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EFFECT OF HYPERCALORIC AND ISOCALORIC DIETS DIFFERENT IN FATTY ACID CONTENT ON THE ENDOCANNABINOID SYSTEM IN PREGNANT DAMS AND THEIR OFFSPRING

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“Signore, dammi la forza”

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Abstract

Lipid molecules are the building blocks of all cell membranes and provide essential secondary metabolites. The central nervous system is enriched in polyunsaturated fatty acids (PUFAs): arachidonic acid (AA) and docosahexaenoic acid (DHA). During brain development, PUFAs play a critical role in determining neuronal structure, particularly axonal outgrowth. In mammals, AA and DHA cannot be synthesized *de novo* and must be obtained largely from dietary sources. Moreover, during pregnancy and lactation, fetuses and infants rely on PUFAs from their mothers through the placenta and breast milk, respectively. Thus, the maternal diet needs to contain an appropriate omega-3/omega-6 PUFAs ratio to help child development. Based on these premises, we investigated the consequences of maternal malnutrition, defined as a shifted dietary ratio of omega-3 or omega-6 PUFAs on the brain of the offspring. In particular, we modeled human relevance by manipulating both the content and time of daily diets, with a keen focus on endocannabinoid (eCB) system given the critical roles of this AA-derived neuromodulator system during pre- and postnatal brain development.

In the first approach of this project we fed female mice with hypercaloric diets, rich in omega-3 or in omega-6 PUFA, for two (short-term diet) or nine weeks (long-term diet) before mating and during gestation. We found that, levels of endocannabinoids (AEA, 2-AG) and AEA-like mediators (PEA, OEA) did not change with short-term diet in pregnant dams. Moreover, only omega-3 diet induced a significant increase of 2-AG levels, after long-term protocol. In embryos, we found that the short-term exposure of two weeks of omega-3 and omega-6 diet feeding is already sufficient to allow alteration of endocannabinoid system, especially FAAH and CB1R. In fact, in female embryos, we found down-regulation of CB1R and increase of FAAH after treatment with both diets. Interestingly, both enzyme and receptor levels are normalized after high-fat diet administration for a long period, where AEA levels were found decreased. By contrast, in males the alterations found in CB1R after the shorter protocol persist also after the prolongation of the treatment. In line with this, these data suggest a relationship among AEA, CB1R and FAAH, and an important link between PUFAs and endocannabinoid system.

The second approach of this project was based on the administration of isocaloric diets, different in omega-3 levels but not in omega-6 PUFAs throughout gestation and until adulthood. We discovered that during gestation, not only the increase but also the decrease of omega-3 levels markedly affected the eCB system in the hippocampus of embryos. Furthermore, our *in vivo* results strongly suggest that omega-3 diets enriched and deficient affect the principal presynaptic marker in embryonic hippocampus but not in adult, where maternal malnutrition leads to long-term behavioral alterations in adult rats characterized by

the presence of recognition memory deficits. As a whole, the second approach of this study supports our hypothesis about a relationship between PUFAs and the endocannabinoid system and provides further evidence on the importance of omega-3 PUFA on hippocampal development and functioning.

All together, our results suggest that changes in dietary omega-3/omega-6 PUFAs ratio during gestation affect the endocannabinoid system in the brain of the offspring and the major effects of diets are present at the beginning of the treatment (i.e during gestation). Moreover, these data suggest that not only lipids but also fat can have a role in these changes.

Introduction

Polyunsaturated Fatty Acids

A variety of fatty acids exists in the diet, in the bloodstream, and in cells and tissues of humans. Fatty acids are energy sources and membrane constituents. They have biological activities to influence cell and tissue metabolism, function, and responsiveness to hormonal and peripheral metabolic signals.

Fatty acids are composed of a hydrocarbon chain with a methyl group and a terminal carboxyl group. A fatty acid is saturated if each carbon is joined to its neighbor by a single bond. If one or more double bond is present, the fatty acid is said to be unsaturated. Unsaturated fatty acids are also classified as monounsaturated (one double bond) or polyunsaturated (more than one double bond). Unsaturated fatty acids can adopt two distinct stereo configurations, denoted as the *cis* and *trans* configurations. *Cis* double bonds have the two hydrogen atoms on the same side of the molecule, whilst in the *trans* configuration, they are on opposite sides of the molecule. Geometrical isomerism has implications for the shape and physical properties of the molecule, whereby *cis*-fatty acids have a kink in the chain and *trans*-fatty acids adopt a configuration and function like saturated fatty acids (Roche, 1999)

There are two main classes of polyunsaturated fatty acids (PUFAs), omega-3 (n-3) and omega-6 (n-6) differing in the position of their final carbon bond at the methyl end (Fig. 1). The omega-3 and the omega-6 PUFA series share the same biosynthetic enzymes, and hence these conversions occur competitively between the two series. The short chain omega-3 fatty acids are converted to long chain forms with an efficiency of less than 5% in human, therefore most of these long chain PUFAs must be obtained directly from the diet.

Linoleic acid (LA; 18:2n-6) and α-linoleic acid (ALA; 18:3n-3) are the principal essential unsaturated fatty acids. Plants and plant oils contain both of these, although most of the α-linoleic acid is in plant oils, notably from soybean, canola, flaxseed and walnut oils. LA and ALA do not perform the same physiological functions in the cell. Indeed, the two fatty acids can serve competing roles. Linoleic acid can be converted to arachidonic acid, (AA; 20:4n-6) from which endocannabinoids are derivatives. On the other hand, α-linoleic acid can be converted in small amounts to two somewhat longer n-3 fatty acids, eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), which both play physiological roles (Kidd, 2007). Both EPA and DHA are found in far higher concentrations in fish and marine mammals eating fish. Thus, fish, especially oily fish (anchovies, sardines, mackerel, salmon, herring), are the main dietary source of these n-3 fatty acids (Crupi et al., 2013).

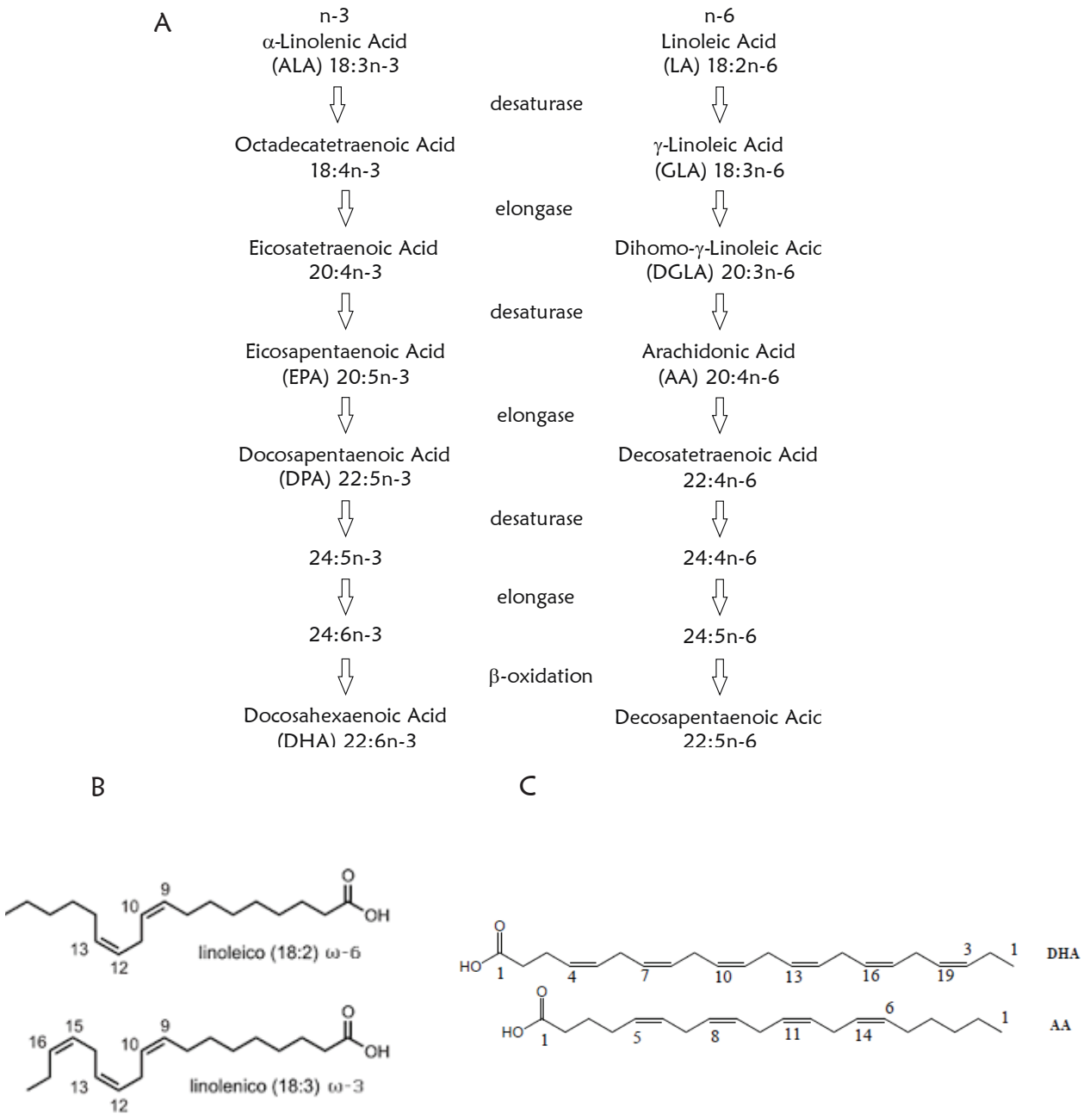


Fig. 1. A Nomenclature of major omega-3 and omega-6 PUFAs. **B** Representative structure for the principal essential unsaturated fatty acids. **C** Example of an omega-3 PUFA (DHA) and an omega-6 PUFA (AA).

PUFAs and diet

Dietary fats are essential nutrients providing energy, essential fatty acids and fat-soluble vitamins. Whilst adequate amounts of dietary fat are readily provided by the Western-type diet, the optimal fatty acid composition of the diet is an important factor which can play a role in health and disease prevention. With the industrial revolution, the dietary intake has drastically changed from an omega-3-rich diet to an almost omega-3-deficient diet accompanied by a sedentary lifestyle. Dietary deficiency or imbalance of key nutrients at critical stages of development can alter normal brain (Dobbing, 1971, Morgane et al., 1993, Coti Bertrand et al., 2006, Crupi et al., 2013, Nyaradi et al., 2013).

Brain contains large amounts of fatty acids, 50% of which are PUFAs; predominantly DHA and AA. Owing to their long carbon chains and high degree of unsaturation, DHA and AA confer specific properties on the lipid bilayer that it is dynamic and flexible, implying that these fatty acids affect brain functions by altering the biophysical properties of cell membranes (Crawford, 1992), transporter, receptor and neurotransmitter functions (Hallahan and Garland, 2005). In fact, an adequate lipid environment is vital for the normal functioning of neuronal membrane proteins such as ion channels, enzymes, ion pumps and receptors. Recent studies show that DHA contributes to the fidelity of cytoplasmic neurotransmission, such as serotonergic, dopaminergic, norepinephrinergic, and acetylcholinergic systems (Fontani et al., 2005, Kidd, 2007).

The fastest stage of neural development is during fetal growth. However, there is also significant development during the first five years of postnatal life. At this time, environmental factors, including nutrient intake, play a critical role in the development of the brain's cytoarchitecture (Levitt, 2003). Moreover, studies have shown evidence for the importance of dietary PUFA during pregnancy and infants (Larque et al., 2002, Helland et al., 2003, Daniels et al., 2004).

During pregnancy, AA and DHA are transported across the placenta into fetal venous blood. During the third trimester of pregnancy, fetuses require approximately 40 to 60 mg of omega-3 PUFA per kilogram of body weight per day (Clandinin et al., 1989). During the last gestational trimester and first postnatal months, there is significant growth of the human brain and a large increase in the cerebral volume of DHA and AA (Helland et al., 2003).

DHA is a nutrient indispensable for the development of the sensory, perceptual, cognitive, and motor neural systems during the brain's growth spurt. The deposition of DHA in human brain phospholipids occurs primarily during the fetal period of active neurogenesis and cell maturation (from the sixth month of pregnancy) and the early postnatal period of development

of intense synaptogenesis, and continues throughout the first two years of life (Guesnet and Alessandri, 2011). The rate of PUFA conversion in placenta and fetus are limited, it is considered that preformed DHA and AA, circulating in the mother's blood, are required for the fetal brain (Haggarty, 2004, Guesnet and Alessandri, 2011). Therefore, after birth and until 6 month of age, breast milk and/or milk replacers are the sole sources of omega-3 and omega-6 fatty acids.

Changes in PUFA intake, for instance by dietary depletion during pre- and post-natal life, but also throughout adulthood correlate with neurochemical alterations, including down-regulation of the vesicular monoamine transporter (VMAT-2) and a depletion of VMAT-associated vesicles in the hippocampus (Chalon, 2006, Kuperstein et al., 2008). A depletion of pre-synaptic vesicles can explain the neurotransmitter depletion in conditions of omega-3 PUFA deficiency (Delion et al., 1994, Delion et al., 1997, Chalon et al., 1998, Zimmer et al., 1998, Chalon, 2006). Moreover, in rat primary hippocampal neurons, DHA stimulates synaptogenesis, synaptic activity and synapsin-1 expression (Cao et al., 2009).

PUFA are present in growth cones and synaptosomal membranes and this location enables them to play a significant role in the dynamics of synapses (Martin and Bazan, 1992, Kearns and Haag, 2002, Haag, 2003). The exact mechanisms are unknown, but likely involve a complex interplay of synergistic effects on neuronal membrane structure and function and gene expression (Yorek et al., 1989, Youdim et al., 2000, Cansev et al., 2008, Cao et al., 2009, He et al., 2009, Bhatia et al., 2011)

Relevant studies suggest that omega-3 PUFA deficiency decreases the mean cell body size of neurons in the hippocampus, hypothalamus, and parietal cortex (Ahmad et al., 2002a, Ahmad et al., 2002b), decreases the complexity of dendritic arborizations on cortical neurons (Wainwright, 2002), and, in culture, DHA enhances neurite outgrowth of hippocampal neurons (Calderon and Kim, 2004, Lafourcade et al., 2011, Crupi et al., 2013). Furthermore, Bertrand P.C. and collaborator have demonstrated that neurogenesis in the embryonic brain is altered by omega-3 PUFAs deficiency (Coti Bertrand et al., 2006). Deficiency at key stages of brain development can have lasting effects on neural function, regardless of later restitution of an adequate diet (Adlard and Dobbing, 1972). Further studies are needed to address the mechanism and sensitive periods during prenatal development when omega-3 PUFAs restriction can affect normal neurogenesis. In this regard, different studies have provided evidence that maternal intake of DHA during pregnancy is associated with higher scores on tests of cognitive test in preschool children (Cheruku et al., 2002, Helland et al., 2003) and a relation between in utero DHA deprivation and several neurologic birth defects has been proposed (Crawford et al., 2003).

Age-related deficits in hippocampal functions, including learning and memory, have recently been reported to be ameliorated by AA and DHA supplementation in aged rats (Gamoh et al., 2001, Kotani et al., 2003, Okaichi et al., 2005). Furthermore, ingestion of AA during postnatal day 2-31 was shown to increase the number of neural stem/progenitor cells in neonatal rats and proliferation and astrogenesis of fetal rat progenitor cells (Maekawa et al., 2009, Katakura et al., 2013)

The Endocannabinoid System

The therapeutic and psychotropic actions of the plant *Cannabis sativa* were first described about 4000 years ago in India. By the 19th century, cannabis extracts had gained widespread use for medicinal purposes. Marijuana extracts has gained widespread use for medicinal purposes until 1937, when concern about the dangers of abuse led to the banning of marijuana for further medicinal use in the United States. However, over the last 50 years, the isolation and characterization of the psychoactive component of C. sativa, Δ^9-tetrahydrocannabinol (Δ^9-THC) represented a challenging research task, awakening renewed interest in its use for pharmacotherapy (Mechoulam et al., 1967, Mechoulam and Gaoni, 1967).

Owing to the lipophilicity and cell membrane-altering action of THC, it was not thought that this compound acted through specific receptors. Only in 1988, the synthesis of a high-affinity cannabinoid ligand allowed the characterization of cannabinoid receptors. (Devane et al., 1988, Di Marzo et al., 1998). Later, other researchers established the functional expression of the cloned cDNA in the brain (Matsuda et al., 1990). This receptor is now known as the type 1 cannabinoid receptor (CB1R) and is the most abundant G protein–coupled receptors (GPCRs). A secondary entity, known as CB2R, was identified by sequence homology and presumed to be mainly present in the immune lineage at the periphery (Fig. 2). Today we now that CB2R is also present in the central nervous system (CNS) particularly astro- and microglia cells (Ashton and Glass, 2007, Benito et al., 2008). Both receptors are coupled to G_i/G_0 proteins through which they inhibit the adenylate cyclase. CB1Rs also modulate ion channels, inducing inhibition of voltage-sensitive Ca^{2+} channels and activation of G-protein-activated inwardly rectifying K^+ channels (Turu and Hunyady, 2010, Castillo et al., 2012). Cannabinoid receptors also modulate several signaling pathways that are more directly involved in the control of cell proliferation and survival, including mitogen-activated protein kinase (MAPK), such as extracellular signal-regulated kinase (ERKs), c-Jun N-terminal kinase (JUN) and p38, phosphatidylinositol 3-kinase (PI3K)/Akt, focal adhesion kinase and synthesis of ceramide (Parolaro and Massi, 2008). In the brain, CB1Rs are found in areas controlling motor, cognitive, emotional and sensory functions (i.e., the hippocampus, basal ganglia, cerebellum, cortex, and olfactory bulb) (Herkenham et al., 1990, Katona et al., 1999). Small nuclei with high density of CB1Rs are also found in other areas, for example those controlling pain, body temperature, sleep-wake cycles, and hormone function, such as the brainstem, the hypothalamus, and the pituitary gland (Herkenham et al., 1991). CB1Rs have also been shown in peripheral tissues such as the eye, muscles, pancreas, heart, lung (Pagotto et al., 2006, Jenkin et al., 2010, Chorvat et al., 2012, O'Keefe et al., 2014), placenta, the fetal

membranes and myometrium (Straiker et al., 1999, Park et al., 2003, Dennedy et al., 2004). Within the brain, the CB2R is only expressed in perivascular microglia cells, vascular endothelial cells, while its presence on certain neuron subpopulations is contention. Increased expression, possibly owing to infiltration of immune cells and activation of microglia, can be observed in certain neurodegenerative disorders (Sagredo et al., 2007). Transcripts for CB2R (Cnr2) have also been identified in the placenta and trophoblasts (Buckley et al., 1998).

The discovery of the CB1Rs led to the rapid identification of a family of lipid transmitters that serve as its natural ligands (Devane et al., 1992, Mechoulam et al., 1995, Sugiura et al., 1995). The subsequent description of a complex biochemical pathway for the synthesis, release (Di Marzo et al., 1994, Cadas et al., 1996), transport (Beltramo et al., 1997) and degradation (Cravatt et al., 1996) of endocannabinoids completed the scaffold of a new signaling system termed the 'endocannabinoid system'.

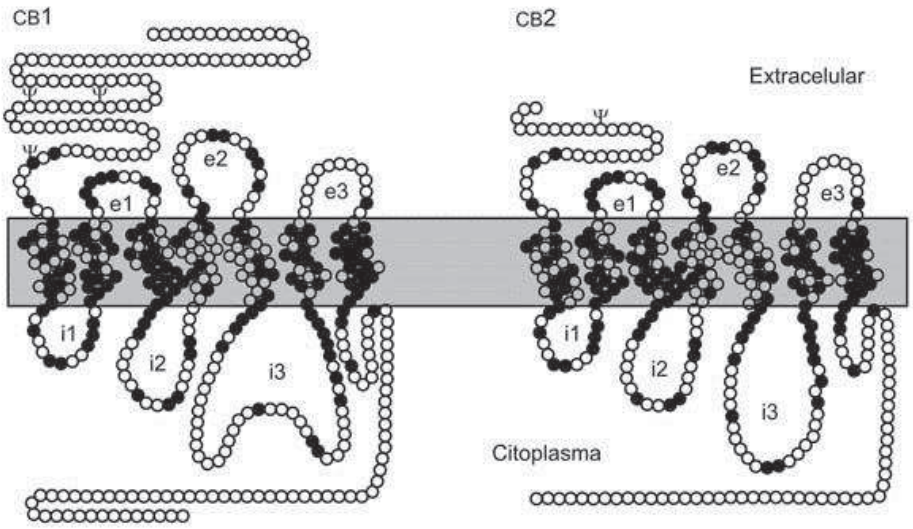


Fig. 2. Representation of CB1 and CB2 receptors structure

Endocannabinoids and the Endogenous Cannabinoid System

The family of endocannabinoids (eCBs) and their structural analogues/potentially includes hundreds of bioactive molecules (Bradshaw and Walker, 2005). The two best-characterized eCBs derivatives of AA are 2-arachidonoylglycerol (2-AG), the most abundant mammalian endocannabinoid that affects synaptic neurotransmission (Gao et al., 2010, Tanimura et al.,

2010), and Anandamide (AEA), which is a mixed endovanilloid and endocannabinoid ligand (Devane et al., 1992). Other eCBs include 2-arachidonylglycerylether (noladin ether), *N*-arachidonyldopamine (NADA), palmitoylethanolamide (PEA), N-oleoylethanolamine (OEA) and *O*-arachidonylethanolamine (virodhamine) (Fig.3).

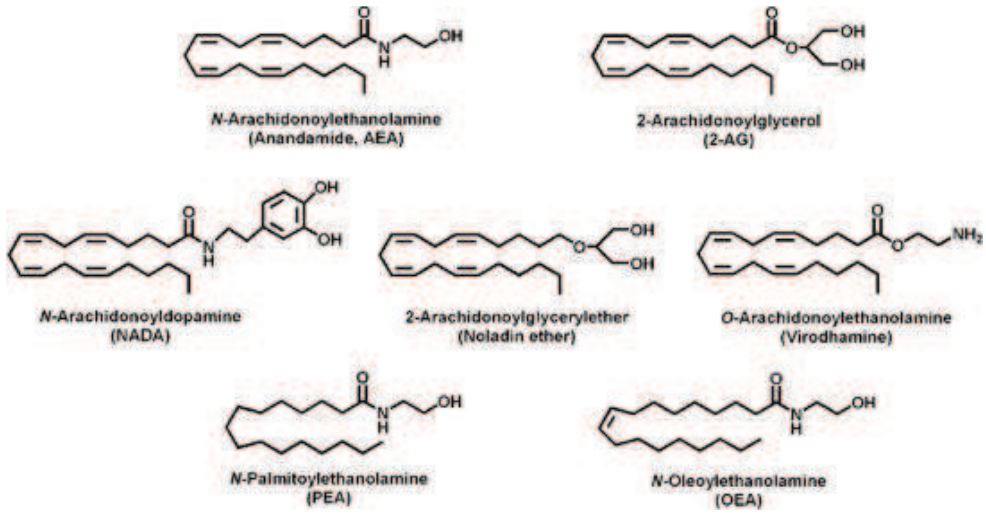


Fig. 3. Chemical structure of endogenous compounds

2-AG, AEA and related lipids can stimulate CB1Rs and CB2Rs. Accumulating evidence suggests the presence of other targets for eCBs like the purported "CB3" receptor GPR55 (Ross, 2009), for AEA and virodhamine (Sharir et al., 2012), and the transient receptor potential vanilloid 1 (TRPV1) ion channel particulary for AEA (Di Marzo and De Petrocellis, 2010). Other eCB targets are the fatty acid receptors peroxisome proliferator-activated receptor-α (PPARα) and PPARγ localized in the nucleus where they shuffle from/to cytosol in a ligand-dependent manner (Pertwee et al., 2010, Pistis and Melis, 2010).

It is widely accepted that eCBs are produced primary "on demand" from membrane lipid precursors by multiple biosynthetic pathways (Fezza et al., 2014). Anandamide is formed by the cleavage of its phospholipid precursor, *N*-arachidonoyl-phosphatidylethanolamine (NArPE). The precursor is synthesized by the enzyme *N*-acyltransferase (NAT), which catalyses the transfer of AA from phosphatidylcholine to the head group of phosphatidylethanolamine (Cadas et al., 1996, Piomelli, 2003). Release of AEA from NArPE is catalyzed by N-acetyl-phosphatidyl-ethanolamine-phosphate D (NAPE-PLD) (Okamoto et al., 2004). Moreover, a series of candidate enzymes with considerable AEA biosynthetic activity has recently been identified, including α/β-hydrolase 4, a lyso-NAPE lipase to form *N*-acyl ethanolamines (Simon and Cravatt, 2006), and PTPN22, a phosphatase cleaving

NAPE-derived phosphor-AEA to yield AEA (Liu et al., 2006). By contrast, sn-1-diacylglycerol lipase (DAGL) α/β are responsible for the synthesis of 2-AG (Bisogno et al., 2003).

Endocannabinoid signaling is terminated by two-step process that includes transport into cells and hydrolysis by two specific enzymatic systems: the fatty acid amide hydrolases (FAAH) for AEA (Cravatt et al., 1996) and monoacylglyceride lipase (MAGL) and ABHD6 for 2-AG (Dinh et al., 2002, Fiskerstrand et al., 2010, Marrs et al., 2010). FAAH is widely distributed throughout the body, with high concentration in the brain and liver. In the brain, FAAH is present in different neurons such as pyramidal cells of the cerebral cortex and hippocampus, Purkinje cells of the cerebellar cortex and mitral cells of the olfactory bulb (Tsou et al., 1998). MAGL is located mainly in the hippocampus, cortex, cerebellum and anterior thalamus, with moderate expression in the extended amygdala, including the shell of the nucleus accumbens (Dinh et al., 2002). Comparison of the distribution of FAAH and MAGL at the cellular level shows that FAAH is primarily a postsynaptic enzyme, whereas MAGL is a presynaptic enzyme (Gulyas, 2004, Rodriguez de Fonseca et al., 2005, Katona and Freund, 2008). Interestingly, it has been found that microglia release 2-AG, and functional MAGL has been described in primary microglial cell cultures, particularly in diseases (Mulder et al., 2011). Alternative to hydrolytic routes, AEA can be oxidized by cyclooxygenase-2 (COX-2), distinct lipoxygenases (LOX) or cytochrome P450. 2-AG by ABDH6 and ABDH12 (Marrs et al., 2010) or it can be hydrolyze by FAAH.

In the adult brain, eCBs are synthesized postsynaptically in an activity-dependent manner and bind presynaptic CB1 receptors on both excitatory and inhibitory afferents thus decreasing neurotransmitter release (Kano et al., 2009). Among the many signaling lipids, eCBs are recognized also for their important roles in neuronal and glial development (Maccarrone et al., 2014). Indeed, during CNS development, eCB signaling seems to regulate fundamental processes such as proliferation, migration, specification and survival of neuronal progenitors (Guzman et al., 2001, Galve-Roperh et al., 2006). Moreover, eCB system has a role in the phenotypic differentiation of neurons and in the control of the synaptic communication (Berghuis et al., 2005, Bernard et al., 2005). The importance of eCB signaling during neuronal development is underscored by the pathogenic impact of maternal marijuana smoking or cannabinoid administration during pregnancy, which cause cognitive, motor and social deficits that last into the adulthood of the offspring.

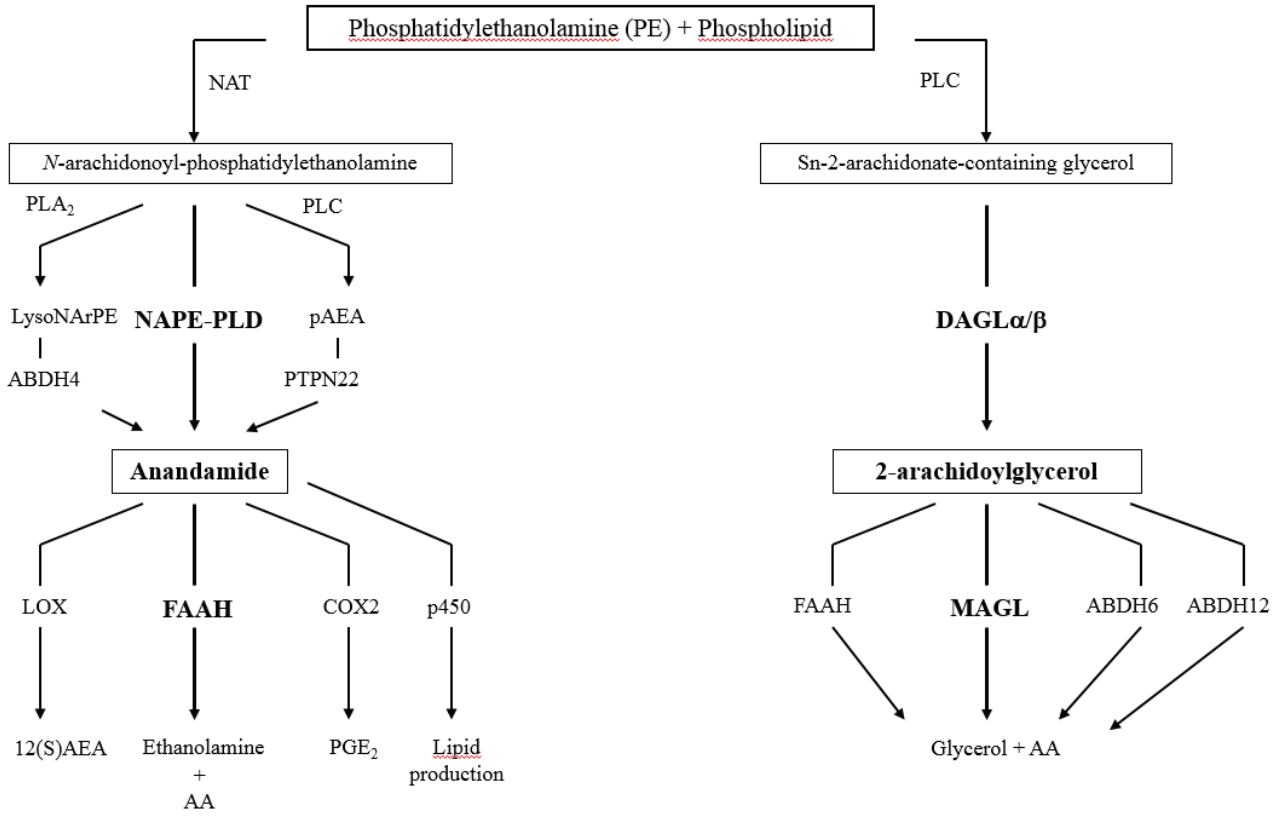


Fig. 4. Synthesis and degradation of AEA and 2-AG. Adapted from Ueda at al 2013

The embryonic period in humans begins at fertilization and continues until the end of the 10th gestation week (GW) (Wilcox et al., 1999). The embryo starts out as a single cell zygote and then divides several times to form a ball of cells called a morula. Further cellular division is accompanied by the formation of a small cavity between the cells. This stage is called a blastocyst. Up to this point, there is no growth in the overall size of the embryo. Each division produces successively smaller cells. The blastocyst reaches the uterus at roughly the fifth day after fertilization (Cockburn and Rossant, 2010, Niakan et al., 2012). It is here that lysis of the zona pellucida occurs. This allows the trophectoderm cells of the blastocyst to come into contact with, and adhere to, the endometrial cells of the uterus. The embryo becomes embedded in the endometrium in a process called implantation. In most successful pregnancies, the embryo implants 6 to 7 days after ovulation (Szekeres-Bartho, 2015).

The development of the nervous system occurs through the interaction of several synchronizes processes, some of which are complete before birth, while others continue into adulthood (Lenroot and Giedd, 2006). The first key event is the formation from ectodermal tissue of the neural plate along the dorsal side of the embryo, which is the source of the majority of neurons and glial cells in the mature human (Lenroot and Giedd, 2006). The processes that contribute to brain development range from the molecular events of gene expression to environmental input (Stiles and Jernigan, 2010, Niakan et al., 2012). Neuron production begins in the embryonic day 42 (E42) (Stiles and Jernigan, 2010) and extends through midgestation in most brain areas. Before that, from the end of gastrulation through E42, brain is rich in neural progenitor (NP) cells (Courchesne et al., 2000, Innocenti and Price, 2005, Lenroot and Giedd, 2006). The intrinsic properties of NP cells are crucial for their final destination into different neuronal phenotypes, which is modulated by extracellular signaling systems and gene expression signature programs that control neural tissue formations (Galve-Roperh et al., 2009). This population of mitotic cells produces two identical NP cells by what is described a “symmetrical” mode of cell division. From E42 the mode of cell division shift from symmetrical to asymmetrical (Wodarz and Huttner, 2003). With the asymmetrical division, neural progenitors are able to produce two different cells. One NP cell that, remains in the proliferative zone and continues to divides and one post-mitotic neuron that leaves the proliferative zone to take its place in the developing cerebrum (Stiles and Jernigan, 2010). After this migration, a period of rapid cell death occurs. The cell bodies of the neurons are primarily found in the gray matter of the brain. Their myelinated axons form white matter. Myelination occurs regionally beginning with the brain stem at 29

week (Inder and Huppi, 2000) Although most major tracts are significantly myelinated by early childhood, axons within the cortex continue to myelinate into the second and third decades of life (Benes, 1989). Another important developmental process is the proliferation and organization of synapses, which begins slightly later around the 20th week of gestation (Lenroot and Giedd, 2006). Synaptic density increases rapidly after birth, reaching by 2-years of age a level approximately 50% greater than the typically seen in adult (Huttenlocher, 1979). Beginning at approximately 15 weeks the surface to the growing brain begins to fold into sulci and gyri, and continue to increase after birth due to changes in cell—packing density and maturation of subcortical tracts (Levine and Barnes, 1999).

A spontaneous abortion, or miscarriage, in the first trimester of pregnancy is usually due to major genetic errors or growth abnormalities in the developing embryo. During this critical period, the developing embryo is also susceptible to toxic exposures such as alcohol, toxins, radiation therapy, nutritional deficiencies and drugs abuse

The Endocannabinoids system during brain development

During the last decade, evidence has been emerging to suggest that the eCB system has adverse effects on gametogenesis, implantation and pregnancy duration (Taylor et al., 2007). During early pregnancy, the development of the pre-implantation embryo and their timely oviductal transport into the uterus occurs simultaneously and are regulated by several endocrine, paracrine and autocrine factors of which the eCB system has been identified as one of the key hormone-cytokine/receptor signaling systems.

- *Endocannabinoids*

The bioavailability of 2-AG and AEA strikingly diverges as the embryo develops (Berrendero, 1998, Berrendero et al., 1999). 2-AG levels gradually increase as tissue differentiation progresses, including in the nervous system (Keimpema, 2010). By contrast, AEA predominates in the blastocyst and early embryonic stages, and is required to define uterine receptivity for embryo implantation and the maintenance of pregnancy (Fig. 5) (Paria, 2001). Indeed, several studies demonstrated that with high levels of AEA, embryos have a delayed and asynchronous development, whereas low levels of AEA are beneficial (Paria et al., 1995, Wang et al., 2003b, Wang et al., 2006b). Animal studies have established that AEA regulates the “window” during which the uterus is receptive (Wang et al., 2004). Studied by Paria et al. have shown that lower AEA levels and elevated levels of FAAH occur in the uterus at implantation sites when compared to the inter-implantation sites, which have much

higher levels of AEA and lower levels of FAAH. These studies suggest that high levels of AEA may be responsible for inhibition of trophoblast proliferation, whereas low levels are required for trophoblastic proliferation (Paria and Dey, 2000). Similar results were found in women who have a successful implantation during an *in vitro* fertilization program.

- *Enzymes*

Control of eCB levels is achieved by the balance between the extent of stimulation of their synthesis by neuronal activity and rate of clearance via uptake and degradation.

DAGLα/β are present in the brain during early development and expression in maintained throughout life (Bisogno, 2003, Berghuis et al., 2007). While total DAGLα protein levels seem constant during brain development, the expression of DAGLβ appears to be most abundant in embryonic brain tissue (Bisogno et al., 2003, Berghuis et al., 2007). Around E14.5, DAGLα/β are expressed on the developing telencephalic axons of pyramidal neurons while by E18.5 DAGLα/β are mainly localized to postsynaptic dendrites of glutamatergic pyramidal cells (Chen et al., 2005, Berghuis et al., 2007, Mulder et al., 2008). This transition may be due to developmental changes in the requirement for 2-AG synthesis from presynaptic to the postsynaptic compartment (Bisogno et al., 2003, Harkany et al., 2007). 2-AG-degrading enzyme, MAGL, is excluded from motile growth cones until synaptogenesis commences (Keimpema et al., 2010, Keimpema, 2013a). Then, MAGL becomes enriched at the presynapse, where it probably functions as a 'stop' signal to limit 2-AG-mediated neurite elongation (Keimpema, 2010). In fact, following neuronal polarization and during directional axonal growth, 2-AG is protected from ligand degradation (Keimpema, 2010) by a mechanism that increases MAGL protein turnover (Keimpema, 2013b) in motile neurite domains, particularly in the growth cone.

Expression of the proposed AEA-synthesizing enzyme NAPE-PLD is low during embryonic development (Chan et al., 2013). It is apparent on axons of glutamatergic and GABAergic pyramidal cells in developing cortex by E18.5 but not at early time (Berghuis et al., 2007). AEA-hydrolysis enzyme FAAH is detected in radial glia during late gestation and throughout the neonatal period (Aguado et al., 2006, Harkany et al., 2007). FAAH also has a role during implantation indeed it has been suggests that the decreased activity and expression of FAAH in peripheral lymphocytes could be used as an early marker for first trimester miscarriage (Maccarrone et al., 2000, Maccarrone et al., 2002).

- *Cannabinoid receptors*

CB1R signaling has been shown to play a crucial role in the oviductal transport of embryos (Wang et al., 2006a, Sun and Dey, 2008). Studies on CB1R knockout mice showed a large number of embryos in the morula or blastocyst stage retained in their oviducts on day 4 of pregnancy (Paria et al., 2001). This suggests that CB1R deficiencies in some woman may have a role to play in tubal pregnancy or female infertility.

CB1R are expressed in progenitor cells grown in neurospheres from different stages of brain development, starting at embryonic day 13.5, to early postnatal radial progenitors (postnatal day 2.5) (Aguado, 2005, Aguado et al., 2007). CB1Rs show a characteristic expression profile during embryonic development and its expression is correlated with neural differentiation (Berghuis et al., 2007, Harkany et al., 2007). In mammals, CB1 receptor expression is characterized by its abundant levels in white matter areas (where the axons of neural cells are present), with their levels progressively increasing from prenatal stages to adulthood in grey matter areas (where mostly occupied by neural cell bodies and dendrites) during neural development (Mulder, 2008). During neocortical development, high CB1Rs levels are distributed along the cortical plate on the surface of distal axons segments of pyramidal cells coursing in the intermediate zone and establishing the fornix pathway of hippocampus (Berghuis et al., 2007, Mulder et al., 2008). Later, CB1Rs are distributed on axons and axonal growth cones in cortical layers I-VI and in both excitatory and inhibitory neurons (Morozov and Freund, 2003, Berghuis et al., 2007) where they facilitate radial migrating of immature pyramidal cells and GABAergic interneurons from subventricular zone (SVZ) and deep migratory stream, respectively (Berghuis et al., 2005). Different studies suggest that in fetal brain there is a preferential limbic expression of CB1Rs with higher levels throughout the cerebral cortex, hippocampus, caudate nucleus, putamen, and cerebellar cortex. During the second trimester of development, it is detectable also in the hippocampus CA region and in the basal nuclear group of the amygdaloid complex. Furthermore, during developing rat brainstem CB1R gradually decreases from gestation day 21 towards adulthood, while in the cerebellum it continuously increases, reaching maximum expression during adulthood (Anavi-Goffer and Mulder, 2009). These changes, which occur in CB1R expression during rat developing brain, are also evident in human brain. In fact, Wang and collaborator (Wang et al., 2003b) showed an intense expression of CB1R mRNA in the hippocampal CA region and basal nuclear group of the amygdaloid complex of fetal brain, whereas, the adult brain showed very high expression throughout the cerebral cortex, caudate nucleus, putamen and cerebellar cortex.

In addition to CB1R, CB2Rs have also been implicated in progenitor cell fate decision and the neural survival (Palazuelos et al., 2012). CB2Rs are present in embryonic undifferentiated NP and stem cells and their levels are strongly reduced in differentiated neuronal cells (Fernandez-Ruiz et al., 2007, Arevalo-Martin et al., 2008). *In vitro* data suggest that both stem cell differentiation and glial specification are affected by pharmacological modulation of CB2Rs, and glia cells may simultaneously express both CB1 and CB2R (Molina-Holgado et al., 2007, Arevalo-Martin et al., 2008). CB2R-mediated NP cell proliferation and neurosphere generation relies on the activation of the extracellular signal-regulated kinase and the PI3K/Akt pathways (Palazuelos et al., 2006). Moreover, CB2R can regulate asymmetric cell division, cell cycle exit and long-range migration throught a machanism which involved DAGLα and DALGβ (Goncalves, 2008, Mulder et al., 2008, Walker et al., 2010). Following commitment to a neuronal fate, a CB2R-to-CB1R switch occurs (Goncalves, 2008): CB1R levels become upregulated at the expense of CB2R levels.
Furthermore, brain inflammation is associate with the inhibition of neurogenesis as a result of immune cell infiltration and excess of pro-inflammatory mediators. Due to the role of CB2Rs in attenuating the activation and recruitment of microglial and peripheral immune cells, CB2R engagement would be expected to contribute to the stimulation of neurogenesis (Palazuelos et al., 2008, Solbrig and Hermanowicz, 2008).

Based on these data and the spatial localization and temporal precision of eCB system, its action emerges as a key regulatory signaling network fundamental to controlling the acquiring of neuronal identity during neurogenesis.

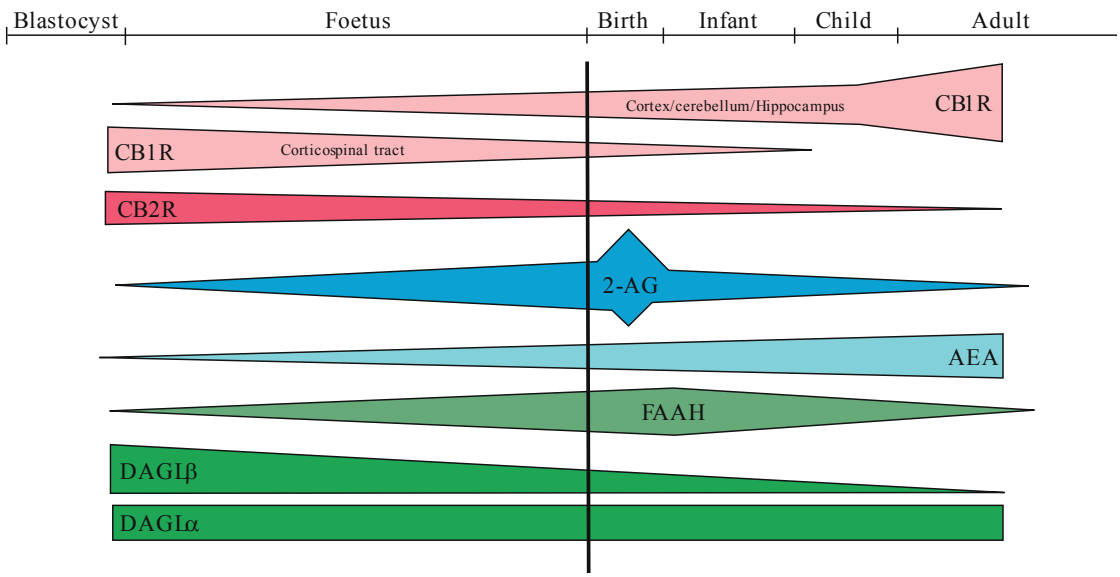


Fig. 5. Temporal changes in available quantitates of eCB system at the indicated developmental stages. Adapted from (Anavi-Goffer and Mulder, 2009).

The endocannabinoids system during neurogenesis

Neuronal development is a finely orchestrated process that leads from a round, post-mitotic cell to a highly polarized neuron, which makes thousands of connections with other neurons in a highly regulated manner, becoming phenotypically and morphologically specialized to conduct, deliver, receive and integrate electrical as well as chemical signals in a functional neuronal network (Valtorta et al., 2011). The process of neuronal development can be subdivided into several steps, including cell migration, establishment of cell polarity, neurite outgrowth, axonal navigation and synapse formation, maturation, stabilization or elimination.

The endocannabinoid system is present at early developmental stages of nervous system formation and can modulate neural proliferation, specification, maturation and the maintenance and survival of differentiated neural cells through the "*on-demand*" recruitment of second messengers such as Src/Stat3, ERK1/2, and PI3K/Akt pathways and the modulation of sphingolipid-derived signaling mediators and cell death pathways (Guzman, 2003). The eCB system plays also a role in the regulation of primary fate decision points of NP by modulating whether neural precursors commit to generate neurons or glia (Harkany, 2007, Harkany et al., 2008). Particulary, CB1R activation on neural progenitors promotes their differentiation into glial cells (Aguado et al., 2006).

In neural stem cells, the expression of CB1R is low, but undergoes robust up-regulation after initial differentiation into a neuron (Galve-Roperh et al., 2007, Harkany et al., 2007). The activation of CB1R and CB2R promote proliferation of NP cells through activation of PI3K/Akt and ERK1/2 signaling pathways *in vitro* (Aguado et al., 2005, Molina-Holgado et al., 2007). Indeed, deletion of CBR or their pharmacological inhibition arrests proliferation in neurospheres *in vitro* and in proliferative zones *in vivo* in both developing and adult brain (Molina-Holgado et al., 2007). Furthermore, neural stem cells express DAGLs and FAAH. Deletion of FAAH and consequent AEA increase induces neurogenesis in proliferative zone in the developing and adult brain (Molina-Holgado et al., 2007, Mulder et al., 2008).

In developing cerebral cortex, pyramidal progenitors are born in VZ/SVZ and they migrate radially to their final destination into the cortical plate (Rakic, 1972). CB1R- and FAAH-deficient mice show aberrant pyramidal distribution at early postnatal stages (Mulder, 2008). Deletion of CB1Rs arrests newly born neurons in deep cortical layers, whereas elevated levels of AEA through deletion of FAAH markedly increases the penetration of newly formed neurons into the cortical plate (Mulder et al., 2008). Furthermore, CB1Rs could have a role in the regulation of long-distance migration in the developing brain (Morozov et al., 2009).

Endocannabinoids signaling, through CB1Rs and multiple signal transduction mechanisms, control cortical neuron specification and morphological differentiation exerting differential

effects on growth cone navigation, axonal elongation, and synaptogenesis of inhibitory interneurons and excitatory (pyramidal) cells in the mammalian cerebrum (Fig.6).

CB1Rs contribute to neuronal polarization by promoting neurite outgrowth through the PI3K/Akt-mammalian target of rapamycin (mTOR) pathway along with several MAPK pathways (Howlett, 2002, Tortoriello, 2014). Neuronal morphology is also regulated by the phosphorylation of Jun, which triggers the rapid degradation of stathmin-2 to alter cytoskeletal stability (Bromberg et al., 2008, Tortoriello et al., 2014). Alternatively, CB1R activation can modulate the activity of RHO-family GTPases, particularly RhoA (Mai et al., 2015) to induce growth cone repulsion and collapse (Berghuis et al., 2007, Argaw et al., 2011).

CB1Rs can also interplay with neurotrophin signaling. Activation of tyrosine kinase receptors such as the fibroblast growth factor receptor (FGFR), and their activity-dependent phosphorylation are thought to induce 2-AG production via sequential activation of phospholipase Cγ (PLCγ) to produce arachidonoyl-containing diacylglycerol (DAG) for conversion to 2-AG by DAGLs and to exert cell-autonomous actions on CB1R in motile growth cones(Williams et al., 2003). Neurotrophins and 2-AG can coincidently activate PI3K-Akt signaling. This, in turn, influences the activity of the transcriptional regulators PAX6 and cyclin AMP response element-binding protein (CREB) and their control of neural progenitor cell proliferation and fate decision (Bromberg et al., 2008) (Fig. 6).

Between embryonic day 14.5 and 18.5 in mice CB1Rs are expressed in several developing axonal trajectories. They guide the elongation of axons to their targets (Mulder et al., 2008, Watson et al., 2008); and the achieving of proper synapse positioning on postsynaptic target cells (Berghuis et al., 2007). The concept of eCB-drives synapse specification is supported by the fact that removal of CB1R from developing axonal tracts impairs with the conclusion of synaptogenesis and the selection of post-synaptic targets (Fernandez-Ruiz et al., 2000, Berghuis et al., 2005, Berghuis et al., 2006).

In immature neurons, DAGLα is localized to the primary neurite (quiescent axon) and the growth cone (Fig. 7-A), and is probably involved in autocrine signaling. Additional data show a subcellular switch of DAGLs during neuronal polarization and synaptogenesis (Keimpema, 2010). Indeed, once synapses are formed DAGLs are redistributed to the somatodendritic axis of neurons (Maccarrone et al., 2014), the down-regulation of DAGLs expression can be viewed as a pivotal step to increase the reliance of postmitotic neurons on extracellular 2-AG produced by pyramidal cells in the cortical plate.

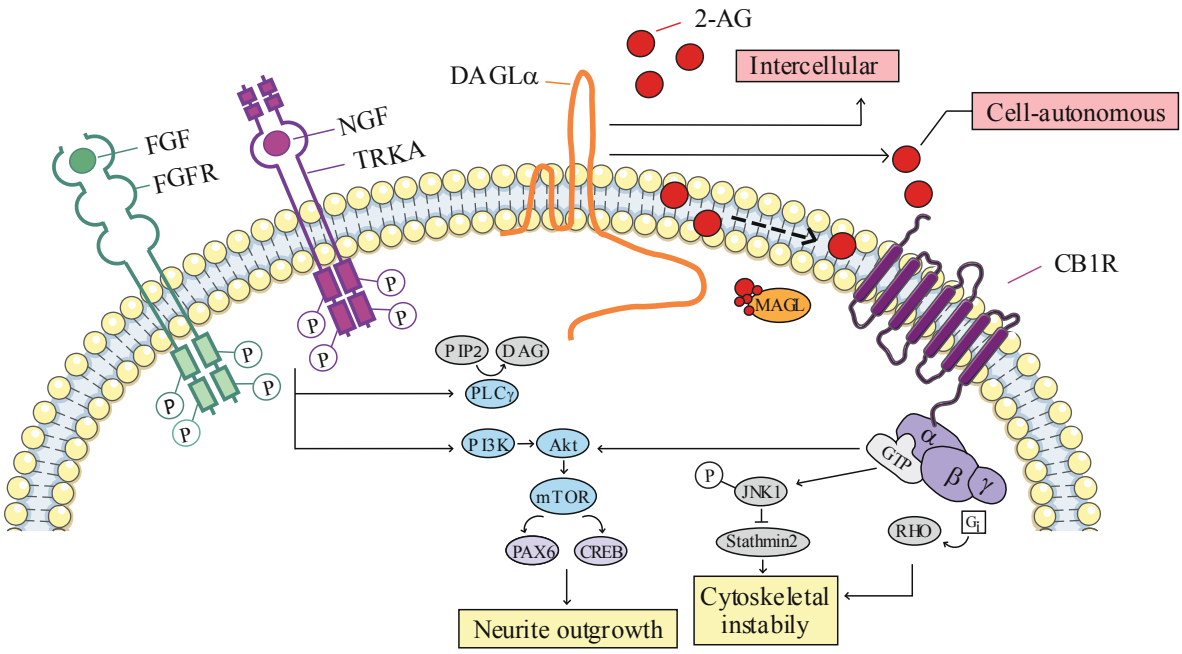


Fig. 6. Design of endocannabinoid signaling during neurite outgrowth and synaptogenesis. Both cell-autonomous and cell-cell interaction of morphogenic signals can modulate NP cell proliferation and asymmetric division. FGFR activation induces phospholipase Cγ (PLCγ) activation which generates arachidonoyl-containing diacylglycerol (DAG). A similar mechanism was more recently described for TRKA signaling (Kaimpema E. et al., 2013) via PI3K pathway, leading to increased expression of DAGL, MAGL and CB1R. CB1R activation can then alter cytoskeletal stability through activation of Rho-family GTPases and phosphorylation of Jun N-terminal kinase (JNK) and consequent degradation of stathmin-2. It can also activate the phosphatidylinositol 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) pathway controlling neuronal progenitor cell proliferation and fate decision. Adapted from Maccarrone M. et al., 2014.

These data suggest that, in differentiating neuronal tissue, the specification and extension of axons towards postsynaptic target areas may require autocrine eCB signaling (Bisogno et al., 2003, Williams et al., 2003), while the precise positioning of synapses on postsynaptic targets, the establishment of cell-to-cell contacts, and the onset of synaptic communication within target regions are controlled by a paracrine action of 2-AG (Berghuis, 2007, Harkany, 2007). Once synapse establishment completes eCB signaling in the embryonic brain can directly translate into retrograde synaptic signaling.

At mature synapses, receptor and enzyme components of the eCB system have distinct subcellular distribution, both intracellularly and extracellularly on presynaptic and postsynaptic neurons, microglia and astrocytes. Indeed, 2-AG can be produced postsynaptically and diffuse in a retrograde manner across the synapse to inhibit synaptic transmission by activating presynaptic CB1Rs. 2-AG is then mainly inactivated by presynaptic MAGL, with a possible contribution from ABHD6 and ABHD12, present in both

post- and presynaptic neurons (Marrs, 2010, Maccarrone et al., 2014). By contrast, AEA not only can be produced postsynaptically like 2-AG, but also presynaptically due to the presence of NAPE-PLD, which then can act at postsynaptic TRPV1 (Ross, 2003, Maccarrone, 2008, Castillo et al., 2012, Puente, 2014). AEA is then inactivated by FAAH. It is interesting to note that perisynaptic astrocytes often contain MAGL. It might suggest that astrocytes form a barrier that may limit 2-AG spread beyond its intended site of action (Maccarrone et al., 2014).

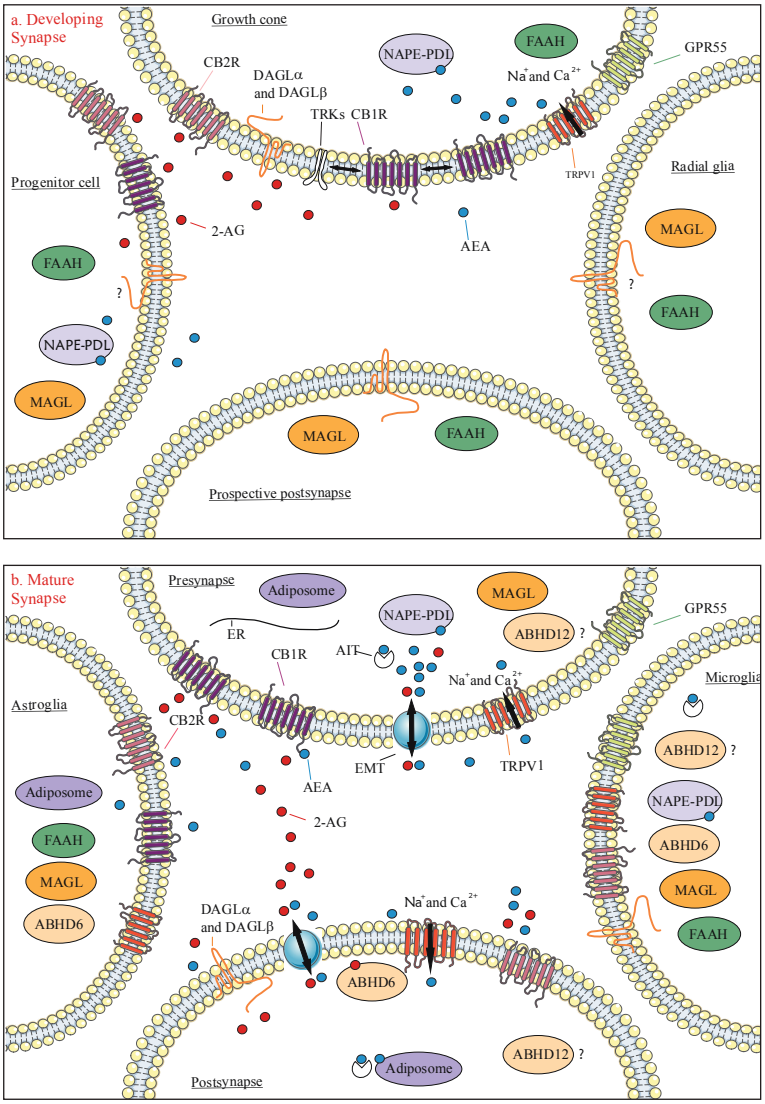


Fig. 7. Molecular architecture of the endocannabinoid system during synaptogenesis and at mature synapse. **A**. At the developing synapse, Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) bind their target receptors: cannabinoid 1 receptor (CB1R), CB2R, G protein-coupled receptor 55 (GPR55) and transient receptor potential cation channel subfamily V member 1 (TRPV1) present during growth cone elongation. CB1 and CB2 receptors are also present on progenitor cells. Their activation promotes neurogenesis through multiple signal transduction mechanisms. The availability of eCBs is determined by biosynthesis and degrading enzymes, NAPLE-PLD and FAAH for AEA, and DAGLα/β and MAGL for 2-AG. **B**. At the mature synapses, in addition to FAAH and MAGL there are other mechanisms involved in the availability of eCBs such as ABHD6, ABHD12, endocannabinoid transmembrane transporter (EMT) and AEA intracellular transporter (AIT). Endocannabinoid signaling is translated into retrograde synaptic signaling. Adapted from Maccarrone M. et al., 2014.

Aims

Lipid molecules are the building blocks of the central nervous system which is particulary rich in omega-3 and omga-6 PUFAs that play a critical role in its development and functions. In mammals, PUFAs cannot be synthesized *de novo* and must be obtained largely from dietary sources. Moreover, endocannabinoids have recently been considered as neurodevelopmental signaling cues that, by targeting the CB1R, exert a modulatory role on the molecular and cellular mechanisms in brain development (Diaz-Alonso et al., 2012).

On these bases the present PhD project was designed with the intent to study if changes in dietary omega-3/omega-6 PUFAs ratio could affect the endocannabinoid system in the embryonic brains from mothers fed with different diets.

Recent evidence indicates that in modern Western diet, there is increased intake of high-calorie high-fat food, characterized by an increased omega-6 and/or a reduced omega-3 PUFA content. Yet, the consequences of maternal malnutrition on eCB system in the brains of their progeny are mostly unknown. Therefore, the first approach of this project was to feed female C57Bl6/J mice with **hypercaloric diets** following two different protocols.

- Short-Term Diet Protocol: animals were fed for two weeks before mating and throughout gestation;
- Long-Term Diet Protocol: animals were fed for nine weeks before mating and throughout gestation.

The diets are the following: omega-3 PUFA enriched diet (containing 32.1% fat, poor in omega-6); omega-6 PUFA enriched diet (containing 34.3% fat, poor in omega-3) and a standard diet containing 10% total fat.

Biochemical analyses were performed on both female and male tissue (cortex/hippocampus) of E18 embryos. The following points were examined:

- eCB levels (AEA, 2-AG, PEA and OEA) were quantified from brain (cortex/hippocampus tissue), plasma and adipose tissue of mothers fed with diets and in brain (cortex/hippocampus tissue) and plasma of E18 embryos.
- qPCR and Western blots were performed on female and male tissue (cortex/hippocampus tissue) of E18 embryos to test if alteration in CB1R and TRPV1 receptors, metabolic enzyme endocannabinoids (NAPE-PLD, FAAH, DAGLα and MAGL) and CRIP1a were present.

It is known that the eCB system has an important role during neurite outgrowth. Our studies have provided novel information on the role of PUFAs on neurogenesis and neuroplasticity,

but the mechanism behind its role remains incompletely established. For these reasons, we evaluated the role of principal omega-3 and omega-6 PUFAs, EPA and DHA, and linoleic acid in neurite development of cultured neurons by determining the changes in neurite length and branching.

Next, given the multiple mechanism by which high fat diets could interfere on eCB system, to better characterize the effect of PUFAs, in the second part of this thesis we focused our attention on omega-3 PUFAs, and we fed Pregnant Sprague–Dawley rats **isocaloric diets** different in omega-3 levels but not in omega-6 (30%) throughout gestation and until adulthood. The diets are the following: omega-3 PUFA deficient diet (containing 6% total fat, rich in linoleic acid); omega-3 PUFA enriched diet (containing 6% fat, rich in alfa-linolenic acid) and a standard diet containing 6% total fat.

Biochemical analyses were performed at embryonic day 18 and postnatal day (PND) 75. The following points were examined:

- Western blot analyses were performed on hippocampus of E18 embryos in order to check for the presence of alterations in CB1 and CB2 receptors, and in metabolic enzymes of the principal endocannabinoids AEA (NAPE-PLD and FAAH) and 2-AG (DAGLα/β and MAGL).
- Given the role of omega-3 PUFAs and the eCB system in neurodevelopment, western blot analyses were performed on hippocampal lysates from embryos and adult animals in order to check for the presence of alterations in some of the most relevant neuronal markers: β-tubulin, GAP43, synaptotagmin, synaptophysin and PSD-95.

Finally, we checked for the presence of cognitive deficits in the adult offspring following diet administration through the classic and spatial versions of the novel object recognition (NOR) test.

Materials and Methods

Animals, dietary treatments and experimental design

Female C57Bl6/J mice were housed in clear plastic cages on a 12-hour light-dark cycle (lights on 08:00h) and in a temperature (22 ± 2°C) and humidity controlled environment (50 ± 10%). All experiments were performed in accordance with guidelines released by the Austrian Ministry of Science and the European Community.

Animals were randomly separated in three groups and fed with hypercaloric diets following two protocols:

- Short-Term Diet (STD) protocol: animals were fed for two weeks before and throughout gestation (Fig. 8-B);
- Long-Term Diet (LTD) protocol: animals were fed for nine weeks before and throughout gestation (Fig. 8-C).

The diets are the following (Fig. 8-A):

- w3 diet: omega-3 PUFA enriched diet, containing 32.1% total fat and poor in omega-6;
- w6 diet: omega-6 PUFA enriched diet, containing 34.3% total fat and poor in omega-3;
- Control diet: containing 10% total fat.

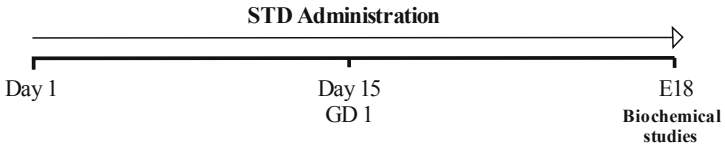
Maternal body weight was recorder 2-3 days a week for the duration of the study. After 18 days of gestation, 5 mice from each group were anesthetized with isoflurane, and blood, adipose tissue and cerebral areas (cortex/hippocampus) were collected, immediately frozen in liquid nitrogen, and stored at -80°C until processing.

Embryos were quickly removed and weighed. Female and male embryos were then separated. Blood samples were collected and cerebral areas (cortex/hippocampus) were obtained by regional dissection on ice, immediately frozen in liquid nitrogen and stored at -80°C until processing.

A

	Diet	Kcal/g	Fat %	W3 Fatty acid %	W6 fatty acid %
1	Control	3,68	10	0,26	1,87
2	w3 Diet	5,13	32,1	7,67	3,05
3	w6 Diet	5,14	34,3	10,39	33,6

B



C

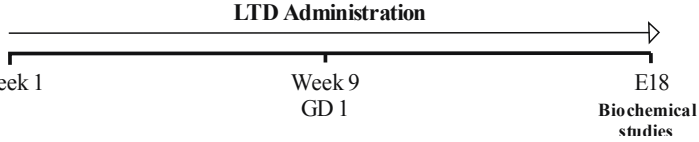


Fig. 8. Diets composition (**A**) and short term diet (**B**) and long tern diet (**C**) administration and experimental time line

Biochemical Studies

Liquid Chromatography-Atmospheric Pressure Chemical Ionization Mass Spectrometry

Extraction, purification, and quantification of 2-AG, AEA, PEA and OEA from tissue of pregnant mice and fetuses fed standard chow and hypercaloric diet, were carried out according to previous published protocols (De Marchi et al., 2003). Briefly, at E18 separate dams were used for the dissection of cerebral areas (cortex/hippocampus), blood and adipose tissue, and embryos were used for cerebral tissue and blood. Plasma samples were obtained after centrifugation of blood after erythrocytes and granulocytes sedimentation at the bottom of the tube. The collected areas were immediately frozen in liquid nitrogen and stored at -80°C.

After lipid extraction and prepurification on silica gel columns, 2-AG, AEA, PEA and OEA levels from three animals per condition were analyzed by isotope dilution using liquid chromatographyatmospheric pressure chemical ionization-mass spectrometry (Shimadzu LCMS-2020). Results were expressed as pmoles per mg or g of tissue.

RNA isolation and Quantitative Polymerase Chain Reaction

E18 embryonic brains (n = 3) were microdissected on ice to isolate cortical and hippocampal regions and snap frozen in liquid N_2 until processing. RNA was extracted using the RNeasy mini kit (Quiagen) with DNase I step performed to eliminate trances of genomic DNA. RNA quality and concentration were measured with Nanodrop spectrophotometer and 1% agarose gel stained with GelGreen™ (Biotium). Total RNA was then reverse transcribed to a cDNA library in a reaction mixture using a high-capacity cDNA reverse transcription kit (Applied Biosystems). The cDNA library was then used for quantitative, real-time PCR (qPCR). SYBR Green Master Mix Kit (Life technologies) including reverse and forward primers (5μM/μl). The primer sequences used in this project are show in Table 1.

Nineteen microliters of master mix were added to wells of a 96-well plate followed by 1 μl of cDNA. Quantitative PCRs were performed after an initial 10 min 95°C denaturation followed by 40 cycles of 95°C for 15 s denaturation, annealing and extension at calculated temperatures (60 s), and a dissociation stage (from 60 to 95° C with 0.5°C steps for 10 s each) MyiQ; Bio-Rad), with primers pair amplifying short fragments for each gene. All samples were run in triplicate, fluorescence emission was detected, and cycle threshold values were calculated automatically. Samples without template served as negative control. Expression levels were normalized to the housekeeping gene encoding glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) or TATA-binding protein (TBP) for every sample in parallel assays.

GeneBank number	Protein	Primer pair
NM_198114	DAGLα	(forward) 5'- TCATGGAGGGGCTCAATAA-3'
		(reverse) 5'-AGCCCTCCAGACTCATCTCA-3'
NM_011844	MGL	(forward) 5'- CAGAGAGGCCAACCTACTTTTC-3'
		(reverse) 5'-ATGCGCCCCAAGGTCATATTT-3'
NM_178728	NAPE-PLD	(forward) 5'- CGTGCTCAGATGGCTGATAA-3'
		(reverse) 5'-ATGAGCTCGTCCATTTCCAC-3'
NM_010173	FAAH	(forward) 5'- TGGAAGCCCTCCAAGAGTCCA-3'
		(reverse) 5'-TGTCCATAAACACAGCTCTTCAG-3'
NM_007726	CB1R	(forward) 5'-TCTTAGACGGCCTTGCAGAT-3'
		(reverse) 5'-AGGGACTACCCCTGAAGGAA-3'
NM_029861.2	CRIP1a	(forward) 5'AGAACCGCACAATCAAGCTG-3'
		(reverse) 5'-TTACCCTTCAGCTCCAGTGG-3'
NM_001001445	TRPV1	(forward) 5'-GACCTGGAGTTCACCGAGAA-3'
		(reverse) 5'-GCATGTTGAGCAGGAGGATG-3'
NM_008084	GAPDH	(forward) 5'-AACTTTGGCATTGTGGAAGG-3'
		(reverse) 5'-ACACATTGGGGGTAGGAACA-3'
NM_013684	TBP	(forward) 5'-ACCCTTCACCAATGACTCCTATG-3'
		(reverse) 5'-ATGACTGCAGCAAATCGCTTGG-3'

Table 1. List of forward and reverse primers used for amplification using Quantitative PCR.

Quantitative Western blotting with total protein normalization

Tissue samples were obtained from E18 embryos that were quickly removed from dams killed by cervical dislocation under anesthesia. Cerebral areas (cortex/hippocampus) were obtained by regional dissection on ice, immediately frozen in liquid nitrogen and stored at -80°C until processing. To fractionate membranes and cytoplasm, samples were homogenized by sonication in HEM buffer (25 mM Hepes pH 7.5, 1 mM EDTA, 6 mM $MgCl_2$) containing 1 mM dithiothreitol (DTT) and a cocktail of protease inhibitors (PI, Complete™, Roche) and centrifuged at 700 g at 4°C for 5 min. The supernatant was centrifuged twice at 14.4k g at 4°C for 30 min used as cytoplasmic lysate and pellet was resuspended with HEM and use as membrane fraction. Protein concentrations were determined by Bradford's colorimetric method (Bradford, 1976).

Total protein labeling was initiated by adding Cy5 dye reagent (GE Healthcare) that had been pre-diluted (1:10) in ultrapure water. Samples were mixed and incubated for 5-30 min at room temperature (RT). The labeling reaction was terminated by adding Amersham WB loading buffer (GE Healthcare; 20 µl/sample) containing 40 mM DTT. Samples were then heated at

95°C for 3 min. Equal amounts (20 μg/40 μl) of each sample were loaded onto an Amersham WB gel card-14, (13.5% or 8%). Electrophoresis (600 V, 42 min) and protein transfer onto polyvinylidine-difluoride membranes (100 V, 30 min) were at default settings in an integrated Amersham WB system (GE Healthcare) for quantitative SDS-PAGE and Western blotting of proteins with fluorescence detection. Membranes were blocked for 1 h at RT in 3% bovine serum albumin (BSA, Sigma-Aldrich) in 1x TBS before incubation with primary antibody at 4°C overnight.

The following primary antibodies were used:

- guinea pig anti-cannabinoid receptor 1 (CB1R) (1:500) (kindly provided from Dr. Masahiko Watanabe)
- rabbit anti-cannabinoid receptor-interacting protein 1a (CRIP1a) (1:500) (Keimpema et al., 2010)
- rabbit anti-monoacylglycerol lipase (MAGL) (1:500, Cayman Chemical)
- rabbit anti-diacylglycerol lipase alpha (DAGLα) (1:1000, Abcam, UK)
- rabbit anti-fatty acid amide hydrolase (FAAH) (1:500, Cayman Chemical)
- rabbit anti-N-acyl phosphatidyl ethanolamine (NAPE-PLD) (1:500, Abcam, UK)
- rabbit anti-transient receptor potential cation channel subfamily V member 1 (TRPV1) (1:500, Santa Cruz, USA)

To demonstrate the specificity of the guinea pig anti-CB1R antibody, we prepared cerebellar homogenates from wild-type (WT) mice and their CB_1R KO littermates. We found specific bands at the expected molecular weight (~53 kDa) in WT mice but not in CB1R KO mice (Fig. 14 and 20).

Antibody binding was detected by using species-specific (anti-rabbit, anti-mouse and anti-guinea pig) carbocyanin (Cy) 3-labeled secondary antibodies (1:1000; GE Healthcare). Membranes were dried before scanning at 560 nm (Cy3) and 630 nm (Cy5) excitation. Automated image analysis was performed in the Amersham WB evaluation software, and optimized by manual editing.

Cortical/Hippocampal neuronal cultures and morphology

Cerebral tissues (cortex/hippocampus) of embryos were isolated at E14.5. Cells were mechanically dissociated into single cell suspension by trypsin digestion (0.1%, 3 min) and plated at a density of 50,000 or $1x10^6$ cells onto poly-D-lysine-coated (PDL; Sigma) 24-well plates for morphometry analysis. Cultured neurons were maintained in Neurobasal medium, supplemented with 1% B27/1% Glutamax/1% penicillin/streptomycin for 2 days *in vitro* as described (Mulder et al., 2008) (DHA, EPA, or LA; 10 μM; Tocris) and then dissolved in Neurobasal medium containing 0.1% fatty acid-free BSA (4% stock concentration, Sigma-Aldrich) and the medium were first added 12 h after cell seeding and replenished every other day.

Immunocytochemistry

Staining of β-tubulin III was performed for morphometric analysis. Cultured cells were fixed with 4% paraformaldehyde for 30 min at RT, washed with 0.1 M phosphate buffered saline (PBS; pH 7.5), blocked with 10% normal donkey serum (Jackson Immuno Reseasrch)/5% BSA (Sigma-Aldrich) in PBS containing 0.3% Triton X- 100 at RT for 1 h, and incubated with primary antibodies mouse anti-β-Tubulin III (1:1000, Promega, USA) at 4°C overnight. The cells were then washed with PBS and incubated with Alexa Fluor 488-conjugated secondary antibody (1:1000) at RT for 1 h.

Images of primary neurons and their neurite were captured by means of laser-scanning microscopy (model 710LSM, Zeiss). Axonal and neurite length and the number of their branches were determined by analyzing calibrated images in the LSM5 image browser software module (v. 3.2.0.115, Zeiss).

Animals, dietary treatments and experimental design

Female Sprague Dawley rats were obtained from Charles River laboratories (Calco, Italy) and were housed in clear plastic cages on a 12 hour light-dark cycle (lights on 08:00h) and in a temperature (22 ± 2°C) and humidity controlled environment (50 ± 10%). All experiments were performed in accordance with the guidelines released by the Italian Ministry of Health (D.L.116/92) and (D.L.111/94-B), and the European Community directives regulating animal research (86/609/EEC).

Animals were randomly separated in three different groups and fed with isocaloric diets different in omega-3 PUFA content but not in omega-6 PUFA (30%) throughout gestation and lactation. After weaning, the offspring were subjected to the same diet until adulthood (Fig. 9).

Biochemical analysis were performed at embryonic day 18 and postnatal day (PND) 75, whereas behavioral analysis were performed at PND 75.

The diets are the following:

- Poor diet (omega-3 PUFA deficient diet - containing 6% total fat in the form of peanut oil, rich in linoleic acid, omega-3/omega-6 1:23.6);
- Rich diet (omega-3 PUFA enriched diet - containing 6% fat in the form of rapeseed oil, rich in alfa-linolenic acid, omega-3/omega-6 1:4.5);
- Standard diet (containing 6% total fat in the form of peanut oil and rapeseed oil – omega-3/omega-6 1:8.6).

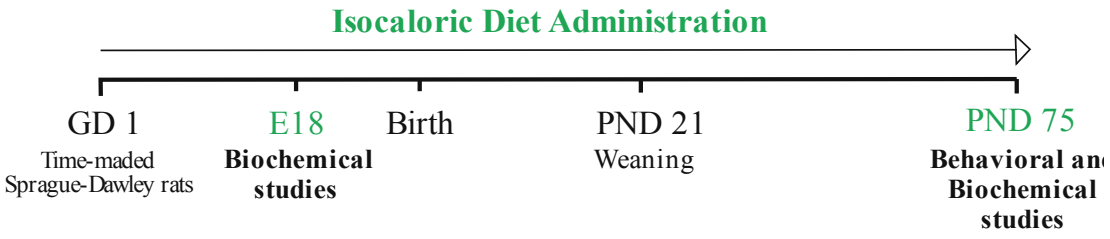


Fig. 9. Isocaloric diet administration and experimental time line

Biochemical Studies

Western blotting

For western blot analyses, rats were decapitated and the cerebral areas (hippocampus) were obtained by regional dissection on ice, immediately frozen in liquid nitrogen and stored at -80°C until processing.

For total protein lysates, each brain region was homogenized in an appropriate volume of ice-cold buffer (10 mM Hepes pH 7.5, 1.5 mM MgCl2, 10 mM KCl, 2 mM DTT, 1 mM PMSF, 1 mM EDTA, 1 mM EGTA, 2 mM sodium orthovanadate, 50 mM NaF, 10 mM sodium pyrophosphate, 0.5% Triton, 5 μg/ml aprotinin, and 5 μg/ml leupeptin) and centrifuged at 13,000 rpm at 4°C for 3 min. The supernatant was used as total protein lysate and protein concentrations were determined according to the Micro-BCA assay kit (Pierce, Rockford, IL). Protein lysates were prepared in boiling sodium dodecyl sulphate (SDS) sample buffer and equal amounts of total protein were run on a 10% or 8% SDS-polyacrylamide gel. The proteins were then transferred to polyvinylidene difluoride (PVDF) membranes, blocked for 2 h at RT in 5% dry skimmed milk in TBS1x 0.1% tween20 before incubation overnight at 4°C with the primary antibody.

The following primary antibodies were used:

- mouse anti-β-Tubulin III (1:10000, Covance, Berkeley, California, US)
- mouse anti-growth associated protein 43 (GAP43) (1:2000, Millipore, Watford, UK)
- mouse anti-synaptophysin (SYP) (1:2000, Abcam, UK)
- mouse anti-synaptotagmin 1 (SYT) (1:1000, Synaptic Systems, Göttingen, Germany)
- rabbit anti-postsynaptic density protein 95 (PSD95) (1:500, Abcam, UK)
- rabbit anti-CB1R (1:500, Cayman Chemical)
- rabbit anti-CB2R (1:500, Cayman Chemical)
- rabbit anti-MAGL (1:500, Cayman Chemical)
- rabbit anti-DAGLα (1:1000, Abcam, UK)
- rabbit anti-DAGLβ (1:1000, Abcam, UK)
- rabbit anti-FAAH (1:500, Cayman Chemical)
- rabbit anti-NAPE-PLD (1:500, Abcam, UK)

Bound antibodies were detected with horseradish peroxidase (HRP) conjugated secondary anti-rabbit or anti-mouse antibody (1:5000-15000; Chemicon International, Temecula, CA) for 1 hour at room temperature and visualized using Clarity Western ECL Substrate (BIO-

RAD). For detection of beta-actin, the blot was stripped with Restore Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL) and re-blotted with anti-beta-Actin antibody anti-beta-actin monoclonal antibody (1:15000-60000; Sigma Aldrich, Italy) overnight at 4°C and visualized as described. For densitometry, images were digitally scanned and optical density of the bands was quantified using Image Pro Plus 7.0 software (MediaCybernetics, USA) and normalized to control. To allow comparison between different films, the density of the bands was expressed as arbitrary units.

Behavioral Analysis

Classic and Spatial Versions of the Novel Object Recognition Test

In order to determine whether lifelong administration of isocaloric diets enriched or deficient in omega-3 PUFAs from gestation could have long-term consequences on cognitive functions we performed the NOR test in the adult offspring (PND 75). The experimental apparatus used for the object recognition test was an open-field box (43 x 43 x 32 cm) made of Plexiglas, placed in a dimly illuminated room. Animals performed each test individually. The experiment was performed and analyzed as previously described (Realini et al., 2011, Zamberletti et al., 2012). Briefly, each animal was placed in the arena and allowed to explore two identical previously unseen objects for 5 minutes (familiarization phase). After an inter-trial interval of 3 min one of the two familiar objects was replaced by a novel, previously unseen object and rats were returned to the arena for the 5-minute test phase. In the spatial variant of the test, both novel and familiar objects were placed in different positions compared to the familiarization phase that is a spatial cue was added in the test. During the test phase the time spent exploring the familiar object (Ef) and the new object (En) was videotaped and recorded separately by two observers blind to the treatment groups and the discrimination index was calculated as follows : [(En-Ef)/(En+Ef)] x 100.

Statistical analyses

Behavioral and biochemical results were analyzed by one-way ANOVA, followed up by Bonferroni's post hoc test. All data were expressed as mean ± S.E.M or as mean ± S.D. The level of statistical significance was set at $p<0.05$.

Results

Effect of diets on maternal body weight and fetal mortality

Maternal body weights were measured every 2-3 days throughout the course of the study. We found no significant changes of mother body weight during treatment with omega-3 diet (green). In contrast, omega-6 high-fat diet (red) significantly increased maternal body weight already after 5 days of treatment (Fig. 10-A). These diets did not induce embryonic mortality. In fact, no changes were observed in the number and weight of embryos at E18 in the treated group (Fig. 10-B and C). The ratio of female/male offspring did not change either (Fig. 10-D).

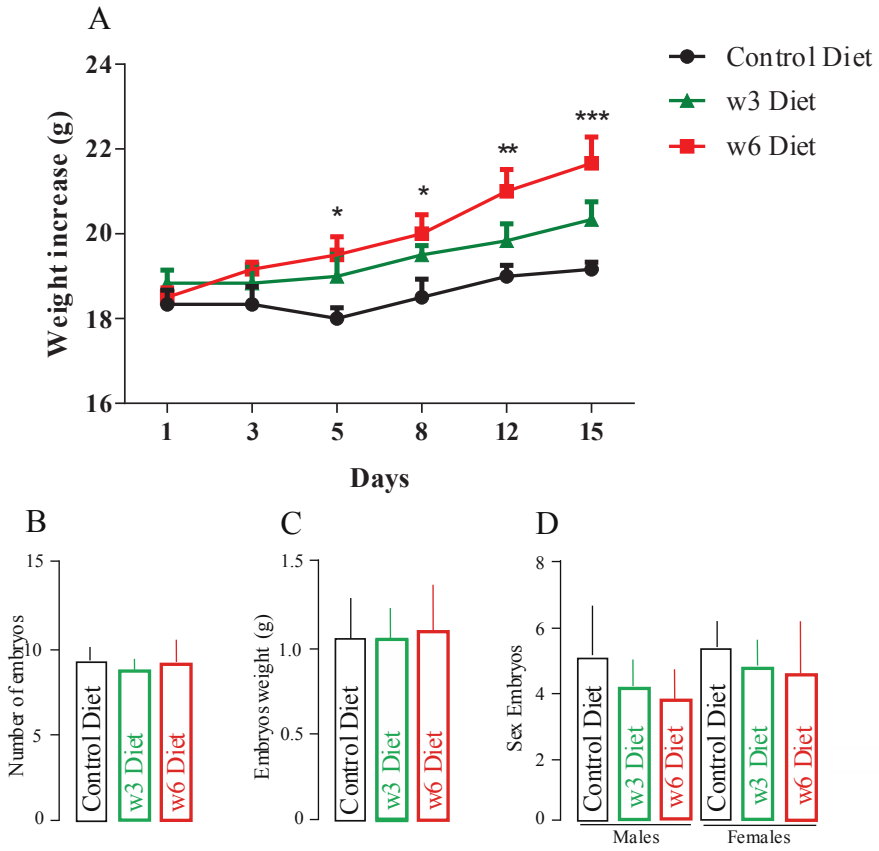


Fig. 10. Effect of short-term diet on maternal body weight (A), number (B) and weight of embryos (C) and on their sex ratio (D). Animals were fed with control diet or omega-3 and omega-6 PUFAs enriched diets for 15 days before mating and throughout gestation. Each value represents the mean ± S.D. of 5 mice per group Statistical analysis was performed using One-way ANOVA followed by Bonferroni's post hoc test.

Characterization of the endocannabinoid system in short-term protocol

2-AG and AEA are the principal ligands for CB1R (Pertwee, 2008) and 2-AG signaling has recently emerged as a molecular determinant of neuronal migration and synapse formation during development (Keimpema et al., 2010). Also the levels of AEA are tightly regulated during embryo development: low concentrations are present in the brain at midgestation, whereas its levels gradually increase throughout the perinatal period. NAPE-PLD and FAAH are the enzymes mainly responsible for AEA synthesis and degradation, while DAGLα/β and MAGL are the enzymes mainly responsible for 2-AG synthesis and degradation respectively.
In order to understand how eCB levels might fluctuate during pregnancy and if hypercaloric diet might affect these lipids, their levels in cerebral tissue (cortex/hippocampus), plasma and adipose tissue of mothers and in cerebral tissue (cortex/hippocampus) and plasma of E18 embryos were measured.

Endocannabinoids levels

In maternal tissue, 2-AG levels were found higher than AEA (Table 2). Moreover, after short-term protocol, both lipids were found higher in plasma tissue respect to cerebral and adipose tissue of control pregnant mice. Interestingly, no changes were observed in AEA, 2-AG levels in cerebral tissue, plasma and adipose tissue of mothers fed with STD protocol (Fig. 11). We also tested for PEA and OEA, AEA-like mediators. The results showed that administration of either hypercaloric diet protocol induced a similar distribution and effect of AEA in pregnant mice.
In embryonic tissues, AEA were not detectable in either plasma or in cerebral tissue because of the very low concentration of eCBs. The same problem was found for 2-AG levels in plasma (Table 2). However, no significant alteration was found in cortical tissue after omega-3 and omega-6 diet administration in either male or female embryos (Fig. 12).

		Mothers			Female embryos			Male embryos		
	Tissue	**control**	**w3 diet**	**w6 diet**	**control**	**w3 diet**	**w6 diet**	**control**	**w3 diet**	**w6 diet**
2-AG pmol/mg ± S.D.	plasma	143,72 ± 30,8	139,05 ± 58,4	207,65 ± 31,09	nd	nd	nd	nd	nd	nd
	Ctx/hippo	16,81 ± 3,5	19,93 ± 4,5	18,05 ± 6,99	16,88 ± 2,57	16,92 ± 3,84	20,6 ± 13,9	21,06 ± 3,14	13,50 ± 4,33	20,32 ± 8,5
	AT	4,69 ± 2,8	3,17 ± 0,89	4,29 ± 2,57						
AEA pmol/mg ± S.D.	plasma	1,35 ± 0,39	nd	1,29 ± 0,1	nd	nd	nd	nd	nd	nd
	Ctx/hippo	0,03 ± 0,02	0,03 ± 0,01	0,04 ± 0,01	nd	nd	nd	nd	nd	nd
	AT	0,01 ± 0,001	nd	0,01 ± 0,001						
PEA pmol/mg ± S.D.	plasma	14,88 ± 1,19	13,38 ± 4,2	16,47 ± 3,7	4,78 ± 0,87	5,79 ± 0,49	4,48 ± 0,13	5,18 ± 0,94	5,51 ± 0,40	4,59 ± 0,41
	Ctx/hippo	0,1 ± 0,01	0,10 ± 0,04	0,10 ± 0,01	0,09 ± 0,03	0,08 ± 0,04	0,09 ± 0,04	0,06 ± 0,02	0,07 ± 0,01	0,07 ± 0,02
	AT	0,24 ± 0,08	0,28 ± 0,12	0,23 ± 0,17						
OEA pmol/mg ± S.D.	plasma	13,16 ± 4,2	12,82 ± 2,8	13,42 ± 4,14	4,29 ± 0,34	3,67 ± 0,31	3,57 ± 0,07	4,67 ± 0,97	4,45 ± 0,35	3,77
	Ctx/hippo	0,19 ± 0,03	0,17 ± 0,06	0,19 ± 0,04	0,06 ± 0,01	0,06 ± 0,03	0,06 ± 0,01	0,04 ± 0,01	0,06 ± 0,02	0,05 ± 0,01
	AT	0,34 ± 0,06	0,29 ± 0,1	0,23 ± 0,1						

Table 2. Average of endocannabinoid (AEA, 2-AG) and AEA-like mediator (PEA, OEA) levels in cerebral tissue (cortex/hippocampus), plasma and adipose tissue (AT) of mothers and E18 male and female embryos after short-term diet protocol. Each value represents the mean ± S.D. of 3 mice per group. Statistical analysis was performed using One-way ANOVA followed by Bonferroni's post hoc test; nd = not detected.

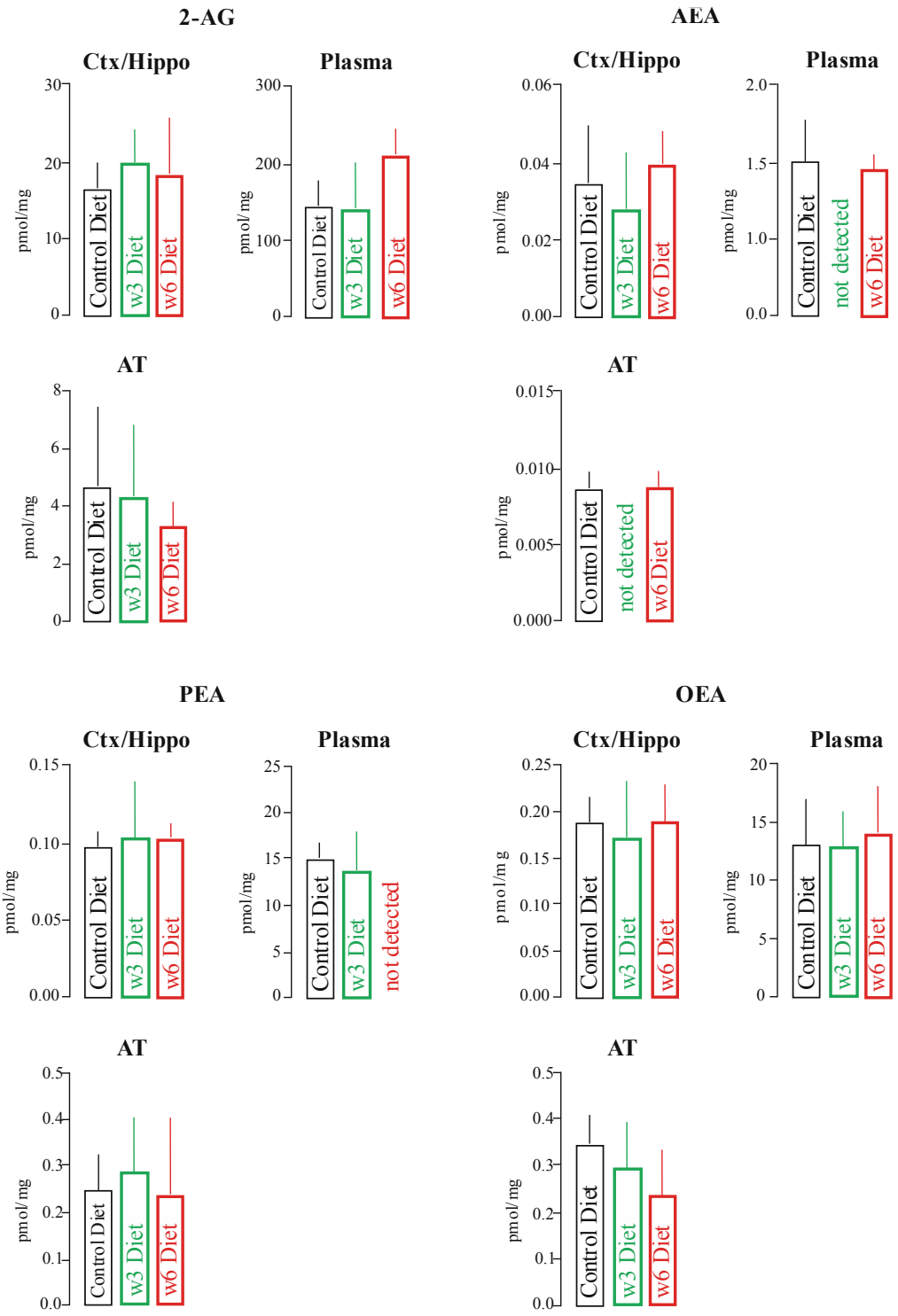


Fig. 11. Endocannabinoids (AEA, 2-AG) and AEA-like mediators (PEA, OEA) levels in maternal cerebral tissue (cortex/hippocampus), plasma and adipose tissue (AT) after short-term diet protocol. Each value represents the mean ± S.D. of 4 mice per group. Statistical analysis was performed using One-way ANOVA followed by Bonferroni's post hoc test.

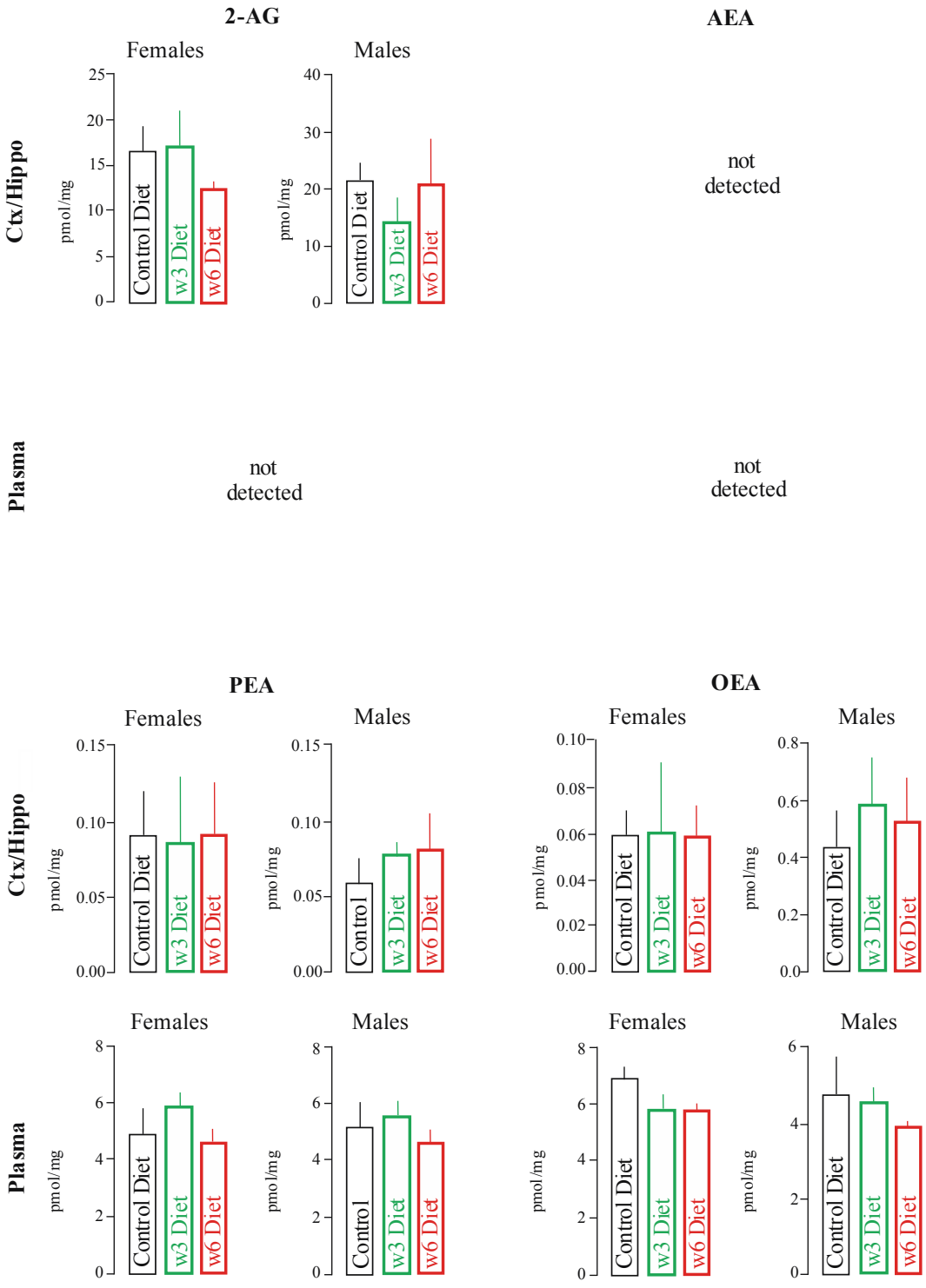


Fig. 12. Endocannabinoid (AEA, 2-AG) and AEA-like mediators (PEA, OEA) levels in cerebral tissue (cortex/hippocampus), plasma and adipose tissue (AT) of E18 female and male embryos after short-term diet protocol. Each value represents the mean ± S.D. of 3 mice per group. Statistical analysis was performed using One-way ANOVA followed by Bonferroni's post hoc test;

Molecular architecture of endocannabinoid system

Next, we demined alterations in mRNA and protein levels of receptor and enzyme components of the eCB system using qPCR and Western blot with total protein normalization.

- ***mRNA levels***

Cortical and hippocampal mRNA levels of E18 embryos were measured by qPCR; gene expression was normalized in independent experiments to the expression level of two functionally different housekeeping genes: GAPDH and TBP.

In brain tissue of E18 *male* and *female* embryos, both mRNA levels of the 2-AG synthesis and degrading enzymes, DAGLα and MAGL respectively, did not change after treatment with omega-3 or omega-6 diets, compared to the control-fed mice (Fig. 13). No significant changes were observed in mRNA levels of the AEA synthesis enzyme NAPE-PLD, in *females* or *male* embryos either, despite a slight increase in the *male* omega-6 group. Interestingly, omega-3 and omega-6 diets induced a significantly increase of the AEA-degrading enzyme mRNA levels (FAAH) in male embryos compared to the control (Fig. 13). This change was gender specific.

We also assessed the expression of CB1Rs and its interacting protein CRIP1a. qPCR measurements revealed that CB1R mRNA level was robustly downregulated both in omega-3 and omega-6 diets in *females*, as compared to controls. There were no differences between the diet groups for CRIP1a in either *female* or *male* embryos. Finally, no alterations were found in mRNA levels of the vanilloid receptor TRPV1 either.

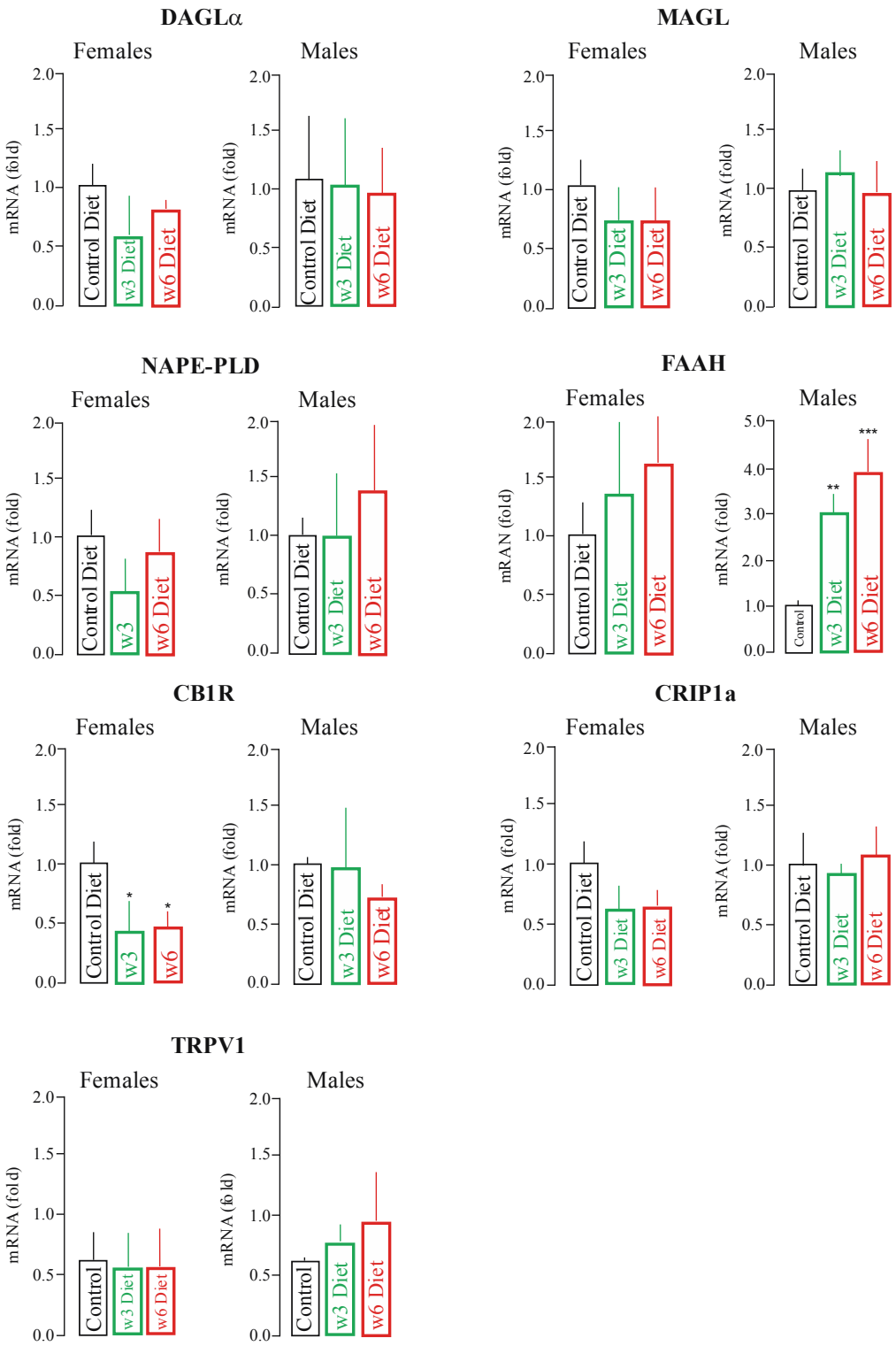


Fig. 13. mRNA expression of endocannabinoids system in brain tissue (cortex/hippocampus) of E18 female and male embryos after short-term diet protocol. mRNA levels were normalized to GAPDH. Each value represents the mean ± S.D. of 3 mice per group. Statistical analysis was performed using One-way ANOVA followed by Bonferroni's post hoc test; * $P < 0.05$ vs control diet; ** $P < 0.01$ vs control diet; *** $P < 0.001$ vs control diet.

- ***Protein expression***

Cortical and hippocampal protein levels of E18 embryos were measured by quantitative Western blotting with total protein normalization (Fig. 14).

In brain tissue of E18 *male* and *female* embryos, protein levels of DAGLα, MAGL and NAPE-PLD did not change after treatment with omega-3 or omega-6 diets compared to the control-fed mice (Fig. 15). FAAH was not detectable in cerebral tissue because of problems with the antibody.

Quantitative Western blot measurements revealed that CB1R protein levels were robustly reduced by about 58% and 66% after both omega-3 and omega-6 diets administration in *female* groups compared to the control. Despite the absence of mRNA alterations, similar alterations in CB1R protein levels were also found in *male* embryos. Indeed, we found a significant increase of CB1R of about 65% and a decrease of 71% respect to the control group.

No changes in CRIP1a and TRPV1 protein levels were observed in any of the experimental groups.

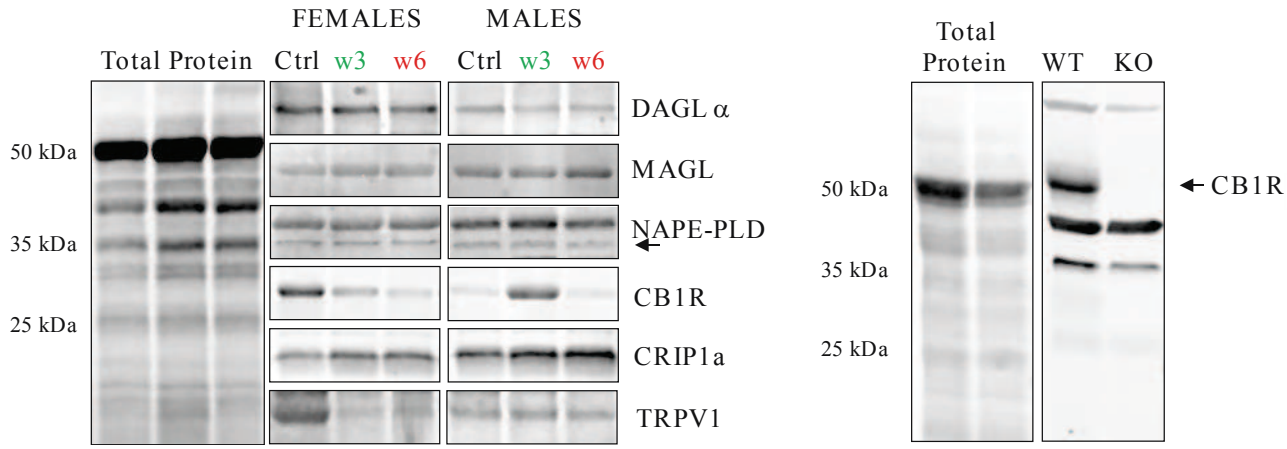


Fig. 14. Immunoblotting of DAGLα, MAGL, NAPE-PLD, CB1R, CRIP1a and TRPV1. Protein expression in cerebral tissue (cortex/hippocampus) of E18 female and male embryos after short-term diet protocol. Cerebellar membranes from adult CB1R knockout (KO) and wild-type mice were used to control the specificity of the guinea pig anti-CB1R antibody

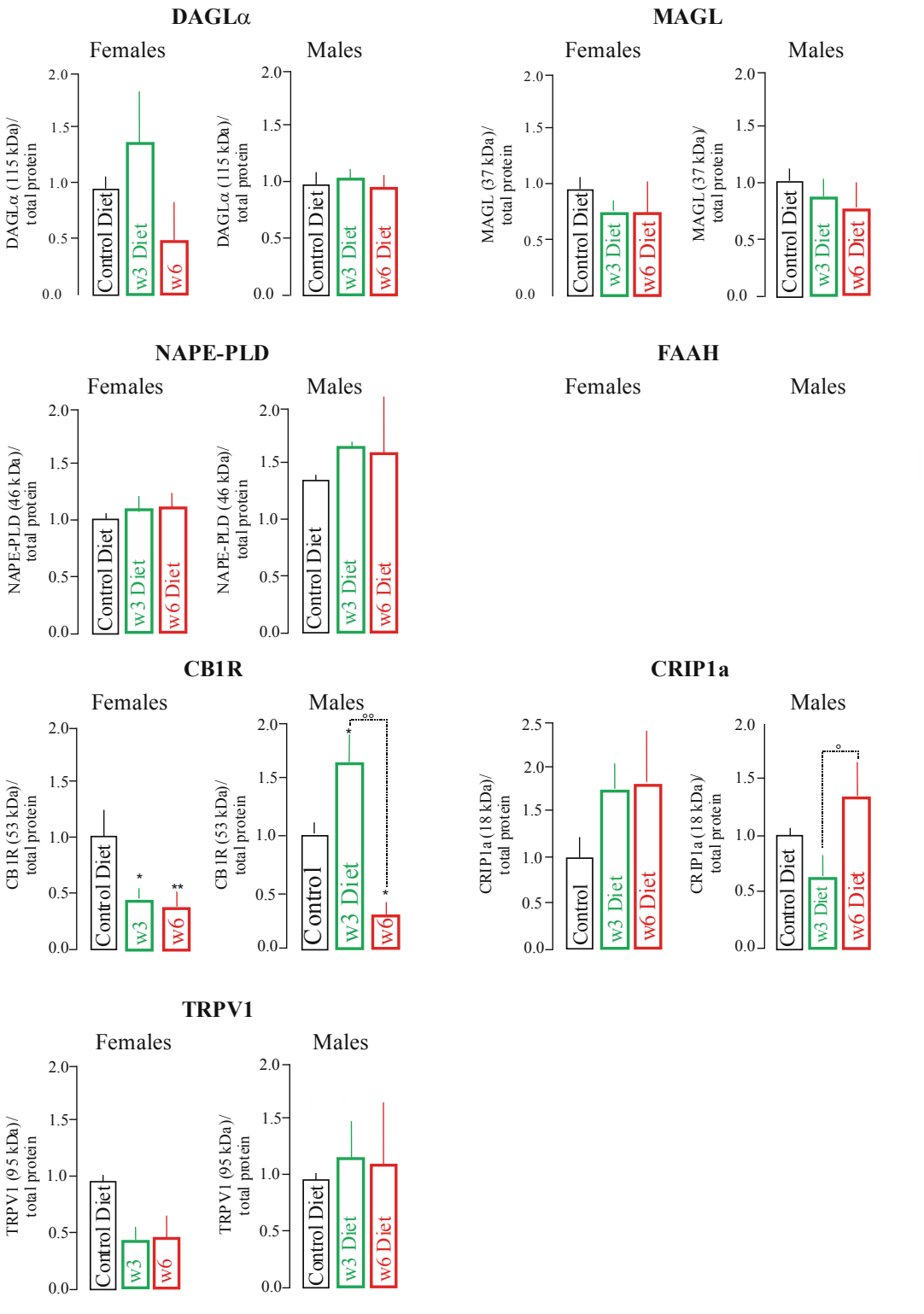


Fig. 15. Protein expression of endocannabinoid system in brain tissue (cortex/hippocampus) of E18 female and male embryos after short-term diet protocol. Each value represents the mean ± S.D. of 3 mice per group. Statistical analysis was performed using One-way ANOVA followed by Bonferroni's post hoc test; * $P < 0.05$ vs control diet; ** $P < 0.01$ vs control diet.

Effect of diets on maternal body weight and fetal mortality

Maternal body weights were measured every 2-3 days throughout the course of the study. We found no significant alteration on maternal body weight during treatment with omega-3 diet (green). In contrast, after six weeks of omega-6 high-fat diet administration we observed a significant increase in maternal body weight (red) with respect to controls diet (Fig. 16-A). Moreover, we found significant changes in number of E18 embryos (Fig. 16-B) and in females/males embryos ratio (Fig. 16-D). These changes are related to the fact that female mice have more problems to get pregnant after long-term protocol with respect to control-fed mice. No changes were observed in weight of E18 embryos (Fig. 16-C). Finally, we also found differences in female/male embryos ratio (Fig. 16-D)

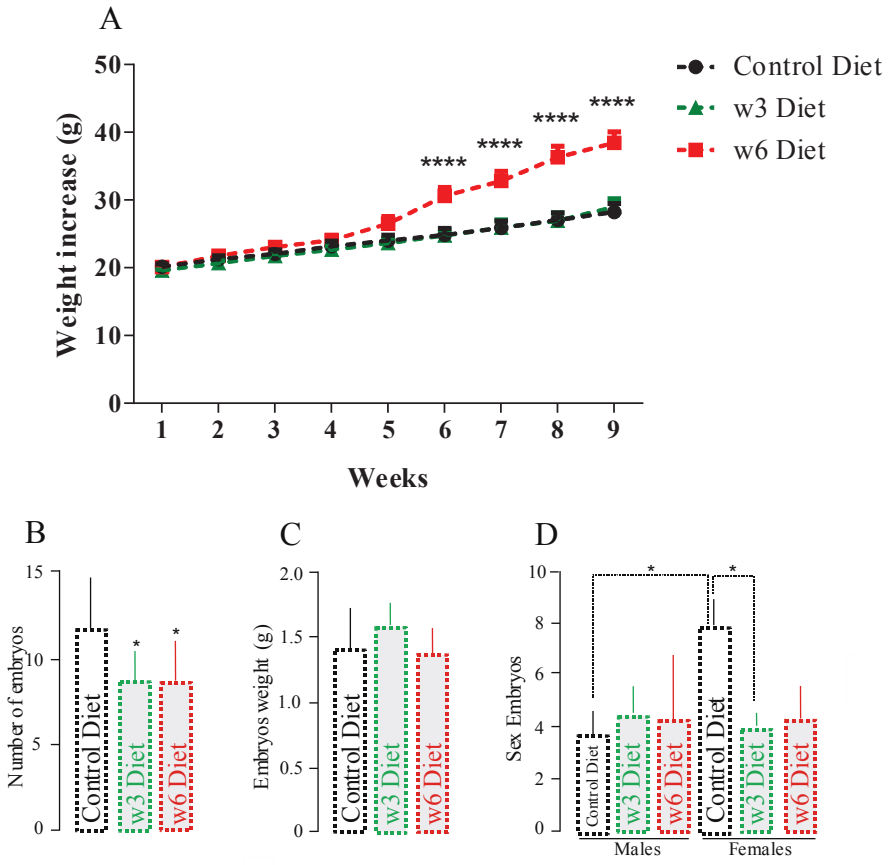


Fig. 16. Effect of long-term diet on maternal body weight (A), number (B) and weight of embryos (C) and on their sex ratio (D). Animals were fed with control diet or omega-3 and omega-6 PUFAs enriched diets for 9 weeks before mating and throughout gestation each value represents the mean ± S.D. of 5 mice per group. Statistical analysis was performed using One-way ANOVA followed by Bonferroni's post hoc test; *P<0.05 vs control diet.

Characterization of the endocannabinoid system in long-term protocol

We checked for alteration in the eCB system preforming the same experimental analysis as for the STD protocol.

Endocannabinoid levels

In maternal tissue, 2-AG levels were higher than those of AEA (Table 3), and their levels were higher in the plasma compared to cerebral and adipose tissue of control pregnant mice. The same distribution was found also for PEA and OEA levels. No changes were observed in AEA, PEA and OEA levels in cerebral tissue, plasma and adipose tissue of mothers fed with long-term protocol. 2-AG levels were found higher in maternal plasma omega-3 diet group compared to the control (Fig. 17).

In embryos, eCBs are expressed at higher levels in the plasma with respect to cerebral tissue, both in *males* and *females* (Table 3). Moreover, plasma PEA levels were higher compared to the other lipids and its concentration was similar in *male* and *female* embryos.

In cerebral tissue of *female* and *male* embryos, omega-6 high-fat diet induced slight but not significant increase of 2-AG levels (Fig. 18), while no differences were found in plasma.

AEA was strongly decreased by about 98% and 85% after both omega-3 and omega-6 diet administration in *female* cerebral tissue. By contrast, in *male* embryos high-fat diets did not induce significant alterations in AEA levels with respect to the control groups (Fig. 18), despite a decrease observed with omega-6 diet.

Finally, we found no significant alterations in PEA levels both in cortex/hippocampus and plasma tissue. In contrast, OEA levels were different in cerebral tissue of *female* embryos in omega-3 groups but not in *male* embryos. Finally, we found no changes of PEA and OEA levels in plasma (Fig. 18).

		Mothers			Female embryos			Male embryos		
	Tissue	control	w3 diet	w6 diet	control	w3 diet	w6 diet	control	w3 diet	w6 diet
2-AG pmol/mg ± S.D.	plasma	65,9 ± 7,9	184,1 ± 21,8	97,4 ± 27,1	10,4 ± 3,6	7,3 ± 2,5	4,0 ± 1,1	4,3 ± 0,6	7,1 ± 2,7	10,4 ± 2,6
	Ctx/hippo	2,8 ± 0,2	2,4 ± 0,3	3,0 ± 0,3	0,9 ± 0,2	0,8 ± 4,33	1,4 ± 0,01	0,9 ± 0,2	0,6 ± 0,1	1,3 ± 0,2
	AT	0,50 ± 0,2	0,40 ± 0,1	0,30 ± 0,1						
AEA pmol/mg ± S.D.	plasma	9,30 ± 0,8	5,01 ± 2,6	9,93 ± 0,2	2,29 ± 0,9	1,62 ± 0,6	2,38 ± 0,3	2,87 ±1,0	0,85 ± 0,2	2,67 ± 0,5
	Ctx/hippo	0,05 ± 0,07	0,06 ± 0,03	0,05 ± 0,03	0,33 ± 0,33	0,01 ± 0,01	0,03 ± 0,04	0,10 ± 0,09	0,13 ± 0,12	0,02 ± 0,03
	AT	0,04 ± 0,01	0,02 ± 0,01	0,03 ± 0,001						
PEA pmol/mg ± S.D.	plasma	98,6 ± 8,6	100,3 ± 0,5	120,1 ± 5,3	40,9 ± 0,8	39,3 ± 2,5	25,2 ± 6,7	44,5 ± 2,8	43,8 ± 1,9	27,9 ± 4,0
	Ctx/hippo	0,40 ± 0,01	0,4 ± 0,1	0,4 ± 0,4	1,2 ± 0,7	0,6 ± 0,01	0,6 ± 0,1	0,5 ± 0,01	0,7 ± 0,01	0,4 ± 0,01
	AT	0,50 ± 0,1	0,70 ± 0,1	0,40 ± 0,1						
OEA pmol/mg ± S.D.	plasma	103,3 ± 11,1	118,6 ± 23,1	109,9 ± 7,7	24,3 ± 3,4	64,1 ± 31,4	13,9 ± 1,0	15,2 ± 5,6	38,6 ± 4,1	17,8 ± 1,4
	Ctx/hippo	0,4 ± 0,1	0,7 ± 0,01	0,5 ± 0,1	0,3 ± 0,1	0,7 ± 0,01	0,3 ± 0,1	0,2 ± 0,1	0,5 ± 0,2	0,3 ± 0,2
	AT	0,95 ± 0,1	0,88 ± 0,03	0,60 ± 0,04						

Table 3. Average of Endocannabinoids (AEA, 2-AG) and AEA-like mediators (PEA, OEA) levels in cerebral tissue (cortex/hippocampus), plasma and adipose tissue (AT) of mothers and E18 male and female embryos after long-term diet protocol. Each value represents the mean ± S.D. of 5 mice per group. nd = not detected; Statistical analysis was performed using One-way ANOVA followed by Bonferroni's post hoc test; Significant data are expressed in red.

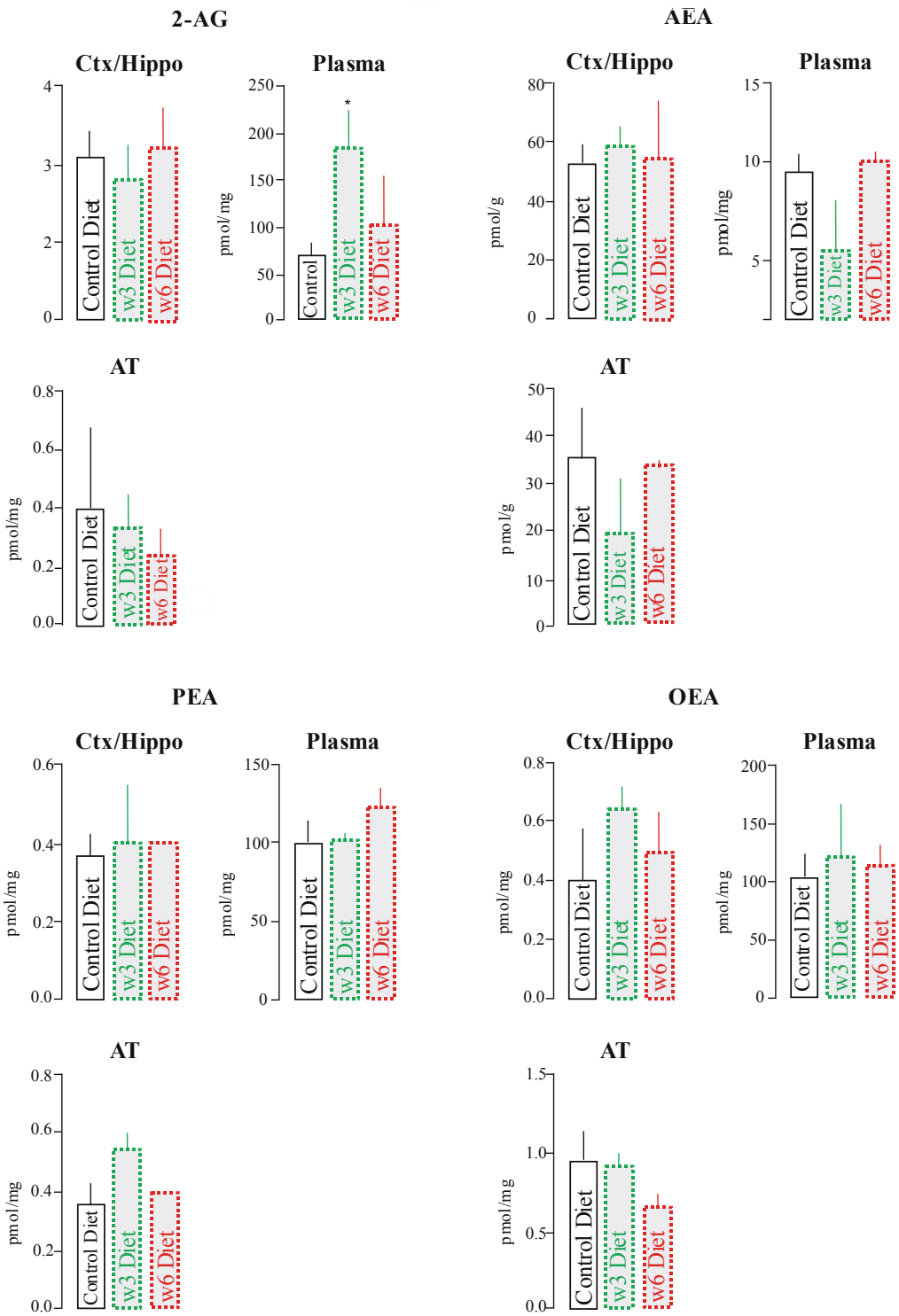


Fig. 17. Endocannabinoids (AEA, 2-AG) and AEA-like mediators (PEA, OEA) levels in mother cerebral tissue (cortex/hippocampus), plasma and adipose tissue (AT) after long-term diet protocol Each value represents the mean ± S.D. of 5 mice per group. Statistical analysis was performed using One-way ANOVA followed by Bonferroni's post hoc test; *P<0.05 vs control diet.

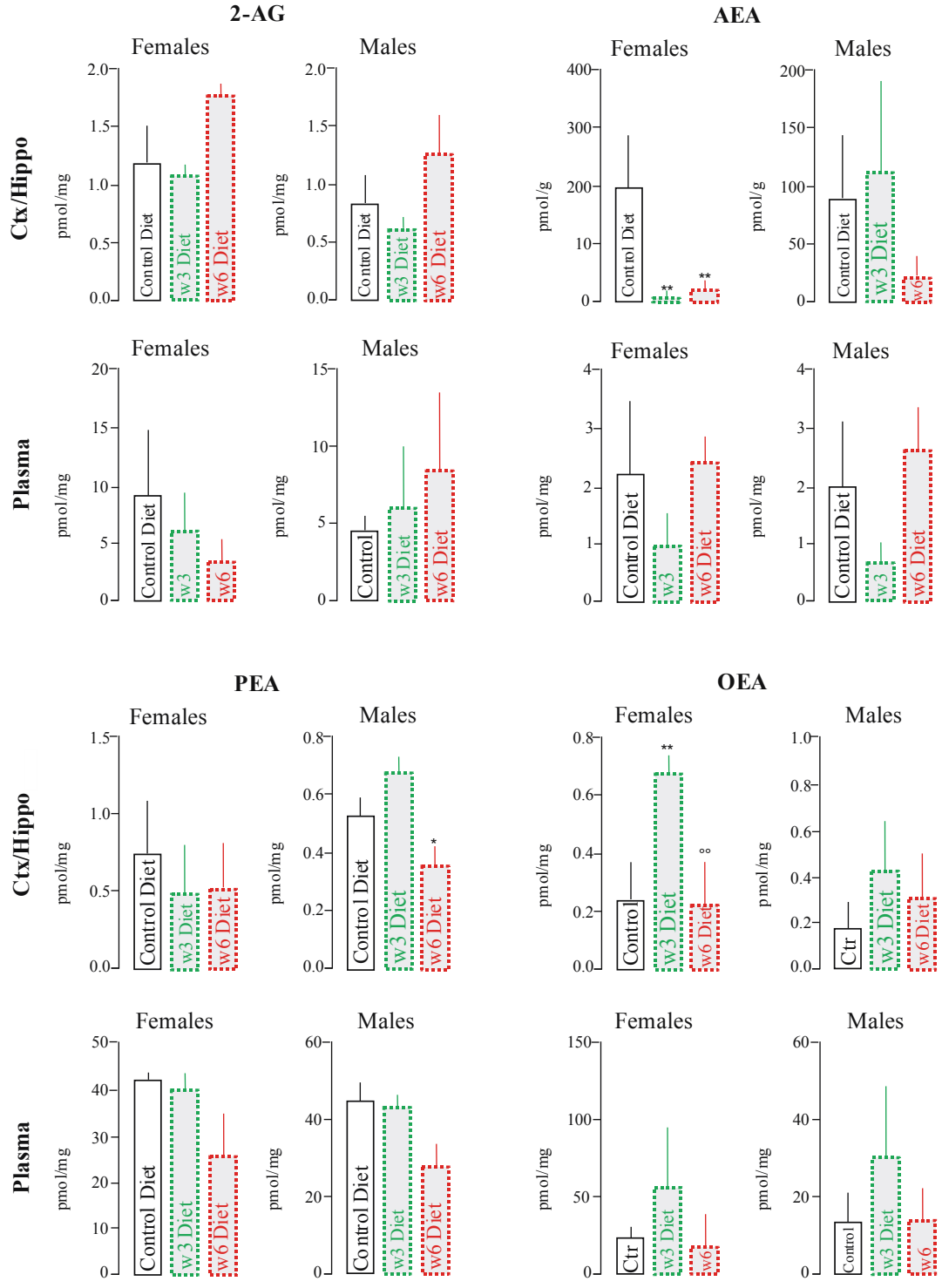


Fig. 18. endocannabinoids (AEA, 2-AG) and AEA-like mediators (PEA, OEA) levels in cerebral tissue (cortex/hippocampus), plasma and adipose tissue (AT) of E18 embryos after long-term diet protocol. Each value represents the mean ± S.D. of 3 mice per group. Statistical analysis was performed using One-way ANOVA followed by Bonferroni's post hoc test; **P<0.01; *P<0.05 vs control diet; °°P<0.01; °P<0.05 vs omega-3 diet.

Molecular architecture of endocannabinoid system

- ***mRNA levels***

In cerebral tissue of E18 *male* and *female* embryos, both DAGLα and MAGL mRNA levels did not change in mice fed with omega-3 or omega-6 diets compared to control-fed mice (Fig. 19). No significant changes were also observed in mRNA levels of the AEA synthesis and degradative enzymes NAPE-PLD and FAAH, both in *female* and in *male* embryos.

We also checked for the presence of alterations in CB1R and CRIP1a. Differently from what has been observed in the short-term protocol, quantitative real-time PCR measurements revealed no changes in CB1R and CRIP1a mRNA level both in omega-3 and omega-6 long-term protocol administration (Fig. 19). Finally, both omega-3 and omega-6 diets significantly decreased TRPV1 mRNA levels in *male* embryos but not in *females* (Fig. 19).

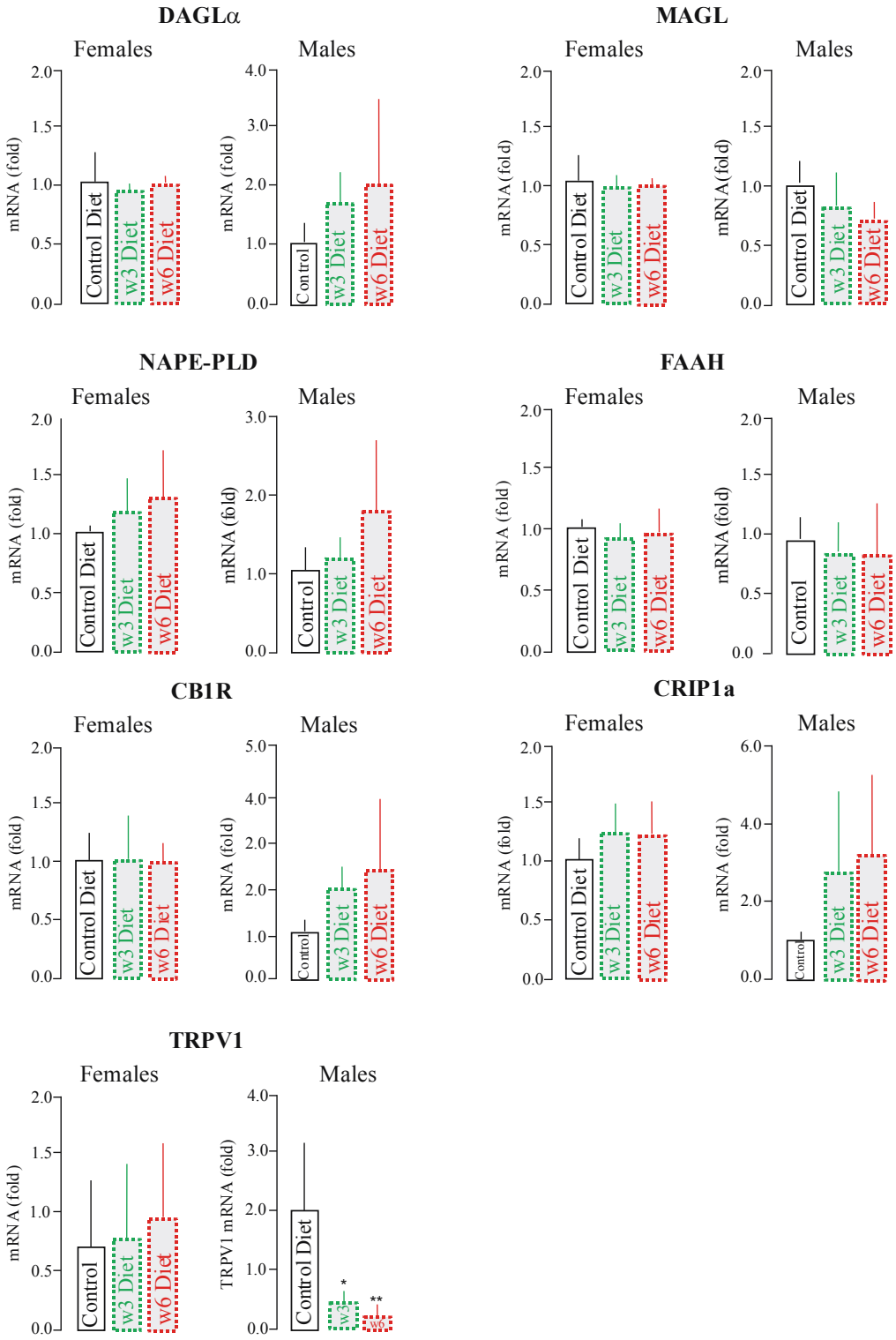


Fig. 19. mRNA expression of endocannabinoids system in brain tissue (cortex/hippocampus) of E18 female and male embryos after long-term diet protocol. mRNA levels were normalized to GAPDH. Each value represents the mean ± S.D. of 3 mice per group. Statistical analysis was performed using One-way ANOVA followed by Bonferroni's post hoc test; * $P < 0.05$; ** $P < 0.01$ vs control diet.

- ***Protein expression***

Figure 20 shows the protein levels of E18 embryos as measured by quantitative Western blotting.

In brain tissue of E18 *male* and *female* embryos, protein levels of DAGLα and MAGL did not change in mice fed with omega-3 or omega-6 diets compared to control-fed mice (Fig. 21). Similarly, no significant changes were observed in the protein levels of NAPE-PLD.

In *male* embryos, protein levels of FAAH did not change. In *females*, FAAH was not detectable because of problems with the antibody.

Interestingly, quantitative western blot measurements revealed that omega-6 diet decreased CB1R protein levels only in in *male* embryos and this was associated to a significant decrease also in CRIP1a protein levels. In contrast, No changes were observed in *female* embryos both in CB1R and CRIP1a protein levels after diet administration.

Finally, we found no significant alterations in TRPV1 protein levels both in *female* and *male* embryos after omega-3 and omega-6 long-term protocol.

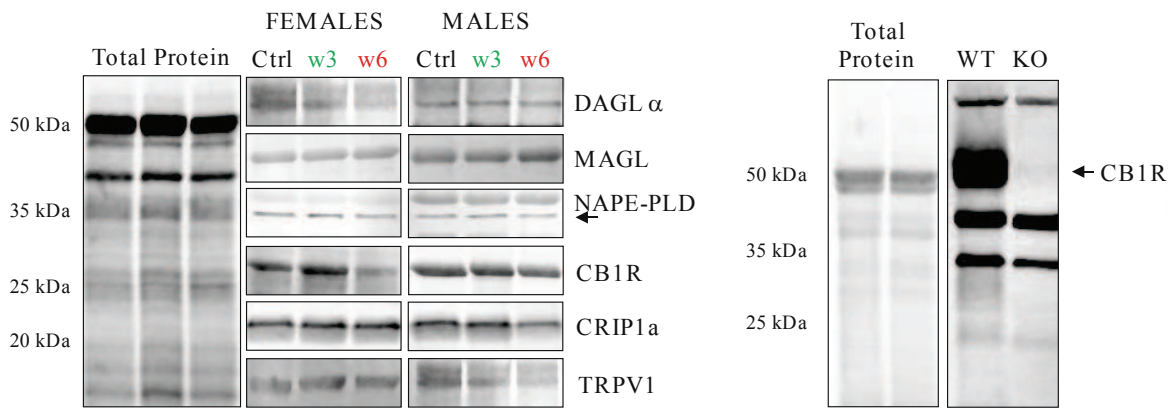


Fig. 20. Immunoblotting of DAGLα, MAGL, NAPE-PLD, CB1R, CRIP1a and TRPV1. Protein expression in brain tissue (cortex/hippocampus) of E18, female and male embryos after long term diet protocol. Cerebellar membranes from adult CB1R knockout (KO) and wild-type mice were used to control the specificity of the guinea pig anti-CB1R antibody

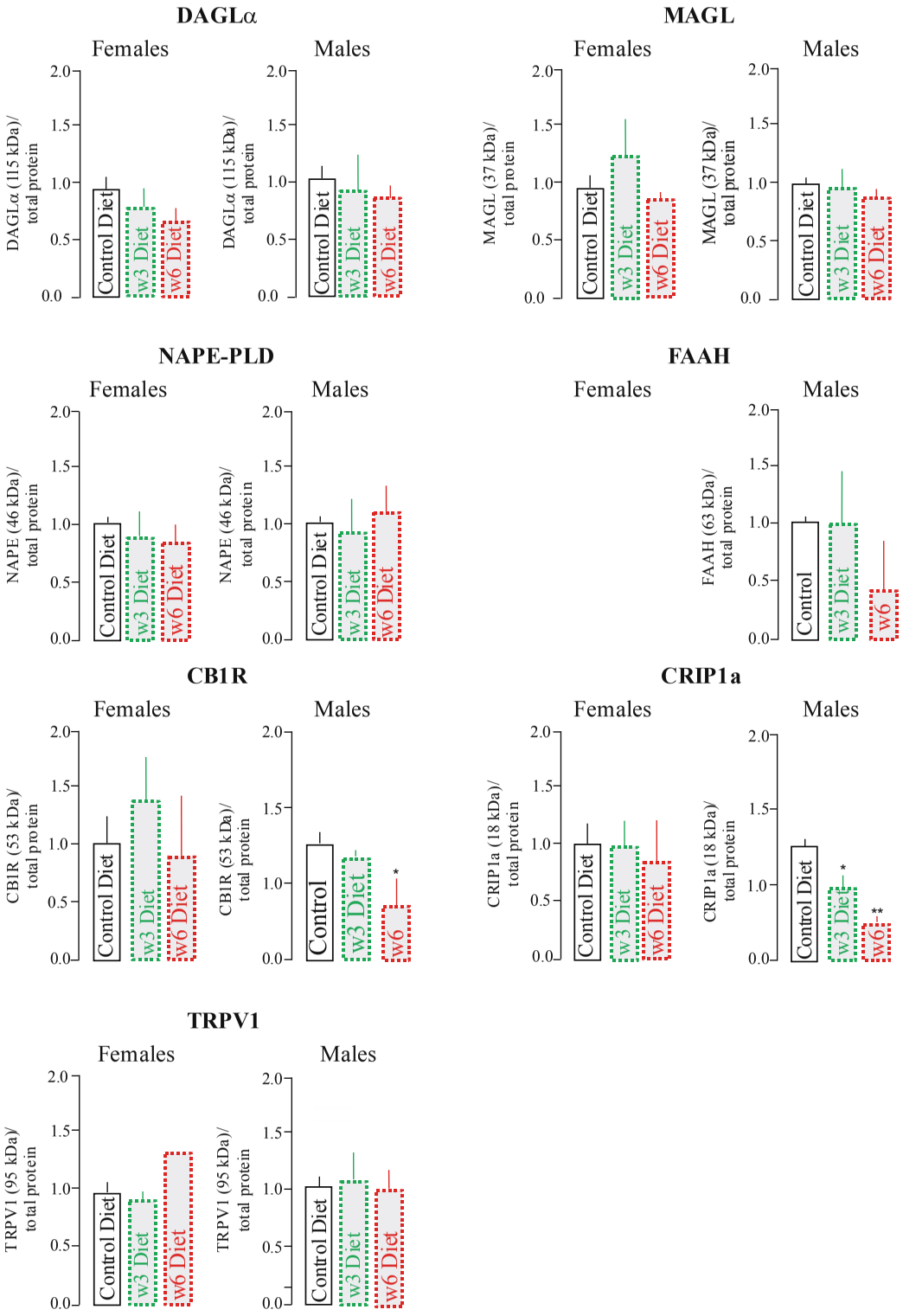


Fig. 21. Protein expression of endocannabinoid system in brain tissue (cortex/hippocampus) of E18 female and male embryos after long term diet protocol. Each value represents the mean ± S.D. of 3 mice per group. Statistical analysis was performed using One-way ANOVA followed by Bonferroni's post hoc test; * $P < 0.05$; ** $P < 0.01$ vs control diet.

Morphometric analysis

To evaluate the effects of PUFAs on the neuronal morphology, we performed immunoicytochemistry using specific neuronal marker β-tubulin III.

Our results suggest that both omega-3 (EPA and DHA) and omega-6 (LA) PUFA supplementation can promote neurite extension (Fig. 22-A). Interestingly, axonal length was found strongly increased by about 130% after treatment with all ligands (Fig. 22-B). Furthermore, our results demonstrated that only EPA increased the number of both primary (Fig. 22-C) and secondary (Fig. 22-D) branches.

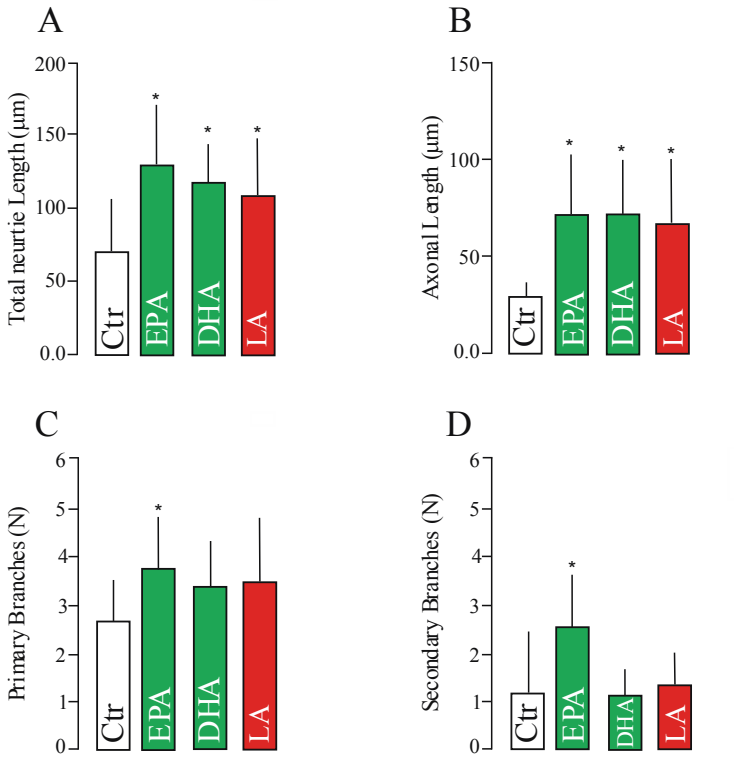


Fig. 22. Effect of EPA and DHA omega-3 PUFAs and LA omag-6 PUFA on the neurite growth (A), axonal length (B) and the number of primary (C) and secondary branches (D) of cortical/hippocampal neurons. Each value represents the mean ± S.D. Statistical analysis was performed using One-way ANOVA followed by Bonferroni's post hoc test; *P<0.05 vs control group.

Effect of diets on maternal body weight and number of embryos

To evaluate the consequences of maternal nutrition on the brains of their offspring, we fed pregnant Sprague–Dawley rats with three diets different in omega-3 levels but not in omega-6 (30%) throughout gestation and lactation. To exclude any toxic effect of diets we monitored maternal body weight. Furthermore, to understand if these different diets might affect embryo mortality we checked the number of embryos at gestation day 18 (GD18) for each group. We found no differences on mother body weight and in the number of fetuses at birth (Fig. 23-B).

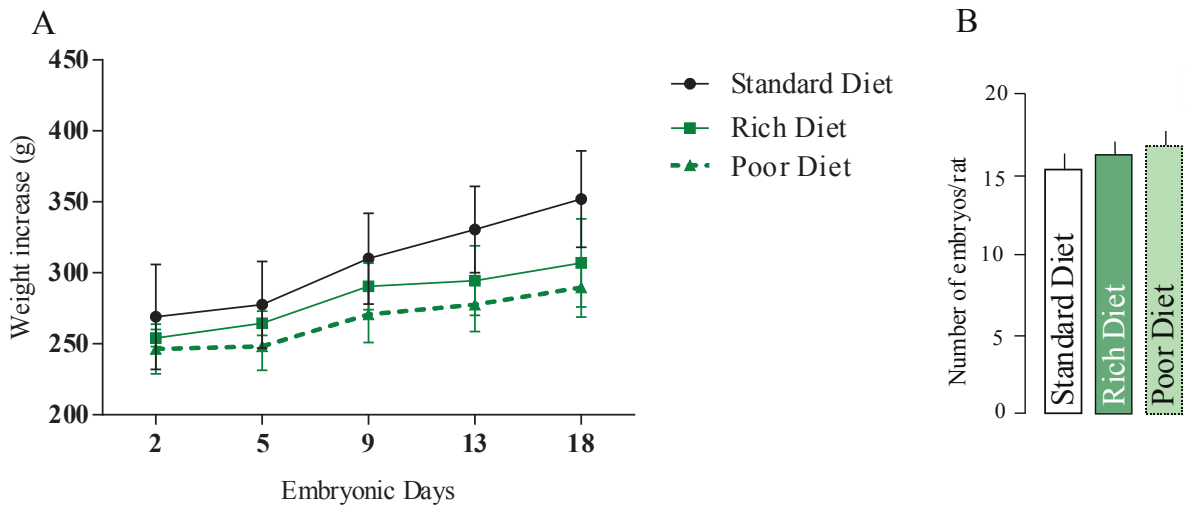


Fig. 23. Effect of omega-3 PUFAs deficiency (poor) or enrichment (rich) diets on maternal body weight (A) and on the number of embryos (B). Animals were fed with standard, rich or poor diets for 18 days. Each value represents the mean ± S.E.M of 5 rats per group. Statistical analysis was performed using One-way ANOVA followed by Bonferroni's post hoc test.

Characterization of the Endocannabinoid System in E18 hippocampal tissue

To assess whether isocaloric diets different in omega-3 PUFA content could modulate the eCB system we focuses our attention on E18 hippocampal tissue obtained from dams fed throughout gestation and we studied the protein levels of the principal components of this system.

In embryonic hippocampus, omega-3 deficiency or enrichment did not alter DAGLα and MAGL protein levels compared to the standard diet (Fig. 24-A and C), whereas DAGLβ protein levels were increased by about 99% after administration of enriched diets (Fig. 24-B). Moreover, we found alteration in both AEA metabolic enzymes expression. NAPE-PLD was reduced by about 47% after administration of enriched diets (Fig. 24-D), whereas, FAAH protein levels were reduced in both experimental groups by about 49% and 35% respectively (Fig. 24-E).

We also checked for the presence of alterations in CB1R and CB2R. Omega-3 deficiency or enrichment diets did not induce significant alterations in both receptors expression with respect to the standard diet (Fig. 24-F and G).

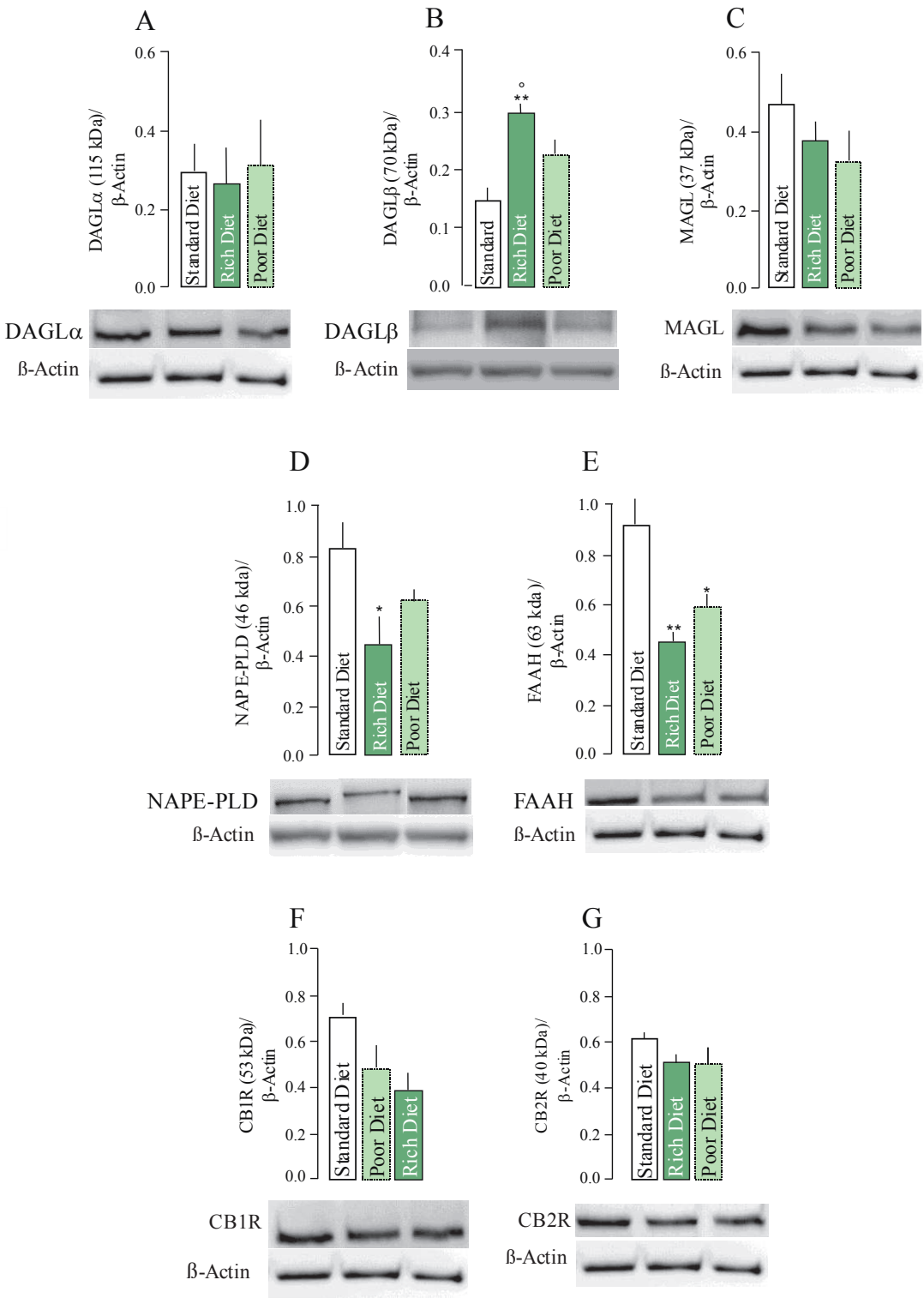


Fig. 24. Effect of omega-3 PUFAs deficiency or enrichment diets on 2-AG metabolic enzymes DAGLα (A), DAGLβ (B) and MAGL (C), on AEA metabolic enzymes NAPE-PLD (D) and FAAH (E) and on cannabinoid receptor 1 (F) and 2 (G) in hippocampus of E18 embryos. Each value represents the mean ± S.E.M of 4 rats per group. Statistical analysis was performed using One-way ANOVA followed by Bonferroni's post hoc test. *P<0.05; **P<0.01 vs standard diet. ° P<0.05 vs poor diet.

Characterization of neuronal markers in E18 hippocampal tissue

No changes were observed in β-tubulin III (Fig. 25-A) whereas, GAP43 expression was reduced by about 60% and 74% after treatment with both enriched and deficient diets compared to control (Dig. 25-B). Interestingly, we showed that expression of pre-synaptic markers is modulated by diets. Indeed, poor diets can reduce by 48% synaptophysin and synaptotagmin protein levels by about 48% and 60% respectively (Fig. 25-C), whereas rich diets reduced synaptotagmin protein levels by about 60% compared to standard diets (Fig. 25-D). In contrast, treatment with isocaloric diets omega-3 PUFAs deficiency or enrichment did not change protein levels of the post-synaptic marker PSD95 (Fig. 25-E).

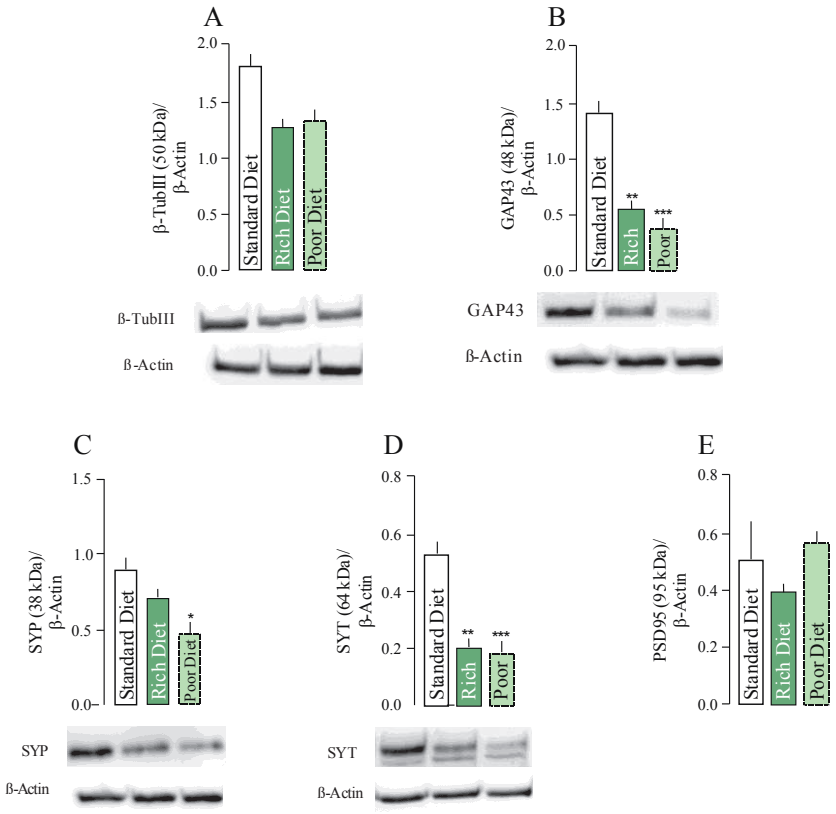


Fig. 25. Effect of omega-3 PUFAs deficiency or enrichment diets on neuronal markers β-tubulin III (A) and GAP43 (B), on pre-synaptic markers synaptophysin (SYP) (C) and synaptotagmin (SYT) (D), and on post-synaptic marker PSD95 (E) in hippocampus of E18 embryos. Each value represents the mean ± S.E.M of 4 rats per group. Statistical analysis was performed using One-way ANOVA followed by Bonferroni's post hoc test. *P<0.05; **P<0.01 vs Standard Diet.

Characterization of synaptic markers in adult hippocampal tissue

To study long-term consequence of dietary intake on neuronal markers, we performed the same biochemical analyses in the adult offspring after lifelong administration of omega-3 enriched and deficient diets. In the adult hippocampus, no significant changes in both pre- and post-synaptic markers were observed after administration of enriched or deficient diets (Fig. 26).

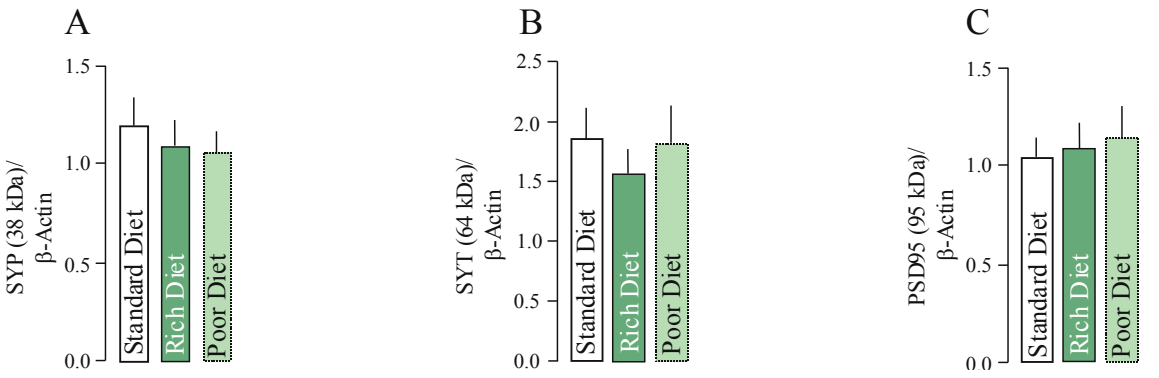


Fig. 26. Effect of omega-3 PUFAs deficiency or enrichment diets on pre-synaptic markers synaptophysin (SYP) (A) and synaptotagmin (SYT) (B), and on post-synaptic marker PSD95 (C) in hippocampus of PND 75 rats. Each value represents the mean ± S.E.M of 4 rats per group. Statistical analysis was performed using One-way ANOVA followed by Bonferroni's post hoc test.

Cognitive impairment in adult rats

Finally, we investigated the possible behavioral consequences of different dietary omega-3 intake in terms of cognitive functions in the adult offspring, as measured through the novel object recognition test

Lifelong administration of both omega-3 deficient and enriched diets resulted in impaired cognitive performance in the classic version of the NOR test in the adult male offspring, as stated by the significant reductions of the discrimination index by about 76% and 60%, respectively, compared to controls fed a standard diet (Fig. 27-A). A similar effect was also observed in the spatial version of the test, the discrimination index being reduced by about 64% and 50% in the two experimental groups (Fig. 27-B).

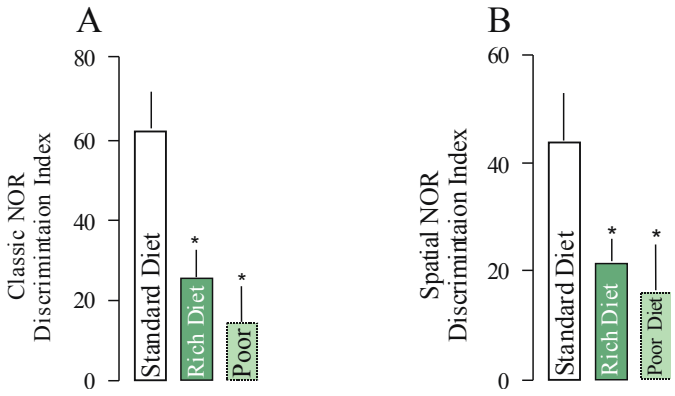


Fig. 27. Effect of omega-3 PUFAs deficiency or enrichment on cognitive functions in the classic (A) and spatial (B) NOR test in the adult offspring. Each value represents the mean ± S.E.M of 8 rats per group. Statistical analysis was performed using One-way ANOVA followed by Bonferroni's post hoc test; *P<0.05 vs Standard Diet.

Discussion

About 50% of dry weight of the adult brain consists of lipids, of which 20-25% is long-chain polyunsaturated fatty acids (PUFAs) (Lauritzen et al., 2001). The major PUFAs, DHA (22:6n-3) and AA (20:4n-6), are formed from the fatty-acid precursor ALA and LA, respectively. It should be noted that the metabolic pathway of the omega-3 fatty acid series shares enzymes with that of the omega-6 pathway. Thus, the two series of PUFAs compete for the same enzymes. As a consequence, high intake of DHA results in a decrease of tissue AA (Innis, 1991, Rioux et al., 2006). PUFA precursors of the omega-3 and omega-6 families are essential nutriments that cannot be generated *de novo* in mammals and must be obtained from the diet (Haggarty, 2002). The fetus and newborn infant are capable to convert ALA into DHA and LA into AA (Uauy et al., 2000, Carnielli et al., 2007). However, the enzymatic systems involved seem unable to supply sufficient PUFAs to meet the high requirements until 16 weeks after term (Lauritzen et al., 2001). Deposition of lipids in the fetus increases exponentially with gestational age, reaching a rate of accretion of around 7 g/day just before term (Boulton et al., 1978). Some of the fatty acid deposited in the fetus will accrue from fetal lipogenesis (Dunlop and Court, 1978) but the great bulk of fetal lipid is derived from the maternal circulation via the placenta and all of the omega-3 and omaga-6 fatty acid structure acquired by the fetus has to cross the placenta. For these reasons, to cope the fatty acid demand, maternal PUFAs in plasma phospholipids increase by 50%. The concentrations of all fatty acids increase, but those of AA and DHA increase relatively less (Al et al., 2000, Hornstra, 2000). The higher concentrations of fatty acids are not brought about by altered dietary behavior, but by an accelerated breakdown of maternal fat depots during the last trimester (Herrera, 2002, Haggarty, 2004) .This suggests that the fetal PUFA supply does not only depend on the PUFA content of the maternal diet during pregnancy but also on PUFAs content of the diet prior to pregnancy, and the maternal diet needs to include an appropriate omega-3/omega-6 PU FAs ratio.

In the United States, about 30% of all pregnancies are carried by obese women that because of changes in diet and lifestyle have led to a dramatically altered ratio of omega-3/omega-6 fatty acid consumption. 1:1-2 is the optimum recommended dietary ratio, but in the modern Western diet, an increase intake of omega-6 PUFAs, and a concomitant decrease in the intake of omega-3 PUFAs, has resulted in an increase ratio of omega-3/omega-6 PUFAs to 1:15-30.

Based on this, we aimed at investigating the consequences of maternal malnutrition on the brain of their offspring; in particular, we modeled animal dietary changing both omega-3 or omega-6 PUFA content and time of administration, and we focused our attention on the endocannabinoid (eCB) system.

Hypercaloric diets

Effect of hypercaloric diets on the eCB system in pregnant dams

In the first part of this project we used hypercaloric diets to mimic the imbalance of essential PUFAs in mice. Families of omega-3 PUFA change membrane phospholipid composition in most organs, including the brain. Feeding long chain omega-3 PUFA, such as EPA and DHA, will increase their concentrations *in vivo*, and to some extent will lower the concentrations of omega-6 PUFA, specifically AA. Thus, remodeling the phospholipid composition of cell membranes and organelles by dietary PUFA is a means to change substrate availability for and the biosynthesis of eCBs (Watkins et al., 2010, Kim et al., 2011, Kim et al., 2014).

The endocannabinoid system consists of cannabinoid receptors, their endogenous lipid ligands and the enzymes for ligand synthesis and degradation. Endocannabinoids are endogenous lipid mediator made from essential omega-6 fatty acids available only from dietary sources (Mechoulam and Parker, 2013). The two best characterized eCBs, 2-AG and AEA, are both metabolic derivate of a single fatty acid precursor, the omega-6 AA in phospholipids (Devane, 1992, Gao, 2010, Tanimura, 2010). Since humans cannot synthesize AA *de novo*, tissue phospholipid concentrations are dependent upon the competition between dietary intake of omega-6 ad omega-3 fats (Lands et al., 1992). Endocannabinoids have emerged as important lipid mediators in various physiological events, including reproduction. Indeed, 2-AG has identified in the uterus and blastocyst and shown to have a similar role to AEA during implantation (Liu et al., 2002, Wang et al., 2003a, Wang et al., 2007, Fride, 2008). It has been reported that 2-AG levels are significant higher than AEA in the uterus (Wang et al., 2007) and in brain (Sugiura et al., 1995, Stella et al., 1997). In accord with this, in our studies, after treatment with hypercaloric diets, 2-AG levels were higher than AEA in pregnant mice. This could be related to the multiple roles of 2-AG in lipid metabolism (Sugiura et al., 2006) or it is a consequence of its role in housekeeping cell functions or activation of signaling pathways (Piomelli, 2003), that finally require higher levels of 2-AG than AEA. Furthermore, after short-term diet protocol, both lipids were higher in plasma relative to cerebral and adipose tissue. While few studies have examined the possible role of 2-AG in pregnancy (Paria et al., 1998, Wang et al., 2007, Fonseca et al., 2010a), high AEA levels are associated with alteration in blastocyst development, oviductal transport and implantation (Maccarrone et al., 2002, Wang et al., 2004, Wang et al., 2006b, Fezza et al., 2008). In humans, low levels of the AEA-degradative enzyme, FAAH, and high levels of AEA have been found in women that eventually spontaneously miscarry (Maccarrone et al., 2002, Taylor et al., 2011). In our studies, we also found a further increase of AEA in plasma after prolonged diet administration, which neither was affected by high-fat omega-3 diet

treatment nor by high-fat omega-6 diet, rich in AEA precursor. Furthermore, we found a decrease of embryos born in both treated groups. By contrast, after nine weeks of treatment, lower concentrations of 2-AG were found in plasma with respect to the short protocol and, more interestingly, omega-3 high-fat diet induced a significant increase of 2-AG levels, relative to controls. These changes could be related to the fat composition of diets. In fact, it is reported, that dietary sources of omega-3 PUFAs, with 13.7% of total calories provide as fat, reduce the concentration of AEA and 2-AG in plasma and brain (Wood et al., 2010). Moreover, circulating increase of both eCBs were shown to be higher in obese compared with lean women (Engeli et al., 2005, Matias et al., 2006).

Studies measuring tissue contents or release of AEA from leukocytes (Bisogno et al., 1997), brain, liver and intestine (Fegley et al., 2005) have so far revealed that production of AEA is often accompanied by substantially higher amounts of *N*-acylethanolamides derived from other fatty acids, particularly *N*-palmitoylethanolamide (PEA; C16:0) and *N*-oleoylethanolamide (OEA; C18:1). Metabolic enzymes of AEA are NAPE-PLD and FAAH, which are responsible for its synthesis and degradation, respectively. Similar biosynthetic pathways using the fatty acids, palmitic acid and oleic acid have also been suggested to produce PEA and OEA, respectively (Hansen et al., 2000, Okamoto et al., 2004). They are endogenous ligands for PPAR-α (Lo Verme et al., 2005). OEA has primary been studied as satiety factor (Rodriguez de Fonseca et al., 2001, Fu et al., 2003), while PEA as an inti-inflammatory factor (Calignano et al., 1998, Jaggar et al., 1998). During pregnancy, plasma and tissue levels of these two AEA-like mediators also fluctuate, suggesting a possible role in pregnancy maintenance and/or success (Fonseca et al., 2010b). The results presented here, about PEA and OEA levels, showed that the administration of both hypercaloric diet protocols induces a similar distribution to AEA in pregnant mice. According to some possible scenarios proposed to describe PEA mechanism of action, this compound may potentiate AEA effect by competing with AEA for FAAH-mediated degradation (LoVerme et al., 2005). Moreover, like for AEA, high-fat diets did not affect this distribution.

In summary, according to the evidence presented, these data underline the relationship between PUFAs and eCBs during gestation but it should be noted that levels of these lipids depend on metabolic enzyme activities in maternal tissue and further investigations are required to elucidate the correlation between endocannabinoid system and high-fat diets.

Effect of hypercaloric diets on the eCB system in E18 embryos

We therefore measured the levels of eCBs in brain and plasma tissue from E18 male and female embryos of mothers fed with hypercaloric diets, and compared then to the transcript and protein levels of eCB metabolizing enzymes. The results revealed that, like in plasma of mothers, 2-AG levels are higher than AEA after long-term protocol and high-fat diets induce slight but not significant changes in their levels. By contrast, in cerebral tissue of both male and female embryos, omega-3 and omeg-6 long-term diets affect AEA concentration. These data, with the qPCR and western blot results, suggest a relationship among AEA, CB1R and FAAH. It is known, in fact, that activation of CB1R may regulate the biosynthesis of its endogenous ligands and low AEA levels were found in hippocampus of CB1R knockout homozygotes mice (Hunter and Burstein, 1997, Di Marzo et al., 2000). Here, we demonstrated that AEA is down-regulated by omega-3 and omega-6 long-term diet treatment in cerebral tissue of female embryos, and *faah* transcript was found up-regulated after short-term protocol, but not after long-term protocol. Moreover, also both transcript and protein levels of CB1R were decreased in both omega-3 and omega-6 diet groups after only short-term protocol. Taken together, these data suggest that high-fat diet administration for a long-period down-regulates AEA concentration through the combination of two different mechanisms. In particular, we presume that the slight increase of *faah* is not sufficient to induce the strongly AEA decrease found in female embryonic brain. Instead, the combination with the changes in CB1R expression could maintain this status until the end of the treatment where both enzyme and receptor levels are normalized. This hypothesis is underscored also by male results. As such, omega-6 diet decrease AEA levels in cerebral tissue after long-term administration and this is correlated with an up-regulation of *faah* transcript levels, and a down-regulation of CB1R still present also after LTD protocol. Moreover, omega-3 diet induced no significant alteration in AEA levels thanks to the up-regulation of both CB1R and *faah* expression. In line with this, should be important evaluate also the protein expression of FAAH.

It is known that OEA and PEA are structurally similar to AEA, and although they exhibit little or no activity at the CB1R, they are thought to be “entourage compounds” that modulate the effects of AEA and these receptors by inhibiting AEA degradation, thereby indirectly prolongation its biological effect (Jonsson et al., 2001, Lambert et al., 2002, Rodriguez de Fonseca et al., 2005, Garcia Mdel et al., 2009). In line with this, in embryos, the high-fat diet effects on AEA concentration were found accompanied by the same, but less strong, alteration in PEA levels. By contrast, we found an increase of OEA levels in omega-3 groups in both

female and male embryos tissues. This is correlated to the composition of the diet which is rich in oleic acid, precursor of this lipid.

There is strong evidence in support of AEA as modulator/activator of TRPV1 receptors in both the peripheral nervous system and the CNS (Ross, 2003, Chavez et al., 2010). This vanilloid receptor is part of a large family of transient receptor potential (TRP) channels, which typically act as molecular detector of noxious signals in primary sensory neurons (Caterina and Julius, 2001, Ramsey et al., 2006). In brain, TRPV1 can be found in prefrontal cortex, amygdala, hypothalamus, cerebellum, hippocampus and dentate gyrus, and functional studies have demonstrated that exogenous activation of TRPV1 facilities transmitter release in different cerebral areas (Sasamura et al., 1998, Yang et al., 1998, Doyle et al., 2002, Marinelli et al., 2002, Marinelli et al., 2003, Musella et al., 2009). Moreover, recent studies showed the interaction between omega-3 PUFAs and TRPV1 (Matta et al., 2007), particularly in obesity (Suri and Szallasi, 2008). Ablation of TRPV1-positive fibers is reported to diminish weight gain and improves glucose tolerance both in mice on a high-fat diet and in rat models of type 2 diabetes (Suri and Szallasi, 2008). In the present study, we demonstrated that while TRPV1 protein levels did not significant change after both experimental diet protocols, the transcript showed a strong decrease in brain of male embryos after both omega-3 and omega-6 long-term diet administration. Therefore, further studies are needed to better understand the mechanism and the consequences of these variations.

The CB1R mediates numerous physiological functions in the CNS (Sim et al., 1996, Eggan and Lewis, 2007) as a pre-synaptic modulator of neurotransmitter release, including, but not limited to energy balance, neuroprotection, pain and cellular differentiation and proliferation (Pertwee, 2006, Bennetzen, 2011). Based on the location and function of CB1R in the CNS, it is no surprise that CB1R provide a potentially promising therapeutic target for a diverse number of diseases and disorders (Seely et al., 2011). The recently identified cannabinoid receptor-interacting protein 1a (*Cnrip1a*, CRIP1a), expressed in the developing rodent forebrain (Keimpema et al., 2010), binds the C-terminal domain of CB1R, a region known to be important for receptor desensitization and internalization (Niehaus et al., 2007, Smith et al., 2010, Smith et al., 2015), indicating a role in modulating receptor activity. Moreover, in their studies, Blume et al. (Blume et al., 2015) reported that CRIP1a attenuated CB1R signaling in neuronal cells. In line with that, our data show that in female embryos, *Cnrip1a* transcript levels did not reveal significant differences. However, the protein profile showed an increase in CRIP1a expression after omega-3 and omega-6 short-term diet treatment, which correlates with the decrease of CB1R protein expression in the same embryonic tissue. Furthermore, in male embryos, after the same experimental protocol, where the CB1R is

down-regulated in omega-6 diet group, CRIP1a was found increased. These findings support the idea of the functional interaction between CRIP1a and CB1R, and hypercaloric diets, rich in omega-3 or in omega-6 PUFAs, could modulate these proteins both in female and in male embryos. Interestingly, the prolongation of diet administration did not affect CRIP1a mRNA and protein levels in female embryos, which correlates with the mechanism explained before. In contrast, in males, we found a significant decrease of both proteins, CRIP1a and CB1R, which allowed us to entertain the possibility that both diets could have a direct effect on cannabinoid receptors and not only through the modulation of its interacting protein.

It can be concluded from the first approach of this study that high fat diets during gestation can induce changes in endocannabinoid system in both female and male embryos. Moreover, the short-term exposure of two weeks of omega-3 and omega-6 diet feeding is already sufficient to allow alteration of eCB system, especially CB1R. These results demonstrate also the link between dietary PUFA intake and eCBs tissue concentration.

Isocaloric diets

To better characterize the effect of PUFAs, in the second part of this thesis we focused our attention on omega-3 PUFAs. The American diet emphasized omega-6 PUFAs at the expense of omega-3 PUFAs. It is known that omega-3 PUFAs are crucial in fetal and neonatal brain and nervous system development (Freeman, 2000) and may have beneficial effects in mood disorders (Haastrup et al., 2012). For this reason, we fed pregnant rats with isocaloric diets (6% fat) rich or poor in omega-3 but not in omega-6 throughout gestation and until adulthood. Different reports showed that diets enriched with long-chain omega-3 PUFA (EPA and DHA) decreased AA levels and resulted in lower eCB levels in the brain (Berger et al., 2001, Artmann et al., 2008, Wood et al., 2010). Nevertheless, our data showed that the effects of feeding omega-3 PUFA to rats are more complex than a simple decrease of AA. Indeed, we discovered that, during gestation, diets rich or poor in omega-3, but with the same content of omega-6 PUFA, markedly affect the eCB system through mechanisms that involve the most important enzymes responsible for the metabolism of its ligands in hippocampus of E18 embryos. In this part of the project, AEA and 2-AG concentrations were not directly measured, however, NAPE-PLD and DAGLβ protein levels were found changed by both isocaloric diets, as well as the degradative enzyme FAAH. Finally, no significant alterations were found in CB1R expression. Interestingly, this is in contrast with what we found after high-fat diets treatment, and suggest the both omega-3 and omega-6 PUFA alteration are necessary to affect this receptor.

One of the brain structure associated with learning and memory as well as mood is the hippocampus. Interestingly, the hippocampus is one of the two structures in the adult brain where the formation of the newborn neurons, or neurogenesis, persists till adulthood. Changes in PUFAs intake, for instance by dietary depletion during pre- and post-natal life, but also throughout adulthood correlate with neurochemical alterations in the hippocampus (Chalon et al., 2001). During embryogenesis, between E14 and E17 synaptogenesis is initiated and coincides with large increases in brain fatty acid accumulation, including DHA (Green et al., 1999). Different studies associated DHA deficient or supplementation to major aspect of synapse development and function, including the expression of pre- and post-synaptic proteins involved in vesicle trafficking and recycling processes as well as synaptic transmission. Moreover, Cao and collaboration (Cao et al., 2009) showed that the gestational DHA-deprivation in fetal hippocampi inhibited neurite outgrowth and synaptogenesis in cultured hippocampus. Our data strongly suggest that omega-3 deficiency or enrichment diets affect the principal pre-synaptic marker of E18 hippocampal tissue, but not those localized postsynapticaly. Interestingly, these alterations were not found in adult rats where maternal malnutrition leads to long-term behavioral alterations characterized by the presence of recognition memory deficits. Beside its important role during gestation, in brain, the spatio-temporal expression of the eCB system guides major developmental processes including neurogenesis, cell differentiation, cell migration, neuronal specification and synaptogenesis (Harkany et al., 2007, Maccarrone et al., 2015). Furthermore, endocannabinoid signaling emerges as a key modulator of emotions and cognition (Campolongo and Trezza, 2012). For these reasons, a dysregulation of this system during a crucial time window of neuronal development, such as embryogenesis, might affect neuronal markers and cause behavioral alterations in adult rats.

As a whole, the second approach of this study supports our hypothesis about a relationship between PUFAs and the endocannabinoid system and provides further evidence on the importance of omega-3 PUFA on hippocampal development and functioning.

Effect of PUFAs *in vitro*

Brain development, is regulated by a variety of extracellular signaling that, together with neuronal activity, orchestrate and regulate progenitor proliferation, differentiation and neuronal maturation. Different studies *in vitro* and *in vivo* have directly related omega-3 and omega-6 to neurogenesis and neurite outgrowth (Calderon and Kim, 2004, Kawakita et al., 2006, Cao et al., 2009, Robson et al., 2010, Crupi et al., 2013), but the mechanism behind its role has yet to be fully established. The formation, guidance and stabilization of neurites, are

the key feature of both the developing and adult nervous system. Here, we provide evidence that cortical/hippocampal neurons supplemented with omega-3 and omega-6 PUFAs increased total neurite length. Moreover, both PUFA series increased both individual neurite length and axonal length. Given the role of the eCB system in neuronal development and the axonal targeting of CB1Rs (Maccarrone et al., 2014) could be interesting evaluate also the expression of this receptor.

All together, our results suggest that changes in dietary omega-3/omega-6 PUFAs ratio during gestation affect the endocannabinoid system in the brain of the offspring and the major effect of diets are present at the beginning of the treatment (i.e during gestation).

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