Università degli Studi di Cagliari

DOTTORATO DI RICERCA

IN NEUROSCIENZE E SCIENZE MORFOLOGICHE

CORSO DI DOTTORATO IN NEUROSCIENZE

Ciclo XXIII

FATTY ACID ETHANOLAMIDES PINPOINT NICOTINIC RECEPTORS AND MODULATE NEURONAL EXCITABILITY THROUGH NUCLEAR RECEPTORS PPARα

Settore/i scientifico disciplinari di afferenza

BIO/14

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Esame finale anno accademico 2009 - 2010

This thesis is based on the following articles:

I. 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.

II. 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.

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

INTRODUCTION

Lipid homeostasis is of particular interest due to high concentration in central nervous system (CNS) second only to adipose tissue. The importance of lipid molecules in cell signalling and tissue physiology is demonstrated by many CNS disorders and injuries that involve their deregulated metabolism (Wenk, 2005; Adibhatla and Hatcher, 2007). Accordingly, lipid imbalances are associated with both neurological disorders (e.g. Alzheimer's Disease, Parkinson's Disease, Multiple Sclerosis, Huntington's Disease, Amyotrophic Lateral Sclerosis, Schizophrenia, Bipolar Disorders and Epilepsy) and CNS injury (e.g. stroke, traumatic brain injury, and spinal cord injury) (Fahy et al., 2005; Adibhatla and Hatcher, 2007).

Phospholipids are important components of all mammalian cells with discrete biological functions ranging from formation of membrane bilayers to be precursors of diverse second messengers as well as unconventional neurotransmitters. Modifications in phospholipids, fatty acid composition and cholesterol content alter membrane fluidity and affect a number of cellular functions. It is, therefore, conceivable that changes in dietary lipids can influence membrane composition and may bear therapeutic potential in diverse disorders, including CNS disorders (Clandinin et al., 1983; Confaloni et al., 1988).

Lipids comprise a large number of chemically different molecules arising from combinations of fatty acids with various backbone structures (Wenk, 2005; Adibhatla and Hatcher, 2007) and are classified into eight categories: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides (Fahy et al., 2005; Adibhatla and Hatcher, 2007). Fatty acid ethanolamides (FAEs) are comprised in the subtype of the fatty acyls.

The present thesis aims to extend our knowledge on the role played by lipid molecules such as FAEs in physiological functions, and to broaden their activity from the periphery to the CNS.

1.1 Fatty acid ethanolamides

FAEs are amides of long-chain fatty acids with ethanolamine (Figure 1). FAEs are a family of naturally occurring lipid signalling molecules, which include the endogenous cannabinoid arachidonoylethanolamide (anandamide), the anorexic mediator oleoylethanolamide (OEA), and the analgesic and anti-inflammatory mediator palmitoylethanolamide (PEA) (Devane et al., 1992; Di Marzo et al., 1994; Calignano et al., 2001). Along with these, stearoylethanolamide and linoleoylethanolamide are the most abundant FAEs in animal tissues with anandamide being a

minor component in comparison with the four above-mentioned FAEs (Hansen et al., 2001; Hansen and Diep, 2009).

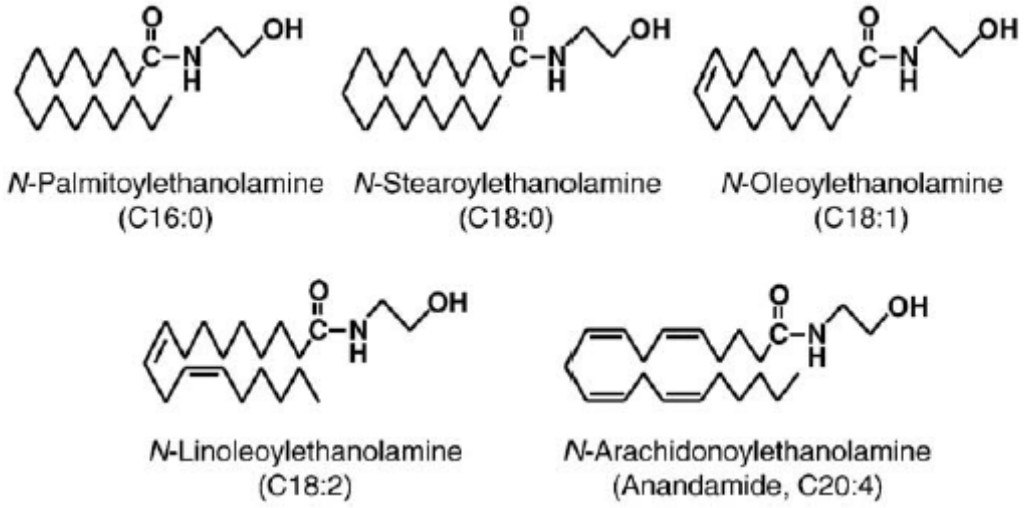

Figure 1. Chemical structures of FAEs.

The study of their pharmacological activity started in the mid-1950s when the anti-inflammatory activity of PEA was discovered in peanut oil, soybean lecithin and egg yolk (Coburn et al., 1954; Long and Martin, 1956). However, the research had significantly slowed down over the years until anandamide (AEA) was identified as an endogenous compound specifically binding to and activating type-1 cannabinoid (CB1) receptors in 1992 (Devane et al., 1992). Subsequently, a second type of CB receptor (CB2), which is expressed mainly in immune tissues and cells, was identified (Showalter et al., 1996). Because of the widespread distribution of CB receptors, their endogenous ligands (i.e. endocannabinoids) display a wide array of functions ranging from pain perception to energy metabolism and neuroprotection (Basavarajappa et al., 2009; de Kloet and Woods, 2009; Richard et al., 2009). Subsequently, it was demonstrated not only that mammalian cells produce a wide range of FAEs besides AEA, but also that FAEs other than AEA display cannabinoid-like activity (Bachur et al., 1965; Sheskin et al., 1997). However, although many of cannabinoid actions are ascribed to activation of CB receptors, studies carried out in knock-out mice have disproved the involvement of these receptors *solo*, thus suggesting the existence of CB receptors other than CB1 and CB2 (Wiley and Martin, 2002) and, therefore, the possibility of new targets for these lipid signalling molecules (Howlett et al., 2004; Pistis and Melis, 2010). Indeed, a certain degree of promiscuity has been ascribed to AEA and some other endocannabinoids, which also bind to other receptors, such as transient receptor potential vanilloid type 1 (TRPV1), and

nuclear receptor peroxisome proliferator-activated receptor-alpha (PPARα) and -gamma (PPARγ) (O'Sullivan, 2007; Starowicz et al., 2007; Sun and Bennett, 2007; Starowicz et al., 2008; Borrelli and Izzo, 2009; Pistis and Melis, 2010).

FAEs are otherwise termed *N*-acylethanolamines (NAEs), which closely relate them to their progenitor N-acyl- phosphatidylethanolamine (NAPE). The formation of NAPE is catalysed by *N*-acyltransferase, a ubiquitous Ca2+-activated enzyme, which transfers the Sn-1 fatty acid to phosphatidylethanolamine from a donor phospholipid (Hansen et al., 2000; Hansen and Diep, 2009). *N*-acyltransferase is not specific for any fatty acids, since it is supposed to catalyse the transfer of any acyl group from the Sn-1 position of donor phospholipids. NAPE, in turn, is converted to NAEs, including AEA, by *N*-acyl-phosphatidylethanolamine-selective phospholipase D (NAPE-PLD) (Okamoto et al., 2004).

NAPE-PLD is expressed in many tissues, including the brain (Morishita et al., 2005; Cristino et al., 2008; Egertova et al., 2008; Suarez et al., 2008). NAPE-PLD cellular localization is particularly remarkable, and it might explain the functional significance of FAEs within the CNS. Indeed, postsynaptic localization of NAPE-PLD might suggest that FAEs may be an autocrine signal acting retrogradely at presynaptic CB1 receptors, whereas the axonal localization might imply that synthesized FAEs may target postsynaptic neurons and modulate synaptic strength. Accordingly, a role for FAEs in the modulation of cognitive processes was recently explored, where the role played by PPARα was pointed out (Mazzola et al., 2009).

FAEs hydrolysis is catalyzed by three enzymes: fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996), FAAH-2 (Wei et al., 2006), and NAE-hydrolyzing acid amidase (NAAA) (Tsuboi et al., 2005). FAAH hydrolyzes all FAEs with high efficiency, and it is expressed in many different tissues and cell types (e.g. brain, small intestine and testis). On the contrary, FAAH-2 is found in multiple primate genomes, marsupials, and other vertebrates, but not in mouse and rat (Wei et al., 2006). NAAA is found in lung, spleen, thymus, and intestine, whose subcellular location is in the lysosomes. Importantly, NAAA hydrolyzes PEA to much greater extent than AEA (Tsuboi et al., 2007).

Since FAAH catalyzes all FAEs, its pharmacological blockade (e.g. by URB597) and genetic deletion not only enhance FAE levels (Cravatt et al., 2001), but also amplify endocannabinoid effects (Kathuria et al., 2003; Fegley et al., 2005) mediated by diverse receptors other than CB receptors (Jhaveri et al., 2008; Melis et al., 2008; Sagar et al., 2008; Mazzola et al., 2009). Noteworthy, the roles played by OEA and PEA are often opposite to those exerted by AEA and, more generally, through activation of CB receptors. Thus, nowadays many of the effects of either pharmacological blockade (e.g. URB597) or genetic deletion of the FAAH are seen both in the

periphery and CNS as the outcome of ups and downs of diverse receptor-mediated pathways, which result in/from an interplay between these parallel endogenous systems where small lipid molecules co-star.

Interestingly, elevation of FAEs through pharmacological inhibition of FAAH (URB597) prevents nicotine-induced elevation of extracellular dopamine levels in the shell of the nucleus Accumbens, development of nicotine-induced conditioned place preference, and acquisition of nicotine self-administration (Scherma et al., 2008). Additionally, URB597 was proved to prevent reinstatement of nicotine seeking (Forget et al., 2009), thus suggesting that targeting FAAH may be a novel strategy to prevent relapse for tobacco smoking.

Tobacco addictive properties are thought to be mediated by activation of midbrain dopamine cells by its active ingredient nicotine (Mereu et al., 1987; Pidoplichko et al., 1997; Balfour, 2004; Changeux, 2010). Acutely, stimulation of mesolimbic DA transmission is considered as a hallmark of all drugs of abuse (Volkow et al., 2004) and nicotine makes no exception to this rule (Pidoplichko et al., 1997). The molecular mechanisms underlying nicotine-induced excitation of midbrain dopamine neurons were begun to be identified in 1997 (Pidoplichko et al.). Shortly after, the intimate knowledge of the nicotinic acetylcholine receptor subunits key in such an effect was discovered through deletion of these latter in transgenic mice (Picciotto et al., 1998). Ever since, nicotine actions have been dissected by examining the role of nAChRs on behaviours such as reward, cognition and locomotion (Picciotto and Corrigall, 2002; Mineur and Picciotto, 2008; Brunzell and Picciotto, 2009; Changeux, 2010).

However, how increased FAE levels might prevent nicotine behavioral effects is still an unsolved issue.

1.2 Nicotinic Acetylcholine Receptors

The nicotinic acetylcholine receptor (nAChR) is the first membrane receptor of a neurotransmitter and ion channel characterized as a protein (Changeux et al., 1970), whose biochemical isolation represents a landmark in the history of pharmacology.

nAChRs are cholinergic receptors forming ligand-gated ion channels in the plasma membranes of neurons and on the postsynaptic side of the neuromuscular junction. Being ionotropic receptors, nAChRs are directly linked to an ion channel (Itier and Bertrand, 2001).

nAChR are present in many tissues and are the best-studied of the ionotropic receptors (Itier and Bertrand, 2001). nAChRs are made up of five subunits, arranged symmetrically around a central pore (Itier and Bertrand, 2001), and are generally classified into two subtypes based on their

expression: muscle-type and neuronal-type nicotinic receptors. Because muscle-type nAChRs have not been the subject of the present thesis, I here focus on the neuronal-type nAChRs.

The neuronal subtypes are various homomeric or heteromeric combinations of twelve different nAChR subunits: α2-α10 and β2-β4. Examples of the neuronal subtypes include the homomeric $(\alpha7)_5$ and the heteromeric $(\alpha4)_3(\beta2)_2$, $(\alpha4)_2(\beta2)_3$ (Figure 2). Homomeric nAChRs show less affinity for agonists than those containing α4β2 subunits, but they desensitize more rapidly than these latter (Changeux, 2010).

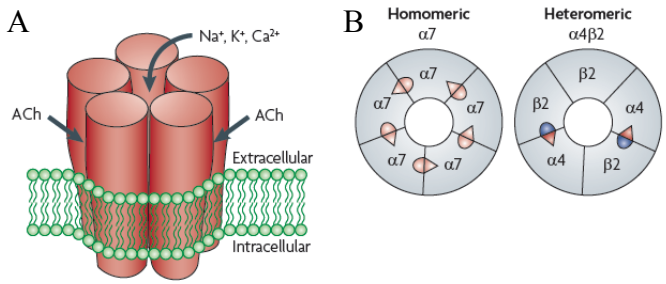

Figure 2. Structure of nicotinic acetylcholine receptors. (**A**) Nicotinic acetylcholine receptors (nAChRs) are transmembrane oligomers consisting of five subunits. (**B**) The two main types of brain nAChRs are the α7 homo-oligomer, characterized by a fast activation, a low affinity and a high calcium permeability, and the α4β2 hetero-oligomer, featured by a high affinity and slow desensitization (Taly et al., 2005; Taly et al., 2009).

Similarly to the other ligand-gated ion channels, the opening of nAChR channel pore requires the binding of the ligand, that is acetylcholine (ACh). Other agonists of the nAChR are nicotine, epibatidine, and choline. The dynamic behavior of this ion channel can be regarded as the independent combination of conformational changes (i.e. gating and desensitization) and two ligand-binding steps in the form of cyclic reaction schemes in the context of a two-state model (Monod et al., 1965; Karlin, 1967; Edelstein and Changeux, 1998) (Figure 3).

Upon binding ACh, the nAChR ion channel is stabilized in the open conformation for several milliseconds, then the open pore of the receptor/channel closes to a resting state or closes to a desensitized state that is unresponsive to ACh or other agonists for many milliseconds or more (Dani, 2001). Consistent with its physiological role, the closed ⇌ open reaction (gating) of the diliganded nAChR is much more favorable (Salamone et al., 1999) than the one of the unliganded receptor (Jackson, 1986; Grosman and Auerbach, 2000).

The affinity of the nAChR for ACh is, therefore, higher in the open than in the closed state. The rate at which nAChRs proceed through the various conformational states as well as the selectivity with which they conduct cations in the open state depend on many factors, including the subunit composition (Dani, 2001). As a result, the extensive nAChR diversity produces many different

responses to agonists (Dani, 2001): particularly, speed of activation, intensity of membrane depolarization, size of the ionic signal, desensitization rate, recovery from desensitization, and pharmacology, they all depend on the subunit composition of nAChRs as well as other local factors.

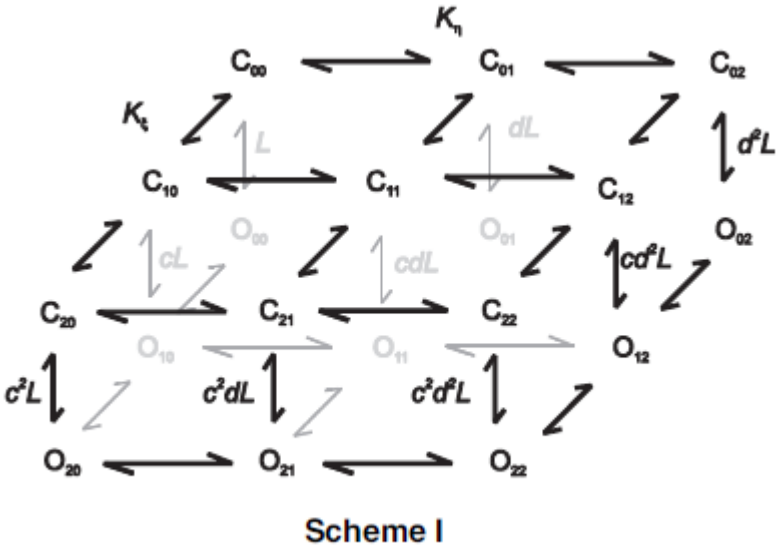

Figure 3. The Wyman Monod Changeux (WMC) model describes allosteric transitions of proteins made up of identical subunits. It was proposed by Jean-Pierre Changeux, and described by Jacques Monod, Jeffries Wyman, and Jean-Pierre Changeux himself. It stands in opposition to the sequential model (Tschudy and Bonkowsky, 1973).
The main idea of the model is that regulated proteins, such as enzymes and receptors, exist in different interconvertible states *in the absence of any regulator*. The ratio of the different conformational states is determined by thermal equilibrium. The regulators merely shift the equilibrium toward one state or another, that is to say an agonist will stabilize the active form of a receptor. Phenomenologically, it looks as if the agonist *provokes* the conformational transition. One crucial feature of the WMC model is the dissociation between the binding function (the fraction of protein bound to the regulator), and the state function (the fraction of protein under the activated state).
In the historical WMC model, each allosteric unit, called a protomer (generally assumed to be a subunit), can exist in two different conformational states - designated 'O' (for open) or 'C' (for closed) states. In any one molecule, all protomers must be in the same state. That is to say, all subunits must be in either the O or the C state. This model does not predict proteins with subunits in different states. The O state has a higher affinity for the ligand than the C state. Because of that, although the ligand may bind to the subunit when it is in either state, the binding of a ligand will increase the equilibrium in favor of the O state.

When an agonist binds to the site, all subunits undergo a conformational change, the channel opens and positively charged ions move across it; particularly, sodium and potassium enter the cell (some subunit combinations are also permeable to calcium) resulting in a net inward flow of positively-charged ions (Itier and Bertrand, 2001). Noteworthy, since some nAChRs are permeable to calcium, they can affect the release of other neurotransmitters (Itier and Bertrand, 2001).

The activation of nAChRs modifies neuronal state through two main mechanisms. First, the net flow of positively-charged ion depolarizes the membrane, resulting in an excitatory postsynaptic

potential, and activates voltage-gated ion channels. Secondly, calcium entry acts either directly or indirectly on discrete intracellular cascades with consequent regulation of the activity of some genes and/or the release of neurotransmitters.

nAChRs are regulated by a phenomenon described by Katz and Thesleff (1957) as ligand-bound desensitisation. This was defined as a decreased responsiveness of the receptor toward a stimulus resulting from prolonged or repeated exposure to the stimulus itself. As already mentioned, kinetic parameters including desensitization depend on the subunit composition, and modulatory processes, such as protein kinases, influence nAChRs subtypes differently and selectively (Huganir and Greengard, 1983; Safran et al., 1987; Pitchford et al., 1992; Paradiso and Brehm, 1998; Fenster et al., 1999c; Fenster et al., 1999b; Fenster et al., 1999a; Charpantier et al., 2005). Notably, desensitised receptors can revert back to a prolonged open state when an agonist is bound in the presence of a positive allosteric modulator (Hurst et al., 2005). Importantly, receptor desensitisation has also been modelled in the context of a two-state mathematical model, that is the WMC model.

To elucidate the contribution of different nAChR subunits to nicotine actions, new strategies have been developed over the years. These include deletions (Picciotto et al., 1995; Picciotto et al., 1998) of almost all known nAChR subunit genes (Greenbaum and Lerer, 2009), and targeted knock-in gene mutations yielding gain-of-function receptor subunits (Orr-Urtreger et al., 2000; Tapper et al., 2004; Klaassen et al., 2006; Zhao-Shea et al., 2010). Re-expression of a deleted gene either using inducible transgenic expression systems (King et al., 2003) or by stereotaxically injecting a lentiviral vector carrying the missing gene (Maskos et al., 2005; Avale et al., 2008; Tolu et al., 2009) or the relevant small interfering RNA (le Novere et al., 1999; Avale et al., 2008) has also been recently developed. Hence, these mutant animals provide the tools to analyze the relative contribution of diverse nAChR subunits to both neuronal firing patterns and behaviours elicited by nicotine (Mameli-Engvall et al., 2006; Maubourguet et al., 2008; Changeux, 2010). Ultimately, these studies provide the key to understand function and dysfunction of nicotinic systems, and how they are significant for brain diseases such as addiction, schizophrenia, Alzheimer's disease, Parkinson’s disease, Tourette's syndrome, and ADHD. In fact, ACh through activation of nAChRs increases arousal, heightens attention, influences REM sleep, produces euphoria, decreases fatigue and anxiety, acts as an analgesic, and influences a number of cognitive functions (Levin, 1992; Everitt and Robbins, 1997; Adler et al., 1999; Marubio et al., 1999). Particularly, ACh, by increasing the signal-to-noise ratio and by helping to evaluate the significance and relevance of stimuli, affects discriminatory processes.

Although cholinergic neurons are widely distributed, we can identify two major cholinergic projections. The first one arises from neurons in the pedunculopontine tegmentum and the laterodorsal pontine tegmentum, thus providing a widespread innervation mainly to the thalamus and midbrain, and also descending innervation reaching the brainstem. The second projection arises in the basal forebrain and makes broad projections throughout the cortex and hippocampus. Since a relatively small number of cholinergic neurons make spare projections, which can reach very large areas, the activity of few cholinergic neurons can influence relatively large neuronal structures (Dani, 2001). Importantly, ACh controls dopamine (DA) neuron firing pattern and frequency through activation of nAChRs (Mameli-Engvall et al., 2006), and an imbalance between DA/ACh function is often associated in diverse brain disorders (Garcia-Rill et al., 1995; Sarter et al., 2006; Avena, 2007; Maskos, 2008; McClernon and Kollins, 2008; Zhao-Shea et al., 2010).

1.3 Dopamine systems

Dopamine (DA) is one of many neurotransmitters found in the CNS, as well as one of the most heavily examined brain chemicals of the past decades. Because of its roles in a part of the brain keenly sensitive to relationships (i.e. pair bonding), in the regulation of mood and affect, as well as in appetitive motivation, learning and reward processes (both natural and maladaptive), the mesolimbic DA pathway has received special attention from neuropsychopharmacologists (Wise, 2004; Young and Wang, 2004).

This circuitry is stimulated in both rewarding interactions such as maternal bonding, mating, social affiliation and cooperation, and aversive experiences such as exposure to aggression or rejection. As a result, when this system works normally one can perceive feelings of pleasure and well-being, while its impairment will cause displacement of these latter feelings with anxiety, anger, low self-esteem, and/or other "bad feelings" (Comings et al., 1996). It has been suggested that such abnormalities in mesolimbic DA transmission might eventually lead to cravings for drugs and/or behaviors aimed at masking or relieving those “bad feelings” such as binge eating, drug abuse or to other addictive behaviors such as compulsive gambling, compulsive sex, or risk taking activities (Green et al., 1999; Comings and Blum, 2000; Blum et al., 2008a; Blum et al., 2008b).

Because the mesolimbic system is associated with feelings of reward and desire, this pathway is profoundly implicated in the neurobiological hypothesis of addiction, schizophrenia, depression and ADHD (Melis et al., 2005; Laviolette, 2007; Friedman et al., 2008; Pitchot et al., 2008; Koob and Volkow, 2010). Particularly, drug addiction is envisaged as the loss of control over drug use or the

compulsive seeking and taking of drugs despite adverse consequences (Baler and Volkow, 2006). Similarly to drug addiction, schizophrenia and depression show similar structural changes within dopaminergic circuitry (Laviolette, 2007; Pitchot et al., 2008). Lastly, evidence from brain imaging studies have shown that brain DA neurotransmission is disrupted in ADHD (Ernst et al., 1999; Lou et al., 2004; Volkow et al., 2007b; Volkow et al., 2007a; Volkow et al., 2009), thus suggesting that these deficits may underlie the core symptoms of inattention (Volkow et al., 2007a) and impulsivity (Rosa Neto et al., 2002). Animal models of these brain disorders have greatly aided our understanding of the mechanisms that regulate affect and emotions, in particular for evolutionarily conserved states such as anxiety and fear. In fact, there is an intriguing overlap between the brain areas involved in addiction and those associated with mood and anxiety disorders. Additionally, DA-related reward regions of the mammal brain, which are active when reward is perceived, show similar activation patterns in adaptive or maladaptive decision-making in human beings (Linnet et al., 2009).

The mesolimbic DA system originates in the ventral tegmental area (VTA) where DA cell bodies projecting to limbic subcortical areas (i.e., NAcc, amygdala, and olfactory tubercle) and to limbic cortices (i.e., medial prefrontal, cingulated, and entorhinal) constitute the mesolimbocortical system (Anden et al., 1966; Ungerstedt, 1971; Lindvall et al., 1974; Bjorklund and Lindvall, 1975; Loughlin and Fallon, 1983) (Figure 4).

This system has been extensively characterized by means of electrophysiological techniques both *in vivo* (Bunney et al., 1973; Bunney and Aghajanian, 1977) and *in vitro* (Grace and Onn, 1989; Lacey et al., 1989; Johnson and North, 1992a, b).

In vivo, VTA DA neurons display a typical firing pattern that is either single spiking or consisting of bursts of action potentials (Bunney et al., 1973; Grace and Bunney, 1984a,b). The bursting mode has been shown to be more efficient in increasing DA outflow in the terminal regions than the single-spike firing mode (Gonon and Buda, 1985; Gonon, 1988; Bean and Roth, 1991; Overton and Clark, 1997); therefore, it might mediate synaptic changes and contribute to reward-related learning processes (Gonon, 1988; Schultz, 1997; Reynolds et al., 2001; Reynolds and Wickens, 2002; Wightman and Robinson, 2002).

In vitro, VTA DA cells are mainly characterized by a regular (pacemaker-like), single-spike spontaneous firing (Johnson and North, 1992a; Lacey, 1993), presumably because of the loss of extrinsic afferents impinging on these neurons. Under these conditions, VTA DA cells can also display burst activity when N-methyl-d-aspartate (NMDA) (Johnson et al., 1992) and the small-conductance Calcium-dependent potassium channel (SK) blocker, apamin, are applied (Seutin et al., 1993). Otherwise, DA cells can switch from the pacemaker-like firing to a bursting mode when

group I mGluRs are activated and SK reduced (Mercuri et al., 1993; Prisco et al., 2002; Zheng and Johnson, 2002). Both types of patterns might bear relevance in information coding and ultimately result in the translation of the glutamatergic signal into the DAergic one onto their target neurons in the forebrain.

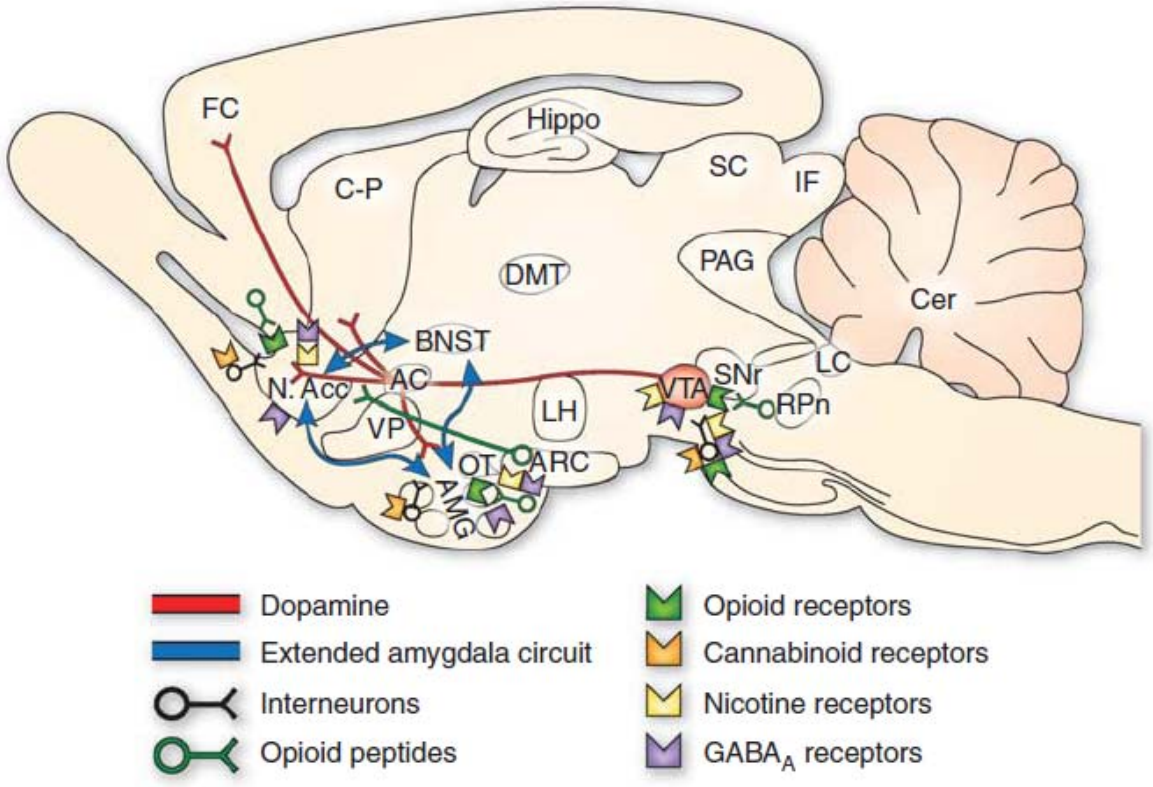

Figure 4. Sagittal section through a representative rodent brain illustrating the pathways and receptor systems implicated in the acute reinforcing actions of drugs of abuse. The blue arrows represent the interactions within the extended amygdala system hypothesized to have a key function in drug reinforcement. The medial forebrain bundle represents ascending and descending projections between the ventral forebrain (nucleus accumbens, olfactory tubercle, septal area) and the ventral midbrain (VTA) (Koob and Volkow, 2010).
AC, anterior commissure; AMG, amygdala; ARC, arcuate nucleus; BNST, bed nucleus of the stria terminalis; Cer, cerebellum; C-P, caudate-putamen; DMT, dorsomedial thalamus; FC, frontal cortex; Hippo, hippocampus; IF, inferior colliculus; LC, locus coeruleus; LH, lateral hypothalamus; N Acc., nucleus accumbens; OT, olfactory tract; PAG, periaqueductal gray; RPn, reticular pontine nucleus; SC, superior colliculus; SNr, substantia nigra pars reticulata; VP, ventral pallidum; VTA, ventral tegmental area.

The striking differences between the characteristics of VTA DA cells recorded *in vivo* and *in vitro* reveal the weight of the inputs on the control of both the spontaneous activity of these neurons (Johnson and North, 1992a) and the somatodendritic DA release (Chen and Rice, 2002), which contributes to the regulation of the burst firing through a network feedback mechanism (Paladini et al., 2003). VTA DA cells possess an additional self-regulatory mechanism that involves the endocannabinoids, retrograde messengers that earned the *status* of unconventional neurotransmitters (Freund et al., 2003). In fact, VTA DA cells release endocannabinoids in an

activity-dependent manner, which depress glutamatergic afferents on mesolimbic DA cells (Melis et al., 2004a) and ultimately their own firing activity and pattern (Melis et al., 2004b). The afferent inputs to VTA DA neurons comprise glutamatergic (Christie et al., 1985; Sesack and Pickel, 1992; Carr and Sesack, 2000; Geisler et al., 2007), GABAergic (Waddington and Cross, 1978), cholinergic (Semba and Fibiger, 1992; Oakman et al., 1995; Garzon et al., 1999), serotonergic (Herve et al., 1987), and noradrenergic fibers (Mejias-Aponte et al., 2009; Sara, 2009). In such a complex picture, changes of the amount of excitatory and inhibitory inputs onto the DA neuron influence neuronal excitability and, therefore, the behavioral actions of DA itself (Melis et al., 2005). Because changes in DA cell activity are reflected in the dynamics of micro-circuits generating altered responses to stimuli/inputs, factors regulating their state are fundamental. Among these, endogenous ligands to the nuclear receptor-transcription factor peroxisome proliferator-activated receptors type-alpha (PPARα) have been recently shown to suppress nicotine-induced responses of midbrain DA neurons *in vivo*.

1.4 Peroxisome Proliferator-Activated Receptors (PPAR)

Nuclear receptor transcription factors peroxisome proliferator-activated receptors (PPARs) have long been known for controlling the expression of genes related to lipid and glucose homeostasis as well as inflammatory responses (Feige et al., 2006; Michalik et al., 2006; Michalik and Wahli, 2006; Bensinger and Tontonoz, 2008; Michalik and Wahli, 2008).

Derangement of PPAR pathways promotes diseases, such as obesity, type 2 diabetes, cardiovascular diseases, cancer, neurodegenerative diseases, hypertension, and chronic inflammation (Michalik et al., 2006; Michalik and Wahli, 2006; Heneka and Landreth, 2007; Heneka et al., 2007; Bensinger and Tontonoz, 2008; Michalik and Wahli, 2008; Aleshin et al., 2009). As a result, PPARs and their downstream pathways are under intensive investigation.

FAEs as well as unsaturated fatty acids are endogenous ligands of PPARs (Michalik et al., 2006). Three isoforms, namely type α, β/δ, and γ, have been described so far (Michalik et al., 2006). Despite their differences in function and distribution, PPARs share the same general structure and molecular mechanism of action (Ferre, 2004). Being members of the nuclear receptor supergene family, their most recognized mechanism of action is regulation of gene transcription (Berger and Moller, 2002). Hence, upon ligand binding, PPARs undergo conformational changes, which promote dissociation of corepressor proteins (which possess histone deacetylation activity), and enable the association of coactivators and coactivator proteins. Subsequently, PPARs form heterodimers with the retinoid X receptor (RXR) and subsequently bind to PPAR response elements

(PPRE) in the regulatory region of target genes (Michalik et al., 2006; Ricote and Glass, 2007). The ultimate result is either an increased or decreased gene transcription, and this mainly depends on the target gene. The above mentioned mechanism represents the most recognized one, hence termed "classical" mechanism. Nonetheless, the very picture of PPAR mechanism of action is composite with several variations of this scenario (Gelman and Auwerx, 1999; Gelman et al., 1999; Escher and Wahli, 2000; Kersten et al., 2000). For example, PPARs can interact with other nuclear receptors, including the thyroid hormone receptor (Feige and Auwerx, 2007), and form non-RXR heterodimers. Alternatively, PPARs participate in the regulation of gene expression in a DNA-binding independent manner. Further, being PPAR a phosphoprotein, whose activity is regulated by phosphorylation in addition to ligand binding, several kinase pathways can affect PPAR activity (Zhang et al., 1996a; Zhang et al., 1996b; Camp and Tafuri, 1997; Chan et al., 2001; Hsi et al., 2001).

Similarly to the other nuclear receptors, PPAR function can be significantly modified by a number of co-activator and co-repressor proteins (Yu and Reddy, 2007). Nowadays, over 200 nuclear receptor cofactors have been discovered for which the functional significance is not clear yet. However, emerging evidence points to key role played by co-regulators in affecting the function of many nuclear receptors including PPARs (McKenna and O'Malley, 2002b, a; Lonard and O'Malley, 2006). Particularly, the binding of co-repressors to nuclear receptors occurs in the unliganded state, and can be stabilized by antagonists. Hence, a ternary complex is formed by PPARα ligand-binding domain bound to the PPARα antagonist and a SMRT co-repressor motif. Consequently, the co-repressor motif adopts changes (i.e. a three-turn α-helix) preventing the receptor from assuming the active conformation (Xu et al., 2002). As a result, these accessory molecules confer to nuclear receptors, including PPARs, a considerable functional flexibility.

Noteworthy, non-genomic actions have also been recently ascribed to PPARs (Gardner et al., 2005; Melis et al., 2008; Ropero et al., 2009). In this scenario, their location is within the cytosol, where peroxisome proliferators bind to PPARs with a consequent conformational change. A non-genomic mechanism would explain the rapid actions observed, which involve signaling pathways similar to those described for many other nuclear receptor ligands (Losel and Wehling, 2003; Losel et al., 2003; Moraes et al., 2007). The hypothesis of a non-genomic mechanism is substantiated by several observations. First, PPARs have been found in human platelets (Akbiyik et al., 2004; Ali et al., 2006), which are anucleated cells and, therefore, represent an excellent experimental model system to study this kind of interactions. Second, PPAR effects are rapid in onset, since occur in a short time scale (2-5 min) (Melis et al., 2008; Ropero et al., 2009; Melis et al., 2010), thus ruling out the possibility of a genomic mechanism of action. Additionally, PPAR activation induced production of

cytosolic effectors, such as reactive oxygen species, and, importantly, PPAR actions were still occurring in the presence of protein and mRNA synthesis inhibitors (Ropero et al., 2009; Melis et al., 2010). Lastly, PPAR effects were abolished by the general inhibitor of tyrosine kinases (i.e. genistein), thus suggesting that PPARs exert their effects through activation of a tyrosine kinase (Melis et al., 2008).

Remarkably, an interplay between PPAR isotypes has been suggested when PPARβ/δ activation resulted in repression of PPARγ- and PPARα-mediated activation of target gene expression (Shi et al., 2002), and when PPARβ/δ -dependent PPARγ-activation was demonstrated (Consilvio et al., 2004). These results indicate that a functional cross-talk between PPARs exists concerning the control of their expression levels. Noteworthy, PPARs can also exert a dichotomous control of diverse phospholipase A(2) isotypes -important players of astrocyte responses to various proinflammatory substances- depending on the conditions: particularly, this control appears to be opposite under physiological rather than pathophysiological circumstances (Sergeeva et al., 2010).

As a result, the general picture emerging from these multiple mechanisms of action and interaction indicates PPARs as metabolic switches, which convergently regulate metabolic pathways through their pleiotropic interactions with nuclear receptors, other transcription factors and surface expressed receptors. Unraveling the mechanisms through which PPARs exert such a powerful control is, therefore, compelling.

2. SPECIFIC AIMS OF THE STUDY

1) To investigate whether FAEs can suppress nicotine-induced stimulation of VTA dopamine (DA) neuron firing rate through the peroxisome-proliferator-activated receptor-α (PPARα) and to elucidate their mechanism of action in an in vitro brain slice preparation.

2) To determine whether there is an interaction between PPARα and nAChRs in VTA DA cells: particularly, to characterize the postsynaptic effects of PPARα modulation on VTA DA neurons, and to examine the contribution of nAChRs in the effects.

3) To study whether PPARα modulation of nAChRs in VTA DA neurons might confer DA cells to access distinct firing patterns and/or to change their firing frequency both in vivo and in vitro.

4) To examine the physiological relevance of PPARα modulation of nAChR stimulation through behavioral analysis.

3. MATERIALS AND METHODS

3.1 Animals

All procedures were performed in accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2004) and EEC Council Directive (219/1990 and 220/1990) and approved by Animalerie centrale and Médecine du travail, Institut Pasteur. We made all efforts to minimize pain and suffering, and to reduce the number of animals used. Animals were housed in groups of three to six in standard conditions of temperature and humidity under a 12h/12h light/dark cycle (with lights on at 7.00 am) with food and water available *ad libitum.*

Male Sprague Dawley rats (Harlan Nossan, San Pietro al Natisone, Italy) at different ages were used for electrophysiologycal experiments: 14-21 and 21-46 d old for whole cell patch clamp, and evoked field potential recordings, respectively. Rats (63-90 d old) were used for and in vivo single unit extracellular recordings and behavioral analysis.

C57BLJ/6 mice (60-180 d; Harlan Nossan, San Pietro al Natisone, Italy) were used for behavioral observations.

$\beta2^{-/-}$, $\beta2^{+/+}$ and β2-DA-VEC mice (see below for detailed information) at different ages were used for electrophysiological experiments: particularly, mice 21-36 and 100-120 d old were used for whole cell patch clamp, and evoked field potential recordings, respectively. $\beta2^{-/-}$, $\beta2^{+/+}$ and β2-DA-VEC mice 100-120 d old were used for behavioral analysis.

3.1.1. Mice

A line expressing Cre recombinase under the control of the dopamine transporter promoter, line DAT-Cre (Tolu et al., 2009), was backcrossed for several generations with the B2-nAChR knock-out line ACNB2. The resulting mice used in this study were heterozygous for the Cre transgene and homozygous for the B2 knock-out, Cre+-; B2 -/-.

Lentivirus stereotaxic injections

Mice (56-60 d) were anaesthetized using 250µl of ketamine 1.5% (Merial, France)/xylazine 17.5% (Bayer Healthcare, France) in PBS. They were introduced into a stereotaxic frame adapted for use with mice. Lentivirus (2µl at 75ng p24 protein per µl) was injected bilaterally at: antero-posterior -3.4mm, lateral ±0.5mm from Bregma and -4.4mm from the surface for VTA injection. The mice were tested beginning after 4 to 6 weeks of viral expression.

Lentiviral reexpression vector

The PDGF-flox LV vector (Tolu et al., 2009) was modified to contain the cDNA of the B2 nAChR subunit instead of the eGFP reporter gene. Injection of the vector into DAT-Cre mice leads to

recombination and subsequent expression of the B2 subunit on a B2-/- background exclusively in DA neurons.

3.2 Drugs and Chemicals

Nicotine ((-)-nicotine hydrogen tartrate), fenofibrate, catalase, sodium-orthovanadate were purchased from Sigma (Italy). OEA, PEA, mAEA, AM281, AM251, capsazepine, WY14643, MK886, GW6471, mecamylamine, methyllycaconitine, dihydro-β-erythroidine, PP2 and genistein were purchased from Tocris. For the *in vivo* experiments WY14643 was dissolved in 10% Tween 80 and 20% DMSO of final volume.

3.3 Electrophysiological experiments

3.3.1. Electrophysiological studies in vitro

Whole cell patch clamp recordings from Sprague Dawley rat and mouse VTA DA cells were as described previously (Melis et al., 2006). Briefly, male Sprague Dawley rats or $\beta2^{-/-}$ and $\beta2^{+/+}$ mice were anesthetized with halothane and killed. A block of tissue containing the midbrain was rapidly dissected and sliced in the horizontal plane (300 and 230 μm for rat and mouse slices, respectively) with a vibratome (VT1000S, Leica) 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 cerebrospinal fluid (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 (two per animal) were allowed to recover for at least 1 hr before being placed (as hemislices) 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 current- and voltage-clamp recordings (one per hemislice) were made by using an Axopatch 200B amplifier (Molecular Devices, CA). Current-clamp experiments were made with electrodes filled with a solution containing the following (in mM): 144 KCl, 10 HEPES, 3.45 BAPTA, 1 $CaCl_2$, 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, CA). Dopamine neurons from the posterior VTA were identified by the presence of a large I_h current (Johnson and North, 1992a) that was assayed immediately after break-in, using a series of incremental 10 mV hyperpolarizing steps from a holding potential of -70 mV. Dopamine cells with long duration action potentials > 3 ms

(measured from the action potential threshold to the maximum after-hyperpolarization period) responded to DA (30 μM, 5 min) with a hyperpolarization (10.4 ±0.4 mV, n=23, data not shown) and were selected for recording (Margolis et al., 2008). Additionally, the second derivative of the action potential of the recorded cells originated the waveform (Lammel et al., 2008) that has been used for cell identification of DA neurons in many *in vivo* extracellular studies (Grace and Bunney, 1983; Melis et al., 2006), including the present one. Each slice received only a single drug exposure. Drugs were applied in known concentrations to the superfusion medium.

Evoked field potential recordings were as described previously (Nugent et al., 2008). A block of tissue containing the midbrain was obtained from male Sprague Dawley rats or $\beta2^{-/-}$, $\beta2^{+/+}$, β2-DA-VEC mice. Field potentials were stimulated at 0.1 Hz (100 ms pulse) using a bipolar stainless-steel stimulating electrode placed 100–300 μm rostral to the recording site in the VTA (Nugent et al., 2008). Extracellular evoked recordings were made with electrodes filled with a solution containing NaCl (2 M). All the drugs were dissolved in DMSO. The final concentration of DMSO was < 0.01 %.

3.3.2. Electrophysiological study in vivo

Male Sprague Dawley rats were anesthetized with chloral hydrate (400 mg/kg, i.p.), their femoral vein was cannulated for i.v. administration of pharmacological agents and they were placed in the stereotaxic apparatus (Kopf, Tujunga, CA, USA) 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 (AP, -6.0 mm from bregma; L, 0.3–0.6 mm from midline) for the placement of a recording electrode. Single unit activity of neurons located in 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, 500–5,000 Hz) and individual spikes were isolated by means of a window discriminator (Digitimer, Hertfordshire,UK), displayed on a digital storage oscilloscope (TDS 3012, Tektronics, Marlow, UK). Experiments were sampled on line and off line with Spike2 software (Cambridge Electronic Design, Cambridge, UK) by a computer connected to CED 1401 interface (Cambridge Electronic Design, Cambridge, UK). Single units were isolated and identified according to the already published criteria (Melis et al., 2006). VTA DA neurons were selected when all criteria for identification were fulfilled: firing rate, <10 Hz; duration of action potential >2.5 ms as measured from start to end, inhibitory responses to hindpaw pinching (Ungless et al., 2004). Bursts were defined as the occurrence of two spikes at interspike interval <80 ms, and terminated when the interspike interval exceeded 160 ms (Grace and Bunney, 1983).

3.4. Behavioral study

3.4.1. Locomotor activity

C57BLJ/6 mice were individually tested for motor activity under standardized environmental conditions with a Digiscan Animal Activity Analyser (Ominitech Electronics, Columbus, Ohio). Each cage (42 cm x 30 cm) had two sets of 16 photocells located at right angles to each other, projecting horizontal infrared beams 2.5 cm apart and 2 cm above the cage floor and a further set of 16 horizontal beams which height could be adapted to the size of the animals. Basal horizontal and vertical activity were measured as total number of sequential infrared beam breaks in the horizontal or vertical sensors, recorded every 5 minutes, beginning immediately after placing the animals in the cage, over a period of 120 minutes. To evaluate the effect of nicotine (0.02 mg/kg, s.c.) on locomotor behaviour, animals were habituated to the apparatus for 60 minutes after administration of WY14643 (40 mg/kg, i.p.) or its vehicle (10 ml/kg, i.p.). Horizontal locomotor activity was assessed every 10 minutes for an additional period of 60 minutes after nicotine administration. All behavioral experiments were conducted between 10:00 am and 3:00 pm.

3.4.2. Prepulse inhibition of the acoustic startle response

Rats were placed in a startle reflex apparatus (PPI) (Med Associates, St Albans, VT, USA) for a 5-min acclimatization period with a 70 dB background noise, which continued for the remainder of the session. Each session consisted of three consecutive sequences of trials. During the first and the third sequence, the rats were presented with five pulse-alone trials of 115 dB. The second sequence consisted of 50 trials in pseudorandom order, including 12 pulse-alone trials, 30 trials of pulse preceded by 73, 76, or 82 dB prepulses (10 for each level of prepulse loudness), and eight no-stimulus trials, where only the background noise was delivered. The duration of pulses and prepulses was 80 and 40 ms, respectively. Prepulse–pulse delay amounted to 100 ms. Intertrial intervals were selected randomly between 10 and 15 s. Percent PPI was calculated with the following formula: 100−[(mean startle amplitude for prepulse–pulse trials/mean startle amplitude for pulse-alone trials) × 100].

The goal of this experiment was to verify the effects of WY14643 (WY, 40mg/kg/2ml, i.p.) on the PPI disruption mediated by apomorphine (APO, 0.25mg/kg/ml, s.c.). Thus, animals received WY or its vehicle (VEH, SAL/DMSO, 1:1, v/v) 30 min before either APO or saline treatment. Immediately after APO or SAL injection, rats were placed in the startle chamber and subjected to behavioral testing (n=6-11 animals per group).

3.5. Biochemical analysis

3.5.1. Measurement of FAE levels.

AEA, PEA, and OEA from midbrain slices (300 μm) were measured as previously described (Di Marzo et al., 2001; Melis et al., 2006) and expressed as percentage of molarity to avoid artifacts due to diverse water content in the tissue.

3.6. Statistical analysis

All the numerical data are given as mean ± S.E.M.. Data were compared and analyzed by utilizing 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 $P<0.05$. For PPI experiments all analyses were conducted using Statistica (Statsoft, Tulsa, USA). The significance of differences between groups was determined by two-way analysis of variance (ANOVA) with pretreatment (WY vs VEH) and treatment (APO vs SAL) as a between-subjects factor, followed by Tukey's test for multiple comparisons.

4. RESULTS

4.1 FAEs suppress nicotine-induced stimulation of VTA dopamine neuron firing rate through the peroxisome-proliferator-activated receptor-α (PPARα) in an in vitro brain slice preparation (Paper I).

Utilizing whole-cell patch-clamp recordings in brain slices containing the mesencephalon we examined the effect of nicotine on posterior VTA DA neurons. Figure 5A (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. 5A, bottom) that has been utilized for cell identification of DA neurons in many *in vivo* extracellular studies (Grace and Bunney, 1983, 1984), and qualitatively corresponds to the typical action potential recorded *in vivo*. 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 (about 40%; Fig. 5B-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. 5F), 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 co-applied with nicotine (1 μM). mAEA was tested at two different concentrations, 30 nM and 1 μM. The lower concentration of mAEA neither significantly affected spontaneous discharge rate of DA neurons nor modulated nicotine-evoked excitation (the peak of nicotine with mAEA was 147.7±13.7 % of baseline firing rate, P=0.58, t-test, versus nicotine alone; Fig. 5E, G). Since this concentration of mAEA might have been too low, we next tested a concentration of 1 μM, which itself significantly 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 CB1 and/or TRPV1 receptors. The CB1 receptor antagonist AM281, at a concentration (500 nM) that fully reverses activation of CB1 receptors by maximal concentrations of agonists (Melis et al., 2004a), 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). On the other hand, 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., 2003). To isolate the agonistic activity of mAEA at CB1 receptors, or possibly at PPARα, 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 as compared to controls (151.4±13.5 % of baseline, *t*=0.77, n=6, P=0.4563, t-test; Fig. 5G), suggesting that CB1 receptor stimulation did not affect nicotine-induced excitation of DA neuron firing and that mAEA did not activate PPARα.

Next, we tested two different non-cannabinoid 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. 5H), without affecting DA neuron spontaneous activity during pre-application (Fig. 5E). The effects of OEA were mimicked by PEA (10 μM, 101.9 ± 3 % of baseline, n=6, *t*=2.66, P=0.01; Fig. 5E, 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. 5F), or PEA (10 μM, 5 min preapplication: -0.6 ± 7.5 pA, N=5, t=4.442, P=0.001, paired t-test; Fig. 5F). 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 PPARα antagonist MK886 would block the actions of OEA and PEA on nicotine-induced excitation. As predicted, when OEA or PEA were co-applied with MK886 (300 nM), nicotine 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 vs PEA alone: $F_{1,228}$=4.84, n=8, P=0.04, two-way ANOVA; Fig.6A, B, C). MK886, when perfused either alone or in combination with OEA/PEA, did not alter spontaneous firing rate of VTA DA neurons (Fig. 6 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. 6B).

Next, we determined whether the synthetic PPARα 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 ± 14.7 % of baseline, n=9, *t*=2.54, P=0.02; Fig. 6D, 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. 6D, E), confirming the role of PPARα in the modulation of DA neuron responses to nicotine.

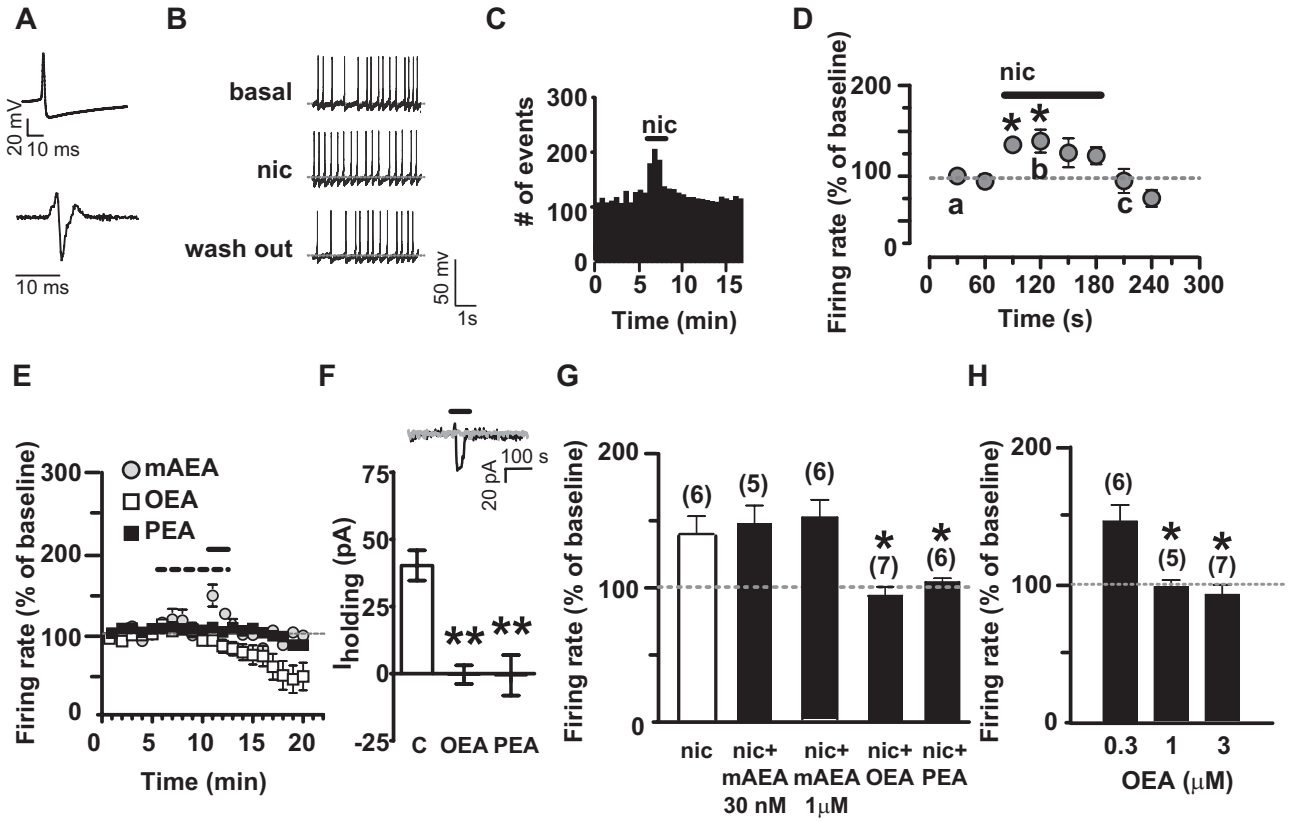

Figure 5. Activation of dopamine neurons by nicotine is prevented by oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) *in vitro*. (**A)** Typical action potential waveform of a dopamine neuron recorded under current-clamp mode (top panel) and its second derivative (bottom panel). (**B)**, Representative traces of a dopamine neuron spontaneous activity during baseline level (top panel), nicotine application (nic, 1 μM for 2 min, middle panel) and wash out (bottom panel). (**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 methanandamide (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 (3μM and PEA 10μM) also blocked nicotine-induced inward currents ($I_{holding}$) when dopamine neurons were recorded under the voltage-clamp mode (V_{hold}= -70mV). 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 (3μM; OEA's effect on $I_{holding}$ is superimposed in light grey 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 curve 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 ± s.e.m. * P< 0.05, ** P< 0.01.

Although it is well established that PPARα 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).

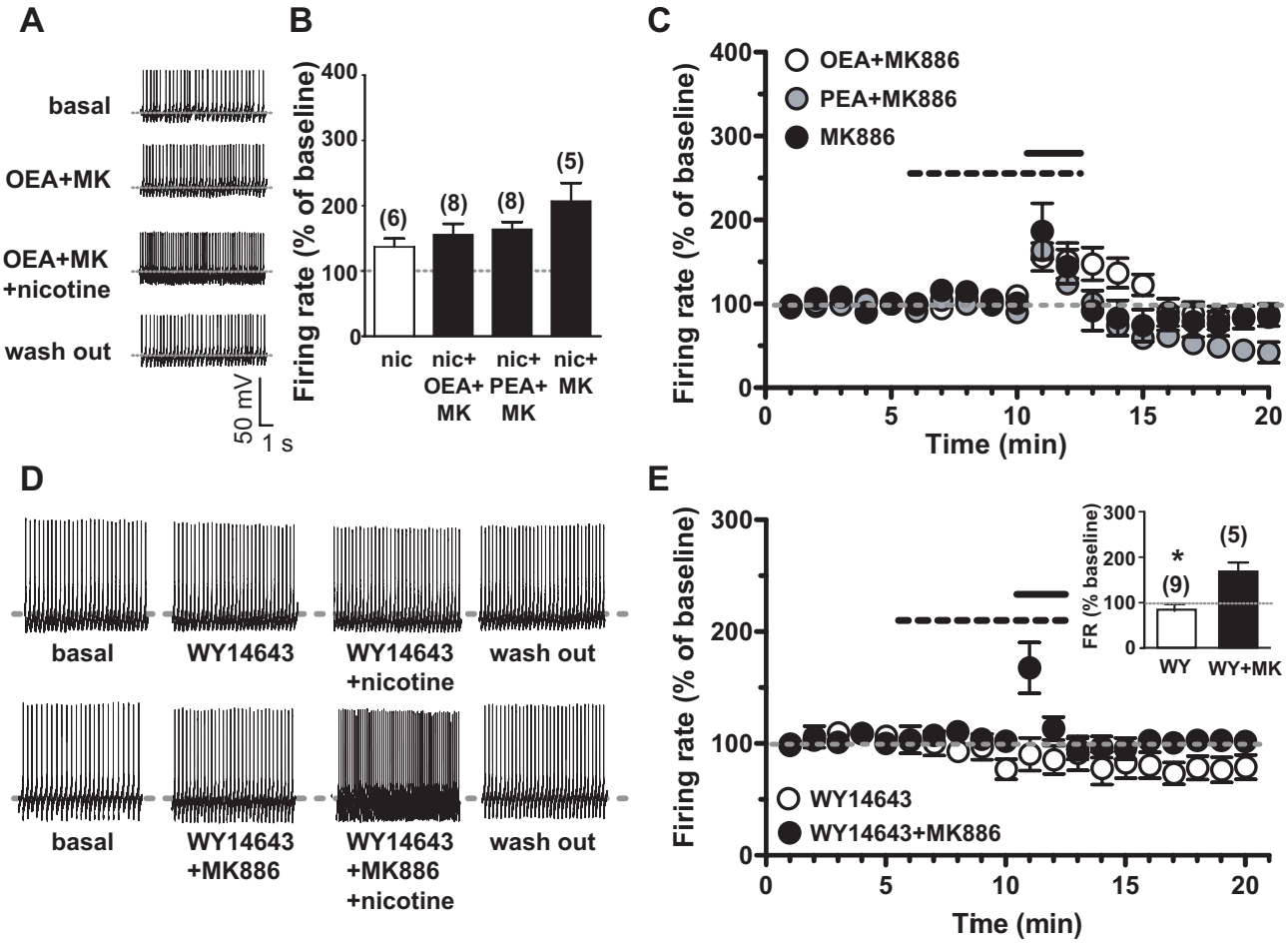

Figure 6. OEA and PEA block nicotine activation of dopamine neurons through a PPAR-α -mediated mechanism. (**A)** Representative traces of the spontaneous activity of a dopamine neuron during baseline (top panel), OEA (3 μM) + the PPAR-α antagonist MK886 (0.3 μM) pre-application (5 min, second panel), subsequent nicotine application (1 μM, 2 min, third panel) and wash out (bottom panel). (**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 co-applied 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) + 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 panel), which was then restored by the co-application of MK886 (bottom panel). (**E)** Time course of the effect of nicotine on dopamine neuron firing rate in the presence of WY14643 (open symbols) or WY14643+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±s.e.m. * P< 0.05.

We hypothesized that phosphorylation of nAChRs could account for PPARα 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. 7A, B), demonstrating that inhibition of tyrosine kinases reverses the effect of PPARα 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). The prediction was 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. 7A, 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. 7C, D), suggesting that SFK is not involved in the negative modulation of nAChRs by PPARα agonists.

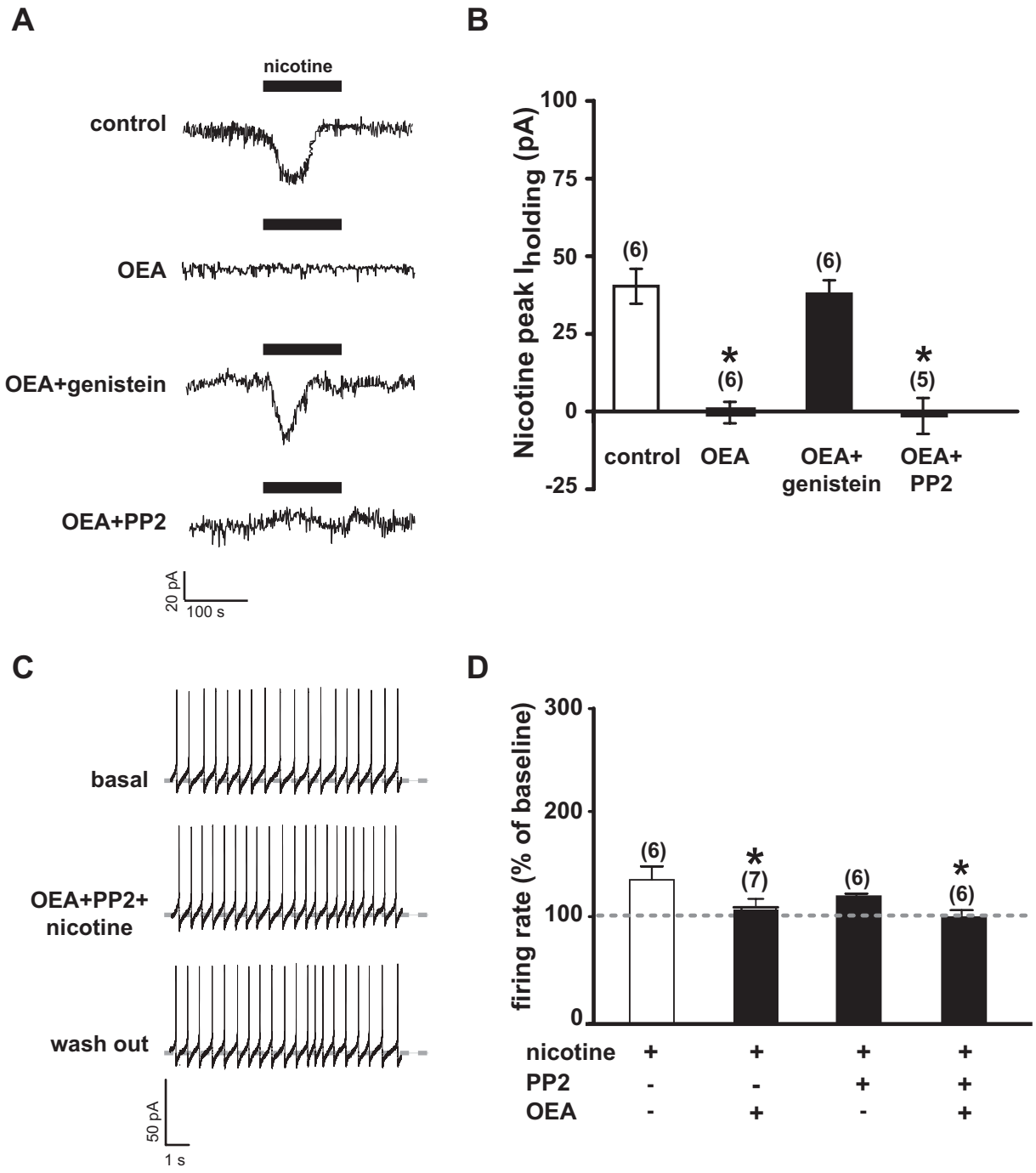

Figure 7. Tyrosine kinases, but not Src kinase, inhibition prevent OEA-induced blockade of nicotine effects. (**A**) In voltage-clamp mode, nicotine (1 μM, 2 min) caused a 43 pA inward current (top panel), that is blunted by OEA pre-application (3 μM, 5 min; second panel). Pre-treatment with the general tyrosine kinase inhibitor genistein (10 μM, 5 min) fully blocked OEA actions by restoring nicotine-induced inward current (third panel). However, pre-treatment with the selective Src kinase inhibitor PP2 (10 μM, bottom panel) 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 panel). (**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±s.e.m. * $P< 0.05$.

4.2. Interaction between PPARα and nAChRs in VTA DA cells: postsynaptic effects of PPARα modulation on VTA DA neurons, and the contribution of nAChRs in PPARα effects (Paper II).

To characterize the postsynaptic effects of PPARα modulation on DA neurons, all experiments were performed in the presence of CNQX (10 μM), D-AP5 (100 μM) and picrotoxin (100 μM), in order to block AMPA-, NMDA- and $GABA_A$-mediated postsynaptic responses. Figure 8 (A, B) shows that bath application of the PPARα antagonist MK886 (500 nM, 8 min) significantly increased the spontaneous activity of VTA DA neurons (about 275% above baseline, n=6; $F_{19,100}$=3.308, P<0.0001, one-way ANOVA). Under voltage-clamp mode ($V_{holding}$= -70 mV), MK886 caused an inward current of 93.3 ± 29.3 pA (Figure 8 C). Both effects were reversible on wash out and blocked by the synthetic PPARα agonist WY14643 (Figure 9A,B; firing rate: WY14643+MK886 vs MK886 alone: $F_{1,171}$=7.86, n=6, P=0.02, two-way ANOVA; $I_{holding}$: 10.0 ± 2.6 pA, t=2.42, P=0.03, unpaired two-tailed t-test), at a dose (300 nM) *per se* ineffective (Fig. 6 D,E). MK886 effect was concentration-dependent (Figure 9C; 100.8 ± 6.8 % and 434.9 ± 69.6% of baseline at 0.3 and 1 μM, respectively), and mimicked by the structurally dissimilar PPARα antagonist GW6471 (0.3-1 μM) (Figure 9C; 0.3 μM: 161.0 ± 15.97 % of baseline; 0.5 μM: 211.5 ± 25.4% of baseline; 1 μM: 338.2 ± 47 % of baseline).

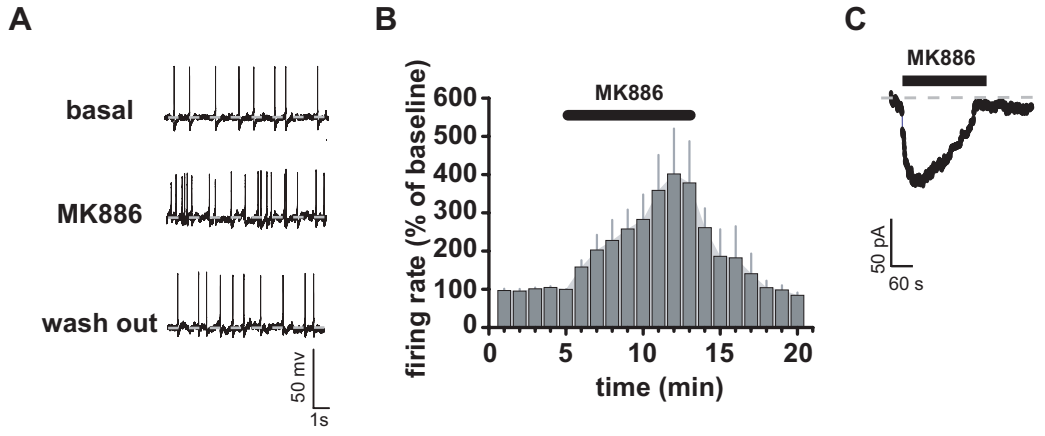

Figure 8. PPARα blockade activates VTA dopamine neurons *in vitro*. (**A)** MK886 application (0.5 μM) increases dopamine neuron spontaneous activity. Current-clamp recording from a dopamine neuron (left panel) and rate histogram depicting MK886 averaged effects (right panel). (**B)** In voltage-clamp mode MK886 caused an inward current (V_{hold}= -70 mV).

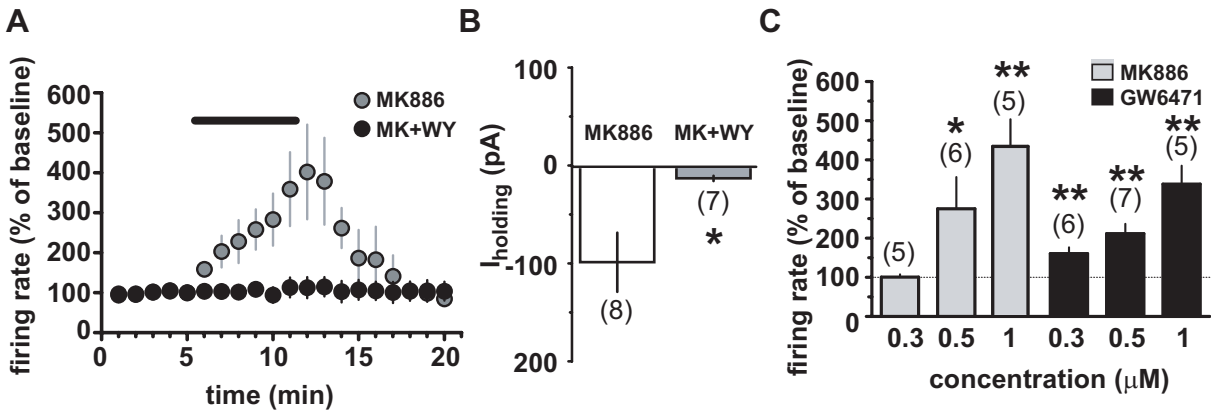

Figure 9. (**A**) WY14643 blocked MK886-induced activation of dopamine neurons and (**B**) the MK886-induced inward current. (**C**) Summary of dose-related effects of PPARα antagonists on dopamine neuronal frequency. Numbers above bars indicate *n* values. Data expressed as mean ± SEM. **p* < 0.05; ***p* < 0.005.

To examine the contribution of nAChRs to the effects of MK886, nAChRs were blocked by bath applying either mecamylamine (MEC, a non-competitive nAChR antagonist), methyllycaconitine (MLA, a specific antagonist for α7 subtype nAChRs) or dihydro-β-erythroidine (DHBE, a competitive antagonist for neuronal α4 containing nAChRs). Figure 10 (A,B) shows that MK886 (0.5 μM) actions on DA neuronal firing rate were prevented by MEC (100 μM, 5 min; $F_{2,285}$=5.7, P=0.01, two-way ANOVA) and DHBE (1 μM, a dose *per se* ineffective on DA cell firing rate (Matsubayashi et al., 2003); 5 min: $F_{1,90}$=4.97, P=0.05, two-way ANOVA), whereas MLA was ineffective (5 nM, 5 min; $F_{1,190}$=0.86, P=0.4, two-way ANOVA). Additionally, DHBE blocked MK886-induced inward currents (Figure 10C; MK886+DHBE: -3.857 ± 18.9 pA, P= 0.018), suggesting that most, if not all, of DA responses to MK886 were due to activation of α4 subunit containing nAChRs. Since the α4 subunit generally assembles with the β2 subunit, we hypothesized that most of the effects would be mediated by postsynaptic α4β2-nAChRs. Therefore, we examined MK886 effects in β2-nAChR knock out ($\beta2^{-/-}$) mice. MK886-induced effects were absent in $\beta2^{-/-}$ mice (Figure 10D; two-way ANOVA $F_{1,152}$=8.65, P=0.018; t=5.1, P=0.0005, unpaired t-test), whereas its effects in $\beta2^{+/+}$ mice were comparable to those observed in juvenile rats.

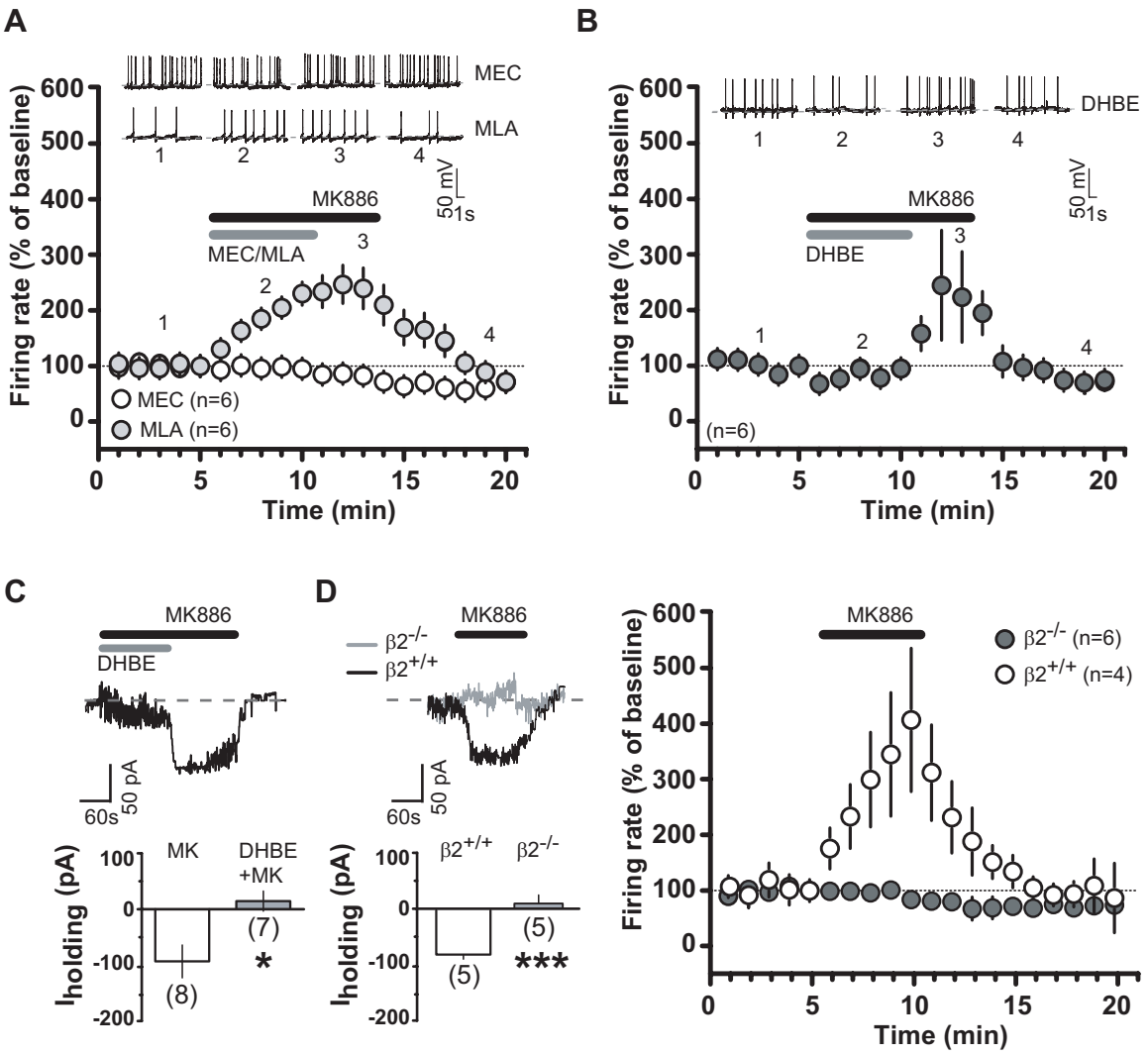

Figure 10. MK886 enhances dopamine neuron activity through α4β2-nAChRs. (**A** and **B**) Time course of MK886 effect, alone or together with mecamylamine (MEC) (100 μM), methyllycaconitine (MLA) (5 nM) or dihydro-β-erythroidine (DHBE) (1 μM), on dopamine neuron activity. Grey and black bars represent time of nAChR antagonist or MK886 application, respectively. Insets show representative traces of dopamine neuron frequency. (**C**) Bar graph illustrating DHBE mean effect on MK886-induced inward current. Inset shows representative DHBE+MK886 effect on dopamine cell. Grey and black bars represent the time of DHBE and MK886 application, respectively. (**D**) MK886 effects on dopamine neurons in $\beta2^{-/-}$ and $\beta2^{+/+}$ mice under voltage-clamp (left panel) and current-clamp (right panel) modes. Inset shows a representative MK886 effect.

To further characterize the relationship between MK886-induced current and membrane potential, 6-s voltage ramps ranging from -120 to +60 mV were applied to voltage-clamped DA neurons (Matsubayashi et al., 2003) in the absence and presence of MK886 (0.5 μM). The MK886-induced net current was observed by subtracting currents in the presence of MK886 from those in the absence of MK886. When the MK886-induced current was compared with the one produced by nicotine (1 μM) under the same conditions, a strong correlation was found between the currents induced by both MK886 and nicotine (Figure 11) at all membrane potentials (Figure 12), particularly at resting membrane potential (i.e. -60 mV), thus suggesting a common site of action.

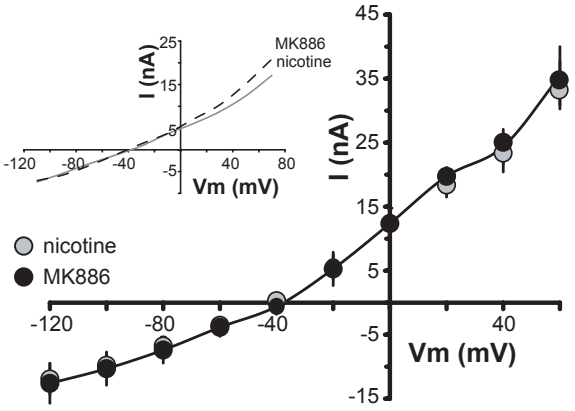

Figure 11. Current-voltage relationships in the absence and presence of either MK886 or nicotine when a voltage ramp was applied. In the voltage-clamp mode, 6 s voltage ramp waves ranging from -120 to 60 mV were applied to a whole cell patched dopamine neuron. The inset shows an example of the traces of net MK886- (dashed line) and net nicotine- (solid line) induced current-voltage relationships.

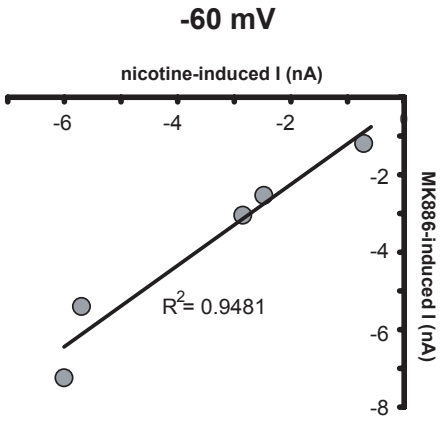

Figure 12. *Left panel,* Magnitude of currents induced by nicotine plotted as function of those induced by MK886 at membrane potential of -60 mV. Data fit by linear regression with $r^2 = 0.9481$ ($p < 0.001$). *Right panel*, R^2 for five cells in *left panel* plotted as a function of voltage membrane.

The functional properties of nAChRs depend on the tyrosine phosphorylation status of the receptors, being the result of a balance between tyrosine kinases and phosphatases, which negatively and positively modulate nAChR-mediated currents, respectively (Charpantier et al., 2005). To investigate the possibility that MK886 was modifying the status of nAChRs, we incubated, and continuously perfused, rat brain slices with the general tyrosine phosphatase inhibitor sodium orthovanadate (OVN, 1 mM), and then applied MK886 (0.5 μM, 5 min). We only performed voltage-clamp experiments because of OVN actions on hyperpolarization-activated cyclic nucleotide-gated channels, which prevented pacemaker-like spontaneous activity (Huang et

al., 2008). OVN prevented MK886-induced inward currents (Figure 13A; -3.714 ± 10.8 pA; t=2.93, P=0.01, unpaired t-test vs MK886 alone) without affecting nicotine-induced inward currents (OVN+nicotine: 27.29 ± 6.2 pA; nicotine: 40.33 ± 5.6; t=1.53, P=0.15, unpaired t-test vs nicotine alone; Fig. 13B), thus indicating that it blocked phosphatases downstream of PPARα. Thus, the present observations suggest that MK886 does not directly act on nAChRs, but increases sensitivity of high-affinity nAChRs to endogenous ACh in midbrain DA neurons.

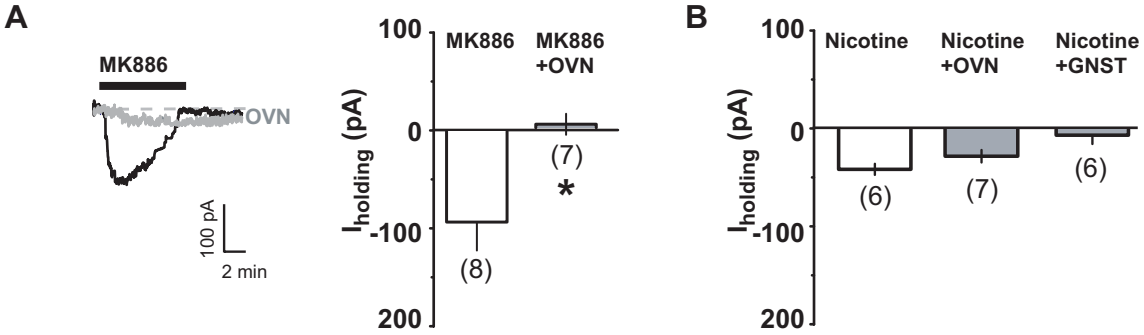

Figure 13. PPARα blockade effects on dopamine neurons involve tyrosine phosphatases. (**A**) Under voltage-clamp mode, MK886 effect on dopamine neuron in presence (grey) or absence (black) of sodium-orthovanadate (OVN). (**B**) Under voltage-clamp mode, nicotine effects on dopamine neuron in the presence of OVN, and genisteine (GNST). Numbers above bars indicate *n* values. Data are expressed as mean ± SEM. **p* < 0.05.

Accordingly, when we enhanced endogenous ACh actions at nAChRs by applying both the ACh esterase inhibitor neostigmine (2 μM) and the muscarinic antagonist atropine (5 μM), we observed an increased DA cell firing rate (Figure 14A; 128.7 ± 7.6 % of basal, t=3.47, P=0.03, paired-t test), which was further enhanced in the presence of an ineffective dose of MK886 (0.3 μM, 5 min) (Figure 14B: 161.3 ± 5.5 % of basal, t=7.53, P=0.008, paired-t test). Consistently, the effects of nicotine (0.1-5 μM) were strongly enhanced in the presence of MK886 (0.3 μM) (Figure 14B: for example, nicotine 0.1 μM: 107.5 ± 10.5 % and 166.2 ± 17.7 % of baseline in the absence or presence of MK886, respectively; n=6 for both groups; Nicotine+MK886 vs Nicotine alone: t=2.85, P=0.008, unpaired t-test; and nicotine 5 μM: 157.7 ± 18.8 % and 306.8 ± 14.5 % of baseline in the absence or presence of MK886, respectively; n=5 for both groups; nicotine+MK886 vs nicotine alone: t=6.27, P=0.0001, unpaired t-test).

To investigate the role of PPARα in more detail, we then tested the effects of the endogenous ligand for PPARα, OEA, on DA cell firing. OEA (10 μM) decreased DA neuronal discharge (Figure 15A,B: $F_{19,60}$=17.03, P<0.0001, one-way ANOVA) in a concentration-dependent fashion (Figure 15D: 3 to 30 μM; 3 μM: 98.2±8.6 %, n=4, ; 10 μM: 27.7±16.5 %, n=4; 30 μM: 20.0±5.8 %, n=5), an effect reversed by MK886 (Figure 15C: 500 nM, 3 min; n=5, $F_{1,133}$=32.40, P=0.0007, two-way ANOVA).

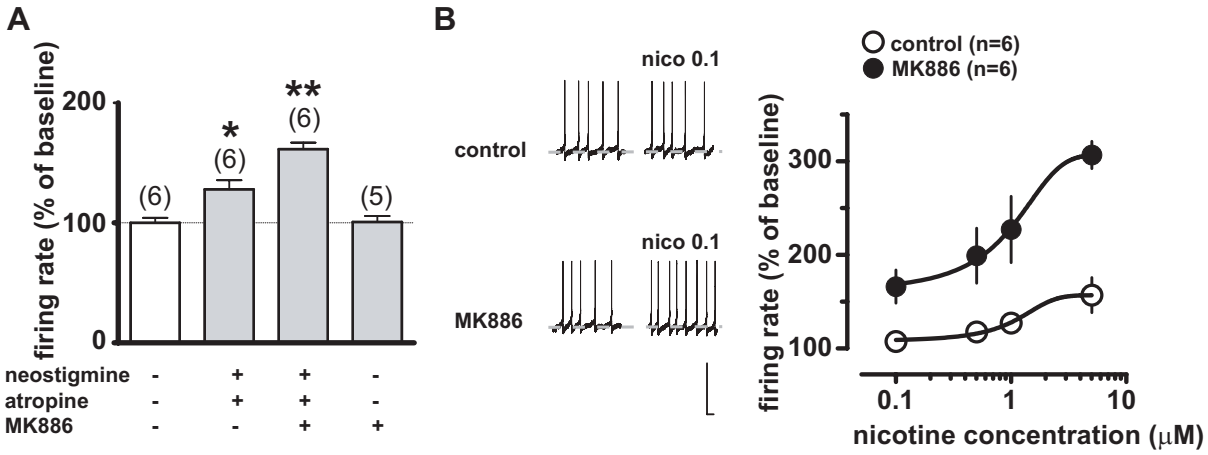

Figure 14. PPARα blockade increases efficacy of nAChR agonists. (**A**) Under current-clamp mode, effects of enhanced endogenous ACh levels acting at nAChRs (neostigmine+atropine) in presence or absence of MK886 (0.3 μM). (**B**) Current-clamp recording of a dopamine neuron (left panel) showing enhanced nicotine response in presence of MK886 (0.3 μM). Dose-response curves depicting averaged effects of nicotine (right panel) on dopamine neurons in presence or absence of MK886. Numbers above bars indicate *n* values. Data are expressed as mean ± SEM. **p* < 0.05, ***p* < 0.005.

The OEA effect was mimicked by two structurally different PPARα agonists, WY14643 (0.3-3 μM) and fenofibrate (1-10 μM) (Figure 15D, F). Fenofibrate was the least potent (1 μM: 134.2±30 %, n=6; 10 μM: 42.1±23.8 %, n=6; 30 μM: 24.3±4.9 %, n=5), and WY14643 the most potent (0.3 μM: 75.8±15.5 %, n=5 ; 1 μM: 17.9±5 %, n=5; 3 μM: 5.9±0.5 %, n=4). In addition, under voltage-clamp mode, the three PPARα agonists also produced outward currents (Figure 15E; OEA 10 μM: 45.4±5.7 pA; WY14643 1 μM: 27.3±5.1 pA; fenofibrate 3 μM: 43.6±7.05 pA), which were blocked by MK886 (Figure 15E). MK886 also reversed the effects produced by the two agonists on DA cell frequency (Figure 15F; WY14643: $F_{1,171}$=7.83, P=0.02, two-way ANOVA; fenofibrate: $F_{1,190}$=5.07, P=0.04, two-way ANOVA).

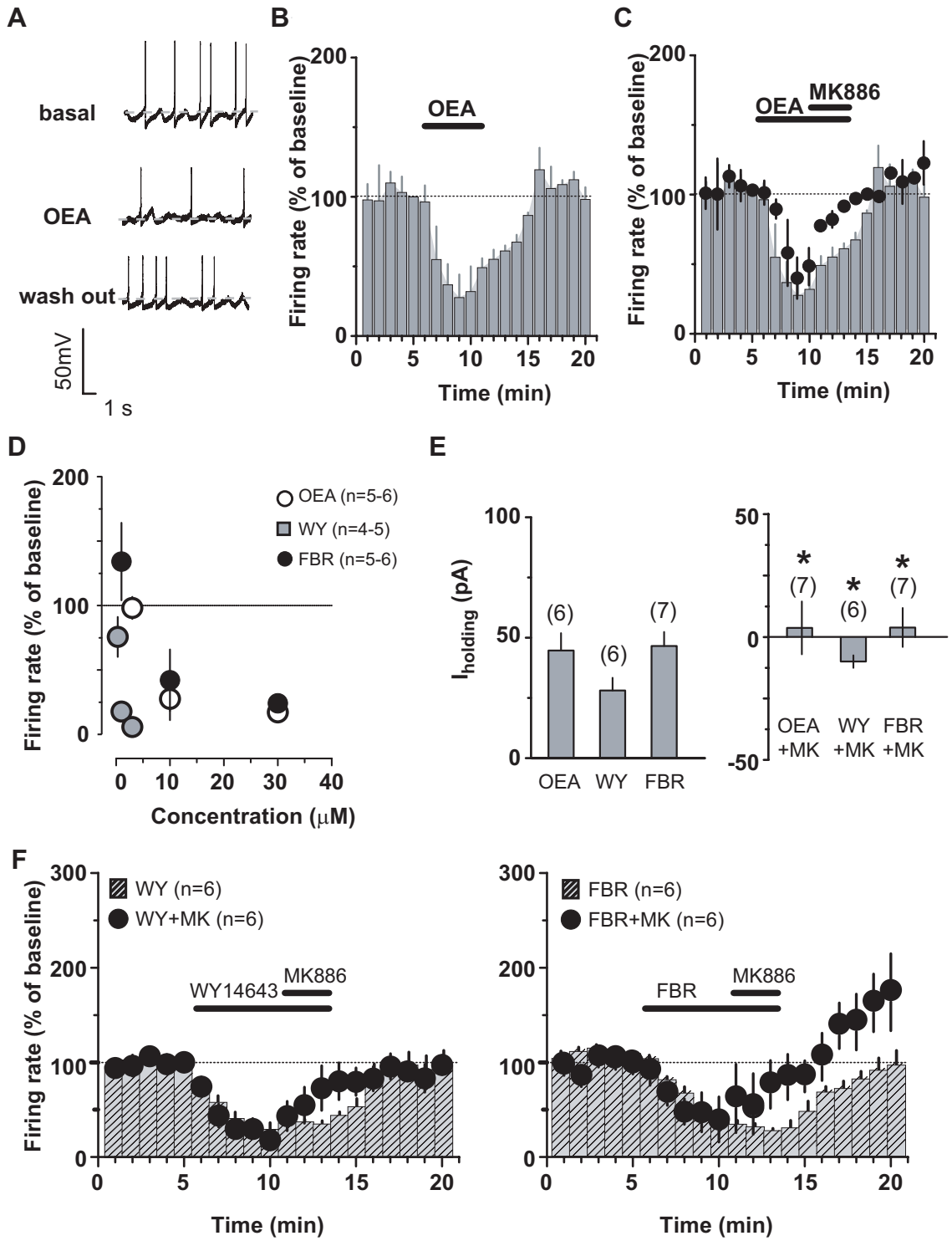

Figure 15. PPARα activation decreases VTA dopamine neuronal activity. (**A**) Current-clamp traces of a dopamine neuron before, during and after oleoylethanolamide (OEA) application (10 μM). Rate histogram depicting averaged OEA effects on dopamine neuron frequency in absence (**B**) and presence (**C**) of MK886 (0.5 μM**). (D),** Dose-response curves depicting averaged effects of PPARα agonists on dopamine neuron frequency. (**E**), Under voltage-clamp mode, effects of OEA (10 μM), WY14643 (WY; 1 μM) and fenofibrate (FRB; 10 μM) on dopamine neurons in absence (left panel) and presence (right panel) of MK886 (0.5 μM). (**F**), Under current-clamp mode, MK886 (0.5 μM) reversed the effects of both WY14643 (left panel) and fenofibrate (right panel) on dopamine neurons. Numbers above bars indicate *n* values. Data expressed as mean ± SEM. **p* < 0.05.

Activation of PPARα has been shown to increase intracellular concentrations of reactive oxygen species (Teissier et al., 2004), such as hydrogen peroxide, which is produced by DA cells in an activity-dependent fashion and depresses firing rate of DA neurons through ATP-sensitive K+ channels (Avshalumov et al., 2005). Because hydrogen peroxide can also activate a tyrosine kinase (Wu et al., 2007), we tested the possibility that it might be involved in PPARα ligand actions by filling the DA cell with catalase (500 IU/ml), to rapidly inactivate hydrogen peroxide, and then bath applying OEA. Figure 16 (A,B) shows that, in the presence of catalase, OEA (10 μM, 5 min) failed to produce any inhibitory effect (OEA vs OEA+catalase: n=5, $F_{1,112}$=16.87, P=0.003, two-way ANOVA), thus indicating hydrogen peroxide as a downstream effector of PPARα. Importantly, because depletion of intracellular hydrogen peroxide by including catalase in the patch pipette increases the spontaneous firing rate of midbrain DA neurons (Avshalumov et al., 2005), we can assume that OEA-induced increase in firing rate, which is comparable to the previously reported in the presence of catalase into DA cell compartment (Avshalumov et al., 2005), might be ascribed to a reduced hydrogen peroxide inhibitory physiological role. Nonetheless, if hydrogen peroxide production is also related to PPARα activation, then one would expect that under these conditions the effects of nicotine on DA cell frequency would be restored. When nicotine (1 μM, 2 min) was applied in the presence of both OEA (the lower dose of 3 μM that is ineffective on firing rate (Fig.5 G, H), 7 min) and catalase (500 IU/ml), nicotine transiently increased the spontaneous activity of VTA DA neurons (Figure 16C,D: nicotine+OEA vs nicotine+OEA+catalase: n=5, t=3.57, P=0.005, two-tailed *t* test; nicotine+OEA+catalase vs OEA+catalase at 10min: P=0.0336, t= 2.493, paired t-test) consistently with previous reports (Pidoplichko et al., 1997)(Fig. 5 B-D). As previously observed (Fig. 6C), here we observed a reduction of spontaneous activity following nicotine application in the presence of OEA. Although at this stage we cannot provide a definite explanation for this phenomenon, it is plausible that nicotine would desensitize β2-nAChRs more rapidly once PPARα are activated, with either a possible consequent internalization or run down of these receptors. Nonetheless, the recovery of nicotine effects on DA cell firing supports the hypothesis that activation of PPARα leads to hydrogen peroxide production and blocks nicotine effects on DA neuronal activity.

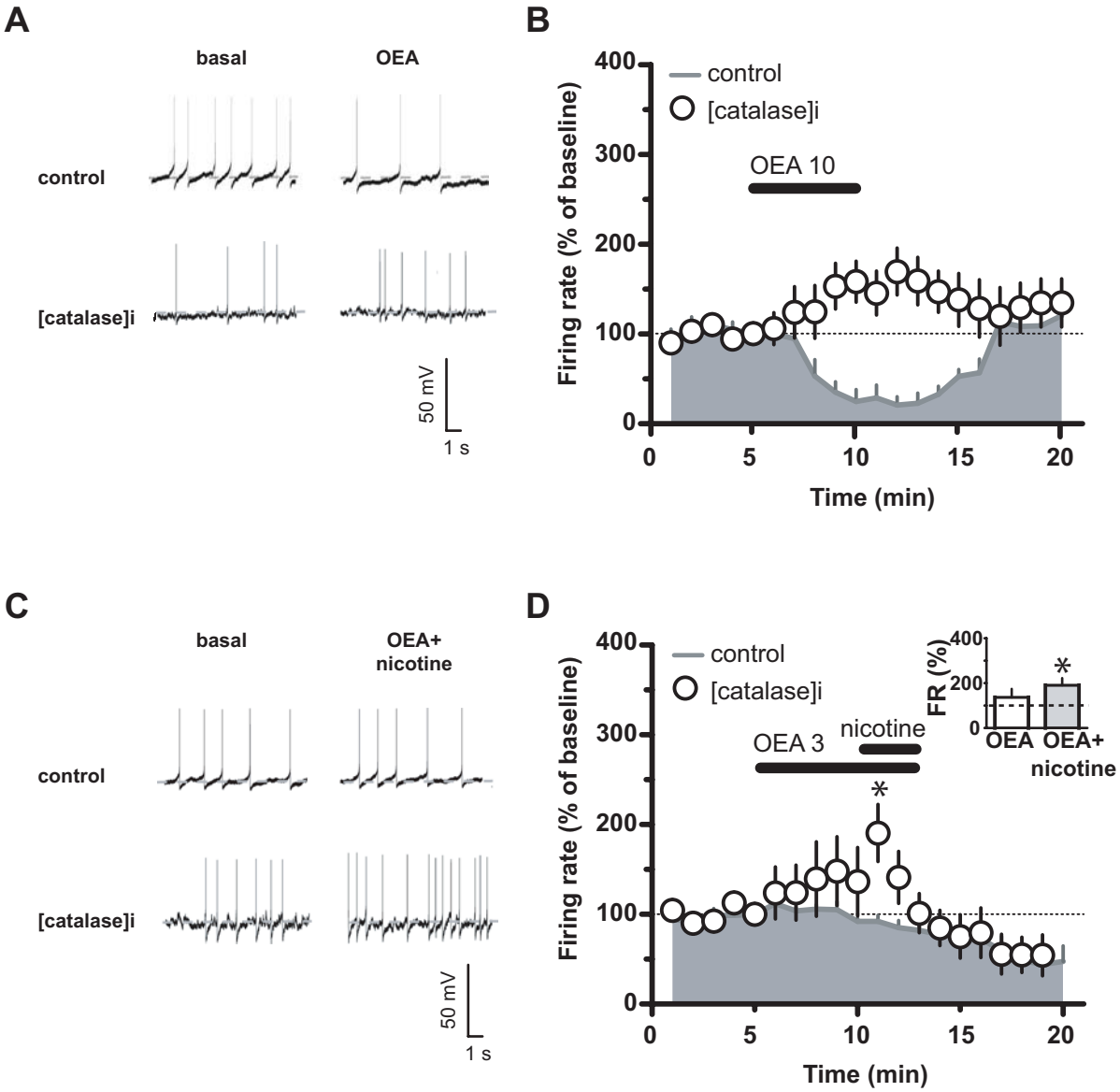

Figure 16. Hydrogen peroxide is downstream effector of PPARα. (A), Current-clamp recording of a dopamine neuron during bath application of OEA (10 μM) in absence or presence of catalase (500 U/I). (B), Time course of averaged effects of OEA (10 μM) on dopamine neuron frequency in the presence (open circles) and absence (grey area) of catalase. (C) Current-clamp recording of a dopamine neuron during nicotine (1 μM) and OEA (3 μM) application in the absence or presence of catalase. (D) Time course of OEA (3 μM) and OEA+nicotine averaged effects on dopamine neuronal activity in the presence (open circles) and absence (grey area) of catalase. The inset shows that nicotine produced an effect on firing rate (FR) in the presence of OEA (3 μM) and catalase. Catalase was applied through the recording pipette. Data expressed as mean ± SEM. *$p < 0.05$.

4.3. PPARα modulation of nAChRs in VTA DA neurons affects net VTA output both in vitro and in vivo (Paper II).

To ensure that PPARα modulation of DA neuronal firing was not restricted to a certain DA subpopulation within the VTA, the effects of both MK886 and WY14643 were also investigated using extracellular evoked field potential recordings in horizontal brain slices (Nugent et al., 2008). Figure 17 (A) shows that MK886 (500 nM, 5 min) induced a significant change in both negative components of the field potential (i.e. N1 and N2) (N1 amplitude at time = 5 min was enhanced by about 25% of baseline, n= 6, t= 2.098, P=0.04, paired t-test; N2 amplitude at time = 5 min was enhanced by about 50% of baseline, n= 6, t= 2.24, P=0.03, paired t-test). Conversely, WY14643 (1 μM, 5 min) significantly reduced both components of the field potential (Figure 17B: N1 amplitude at time = 5 min was reduced by about 10% of basal, n=6, t=3.82, P=0.006, paired t-test; N2 amplitude at time = 5 min was reduced by about 15% of basal, n=6, t=3.81, P=0.01, paired t-test). Although both components are similarly affected by PPARα modulation, in the next set of experiments we measured only the second negative potential (i.e. N2) because it has been shown to largely represent the postsynaptic responses of DA neurons (Zheng et al., 2006). Nonetheless, it should be noted that N2 component is the summation of stimulation-evoked postsynaptic responses of DA cells and of antidromic action potentials (Zheng et al., 2006), and, therefore, represents both synaptic and population spike components.

To further examine the role played by nAChRs in the effect of PPARα activation, we bath applied WY14643 (1 μM, 5 min) in $\beta2^{-/-}$ mice. Figure 17 (C) shows that WY14643 failed to produce any effect on N2 amplitude when compared with $\beta2^{+/+}$mice ($F_{1,133}$=6.61, P=0.03, two-way ANOVA). Next, we took advantage of the $\beta2^{-/-}$ mice in which the Cre-inducible switching on for β2-nAChR gene was allowed only in DA neurons, thus selectively re-expressing the corresponding nAChR subunit exclusively in VTA DA cells by stereotaxically injecting a lentiviral vector (β2-DA-VEC mice) (Tolu et al., 2009). Importantly, in β2-DA-VEC mice WY14643 effect was restored (Figure 17C: N2 amplitude at time = 5 min was reduced by about 25 % of basal, $F_{4,76}$=18.30, P<0.0001, one-way ANOVA), and similar to its effect in $\beta2^{+/+}$ mice ($F_{1,133}$=0.01, P=0.998, two-way ANOVA). Remarkably, this gene-target strategy allows not only to dissect the functional role of β2-nAChRs on DA cells of the VTA (Tolu et al., 2009), but also to discriminate the contribution of DA neuronal activity to net VTA output (Zheng et al., 2006), and, ultimately, of PPARα.

Because β2-nAChRs are key in controlling the firing rate/pattern of VTA DA cells (Mameli-Engvall et al., 2006), we next examined whether PPARα activation affected the number of spontaneously active VTA DA neurons *in vivo*.

Neuronal sampling of the VTA was performed in anesthetized rats following administration of either WY14643 (40 mg/kg i.p., 15 min before the experiment) or vehicle. Acute administration of WY14643 reduced the number of spontaneously active DA neurons, since the mean (± S.E.M.) number of DA neurons encountered in the VTA was 1.51±0.2 and 0.97±0.2 cell/track in vehicle- and WY14643-treated animals, respectively (Figure 17D: t=2.21, P=0.02, unpaired t-test). The average spontaneous firing rate of DA cells was not different in vehicle- and WY14643-treated rats (Figure 17D; t=0.29, P=0.38, unpaired t-test), in agreement with the ineffectiveness of the dose tested on firing frequency (Melis et al., 2008).

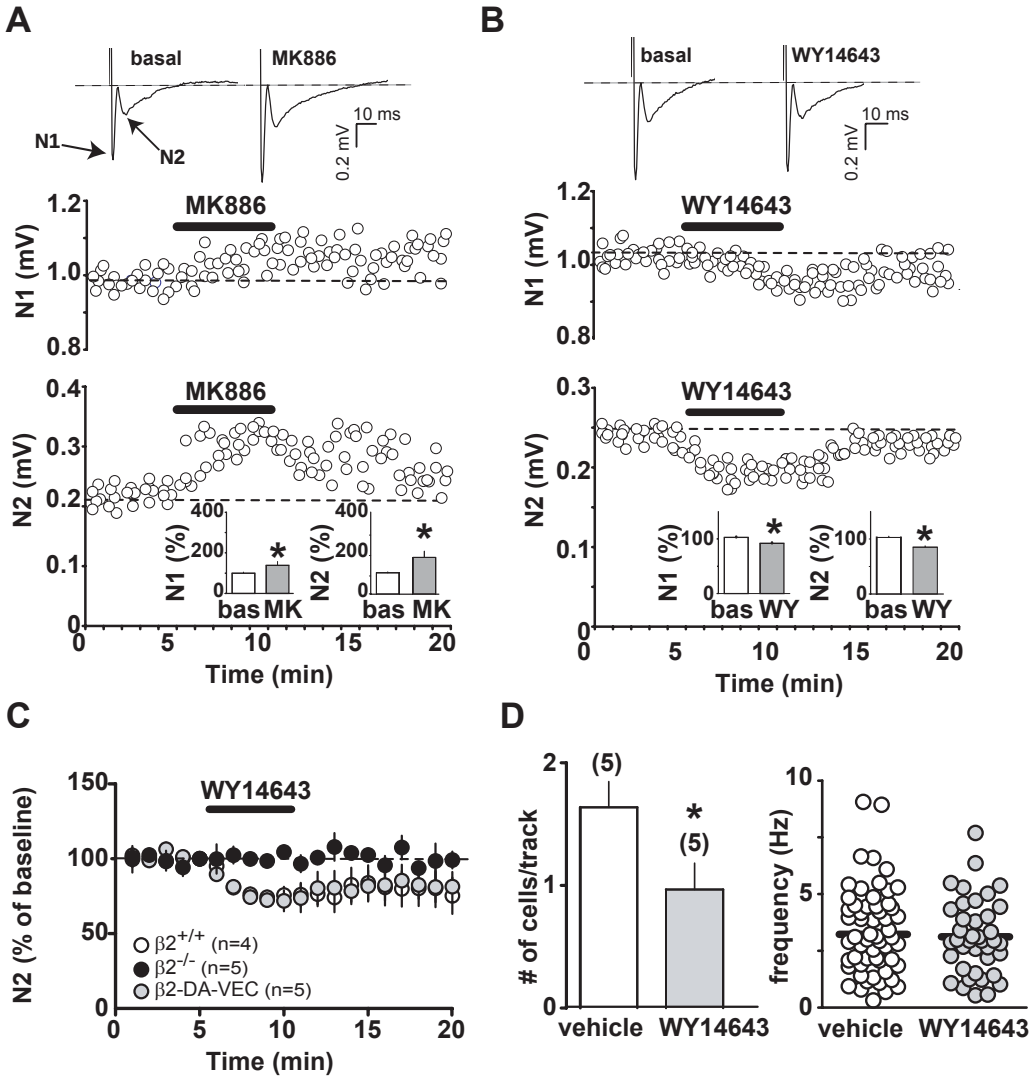

Figure 17. PPARα activation reduces VTA output i*n vitro* and *in vivo*. (**A** and **B**). Typical evoked field potential recordings showing effects of MK886 (0.5 μM, **A**) and WY14643 (1 μM, **B**) on field potential amplitude. Bin= 10 s. Traces from typical experiments (top), time-courses of the effects of MK886 and WY14643 on N1 (middle) and N2 (bottom) components, and the mean averaged responses (bar graph in insets) are shown. (**C**) Averaged N2 amplitude from the VTA of $\beta2^{-/-}$, $\beta2^{+/+}$ and β2-DA-VEC mice in response to WY14643 (1 μM). Bin= 1 min. (**D**) WY14643 (40 mg/kg i.p.) decreases the number of VTA DA cells encountered during neuronal sampling in anesthetized rats (left panel), but not averaged firing frequency (right panel). Data expressed as mean ± SEM. *$p < 0.05$.

4.4. Behavioral correlate of PPARα modulation of nAChR stimulation: physiological relevance (Paper II and unpublished data).

Since midbrain DA neuron activity is thought to be a substrate for nicotine-induced increases in spontaneous activity, we investigated whether activation of PPARα was involved in control of locomotion. Nicotine (0.02 mg/kg s.c.) was tested for its effects on locomotor activity of vehicle- and WY14643-treated mice. Figure 18 shows that in WY14643-treated mice (40 mg/kg i.p., 60 min before nicotine) nicotine-induced locomotor stimulation was markedly reduced ($F_{1,112}$=11.60, P=0.0043, two-way ANOVA).

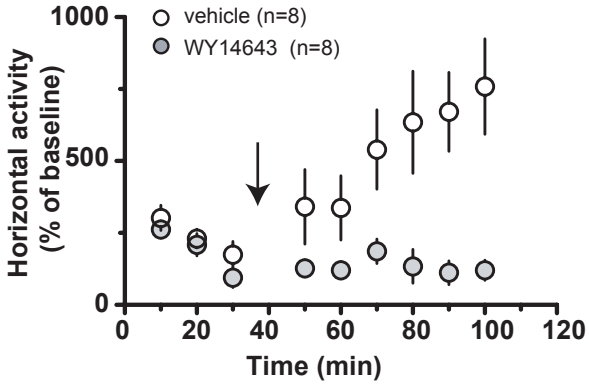

Figure 18. PPARα activation reduces nicotine-induced effects on locomotion. Time-course curve of nicotine (0.02 mg/kg s.c.) effects on locomotor activity in WY14643- and vehicle- treated mice. Arrows indicate time of nicotine administration. Data expressed as mean ± SEM.

Because β2-nAChRs on VTA DA neurons are critical for nicotine-induced locomotor activation (King et al., 2004; Mineur et al., 2009), nicotine effects were tested on locomotion in $\beta2^{-/-}$ and β2-DA-VEC mice. Figure 19 shows that re-expression of β2-nAChRs limited to DA cells of the VTA is sufficient to restore nicotine-dependent locomotor activation, which was absent in $\beta2^{-/-}$ mice ($F_{1,96}$=9.16, P=0.01, two-way ANOVA). Remarkably, when we investigated whether PPARα activation was involved in control of locomotion by β2-nAChRs, we observed that in WY14643-treated β2-DA-VEC mice nicotine-induced locomotor stimulation was markedly reduced ($F_{1,96}$=5.02, P=0.04, two-way ANOVA, Fig.19) and comparable with $\beta2^{-/-}$ mice ($F_{1,96}$=0.49, P=0.5, two-way ANOVA, Fig. 19). Notably, when WY14643 (40 mg/kg i.p.) was tested for its effects on

spontaneous locomotor activity, we observed no effect on this behaviour (40 mg/kg i.p. vs vehicle: $F_{1,70}$=2.41, P=0.1427, two-way ANOVA; data not shown).

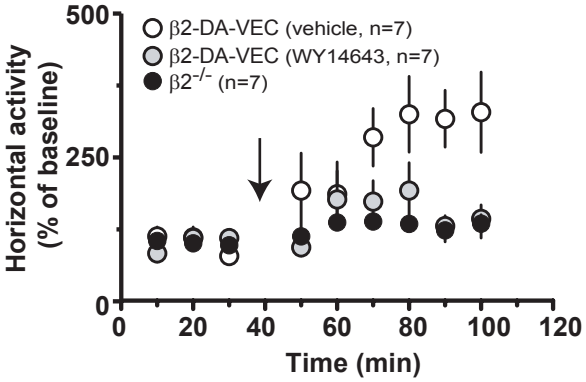

Figure 19. PPARα modulation of β2-nAChRs on VTA DA cells reduces nicotine-induced stimulation of locomotion. Time-course curve of nicotine (0.02 mg/kg s.c.) effects on locomotor activity in WY14643- and vehicle- treated β2-DA-VEC mice compared with $\beta2^{-/-}$ mice. Arrows indicate time of nicotine administration. Data expressed as mean ± SEM.

Prepulse inhibition of the acoustic startle response (PPI) has been proposed to act as a filtering mechanism to modulate responses to non-salient stimuli (Braff et al., 1995), and deficits in PPI appear to be related to stress (Grillon and Davis, 1997) as a result from an increased general level of alertness facilitating the processing of the prepulse.

Because β2-nAChRs play a crucial role in mediating the switch from "basal" to "excited" states of VTA DA neurons (Mameli-Engvall et al., 2006), PPARα activation might make DA neurons less sensitive to external information. To test whether PPARα activation would protect animals by sensorimotor gating deficits elicited pharmacologically by a dopaminergic drug (apomorphine, APO 0.25mg/kg/ml s.c.), we administered WY14643 (40 mg/Kg i.p.) 30 min before either APO or saline treatment. Figure 20 shows that WY14643 robustly attenuated PPI in rats, relative to vehicle-treated controls (WY+APO vs APO, $F_{1,8}$=8.43, P=0.019, two-way ANOVA; VEH+APO vs VEH+VEH, $F_{1,5}$=87.13, P=0.0002, two-way ANOVA).

But how PPARα signaling might modulate PPI is a critical issue. In fact, the time lag of the behavioral observations (30 min after systemic administration) does not necessarily rule out the involvement of transcriptional changes. However, since in principle the effects occurring after 10-20 min can be attributed to non genomic mechanisms, we measured FAE levels in midbrain slices during exposure (15-60 min) to WY14643 (3 μM). Figure 21 (A,B) shows that PPARα activation leads to increased levels of OEA and PEA (OEA: $F_{3,8}$=20.35, P=0.0004, one-way ANOVA; PEA:

$F_{3,8}$=19.19, P=0.0005, one-way ANOVA), but not AEA ($F_{3,8}$=2.84, P=0.1, one-way ANOVA; Fig. 22). The effect is time dependent and could involve a positive feedback (Barbier et al., 2004), where activation of PPARα leads to increased synthesis of its endogenous ligands (Fiedler et al., 2001), which would result in an enhanced negative modulation of β2-nAChRs and might help explain the consequent relevant behavioral changes.

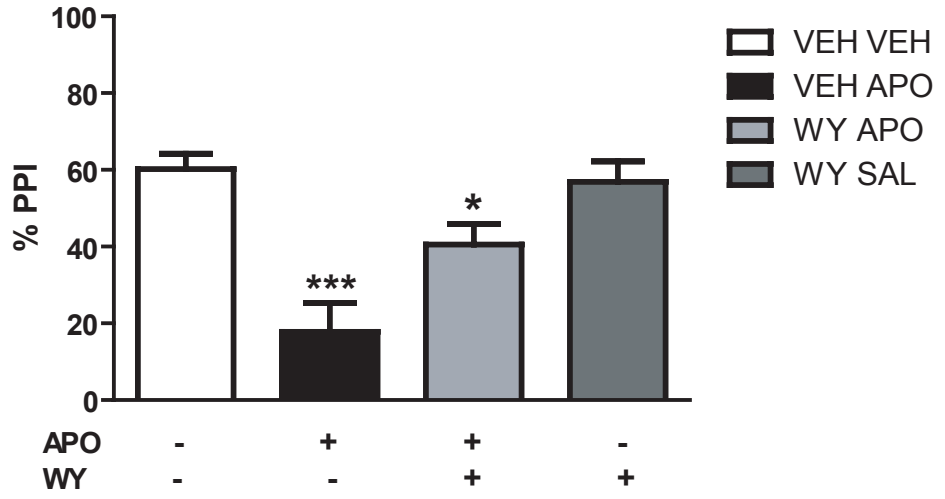

Figure 20. PPARα prevent apomorphine-induced disruption of PPI. Acute systemic administration of the PPARα synthetic agonist, WY14643 (WY 40 mg/kg), attenuates PPI in Sprague Dawley rats when administered as a single, intraperitoneal injection. Drug was administered 30 min before behavioral testing. Mean percentage prepulse inhibition (PPI) are represented in bars ± SEM. **p* <0.05, *** *p* <0.0005 (two-way ANOVA).

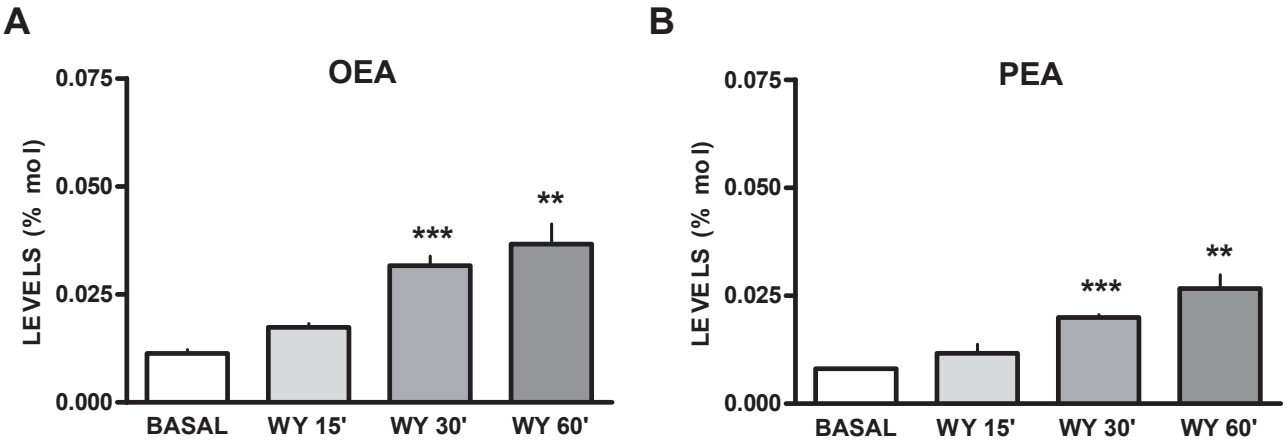

Figure 21. Time course of OEA and PEA levels in horizontal midbrain slices following incubation of WY14643 (3 μM). Values (mean ± SEM) are expressed as percentage molarity (nmol of FAE/nmol total lipids). **p* < 0.05 one-way ANOVA.

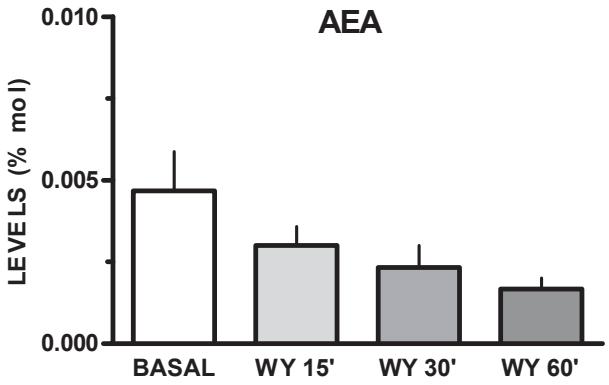

Figure 22. Time course of AEA levels in horizontal midbrain slices following incubation of WY14643 (3 μM). Values (mean ± SEM) are expressed as percentage molarity (nmol of AEA/nmol total lipids). $p > 0.5$ one-way ANOVA.

5. DISCUSSION

The main finding of the present thesis is that FAEs by engaging the nuclear receptors PPARα significantly contribute to the effects of endogenous cholinergic transmission mediated by β2-nAChRs on DA neuron excitability. As a result, activation of PPARα by FAEs can prevent the effects of nicotine on both DA neuron firing rate and locomotor activity, as well as modulate sensorimotor gating as assayed by PPI.

In 2008 this was, to our knowledge, the first evidence of an important functional role of this family of nuclear receptor transcription factors in the brain. Importantly, the findings of the present thesis also highlight the role of those FAEs devoid of cannabinoid actions (e.g. OEA, PEA) in the regulation of neuronal functions. In fact, the roles of these nuclear receptors in neuronal physiology and/or in the pathophysiological mechanisms of brain disorders are still largely unknown, and have long been overlooked, although they are ubiquitously distributed in the CNS (Moreno et al., 2004).
Noteworthy, the discovery that both OEA and PEA, but not mAEA, block the effects of nicotine on DA neurons *in vitro,* and that these results were mimicked by the synthetic agonist of PPARα (i.e. WY14643) and prevented by the PPARα antagonist (i.e. MK886), shed the light into the many actions played by the noncannabinoid FAEs. More interestingly, the finding that the effects of the tyrosine kinase inhibitor genistein, similarly to catalase, restored neuronal responses to nicotine suggested that nAChR phosphorylation and hydrogen peroxide production could account for the blockade operated by PPARα activation. Additionally, the present thesis supports the idea of a constitutive interaction between PPARα and tyrosine kinases, which in turn may tonically control the ratio of phosphorylated/dephosphorylated nAChRs (Figure 23).
Although the present thesis does not provide direct evidence for β2-nAChRs to be phosphorylated following PPARα activation, this hypothesis is substantiated by pharmacological and genetic manipulations, which, although indirectly, support that PPARα negatively modulates β2-nAChRs in VTA DA cells through phosphorylation/dephosphorylation processes operated by tyrosine kinases and phosphatases. Hence, the whole cell patch clamp experiments show that PPARα blockade produces both an increased firing rate and an inward current in DA cells, which are blocked by DHBE, are absent in $\beta2^{-/-}$ mice and in the presence of sodium-orthovanadate, the general tyrosine phosphatase inhibitor. Conversely, a global decreased VTA DA cell excitability were also reported in $\beta2^{-/-}$ mice, thus supporting the hierarchical control by β2-nAChR of DA neuron activity (Mameli-Engvall et al., 2006). Accordingly, the present electrophysiological experiments show a decreased VTA net output following PPARα activation in juvenile rats and $\beta2^{+/+}$ mice, but not in $\beta2^{-/-}$ mice.

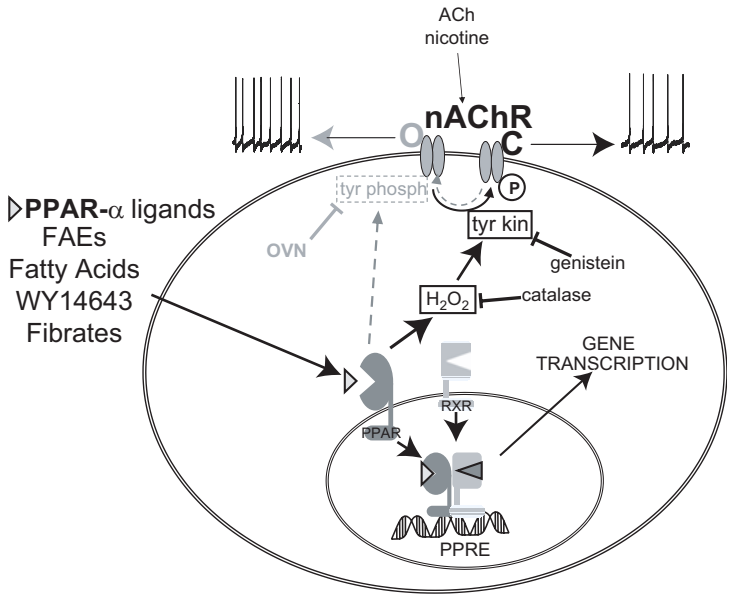

Figure 23. Schematic diagram illustrating the proposed mechanism of PPARα activation/blockade, and modulation of responses of DA neurons to nAChR agonists through a non-genomic mechanism. PPARα activation by endogenous and synthetic PPARα agonists (i.e. WY14643, fibrates) stimulate hydrogen peroxide production and subsequent activation of a tyrosine kinase (Tyr Kin). Tyrosine kinases, in turn, phosphorylate (P) of nAChRs, with a reduced response to the agonists and/or rapid internalization of nAChRs (C=closed state). Genistein, a general tyrosine kinase inhibitor, blocks the effects of PPAR-α activation. Catalase prevents downstream hydrogen peroxide actions. On the other hand, PPARα blockade (i.e. MK886, GW6471) prevents endogenous ligands (e.g. OEA, PEA) to act. Consequently, the balance between phosphorylated/dephosphorylated status of nAChRs is shifted towards "dephosphorylated nAChRs" through activation of a tyrosine phosphatase (tyr phosph) with a resulting enhanced response to nAChR agonists (i.e. Ach, nicotine) and/or promote their rapid expression on cell surface (O= open state)). Sodium Orthovanadate (OVN), a general tyrosine phosphatase inhibitor, prevents the effects of PPAR-α blockade. ↓ : activation, ⊥: inhibition. Dashed grey arrows: inhibited pathway (adapted from Pistis and Melis, 2010).

Remarkably, restoration of β2-nAChR only in the DA cells within the VTA re-established effects of PPARα activation, thus indicating β2-nAChRs as the ultimate targets of FAEs through PPARα in VTA DA neurons. Additionally, nicotine effects can be blocked and restored by inhibiting tyrosine kinase and phosphatase, respectively. Also, in agreement with the hypothesis that the number of functional cell surface nAChRs can be indirectly controlled through processes involving tyrosine phosphorylation (Wiesner and Fuhrer, 2006), we found that when the balance between active and inactive PPARα was shifted towards the inactive, both endogenous ACh and nicotine responses were enhanced, as if the β2-nAChRs were in an open state.

The conclusion that FAEs through PPARα activation might help in controlling state and/or number of β2-nAChRs is compatible with the distribution of PPARα in the midbrain (Kainu et al., 1994; Cullingford et al., 1998; Galan-Rodriguez et al., 2009), and provides a functional correlate for their cytoplasmic expression (Galan-Rodriguez et al., 2009). Indeed, although belonging to the family of nuclear receptor transcription factors (Zhu et al., 2000), non-genomic actions of PPARα activation have also been described (Barbier et al., 2004; Gardner et al., 2005; Scatena et al., 2007;

Melis et al., 2008; Ropero et al., 2009), and likely mediate the rapid-onset effects described in the present thesis. It can be, therefore, speculated that OEA and PEA -and more generally noncannabinoid FAEs- 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). We, therefore, are tempted to describe an intriguing scenario where acetylcholine and FAEs reciprocally control each other. Thus, this novel mechanism of regulation of β2-nAChRs by FAEs through activation of PPARα may represent a new therapeutic avenue for the discovery of medications involving unbalance between the DA and cholinergic systems.

Our hypothesis is substantiated in the present thesis by the changes in number of spontaneously active DA neurons *in vivo* following PPARα activation. This latter is consistent with both the decreased spontaneous activity of individual VTA DA neurons and the diminished VTA net output observed *in vitro*. Additionally, a functional consequence of modulation of DA neuron excitability by PPARα was the reversal of nicotine-induced increased locomotor activity by administration of a synthetic PPARα ligand as well as the blockade of nicotine-induced excitation of DA cells *in vitro*. Lastly, in the PPI assay, animals pretreated with PPARα agonist recovered significantly from the apomorphine-induced disruption of PPI. Taken altogether, these observations could even lead to conjecture that engagement of PPARα may have protective effects against the behavioral changes produced by DA agonist, and that PPARα agonists may elicit antipsychotic effects. While drawing a similar deduction might sound extreme, one cannot ignore that unexpected resolution of longstanding schizophrenic symptoms have been reported after starting a ketogenic diet (KD: high fat/adequate protein/low carbohydrate) (Pacheco et al., 1965; Kraft and Westman, 2009). Remarkably, both brain and peripheral tissue levels of FAEs change with high fat diets (Artmann et al., 2008; Hansen et al., 2009). Particularly, a 30% fat diet enriched in olive oil directly raises rat brain OEA (Artmann et al., 2008). Thus, one can predict that changes to fatty acid profile following KD may also directly modify FAE levels, which in turn would behave like brain PPARα-activating molecules (Cullingford, 2008). Accordingly, dietary fat, rather than protein or carbohydrate, stimulates OEA production, thus translating lipid nutrients into cellular lipid signals (Schwartz et al., 2008). This might finally provide one plausible mechanism, among others, for the hitherto unsolved issue of how KD exerts anticonvulsant and antiepileptogenic actions. Hence, KD has been shown to diminish neuronal excitability of dentate gyrus and proved to be both anticonvulsant and antiepileptogenic (Bough et al., 2003; Bough and Rho, 2007). Further, both fenofibrate and KD exerted anticonvulsive properties, thus suggesting a common action on a PPARα-driven pathway (Porta et al., 2009). Accordingly, whole-cell patch clamp recordings from rat layer II/III cortical pyramidal cells revealed a protective effect of PPARα ligands in the nicotine-enhanced frequency

of spontaneous inhibitory postsynaptic current (sIPSC) (Melis and Pistis, unpublished observations), thus suggesting a plausible therapeutic application in the model of epileptogenesis in which acetylcholine significantly enhances cortical GABAergic transmission (Xiang et al., 1998; Klaassen et al., 2006). Negative modulation of α4β2-nAChRs by PPARα activation would, thus, prove particularly beneficial in this latter model (Klaassen et al., 2006), which is featured by asynchronously firing of layer II/III pyramidal cells synchronized after recovery from a large GABAergic inhibition triggered by cholinergic activation of "hyperactive" α4β2-nAChRs.

The physiological relevance of the present findings lies in the dynamic interplay between the dopaminergic and cholinergic systems in major psychiatric disorders (Maskos, 2008; Rahman et al., 2008; Terry, 2008). Our current hypothesis is that FAEs, acting as small bioactive lipid molecules at PPARα, with consequent hydrogen peroxide production and activation of tyrosine kinase(s), are ultimately responsible for phosphorylation of β2-nAChRs. Accordingly, the cellular effects resulting from PPARα activation, such as decreased DA cell spontaneous activity and blockade of nicotine-induced excitation of DA neurons, are fully prevented when hydrogen peroxide half-life is reduced by raising the intracellular levels of catalase. Importantly, PPI was also acutely attenuated by the potent and highly selective ligand to PPARα, WY14634. But how might PPARα signaling modulate PPI is currently under investigation. Notably, the time lag of our behavioral observations does not rule out the involvement of transcriptional changes. Noteworthy, it has been previously reported that in rodents treatment with synthetic PPARα activators, such as WY14634, results in enhanced levels of endogenous PPARα activators in tissues such as liver, kidney and heart (Lee et al., 1995; Fiedler et al., 2001; Johnson et al., 2002; Barbier et al., 2004). Therefore, the behavioral changes should be interpreted with caution as regards the mechanism of action, since PPARα activation controls the synthesis of enzymes catalyzing its endogenous ligand synthesis only thorugh its "classic" mechanism. Nonetheless, given that β2-nAChRs play a crucial role in mediating the switch from "basal" to "excited" states of VTA DA neurons (Mameli-Engvall et al., 2006), the present thesis supports the role played by these receptors as a gate enabling DA cells to respond to excitatory afferents and, thereby, to switch between tonic and phasic activity (Maskos, 2008). Thus, we currently hypothesize that engagement of PPARα by FAEs, by making DA neurons less sensitive to external information, might translate into prevention of an erroneous attribution of saliency to otherwise irrelevant stimuli/events. Ultimately, FAEs through PPARα activation might modulate the mesolimbic DA pathway by decreasing the number of spontaneously active VTA DA neurons and, therefore, prove beneficial in those pathophysiological conditions,

such as stress or psychiatric disorders such as schizophrenia, attention-deficit hyperactivity disorder and binge eating, where a dysfunctional DA system plays a prominent role.

Targeting PPARα might, thus, represent a promising therapeutic approach to prevent relapse to nicotine, which is often related to stress and/or incentive stimuli, which become conditioned once associated with previous drug use. Notably, a trend for the common NAPE-PLD haplotype has been recently reported to be protective not only for severe obesity but also for smoking (Wangensteen et al., 2010). It can be, therefore, speculated that the common NAPE-PLD haplotype might confer allostatic changes to adjust the set point and to grant less vulnerability to both severe obesity and tobacco smoking. Notably, Wangensteen et al (2010) did not consider noncannabinoid FAEs acting through PPARα as responsible for the significant clinically relevant genetic protective factor for both severe obesity and nicotine dependence. However, to our knowledge, OEA has been recognized as an important satiety factor (Rodriguez de Fonseca et al., 2001; Fu et al., 2003; Gaetani et al., 2003; Oveisi et al., 2004; Lo Verme et al., 2005; Fu et al., 2007; Schwartz et al., 2008; Gaetani et al., 2010) and proposed as a valuable medication for the treatment of tobacco dependence (Melis et al., 2008; Mascia et al., 2010).

Finally, the present thesis not only demonstrates that the actions of FAEs are not restricted to the periphery, but also suggests that modulation of neuronal responses to nicotine by noncannabinoid FAEs may represent an interesting extension of their peripheral biological properties. In fact, their central effects have long been neglected due to their poor ability to cross the blood brain barrier (but see Campolongo et al. 2009) and to their instability (Kilaru et al., 2010). Though additional research is needed to confirm the association between dietary change, metabolic consequences and the amelioration of symptoms in nicotine smokers and schizophrenic patients, we can no longer deny the fact that changes in brain lipid metabolism and/or composition, as well as variations of the levels of endogenous lipid signaling molecules exert unsuspected actions on neurotransmitter functions, which might be exploited therapeutically through nutritional and pharmacological strategies.

6. ACKNOWLEDGMENTS

I would like to express my gratitude to the many people who contributed to this thesis.

I wish to thank:

My supervisor Professor Marco Pistis for sharing ideas and providing me with the perfect working conditions, exciting discussions, endless optimism during our struggles with the publication of the papers, and for his bright, enduring and friendly support and contributions to my work. I appreciated his advice, comments, and readiness to discuss any questions or ideas that I have had.

My mentor Professor Gian Luigi Gessa, who, with his long lasting enthusiasm for science blended with ambition, geniality, generosity and great sense of humour, has taught me the Dos and Don'ts in science as well as to challenge conservative ideas.

Past and present lab-mates of the Pistis lab for keeping up the wonderful atmosphere of the lab. Particularly, Anna Lisa Muntoni, as a stand-in big sister figure, for her friendly support and patience, for honoured me wisdom, for teaching me to not be afraid of being late, and for the many fascinating scientific and less scientific, though insightful, discussions. Stefano Carta, Salvatore Lecca and Antonio Luchicchi for providing a stimulating and fun work environment as well as for their routine mutual aid. It has been a pleasure to share with them the doctoral studies along with the glory and sadness of day-to-day research.

Researchers at the Department of Neuroscience for making work more enjoyable. Particularly, Liana Fattore and Roberto Frau for their trust and friendship, and for sharing enthusiasm for my work, to which they contributed with their behavioral observations.

Professor Sebastiano Banni, who provided me with the FAE measurement data that I regard as so important to add them in the present thesis. With his enthusiasm and great efforts to explain things clearly and simply, he helped to make the world of fatty acids fun for me.

My parents, whose foresight and values paved the way for a privileged education, for their unconditioned love and unwavering faith in me at all the times. Particularly, my dad for teaching me to not be afraid of challenging conservative theories and of their supporters, and my mom for teaching me to always look at the half full cup.

My family William T., Eleonora and William for their love and support, and for brightening up the day when it was needed. To William T. Dunn III I dedicate this thesis.

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