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RATIONALE FOR AN ADJUNCTIVE THERAPY WITH FENOFIBRATE IN PHARMACORESISTANT NOCTURNAL FRONTAL LOBE EPILEPSY (FNLE).

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Summary

Abstract

Introduction: Nocturnal Frontal Lobe Epilepsy (NFLE) is characterized by onset during infancy or childhood with persistence in adulthood, family history of similar nocturnal episodes simulating non-REM parasomnias (sleep terrors or sleepwalking), general absence of morphological substrates, often by normal interictal electroencephalographical recordings (EEGs) during wakefulness. Neurological examination is generally normal. A symptomatic aetiology is present in only 13% of NFLE cases, whereas the vast majority remains cryptogenic. A family history of epilepsy may be present and a clear Mendelian autosomal dominant inheritance has been described in some families. Recent studies indicate the involvement of neuronal nicotinic acetylcholine receptors (nAChRs) in the molecular mechanisms of NFLE. In particular, mutations in the genes encoding for the α4 (CHRNA4) and ß2 (CHRNB2) subunits of the nAChR induce changes in the biophysical properties of nAChR, resulting generally in a "gain of function". Preclinical studies report that activation of a nuclear receptor called type peroxisome proliferator-activated receptor (PPAR-α) by endogenous molecules or by medications (e.g. fenofibrate) reduces the activity of the nAChR and, therefore, may decrease the frequency of seizures. Thus, we hypothesize that negative modulation of nAChRs might represent a therapeutic strategy to be explored for pharmacological treatment of this form of epilepsy, which only partially responds to conventional antiepileptic drugs. In fact, carbamazepine, the current medication for NFLE, abolishes the seizures only in one third of the patients.

The aim of the project is:

to verify the clinical efficacy of adjunctive therapy with fenofibrate in pharmacoresistant NFLE and ADNFLE patients; focousing on the analysis of the polysomnographic action of the PPAR- agonist (fenofibrate).

Furthermore, to demonstrate the subtended mechanism of efficacy by means of electrophysiological and behavioral experiments in an animal model of the disease: particularly, transgenic mice carrying the mutation in the nAChR 4 subunit (Chrna4S252F) homologous to that found in the humans. Considering that neocortical networks and thalamocortical cells have a major role in the generation of ictal activity and synchrony, the neurons located in these two regions (i.e. frontal cortex and thalamus) has been recorded by performing patch-clamp experiments in brain slices. Behavioral experiments and subdural EEG also been performed to monitor the efficacy of PPAR- **α** agonists on spontaneous or evoked seizures.

Given that PPAR-α agonists (e.g. fenofibrate) are already clinically utilized for lipid

metabolism disorders, the study provides new evidence for a novel therapeutic indication of these drugs, and be suggestive of a promising therapeutic avenue in the treatment of NFLE\ADNFLE.

Methods:

1) 16 patients affected by ADFNLE & FNLE and pharma-coresistant under treatment with several anti-epileptic drugs will be selected among 2756 outpatients attending the Epilepsy Diagnostic and Treatment Centre of Cagliari (Italy). After informed consent is obtained, patients had been enrolled into the protocol and clinical and instrumental recognition applied before add-on therapy with fenofibrate.

Fenofibrate is a long time used drug aimed at dismetabolic disease of the lipids, which has been recently shown to be an agonist at the peroxisome-proliferator-activated receptor (PPAR-alfa), a family of nuclear receptor transcription factors, which in turn modulates the nAChR involved in ADFNLE.

2) The EEG methods employed include repeated Video- EEG (VEEG), twenty-four hours Video-digital EEG, scrutiny of several previously recorded EEGs, all of them recorded in digital format. Digital recordings acquired in 11 FNLE epileptic subjects after 6 months of adjunctive fenofibrate therapy will be compared with baaseline.

Expected Results: The present study, based both on recent advances in basic research issued by our laboratories and clinical observations described in recent studies, pursued a pragmatic investigation on such new categories of antiepileptic drugs based on nicotinic role in epilepsy.

It is expected:

1) to obtain a significant reduction of seizure frequency in a selected population affected by FNLE non responders to traditional therapy by means of adjunctive therapy with fenofibrate.

2) to demonstrate the efficacy of ppar-alpha agonist in acute and chronic administration of fenofibrate in a farmacological animal models whitihin in vivo and in vitro experiments,

3) to demonstrate the efficacy of ppar-alpha agonist in chronic administration of fenofibrate in a genetic mouse model of NFLE in vivo & in vivo experiments.

Discussion and Conclusion: Hence, if treatment necessity is undoubtful to eliminate nocturnal seizures and turn sleep into a restful experience (excessive daytime sleepiness is a frequent symptom in NFLE), the pharmacological approach remains empirical based on a restricted spectrum of classical antiepileptic agents. Carbamazepine, the most efficacious drug in NFLE,

completely abolishes the seizures in ~20% of the cases and reduces the seizures in another 48%, leaving approximately one third of patients resistant to therapy. The recently discovered novel mechanism of regulation of nAChRs by PPAR-α may represent a new therapeutic avenue formedications aimed at diseases such as NFLE and ADNFLE, where "a gain of function" of nAChR is responsible for the cardinal symptoms. The use of available animal models of ADNFLE, together with advanced neurophysiological techniques in humans, offered an extraordinary opportunity to develop an important therapeutic strategy that, noteworthy, may rely on drugs already clinically utilized in humans.

The present project is realised in cooperation with the Medicine Sleep Centre of the University of Parma and the Medicine Sleep Centre of Cagliari.

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Rationale of the work.

Epilepsy is a common neurological disorder affecting1% of the population worldwide. Over the past decade, several idiopathic epilepsies have been identified that show single-gene inheritance, such as autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE). Considerable clinical and genetic evidences now provide a strong link between the ADNFLE syndrome and mutations located within the second pore-forming transmembrane domain of the nAchR sub-units. Given the similarities in clinical symptoms, it has been suggested that a common functional anomaly of mutant α4- and ß2-subunits, containing nAchRs, underlies ADNFLE. The key pathogenetic mechanism underlying this inherited form of epilepsy is the "gain of function" of nAChRs containing the mutated α4 or ß2 subunits. Enhanced activity of the mutated nAChRs translates into abnormal cholinergic inputs to pyramidal cells or to GABAergic afferents in the frontal cortex (FC), such as enhanced nicotine-evoked IPSCs, which have been hypothesized to trigger network synchrony and ictal activity. Theoretically, blockade of nAChRs could provide a therapeutic approach to treat the disease. As described in Melis et al 2008, the suppression of neuronal nicotine's effect by PPAR-α involves a non-genomic mechanism, due to the rapid onset of agonist actions. Tyrosine phosphorylation of nAChRs modulates their functional properties in neurons; nAChRs are rapidly phosphorylated and dephosphorylated, and the dephosphorylated form of the receptor is more active. The balance between tyrosine kinases and phosphatases determines the net phosphorylation status of nAChRs, constituting an important regulatory mechanism for their activity at the plasma membrane, and offering an additional capacity for modulation of neuronal network functions. On these bases, we hypothesize that **negative regulation of nAChRs by enhancing the activity of tyrosine kinase, should provide a mean to negatively regulate nAChRs in ADNFLE**. The potent agonistic properties of PPAR-α open up a new role for these nuclear receptors far beyond their well-known effects on energy homeostasis and lipid metabolism.

Based onour working hypothesis, we expect that either an enhancement of FAE levels (and therefore PPAR-α activation), or direct PPAR-alfa stimulation, may be beneficial in the treatment of ADNFLE.

PPAR-α agonists, such as **fibrates**, are already well-established therapeutic options for the treatment of hyperlipidemia. Recent studies have suggested that a long time used dietetic add-on treatment of epilepsy, represented by the ketogenic diet, share properties similar to those showed by fenfibrates, a class of drugs aimed at controlling and modulating lipid metabolism which has

also shown a antiepileptic effects on experimental models of epilepsy.

The modulatory effects of such a class of drugs, spurs the rationale attempt to use, as add-on **FENFIBRATE** in NFLE treatment, given their modulation specifically upon the nicotinic mutated receptor.

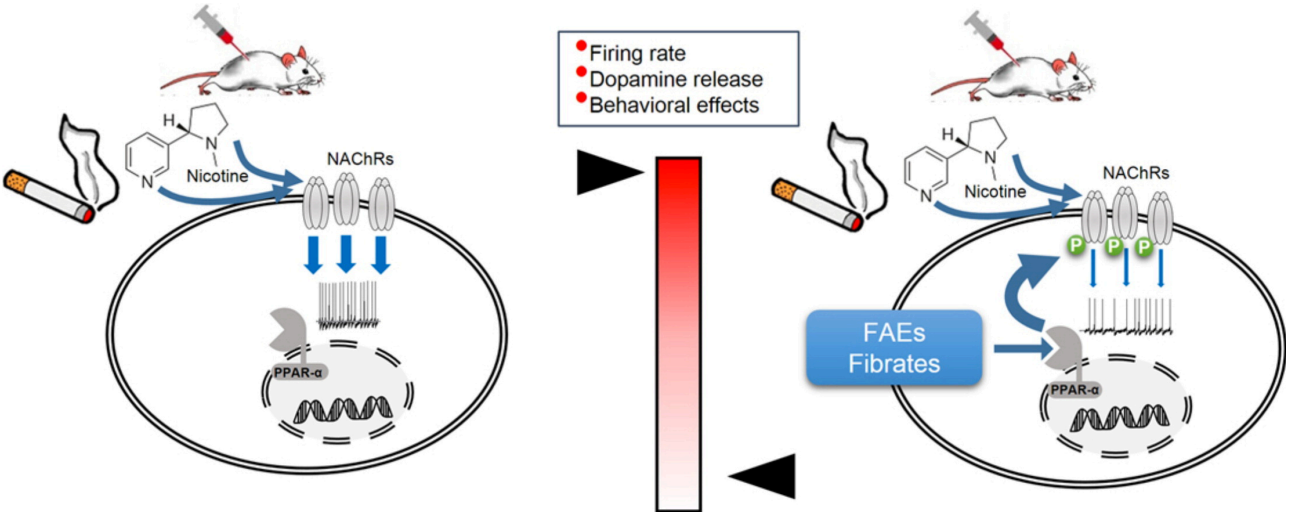
Ergo, we propose to explore whether the modulation of nAChRs by PPAR-alpha may be a feasible therapeutic strategy for ADNFLE\NFLE.

To that, we performed electrophysiological and behavioral experiments first in a pharmacological epileptic animal model of the disease, than in a transgenic mouse model carrying a mutation homologous to that found in the human disease.

We finally verified the clinical efficacy of an adjunctive therapy with fenofibrate in pharmacoresistant NFLE and ADNFLE patients.

INTRODUCTION

Physiological Role of Peroxisome Proliferator-Activated Receptors Type Alpha and nicotine receptors: bases of the therapeutic target.



Miriam Melis , Marco Pistis

Targeting the interaction between fatty acid ethanolamides and nicotinic receptors: Therapeutic perspectives

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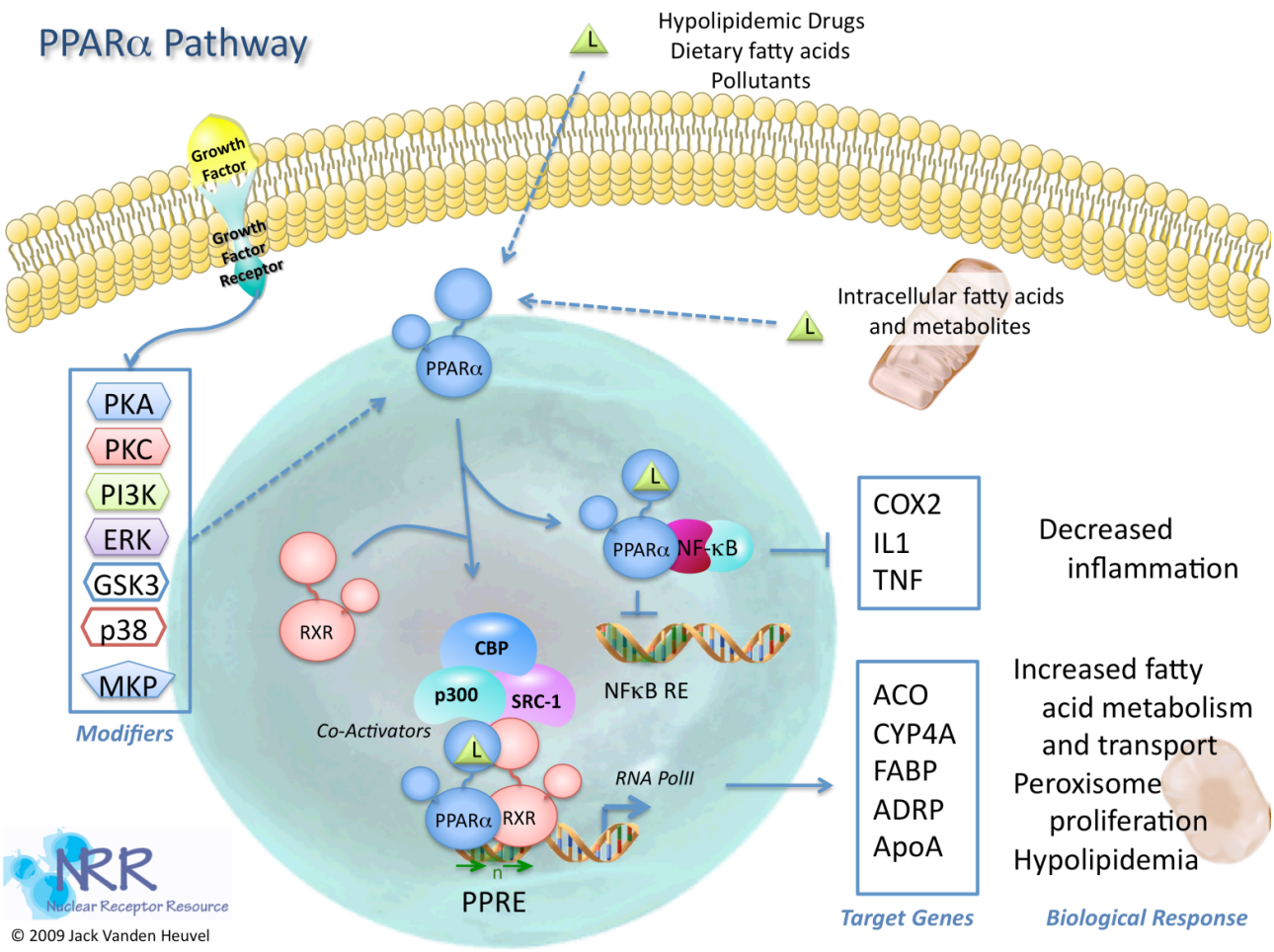
Fatty acids have a different role in the brain as compared to their effects in peripheral tissues in terms of energy stores and production in situ. Although brain cells possess the enzyme machinery for fatty acid mitochondrial -oxidation, to generate ATP they mainly rely on glucose. Alternatively, under extreme physiological conditions they depend on ketone bodies [1]. In addition, because progressive brain damage is not a feature of mitochondrial -oxidation defects, mitochondrial fatty acid -oxidation most likely plays only aminor role in central nervous system (CNS) energy metabolism [2]. Peroxisomal oxidation upon nuclear receptor peroxisome proliferator-activated type alpha (PPAR-α) activation participates also in hydrogen peroxide production as well as in other facets of fatty acid metabolism. Conversely, peroxisomal -

oxidation dysfunction has been linked to neurodegeneration in human diseases [3, 4]. Therefore, brain fatty acid -oxidation (both mitochondrial and peroxisomal) has an important role in signaling pathways and catabolism of pro-inflammatory molecules such as eicosanoids. Palmitoylethanolamide (PEA) is a naturally occurring Nacylethanolamine (NAE), a saturated fatty acid (palmiticacid) derivative. NAEs are bioactive lipids, whose functions and properties were discovered first in 1957, when PEA (from soybeans, peanuts, and egg yolk) exerted antiinflammatory activity in guinea pigs [5]. High levels of PEA were also measured in mammalian tissues, especially in the brain [6].

The role of PEA, as well as of other NAEs, in the brain has been elucidated only recently, when the interest in lipid messengers was rekindled by the discovery that arachidonoylethanolamide (AEA, or anandamide), an NAE with an arachidonic acid (ARA) moiety, is an endogenous ligand to cannabinoid receptors [7]. NAEs, including AEA and the non-cannabinoid PEA and oleoylethanolamide (OEA), are lipid messengers produced "on demand". In fact, they are not stored in vesicles, but released from membrane phospholipids (N-acylphosphatidylethanolamines, NAPEs)[8] when cells are subjected to potentially harmful stimuli, such as membrane depolarization or increase of cytoplasmic Ca2+ levels. OEA and PEA lack binding affinity at cannabinoid receptors [9,10] and are considered being endogenous agonists at PPAR-α and other non-cannabinoid targets. The dopaminergic system consist of several pathways originating in the midbrain, particularly within the ventral tegmental area (VTA, A10) and the pars compacta of the substantia nigra (SNpc, A9), where dopamine synthesizing cells are located [11,12]. Dopamine cells project via their axons to their target regions in both cortical (medial prefrontal, cingulated, and entorhinal cortices) and other forebrain areas (striatum and nucleus accumbens, amygdala, olfactory tubercle) where dopamine is released [13,14,15].



The different distribution of dopamine projections in these areas stemming from either the VTA or SNpc names the systems mesocorticolimbic and mesostriatal, respectively. Given that these systems are involved not only in motor control and movement disorders (e.g. Parkinson disease), but also in "higher" brain functions (e.g. emotional and cognitive tasks) and dysfunctions (e.g. schizophrenia, drug addiction) [16], their individual cellular properties [17], as well as their ability to change in response to altered functional demands are now considered extremely important.

Despite the fact that evidence of the role of NAEs in the modulation of dopamine functions was shown [18-20], it is only with the discovery that these lipid molecules prevent nicotine-induced excitation of midbrain dopamine neurons via PPAR-α [21] that their role as fine regulators of the brain reward system emerged.

Hence, these findings opened a potential new avenue for the pharmacological treatment of nicotine addiction. Indeed, synthetic PPARα ligands, such as lipid lowering fibrates, prevent nicotine-induced excitation of dopamine neurons, and increased extracellular dopamine levels in the shell of the NAc [22], both of which represent a hallmark of its acute rewarding properties. PPARα activation can be achieved directly and indirectly. Hence, PPARα synthetic agonists such as fibrates are effective and safe marketed drugs for their lipid lowering pharmacological properties. In addition, PPARα activation can be attained by pharmacologically manipulating (i.e. enhancing) intracellular levels of its endogenous ligands. Hence, increased levels of FAEs in the midbrain can be obtained not only by preventing their degradation (e.g. URB597), but also by promoting their synthesis [23] and [24]. This is particularly relevant since increasing levels of PPARα endogenous ligands such as FAEs, but also free fatty acids, are able to influence neuronal activity of midbrain dopamine cells and to prevent their nicotine-induced excitation [25]. In the midbrain, FAE synthesis depends upon raises of intracellular Ca2+ and activation of α7-nAChRs [26]. Hence, either a hypercholinergic drive and direct activation of α7-nAChRs can powerfully increase OEA and PEA levels, and prevent nicotine-induced stimulating effects both in vitro and in in vivo [27]. In addition, although modifying brain fatty acid composition through changes in the diet is rather difficult, low concentrations of added fatty acids can affect FAE biosynthesis within the midbrain, an effect that most likely occurs through activation of PPARα [100]. Whether or not this effect can prevent nicotine actions on dopamine system function remains to be explored yet. Nonetheless, since PEA and OEA can modulate dopamine cell excitability [28] and [29], it is tempting to speculate that a nutritional approach might powerfully regulate their activity and metabolism via PPARα induction.

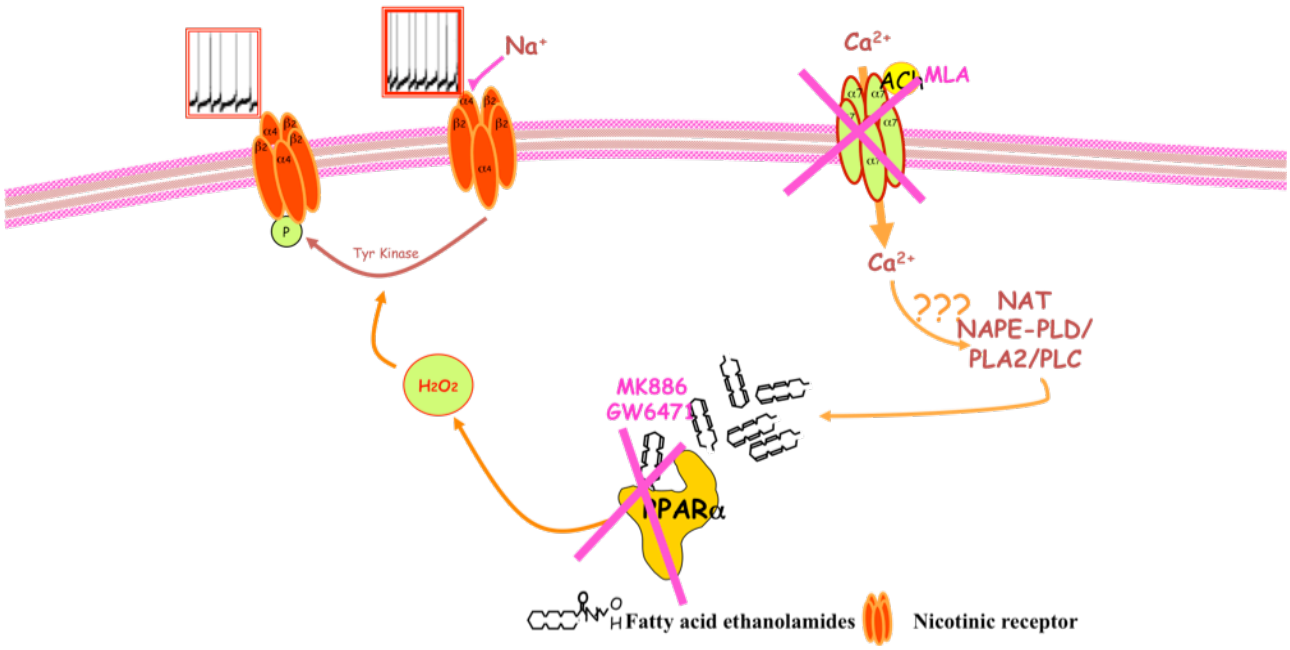
Importantly, OEA and PEA, by inducing mitochondrial enzymes and/or peroxisomal β-oxidation [30], may also play a role in guaranteeing steady state levels of hydrogen peroxide required to regulate dopaminergic neuronal activity [31]. Accordingly, the cellular effects resulting from PPARα activation, such as decreased spontaneous activity of dopamine cells and blockade of nicotine-induced excitation of these neurons, are fully prevented when hydrogen peroxide half-life is reduced by raising the intracellular levels of catalase, and require β2*nAChRs [32]. Thus, FAEs as PPARα ligands act as intrinsic modulators of cholinergic transmission and alter dopamine cell excitability, contributing to acetylcholine effects on dopamine system. The findings that activation of α7-nAChRs activation on dopamine cells can selectively increase FAE levels to prevent their own aberrant excitation in response to high cholinergic drive suggested their involvement in other disorders characterized by an unbalance

between dopamine and acetylcholine systems [33]. Hence, since FAEs via PPARα seem to act as possible switches in the tonic/phasic transition regulated by β2*nAChRs, their role in mood control was suggested [34]. Accordingly, both PEA [115] and FAAH inhibitors display antidepressant-like activity in rodents [35-37].

Remarkably, FAEs may also play a role in the etiopathogenesis of schizophrenia. Accordingly, activation of either PPARα or α7-nAChRs has proven to be effective in preclinical models of schizophrenia [40-43], thus supporting this interplay in the pathophysiology of this disease [44-46]. Noteworthy, the role played by FAEs in the pathophysiology of dopamine systems is further supported by a population study of the gene encoding FAAH [47-48] in which, by logical extension, the homozygosis for this FAAH missense polymorphism was also found associated with overweight and obesity [49]. Indeed, in the brain overlapping reward and reinforcement pathways likely modulate natural and artificial rewards, such as food and illicit drugs, respectively. Thus, the effects of the FAAH 385 A/A polymorphism could result in the amplification of those reward seeking behaviors such as the consumption of palatable and sweet foods that are usually associated with overweight and obesity [48].

Finally, yet importantly, altered functional properties of nAChRs are implicated in the pathogenesis of nocturnal frontal lobe epilepsy, linked to mutations of α4 or β2 subunits. Notably, PEA has proven to have antiepileptic effects in kindled rats, and displayed anticonvulsant activity in mice [54]. Accordingly, acute and chronic PPARα agonists are able to reduce nicotine-induced seizures, and to abolish nicotine-induced generation of ictal activity and synchrony in the frontal cortex. Remarkably, these latter observations were accompanied by an increased ratio of phosphorylated/dephosphorylated β2 subunits in the frontal cortex following acute PPARα ligand treatment, whereas the chronic regimen induced a threefold increase in OEA levels in the same brain region. Noteworthy, these findings may be extended to other models of epilepsy, since chronic fenofibrate was shown to be effective as anticonvulsant in pentylentetrazole-induced seizures and on latencies to the onset of status epilepticus induced by lithium–pilocarpine [49-55]. the role played by the FAEs, endogenous PPARα ligands, which act as endogenous modulators of cholinergic transmission, and alter intrinsic dopamine cell excitability, and dependent behavior. The reduced response to acetylcholine impinging on the same dopamine neuron releasing endogenous PPARα ligands might, thus, counterbalance the excessive cholinergic drive, and provide fine modulation of dopamine pathways at single-cell level, where phosphorylation of β2*nAChRs is the effector mechanism. Consequently, PPARα

activation by FAEs may be suited to help resolve disruption of dynamic balance of dopamine-acetylcholine systems, and prove beneficial in those disorders associated with dysfunction of such interplay.



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Nocturnal Frontal Lobe Epilepsy: clinical and genetic features

Nocturnal frontal lobe epilepsy (NFLE) is caracterized by seizures that are almost exclusively sleep related. In the last few decades, the spread and refinement of video–polysomnographic (V-PSG) technology has allowed NFLE to be fully defined and described, so that it is now considered a distinct epileptic syndrome (Provini et al. 1999).

It is now recognized that NFLE is not a homogeneous disease as familial, idiopathic, sporadic, cryptogenetic, or symptomatic forms do exist. In addition, a genetic heterogeneity is also evident within the familial type.

No epidemiologic data are available on NFLE prevalence. The frequency of pure sleep-related seizures, defined as "seizures occurring exclusively or predominantly (>90 %) from sleep" clusters around 12 % of people with epilepsy, the majority of patients being affected by focal epilepsy. NFLE is probably not rare, accounting for 13 % of polysomnographic recordings for nocturnal motor disorders in a retrospective study performed at a tertiary centre in Italy and for 6.3 % of a large series of focal drug-resistant epileptic patients. Because many cases of NFLE are misdiagnosed as parasomnias, the clinical relevance of NFLE is underestimated, especially in children(Yaqub, Waheed, and Kabiraj 1997)(Fernández and Salas-Puig 2007).

The genetic origin of the disease was firstly confirmed by a linkage study in Australian kindred. More specifically, a locus was identified at chromosome 20q13.2-q13.3 and two different mutations were detected in the gene coding the α4 subunit of the neuronal nicotinic acetylcholine receptor (nAChR) (CHRNA4). Both mutations were shown to have major effects on receptor function in vitro. With the identification of these mutations, subsequently confirmed in families of different origins, ADNFLE became the first epilepsy in which genetic bases were detected, and was quickly recognized as a genetically heterogeneous disorder showing mutations in the CHRNA4 gene and in the the β2 subunit of the nACh receptor (CHRNB2).

For detailed description of genetic mutation see Nobili et al 2014 Curr Neurol Neurosci Rep. 2014.

Clinical Features and Sleep traits of NFLE

ADNFLE and sporadic NFLE show similar clinical and electroencephalographic features. In the high majority of NFLE patients, seizures begin before the age of 20 years, with a peak during

childhood, although onset during adulthood has been also reported. Seizure frequency is usually high, and patients generally experience many seizures a night, although the frequency may diminish during adulthood. Of note, occasional seizures during wakefulness can occur (Oldani et al. 1998).

The broad spectrum of clinical manifestations in NFLE includes motor phenomena of increasing complexity and duration ranging from major attacks (asymmetric tonic–dystonic posturing, hyperkinetic seizures, or prolonged epileptic nocturnal wandering), to paroxysmal arousals (frequent, abrupt, and brief arousals associated with stereotyped movements of the trunk and/or head elevation), and minor motor events, in the form of brief (2–4 s) stereotyped movements involving the limbs, the axial musculature, and/or the head (Terzaghi et al. 2008).

Clinical manifestations of NFLE are strongly influenced by circadian sleep-wake alternation and by physiologic components of sleep structure. Regardless of their semiology, the majority of NFLE seizures arise during nonrapid eye movement (NREM) sleep and are followed by a sudden transition to a more superficial stage or a frank awakening. Furthermore, changes in autonomic cardiac control presumed to precede SO, represent part of the arousal response, which could be implicated in the occurrence of both seizure and arousal motor manifestations (Terzaghi et al. 2007).

The epileptic fragmentation of the first part of the night can produce a significant sleep disruption with a consequent increase of wake after sleep onset (WASO) and rapid eye movement (REM) sleep latency but without alterations of slow-wave sleep (SWS), which even increases. Moreover, the temporal development of epileptic events across sleep period seems to be modulated by the homeostatic process of deep NREM sleep. Furthermore, changes in autonomic cardiac control associated with motor activity (phases of transitory activation) presumed to precede sleep onset, could represent part of the arousal response, which could be implicated in the occurrence of both seizure and arousal motor manifestations (Calandra et al. Sleep Med. 2012).

The cyclic alternating pattern (CAP), the marker of unstable NREM sleep (Terzano et al. 2001) plays a primary role in the activation of epileptic events in NFLE patients (Parrino et al. 2006)(Parrino, De Paolis, et al., 201)(Parrino, Ferri, et al. 2012).

In NFLE patients, CAP is a powerful triggering condition for the occurrence of both ictal and interictal epileptic events that arise in concomitance with phase A. In turn epileptic manifestations act as a subcontinuous sleep disturbance that induces a significant increase of sleep instability. This enhancement of unstable NREM sleep together with the alterations of conventional sleep measures can define distinctive polysomnography (PSG) features in NFLE

patients and can be responsible for the poor sleep quality and the excessive daytime sleepiness, which has already been described in these patients(Parrino, De Paolis, et al. 2012).

Because of the almost exclusive recurrence of seizures during sleep, NFLE patients often are scarcely aware of the presence, complexity, and frequency of attacks. Moreover, a reliable description of epileptic motor events occurring during the night often are difficult to collect from a witness or sleep partner, as observers may be absent, or if present, not fully awake or reliable. Lastly, MMEs often are difficult to be clearly qualified.

For these reasons, nocturnal video-EEG features and PSG metrics can provide objective data of the effects of antiepileptic therapy, especially on minor seizures and sleep parameters.

The discrepancy between an adequate subjective seizures outcome and PSG findings in treated NFLE subjects can open up the discussion to the identification of clinical and sleep measures, which can define a more objective response to treatment. The subjective effectiveness of antiepileptic treatment in NFLE subjects could be mostly related to the partial reduction of the longer and more complex nocturnal seizures that are more likely to be perceived. In contrast the persistence of residual objective PSG epileptic phenomena (both MAs and MMEs) and a high level of unstable NREM sleep indicate a partial resistance of both seizures and disturbed arousal system to the therapeutic action of the antiepileptic treatment (De Paolis et al. 2013).

So far the principle target of antiepileptic treatment is to eliminate or decrease major epileptic events. However, despite the reduction of nocturnal seizures, the normalization of SE and WASO, and the high values of SWS in our NFLE subjects, daytime sleepiness is still present under drug treatment. The persistence of high values of CAP rate even under therapy could be a partial explanation of diurnal concerns**. In this perspective, the goals of therapy in NFLE should include the treatment of structural sleep alterations.**

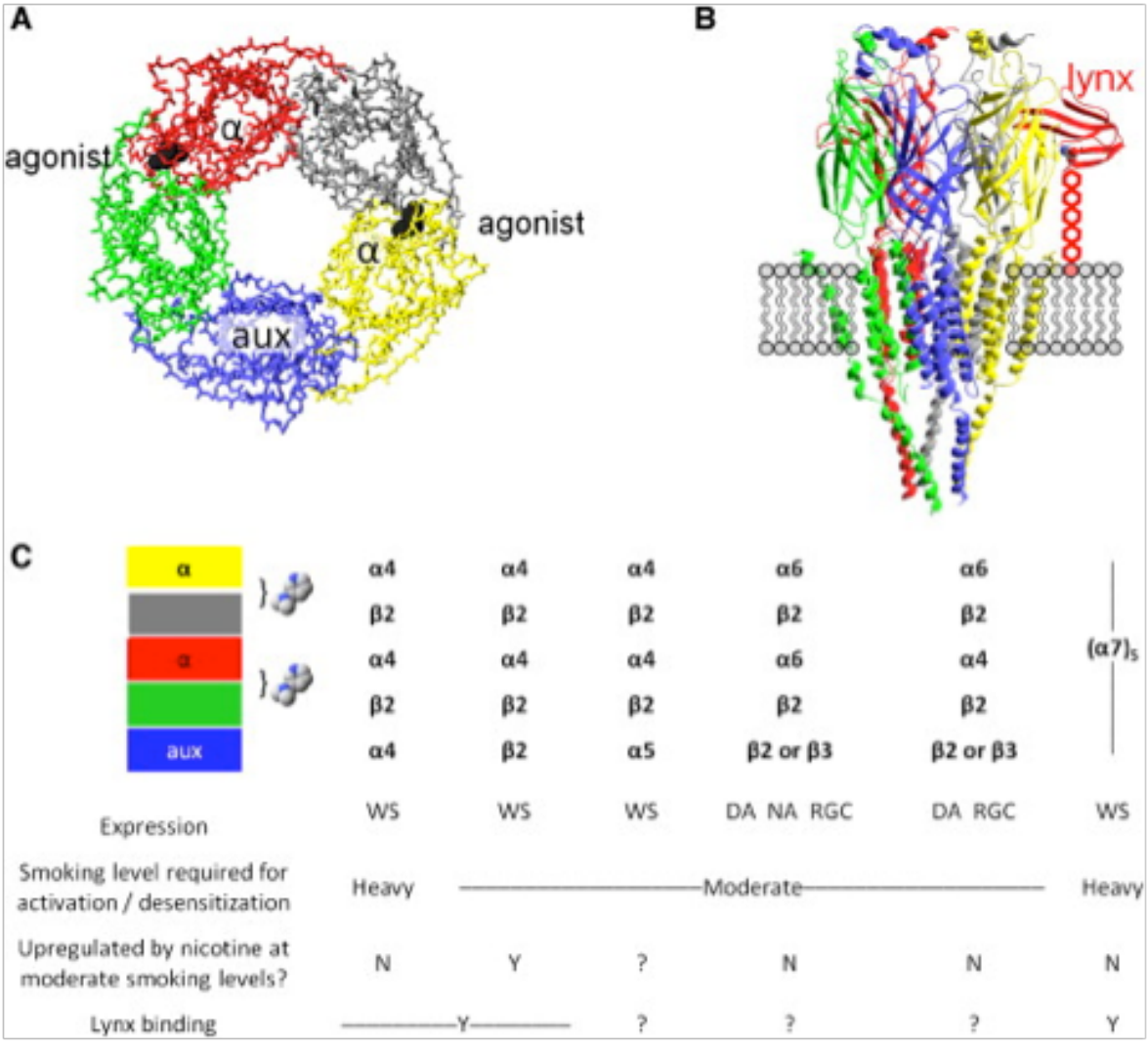
*The Role of the neuronal nicotinic acetylcholine receptors (nAChRs)**

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand gated cationic channels that are composed of various combinations of five subunits arranged quasi-symmetrically around a central channel. They are encoded by nine α (α2-α10) and three β (β2-β4) subunit genes that show distinct patterns of expression both in neuronal and non-neuronal tissues. Each subunit is composed of three different parts, which include the extracellular, cytoplasmatic and transmembrane portions. The NH2-terminus is the largest extracellular domain, contains several glycosylation sites and, in α subunits, contributes the main components of the high-affinity ligand binding sites that are located at the interface between neighbouring subunits. This domain is important for receptor assembly and expresses the main immunogenic region. The four transmembrane regions (TM) probably have a mixed α-helical/non-helical secondary structure with their helical segments forming both an inner ring (TM2) that shapes the pore and an outer shell (TM3-TM4) that shields the inner ring from membrane lipids. The different subunits assemble to build two classes of nAChRs, the homomeric or heteromeric α-Bungarotoxin-sensitive receptors that are composed solely from α subunits (α7-α10), and the heteromeric α-Bungarotoxin-insensitive receptors that consist of various combinations of α and β subunits (α2-α5, β2-β4).

The mechanisms of nAChR activation, ion pore opening and closure and desensitisation are determined by highly conserved amino acid positions within the subunits. The function of the different neuronal nAChR subtypes depends on their subunit composition, their distribution within the brain as well as on their cellular location. Postsynaptic nAChRs often contribute to fast excitatory transmission, while preterminal and presynaptic nAChRs enhance neurotransmitter release (which, depending on the released transmitter, either increases inhibitory or exitcatory synaptic impulses), and nonsynaptic nAChRs are likely to modulate neuronal excitability.

The nAChRs are involved in brain development and plasticity and participate in many different brain functions, including modulation of sensory inputs, locomotor activity, analgesia, attention, learning, memory and reward mechanisms. It is therefore not surprising that genetic variants in nAChR subunit genes were found to be associated with different common disorders and behaviours, including Alzheimer disease, schizophrenia and smoking related endophenotypes. However, the only monogenic disorder so far known to be caused by high penetrance mutations in neuronal nAChR is ADNFLE.

ADNFLE mutations in the nAChR subunits appear to modify the number and distribution of α4β2 nicotinic receptors in the living human brain. The distribution of α4β2 nicotinic receptors has been studied in eight ADNFLE patients carrying nicotinic receptor mutations (causative mutations: four



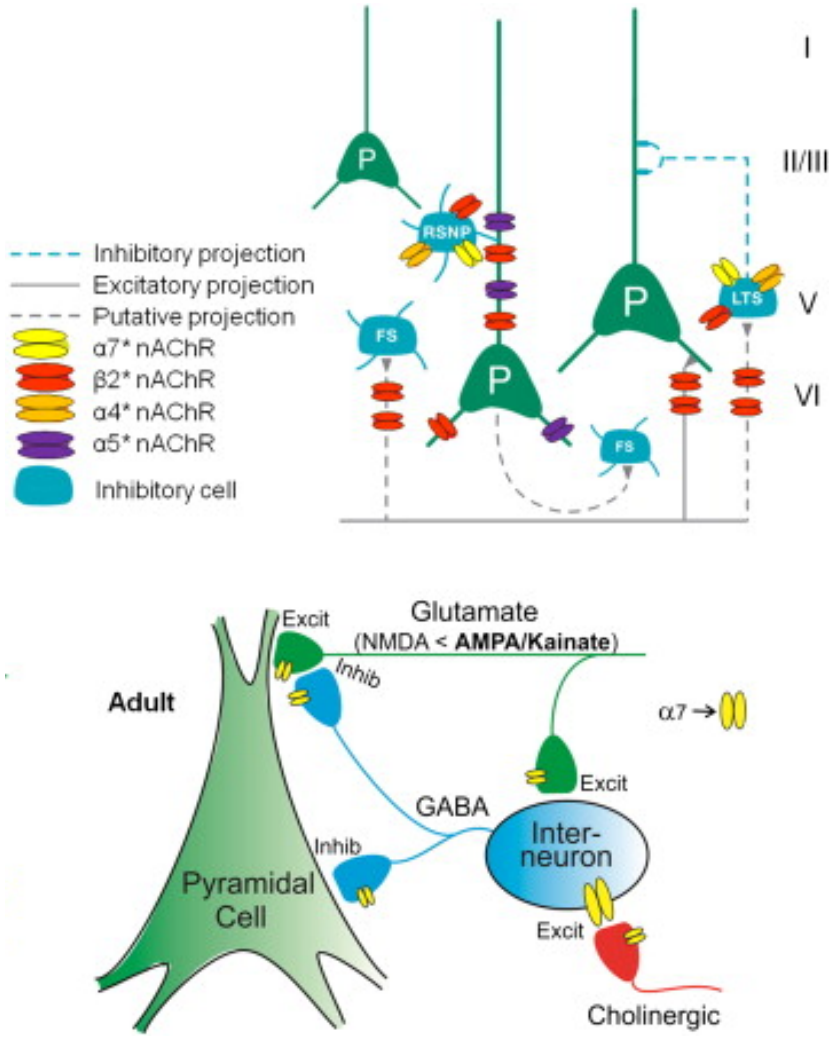
Neural systems governed by nicotinic acetylcholine receptors: emerging hypotheses.
Miwa JM1, Freedman R, Lester HA. Neuron. 2011 Apr 14;70(1):20-33

patients with α4-S280F (numbering according to reference sequence NP_000739.1) (mutation is also named S248F 2); two with α4-S284L (also named S252L 26); one with α4- T293I (also named T265I 28); and two with β2-V287L), by a PET-scan using [18F]-F-A-85380, a

radioligand with a high affinity for α4β2 nAChRs.
This PET study demonstrated a regional nAChR density decrease in the prefrontal cortex, an observation consistent with partial epilepsy involving the frontal lobe. The increase of nAChR density in the mesencephalon suggests that these brain structures are involved in the pathophysiology of ADNFLE through the role of brainstem ascending cholinergic systems in arousal. Electrophysiological studies of the receptors carrying the different mutations identified a common alteration in their properties, corresponding to a gain of function.

The theory of the "Gain of function".

Mostly, seizures are characterized by short bursts of abnormal neuronal activity that, by their origin and spreading pattern in brain, determine the clinical phenotype of the epilepsy; arise from a state of hypersynchronisation that causes groups of neurons or whole neuronal networks to fire simultaneously. In ADNFLE these episodes are usually occur with NREM sleep phases that can be subdivided into two main phases, one that consists of transient arousals and one that is characterised by tonic activities in the EEG. The association between the seizures and NREM phases suggests that sleep-controlling brain structures are involved in the pathogenesis of ADNFLE. One of the main regulators of sleep is the central cholinergic system that controls both the circadian clock and the sleep-wake cycle. Two major cholinergic cell groups are responsible for the cerebral activation that accompanies wakefulness and paradoxical sleep. One is located within the pontomesencephalic tegmentum that projects rostrally into the non-specific thalamo-cortical relay system and the other is placed within the basal forebrain that receives input from the brainstem reticular formation and projects as the ventral, extrathalamic relay upon the cerebral cortex.
Functional changes in nAChRs as those induced by ADNFLE mutations might affect these cholinergic projections and cause aberrations in sleep stages that, for unknown reasons, render certain neuronal networks more vulnerable for sudden bursts of hyperactivity during NREM sleep. An older hypothesis postulates that seizures are the result of reduced inhibition that causes an increase in excitatory neuronal activity. More recently it has been shown that the opposite mechanism is at least as plausible, and is the one most likely involved in seizures caused by nAChR subunits. The basic principle underlying this mechanism is an enhancement of neuronal synchrony by inhibition. It has been shown that pyramidal cells that are connected to the same GABAergic interneuron synchronise their firing when released from the interneuron's inhibitory effect.



Neural systems governed by nicotinic acetylcholine receptors: emerging hypotheses.
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Interneurons innervating layer II/III pyramidal cells are known to carry α4β2 nAChRs. Triggered by the gain-of-function effect ADNFLE mutations exhibit on these receptors they could activate larger numbers of GABAergic interneurons that in turn could easily synchronise enough pyramidal cells to change local cortical activity from synchronisation to hypersynchronisation. Spreading of the hypersynchronisation into the motor control area would than be likely to cause the type of seizures typically associated with ADNFLE. Such a mechanism in which a gain-of-function of certain nAChR subunits causes hypersynchronisation by increasing GABAergic inhibition is supported by animal models studies. GABA antagonists such as picrotoxin have shown to normalize the EEG and suppress spontaneous seizures in genetically altered pS280F-KM mice.

*From:

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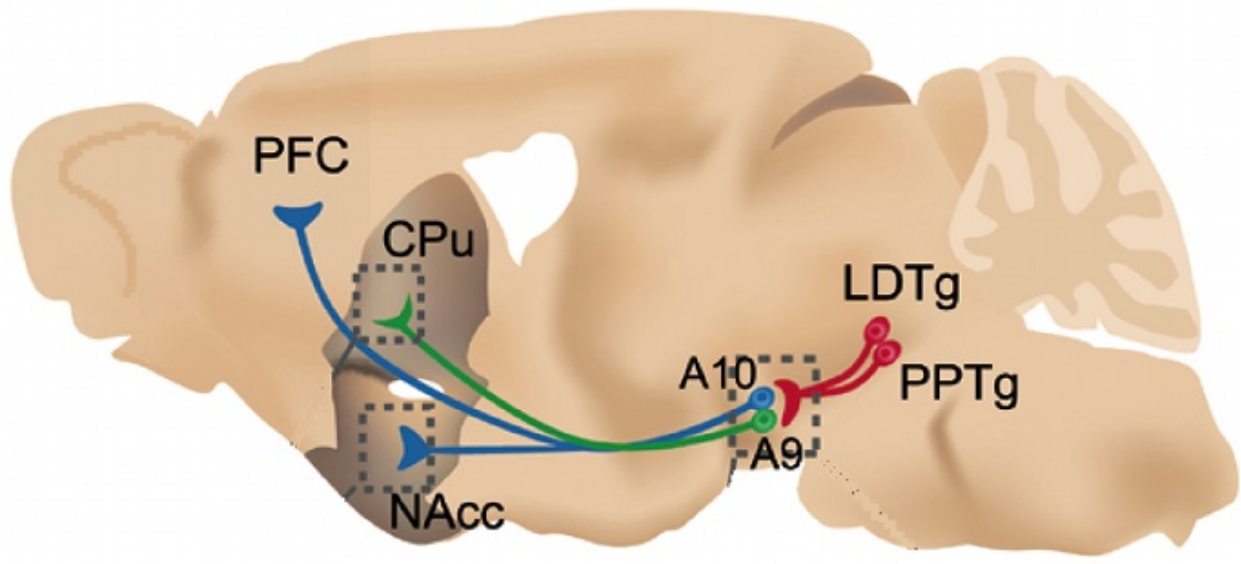
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PPAR-ALPHA AGONISTS AS NOVEL ANTIEPILEPTIC DRUGS: PRECLINICAL FINDINGS.

(Puligheddu et al. Plos One 2013)

Abstract

Nicotinic acetylcholine receptors (nAChRs) are involved in seizure mechanisms. Hence, nocturnal frontal lobe epilepsy was the first idiopathic epilepsy linked with specific mutations in α4 or β2 nAChR subunit genes. These mutations confer gain of function to nAChRs by increasing sensitivity toward acetylcholine. Consistently, nicotine elicits seizures through nAChRs and mimics the excessive nAChR activation observed in animal models of the disease. Treatments aimed at reducing nicotinic inputs are sought as therapies for epilepsies where these receptors contribute to neuronal excitation and synchronization. Previous studies demonstrated that peroxisome proliferator-activated receptors-α (PPARα), nuclear receptor transcription factors, suppress nicotine-induced behavioral and electrophysiological effects by modulating nAChRs containing β2 subunits. On these bases, we tested whether PPARα agonists were protective against nicotine-induced seizures. To this aim we utilized behavioral and electroencephalographic (EEG) experiments in C57BL/J6 mice and in vitro patch clamp recordings from mice and rats. Convulsive doses of nicotine evoked severe seizures and bursts of spike-waves discharges in ~100% of mice. A single dose of the synthetic PPARα agonist WY14643 (WY, 80 mg/kg, i.p.) or chronic administration of fenofibrate, clinically available for lipid metabolism disorders, in the diet (0.2%) for 14 days significantly reduced or abolished behavioral and EEG expressions of nicotine-induced seizures. Acute WY effects were reverted by the PPARα antagonist MK886 (3 mg/kg, i.p.). Since neocortical networks are crucial in the generation of ictal activity and synchrony, we performed patch clamp recordings of spontaneous inhibitory postsynaptic currents (sIPSCs) from frontal cortex layer II/III pyramidal neurons. We found that both acute and chronic treatment with PPARα agonists abolished nicotine-induced sIPSC increases. PPARα within the CNS are key regulators of neuronal activity through modulation of nAChRs. These effects might be therapeutically exploited for idiopathic or genetically determined forms of epilepsy where nAChRs play a major role.

Introduction

Binding of nicotine to nicotinic acetylcholine receptors (nAChRs) elicits in laboratory animals dose-dependent effects that begin with hypermotility and culminate with clonic-tonic seizures and death at high doses [1]. Investigation on the mechanisms underlying nicotine-induced seizures might help to understand how nAChRs participate in the mechanisms of epilepsy. Interest for nAChR in several epileptic syndromes previously considered "idiopathic" was rekindled by the finding that altered functional properties of nAChR are implicated in the pathogenesis of nocturnal frontal lobe epilepsy (NFLE), and that seizures induced by nicotine in rodents model nAChR-related epilepsy. NFLE is linked with mutations of the α4 or β2 subunits [2], [3], [4], [5], [6], [7], the most abundantly expressed subunits in the CNS [8]. Though in human NFLE, or in genetically engineered mice that model the disease, functional properties of nAChRs are intimately altered, nicotine evokes seizures by over-activating a healthy system. However, several neurophysiological events ultimately leading to seizures may share common steps between these conditions. Indeed, the common trait in enhancing the epileptogenesis is the over-activation of cholinergic systems, either pharmacologically induced or mediated by the gain of function that mutated nAChRs exhibit toward their ligands [9]. These effects might be based on the extensive expression of nAChRs, particularly those containing the β2 subunit (β2*nAChRs) in thalamo-cortical, hippocampal and frontal regions [10]. Indeed, altered cholinergic activation of neocortical and/or thalamocortical networks plays a central role in the generation of both nicotine-induced and NFLE seizures, which originate in the frontal cortex (FCx) [11] and specifically affect $GABA_A$-mediated inhibitory inputs to pyramidal neurons [12].

Based on this evidence, negative regulation of nAChRs might represent a potential therapeutic approach in nAChR-related forms of epilepsies.

We previously discovered that nicotine-evoked excitation of dopamine neurons both in vivo and in vitro, as well as nicotine addictive properties in rats and monkeys, are suppressed by ligands to the peroxisome-proliferator-activated receptor-α (PPARα) [13], [14], [15], [16]. PPARα is one of three subtypes of the nuclear receptor PPAR family [17], [18], [19], expressed by neurons in many brain regions [20], [21], and activated by endogenous ligands, the N-acylethanolamines oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) [22], and by synthetic ligands such as hypolipidemic fibrates [16].

Evidence points to a non-transcriptional interaction between PPARα and nAChR, via phosphorylation [13], [14], [23]. This mechanism might account for the blockade of neuronal and behavioral responses to nicotine [13], [15], [16].

Building upon these findings, we postulated that PPARα agonists might display anticonvulsant properties. Thus, we first investigated whether acutely or chronically administered PPARα ligands reduce the severity of nicotine-induced seizures. Next, we assessed whether these compounds regulate the phosphorylation status of the β subunit of the nAChR and abolish nicotine-induced enhancement of inhibitory currents on pyramidal neurons in layers II/III of the frontal cortex (FCx) in mice and rats.

Materials and Methods

Animals

For behavioral experiments, electroencephalographic (EEG) and patch-clamp recordings male C57BL/6J mice (Harlan, San Pietro al Natisone, Italy) (n = 80; weight: 25–30 g each) were used. For immuno-blotting experiments and patch-clamp recordings, we utilized male Sprague-Dawley rats (250–300 g and 14–21 d, respectively, n = 30, Harlan, San Pietro al Natisone, Italy) Mice and rats were housed six per cage under a 12 h light/dark cycle (light on at 7:00 AM), in conditions of constant temperature (21±2°C) and humidity (60%), with food and water ad libitum.

Ethics Statement

Experiments were performed in strict accordance with the EEC Council Directive of 24 November 1986 (86/609). All efforts were made to minimize pain and suffering and to reduce the number of animals used. The experimental protocols were also approved by the Animal Ethics Committee of the University of Cagliari.

Treatments

Animals were randomly assigned to the experimental groups undergoing behavioral seizure scoring or EEG recordings. In the first set of experiments a dose-response curve for nicotine (5, 7, 10 mg/kg) was carried out. Each mouse received a single dose of nicotine.

Subsequent acute experiments, aimed to assess the effect of the PPARα agonist WY14643 or the antagonist MK886, were carried out with the 10 mg/kg dose of nicotine. In these cases, mice received two injections, spaced by 10 min intervals, before the final nicotine administration after 15 min: we first injected the antagonist MK886 (3.0 mg/kg, i.p.) or its vehicle followed by the administration of WY14643 (80 mg/kg, i.p.) or its vehicle.

For chronic studies, one week after EEG electrode implants (see below) mice were divided into diet treatment groups: (i) a standard diet (control group, Harlan Teklad Global 2016); (ii) a 0.2% fenofibrate diet (Fenofibrate from Sigma-Aldrich+Harlan Teklad Global 2016). Mice were fed diets for 14 days. On the day of the experiment, mice received one injection [MK886 (3.0 mg/kg, i.p.)] or its vehicle before nicotine (10 mg/kg) administration after 15 min.

Seizure Scoring

Immediately after nicotine injection, mice were placed in a regular mouse cage with bedding,

and behavioral responses were recorded for 5 min. The symptoms were scored independently by two experimenters blind to the treatment on an arbitrary scale from 0 to 6 (modified from Franceschini et al., 2002) [24] as follows: 0, no visible effects; 1, locomotor effects including increased exploring activity and/or sedation; 2, tachypnea, tremors, back arching; 3, any combination of the symptoms in 1 and 2 plus rapid movements of the legs, wild running, or partial loss of righting reflex; 4, any combination of the previous symptoms plus complete loss of righting reflex, clonic seizures, 5, any combination of the preceding symptoms plus tonic seizures; 6, death, with or without hyperextension of the limbs along the axis of the body (soldier position).

EEG Recordings

Mice were anesthetized with Equithesin (5 ml/kg, i.p.) and placed in a stereotaxic apparatus (David Kopf, mod. 900). The skull was exposed and perforated, the holes aimed at the following positions: one located above the (either left or right) sensorimotor cortices (FPr and FPl), [coordinates from bregma (mm): anteroposterior +2, lateral 3, ventral to skull surface 1.5], two targeted to the dorsal hippocampus with bipolar leads glued together [coordinates from bregma (mm): anteroposterior +2, lateral 1 and 2, ventral 1.3 and 2] and one on the skull over the cerebellum as a reference [coordinates from bregma (mm): anteroposterior −5.9, lateral 1.5] [25]. A four-pin male socket was positioned into the holes, secured to the skull with epoxy resin and covered with acrylic cement to improve retention.

One week following surgical preparation of the animals (or three to five weeks for chronic studies), experiments started according to the protocol described above, electrical potentials were acquired and the signals amplified, bandpass-filtered and recorded on a portable digital EEG polygraph (BQS 98 System Micromed). In addition, in view of the possibility that digital data generated by the above mentioned experimental settings may need unconventional processing, further home-made software analysis rewritten on a Matlab® platform was used (e.g. signal analysis with wavelets versus traditional fast Fourier transform (FFT) processing).

Electrode impedance was maintained at <5 kΩ. The amplified signals, processed with a band-pass filter (0.02 to 70 Hz), was stored on the hard disk at a sampling rate of 256/s.

The behavior of the animals was video-recorded for the entire duration of the experiment.

In vitro Electrophysiology

The preparation of FCx slices and whole-cell patch clamp recordings from layer II/III pyramidal

neurons was as described previously [12]. Briefly, male mice (10–25 d, Harlan) and Sprague Dawley rats (14–21 d, Harlan) were anesthetized with halothane and killed. A block of tissue containing the FCx was sliced in the coronal plane (300 μm) with a vibratome (Leica, Nussloch, Germany) in ice-cold low Ca2+ artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 2.5 KCl, 2 CaCl2, 2 MgCl2, 1.25 NaH2PO4, 26 NaHCO3, and 10 D-glucose (pH 7.3–7.4). Slices (two per animal) were transferred in a holding chamber and allowed to recover for at least 1 hr before being placed in the recording chamber and superfused with ACSF (32–34°C) saturated with 95%O2/5%CO2:

FCx layer II/III pyramidal cells were identified visually with an upright microscope with infrared illumination, and whole-cell voltage-clamp recordings were made by using an Axopatch 200B amplifier (Molecular Devices, CA). All GABAA IPSC recordings were made with electrodes filled with an internal solution containing the following (mM): 140 cesium-methylsulfonate, 0.2 EGTA, 5 NaCl, 10 HEPES, 2 Mg2ATP, 0.25 Mg2GTP, pH 7.2–7.4. Experiments were begun only after series resistance had stabilized (typically 15–40 MΩ). Series resistance and input resistance were monitored continuously on-line with a 4 mV depolarizing step (25 ms). Data were filtered at 2 KHz, digitized at 10 KHz, and collected on-line with acquisition software (Clampex 8.2, Molecular Devices, CA). Neurons were voltage-clamped at a membrane potential of 0 mV. All GABAA spontaneous IPSCs were recorded in presence of 2-amino-5-phosphonopentanoic acid (AP5; 100 μM), 6-cyano-2,3-dihydroxy-7-nitro-quinoxaline(CNQX; 10 μM), to block N-methyl-D-aspartate- (NMDA), α-amino-3-hydroxy-5-methyl-isoxazoleprop ionicacid- (AMPA) mediated synaptic currents, respectively. As already described [12], there was no effect of this solution on the holding current of the pyramidal cells.

N-acylethanolamine Quantification

Male C57BL/6J mice (Harlan, San Pietro al Natisone, Italy) were fed, as described above, with a standard diet or a 0.2% fenofibrate diet. After 14 days, mice were killed and brain rapidly removed. Frontal cortex slices were obtained and immediately frozen. Frozen slices were homogenized and extracted with chloroform/methanol/Tris-HCl 50 mM pH 7.5 (2:1:1, v/v) containing internal deuterated standards for PEA and OEA quantification by isotope dilution ([2H]4 PEA, [2H]4 OEA; Cayman Chemicals, MI, USA). The lipid-containing organic phase was dried down, weighed and pre-purified by open bed chromatography on silica gel. Fractions were obtained by eluting the column with 90:10 (v/v) chloroform/methanol. PEA and OEA were quantified by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) [(Agilent 1100 HPLC system (Agilent, Palo Alto, CA, USA)

equipped with MS Detector 6110 single quadruple)], and using selected ion monitoring at M +1 values for the four compounds and their deuterated homologues, as previously described [26], [27].

Phosphorylation of nAChRs

Phosphorylation of the β2*-nAChRs by the PPARα agonist was assessed ex vivo by immunoblotting in rat brain homogenates. Rats (250–300 g) were treated with the PPARα agonist WY14643 (40 mg/kg, i.p.) or vehicle and killed after 15 min. Brains were rapidly removed and the FCx was immediately frozen in liquid nitrogen. The tissue was then sonicated in cell lysis buffer (50 mM TRIS, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, 1% Triton X-100, 0.02% NaN3) containing1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail. Protein concentrations of the lysates were measured by the Bio-Rad Dc Protein Assay.

β2 subunit protein was immunoprecipitated from whole-cell lysates using a rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 342–433 of the human β2 subunit (sc-11372, Santa Cruz Biotechnology). Antibodies were coupled to protein A Dynabeads (Invitrogen) using 5 μg anti-β2 antibody by rotating the mixture for 10 min at room temperature. Beads were washed twice in PBS and then the antibody-conjugated beads were incubated with 500 μg of protein lysate for 10 min at 4°C, followed by 3 washing steps in PBS supplemented with 0.1% Tween 20 (Sigma-Aldrich). Bound protein was eluted with 4× XT Sample buffer (Bio-Rad) and Reducing agent (Bio-Rad).

The immuno-precipitates were separated on an XT Criterion 10% gel (Bio-Rad, Copenhagen, Denmark) with 1× XT MOPS running buffer (Bio-Rad) for 1 hour at 175 V (constant) and subsequently electro-transferred to a nitrocellulose membrane at 400 mA (constant) for 1 hour. The membranes were incubated with 4G10 anti-phosphotyrosine (PY) antibodies (Upstate Biotechnology).

Chemiluminescence was detected and quantified with the Versa Doc 1000 Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). Samples from control and treated rats were run on the same immunoblots and then analyzed together. Values obtained from treated rats were calculated as percentage of control values.

Drugs

Nicotine [(−)-nicotine hydrogen tartrate)] and fenofibrate were purchased from Sigma (Italy). Nicotine was dissolved in 0.9% NaCl solution and the pH was adjusted to 7.0 with 0.1 M NaOH. Concentrations were adjusted for the mouse to receive 10 µl/g of nicotine solution. WY14643 and MK886 were purchased from Tocris. WY14643 and MK886 were dissolved in a solution containing 10% Tween80, 20% dimethyl sulfoxide (DMSO) and 70% distilled water. All drugs for patch-clamp experiments were dissolved in DMSO as stock solutions and then diluted to the final volume in ACSF (final concentration <0.01%).

Statistical Analysis

Seizure scores (expressed as mean ±95% confidence interval, C.I.) were analyzed by utilizing Kruskal-Wallis test for non-parametric data, and Dunn's test as a post-hoc.

Electrophysiological experiments were sampled on line and off line with data analysis electrophysiology software by computers connected to specific interface. Drug-induced changes in sIPSCs were calculated by averaging the effects following drug administration and normalized to the pre-drug baseline.

Data were analyzed utilizing parametric one-way ANOVA or Student's t-test, when they had equal variance and were normally distributed; nonparametric t-test was utilized in all other circumstances. Post hoc multiple comparisons were made using the Dunnett's or Newman-Keuls'test when appropriate. Western blot data were analyzed using Student's t-test. Contingency tables were analyzed using Fisher's exact test. Alpha was set at $P<0.05$. All analyses were performed using the software Statistica 6 (StatSoft inc. Tulsa, OK, USA).

Results

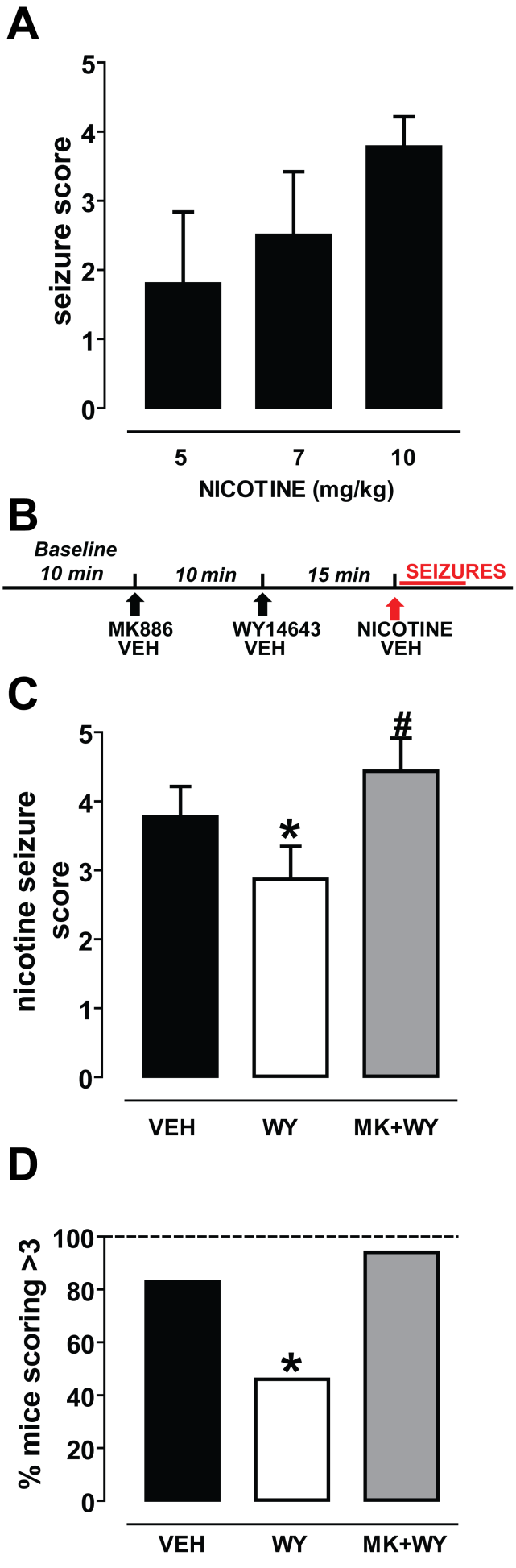
Measurement of Nicotine-induced Seizures

Nicotine was subcutaneously administered at the doses of 5 (n = 4), 7 (n = 5), and 10 mg/kg (n = 18) in mice and its effects were observed during 5 min after injection by two observers blind to the treatment. Data for behavioral observations were also obtained from mice carrying electrodes for EEG recordings, and were pooled with those obtained from non-implanted animals.

After a first exploratory phase, mice displayed impaired locomotor activity with shakes. A few seconds later they developed strong tachypnea with tremors, back arching, and partial loss of righting reflex. At higher doses, the previous symptoms were followed by increased locomotor activity, with rapid movements of the legs and wild running. Clonic and tonic seizures occurred after complete loss of righting reflex. Fig. 1A shows that nicotine elicited seizures with dose-dependent severity ($r2 = 0.76$, $P<0.001$). The calculated ED50 for nicotine was 5.08±0.07 mg/kg, similar to that already reported in the literature for the same mouse strain [24]. Only the dose of 10 mg/kg was able to induce severe symptoms, which scored higher than 3. In this case, the number of animals which scored 4 or 5 was 15 out of 18 (83%) and, therefore, we chose this dose for the subsequent experiments.

Figure 1. The PPARα agonist WY14643 reduces nicotine-induced seizures.

(A) Graph displaying the dose-dependency of the severity of nicotine-induced seizures in C57BL/6 mice. Nicotine was administered subcutaneously at 5, 7 and 10 mg/kg (n = 4, n = 5, n = 18, respectively). (B) Schematic representation of the experimental protocol of experiments in (C) and (D). (C) The PPARα agonist WY14643 (WY, 80 mg/kg, i.p., n = 23) reduced the severity of nicotine-induced seizures (nicotine dose: 10 mg/kg, s.c) (*P<0.05 vs. vehicle, Dunn's-test). This effect was abolished by the selective PPARα antagonist MK886 (MK, 3 mg/kg, i.p.). (n = 16, # P<0.001 vs. WY, Dunn's-test). (D) This graph shows that the percentage of mice undergoing severe nicotine-induced seizures (indexed by scores >3) is significantly attenuated after WY14643 pretreatment (*P<0.05 vs. vehicle, Fisher's test). MK886 reversed this effect (P>0.05 vs. vehicle, Fisher's test). Data are expressed as mean±95% C.I.


A
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0
1
2
3
4
5
5
7
10
NICOTINE (mg/kg)
B
Baseline
10 min
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SEIZURES
MK886
VEH
WY14643
VEH
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VEH
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nicotine seizure score
0
1
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0
20
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100
*
VEH
WY
MK+WY

Fig 1

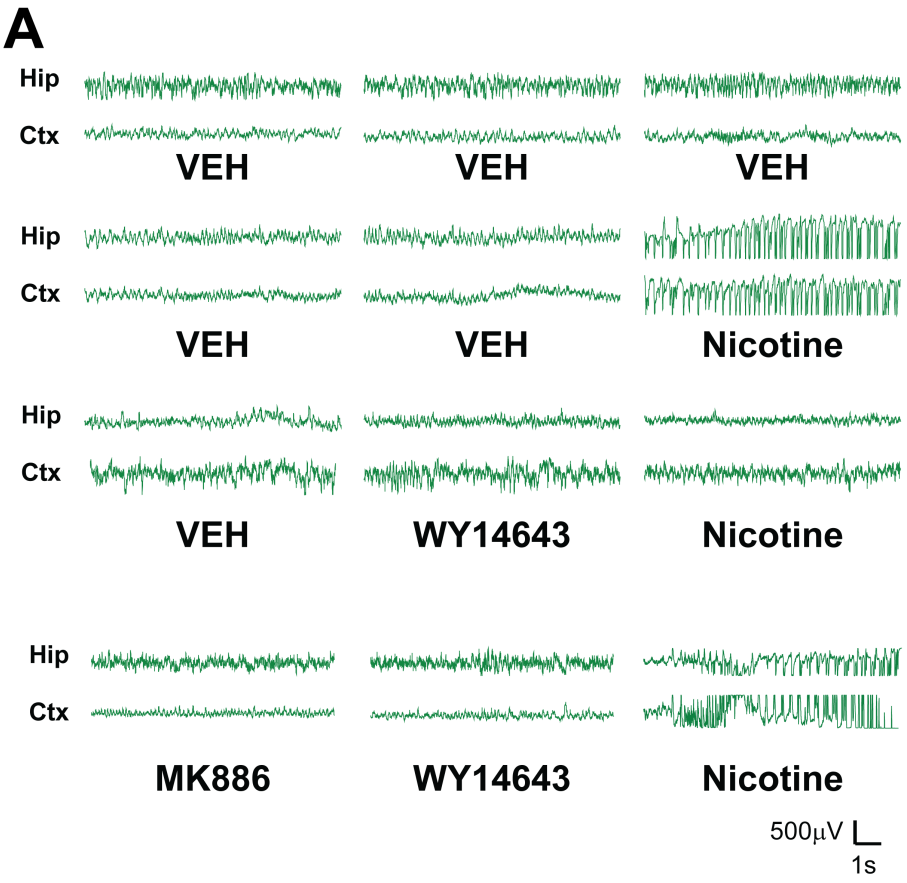
The PPARα agonist WY14643 Acutely Reduces the Severity of Nicotine-induced Seizures

To test the effect of the PPARα agonist WY14643 (WY, 80 mg/kg, i.p.) and the antagonist MK886 (MK, 3 mg/kg, i.p.) on nicotine-induced seizures, mice were randomly assigned to three groups: i) the vehicle-vehicle-nicotine (VEH group), ii) the vehicle-WY-nicotine group (WY group), and iii) the MK886-WY-nicotine group (MK-WY group). Each animal received the three injections spaced 10 and 15 min (Fig. 1B).

When scores were evaluated, non-parametric ANOVA yielded a highly significant difference among groups ($P<0.0001$, Kruskal-Wallis test) (Fig. 1C). Post-hoc analysis revealed that mice pre-treated with the PPARα agonist WY14643 were significantly protected against nicotine-induced seizures when compared with vehicle pre-treated animals. The difference in the average score was statistically significant (VEH mice: 3.8±0.4, n = 18; WY mice: 2.9±0.5, n = 24; $P = 0.019$, Dunn's test) (Fig. 1C). Additionally, WY mice which obtained a score of 4 or 5 were 46% of all treated mice (11 out of 24). The difference was statistically significant when compared with the VEH-group (15/18, 83%) ($P<0.05$, Fisher's test, Fig. 1D). The protective effect of WY was reverted by the PPARα antagonist MK. Hence, post-hoc analysis indicated that in the MK-WY-group the severity of nicotine-induced seizures was restored, since the average score was significantly higher than that assigned to the WY mice (4.4±0.5, n = 16, $P = 0.001$, Dunn's test, Fig. 1C), but not different from that of VEH-mice ($P>0.05$, Dunn's test) (Fig. 1C). Consistently, MK-WY mice receiving a score of 4 or 5 were 15 out of 16 (94%) ($P = 0.6$ vs. VEH mice) (Fig. 1D).

In vivo electrophysiological recordings revealed that high doses of nicotine elicited seizures that originate in the hippocampus [28] and in the thalamo-cortical pathways [11]. These neural circuits may also be involved in NFLE (for review, see [29]). For this reason, one EEG electrode was placed in the sensorimotor cortex and two in the dorsal hippocampus. Of the two electrodes positioned in the hippocampus the one with the best signal was chosen.

As depicted in Fig. 2, nicotine-induced (10 mg/kg, s.c.) convulsive activity was paralleled by a synchronous spiking discharge pattern both in the hippocampus and in the cortex in 7 out of 7 (100%) treated mice (Fig. 2A, B). Spiking activity had an onset of 130±7 s after nicotine injection and a duration of 52±10 s (n = 7). No spike/wave discharge events were recorded in vehicle treated mice (Fig. 2 A, B).



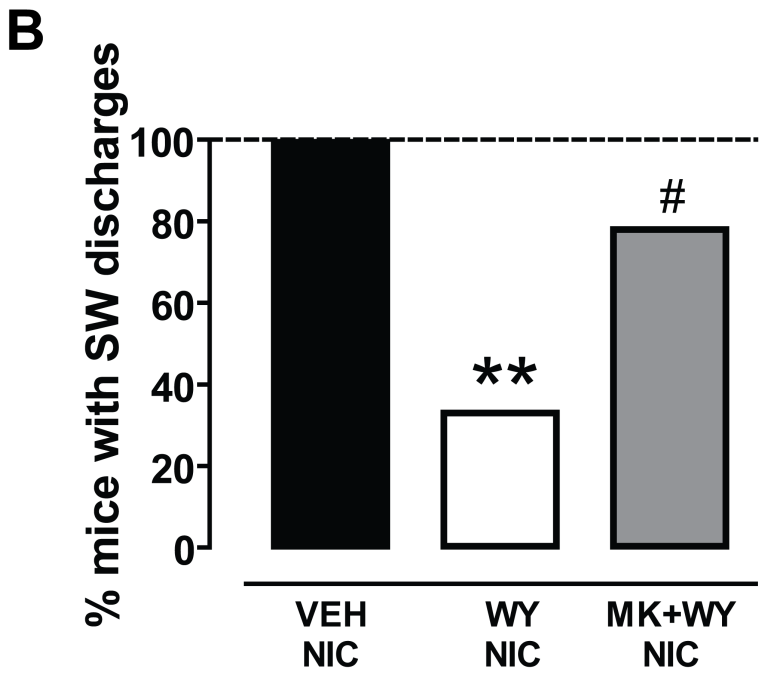


Figure 2. The PPARα agonist WY14643 suppresses nicotine-induced spike-wave activity.

(A) Representative traces of EEG recordings from hippocampal (Hip) and sensorimotor cortical (Ctx) electrodes chronically implanted in mice. Following the administration of vehicle (VEH) and 10 mg/kg nicotine, bursts of synchronous spike-wave (SW) activity with high-amplitude and low-frequency (most in the delta rhythm range) were recorded. This activity was suppressed when animals were pretreated with the PPARα agonist WY14643 (WY, 80 mg/kg), which *per se* did not change baseline EEG activity. The PPARα antagonist MK886 (MK, 3 mg/kg, i.p.) restored nicotine-induced SW discharges. (B) The graph shows the percentage of mice presenting SW discharges

following the three treatment protocols. Vehicles treated mice did show SW activity, whereas 100% of nicotine treated mice displayed bursts of SW activity. The effects of nicotine were blocked in the majority of WY treated mice, since SW burst were recorded only in 33% of treated animals (**P<0.01 vs. vehicle, Fisher's test). Conversely, when MK was administered 15 before WY nicotine-induced SW activity was recorded in 78% of MK+WY treated mice ($^{\#}$P<0.05 vs. WY, Fisher's test).

Pretreatment with WY significantly attenuated the effects of nicotine, since only 3 out of 9 (33.3%) mice displayed spiking activity when compared with vehicle-treated mice (P<0.05, Fisher's test, Fig. 2B). WY pretreatment, however, did not change either the onset or the duration of spike-wave activity in those animals not responding to the treatment (114±66 s and 32±18 s, respectively, n = 3). Consistent with behavioral experiments, MK prevented WY-induced protection from seizures, since nicotine's effect was restored in 7 out of 9 mice (77.8%) (Fig. 2A, B).

Chronic Administration of the Clinically Available PPARα agonist Fenofibrate Attenuates the Severity of Nicotine-induced Seizures.

The results of the previous experiments prompted us to assess the efficacy of the clinically available PPARα agonist fenofibrate. Fenofibrate does not cross the blood-brain barrier rapidly enough to allow acute studies in vivo; therefore, we chronically treated the animals with a diet containing 0.2% w/w fenofibrate [30]. Mice were randomly divided into three groups: (i) fenofibrate diet (FBR), (ii) control diet (CTRL) and (iii) fenofibrate washout diet (FBR-WO). The fenofibrate containing diet was administered ad libitum for 14 days. FBR mice consumed a daily average of 3.6±0.1 g of food pellets, which approximately corresponds to 28–30 mg/kg fenofibrate per day. FBR mice were divided in two additional groups at the end of the treatment: one group received the PPARα antagonist MK886 15 min before the nicotine challenge, and the other received its vehicle. CTRL mice were fed with the control diet, identical and equicaloric but without fenofibrate. FBR-WO mice were fed for 14 days with a fenofibrate-containing diet, as FBR mice, and then fed with a control diet for additional 14 days to assess the effect of fenofibrate washout. In each animal EEG electrodes were implanted 7 days before the beginning of the treatments and nicotine was tested on the 15th day from the beginning of treatment (for FBR and CTRL mice) or on the 30th day (for FBR-WO mice).

When nicotine-induced seizures were scored, non-parametric ANOVA yielded a highly significant difference among groups (P<0.0001, Kruskal-Wallis test) (Fig. 3A). Post-hoc analysis revealed that FBR mice were significantly protected against nicotine-induced seizures when compared with CTRL animals. The difference in the average score was statistically significant (CTRL mice: 3.4±0.7, n = 14; FBR mice: 2.1±0.3, n = 8; P<0.001, Dunn's test) (Fig. 3A). Additionally, none among the FBR mice obtained a score of 4 or 5 (0 out of 8) (Fig. 3 B). The

difference was statistically significant when compared with the CTRL group (11/14, 78%) (P<0.001, Fisher's test, Fig. 3B). The protective effect of the fenofibrate diet was not reverted by the PPARα antagonist MK. Hence, post-hoc analysis indicated that in the MK-treated FBR mice the severity of nicotine-induced seizures was not restored, since the average score was significantly lower than that assigned to CTRL mice (2.4±0.4, n = 8, P<0.001, Dunn's test, Fig. 3A), but not different from that of FBR-mice (P>0.05, Dunn's test) (Fig. 3A). Consistently, no MK-treated FBR mice received a score of 4 or 5 (Fig

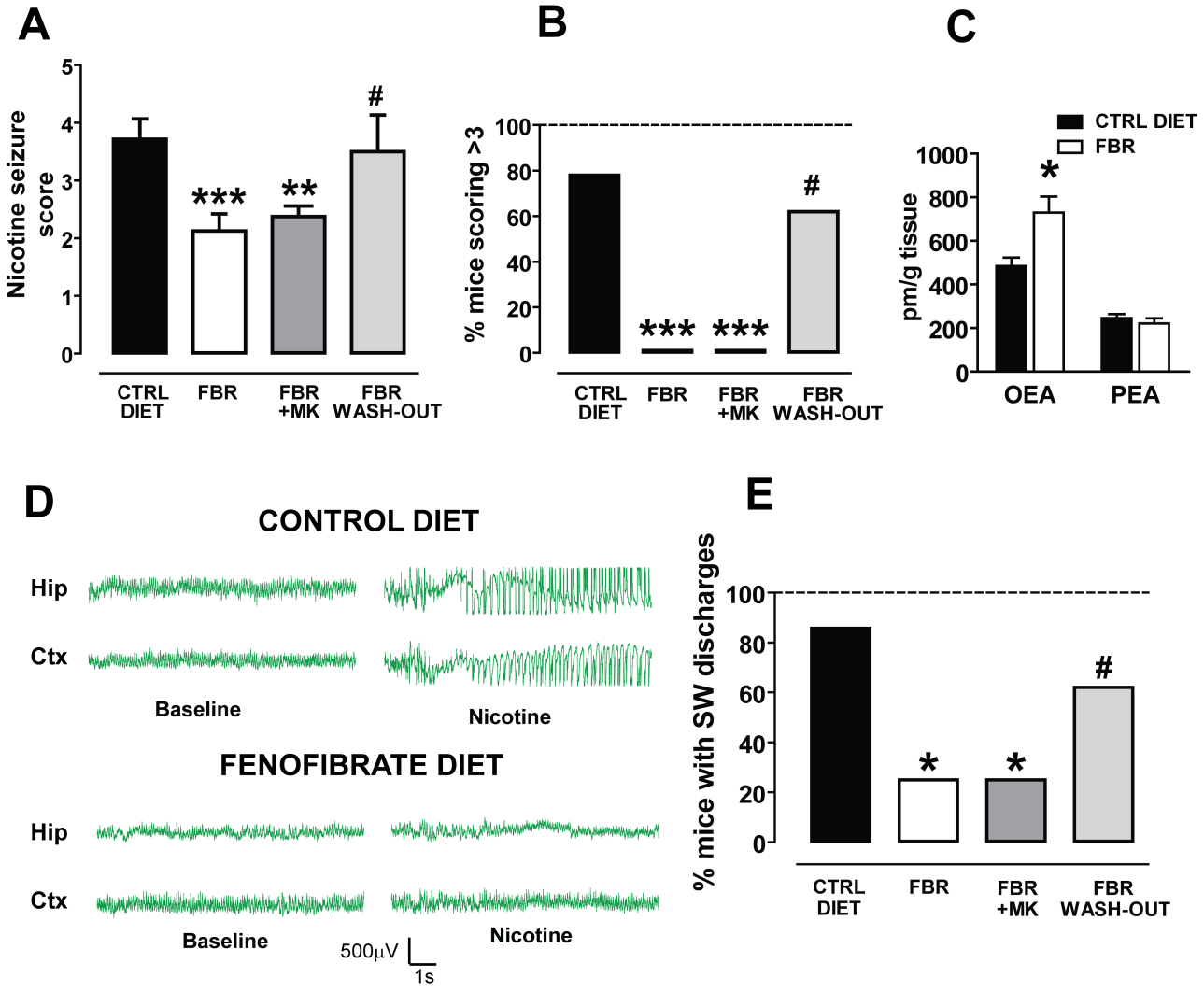


Figure 3. The clinically used PPARα agonist fenofibrate chronically administered with food reduces nicotine-induced seizures and spike-wave activity, and increases the OEA levels in the frontal cortex.
(A) Graph displaying that fenofibrate (n = 8, FBR, 0.3% w/w in the diet for 14 days) reduced the severity of nicotine-induced seizures (nicotine dose: 10 mg/kg, s.c) (***P<0.001 vs. control diet, CTRL DIET, Dunn's-test). This effect was not abolished by the selective PPARα antagonist MK886 (FBR+MK, 3 mg/kg, i.p.) (n = 8, **P<0.01 vs. CTRL DIET, Dunn's-test) but by withdrawal of fenofibrate treatment for 14 days (FBR WASH-OUT, n = 8, # P<0.05 vs. FBR and P>0.05 vs. CTRL DIET, Dunn's test). (B) This graph shows that the percentage of mice undergoing severe nicotine-induced seizures (indexed by scores >3) is significantly attenuated after chronic fenofibrate treatment (***P<0.001 vs. control diet, Fisher's test) and restored after fenofibrate withdrawal for 14 days (#P>0.05 vs. control diet). MK886 did not reverse this effect (***P<0.001 vs. control diet, Fisher's test). (C)

Chronic activation of PPARα by fenofibrate changes oleoylethanolamide (OEA), but not palmitoylethanolamide, levels within frontal cortex. Concentrations of these endogenous PPARα ligands are expresses as pmol per gram of tissue. Error bars depict S.E.M. (*P<0.05, Student's t-test). (D) Representative traces of EEG recordings from hippocampal (Hip) and sensorimotor cortical (Ctx) electrodes chronically implanted in mice. In mice fed with control diet 10 mg/kg nicotine elicits bursts of synchronous spike-wave (SW) activity with high-amplitude and low-frequency (most in the delta rhythm range). This activity was suppressed in animals fed with fenofibrate in food pellets. (E) The graph shows the percentage of mice presenting SW discharges following the four treatment protocols. 86% of control diet fed mice (6 out of 7) did show nicotine-induced SW activity. The effects of nicotine were fully blocked in all fenofibrate treated mice, since SW burst were recorded only in none of treated animals (***P<0.01 vs. control diet, Fisher's test). MK, administered 15 before nicotine, did not restore nicotine-induced SW activity (***P<0.001 vs. control diet, Fisher's test), whereas fenofibrate washout did (#P>0.05 vs. control diet, Fisher's test). Data are expressed as mean±95% C.I.

The lack of effect by MK could be explained by the fact that PPARα activation induces mitochondrial activity, peroxisomal β-oxidation and the biosynthesis of endogenous PPARα ligands, the N-acylethanolamines OEA and PEA. These combined effects might trigger a feed-forward mechanism to further sustain PPARα activity [31]. To test this hypothesis we measured the levels of OEA and PEA in the FCx from FBR and CTRL mice. As predicted, in FBR mice OEA levels were significantly increased in the FCx when compared to CTRL mice (729.5±73.4 pm/g vs. 484.3±38.4 pm/g; n = 5; P<0.05, Student's t-test) (Fig. 3C). No changes of PEA levels were detected (FBR: 221.7±23.0 pm/g; CTRL: 245.6±18.4 pm/g; n = 5; P = 0.44 Student's t-test) (Fig. 3C).

Fenofibrate washout for 14 days abolished the protective effects of the drug, since in FBR-WO mice the severity of nicotine-induced seizures was restored (Fig. 3A). FBR-WO mice received a score of 3.5±0.6 (n = 8), significantly higher that FBR mice (P<0.05, Dunn's test) but not dissimilar from CTRL mice (P>0.05, Dunn's test) (Fig. 3A).

EEG recordings revealed that nicotine-induced (10 mg/kg, s.c.) convulsive activity was paralleled by a synchronous spiking discharge pattern in both the hippocampus and the cortex in 6 out of 7 (86%) CTRL mice (Fig. 3D, E). Spiking activity had an onset of 148±8 s after nicotine injection and a duration of 53±9 s (n = 6).

The effect of nicotine was significantly attenuated in FBR mice, since only 2 out of 8 (25%) mice displayed spiking activity, when compared with CTRL mice (P<0.05, Fisher's test, Fig. 3E). In FBR mice, however, neither the onset nor the duration of spike-wave activity changed in those animals not responding to the treatment (179.5±25 s and 27±2 s, respectively, n = 2).

The effects of nicotine were restored in FBR-WO mice: spiking activity was indeed present in 5 out of 8 mice (P>0.05 vs. CTRL mice, Fisher's test) (Fig. 3E). Consistent with behavioral experiments, MK did not block FBR-induced protection from seizures, since nicotine's spike-wave activity was assessed in 2 out of 8 mice (25%) (Fig. 3E).

PPARα Agonists Suppress Nicotine-induced sIPSCs in Layer II–III Pyramidal Neurons in

the Mouse and Rat Frontal Cortex

Nicotine-induced seizures, as well as those spontaneously occurring in transgenic mice carrying the NFLE mutations, might be primarily generated within FCx circuits [12], [29]. Since excitatory transmission on FCx pyramidal neurons is not affected by NFLE mutations or by nicotine [12], we carried out whole-cell recordings of spontaneous inhibitory postsynaptic currents (sIPSCs) from layer II/III pyramidal cells of mouse and rat coronal slices.

Under voltage-clamp mode (Vholding = 0 mV to isolate sIPSCs [12]), nicotine (5 μM, 30 s) significantly increased both frequency and amplitude of sIPSCs in mouse pyramidal cells. sISPC frequency was enhanced to 133.2±2.9% of baseline ($P<0.0001$, n = 23, paired t-test), the amplitude to 116.5±7.5% of baseline ($P<0.05$, n = 23, paired t-test) (Fig. 4, Table 1). In marked contrast, pretreatment with the PPARα agonists WY (1 μM, 5 min) and fenofibrate (10 μM, 5 min) dramatically blunted the effect of nicotine on sIPSCs. During nicotine application in the presence of WY, sIPSC frequency was 91.8±10.2% of baseline and amplitude was 91.8±4.7% of control values (for both parameters, $P>0.05$ vs. baseline, n = 7, paired t-test) (Fig. 4A, Table 1). Consistently, the structurally different PPARα agonist fenofibrate also suppressed nicotine-induced increase of sIPSC frequency and amplitude (for both parameters, $P>0.05$ vs. baseline, n = 6, paired t-test) (Fig. 4B, Table 1).

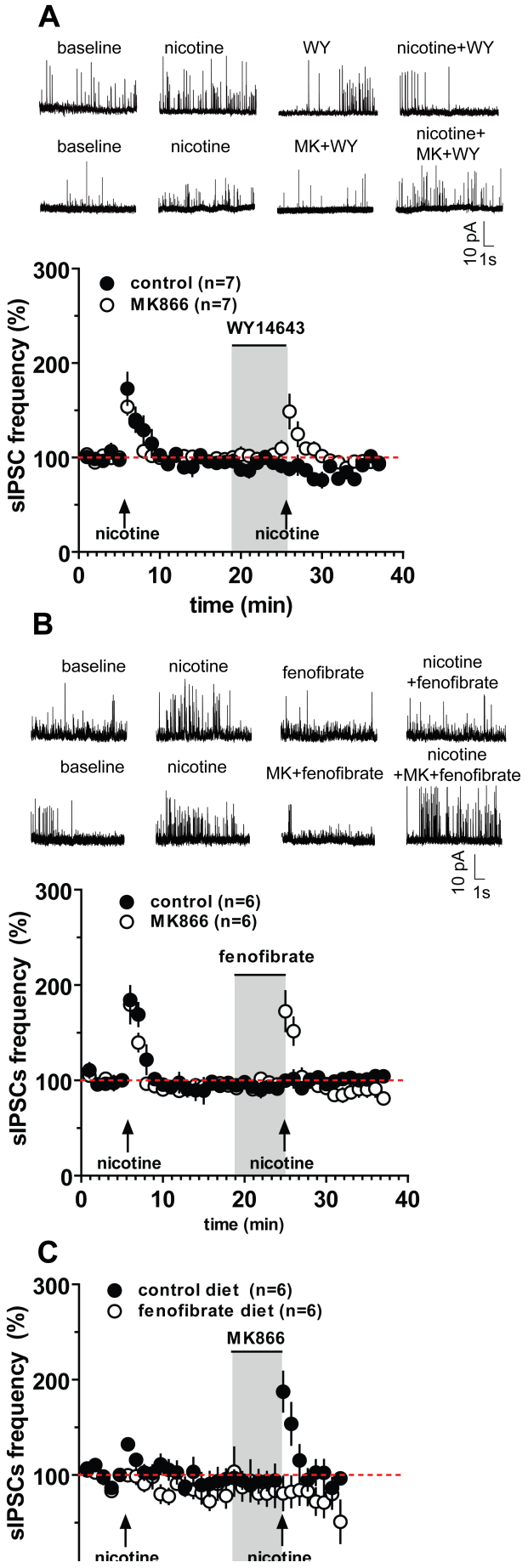


Figure 4. The PPARα agonists WY14643 and fenofibrate suppress nicotine-induced increase of spontaneous inhibitory postsynaptic currents (sIPSC) in frontal cortex (FCx) pyramidal neurons.
The graphs illustrate that in mouse FCx slices, nicotine (5 μM perfused at arrows for 30 s) increases sIPSCs frequency in layer II/III pyramidal neurons. Acutely, the PPARα agonists WY14643 (1 μM, WY) (A), and fenofibrate (10 μM) (B) fully suppressed nicotine-induced increase in sIPSC frequency. Similarly, chronic fenofibrate (0.2% w/w for 14 days in food) fully suppressed nicotine-induced increase in sIPSC frequency (C). The gray box represents the time of PPARα agonist (+/− antagonist) perfusion. The PPARα antagonist MK886 (0.3 μM) (open symbols) blocked the effects of acute WY (A) and fenofibrate (B), but not the one of chronic fenofibrate (C), and restored nicotine-induced increase in sIPSCs. Representative recordings of the effect of nicotine, and of PPARα agonists and antagonist, on spontaneous IPSCs from pyrami

	Baseline	Nicotine	WY +Nicotine	FBR +Nicotine	WY+MK+Nicotine	FBR+MK+Nicotine
C57BL/6J mice						
Frequency, Hz	46.2±1.0	61.2±1.1***	35.1±6.6	40.0±1.6	68.7±12.2*	79.7±16.7*
Amplitude, pA	17.8±0.8	19.8±0.8*	18.3±2.7	18.1±1.6	21.5±1.9*	20.3±1.5*
Rats						
Frequency, Hz	15.3±3.2	22.2±4.7***	15.8±11	11.5±0.6	22.5±5.7**	22.5±7.7*
Amplitude, pA	16.4±0.7	21.3±1.5***	13.4±0.8	14.0±1.3	25.1±5.4*	22.8±1.9*

Nicotine (5 μM) significantly increases both frequency and amplitude of sIPSC (*P<0.05, 01, ***P<0.001 vs baseline, paired t-test). Both WY14643 (WY 1 μM) and fenofibrate (FBR, 10 μM) suppressed nicotine's effects on sIPSCs frequency and amplitude. The PPARα antagonist MK886 restored nicotine's effects on sIPSC frequency (*P<0.05, **P<0.01 vs baseline, paired t-test).

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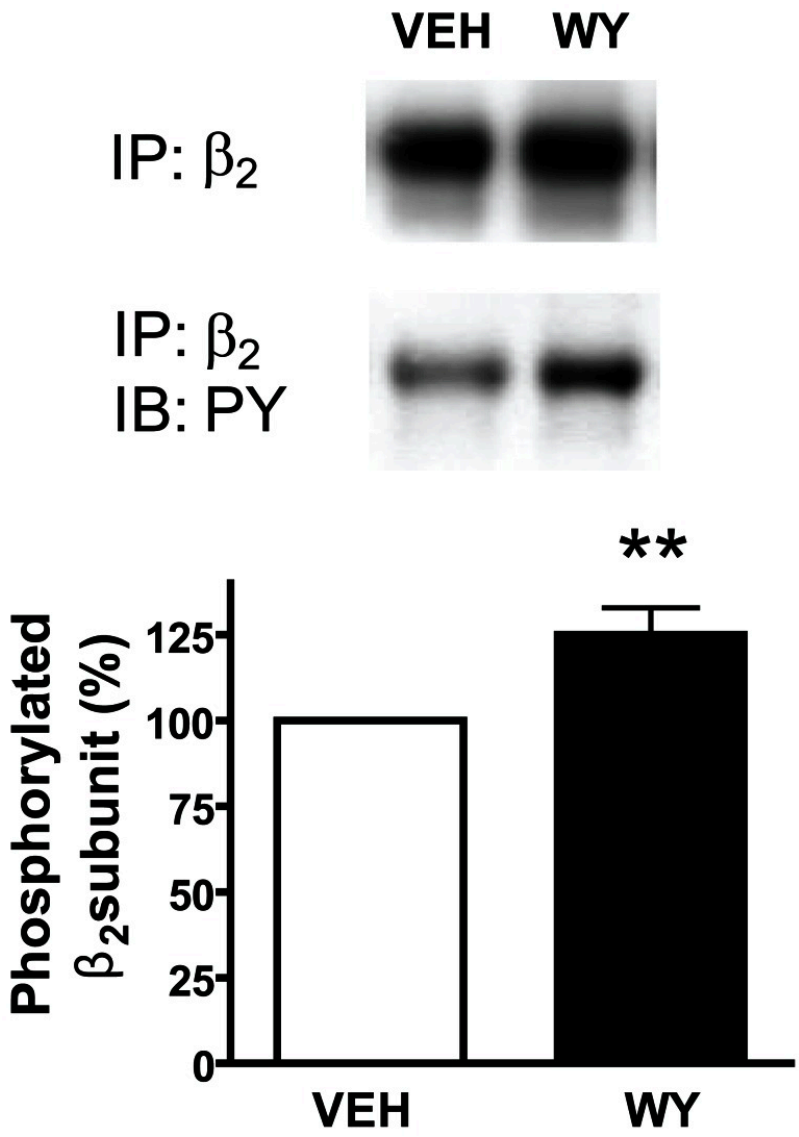
The effects of both PPARα agonists were blocked by the synthetic PPARα antagonist MK. In the presence of MK (0.3 μM, 5 min) and either PPARα agonist, nicotine effects on sIPSCs frequency and amplitude were fully restored. Hence, in slices perfused with WY+MK or fenofibrate+MK, nicotine induced an increase in sIPSC frequency to 141.7±19.3% and to 172.5±20.9% of baseline, respectively (P>0.05vs. nicotine alone, n = 6–7, paired t-test; Table 1,Fig. 4A,B). Table 1 shows that sIPSC amplitude in the presence of nicotine alone was not significantly different from the amplitude in the presence of nicotine+WY+MK or nicotine+fenofibrate+MK(P>0.05, n = 6, paired t-test).

To assess whether suppression of nicotine effects by acute PPARα agonists was species-specific we replicated the experiments described above in Sprague Dawley rats. The results (Supporting Results S1) confirmed that both WY and fenofibrate abolished nicotine-induced effects in both species (Table 1; Figure S1).

Experiments with mice chronically fed with fenofibrate (0.2% w/w for 14 days) confirmed that this drug abolishes nicotine-induced increases in sIPSC frequency in FCx pyramidal neurons. Hence, nicotine-induced increase in sIPCS frequency was 132.2±4.6% of baseline (n = 6) in CTRL mice vs. 99.7±5.6% of baseline (n = 6) in FBR mice (P<0.05, Student's t-test) (Fig. 4C). Consistent with behavioral experiments, MK was unable to revert the effect of fenofibrate, since in FBR mice MK did not restore the effects of nicotine (sIPSC frequency in control animals was 187.5±20.9% vs. 80.7±17.6% in FBR mice).

The PPARα Agonist Enhances Phosphorylation of the β2 Subunit of nAChRs

Consistent with our previous reports [13], [14], these findings led us to hypothesize that PPARα might regulate the balance between phosphorylated and dephosphorylated β2*nAChRs. To test this hypothesis, we analyzed phosphorylation of β2 subunits with Western blot after *in vivo*exposure to the synthetic PPARα agonist WY. Rats were administered WY (40 mg/kg, i.p.) and after 15 min, brain were rapidly removed and the FCx were dissected. As expected, in FCx total homogenates an increased immunoreactivity for phosphorylated β2 subunits was observed in WY-treated animals (125.0±7.9% of controls, n = 7, p<0.01, Student's t-test) (Fig. 5).



The PPARα agonist WY14643 enhances phosphorylation of β2 subunits of the nicotinic acetylcholine receptors.

Discussion

Our study shows that acute and chronic PPARα agonists, including the clinically available fenofibrate, reduce nicotine-induced behavioral and EEG seizure expression and abolish nicotine-induced enhancement of sIPSCs in FCx pyramidal neurons.

We presume, based on the present results and previous extensive work by our group, that the mechanism whereby PPARα exerts these effects involves phosphorylation of specific nAChR subunits [13], [14], [22], [23]. Indeed, nAChR channel properties depend on its tyrosine phosphorylation status, being the result of a balance between tyrosine kinases and phosphatases [32], which negatively or positively modulates nAChR-mediated currents, respectively, and controls the number of functional surface receptors [33].

We specifically measured the ratio of phosphorylated/dephosphorylated β2 subunits, which is increased in the FCx by acute PPARα ligand treatment, but it is difficult to quantify this effect in subunit combinations less abundantly expressed in the CNS. Therefore, we cannot exclude that subunits other than the β2 might also be targeted by PPARα. However, it must be pointed out that β2*nAChRs play a major role, yet not exclusive, in nicotine-induced seizures [10], [34]. In fact, the α4β2 subunit combination is the most abundantly expressed in the whole brain and particularly in regions relevant to ictogenesis, such as thalamo-cortical circuits and FCx (see [12] and references therein). Furthermore, it was shown by Klaassen et al. [12] that nicotine-induced sIPSC in layer II/III of the FCx are mediated by α4β2 nAChRs, as they are insensitive to the α7-selective blocker methyllycaconitine but fully blocked by the α4β2-selective antagonist dihydro-β-erythroidine. However, it must be pointed out that other nAChR subunits are implicated in nicotine-evoked seizures. In particular, their severity is reduced in mice lacking α3, α5, and β4 [35], [36], but not α7 [24]. The role of α7, however, is still controversial, since pharmacological experiments with selective α7 agonists and antagonists yielded contradictory results [10], [34].

Irrespectively of the subunits involved other than β2, the PPARα agonists reduced both the severity of nicotine-induced effects and the number of animals undergoing severe symptoms. These behavioral effects were paralleled by a significant decrease in the number of mice that showed bursts of spike-wave discharges. The pharmacological specificity was confirmed by the finding that pre-treatment with the PPARα antagonist prevented the protection exerted by the agonist, with the exception of chronic fenofibrate treatments. The latter finding might be explained with the finding that PPARα activation induces fatty acid metabolism [31] and NAE biosynthesis with increased production of OEA. Notably, OEA would further sustain PPARα activation by triggering a feed-forward mechanism difficult to be antagonized by a single administration of the PPARα antagonist MK. A second possible explanation is that changes induced by this treatment regimen, possibly on function, number and phosphorylation status of nAChRs, are not promptly reversed by an acute treatment with the PPARα antagonist. Indeed, a reversal of fenofibrate-induced protection is observed following a 14-days washout of the drug, achieved by replacing fenofibrate-containing food pellets with control food.

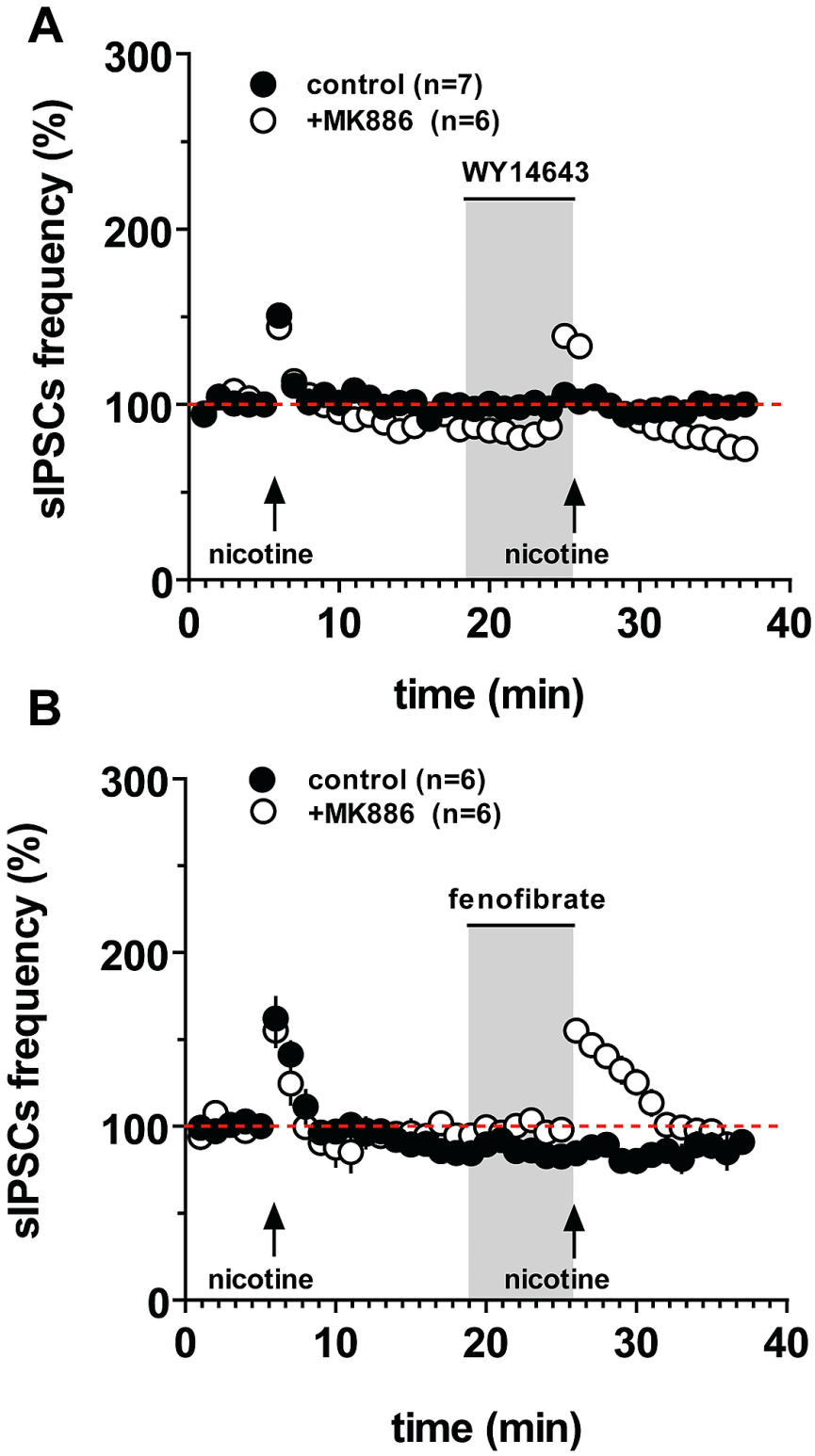
The present data, while confirming the relationship between nAChRs and epileptogenesis, give support to the role played by nuclear receptors PPARα in the modulation of nAChR function in the CNS. Hence, in our previous studies, we demonstrated that both endogenous and synthetic PPARα agonists suppressed nicotine-induced electrophysiological effects on dopamine neurons and prevented behavioral effects of nicotine predictive of its addicting properties by regulating β2* nAChRs specifically [13], [14], [15], [16]. Here we extend those results to the seizure-inducing action of nicotine.

Nuclear and cytoplasmic immunostaining for PPARα is present in neurons distributed in all layers of the FCx [20], [21], whereas cholinergic afferents, arising principally from the basal forebrain, innervate neurons in all layers of the rodent FCx [37]. Several subtypes of GABA cortical interneurons express functional α4β2* nAChRs [38], [39] and have been involved in nicotine-induced seizures [10], [12], [34], [38] since they innervate adjacent pyramidal cells. Consistently, we show that nicotine induced an increase in the frequency of GABAA-mediated sIPSCs, possibly by depolarizing presynaptic GABAergic terminals impinging on pyramidal neurons. According to behavioral observations, these effects were suppressed by PPARα agonists, suggesting that PPARα regulate the functions of nAChRs in cortical interneurons,

similarly to dopamine neurons.

Notably, the relevance of our results might extend beyond nicotine-induced seizures or NFLE, since chronic fenofibrate was effective in rats as anticonvulsant in pentylentetrazole-induced seizures and on latencies to the onset of status epilepticus induced by lithium–pilocarpine [30]. Additionally, recent studies demonstrated that the antiepileptic drugs carbamazepine and lamotrigine, which are employed effectively also in NFLE patients, negatively modulate the activity of α4β2 nAChRs [40], [41]. This effect might contribute to their mechanisms of action since these receptors control neuronal excitability and both glutamate and GABA release in the hippocampus and the thalamo-cortical system [42], [43], [44], [45], [46].

In conclusion, we provided evidence that PPARα within the CNS might be a key regulator of neuronal activity by the modulation of functional properties of nAChRs. These effects might be therapeutically exploited not only when nicotine addiction is concerned [15], but also for idiopathic or genetically determined forms of epilepsy where nAChRs play a major role.



The PPARα agonists WY14643 and fenofibrate suppress nicotine-induced increase of spontaneous inhibitory postsynaptic currents (sIPSC) in rat frontal cortex (FCx) pyramidal neurons. The graphs illustrate that in rat FCx slices, nicotine (5 μM perfused at arrows for 30 s) increases sIPSCs frequency in layer II/III pyramidal neurons. The PPARα agonists WY14643 (1 μM, WY) (**A**) and fenofibrate (10 μM) (**B**) (n = 6–7; closed symbols) fully suppressed nicotine-induced increase in sIPSC frequency. The gray box represents the time of PPARα agonist (+/− antagonist) perfusion. The PPARα antagonist MK886 (0.3 μM) (open symbols) blocked the effects of WY (**A**) and fenofibrate (**B**) (n = 6) and restored nicotine-induced increase in sIPSCs. Symbols represent the mean±SEM.

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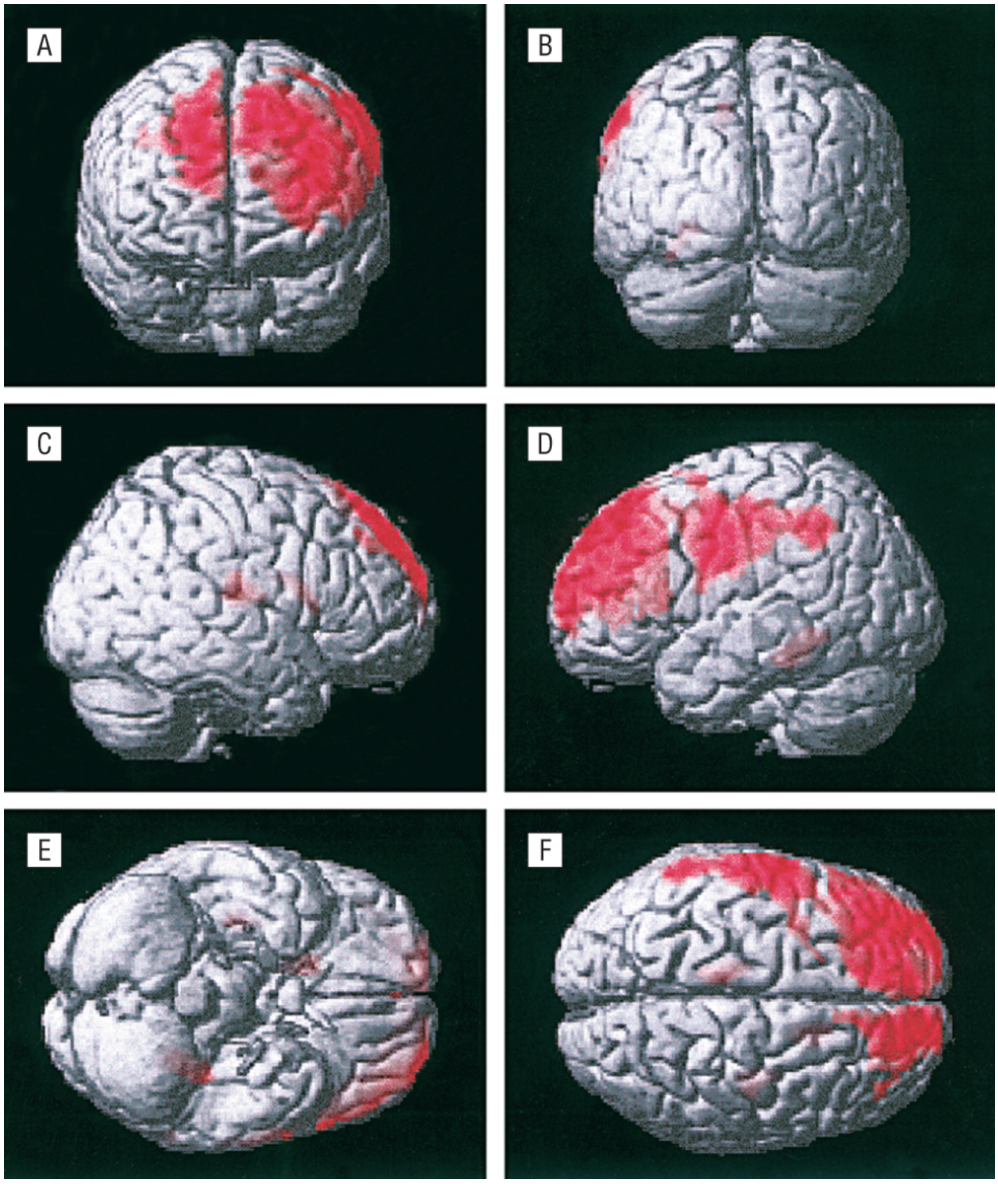


Yong-Won Cho, Arch Neurol. 2003

Peroxisome Proliferator-Activated Receptor type alpha agonists as new therapeutic target for epilepsy: the translational study

Puligheddu et al – in preparation

Abstract:

Nocturnal frontal lobe epilepsy (NFLE) is an idiopathic partial epilepsy with a family history in about 25% of cases with autosomal dominant inheritance [nocturnal frontal lobe epilepsy (ADNFLE)]. One of the key pathogenetic mechanisms is a "gain of function" of neuronal nicotinic acetylcholine receptors (nAChRs) containing the mutated α4 or β2 subunits. Fenofibrate, a common lipid-regulating drug, is an agonist at peroxisome proliferator-activated receptor alpha (PPAR-α) that is a ligand-activated transcription factor which negatively modulates β2*nAChR function. To test the clinical efficacy of adjunctive therapy with fenofibrate in pharmaco-resistant ADFNLE\FNLE patients, we first demonstrated the effectiveness of fenofibrate in a mutated (CHRNA4-S252F) mouse model displaying the disease genotype and phenotype in both in vitro and in vivo experiments. An add-on clinical protocol was subsequently implemented in a clinical setting. Here we show that a chronic fenofibrate diet markedly reduced the frequency of large IPSCs recorded from cortical pyramidal neurons in CHRNA4-S252F mice, and prevented nicotine-induced increase of IPSC frequency. Moreover, fenofibrate diet eliminated the differences observed between genotypes in the frequency of arousals from sleep under basal conditions in vivo. Accordingly, patients affected by FNLE, which were non-responders to traditional therapy, by means of adjunctive therapy with fenofibrate were found to display a significant reduction of seizure frequency. Furthemore, digital Videopolysomnographic recordings acquired in FNLE-subjects after 6 months of adjunctive fenofibrate therapy showed significant effects on motor-behavioural seizures control. Altogether, these observations pinpoint dysfunctional nicotinic receptors in NFLE, and provide a new perspective in both the investigation and the treatment of epilepsy.

Introduction

Epilepsy is a common neurological disorder affecting 1% of the population worldwide [1]. Over the past decade, several idiopathic epilepsies have been shown single-gene inheritance, such as autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) [2]. Clinical features of ADNFLE are clusters of stereotypic episodes of arousal from sleep associated with dystonic neck, limb, and trunk movements that occur during stages 2-4 of non-rapid eye movement (non-REM) sleep [3] [4], and are accompanied by severe sleep instability [5]. One of the key pathogenetic mechanisms underlying this inherited form of epilepsy is the "gain of function" of the neuronal nicotinic acetylcholine receptors (nAChRs) containing the mutated α4 or β2 subunits. Consequently, a blockade of nAChRs might theoretically provide a therapeutic approach to treat ADNFLE.

PPAR-α activation leads to phosphorylation of β2*-nAChRs with a consequent blockade of nicotine-induced activation of discrete neuronal populations in rodents [6] [7] [Puligheddu et al., 2013]. Synthetic PPAR-α agonists such as fibrates are already well established therapeutic options for the treatment of hyperlipidemia. Notably, fibrates exhibit similar anticonvulsant properties [Puligheddu et al., 2013] to traditional ketogenic diet in an experimental model of epilepsy [9]. Indeed, we observed that PPAR-α agonists are protective against nicotine-induced seizures in behavioral and electroencephalographic (EEG) experiments in vivo, and in vitro abolish nicotine-induced spontaneous inhibitory post-synaptic currents (sIPSC) increased frequency in rodents (Puligheddu et al., 2013).

Altogether, these observations prompted us to test both in vitro and in vivo the efficacy of a chronic diet with fenofibrate in a mutated mouse model displaying both disease genotype and phenotype. To optimize these results experiments were carried out in a previously described mouse model of NFLE (CHRNA4-S252F) [10]. Next, we set a clinical study based on the viability of fenofibrate in both ADFNLE and FNLE drug-resistant patients. To this aim, an add-on protocol was implemented in a clinical setting. Based on our working hypothesis, we expect that fibrates via PPAR-α activation may be beneficial to the treatment of epilepsy.

Materials and Methods

Rationale and aim of the study

The overall objective of this study was to test the efficacy of fenofibrate as an add-on therapy on

nocturnal frontal lobe epilepsy. In a mutated mouse model displaying the disease genotype and phenotype (CHRNA4-S252F) (10) we characterized electrophysiological and behavioral effects of a chronic diet with fenofibrate. Finally, we applied insights gained through this approach to examine the viability of fenofibrate in both ADFNLE and FNLE drug-resistant patients. To this aim, an add-on therapeutic protocol was implemented in a clinical setting. Methods used in this study that have been published previously in detail are included in the Supplementary Materials.

2.1.Neurophysiology and behavior of NFLE mouse model (CHRNA4-S252F)

Animals

For all the experiments we used male, age-matched, wild type and heterozygous mice model of NFLE (CHRNA4-S252F) (10). The colony founders were obtained from the Mutant Mouse Regional Resource Center (MMRRC) and were donated to the MMRRC by Dr. Jim Boulter of UCLA. Mice were housed six per cage under a 12 h light/dark cycle (light on at 7:00 AM), in conditions of constant temperature (21 ±2°C) and humidity (60%), with food and water ad libitum. Animals were randomly assigned to the different experimental protocols: in vitro electrophysiology or electroencephalographic (EEG) recordings coupled with behavioral observations.

Ethics Statement

Experiments were performed in strict accordance with the EEC Council Directive of 24 November 1986 (86/609). All efforts were made to minimize pain and suffering and to reduce the number of animals used. The experimental protocols were also approved by the Animal Ethics Committee of the University of Cagliari and by the Italian Ministry of Health.

Chronic diet

Both wild type (wt) and heterozygous Chrna4-S252F (mut) mice were randomly divided into diet treatment groups: (i) a standard diet (control group, Harlan Teklad Global 2016); (ii) a 0.2% fenofibrate diet (Fenofibrate from Sigma-Aldrich + Harlan Teklad Global 2016) (9)(8). Mice were fed diets for 14 days. For EEG and observational studies, diets started one week after electrode implantation (see below).

Chronic EEG recordings

Adult mice (PND > 90) were anesthetized with Equithesin (5 ml/kg, i.p.) and placed in a

stereotaxic apparatus (David Kopf, mod. 900). The skull was exposed and coated stainless steel electrodes were implanted in the right prefrontal cortex (from bregma (mm): anteroposterior +2.8, lateral 1.5, ventral to skull surface 1.5), and dorsal hippocampus (from bregma (mm): anteroposterior +2, lateral 2, ventral 2) (13).Ground and reference electrodes were positioned in the skull over the cerebellum. Electromyography (EMG) signals were recorded from two electrodes inserted in the neck musculature. All electrodes were connected to a five pin female socket secured to the skull with epoxy resin and covered with acrylic cement to improve retention.

Baseline recordings were performed one week following surgical preparation. On day 15th after diet onset, the mice were connected to the recording system with a light-weight cable and a swivel allowing free movements in the cage. Digitally synchronized video/EEG recordings were acquired for 24 h. The signals were sampled at 256 Hz, bandpass-filtered (0.02-70 Hz) and recorded on a portable digital EEG polygraph (BQS 98 System Micromed).

The digital data generated by the experimental settings were processed in Spike2 software analysis platform. Hippocampal activity was used, together with the EMG, to score an animal as being in wake, or sleeping in rapid eye movement (REM) or non-REM (14). For sleep-related micro-movement detection, the Root Mean Square of EMG signal was calculated(14). To minimize artifacts, the EMG signal peaks which fell on sleep states (non-REM, REM) were confirmed as sleep-related micro-movements with video-recording analysis carried out by an experimenter blind to mice genotype and treatment.

2.2 In vitro electrophysiology

The preparation of FCx slices and whole-cell patch clamp recordings from layer II/III pyramidal neurons was as described previously [Klaassen et al. 2006, Puligheddu et al. 2013]. Briefly, male mice (PND 70-75 d were anesthetized with halothane and killed. A block of tissue containing the frontal cortex (FCx) was sliced in the coronal plane (300 μm) with a vibratome (Leica, Nussloch, Germany) in ice-cold low Ca2+ artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 2.5 KCl, 2 CaCl2, 2 MgCl2, 1.25 NaH2PO4, 26 NaHCO3, and 10 D-glucose (pH 7.3–7.4). Slices (two per animal) were transferred in a holding chamber and allowed to recover for at least 1 hr before being placed in the recording chamber and superfused with ACSF (32–34°C) saturated with 95%O2/5%CO2: FCx layer II/III pyramidal cells were identified visually with an upright microscope with infrared illumination, and whole-cell voltage-clamp recordings were made by using an Axopatch 200B amplifier (Molecular Devices, CA). All GABAA IPSC recordings were made with electrodes filled with an internal solution containing

the following (mM): 140 cesium-methylsulfonate, 0.2 EGTA, 5 NaCl, 10 HEPES, 2 Mg2ATP, 0.25 Mg2GTP, pH 7.2–7.4. Experiments were begun only after series resistance had stabilized (typically 15–40 MΩ). Series resistance and input resistance were monitored continuously on-line with a 4 mV depolarizing step (25 ms). Data were filtered at 2 KHz, digitized at 10 KHz, and collected on-line with acquisition software (Clampex 8.2, Molecular Devices, CA). Neurons were voltage-clamped at a membrane potential of 0 mV. All GABAA spontaneous IPSCs (sIPSCs) were recorded in presence of 2-amino-5-phosphonopentanoic acid (AP5; 100 μM), 6-cyano-2,3-dihydroxy-7-nitro-quinoxaline(CNQX; 10 μM), to block N-methyl-D-aspartate-(NMDA), α-amino-3-hydroxy-5-methyl-
isoxazolepropionicacid- (AMPA) mediated synaptic currents, respectively. As already described [Klaassen et al., 2006], there was no effect of this solution on the holding current of the pyramidal cells.

2.3 Clinical study

Patients: Twelve subjects affected by drug-resistant ADFNLE and FNLE have been enrolled for the purpose of this study from outpatients attending the Epilepsy Diagnostic and Sleep Disorders Center of Cagliari and Parma (Italy). Diagnosis were obtained on the basis of reports of patients or family members witnessing the event, from serial EEG, digital video-EEG plus Holter-EEG recordings, and genetic test when available. Only primary NFLE patients, both sporadic and genetic (with genetic test when available), were considered for the study; secondary forms were excluded by means of a high-resolution MRI, customized according to main electro-clinical information employing a 1.5-tesla ACS-NT unit (Philips Medical Systems, Best, The Netherlands). Drug resistance refers to the ILAE (International League Against Epilepsy) definition of DRE (Drug Resistant Epilepsy) to achieve sustained seizure freedom (15). Informed consent, approved by Institutional Review Board, has been obtained from patients after a full explanation of the procedure. Recruited subjects underwent adjunctive therapy with fenofibrate. The chemical name for fenofibrate is 2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl-propanoic acid, 1-methylethyl ester. Fenofibrate is usually indicated as adjunctive therapy to diet to reduce elevated LDL-Colestherol, Triglycerides, and Apo B, and to increase HDL-C in adult patients with primary hyperlipidemia or mixed dyslipidemia. In this protocol, fenofibrate is orally administered off label at 600 mg per day, three times a day, and for 6 months. Efficacy and safety adverse reactions have been documented and reported. Since changes in liver function are mostly the cause of discontinuation of fenofibrate treatment in 1.6% of patients in double-blind trials, blood examinations have been carried out every two months during the total observation

time. Furthermore, for safety survey both cardiologic examination and electrocardiogram (ECG) have been performed at the beginning (T0) and at the end (T1) of the study. Intermediate clinical observations (i.e., nocturnal seizure and sleep diary examination) have been performed every two months. Finally, in order to evaluate subjective clinical effects of fenofibrate, epileptic quality of Life questionnaire (EPI-QoL) (16) and Visual Analogic Scale (VAS) quality of life have been filled by patients at T0 and T1.

VPSG has been manually, double blinded, examined by two neurophysiologists expert in epilepsy and sleep disorders (GM and MP) and in agreement with clear-cut ictal epileptiform activity and stereotyped motor pattern, each VPSG motor event was classified as Minor Motor Events (MMEs), while paroxysmal arousals, tonic-dystonic seizures, hyperkinetic seizures, and prolonged motor behaviors were classified as Major Events (MEs) (5). In both condition (T0 and T1) the total distribution of MEs and MMEs was analyzed throughout total sleep time (NREM and REM sleep).

Statistical analysis

Due to non-parametric distribution of data, the analysis of seizure diary and VPSG epileptic events in NFLE patients before and after add-on FEN treatment was assessed by the non-parametric Wilcoxon two-tailed signed rank test with paired comparison.

Questionnaire Scores: Epileptic quality of Life questionnaire (EPI-QoL) and Visual Analogic Scale (VAS) scores acquired at T0 and T1 have been analyzed by the non-parametric Wilcoxon two-tailed signed rank test with paired comparison. Cross-correlation between the variation of seizure frequency and quality of life scores after six months of add-on FEN treatment has been calculated in order to evaluate an interdependency between this two conditions.

Statistical significance was set at $P < 0.05$ (*) and test was performed using the calculation software GraphpadPRISM®.

Table 1. Clinical history of recruited subjects
Demography, clinical and instrumental survey and traditional antiepileptic therapy of recruited subjects.

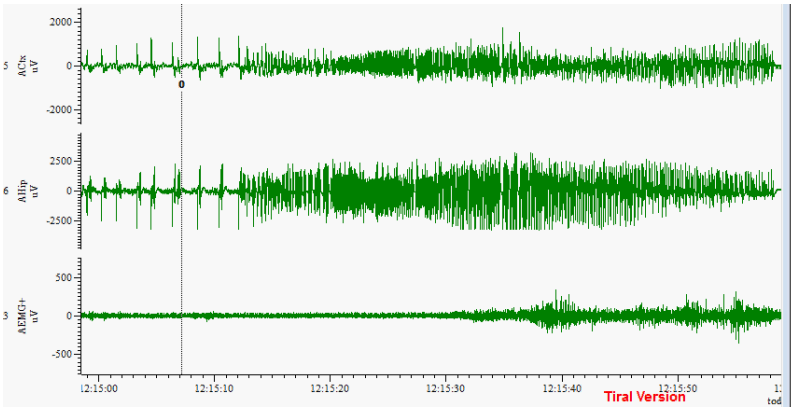
Legend: ME major events, MME minor motor events, PA paroxysmal arousal, ENW epileptic nocturnal wondering. CBZ carbamazepine, LVT levetiracetam, OXC oxcarzepine, LTG lamotrigine, VPA valproic acid.

Subject	Gender	Age (y)	Anamnestic nocturnal events	Seizure onset age (years)	Seizure frequency	Cerebral NMR	AED	Routine wake–EEG findings
Pz1-BM	M	34	ME, MME,PA,ENW	4	Several time\day	Normal	VPA-Mg 750 mg CBZ 600 mg	Sharp waves on right frontotemporal region
Pz2-GL	F	32	ME,MME, PA	10	Several time\day	Normal	LTC 3000 mg VPA 1000 mg	Bilateral frontal high amplitude sharp waves
Pz3-PP	F	47	ME,MMEs	18	Several time\day	Normal	LVT 1000 mg	Sharp waves on right frontotemporal region
Pz4-VP	M	29	ME,MMEs	10	Several times/wk	Normal	LTG 200 mg	Bilateral aspecific fronto-temporal slow waves
Pz5-GR	M	29	ME,PA,ENW MMEs	23	Several times/wk	Normal	CBZ mg	Bilateral aspecific fronto-central slow waves

Pz6-PR	M	41	ME,MMEs,ENW, PA	5	Several time\day	Normal	OXC 600 mg	Bilateral frontal high amplitude sharp waves
Pz7-BS-ADNFLE	F	24	ME,MMEs, PA	4	Several time\day	Normal	OXC 600 mg	ndr
Pz8-CT-ADNFLE	F	52	ENW,MMEs,PA	6	Several time\day	Normal		ndr
Pz9-AP-ADNFLE	F	23	ME,ENW,MMEs, PA	4	Several time\mo	Normal	OXC 600 mg	Bilateral aspecific fronto-temporal slow waves
Pz10-MZ-ADFNLE	F	52	ME,ENW,MMEs, PA	6	Several time\day	Normal		ndr
Pz11-CM	M	35	ME, ENW,PA,MME	10	Several times/wk	Normal	CBZ RM 600 mg	Bilateral frontal high amplitude sharp waves
PZ12-IC	F	29	ME,ENW,PA,MME	7	Several times/mo	Normal	CBZ 400 mg	ndr
Pz 13-BC	M	37	ME,ENW,PA,MME	6	Several time\day	Normal	LVT 750 mg	ndr

3. Results

3.1. Neurophysiology and behavior of NFLE mouse model (CHRNA4-S252F)

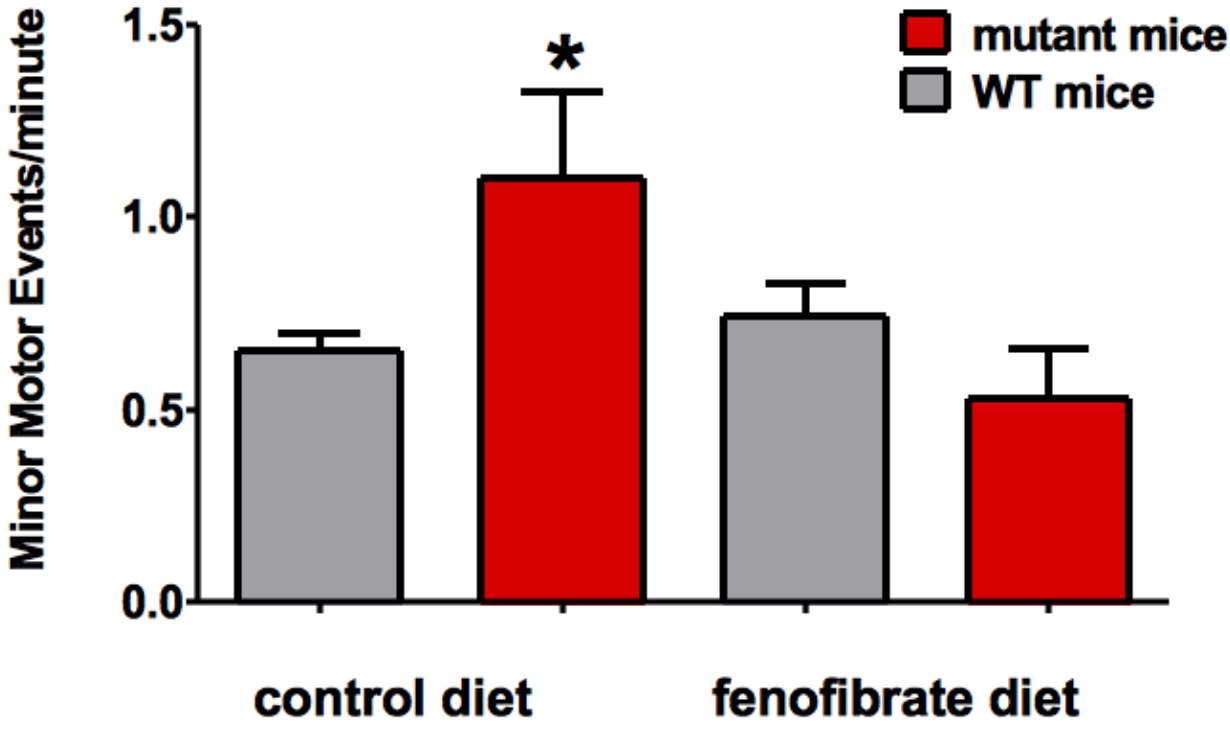


Chronic Administration of the Clinically Available PPARα agonist Fenofibrate reduced minor motor nocturnal events.

The results of the previous experiments prompted us to assess the efficacy of the clinically available PPARα agonist fenofibrate. Mice were randomly divided into three groups: (i) fenofibrate diet (FBR), (ii) control diet (CTRL) and (iii) fenofibrate washout diet (FBR-WO).

The fenofibrate containing diet was administered ad libitum for 14 days. FBR mice consumed a daily average of 3.6±0.1 g of food pellets, which approximately corresponds to 28–30 mg/kg fenofibrate per day. FBR mice were divided in two additional groups at the end of the treatment: one group received the PPARα antagonist MK886 15 min before the nicotine challenge, and the other received its vehicle. CTRL mice were fed with the control diet, identical and equicaloric but without fenofibrate. FBR-WO mice were fed for 14 days with a fenofibrate-containing diet, as FBR mice, and then fed with a control diet for additional 14 days to assess the effect of fenofibrate washout. In each animal EEG electrodes were implanted 7 days before the beginning of the treatments and nicotine was tested on the 15th day from the beginning of treatment (for FBR and CTRL mice) or on the 30th day (for FBR-WO mice).

When nocturnal motor events were scored, two-way ANOVA yielded a significant difference among groups ($P<0.017$) (Fig. 2). Post-hoc analysis revealed that nocturnal minor motor events FBR mice were significantly protected seizures when compared with CTRL animals ($p< 0.05$).



3.2 Electrophysiology.

It has been previously shown that the electrophysiological mechanism underlying epileptogenesis at the level of cortical circuits involves changes in synaptic activity onto pyramidal cells as key source of cortical EEG activity. We, therefore, examined the effect of acute activation of PPAR-α on inhibitory synaptic events of layer II/III pyramidal cells of the frontal cortex (FCx) in a mouse model of NFLE (CHRNA4-S252F) (1). Whole-cell voltage-clamp recordings of spontaneous inhibitory post-synaptic currents (sIPSCs) were carried out from layer II/III pyramidal cells of wild type (wt) and heterozygous (mut) Chrna4-S252F mouse brain slices (1). Under basal conditions (Vholding=0 mV to isolate sIPSCs(1)), sIPSCs in wt and mut mouse pyramidal cells displayed a difference in the frequency of events larger than 50 pA (Figure 1A supplementary material; $P < 0.05$, unpaired t test with Welch's correction). We next examined the effects of nicotine (5 μM, 30 s) on sIPSCs recorded from wt and mut mouse pyramidal cells and, as previously described (1), we found a larger and longer effect in mut when compared to wt mice (Figure 1B supplementary material, $P < 0.01$, $F_{30,180}=6.62$, two way RM ANOVA followed by Bonferroni). However, irrespective of the genotype, nicotine-induced effects were absent following an acute bath application of synthetic PPAR-α agonist WY14643 (3 μM, 5 min, Figure 1B supplementary material, $P > 0.05$, $F_{1,180}=3.46$, two way RM ANOVA followed by Bonferroni) in agreement with previous reports (2-4). We next chronically treated the mice with clinically available PPAR-α agonist, i.e. fenofibrate. Both wt and mut mice were fed a diet containing 0.2% w/w fenofibrate (4, 5) and were randomly divided into four groups, i.e. fenofibrate diet wt and mut mice, and control diet wt and mut mice. The fenofibrate diet was administered ad libitum for 14 days. Fenofibrate wt and mut mice consumed a daily average of 4.61± 0.22 and 4.39 ± 0.13 g of food pellets, respectively, which corresponds to approximately 300 mg/kg of fenofibrate per day. Experiments with mice chronically fed with fenofibrate confirmed that PPAR-α activation abolishes nicotine-induced increases in sIPSC frequency in FCx pyramidal neurons in both genotypes (Figure 1A, $P > 0.05$, $F_{1,140}=0.64$, two way RM ANOVA followed by Bonferroni). Hence, nicotine-induced increase in sIPCS frequency was 105.6±10.9% of baseline in fenofibrate wt mice ($n = 7$) and 84.6±10.9% of baseline in fenofibrate mut mice ($n = 8$). Remarkably, fenofibrate diet abolished the differences observed between genotypes in the frequency of IPSCs > 50 pA under basal conditions (Figure 1B, $P > 0.05$, unpaired t test with Welch's correction). Hence, the frequency of sIPSCs > 100 pA recorded from fenofibrate mut mice was similar to control wt mice (Figure 1B, $P > 0.05$, unpaired t test with Welch's correction).

Figure 1. Effect of chronic diet with PPAR-α agonist fenofibrate in a mouse model of ADNFLE Voltage clamp recordings obtained from wt and mut mouse cortical layer II/III pyramidal cells. (A) Chronic fenofibrate (0.2% w/w for 14 days in food, FBR) fully suppressed nicotine-induced (5 μM perfused at arrow for 30 s) increase in sIPSC frequency in both wild type (wt, gray circles) and mutant Chrna4S252F (mut, red circles) mice. Notably, in FCx slices from mice fed a control diet (CTRL), nicotine increases sIPSCs frequency in layer II/III pyramidal neurons irrespective of the genotype. (B) The cumulative distribution plot of sIPSC frequency of neurons revealed that chronic fenofibrate reduced the occurrence of large sIPSCs in mut (mut FBR, dashed red line) mice with respect to mut mice fed a control diet (mut CTRL, solid red line). Notably, the frequency of larger sIPSCs in mut FBR mice was comparable to wt CTRL mice (solid gray line). Representative recordings of the effect of chronic fenofibrate diet on sIPSCs from pyramidal cells at Vh = 0 mV are depicted on the upper part of panel A. Symbols represent the average±S.E.M. two- way ANOVA, *P<0.05.

Figure 1

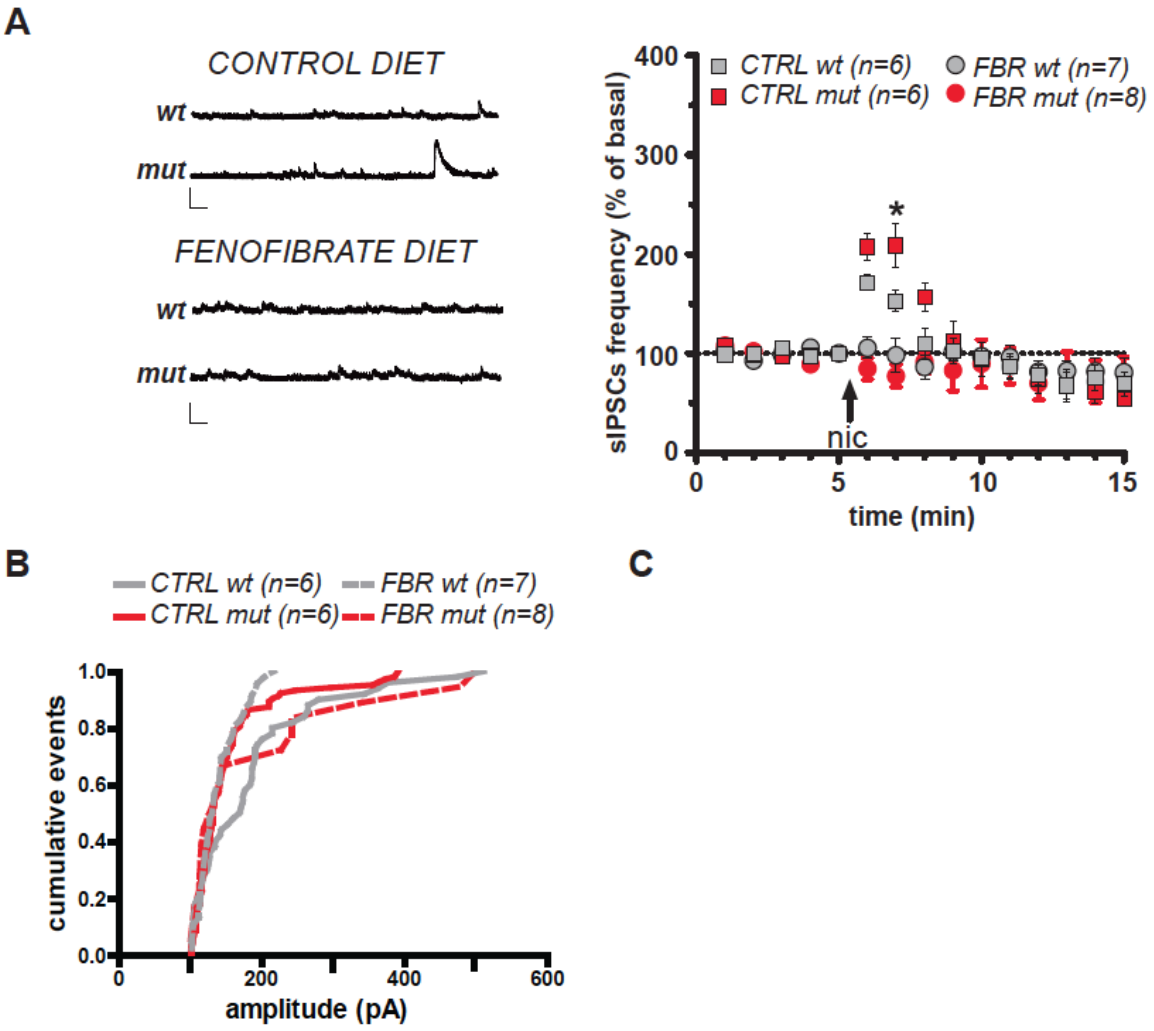
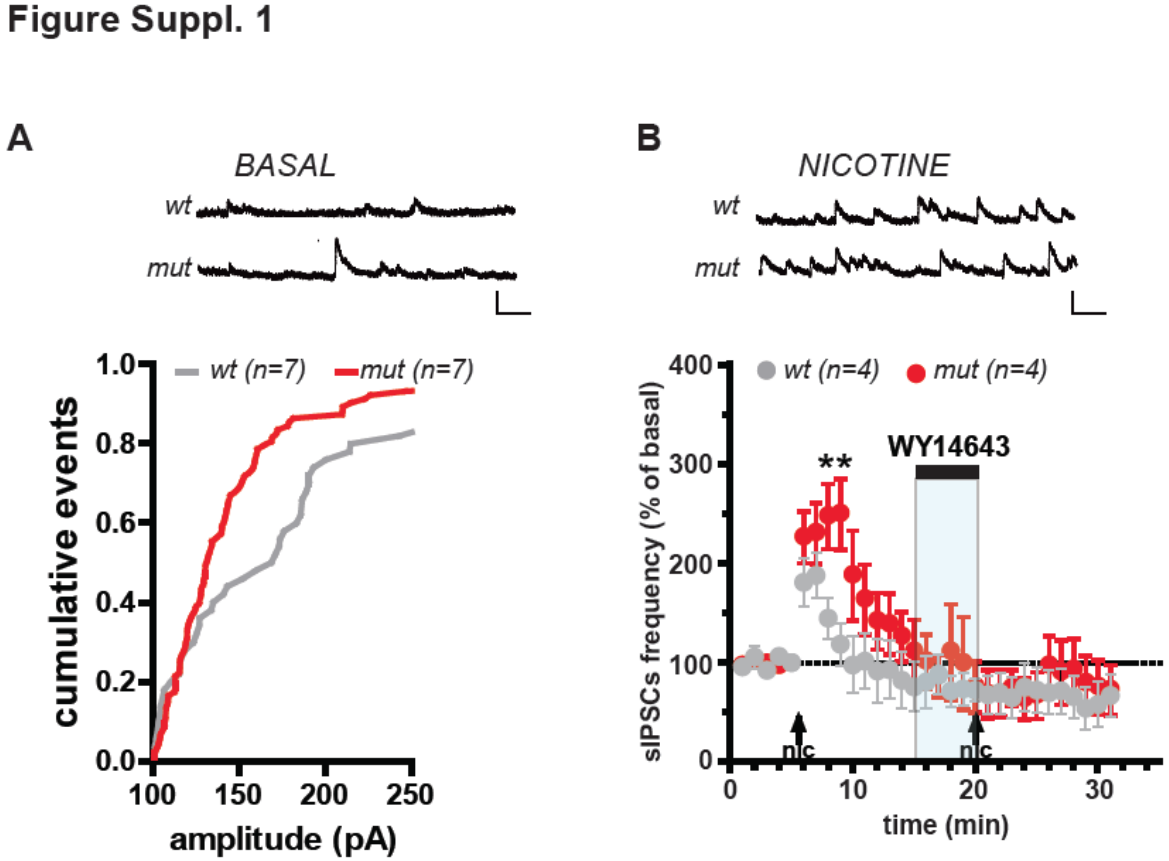


Fig. S1. Cellular electrophysiology in a mouse model of ADNFLE: acute effects of a synthetic PPAR-α agonist.
(A) Voltage clamp recordings obtained from wild type (wt) and mutant Chrna4S252F (mut) mouse cortical layer II/III pyramidal cells revealed an increased frequency of large sIPSCs in mut mice with respect to wt mice. The graph illustrates a nonlinear fit of Gaussian histogram of frequency distribution of events larger than 50 pA in wt and mut mice. In the inset the cumulative distribution plot of sIPSC frequency of neurons shows an increased frequency of events larger than 100 pA in mut mice when compared to wt mice. (B) The graph illustrates that in mouse FCx slices, nicotine (5 μM perfused at arrows for 30 s) increases sIPSCs frequency in layer II/III pyramidal neurons. Acutely, the PPARα agonists WY14643 (1 μM) fully suppressed nicotine-induced increase in sIPSC frequency in both genotypes. The light blue box represents the time of PPARα agonist perfusion. Representative recordings of the effect of nicotine, and of PPARα agonist, on spontaneous IPSCs from pyramidal cells at Vh = 0 mV are depicted on the upper part of panels A and B. Symbols represent the average ± S.E.M. two- way ANOVA, *P<0.05.



3.2. Clinical protocol for drug-resistant ADNFLE\NFLE patients

In order to study the effect of an adjunctive therapy with fenofibrate, patients affected by ADNFLE\FNLE, and non-responders to traditional therapy, were recruited. Clinical data, brain nuclear magnetic resonance, and routine wake–EEG findings of NFLE subjects under traditional antiepileptic treatment are summarized in Table S1. Eight subjects presented nocturnal motor events since childhood, while the mean duration of paroxysmal motor events during sleep was approximately 3 years before diagnosis in the remaining subjects. Four subjects reported a family history of epilepsy, with confirmed α2β2 mutation, and eight subjects described parasomnias or nocturnal motor events in first-grade relatives. None of the subjects displayed either perinatal hypoxia, or childhood febrile convulsions, or mild head injury and diurnal seizures were rarely reported. Neurologic examinations were unremarkable and customized MRI were normal for all subjects.

Protocol

The fenofibrate add-on therapy consisted of 600 mg per day for six months. Among the patients, two (ADNFLE) subjects refused (signed declaration) traditional antiepileptic (AE) therapy, and fenofibrate was administered as monotherapy. Patient 12 (BC) dropped out from the study because of increased hepatic enzymes above normality range during the first month of therapy, though they immediately returned to normal ranges after interruption. After six months of add-on therapy (T1-FEN), results of quality of life tests and daily seizures diaries were collected. According to subjective seizure diaries, which were based upon subject and bed-partner reports, eight out of eleven subjects were seizure free, whereas the remaining 3 reported a reduction larger than 75% of major events (Figure 2A, P = 0,002). Table 1 shows seizures rate change calculated according to Labar rule (Labar, Murphy, & Tecoma, 1999). Figure 2 (B-C) displays the Wilcoxon test results with regard to Visual Analogic Scale (VAS) and epileptic quality of Life questionnaire (EPI-QoL) . Although none of subjects displayed a severe discomfort in relation to their pathological condition, both the scores showed a significant improvement after six months of add-on therapy (P =0,0186 and P=0,022 respectively) (Table 1 and Figure 2 B-C). Nonetheless, no significant correlation between EPI-QoL improvement and reduction in seizures frequency was unveiled (data not shown).

3.3.2 Videopolisomnograpy (VPSG) recordings at T1-FEN .

A total of 328 epileptic motor events were counted during VPSG recordings of NFLE subjects at T0. The prevalence of seizures (approximately 85%) was recorded during NREM sleep in agreement with previous reports (Parrino et al., 2012). The 225 epileptic events (68% of total NREM sleep seizures) were classified as Minor Motor Events (MMEs), and they mainly

occurred during stage 2 of NREM sleep (N2). The remaining seizures (32% of total NREM sleep seizures) were classified as Major Events (MEs) (28 events).

After 6 month of fenofibrate add-on therapy (T1-FEN), VPSG recording displayed a significant decrease of the total number NREM sleep seizures (Figure 2 D, P=0,002). In detail, a reduction of MEs (Figure 2E, P=0,021) and MMEs (Figure 2F p=0,0087) was evident . Table 2 shows the hourly indexes of all VPSG epileptic events and of the ME and MME subgroups at T0 and T1 .

Tab1 Effect of add-on Fenofibrate treatment on reported seizures and quality of life tests.

Single subjects seizures report (T0) and Labar seizures rate change after 6 month of add-on fenofibrate treatment ((T0 and TT1). Subjects were administered a Visual Analogic Scale (VAS) and epileptic quality of life questionnaire (EPI-QoL) and the relative score change is displayed after treatment. Wilcoxon two-tailed signed rank test with paired comparison (*P<0.05; ** P<0,001)
M: male; F: female

Subject	Gender	Age (y)	N. seizures (reported diary) [% change after six month off add-on FEN]	EPIQoL T0 [% change after six month of add-on FEN]	VAS T0 [% change after six month of add-on FEN]
Pz1-BM-NFLE	M	35	92(-97,83)	184(19,02)	5(28,6)
Pz2-GL-NFLE	F	32	25(-96)	124(62,10)	5(0)
Pz3-PP-NFLE	F	47	36(-100)	193(25,90)	5(37,5)
Pz4-VP.NFLE	M	29	104(-98,07)	216(12,03)	5(33,3)
Pz5-GR-NFLE	M	29	38(-89,47)	216(15,74)	5(44,4)
Pz6-PR.NFLE	M	41	93(-100)	189(20,63)	2,5(58,3)
Pz7-BS-ADNFLE	F	24	252(-90,48)	196(20,41)	5(33,3)
Pz8-CT-ADNFLE	F	52	24(-87,5)	236(3,28)	5(0)
Pz9-AP-ADNFLE	F	23	168(-98,21)	240(-5,91)	9(0)
Pz10-MZ-ADFNLE	F	52	24(-100)	220(-3,48)	5(28,6)
Pz11-CM.NFLE	M	35		240(0,5)	5(0)
Wilcoxon test			p =0,002**	p=0,0186 *	p=0,022 *

Tab2 Effect of add-on Fenofibrate treatment on VPSG recorded seizure.

Subjects	Gender	Age (y)	VPSG T0 total N of Seizures	VPSG T1-FEN total N of Seizures	VPSG T0 N of MEs	VPSG T1-FEN N of MEs	VPSG T0 N of MMEs	VPSG T1FEN N of MMEs
Pz1-BM-NFLE	M	35	48	18	1	0	47	18
Pz2-GL-NFLE	F	32	37	5	1	0	36	5
Pz3-PP-NFLE	F	47	35	16	6	0	24	14
Pz4-VP.NFLE	M	29	36	17	0	0	36	17
Pz5-GR-NFLE	M	29	10	3	1	0	6	1
Pz6-PR.NFLE	M	41	63	31	0	0	59	30
Pz7-BS-ADNFLE	F	24	36	31	15	3	19	27
Pz8-CT-ADNFLE	F	52	25	14	0	0	18	9
Pz9-AP-ADNFLE	F	23	33	22	2	0	26	15
Pz10-MZ-ADFNLE	F	52	14	1	0	0	10	1
Pz11-CM-NFLE	M	35	8	2	2	0	6	2
Wilcoxon test				p=0,002*		p=0,021*		p= 0,0087**

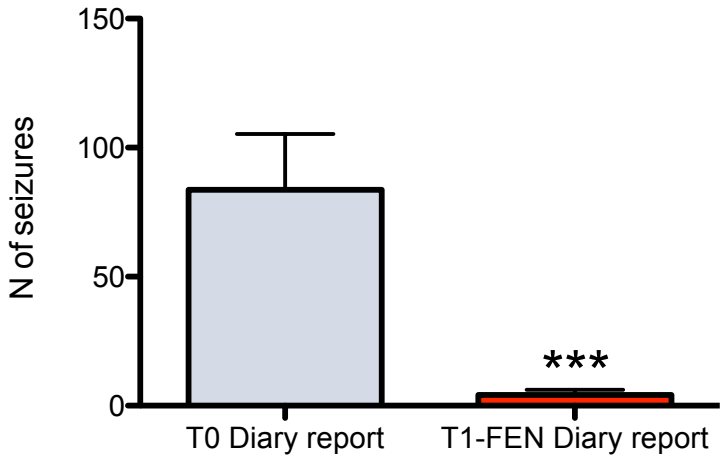


Fig 4A. Effect of fenofibrate add-on teraphy on seizures reported diary three months before (T0) e after treatment (T1-FEN).

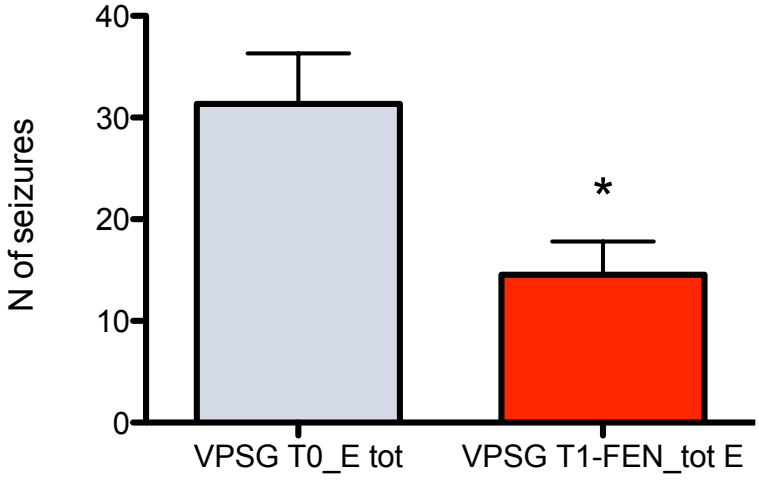


Fig 4B. Effect of fenofibrate add-on teraphy on total seizures recorderd during VPSG before (T0) e after treatment (T1-FEN).

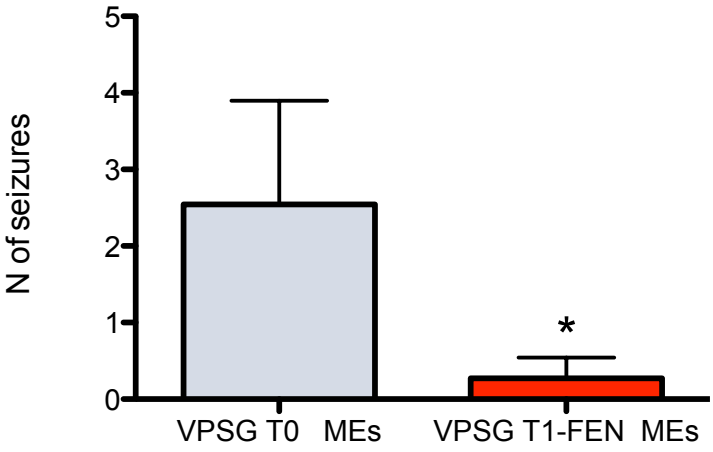


Fig 4C. Effect of fenofibrate add-on teraphy on Major Events recorderd during VPSG before (T0) e after treatment (T1-FEN).

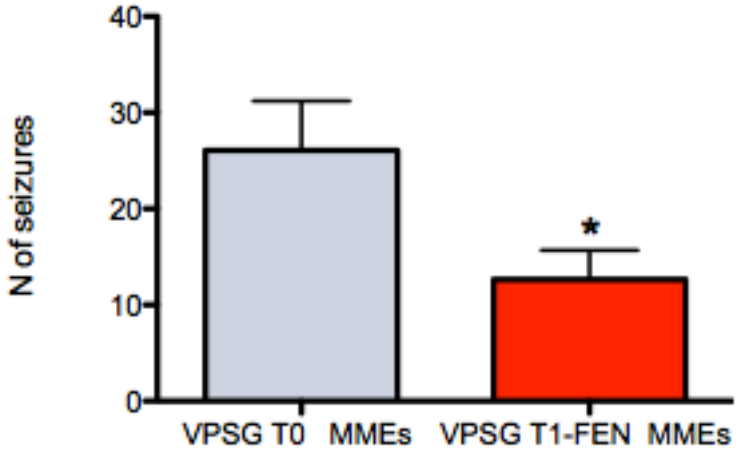
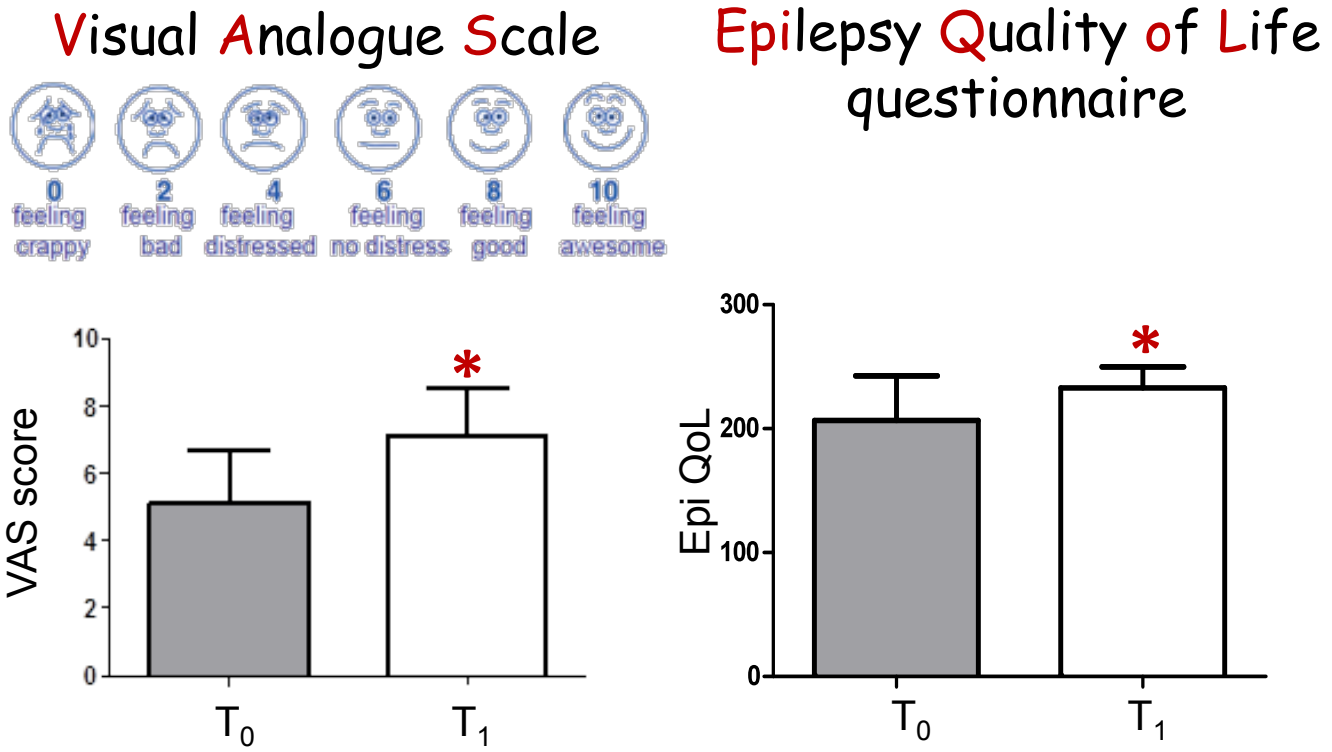


Fig 4D. Effect of fenofibrate add-on teraphy on Minor Motor Events recorderd during VPSG before (T0) e after treatment (T1-FEN).

Fig. 5 results of visual Analogic scale and EpiQoL before (T0) e after treatment (T1-FEN).



Discussion

Nowadays, a beneficial therapy for Nocturnal Frontal Lobe Epilepsy (FNLE) still represents a significant clinical need, as carbamazepine, the most efficacious drug for FNLE, completely abolishes the seizures only in ~20% of patients, and reduces the seizures only in 48% [12], thus leaving approximately one third of patients without therapeutic response. The major finding of the present study is the clinical efficacy of the PPAR-alpha agonist fenofibrate add-on therapy in NFLE patients, which exhibited a remarkable seizures reduction with a good control of the disease. We also extend the evidence for the action of PPAR-alpha as negative modulator of nAChRs to a mutated mouse model of NFLE, which displays both the genotype and the phenotype of the human disease.

Several observations support our conclusions. First, the patients and their bed-partners reported in daily diary of seizures a significant reduction of these events together with a consistent improvement in their quality of life, as indexed by EpiQoL test and VAS. Secondly, the instrumental VPSG nocturnal recordings supported that the efficacy of the add-on therapy is evident not only for major motor events, but also for minor motor events. Notably, minor motor events seriously affect the development of the disease and disrupt sleep-architecture, though they are totally unperceived by the subjects. The observation that a single instrumental analysis, though compared with a single night under basal conditions, substantiated the six month daily reports in the diary of seizures filled by the patients and their bed-partners strongly underscores the efficacy of this add-on therapy. In addition, while traditional antiepileptic treatment for this type of epilepsy determines a partial (about 25%) reduction of objective VPSG seizures, sleep instability remains pathologically enhanced, and is associated with persistence of daytime sleepiness [2]. Third, a significant reduction of minor motor events is also evident during the overnight recordings in mutated mice chronically fed with the fenofibrate diet. Notably, minor motor events are related to a loss of cortical inhibition, which is secondary to arousal triggered by internal epileptiform stimuli of innate motor patterns generated by central pattern generators [14-15] and might occur in either presence or absence of an epileptiform discharge [15]. The observation that fenofibrate diet reduces minor motor events and arousability induced by epileptic stimuli in the mouse model of the disease demonstrates a helpful control on seizure activity, epileptiform internal stimuli and, consequently, ameliorates sleep instability by reducing the triggered arousability. Fourth, ex vivo both acute and chronic PPAR-alpha activation reduces nicotine-induced enhancement of frequency of sIPSCs in FCx pyramidal neurons irrespective of the genotype. This increase has been hypothesized to trigger network synchrony and ictal activity [3]. Notably, the observation that mutated mice exhibit an augmented frequency of larger

sIPSCs (> 100 pA amplitude), which following the fenofibrate diet becomes comparable to wild type mice, supports the hypothesis that network synchrony and ictal activity might account for cellular and behavioural expressions of the disease. Lastly, these observations support our hypothesis that PPAR-alpha activation might prevent/reduce the loss of cortical inhibition, as previously demonstrated in a pharmacological model of the disease (chapter 2 and ref [13].

The conclusions that PPAR-alpha might be a key regulator of neuronal activity through the modulation of nAChR functional properties, and that these effects might be therapeutically exploited, are in agreement with the fact that nAChRs play a major role in diverse idiopathic and/or genetically determined forms of epilepsy. Remarkably, a dysfunction of the brain cholinergic systems is hypothesized to underlie diverse disorders, including insomnia, mania and depression [15][16][17]. In agreement, we observed a significant improvement of quality of life in our group of NFLE patients. Remarkably, the finding that such an effect does not correlate with seizure control might support not only the cholinergic hypothesis of mood disorders, but also the therapeutic potential of PPAR-alpha agonists as antidepressant-like drugs [18][19].

Nevertheless, given that the patients were not aware of any possible antidepressant-like property of fenofibrate add-on therapy, which would have contributed to a placebo effect, the significant efficacy score reported, and finally yet importantly, the patient determination to continue taking the fenofibrate even after the completion of the study, support the preclinical findings suggestive of an independent role of fenofibrate on mood regulation.

Further studies on macro and micro- sleep architecture are certainly required in order to verify this final hypothesis.

Final Conclusion and "take home" messages.

A therapy for NFLE and ADNFLE represents a significant clinical need.

The recently discovered novel mechanism of regulation of nAChRs by PPAR-alfa may represent a new therapeutic avenue for medications aimed at diseases such as NFLE and ADNFLE, where "a gain of function" of nAChR is responsible for the cardinal symptoms.

The use of available animal models of ADNFLE together with advanced neurophysiological and brain-imaging techniques in humans offered an extraordinary opportunity to study a potentially important therapeutic strategy, which, noteworthy, may rely on drugs already clinically utilized in humans.

The conclusions that PPAR-alpha might be a key regulator of neuronal activity through the modulation of nAChR functional properties, and that these effects might be therapeutically exploited, are in agreement with the fact that nAChRs play a major role in diverse idiopathic and/or genetically determined forms of epilepsy.

Remarkably, a dysfunction of the brain cholinergic systems are hypothesized to underlie diverse disorders, including insomnia, mania and depression In agreement, we observed a significant improvement of quality of life in our group of NFLE patients. Indeed, the finding that such an effect does not correlate with seizure control might support not only the cholinergic hypothesis of mood disorders, but also the therapeutic potential of PPAR-alpha agonists as antidepressant-like drugs

Furthermore, one of the specific features of NFLE is the relationship with sleep rhythms and seizures. Our results will also support our working hypothesis that fibrates might represent an innovative therapeutic avenue not only for FNLE and ADNFLE, but also for other sleep disorders.

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