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Peroxisome proliferator-activated receptors- α modulate dopamine cell activity through nicotinic receptors

Miriam Melis¹, Stefano Carta¹, Liana Fattore², Stefania Tolu³, Sevil Yasar⁴, Steven R. Goldberg⁵, Walter Fratta^{1,2}, Uwe Maskos³, and Marco Pistis¹

¹B.B. Brodie Department of Neuroscience, University of Cagliari, 09042 Monserrato, Italy

²CNR, Neuroscience Institute, Cagliari, Italy

³Unité Neurobiologie intégrative des systèmes cholinergiques, CNRS URA 2182, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15, France

⁴Division of Geriatric Medicine and Gerontology, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21224, USA

⁵Preclinical Pharmacology Section, Behavioral Neuroscience Research Branch, Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Department of Health and Human Services, Baltimore, MD 21224, USA

Abstract

Background—Modulation of midbrain dopamine neurons by nicotinic acetylcholine receptors (nAChRs) plays an important role in behavior, cognition, motivation and reward. Specifically, nAChRs containing $\beta 2$ subunits ($\beta 2$ -nAChRs) switch dopamine cells from a resting to an excited state. However, how $\beta 2$ -nAChRs can be modulated and thereby dopamine firing activity be affected is still elusive. Because changes in dopamine 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- α (PPAR α) have been recently found to suppress nicotine-induced responses of dopamine neurons.

Methods—We used both *in vitro* and *in vivo* electrophysiological techniques together with behavioral analysis to investigate on the effects of modulation of PPAR α in Sprague Dawley rat and C57BLJ/6 mouse dopamine neurons, and their interactions with $\beta 2$ -nAChRs. To this aim, we took advantage of a selective re-expression of $\beta 2$ -nAChR exclusively in dopamine cells by stereotactically injecting a lentiviral vector in the mouse ventral tegmental area.

Results—We found that activation of PPAR α decreases *in vitro* both dopamine cell activity and ventral tegmental area net output through negative modulation of $\beta 2$ -nAChRs. Additionally, PPAR α activation *in vivo* reduces both the number of spontaneously active dopamine neurons and nicotine-induced increased locomotion.

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Corresponding authors: Miriam Melis, “B.B. Brodie” Department of Neuroscience, University of Cagliari, Cittadella Universitaria, 09042 Monserrato (CA), Italy, myriam@unica.it, **Phone:** +390706754340, **Fax:** +390706754320.

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Conclusions—Our combined findings suggest PPAR α ligands as important negative modulators of β 2-nAChRs on dopamine neurons. Thus, PPAR α ligands might prove beneficial in treating those disorders where dopamine dysfunction plays a prominent role, such as schizophrenia and nicotine addiction.

Keywords

acetylcholine; dopamine neuron; nicotine; patch-clamp; peroxisome proliferator-activated receptor; ventral tegmental area

Introduction

Dopamine (DA) neurons of the ventral tegmental area (VTA) project to subcortical and cortical structures to form the mesocorticolimbic pathway. VTA DA neurons detect primary rewards, novel and reward-predicting stimuli (1,2), and respond to these with changes in their firing activity (3), resulting in phasic DA transmission in target regions where the signal is integrated and translated into learned appetitive behaviors (4). VTA DA cell function is shaped in response to both behaviorally relevant events and drugs of abuse, including nicotine. VTA DA cell firing pattern is controlled by extrinsic afferents, among which the excitatory inputs play a major role (5,6). The excitatory projections to the VTA originate from the prefrontal cortex, the bed nucleus of the stria terminalis, and the cholinergic laterodorsal and pedunculopontine tegmental nuclei (7–9). These latter make synaptic contacts with VTA DA cells (10), which express both muscarinic and nicotinic receptors (11,12), and whose activation leads to increases in DA neuron activity (13,14). Importantly, endogenously produced acetylcholine (ACh) controls DA neuron firing pattern and frequency through activation of nicotinic receptors (nAChRs) (15), and an imbalance between DA/ACh function is often associated in diverse brain disorders (7,16–19).

Nicotine-induced increase in DA neuron firing rate can be suppressed by ligands for the peroxisome-proliferator-activated receptor- α (PPAR α) (20), a family of nuclear receptor transcription factors involved in the modulation of various peripheral physiological responses, such as lipolysis, inflammation and energy balance (21). PPAR α -induced phosphorylation of nAChRs accounts for the suppression of DA neuron responses to nicotine (20). Because the effects of nicotine on DA neurons were enhanced when PPAR α were blocked (20), we hypothesized a constitutive interaction between PPAR α and tyrosine kinases regulating the function of nAChRs in VTA DA cells. Thus, tonic control of the ratio of phosphorylated/dephosphorylated nAChRs might allow DA neurons to have access to distinct firing patterns and/or to change their firing frequency.

To explore the possible role of PPAR α in the regulation of DA neuron excitability by endogenous acetylcholine, we investigated interactions between nuclear PPAR α and surface nAChRs in VTA DA cells both *in vitro* and *in vivo*, and the functional consequences of PPAR α activation on nicotine-induced increases in spontaneous locomotion, a DA-mediated behavior.

Methods and Materials

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), 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.

Electrophysiological studies in vitro

Whole cell patch clamp recordings from VTA DA cells were as described previously (22) and Supplement 1. Briefly, male Sprague Dawley rats (Harlan Nossan, San Pietro al Natisone, Italy) or $\beta 2^{-/-}$ and $\beta 2^{+/+}$ mice (see below for detailed information) were anesthetized with halothane and killed. Recordings were made from horizontal slices superfused with artificial cerebrospinal fluid (ACSF, 37° C) saturated with 95% O₂ and 5% CO₂ containing (in mM): 126 NaCl, 1.6 KCl, 1.2 NaH₂PO₄, 1.2 MgCl₂, 2.4 CaCl₂, 18 NaHCO₃, and 11 glucose. Evoked field potential recordings were as described previously (23) and Supplement 1.

All the drugs were dissolved in DMSO. The final concentration of DMSO was < 0.01 %.

Electrophysiological study in vivo

Extracellular single unit recordings from male Sprague Dawley rat VTA DA neurons were performed as described previously (24) and Supplement 1. Single unit activity of DA neurons located in VTA was recorded extracellularly with glass micropipettes filled with 2% pontamine sky blue dissolved in 0.5 M sodium acetate. Single units were isolated and identified according to the already published criteria (20).

Mice

A line expressing Cre recombinase under the control of the dopamine transporter promoter, line DAT-Cre (Tronche), 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^{−/−}.

Lentiviral expression vector and stereotaxic procedure

pTripPDGF lentivector (25) was modified by replacing the eGFP with the $\beta 2$ nAChR sequence. Lentivirus was bilaterally injected (26) at a dose of 230ng p24 protein/2 μ L into the VTA of $\beta 2^{-/-}$ /DAT-Cre transgenic mice, generated by backcrossing $\beta 2^{-/-}$ with DAT-Cre mice (27). Injection of the vector into $\beta 2^{-/-}$ /DAT-Cre mice leads to recombination and subsequent expression of the $\beta 2$ subunit exclusively in DA neurons (25).

Behavioral study

Mice were pretreated with either WY14643 (40 mg/kg, i.p.) or its vehicle (10 ml/kg, i.p.) 60 min before nicotine administration (0.02 mg/kg, s.c.) and individually tested for motor activity under standardized environmental conditions as previously described (28) and Supplement 1.

Statistical analysis

Numerical data are given as mean $\pm$ S.E.M.. Data were compared and analyzed through two-way ANOVA for repeated measures, or one-way ANOVA or Student's t-test, when appropriate.

Results

The PPAR α antagonist MK886 enhances DA neuron spontaneous activity

To characterize the postsynaptic effects on DA neurons, all experiments were performed in the presence of CNQX (10 μ M), D-AP5 (100 μ M) and picrotoxin (100 μ M), in order to block AMPA-, NMDA- and GABA_A-mediated postsynaptic responses. Whole-cell current- and voltage- clamp recordings were performed from medial posterior VTA DA neurons in rat horizontal slices containing the midbrain. DA cells displayed electrophysiological

characteristics that facilitated their identification (29). Figure 1(A) shows that bath application of the PPAR α 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 ($V_{\text{holding}}=-70$ mV), MK886 caused an inward current of 93.3 ± 29.3 pA (Figure 1B). Both effects were reversible on wash out and blocked by the synthetic PPAR α agonist WY14643 (Figure 1B,C; 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) that is ineffective (20). MK886 effect was concentration-dependent (Figure 1D; 100.8 ± 6.8 % and 434.9 ± 69.6 % of baseline at 0.3 and 1 μM , respectively), and mimicked by the structurally dissimilar PPAR α antagonist GW6471 (0.3–1 μM) (Figure 1D; 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).

PPAR α blockade induces dephosphorylation of nAChRs and increases efficacy of nAChR agonists

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 $\alpha 7$ subtype nAChRs) or dihydro- β -erythroidine (DHBE, a competitive antagonist for neuronal $\alpha 4$ containing nAChRs). Figure 2(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 that is ineffective on DA cell firing rate (30); 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 2C; MK886+DHBE: -3.857 ± 18.9 pA, $P=0.018$), suggesting that most, if not all, of responses in DA neurons to MK886 were due to activation of $\alpha 4$ subunit containing nAChRs. Since the $\alpha 4$ subunit generally assembles with the $\beta 2$ subunit, we hypothesized that most of the effects would be mediated by postsynaptic $\alpha 4 \beta 2$ -nAChRs. Therefore, we examined MK886 effects in $\beta 2$ -nAChR knock out ($\beta 2^{-/-}$) mice. MK886-induced effects were absent in $\beta 2^{-/-}$ mice (Figure 2D; two-way ANOVA $F_{1,152}=8.65$, $P=0.018$; $t=5.1$, $P=0.0005$, unpaired t-test), whereas its effects in $\beta 2^{+/+}$ mice were comparable to those observed in rats. 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 (30) 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 we compared the MK886-induced current with the one produced by nicotine (1 μM) under the same conditions, we found a strong correlation between the currents induced by both MK886 and nicotine at all membrane potentials (Figure 2E), particularly at resting membrane potential (i.e. -60 mV), thus suggesting a common site of action.

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 (31). 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 (32). OVN prevented MK886-induced inward currents (Figure 3A; -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; data not shown), thus indicating that it blocked

phosphatases downstream of PPAR α . Thus, our observations suggest that MK886 does not directly act on nAChRs, but increases sensitivity of high-affinity nAChRs to endogenous ACh in midbrain DA neurons. Accordingly, when we enhanced endogenous ACh actions at nAChRs by applying both the ACh esterase inhibitor neostigmine (2 μ M) and the muscarinic antagonist atropine (5 μ M), we observed an increased DA cell firing rate (Figure 3B; 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 μ M, 5 min) (Figure 3B: 161.3 ± 5.5 % of basal, $t=7.53$, $P=0.008$, paired-t test). Consistently, the effects of nicotine (0.1–5 μ M) were strongly enhanced in the presence of MK886 (0.3 μ M) (Figure 3C: for example, nicotine 0.1 μ M: 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 μ M: 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).

Activation of PPAR α modulates DA neuronal activity through hydrogen peroxide production

To investigate the role of PPAR α in more detail, we then tested the effects of an endogenous ligand for PPAR α oleoylethanolamide (OEA) on DA cell firing. OEA (10 μ M) decreased DA neuronal discharge (Figure 4A,B: $F_{19,60}=17.03$, $P<0.0001$, one-way ANOVA) in a concentration-dependent fashion (Figure 4D: 3 to 30 μ M; 3 μ M: 98.2 ± 8.6 %, $n=4$; 10 μ M: 27.7 ± 16.5 %, $n=4$; 30 μ M: 20.0 ± 5.8 %, $n=5$), an effect reversed by MK886 (Figure 4C: 500 nM, 3 min; $n=5$, $F_{1,133}=32.40$, $P=0.0007$, two-way ANOVA). The OEA effect was mimicked by two structurally different PPAR α agonists, WY14643 (0.3–3 μ M) and fenofibrate (1–10 μ M) (Figure 4D). Fenofibrate was the least potent (1 μ M: 134.2 ± 30 %, $n=6$; 10 μ M: 42.1 ± 23.8 %, $n=6$; 30 μ M: 24.3 ± 4.9 %, $n=5$), and WY14643 the most potent (0.3 μ M: 75.8 ± 15.5 %, $n=5$; 1 μ M: 17.9 ± 5 %, $n=5$; 3 μ M: 5.9 ± 0.5 %, $n=4$). In addition, under voltage-clamp mode, the three PPAR α agonists also produced outward currents (Figure 4E; OEA 10 μ M: 45.4 ± 5.7 pA; WY14643 1 μ M: 27.3 ± 5.1 pA; fenofibrate 3 μ M: 43.6 ± 7.05 pA), which were blocked by MK886 (Figure 4E). MK886 also reversed the effects produced by the two agonists on DA cell frequency (Figure 4F; WY14643: $F_{1,171}=7.83$, $P=0.02$, two-way ANOVA; fenofibrate: $F_{1,190}=5.07$, $P=0.04$, two-way ANOVA). Activation of PPAR α has been shown to increase intracellular concentrations of reactive oxygen species (33), 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 (34). Because hydrogen peroxide can also activate a tyrosine kinase (35), we tested the possibility that it might be involved in PPAR α ligand actions by filling the DA cell with catalase (500 IU/ml), to rapidly inactivate hydrogen peroxide, and then bath applying OEA. Figure 5 (A,B) shows that, in the presence of catalase, OEA (10 μ M, 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 PPAR α . Importantly, because depletion of intracellular hydrogen peroxide by including catalase in the patch pipette increases the spontaneous firing rate of midbrain DA neurons (34), 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 (34), might be ascribed to a reduced hydrogen peroxide inhibitory physiological role. Nonetheless, if hydrogen peroxide production is also related to PPAR α activation, then one would expect that under these conditions the effects of nicotine on DA cell frequency would be restored. When nicotine (1 μ M, 2 min) was applied in the presence of both OEA (the lower dose of 3 μ M that is ineffective on firing rate (20), 7 min) and catalase (500 IU/ml), nicotine transiently increased the spontaneous activity of VTA DA neurons (Figure 5C,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 (14, 20). As previously reported (20), 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 $\beta 2$ -nAChRs more rapidly once PPAR α 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 PPAR α leads to hydrogen peroxide production and blocks nicotine effects on DA neuronal activity.

The effects of PPAR α on population spike amplitude in the VTA require the expression of $\beta 2$ -containing nAChRs in DA neurons

To ensure that PPAR α 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 (23). Figure 6 (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 μ M, 5 min) significantly reduced both components of the field potential (Figure 6B: 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 PPAR α 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 (36). Nonetheless, it should be noted that N2 component is the summation of stimulation-evoked postsynaptic responses of DA cells and of antidromic action potentials (36), and, therefore, represents both synaptic and population spike components. To further examine the role played by nAChRs in the effect of PPAR α activation, we bath applied WY14643 (1 μ M, 5 min) in $\beta 2^{-/-}$ mice. Figure 6 (C) shows that WY14643 failed to produce any effect on N2 amplitude when compared with $\beta 2^{+/+}$ mice ($F_{1,133}=6.61$, $P=0.03$, two-way ANOVA). Next, we took advantage of the $\beta 2^{-/-}$ mice in which the Cre-inducible switching on for $\beta 2$ -nAChR gene was allowed only in DA neurons, thus selectively re-expressing the corresponding nAChR subunit exclusively in VTA DA cells by stereotactically injecting a lentiviral vector ($\beta 2$ -DA-VEC mice)(25). Importantly, in $\beta 2$ -DA-VEC mice WY14643 effect was restored (Figure 6C: 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 $\beta 2^{+/+}$ 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 $\beta 2$ -nAChRs on DA cells of the VTA (25), but also to discriminate the contribution of DA neuronal activity to net VTA output (36), and, ultimately, of PPAR α .

PPAR α activation reduces the number of spontaneously active DA neurons in vivo

Because $\beta 2$ -nAChRs are key in controlling the firing rate/pattern of VTA DA cells (15), we examined whether PPAR α activation affected the number of spontaneously active VTA DA neurons *in vivo*. We performed neuronal sampling of the VTA 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 ($\pm$ 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 6D: $t=2.21$, $P=0.02$, unpaired t-test). The average spontaneous firing rate of DA cells was not

different in vehicle- and WY14643-treated rats (Figure 6D; $t=0.29$, $P=0.38$, unpaired t-test), in agreement with the ineffectiveness of the dose tested on firing frequency (20).

The PPAR α agonist blocks nicotine-induced locomotor activity

Since midbrain DA neuron activity is thought to be a substrate for nicotine-induced increases in spontaneous activity, we investigated whether activation of PPAR α was involved in control of locomotion. We tested nicotine (0.02 mg/kg s.c.) for its effects on locomotor activity of vehicle- and WY14643-treated mice. Figure 6 (E) 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). Because $\beta 2$ -nAChRs on VTA DA neurons are critical for nicotine-induced locomotor activation (37, 38), we tested nicotine effects on locomotion in $\beta 2^{-/-}$ and $\beta 2$ -DA-VEC mice. Figure 6 (F) shows that re-expression of $\beta 2$ -nAChRs limited to DA cells of the VTA is sufficient to restore nicotine-dependent locomotor activation, which was absent in $\beta 2^{-/-}$ mice ($F_{1,96}=9.16$, $P=0.01$, two-way ANOVA). Remarkably, when we investigated whether PPAR α activation was involved in control of locomotion by $\beta 2$ -nAChRs, we observed that in WY14643-treated $\beta 2$ -DA-VEC mice nicotine-induced locomotor stimulation was markedly reduced ($F_{1,96}=5.02$, $P=0.04$, two-way ANOVA, Fig. 6F) and comparable with $\beta 2^{-/-}$ mice ($F_{1,96}=0.49$, $P=0.5$, two-way ANOVA, Fig. 6F). 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).

Discussion

Here we show that PPAR α significantly contribute to the effects of endogenous cholinergic transmission mediated by $\beta 2$ -nAChRs on DA neuron excitability, and to the effects of nicotine on locomotor activity, a DA associated behavior. The changes in number of spontaneously active DA neurons *in vivo* following PPAR α activation are consistent with both the decreased spontaneous activity of individual VTA DA neurons and the diminished VTA net output observed *in vitro*. A functional consequence of modulation of DA neuron excitability by PPAR α was the reversal of nicotine-induced increased locomotor activity by administration of a synthetic PPAR α ligand in $\beta 2$ -DA-VEC mice. Results obtained with selective antagonists for $\alpha 4$ versus $\alpha 7$ nAChRs and from mice lacking the $\beta 2$ subunit further show that modulation of cholinergic transmission by PPAR α , and the resulting modulation of DA neuron excitability, is mediated by $\alpha 4\beta 2$ -nAChRs.

Our observations support a constitutive interaction between PPAR α and $\beta 2$ -nAChRs in VTA DA cells. Particularly, our behavioral experiments show that $\beta 2$ is a critical subunit for PPAR α effects. Indeed, re-expression of $\beta 2$ -nAChRs exclusively in VTA DA cells is sufficient to rescue nicotine effects on locomotion and, thereby, PPAR α actions. Accordingly, our electrophysiological experiments show a decreased VTA net output following PPAR α activation in rats, but not in $\beta 2^{-/-}$ mice. Remarkably, restoration of $\beta 2$ -nAChR only in the DA cells within the VTA re-established effects of PPAR α activation, thus indicating $\beta 2$ -nAChRs as targets of PPAR α ligands in VTA DA neurons. Also, our results confirm the versatility and reliability of the genetarget system to achieve lentiviral gene targeting exclusively in DA cells of the VTA (25). Additionally, our whole cell patch clamp experiments show that MK886 produces both an increased firing rate and an inward current in DA cells, which are blocked by DHBE, and are absent in $\beta 2^{-/-}$ mice. Conversely, a global decreased VTA DA cell excitability were also reported in $\beta 2^{-/-}$ mice, thus, suggesting a hierarchical control by $\beta 2$ -nAChR of DA neuron activity (15). Lastly, in agreement with the hypothesis that the number of functional cell surface nAChRs can be indirectly controlled through processes involving tyrosine phosphorylation (39), we found

that when the PPAR α were blocked, both endogenous ACh and nicotine responses were enhanced (Figure 3C,D).

The conclusion that PPAR α activation might help in controlling state and/or number of β 2-nAChRs is compatible with the distribution of PPAR α in the midbrain (40–42), and provides a functional correlate for their cytoplasmic expression (42). Indeed, although belonging to the family of nuclear receptor transcription factors (21), non-genomic actions of PPAR α activation have also been described (20,43,44), and likely mediate the rapid-onset effects described in the present study. Based on our findings, we propose both endogenous and synthetic (e.g. fibrates) PPAR α ligands as important negative modulators of β 2-nAChRs in the VTA. Endogenous ligands for PPAR α are endocannabinoids and their cognate compounds, specifically belonging to N-acyl-ethanolamine (NAE) group, such as OEA and palmitoylethanolamide (45), which are signaling molecules involved in the regulation of diverse physiological functions in both CNS and peripheral tissues. Like endocannabinoids, NAEs are synthesized *on demand* (46,47) and rapidly degraded (48). Both brain and peripheral tissue levels of NAEs change with high fat diets (49,50). Particularly, a 30% fat diet enriched in olive oil directly raises rat brain OEA (49). Thus, one can predict that changes to fatty acid profile following ketogenic diet (KD: high fat/adequate protein/low carbohydrate) may also directly modify NAE levels, which in turn would behave like brain PPAR α -activating molecules (51). Accordingly, KD diminished neuronal excitability of dentate gyrus and proved to be both anticonvulsant and antiepileptogenic (52). Further, both fenofibrate and KD exerted anticonvulsive properties, thus suggesting a common action on a PPAR α -driven pathway (53).

The physiological relevance of the present findings lies in the dynamic interplay between the dopamine and cholinergic systems in major psychiatric disorders (7,54,55). Our current hypothesis is that small bioactive lipid molecules acting at PPAR α , with consequent hydrogen peroxide production, are ultimately responsible for negative modulation of β 2-nAChRs. Hence, the cellular effects resulting from PPAR α 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.

β 2-nAChRs play a crucial role in mediating the switch from “basal” to “excited” states of VTA DA neurons (15), and serve as a gate enabling these cells to respond to excitatory afferents and, thereby, to switch between phasic and tonic activity (7). Thus, PPAR α activation, by making DA neurons less sensitive to external information, might translate into prevention of an erroneous attribution of saliency to otherwise irrelevant stimuli/events. Negative modulation of β 2-nAChRs located on VTA DA neurons might, 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. Additionally, targeting PPAR α might represent a promising therapeutic approach to prevent relapse to nicotine, which is often related to stress and/or conditioned stimuli associated with previous drug use.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

PPAR α 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 ($V_{\text{hold}} = -70$ mV) blocked by the PPAR α agonist WY14643 (0.3 μM). (C) WY14643 blocked MK886-induced activation of dopamine neurons. (D) Summary of dose-related effects of PPAR α antagonists on dopamine neuronal frequency. Numbers above bars indicate n values. Data expressed as mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.005$.

Figure 2.

MK886 enhances dopamine neuron activity through $\alpha 4\beta 2$ -nAChRs. (**A** and **B**) Time course of MK886 effect, alone or together with mecamylamine (MEC) (100 μ M), methyllycaconitine (MLA) (5 nM) or dihydro- β -erythroidine (DHBE) (1 μ M), 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 $\beta 2^{-/-}$ and $\beta 2^{+/+}$ mice under voltage-clamp (left panel) and current-clamp (right

panel) modes. Inset shows a representative MK886 effect. (E) *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 f plotted as a function of voltage membrane. Numbers above bars indicate n values. Data expressed as mean $\pm$ SEM. $*p < 0.05$, $***p < 0.0005$.

Figure 3.

PPAR α blockade increases efficacy of nAChR agonists. (A) Under voltage-clamp mode, MK886 effect on dopamine neuron in presence (grey) or absence (black) of sodium-orthovanadate (OVN). (B) Under current-clamp mode, effects of enhanced endogenous ACh levels acting at nAChRs (neostigmine+atropine) in presence or absence of MK886 (0.3 μ M). (C) Current-clamp recording of a dopamine neuron (left panel) showing enhanced nicotine response in presence of MK886 (0.3 μ M). 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 $\pm$ SEM. **p* < 0.05, ***p* < 0.005.

Figure 4.

PPAR α activation decreases VTA dopamine neuronal activity. (A) Current-clamp traces of a dopamine neuron before, during and after oleoylethanolamide (OEA) application (10 μ M). Rate histogram depicting averaged OEA effects on dopamine neuron frequency in absence (B) and presence (C) of MK886 (0.5 μ M). (D), Dose-response curves depicting averaged effects of PPAR α agonists on dopamine neuron frequency. (E), Under voltage-clamp mode, effects of OEA (10 μ M), WY14643 (WY; 1 μ M) and fenofibrate (FRB; 10 μ M) on dopamine neurons in absence (left panel) and presence (right panel) of MK886 (0.5 μ M). (F), Under current-clamp mode, MK886 (0.5 μ M) 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 $\pm$ SEM. $*p < 0.05$.

Figure 5.

Hydrogen peroxide is downstream effector of PPAR α . (A), Current-clamp recording of a dopamine neuron during bath application of OEA (10 μ M) in absence or presence of catalase (500 U/I). (B), Time course of averaged effects of OEA (10 μ M) 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 μ M) and OEA (3 μ M) application in the absence or presence of catalase. (D) Time course of OEA (3 μ M) 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 μ M) and catalase. Catalase was applied through the recording pipette. Data expressed as mean $\pm$ SEM. * $p < 0.05$.

Figure 6. PPAR α activation reduces VTA output and nicotine induced stimulation of locomotion. (**A** and **B**). Typical evoked field potential recordings showing effects of MK886 (0.5 μ M, **A**) and WY14643 (1 μ M, **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 $\beta 2^{-/-}$, $\beta 2^{+/+}$ and $\beta 2$ -DA-VEC mice in response to WY14643 (1 μ M). 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). (**E**) Time-course curve of nicotine

(0.02 mg/kg, s.c.) effects on locomotor activity in WY14643- and vehicle- treated mice. (F) Time-course curve of nicotine (0.02 mg/kg, s.c.) effects on locomotor activity in WY14643- and vehicle- treated β 2-DA-VEC mice compared with β 2^{-/-} mice. Arrows indicate time of nicotine administration. Data expressed as mean $\pm$ SEM. * p < 0.05.