



EVALUATION OF ACYLETHANOLAMIDE-BASED NEUROPROTECTANTS IN ANIMAL MODELS OF PERINATAL AND ADULT BRAIN HYPOXIA

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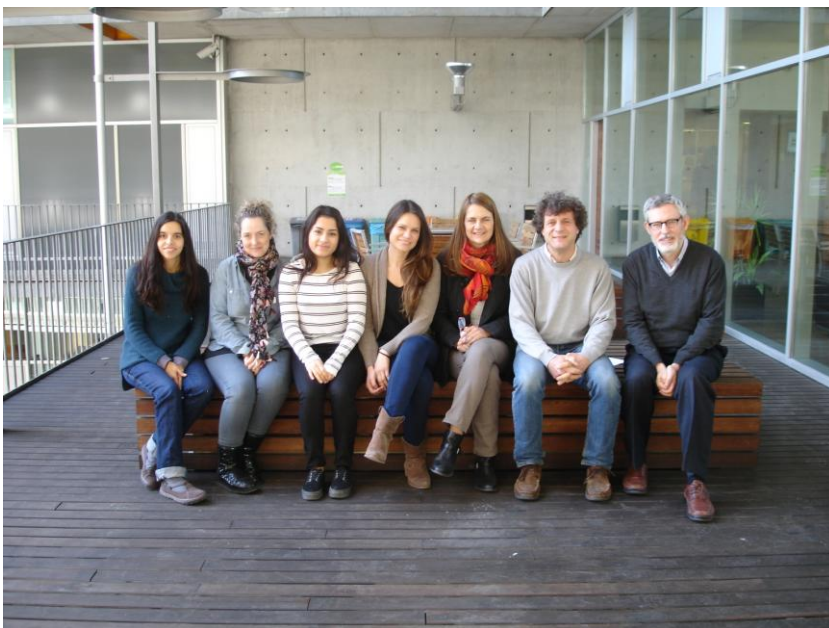
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1. Abstract

The acylethanolamides oleoylethanolamide and palmitoylethanolamide are lipid compounds with neuroprotectant properties in animal models of neurodegenerative diseases. However, their role as neuroprotectants in brain damage derived from acute hypoxia remains to be fully determined. In the present project we intend to evaluate the roles of these lipids in a) animal models of perinatal or adult hypoxia-ischaemia stroke, that is acute hypoxia derived of carotid artery clamps followed by hypoxic stress, and b) a model of perinatal hypoxia that resembles cerebral palsy produced by dystocic delivery in humans. Studies will evaluate the effects of pre-treatment and post-treatment with acylethanolamides on the behavioural and physiological outcomes of experimental subjects suffering either of the two models of hypoxia. Studies will be performed in normal rats and mice, as well as in genetically modified animals lacking the receptors for the acylethanolamides, mainly PPAR-alpha and TRPV1. Additionally, the consortium will evaluate the pharmacological efficacy of analogues of acylethanolamides. To this end, we will use sulfamoyl derivatives patented by the consortium that displayed neuroprotectant activity. The final aim of the project is to understand the role of these important lipids in brain hypoxia and take advantage of their protective role for developing efficient therapies that may attenuate the tremendous impact of hypoxia on brain function, both in adults and in neonates.

The **specific objectives** of the project are:

1. To evaluate the roles of OEA and PEA in animal (rat and mice) models of perinatal hypoxia-ischaemia stroke, that is acute hypoxia derived from carotid artery clamps followed by hypoxic stress. Studies will evaluate the effects of pre-treatment and post-treatment with these acylethanolamides on the behavioural and physiological outcomes of experimental subjects.

2. To evaluate the roles of OEA and PEA in a rat model of perinatal hypoxia that resembles cerebral palsy produced by dystocic delivery in humans.

Studies will evaluate the effects of pre-treatment and post-treatment with acylethanolamides on the behavioural and physiological outcomes of experimental subjects.

3. To evaluate the targets of OEA and PEA for their effects on perinatal hypoxia-ischaemia stroke. This will be addressed by studying the effects of these acylethanolamides on genetically modified animals lacking the receptors for the acylethanolamides, including PPARalpha, TRPV1 and CB1 receptors, as well as the OEA/PEA degrading enzyme FAAH.

4. To evaluate the pharmacological efficacy of analogues of acylethanolamides, mainly the neuroprotectant octadecylpropyl sulfamide, a sulfamoyl derivative of OEA with potent PPARalpha agonistic properties.

The **final aim** of the project is to generate a scientific evidence-based comprehensive report of the role of these important lipids in brain hypoxia and to define their protective role for developing efficient therapies that may attenuate the tremendous impact of hypoxia on brain function, both in adults and in neonates.

2. Results

- **Palmitoylethanolamide (PEA) is neuroprotectant in a rat model of neonatal hypoxia-ischaemia.** Deficits in recognition memory and spatial reference memory observed following rat neonatal hypoxia-ischaemia were prevented by treatment with 10 mg/kg of PEA administered one hour after the insult. This treatment also reversed the pathological changes in the brain associated with the insult.
- **The neuroprotective efficacy of oleoylethanolamide (OEA) has been ascertained in a mouse model of perinatal hypoxia-ischaemia.** Motor dysfunctions such as hyper-reflexia and hyperlocomotion observed following the hypoxia-ischaemia insult were reduced by treatment with 5 mg/kg OEA administered 30 min before the procedure. This dose also reduced the infarct volume in the hippocampus and motor cortex.
- **Expression of the PPARalpha-NAPE-PLD system in asphyctic brains.** In the rat perinatal asphyxia model, the data revealed the existence of clear effect of the hypoxic insult on the expression of the NAPE-PLD-PPARalpha-FAAH system, mainly in the hippocampus, associated with behavioural alterations. However, neither the administration of PEA nor that of OEA reversed the damage observed completely.

- **A new nanoemulsion has been patented and published (nanomedicine) that allows the oral administration of OEA.** OEA is a lipid mediator that acts as a satiety factor. The main limiting factor for its administration is its poor water solubility. We designed and characterized new nanoemulsions as delivery system for hydrophobic compounds such as OEA. The nanoemulsion components and preparation methods were selected in order to achieve the desired final properties. Then, we evaluated the in vivo properties of the nanoemulsions as drug delivery systems testing the anorectic effects of OEA in rats after both intragastric and intraperitoneal administration. The in vivo toxicity of the nanoemulsions was evaluated after a 3-week treatment. Nanoemulsions proved to be stable and non-toxic, and had no effect on feeding behaviour when administered without OEA. The effects of OEA were observable after its oral and parenteral administration with the nanoemulsions to 24-h fasted rats, finding a better efficacy compared with a vehicle containing Tween 20 after oral administration. These results support the efficacy of these nanoemulsions to deliver highly hydrophobic bioactive drugs.
- **A full characterization of octadecyl-propylsulfamide has been performed, confirming its characteristics as PPAR α agonist.** Our results suggest that OEA activity on the PPAR α receptor (e.g. lipid metabolism and feeding behaviour) may be dissociated from other actions at alternative targets (e.g. pain) because other non-cannabimimetic ligands that interact with PPAR α , such as CC7, do not reproduce the full spectrum of the pharmacological activity of OEA. These results provide new opportunities for the development of specific PPAR α -activating drugs focused on sulfamide derivatives with a long alkyl chain for the treatment of metabolic dysfunction.
- **Octadecylpropyl sulfamide reverses the memory deficits induced by hypoxia-ischaemia (HI) in mice.** HI-induced brain lesions were observed in the ipsilateral hippocampus and cortex of mice treated with vehicle and sulfamide. However, sulfamide treatment showed a tendency to decrease infarct size. Importantly, sulfamide significantly reversed the memory deficits observed in the object recognition test 7 days following HI. No significant effects were observed between groups in the other behavioural tests. These data reveal for the first time the neuroprotectant properties of octadecylpropyl sulfamide, and suggest that post-

treatment with this compound after HI injury can be advantageous for preventing concomitant memory alterations.

- **A crucial molecular mechanism involved in the neuroprotective role of cannabinoid CB2 receptors (CB2R) following hypoxia-ischaemia (HI) has been demonstrated.** We evaluated the involvement of CB2R in the behavioural and biochemical underpinnings related to brain damage induced by HI in adult mice. HI-induced brain damage ipsilateral to the carotid ligation was observed in WT mice in hippocampal areas and in the sensory, entorhinal and piriform cortices. In KO mice, more extensive brain injury was observed. Behavioural deficits in the Irwin test were observed in both genotypes. However, while WT mice showed progressive recovery by day 7, KO mice did not. Only KO mice showed alterations in motor learning, coordination and balance, and did not recover over time. Profound memory deficits in the object recognition test were observed 72 h following HI in KO and WT mice, and no recovery was present in either genotype, suggesting a ceiling effect in this function. Accordingly, both WT and KO mice showed widespread microglia expression in lesioned areas and around the penumbra of injured zones as well as in non-lesioned areas. However, in KO mice the expression of microglia in non-injured sites was more extensive. TIM-3 expression in WT mice was observed in lesioned areas associated with activated microglia, and in non-lesioned areas such as the striatum and motor cortex. In KO mice TIM-3 expression was exacerbated in lesioned areas, and it extended to other non-lesioned areas such as the thalamus, the lateral septum, and olfactory tubercle. Our results indicate that CB2 receptors may have a crucial neuroprotective role following HI insult through the modulation of the inflammatory-related protein TIM-3 in microglia.

- **Cannabinoid CB1 receptors (CB1R) are specifically involved in neuroprotection of recognition memory following brain lesions induced by hypoxia-ischaemia (HI).** CB1R knockout (KO) mice and control littermates (WT) underwent permanent ligation of the left common carotid artery and hypoxia. HI-induced brain lesions were observed in both WT and KO mice in the ipsilateral hippocampus and cortex, and no significant differences were found between genotypes. In behavioural studies, WT and KO mice showed memory deficits in the object recognition test 72 h following HI. However, WT mice recovered performance 7 days after the insult, while KO mice did not. Functional and motor learning deficits were

observed in both genotypes 24 h, but recovered 7 days after HI. These findings suggest that CB1R may specifically modulate hippocampal-dependent memory deficits associated with HI-induced brain damage.

3. Relevance and possible impact

- PEA is neuroprotectant in a rat model of neonatal hypoxia-ischaemia, reducing the memory deficits and the pathological changes in the brain associated with the insult. In the mouse model of perinatal hypoxia-ischaemia, OEA may have neuroprotective effects. PEA is currently commercialized for neuropathic pain and these results might facilitate its implementation in the clinics.
- In the rat perinatal asphyxia model, the data revealed the existence of clear effect of the hypoxic insult on the expression of the NAPE-PLD-PPARalpha-FAAH system, mainly in the hippocampus, associated with behavioural alterations. However, neither the administration of PEA nor that of OEA reversed the damage observed completely.
- The newly developed and characterized OEA-PEA analogues are promising therapeutic targets for ischaemia-induced brain damage.
- In the adult hypoxia-ischaemia model, CB1 agonists and octadecylpropyl sulfamide are potential therapeutic candidates for neuroprotection against memory deficits associated with brain damage following HI.
- The neuroprotective role of CB2R has been associated with a better functional outcome and lower deficits in motor learning, coordination and balance following HI. Combined therapy with CB2R agonists and TIM-3 antagonists could be novel targets for decreasing inflammation during HI and reducing related functional and motor deficits.

4. Publications

Scientific articles

1. Perez Martin, M; Rivera P, Blanco E, Lorefice, C.; Decara, J; Pavón FJ, Serrano, A., Rodriguez de Fonseca, F.; Suarez J, (2016). Environmental Enrichment, Age, and PPARalpha interact to regulate neurogenic niches. **Front Neurosci.** (in press, 2016) doi: 10.3389/fnins.2016.00089.
2. Blanco E, Galeano P, Holubiec MI, Romero JI, Logica T, Rivera P, Pavón FJ, Suarez J, Capani F, Rodríguez de Fonseca F (2015). Perinatal asphyxia results in altered expression of the hippocampal acylethanolamide/endocannabinoid signaling system associated to memory impairments in postweaned rats. **Front Neuroanat.** 9:141. doi: 10.3389/fnana.2015.00141.
3. Galeano P, Blanco E, Logica Tornatore TM, Romero JI, Holubiec MI, Rodríguez de Fonseca F, Capani F. Life-long environmental enrichment counteracts spatial learning, reference and working memory deficits in middle-aged rats subjected to perinatal asphyxia. **Front Behav Neurosci.** 2015 Jan 5; 8:406. doi: 10.3389/fnbeh.2014.00406. eCollection 2014.
4. Fresno N, Macías-González M, Torres-Zaguirre A, Romero-Cuevas M, Sanz-Camacho P, Elguero J, Pavón FJ, Rodríguez de Fonseca F, Goya P, Pérez-Fernández R. (2015) Novel Oxazolidinone-Based Peroxisome Proliferator Activated Receptor Agonists: Molecular Modeling, Synthesis, and Biological Evaluation. **J Med Chem.** 2015 Aug 27; 58(16):6639-52. doi: 10.1021/acs.jmedchem.5b00849. Epub 2015 Aug 12. PubMed PMID: 26226490.
5. Galeano P, Romero JI, Luque-Rojas MJ, Suárez J, Holubiec MI, Bisagno V, Santín LJ, De Fonseca FR, Capani F, Blanco E. (2013). Moderate and severe perinatal asphyxia induces differential effects on cocaine sensitization in adult rats. **Synapse.** 67(9): 553-67. doi: 10.1002/syn.21660.
6. Moreno-Santos I, Pavón FJ, Romero-Cuevas M, Serrano A, Cano C, Suardiáz M, Decara J, Suarez J, de Fonseca FR, Macías-González M (2014). Computational and biological evaluation of N-octadecyl-N'-propylsulfamide, a selective PPAR α agonist

structurally related to N-acylethanolamines. **PLoS One**. 20;9(3): e92195. doi: 10.1371/journal.pone.0092195.

7. Rivera P, Arrabal S, Vargas A, Blanco E, Serrano A, Pavón FJ, Rodríguez de Fonseca F, Suárez J (2014). Localization of peroxisome proliferator-activated receptor alpha (PPAR α) and N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) in cells expressing the Ca(2+)-binding proteins calbindin, calretinin, and parvalbumin in the adult rat hippocampus. **Front. Neuroanat**. 8:12. doi: 10.3389/fnana.2014.00012.

8. Wulff-Pérez M, Pavón FJ, Martín-Rodríguez A, de Vicente J, Alen F, de Fonseca FR, Gálvez-Ruiz MJ, Serrano A (2014). Preparation, characterization and in vivo evaluation of nanoemulsions for the controlled delivery of the antiobesity agent N-oleoylethanolamine. **Nanomedicine (Lond)** 9(18): 2761-72. doi:10.2217/nnm.14.35.

9. Kossatz E, Maldonado R and Robledo P. Exacerbated glial activation and TIM-3 expression of CB2 receptor knockout mice following hypoxia-ischemia insult. March 2016. Submitted to **European Neuropsychopharmacology**

10. Galeano P, Romero JI, Holubiec MI, Herrera I, Logica Tornatore T, Suárez J, Capani F., Blanco E, Rodríguez de Fonseca F (2016). Palmitoylethanolamide exerts neuroprotectant effects in neonatal ischemia by reducing hippocampal inflammation that seems to be mediated by PPAR α . *Manuscript in preparation*

11. Kossatz E, Maldonado R and Robledo P. Memory deficits induced by hypoxia-ischemia insult in adult mice are aggravated in CB1 receptor knockout mice. *Manuscript in preparation*

12. Kossatz E, Maldonado R and Robledo P. Role of octadecylpropyl sulfamide in neuroprotection following hypoxia-ischemia insult. *Manuscript in preparation*

Communications

Kossatz E, Maldonado R, Robledo P. Role of cannabinoid CB2 receptors in brain damage following hypoxia-ischemia in adult mice. *16th SEIC Annual Meeting 22–23 September 2015, Granada, Spain. Poster.*

Kossatz E, Maldonado R, Robledo P. Role of cannabinoid CB2 receptors in brain damage following hypoxia-ischemia in adult mice. *SENC and RTA Scientific Meeting*. 22–23 September 2015, Granada, Spain. Oral presentation.

Kossatz E, Maldonado R, Robledo P. Development of an experimental model of hypoxia-ischemia in adult mice. *SENC and RTA Scientific Meeting*. 24-25 September 2013, Oviedo, Spain. Poster and oral presentation.

Kossatz E, Peñalba B, Maldonado R, Robledo P. Development of an experimental model of hypoxia-ischemia in adult mice. *12th FELASA SECAL Congress*. 10-13 June 2013, Barcelona, Spain. Poster.

López MV, Rodríguez de Fonseca F, Robledo P, Maldonado R, Fernández Espejo E. Oleoylethanolamide and palmitoylethanolamide exert neuroprotection of culture cortical neurons subjected to hypoxia. *14th SEIC Annual Meeting*. 28-30 November 2013, Barcelona, Spain. Oral presentation.

Kossatz E, Fernández Espejo E, Rodríguez de Fonseca F, Robledo P, Maldonado R. Role of cannabinoid CB1 and CB2 receptors in brain damage following hypoxia-ischemia in adult mice. *14th SEIC Annual Meeting*. 28-30 November 2013, Barcelona, Spain. Poster.

Patents

Authors: Ruth Pérez, Nieves Fresno, Jose Elguero, Pilar Goya, Ana Belen Torres, Francisco Javier Pavon, Miguel Romero Cuevas, Fernando Rodríguez de Fonseca, Manuel Macias.

Title: Derivados de Oxazolidinona como ligandos PPAR

Application Nr.: (ES) P201331058

Priority date: 11/07/2013

Priority country: International

Holding institution: Fundación IMABIS y CSIC

Situation: submitted