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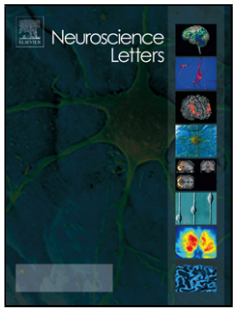

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Acylethanolamides and endocannabinoid signaling system in dorsal striatum of rats exposed to perinatal asphyxia

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Running title: Perinatal asphyxia and acylethanolamide/endocannabinoid signaling

Highlights

- Perinatal asphyxia results in astrogliosis in the dorsal striatum
- Perinatal asphyxia induces a decrease in the expression of NAPE-PLD in dorsal striatum
- Perinatal asphyxia decreases the expression of PPARα receptor in dorsal striatum
- Acylethanolamides and the endocannabinoid system play a key role in perinatal asphyxia

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Abstract

Endocannabinoids (eCBs) and acylethanolamides (AEs) have lately received more attention due to their neuroprotective functions in neurological disorders. Here we analyze the alterations induced by perinatal asphyxia (PA) in the main metabolic enzymes and receptors of the eCBs/AEs in the dorsal striatum of rats. To induce PA, we used a model developed by Bjelke et al. (1991). Immunohistochemical techniques were carried out to determine the expression of neuronal and glial markers (NeuN and GFAP), eCBs/AEs synthesis and degradation enzymes (DAGLα, NAPE-PLD and FAAH) and their receptors (CB1 and PPARα). We found a decrease in NAPE-PLD and PPARα expression. Since NAPE-PLD and PPARα take part in the production and reception of biochemical actions of AEs, such as oleoylethanolamide, these results may suggest that PA plays a key role in the regulation of this system. These data agree with previous results obtained in the hippocampus and encourage us to develop further studies using AEs as potential neuroprotective compounds.

Abbreviations

2-AG: 2-arachidonoyglycerol

Keywords: perinatal asphyxia; DAGLα; NAPE-PLD; CB1; PPARα; dorsal striatum.

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1. Introduction

The consequences of perinatal asphyxia (PA) lead to metabolic dysfunctions [1] and the central nervous system (CNS) is especially vulnerable to the oxidative damage caused by PA [2]. It has been reported that 25% of those newborns who survive PA develop different neurological disorders [3].

In the present work we employed a model developed by Bjelke et al. [4] that has been broadly employed [5-11]. Previous results show that PA can cause significant damage in different areas of the CNS, among them the striatum [4-6,10,11], such as astrogliosis, decreased phosphorylation of high and medium molecular weight neurofilaments, and synaptic alterations [6-8].

The endogenous cannabinoid system (ECS) is comprised of cannabinoid receptors, endocannabinoids (eCBs) and enzymes responsible for these lipids' synthesis, transport and inactivation [12]. The ECS is quite known for its regulation over different neurophysiological processes through the activation of cannabinoid receptors 1 and 2 (CB1, CB2) [12-14].

CB1 is a protein-G coupled receptor highly expressed in the CNS [15,16]. eCBs are divided into two groups that present specific structures, acylethanolamides (AEs) and monoacylglycerols (MAGs) [12,17]. AEs are synthesized from glycerophospholipids in a reaction comprising 2 steps: 1) N-acyl-phosphatidylethanolamine (NAPE) is synthesized trough the action of the N-acyl transferase; 2) the AE is released from NAPE by the NAPE-hydrolyzing phospholipase D (NAPE-PLD). AEs are degraded by the fatty acid amide hydrolase (FAAH) [12,18-20]. Concerning MAGs, the main synthetic enzyme

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involved in its biosynthetic pathway is the diacylglycerol lipase (DAGL), while they are inactivated through the action of the MAG lipase (MAGL) [12,19,20].

Palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) are AEs that share biosynthetic and degradative pathways with the acylethanolamide AEA, but do not bind to CB1 or CB2 [21,22]. Nevertheless, they present neuromodulatory properties as endogenous agonists of peroxisome proliferator-activated receptor alpha (PPARα). PPARs are ligand-activated transcription factors [23] that play an important neuroprotective role in several diseases [24].

Although it has been previously demonstrated that the ECS plays a neuroprotective role in models of neonatal and adult cerebral ischemia [13,25,26], this effect has not yet been tested in the PA model developed by Bjelke et al. [4]. In former works, OEA and PEA have been shown to present neuroprotective effects in adult cerebral ischemia [27,28], but they have not been tested in neonatal hypoxia-ischemia models. Consequently, our aim was to analyze how the eCBs/AEs signaling system is affected in the rodent model of PA developed by Bjelke et al. [4]. We studied the expression of DAGLα, NAPE-PLD, CB1, PPARα and FAAH in the dorsal striatum of control (CTL), cesarean delivery (C+) and asphyctic (PA) 30-day-old rats.

2. Material and methods

2.1. Ethics statements

Previously approved procedures (Institutional Animal Care and Use Committee at the University of Buenos Aires and the Committee on Ethics of the Hospital R. U. de Málaga) were carried out under strict adherence to the European Directive 2010/63/EU on the

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protection of animals used for experimentation, as well as with Spanish regulations (RD 53/2013 and 178/2004). All efforts were made to reduce the number of animals used and to minimize their suffering.

2.2. Animals, cesarean section and perinatal asphyxia procedures

Animal procedures and a detailed description of the PA model employed can be found in Supplementary Material (sections 1 and 2).

2.3. Sample processing and immunohistochemistry

Methods used are described in sections 3 and 4 of the Supplementary Material.

2.4. Immunostaining quantification and NeuN stereological analysis

We studied five to seven coronal sections acquired from 5-6 animals per group. These sections were obtained from Bregma levels 1.2 mm to 0.2 mm (dorsal striatum) [29]. Both densitometry (NeuN, NAPE-PLD, FAAH, DAGLα, and CB1) and cell counting (GFAP and PPARα) were performed using Image J 1.38X (NIH, USA). Stereological analysis of NeuN-positive cells was performed as previously described [5]. Detailed descriptions can be found in Supplementary Material (sections 5 and 6).

2.5. Statistical analysis

Data were examined by one-way ANOVA tests followed by *post-hoc* tests (Bonferroni's correction) using the SSPS 15.0 (SPSS Inc., Chicago, IL, USA). A probability was considered as significant $\leq 5\%$ (two-tailed).

3. Results

3.1. NeuN and GFAP immunostaining in dorsal striatum

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The number of total NeuN-positive cells in dorsal striatum did not differ between groups ($F_{(2, 15)}$ = 2.01, p = n.s.; Fig.1). However, the PA group showed an increment of the number of GFAP-positive cells compared to CTL and C+ groups ($F_{(2, 15)}$ = 5.75, p = 0.01; Post-hoc comparisons: p< 0.05; Fig.1).

3.2. Immunohistochemical expression of DAGLα and NAPE-PLD

The C+ group showed a higher mean optical density for the DAGLα immunostaining than CTL and PA groups ($F_{(2, 14)}$ = 7.13, p< 0.01; Post-hoc comparisons: p< 0.05; Fig.2). Regarding NAPE-PLD immunostaining, the PA group displayed a lower mean optical density than the other two groups. ($F_{(2, 15)}$ = 6.73, p< 0.01; Post-hoc comparisons: p< 0.05; Fig. 2).

3.3. Immunohistochemical expression of CB1, PPARα and FAAH.

In both striatal subregions (lateral and medial), the PA group showed higher mean optical densities for the CB1 immunostaining compared to CTL group (Lateral: $F_{(2, 15)}$ = 4.50, p< 0.05; Medial: $F_{(2, 15)}$ = 5.40, p< 0.05; Post-hoc comparisons: p< 0.05; Fig.3). No differences were found between C+ and PA and between CTL and C+ groups (p=n.s.; Fig 3). Regarding PPARα, the PA group displayed a lower number of PPARα-positive nuclei than the other two groups ($F_{(2, 15)}$ = 7.41, p< 0.01;. Post-hoc comparisons: p< 0.05 vs. CTL and p< 0.01 vs. C+; Fig. 3). Finally, the levels of FAAH were not different between groups ($F_{(2, 15)}$ < 1; Fig. 4).

4. Discussion

A growing evidence indicates that modulation of the ECS has neuroprotective effects in hypoxia-ischemia [13,25,26]. Regarding AEs that do not bind to CB receptors, it has been

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demonstrated that OEA administration before ischemic brain injury induces an increase in PPARα expression and reduces infarct volume and brain edema in mice [28]. In the present work, we have found a reduction in NAPE-PLD levels along with a reduced expression of the OEA receptor, PPARα, which may suggest that the potential neuroprotective role of this system is impaired.

4.1. Asphyctic rats show astrogliosis in the striatum with the absence of neuronal loss

The striatum is one of the most affected areas after a hypoxic-ischemic event [30]. Kruse et al. [31] demonstrated that organotypic striatum cultures exposed to hypoxia present an increase in GFAP levels. It has been also shown that this tissue presents astrogliosis after hypoxia-ischemia in a rodent model [32]. As expected, we observed a higher number of GFAP-positive cells in the striatum of asphyctic animals (see Fig. 1). This was not accompanied by neuronal loss as previously reported [6].

The damage cause in the striatum may cause behavioral changes. Laviola et al. [33] has suggested that changes in the rodent striatum may be related to susceptibility to stereotyped behavior and irregular reactions to social and environmental novelty. Moreover, damage in the striatum caused by perinatal asphyxia is associated with deficits in motor performance [34].

4.2. Perinatal asphyxia is associated with a reduced expression of NAPE-PLD and PPARα

In the present work, we were able to observe a reduction in the expression of NAPE-PLD and PPARα in the striatum (Figs. 2 and 3), similarly to what we have previously reported in the hippocampus [35]. Since NAPE-PLD is responsible for the production of AEs, these

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and previous results lead us to hypothesize that the levels of the endocannabinoid AEA and other non-cannabinoid lipids, such as OEA and PEA, suffer a decrease in hippocampus and dorsal striatum following PA. Moreover, the lack of differences in FAAH levels (Fig. 4) could indicate that AEs degradation is stable in all groups. These results discard the possibility that a NAPE-PLD decrement could be compensated by a change in the degradation through FAAH, and gives more evidence to the hypothesis that NAPE-PLD changes are coupled to changes in AEs. Regarding the reduction in PPARα expression, this finding leads our attention toward its ligands, OEA and PEA, since it has been shown that PPARα plays a key role in the neuroprotective effect that non-cannabinoid AEs present after an ischemic event [27,28]. O' Sullivan et al. [36] and Sun et al. [37] have shown that OEA, not only bounds to PPARα, but that it also positively regulates its transcriptional activity. Thus a decrease in OEA production may induce a reduction in PPARα. These results may indicate that the production and actions of AEs that present no endocannabinoid activity are affected due to PA in the striatum, which holds relevance due to the fact that OEA and PEA present neuroprotective effects in cerebral ischemia [27,28].

Furthermore, DAGLα was increased in the C+ group (see Fig. 2). We can not draw a conclusion about the role that this enzyme plays in cesarean delivery and further studies are needed to address this issue properly, however, a previous work has shown that there is an increase in different AEs in newborns delivered by C-section compared to vaginal delivery, while there are no changes in 2-AG concentrations [38]. Thus, the increase in DAGLα levels may be related to the maintenance of 2-AG levels.

Regarding the changes in CB1 expression, these should be interpreted taking into account that this receptor may be involved in matters such as female reproduction [39]. In this

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work, we observed an increase in CB1 expression in the PA group compared to the CTL group, however there were no differences between PA and C+ groups. Indeed, these changes may be occurring due to the effect of the cesarean procedure to which these animals are subjected in the present experimental model. Animals subjected to C-section presented higher levels of CB1 than the control ones, while those that were exposed to asphyxia showed an increase compared to the C+ animals. Nevertheless, these changes were not significant. However, they can contribute to the significant change observed between the AP and CTL groups. In order to study these changes more closely further experiments focusing on cesarean birth should be carried out.

5. Conclusions

In the present work, we have observed a reduced expression of NAPE-PLD and PPARα in striatum of asphyctic animals. These reductions were accompanied by astrogliosis without neuronal loss. Overall, these results may indicate a dysregulation of the signaling pathway of non-cannabinoid AEs in the striatum of postweaned asphyctic rats and encourage us toward conducting future studies in order to determine the potential neuroprotective effects of AEs in animal models of PA.

Author Contributions

EB, PG, FC and FRF conceived and designed the study; EB, PG, MIH, JIR, TLT and JS acquired and analyzed the data; EB, PG, JS, MIH, FC and FRF interpreted the data; EB, PG, MIH, FC and FRF wrote the manuscript. All authors approved the final version of the manuscript.

Acknowledgments

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Figure legends

Fig 1. GFAP immunostaining is increased in dorsal striatum of perinatally asphyctic rats. (A) Representative photomicrographs showing NeuN-positive cells (upper panel) and GFAP-positive cells (lower panel) in dorsal striatum. Scale bars = 30 µm (insets: 5 µm). Red arrows indicate astrocytes. **(B)** Quantification of the number of total NeuN-positive cells (left panel) and GFAP-positive cells per mm^2 (right panel). No changes in the total number of NeuN+ cells are observed while the number of GFAP+ cells is increased in the PA group. Bars show the mean + SEM. *$p < 0.05$ vs. CTL; $^{\#}p < 0.05$ vs. C+.

Fig. 2. Effects of cesarean section and perinatal asphyxia on DAGLα and NAPE-PLD expression in dorsal striatum. (A) Representative photomicrographs showing DAGLα (upper panel) and NAPE-PLD (lower panel) immunostaining in dorsal striatum. Scale bars = 75 µm. **(B)** Densitometric quantification of DAGLα (left panel) and NAPE-PLD (right panel) immunostaining. In the C+ group DAGLα expression is increased, while NAPE-PLD immunostaining is reduced in the PA group. Bars show the mean + SEM. *$p < 0.05$ vs. CTL; $^{\#}p < 0.05$ vs. C+; $^{\&}p < 0.05$ vs. PA.

Fig. 3. Effects of perinatal asphyxia on CB1 and PPARα expression in dorsal striatum. (A) Representative photomicrographs showing CB1 (upper panel) and PPARα (lower panel) immunostaining in dorsal striatum. In the upper panel the medial and lateral dorsal striatum are delimited. Scale bars = 30 µm (insets: 5 µm). **(B)** Densitometric quantification of the CB1 immunostaining (left panel) and the number of PPARα-positive nuclei per mm^2. CB1 immunostaining is increased in the PA group compared only to the CTL group, both in lateral and medial subregions, while the number of PPARα+ nuclei per mm^2 is decreased in the PA

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group compared to the other two groups. Bars show the mean + SEM. *$p < 0.05$ vs. CTL; $^{\#\#}p < 0.01$ vs. C+.

Fig. 4. Immunohistochemical expression of FAAH in dorsal striatum. (A) Representative photomicrographs showing FAAH immunostaining in dorsal striatum, Scale bars = 30 μm. **(B)** Densitometric quantification of the FAAH immunostaining. No changes are observed in FAAH expression between the groups. Bars show the mean + SEM.

Fig. 5. Schematic summary of the effects that PA exerts over the ECS and AEs. AEs and MAGs are produced in neurons through the action of NAPE-PLD (red) and DAGLα (green) respectively. AEs, such as AEA, bind to CB1 (blue) receptors while others such as OEA bind to PPARα (yellow) and elicit a number of different cellular responses. AEs are degraded through the action of FAAH (purple) in neurons. After a PA event (bright red) levels of NAPE-PLD are decreased, probably leading to a lower production of AEs. Since the transcription of PPARα is in part regulated by OEA, lower levels of this AE may also be causing a decrease of PPARα.

ACCEPTED MANUSCRIPT

AEA: arachidonoylethanolamide
AEs: acylethanolamides

CB1: cannabinoid receptor 1

CB2: cannabinoid receptor 2

DAGL: diacylglycerol lipase

eCBs: endocannabinoids

ECS: endogenous cannabinoid system

FAAH: fatty acid amide hydrolase

MAGL: MAG lipase

MAGs: monoacylglycerols

NAPE: N-acyl-phosphatidylethanolamine

NAPE-PLD: NAPE-hydrolyzing phospholipase D

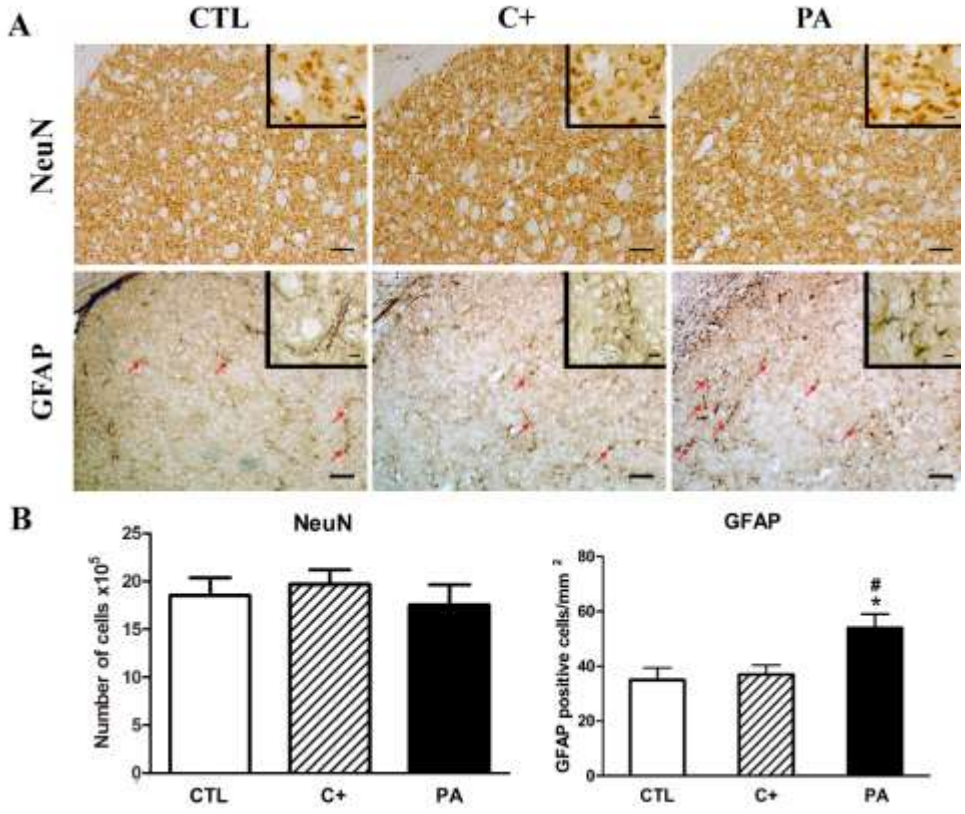
OEA: oleoylethanolamide

PA: Perinatal asphyxia

PEA: palmitoylethanolamide

PPARα: peroxisome proliferator-activated receptor alpha

ACCEPTED MANUSCRIPT



Figr-1

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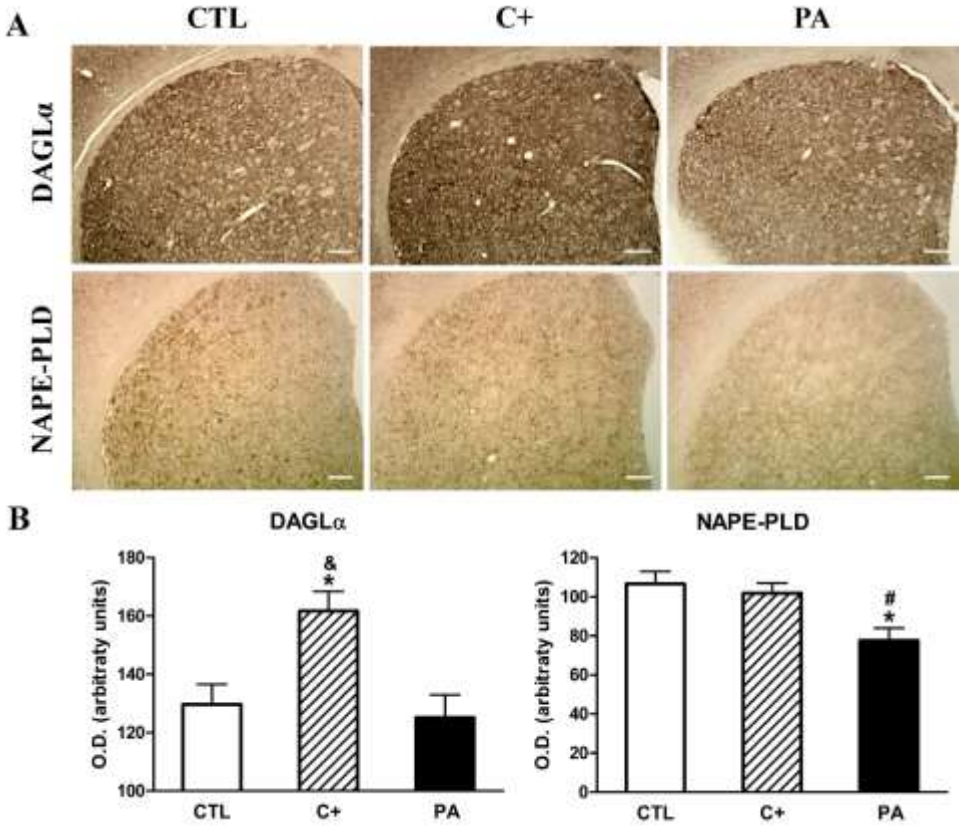

Figr-2

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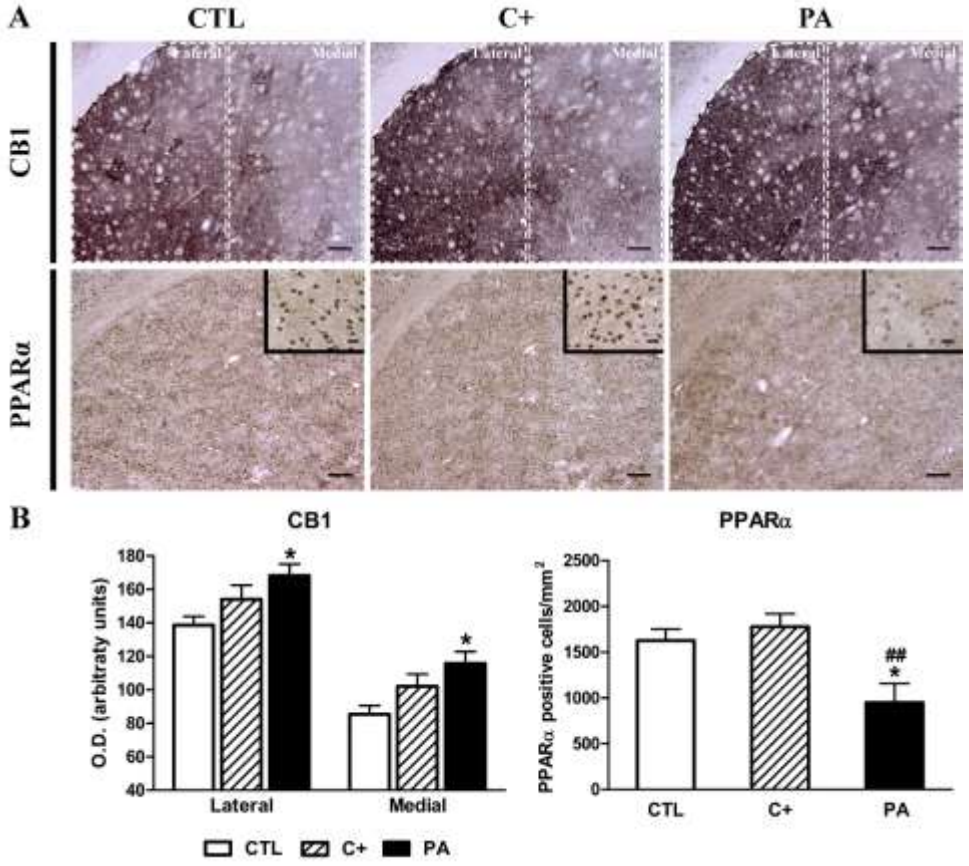

Figr-3

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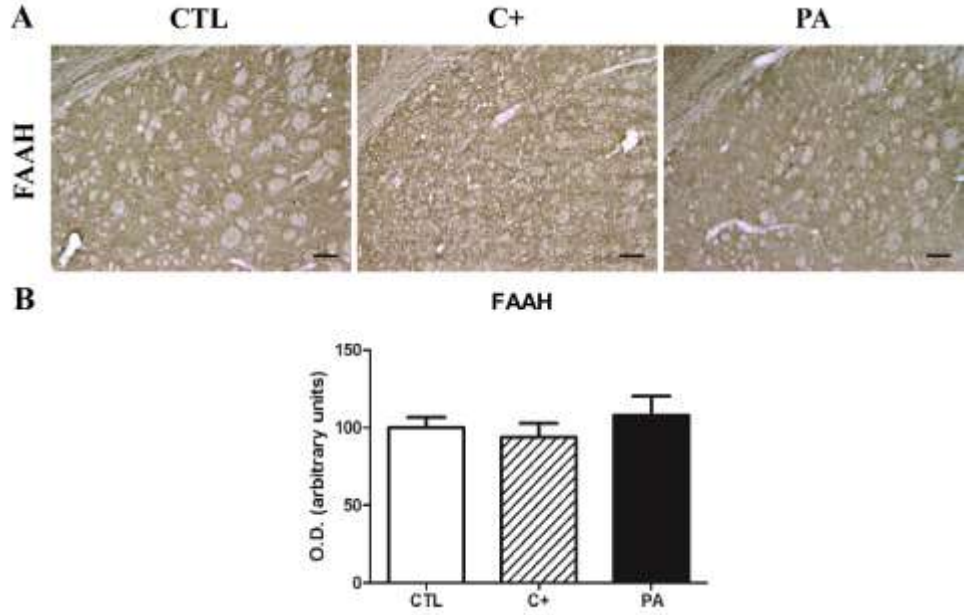

Figr-4

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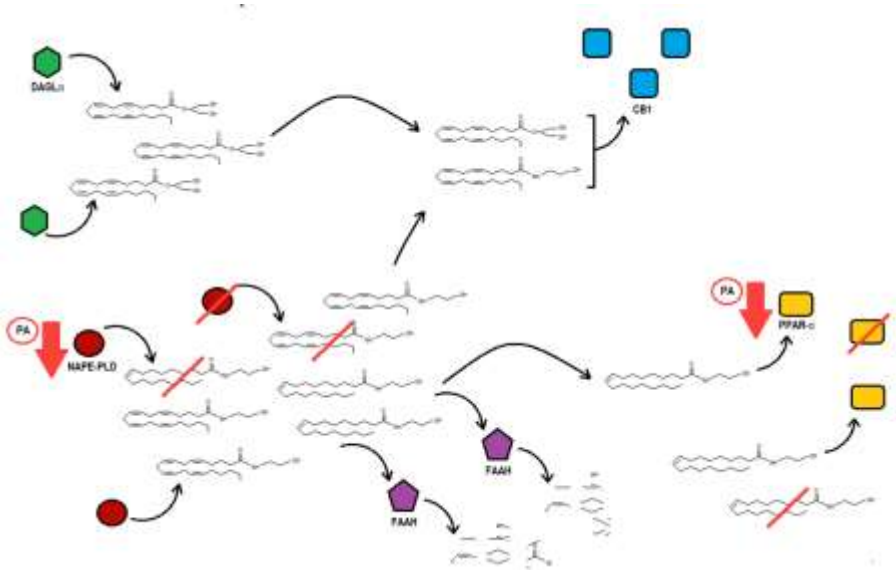

Figr-5