from the cortical surface) was recorded extracellularly with glass micropipettes filled with 2% pontamine sky blue dissolved in 0.5 M of sodium acetate (impedance 2–5 MΩ). Single-unit activity was filtered (band pass 500–5000 Hz), and individual spikes were isolated by means of a window discriminator (Neurolog Instruments, Digitimer, Herts, UK), displayed on a digital

storage oscilloscope (TDS 3012, Tektronics, Marlow, UK) and digitally recorded. We sampled experiments on-line and off-line with Spike2 software (Cambridge Electronic Design, Cambridge, UK) by a computer connected to CED 1401 interface (Cambridge Electronic Design). Single units were isolated and identified according to already published criteria (Grace & Bunney 1983, 1984; Ungless, Magill & Bolam 2004). Because we recorded only one cell per rat, we selected VTA DA neurons when all criteria for identification were fulfilled: firing rate $\leq$ 10 Hz, duration of action potential $\geq$ 2.5 ms and inhibitory responses to hindpaw pinching. We defined bursts as the occurrence of two spikes at an interspike interval $\leq$ 80 ms. Bursts terminated when the interspike interval exceeded 160 ms (Grace & Bunney 1983). At the end of each recording section, direct current (10 mA for 15 minutes) was passed through the recording electrode to eject pontamine sky blue, which allowed the identification of the recorded cells. Brains were removed and fixed in 8% formalin solution. We identified the position of the electrodes microscopically on serial sections (60 μm) stained with cresyl violet.

Experiments in the ShNAc

We recorded extracellularly single-unit activity of neurons located in the medial part of the NAc (shell) (1.5 mm anterior from bregma, 0.8–1.3 mm lateral from the midline, 6.5–7.0 mm ventral from cortical surface) using the same instruments previously described for the VTA experiments. In addition, because MSNs of the ShNAc do not fire spontaneously in anesthetized animals, we delivered electrical stimuli in the basolateral amygdala (BLA) to evoke spike firing in the NAc cell. For this reason, we inserted a formvar-coated stimulating stainless steel bipolar electrode with an inclination of 15° anteroposterior on the coronal plane (250-μm tip diameter) in the ipsilateral BLA (3.2 mm posterior from bregma, 5.0 mm lateral from the midline, 7.0 mm ventral from the cortical surface), which is a major excitatory projecting area to the NAc.

After the glass electrode had been positioned to the dorsal limit of the NAc, we searched cells that responded to the stimulation of the BLA. Stimuli (~0.5 mA) were delivered to the BLA at 1-second intervals, while the microelectrode was lowered incrementally through the NAc. Once we detected a cell, we adjusted the position of the microelectrode in order to maximize the spike amplitude relative to background noise. We identified neurons that responded to BLA stimulation by their robust excitatory response (latency range 10–25 ms). We did not include in this study cells whose latencies were longer than 26 ms following BLA stimulation because they could exhibit a polysynaptic response component (Mulder, Hodenpijl & Lopes da Silva 1998).

The experimental protocol was essentially that published by Floresco *et al.* (2001) with some modifications (Pistis *et al.* 2002). Once we isolated a cell, we adjusted stimulation currents to approximately half-maximal intensity, such as ~50% of electrical stimuli (1 Hz) in the BLA elicited an action potential in the recorded cell. We calculated evoked spike probability by dividing the number of action potentials observed by the number of stimuli administered in 100-second periods.

Once stable levels of evoked spike probability were achieved (< 10% changes over 10–15 minutes), we administered drugs intravenously and assessed spike probability every 100 seconds. Changes in spike probability were an index of changes induced by the studied compounds over the excitation of NAc cells evoked by BLA stimulation. As well as for the VTA experiments, we recorded only one cell per rat.

Statistical analysis

For VTA experiments, we calculated drug-induced changes in firing rate and pattern by averaging the effects after drug administration (2 minutes) and normalizing to the predrug baseline.

For ShNAc experiments, we determined predrug spike probability baseline as the mean of at least three consecutive assessments (100 seconds) over 10 minutes before drug administration. We generated peristimulus time histograms (1-ms bins, 100 cumulative sweeps) by CED Spike2 software (Cambridge Electronic Design). Following drug administration, we calculated spike probability every 100 seconds and normalized it to the predrug baseline.

All the numerical data are given as mean ± standard error of the mean. Data were compared and analyzed by using two-way analysis of variance (ANOVA) for repeated measures (treatment × time), or one-way ANOVA or Student's *t*-test for repeated measures, when appropriate. *Post hoc* multiple comparisons were made using the Dunnett's or Bonferroni's tests. We performed statistical analysis by means of the NCSS program (Kaysville, UT, USA). The significance level was established at $P < 0.05$.

Drugs

Nicotine [(−)-nicotine hydrogen tartrate] was purchased from Sigma (St. Louis, MO). Morphine chloridrate and cocaine chloridrate were purchased from S.a.l.a.r.s (Como, Italy) and Akzo Pharmadivision Diosynth (Oss, the Netherlands). SCH 23390 was purchased from Sigma/RBI, and L-sulpiride was purchased from Ravizza (Latina, Italy). Rimonabant (SR141716A) was a generous gift of Sanofi-Aventis Recherche (Montpellier, France). URB597 was purchased from Alexis (Lausen, Switzerland). MK886 was purchased from Tocris (Bristol,

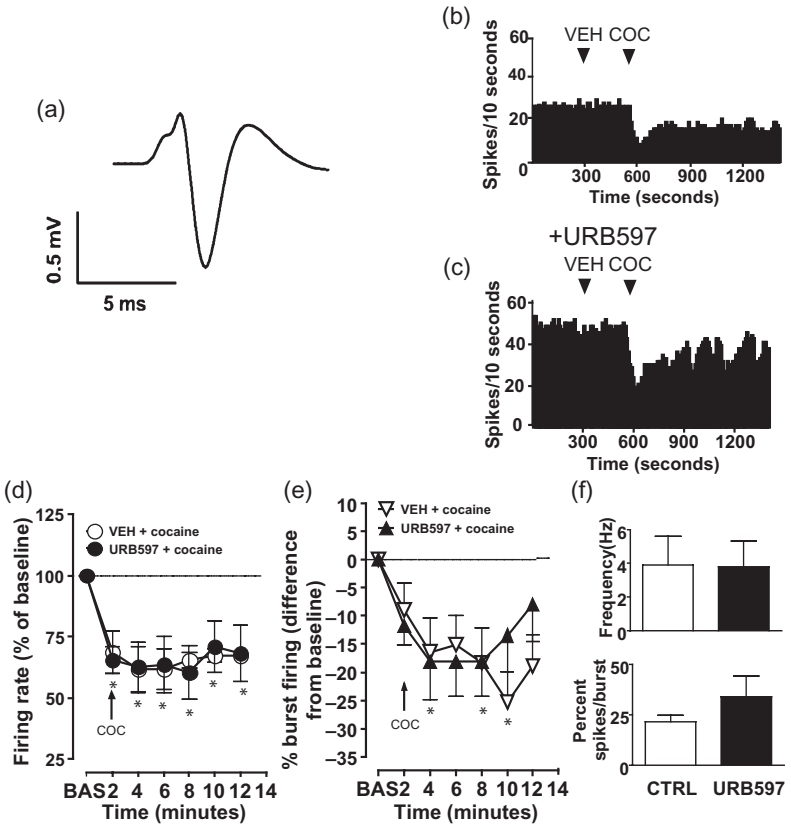


Figure 1 Effects of URB597 on the responses of ventral tegmental area (VTA) dopamine (DA) neurons to cocaine. (a) Average trace, acquired from a digital storage oscilloscope, showing the typical broad, notched waveform of an isolated VTA DA neuron recorded from an anesthetized rat. (b) Representative firing rate histogram showing the decrease in firing rate of an individual VTA DA neuron produced by intravenous cocaine (COC, 1 mg/kg injected at arrowheads) in control conditions. The injection of vehicle (VEH) is ineffective. (c) This rate histogram displays that URB597 pre-treatment (0.1 mg/kg, i.v.) does not alter cocaine's depression of firing rate of a VTA DA neuron. (d,e) Graphs illustrating the time course of cocaine's effects on firing rate and burst firing of VTA DA neurons with and without URB597 pre-treatment. Pre-treatment with URB597 (0.1 mg/kg, i.v.) does not affect the inhibition of VTA DA neurons induced by cocaine (COC, 1 mg/kg, i.v.; arrow) either in firing rate (d) or burst firing (e). (f) These histograms show that the pre-treatment with URB597 did not affect baseline firing activity (top) or burst firing (bottom) of recorded VTA DA neurons ($P > 0.05$, Student's *t*-test). Results are means, with vertical bars representing the standard error of the mean of firing rate and burst firing, expressed as a percentage of, or difference from, the baseline (BAS). *$P < 0.01$ versus baseline, one-way analysis of variance and Dunnett's test

UK). We diluted nicotine, SCH 23390, L-sulpiride, cocaine and morphine in saline. We adjusted nicotine solution to pH = 7 with NaOH. We emulsified rimonabant in 1% Tween80 (Sigma, St Louis, MO, USA), and then, we diluted in saline and sonicated. We dissolved URB597 in dimethylsulfoxide (DMSO) (100 µg/µl) and diluted to the final concentration in saline. The final concentration of DMSO was 0.1%. We emulsified MK886 in 10% of Tween80, dissolved in 20% of DMSO and then diluted to the final concentration in distilled water.

RESULTS

FAAH inhibition does not affect morphine and cocaine effect on VTA DA neurons

We first assessed whether FAAH inhibition modulates responses of VTA DA neurons to cocaine and morphine. We recorded from VTA DA neurons ($n = 22$) only when they fulfilled all criteria reported in the literature (see methods). A typical waveform of a DA neuron action potential is graphically depicted in Fig. 1a. When we isolated a neuron, we recorded its basal activity for at least 5 minutes before administration of vehicle, and, after an additional 4 minutes, we injected morphine or cocaine. The vehicle used for these experiments had no effect on VTA DA neurons.

In line with previous studies (Einhorn, Johansen & White 1988), cocaine (1.0 mg/kg, i.v.) inhibited firing rate (61.62 ± 9.35% of baseline; $F_{(5;30)} = 5.996$; $n = 6$; $P < 0.001$; one-way ANOVA for repeated measures and Dunnett's test) (Fig 1b,d) and burst firing (−16.42 ± 6.14 of baseline level; $F_{(5;25)} = 4.659$; $n = 6$; $P < 0.01$; one-way ANOVA for repeated measures and Dunnett's test) (Fig. 1e) of VTA DA neurons. As reported in literature (Matthews & German 1984), morphine

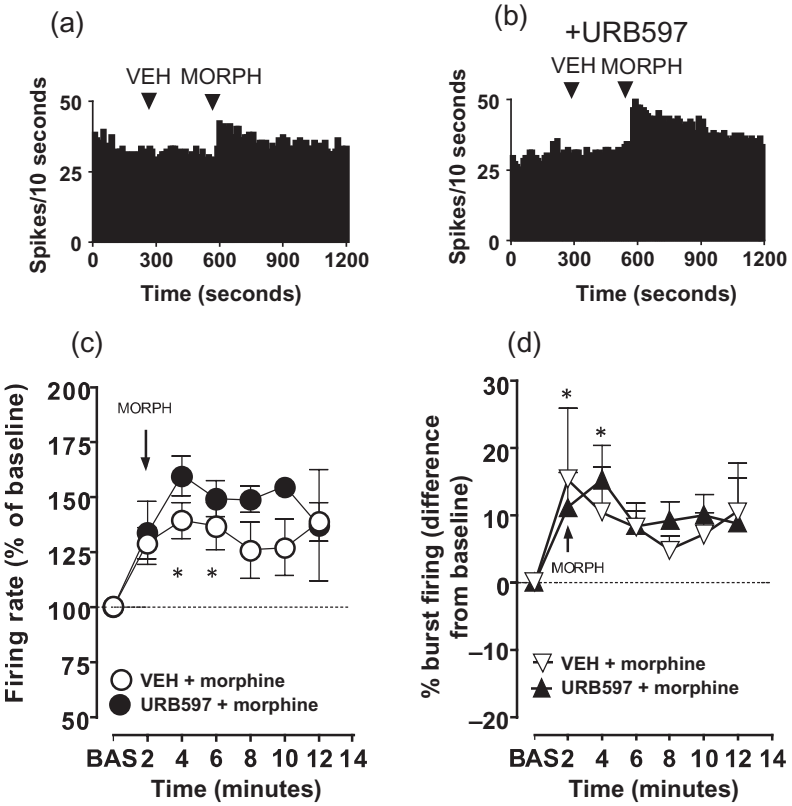


Figure 2 Lack of effect of URB597 on morphine-induced increases in firing rate and burst firing of ventral tegmental area (VTA) dopamine (DA) neurons. (a) Representative firing rate histogram showing that intravenous injection of morphine (MORPH, 4 mg/kg) enhances firing rate of VTA DA neurons in control conditions. (b) This exemplificative rate histogram displays that the administration of URB597 (0.1 mg/kg, i.v., 2 hours before the recordings) did not affect morphine-induced enhancement of firing rate in a VTA DA neuron. (c,d) Graphical depiction of the time course of firing rate (c) or burst firing (d) of VTA DA neurons following intravenous administration of morphine (MORPH, 4 mg/kg). Pre-treatment with URB597 (0.1 mg/kg, i.v.) did not alter the effects of morphine either on firing rate or burst activity of VTA DA neurons. Results are means, with vertical bars representing the standard error of the mean of firing rate and burst firing, expressed as a percentage of, or difference from, the baseline (BAS). *$P < 0.05$ versus baseline, one-way analysis of variance and Dunnett's test

(4.0 mg/kg, i.v.) stimulated firing rate (Fig. 2a,c) and burst firing (Fig. 2d) (139.46 ± 8.17% of baseline level; $F_{(4;20)} = 3.299$; $n = 5$; $P < 0.05$; one-way ANOVA for repeated measures and Dunnett's test) of VTA DA neurons. In a separate group of rats, we administered the FAAH inhibitor URB597 (0.1 mg/kg, i.v., 1–2 hours before recordings), which persistently (> 6 hours) increases brain levels of anandamide (Kathuria *et al.* 2003). URB597 pre-treatment did not change cocaine effects on firing rate (URB + cocaine: 66.5 ± 9.98% of baseline level; $F_{(1;60)} = 0.0003$; $n = 6$, $P > 0.05$ versus vehicle + cocaine) (Fig. 1c,d) and burst firing (URB + cocaine: −18.13 ± 6.68 of baseline level; $F_{(1;60)} = 0.15$; $n = 6$, $P > 0.05$ versus vehicle + cocaine) (Fig. 1e). URB597 pre-treatment slightly enhanced the excitatory action of morphine on firing rate, but this effect did not reach statistical significance (URB + morphine: 159.63 ± 9.06 of baseline level; $F_{(1;40)} = 2.76$; $n = 5$, $P = 0.13$ versus vehicle + morphine; two-way ANOVA and Bonferroni's test) (Fig. 2b,c). The effect of morphine on burst firing was similar between controls and URB597-treated animals (URB + morphine: +15.34 ± 5.13 of baseline level; $F_{(1;40)} = 0.12$; $n = 5$, $P > 0.05$ versus vehicle + morphine; two-way ANOVA and Bonferroni's test) (Fig. 2d).

URB597 had no significant effect on either the frequency or burst firing of DA neurons; the mean baseline frequency of VTA DA neurons recorded was 3.9 ± 1.7 Hz in control animals and 3.8 ± 1.53 Hz in URB597-pre-treated animals ($n = 11$, $P > 0.05$ versus controls; Student's *t*-test) (Fig. 1f). There was also no significant change in the percent of spikes in bursts after URB597 (21.5 ± 3.32% for control rats and 33.9 ± 10.22% for URB597-pre-treated animals, $n = 11$, $P > 0.05$; Student's *t*-test) (Fig. 1f). Thus, there was no effect of URB597 on cocaine or morphine-induced actions on DA neurons in the VTA, in contrast to our previous findings with nicotine (Melis *et al.* 2008), demonstrating a selective blockade by FAAH inhibition of nicotine-induced alterations in VTA DA neuron excitability.

FAAH inhibition blocks the effects of nicotine on MSNs of the ShNAc

The ShNAc plays a pivotal role in the mechanisms underlying the primary reinforcing effects produced by natural stimuli and by drugs of abuse, as well as in reinstatement of drug-seeking behavior in abstinent animals. We next assessed whether FAAH inhibition modulates responses of MSNs in the ShNAc to nicotine.

All recorded MSNs ($n = 59$) were quiescent, responded to BLA stimulation and were located in the medial part of the ShNAc. BLA stimulation evoked firing in MSNs of the ShNAc with a mean latency of 18.4 ± 0.7 ms (Fig. 3a,b). The average baseline spike probability

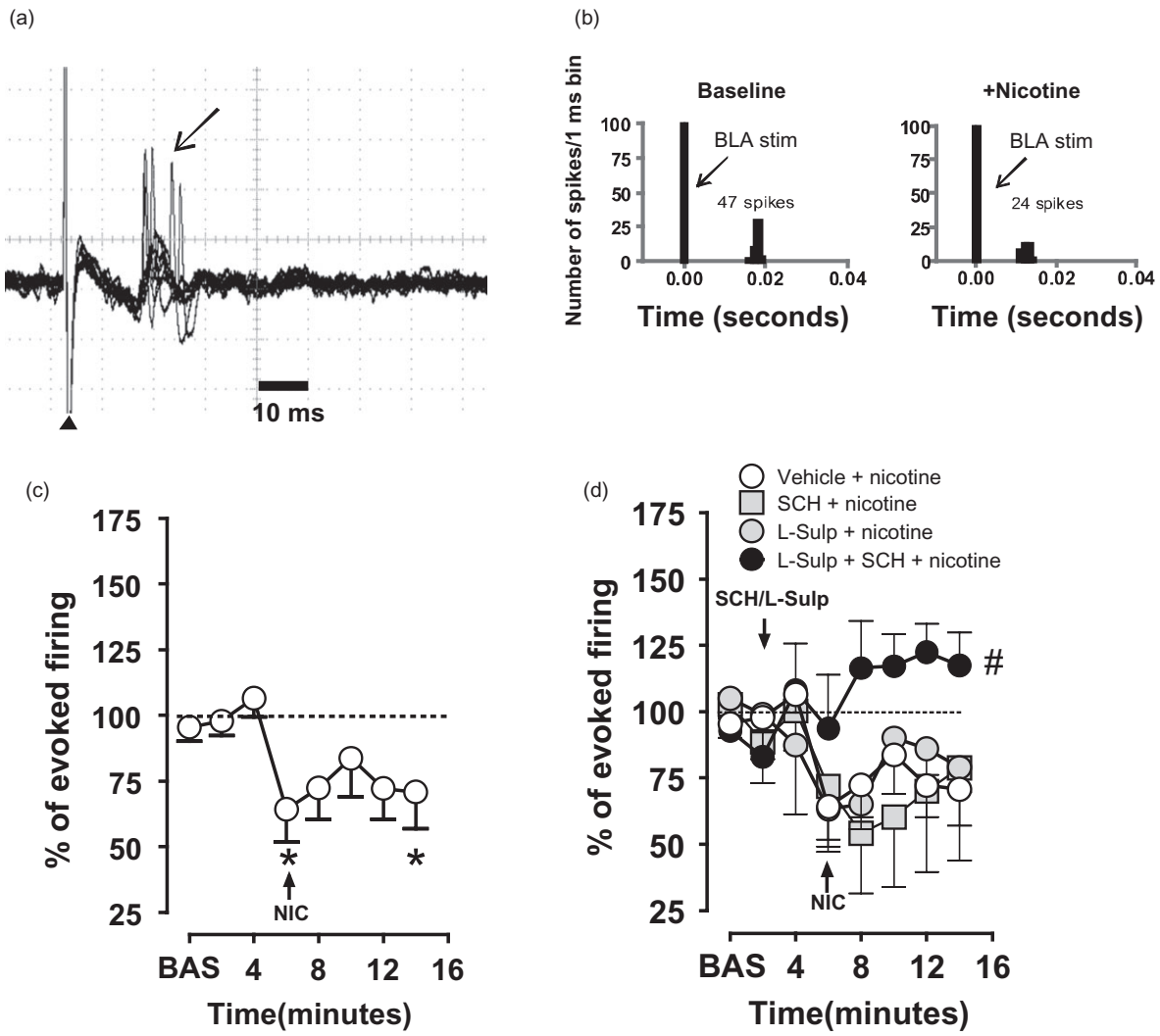


Figure 3 Nicotine depresses the excitability of medium spiny neurons (MSNs) in the shell of the nucleus accumbens (ShNAc). (a) Superimposed traces acquired from a digital storage oscilloscope showing a relatively constant latency of the orthodromic responses of a representative MSN after basolateral amygdala (BLA) stimulation. The arrowhead indicates the artifacts produced by BLA stimulation; the arrow shows evoked action potentials of a MSN. Once a cell was isolated, the current applied to the BLA was adjusted to obtain ~50% of probability to elicit an action potential after a single pulse stimulation. (b) Representative peristimulus time histograms displaying the typical inhibitory response of a MSN in the ShNAc after BLA stimulation and injection of nicotine (0.2 mg/kg, i.v.). (c) Graph showing the time course of nicotine-induced inhibition of spike firing of MSNs. (d) Graphical depiction illustrating that nicotine-induced inhibition was prevented by the combined administration (at arrow), but not by the separate injection, of the dopamine D1 receptor antagonist SCH23390 (SCH, 1 mg/kg, i.v.) and the D2 receptor antagonist L-sulpiride (L-Sulp, 10 mg/kg, i.v.). Results are means, with vertical bars representing the standard error of the mean of evoked spike firing, expressed as a percentage of the baseline (BAS). *$P < 0.05$ versus baseline, one-way analysis of variance (ANOVA) and Dunnett's test; #$P < 0.05$ versus vehicle + nicotine, two-way ANOVA and Bonferroni's test

following BLA stimulation was 46.3 ± 1.5%. We recorded evoked activity of MSNs of the ShNAc for 300 seconds before the administration of nicotine, morphine or cocaine. As previously reported (Hakan, Hart & Eyl 1993), nicotine (0.2 mg/kg, i.v.) depressed the excitability of MSNs in the ShNAc, as measured by their response to BLA stimulation (64 ± 12% of baseline level; $F_{(5;40)} = 3.44$, $n = 6$, $P < 0.01$; one-way ANOVA for repeated measures and Dunnett's test) (Fig. 3b,c). This effect required the joint activation of DA receptors by nicotine-induced release of DA, because combined administration of the DA D1 receptor antagonist SCH23390 (1.0 mg/kg, i.v.) and the DA D2 receptor antagonist L-sulpiride (10 mg/kg, i.v.), 4 minutes before nicotine, fully prevented the depression of MSN excitability by nicotine (122.5 ± 10.6% of baseline level; $F_{(1;80)} = 14.09$; $n = 6$; $P < 0.001$ versus control; two-way ANOVA and Bonferroni's test) (Fig. 3d). Neither L-sulpiride nor SCH23390, when administered separately, were able to prevent nicotine-induced depression of MSN excitability (SCH23390: $F_{(1;70)} = 0.05$, $n = 6$,

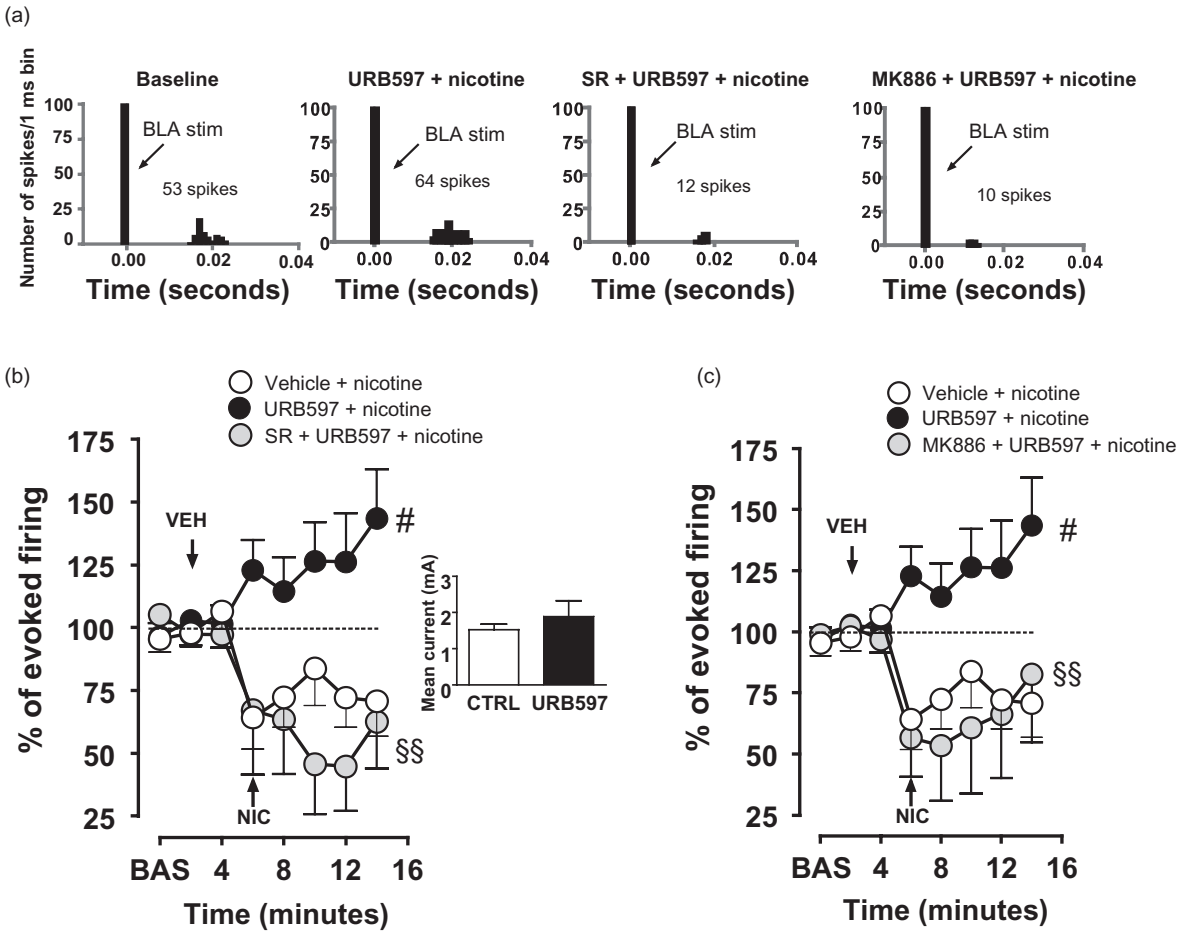


Figure 4 URB597 suppresses nicotine's action on medium spiny neurons (MSNs) in the shell of the nucleus accumbens (ShNAc). (a) Exemplificative peristimulus time histograms showing that nicotine-induced decrease of MSN excitability is reversed by URB597, whereas the CB1-receptor antagonist rimonabant (SR, 0.5 mg/kg) and the peroxisome proliferator-activated nuclear receptor-α antagonist MK886 (3 mg/kg), administered 15 minutes before URB597, prevented the effects of the fatty acid amide hydrolase inhibitor and restored nicotine-induced inhibition of MSNs' responses to basolateral amygdala (BLA) stimulation in the ShNAc. (b,c) Graphical depiction illustrating that URB597 pre-treatment prevented nicotine-induced inhibition of MSNs and that this inhibition by nicotine was reversed by rimonabant (SR, 0.5 mg/kg, i.v.) (b) or MK886 (3 mg/kg, i.p.) (c). The histogram in the inset displays that the mean current administered in the BLA to evoke spike firing in MSNs was not different between controls (CTRL) and URB597-pre-treated animals. Results are means, with vertical bars representing the standard error of the mean of evoked spike firing, expressed as difference percentage of the baseline (BAS). #$P < 0.05$ versus vehicle + nicotine, §§$P < 0.001$ versus URB + nicotine, two-way analysis of variance and Bonferroni's test

$P > 0.05$; L-sulpiride: $F_{(1;70)} = 0.02$, $n = 6$, $P > 0.05$; two-way ANOVA for repeated measures and Dunnett's test) (Fig. 3d).

Pre-treatment with URB597 (0.1 mg/kg, i.v., 1–2 hours before recordings) blocked nicotine's depression of MSNs in the ShNAc (Fig. 4a,b) (126.6 ± 15.6% of baseline level, $n = 6$, $F_{(1;70)} = 9.03$, $P < 0.01$ versus control; two-way ANOVA and Bonferroni's test). Consistent with the data obtained in the VTA, URB597 had no significant effect by itself; the mean current administered for spike firing evoked by BLA stimulation in MSNs in the ShNAc was not different between controls and URB597-pre-treated animals (1.52 ± 0.16 mA versus 1.9 ± 0.4 mA, respectively; $n = 6$; $P > 0.05$; Student's t-test) (Fig. 4b, inset). Interestingly, after URB597 treatment, nicotine increased, rather than depressed, firing evoked by BLA stimulation in MSNs ($F_{(8;40)} = 3.32$, $n = 6$, $P < 0.01$; one-way ANOVA for repeated measures and Dunnett's test) (Fig. 4a,b). These results indicate that FAAH inhibition prevents the inhibitory effects of nicotine on MSNs in the ShNAc.

URB597 blocks nicotine's effects in the ShNAc via CB1-receptor- and PPAR-α-dependent mechanisms

Next, we explored the mechanism by which URB597 blocks nicotine-induced inhibition of MSNs in the ShNAc. In fact, it is well established that URB597 elevates brain

levels of both anandamide, an endogenous CB1-receptor ligand, and also of non-cannabinoid NAEs, such as OEA and PEA, which show no affinity for CB1-receptors but are agonists at PPAR-α (Fu *et al.* 2003; Fegley *et al.* 2005). To explore the possible contributions of these two receptor systems to URB597's blockade of nicotine's effects on MSNs in the ShNAc, we treated one group of rats with both URB597 (0.1 mg/kg, i.v.) and the CB1-receptor antagonist rimonabant (SR141716A; 0.5 mg/kg, i.v.) 1–2 hours before recording and treated another group with URB597 together with the selective PPAR-α antagonist MK886 (Kehrer *et al.* 2001) (3 mg/kg, i.p., 15 minutes before URB597). Interestingly, both rimonabant and MK886 fully reversed URB597's blockade of nicotine's effects in MSNs of the ShNAc (rimonabant + URB597 versus URB597: 63.5 ± 21.8% of baseline level; $F_{(1;63)} = 10.3$, $n = 5$, $P < 0.05$, MK886 + URB597 versus URB597: 56.8 ± 16% of baseline level; $F_{(1;70)} = 5.462$, $n = 6$, $P < 0.05$, two-way ANOVA and Bonferroni's test) (Fig. 4a–c).

FAAH inhibition prevents cocaine's action on MSNs in the ShNAc

We then studied the effects of cocaine and morphine on MSN excitability in the ShNAc, and the consequences of FAAH inhibition by URB597. Cocaine (1.0 mg/kg, i.v.), in agreement with previous studies (White *et al.* 1993), depressed the excitability of MSNs of the ShNAc (37.06 ± 10.6% of baseline level; $F_{(6;48)} = 7.28$, $n = 7$, $P < 0.001$, one-way ANOVA for repeated measures and Dunnett's test) (Fig. 5a,b), as measured by their response to BLA stimulation. When we studied the effect of morphine (4.0 mg/kg, i.v.), the effects were highly variable (data not shown) and, overall, did not reach statistical significance, in line with other studies (Hakan & Henriksen 1987). For this reason, we did not further characterize the effect of URB597 on morphine-induced effects on MSNs. Pre-treatment of rats with URB597 (0.1 mg/kg, i.v., 1–2 hours before recordings) prevented cocaine-induced depression of MSNs in the ShNAc (95.3 ± 15.1% of baseline level; $F_{(1;77)} = 11.97$, $n = 6$, $P < 0.01$ versus control, two-way ANOVA and Bonferroni's test) (Fig. 5a,b).

URB597 blocks cocaine's effects in the ShNAc via a PPAR-α-dependent mechanism

Finally, we explored the mechanism by which URB597 blocks cocaine-induced inhibition of excitability of MSNs in the ShNAc. When we coadministered URB597 and the CB1-receptor antagonist rimonabant (SR141716A; 0.5 mg/kg, i.v.), we found that URB597's actions were not reversed by CB1-receptor blockade (98.34 ± 18.45% of baseline; $F_{(1;70)} = 0.04$, $n = 6$, $P > 0.05$ versus URB597-pre-treated animals, two-way ANOVA and Bonferroni's test) (Fig. 5c), suggesting that CB1-receptors were not involved. However, when we pre-treated rats with MK886 (3.0 mg/kg, i.p., 15 minutes before URB597 administration), URB597's blockade of cocaine's inhibition of MSNs was completely prevented, and cocaine exerted an inhibitory effect similar to that observed under control conditions (58.02 ± 15.59% of baseline level; $F_{(1;70)} = 7.028$, $n = 6$, $P < 0.05$ versus URB597–pre-treated animals, two-way ANOVA and Bonferroni's test) (Fig. 5d). These data suggest that endogenous PPAR-α ligands modulate the effects of cocaine in the ShNAc.

DISCUSSION

In this study, FAAH inhibition by URB597 blocked the acute inhibitory effects of both nicotine and cocaine on firing of MSNs in the ShNAc that was evoked by BLA stimulation in anesthetized rats. Pharmacological blockade of the target receptors for endogenous lipids that are the primary substrates for FAAH (CB1-receptors and PPAR-α) showed that URB597's blockade of nicotine's inhibition of MSN excitability was because of the combined activation of both surface CB1-receptor and nuclear PPAR-α, while URB597's blockade of cocaine's inhibition of MSN excitability was due only to activation of PPAR-α and did not involve CB1-receptors.

In the VTA, URB597 did not prevent the decreases in firing rate and burst firing produced by cocaine, or the increases in firing rate and burst firing produced by morphine, in DA neurons of anesthetized rats. These results extend our previous findings, where FAAH inhibition by URB597 completely abolished nicotine-induced increases in firing rate and burst firing of VTA DA neurons in anesthetized rats (Melis *et al.* 2008) and nicotine-induced neurochemical and behavioral effects in rats (Scherma *et al.* 2008). In that study, URB597 blocked nicotine-induced increases in DA levels in the ShNAc and blocked the development of nicotine self-administration and nicotine-induced conditioned place preferences (Scherma *et al.* 2008).

The present finding that nicotine-induced depression of MSN excitability requires the combined activation of D1 and D2 DA receptors suggests that DA release in the ShNAc plays a crucial role in this effect. Moreover, because URB597's blockade of nicotine's inhibition of MSN excitability in the ShNAc was reversed by either the selective CB1-receptor antagonist/inverse agonist rimonabant or by the PPAR-α antagonist MK886, the blockade of nicotine's effects by URB597 appears to involve both surface CB1-receptors and nuclear PPAR-α. Following URB597 administration, we found that nicotine became excitatory on MSNs in the ShNAc. The

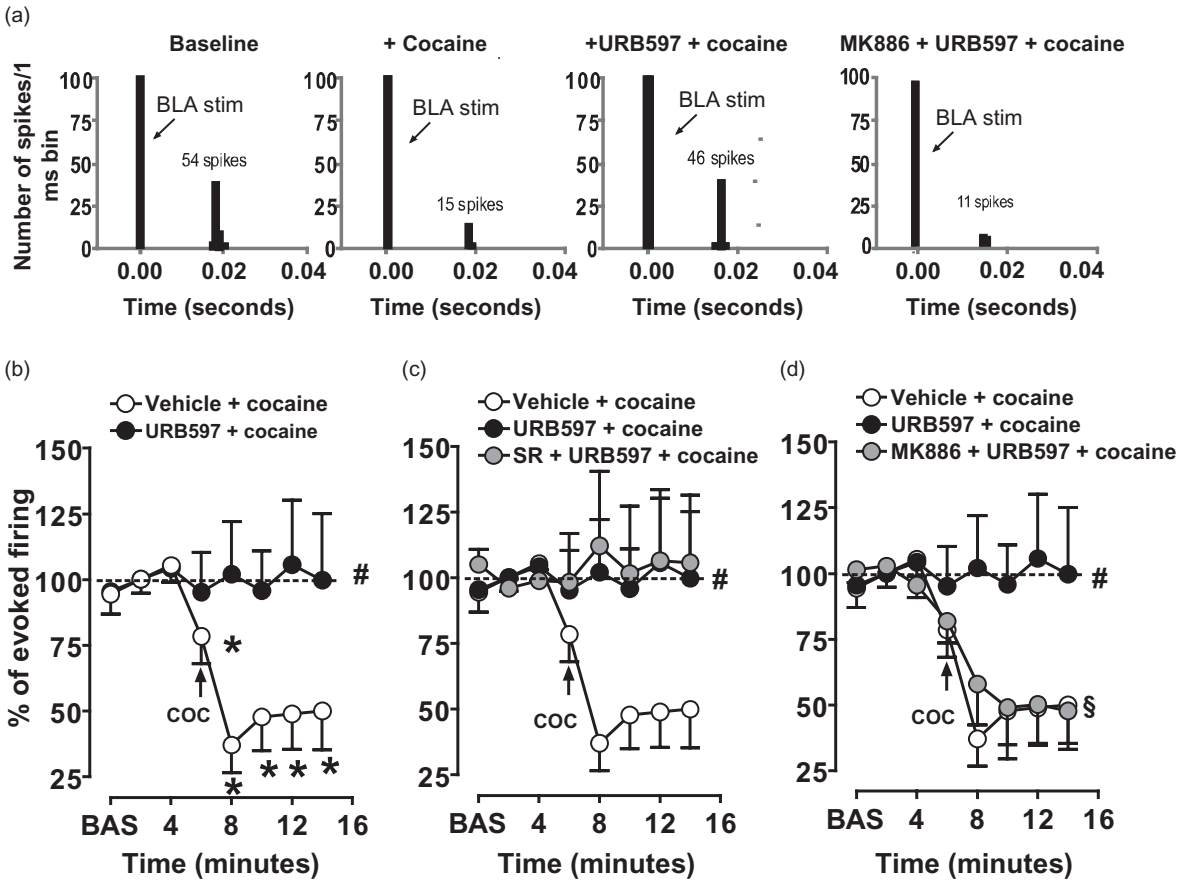


Figure 5 URB597 suppresses cocaine's action on medium spiny neurons (MSNs) of the shell of the nucleus accumbens (ShNAc). (a) Representative peristimulus time histograms showing the response of recorded ShNAc MSNs after basolateral amygdala (BLA) stimulation. The probability of evoking MSN responses after BLA stimulation decreased after cocaine administration. Pre-treatment with URB597 reversed cocaine-induced inhibition of MSNs. The peroxisome proliferator-activated nuclear receptor-α antagonist MK886 blocked URB597's effect and restored cocaine-induced inhibition of MSNs. (b–d) Graphical depictions of the time course of cocaine's effects on MSN excitability in the ShNAc. Cocaine depresses the excitability of MSNs in a long-lasting manner (b). This effect was blocked by URB597, which fully prevented cocaine-induced inhibition (b). Pre-treatment with the CB1-receptor antagonist rimonabant (SR; 0.5 mg/kg, i.v.) did not alter URB597's blockade of cocaine's actions (c), whereas MK886 (3 mg/kg, i.p.) (d) completely prevented URB597's blockade of cocaine's actions and restored cocaine-induced inhibition of MSNs. Results are means, with vertical bars representing the standard error of the mean of evoked spike firing, expressed as a percentage of the baseline (BAS). $^{*}P < 0.05$ versus baseline, one-way analysis of variance (ANOVA) and Dunnett's test; $\#P < 0.05$ versus vehicle + cocaine, $\S P < 0.05$ versus URB597 + cocaine, two-way ANOVA and Bonferroni's test

reason for this reversal of nicotine's effect is not clear. One possibility is that it might be the result of a combination of factors: (1) the depression of nicotine-induced DA release (Scherma *et al.* 2008) and (2) activation of CB1-receptors in the NAc by anandamide and depression of nicotine-induced GABA release. These effects may ultimately unmask the enhancement of glutamate release induced by nicotine (Reid *et al.* 2000) and the consequent excitation of MSNs.

Although URB597 did not affect the inhibitory actions of cocaine on VTA DA neurons, it completely prevented cocaine-induced inhibition of MSN in the ShNAc. These findings add some complexity to the controversial issue of interactions between the endocannabinoid system and the effects of cocaine or, more generally, of psychostimulants (Wiskerke *et al.* 2008). For example, it has been demonstrated that CB1-receptor knockout mice will self-administrate cocaine (Cossu *et al.* 2001) and that rimonabant does not modify the development of cocaine-induced conditioned place preference (Martin *et al.* 2000). Moreover, URB597 does not alter cocaine self-administration by squirrel monkeys under a fixed ratio schedule (Justinova *et al.* 2008). In contrast, other studies have shown that rimonabant prevents cocaine-induced increases in DA levels in the NAc (Cheer *et al.* 2007) and increases the breakpoint for cocaine self-administration under a fixed ratio schedule in rats with extended access to the drug (Orio *et al.* 2009). Thus, the present finding that URB597 blocks the inhibitory effects of cocaine on MSN

excitability in the ShNAc, although not the inhibitory effects of cocaine on DA neurons in the VTA, was unexpected, particularly because we previously found that URB597 had no effect on cocaine self-administration by squirrel monkeys (Justinova *et al.* 2008). However, besides the difference in experimental subjects (squirrel monkeys versus rats), there was a difference in the protocols of cocaine administration in these two studies. Unlike the self-administration studies in monkeys, where intravenous injections of cocaine were repeatedly self-administered during 1-hour sessions and the effects of URB597 were examined over three consecutive daily sessions, in the present experiments, the electrical activity of neurons was examined in a single brain area following a single acute intravenous injection of cocaine. It is possible that URB597's effect could impair the drug-induced acute responses of a specific neuronal population without significantly affecting behavior induced by chronic administration of the same drug. Thus, this piece of evidence might reveal that, at least for cocaine, FAAH inhibition might prevent the initial acute effects of cocaine administration. In support of this hypothesis, a recent study suggests that FAAH inhibitors do not affect cocaine self-administration but significantly reduced cocaine-induced reinstatement in abstinent animals (Adamczyk *et al.* 2009).

In the present experiments, URB597 blocked the inhibitory effect of cocaine on MSN excitability in the ShNAc through a non-CB1-receptor-dependent mechanism, because MK886, but not rimonabant, completely reversed URB597's blockade of cocaine's inhibition of MSNs in the ShNAc. This result further supports the conclusion that the effects of FAAH inhibition on the actions of addicting drugs are often because of a combination of different mechanisms, involving both surface CB1-receptors for the endocannabinoid anandamide and PPAR-α nuclear receptors for the non-cannabinoid OEA and PEA. In line with other studies, however, the lack of effect of rimonabant in the present experiments with cocaine indicates that CB1-receptors are probably not primarily involved in the acute reinforcing effects of psychostimulants (Maldonado *et al.* 2006; Wiskerke *et al.* 2008).

How PPAR-α modulated acute neuronal responses to cocaine in the present experiments is not known. Among possible explanations, a conservative hypothesis may involve a negative modulation exerted by PPAR-α agonists, such as OEA and PEA, on cholinergic transmission within the ShNAc. In fact, cholinergic interneurons of the NAc were shown to modulate the response of MSNs (de Rover *et al.* 2002). In that study, the authors hypothesized that this effect occurred through an increase of GABAergic interneuron activity within the ventral striatum. These neurons receive inputs from the cholinergic neurons mediated by nAChRs, and their synapses impinge directly to MSNs. Moreover, other studies have demonstrated an increase in acetylcholine release in the NAc after psychostimulant exposure (Guix, Hurd & Ungerstedt 1992; Imperato *et al.* 1992; Bickerdike & Abercrombie 1997). In our previous *in vitro* studies in brain slices, we identified a mechanism by which PPAR-α, activated by endogenous agonists OEA and PEA, specifically modulates nAChRs by inducing their inactivation through phosphorylation (Melis *et al.* 2008). By analogy, it is likely that PPAR-α activation within the NAc might modulate cocaine's response through inactivation of nAChRs in GABAergic interneurons. This should result in an impairment of GABA transmission to the MSNs that could explain the lack of inhibitory effect of cocaine on MSNs after URB597 pre-treatment. Interestingly, interactions between OEA and PEA, and acetylcholine transmission might be bidirectional, given that their biosynthesis is increased after stimulation of muscarinic receptors (Stella & Piomelli 2001), which are present in the terminal regions of GABAergic interneurons (de Rover *et al.* 2002).

It must be pointed out that, since all drugs were administered systemically, we were unable to determine whether the observed effects of PPAR-α agonists were because of activation of nuclear receptors within the NAc or other brain regions. However, relatively high levels of PPAR-α binding was detected in the rodent striatum (Moreno, Farioli-Vecchioli & Ceru 2004), coexpressed with tyrosine hydroxylase (Plaza-Zabala *et al.* 2009), thus a direct action of PPAR-α agonists in this brain region is likely.

Endocannabinoids have been involved in the modulation of forms of synaptic plasticity that occur in the NAc or in the VTA early after the administration of addictive substances belonging to different classes (Hoffman *et al.* 2003; Mato *et al.* 2004; Pan, Hillard & Liu 2008). It is likely that, besides 'classical' endocannabinoids, endocannabinoid-like lipid messengers might also modulate acute effects of addicting substances. Indeed, irrespective of mechanisms involved, pharmacological inhibition of FAAH might represent an opportunity to reveal how homeostatic signals, such as the endocannabinoid anandamide and the non-cannabinoid acetylethanolamides OEA and PEA, and their respective target receptors, might be recruited to regulate the effects of addicting drugs within brain reward pathways, and might represent a potential new approach to the treatment of drug addiction.

Acknowledgements

This study was supported in part by a grant from Philip Morris USA and Philip Morris International; by the

Division of Geriatric Medicine and Gerontology of Johns Hopkins University School of Medicine; and by the Intramural Research Program of the National Institute on Drug Abuse, National Institutes of Health, Department of Health and Human Services.

Authors Contribution

MP and SRG were responsible for the study concept and design. AL, SL, SC and GP carried out the experiments, and performed data analysis and interpretation of findings. AL and ALM drafted the manuscript. SY, ALM and SRG provided critical revision of the manuscript for important intellectual content. All authors critically reviewed content and approved final version submitted for publication.

References

Adamczyk P, McCreary AC, Przegalinski E, Mierzejewski P, Bienkowski P, Filip M (2009) The effects of fatty acid amide hydrolase inhibitors on maintenance of cocaine and food self-administration and on reinstatement of cocaine-seeking and food-taking behavior in rats. J Physiol Pharmacol 60:119–125.

Bickerdike MJ, Abercrombie ED (1997) Striatal acetylcholine release correlates with behavioral sensitization in rats withdrawn from chronic amphetamine. J Pharmacol Exp Ther 282:818–826.

Carlezon WA, Jr., Thomas MJ (2009) Biological substrates of reward and aversion: a nucleus accumbens activity hypothesis. Neuropharmacology 56 (Suppl. 1):122–132.

Cheer JF, Wassum KM, Sombers LA, Heien ML, Ariansen JL, Aragona BJ, Phillips PE, Wightman RM (2007) Phasic dopamine release evoked by abused substances requires cannabinoid receptor activation. J Neurosci 27:791–795.

Committee on Guidelines for the Use of Animals in Neuroscience and Behavioral Research (2003) Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research. National Research Council. Washington D.C., USA: The National Academies Press. Available from http://grants.nih.gov/grants/olaw/National_Academies_Guidelines_for_use_and_care.pdf (accessed 28 April 2010).

Cossu G, Ledent C, Fattore L, Imperato A, Bohme GA, Parmentier M, Fratta W (2001) Cannabinoid CB1 receptor knockout mice fail to self-administer morphine but not other drugs of abuse. Behav Brain Res 118:61–65.

Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB (1996) Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. Nature 384:83–87.

Cravatt BF, Lichtman AH (2002) The enzymatic inactivation of the fatty acid amide class of signaling lipids. Chem Phys Lipids 121:135–148.

de Rover M, Lodder JC, Kits KS, Schoffelmeer AN, Brussaard AB (2002) Cholinergic modulation of nucleus accumbens medium spiny neurons. Eur J Neurosci 16:2279–2290.

Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, 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 258:1946–1949.

Dinh TP, Carpenter D, Leslie FM, Freund TF, Katona I, Sensi SL, Kathuria S, Piomelli D (2002) Brain monoglyceride lipase participating in endocannabinoid inactivation. Proc Natl Acad Sci U S A 99:10819–10824.

Einhorn LC, Johansen PA, White FJ (1988) Electrophysiological effects of cocaine in the mesoaccumbens dopamine system: studies in the ventral tegmental area. J Neurosci 8:100–112.

Fegley D, Gaetani S, Duranti A, Tontini A, Mor M, Tarzia G, Piomelli D (2005) Characterization of the fatty acid amide hydrolase inhibitor cyclohexyl carbamic acid 3′-carbamoyl-biphenyl-3-yl ester (URB597): effects on anandamide and oleoylethanolamide deactivation. J Pharmacol Exp Ther 313:352–358.

Floresco SB, Blaha CD, Yang CR, Phillips AG (2001) Modulation of hippocampal and amygdalar-evoked activity of nucleus accumbens neurons by dopamine: cellular mechanisms of input selection. J Neurosci 21:2851–2860.

Forget B, Coen KM, Le Foll B (2009) Inhibition of fatty acid amide hydrolase reduces reinstatement of nicotine seeking but not break point for nicotine self-administration—comparison with CB(1) receptor blockade. Psychopharmacology (Berl) 205:613–624.

Fu J, Gaetani S, Oveisi F, Lo Verme J, Serrano A, Rodriguez De Fonseca F, Rosengarth A, Luecke H, Di Giacomo B, Tarzia G, Piomelli D (2003) Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha. Nature 425:90–93.

Grace AA, Bunney BS (1983) Intracellular and extracellular electrophysiology of nigral dopaminergic neurons—1. Identification and characterization. Neuroscience 10:301–315.

Grace AA, Bunney BS (1984) The control of firing pattern in nigral dopamine neurons: burst firing. J Neurosci 4:2877–2890.

Guix T, Hurd YL, Ungerstedt U (1992) Amphetamine enhances extracellular concentrations of dopamine and acetylcholine in dorsolateral striatum and nucleus accumbens of freely moving rats. Neurosci Lett 138:137–140.

Hakan RL, Hart C, Eyl C (1993) Specific neurophysiological effects of systemic nicotine on neurons in the nucleus accumbens. Synapse 15:191–197.

Hakan RL, Henriksen SJ (1987) Systemic opiate administration has heterogeneous effects on activity recorded from nucleus accumbens neurons in vivo. Neurosci Lett 83:307–312.

Hillard CJ, Jarrahian A (2000) The movement of N-arachidonoylethanolamine (anandamide) across cellular membranes. Chem Phys Lipids 108:123–134.

Hoffman AF, Oz M, Caulder T, Lupica CR (2003) Functional tolerance and blockade of long-term depression at synapses in the nucleus accumbens after chronic cannabinoid exposure. J Neurosci 23:4815–4820.

Imperato A, Obinu MC, Demontis MV, Gessa GL (1992) Cocaine releases limbic acetylcholine through endogenous dopamine action on D1 receptors. Eur J Pharmacol 229:265–267.

Justinova Z, Mangieri RA, Bortolato M, Chefer SI, Mukhin AG, Clapper JR, King AR, Redhi GH, Yasar S, Piomelli D, Goldberg SR (2008) Fatty acid amide hydrolase inhibition heightens anandamide signaling without producing reinforcing effects in primates. Biol Psychiatry 64:930–937.

Kano M, Ohno-Shosaku T, Hashimotodani Y, Uchigashima M, Watanabe M (2009) Endocannabinoid-mediated control of synaptic transmission. Physiol Rev 89:309–380.

Kathuria S, Gaetani S, Fegley D, Valino F, Duranti A, Tontini A, Mor M, Tarzia G, La Rana G, Calignano A, Giustino A, Tattoli

M, Palmery M, Cuomo V, Piomelli D (2003) Modulation of anxiety through blockade of anandamide hydrolysis. Nat Med 9:76–81.

Kehrer JP, Biswal SS, La E, Thuillier P, Datta K, Fischer SM, Vanden Heuvel JP (2001) Inhibition of peroxisome-proliferator-activated receptor (PPAR)alpha by MK886. Biochem J 356:899–906.

Maldonado R, Valverde O, Berrendero F (2006) Involvement of the endocannabinoid system in drug addiction. Trends Neurosci 29:225–232.

Martin M, Ledent C, Parmentier M, Maldonado R, Valverde O (2000) Cocaine, but not morphine, induces conditioned place preference and sensitization to locomotor responses in CB1 knockout mice. Eur J Neurosci 12:4038–4046.

Maskos U, Molles BE, Pons S, Besson M, Guiard BP, Guilloux JP, Evrard A, Cazala P, Cormier A, Mameli-Engvall M, Dufour N, Cloez-Tayarani I, Bemelmans AP, Mallet J, Gardier AM, David V, Faure P, Granon S, Changeux JP (2005) Nicotine reinforcement and cognition restored by targeted expression of nicotinic receptors. Nature 436:103–107.

Mato S, Chevaleyre V, Robbe D, Pazos A, Castillo PE, Manzoni OJ (2004) A single in-vivo exposure to delta 9THC blocks endocannabinoid-mediated synaptic plasticity. Nat Neurosci 7:585–586.

Matthews RT, German DC (1984) Electrophysiological evidence for excitation of rat ventral tegmental area dopamine neurons by morphine. Neuroscience 11:617–625.

Mazzola C, Medalie J, Scherma M, Panlilio LV, Solinas M, Tanda G, Drago F, Cadet JL, Goldberg SR, Yasar S (2009) Fatty acid amide hydrolase (FAAH) inhibition enhances memory acquisition through activation of PPAR-alpha nuclear receptors. Learn Mem 16:332–337.

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

Melis M, Pillolla G, Luchicchi A, Muntoni AL, Yasar S, Goldberg SR, Pistis M (2008) Endogenous fatty acid ethanolamides suppress nicotine-induced activation of mesolimbic dopamine neurons through nuclear receptors. J Neurosci 28:13985–13994.

Merritt LL, Martin BR, Walters C, Lichtman AH, Damaj MI (2008) The endogenous cannabinoid system modulates nicotine reward and dependence. J Pharmacol Exp Ther 326:483–492.

Moreno S, Farioli-Vecchioli S, Ceru MP (2004) Immunolocalization of peroxisome proliferator-activated receptors and retinoid X receptors in the adult rat CNS. Neuroscience 123:131–145.

Mulder AB, Hodenpijl MG, Lopes da Silva FH (1998) Electrophysiology of the hippocampal and amygdaloid projections to the nucleus accumbens of the rat: convergence, segregation, and interaction of inputs. J Neurosci 18:5095–5102.

Orio L, Edwards S, George O, Parsons LH, Koob GF (2009) A role for the endocannabinoid system in the increased motivation for cocaine in extended-access conditions. J Neurosci 29:4846–4857.

Pan B, Hillard CJ, Liu QS (2008) Endocannabinoid signaling mediates cocaine-induced inhibitory synaptic plasticity in midbrain dopamine neurons. J Neurosci 28:1385–1397.

Paxinos G, Watson C (1997) The Rat Brain in Stereotaxic Coordinates. 3rd edn. San Diego, CA: Academic Press.

Pistis M, Muntoni AL, Pillolla G, Gessa GL (2002) Cannabinoids inhibit excitatory inputs to neurons in the shell of the nucleus accumbens: an in vivo electrophysiological study. Eur J Neurosci 15:1795–1802.

Plaza-Zabala A, Berrendero F, Suarez J, Bermudez-Silva FJ, Fernandez-Espejo E, Serrano A, Pavon FJ, Parsons LH, De Fonseca FR, Maldonado R, Robledo P (2009) Effects of the endogenous PPAR-alpha agonist, oleoylethanolamide on MDMA-induced cognitive deficits in mice. Synapse 64:379–389.

Reid MS, Fox L, Ho LB, Berger SP (2000) Nicotine stimulation of extracellular glutamate levels in the nucleus accumbens: neuropharmacological characterization. Synapse 35:129–136.

Riegel AC, Lupica CR (2004) Independent presynaptic and postsynaptic mechanisms regulate endocannabinoid signaling at multiple synapses in the ventral tegmental area. J Neurosci 24:11070–11078.

Rodriguez de Fonseca F, Navarro M, Gomez R, Escuredo L, Nava F, Fu J, Murillo-Rodriguez E, Giuffrida A, LoVerme J, Gaetani S, Kathuria S, Gall C, Piomelli D (2001) An anorexic lipid mediator regulated by feeding. Nature 414:209–212.

Scherma M, Panlilio LV, Fadda P, Fattore L, Gamaleddin I, Le Foll B, Justinova Z, Mikics E, Haller J, Medalie J, Stroik J, Barnes C, Yasar S, Tanda G, Piomelli D, Fratta W, Goldberg SR (2008) Inhibition of anandamide hydrolysis by cyclohexyl carbamic acid 3′-carbamoyl-3-yl ester (URB597) reverses abuse-related behavioral and neurochemical effects of nicotine in rats. J Pharmacol Exp Ther 327:482–490.

Solinas M, Goldberg SR, Piomelli D (2008) The endocannabinoid system in brain reward processes. Br J Pharmacol 154:369–383.

Solinas M, Yasar S, Goldberg SR (2007) Endocannabinoid system involvement in brain reward processes related to drug abuse. Pharmacol Res 56:393–405.

Stella N, Piomelli D (2001) Receptor-dependent formation of endogenous cannabinoids in cortical neurons. Eur J Pharmacol 425:189–196.

Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, Yamashita A, Waku K (1995) 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. Biochem Biophys Res Commun 215:89–97.

Ungless MA, Magill PJ, Bolam JP (2004) Uniform inhibition of dopamine neurons in the ventral tegmental area by aversive stimuli. Science 303:2040–2042.

White FJ, Hu XT, Henry DJ (1993) Electrophysiological effects of cocaine in the rat nucleus accumbens: microiontophoretic studies. J Pharmacol Exp Ther 266:1075–1084.

Wiskerke J, Pattij T, Schoffelmeer AN, De Vries TJ (2008) The role of CB1 receptors in psychostimulant addiction. Addict Biol 13:225–238.

