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Nutritional omega-3 deficiency abolishes endocannabinoid-mediated neuronal functions

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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_1Rs) normally responding to endocannabinoids were uncoupled from their effector $G_{i/o}$ proteins. Finally, the dietary-induced reduction of CB_1R functions in mood-controlling structures was associated with impaired emotional behavior. These findings identify a plausible synaptic substrate for the behavioral alterations caused by the n-3 PUFAs deficiency that is often observed in western diets.

Human deficiency diseases are the results of 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 is often a corollary of obesity. In the United States, about 30% 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 CNS. In contrast with other tissues, the CNS and retina are enriched in PUFAs: arachidonic acid (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, the precursor of arachidonic acid) and α-linolenic acid (18:3n-3, the precursor of DHA) are not synthesized *de novo* by mammals and a balanced diet containing appropriate amounts of these precursors is necessary to maintain sufficient brain levels of long chain PUFAs[1,2]. Brain and retina DHA decrease 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 rely exclusively on their mother's diet for their intake of PUFAs. 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.

Given 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. 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 markedly diminished the synaptic and behavioral functions of the cannabinoid CB_1R.

RESULTS

Effect of n-3 dietary deficiency on brain lipids

To evaluate the consequences of maternal malnutrition on the brains of their offspring, we fed female C57BL6/J mice a diet containing 6% fat in the form of African peanut oil rich in linoleic acid (n-3–deficient diet) or an isocaloric diet composed of African peanut oil and rapeseed oil naturally rich in α-linolenic acid (n-3 diet) throughout gestation and lactation[6]. After weaning, the offspring received 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 effect on total brain PUFAs levels (**Fig. 1a**,**b**). The n-3–deficient diet did not change saturated or mono-unsaturated fatty acids levels (data not shown), but it specifically decreased the brain levels of the major n-3 PUFAs, such as DHA, docosapentaenoic acid (22:5n-3) and eicosapentaenoic acid (20:5n-3, EPA), and it increased n-6 PUFAs such as docosapentanoic acid (22:5n-6; **Fig. 1**)[6].

n-3 deficiency ablates endocannabinoid synaptic plasticity

How could synaptic functions be perturbed by those changes in brain PUFA? Dietary PUFA disproportion modifies the brain lipid

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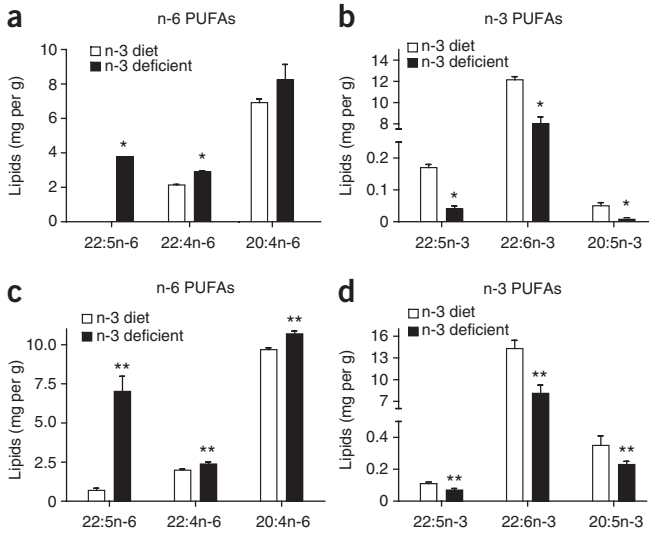

Figure 1 n-3/n-6 PUFA dietary imbalance alters PUFAs level in mouse brain. Fatty acids are expressed as percentage of total lipids (mg per 100 g of lipids). (**a**) The n-3 deficient diet significantly increased brain levels of the n-6 PUFAs 22:5n-6 (docosapentaenoic acid; n-3 diet, 0.14 ± 0.03; n-3–deficient diet, 3.8 ± 1.25) and 22:4n-6 (tetraenoic acid; n-3 diet, 2.14 ± 0.03; n-3–deficient diet, 2.91 ± 0.05) compared with the n-3 diet group. The increase of arachidonic acid did not reach statistical significance (n-3 diet, 6.92 ± 0.21; n-3–deficient diet, 8.24 ± 0.905; $^{*}P = 0.186$). $^{*}P < 0.05$. (**b**) n-3 deficiency decreased the brain levels of three crucial n-3 PUFAs (22:5n-3 (docosapentaenoic acid): n-3 diet, 0.17 ± 0.01; n-3–deficient diet, 0.04 ± 0.1; 22:6n-3 (DHA): n-3 diet, 12.15 ± 0.3; n-3–deficient diet, 8.03 ± 0.62; 20:5n-3 (EPA): n-3 diet, 0.05 ± 0.01; n-3–deficient diet, 0.01 ± 0.003). (**c**,**d**) Analysis of fatty acids in the PFC ($n = 6$ in each group). The n-3–deficient diet augmented PFC levels of the n-6 PUFAs 22:5n-6 (n-3 diet, 0.69 ± 0.14; n-3–deficient diet, 7.02 ± 0.96) and 22:4n-6 (n-3 diet, 1.98 ± 0.07; n-3–deficient diet, 2.38 ± 0.13) and arichdonic acid (n-3 diet, 9.68 ± 0.12; n-3–deficient diet, 10.69 ± 0.18) compared with the n-3 diet group (**c**). $^{**}P < 0.005$. Dietary n-3 deficiency reduced n-3 PUFAs levels in the PFC (docosapentaenoic acid: n-3 diet, 0.11 ± 0.01; n-3–deficient diet, 0.07 ± 0.1; DHA: n-3 diet, 14.3 ± 1.13; n-3–deficient diet, 8.12 ± 1.13; EPA: n-3 diet, 0.35 ± 0.06; n-3–deficient diet, 0.23 ± 0.02; **d**). Error bars represent s.e.m.

biochemistry[1], affects monoaminergic neurotransmitter and glutamate receptors, and impairs rodents' behavior (reviewed in refs. 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 the G protein–coupled receptor (GPCR) CB_1R[8]. The eCB system is involved in 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 be involved in emotional behavior and the pathophysiology of depression[13,14]. We focused our attention on the PFC. 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 an n-3 diet, tetanic stimulation induced a robust eCB-mediated long-term depression (eCB-LTD) of excitatory synapses onto layer V/VI pyramidal neurons in the prelimbic area (PrPFC) (**Fig. 2a**)[15]. This form of synaptic plasticity, normally mediated by eCB, was totally ablated in mice that were fed 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. Notably, eCB-LTD was initially described in the accumbens[16] and synaptic dysfunctions in the accumbens may contribute to the etiology of mood disorders[13,14]. As in the PrPFC, eCB-LTD was lacking in the accumbens of mice on the n-3–deficient diet (**Fig. 2c**), indicating that n-3 deficiency may abolish eCB-mediated plasticity in different areas.

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

We next asked whether the nutritional-induced impairment of eCB-LTD generalized to other forms of plasticity at PrPFC synapses. Input-output relationships were similar in the two mice groups, ruling out the possibility that the inability of mice on the n-3–deficient diet to trigger eCB-LTD was caused by decreased excitability of glutamatergic PrPFC synapses (**Supplementary Fig. 1**). The ratio between AMPA receptor (AMPAR)-mediated and NMDA receptor (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 that is triggered by mGluR2/3 receptors[19] and is 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 suggest that the deleterious effects of the unbalanced diet

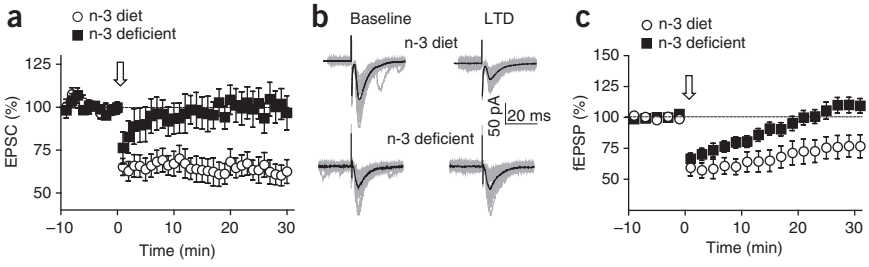

Figure 2 Endocannabinoid-dependent synaptic plasticity is absent in n-3–deficient mice. (**a**) In n-3 diet mice, stimulation of deep-layer PrPFC synapses (arrow, 10 min at 10 Hz) induced normal eCB-mediated LTD (white circles, $n = 15$). In contrast, LTD was completely ablated in n-3–deficient mice (black 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 on the n-3 diet (top) and impaired plasticity in a cell from a mouse on the n-3–deficient diet (bottom). (**c**) eCB-mediated LTD was abolished in the accumbens of mice on the n-3–deficient diet (black squares, $n = 5$) compared with n-3 diet mice (white circles, $n = 7$). Error bars represent s.e.m.

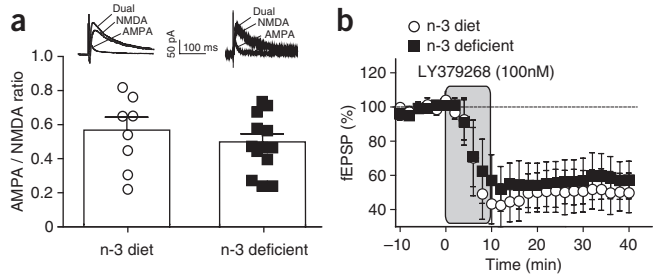

Figure 3 Nutritional n-3 deficiency did not alter other forms of synaptic plasticity. (**a**) n-3 deficiency neither enhanced nor depressed basal synaptic efficacy; the AMPAR/NMDAR ratio was similar in both groups (n-3 diet, white circles, $n = 8$; n-3–deficient, black squares, $n = 13$). (**b**) Bath application of the $mGluR_{2/3}$-specific agonist LY379268 (100 nM) triggered similar LTD in both groups (n-3 diet, white circles, $n = 7$; n-3-deficient, black squares, $n = 8$). Error bars represent s.e.m.

are not the results of unspecific or generalized alteration of synaptic transmission and plasticity, but instead result from the specific ablation of eCB-mediated synaptic plasticity.

n-3 deficiency desensitizes and uncouples CB_1Rs

A number of different mechanisms may explain the observed lack of eCB plasticity in mice on the n-3–deficient diet. Altered PUFA environment may directly impair CB_1Rs functionality. Previously, we found that the eCB system is exquisitely sensitive to agonist-induced desensitization of CB_1R *in vivo* and *in vitro*[21,22]. To determine the effects of n-3 deficiency on CB_1Rs presynaptic functions, we built dose-response curves for the cannabinoid agonist CP55,940 in the two groups. In mice on the n-3 diet, CP55,940 inhibited synaptic transmission similar to what we previously found in mice fed with standard chow[15]. In contrast, there was a massive reduction in the maximum inhibitory effects of CP55,940 in the n-3–deficient group (**Fig. 4a**). We tested whether this impairment was associated with reduced coupling of CB_1Rs to their effector $G_{i/o}$ proteins using [^{35}S]GTPγS autoradiography[23]. Basal [^{35}S]GTPγS binding levels in PrPFC were similar between mice on the n-3 diet and those on the n-3–deficient diet (data not shown). However, the ability of the cannabinoid agonist WIN55,212-2 (WIN) to stimulate [^{35}S]GTPγS binding was substantially reduced in PrPFC layers V-VI of mice on the n-3–deficient diet (**Fig. 4b** and **Supplementary Fig. 2**).

To determine whether the n-3–deficient diet impairs CB_1R coupling in different areas, we analyzed WIN-induced stimulation of [^{35}S]GTPγS binding in the motor cortex in the same brain sections (**Fig. 4b**). We detected no difference in the ability of CP55,940 to stimulate [^{35}S]GTPγS binding in motor cortex between mice on the n-3 diet and those on the n-3–deficient diet. Thus, brain region–specific uncoupling of CB_1R to $G_{i/o}$ proteins may underlie the impairment of CB_1R function in PrPFC of mice on the n-3 deficient diet. Desensitization of CB_1Rs at the $G_{i/o}$ protein level may ensue from receptor down-regulation[24]. Thus, we analyzed the effects of n-3–deficient diet on CB_1R expression in the PFC by means of receptor autoradiography and western blot. The density of CB_1Rs, measured by specific binding of [^{3}H]CP,55940 in the prelimbic and motor cortices, was similar between mice on the n-3 diet and those on the n-3–deficient diet (**Fig. 4c**). Consistently, total protein levels of CB_1R in PFC remained unaffected by the diet (0.090 ± 0.005, $n = 8$ in the n-3 diet, and 0.085 ± 0.009, $n = 9$ in n-3–deficient diet). Collectively, our data strongly argue against the idea that reduced CB_1R expression levels underlie the diminished CB_1R function seen in mice on the n-3–deficient diet (**Fig. 4d**).

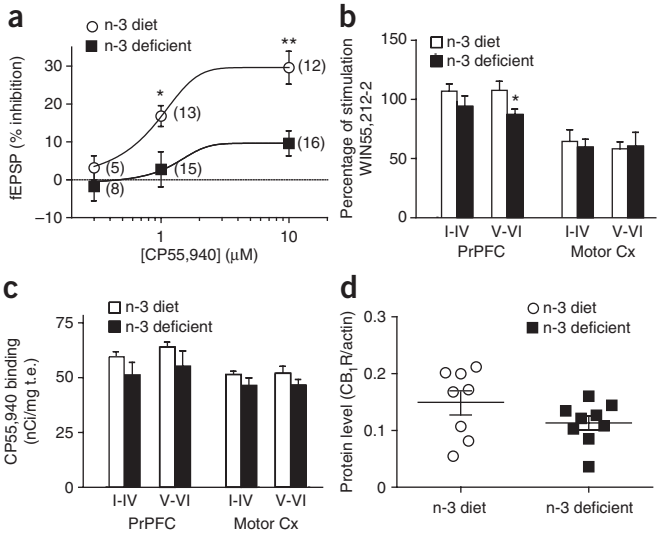

Figure 4 Nutritional n-3 deficiency reduces synaptic CB_1R efficiency and CB_1R coupling to $G_{i/o}$-proteins. (**a**) Concentration-response curve for CB_1R-dependent inhibition of synaptic transmission in PFC slices from n-3 and n-3–deficient mice. There was a marked reduction in the ability of the cannabinoid agonist CP55,940 to inhibit EPSC size in the PrPFC of n-3–deficient mice. $^*P < 0.05$, $^{**}P < 0.005$. (**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 CP55,940, was similar in n-3 diet and n-3–deficient mice. (**d**) n-3 deficiency did not alter CB_1R protein levels in the PFC compared with the normal n-3 diet. Error bars represent s.e.m.

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

As membrane-bound PUFAs are the precursors of eCB, it is possible that dietary PUFAs elevate circulating eCB levels, which in turn saturate and/or desensitize CB_1Rs[25]. However, the bulk levels of AEA and 2-AG in the brain were similar in both groups (**Supplementary Fig. 3**). The apparent discrepancy with previous data showing interactions between diets and circulating eCBs could be a result of 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 CB_1R antagonist AM251 on baseline excitatory postsynaptic currents (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 mice on the n-3 diet (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, $P = 0.0358$). These data suggest that, in mice on the n-3–deficient diet, the inability to induce eCB plasticity is a result of the partial occupation of CB_1R by enhanced eCB levels near synapses, combined with 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

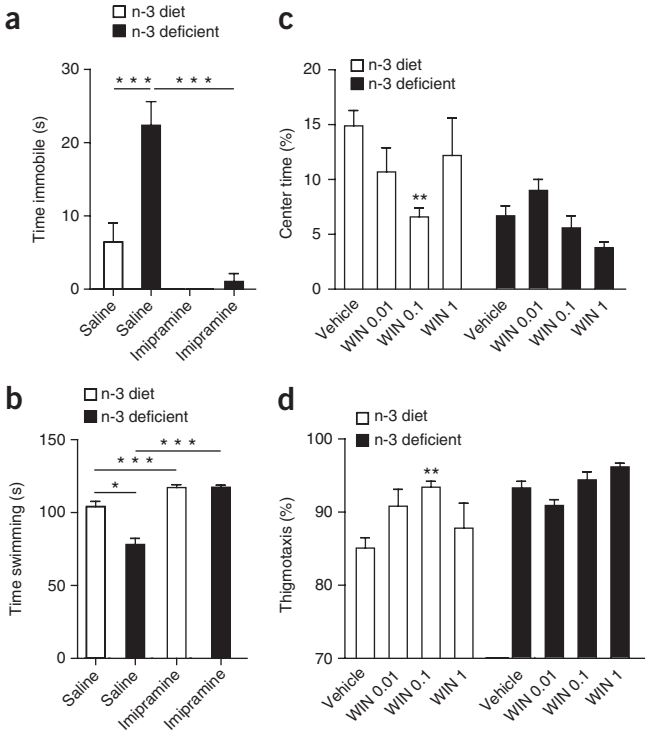

Figure 5 Anxiogenic and prodepressant-like effects of dietary n-3 deficiency. (**a**,**b**) n-3–deficient mice ($n = 24$) spent more time immobile (**b**) and less time swimming (**b**) than n-3 diet mice ($n = 19$). Imipramine (20 mg per kg, intraperitoneal) induced antidepressant-like effects in both groups: decreased 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 × 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 swam less ($P < 0.05$) than n-3 diet mice in the saline group. Imipramine decreased immobility ($P < 0.001$) and increased swimming ($P < 0.001$) time in the n-3–deficient mice. $^{*}P < 0.05$, $^{***}P < 0.001$. (**c**,**d**) WIN (0.01, 0.1, 1.0 mg per kg, intraperitoneal) 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 per kg) significantly decreased the time spent in the center ($P < 0.01$) and increased thigmotaxis ($P < 0.01$) in n-3 diet mice, in contrast with 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, $n = 5$). ** $P < 0.005$. Error bars represent s.e.m.

and PrPFC/accumbens is their involvement in mood disorders and emotional behaviors[5,10,13,14,29]. Indeed, rats subjected to n-3 PUFA deficiency during 15 weeks from weaning[30] display higher depressive-like symptoms in the Porsolt forced swimming test (FST)[31].

We confirmed these data[31] 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. Building on previous findings[30], we found 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 mice lacking the CB_1R[32,33]. We found that n-3 deficiency decreased the number of social exploration (that is, new congener investigation; n-3 diet, 77.1 ± 1.3 per 5 min, $n = 28$; n-3–deficient diet, 60.2 ± 1.9, $n = 16$; $P < 0.001$) and increased the number of litter scratching, an index of anxiety (n-3 diet, 16.9 ± 1.4 per 5 min; n-3–deficient diet, 44.2 ± 3.7; $P < 0.001$) with no significant effect on locomotion (n-3 diet, 2,958.3 ± 61.9 cm; n-3–deficient diet, 2,945.8 ± 73.0; $n = 16$; $P = 0.999$). 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 mice on the n-3–deficient diet than in mice on the n-3 diet (n-3 diet, 88.8 ± 0.6% of total time; n-3–deficient diet, 91.8 ± 0.5% of total time; $P < 0.01$). Increased despair behavior was not linked to impaired total motor locomotion (total distance explored: n-3 diet, 2,814.1 ± 57.4 cm; n-3–deficient diet, 2,757.5 ± 135.1 cm).

We then evaluated whether the behavioral effect of a cannabinoid agonist was altered in n-3–deficient mice. We tested the effect of WIN (0.01–1 mg per kg of body weight) 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 per kg) reduced the time spent in the center of the arena and increased thigmotaxis. This anxiogenic effect of WIN (0.1 mg per kg) was ablated in n-3–deficient mice, consistent with 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, particularly 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 (linoleic acid) and 18:3n-3 (α-linolenic acid) and are metabolized by elongations and desaturations into arachidonic acid, 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]. Because 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 in industrialized countries, leading to a significant deficiency in n-3 PUFAs[4]. Modern western diets are characterized by 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], but 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 markedly reduced the function of the most abundant G protein–coupled receptor of the CNS, the cannabinoid CB_1R receptor. At the mechanistic level, our data indicate 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 had functional consequences and

was reflected by behavioral modifications: the anxiogenic effect of a cannabinoid agonist, WIN, did not occur in n-3–deficient mice.

Dietary n-3 deficiency modifies CB_1R functions in a way that is reminiscent of 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 our 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, particularly 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 that have been implicated in emotional behavior and mood disorders: the PrPFC and the accumbens[13,14]. Our data strongly suggest that the deleterious effect of n-3 deficiency on eCB plasticity does not generalize to other forms of synaptic plasticity, including mGluR2/3-LTD, that share the signaling pathway of CB_1R-LTD. Furthermore, in contrast with our observations following *in vivo* agonist desensitization of CB_1R, there is no homeostatic rescue of LTD by autocrine activation of presynaptic mGluR2/3 (ref. 37).

Preclinical data indicate that CB_1R and the eCB system are important for the control of mood and emotionality[10–12,29]. In many aspects, the emotional disturbances observed in n-3 PUFA–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 with wild-type littermates[38]. Consistent with this, 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 with n-3 PUFA–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]. The fact that we found that n-3 deficiency profoundly affects emotional behaviors in rodents 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, to help define whether dietary n-3 PUFA deficiency in rodents are responsible for behaviors underlying depression, further evaluation in a number of additional depression-related animal procedures is required[13]. Thus, whether n-3 PUFA CB_1R reported here could be targeted with specific dietary supplementation to improve mood disorders 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 that are frequently observed in western diets.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

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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, conducted the data analyses and contributed to the design of the experiments. S.M. performed the cannabinoid biochemical experiments, conducted the data analyses and contributed to the design of the experiments. M.S. participated in the electrophysiology experiments. R.R.-P. and C.M. participated in the cannabinoid 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. V.F.L. participated in the behavioral experiments. L.B. performed the lipid biochemistry experiments and conducted the data analyses. S.L. and O.J.M. equally supervised the project and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE 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; the n-3–deficient diet) or rapeseed oil (rich in α-linolenic acid, 18:3n-3; the n-3 diet) throughout gestation and lactation[6]. After weaning, the offspring were subjected 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-week-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). 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 at 60 °C for 1 min, increased to 85 °C at a rate of 3 °C min^{-1}, increased to 190 °C at a rate of 20 °C min^{-1} and then 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) and maintained in physiological saline (4 °C). Slices were stored for 30 min at 32–35 °C in artificial cerebrospinal fluid (ACSF) containing 126 mM NaCl, 2.5 mM KCl, 2.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 18 mM $NaHCO_3$, 1.2 mM NaH_2PO_4 and 11 mM glucose, equilibrated with 95% O_2/5% CO_2. Slices were stored at 22 ± 2 °C until recording. For recording, slices were superfused (2 ml min^{-1}) 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-µs stimuli were delivered at 0.1 Hz through an 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 (data 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 128 mM cesium methane-sulfonate (CH_3O_3SCs) or potassium gluconate, 20 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.3 mM $CaCl_2$, 2 mM Na^{2+}-ATP, 0.3 mM Na^+-GTP, 10 mM glucose buffered with 10 mM HEPES, pH 7.3, osmolarity 290 mOsm. Electrode resistance was 4–6 MOhm. If access resistance (no compensation, <25 MOhm) changed by > 20%, the experiment was rejected. The potential reference of the amplifier was adjusted to zero before 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 ratio of AMPAR-mediated to NMDAR-mediated EPSC amplitude was measured from EPSC at +40 mV[44]. The AMPAR component of the EPSC was isolated with the NMDAR antagonist DL-AP5 (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 and the brains were 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.

For CB_1R autoradiography, slides were preincubated (30 min, 22 ± 2 °C) in a buffer containing 50 mM Tris-HCl, 5% bovine serum albumin (BSA, vol/vol), pH 7.4, and incubated (2 h, 37 °C, same buffer) with 3 nM [^{3}H]CP55,940 (specific activity, 174.6 Ci $mmol^{-1}$). Nonspecific binding was determined in adjacent sections by co-incubation with 10 µM WIN55,212-2. Unbound radioligand was removed by washing twice (2 h each, 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 d at 4 °C to ^{3}H-sensitive films (Kodak BioMax MR, Sigma).

For CB_1R agonist-stimulated [^{35}S]GTPγS autoradiography, sections were pre-incubated (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^{-1} adenosine deaminase and 0.04 nM [^{35}S]GTPγS. Consecutive sections were incubated with 1 µM of the cannabinoid receptor agonist WIN55,212-2. Nonspecific binding was determined in the presence of 10 µM guanosine-5-*O*-(3-thio) triphosphate (GTPγS). Sections 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) with ^{14}C-polymer standards (Amersham, 2 d, 4 °C).

Western blot. PFC was homogenized in lysis buffer and protein concentration was determined using a BCA assay kit (Uptima). Equal amount of proteins (60 µg) were loaded onto SDS-PAGE gels (10%) and transferred onto PVDF membranes (Millipore). Membranes were incubated overnight (4 °C) with antibodies to CB_1R (1:1,000, kindly provided by K. Mackie, University of Bloomington) or actin antibodies (1:2,500, Sigma). After washing, membranes were incubated 1 h with peroxidase-conjugated secondary antibody to rabbit (1:5,000, Jackson ImmunoResearch). Between each revelation, membranes were incubated (10 min, 70 °C) in stripping buffer (0.065 M Tris, 1%, SDS (vol/vol), 0.7% β-mercaptoethanol (vol/vol), pH 6.7) to remove the previous antibody. Staining was revealed with ECL-Plus western blotting system (Perkin Elmer). 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, vol/vol) 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 and 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 atmospheric pressure chemical ionization source 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 4 h of the light period to avoid low spontaneous activity. Behavioral sessions were video recorded and analyzed using The Observer (Noldus).

For the Porsolt FST[31], mouse were individually placed into a dark gray polyvinylchloride cylinder (15 cm in 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 2 min of the test was used as an index of despair-like behavior. Climbing and swimming were also measured. A mouse was considered to be immobile when it made only minimal movements to keep its head above water. Imipramine (Sigma) was dissolved in saline (0.9% NaCI (vol/vol), 20 mg per kg) and administered intraperitoneal in 0.1 ml per 10 g of mouse 30 min prior to experiment.

For the open field test, the apparatus consisted of a white Plexiglas-covered sawdust bedding (40 × 40 cm) with 16-cm-high walls. The floor was divided into 16 green lines squares (10 × 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 the end of the trial, mice were returned to their home cages and test boxes were 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.

For social investigation, group-housed experimental subjects (four per cage) were separated and transferred to a new cage (40 × 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 × treatment) followed by the appropriate *post hoc* test (Bonferroni). Nonpaired 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 per slices analyzed, with at least five animals included in each condition. Statistical significance between groups was tested using one-way ANOVA or the Mann-Whitney test. In [^{35}S]GTPγS autoradiographic experiments, the effect of the cannabinoid agonist was expressed as percentage of stimulation over basal activity ((% = agonist binding × 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 in prelimbic and motor cortices was carried out using ImageJ (US National Institutes of Health). Statistical comparison of experimental groups was made using a nonpaired Student's *t* test, where *n* corresponds to the number of animals analyzed. All statistical tests were performed with GraphPad Prism (GraphPad Software) using a critical probability of $P < 0.05$.

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