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DHA prevents altered 5-HT1A, 5-HT2A, CB1 and GABAA receptor binding densities in the brain of male rats fed a high-saturated-fat diet

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Abstract
Low levels of docosahexaenoic acid (DHA) have been linked to a number of mental illnesses such as memory loss, depression and schizophrenia. While supplementation of DHA is beneficial in improving memory and cognition, the influence of dietary fats on the neurotransmitters and receptors involved in cognitive function is still not known. The aim of this study was to investigate serotonin receptor (5-HT_{1A} and 5-HT_{2A}), cannabinoid receptor (CB1) and gamma-aminobutyric acid type A ($GABA_A$) receptor binding densities in the brain of male rats fed a high-saturated-fat (HF) diet, as well as the effect of DHA supplementation on HF diet. Alterations of these receptors in the post-mortem rat brain were detected by [^{3}H]-WAY-100635, [^{3}H]-ketanserin, [^{3}H]-CP-55,940 and [^{3}H]-muscimol binding autoradiography, respectively. In the hippocampus, the 5-HT_{1A}, CB1 and $GABA_A$ receptor binding densities significantly increased in response to an HF diet, while in the hypothalamus, 5-HT_{1A} and CB1 binding densities significantly increased in HF-fed rats. Importantly, DHA supplementation prevented the HF-induced increase of receptors binding density in the hippocampus and hypothalamus. Furthermore, DHA supplementation attenuated 5-HT_{2A} receptor binding density in the caudate putamen, anterior cingulate cortex and medial mammillary nucleus, which was also increased in HF group. This study showed that an HF diet increased 5-HT_{1A}, 5-HT_{2A}, CB1 and $GABA_A$ receptor binding densities in the brain regions involved in cognitive function and that dietary DHA can attenuate such alterations. These findings provide insight into the mechanism by which DHA supplementation ameliorates reduced cognitive function associated with an HF diet.

Keywords
fat, saturated, high, fed, rats, male, brain, densities, diet, binding, dha, receptor, gabaa, cb1, ht2a, ht1a, 5, prevents, altered

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Title: DHA prevents altered 5-HT1$_A$, 5-HT2$_A$, CB1 and GABA$_A$ receptor binding densities in the brain of male rats fed a high-saturated fat diet

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Abstract

Low levels of docosahexaenoic acid (DHA) have been linked to a number of mental illnesses such as memory loss, depression and schizophrenia. While supplementation of DHA is beneficial in improving memory and cognition, the influence of dietary fats on the neurotransmitters and receptors involved cognitive function is still not known. The aim of this study was to investigate serotonin receptor (5-HT_{1A} and 5-HT_{2A}), cannabinoid receptor (CB1) and gamma-aminobutyric acid type A ($GABA_A$) receptor binding densities in the brain of male rats fed a high-saturated fat (HF) diet, as well as the effect of DHA supplementation on HF diet. Alterations of these receptors in the post-mortem rat brain were detected by [^{3}H]-WAY-100635, [^{3}H]-Ketanserin, [^{3}H]-CP-55,940 and [^{3}H]-Muscimol binding autoradiography, respectively. In the hippocampus, the 5-HT_{1A}, CB1 and $GABA_A$ receptor binding densities significantly increased in response to a HF fat diet. While in the hypothalamus, 5-HT_{1A} and CB1 binding densities significantly increased in HF fed rats. Importantly, DHA supplementation prevented the HF induced increase of receptors binding density in the hippocampus and hypothalamus. Furthermore, DHA supplementation attenuated 5-HT_{2A} receptor binding density in the caudate-putamen, anterior cingulate cortex and medial mammillary nucleus, which was also increased in HF group. This study showed that a high-saturated fat diet increased 5-HT_{1A}, 5-HT_{2A}, CB1 and $GABA_A$ receptor binding densities in the brain regions involved in cognitive function, and that dietary DHA can attenuate such alterations. These findings provide insight into the mechanism by which DHA supplementation ameliorates reduced cognitive function associated with a high-saturated fat diet.

Keywords: DHA, high-saturated fat, serotonin receptor, CB1 receptor, $GABA_A$ receptor

Introduction

Different types of dietary fats affect body metabolism and cognitive function differently [1]. Studies have shown that a diet high in saturated fat promotes fat deposition and impairs memory and learning, and even contributes to the development of depression [2-4]. Conversely, a diet high in n-3 polyunsaturated fat, especially docosahexaenoic acid (DHA), can have the opposite effect [2-4]. A growing body of clinical findings implicates low DHA status with being overweight [5], impaired cognitive function, and depression [6-8]. Plasma DHA was lowered in elderly subjects with depressive disorders compared to individuals without depression [8]. The tissue DHA content of the orbitofrontal cortex and cingulate cortex was also found to be lower in individuals with major depression [6, 7]. Beneficial effects of DHA by improving cognition and anti-depressive effects have been described in clinical trials and animal studies. There is evidence that DHA supplementation improves cognition [9], enhances memory [10] and induces an anti-stress response [11], however, the underlying mechanisms remain unclear. Certain brain areas such as the hippocampus and cingulated cortex are important for cognitive function. However, there is little information on how dietary fat influences key receptors in these brain regions, which are important in the regulation of cognitive and metabolic function.

The neurotransmitter serotonin (5-HT) acts via 5-HT_{1A} and 5-HT_{2A} receptors and has an important role in various central functions including control of energy intake, obesity, memory and learning [12-14]. 5-HT_{1A} receptors are distributed throughout the brain and are located either pre or post-synaptically, where they regulate various brain functions [12, 15]. As presynaptic autoreceptors, the 5-HT_{1A} receptors are found in dorsal and median raphe nuclei and negatively regulate 5-HT synthesis. A highly palatable diet in rats increases the density of 5-HT_{1A} pre-synaptic receptor in these regions, suggesting a decrease in synthesis

and consequently a decreased release of 5-HT [16]. 5-HT_{1A} receptors as post-synaptic receptors have a wide distribution in the brain with high density in the cortical and limbic areas, especially in the hippocampus and cortex, and low expression in other brain regions such as the hypothalamus, striatum and amygdala [17]. Clinical studies have shown that 5-HT_{1A} receptor expression is negatively associated with memory function [18]. Postsynaptic 5-HT_{2A} receptors can be found in high levels in cerebral cortical areas and at intermediate levels in the hypothalamus, striatum and hippocampus [19, 20]. Using [^{125}I] DOI binding autoradiography, a high-saturated fat diet increased 5-HT_{2A} binding density in the ventromedial hypothalamic nucleus and anterior olfactory nucleus in diet induced obese mice, but not in mice resistant to obesity development [21]. Furthermore, using [^{3}H]-Ketanserin autoradiography, 5-HT_{2A} receptor binding densities were significantly increased in post-mortem tissue from the temporal cortex of patients with dementia [22]. Based on the accumulated evidence of clinical trials, blockade of 5-HT_{2A} receptor ameliorates both the positive and negative symptoms, and to some extent the cognitive deficits in schizophrenia [23, 24]. The highly selective 5-HT_{2A} antagonists MDL 100907 and EMD 281014, both developed as anti-psychotics, have also been shown to enhance cognitive function in animal models [25, 26].

The cannabinoid CB1 receptor plays an important role in various aspects of neural functions including learning and memory, anxiety, depression, addiction, appetite and feeding behaviour. Both CB1 knockout mice and CB1 antagonist (SR141716)-treated wild-type mice exhibited deficits in extinction of spatial memory [27, 28]. The systemic administration of the CB1 agonist WIN55,212-2 in rats impaired the acquisition of contextual fear conditioning [29], which is known to depend on the hippocampus [30]. GABA is the major inhibitory neurotransmitter in the brain. There are two receptors that mediate GABA neurotransmission

in the brain; $GABA_A$ and $GABA_B$. The inhibitory function of $GABA_A$ is increasingly being recognised as important in the regulation of cognition, emotion, memory and obesity. It has been reported that the density of $GABA_A$ receptors was increased in the cortex of schizophrenia patients in order to compensate for the lowered levels of GABA [31, 32]. Allelic variants in the $GABA_A\alpha6$ receptor subunit gene (GABRA6) were also associated with abdominal obesity [33]. Furthermore, the majority of leptin's antiobesity effects were mediated by GABAergic neurons reducing inhibitory tone to postsynaptic anorexigenic POMC neurons in the hypothalamus [34].

The effect of a DHA supplemented high-saturated fat diet on these receptor binding densities in brain regions associated with cognition has not been thoroughly investigated. To address this issue, we have used multiple ligands including [^{3}H]-WAY-100635, [^{3}H]-Ketanserin, [^{3}H]-CP-55,940 and [^{3}H]-Muscimol to examine the regional changes of 5-HT_{1A}, 5-HT_{2A}, CB1 and $GABA_A$ receptor in the rat brain. Rats were fed either high-saturated fat diet, DHA supplement in high-statured fat diet or low-fat diet for 4 weeks. We examined alterations in receptor expression in response to a high-saturated fat diet, and if these alterations could be prevented by a supplementation of dietary DHA.

Experimental procedure

Animals and dietary treatments

Thirty male Wistar rats (300-320g) were obtained from the Animal Resources Centre (Perth, Western Australia, Australia) and housed in environmentally controlled conditions (22°C, 12 hr light–dark cycle with light cycle from 06:00 to 18:00 h and dark cycle from 18:00 to 06:00 h) with *ad libitum* access to standard laboratory chow and water. Rats were allowed 1 week to adapt to their new environment before experiments began. They were randomized into three

groups with different diets: (1) standard laboratory chow as the low-fat control (LF, fat content 10% in kcal, saturated fat 1%), (2) high-fat diet (HF, 25% in kcal, saturated fat 10%), (3) high-fat diet + 0.5% DHA. The dose of DHA supplementation used in this study was based on the dose recommended for humans at 250mg/70kg/day (European Food Safety Authority) [35]. After four weeks of dietary treatment, rats were sacrificed by rapid CO_2 asphyxiation between 07:00 and 09:00 hrs in order to minimize the impact of circadian variation, and the brains were immediately removed and frozen in liquid nitrogen. Five rats per group were used to examine [^{3}H]-WAY-100635, [^{3}H]-Ketanserin, [^{3}H]-CP-55,940 and [^{3}H]-Muscimol binding in the brain. The study was approved by the University of Wollongong Animal Ethics Committee and all animal experiments were conducted in compliance with the National Health and Medical Research Council Australian, Code of Practice for the Care and Use of Animals for Scientific Purposes (2004).

Histological procedures

Coronal brain sections (14 μm) were cut in a cryostat at −18 °C from the level of Bregma -0.24mm to -5.16mm [36], thaw-mounted onto poly-L-lysine coated microscope slides (Polysine™, Menzel GmbH & Co, KG) [37] and stored at -20 °C.

[^{3}H]-WAY-100635, [^{3}H]-Ketanserin, [^{3}H]-CP-55,940 and [^{3}H]-Muscimol binding autoradiography

[^{3}H]-WAY-100635 autoradiography was performed to examine 5-HT_{1A} receptor binding density following procedures as described in previous work from our laboratories [38]. Brain sections were warmed to room-temperature and pre-incubated in 50 nM Tris–HCl buffer (pH 7.4) for 30 min. The sections were then incubated with 5 nM [^{3}H]-WAY-100635 (specific activity 83.0 Ci/mmol, Amersham Biosciences, UK Limited) at room temperature for 2.5 hrs

in 50 mM Tris–HCl (pH 7.4) containing 10 μM pargyline (Sigma). Non-specific binding was determined by incubating consecutive sections exposed to 10 μM 5-HT. All sections were washed for 2 min and then 3 min in ice-cold 50 mM Tris–HCl buffer.

[^{3}H]-Ketanserin autoradiography was performed as described previously [19]. Binding of [^{3}H]-Ketanserin (67.0Ci/mmol; PerkinElmer Life Sciences, Boston, MA, USA) to 5-HT_{2A} receptors was measured by preincubating sections in 170 mM Tris-HCl buffer (pH 7.4) for 15 min at room temperature. Sections were then incubated for 120 min at room temperature in buffer containing 2 nM [^{3}H]-Ketanserin. Nonspecific binding was determined by the addition of 2 μM spiperone to consecutive sections. Sections were washed in ice-cold buffer (2 × 10 min), dipped in distilled water and dried.

Binding of [^{3}H]-CP-55,940 was used to assess binding density of CB1 receptor [39]. Sections were allowed to defrost and then preincubated for 30 min in Tris-HCl buffer (5% bovine serum albumin (BSA), 50 mM Tris-HCl, pH 7.4) at room temperature. The binding sites of CB1 receptor were defined by incubation with 10 nM [^{3}H]-CP-55,940. Nonspecific binding was determined in the presence of 10 μM CP-55,940. Following incubation for 2 hrs at room temperature, slides were washed firstly for 1 hr and then 3 hrs in ice-cold buffer (1% BSA, 50 mM Tris–HCl, pH 7.4), and then finally washed for a further 5 min in buffer containing no BSA. Slides were then dipped briefly in ice-cold distilled water and dried under a gentle stream of cool air.

[^{3}H]-Muscimol binding was performed to examine $GABA_A$ receptor binding density based on the method described in previous work from our laboratories [31]. Briefly, all sections underwent three 5 min pre-incubations at 4 °C in 50 mM Tris-citrate (pH 7.0). Sections were

then incubated for 45 min at 4 °C in the same buffer containing 3 nM [^{3}H]-Muscimol (specific activity 29.5 Ci/mmol, PerkinElmer, USA). Non-specific binding was determined by incubating adjacent sections in [^{3}H]-Muscimol plus 100 μM GABA. Following incubation, sections were rinsed four times for 2s each in 4 °C buffer.

Quantification and statistical analysis

Quantification of binding sites was performed on a high-resolution Beta Imager (BioSpace, Paris, France) according to our previous study [40]. Briefly, sections were placed in a sample holder inside the detection chamber of the Beta Imager. The levels of bound radioactivity in the brain sections were directly determined by counting the number of β-particles emerging from the tissue sections. The Beta Vision Plus program (BioSpace, France) was used to measure the activities in the regions of interest. Radioligand binding signal was expressed in counts per minute per square millimetre (cpm/mm^2), and with the use of standards was converted to fmol/mg tissue equivalents. The receptor density in various brain regions was quantified by measuring the average density of each region in three to five adjacent brain sections. Different brain regions were identified by reference to a standard rat brain atlas [36]. Data was expressed as mean ± SEM. [^{3}H]-WAY-100635, [^{3}H]-Ketanserin, [^{3}H]-CP-55,940 and [^{3}H]-Muscimol binding densities for each brain region were analyzed using a one-way ANOVA followed by a post-hoc Tukey–Kramer–HSD test using the SPSS 15.0 program (Chicago, IL). *P* values of less than 0.05 were regarded as statistically significant, and *P* values of less than 0.10 as a statistically significant trend.

RESULTS

5-HT$_{1A}$ receptor binding

The 5-HT$_{1A}$ receptor was widely distributed throughout the rat brain (Table 1). High 5-HT$_{1A}$

receptor density was observed in the hippocampus, anterior cingulated cortex (ACC), lateral septal nucleus, primary motor cortex, and medial posterodorsal amygdala. Binding to 5-HT_{1A} receptor was also observed in the ventromedial hypothalamus (VMH) and piriform cortex in lower levels.

Within the hippocampus there was a significant effect of dietary intervention on 5-HT_{1A} receptor density ($F_{(2, 12)}$=11.641, *P* =0.002) (Table 1). The rats on HF diet had significantly higher 5-HT_{1A} binding density (+54%, *P* =0.006), compared to rats on LF diet. For the DHA supplemented group, 5-HT_{1A} binding density was significantly lower than the HF group (-40%, *P* =0.002), but there was no significant difference in 5-HT_{1A} binding density in the hippocampus between DHA group and LF group (Fig 1A, Fig 2).

A dietary effect was also observed on 5-HT_{1A} receptor density within the VMH ($F_{(2, 12)}$=8.222, *P* =0.006) (Table 1). Rats maintained on HF diet had significantly higher 5-HT_{1A} receptor expression in VMH than rats on LF diet (+58%, *P* =0.007). In addition, dietary intervention by the addition of DHA to the HF diet significantly decreased receptor densities compared to the rats on HF diet (-31% decrease, *P* =0.022), but there was no significant difference in 5-HT_{1A} receptor expression in the VMH between the DHA and LF group (Fig 3A, Fig 2).

5-HT2A binding density

There was abundant binding of [^{3}H]-Ketanserin to 5-HT_{2A} receptors in the ACC, caudate putamen, medial mammillary nucleus (MM), primary motor cortex, piriform cortex, medial posterodorsal amygdala and VMH. 5-HT_{2A} receptor expression was also observed at lower levels in the hippocampus (Table 2).

5-HT_{2A} binding density in the ACC differed between the various diet treatment groups in this study ($F_{(2, 12)}$=12.474, *P* =0.001) (Table 2). The 5-HT_{2A} binding density was significantly higher in the HF group than the LF or HF + 0.5% DHA group (+71%, *P* =0.003 and +75%, *P*

234 =0.002 respectively) (Fig 4A and Fig 5). There was no significant difference between DHA
235 and LF group.
236 Within the caudate putamen dietary intervention had a significant effect on 5-HT_{2A} binding
237 density ($F_{(2, 12)}$=11.179, *P* =0.002) (Table 2). Rats fed the HF diet had significantly higher 5-
238 HT_{2A} binding density (+43%, *P* =0.001) compared to rats on the LF diet. The DHA
239 supplemented group had significantly lower 5-HT_{2A} binding density compared with the HF
240 group (-19% lower, *P* =0.026), while there was no significant difference between DHA group
241 and LF group (Fig 4B and Fig 5).
242 This study also demonstrated differences between diet treatment groups in 5-HT_{2A} receptor
243 density in the MM ($F_{(2, 12)}$=6.857, *P* =0.010) (Table 2). In the HF group 5-HT_{2A} binding
244 density was 47% higher than the LF group (*P* =0.026) and 55% higher than the DHA
245 supplemented group (*P* =0.015). No difference was observed between LF and DHA groups
246 (Fig 4C and Fig 6). A similar pattern of receptor expression in response to diet treatment was
247 also observed in the anterior amygdaloid area.
248 **CB1 receptor binding density**
249 Diet affected the expression of CB1 receptor within the hippocampus ($F_{(2, 12)}$=2.960, *P*
250 =0.048) (Table 3). The rats on HF diet had 43% elevated CB1 receptor density compared with
251 rats on LF diet (*P* =0.007) (Fig 1B, Fig 7). DHA supplemention significantly lowered CB1
252 receptor binding density compared with the HF group (-22%, *P* =0.041), but there was no
253 significant difference in hippocampal CB1 receptor density between the DHA and LF groups.
254 There was also a significant effect by dietary intervention on CB1 receptor density in the Arc
255 ($F_{(2, 12)}$=37.138, *P* <0.001) (Table 3). In this region, rats on the HF diet had significantly
256 higher CB1 receptor density than the rats on LF diet (+64%, *P* <0.001) (Fig 3B, Fig 7). The
257 supplementation of DHA in the HF diet significantly decreased receptor expression compared
258 to the rats on HF diet (-39%, *P* <0.001), but no difference was observed between DHA and

LF groups.

Furthermore, HF diet significantly increased CB1 receptor density in the substantia nigra (SN), ventral tegmental area (VTA), and amygdala compared with LF diet (SN: +37%, *P* =0.003; VTA: +15%, *P* =0.020; amygdala: +20%, *P* =0.045) (Table 3). CB1 receptor binding density was decreased with DHA supplementation compared with the HF group in these brain areas. There was no effect of dietary intervention on CB1 in the VMH, caudate putamen, piriform cortex, primary motor cortex and ACC.

$GABA_A$ binding density

$GABA_A$ receptor binding density in the hippocampus was affected by the different diets utilised in this study ($F_{(2, 12)}$=4.386, *P* =0.040) (Table 4). Hippocampal $GABA_A$ receptor density was increased 53% in the HF group compared to the LF group (*P* =0.021) (Fig 1C, Fig 8), while DHA supplementation significant lowered the HF induced elevation in $GABA_A$ receptor binding density by 42% (*P* =0.038). There was also a positive correlation between CB1 and $GABA_A$ receptor binding density in the hippocampus (R=0.593, *P* =0.025) (Fig 9).

In the thalamus and posterior cingulated cortex (PCC), HF diet significantly decreased $GABA_A$ receptor density compared with LF diet (thalamus, -41%, *P* =0.020; PCC -60%, *P* =0.011) (Table 4). While $GABA_A$ receptor density was significantly increased by DHA supplementation compared with HF group in these brain areas (thalamus, +77%, *P* =0.011; +PCC 154%, *P* =0.009). There was no significant effect of dietary intervention on $GABA_A$ receptor density in the ACC.

Energy intake, body weight, and plasma leptin level of rats with dietary intervention

The average of energy intake during the dietary treatment was significantly different among the three groups (*P* =0.010, HF: 94.38±2.69 kcal/24hours; LF: 84.69±1.56 kcal/24hours; HF + 0.5% DHA: 90.81±1.86 kcal/24hours), in which HF group was significantly higher than LF group (*P* =0.007). No significant difference was found between other groups. The four week

accumulative energy intake was also significantly higher in HF group than the LF group (11.44%, P = 0.012). There was no significant difference in body weight changes among three groups (P =0.503, HF: 84.80±5.48g; LF: 81.78±6.05g; HF + 0.5% DHA: 83.00±6.04g). The plasma level of leptin in HF diet fed rats (11.47±2.17ng/ml) was significantly higher than that of the LF group (4.72±0.73ng/ml) (P =0.005). DHA supplementation decreased the plasma leptin level (7.21±1.01ng/ml) of rats compared with HF group in statistically significant trend (P =0.070), while there was no significant difference in plasma leptin between DHA and LF group (P =0.290).

DISCUSSION

Serotonin, cannabinoids and GABA systems play an important role in cognitive function [14, 29, 31], and a chronic high-saturated fat diet has been shown to affect memory and learning [2]. Therefore, the effects of high-saturated fat diets on these neurotransmitter systems are of interest. This study showed that a high-saturated fat diet increased the density of 5-HT_{1A} receptor in the hippocampus and VMH, 5-HT_{2A} receptor in the ACC, caudate putamen and MM, CB1 receptor in the hippocampus, Arc, SN, VTA and amygdale, and $GABA_A$ receptor in the hippocampus. These regions are primarily limbic structures associated with the regulation of cognition. In addition, these HF diet induced changes in receptor density can be prevented by dietary supplementation of 0.5% DHA.

A number of changes in receptor expression have been observed in the brain of individuals with abnormal cognitive function. It has been reported that 5-HT_{1A} receptor binding density in the human hippocampus is negatively correlated with memory [18]. Furthermore, 5-HT_{1A} and 5-HT_{2A} receptor binding densities are significantly increased in the temporal cortex of

patients with dementia [22]. Both $GABA_A$ and CB1 receptor densities are increased in the posterior cingulated cortex of schizophrenia [41, 42]. This study similarly found alterations in receptor density in response to a high-saturated fat diet, specifically increased 5-HT_{1A}, 5-HT_{2A}, $GABA_A$ and CB1 receptor densities in a number of brain regions, particularly in the limbic structures. Although the mechanism for the alteration of receptor binding densities is unclear, such effects could be due to the high-saturated fat diet decreasing the level of the respective neurotransmitters in the limbic regions. This is supported by a study showing that a high-fat diet (20% corn oil) for six weeks significantly decreased 5-HT levels in the brainstem of rats [43]. In addition, maternal high-fat consumption results in a significant decrease in CSF 5-HT content leading to 55% of offspring with increased anxiety as assessed by the novel object tests, and 78% with aberrant behavior (anxious and/or aggressive) [44].

We found that hippocampal 5-HT_{1A} binding density was increased in rats fed a high-saturated fat diet. Hippocampal circuits play an important role in learning and memory, but also in the hedonic aspects of eating [18, 45]. 5-HT_{1A} receptors in the hippocampus are negatively associated with memory function in clinical and animal studies [18, 46]. Using positron emission tomography (PET), a significant negative correlation was found between explicit memory function and 5-HT_{1A} receptor expression localized in the bilateral hippocampus of healthy subjects. Furthermore, administration of the 5-HT_{1A} agonist tandospirone dose-dependently impaired explicit verbal memory [18]. In a rat study, injection of the 5-HT_{1A} agonist 8-OH-DPAT into hippocampus resulted in memory and learning impairment [46]. Conversely, administration of WAY 100635, a 5-HT_{1A} antagonist, into the hippocampus of rats prevented the deficit of spatial learning induced by administration of CPP, a NMDA receptor antagonist [47]. Recent findings indicate that dietary factors which promote excessive food intake and weight gain can also interfere with hippocampal functioning. For

example, epidemiological and animal studies show that intake of diets high in saturated fat are associated with memory deficits and microglial activation (indicating inflammation and/or gliosis) in the hippocampus [2, 3]. Therefore, the high-saturated fat diet induced increase in hippocampal 5-HT_{1A} receptor expression observed in this study may be involved in impairment of hippocampus function associated with learning and memory which in turn contributes to an increased energy intake.

Furthermore, we found that both CB1 and $GABA_A$ receptor density were increased in the hippocampus of rats fed high-saturated fat diet. It is known that CB1 receptors are highly expressed in the hippocampus and are involved in memory function in this brain region. An intrahippocampal administration of rimonabant, a CB1 antagonist, completely attenuated the memory disruptive effects of cannabinoid induced memory impairment [48]. Systemic and intrahippocampal administration of cannabinoid agonists have been shown to impair hippocampal-dependent memory tasks [48, 49]. Oral administration of a CB1 inverse agonist, SLV319, inhibits the CB1 receptor-mediated catalepsy induced by HU-210 ip injection in mice [50]. In the present study, the elevated CB1 receptor binding density suggests that activation of CB1 in the hippocampus may contribute to high-saturated fat associated memory deficits. Endocannabinoid (eCB) ligands have been shown to act on the CB1 receptor to inhibit the release of GABA in the rat hippocampus [51]. In this study the increased $GABA_A$ receptor expression in response to high-fat diet may reflect decreased GABA as a consequence of CB1 receptor activation in the hippocampus. This is supported by our observation that CB1 receptor density is positively correlated with $GABA_A$ receptor density. Furthermore, a previous study showed that in high-fat diet induced obese mice CB1 receptor immunoreactivity and the eCBs, anandamide and 2-arachidonoyl glycerol (2-AG) were increased in the hippocampus [52]. In this study CB1 receptor binding density in the

hippocampus was increased even without any changes in body weight. This suggests that high-fat diet alone rather than obesity increases CB1 binding.

Both clinical trials and animal studies have shown that DHA supplementation can improve learning and memory [53, 54]. Conversely, depletion of DHA in rat brain was found to increase 5-HT_{1A} expression in the hippocampus and was associated with impairment of spatial learning and memory [55, 56]. In our study, addition of DHA to the diet prevented the increase of hippocampal 5-HT_{1A} density in rats induced by a high-saturated-fat diet. DHA supplementation is also able to prevent increased CB1 and $GABA_A$ receptor densities induced by high-fat diet, as shown in this study. These findings suggest the effect of DHA supplementation on improving learning and memory may be via its influence on hippocampal 5-$HT_{1A,}$ CB1 and $GABA_A$ systems.

The hypothalamus is well recognised as a critical centre in the regulation of energy balance. Hypothalamic 5-HT_{1A} receptors are involved in the control of negative energy balance. A negative relationship has been reported between the 5-HT content in the hypothalamus and amount of fat and food intake in rodents. For example, an infusion of 5-HT into the hypothalamus can lead to a dose-related decrease in the amount of fat intake in either fat- or carbohydrate- preferring rats [57]. The intrahypothalamic injection of a 5-HT_{1A} agonist, 8-OH-DPAT, decreases food intake and promotes satiety [58]. Conversely, intra-hypothalamic injection of WAY-100635, a 5-HT_{1A} antagonist, blocks the anorexic effect induced by 5-HT [59]. The present study showed that rats fed a high-fat diet had increased 5-HT_{1A} receptor expression in the ventromedial hypothalamus (VMH). This finding supports the assertion that a high-fat diet significantly decreases central 5-HT levels in rats [43]. Moreover, in the present study DHA supplementation prevented the increase in VMH 5-HT_{1A} receptor density

induced by a high-saturated fat diet, which is in agreement with various reports in the literature. Previous studies have shown that n-3 PUFA/DHA intake influences 5-HT levels in the brain. A positive association has been reported between the amount of dietary DHA and brain 5-HT in piglets [60]. While rats maintained on a n-3 deficient diet have a low response to fenfluramine induced 5-HT stimulation [61]. Finally, n-3 PUFA supplementation in mice reverses the stress-induced reduction in 5-HT levels [62].

CB1 receptor expression was also increased in the Arc of the hypothalamus as a result of 4 weeks of high-saturated fat diet, and this was prevented by dietary DHA supplementation. Hypothalamic eCBs and the CB1 receptor are involved in food intake and the response to peripheral feeding signals. Intravenous injection of leptin reduces the levels of the eCBs anandamide and 2-AG in the hypothalamus of normal rats and ob/ob mice [63]. High-saturated fat diets increase plasma leptin thereby downregulating eCBs in the Arc, which may have led to the upregulation of Arc CB1 receptor density observed in this study. Moreover, the prevention of hyperleptinemia in high-saturated fat fed rats supplemented with DHA may have played a role in maintaining CB1 receptor binding density at levels similar to LF rats.

In the present study a high-saturated fat diet increased 5-HT_{2A} receptor binding density in the caudate putamen (striatum), ACC and MM of rats. The striatal serotonergic (5-HT) system is involved in reward behaviour; elevated 5-HT neurotransmission increases reward (positive feedback) sensitivity and decreases negative feedback sensitivity in rats [64, 65]. Rats fed a high-saturated fat diet have lowered levels of 5-HT release from striatal slices compared to rats fed a low-fat diet [64]. High saturated-fat diet induced obesity has been considered as a compulsive disorder reflecting a “reward deficiency syndrome” [66]. Therefore, the increase in striatal 5-HT_{2A} receptor binding density observed in this study may contribute to deficits in

the reward system. The ACC and MM are involved in cognitive and memory function [67, 68]. Studies with functional neuroimaging techniques, including PET and functional magnetic resonance imaging (fMRI), have ascribed the ACC with cognitive function and working memory [69]. Rodents with lesions of the MM are impaired on tests of spatial memory tasks and working memory [70, 71]. When rats are fed a high-fat diet they show a reduction in their cognitive ability and a decline in working memory after just nine days [72]. The 5-HT_{2A} receptor plays an important role in cognitive abilities and working memory process [13, 73]. In the present study, 5-HT_{2A} receptor binding density increased in brain regions related to cognition and memory (ACC and MM).

Decreased DHA content in the brain is associated with increased density of cortical 5-$HT2_A$ receptors and altered serotonergic neurotransmission [74, 75]. Perinatal DHA-deficient rats have significantly lowered 5-HT content in the prefrontal cortex [74]. Moreover, a n-3 PUFA-supplemented diet reverses decreased brain 5-HT levels in mice subjected to chronic mild stress [76]. In the present study adding DHA into the high-saturated fat diet of rats prevents increased levels of 5-$HT2_A$ binding density in the striatum, ACC and MM. The previously discussed ability of DHA supplementation to maintain central 5-HT levels is a potential mechanism by which DHA prevents 5-$HT2_A$ receptor upregulation. In addition, DHA content influences the physicochemical properties of neuronal membranes, and thus modulates the function of membrane bound proteins, such as receptors [77, 78]. Alterations in the fatty acid composition of neural membranes with DHA supplementation may result in changes in the affinity of neuronal receptors towards their neurotransmitter [77]. Therefore it is also possible that DHA directly affects the 5-$HT2_A$ receptor by increasing affinity to its neurotransmitter, negating the need for an increase in expression to cope with reduced 5-HT levels. DHA can affect gene expression as well as mRNA stability [77]. It is therefore also

possible that DHA exerts its effects on the 5-HT2_A receptor at a transcriptional level. However, the exact mechanism by which DHA influences this receptor requires further research.

In summary, we found that a high-saturated fat diet significantly increased 5-HT$_{1A}$, CB1 and GABA$_A$ receptor binding densities in various rat brain regions, especially in limbic structures such as hippocampus and hypothalamus, which are important in the regulation of energy balance, learning, memory and cognitive functions. Furthermore, 5-HT2_A receptor binding was increased in the caudate putamen, anterior cingulated cortex and medial mammillary nucleus of rats fed a high-saturated fat diet. The anatomical distributions of these receptor alterations suggest serotonin, cannabinoid and GABA receptor contribute at least partially to cognitive dysfunctions and abnormal energy balance induced by high-saturated fat diet, which is well supported by current literature. Importantly, the addition of dietary DHA prevented alteration of these receptor binding densities in rats induced by high-fat diet. The present findings point to DHA acting on numerous receptor systems in various areas of the brain. Furthermore, our results support the assertion that DHA supplements have beneficial effects on improving memory and cognition. Therefore, potential strategies to improve mental function against the adverse effects of high-saturated fat diets include targeting the serotonin, CB1 and GABA receptor systems, as well the proper application of molecular nutrition using supplements such as DHA.

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673 **Figure Legends:**
674
675 Fig 1. The effect of dietary intervention on [^{3}H]-WAY-100635 (A), [^{3}H]-CP55940 (B) and
676 [^{3}H]-Muscimol (C) binding (nCi/mg tissue) in the hippocampus of the rat brain. Data are
677 expressed as mean ± SEM. Abbreviations: LF, low-fat diet; HF, high-saturated fat diet; DHA,
678 n-3 polyunsaturated docosahexaenoic acid; Hip: hippocampus. *P <0.05 vs. HF.
679
680 Fig 2. Autoradiograph depicting [^{3}H]-WAY-100635 binding in the hippocampus and
681 ventromedial hypothalamus (VMH) of rats fed a LF (B), HF (C) and HF+DHA diet (D).
682 Panel A is from a rat brain atlas. The density of [^{3}H]-WAY-100635 binding was significantly
683 increased in the hippocampus and VMH by HF diet, whereas the DHA supplement prevented
684 the increase of [^{3}H]-WAY-100635 binding by HF diet. LF, low-fat diet; HF, high-saturated
685 fat diet; DHA, n-3 polyunsaturated docosahexaenoic acid.
686
687 Fig 3. The effect of dietary intervention on [^{3}H]-WAY-100635 (A) and [^{3}H]-CP55940 (B)
688 binding (nCi/mg tissue) in the hypothalamus of the rat brain. Data are expressed as mean ±
689 SEM. Abbreviations: LF, low-fat diet; HF, high-saturated fat diet; DHA, n-3 polyunsaturated
690 docosahexaenoic acid; VMH, ventromedial hypothalamus; Arc, hypothalamic arcuate
691 nucleus. *P <0.05 vs. HF.
692 Fig 4. The effect of dietary intervention on [^{3}H]-Ketanserin binding density (nCi/mg tissue)
693 in the rat brain. Data are expressed as mean ± SEM. Abbreviations: MM, medial mammillary
694 nucleus; CPu, caudate putamen; ACC, anterior cingulate cortex; LF, low-fat diet; HF, high-
695 saturated fat diet; DHA, n-3 polyunsaturated docosahexaenoic acid. *P <0.05 vs. HF.
696
697 Fig 5. Autoradiograph depicting [^{3}H]-Ketanserin binding in the anterior cingulae cortex and
698 caudate putamen of rats on LF (B), HF (C) and HF+DHA diet (D). Panel (A) is from a rat

699 brain atlas. The density of [^{3}H]-Ketanserin binding was significantly increased in the anterior
700 cingulae cortex and caudate putamen by HF diet whereas the DHA supplement prevented the
701 increase of [^{3}H]-Ketanserin binding by HF diet. LF, low-fat diet; HF, high-saturated fat diet;
702 DHA, n-3 polyunsaturated docosahexaenoic acid.
703
704 Fig 6. Autoradiograph depicting [^{3}H]-Ketanserin binding in the medial mammillary nucleus
705 of rats on LF (B), HF (C) and HF+DHA diet (D). Panel (A) is from a rat brain atlas. The
706 density of [^{3}H]-Ketanserin binding was significantly increased in the medial mammillary
707 nucleus induced by HF diet, whereas the DHA supplement prevented the increase of [^{3}H]-
708 Ketanserin binding by HF diet. LF, low-fat diet; HF, high-saturated fat diet; DHA, n-3
709 polyunsaturated docosahexaenoic acid.
710
711 Fig 7. Autoradiograph depicting [^{3}H]-CP-55,940 binding in the hippocampus (A-C) and
712 hypothalamic arcuate nucleus (D-F) of rats on LF (A and D), HF (B and E) and HF+DHA
713 diet (C and F). The density of [^{3}H]-CP-55,940 binding was significantly increased in the
714 hippocampus and hypothalamic arcuate nucleus by HF diet, whereas the DHA supplement
715 prevented the increase of [^{3}H]-CP-55,940 binding by HF diet. LF, low-fat diet; HF, high-
716 saturated fat diet; DHA, n-3 polyunsaturated docosahexaenoic acid.
717
718 Fig 8. Autoradiograph depicting [^{3}H]-Muscimol binding in the hippocampus of rats on LF
719 (A), HF (B) and HF+DHA diet (C). The density of [^{3}H]-Muscimol binding was significantly
720 increased in the hippocampus by HF diet, whereas the DHA supplement prevented the
721 increase of [^{3}H]-Muscimol binding by HF diet. LF, low-fat diet; HF, high-saturated fat diet;
722 DHA, n-3 polyunsaturated docosahexaenoic acid.
723

Fig 9. There was significant correlation between [^{3}H]-CP55940 and [^{3}H]-Muscimol binding (nCi/mg tissue) in the hippocampus of rat brain.

Table 1. Specific [^{3}H]-WAY-100635 binding (nCi/mg tissue; mean ± SEM) in different brain regions following 4 weeks of dietary intervention

	Mean ± SEM.			One-way ANOVA		*P* value, Tukey's HSD post hoc		
	LF (n=5)	HF (n=5)	HF+DHA (n=5)	*F* (2, 12)	*P* value	HF vs. LF	HF vs. DHA	DHA vs. LF
Hip	2.14±0.21	3.29±0.16	1.97±0.24	11.641	***0.002***	***0.006***	***0.002***	0.827
VMH	0.74±0.06	1.17±0.10	0.81±0.08	8.222	***0.006***	***0.007***	***0.022***	0.790
M1	1.66±0.09	1.58±0.09	2.05±0.07	1.167	0.344	–	–	–
ACC	1.64±0.12	1.41±0.15	1.48±0.17	0.635	0.547	–	–	–
LSD	2.89±0.32	2.65±0.24	3.43±0.42	0.043	0.958	–	–	–
MeP	1.46±0.07	1.67±0.22	1.69±0.13	0.654	0.538	–	–	–
Pir	1.09±0.08	1.04±0.05	1.11±0.10	0.192	0.828	–	–	–

Abbreviations: VMH, Ventromedial hypothalamus; Hip, Hippocampus; M1, primary motor cortex; ACC, anterior cingulate cortex; LSD, lateral septal nucleus; MeP, Medial posterodorsal amygdala; Pir, Piriform cortex; LF, low-fat diet; HF, high-fat diet; DHA, n-3 polyunsaturated docosahexaenoic acid.

Table 2. Specific [^{3}H]-Ketanserin binding (nCi/mg tissue; mean ± SEM) in different brain regions following 4 weeks of dietary intervention

	Mean ± SEM.			One-way ANOVA		*P* value, Tukey's HSD post hoc		
	LF (n=5)	HF (n=5)	HF+DHA (n=5)	*F* (2, 12)	*P* value	HF vs. LF	HF vs. DHA	DHA vs. LF
ACC	1.92±0.25	3.29±0.21	1.88±0.21	12.474	***0.001***	***0.003***	***0.002***	0.99
CPu	2.13±0.20	3.04±0.07	2.45±0.10	11.179	***0.002***	***0.001***	***0.026***	0.276
MM	2.44±0.39	3.58±0.20	2.31±0.14	6.857	***0.010***	***0.026***	***0.015***	0.943
AA	2.50±0.19	3.28±0.14	2.39±0.13	9.660	***0.003***	***0.006***	***0.002***	0.888
Hip	0.98±0.05	0.97±0.06	0.89±0.04	0.916	0.426	–	–	–
VMH	1.36±0.07	1.35±0.14	1.34±0.09	0.016	0.984	–	–	–
MeP	1.82±0.05	1.76±0.11	1.82±0.16	0.116	0.891	–	–	–
Pir	3.20±0.22	3.48±0.30	3.73±0.39	0.698	0.517	–	–	–
M1	4.45±0.50	4.81±0.43	4.92±0.39	0.303	0.744	–	–	–

Abbreviations: MM, Medial mammillary nucleus; ACC, Anterior cingulated cortex; AA, Anterior amygdaloid area; CPu, Caudate putamen; Hip, hippocampus; M1, primary motor cortex; MeP, Medial posterodorsal amygdala; Pir, Piriform cortex; VMH, Ventromedial hypothalamus; LF, low-fat diet; HF, high-fat diet; DHA, n-3 polyunsaturated docosahexaenoic acid.

Table 3. Specific [^{3}H]-CP55940 binding (nCi/mg tissue; Mean±SEM) in different brain regions following 4 weeks of dietary intervention

	Mean±SEM			One-way ANOVA		*P* value, Tukey's HSD post hoc		
	LF	HF	HF+DHA	F (2, 12)	p- value	HF vs. LF	HF vs. DHA	DHA vs. LF
Hip	82.92±7.39	118.49±14.70	92.47±4.83	2.960	***0.048***	***0.007***	***0.041***	0.778
Arc	37.15±1.72	61.01±3.01	37.27±1.62	37.138	***<0.001***	***<0.001***	***<0.001***	0.999
VMH	65.99±4.41	75.06±4.06	61.45±3.02	3.202	0.077	–	–	–
Amg	47.89±1.32	57.55±2.56	45.38±3.25	6.559	***0.012***	***0.045***	***0.013***	0.764
SN	37.68±1.81	51.73±4.61	34.75±2.93	7.465	***0.008***	***0.003***	***0.001***	0.810
VTA	40.12±1.55	47.34±1.57	38.30±1.10	11.260	***0.002***	***0.020***	***0.005***	0.651
CPu	54.10±6.20	56.78±4.10	51.53±2.66	0.331	0.725	–	–	–
Pir	56.13±3.20	65.76±7.12	54.34±2.87	1.635	0.236	–	–	–
M1	65.14±6.95	63.59±5.15	61.87±6.21	0.071	0.932	–	–	–
ACC	57.30±6.91	60.84±6.21	55.27±3.41	0.244	0.787	–	–	–

Abbreviations: Arc, hypothalamic arcuate nucleus; SN, Substantia nigra; VTA, Ventral tegmental area; Hip, hippocampus; VMH, Ventromedial hypothalamus; Amg, Amygdala; CPu, Caudate putamen; Pir, Piriform cortex; M1, Primary motor cortex; ACC, anterior cingulate cortex; HF, high-fat diet; LF, low-fat diet; DHA, n-3 polyunsaturated docosahexaenoic acid.

Table 4. Specific [^{3}H]-Mmuscimol binding (nCi/mg tissue; mean ± SEM) in different brain regions following 4 weeks of dietary intervention

	Mean ± SEM.			One-way ANOVA		*P* value, Tukey's HSD post hoc		
	LF (n=5)	HF (n=5)	DHA (n=5)	*F* (2, 12)	*P* value	HF vs. LF	HF vs. DHA	DHA vs. LF
Hip	3.43±0.47	5.25±0.57	3.05±0.67	4.386	***0.040***	***0.021***	***0.038***	0.656
PCC	3.23±0.68	1.23±0.34	3.12±0.32	6.923	***0.011***	***0.012***	***0.009***	0.878
Thalamus	5.10±0.58	3.01±0.60	5.35±0.47	5.375	***0.022***	***0.020***	***0.011***	0.760
ACC	2.23±0.21	1.96±0.34	2.59±0.47	0.733	0.502	–	–	–

Abbreviations: ACC, Anterior cingulated cortex; Hip, hippocampus; PCC, posterior cingulated cortex; LF, low-fat diet; HF, high-fat diet; DHA, n-3 polyunsaturated docosahexaenoic acid.

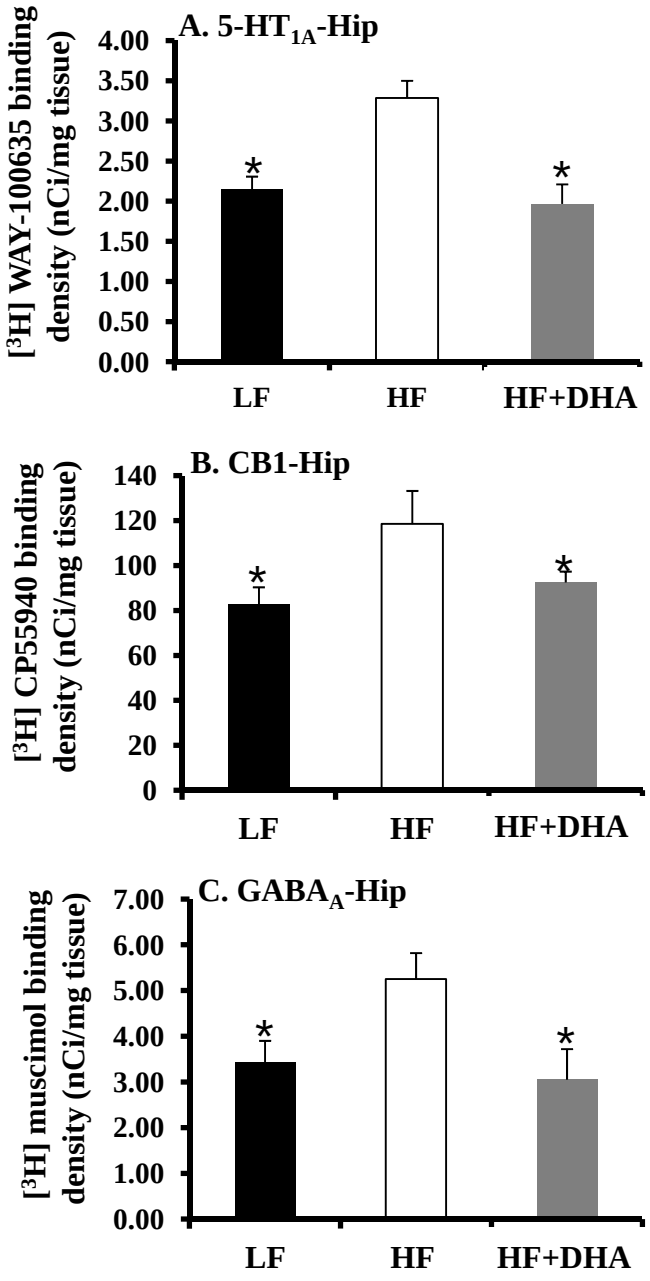
A. 5-HT1A-Hip
[3H] WAY-100635 binding density (nCi/mg tissue)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
LF
HF
HF+DHA
B. CB1-Hip
[3H] CP55940 binding density (nCi/mg tissue)
140
120
100
80
60
40
20
0
LF
HF
HF+DHA
C. GABAA-Hip
[3H] muscimol binding density (nCi/mg tissue)
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
LF
HF
HF+DHA

Fig 1.

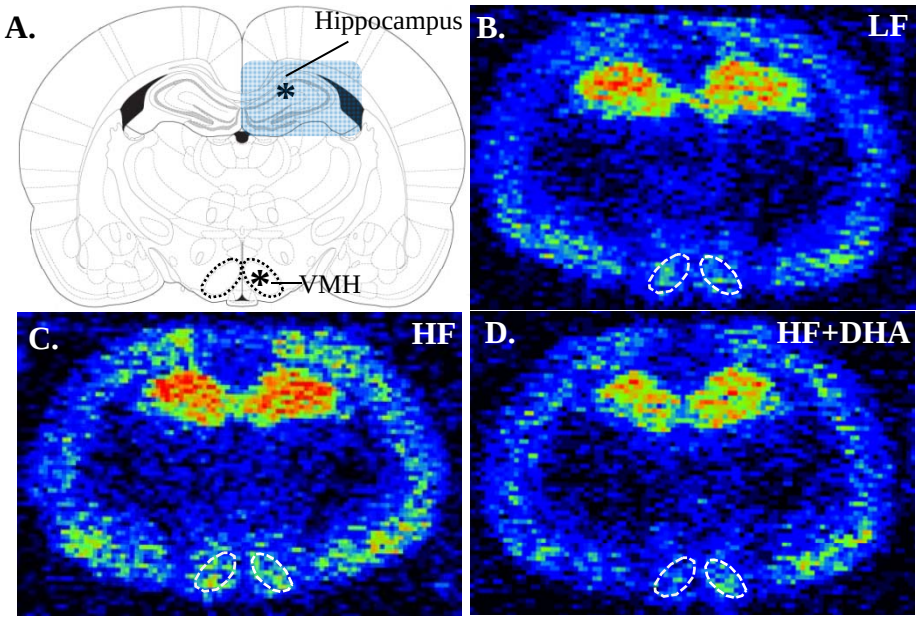

Fig 2.

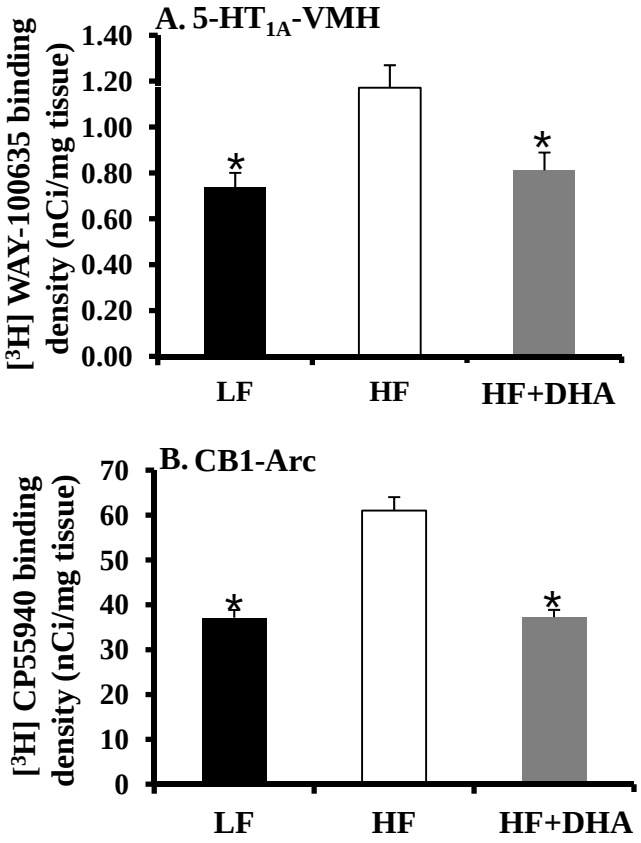
A. 5-HT1A-VMH
[3H] WAY-100635 binding density (nCi/mg tissue)
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
*
*
LF
HF
HF+DHA
B. CB1-Arc
[3H] CP55940 binding density (nCi/mg tissue)
70
60
50
40
30
20
10
0
*
*
LF
HF
HF+DHA

Fig 3.

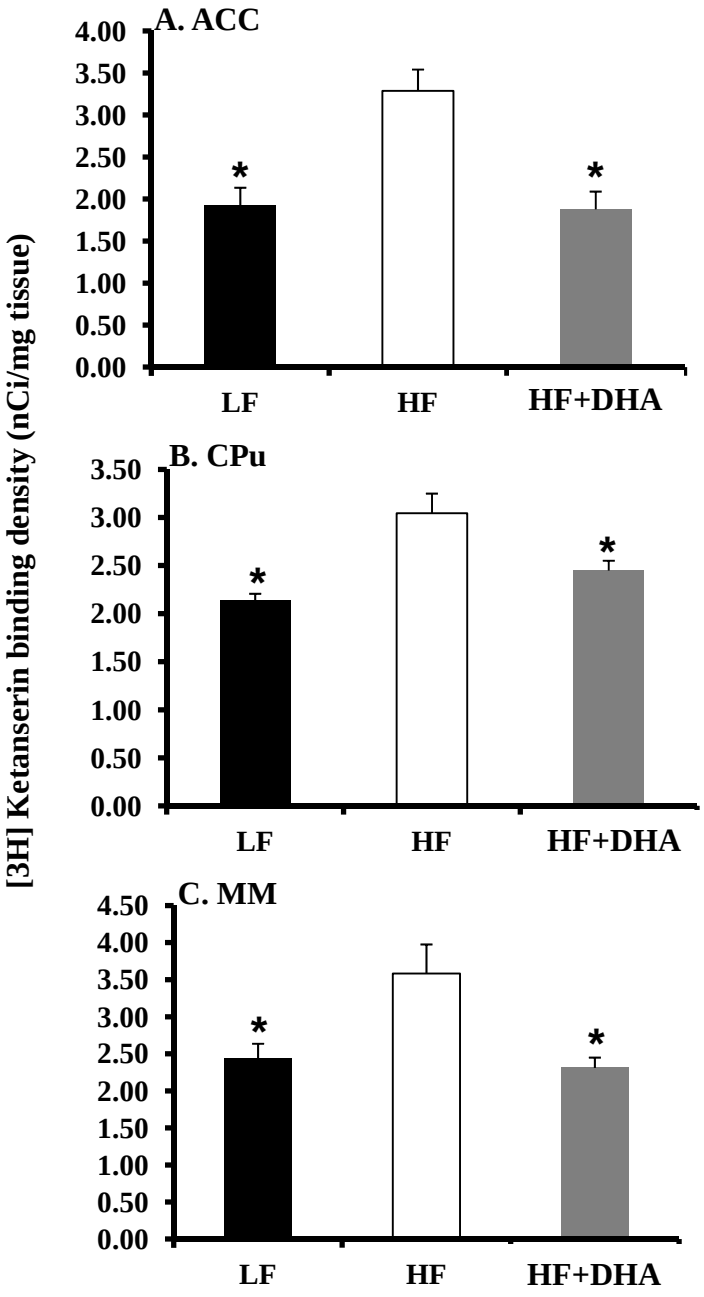
A. ACC
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
*
*
LF
HF
HF+DHA
B. CPu
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
*
*
LF
HF
HF+DHA
C. MM
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
*
*
LF
HF
HF+DHA
[3H] Ketanserin binding density (nCi/mg tissue)

Fig 4.

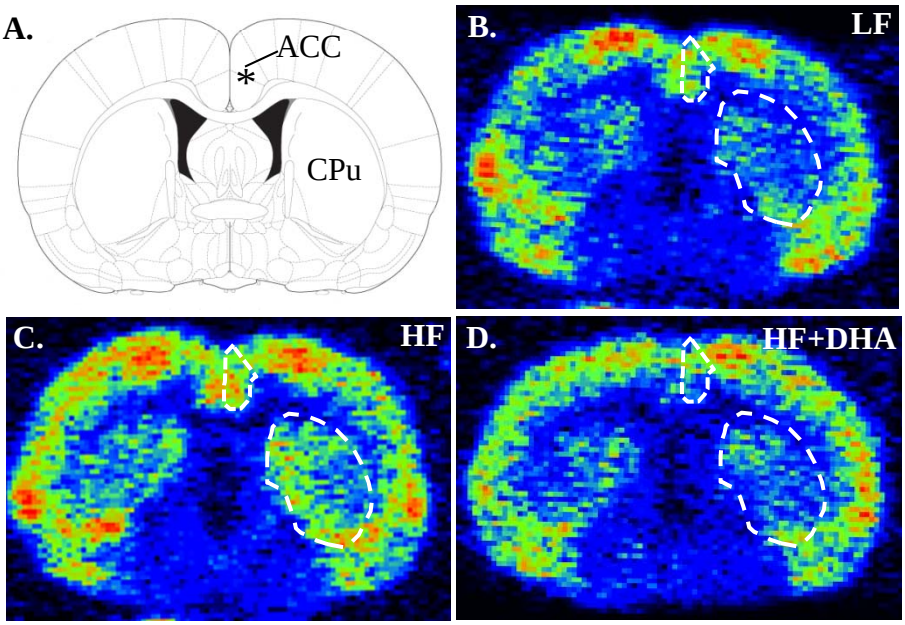

Fig 5.

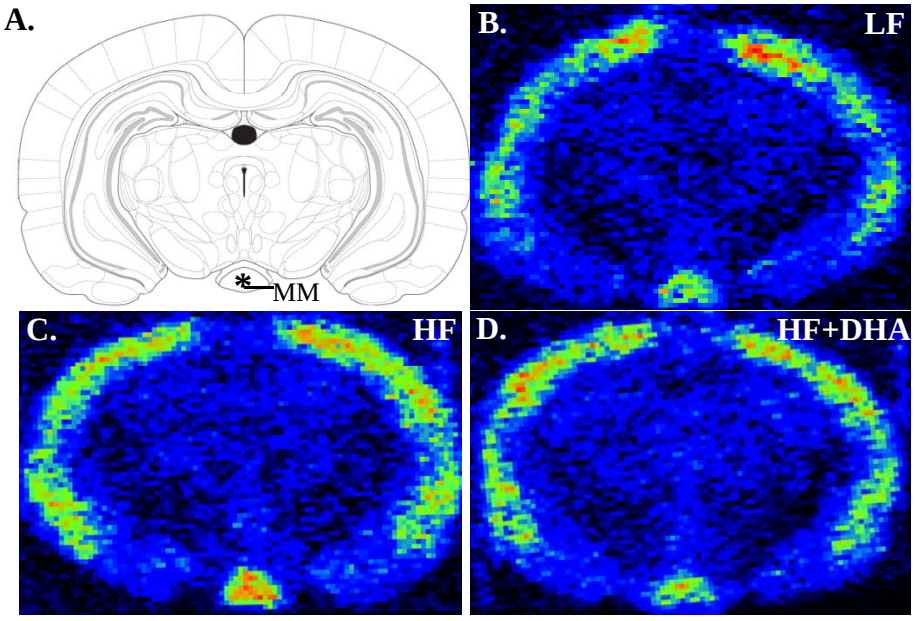

Fig 6.

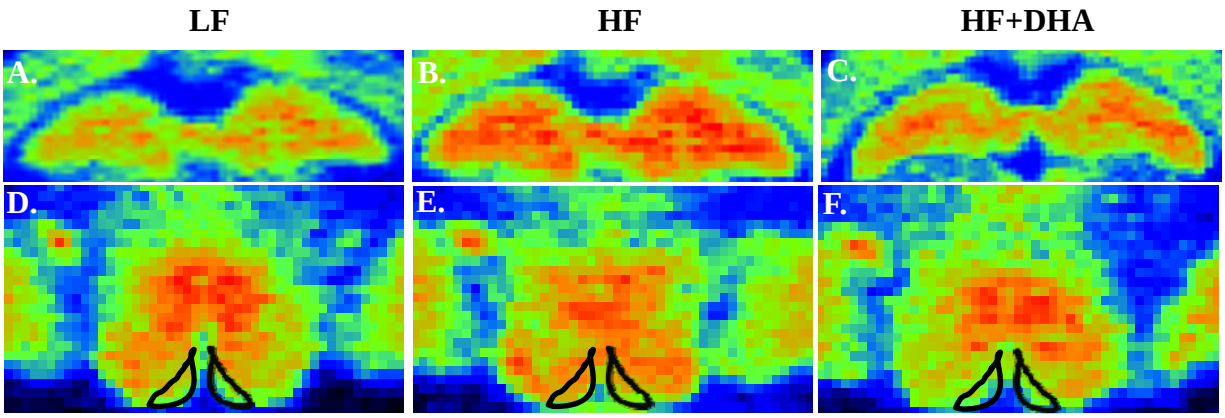

Fig 7.

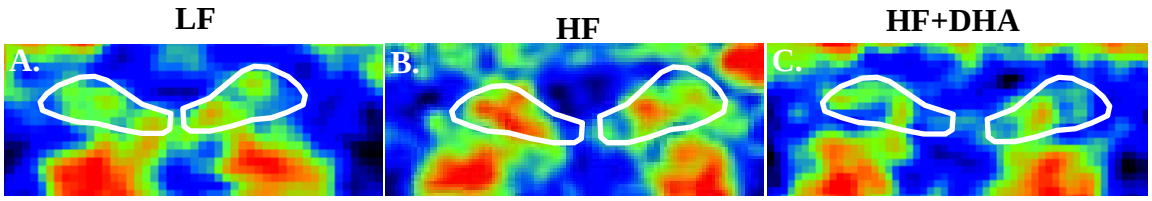

Fig 8.

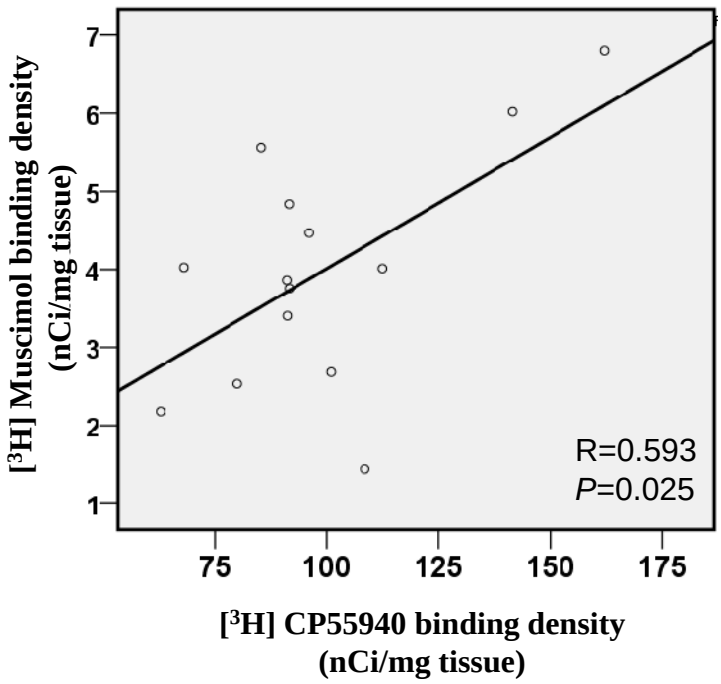
[3H] Muscimol binding density
(nCi/mg tissue)
7
6
5
4
3
2
1
R=0.593
P=0.025
75
100
125
150
175
[3H] CP55940 binding density
(nCi/mg tissue)

Fig 9.