STUDY ON NEW THERAPEUTIC TARGETS FOR THE DEVELOPMENT OF NEUROPROTECTANT DRUGS

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Duration: 3 years
1. Summary of the project

Acylethanolamides, anandamide (AEA), oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) are endocannabinoids acting at CB1 cannabinoid (AEA) or peroxisome proliferator-activated-α (PPAR-α) (OEA, PEA) receptors. Neuroprotective effects of these compounds have been suggested as they accumulate in the brain following ischemia or neurotoxicity, being thus relevant in the control of neuronal survival and plasticity. AEA-mediated neuroprotection has been reported, but not for OEA or PEA. This proposal will evaluate whether these transmitters modify the neurotoxicity induced by 6-hydroxydopamine (6-OHDA) in rats, and by MDMA in wild-type and knockout mice lacking the PPAR-α receptor. Specifically, we will investigate whether the administration of OEA/PEA modifies the time-course and the intensity of 6-hydroxydopamine- and MDMA-induced neurotoxicity. A multidisciplinary approach will be taken to measure several parameters of toxicity. In behavioural studies, motor and cognitive effects will be evaluated. Anatomical correlates of dopaminergic neurodegeneration will be used to assess the expression of tyrosine hydroxylase and the induction of apoptosis. The integrity of the dopaminergic transmission will be evaluated measuring changes in binding of dopamine transporters and the induction of neurodegeneration-related genes, including oxidative stress and antioxidant enzymes. In vitro studies with dopamine neuron cultures will be also done to assess the protective effects of acylethanolamides. The participation of these transmitters in the above-mentioned effects will be verified by pharmacological methods. New, more potent and longer-acting analogues of OEA and PEA will be developed. The ultimate goal of this project is to evaluate whether these lipid mediators could offer new therapeutic strategies for alleviating neuronal injury.

The proposed objectives are the following:

1. The first hypothesis is that oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) given before the neurotoxin 6-hydroxydopamine (6-OHDA) could rescue or protect dopaminergic neurons from neurotoxicity in rats, as well as protect cultured
mesencephalic dopamine neurons from toxicity. Synthetic PPAR-α agonists should have a similar neuroprotective effect than OEA and/or PEA.

2. The second hypothesis is that OEA and PEA given before the neurotoxin 3,4-methylenedioxymethamphetamine (MDMA, “ecstasy”) could rescue or protect dopaminergic neurons from neurotoxicity in mice. Synthetic PPAR-α agonists should have a similar neuroprotective effect to OEA and/or PEA. Besides, PPAR-α knockout animals should present a higher sensitivity to the neurotoxic effects of MDMA than wild-type mice, whilst the neuroprotective effects of OEA and/or PEA should disappear in these genetically-modified animals. PPARα knockout mice will also be useful to identify the involvement of this transcription factor in the neuroprotective effects of AEA, which primarily acts in a different target, the CB1 cannabinoid receptor.

3. The pharmacological profile of OEA/PEA will be analysed on the expression of genes related to neurodegeneration in the dorsal striatum and ventral mesencephalon of rats and mice treated either with OEA/PEA previous to a neurotoxic dose of 6-OHDA or MDMA. The study will be done using whole genome scans followed by quantitative real-time PCR of candidate genes. In addition, protein expression of selected candidates will be studied in the dorsal striatum and ventral mesencephalon of rats or mice treated either with OEA/PEA previous to a neurotoxic dose of 6-OHDA or MDMA. Western blot, and when appropriate, immunohistochemistry, will be performed.

4. New acylethanolamide analogs based on sulfamoyl structures will be designed and evaluated. The ability of these new compounds for the regulation of gene expression though the activation of PPAR-α receptors will be validated in vitro and they will also be tested as neuroprotectants using the techniques described in objectives 1 and 2.
2. Results

The main results obtained to achieve the different objectives are described below.

**Objective 1. Neuroprotective effects of oleoylethanolamide and palmitoylethanolamide against 6-OHDA** (Subprojects 1 and 3)

The potential neuroprotective efficacy of systemic OEA administration has been tested *in vivo* and in parkinsonian models based on 6-OHDA (rats) and MPTP-induced degeneration of substantia nigra dopamine neurons (mice). The findings of in vivo studies in rats revealed that peripheral administration of the acylethanolamide OEA (0.5, 1, and 5 mg/kg) is followed by a rapid cross of the blood-brain barrier and by a rapid clearance of the compound. Functional studies indicated that all functional deficits were reduced in those animals treated with OEA (5 mg/kg). Besides, 6-OHDA-induced reduction of striatal and nigral tyrosine-hydroxylase immunoreactivity (TH-ir) density as well as striatal synaptophysin expression were significantly antagonized by OEA (5 mg/kg). The oxidative response within the striatum to toxin injury, as measured through heme oxygenase-1 expression, was also reduced after OEA (5 mg/kg) administration. In summary, OEA crosses the blood-brain barrier after systemic administration, and dose-dependently protects the nigrostriatal circuit from 6-OHDA-induced toxicity. Wild-type and PPAR-α knockout C57BL/6 mice rendered parkinsonian with a toxic dose of MPTP (40 mg/kg) that induces 70-80% cell death in the substantia nigra were treated with systemic OEA (0, 0.5, 1, and 5 mg/kg) before and after MPTP administration. OEA (5 mg/kg) significantly reduced loss of striatal TH-ir and nigral cell death. Interestingly, this neuroprotective effect on striatal TH density, but not in the number of dopamine nigral cells, was also found in PPAR-α knockout mice, suggesting that OEA protective effects on dorsal striatum are not mediated by its agonistic action upon PPAR-α receptors. In summary, systemic OEA (5 mg/kg) exerts partial neuroprotective effects in parkinsonian animals, both in 6-OHDA and MPTP models, and the striatal effect does not seem to be mediated by PPAR-α receptors (Galán-Rodriguez et al., Neuropharmacology, 2009)
**In vitro studies** of the effects of the acylethanolamide **OEA** in cultured cells after inducing oxidative stress through 6-OHDA were also carried out in primary dopamine neurons and chromaffin extra-adrenal cells. Protective effects were measured through the lactate dehydrogenase (LDH) and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tests. OEA (0.5 and 1 µM) given before and after toxin insult exerted significantly neuroprotective effects on cultured neurons. However, OEA given before 6-OHDA was ineffective, although given after 6-OHDA (OEA 1 µM) was effective. OEA effects followed U-shaped dose-response curves. This type of response could suggest toxicity due to high drug concentration or that opposing intracellular pathways are activated by different drug doses. OEA-induced protective effects were abolished after adding low doses of of the TRPV1 receptor agonist capsaicin (0.1-1 µM) or the adenylyl cyclase inhibitor MDL-12,330A (1 and 10 µM), but not by adding the blocker of PPAR-α receptor GW6471. These findings suggest that the protective effects of OEA on dopamine neurons cells are mostly mediated by blockade of TRPV1 receptors and activation of the cAMP pathway. In a separate experimental series the in vitro protective properties of **PEA** and WY14643, a selective agonist of PPAR-α receptors, was evaluated in dopaminergic substantia nigra neurons and noradrenergic extra-adrenal chromaffin cells using a similar approach to that described above. PEA exerted neuroprotective effects in substantia nigra cells in the LDH test at all doses tested (0.5, 1 and 5 µM), although only the dose of 5 µM of PEA was effective in the MTT test. WY14643 did not induce neuroprotective effects in these tests when administered at the dose of 1, 5 and 10 µM. These data indicate that PEA induces similarly neuroprotectant effects on dopamine neurons than OEA, although the PPAR-α agonist WY14643 is devoid of these protectant effects (Galán-Rodriguez et al., Neuropharmacology, 2009).

Moreover, we were interested to discern whether **dopamine neurons of the substantia nigra pars compacta** (SNpc) are affected in PPAR-α knockout mice and if MPTP-induced toxic effects are modified in these mutant mice. First, the data revealed that PPAR-α knockout mice present a reduced SNpc in comparison with wild-type mice both in volume and in number of dopamine neurons. This result suggests that PPAR-α receptors
are necessary to protect nigral dopamine neurons from normal oxidative damage. The lateral part of the SNpc was the most damaged in the PPAR-α knockout mice. At a biochemical level, a substantial decrease in anti-oxidant molecules was found in SNpc of knockout mice, which could account for the reduced protection against normal oxidative damage. As expected, MPTP caused rapid onset of Parkinsonism in wild-type mice, linked to a strong reduction in the number of nigral dopamine neurons (74%). However, PPAR-α knockout mice were more resistant than wild-type mice to MPTP, since this toxic induced a less intense reduction of nigral dopamine neurons (36%). MPTP induced an increase in nitric oxide in the substantia nigra pars compacta in wild-type mice, but nitric oxide production was reduced in knockout mice, suggesting that lack of PPAR-α receptors downregulates nitric oxide synthase activity and nitric oxide production after MPTP insult (Gonzalez-Aparicio et al., Neuroscience, 2011).

The involvement of PPAR-α receptors in the motor sensitization produced by morphine and cocaine was also investigated in PPAR-α knockout mice and by using a selective PPAR-α agonist, WY14643. The findings in knockout mice indicate that PPAR-α plays an inhibitory role in the expression, but not in the induction, of motor sensitization to morphine. However, sensitization to cocaine was not modified in these mutant mice, suggesting that this nuclear receptor participates in motor activating effects of opiates but not of psychostimulants. Furthermore, brain PPAR-α expression was upregulated after the highest dose of repeated morphine administration, but not chronic cocaine, suggesting that this receptor could play a compensatory role to maintain the homeostasis after chronic opioid administration. In accordance, systemic WY14643 blocked sensitization to morphine, confirming that PPAR-α plays a compensatory role opposing morphine-induced motor sensitization, probably through a reduction of inflammation-associated changes (Fernandez-Espejo et al., Neuroscience, 2009).

The anatomical distribution of PPRA-α receptors in rat brain areas related to dopaminergic transmission as well as the expression of specific genes related to dopamine transmission and acylethanolamide neuromodulation by real time PCR has also been studied. Analysis of the
effects of OEA in rat brain is being performed and compared to that of 6-OHDA-treated rats. Permeability through the blood brain barrier of both PEA and OEA has been demonstrated (Plaza-Zabala et al., Synapse 2010). Finally, the potential changes induced by systemic administration of OEA in diencephalic neuropeptide networks has been studied (Serrano et al., Neuropharmacology 2011). This study revealed that OEA induced substantial changes in peripheral signals that target the hypothalamus, which seem to play a more important role than the direct effect on hypothalamic peptidergic neurons.

**Objective 2. Neuroprotection against 3,4-methylenedioxymethamphetamine (MDMA)-induced neurotoxicity**

(Subproject 1)

The dopamine transporter (DAT) functionality was evaluated following a neurotoxic regimen of MDMA in mice and the possible neuroprotective effects of OEA were investigated. The acute administration of MDMA at the dose of 10 mg/kg increased the extracellular levels of striatal dopamine with respect to baseline in mice treated repeatedly with saline or with 3 mg/kg of MDMA. In contrast, dopamine levels were not increased in mice treated with the dose of 30 mg/kg of MDMA. These results show that repeated treatment with MDMA at the dose of 30 mg/kg decreases the functional activity of DAT suggesting possible neurotoxic effects. The apoptotic reaction following a neurotoxic regimen of MDMA in mice was also studied. Repeated administration of high doses of MDMA (30 mg/kg) in mice did not induce apoptosis in the hippocampus, frontal cortex or striatum as measured at 24 hours of the last injection. Indeed, no programmed cell death was observed in these brain structures 24 hours after the last injection of a repeated high dosing regimen of MDMA. On the other hand, the administration of MDMA diminishes DAT in the striatum and produces a decrease in learning the active avoidance task. Interestingly, pre-treatment with OEA (5 mg/kg) prevents DAT reduction and delays the cognitive deficits induced by 5 mg/kg MDMA (Plaza-Zabala et al., Synapse, 2010).

In a second experiment, we found that the different doses of MDMA tested (0, 0.03, 0.06, 0.125 and 0.25 mg/kg/infusion) were unable to maintain a
self-administration behaviour in knockout mice deficient in the serotonin transporter (SERT), whilst wild-type animals acquired such a behaviour. The administration of MDMA increased the extracellular levels of striatal dopamine with respect to baseline both in wild-type and SERT knockout mice. However, the extracellular concentration of 5-HT was increased only in the wild-type genotype, thus indicating a specific role of SERT in the reinforcing properties of MDMA (Trigo et al., Biological Psychiatry, 2007).

On the other hand, the hyperthermic effects of MDMA (1, 5 i 25 mg/kg) are abolished in PPAR-α knockout mice, thus revealing a crucial role for these receptors in the alterations in body temperature produced by MDMA. Moreover, we obtained data showing that the hyperlocomotor effects of MDMA (20 mg/kg) were also diminished in PPAR-α knockout mice suggesting an involvement of these nuclear receptors in the psychostimulant properties of MDMA. We also revealed that another cannabinoid, THC, interacts with MDMA in terms of learning and memory processing in a dose-specific manner. Thus, THC (0.3 and 1 mg/kg) may enhance the effects of low doses of MDMA (3 mg/kg) to produce a cognitive impairment, while it may induce protective effects only at a dose of THC 1 mg/kg against the MDMA-induced alterations observed at high doses (10 mg/kg) (Tschon et al., 4th European Workshop on Cannabinoid Research, Madrid, 2009).

The anatomical description of PPRA-α receptors in dopaminergic neurons of the mouse has also been performed and its role on neurogenesis is being evaluated in young and aged animals.

Objective 3. Molecular and gene studies (Subprojects 1 and 2)

Using a yoked-control operant intravenous self-administration paradigm, which is the most relevant animal model to study the addictive potential of drugs of abuse in humans, we showed that repeated exposure to MDMA induces the expression of genes related to inflammatory and immunological responses in several brain structures including the ventral striatum, frontal cortex, dorsal raphe nucleus and hippocampus. Among them, the four genes whose differential expression was validated by qRT-PCR, Camk2a,
Kalrn, Ddn and Egr3, have predicted binding sites for pancreatic duodenal homeobox-1 (Pdx1). In addition, the gene expression changes identified in the dorsal raphe nucleus following MDMA self-administration suggest that this brain region may be involved in motivated learning associated with active MDMA seeking behaviour (Fernández-Castillo, Genes Brain and Behavior, 2011).

On the other hand, the expression of genes related to neurodegeneration in the dorsal striatum and ventral mesencephalon of rats and mice treated either with OEA or PEA previous to a neurotoxic dose of 6-OHDA (rats) or MDMA (mice) was studied in order to carry out the pharmacological profile of OEA/PEA in terms of gene expression. Studies have been initiated and the results will be available soon. The pharmacological profile of OEA is under evaluation by analyzing the expression of genes in the dorsal striatum, ventral mesencephalon and hypothalamus of rats and mice. Additionally, the expression of selected genes in mice brain after treatment with OEA ± the neurotoxin MPTP has been achieved, although the final results are not yet available. Specific studies on the effects of OEA in hypothalamic peptides and neurotransmitters have been performed and published (Serrano et al, Neuropharmacology 2011). A pilot study of OEA-protecting effects against oxidative-stress associated damage in the liver has been also performed and published (González-Aparicio et al., Psychoparmacology, 2011). In addition, a method for evaluating the levels of OEA in brain tissue by means of microdialysis coupled to mass spectrometry has been developed in association with the Scripps Research Institute (La Jolla, California, USA).

**Objective 4. Synthesis of new acylethanolamide analogs** (Subproject 2)

The structural requirements for the binding of OEA to the PPAR-α receptor have been studied through docking analysis (Moreno-Santos et al., Journal of Biological Chemistry, submitted). Based on this information, new acylethanolamide analogs have been designed as potential neuroprotectants. Of them, those based on sulfamoyl structures have been found to be neuroprotectant in vitro. Additional PPAR-α agonists derived of oleyl derivatives of pyrazoles and triazole moieties have been
synthesized. The ability of these new compounds to regulate gene expression though the activation of PPAR-α receptors has been validated in vitro, and they have been validated and tested as neuroprotectants using the techniques described in objectives 1 and 2. One patent (Rodriguez de Fonseca et al., WO2009/050318 A1) and three publications (Alvarado et al., Bioorganic & Medicine Chemistry, 2008; Cano et al., European Journal of Medicinal Chemistry, 2009; Almeida et al., ChemMedChem, 2010) have been generated and a second patent (Spanish Patent 201130486) and a manuscript (Moreno-Santos et al., Journal of Biological Chemistry, submitted) are under evaluation.

3. Relevance and possible clinical applicability of the final results

The multidisciplinary approaches used in this research project made it possible to achieve the different objectives, and provided information of interest for the development of new therapeutic approaches for neuroprotection. The main results of the project that could have potential clinical applications can be summarized as follows:

1. **The identification of the neuroprotective effects of two new compounds, oleoylethanolamide and palmitoylethanolamide** in different animal models. These neuroprotective effects have been investigated in two complementary models of neurotoxicity: the neurotoxic effects produced by 6-OHDA in rats that promotes a degeneration of the dopaminergic fibers, and the neurotoxicity promoted by the repeated administration of MDMA in mice that also produces dopaminergic neurotoxicity. The neuroprotective effects on these animal models for dopamine neurons suggest a potential therapeutic interest of these new compounds for Parkinson disease and/or for preventing the deleterious consequences of the consumption of synthetic drugs of abuse. Clinical trials with these compounds should be carried out to confirm the interest of these findings.

2. **The identification of the specific mechanisms involved in the neuroprotective effects of oleoylethanolamide and palmitoylethanolamide.** The selective involvement of PPAR-α receptors in
some of the neuroprotective effects of these compounds identifies a new target for the development of new neuroprotective agents. OEA and PEA have been the specific pharmacological tools used in the present project for the identification of this new mechanism underlying the neuroprotective effects. However, the pharmacokinetic and pharmacodynamic properties of these lipids can be improved in order to obtain more appropriate compounds to target in vivo the PPAR-α receptors, and to obtain new neuroprotective agents. Moreover, neuroinflammation seems to participate in sensitizing effects of drugs of abuse, and the nuclear receptor PPAR-α plays a prominent role in these inflammatory responses. This nuclear receptor participates in motor activating effects of opiates and it could be useful for the design of new drugs aimed at treating opiate abuse.

3. **The characterization of the changes in gene expression occurring in different brain structures during these neurodegenerative processes.** We have identified specific changes on gene expression that could play a major role in these neuroprotective effects by using whole genome scan techniques followed by quantitative real-time PCR of candidate genes. Therefore, these candidate genes are of interest to further understand the mechanisms involved in these neuroprotective effects and to promote further research activities in order to identify new potential neuroprotective agents.

4. **The generation of new acylethanolamide analogues that could be of interest as new neuroprotective agents.** These acylethanolamide analogues can improve the pharmacokinetic and pharmacodynamic properties of OEA and PEA and should therefore be more suitable than the original lipids for their possible application as neuroprotective agents, as they show similar neuroprotective properties that are patentable and are dependent on PPAR-α receptor.

**In summary**, this project has identified the **neuroprotective effects of oleoylethanolamide (OEA) and palmitoylethanolamide (PEA)** as well as the **neurobiological target** for these pharmacological responses. The **new analogue compounds** that have been generated can be of potential interest as **new neuroprotective agents**. In addition, the **candidate**
genes identified can be of potential interest for developing new research strategies.

4. Publications

Trigo JM, Renoir T, Lanfumey L, Hamon M, Lesch KP, Robledo P, Maldonado R.


Fernandez-Espejo E, Ramiro-Fuentes S, Rodriguez de Fonseca F.
Synthesis and pharmacological evaluation of sulfamide-based analogues of anandamide.

Effects of the endogenous PPAR-alpha agonist, oleoylethanolamide on MDMA-induced cognitive deficits in mice.

Orejarena MJ, Lanfumey L, Maldonado R, Robledo P.
Involvement of 5-HT2A receptors in MDMA reinforcement and cue-induced reinstatement of MDMA-seeking behaviour.

Synthesis of fatty acid amides of catechol metabolites that exhibit antiobesity properties.

Serrano A, Pavón FJ, Tovar S, Casanueva F, Señarís R, Diéguez C, de Fonseca FR.
Oleoylethanolamide: effects on hypothalamic transmitters and gut peptides regulating food intake.


**Patents coming out from this project**


Immunofluorescence images of OX6 expression in striatal tissue 96 hours after bilateral 6-OHDA infusion and OEA pretreatment. OEA was infused into the right striatum at 5 µM dose. These pictures show that microglial reaction was present in both damaged striata, but the number of reactive microglial cells (ramified cells) was lower in the OEA treated striatum (inboxes, amplified images showing dispersed OX6+ microglia with ramified morphology). Abbrev.: str, striatum; cc, corpus callosum
TUNNEL immunostaining in the hippocampus and frontal cortex (A, B and C) and in the striatum (D, E and F). A and D show DNA fragmentation in a positive control experiment. B and E show no DNA fragmentation in a negative control experiment. C and F show immunostaining in the brain of mice treated repeatedly with MDMA (30 mg/kg).

OEA and its analog oleyl-propyl-sulfamide interacts with the LBD of PPAR-α receptor
### Table 1: Chemical structures and pharmacological properties of OEA (R1) and the synthetic analogues KDS3103 (R2), N-(propyl sulfonamido)heptadecan-1-amine (R3), N-(1-decyloxy-5-chlorophenyl)-1-phenyl-1H-pyrazole-3-carboxamide (R4), N-(1C3,4-dihydroxyphenyl)propyl-2-yloxaide (R5), and PSN632408 (R6)

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*PPAR-α activation, EC50 values in nM.
*GPR1 IP agonist, EC50 values in µM.

Structural analogs of OEA generated throughout the project