Master's thesis

NTNU
Norwegian University of Science and Technology
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Effect of omega-3 fatty acids in regulating synaptic plasticity-associated proteins

Master's thesis in Neuroscience
Supervisor: Svend Davanger
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Abstract

With an increasing number of the population reaching older age, it is important to find lifestyle interventions that may counteract cognitive decline and dementia. Enrichment of diet with omega-3 fatty acids (n-3 PUFAs) has shown promising effects, but little is known about the cellular and molecular mechanisms in the brain. In this project, I have studied the effects of a n-3 PUFA rich diet on the concentrations of functionally important synaptic and glial proteins in normal mice (C57bl/6). This is part of a bigger project where we investigate the neuroprotective mechanisms of n-3 PUFAs on synapses in normal mice and on a mouse Alzheimer model (5xFAD). I have selected 14 different neuronal and glial proteins that are important for synaptic plasticity, and investigated any diet-based changes in their concentrations in the cerebral cortex and the hippocampus. A comparison of the level of these proteins was performed with the Western blot technique on homogenates from hippocampus and cortex homogenates of mice raised on a diet which was either deficient or rich in the two common omega-3 fatty acids, DHA and EPA. First of all, we observed higher levels of the glutamate receptor subunits: GluA2 and GluN2B in mice fed with n-3 rich PUFA rich diet in both hippocampus and cortex. The GluA1 subunit, however, was reduced in the cortex but not in the hippocampus with the n-3 PUFA rich diet. Furthermore, in the cortex but not in the hippocampus, the synaptic vesicle proteins synaptophysin and synaptotagmin-1 showed higher concentrations in the n-3 PUFA rich group. Lastly, the postsynaptic scaffolding protein, PSD-95, the immediate early gene, Arc/Arg3.1, and the microglia/macrophage-specific calcium-binding protein, Iba1, are all significantly higher in the cortex of omega-3 fatty acid-deficient group, while the astrocyte marker, GFAP, was increased in the cortex of the n-3 PUFA enriched group. There were no significant changes in the glutamate receptor subunits GluN1, or GluN2A, in the neuronal growth factor BDNF, or in the microglial cell marker CD68. Taken together, my results indicate that dietary n-3 PUFAs may play a role in regulating the expression of a number of neuronal and glial proteins that are important for synaptic plasticity.

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Abbreviations

3-PUFA	n-3 polyunsaturated fatty acids
ALA	α-linolenic acid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
Arc1	Activity-regulated cytoskeleton associated protein 1
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CaMKII	Calcium-calmodulin-dependent protein kinase II
CD68	Cluster of Differentiation 68
CNS	Central Nervous System
COX-2	Cyclooxygenase-2
CREB	cAMP response element-binding protein
CSF	Cerebrospinal fluid
DBS	Dry blood spot
DHA	Docosahexaenoic acid
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
GFAP	Glial fibrillary acidic protein
GluA1	AMPA receptor subunit 1
GluA2	AMPA receptor subunit 2
GluA3	AMPA receptor subunit 3
GluA4	AMPA receptor subunit 4
GluN1	NMDA receptor subunit 1
GluN2A	NMDA receptor subunit 2A
GluN2B	NMDA receptor subunit 2B
GluN2C	NMDA receptor subunit 2C
GluN2D	NMDA receptor subunit 2D
GluN3A	NMDA receptor subunit 3A
GluN3B	NMDA receptor subunit 3B
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horse radish peroxidase
Iba-1	Ionized calcium binding adaptor molecule 1

IEG	Immediate-early genes
IFNγ	Interferon-gamma
IgG	Immunoglobulin G
iGluR	Ionotropic glutamate receptor
LC-PUFA	Long-chain n-3 polyunsaturated fatty acids
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
LPS	Lipopolysaccharide
LTD	Long term depression
LTP	Long term potentiation
mEPSC	Miniature excitatory postsynaptic current
MHC	Major histocompatibility complex
NaPi	Sodium phosphate (inorganic) buffer
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
OPC	Oligodendrocyte progenitor cell
PKC	Protein kinase C
PNS	Peripheral nervous system
PPI	Protein phosphatase I
PSD	Postsynaptic density
PSD-95	Postsynaptic density protein of 95 kDa
PVDF	Polyvinylidene fluoride
S100β	S100 calcium-binding protein β
SDS	Sodium dodecyl sulphate
TNF-α	Tumour necrosis factor alpha
tBHQ	Tert-butylhydroquinone

1. Introduction

“As the world's population increases in age, the number of people living with dementia grows, and this figure is projected to continue to rise,” wrote Livingston et al. (Livingston et al., 2017) almost three years ago. The authors pointed to the need for lie-style interventions, including diet, in order to curb this development. However, in spite of initially promising findings, the potential role of dietary omega 3 fatty acids, n-3 PUFAs, in preventing Alzheimer’s disease or other forms of dementia has not been unequivocally confirmed. N-3 PUFAs have been shown to be involved in lowering the risk of cognitive impairment in individuals without dementia (Fotuhi et al., 2009). However, the results of other clinical trials have been less conclusive (Bhatti et al., 2020). One reason for discrepancies in this field of science, may be that we still do not know sufficient details about the mechanisms with which n-3 PUFAs interact with important brain proteins, especially those that regulate synaptic plasticity, in the normal brain. In this master’s thesis, I have investigated the effect of a dietary intervention with the n-3 PUFAs DHA and EPA on neuronal and glial proteins that play important roles in synaptic plasticity.

Memory is the encoding, storage, and retrieval in the human mind of past experiences (Britannica, 2020a). To perform these functions, we use the brain. Evidence suggests that data about past experiences are stored as long-lasting changes in synaptic connections (Mayford et al., 2012). In order for this to happen, synapses have the ability to change the strength of their connections, this capacity is known as synaptic plasticity (Citri & Malenka, 2008). The concept of changes in synaptic structure and function is thus part of a wider phenomenon that may be called neuroplasticity, i.e., the capacity of neurons and neural networks in the brain to change their connections and behaviour in response to new information, sensory stimulation, development, damage, or dysfunction (Britannica, 2020b). A large number of cellular and molecular mechanisms contribute to modify synaptic structure and function, and a wide range of intrinsic and extrinsic factors modulate them, leading to a highly regulated system. These changes can result in an increased efficacy called potentiation, or in a decrease synaptic efficacy, called depression.

On one hand, extrinsic factors such as lifestyle can affect synaptic plasticity, for instance, exercise, smoking, alcohol or diet (Livingston et al., 2017). In the latter case, some of the components to take into account are dietary antioxidants and omega 3 fatty acids, or n-3

PUFA (Mazza et al., 2007). This project will be focused on the effects of n-3 PUFA. The rationale behind this choice was based in the recently published results that show that dietary supplementation with this component might modify n-3 PUFA content in hippocampal synaptosomes, change the concentration of post-synaptic glutamate receptors, and modify long term potentiation in the hippocampus CA1 area (Aryal et al., 2019).

On the other hand, a wide range of intrinsic factors play a key role in synaptic modulation. Glial cells are well known to be important in metabolic maintenance and homeostasis in the brain. But increasing evidence supports also the role of the tripartite synapse (glia, presynaptic terminal and postsynaptic density) in regulating synaptic function (Beattie et al., 2002; Ben Achour & Pascual, 2010; Todd et al., 2006). Within glial cells, I will focus on astrocytes and microglia and the impact n-3 PUFA has on them.

Nonetheless, the mechanisms that underlie n-3 PUFA effect on these factors are yet unknown. In the present study, I hypothesize that n-3 PUFA-deficient diet has an adverse influence in the adult mammalian brain. For this, differences in concentrations of molecular markers of synaptic plasticity, glial cells and glutamate receptors were studied in different brain regions of mice raised on a diet which is either deficient or rich in the two common omega-3 fatty acids, DHA and EPA.

1.1. Synaptic plasticity

Synapses consist of a presynaptic terminal containing vesicles, some of which are docked to the active zone of the plasma membrane, a synaptic cleft, and a postsynaptic element of the receiving cell, where the plasma membrane corresponding to the synaptic cleft is endowed with a postsynaptic density (PSD). PSD is an electron-dense zone that holds a high number of transmitter receptors and protein complexes. Scaffold proteins constitute a large proportion of PSD proteins. Within these scaffold proteins, PSD-95 is found (Chen et al., 2011). PSD scaffold proteins regulate cytoskeletal structures and participate in the trafficking, anchoring and clustering of glutamate receptors (Feng & Zhang, 2009).

On one hand, we define synaptic plasticity as any change in synapses with an effect on or derived from changes in synaptic transmission, i.e., either strengthening or weakening synapses in response to a stimulus. Depending upon the temporal properties of these synaptic modifications, synaptic plasticity can be seen as long-term or short-term. These

changes may be expressed at different levels: physiological, structural or molecular; which in most cases are intertwined. On the other hand, physiologist will often state that the classical long-term potentiation (LTP) (described on the section *1.1.2 Long-Term potentiation (LTP)*) as measured with electrophysiological methods is the quintessential synaptic plasticity.

In either case, synaptic plasticity plays a key role in the development of the nervous system as well as in the adult brain being shaped and remodelled by experience, making it one of the most important areas of neuroscience. Changes in the strength of synaptic plasticity allow, among other things, learning and the formation and storage of memories (Abraham et al., 2019).

Synaptic function is often studied through the activation of glutamate receptors placed in dendritic spines; small protrusions localized in dendrites.

1.1.1. Ionotropic glutamate receptors

Glutamate is the major excitatory neurotransmitter in the mammalian nervous system. This neurotransmitter is storage in synaptic vesicles which content is released to the synaptic cleft through exocytosis. There are several proteins that assists the anchoring and exocytosis of these synaptic vesicles, among them, synaptophysin and synaptotagmin-1 (Brose et al., 1992; Kwon & Chapman, 2011). Once it is released, glutamate can act on diverse membrane receptors, including ionotropic glutamate receptors (iGluRs), which form cation channels. Glutamate's principal ionotropic receptors are the AMPA and NMDA receptors, named after their experimental agonists (Figure 1). Activation of these receptors is an important tool in research on synaptic plasticity (Riedel, 2003).

AMPA receptors are heterotetrameric, formed by different combinations of four types of subunits: GluA1, GluA2, GluA3 and GluA4. This type of receptor is permeable to Na^+ and K^+, and when the GluA2 subunit is included, it also allows the influx of Ca^{2+} ions. Furthermore, GluA2-containing receptors play a key role in the growth and maintenance of dendritic spines (Passafaro et al., 2003). Thus, this subunit is thought to have an important role in information processing and that it is in charge of signal transmission in the synapses.

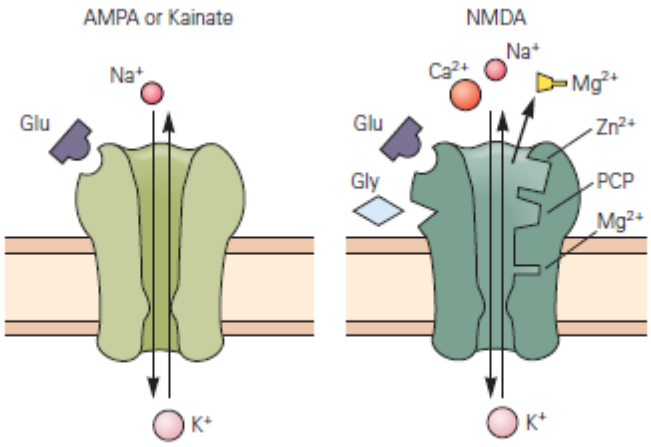


Figure 1. Different types of ionotropic glutamate receptors. A) The AMPA receptor binds the glutamate agonists AMPA. This receptor has a permeable channel to Na^+ and K^+. B) The NMDA receptor, which binds the glutamate agonist NMDA, contains a channel permeable to Ca^{2+}, K^+, and Na^+. It also has binding sites for glycine, glutamate, Mg^{2+}, Zn^{2+} and phencyclidine (PCP), each of which modulates the activity of the channel in different ways. Figure adapted from Kandel et al., 2013, p. 215.

NMDA receptors are both ligand- and voltage-gated. Their activation depends not only on the binding of glutamate but also on a partial depolarization of the postsynaptic cell that allows the removal of the Mg^{2+}-ion that blocks the receptor's ion channel. In this way, NMDA receptors alter the physiological strength of the synaptic response. NMDA-receptors are a combination of the different subunits: GluN1, GluN2A-GluN2D, GluN3A and GluN3B, which confer unique characteristics of permeability. Especially, GluN2B has been the subject of numerous studies due to its possible role in information processing (Hrabetova et al., 2000). It's been observed that an overexpression of this subunit in mouse forebrain leads to better results in learning and memory tasks (Cui et al., 2011). The NMDA receptor has several binding points also for other molecules than glutamate. These molecules can act as modulators of their activity and interact with structural and scaffold-proteins.

1.1.2. Long-term potentiation

In some forms of synaptic plasticity, the biochemical and structural reactions in the synapse lead to long-lasting changes in synaptic strength, this is a set of mechanisms known as long-term potentiation (LTP). LTP was first described by Bliss and Lømo (Bliss & Lomo, 1973). In this study, they showed a long-lasting potentiation of the performant path in the hippocampus as a result of a brief high-frequency stimulation.

AMPA receptors are ionotropic transmembrane receptors that perform fast synaptic transmission in the CNS. Thus, the binding of glutamate to AMPA receptors allow a flux

of Na^+ ions into the post-synaptic neuron promoting a local depolarization of the postsynaptic membrane. In the post-synaptic membrane, there are also voltage-gated NMDA receptors. The ion channel of these receptors is blocked by an Mg^{2+}-ion, that can only be displaced when the depolarization of the postsynaptic membrane exceeds a certain level. When this depolarization threshold is reached due to the opening of AMPA receptor ion channels or by theta-burst stimulation, and the binding of glutamate molecules NMDAR receptors also occur, NMDAR are activated leading to an influx of both Na^+ and Ca^{2+} into the cell (Larson & Munkácsy, 2015). Therefore, the increasing levels of Ca^{2+} in the postsynaptic neuron is a sign of NMDA receptor activation what constitutes the first step in LTP.

The course of LTP can be divided into two phases: an *early LTP* characterized by rapid changes of just a few hours affecting only existing proteins; and a *late LTP* involving the activation of transcription factors, synthesis of new proteins and structural changes (Figure 2).

With the activation of NMDAR, Ca^{2+} influx activates signalling pathways that will eventually lead to synaptic modifications. During the *early LTP,* the increase of $[Ca^{2+}]$ in the postsynaptic neuron activates two protein kinases and consequently, their signalling pathways: PKC and CaMKII (Hu et al., 1987; Lisman, 1994). These enzymes phosphorylate the GluA1 subunit of the AMPAR, which modifies its configuration and/or function. Also, this phosphorylation will allow the insertion into the postsynaptic plasma membrane of pre-existing AMPAR in post-synaptic vesicles by exocytosis (Bredt & Nicoll, 2003). That is to say, that during the *early LTP,* both an enhancement of AMPAR efficacy and increase in number occurs. Meanwhile, the development of new synapses occurs during the *late LTP* that is characterized by changes in the genetic expression and hence, by long-lasting modifications. The activation of the previously mentioned protein kinases will lead also to the activation of transcription factors such as CREB and IEG (Malenka & Bear, 2004) (Figure 2). In this way, LTP activity communicates with the nucleus. At the end, the activation of such transcription factors leads to an increase synthesis of AMPAR.

These LTP characteristics provide evidence for the Hebbian postulate that states: “When an axon of a cell A… excites cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells so that A’s efficiency as one of the cells firing B is increased” (Hebb, 1949).

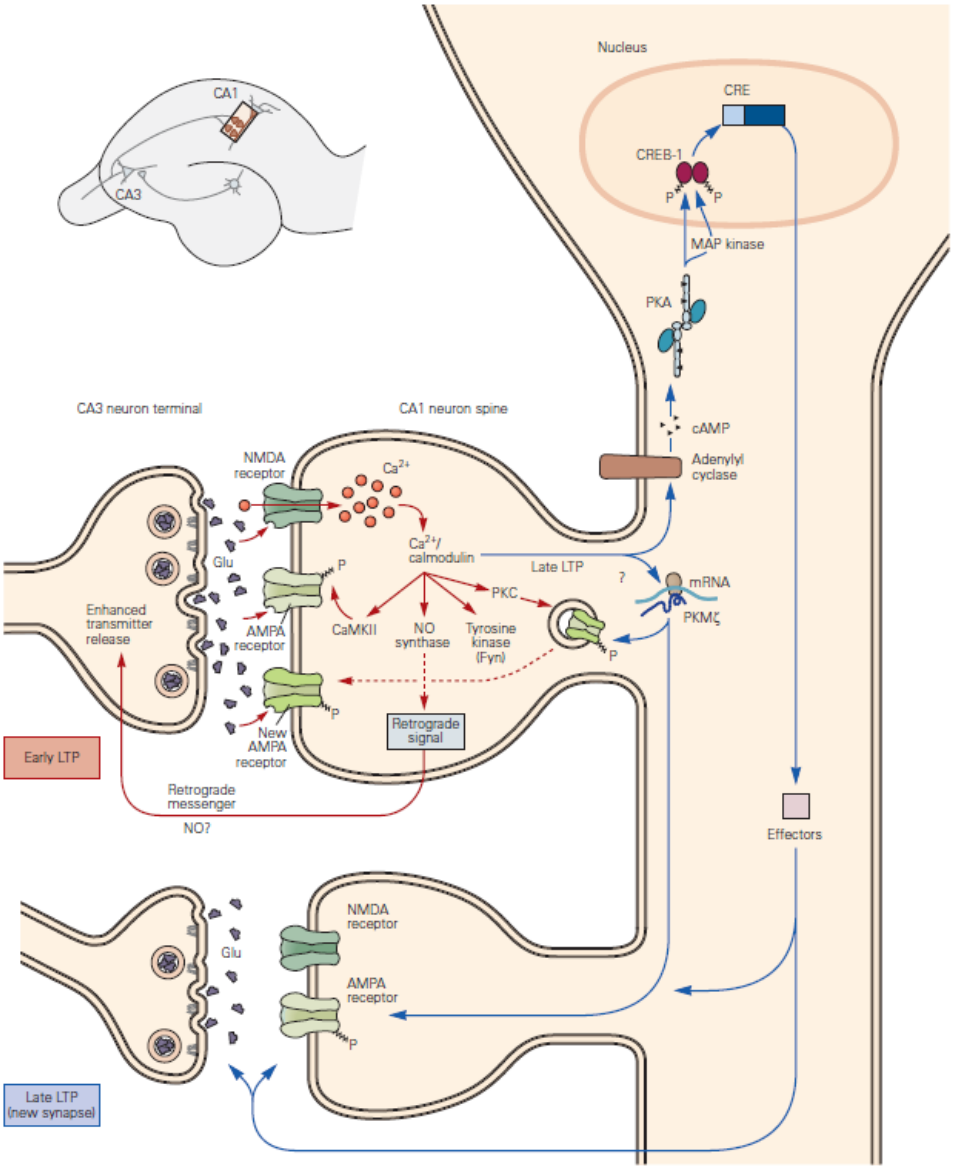


Figure 2. A model for the molecular mechanisms of early and late phases of long-term potentiation. Activation of NMDAR induces early LTP which causes an influx of Ca^{2+} in the postsynaptic dendrite. The increase in the concentration of Ca^{2+} activates CAMKII, which phosphorylates AMPARs, modifying their efficacy**.** Continuous stimulation also activates adenylyl cyclase generating cAMP which activates PKA. This leads to the activation of MAP kinase, which travel to the nucleus where it phosphorylates CREB-1. CREB-1 activates the transcription of certain genes that are thought to be related with the growth of new synaptic connections. Repeated stimulation activates the production of PKMζ in the dendrites which increases the number of AMPA receptors in the postsynaptic membrane. Image obtained from Kandel et al., 2013, p. 1502.

As mention above, synaptic plasticity and LTP underlie memory formation (Lynch, 2004). During the *early LTP*, where only transient changes on preexisting proteins occur, short-term memory is formed. While long-term memory results from *late LTP*, where long-lasting changes occur due to the change in the genetic expression pattern and the formation of new synapses.

1.1.3. Long-term depression

A low frequency but long-lasting stimulus will evoke a weak or modest postsynaptic response causing a synaptic weakening in the active synapses. This is called long-term depression (LTD). In this case, the lower but long-lasting influx of Ca^{2+} leads to a depolarization that is not enough to remove Mg^{2+} from NMDA receptors, so they remain partially blocked, allowing just a small flow of Ca^{2+} into the postsynaptic cell. This mild and prolonged increase of $[Ca^{2+}]$ will activate protein phosphatases such as PPI, instead of kinases as in LTP, which results in the dephosphorylation of AMPA receptors. This drives to the internalization of these receptors and a decreased efficiency of synaptic transmission (Beattie et al., 2000). Endocytosis of AMPA receptors is modulated by large number of proteins, for instance Arc/Arg3.1. Arc/Arg3.1 is an immediate-early gene who is rapidly transcribed and induced after synaptic activity, after what, it is targeted to dendritic spines (Chowdhury et al., 2006). Furthermore, LTD is associated with the retraction of existing spines (Zhou et al., 2004).

It can be inferred that the level of NMDAR activation differentiates between LTP and LTD, through its control over the two-way process of phosphorylation and dephosphorylation of AMPAR (Henley & Wilkinson, 2013). LTP and LTD are balanced by means of the so-called metaplasticity. This mechanism regulates synaptic plasticity across time and space in order to maintain homeostasis in the healthy brain (Abraham & Bear, 1996). This physical modification of synapses due to changes in the input-output relationship of neurons leads to storage of memories in neuronal networks. As a result, the cells' response to future inputs is altered due to the neural activity and changes in the weighting of synapses.

1.1.4. Structural plasticity: formation and stabilization of dendritic spines and synaptogenesis

In addition to these physiological and molecular changes, synaptic plasticity may also be linked to structural modifications in the brain. In the adult brain, large-scale structural changes, such as the complete retraction or *de novo* formation of dendritic branches, is extremely uncommon, with the exception of the formation of dendritic trees during adult neurogenesis. Nevertheless, small adjustments of the neural networks might be still possible through the growth or shrinking of dendritic spines (Holtmaat & Svoboda, 2009). Dendritic spines are small protrusions of the dendritic shaft typically consisting of a head

and a thin neck. The volume of the spines can range from 0.001 to 1 µm and the head can adopt diverse shapes: thin, stubby, mushroom and cup shaped spines (Hering & Sheng, 2001) (Figure 3). They are mainly found on excitatory neurons, where their principal function is to increase the surface area of membrane available. A PSD is typically found at the top of these dendritic spines, and there is a correlation between PSD area and spine head volume in the order that the greater the dendritic spine volume the greater PSD area and the greater number of AMPA and NMDA receptors in the synapse (Arellano et al., 2007; Harris et al., 1992; Takumi et al., 1999).

There appears to be a correlation between spine shape and synaptic strength, and abnormalities ins spine number and morphology have been found in several neurological disorders (Menna et al., 2013). Spine shape as well as the number of receptors found in each dendrite is modulated by age but mainly by activity, suggesting they are a substrate for neuroplasticity (Sala & Segal, 2014).

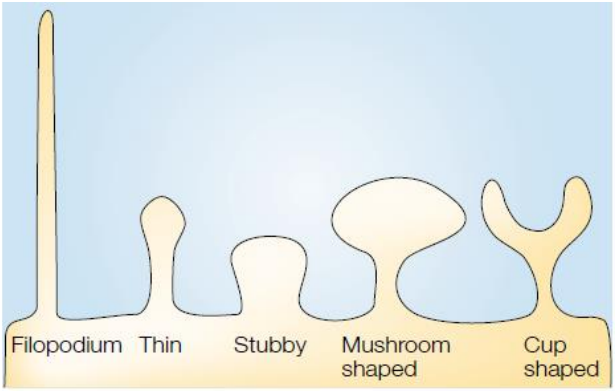


Figure 3. Different shapes of dendritic spines. Image adapted from Hering & Sheng, 2001.

1.1.4.1. Formation and stabilization of dendritic spines

The formation of dendritic spines starts with the generation of filopodia. Later on, these emerging spines adopt the shape of stubby structures, that with further development will disappear being the mushroom-shaped spines predominating in the adult brain. This can be seen as a stabilization of the dendritic spines, turning from transient structures into persistent spines resembling the strengthening of synaptic contacts. Transient spines are continuously appearing and disappearing while persistent spines remain over time (Engert & Bonhoeffer, 1999; Matsuzaki et al., 2004).

The formation of dendritic spines is correlated with the induction of LTP. However, spines appear with a delay of 20-30 minutes, which cannot explain the rapid onset of

synaptic strength (Maletic-Savatic et al., 1999). A reason for this could be a transient physiological enhancement of synaptic strength at the beginning of LTP, which later on is replaced by the formation of new synaptic sites.

Not only the growth of new synaptic dendrites has been related with synaptic plasticity events in learning and memory formation and storage, but many studies also suggest that morphological changes in already existing dendritic spines can be linked to synaptic plasticity (Matsuzaki et al., 2001). Different shapes and sizes of spines might affect the expression of functional AMPA receptors (Fukazawa et al., 2003). These changes either in the shape or the size of the spine, are dependent on F-actin and scaffolding proteins (Jin, 2005).

All changes in dendritic and spine structure mentioned above, alter the synaptic connectivity contributing to functional changes in the brain. This structural plasticity may have great impact on increasing the memory storage capacity of the brain, compared with changes in the synaptic plasticity alone.

1.1.4.2. Synaptogenesis

Synaptogenesis is the process involving the generation of synapses. That is to say, the formation of a neurotransmitter release site in the presynaptic neuron and a post-synaptic receptive field, both of them precisely aligned (Trachtenberg et al., 2002). Synaptogenesis in the CNS occurs not only during neurogenesis but also throughout life due to activity-dependent structural changes (Bear, 2016).

Under the microscope, neural tissue culture shows a continuous formation and retraction of filopodia seeking for contacts. The process of synaptogenesis starts once this contact between the filopodia and a presynaptic site is established (Knott et al., 2006). These new spines connect with already existing boutons (Trachtenberg et al., 2002), and they are especially responsible for the generation of new synapses leading to the experience-dependent reshape of neuronal networks in the adult brain (Colón-Ramos, 2009). This interaction is the first step (synaptic specificity) in synaptogenesis that will lead to the onset of a presynaptic active zone at the site of contact followed by the recruitment of neurotransmitter receptors to the postsynaptic membrane. The second step is known as synaptic assembly when adhesion molecules are expressed in order to align pre- and post-synaptic structures (Bear, 2016).

Besides, it has been proposed that astrocytes as well as microglia regulate synaptic formation and removal and may thus contribute to synaptic plasticity (Baldwin & Eroglu, 2017; Sominsky et al., 2018). For instance, when neurons are co-cultured with astrocytes, there is an increase in the generation of new synapses (Pfrieger & Barres, 1997).

Bringing everything together, plasticity mechanisms such as LTP, LTD, and structural plasticity are the main procedures that lead to long-lasting changes in synaptic activity. Therefore, they take part in the processes of formation and storage of memories as well as the process of learning. Consequently, these types of plasticity are a target to study the effect of ageing and other diseases in cognitive capacity.

1.1.5. Glial cells modulation of synaptic plasticity

The adult brain contains approximately 100 billion neurons supported by about the same number of glial cells (von Bartheld et al., 2016). Glial cells or neuroglia are non-neuronal cells found in the central nervous system (CNS) and peripheral nervous system (PNS). In the CNS, different types of glial cells can be found: astrocytes, microglia, oligodendrocytes and oligodendrocyte progenitor cells (OPCs). The main function of glial cells is to assist neurons, they help to define neuronal connections and to maintain the signalling activity. With this aim, they respond to extracellular signals and changes in the physiological state releasing a wide range of factors.

In this way, even if glial cells do not participate in the production of electrical signals or not in direct synaptic transmission, they play a key role in the development of synapses and the neuronal connections shaping process. Within the different types of glial cells mentioned above, mainly astrocytes and microglia take part in this. Astrocytes and microglia are ramified cells with a large number of connective points with both pre and postsynaptic cells in the perisynapsis (near the synapsis), which has given rise to the 'tripartite' and/or 'quad-partite' synapse models (Schafer et al., 2013). Furthermore, reactive microglia along with astrocytes release proinflammatory cytokines, including IL-1β, IFNγ, COX-2, IL-6, and TNF-α noted as being modulators of neural plasticity (Beattie et al., 2002; Schneider et al., 1998; Yirmiya & Goshen, 2011).

1.1.5.1. Microglia

Microglia are the immune cells of the brain and they constitute about 10 % of CNS cells (von Bartheld et al., 2016). As immune cells, microglia are responsible for surveillance of the brain, the protection against pathogens, the response to CNS damage and the

production of inflammatory mediators contributing to brain homeostasis (Li & Barres, 2018). Thus, microglia maintain the sterile integrity of the brain. Moreover, it is considered to be dynamic, as it receives inputs from the local environment, processes the information obtained and it sends outputs in order to maintain brain homeostasis. With this aim, microglia maintain plenty of contact points with neurons (Szepesi et al., 2018).

Microglial cells are produced during neuronal development and probably, shortly after birth, when erythro-myeloid progenitor (EMP) cells are produced and will differentiate later on into microglia (Ginhoux et al., 2010; Gomez Perdiguero et al., 2015). On the contrary, in the adult brain, this cell type is not renewed unless damage in the CNS occurs, after which bone marrow-derived blood monocytes migrate to the CNS travelling through the damaged BBB (Corraliza, 2014).

Finally, as mentioned before evidence suggests that inflammation cytokines as Il-1β and Tnf-α released mainly by microglia can modulate synaptic plasticity (Beattie et al., 2002; Schneider et al., 1998). In particular, levels of Il-1β are increased after induction and during the maintenance of LTP (Rizzo et al., 2018). In addition to cytokines, microglia also release other soluble factors that regulate synaptic function, for instance the trophic factors BDNF and NGF (Patterson, 2015). Altogether, these data emphasized the idea of the key role of microglia in neuronal plasticity.

Immunodetection of a variety of proteins can be used to study microglia in tissue (Hoogland et al., 2015). For example, CD68 is a high glycosylated transmembrane protein expressed by macrophages and activated microglia, also in resting microglia but in lower levels (Zotova et al., 2013). CD68 is found on the plasma membrane, as well as endosomal and lysosomal membranes. Another highly used antibody for microglia is Iba1. Iba1 is a microglia/macrophage-specific calcium-binding protein found mainly in activated microglia (Imai et al., 1996).

1.1.5.2. Astrocytes

Astrocytes are the most abundant glial cell type. These cells have a great energy requirement due to the formation of extensive dendritic trees and long-range projecting branches to establish communication with each other glial cells, neurons and capillaries (Fields & Stevens-Graham, 2002)

This highly connected network is crucial for the preservation of healthy brain functions as it ensures normal neuronal excitability. This is due to their role in maintaining

extracellular ion homeostasis through clearing potassium ions from the perisynapsis (Bellot-Saez et al., 2017). They also regulate the uptake, release and synthesis of several neurotransmitters (Mahmoud et al., 2019). Moreover, the position of these cells in relation to neurons allow them to sense any changes in neural activity and they adjust their level of glycolysis to produce lactate that will be exported to neurons as an energy substrate (Nortley & Attwell, 2017).

Astrocytes seem to regulate synapses by direct contact and by secreting gliotransmitters that target pre- and postsynaptic sites. Gliotransmitters give feedback to neurons and numerous studies support the idea that they modulate synaptic transmission, LTP and LTD, in part through metaplasticity, called to the mechanisms that balances LTD and LTP in time and space, and therefore, they are thought to participate in memory formation (Ota et al., 2013). This connexion between astrocytes and neurons led to the concept of the "tripartite synapse". It is important to mention that astrocytes are able to communicate bi-directionally with neurons, which means that neurotransmitters released from neurons may bind also to adjacent astrocytes, activating and modulating its activity (Szepesi et al., 2018).

A variety of proteins can be used as markers to study astrocytes in tissue samples. Within them, glial fibrillary acidic protein (GFAP), an intermediate filament protein part of the cytoskeleton, is one of the most common astrocytic markers (Zhang et al., 2019). Another commonly used astrocytic marker is S100β for the reason that the highest expression levels of this protein are found mainly in the cytoplasm and nucleus,of astrocytes. S100β is a Ca^{2+} binding peptide exerting both intra- and extracellular functions (Donato et al., 2009).

1.2. N-3 PUFA impact on the brain

Substantial evidence suggests that diet plays a key role in maintaining a healthy brain throughout the lifespan of an individual (Drevon, 1992). In particular, this project will be focused on the role of omega 3 fatty acids (n-3 PUFA). The two major members of the n-3 PUFA family are DHA (22:6 n-3) and EPA (20:5 n-3) (Hussein et al., 2005) (Figure 4).

Brain fatty acid composition can be affected by external factors such as nutrition. Both omega-3 and omega-6 fatty acids are considered to be essential due to the incapacity of the body to synthesize them *de novo* (Cutuli, 2017), although some conversion by

elongation and desaturation of plant-based ALA (18:3 n-3) to DHA is possible even if this process is limited in human beings (Alashmali et al., 2016; Joffre et al., 2019). This is the reason why it should be mainly obtained from the diet. Fatty fish such as salmon, herring, sardines and tuna represent the best dietary sources of DHA. Current human diets usually contains enough content of omega-6, but insufficient ALA and DHA (Simopoulos, 2016).

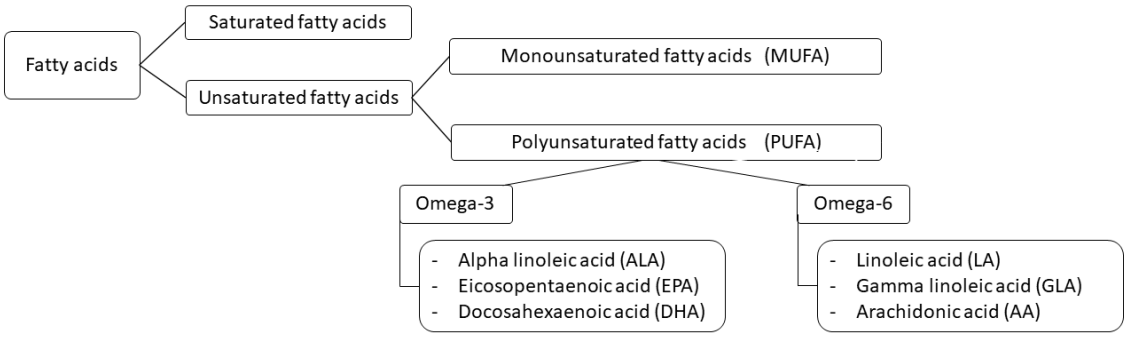


Figure 4. Classification of fatty acids. Fatty acids are classified based on the number of double bonds within the FA chain: saturated fatty acids have no double bonds, while unsaturated fatty acids can have one (MUFA) or more (PUFA). Within the latter, we found omega-6 and omega-3.

DHA is found in high concentrations in the mammalian brain and is considered to be the n-3 PUFA most relevant for brain function. DHA is crucial in several processes implicated in brain development, neurogenesis, neuroplasticity, neuron differentiation and survival, and membrane integrity, neurotransmitter and receptor densities, and fluidity (Calder, 2016; Calderon & Kim, 2004; Cao et al., 2009; Kim et al., 2011; Lauritzen et al., 2016). Consequently, a dietary deficit of this compound can cause severe impairments in the normal brain function, in fact, n-3 PUFA deficiency in rodents have been lately associated with memory dysfunction and reduction of synaptic plasticity, while supplementation with n-3 PUFA may result in improved memory and learning skills, as well as neurogenesis (Alashmali et al., 2016; Joffre et al., 2019). Furthermore, some studies have shown that n-3 PUFA dietary deficiency can affect glutamatergic, dopaminergic and serotoninergic systems.

Although the DHA level in the brain is affected by its ingestion in the diet (Alashmali et al., 2016; Joffre et al., 2019), it can also be affected by other factors like age. With ageing, DHA concentration in the hippocampus decreases, which coincides with a decrease in normal brain function and neuroplasticity.

1.2.1. Docosahexaenoic acid as anti-inflammatory mediators in the brain

Several studies have revealed that excessive activation of inflammatory signalling cascades in the brain may be involved in different neurological disorders. N-3 PUFAs have been demonstrated to have anti-inflammatory properties (Calder, 2016, 2017). Supplementation with LC-PUFA prevents the increase of pro-inflammatory cytokines IL-1β and TNF-α in rats and mice brains in response to lipopolysaccharide (LPS) (Calderon & Kim, 2004; Cao et al., 2009; Kim et al., 2011). Some studies also report that DHA increases the concentration of the anti-inflammatory cytokines (Rey et al., 2019). It has also been observed that in organotypic hippocampal slice cultures, during inflammation processes the spine density decreases as well as the mEPSC frequency what can be counteracted by the addition of DHA which suppresses inflammation (Chang et al., 2015).

These effects of DHA on cytokines and other proteins during inflammatory events suggest alterations in the gene expression encoding those proteins. Nuclear factor kappa B (NF-κB) is one of the main transcription factors involved in up-regulation of the genes encoding proteins involved in inflammation including many cytokines, adhesion molecules and COX-2. Extracellular inflammatory stimuli activate signalling cascades that will trigger the translocation of NF-κB from the cytosol to the nucleus where it binds to response elements and up-regulates inflammatory gene expression. In line with this, DHA decreased the activation of NF-κB in human monocytes, and this was associated with decreased translocation to the nucleus (Perkins, 2007).

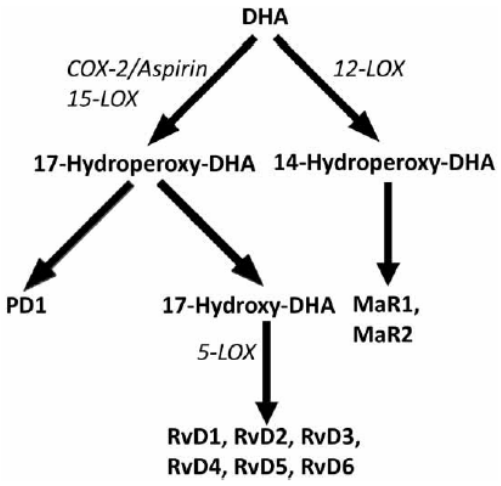


Figure 5. Synthesis pathway of the anti-inflammatory molecules from DHA. DHA is converted into protectins (PD1), resolvins (RvD1, RvD2, RvD3, RvD4, RvD5 and RvD6) and maresins (MaR1 and MaR2), through the action of COX-2, 15-LOX, 12-LOX and 5-LOX enzymes. Figure adapted from Calder, 2017.

A diet rich in DHA has also shown that increased synthesis of resolvins, protectins and maresins have anti-inflammatory effects. They performed this effect through their conversion into specialized pro-resolving mediators (SPMs) that exert the beneficial effects associated with their precursors (Calder, 2017) (Figure 5).

1.2.2. The importance of n-3 PUFA in synaptic plasticity

Evidence is accumulating regarding the beneficial effects of n-3 PUFA supplementation on synaptic functions. Cultures of hippocampal neurons supplemented with DHA show an increase in the neurite length and the number of branches increased significantly compared with those not supplemented (Calderon & Kim, 2004; Cao et al., 2009), this is related with a higher amount of synaptic connections (Jan & Jan, 2010). Moreover, the development and stabilization of these structures are essential for synaptic remodeling during memory consolidation and learning (Cline, 2001). DHA supplementation in embryonic hippocampal cultures significantly promotes synaptogenesis and it increases the expression of pre- and post-synaptic proteins, key participants during the processes of synaptic transmission and LTP (Calderon & Kim, 2004; Cao et al., 2009; Kim et al., 2011).

Recently, it has been shown that dietary n-3 PUFA might increase synaptic density (Cutuli, 2017), modify n-3 PUFA content in hippocampal synaptosomes, change the concentration of post-synaptic glutamate receptors, and modify long term potentiation in the hippocampus CA1 area (Aryal et al., 2019). Nonetheless, the mechanism that underlies these effects are still unknown.

1.2.3. Role of n-3 PUFA deficit in cognitive decline

In the last years, the effects of n-3 PUFA on brain and cognition have been in the spotlight of many studies of neurodegeneration. There is now ample evidence, however, from both animal and human studies to claim that n-3 PUFA is essential for normal brain development as well, but the connections between n-3 PUFA and the mechanisms of brain activity still remains elusive.

It has been reported that early dietary supplementation of n-3 PUFA improves later cognitive function in human infants (Birch et al., 2000; Uauy et al., 2003; Willatts et al., 1998), and memory-related learning ability in young rats (Gamoh et al., 2001). Conversely, n-3 PUFA deficiency during development, which lowers DHA in the brain,

has been shown to induce cognitive deficit in experimental animals (Catalan et al., 2002; Moriguchi et al., 2000). During ageing, higher intakes of n-3 PUFA have been described by several studies as preventive against age-related cognitive decline, dementia, and Alzheimer's disease (Dyall et al., 2007; Gamoh et al., 2001; Morris et al., 2003). In line with this, a low level of DHA in the blood is associated with cognitive decline during ageing (Cardoso et al., 2016). But even those age-related impairments may be reversed with a diet rich in n-3 PUFA. For instance, impairments in LTP caused by age in old rats have been observed to decrease after the administration of n-3 PUFAs (McGahon et al., 1999).

1.2.4. Distribution of docosahexaenoic acid in the brain

N-3 PUFA content varies between different regions in the brain as well as between different cellular types. Therefore, a diet deficient in n-3 PUFAs decreases the content of that compound in all brain structures, but the impact of this decrease will differ depending on the brain region.

For instance, the cerebral cortex regions and hippocampus are found among the regions with highest n-3 PUFA content whereas the hypothalamus and brain stem show the lowest values (Carrié et al., 2000; Joffre et al., 2019; Xiao et al., 2005). Furthermore, the impact of a deficient diet on n-3 PUFA will also differ between these regions. Its levels will decrease in all brain regions but the cortex and hippocampus found with the highest levels of n-3 PUFA also show a higher decrease of n-3 PUFA (Carrié et al., 2000; Joffre et al., 2019; Xiao et al., 2005). Regarding the cellular differences: neurons, astrocytes, oligodendrocytes, and microglial cells also contain n-3 PUFA in different proportions, being higher in astrocytes than in the other cell types (Bourre et al., 1984).

1.3. Effects of ageing in the brain

As CNS is by far the most complex system in the mammalian body, any disorder that has an impact on it is considered a great threat for the individual. Medical and public health achievements in reducing the impact of major disorders, as cardiovascular diseases or cancer, have resulted in increased life expectancy (Lunenfeld & Stratton, 2013). That is to say, there is a larger number of individuals in developed countries living longer and ageing further. Ageing leads to decreased cognitive performance even in the absence of

a disease condition and increase the odds of being prone to neurodegeneration. The reason for this is that neurodegeneration usually manifests in the later stages of life, which makes the time a key factor in the pathogenesis of these disorders (Livingston et al., 2017).

Ageing is a natural process associated with physical, physiological and social impairments. In particular, the main manifestation of ageing in the brain is cognitive decline. This can occur both in normal ageing and pathological conditions, such as neurodegenerative disorders (Wilson et al., 2010). However, the manifestation of this decline can differ substantially between individuals since it is influenced by several factors such as education, intelligence, and mental stimulation (Livingston et al., 2017). Differences in the evolution of these factors influence how the brain adapts to pathological damage and maintain cognitive function.

Several cellular changes are likely to contribute to the structural and functional changes that occur in the brain with age. Decreased functional machinery in the cell is one of these changes, which causes misfolding proteins, imbalance in the intracellular redox state and decreased neurotrophic support. This will bring the outcome of oxidative damage, compromise protein quality and alteration of gene expression patterns, ending up on excitotoxicity as well as cellular senescence (Foster & Kumar, 2002) (Figure 6).

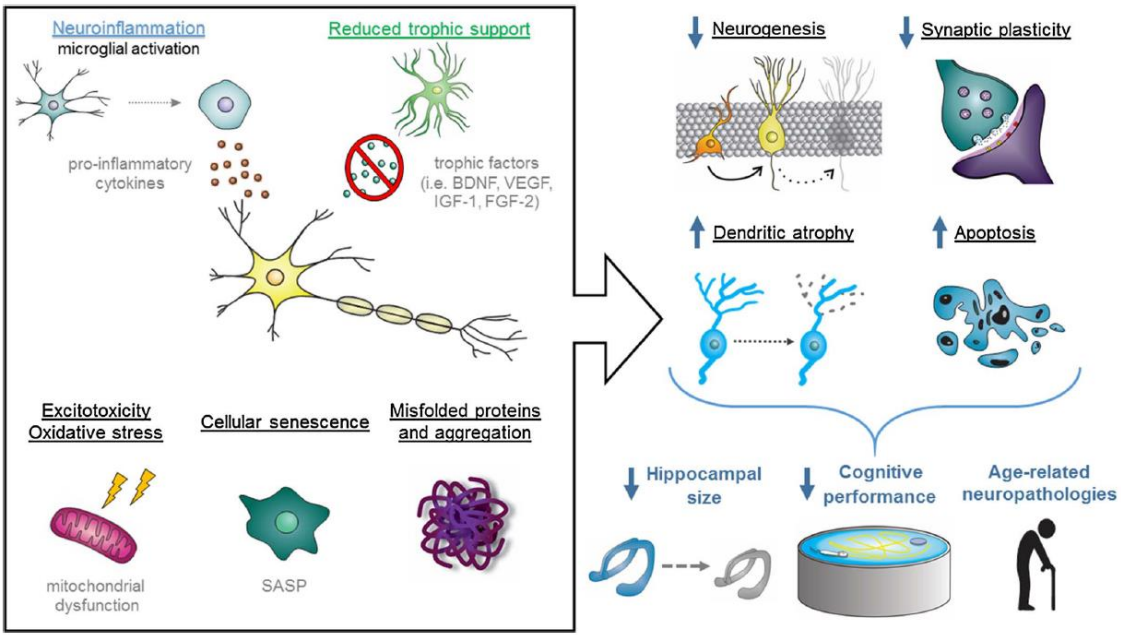


Figure 6. Mechanisms underlying cognitive decline observed during ageing. Neuroinflammation reduced trophic support, excitotoxicity, oxidative stress, cellular senescence and misfolded proteins and aggregation are processes observed in the ageing brain that lead to decreased neurogenesis and synaptic plasticity, increasing dendritic atrophy and apoptosis. As a result, aged individuals might present decreased hippocampal size and cognitive performance, what can lead to age-related neuropathologies. Figure obtained from Bettio et al. (2017).

Ageing also leads to changes in synaptic plasticity, driving to deficits in LTP and increase susceptibility to LTD, with changes in the glutamate receptors composition (Henley & Wilkinson, 2013; Kumar & Foster, 2019). As noted above, the number of spines is modulated, within other factors, by age and a decreased spine density has been reported in aged rats, primates and humans (Hering & Sheng, 2001).

Together, all these factors lead to a reduce hippocampal size, decrease cognitive performance and development of age-related neuropathologies (Bettio et al., 2017).

1.3.1. Astrogliosis

The normal activity of astrocytes is crucial for brain health; however, aberrant activity might suppose a risk and it might also enhance the pathophysiology of some diseases (Singh & Abraham, 2017). This fact can be aggravated during ageing, whereby the capacity of astrocytes to respond to pathology is impaired (Cohen & Torres, 2019). Furthermore, during aging and neurodegenerative diseases astrogliosis is observed. Astrogliosis is the abnormal increase in the number of astrocytes caused by the death of neighbouring neurons (Singh & Abraham, 2017). The disruption of astrocyte-neuron communication impairs synaptic connectivity and triggers a cascade of neuronal injury (Brambilla et al., 2013). Accumulating evidence suggests that astrogliosis and the dysfunction in the astrocyte-neuron signalling could be associated with LTP deficits and cognitive impairment (Singh & Abraham, 2017).

1.3.2. Neuroinflammation and microglia activation during ageing

Inflammation is one of the first mechanisms activated during the innate immunity against any threat to the organism. It also facilitates tissue repair, regeneration and maintenance of homeostasis by recruitment of immune cells to a compromised area. It is widely accepted that neuroinflammation plays a key role in the modulation of learning, memory, neural plasticity and neurogenesis mainly through the action of inflammatory cytokines, including interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) (Delpech et al., 2015; Yirmiya & Goshen, 2011). However, if uncontrolled or prolonged in time, it can lead to tissue damage and loss of function (López-Vicario et al., 2016) which may be linked to several neuronal pathologies, including neurodegenerative diseases and also to ageing processes (Laye et al., 2018; Solito &

Sastre, 2012). We need, therefore, to understand more of the role of microglia in neuroinflammation.

In the brain, neuroinflammation processes are performed by astrocytes, but mainly microglia. Astrocytes are not considered immune cells, but they have some immune-like properties. Like microglia, astrocytes release and respond to inflammatory cytokines, thereby exacerbating the neuroinflammatory response. And under certain circumstances, astrocytes may also participate in synapse elimination in the adult brain (Chung et al., 2013). Meanwhile, microglia turn into an active form in response to injury or immunologic stimulus undergoing morphological changes. This is known as reactive gliosis. Microglia are considered to be the first line of defence in the brain (Ransohoff, 2016). Once activated, it migrates towards the site of injury where it will be in charge of releasing many soluble factors (mainly proinflammatory mediators) and phagocytose any foreign bodies or cell-debris.

On the other hand, an overactivation of microglial cells can result in a chronic proinflammatory state. A persistent microglial activation triggers a cascade of neurotoxic changes, which involves up-regulation and overactivation of proinflammatory factors, as well as oxidative stress and eventually neuroinflammation (Block et al., 2007). This dysregulation of microglia has been related to several neurodegenerative disorders due to neuronal hyperexcitability, hormonal imbalance, decreased production of neurotrophic factors and disruption of neurogenesis (Colonna & Butovsky, 2017).

In this way, microglia can exert either neuroprotective or neurotoxic activity, and the balance between these two events as well as between the release of neuroprotective and neurotoxic factors will determine the role of microglia in the brain. For example, during ageing, degenerated neurons release several signals that activate microglia, but microglia's responsiveness decreases with age, becoming less dynamic and slower. This can in turn lead to misregulation of the immune response (von Bernhardi et al., 2015). These factors contribute to the cognitive decline and pathological changes linked to many age-related disorders of the CNS.

Several studies have as a target the reduction or avoidance of the neurotoxic activity exert by microglia. For instance, there is increasing evidence that omega-3 fatty acids (n-3 PUFA) may have anti-inflammatory effects and prevents microglia from changing its

morphology to an activation state during neuroinflammatory processes (Chang et al., 2015).

1.3.3. Dementia

Dementia is a neurodegenerative disease characterized by decrease cognitive performance, with increasing loss of synapses and neurons. Recently, the role of glial cells in preventing or promoting the loss of synapses and neurons has been highlighted (Gomez-Pinilla & Tyagi, 2013). Furthermore, structural changes in shape and number of dendrites and synapsis similar to the ones observed in aging brains are also found in dementia (Bettio et al., 2017). In this way, it is thought that the cognitive decline linked to dementia could be a result of disruption of synaptic function and neurotransmission.

One approach to prevent the onset of dementia targets the minimization of the impact of aging in this brain structures by changes in the lifestyle. In this way, many of dementia's manifestations could be manageable, and while generally, they are not curable, some of its symptoms might be preventable or reduced with good lifestyle (Livingston et al., 2017).

1.4. Aims and hypotheses

Despite all the advances, the underlying mechanisms of n-3 PUFA actions in the brain are still not well understood. My overall aim is to contribute to ongoing research on how dietary n-3 PUFAs may positively affect brain function. This field of research may in turn shed light on molecular mechanisms in the interaction between n-3 PUFAs and synaptic plasticity, which in turn may be important for the development of new dietary interventions for preventing cognitive decline and dementia in elderly people. The starting aim of this thesis is to optimize the conditions and protocols to follow later on to test my overall and specific hypotheses.

My overall hypothesis is that dietary n-3 PUFAs, DHA and EPA, will lead to changes in the concentration in the brain of proteins that are important for synaptic transmission and plasticity. I will test this hypothesis in homogenates from the cerebral cortex and the hippocampus of normal (C57/BL6) mice.

My specific hypotheses are:

A) Non-receptor synaptic proteins (PSD-95, synaptophysin, synaptotagmin) with established functional roles in synaptic transmission in glutamatergic synapses, will be increased in mice on a n-3 PUFA enriched diet. (“Increased number or size of glutamatergic synapses”)
B) Glutamate receptor subunits, from AMPA- and NMDA-receptors (GluA1, GluA2, GluN1, GluN2A, GluN2B), will be increased in mice on a n-3 PUFA enriched diet. (“Increased efficacy of synaptic transmission”)
C) Synaptic plasticity-related proteins (Arc/Arg3.1) will be increased in mice on a n-3 PUFA enriched diet. (“Increased synaptic plasticity”)
D) Glial cell proteins (CD68, GFAP, Iba1), specific for astrocytes and microglia, respectively, will be decreased in mice on a n-3 PUFA enriched diet. (“Decreased neuroinflammation”)

2. Methods

2.1. Optimization procedure

2.1.1. Homogenization of brain tissue

Procedure 1 – The tissue was teased into small pieces under a microscope with the help of a scalpel. For this, and to maintain the integrity of the proteins the sample was placed in 10% SDS iced cold. Afterwards, cycles of boiling (3 min), sonication (2 min) and pipetting with a glass pipette (10 times, in order to increase the mechanical stress) were performed. Three glass pipettes were modified with heat to obtain different diameters of the aperture, the samples were treated starting with the one of bigger diameter and ending with the smallest one. This cycle was performed repeatedly until no strands were observed and the solution was not viscous.

Procedure 2 – The tissue was treated like in Procedure 1 but the glass pipette was substituted by a pipette with a P20 tip. This was repeated until, as before, the solution showed no viscosity.

Procedure 3 – In this procedure, after the sample was treated as described in Procedure 2, the homogenate was shortly centrifuged in a mini centrifuge and the supernatant was collected and used for further analysis.

Procedure 4 – Procedure performed in following experiments and described in *2.5.2. Homogenization*.

The homogenates obtained from these procedures were analysed by Western blot (as described in the protocol below) using the primary antibody synaptophysin-1 (1:5000, #101002, Synaptic Systems GmbH, Germany). The results were compared with the one obtained from whole pig brain homogenate previously used and validated in the group.

2.1.2. Protein amount for Western blot

The amount of protein from homogenate samples that is recommended to load into a gel to perform Western blot is in the range of 10-50 µg. In order to optimize this value to both have a high efficiency of the technique and use as little amount of sample as possible, three protein concentrations are tested: 10, 20 and 30 µg. These amounts were tested to

decide the one that would be used in the rest of the project. For this, Western blot procedure was performed as described in section *2.2.6. Western Blot.* The primary antibody synaptophysin 1:5000 with a secondary antibody from donkey HRP-linked anti-rabbit IgG (GE Healthcare, Little Chalfont, U.K, NA934-1ML, 1:15.000) were used.

2.1.3. Antibodies concentration

Prior to the analysis of the samples of interest, literature research was carried out to determine which antibodies were the appropriate ones to study the effects of n-3 PUFA in synaptic plasticity and microglia function. Once the list of antibodies was made, the providers were chosen and following their recommendations, several concentrations of each antibody were tested to find the optimal one to be used later in Western blot (Table 1).

Nevertheless, I observed that for some antibodies unspecific bands did not disappear after lowering the concentration of the antibody. Consequently, changes in the blocking buffer were conducted. GluA2, synaptophysin and synaptotagmin-1 were incubated in 10 and 5 % skimmed milk in TBST-T as blocking buffer and incubated with primary and secondary antibodies in 5 % skimmed milk in TBS-T instead of gradually decreasing the percentage of skimmed milk.

2.1.4. Dot blot

Low molecular weight proteins were not giving any signal in previous Western blots. In order to assure that these proteins were in the homogenate, the dot blot technique was performed.

Stripes of 1 cm of PVDF Membrane (Millipore, Prod. No. IPV H00010, pore size 0.45 µm) were pre-wetted for 15 s in 100 % methanol to allow membrane activation. Afterwards, they were soaked 30 s in TBS-T (20 mM Tris, 150 mM NaCl, 0.05 % Tween 20, pH 7.5) for equilibration. 10 µg of protein were spotted within a pre-marked grid. The membrane was then left to dry to fix the proteins to it for 30 minutes at RT.

Table 1. Antibodies list. List of antibodies used in this project with the reference number and the dilutions tried for each one. Abbreviations: WB = Western blot.

Standard name	Name given by the providers	Reference number	Recommended dilution WB	Trial dilutions			
				Dilution 1	Dilution 2	Dilution 3	Dilution 4
β-actin	Beta Actin	20536-1-AP	1:2000-1:5000	1:1.000	1:3.000		
β-tubulin	Beta Tubulin	10094-1-AP	1:1000-1:5000	1:2.000	1:5.000	1:15.000	
Arc	ARC/ARG3.1	16290-1-AP	1:500 - 1:2000	1:500	1:2.000		
BDNF	BDNF	28205-1-AP	1:500 - 1:2000	1:500	1:1.000	1:3.000	
CD68	CD68	25747-1-AP	1:200 - 1:1000	1:1.000	1:5.000		
GFAP	GFAP	16825-1-AP	1:3000 - 1:10000	1:3.000	1:10.000	1:20.000	
GluA1	GluA1	GTX132945	1:500 - 1:3000	1:1.000	1:3.000	1:15.000	
GluA2	Glutamate receptor 2	11994-1-AP	1:500 - 1:2000	1:500	1:1.000	1:10.000	
Iba-1	IBA1	10904-1-AP	1:100 - 1:3000	1:500	1:1.500	1:3.000	
GluN1	NMDAR1	GTX133097	1:500 - 1:3000	1:1.000	1:3.000	1:10.000	
GluN2A	NMDAR2A	19953-1-AP	1:500 - 1:1000	1:500	1:1.000	1:3.000	
GluN2B	GRIN2B	21920-1-AP	1:500 - 1:4000	1:1.000	1:3.000	1:10.000	
PSD95	PSD95	GTX133091	1:500 - 1:3000	1:1.000	1:3.000	1:10.000	
S100 beta	S100 beta	15146-1-AP	1:200 - 1:1000	1:500	1:1.000		
Synaptophysin	Synaptophysin	17785-1-AP	1:5000 - 1:50000	1:5.000	1:15.000	1:20.000	1:40.000
Synaptotagmin-1	Synaptotagmin-1	14511-1-AP	1:500 - 1:1000	1:500	1:1.000	1:5.000	1:20.000

After the membrane was completely dry, it was incubated with blocking solution (TBS-T with 2 % low-fat milk powder) for 1 hour at RT in agitation. Each membrane was then incubated for 1 hour in agitation with the primary antibody in TBS-T containing 2.5% milk powder: Iba-1 (1:1.000), MHC-II (1:1.000, #14-5321-82, ThermoFisher, Waltham, Massachusetts, USA), TREM2 (1:1.000, #MABN755, Sigma-Aldrich, Missouri, USA) and GluN2B (1:3.000). This was followed by 3 x 5 min wash with TBS-T. Secondary antibody HRP-conjugated (1:10.000) was incubated in 1.25 % milk powder for 1 hour at RT. Finally, the membrane was washed with TBS-T 3 x 5 min before reaction development was performed using ECL kit.

2.1.5. Western blot for low molecular weight proteins

Western blot for proteins with a molecular weight under 35 kDa was carried out as described in section *2.2.6. Western blot,* but with some variations. The electrophoresis running time was decreased to 25 min to avoid small proteins running out of the gel and the PVDF membrane used in the blotting was changed to a size pore of 0.2 µm.

2.2. Study of n-3 PUFA effect in modulating synaptic plasticity

2.2.1. Ethics statement

This study was approved by the Norwegian national ethics committee, Mattilsynet, FOTS and Norwegian Food Safety Authority for appropriate use and care of the animals. All efforts were made to minimize the number of animals and their suffering, implementing the 3 R's (reduce, reuse, refine) and giving the animals humane ending points.

2.2.2. Diet

10-14 days old C57bl/6 mice, with their mother, were purchased. They were weaned 7 - 10 days later, after which they were separated from the mother and started on the experimental diet. 70 male and female mice were raised under 22 ± 1°C, 50 ± 10% humidity, and 7 a.m.–7 p.m. light cycles, with *ad libitum* access to food and water.

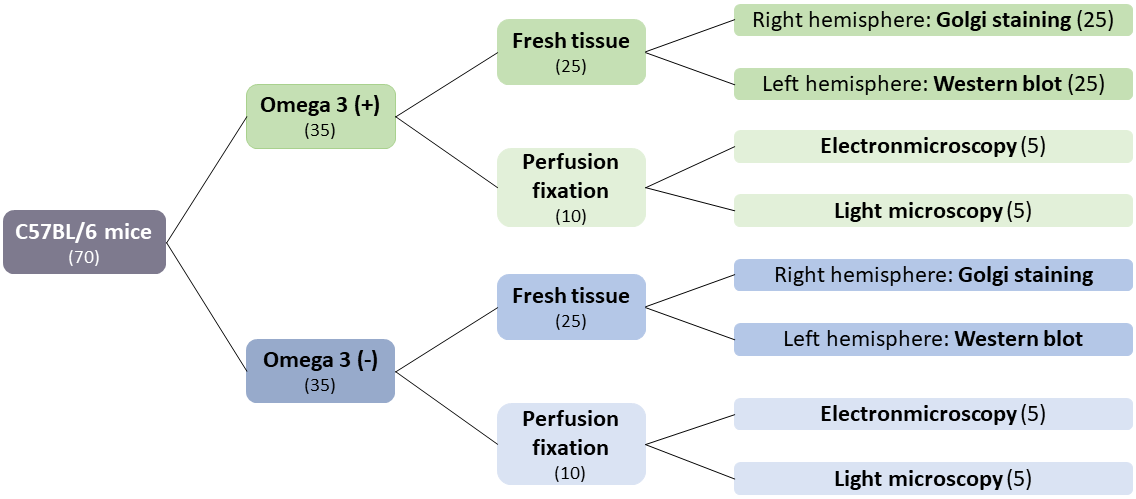


Figure 7. Study design. The number of mice planned for use in each technique is indicated between brackets.

They were fed with either a n-3 PUFA-rich or a n-3 deficient diet for 5 months (this was performed by Dinia Saraj). The diet rich in n-3 PUFA contains 10 % safflower oil and 4 % EPAX (concentrate produced by EPAX, Ålesund (Norway)) while the n-3 PUFA deficient diet contains 14 % (w/w) safflower oil. Other diet constituents were identical, as was the overall amount of fat, carbohydrate and protein (Table 2). Both diets were purchased from Research Diets, InC., USA. Meanwhile, the mothers of these mice were fed with a regular diet.

Table 2. Diet composition (g/kg diet).

Class description	EPAX	non-EPAX
Protein		
Casein	172	172
L-Cystine	2,58	2,58
Carbohydrate		
CornStarch	341,84	341,84
Lodex 10	113,52	113,52
Sucrose	86	86
Solka Floc	43	43
Soybean Oil	60,2	60,2
Mineral Mix S10022G *	30,1	30,1
Vitamin Mix V10037 *	8,6	8,6
Choline Bitartrate	2,15	2,15
Safflower oil	100	140
EPAX	40	0
Total	1000	1000
Fatty acid composition:		
Saturates	0.40	0.23

C18:3, n-3 (ALA)	0.02	0.02
C20:5, n-3 (EPA)	0.08	Not detectable
C22:6, n-3 (DHA)	0.28	Not detectable

(*) Composition described in Appendix I.

2.2.3. Fatty acid analysis

Blood samples from the femoral arteries of 10 mice (5 months of age) were taken for analysis of fatty acid levels (extraction performed by Dinia Saraj). Afterwards, they were decapitated and their brains were extracted as described in section *2.2.4. Dissection*.

Further analyses of the blood samples were performed using the technique of dried blood spots (DBS) in order to know the amount of EPA and DHA in blood serum (reported as g/100 g Fatty acid methyl esters *(*FAME)).

For this measurement, blood was spotted onto filter paper and allowed to dry. These dried blood spots were methylated with 3N methanolic HCl performed by the contract laboratory Vitas Ltd (www.vitas.no). After incubation for 20 min at 50° C, FAME were extracted with hexane and analysed on a gas chromatograph performed on a Supelco SP-2380 (30 m × 0.25 mm × 0.25 μm) column with a flame ionisation detector (GC-FID) (Agilent Technologies, Palo Alto, CA, USA).

2.2.4. Dissection

In order to obtain the tissue samples for western blot (left hemisphere) and Golgi staining (right hemisphere), 45 female mice were anaesthetized with isoflurane (Baxter, Illinois, USA) and decapitated.

A midsaggital incision was made on the skin to be able to remove it from the skull. Afterwards, a scissor was inserted in the foramen magnum and following the sagittal suture, the cranium was cut. With the help of small forceps, the skull was removed gently and the brain obtained. This process had to be performed quickly, within 3 minutes, before the brain was briefly rinsed with NaPi.

Finally, from the left hemispheres the cerebellum, brain stem (pons and medulla oblongata), cortex (small strip from the superior medial cortex) and hippocampus were obtained separately and immerse in ice-cold isopentane (#277285, 2-Methylbutane, Sigma-aldrich, St. Louis, MO, USA) to freeze them before storage at -80 ºC and

subsequently further analysis with Western blot. The right hemisphere was put right away in Golgi solution for further analysis.

2.2.5. Homogenization

The tissue samples from hippocampus and cortex were homogenized in homogenization buffer (0.32 M sucrose, 10 mM HEPES, 1mM EDTA) and protease inhibitor cocktail (cOmplete ULTRA Tablets, Mini, EASYpack, Roche) that was added immediately before homogenization. Once the buffer and the sample were placed in a loose-fitting Teflon glass grinder of 1 ml (Thermo Fisher Scientific, Massachusetts, USA), the homogenization was performed with ten strokes with a drill (Art. 17-376, Biltema, Sweden).

The protein concentration in the resulting samples was estimated using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Massachusetts, USA), where BSA was used as a standard. The samples were stored at -80 ºC until Western blot analysis was performed.

2.2.6. Western blot

An amount of 10 µg of protein from each hippocampus and cortex (n-3 PUFA rich: n = 23, n-3 PUFA poor: n = 21) was mixed with loading buffer 6X (50 mM Tris-HCl pH 6.8, 2 % SDS, 10 % glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA and 0.02 % bromophenol blue). The mixture was boiled for 3 min before loading onto the gel. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 4–20 % SDS-acrylamide gradient gel (4–20% Criterion™ TGX™ Precast Midi Protein Gel, 26 well, 15 µl #5671095) using running buffer (192 mM glycine, 25 mM Tris, pH 8.3, 1 % (p/v) SDS) and Criterion™ Vertical Electrophoresis Cell (Bio-Rad Laboratories, Hercules, California, USA) for 50 min at 200 V. Afterwards, proteins were transferred into a 0.2 or 0.45 µm Immuno-Blot PVDF membrane depending on the molecular weight of the protein of interest. PVDF membrane must be activated in 100 % methanol and the transference was performed by semi-wet blotting in blotting buffer (25 mM Tris, 192 mM Glycine) at 0.4 A for 30 min in a Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories, Hercules, California, USA).

Once the transference was completed, the membrane was blocked with 5% (w/v) skimmed milk powder in TBS-T buffer (20 mM Tris, 137 mM NaCl, 0,05% Tween 20)

for 1 hour and probed overnight against the following primary antibodies: ARC/ARG3.1 (1:1.000), BDNF (1:3.000), CD68 (1:5.000), GFAP (1:20.000), GluA1 (1:15.000), GluA2 (1:10.000), GluN1 (1:5.000), GluN2A (1:1.000), GluN2B (1:10.000), Iba-1 (1:1.000), PSD95 (1:10.000), s100β (1:500), Synaptophysin (1:40.000) and Synaptotagmin-1 (1:40.000). The membranes were then washed 4 times with TBS-T during 15 min each and incubated for 1 hour with a secondary antibody from donkey HRP-linked anti-rabbit IgG (GE Healthcare, Little Chalfont, U.K, NA934-1ML).

The bands were revealed using SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Massachusetts, USA, Cat#: 34580), captured on the ChemiDoc™ Touch Imaging System (BioRad).

After film exposure, without letting it to dry, the membrane was washed four times for 5 minutes each in TBS-T, before incubation as described previously for primary antibodies, with either β-actin 1:3000 or β-tubulin 1:15.000, followed by the secondary antibody.

The quantification of the bands was performed with Image Studio™ Lite, an image analysis software from LI-COR Biosciences (Nebraska, USA).

2.2.7. Normalization of data and statistical analysis

To normalize the bands' intensity within the same membrane and between membranes, first, a lane normalization factor was calculated (Equation 1). Afterwards, each band of the blot was divided by its corresponding factor (Equation 2), with which all the values in each membrane were normalized.

$$Lane\ normalization\ factor = \frac{Signal\ of\ housekeeping\ protein\ for\ each\ lane}{Highest\ signal\ of\ housekeeping\ protein\ on\ the\ blot} \quad (1)$$

$$Normalized\ sample\ signal = \frac{Observed\ sample\ signal}{Lane\ normalization\ factor} \quad (2)$$

In order to normalize the values between membranes, an internal control was used. This internal control is a WT brain homogenate that was loaded in the same concentration in both gels. The normalized values of these samples are divided by the highest normalized value of the control (Equation 3), obtaining an intermembrane factor that will be used to normalize the values between membranes (Equation 4).

$$Intermembrane\ normalization\ factor = \frac{Observed\ control\ signal}{Highest\ normalized\ signal\ of\ the\ control\ lanes} \quad (3)$$

$$Intermembrane\ normalized\ sample\ signal = \frac{Normalized\ sample\ signal}{Intermembrane\ normalization\ factor} \quad (4)$$

Statistical tests were performed using IBM SPSS Statistics for Windows (IBM Corp. Released 2017, Version 25.0. Armonk, NY: IBM Corp). Comparisons between two groups were performed using Student's *t*-test if the data met the assumption of normality through the Shapiro-Wilk test. If not, an independent-Samples Mann-Whitney U test was performed. All values were presented as the mean ± SEM. This is presented along with the *p*-values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$), considering statistically significant those values of $p < 0.05$.

In order to remove outliers from the data, those values being 1.5 IQRs away from a boxplot were considered as outliers. In other words, values below Q_1 - 1.5 * IQR are considered low outliers and values above Q_3 + 1.5 * IQR are high outliers.

This statistical procedure has been reviewed by Jo S Stenehjem (Oslo Centre for Biostatistics and Epidemiology, Dept of Biostatistics, University of Oslo and Dept of Research and Development, Div of Emergencies and Critical Care, Oslo University Hospital).

3. Results

3.1. Homogenization

The process of homogenization of the different brain regions had been optimized through changes in the technique as well as in the reagents. The aim was to obtain a complete tissue homogenization, while avoiding degradation of proteins. This had been tested through the western blot technique that allowed observing if all the tissue was entering the gel and running properly.

Four different procedures of homogenization were tested in this project. For procedures 1 to 3 (Figure 8 C, D and E) not all the sample was entering the gel as it can be appreciated in the upper part of the membrane. Moreover, a non-specific band appeared at around 75 kDa. In procedure number 4 (Figure 8F), a clear band was obtained similar to the one obtained in whole brain homogenates made previously in the group by Håvard Ringsevjen (Figure 8B). In this case (Figure 8F), no unspecific bands or tissue not digested unable to enter the gel were observed.

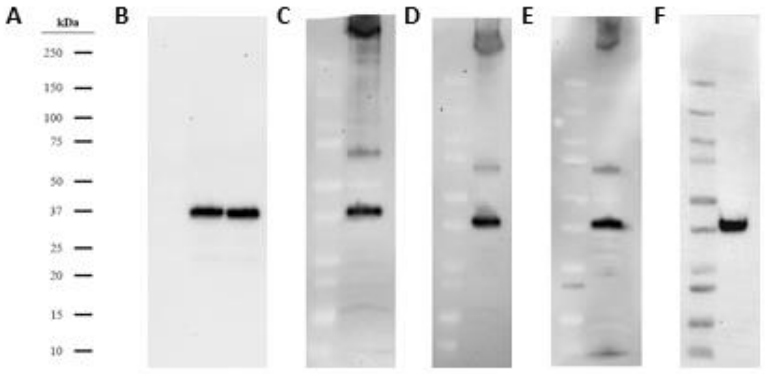


Figure 8. Process of optimization of the homogenization protocol. Western blot with synaptophysin-1 antibody for A) molecular weight standard, B) whole brain homogenate control (Ringsevjen); from C to F, different homogenization protocols: C) procedure 1, D) procedure 2, E) procedure 3 and F) procedure 4. The latter one is the one to be performed in the rest of the project as is the one where all the protein enters the gel and there is just one clear band with little background. (For description of procedures 1-4, see the Methods chapter).

3.2. Optimization of western blot technique

In order to optimize the technique of western blot for our purpose, the following parameters were tested in different variations: protein concentration, antibody concentration, milk concentration in blocking buffer, and pore diameter of PVDF

membranes. I pursued strong bands of interest in the expected location, avoiding non-specific bands, background noise and smear.

The first step was to determine the amount of protein to load into the gel. It is recommended to use between 10 and 50 µg for homogenates. Three different protein concentrations were tested 10, 20 and 30 µg (Figure 9), as the tissue available for each sample was limited, the highest value of 50 µg was omitted. After Western blot, in all of the lanes, a strong band with a little background was obtained.

The following step after the selection of the antibodies to use in the project was to assess the optimal concentration for each one to perform with Western blot. For this purpose, several ratios of each antibody were tested depending on the recommendations provided by the manufacturer (Figure 10).

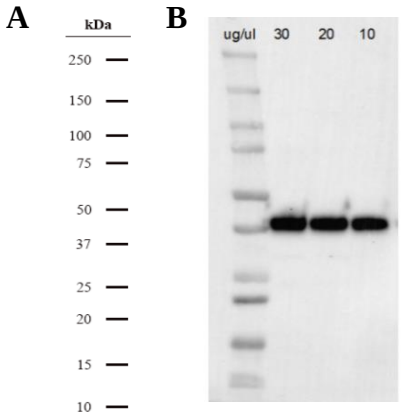
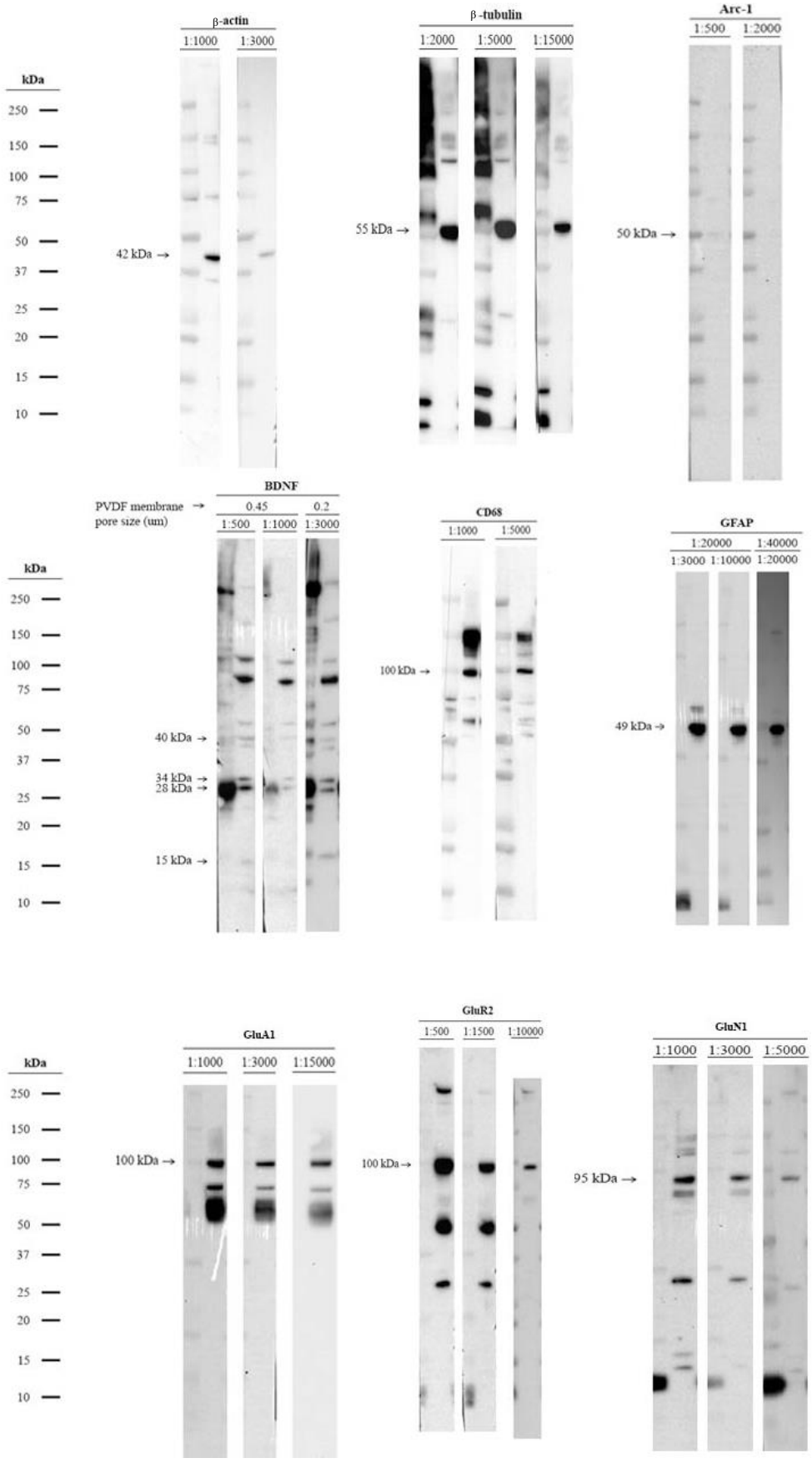


Figure 9. Testing of different amounts of protein loaded into the gel. A) molecular weight standard. B) Concentrations loaded into the gel from left to right: 30, 20 and 10 µg/µl tested with synaptophysin-1 antibody. Clear bands with a little background are obtained in all of them.

In many of the concentrations, several unspecific bands were observed as well as high background and smear. I tried to lower the antibody concentration to avoid bands due to cross-reactivity and make sure it was not degradation products of the same protein that was causing them. For some of the antibodies, this approach was enough, although for others it was necessary to check with the information provided from the manufacturer to reassure those bands were not produced by any technical difficulty from my side.


β-actin
1:1000
1:3000
kDa
250
150
100
75
50
37
25
20
15
10
42 kDa →
β-tubulin
1:2000
1:5000
1:15000
55 kDa →
Arc-1
1:500
1:2000
50 kDa →
BDNF
PVDF membrane pore size (um)
0.45
0.2
1:500
1:1000
1:3000
40 kDa →
34 kDa →
28 kDa →
15 kDa →
CD68
1:1000
1:5000
100 kDa →
GFAP
1:20000
1:40000
1:3000
1:10000
1:20000
49 kDa →
GluA1
1:1000
1:3000
1:15000
100 kDa →
GluR2
1:500
1:1500
1:10000
100 kDa →
GluN1
1:1000
1:3000
1:5000
95 kDa →

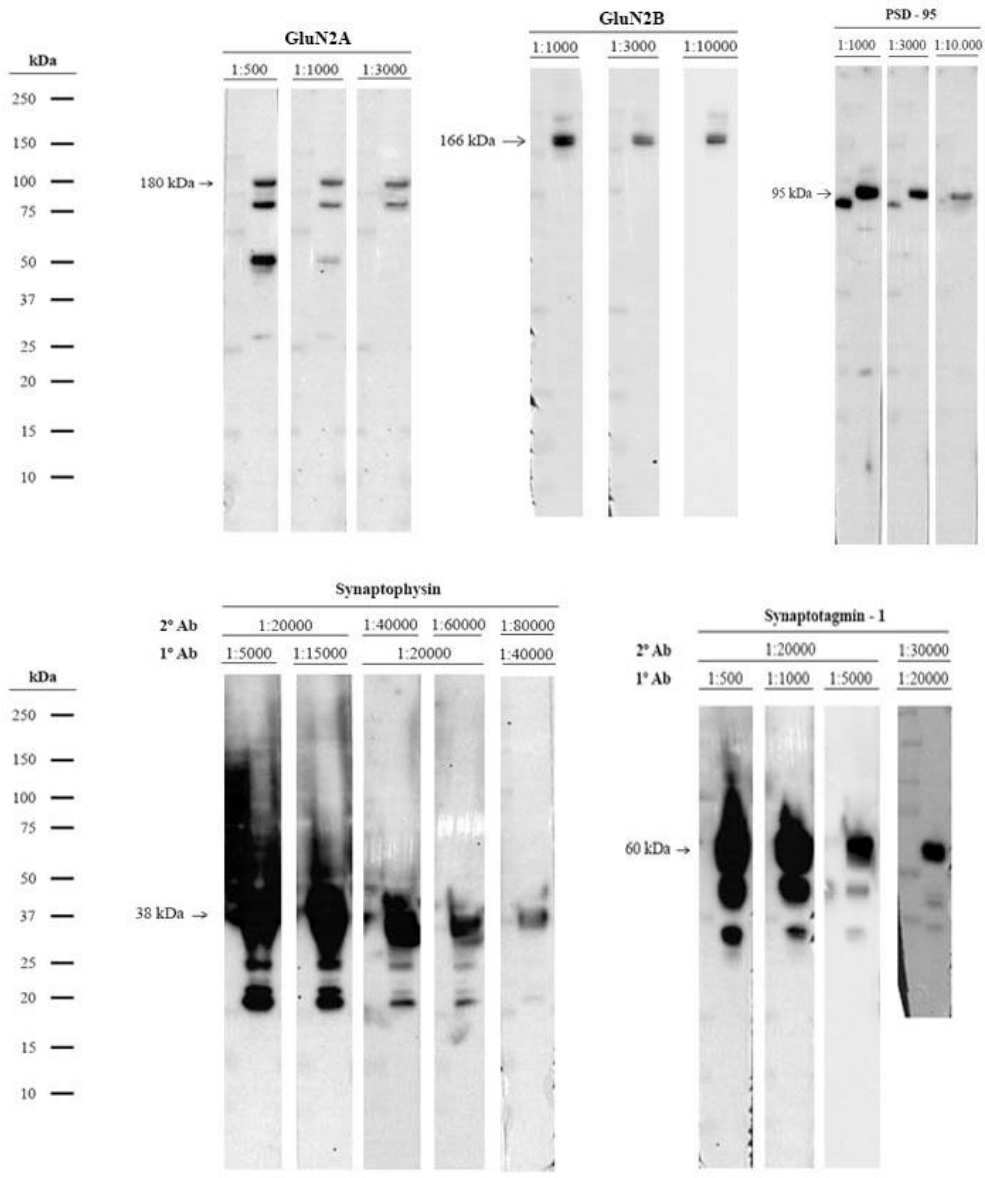


Figure 10. Testing of antibodies concentration. On the left side, the bands that appear in the molecular-weight size marker are shown. On the top of each western blot, the dilution of each antibody is specified as well as the expected band(s) pointed with their molecular weight on the left side.

Nonetheless, some of the antibodies, even at a low concentration, still produced high background and smear. In order to reduce this problem, the milk percentage in blocking buffer was increased in two different ways. On the one hand, 10 % skimmed milk TBS-T as was used as blocking buffer and 5 % skim milk in TBS-T as antibody incubation solution (Figure 11B), and on the other hand, 5 % skimmed milk in TBS-T was used in all of the steps (Figure 11A). While for GluA2 the same unspecific bands appeared in both approaches, for synaptophysin the smear was reduced after blocking with 10 % skimmed milk. On the contrary, synaptotagmin-1 specific band almost disappeared after blocking with 10 % skimmed milk. While the unspecific bands that appear in Figure 11

were not shown after incubation of the antibodies in 5 % skimmed milk in TBS-T, instead of gradually decreasing the percentage as described in *2.2.6. Western blot* (2.5 % in TBS-T for primary antibody and 1.25 % in TBS-T for secondary antibody).

Still, after all these trials, there were some antibodies that did not show any band even at high concentrations (1:150 and 1:500) (Figure 12B and C). All these antibodies have in common that they are against proteins of low molecular weight (< 35 kDa). First, the dot blot technique was performed to confirm the presence of these proteins in the homogenate, obtaining weak signals from all the antibodies tested (Figure 12D).

Once the presence of these proteins in the homogenate was confirmed, the next step was to change the conditions of the western blot technique. For this, both the electrophoresis running time was reduced and the pore size of the PVDF membrane was changed to 0.2 µm (Figure 12E) instead of 0.45 µm (Figure 12B). In this way, both Iba1 and s100β were finally retained into the membrane, while I could not obtain any band for MHC-II and TREM-2 so I decided to omit them from further analyses in the project (Supplementary figure IV.1).

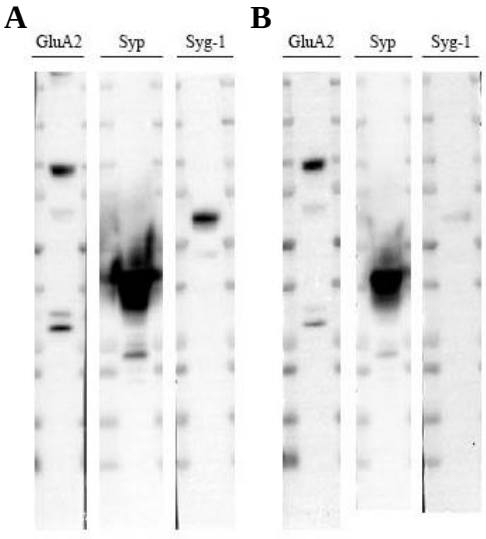


Figure 11. Different percentage of skim milk in blocking buffer and antibody incubation solution. GluA2, synaptophysin and synaptotagmin-1 were incubated with A) 5 % skim milk TBS-T as blocking buffer and 5 % skim milk in TBS-T as antibody incubation solution and B) 10 % skim milk in TBS-T as blocking buffer and 5 % skim milk in TBS-T as antibody incubation solution. Abbreviations: Syp = synaptophysin; Syg-1 = synaptotagmin-1.

Lastly, I chose to continue the project with those antibody concentrations and protocol conditions that made it possible to observe the desired bands with a clear signal and wherein all the bands observed were identified.

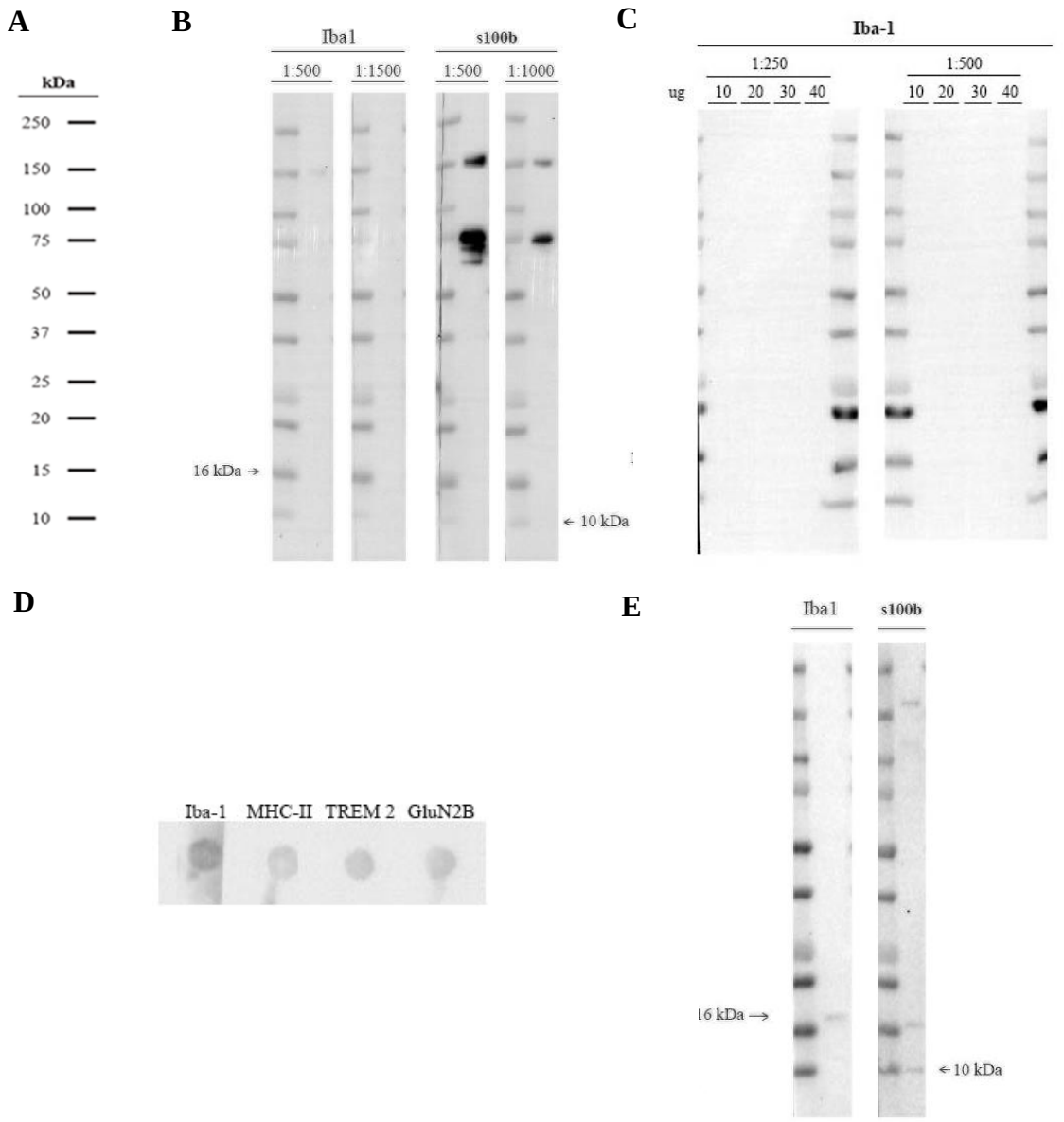


Figure 12. Western blot for low molecular weight proteins. A) Molecular weight marker. B) Western blot of Iba1 and s100β using PVDF membranes of 0.45 µm. C) Western blot of different amount of protein loaded into the gel (10, 20, 30 and 40 µg) incubated with Iba-1 at concentrations of 1:250 and 1:500. D) Dot blot to prove the presence of low molecular weight proteins. Iba1, MHC-II and TREM 2, all of them with molecular weights under 35 kDa were incubated with their respective antibodies to verify their presence in the sample next to a positive control, GluN2B. C) Western blot of Iba-1 and s100β using PVDF membranes of 0.2 µm pore size. The expected molecular weight for each protein is indicated on the sides of the membranes.

3.3. N-3 PUFA concentration in blood changes with diet

In order to test the availability of n-3 PUFA in the body after the different diets, an analysis of both EPA and DHA levels in the blood was performed. Blood samples were obtained from four of the mothers as control and from 10 mice after five months on both n-3 PUFA rich and deficient diets (Figure 13). Blood levels of n-3 PUFA expressed in

g/100 g FAME (represented as mean ± SEM) in the mothers showed values for EPA of 0.34 ± 0.03 and for DHA of 4.86 ± 0.25 (Figure 13A). Serum concentrations of both EPA and DHA (represented as mean ± standard deviation) were lower in mice fed with n-3 PUFA deficient diet (EPA: 0 ± 0; DHA: 0.71 ± 0.03) in comparison with mice fed with n-3 PUFA rich diet (EPA: 2.97 ± 0.12; DHA: 10.55 ± 0.28) (independent two-tailed *t*-test; EPA: t (9) = 24.00, $p < 0.001$; DHA: t (9) = 34.58), $p < 0.001$) (Figure 13B).

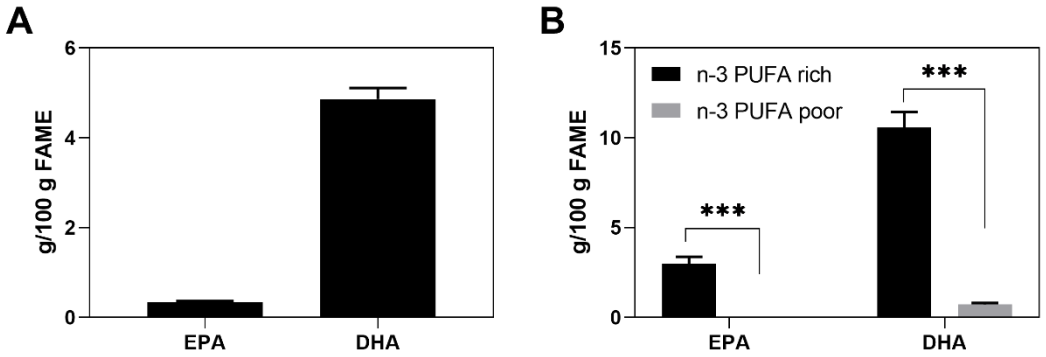


Figure 13. Increased DHA levels in blood serum after n-3 PUFA rich diet. Blood samples analysis of A) the mothers (n = 4) and B) female mice of 5 months of age (n = 10 for each group). g/100 g FAME omega 3 fatty acids: DHA and EPA in blood serum are represented. Independent two-tailed t-test: *** $p < 0.001$ Error bars indicate S.E.M.. FAME = fatty acid methyl esters.

3.4. Changes in the concentration of non-receptor synaptic proteins in the brain influenced by diet

To study the impact at a molecular level of dietary n-3 PUFA on synapses, I performed a comparison of protein concentrations of two presynaptic and one postsynaptic proteins common to the synaptic transmission molecular machinery: synaptophysin and synaptotagmin-1 (both coupled to presynsptic vesicles), and PSD-95 (part of the postsysnaptic density). Their relative concentrations were quantified by western blot analysis in two different parts of the brain: hippocampus and cortex (Figure 14).

In the results obtained for the *hippocampal* samples, no significant difference were found on non-receptor synaptic proteins between the two diets: synaptophysin (n-3 PUFA rich: 37718.21 ± 2410.27; n-3 PUFA poor: 44841.23 ± 1630.08) (independent-Samples Mann-Whitney U Test: $p = 0.467$), synaptotagmin-1 (n-3 PUFA rich: 31702.82 ± 3479.36; n-3 PUFA poor: 27766.87 ± 3270.84) (independent two-tailed *t*-test: $p = 0.569$) and PSD-95 (n-3 PUFA rich: 15975.03.21 ± 1191.25; n-3 PUFA poor: 17128.88 ± 1771.41) (independent two-tailed *t*-test: $p = 0.916$).

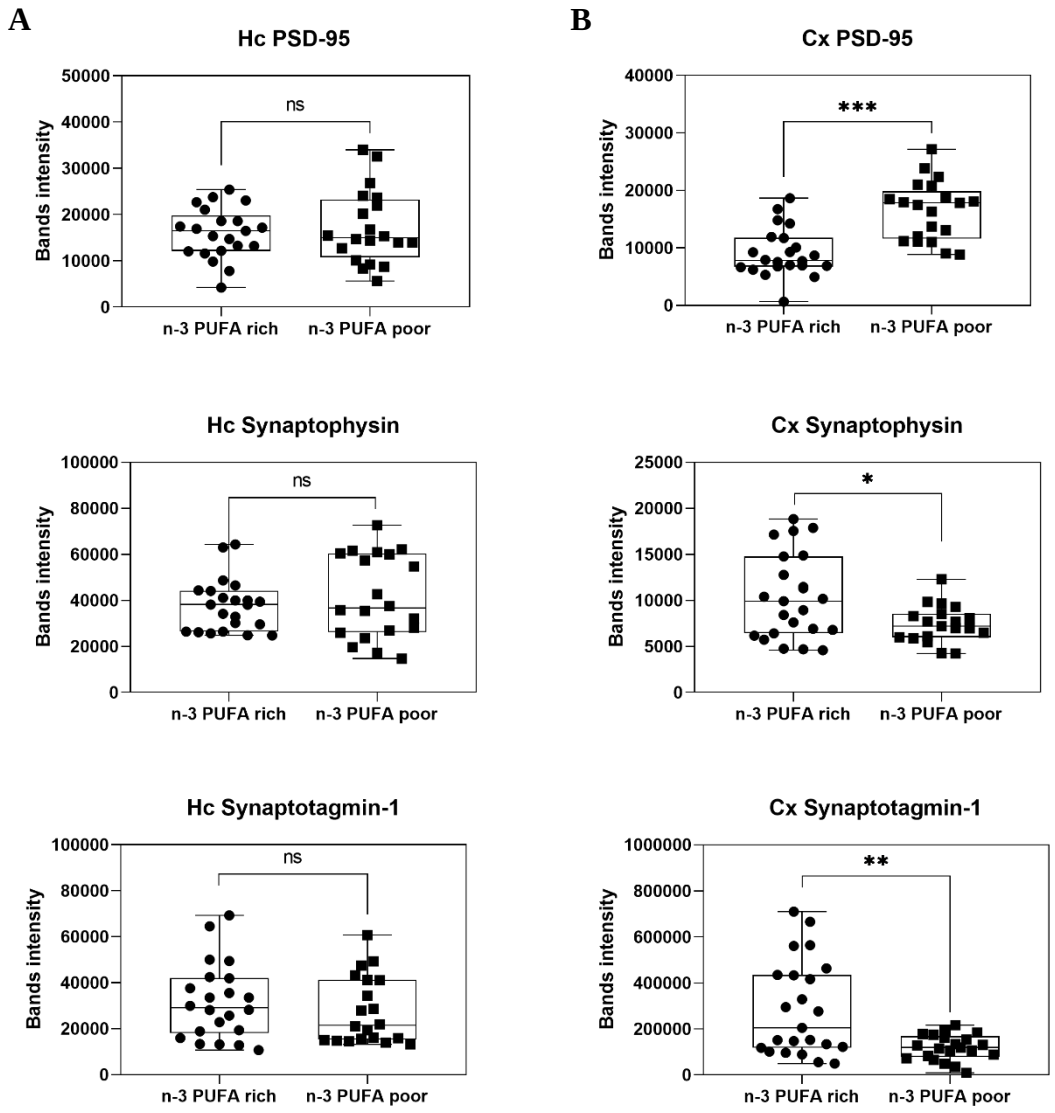


Figure 14. Non-receptor synaptic proteins levels change in the cortex after supplementation with n-3 PUFA. Representative data from one western blot experiment are shown. Proteins analysed in A) hippocampus and B) cortex. ****p* < 0.001, independent two-tailed t-test for Hc synaptophysin and synaptotagmin-1, and independent-Samples Mann-Whitney U Test for the rest of the graphs. Error bars indicate S.E.M.. *N-3 PUFA rich*: n = 21 in Hc PSD-95; n = 22 in Hc synaptophysin, Hc synaptotagmin-1 and Cx PSD-95; n = 23 in Cx synaptotagmin-1 and Cx synaptophysin. *N-3 PUFA poor:* n = 19 in Cx synaptophysin; n = 20 in Hc PSD-95 and Hc synaptotagmin-1; n = 21 in Cx synaptotagmin-1 and Cx PSD-9; n = 22 in Hc synaptophysin. Hc = hippocampus. Cx = cortex.

Meanwhile in the *cortex*, the diet had effect on all of them. In one hand, synaptophysin (n-3 PUFA rich: 10361.13 ± 956.77; n-3 PUFA poor: 7421.70 ± 458.68) (independent-Samples Mann-Whitney U Test: $p < 0.05$*) and synaptotagmin-1 (n-3 PUFA rich: 285453.17 ± 42844.34; n-3 PUFA poor: 119136.09 ± 12080.41) (independent-Samples Mann-Whitney U Test: $p < 0.01$**) showed significantly higher values after n-3 PUFA rich diet. On the other hand, PSD-95 had lower levels in such diet (n-3 PUFA rich:

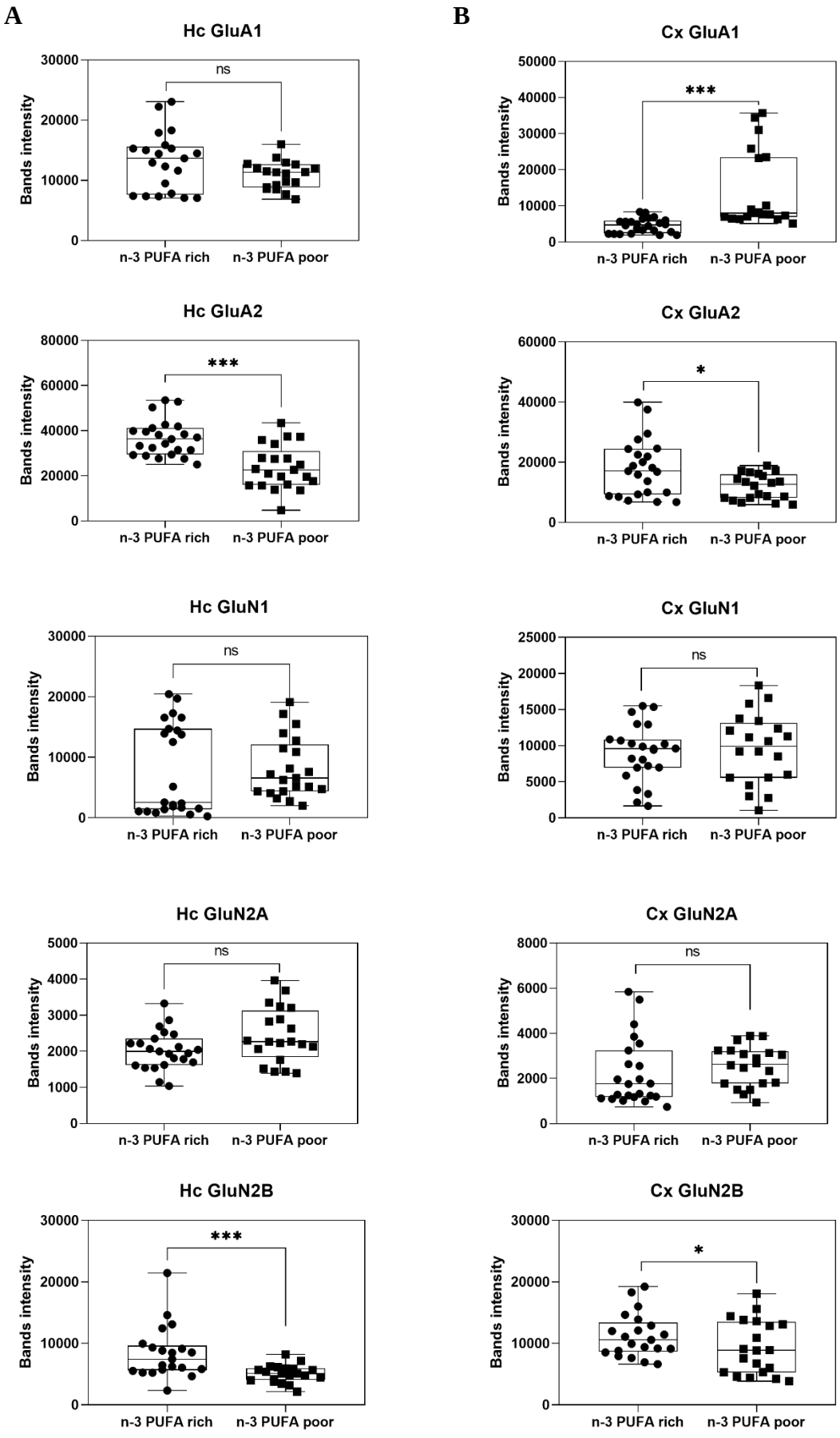
9579.89 ± 799.82; n-3 PUFA poor: 16616.20 ± 1095.71) (independent-Samples Mann-Whitney U Test: $p < 0.001$***) (Figure 14).

3.5. Variations of postsynaptic glutamate receptors subunits in the brain influenced by dietary n-3 PUFA

Glutamate receptors play a key role in glutamatergic transmission as well as in the generation and maintenance of synaptic plasticity. The different subunit combinations that can build them up change their capacity and effectiveness (Henley & Wilkinson, 2016; Kumar & Foster, 2019). This is the reason why several subunits from these receptors had been studied independently in both hippocampus and cortex to analyse the impact of dietary n-3 PUFA on ionotropic glutamate receptors (Figure 15).

On one hand, the AMPAR receptor subunit GluA1 showed no significant difference between diets in the *hippocampus* (n-3 PUFA rich: 13139.85 ± 1061.02; n-3 PUFA poor: 10445.70 ± 660.48; independent-Samples Mann-Whitney U Test: $p = 0.062$) while GluA2 was significantly decreased in the presence of low dietary levels of n-3 PUFA (n-3 PUFA rich: 36623.46 ± 1660.71; n-3 PUFA poor: 23902.31 ± 2025.14; independent-Samples Mann-Whitney U Test: $p < 0.001$***). A similar difference was also observed for the AMPA receptor subunit GluA2 in *cortex* (n-3 PUFA rich: 18087.26 ± 1977.72; n-3 PUFA poor: 11879.39 ± 953.26) independent-Samples Mann-Whitney U Test: $p < 0.05$*). The other way around, GluA1 showed higher values after n-3 PUFA deficiency in the cortex (n-3 PUFA rich: 4665.91 ± 406.58; n-3 PUFA poor: 14209.29 ± 2465.82) (independent-Samples Mann-Whitney U Test: $p < 0.001$***) (Figure 15).

On the other hand, NMDA receptor subunits GluN1, GluN2A and GluN2B showed the same trend in both *hippocampus* and *cortex* (Figure 15). GluN1 (hippocampus: n-3 PUFA rich: 7937.08 ± 1557.36, n-3 PUFA poor: 8689.49 ± 1110.13, independent-Samples Mann-Whitney U Test: $p = 0.254$; cortex: n-3 PUFA rich: 8979.07 ± 819.97, n-3 PUFA poor: 9549.09 ± 1090.83, independent-Samples Mann-Whitney U Test: $p = 0.661$) and GluN2A (hippocampus: n-3 PUFA rich: 2025.36 ± 111.22, n-3 PUFA poor: 2440.38 ± 171.46, independent-Samples Mann-Whitney U Test: $p = 0.155$; cortex: n-3 PUFA rich: 2238.65 ± 308.65, n-3 PUFA poor: 2541.04 ± 195.49, independent-Samples Mann-Whitney U $p = 0.103$) did not show significantly different values between the diets.


A
Hc GluA1
Bands intensity
30000
20000
10000
0
ns
n-3 PUFA rich
n-3 PUFA poor
Hc GluA2
80000
60000
40000
20000
0
***
Hc GluN1
30000
20000
10000
0
ns
Hc GluN2A
5000
4000
3000
2000
1000
0
ns
Hc GluN2B
30000
20000
10000
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***
B
Cx GluA1
50000
40000
30000
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Cx GluA2
60000
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Cx GluN1
25000
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10000
5000
0
ns
Cx GluN2A
8000
6000
4000
2000
0
ns
Cx GluN2B
30000
20000
10000
0
*

Figure 15. Glutamate receptors subunits are affected by n-3 PUFA levels in the diet. Representative data from one western blot experiment are shown. Proteins analysed in A) hippocampus and B) cortex. *N-3 PUFA rich:* n = 21 in Hc GluA1, Hc GluN2B and Cx GRIN2B; n = 23 in Hc GluA2, Hc GluN1, Hc GluN2A, Cx GluA1, Cx GluA2, Cx GluN1 and Cx GluN2A. *N-3 PUFA poor:* n = 19 in Cx GluR1; n = 20 in Hc GluA1, Hc GluN2A, Hc GluN2B, Cx GluA2, Cx GluN1, Cx GLuN2A and Cx GluN2B; n = 21 in Hc GluN1; n = 22 in Hc GluA2. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, independent-Samples Mann-Whitney U Test. Error bars indicate S.E.M.. Hc = Hippocampus; Cx = cortex.

Contrary to this, GluN2B showed significant differences, having higher values in mice fed for 6 months with a diet rich in n-3 PUFA (hippocampus: n-3 PUFA rich: 8414.61 ± 924.66, n-3 PUFA poor: 5069.76 ± 314.93, independent-Samples Mann-Whitney U Test: $p < 0.001$***) (cortex: n-3 PUFA rich: 12601.83 ± 1171.94, n-3 PUFA poor: 9368.06 ± 980.86, independent-Samples Mann-Whitney U Test, $p < 0.05$*).

Taken together, the results from all synaptic proteins so far, indicate that GluA1 and PSD-95 were increased in the cortex after n-3 PUFA deficiency, and synaptophysin and synaptotagmin-1 were decreased. While increased values of GluA2 and GluN2B were observed after n-3 PUFA supplementation in both hippocampus and cortex.

3.6. N-3 PUFA does not have a big impact on two synaptic plasticity markers

Next, I questioned whether two markers of brain plasticity, BDNF and Arc/Arg3.1, were affected by the different diets. These proteins are rapidly and selectively regulated in specific brain regions associated with learning and memory (Bramham & Messaoudi, 2005; Tzingounis & Nicoll, 2006).

On the one hand, BDNF levels showed no difference between diets neither in the *hippocampus* (n-3 PUFA rich: 24465.66 ± 3156.83; n-3 PUFA poor: 40128.10 ± 2924.10) (independent-Samples Mann-Whitney U Test: $p = 0.404$) nor in the *cortex* (n-3 PUFA rich: 23110.53 ± 2049.21; n-3 PUFA poor: 17924.41 ± 1243.61) (independent-Samples Mann-Whitney U Test: $p = 0.113$) (Figure 16).

On the other hand, Arc remained also unchanged in the *hippocampus* (Hc: n-3 PUFA rich: 47196.54 ± 4921.99; n-3 PUFA poor: 68134.23 ± 8617.02) (independent-Samples Mann-Whitney U Test: $p = 0.088$) while in the *cortex*, mice fed with a n-3 PUFA-deficient diet showed significantly higher values of this protein (Cx: EPAX: 9045.67 ± 967.89; n-3 PUFA poor: 14510.36 ± 1829.83) (independent-Samples Mann-Whitney U Test: $p < 0.05$*) (Figure 16).

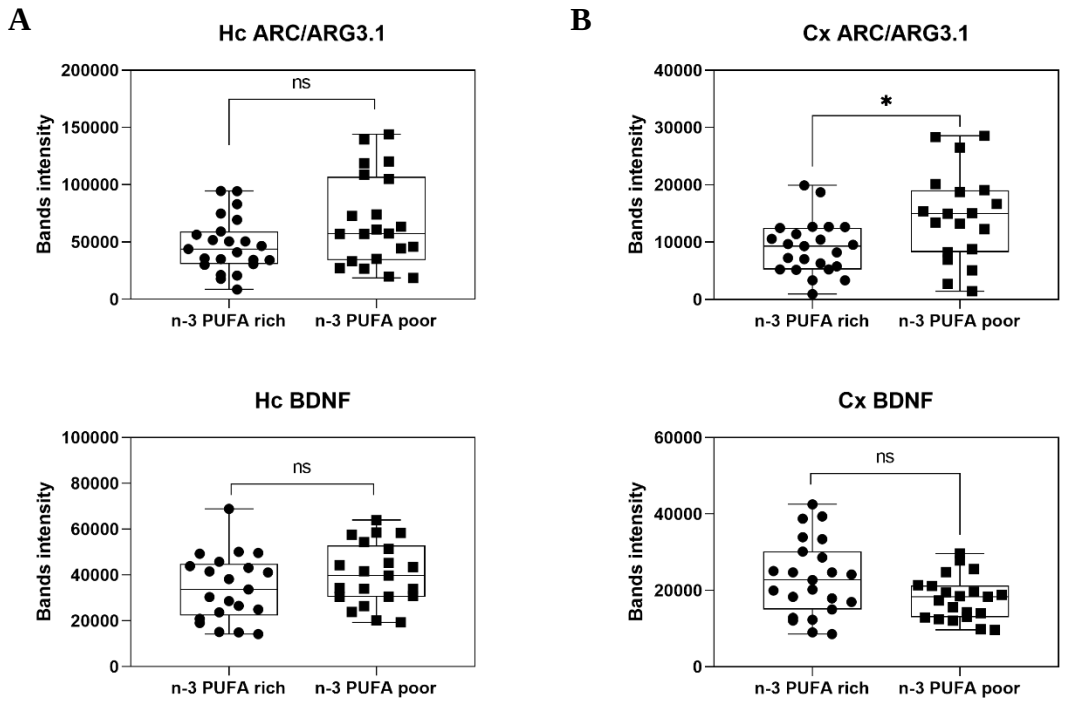

Figure 16. n-3 PUFA deficiency has almost no effect on synaptic plasticity markers Arc/Arg3.1 and BDNF. Representative data from one western blot experiment are shown. Proteins analysed in A) hippocampus and B) cortex. *N-3 PUFA rich:* n = 21 in Hc BDNF; n = 23 in Hc Arc/Arg3.1, Cx Arc/Arg3.1 and Cx BDNF. *N-3 PUFA poor:* n = 19 in Cx Arc/Arg3.1; n = 21 in Hc Arc/Arg3.1, Hc BNDF and Cx BDNF. *p < 0.05, independent-Samples Mann-Whitney U Test. Error bars indicate S.E.M.. Hc = Hippocampus; Cx = cortex.

Altogether, the only diet-dependent difference for these synaptic plasticity markers was for Arc/Arg3.1, which showed lower values in the n-3 PUFA enriched group.

3.7. Effect of diet in glial cells activation

Since microglia activation and neuroinflammation have been related to ageing (Plaza-Zabala et al., 2017), and astrocytes have an important role supporting neurons and maintaining brain homeostasis (Singh & Abraham, 2017), I wanted to assess the impact of n-3 PUFA supplementation on these cellular types.

My results showed that the microglia marker CD68 had no significant difference between the n-3 PUFA rich or poor diets in neither *hippocampus* nor *cortex* (hippocampus: n-3 PUFA rich: 16262.28 ± 2135.69, n-3 PUFA poor: 14544.51 ± 2144.63, independent-Samples Mann-Whitney U Test: p = 0.647) (cortex: n-3 PUFA rich: 1572.40 ± 114.32, n-3 PUFA poor: 1556.30 ± 107.66, independent-Samples Mann-Whitney U Test: p = 0.979). Furthermore, the microglial activation marker Iba1 showed no significant differences between diets in the *hippocampus* (n-3 PUFA rich: 10707.25 ± 1061.72; n-3

PUFA poor: 8885.88 ± 1123.11) (independent-Samples Mann-Whitney U Test: p = 0.794), although it appeared in higher levels in the *cortex* after a n-3 PUFA-deficient diet (n-3 PUFA rich: 8096.34 ± 700.80; n-3 PUFA poor: 21075.30 ± 1762.34) (independent-Samples Mann-Whitney U Test: p < 0.001***) (Figure 17).

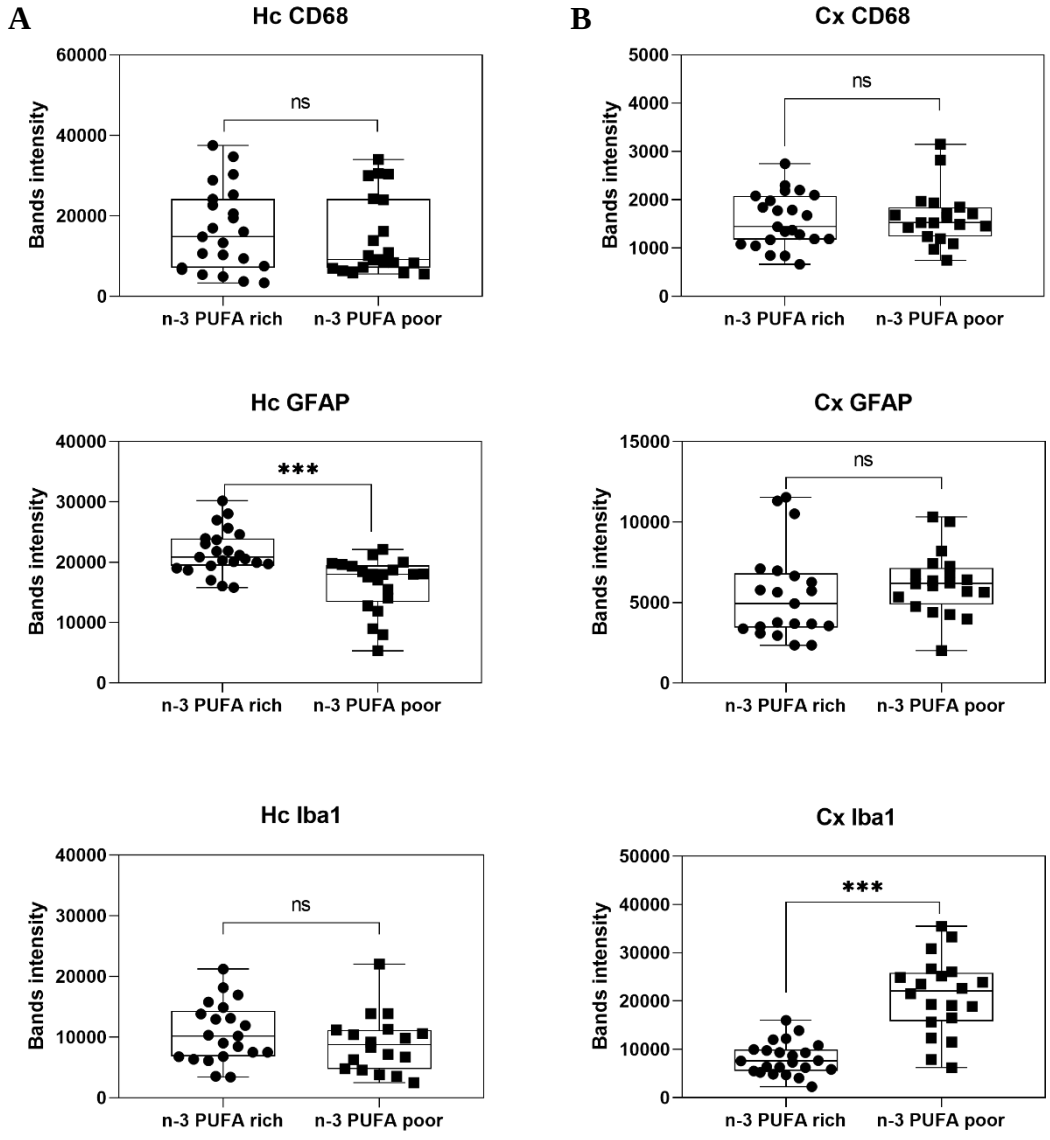

Figure 17. N-3 PUFA regulation of glial activation. Representative data from one western blot experiment are shown. Proteins analysed in A) hippocampus and B) cortex. *N-3 PUFA rich:* n = 21 in Hc Iba1, Cx GFAP; n = 23 in Hc CD68, Hc GFAP, Cx CD68 and Cx Iba1. *N-3 PUFA poor:* n = 18 in Hc Iba1 and Cx CD68; n = 19 in Cx ARC; n = 20 in Cx GFAP; n = 21 in Hc CD68 and Hc GFAP. ****p* < 0.001, independent-Samples Mann-Whitney U Test. Error bars indicate S.E.M.. Hc = Hippocampus; Cx = cortex.

With regard to astrocytes, GFAP, a marker for reactive astrocytes, appeared in higher levels in *hippocampus* after supplementation with n-3 PUFA (n-3 PUFA rich: 21675.79 ± 768.41; n-3 PUFA poor: 16312.05 ± 987.50) (independent-Samples Mann-Whitney U Test: p < 0.001***), while in *cortex* no difference was observed between the diets (n-3

PUFA rich: 5469.41 ± 610.41; n-3 PUFA poor: 6204.82 ± 434.87) (independent-Samples Mann-Whitney U Test: p = 0.130) (Figure 17).

From s100β the bands merged at the end of the membrane and clear bands were not obtained so the analysis was not performed (Supplementary figure IV.2).

In essence, GFAP did not differ between diets, while Iba-1 did but only in the *cortex* where it showed higher values after a deficit of n-3 PUFA.

4. Discussion

My findings provide evidence that an n-3 PUFA deficit for 6 months after weaning effectively results in low levels of n-3 PUFA in blood, in contrast to a diet rich in n-3 PUFA, where high levels of the fatty acids are found in blood. In *hippocampal* and *cortical* tissue this deficiency leads to down-regulation of the glutamate receptor subunits GluA2 and GluN2B. Furthermore, in the *cortex* the markers for synaptic plasticity Arc/Arg3.1 and microglia activation Iba-1, are upregulated, as well as the glutamate receptor subunit GluA1 in mice fed with n-3 PUFA deficient diet. While, synaptophysin and synaptotagmin-1 are increased after supplementation with n-3 PUFA. These provide insights into the role of n-3 PUFA in synaptic plasticity and ageing.

4.1. Methodological considerations

The C57BL/6 inbreed line has been widely used in research due to its physical condition, the capacity of learning and frequency of breeding. As it is an inbred line all the individuals are genetically identical, minimizing the variations due to the genetic background of the individuals. This makes this type of line suitable to test the effect of n-3 PUFA in the diet. Meanwhile, the environmental factors had been kept in the same conditions just altering the diet to minimize the impact of any external factor. Even so, variations between individuals can arise and impact the results. For this reason, a sample population of 25 mice on each diet (even though some animals were lost along the process) was used to diminish the impact of these variations in the final outcome. Furthermore, each mouse had been studied independently from the others.

The number of samples analysed was high, controls must be included to ensure a proper process of data normalization. The first control measure that was taken, was for the quantification of protein concentration in each sample, to ensure the same amount of protein was always loaded into the gel. Nonetheless, a second control was added to ensure the results obtained are not due to loading or protein transfer errors. For this purpose, the housekeeping proteins β-actin and β-tubulin were chosen as they maintain their values unaltered between the different situations tested. The housekeeping control was used to normalize the values of the protein of interest in each band and normalized the values within the same membrane. This will help to remove unavoidable difficulties during the

western blot technique that drive to a non-even process along the membrane. Finally, I used an inter-membrane control, I loaded the same sample in all the gels what allow us to normalized the values between membranes. This is due to the fact that even if the membranes are treated the same way, some variability always occurs.

The behaviour of the antibodies is another aspect I had to take into account. It is important to know the specificity of the antibodies I used to be able to perform an accurate analysis. Extra precaution had been taken to understand where the bands for each antibody appear to avoid cross-reactivity and miss-identification of the proteins. Even though, sometimes difficulties in the antibodies binding have been faced requiring repetition of the blots or it has challenged the analysis. Furthermore, sometimes the binding of the antibodies is not homogeneous along the membrane leading to bias in the results. For instance, some antibodies may had bound to the molecular weight marker, causing the molecular weight band to increase in intensity and partly merge with and interfere with the one from the sample right next to it. Also, in many cases, the samples in the borders of the membrane had a higher signal than the rest. (Supplementary figure IV.3 and IV.4). An outlier analysis has been carried out, and as no differences with the outcome obtained without them were observed, I continue the statistical analysis without these outlier data (Appendix III).

With all these precautions, I tried to get rid of statistical anomalies in the values that could bias the data analysis. But even after all these steps, outliers appear and also some segregation in the data was observed, wherein some groups there appeared to be two populations behaving differently. These technical difficulties are hard to avoid, and with only one experiment, the risk of having biased data is which may affect the outcome cannot be excluded.

4.2. Omega-3 fatty acid concentrations in blood are dependent on the composition of the diet

The difference observed in n-3 PUFA levels in blood serum is coherent with the diet administered. The animals that were supplemented with n-3 PUFA showed significantly higher levels of DHA and EPA in the blood (Figure 13B). While the mothers, which were given a standard animal facility diet are considered as control values for DHA and EPA levels in blood serum (Figure 13A).

4.3. N-3 PUFA effects on synaptic plasticity

Brain ageing is associated with alterations in synaptic function. It is well known that synaptic plasticity decreases with ageing, and that this is associated with a decline in cognitive performance (Bettio et al., 2017). Glutamate receptors are key factors in the generation of LTP, which is a prototype of synaptic plasticity that is integral to learning and memory (Riedel, 2003). Several studies have tested the impact of n-3 PUFA on synaptic plasticity and in particular on the levels of glutamate receptor subunits, both AMPAR and NMDAR. But there are many other proteins that allow synaptic plasticity to happen as vesicle-trafficking proteins, neurotrophic factors, and scaffolding proteins and their associated proteins. I not only show the effect of n-3 PUFA supplementation on different classes of synaptic proteins in the normal brain but also how this may affect different brain regions.

4.3.1. Synaptic proteins in the cortex are affected by changes of dietary n-3 PUFA but not in the hippocampus

Synaptophysin and synaptotagmin-1, synaptic vesicle trafficking proteins, and PSD-95, a postsynaptic scaffold protein, did not show any change related to the diet in the *hippocampus* (Figure 14A). These results contrast with the ones found by Aryal et al. (2019) where they showed a reduction of synaptophysin and synaptotagmin-1 in hippocampal synaptosomes from mice with a n-3 PUFA deficient diet. In the Aryal study, there were no observabel changes in PSD-95. Although in other studies on the hippocampus and whole brain homogenates PSD-95 was observed in higher values after supplementation with n-3 PUFA (VanGuilder et al., 2010; Sidhu et al., 2016). The results regarding this protein are diverse, but this can be due to the different approaches of each study regarding the whole brain or brain regions and treatments of the samples. For instance, the difference observed with the study of Aryal et al. (2019) despite the resemblance, might be due to the use of synaptosomes, an isolated synaptic terminal from a neuron, to study the protein concentration while we used hippocampus and cortex homogenates with no further purification of synaptosomes. Synaptosomes may contain specifically the functionally active proteins, while whole brain homogenates, as in the present study, may reflect also changes in gene expression. Most “synaptic” proteins are present in large quantities throughout the cell, with only a small fraction functionally present in the synapse.

In the *cortex*, on the contrary, synaptotagmin-1 and synaptophysin show lower values after a deficit of n-3 PUFA (Figure 14B). Synaptotagmin-1 is Ca^{2+}-sensor regulating vesicle exocytosis in synapses (Brose et al., 1992) and it has recently been found to regulate also postsynaptic vesicle exocytosis of glutamate receptors (Hussain et al., 2017). Synaptophysin is also involved in the regulation of trafficking vesicles, specially endocytic processes (Kwon & Chapman, 2011). Furthermore, PSD-95 has higher values after a n-3 PUFA poor diet. PSD-95 is a scaffold protein in charge of regulating the localization of many receptors and signalling proteins (Chen et al., 2011).

4.3.2. N-3 PUFA changes the levels of glutamate receptor subunits

Our study strongly indicates that a shortage of dietary n-3 PUFA leads to a reduction of several glutamate receptor subunits. GluA2 and GluN2B showed lower levels in both hippocampus and cortex after 6 months of a n-3 PUFA deficient diet (Figure 15). These two are in some ways the most interesting AMPAR and NMDAR subunits, respectively, since GluA2 has a major role in the regulation of AMPAR Ca^{2+} permeation and voltage rectification (SalPietro et al., 2019), and GluN2B is strongly coupled to LTP and synaptic plasticity (Shipton & Paulsen, 2013). Moreover, increased values of GluA1 in the cortex in n-3 PUFA deficient samples were observed too, which may indicate that there is a net increase in AMPA receptors in the cortex under n-3 PUFA deficiency, though we do not know whether the increase in GluA1 subunits are actually present in the synapses themselves.

Nevertheless, the reduction of GluA2 and GluN2B influenced by an n-3 PUFA deficit is in accordance with the ones previously obtained by several studies. Aryal et al. (2019) also showed increased values of these subunits in synaptosomes from n-3 PUFA enriched mice. Dyal et al. (2006) as well observed increased levels of GluA2 and GluN2B, but also of GluA1, in the adult forebrain after diet supplementation with DHA. Change was also observed in aged mouse brains with n-3 PUFA deficit (Sidhu et al. 2016). Several studies have observed lower levels of GluA2 subunit of AMPAR with ageing, modifying the calcium cellular dynamics (Dyall et al., 2007; Hara et al., 2012; Yu et al., 2011), which might lead to the neurodegeneration associated with ageing (Foster & Kumar, 2002).

Other projects focused on the impact of n-3 PUFA deficit during development had results in line with ours. N-3 PUFA-deficient cultures showed decreased levels of the glutamate receptors GluA1, GluA2 and GluN2B in dissociated hippocampal cultures, but also in the

rest of subunits as GluN1 and GluN2A (Cao D et al. 2009). A limitation of this study is that they only looked at cultured neurons in young animals, so long term effects on living mice as ours was not investigated.

Furthermore, no difference was observed in GluN1 or GluN2A (Figure 15). NMDARs are heterotetramers constituted by two obligatory subunits of GluN1 and two other subunits of GluN2, that vary depending on the cell type they are expressed in and on the developmental stage of the individual (Wyllie et al., 2013). The different combinations of GluN2 subunits control the electrophysiological properties of the NMDAR (Shipton & Paulsen, 2013). This is to say, GluN1 can be taken as a measure of the total amount of NMDAR. In our study, we do not observe any change in the amount of NMDAR between diets, but its constitution changes as we observed more GluN2B subunits after n-3 PUFA supplementation, therefore the efficiency of the receptor is changed. It has been observed that ageing in rodents is accompanied by a reduction in GluN2B which correlates with a decline in learning (Clayton & Browning, 2001; Dyall et al., 2007).

The decrease I observed in the number of both GluN2B and GluA2 subunits in n-3 PUFA deficient mice might be key factors behind a reduction in synaptic plasticity in the aged brain, and supports our hypothesis of the protective function of n-3 PUFA especially during ageing, what can increase memory delay in neurodegenerative diseases as AD making neurons less susceptible to neurotoxicity.

4.3.3. Synaptic plasticity markers are slightly affected by n-3 PUFA levels in the diet

Arc is an immediately early gene crucial for memory consolidation (Guzowski et al., 2000). After activation, Arc is targeted to the PSD of active dendritic spines (Moga et al., 2004) where it contributes to AMPAR endocytosis, regulating synaptic strength. If disruption of its function can lead to significant loss of dendritic spines and synaptic activity, leading to synaptic dysfunction similar to the one observed in some neurological disorders as AD (Hsieh et al., 2006). Reduced Arc expression in hippocampus interferes with learning and memory (Guzowski et al., 2000). But increased levels of Arc lead to lower levels of AMPAR, limiting the level of neuroexcitation. We observed a trend in the *hippocampus* where the values of this proteins are higher in n-3 PUFA poor diet, but it does not reach the significance value, while in the *cortex* the levels of Arc are significantly higher in n-3 PUFA poor diet (Figure 16). It is likely that the decreased concentration level of GluA2, as shown in this work, follows the increase in the concentration of Arc in

the PSD, as Arc is involved in the endocytosis of AMPARs (Chowdhury et al., 2006). Previous studies, contradict this result as Talamonti et al. 2019 observed that in mice with a deficient synthesis of DHA the expression of both Arc-1 and BDNF is significantly lower (Talamonti et al., 2020). We do not confirm these latter results in our experiments (Figure 16).

We also tested BDNF. BDNF plays a critical role in neural development and neuroprotection (Huang & Reichardt, 2001). In addition, BDNF is involved in LTP and consolidation of memories in the hippocampus (Tyler et al., 2002). No clear effect of n-3 PUFA on BDNF is observed, even though there is some trend towards higher values of BDNF in n-3 PUFA rich diet in cortex, but it does not reach a significant level (Figure 16).

Briefly, the small differences observed do not allow us to conclude strongly on the effect of n-3 PUFA on these synaptic plasticity markers.

4.4. N-3 PUFA alters the microglia response without affecting astrogliosis

Novel insights into the pathogenesis of neurodegeneration, highlight the role of inflammation and glial cell activation (Patterson, 2015). Furthermore, the role of n-3 PUFA as an anti-inflammatory compound and its impact on glial cells have gained importance (Ahmad et al., 2019).

Mice with a deficient synthesis of DHA show increase microglial activation without affecting astrogliosis (Talamonti et al., 2020). We observed also these changes in *cortex* influenced just by the n-3 PUFA dietary composition, where higher values of Iba-1 in mice with n-3 PUFA poor diet were observed (Figure 18B). Iba-1 is a microglia specific calcium-binding protein that participates mainly in the process of phagocytosis in activated microglia. Our result, then, probably points to a higher activation of individual microglia, as the total number of microglial cells did not increase, as shown by no difference in the levels of CD68. However, in the *hippocampus*, n-3 PUFA did not affect the anti-inflammatory response of microglia nor the activation of astrocytes (Figure 17A).

Furthermore, the astrocytic activation marker, GFAP (Eng & Ghirnikar, 1994), showed no influence of diet neither in hippocampus nor cortex (Figure 17). We could not analyse s100β as the bands we obtained after Western blot were merged (Supplementary figure

IV2). This made it not possible to differentiate between the samples. s100β is a Ca^{2+} binding peptide exerting both intra- and extracellular functions (Donato et al., 2009). It is mainly found in the cytoplasm, and nucleus of astrocytes, thus this marker would have given information about astrocytes' abundance.

4.5. Different impact of n-3 PUFA on hippocampus and cortex

I have obtained different outcomes for several antibodies between hippocampus and cortex. One explanation for this could be the different levels of n-3 PUFA in those tissues. Each brain region has different amounts of n-3 PUFA therefore, changes on the levels of this compound in diet affect them in different degrees (Carrié et al., 2000; Joffre et al., 2019; Xiao et al., 2005). N-3 PUFA is found in the cellular membranes of neurons and other cell types in different concentrations (Bourre et al., 1984), thus dietary n-3 PUFA will have a varying effect among them. Another explanation could be the different functions these brain regions performed.

4.6. Limitations

One major limitation of this study was the number of trials conducted. Initially, we designed this study to be repeated at least three times in 4 different brain areas, however, due to time constraints this could not be completed. Although the results obtained are both interesting in their own right, and promising, these repetitions are necessary in order to strengthen their reliability. Western blot procedure carries some technical difficulties that might bias the results as for instance gels running differently along their surface, blotting variations in the same membrane or fainted bands after ECL incubation. The impact of these variabilities can be minimized by using the mean of several replicas. Therefore, we have strong intentions of expanding this study in the near future.

The uptake of n-3 PUFA in the brain and within it, across brain areas, might differ. This can be an explanation to the different concentrations of protein found among these areas, reason why further studies in this field should perform analysis of fatty acids composition in the brain in order to detect changes in n-3 PUFA uptake by different areas or cell types.

Another problem we have to face was the fact that some antibodies were not working as expected. After all the trials there, we unfortunately had to exclude MHC-II, TREM2 and s100β, which are all important markers of microglial cells.

Another limitation of this study is that we just tested the impact of a poor and rich n-3 PUFA diet, while no control diet was used. I would use in the future some diet controls to have better insights into the role of n-3 PUFA in synaptic plasticity and microglial cells. As of now the only conclusion obtained is that there is a difference between poor and rich n-3 PUFA diet but it would be also interesting to know if these values improve or decrease in reference to a standard diet.

Finally, proteins found in the whole neuron have been studied in the present investigation, instead of using synaptosomes where only proteins found in the synaptic terminal are found. This fact can lead to results not directly related to synaptic plasticity as proteins found in other regions of the cell are also quantified. We could not perform the preparation of synaptosomes due to limitations with the amount of sample obtained per hippocampus or cortex. The advantage, however, is that we probably get a truer picture of gene expression changes.

4.7. Translational value and future implications

Ageing is a complex process that can be studied from different points to shed light on potential therapeutic interventions to prevent some of its more unfortunate manifestations and to gain insights from its mechanism of action. Decreased cognitive performance is one of the manifestations observed in ageing people even in the absence of any pathological condition. Since life expectancy has increased considerably in the last few years, identifying potential ways to minimize the impact of age is important.

The present thesis focuses on a specific dietary intervention in order to study its impact on aspects of synapses in the normal brain. My further aim, howver, is to shed light also on mechanisms that are important for the ageing brain. My results seem to indicate that an n-3 PUFA rich diet may be beneficial for protecting synaptic function and plasticity through increased levels of glutamate receptors subunits GluA2 and GluN2B as well as protecting the brain from neuroinflammation, with lowering microglia activation.

These two aspects are compromised in Alzheimer's disease (AD). There is strong evidence that AD is accompanied by increased activity of inflammatory mechanisms in

the brain (Heneka et al., 2015). Moreover, patients with this disease are characterized by decreased cognitive performance. Dietary interventions in AD patients have been widely studied ad a way of delaying or preventing the onset of the symptoms (Calon et al., 2004; Cole & Frautschy, 2006; Engelborghs et al., 2014; van der Beek & Kamphuis, 2008). Our group has already started on a follow up to this present study where we will investigate how changes in dietary n-3 PUFAs might up- or down-regulate proteins associated with synaptic plasticity and neuroinflammation in an AD model.

5. Conclusions

1. Non-receptor synaptic plasticity proteins are diversely regulated by n-3 PUFA in diet, having n-3 PUFA more impact on cortex than on hippocampus.

2. N-3 PUFA in the diet has an effect on glutamate subunits receptors GluA2 and GluN2B, which are crucial for learning and memory.

3. Synaptic markers, Arc-1 and BDNF are not significantly influenced by the dietary content of n-3 PUFA.

4. N-3 PUFA does not lead to any changes in astrocytic function, while it may have anti-inflammatory effects in the cortex, as observed with a reduction in Iba1.

5. The impact of n-3 PUFA differs between cortex and hippocampus, being stronger in the cortex. This suggests different absorption and/or metabolism of these compound in the different brain regions.

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Appendix I: Animal diet composition

Supplementary table I.1. Mineral Mix S10022G composition.

Class description	Ingredient	Grams (g)
Carbohydrate	Sucrose, Fine Granulated	221,03
Mineral	Calcium Carbonate, Light, USP	357,00
Mineral	Potassium Phosphate, Monobasic	196,00
Mineral	Sodium Chloride	74,00
Mineral	Potassium Citrate, Monohydrate	70,78
Mineral	Potassium Sulphate	46,60
Mineral	Magnesium Oxide, Heavy, DC USP	24,00
Mineral	Ferric Citrate	6,06
Mineral	Zinc Carbonate	1,65
Mineral	Sodium Metasilicate	1,45
Mineral	Manganese Carbonate Hydrate	0,63
Mineral	Copper carbonate	0,30
Mineral	Chromium Potassium Sulphate	0,28
Mineral	Boric Acid	0,08
Mineral	Sodium Fluoride	0,06
Mineral	Nickel (II) Carbonate	0,03
Mineral	Lithium Chloride, Anhydrous	0,02
Mineral	Sodium Selenate	0,01
Mineral	Potassium Iodate	0,01
Mineral	Ammonium Molybdate Tetrahydrate	0,01
Mineral	Ammonium (meta)vanadate	0,01
	Total:	1000,00

Supplementary table I.2. Vitamin Mix V10037 composition.

Class description	Ingredient	Grams (g)
Carbohydrate	Sucrose	971,93
Vitamin	Vitamin E Acetate 50 %	15
Vitamin	Niacin (a.k.a. B3)	3
Vitamin	Vitamin B12	2,5
Vitamin	Biotin	2
Vitamin	Pantothenic Acid, D, Calcium (a.k.a B5)	1,6
Vitamin	Vitamin D3	1
Vitamin	Vitamin A Acetate	0,8
Vitamin	Pyridoxine HCL (a.k.a. B6)	0,7
Vitamin	Riboflavin (a.k.a. B2)	0,6
Vitamin	Thiamine HCl (a.k.a. B1)	0,6
Vitamin	Folic Acid	0,2
Vitamin	Phylloquinone (a.k.a. Vitamin K1)	0,08
	Total:	1000

Appendix II: Protein concentration of homogenates

Supplementary table II.1. The protein concentration of homogenates. The concentration measured with PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific, Massachusetts, USA) expressed in µg/µl. Cx = Cortex, Hc = Hippocampus, PUFA = polyunsaturated fatty acids.

n-3 PUFA poor			n-3 PUFA rich		
Mouse	**Hc**	**Cx**	**Mouse**	**Hc**	**Cx**
1-1	3,075	3,140	**1-1**	2,213	5,813
1-6	2,642	2,268	**1-3**	3,339	4,049
1-7	2,866	7,198	**1-4**	3,286	3,463
1-8	3,157	4,024	**1-5**	3,581	6,064
2-4	3,223	3,293	**1-6**	3,352	2,646
3-1	0,000	2,835	**1-7**	3,585	4,322
3-4	3,553	4,948	**2-2**	2,207	2,591
4-8	2,412	1,301	**3-9**	1,692	1,868
2-5	2,671	4,698	**3-7**	3,778	0,928
2-9	2,869	7,344	**4-4**	2,467	3,182
4-2	3,624	5,389	**4-6**	2,202	3,116
3-2	3,730	5,077	**2-3**	3,403	3,635
4-3	2,526	4,574	**3-2**	3,787	4,472
4-4	3,101	4,884	**4-8**	2,270	1,944
2-1	3,780	5,758	**4-1**	3,891	4,420
1-2	3,437	5,015	**1-2**	4,166	6,242
1-3	4,324	3,861	**4-3**	2,984	4,260
1-4	3,239	3,524	**2-4**	3,789	6,510
1-5	3,825	3,650	**4-5**	3,411	4,157
3-7	4,089	2,575	**3-5**	2,818	5,702
3-8	3,630	3,368	**2-7**	3,249	3,005
4-9	4,525	2,349	**1-8**	3,241	5,267
			2-9	3,343	2,284

Appendix III: Statistical analysis with and without outliers

A. Statistical analysis from the hippocampus

Supplementary table III.1. Tests of normality Shapiro Wilk for hippocampus samples. Significance level: $p < 0.05$. Df = degrees of freedom.

		All data points		Data without outliers	
	Group	**df**	**p-value**	**df**	**p-value**
ARC	n-3 PUFA poor	18	0,158	14	0,049
	n-3 PUFA rich	22	0,419	16	0,522
BDNF	n-3 PUFA poor	18	0,414	14	0,646
	n-3 PUFA rich	22	0,000	16	0,285
CD68	n-3 PUFA poor	18	0,004	14	0,004

	n-3 PUFA rich	22	0,089	16	0,237
GFAP	n-3 PUFA poor	18	0,086	14	0,025
	n-3 PUFA rich	22	0,607	16	0,218
GluA1	n-3 PUFA poor	18	0,504	14	0,301
	n-3 PUFA rich	22	0,006	16	0,177
GluA2	n-3 PUFA poor	18	0,595	14	0,177
	n-3 PUFA rich	22	0,106	16	0,192
GluN1	n-3 PUFA poor	18	0,075	**14**	**0,006**
	n-3 PUFA rich	22	0,001	**16**	**0,001**
GluN2A	n-3 PUFA poor	18	0,362	14	0,752
	n-3 PUFA rich	22	0,931	16	0,997
GluN2B	n-3 PUFA poor	18	0,972	14	0,935
	n-3 PUFA rich	22	0,001	16	0,078
Iba-1	n-3 PUFA poor	**18**	**0,003**	14	0,658
	n-3 PUFA rich	**22**	**0,000**	16	0,605
PSD95	n-3 PUFA poor	18	0,096	14	0,156
	n-3 PUFA rich	22	0,000	16	0,903
Synaptophysin	n-3 PUFA poor	18	0,182	14	0,304
	n-3 PUFA rich	22	0,024	16	0,157
Synaptotagmin-1	n-3 PUFA poor	**18**	**0,002**	14	0,170
	n-3 PUFA rich	**22**	**0,014**	16	0,831

Supplementary table III.2. Hypothesis Test Summary Independent-Samples. *** p-value < 0.001. NS = no significant, Syp = synaptophysin, syg-1 = synaptotagmin-1.

	All data points			Data without outliers		
	Statistical test	p-value	Sig.	Statistical test	p-value	Sig.
ARC	Mann-Whitney U	0,088	NS	Mann-Whitney U	0,088	NS
BDNF	Mann-Whitney U	0,155	NS	Mann-Whitney U	0,404	NS
CD68	Mann-Whitney U	0,647	NS	Mann-Whitney U	0,647	NS
GFAP	**Mann-Whitney U**	**0,000**	***	**Mann-Whitney U**	**0,000**	***
GluA1	Mann-Whitney U	0,081	NS	Mann-Whitney U	0,062	NS
GluA2	**Mann-Whitney U**	**0,000**	***	**Mann-Whitney U**	**0,000**	***
GluN1	T-test for Equality of Means	0,696	NS	Mann-Whitney U	0,254	NS
GluN2A	Mann-Whitney U	0,080	NS	Mann-Whitney U	0,155	NS
GluN2B	**Mann-Whitney U**	**0,001**	***	**Mann-Whitney U**	**0,001**	***
Iba1	Mann-Whitney U	0,269	NS	Mann-Whitney U	0,794	NS
PSD95	Mann-Whitney U	0,917	NS	T-test for Equality of Means	0,916	NS
Syp	Mann-Whitney U	0,467	NS	Mann-Whitney U	0,467	NS
Syg-1	Mann-Whitney U	0,465	NS	T-test for Equality of Means	0,569	NS

B. Statistical analysis from the cortex

Supplementary table III.3. Tests of normality Shapiro Wilk for cortex samples. Significance level: p < 0.05. Df = degrees of freedom.

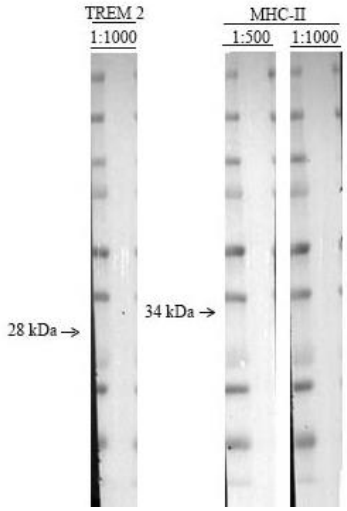
		All data points		Data without outliers	
	Group	**df**	**p-value**	**df**	**p-value**
ARC	n-3 PUFA poor	17	0,000	12	0,452
	n-3 PUFA rich	22	0,364	18	0,807
BDNF	n-3 PUFA poor	17	0,768	12	0,995
	n-3 PUFA rich	22	0,578	18	0,647
CD68	n-3 PUFA poor	17	0,193	12	0,473
	n-3 PUFA rich	22	0,487	18	0,444
GFAP	n-3 PUFA poor	**17**	**0,007**	12	0,621
	n-3 PUFA rich	**22**	**0,005**	18	0,019
GluA1	n-3 PUFA poor	17	0,001	12	0,003
	n-3 PUFA rich	22	0,326	18	0,225
GluA2	n-3 PUFA poor	17	0,002	12	0,063
	n-3 PUFA rich	22	0,061	18	0,027
GluN1	n-3 PUFA poor	17	0,703	12	0,417
	n-3 PUFA rich	22	0,594	18	0,555
GluN2A	n-3 PUFA poor	**17**	**0,003**	12	0,215
	n-3 PUFA rich	**22**	**0,000**	18	0,002
GluN2B	n-3 PUFA poor	17	0,101	12	0,098
	n-3 PUFA rich	22	0,004	18	0,275
Iba-1	n-3 PUFA poor	17	0,511	12	0,998
	n-3 PUFA rich	22	0,205	18	0,347
PSD95	n-3 PUFA poor	17	0,882	12	0,894
	n-3 PUFA rich	22	0,170	18	0,015
Syp	n-3 PUFA poor	17	0,106	12	0,589
	n-3 PUFA rich	22	0,049	18	0,084
Syg1	n-3 PUFA poor	17	0,983	12	0,482
	n-3 PUFA rich	22	0,012	18	0,020

Supplementary table III.4. Hypothesis Test Summary Independent-Samples. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001. NS = no significant, Syp = synaptophysin, syg-1 = synaptotagmin-1.

	All data points			Data without outliers		
	Statistical test	p-value	Sig.	Statistical test	p-value	Sig.
ARC	**Mann-Whitney U**	**0,005**	****	**Mann-Whitney U**	**0,016**	*
BDNF	Mann-Whitney U	0,113	NS	Mann-Whitney U	0,113	NS
CD68	Mann-Whitney U	0,431	NS	Mann-Whitney U	0,979	NS
GFAP	Mann-Whitney U	0,359	NS	Mann-Whitney U	0,130	NS
GluA1	**Mann-Whitney U**	**0,000**	***	**Mann-Whitney U**	**0,000**	***
GluA2	**Mann-Whitney U**	**0,033**	*	**Mann-Whitney U**	**0,015**	*
GluN1	T-test for Equality of Means	0,650	NS	Mann-Whitney U	0,661	NS

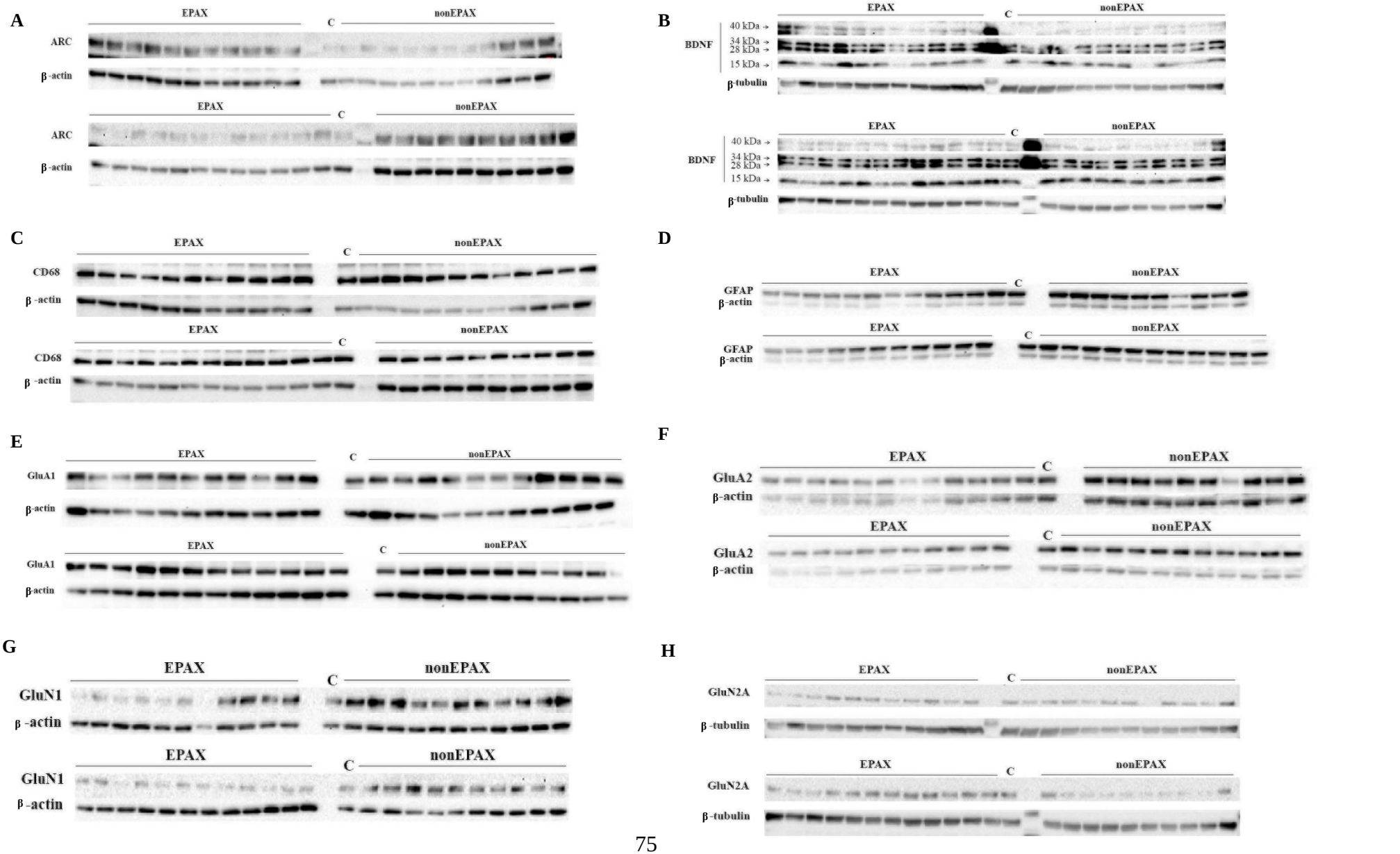
GluN2A	Mann-Whitney U	0,517	NS	Mann-Whitney U	0,103	NS
GluN2B	**Mann-Whitney U**	**0,044**	*	**Mann-Whitney U**	**0,044**	*
Iba1	**Mann-Whitney U**	**0,000**	***	**Mann-Whitney U**	**0,000**	***
PSD95	**Mann-Whitney U**	**0,000**	***	**Mann-Whitney U**	**0,000**	***
Syp	Mann-Whitney U	0,148	NS	**Mann-Whitney U**	**0,047**	*
Syg-1	**Mann-Whitney U**	**0,008**	**	**Mann-Whitney U**	**0,008**	**

Appendix IV: Western blots



Supplementary figure IV.1. Empty blots from TREM2 and MHC-II. In the left side of the blots, the molecular weight where each antibody is expected to be found is indicated. On top of each blot, the dilution used for each antibody is shown.

Supplementary figure IV.2. Blot from s100β. Merged bands after incubation of the blots with s100β antibody are observed.


A
EPAX
C
nonEPAX
ARC
β-actin
EPAX
C
nonEPAX
ARC
β-actin
B
EPAX
C
nonEPAX
BDNF
40 kDa
34 kDa
28 kDa
15 kDa
β-tubulin
EPAX
C
nonEPAX
BDNF
40 kDa
34 kDa
28 kDa
15 kDa
β-tubulin
C
EPAX
C
nonEPAX
CD68
β -actin
EPAX
C
nonEPAX
CD68
β -actin
D
EPAX
C
nonEPAX
GFAP
β-actin
EPAX
C
nonEPAX
GFAP
β-actin
E
EPAX
C
nonEPAX
GluA1
β-actin
EPAX
C
nonEPAX
GluA1
β-actin
F
EPAX
C
nonEPAX
GluA2
β-actin
EPAX
C
nonEPAX
GluA2
β-actin
G
EPAX
C
nonEPAX
GluN1
β -actin
EPAX
C
nonEPAX
GluN1
β -actin
H
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C
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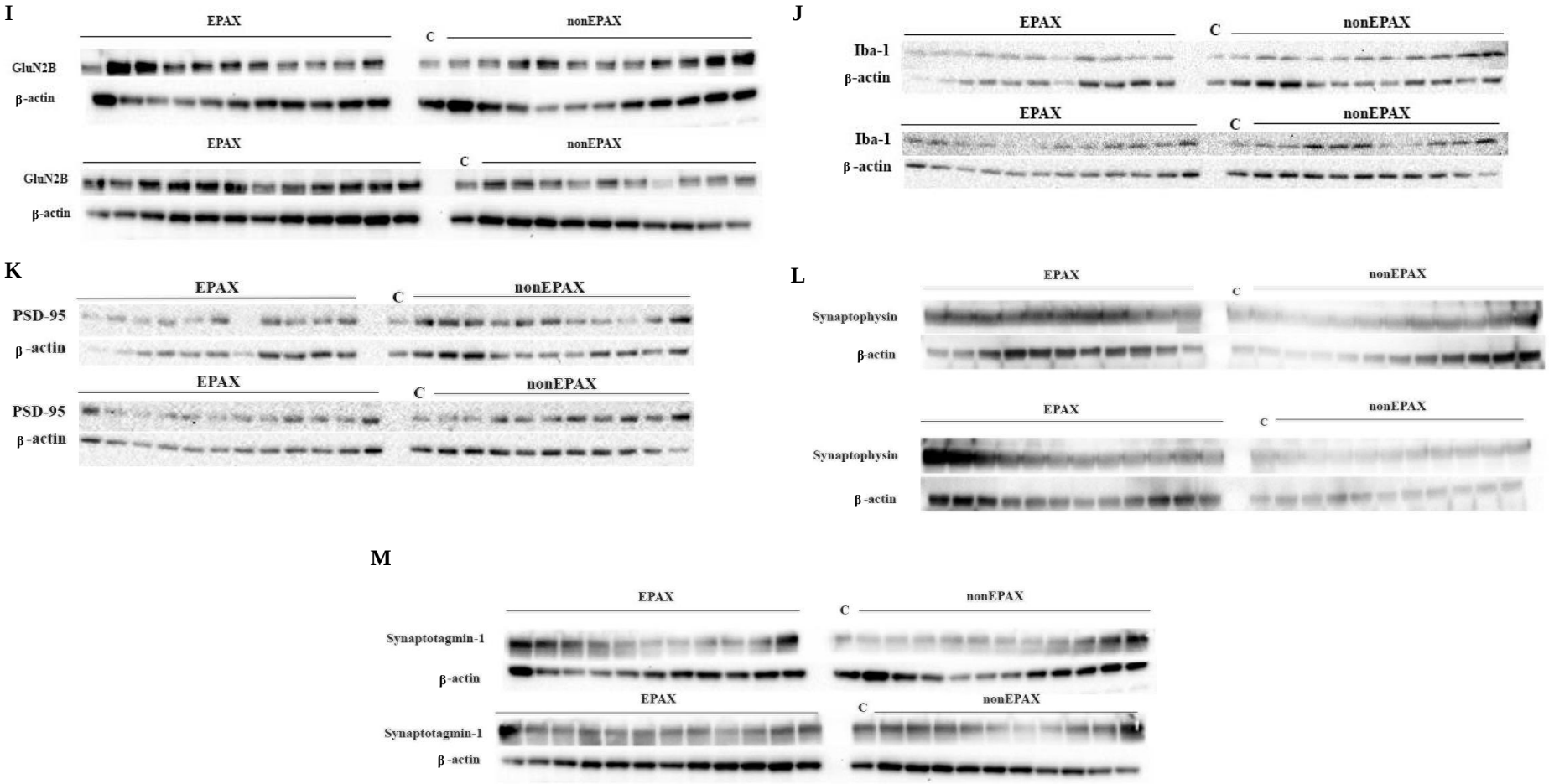
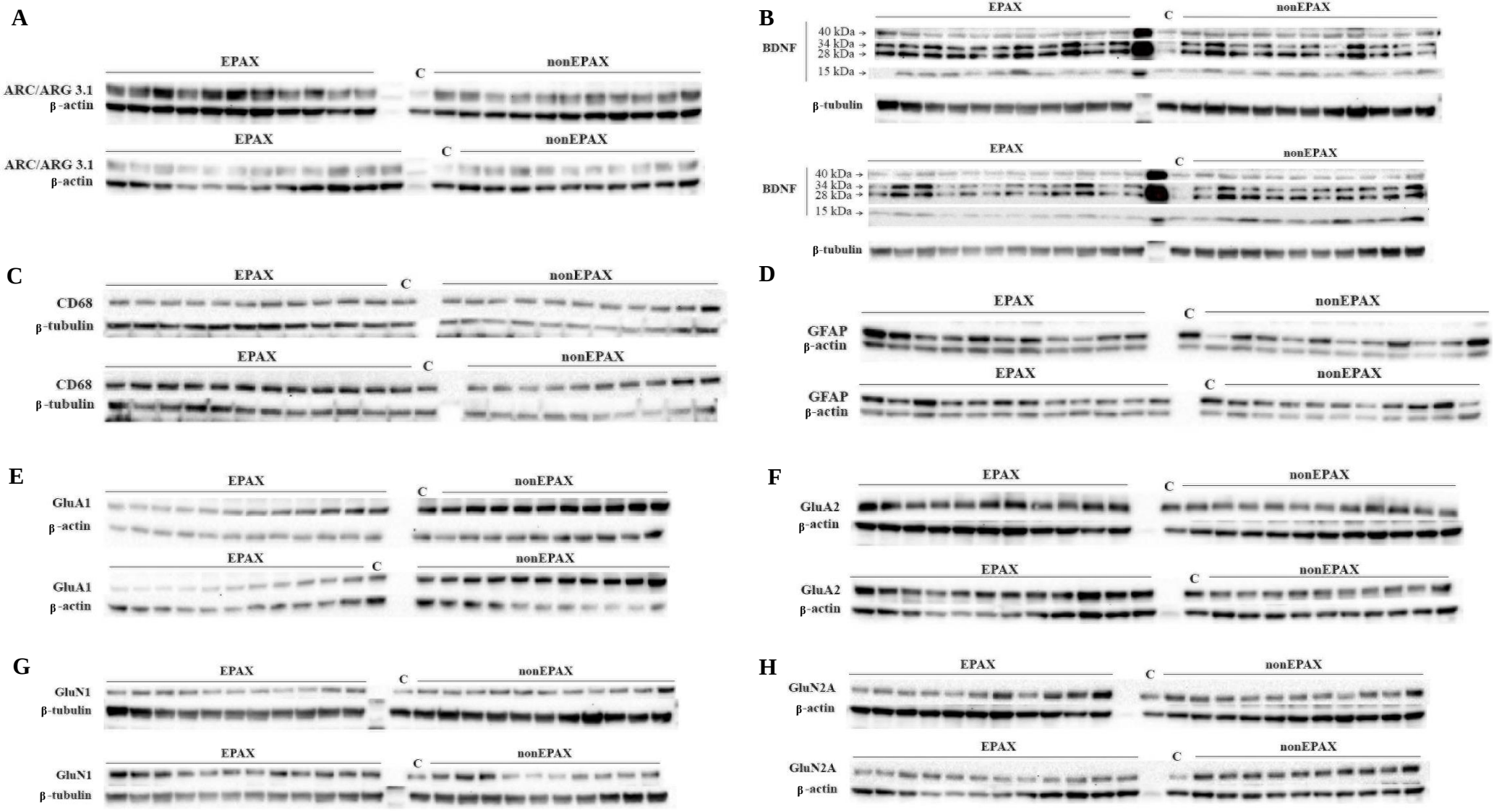
EPAX
C
nonEPAX
GluN2A
β-tubulin



Supplementary figure IV.3. Blots from hippocampal samples. Each figure is composed of two blots, each blot was loaded half with EPAX (n-3 PUFA rich diet) samples and the other half with nonEPAX (n-3 PUFA deficient diet) samples. These two halves are separated by a gap, where the molecular weight standard was placed. The first stripe in each figure is the protein of interest and the second stripe is the housekeeping control. The same sample was loaded in all the blots as an intermembrane control labelled with the letter "C".


A
EPAX
C
nonEPAX
ARC/ARG 3.1
β-actin
EPAX
C
nonEPAX
ARC/ARG 3.1
β-actin
B
EPAX
C
nonEPAX
40 kDa
34 kDa
28 kDa
BDNF
15 kDa
β-tubulin
EPAX
C
nonEPAX
40 kDa
34 kDa
28 kDa
BDNF
15 kDa
β-tubulin
C
EPAX
C
nonEPAX
CD68
β-tubulin
EPAX
C
nonEPAX
CD68
β-tubulin
D
EPAX
C
nonEPAX
GFAP
β-actin
EPAX
C
nonEPAX
GFAP
β-actin
E
EPAX
C
nonEPAX
GluA1
β-actin
EPAX
C
nonEPAX
GluA1
β-actin
F
EPAX
C
nonEPAX
GluA2
β-actin
EPAX
C
nonEPAX
GluA2
β-actin
G
EPAX
C
nonEPAX
GluN1
β-tubulin
EPAX
C
nonEPAX
GluN1
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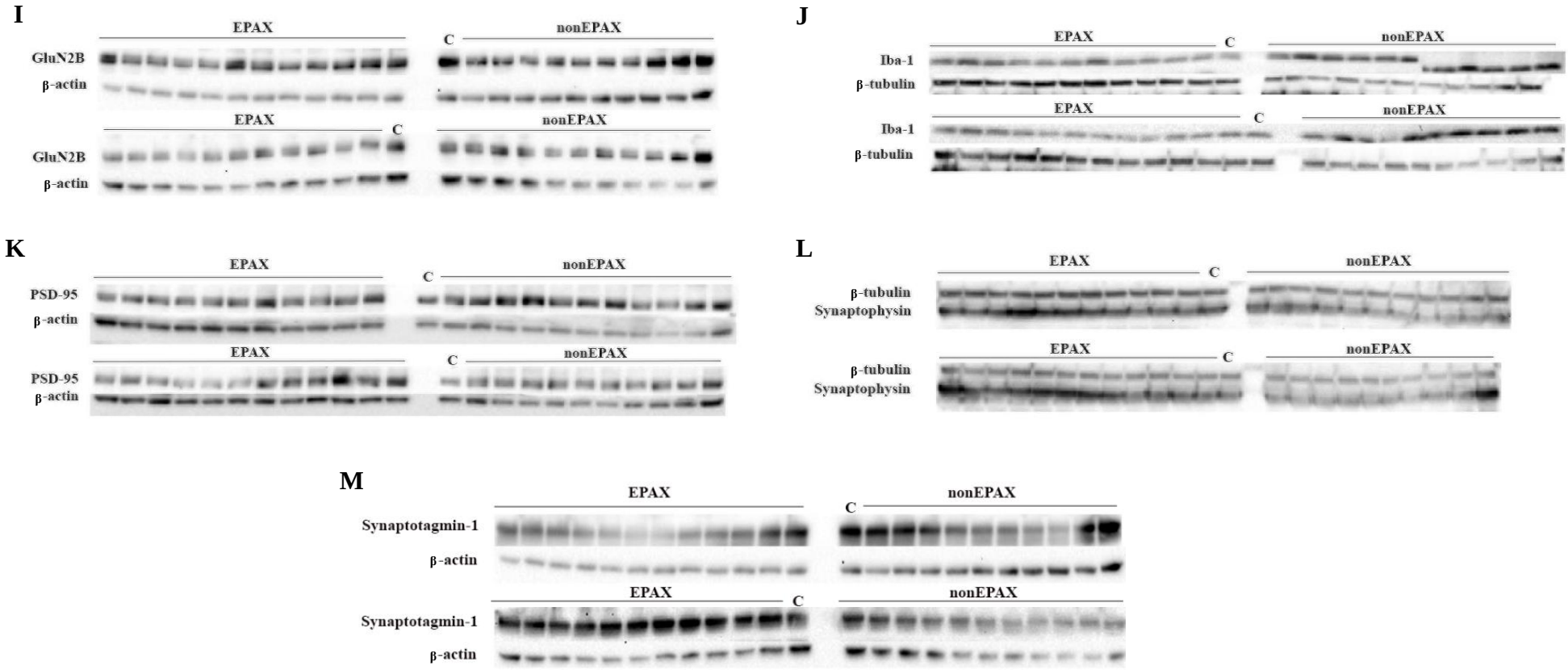
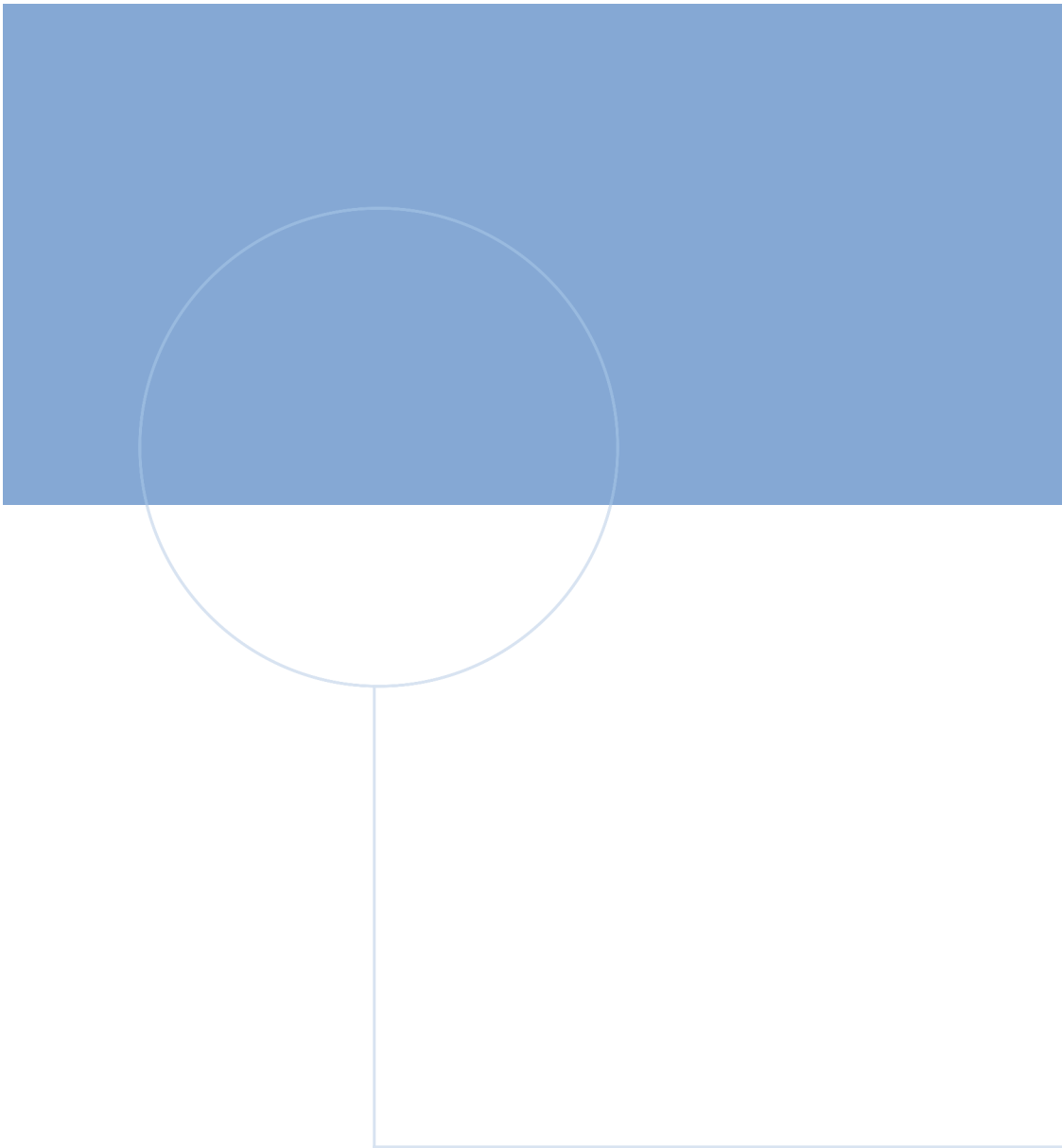
β-actin



Supplementary figure IV.4. Blots with samples from cortex. Each figure is composed by two blots, each blot was loaded half with EPAX (n-3 PUFA rich diet) samples and the other half with nonEPAX (n-3 PUFA deficient diet) samples. These two halves are separated by a gap, where the molecular weight standard was placed. The first stripe in each figure is the protein of interest and the second stripe is the housekeeping control. The same sample was loaded in all the blots as an intermembrane control labelled with the letter "C".



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