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**Mathieu Lafourcade[1,3#], Thomas Larrieu[2,3#], Susana Mato[4#], Anais Duffaud[2,3], Marja Sepers[1,3], Isabelle Matias[1,3], Veronique De Smedt[2,3], Virginie Labrousse[2,3], Lionel Bretillon[6], Carlos Matute[4], Rafael Rodríguez-Puertas[5], Sophie Layé[2,3,¶,°] and Olivier J. Manzoni[1,3,7,8,9, ¶,°]**

[1] INSERM U862, Physiopathology of Synaptic Plasticity Group, Neurocentre Magendie, 146 Rue Léo—Saignat, F 33077 Bordeaux Cedex, France.

[2] INRA UMR 1286, CNRS UMR 5226, PsyNuGen, F 33077 Bordeaux Cedex, France.

[3] University of Bordeaux, Bordeaux, F 33077, France.

[4] Department of Neuroscience and [5] Department of Pharmacology, University of the Basque Country, 48940 Leioa, Bizkaia, Spain.

[6] UMR1324 CGSA, INRA, 17 Rue Sully, 21065 Dijon, France.

[7] INSERM U901, Marseille, 13009, France.

[8] Université de la Méditerranée UMR S901 Aix-Marseille 2, France.

[9]INMED, Marseille, 13009, France.

[#] These authors equally contributed to this study.

[¶] These authors equally contributed to this study.

° Corresponding authors.

**Abstract:**

**The corollaries of the obesity epidemic that plagues developed societies are malnutrition and resulting biochemical imbalances. Low levels of essential n-3 polyunsaturated fatty acids (n-3 PUFAs) have been linked to neuropsychiatric diseases, but the underlying synaptic alterations are mostly unknown. We found that lifelong n-3 PUFAs dietary insufficiency specifically ablates long-term synaptic depression mediated by endocannabinoids in the prelimbic prefrontal cortex and accumbens. In n-3 deficient mice, presynaptic cannabinoid $CB_1$ receptors ($CB_1$Rs) normally responding to endocannabinoids were uncoupled from their effector $G_{i/o}$—proteins. Finally, the dietary-induced reduction of $CB_1$R functions in mood controlling structures is associated with impaired emotional behavior. These findings identify a plausible synaptic substrate for the behavioral alterations caused by the n—3 PUFAs deficiency often observed in western diets.**

Human deficiency diseases are due to a lack of essential nutrients in the diet. In developed countries, inexpensive high—calorie high fat foods are also low in essential nutrients, and malnutrition often is a corollary of obesity. In the United States about 30 percent of all pregnancies are carried by obese women but the functional long—term consequences of maternal malnutrition on the brains and behavior of their progeny are mostly unknown.

Lipid molecules are the building blocks of the central nervous system (CNS). In contrast to other tissues, the CNS and retina are enriched in polyunsaturated fatty acids (PUFAs): arachidonic acid (AA, 20:4n—6) and docosahexaenoic acid (DHA, 22:6n—3). These long chain PUFAs are indispensable to the normal development and function of the CNS [1]. Linoleic acid (18:2n—6, LA, the precursor of AA) and α—linolenic acid (18:3n—3, ALA, the precursor of DHA) are not synthesized de novo by mammals and a balanced diet containing appropriate amounts of these precursors is obligatory to maintain sufficient brain levels of long chain PUFAs [1,2]. Brain and retina DHA decreasee resulting from nutritional deficiency or aging are associated with reduced cognitive ability, increased emotional behavior and decreased vision [3].

Despite their high caloric contents, western diets are poor in essential nutrients and notorious for their low levels of n—3 and high levels of n—6 PUFAs [4]. Fetuses and newborns exclusively rely on their mother's diet for their PUFAs intake. Indeed, major structural fatty acids such as DHA that cannot be efficiently metabolized by developing babies must be received from the mother through the placenta during pregnancy and through breast milk during nursing. Finally, the deleterious consequences of maternal malnutrition on the progeny are further aggravated by a lifetime of nutrient poor diet.

On the basis of the epidemiological and clinical data linking n—3 PUFAs deficiency and mood disorders [5], we hypothesized that lifelong malnutrition may influence synaptic functions in brain areas controlling mood.

Here, we used a specific diet to mimic lifelong n—3/n—6 imbalance of essential PUFAs in mice and discovered that reducing n—3 levels dramatically diminished the synaptic and behavioral functions of the cannabinoid $CB_1R$.

**Results:**

**Impact of n-3 dietary deficiency on brain lipids:**

To evaluate the consequences of maternal malnutrition on the brains of their offspring, mice C57BL6/J females were fed with a diet containing 6% fat in the form of African peanut oil rich in 18:2n—6 called "n—3 deficient diet" or an isocaloric diet composed of African peanut oil and rapeseed oil naturally rich in 18:3n—3 called "n—3 diet" throughout gestation and lactation [6]. After weaning, the offspring was submitted to the same diet throughout the rest of their life to model a lifetime of malnutrition.

We found that lifelong dietary imbalance in the ratio of n—3/n—6 PUFAs had a major impact on total brain PUFAs levels (**Fig. 1a**—**b**). The n—3 deficient diet did not change saturated or mono—unsaturated fatty acids levels (not shown) but it specifically decreased the brain levels of the major n—3 PUFAs such as DHA (22:6n—3), docosapentaenoic acid (22:5n—3, DPA) and eicosapentaenoic acid (20:5n—3, EPA), while at the same time it increased n—6 PUFAs such as docosapentanoic acid (22:5n—6, DPA; **Fig. 1**) [6].

**n-3 deficiency ablates endocannabinoid synaptic plasticity:**

How could synaptic functions be perturbed by those changes in brain PUFA? Dietary PUFAs disproportion modifies the brain lipid biochemistry [1], impacts on monoaminergic neurotransmitter, glutamate receptors and impairs rodents' behavior (reviewed by [1,7]). Furthermore, convergent epidemiological and clinical studies have linked deficits in dietary n—3 PUFAs and mood disorders [5].

The endocannabinoid (eCB) system is in a unique position to link food lipids, synaptic activity and behavior. The two principal eCBs, anandamide (AEA) and 2—arachidonoylglycerol (2—AG), are signaling lipids produced from membrane long—chain fatty acids in response to neuronal activity that bind a G—protein coupled receptor (GPCR) named $CB_1$ receptor ($CB_1R$) [8]. The eCB system is a major actor of synaptic plasticity [9] and its deregulation has been postulated to contribute to the etiology of mood disorders [10—12].

The prefrontal cortex (PFC) is normally implicated in executive tasks and reward and has recently been proposed to participate to emotional behavior and the pathophysiology of depression [13,14]. Thus we focused our attention on the PFC in the present study.

After verifying that n—3 deficiency modified PUFAs levels in the PFC (**Fig. 1c-d**), we evaluated how dietary PUFAs influence eCB—mediated synaptic plasticity in our animal model. In PFC slices prepared from mice that had received a n—3 diet, tetanic stimulation induced a robust eCB—mediated long—term depression (eCB—LTD) of excitatory synapses onto layer V/VI pyramidal neurons within the prelimbic area (PrPFC) (**Fig. 2a**)[15]. This form of synaptic plasticity normally mediated by eCB was totally ablated in mice fed with the n—3 deficient diet (**Fig. 2a**,**b**).

The medial PrPFC sends major excitatory projections to the accumbens, an anatomical substrate for reward and motivation. Noteworthy, eCB—LTD was initially described in the accumbens [16] and synaptic dysfunctions in the accumbens may participate to the etiology of mood disorders [13,14].

As in the PrPFC, eCB—LTD was lacking in the accumbens of n—3 deficient mice (**Fig. 2c**), indicating that n—3 deficiency may abolishe eCB—mediated plasticity in different areas.

**n-3 deficiency does not perturb other types of synaptic plasticity:**

We next evaluated if the nutritional—induced impairment of eCB—LTD had generalized to other forms of plasticity at PrPFC synapses.

Input—output relationships were statistically indistinguishable in the two mice groups, ruling out the possibility that the incapacity to trigger eCB—LTD in n—3 deficient mice was caused by decreased excitability of glutamatergic PrPFC synapses (**Supplementary Fig. 1**). The ratio between AMPAR mediated and NMDAR mediated components of evoked excitatory currents is an indicator of previous long—term synaptic plasticity [17,18]. This index was indistinguishable between the two groups of mice, showing that the conventional long—term potentiation or LTD mechanisms have not been expressed in the PrPFC following n—3 deficiency (**Fig. 3a**).

To determine whether n—3 deficiency acted on the transduction pathways downstream of presynaptic $CB_1R$, we took advantage of the existence of another presynaptic type of LTD triggered by mGluR2/3 receptors [19] and mediated by the same presynaptic transduction pathways [19,20]. The LTD induced by the $mGluR_{2/3}$ agonist LY379268 (100 nM) was normal in the n—3 deficient group (**Fig. 3b**), strongly suggesting that n—3 deficiency had no effect on the signaling cascades downstream of $mGluR_{2/3}$ or $CB_1R$.

Taken together these data show that the deleterious effects of the unbalanced diet are not due to unspecific or generalized alteration of synaptic transmission and plasticity, but rather to the specific ablation of eCB—mediated synaptic plasticity.

**n-3 deficiency desensitizes and uncouples $CB_1$ receptors:**

A number of different mechanisms may explain the observed lack of eCB—plasticity in n—3 deficient mice. Altered PUFA environment may directly impairs $CB_1$Rs functionality. Previous work from our laboratory has demonstrated the exquisite sensitivity of the eCB system to agonist—induced desensitization of $CB_1$R *in vivo* and *in vitro* [21,22]. To interrogate the effects of n—3 deficiency on $CB_1$Rs presynaptic functions we built dose—response curves for the CB agonist CP55,940 in the two groups. In n—3 diet mice, the CB agonist inhibited synaptic transmission similarly to what we previously reported in mice fed with standard chow [15]. In striking contrast, there was a massive reduction in the maximum inhibitory effects of the CB agonist in the n—3 deficient group (**Fig. 4a**). We tested whether this impairment was associated to reduced coupling of $CB_1$Rs to their effector $G_{i/o}$ proteins using [$^{35}$S]GTPγS autoradiography [23]. Basal [$^{35}$S]GTPγS binding levels in PrPFC were similar between n—3 and n—3 deficient mice (data not shown). However, the ability of the cannabinoid agonist WIN55,212—2 (WIN) to stimulate [$^{35}$S]GTPγS binding was significantly reduced in PrPFC layers V—VI of n—3 deficient mice (**Fig**. **4b** and **Supplementary Fig. 2**).

To determine if n—3 deficient diet impaired $CB_1$R coupling in different areas, we analyzed WIN—induced stimulation of [$^{35}$S]GTPγS binding in the motor cortex within the same brain sections (**Fig. 4b**). We detected no difference in the ability of the CB agonist to stimulate [$^{35}$S]GTPγS binding in motor cortex between n—3 and n—3 deficient mice. Thus, brain region specific uncoupling of $CB_1$R to $G_{i/o}$ proteins may underlie the impairment of $CB_1$R function in PrPFC of n—3 deficient mice. Desensitization of $CB_1$Rs at the $G_{i/o}$ protein level may ensue from receptor down—regulation [24] Thus, the effect of n—3 deficient diet on $CB_1$R expression in the PFC was analyzed by means of receptor autoradiography and western blot. The density of $CB_1$Rs, measured by specific binding of [$^3$H]CP,55940 in the prelimbic and motor cortices, was similar between n—3 and n—3 deficient mice (**Fig. 4c**). Consistently, total protein levels of $CB_1$R in PFC remained unaffected by the diet (0.090 ± 0.005, n=8 and 0.085±0.009, n=8 in n—3 and n=9 in n—3 deficient diet, respectively).

Collectively, our data strongly argue against the idea that reduced $CB_1R$ expression levels underlie diminished $CB_1R$ function in n—3 deficient mice (**Fig. 4d**).

**Effects of n-3 deficiency on circulating and synaptic eCB levels:**

Because membrane bound PUFAs are the precursors of eCB, dietary PUFAs could elevate circulating eCB levels that in turn would saturate and/or desensitize $CB_1R$ [25]. However, the brain bulk levels of AEA and 2—AG were similar in both groups (**Supplementary Fig. 3**). The apparent discrepancy with previous data showing interactions between diets and circulating eCBs could be due to homeostatic adaptations restoring normal eCB during a lifetime of n—3 deficiency [26—28]. An alternative explanation is that bulk measurements cannot accurately report the eCB concentration near synapses. To estimate the synaptic levels of eCB we compared the effects of the specific CB1R antagonist AM251 on baseline EPSCs in both groups. Bath application of the selective $CB_1R$ antagonist AM—251 (4 μM, 30 min) did not increase baseline synaptic transmission in the PrPFC of n—3 diet mice (99.31% of control, n=13). In contrast, the same treatment caused a small but significant increase in the n—3 deficient group (112.5%, n=11; unpaired t—test, $p$ = 0.0358). Thus, as a whole, the data suggest that, in n—3 deficient mice, the inability to induce eCB plasticity results from the partial occupation of $CB_1R$ by enhanced eCB levels near synapses, combined to $CB_1R$ desensitization.

**Behavioral correlates of diet-induced $CB_1R$ functional antagonism:**

What are the behavioral correlates of the diet—induced $CB_1R$ functional antagonism and the associated ablation of eCB mediated synaptic plasticity? The most obvious denominator between n—3 PUFAs, $CB_1R$ and PrPFC/accumbens is their involvement in mood disorders and emotional behaviors [5,10,13,14,29]. Indeed, rats submitted to n—3 PUFA deficiency during 15 weeks from weaning [31] display higher depressive—like symptoms in the Porsolt forced swimming test (FST) [30]

We confirmed these data [30] in n—3 deficient mice. There was an increase in the time spent immobile (**Fig. 5a**) and a reduction of the swimming time (**Fig. 5b**) in n—3 deficient diet mice compared to the n—3 diet group. We extended a previous report [31] and showed that the tricyclic antidepressant imipramine reversed immobility in both groups (**Fig. 5a**,**b**).

Exploratory behaviors with emotional load (open—field and social exploration) are impaired in $CB_1R$ deleted mice [32,33]. We found that n—3 deficiency decreases the number of social exploration (i.e. new congener investigation, 77.1 ± 1.3 n = 28 and

60.2 ± 1.9 n=16, in n—3 diet and n—3 deficient mice, respectively; $p < 0.001$) while increasing the number of litter scratching, an index of anxiety (16.9 ± 1.4 and 44.2 ± 3.7 in n—3 diet and n—3 deficient mice respectively; $p < 0.001$) with no significant effect on locomotion (2958.3 ± 61.9 cm n—3 diet and 2945.8 ± 73.0, n—3 deficient diet n=16).

Furthermore, in the open—field test, n—3 deficient mice spent significant less time in the center on the arena (11.0 ± 0.6 % of total time, n=26) than n—3 diet mice (8.0 ± 0.5 % of total time, n=16, $p < 0.01$). In addition, thigmotaxis, the tendency to remain close to the wall, a widely accepted index of anxiety [34], was significantly higher in n—3 deficient mice compared to n—3 mice (88.8 ± 0.6 % and 91.8 ± 0.5 % of total time, n—3 diet and n—3 deficient mice respectively; $p < 0.01$). Increased despair behavior was not linked to impaired total motor locomotion (total distances explored were 2814.1 ± 57.4 cm and 2757.5 ± 135.1 cm, in n—3 diet and n—3 deficient diet groups, respectively).

We then evaluated whether the behavioral effect of a CB agonist, was altered in n—3 deficient mice. We tested the effect of the administration of the cannabinoid agonist WIN (0.01-1 mg/kg) on emotional behavior, measured in the open—field test [35], immediately after the injection (**Fig. 5c—d**). In n—3 mice, WIN (0.1 mg/kg) reduced the time spent in the center of the arena and increased thigmotaxis. This anxiogenic effect of WIN (0.1 mg/kg) was ablated in n—3 deficient mice, corroborating our biochemical and synaptic data showing impaired $CB_1R$ functionality in n—3 deficient mice.

**Discussion:**

There is a considerable interest in understanding how food participates to health and disease. The obesity epidemic illustrates how food, in particular low—cost food with high sugar and fat contents and low essential nutrients levels, shapes our bodies and determines our general health status.

PUFA precursors of the n—3 or n—6 families are essential nutriments that cannot be generated *de novo* in mammals. They exist in plants as precursors 18:2n—6, LA and 18:3n—3, ALA and are metabolized by elongations and desaturations into AA, EPA and DHA in mammals [2]. The conversion of these precursors into long—chain PUFAs is mostly hepatic, although other organs such as the brain express the necessary enzymatic equipment [2]. Since the two series of PUFAs compete for their biosynthetic enzymes, and because they have distinct physiological properties, the dietary n—6/n—3 ratio is of fundamental importance.

In contrast to hunting and gathering food products rich in n—3 PUFAs that composed human diet in the past, modern diets are notoriously poor in these nutrients. Most notably, since the dawn of the industrial revolution, the ratio of n—6/n—3 PUFAs in the diet has steadily increased from 1 to almost 15 within industrialized countries, leading to a significant deficiency in n—3 PUFAs [4]. Modern western diets are characterized by their high saturated fat and sugar contents associated with low n—3 PUFAs levels. Such dietary customs critically contribute to the high prevalence of chronic diseases such as obesity and metabolic disorders in westernized countries. Obesity is associated with psychological morbidity, including major depression [36], yet the underlying pathophysiological mechanisms remain poorly understood.

Here, we used a specific diet to mimic lifelong n—3/n—6 imbalance of essential PUFAs in mice. We discovered that reducing the n—3 levels dramatically reduces the function of the most abundant GPCR of the CNS, the cannabinoid $CB_1R$ receptor. At the mechanistic level, our data revealed that $CB_1R$ functional antagonism [25] results from its region—specific uncoupling from effector $G_{i/o}$—proteins, and its partial occupation by enhanced eCB levels near synapses. This impairment has functional consequences and is reflected by behavioral modifications: the anxiogenic effect of a cannabinoid agonist, WIN, does not occur in n—3 deficient mice.

Hence, dietary n—3 deficiency modifies $CB_1R$ functions in a way reminiscent to that of classical agonist desensitization [21,22,24]. Although we were not able to detect changes in circulating eCB levels in the brains of the adult mice used in the

physiology and behavioral experiments, one cannot exclude the possibility that durable modifications in $CB_1R$ coupling were caused by transient but sizeable elevation of eCB levels at earlier stages. Previous reports of enhanced eCBs levels, in particular 2—AG (the natural full agonist of $CB_1R$) in response to short—term diets, as well as on the ability of chronically elevated levels of this eCB to induce functional antagonism of brain $CB_1R$ via uncoupling from $G_{i/o}$ proteins [25], favor such a scenario [26—28].

Functionally, an important consequence of $CB_1R$ desensitization is to abolish eCB—mediated synaptic plasticity in two brain regions implicated in emotional behavior and mood disorders, the PrPFC and the accumbens [13,14]. Our data strongly suggest that the deleterious impact of n—3 deficiency on eCB plasticity does not generalize to other forms of synaptic plasticity, including mGluR2/3—LTD that shares the signaling pathway of $CB_1R$—LTD. Furthermore, in contrast to what we observed following in vivo agonist—desensitization of $CB_1R$, there is no homeostatic rescue of LTD by autocrine activation of presynaptic mGluR2/3 [37].

Preclinical data point to an important role of $CB_1R$ and the eCB system in the control of mood and emotionality [10—12,29]. In many aspects, the emotional disturbances observed in n—3 PUFAs deficient mice resemble the ones measured in $CB_1R$ null mice: increased thigmotaxis in the open—field test and lower social interactions were measured in $CB_1R$ null mice as compared to wild—type littermates [38]. In agreement, the floating time measured in the FST was higher in $CB_1R$ null mice [39]. However, it should also be noted that some studies report no significant differences in immobility in the Porsolt FST between $CB_1R$ knockout and wild—type controls except when mice were exposed several times to the test [38]. Finally, mice lacking $CB_1R$ in cortical glutamatergic neurons showed decreased immobility in the FST, in contrast to n—3 PUFAs deficient mice, whereas mice lacking $CB_1R$ in GABAergic neurons display the same immobility time as littermate controls [40]. Fundamental biochemical differences between $CB_1R$ knockouts and n—3 deficient mice may explain these behavioral discrepancies.

The selling and clinical trials of Rimonabant, a $CB_1R$ antagonist and anorectic anti—obesity drug was suspended because of the increased risks of serious psychiatric problems including depression [37]. Thus, our data showing that in rodents, n—3 deficiency profoundly impacted on emotional behaviors might not come as a surprise.

Rather, our findings further support the clinical and epidemiological associations between n—3/n—6 imbalance and mood disorder [5].

In all cases, in order to help define if dietary n—3 PUFA deficiency in rodents are responsible for behaviors underlying depression, further evaluation in a number of additional depression—related animal paradigms is required [13]. Thus, whether n—3 PUFA— $CB_1R$ reported here could be targeted with specific dietary supplementation to improve mood disorders still remains a subject of debate [41].

In conclusion, by linking diet to altered synaptic functions of $CB_1R$ in relevant brain areas, our data provide the first synaptic substrate for the impairment of emotional behavior, including depression, associated with the low levels of n—3—PUFAs frequently observed in western diets.

**Author contributions**:

M.L. performed the electrophysiology experiments, conducted the data analyses, and contributed to the design of the experiments. T.L and A.D performed the behavioral experiments and conducted the data analyses. S.M. performed the CB biochemical experiments, conducted the data analyses and contributed to the design of the experiments. M.S. participated to the electrophysiology experiments. R.R.P. and C.M. participated to the CB biochemical experiments. I.M. performed the endocannabinoid measurements and conducted the data analyses. V.D.S. performed the western blot experiments and conducted the data analyses. L.B. performed the lipid biochemistry experiments and conducted the data analyses. S.L. and O.J.M. supervised the entire project and wrote the manuscript.

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**Competing interests statement:** The authors declare that they have no competing financial interests.

**Methods:**

**Animal treatment and diet**

All animal experiments were performed according to the criteria of the European Communities Council Directive (86/609/EEC) and the United States National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

After mating, C57BL6/J females were fed with a diet containing 6% fat in the form of African peanut oil (rich in linoleic acid 18:2n—6) called "n—3 deficient diet" or rapeseed oil (rich in α—linolenic acid 18:3n—3) called "n—3 diet" throughout gestation and lactation [6]. After weaning, the offspring was submitted to the same diet throughout life. The mice were housed, grouped and had *ad libitum* food and water access. The experiments were performed in 6–16 weeks old mice.

**Analysis of fatty acids in brain lipids**

Lipids from total brain and PFC were extracted [42] and fatty acids were transmethylated [43]. Fatty acid methyl esters were analyzed on a Hewlett—Packard 5890 series II gas chromatograph and a CPSil88—silica capillary column (100 m×0.25 mm internal diameter, film thickness 0.20 µm, Varian, Les Ulis, France). The injector and the detector were at 250°C and 280°C, respectively. Hydrogen was used as a carrier (inlet pressure 210 kPa). The oven was fixed 60°C for 1 min, increased to 85°C at a rate of 3°C/min and then to 190°C at a rate of 20°C/min and left at this temperature for 65 min. Fatty acid methyl esters were identified by comparison with commercial standards.

**Slice preparation and electrophysiology**

Whole cell patch—clamp and extracellular field recordings were made from pyramidal cells in coronal slices of mouse PrPFC and nucleus accumbens medium spiny neurons [15,21]. Mouse were anesthetized with isoflurane and decapitated. The brain was sliced (300 µm) in the coronal plane (Integraslice, Campden Instruments, Loughborough, UK) and maintained in physiological saline (4°C). Slices were stored for 30 min at 32–35°C in artificial cerebrospinal fluid (ACSF) containing (mM): 126 NaCl, 2.5 KCl, 2.4 $MgCl_2$, 1.2 $CaCl_2$, 18 $NaHCO_3$, 1.2 $NaH_2PO_4$, and 11 Glucose, equilibrated with 95% $O_2$/5% $CO_2$. Slices were stored at room temperature until recording. For recording, slices were superfused (2 ml/min) with ACSF. All experiments were done at 32–35°C. The ACSF contained picrotoxin (100 µM) to block $GABA_A$ receptors. Drugs were added at the final concentration to the ACSF. To evoke synaptic currents, 100–150 µsec stimuli were delivered at 0.1 Hz through a

ACSF filled glass electrode placed either in layer II—III or in layer V—VI. There was no difference between the two sites and data were pooled together. EPSC area and amplitude were measured (graphs depict amplitudes). For extracellular field experiments, the recording pipette was filled with ACSF. The field excitatory postsynaptic potential (fEPSP) area was measured. The glutamatergic nature of the fEPSP was confirmed at the end of the experiments using the ionotropic glutamate receptor antagonist DNQX (20 μM), that specifically blocked the synaptic component (not shown).

Pyramidal neurons in PrPFC layer V—VI and accumbens neurons were visualized using an infrared microscope. Whole cell patch—clamp experiments were made with electrodes containing (mM): Cesium Methane—Sulfonate ($CH_3O_3SCs$) or $K^+$Gluconate 128, NaCl 20, $MgCl_2$1, EGTA 1, $CaCl_2$ 0.3, $Na^{2+}$—ATP 2, $Na^+$—GTP 0.3, Glucose 10 buffered with Hepes 10, pH 7.3, osmolarity 290 mOsm. Electrode resistance was 4–6 MOhms. If access resistance (no compensation, <25 MOhms) changed by >20%, the experiment was rejected. The potential reference of the amplifier was adjusted to zero prior to breaking into the cell. Data were recorded on an Axopatch—1D, filtered at 1–2 kHz, digitized (5 kHz, DigiData 1200), collected using Clampex 9.2 and analyzed using Clampfit 9.2 (all from Molecular Device, Sunnyvale, USA).

The AMPA receptors (AMPAR)—mediated to NMDA receptors (NMDAR)—mediated EPSCs amplitudes ratio was measured from EPSC at +40 mV[44]. The AMPAR component of the EPSC was isolated with the NMDAR antagonist DL—AP—V (100 μM). The NMDAR—mediated EPSC was obtained by digital subtraction of the AMPAR EPSC from the dual component EPSC.

**Autoradiographic studies**

Mice were decapitated, brains rapidly removed and stored at —80°C. Coronal brain sections (20 μm thick) containing prelimbic and motor cortices were cut using a microtome cryostat, thaw—mounted in gelatinised slides and stored at —20°C.

*$CB_1R$ autoradiography*

Slides were preincubated (30 min, room temperature) in a buffer containing 50 mM Tris—HCl, 5% BSA, pH 7.4, and incubated (2 h, 37°C, same buffer) with 3 nM [$^3$H]CP55,940 (specific activity, 174.6 Ci/mmol). Non—specific binding was determined in adjacent sections by coincubation with 10 μM WIN55,212—2. Unbound radioligand was removed by washing twice (2 h ech, 4°C) in a buffer

containing 50 mM Tris—HCl and 1% BSA, pH 7.4. After drying, autoradiograms were generated by apposing the tissues for 21 days at 4°C to $^{3}$H—sensitive films (Kodak BioMax MR, Sigma, Spain).

*$CB_1R$ agonist—stimulated [$^{35}$S]GTPγS autoradiography*

Sections were preincubated (30 min, room temperature) in a buffer containing 50 mM Tris–HCl, 0.2 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, 1 mM dl—dithiothreitol and 2 mM GDP, pH 7.7, to eliminate endogenous ligands. Sections were incubated for 120 min in the same buffer containing 0.5% BSA, 3 mU/ml adenosine deaminase and 0.04 nM [$^{35}$S]GTPγS. Consecutive sections were incubated with 1 μM of the cannabinoid receptor agonist WIN55,212—2. Non—specific binding was determined in the presence of 10 μM guanosine—5—*O*—(3—thio) triphosphate (GTPγS). Ssections were then washed twice (15 min) in 50 mM Tris—HCl buffer (pH 7.4) at 4 °C, rinsed, dried and exposed to $^{14}$C—sensitive films (Kodak BioMax MR, Sigma, Spain) with $^{14}$C—polymer standards (Amersham, Switzerland, 2 days, 4 °C).

**Western blot**

PFC were homogenized in lysis buffer and protein concentration determined using a BCA assay kit (Uptima, Montlucon, France). Equal amount of proteins (60 μg) were loaded on SDS/PAGE gels (10%) and transferred onto PVDF membranes (Millipore, Billerico, MA, USA). Membranes were incubated overnight (4ºC) with anti— $CB_1R$ (1/1000, kindly provided Dr. K. Mackie, University of Bloomington, Indiana, USA) or anti—actin antibodies (1/2500, Sigma). After washing, membranes were incubated 1 h with peroxidase—conjugated secondary anti—rabbit (1/5000, Jackson ImmunoResearch, Westgrove, PA, USA). Between each revelation, membranes were incubated (10 min, 70ºC) in stripping buffer (0.065 M Tris, 1%, SDS, 0.7% β—mercapto—ethanol, pH 6.7) to remove the previous antibody. Staining was revealed with ECL—Plus Western blotting system (Perkin Elmer, Forest City, CA). Chemiluminescence was captured and quantified by Gene Tools (Syngene).

**Purification and quantification of anandamide and 2-arachidonoylglycerol**

These experiments were performed as described previously [45—47]. Tissues were homogenized and extracted with chloroform/methanol/Tris—HCl 50 mM, pH 7.5 (2:1:1, v/v) containing internal deuterated standards (AEA—$d_4$ and 2—AG—$d_5$). The dried lipid extract was pre—purified by open bed chromatography on silica columns eluted with increasing concentrations of methanol in chloroform. Fractions for AEA and 2—AG measurement were obtained by eluting the column with 9:1 (by vol.)

chloroform/methanol and were concentrated on an N2 stream evaporator. Samples were subjected to isotope—dilution liquid chromatography—chemical ionization—tandem mass spectrometric analysis. Tandem mass spectrometry, in the form of LC—MS—MS, has been used to identify AEA but also 2—AG in the picomol/gram range in both human and rat brain regions [48,49]. Mass spectral analyses were performed on a TSQ Quantum triple quadrupole instrument (Thermo—Finnigan) equipped with an APCI source (atmospheric pressure chemical ionization) and operating in positive ion mode. The Quantum triple quadrupole instrument was used in conjunction with a Surveyor LC Pump Plus (Supelco C18 Discovery Analytical column equipped with a Phenomenex Analytical Guard Cartridge System) and cooled autosampler. The amounts of AEA and 2—AG are expressed as pmol or fmol per mg of weight tissue.

**Behavioral tests**

Mice were regularly handled and weighed before and during behavioral experiments. All tests were conducted in a sound—attenuated separate experimental room, during the last four hours of the light period to avoid low spontaneous activity. Behavioral sessions were video recorded and analyzed using The Observer (Noldus, Netherlands).

*Porsolt forced swim test* (FST)[30].

Mouse were individually placed into a dark gray polyvinylchloride cylinder (15 cm diameter, 30 cm high) half—filled with water (25±1°C), so that it would neither reach the base nor the edge of the cylinder. The water was changed between subjects. Time spent immobile during the two minutes of the test was used as an index of despair—like behavior. Climbing and swimming were also measured. A mouse was considered immobile when it made only minimal movements to keep its head above water. Imipramine (Sigma, St Louis, MO, USA) was dissolved in saline (0.9%NaCl, 20 mg/kg) and administered i.p. in 0.1 ml/10 g of mouse 30 min priors to experiment.

*Open field test*

The apparatus consisted of a white Plexiglas—covered sawdust bedding (40 x 40 cm) with 16 cm high walls. The floor was divided into 16 green lines squares (10 x 10 cm). Each mouse was placed facing a corner and allowed to explore freely for 10 min. A video tracking system (SMART, Panlab) recorded the exact track of each mouse as well distance (cm), time spent in the inner region (central squares) and the outer region (12 squares along the perimeter of the floor), the number of outer and

inner crossing and the total distance traveled. At trial end, mice were returned to their home cages, and test boxes cleaned. The percentage of time spent in the center and the time spent along the walls normalized to the total time spent in the apparatus (thigmotaxis) and total distance (in cm) were measured. All tests were conducted during the light period of the light – dark cycle.

*Social investigation*

Group—housed experimental subjects (4/cage) were separated and transferred to a new cage (40 x 40 cm) with fresh bedding material before the experiment. A social exploration session comprised 5 min exposure of an adult conspecific of the same age and same sex enclosed in a wire mesh cage placed in the corner of the cage [50]. Active investigatory behavior (mainly sniffing the anogenital region, mouth, ears, trunk and tail of the adult), anxiety behavior (scraping bedding) and locomotor activity (distance in cm) were recorded.

**Statistics**

All values are given as mean ± S.E.M. Results obtained in the FST test and the open—field with WIN were analyzed by a two—way analysis of variance (ANOVA, Diet x Treatment) followed by the appropriate post—hoc test (Bonferroni). Non—paired Student's *t* test was used to analyze results obtained in the social investigation and open—field tests. For electrophysiological experiments, *n* corresponds to the number of individual cells/slices analyzed, with at least 5 animals included in each condition. Statistical significance between groups was tested using one—way ANOVA or the Mann—Whitney test, as corresponding. In [$^{35}$S]GTPγS autoradiographic experiments the effect of the cannabinoid agonist was expressed as percentage of stimulation over basal activity (% = agonist binding x 100)/basal binding). For autoradiographic data, serial coronal sections were bilaterally examined between levels 2.4 and 2.0 according to a mouse brain atlas. Densitometry measurements of external (I—IV) and internal (V—VI) layers within prelimbic and motor cortices was carried our using NIH Image J software. Statistical comparison of experimental groups was made using a non—paired Student's *t* test, where *n* corresponds to the number of animals analyzed. All statistical tests were performed with GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) using a critical probability of $p < 0.05$ ($^{*}p < 0.05$, $^{**}p < 0.005$).

## Figure legends

**Figure 1: n-3/n-6 PUFA dietary imbalance alters PUFAs level in mouse brain.**
Fatty acids are expressed as percentage of total lipids (mg/100g of lipids). (A) The n—3 deficient diet significantly increased brain levels of the n—6 PUFAs 22:5n—6 (Docosapentaenoic acid: 0.14±0.03 and 3.8±1.25 in n—3 and n—3 deficient diet, respectively) and 22:4n—6 (Tetraenoic acid: 2.14 ± 0.03 and 2.91±0.05 in n—3 and n—3, deficient diet respectively) compared to the n—3 diet group. The increase of 20:4n—6 (Arachidonic acid) did not reach statistical significance (6.92±0.21 and 8.24±0.905 in n—3 and n—3 deficient diet, respectively). (B) n—3 deficiency decreased the brain levels of three crucial n—3 PUFAs: 22:5n—3 (Docosapentaenoic acid, DPA), 22:6n—3 (Docosahexanaenoic acid, DHA) and 20:5n—3 (Eicosapentaenoic acid, EPA): 0.17±0.01 and 0.04±0.1; 12.15±0.3 and 8.03±0.62; 0.05±0.01 and 0.01±0.003 in mice fed with the n—3 diet or n—3 deficient diet, respectively. (C—D) Analysis of fatty acids in the PFC (n=6 in each group). (C) The n—3 deficient diet augmented PFC levels of the n—6 PUFAs 22:5n—6 (0.69±0.14 and 7.02±0.96 in n—3 and n—3 deficient diet, respectively) and 22:4n—6 (1.98±0.07 and 2.38±0.13 in n—3 and n—3 deficient diet, respectively) and 20:4n—6 (9.68±0.12 and 10.69±0.18 in n—3 diet and n—3 deficient diet, respectively) compared to the n—3 diet group. (D) Dietary n—3 deficiency reduced n—3 PUFAs levels in the PFC: DPA: 0.11±0.01 and 0.07±0.1; DHA: 14.3±1.13 and 8.12±1.13; EPA: 0.35±0.06 and 0.23±0.02 in mice fed with the n—3 or n—3 deficient diet, respectively. Error bars represent s.e.m.

**Figure 2: Endocannabinoid-dependent synaptic plasticity is absent in n-3 deficient mice.**
(A) In n—3 diet mice, stimulation of deep layer PrPFC synapses (arrow: 10 min at 10 Hz) induced normal eCB—mediated long—term depression (LTD, blue circles, n=15). In contrast, LTD was completely ablated in n—3 deficient mice (red squares, n=12). (B) Representative current traces showing normal LTD in a voltage—clamped (—70 mV) PrPFC layer V—VI pyramidal neuron from a mouse fed with the n—3 balanced diet (top, blue traces), and impaired plasticity in a cell from a mouse fed with the n—3 deficient diet (bottom, red traces). (C) eCB—mediated LTD was

abolished in the accumbens of mice fed with n—3 deficient diet (red squares, n=5) compared to n—3 diet mice (blue circles, n=7). Error bars represent s.e.m.

**Figure 3: Nutritional n-3 deficiency did not alter other forms of synaptic plasticity**

(A) n—3 deficiency did neither enhanced nor depressed basal synaptic efficacy: the AMPAR/NMDAR ratio was similar in both groups (n—3 diet, blue circles: n=8; n—3—deficient, red squares: n=13). (B) Bath application of the $mGluR_{2/3}$ specific agonist LY379268 (100 nM) triggered similar LTD in both groups (n—3 diet, blue circles: n=7; n—3—deficient, red squares: n=8). Error bars represent s.e.m.

**Figure 4: Nutritional n-3 deficiency reduces synaptic $CB_1R$ efficiency and $CB_1R$ coupling to $G_{i/o}$-proteins.**

(A) Concentration—response curve for $CB_1R$—dependent inhibition of synaptic transmission in PFC slices from n—3 and n—3 deficient mice. There was a marked reduction in the ability of the cannabinoid agonist CP55,940 to inhibit EPSC size in the PrPFC of n—3 deficient mice $p < 0.05$. (B) n—3 deficiency uncoupled $CB_1R$ from $G_{i/o}$ proteins, in PrPFC layer V—VI. The efficacy of the cannabinoid agonist WIN55,212—2 (1 μM) to stimulate [$^{35}$S]GTPγS binding was reduced in the n—3 deficient group (n—3 diet: n=5; n—3—deficient: n=5; $p < 0.05$). (C) $CB_1R$ density in prelimbic and motor cortices, measured as the specific binding of the CB agonist [$^3$H]CP55,940, was similar in n—3 and n—3 deficient mice. (D) n—3 deficiency did not alter $CB_1R$ protein levels in the PFC compared with normal n—3 diet. Error bars represent s.e.m.

**Figure 5. Anxiogenic and prodepressant-like effects of dietary n-3 deficiency**

(A–B) n—3 deficient mice (n=24) spent more time immobile (A) and less time swimming (B) than n—3 diet mice (n=19). Imipramine (20 mg/kg, ip) induced antidepressant—like effects in both groups: decreased in immobility time (A) and increased time swimming (B). There was a diet effect on immobility (diet, $F(1,42)=7.03$, $p < 0.05$) and swimming time (diet, $F(1,41)=10.027$, $p < 0.01$), and a treatment effect on immobility (imipramine, $F(1,42)=18.45$, $p < 0.001$) and swimming (imipramine, $F(1,41)=39.094$, $p < 0.001$) with an interaction (diet x treatment on immobility $F(1,42)=5.37$, $p < 0.05$) and on swimming $F(1,41)=7.65$, $p < 0.01$). n—3

deficient mice spent more time immobile ($p < 0.001$) and swim less ($p < 0.05$) than n—3 mice in the saline group. Imipramine decreased immobility ($p < 0.001$) and increased swimming ($p < 0.001$) time in the n—3 deficient mice.

(C,D) WIN (0.01, 0.1, 1.0 mg/kg, i.p.) differentially affected time spent in the center and thigmotaxis. There was a significant interaction between the treatment and diet factors ($F(3,27)=3.799$, $p < 0.05$) for the time spent in the center and thigmotaxis. WIN 0.1 mg/kg significantly decreased the time spent in the center ($p < 0.01$) and increased thigmotaxis ($p < 0.01$) in n—3 mice, in contrast to the n—3 deficient group. n—3/vehicle (n=3), n—3/WIN 0.01 (n=5), n—3/WIN 0.1 (n=4), n—3/WIN 1 (n=4), n—3 deficient/vehicle (n=4), n—3 deficient/WIN 0.01 (n=5), n—3 deficient/0.1 (n=5), n—3 deficient/WIN 1 (n=5). Error bars represent s.e.m.

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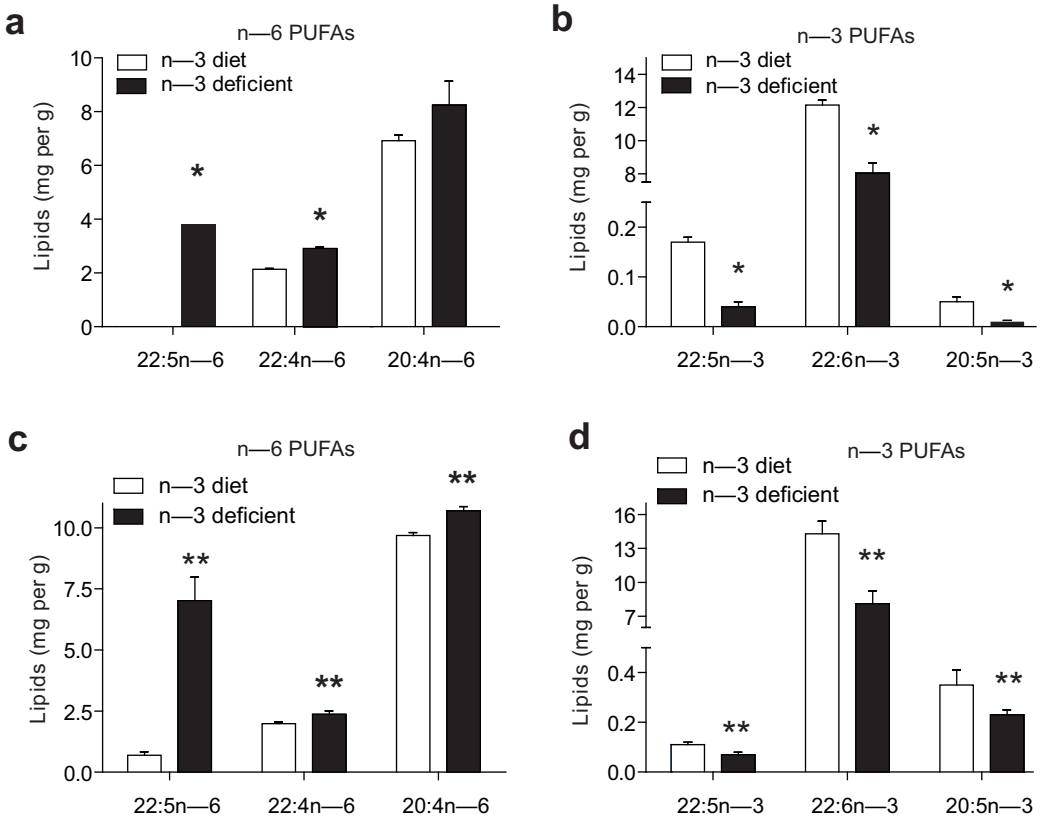
# Supplementary Information Titles

| **Journal:** Nature Neuroscience |
|---|

| **Article Title:** | Nutritional Omega-3 deficiency abolishes endocannabinoid mediated neuronal functions |
|---|---|
| **Corresponding Authors:** | Sophie Layé<br>Olivier J. Manzoni |
| | |

| Supplementary Item & Number<br>**(add rows as necessary)** | |
|---|---|
| Supplementary Figure 1 | Dietary n—3 deficiency does not change the input/output relationship at PrPFC synapses. |
| Supplementary Figure 2 | Reduced CB1R—mediated stimulation of [$^{35}$S]GTPγS binding in the PrPFC of n-3 deficient mice. |
| Supplementary Figure 3 | Nutritional n-3 deficiency does not change eCB levels. |
| | |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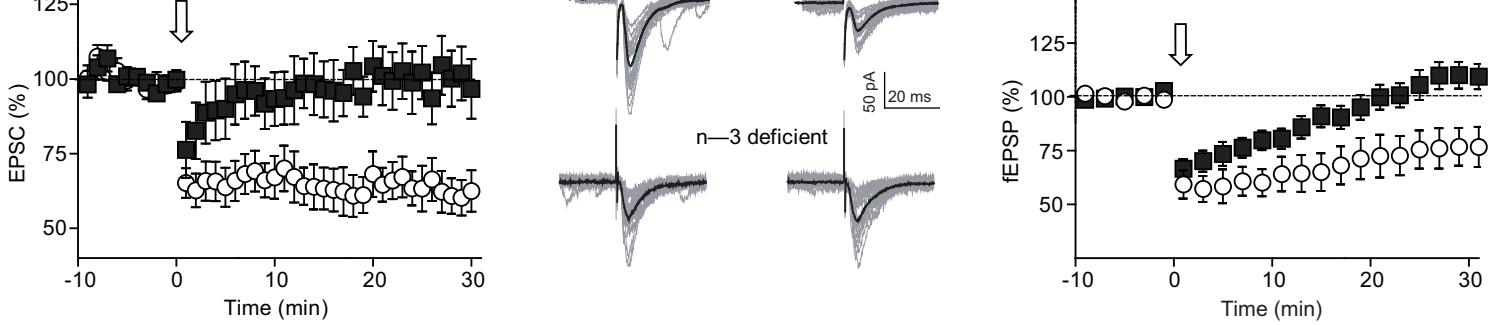
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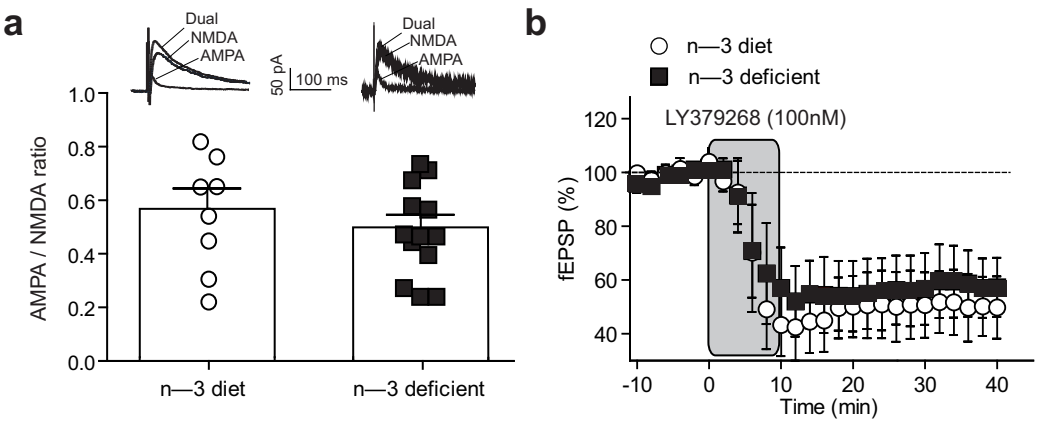
Time (min)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n—3 diet
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b
n—3 diet
n—3 deficient
LY379268 (100nM)
fEPSP (%)
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100
80
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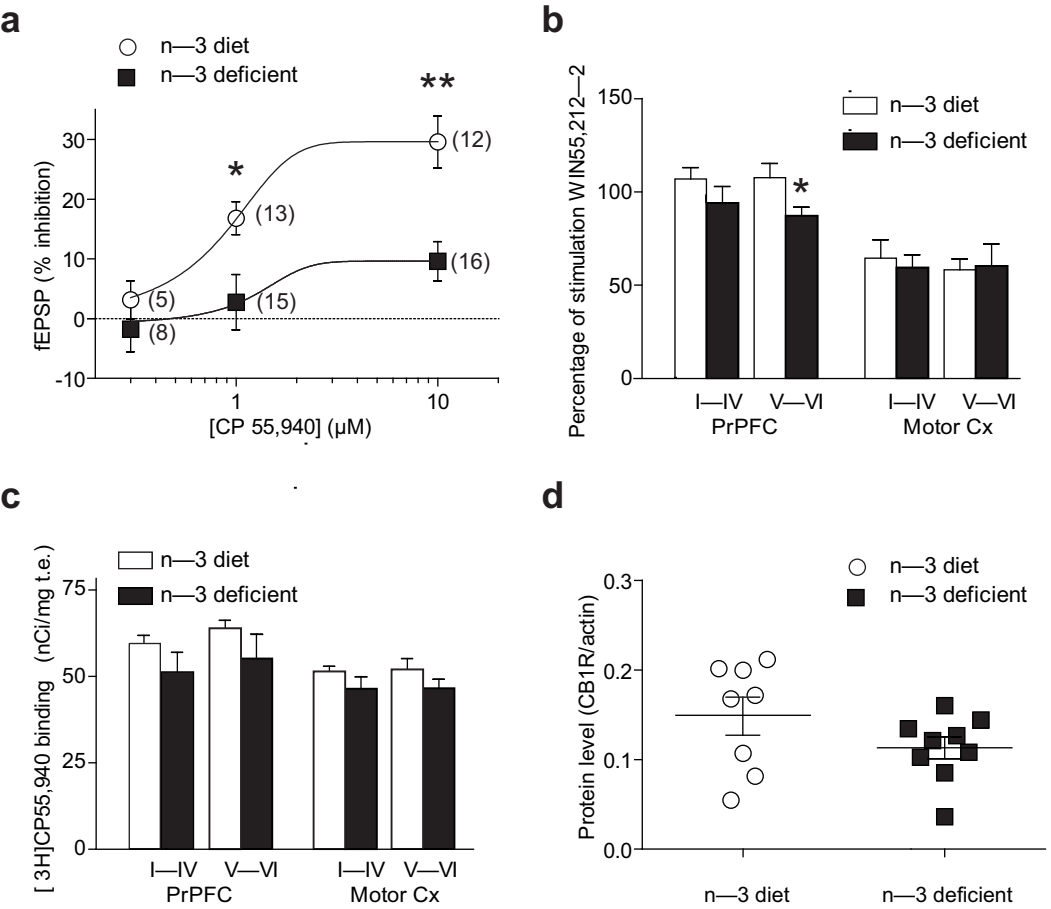
Time (min)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b
Percentage of stimulation WIN55,212—2
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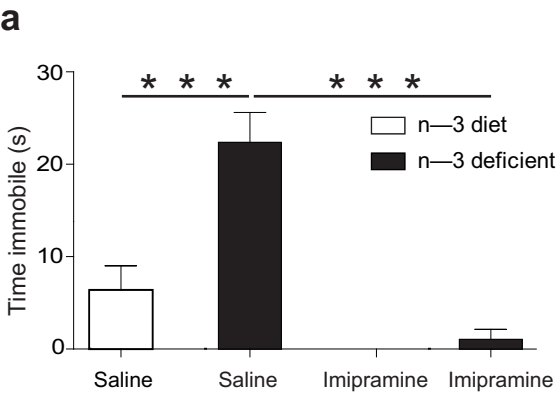
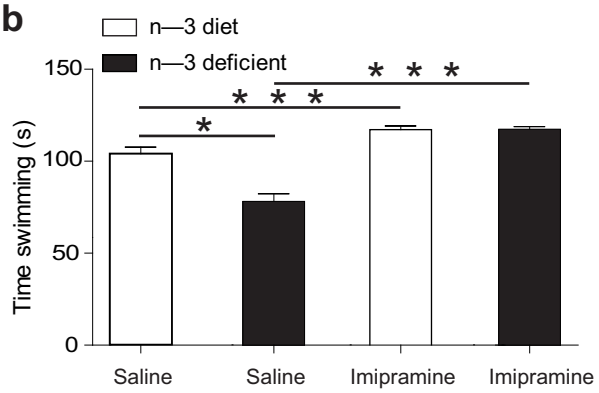
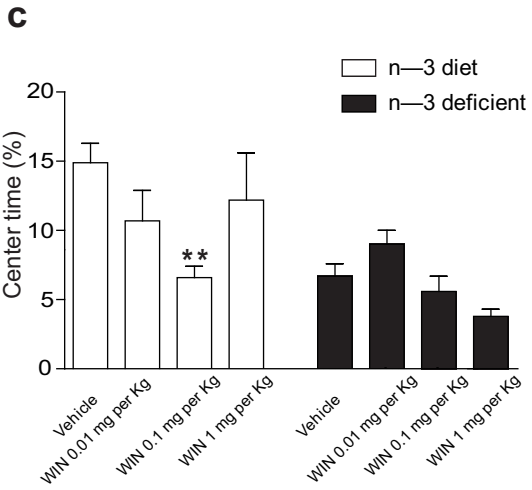
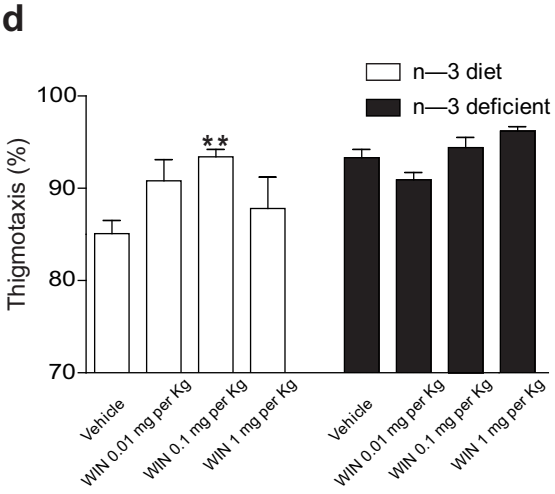
V—VI
PrPFC
Motor Cx
c
[ 3H]CP55,940 binding (nCi/mg t.e.)
n—3 diet
n—3 deficient
I—IV
V—VI
PrPFC
Motor Cx
d
Protein level (CB1R/actin)
n—3 diet
n—3 deficient


a
Time immobile (s)
30
20
10
0
* * *
* * *
n—3 diet
n—3 deficient
Saline
Saline
Imipramine
Imipramine


b
n—3 diet
n—3 deficient
Time swimming (s)
150
100
50
0
* * *
* * *
*
Saline
Saline
Imipramine
Imipramine


c
n—3 diet
n—3 deficient
Center time (%)
20
15
10
5
0
**
Vehicle
WIN 0.01 mg per Kg
WIN 0.1 mg per Kg
WIN 1 mg per Kg
Vehicle
WIN 0.01 mg per Kg
WIN 0.1 mg per Kg
WIN 1 mg per Kg


d
n—3 diet
n—3 deficient
Thigmotaxis (%)
100
90
80
70
**
Vehicle
WIN 0.01 mg per Kg
WIN 0.1 mg per Kg
WIN 1 mg per Kg
Vehicle
WIN 0.01 mg per Kg
WIN 0.1 mg per Kg
WIN 1 mg per Kg