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Dietary long chain n-3 polyunsaturated fatty acids prevent impaired social behaviour and normalize brain dopamine levels in food allergic mice

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ABSTRACT

Allergy is suggested to exacerbate impaired behaviour in children with neurodevelopmental disorders. We have previously shown that food allergy impaired social behaviour in mice. Dietary fatty acid composition may affect both the immune and nervous system. The aim of this study was to assess the effect of n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA) on food allergy-induced impaired social behaviour and associated deficits in prefrontal dopamine (DA) in mice. Mice were fed either control or n-3 LCPUFA-enriched diet before and during sensitization with whey. Social behaviour, acute allergic skin response and serum immunoglobulins were assessed. Monoamine levels were measured in brain and intestine and fatty acid content in brain. N-3 LCPUFA prevented impaired social behaviour of allergic mice. Moreover, n-3 LCPUFA supplementation increased docosahexaenoic acid (DHA) incorporation into the brain and restored reduced levels of prefrontal DA and its metabolites 3,4-dihydroxyphenylacetic acid, 3-methoxytyramine and homovanillic acid in allergic mice. In addition to these brain effects, n-3 LCPUFA supplementation reduced the allergic skin response and restored decreased intestinal levels of serotonin metabolite 5-hydroxyindoleacetic acid in allergic mice. N-3 LCPUFA may have beneficial effects on food allergy-induced deficits in social behaviour, either indirectly by reducing the allergic response and restoring intestinal 5-HT signalling, or directly by DHA incorporation into neuronal membranes, affecting the DA system. Therefore, it is of interest to further investigate the relevance of food allergy-enhanced impairments in social behaviour in humans and the potential benefits of dietary n-3 LCPUFA supplementation.

1. Introduction

Food allergy affects about 6% of young children, causing symptoms that include gastrointestinal and pulmonary distress and atopic dermatitis (Wang and Sampson, 2011). Food allergy is provoked by abrogation of immune tolerance to harmless food antigens, resulting in Th2 polarization of the immune response. The enteric nervous system mediates intestinal immune responses, including food allergic reactions. Inflammatory signalling molecules such as cytokines, neuropeptides, and serotonin (5-hydroxytryptamine; 5-HT) can directly and indirectly activate afferent nerves, which signal to the brain to activate the HPA-axis or the cholinergic anti-inflammatory pathway (Van Der Zanden et al., 2009; Tracey, 2009). Allergy-induced activation of the nervous system has recently been reviewed comprehensively (Undem and Taylor-Clark, 2014). Expression of the IgE receptor on vagal afferents is enhanced in food allergic mice and partial removal of the vagus nerve suppressed Th2-mediated inflammation in the intestines (Liang et al., 2011). Vagal afferents mediate visceral

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nociception during food allergy via 5-HT receptor signalling (Chen et al., 2009). 5-HT in the intestines is produced by enterochromaffin cells, enteric neuronal cells and various immune cells, most predominantly mast cells. Both enterochromaffin cells (Phillips et al., 2010) and mast cells (Williams et al., 1997) release 5-HT in close proximity to afferent nerves, causing receptor binding, neuronal depolarization and signalling to brain regions including the nucleus tractus solitarius (NTS) and hypothalamic paraventricular nucleus (PVN) (Mazda et al., 2004).

Interestingly, these brain regions are important in emotional and social behaviour and increasing evidence shows that intestinal allergic reactions may affect behavioural responses (Kennedy et al., 2012). Indeed, sensitization to ovalbumin in mice resulted in neuronal activation of the PVN and central nucleus of the amygdala and increased anxiety was observed (Costa-Pinto et al., 2006). Furthermore, food allergy increased neuronal activation in the NTS and PVN in rats, which was diminished after blockade of 5-HT3 receptors and vagotomy (Castex et al., 1995), suggesting that food allergy-induced signalling to the brain is mediated by 5-HT binding to receptors on vagal afferents. In humans, food allergy has been suggested to be one of the intestinal triggers that contribute to the expression of various psychological and psychiatric traits, including anxiety, depression (Addolorato et al., 1998), migraine (Alpay et al., 2010), schizophrenia (Severance et al., 2012), attention-deficit hyperactivity disorder (ADHD) (Pelsser et al., 2009) and autism spectrum disorder (ASD) (Jyonouchi, 2009; Chaidez et al., 2013; de Theije et al., 2014a). Recently, it was demonstrated that food allergy in the first year of life was associated with abnormal neurodevelopmental outcomes related to social behaviour (Meldrum et al., 2012). Moreover, intestinal problems are often reported in children with ASD (de Theije et al., 2011; Smith et al., 2009) and milk intake was found to be a predictor of constipation in these patients (Afzal et al., 2003). A (gluten and) milk protein free diet is suggested to improve autistic behaviour (Millward et al., 2008; Whiteley et al., 2010; Lucarelli et al., 1995) and to restore the increased intestinal permeability observed in children suffering from ASD (de Magistris et al., 2010).

Long chain n-3 polyunsaturated fatty acids (n-3 LCPUFA) may have a role in the prevention of allergic diseases (van den Elsen et al., 2012). Populations consuming high levels of n-3 LCPUFA from seafood, such as Inuit, were shown to have low prevalence of atopic diseases (Krause et al., 2002). In addition to its potential immunomodulatory effects, n-3 LCPUFA have been suggested to modulate neuronal function as well. LCPUFA, predominantly docosahexaenoic acid (DHA, C22:6 n-3), arachidonic acid (AA, C20:4 n-6) and docosatetraenoic acid (DTA; 22:4 n-6), are important components of the neuronal cell membrane (Schuchardt et al., 2010) and are essential throughout life for maintaining normal brain function (Bourre et al., 1991; Uauy and Dangour, 2006). Numerous observational studies have shown a link between peripheral n-3 and n-6 LCPUFA imbalances and neurodevelopmental disorders. For instance, ADHD (Colter et al., 2008), schizophrenia (Hoen et al., 2013), and ASD (El-Ansary et al., 2011) have been associated with a relative lack of n-3 LCPUFA in peripheral blood cells or plasma. Some clinical trials have been conducted on the beneficial effect of dietary n-3 LCPUFA supplementation on behaviour in various neurodevelopmental disorders, including ASD (James et al., 2011), but trials with larger sample size are required and are currently in progress (Schuchardt et al., 2010).

Recently, we have shown that an allergic reaction to orally ingested cow's milk protein whey reduced social interaction in mice (de Theije et al., 2014b). Moreover, we showed that these behavioural abnormalities were associated with reduced dopaminergic activity in the prefrontal cortex (PFC). In this study, it was assessed whether dietary supplementation of fish oil, high in n-3 LCPUFA DHA, can prevent food allergy-induced abnormalities in social behaviour and in prefrontal dopamine (DA) and metabolite levels in mice. Moreover, we investigated the effect of n-3 LCPUFA supplementation on allergic sensitization and 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) levels in ileum of food allergic mice.

2. Materials and methods

2.1. Diets

Semi-purified cow's milk protein-free AIN-93G-based diets were composed of either 10% soybean oil (control diet) or 4% soybean oil combined with 6% tuna oil (n-3 LCPUFA-enriched diet) and prepared at Research Diet Services (Wijk bij Duurstede, The Netherlands). The fat percentage of AIN-93G was enhanced from 7 % to 10 % at the expense of cornstarch (Table 1A), as described before (van den Elsen et al., 2013). The ratio n-3: n-6 LCPUFA was 1:9.5 for the control diet and 1:1 for the n-3 LCPUFA-enriched diet. Tuna oil (Table 1B) was a kind gift from Bioriginal (Den Bommel, The Netherlands). Diets were stored at −20 °C prior to use to prevent fatty acid oxidation.

2.2. Animal experiments

Three-week-old, specific pathogen free, male C3H/HeOuJ mice, purchased from Charles River Laboratories (L'Arbresle Cedex, France) were housed at the animal facility of the Utrecht University on a 12 h light/dark cycle with access to food and water *ad libitum*. Mice were fed either the control or n-3 LCPUFA-enriched fish oil diet, starting two weeks prior to first sensitization and continued during the entire experiment. Mice were sensitized intragastrically (i.g.) with 20 mg whey (DMV International, Veghel, The Netherlands) in 0.5 mL PBS containing 10 μg cholera toxin (CT; List Biological Laboratories, Campbell, CA, USA) as an adjuvant. Sham-sensitized mice received CT alone. Mice were sensitized once a week for 5 consecutive weeks as previously described (Schouten et al., 2010). One week after the last sensitization, sham and whey-sensitized mice were challenged i.g. with 50 mg whey/0.5 mL PBS and a social interaction test was conducted the next morning. In the first

Table 1
Diet composition of n-3 LCPUFA-enriched diet.

	Control diet (g/kg diet)	n-3 LCPUFA enriched diet (g/kg diet)
A. Diet composition of chow, based on AIN-93G		
Carbohydrates		
Cornstarch	367.5	367.5
Dextrinized cornstarch	132.0	132.0
Sucrose	100.0	100.0
Cellulose	50.0	50.0
Protein		
Soya	200.0	200.0
Methionine	3.0	3.0
Fat		
Soybean oil	100.0	40.0
Tuna oil	0	60.0
Others		
Mineral mix AIN-93G	35.0	35.0
Vitamine mix AIN-93VX	10.0	10.0
Choline bitartrate	2.5	2.5
Tert-butylhydroquinone	0.014	0.014

	Soybean oil (%)	Tuna oil (%)
B. Fatty acid composition of lipid source		
Fatty acid		
Saturated	15.1	28.9
Monounsaturated	24.9	22.8
Polyunsaturated	59.1	44.5
n-6	*53.1*	*5.5*
C18:2 LA	*53.1*	*1.3*
C20:4 AA		*1.8*
C22:5		*1.6*
n-3	*5.6*	*38.5*
C18:3 ALA	*5.6*	*0.5*
C20:5 EPA		*7.0*
C22:5 DPA		*1.4*
C22:6 DHA		*27.8*
Minor components	0.9	3.8

LA: linoleic acid, AA: arachidonic acid, ALA: a-linolenic acid, EPA: eicosapentaenoic acid, DPA: docosapentaenoic acid, DHA: docosahexaenoic acid.

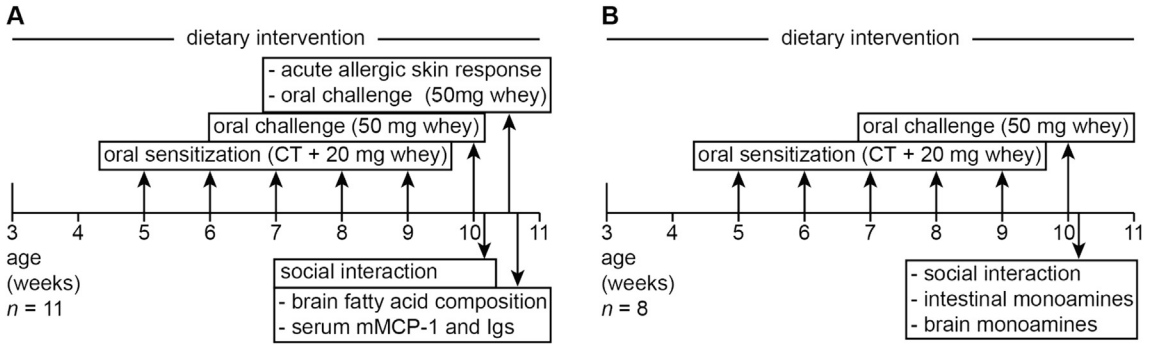

Fig. 1. Schematic overview of the two experiments. (A) In the first experiment, ($n = 11$ mice per group) the acute allergic skin response was measured four days after the social interaction test. Mice underwent a second oral challenge and the next morning, serum was collected for measuring levels of immunoglobulins (Igs) and mouse mast cell protease-1 (mMCP-1) and brain was collected to determine fatty acid composition. (B) In the second experiment, mice ($n = 8$ per group) were sacrificed after the social interaction test to measure monoamines.

experiment, ($n = 11$ per group), the allergic skin response was measured 4 days after the social interaction test (Fig. 1A). Mice received a second oral whey challenge (50 mg whey/0.5 mL PBS) and were decapitated the next morning to measure levels of fatty acids in the brain and immunoglobulins in serum. In the second experiment, mice ($n = 8$ per group) were sacrificed after the social interaction test to measure monoamines in brain and intestine (Fig. 1B). All animal procedures were approved by and conducted in accordance with the guidelines of the Animal Ethics Committee of Utrecht University (approval number: DEC2012.I.04.053).

2.3. Acute allergic skin response

Ear thickness ($n = 11$ per group) was measured in duplicate for each ear using a digital micrometre (Mitutoyo, Veenendaal, The Netherlands) prior to and 1 h after intradermal (i.d.) whey challenge (10 μg/20 μL PBS) in the ear. Isoflurane was used for inhalational anaesthesia during measurements. Ear swelling was calculated by subtracting the mean basal thickness of duplicates per ear from the mean thickness measured after i.d. challenge, to express ear swelling as delta μm. Mean of left and right delta ear swelling were calculated for each mouse.

2.4. Social interaction test

The behavioural assessment was adapted from a previous description (de Theije et al., 2014b; Liu et al., 2012). The morning after oral challenge mice were exposed to a social interaction test ($n = 11$ per group). Mice were placed in a 45 × 45 cm open field with a small perforated Plexiglas cage (10 cm diameter) located against one wall allowing visual, olfactory and minimal tactile interaction (Fig. 4A). Mice were habituated to the open field for 5 min and an age- and gender-matched unfamiliar target mouse was introduced in one of the cages for an additional 5 min. Open fields were cleaned with water followed by 70% ethanol after each test. By using video tracking software (EthoVision 3.1.16, Noldus, Wageningen, The Netherlands) an interaction zone around the cage was digitally determined. Time spent in the interaction zone, latency until first occurrence in the interaction zone and total distance moved were measured. Data from the first experiment was presented, but comparable results were obtained from the second experiment (data not shown).

2.5. Measurements of whey-specific immunoglobulins and mouse mast cell protease-1

Blood ($n = 11$ per group) was collected 16 h after oral challenge, centrifuged for 15 min at 14,000 rpm and serum was stored at −70 °C. Serum concentrations of whey-specific IgE, IgG1 and IgG2a were measured by means of ELISA. Microlon plates (Greiner, Alphen aan de Rijn, The Netherlands) were coated with 20 μg/mL whey in carbonate/bicarbonate buffer (0.05 M, pH = 9.6; Sigma–Aldrich, Zwijndrecht, The Netherlands) overnight at 4 °C. Plates were blocked in ELISA buffer (50 mM Tris, 137 mM NaCl, 2 mM EDTA, 0.05% Tween-20 and 0.5% BSA in PBS) and serum samples were incubated for 2 h. Plates were incubated with biotinylated rat anti-mouse IgE, IgG1 and IgG2a (1 μg/mL; BD Biosciences, Alphen aan de Rijn, The Netherlands) for 2 h and subsequently with streptavidin-horse radish peroxidase (0.5 μg/mL; Sanquin, Amsterdam, The Netherlands) for 1 h. Plates were developed using *o*-phenylendiamine (Sigma–Aldrich) and reaction was stopped after 15 min with 4 M H_2SO_4. Absorbance was measured at 490 nm on a microplate reader (Bio-Rad, Veenendaal, The Netherlands). Results were expressed as arbitrary units (AU), composed using a titration curve of pooled sera from whey-alum i.p. immunized mice serving as an internal standard. Concentration of mouse mast cell protease-1 (mMCP-1) in serum was determined using commercially available ELISA kits (BD Biosciences) according to the manufacturer's protocol.

2.6. HPLC for analysis of monoamines and metabolites in brain and intestines

After decapitation, brains ($n = 8$ per group) were rapidly removed, frozen in isopentane (Sigma–Aldrich) and brain and 1 cm of distal ileum were stored at −70 °C until further analysis. PFC was isolated with 500 μm coronal sections using a cryostat (Model 700, Lameris Instruments, Utrecht, The Netherlands). Brain and intestinal tissues were sonicated in 50–200 μL ice-cold solution containing 5 μM clorgyline and 0.6 μM N-methylserotonin (NMET, internal standard). To 50 μL tissue homogenate, 12.5 μL 2 M HClO4 was added. After 15 min in ice water, the homogenates were centrifuged for 10 min at 15,000 g (4 °C). The mobile phase solution consisted of 50 mM citric acid, 50 mM phosphoric acid, 0.1 mM EDTA, 45 μL/L dibutylamine, 77 mg/L 1-octanesulfonic acid sodium salt, 10% methanol (pH = 3.4). Separation was performed at 45 °C using a flow rate of 0.7 mL/min. The concentration of each compound was calculated by comparison with both the internal and the external standards. The limit of detection (signal/noise ratio 3:1) was 0.3 nM. Settings of HPLC with electrochemical detection using an Alexys 100 LC-EC system (Antec, Lelystad, The Netherlands) were described elsewhere (de Theije et al., 2014b).

2.7. Fatty acid composition brain

Brain was removed ($n = 11$ per group) and stored at −70 °C until analysis. Whole brains were weighted and homogenized in ice cold PBS (25 mg/mL). Brain lipids were extracted as described by Bligh and Dyer (Bligh and Dyer, 1959) and the membrane fatty acid composition was assessed using gas chromatography as previously described (Levant et al., 2007). LCPUFA content was expressed as percentage of total fatty acids (% FA).

2.8. Statistical analysis

Experimental results are expressed as mean ± S.E.M, or Box-and-Whisker Tukey plot when data were not normally distributed. Differences between groups were statistically determined with a two-way ANOVA followed by a Bonferroni's multiple comparisons test. For serum immunoglobulin levels and intestinal 5-HT turnover, log transferred data were used to obtain normality for two-way ANOVA. Latency until first occurrence in the interaction was statistically analysed with a Kruskal–Wallis test followed by a Dunn's multiple comparisons test. Results were considered statistically significant when $P < 0.05$. Analyses were performed using GraphPad Prism, version 6.02.

3. Results

3.1. N-3 LCPUFA supplementation reduces the acute allergic skin response in whey sensitized food allergic mice

One hour after dermal challenge, ear thickness was measured to assess the effect of n-3 LCPUFA supplementation on the acute allergic skin response. The delta ear swelling in whey-sensitized allergic mice (120.5 ± 16.45 μm) was increased compared to sham-sensitized control mice (24.50 ± 4.724 μm, $P < 0.0001$, Fig. 2A). Whey-sensitized mice fed the n-3 LCPUFA-enriched diet showed a reduced allergic skin response (63.18 ± 7.973 μm) when compared to whey-sensitized allergic mice fed the control diet ($P < 0.001$). Mucosal mast cell degranulation in the intestine, determined by the concentration of mMCP-1 in serum, was

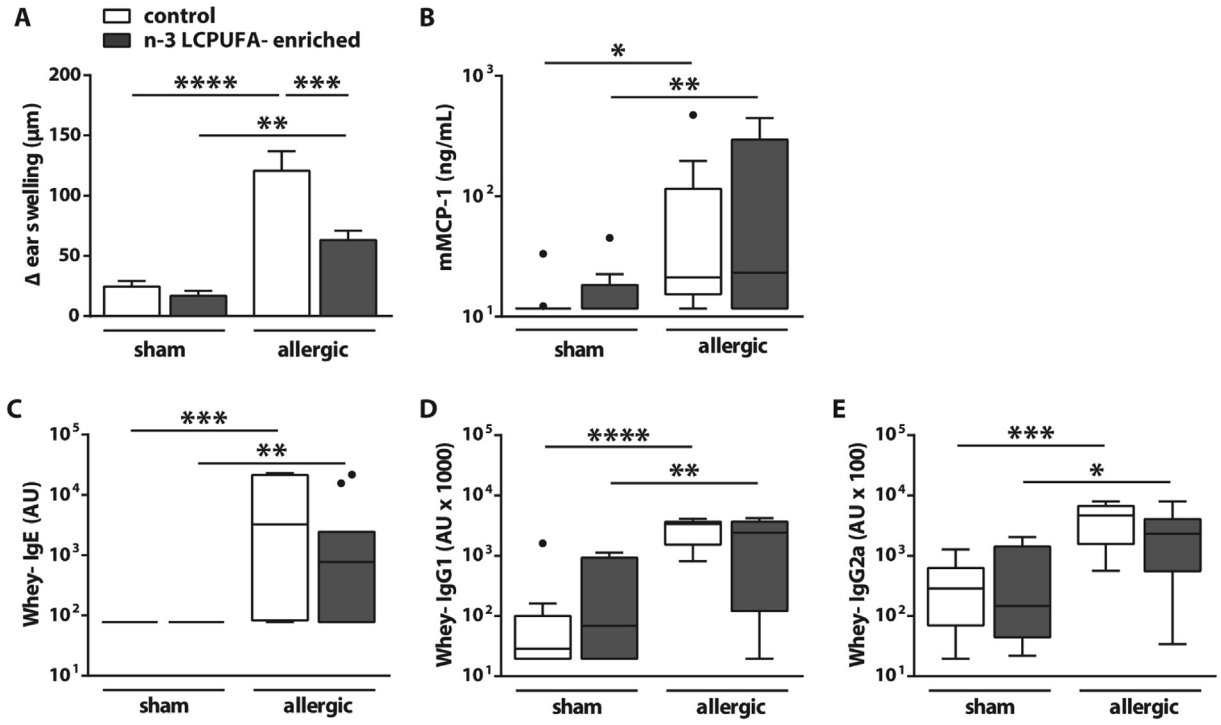

Fig. 2. The effect of n-3 LCPUFA supplementation on the allergic skin and humoral response to whey challenge. (A) The acute allergic skin response (delta ear swelling) after intradermal challenge (sensitization: $P < 0.0001$, diet: $P < 0.001$, interaction: $P < 0.05$). (B) Mouse mast cell protease-1 (mMCP-1) levels (sensitization: $P < 0.001$, diet: ns, interaction: ns). Levels of whey-specific immunoglobulins (C) IgE, (D) IgG1 and (E) IgG2a (sensitization: $P < 0.0001$, diet: ns interaction: ns). Two-way ANOVA followed by Bonferroni's multiple comparisons test. Data are presented as mean ± S.E.M. for ear swelling and as Box-and-Whisker Tukey plots on a log scale for mMCP-1 and immunoglobulins. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, ns: not significant, $n = 11$ per group.

increased in allergic mice fed the control diet (90.07 ± 41.89 ng/mL, $P < 0.05$, Fig. 2B) and the n-3 LCPUFA-enriched diet (118.1 ± 46.84 ng/mL, $P < 0.01$), when compared to their respective sham-sensitized control mice fed either the control diet (13.71 ± 1.954 ng/mL) or the n-3 LCPUFA-enriched diet (16.32 ± 3.075 ng/mL). In addition, serum levels of antigen-specific immunoglobulins were also elevated in allergic mice, regardless of the consumed diet. More specifically, levels of whey-specific IgE (Fig. 2C), IgG1 (Fig. 2D), and IgG2a (Fig. 2E) were increased in allergic mice fed the control diet (IgE: 7386 ± 2938 AU, $P < 0.001$; IgG1: $2.762 \times 10^6 \pm 3.507 \times 10^5$ AU, $P < 0.0001$; IgG2a: $4.607 \times 10^5 \pm 9.591 \times 10^4$ AU, $P < 0.001$) and the n-3 LCPUFA-enriched diet (IgE: 3992 ± 2254 AU, $P < 0.01$; IgG1: $2.049 \times 10^6 \pm 5.033 \times 10^5$ AU, $P < 0.01$; IgG2a: $3.463 \times 10^5 \pm 1.343 \times 10^5$ AU, $P < 0.05$), compared to sham-sensitized mice fed the control diet (IgE: 78.00 ± 0.0 AU; IgG1: $1.978 \times 10^5 \pm 1.412 \times 10^5$ AU; IgG2a: $3.618 \times 10^4 \pm 1.185 \times 10^4$ AU) or the n-3 LCPUFA-enriched diet (IgE: 78.00 ± 0.0 AU; IgG1: $3.385 \times 10^5 \pm 1.411 \times 10^5$ AU; IgG2a: $6.553 \times 10^4 \pm 2.417 \times 10^4$ AU), respectively.

3.2. N-3 LCPUFA supplementation prevents reduced breakdown of 5-HT in the intestine of food allergic mice

As it was previously shown that intestinal levels of 5-HT were increased and levels of 5-HIAA were decreased in food allergic mice (James et al., 2011), the effect of n-3 LCPUFA supplementation on the serotonergic response was determined in distal ileum

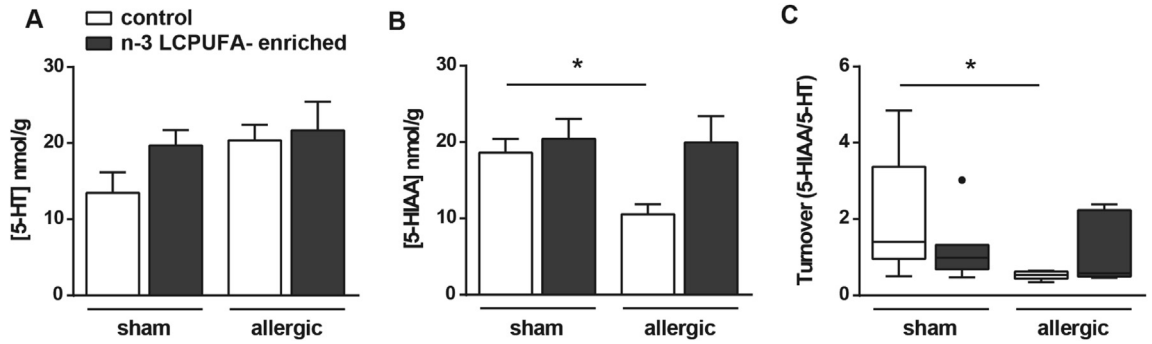

Fig. 3. Levels of serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in ileum homogenates of whey-sensitized allergic and sham-sensitized control mice, fed the control or n-3 LCPUFA-enriched diet. (A) Levels of intestinal 5-HT (sensitization: ns, diet: ns, interaction: ns). (B) Levels of intestinal 5-HIAA (sensitization: $P < 0.05$, diet: $P < 0.05$, interaction: ns). (C) 5-HT turnover (sensitization: ns, diet: $P < 0.05$, interaction: $P < 0.05$). Two-way ANOVA followed by Bonferroni's multiple comparisons test. Data are presented as mean ± S.E.M. for concentrations of 5-HT and 5-HIAA and Box-and-Whisker Tukey plots for turnover rate. $^{*}P < 0.05$, $^{**}P < 0.01$, ns: not significant, $n = 8$ per group.

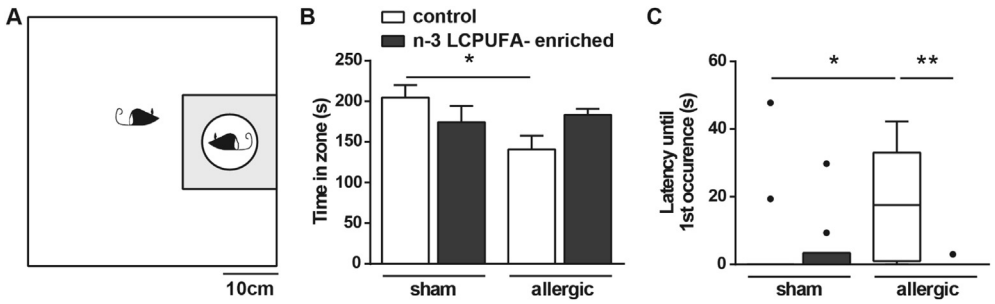

Fig. 4. Social interaction of whey-sensitized allergic and sham-sensitized control mice fed the control or n-3 LCPUFA-enriched diet. (A) Schematic representation of the social interaction test, illustrating the interaction zone (grey rectangle) and the cage (white circle) in which a target mouse was placed. (B) Time spent in the interaction zone (sensitization: ns, diet: ns interaction: $P < 0.05$). (C) Latency of first occurrence in the interaction zone (KW: $P < 0.01$). One allergic mouse on control diet was excluded as significant outlier (209 s, Grubb's test). (B) Two-way ANOVA followed by Bonferroni's multiple comparisons test, mean ± S.E.M. (C) Kruskal–Wallis (KW) test followed by Dunn's multiple comparisons test, Box-and-Whisker Tukey plots. $^{*}P < 0.05$, $^{**}P < 0.01$, ns: not significant, $n = 11$ per group.

homogenates. The increase in intestinal 5-HT levels in food allergic mice (20.34 ± 2.074 nmol/g) was not significantly different from control mice (13.46 ± 2.680 nmol/g, Fig. 3A). However, levels of 5-HIAA were decreased in food allergic mice (10.53 ± 1.314 nmol/g) compared to control mice (18.58 ± 1.819 nmol/g) when fed a control diet ($P < 0.05$, Fig. 3B) and the n-3 LCPUFA-enriched diet was able to prevent this reduction in 5-HIAA levels in ileum of allergic mice (19.95 ± 3.448 nmol/g). Ratio of 5-HIAA/5-HT was used as an index of 5-HT turnover. The turnover was reduced in food allergic mice (0.5250 ± 0.04440) compared to control mice (2.023 ± 0.5364) when fed the control diet ($P < 0.05$, Fig. 3C), but not when fed the n-3 LCPUFA-enriched diet (1.264 ± 0.3531).

3.3. Social interaction is reduced in food allergic mice and restored in food allergic mice fed the n-3 LCPUFA-enriched diet

The morning after oral challenge, mice were exposed to a social interaction test (Fig. 4A). Social interaction was determined by the amount of time that an experimental mouse spent in the interaction zone near an age- and gender-matched, unfamiliar mouse. Whey-sensitized allergic mice fed the control diet spent less time in the interaction zone (140.8 ± 16.87 s) compared to sham-sensitized control mice (204.6 ± 15.29 s, $P < 0.05$, Fig. 4B), and the n-3 LCPUFA-enriched diet was able to prevent reduced social interaction (183.3 ± 7.565 s). Furthermore, latency of first approach to the social target was increased in allergic mice (35.60 ± 19.84 s) compared to control mice fed the control diet (6.109 ± 4.523 s, $P < 0.05$, Fig. 4C). The n-3 LCPUFA-enriched diet prevented increased latency of first approach in allergic mice (0.3 ± 0.3 s, $P < 0.01$). Of note, locomotor activity during habituation in the open field was not different between groups (sham/control: 1848 ± 150.6 cm; sham/n-3 LCPUFA: 1890 ± 150.2 cm; allergic/control: 1775 ± 78.30 cm; allergic/n-LCPUFA: 1868 ± 161.3 cm, data not shown).

3.4. N-3 LCPUFA supplementation increases DHA content at the expense of AA and DTA

Both in allergic (18.50 ± 0.1020% FA) and in sham-sensitized mice (18.28 ± 0.2575% FA), dietary supplementation of n-3 LCPUFA increased DHA content in whole brain homogenates compared to consumption of the control diet (allergic: 16.06 ± 0.1106% FA; sham: 16.27 ± 0.1670% FA, $P < 0.0001$, Fig. 5A). Incorporation of n-3 LCPUFA DHA in brain membranes occurred mainly at the expense of n-6 LCPUFA AA and DTA. Decreased levels of AA (allergic: 7.549 ± 0.1182% FA; sham: 7.550 ± 0.1148% FA, $P < 0.0001$, Fig. 5B) and DTA (allergic: 2.178 ± 8.615 × 10^{-3} % FA; sham: 2.192 ± 0.02415% FA, $P < 0.0001$, Fig. 5C) were observed when mice were fed the n-3 LCPUFA-enriched diet compared to control diet (AA: allergic: 9.118 ± 0.04676% FA; sham: 9.016 ± 0.1259% FA; DTA: allergic: 3.101 ± 0.01436% FA; sham: 3.065 ± 0.04970% FA). No differences were observed between fatty acid content in brain of whey-sensitized allergic mice compared to sham-sensitized control mice.

3.5. N-3 LCPUFA supplementation prevents decreased levels of dopamine metabolites in PFC of food allergic mice

When fed the control diet, reduced levels of DA were observed in the PFC of allergic mice (4.201 ± 1.429 nmol/g) compared to

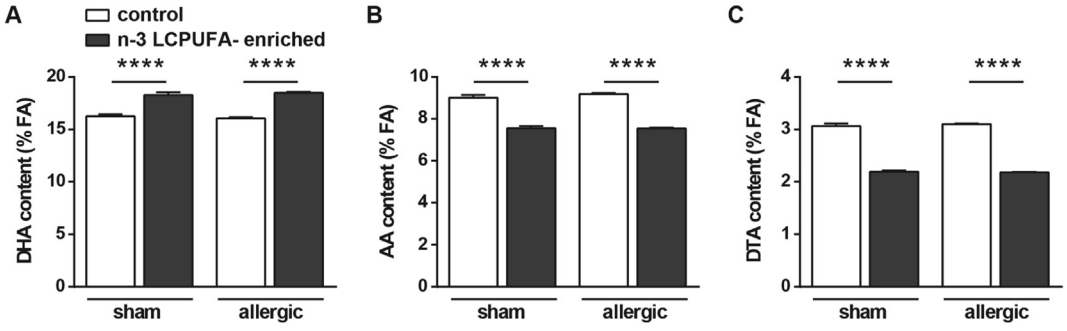

Fig. 5. The effect of n-3 LCPUFA supplementation on n-3 and n-6 LCPUFA content in brain homogenates of whey-sensitized allergic and sham-sensitized control mice. (A) N-3 LCPUFA docosahexaenoic acid (DHA) content (sensitization: ns, diet: $P < 0.0001$, interaction: ns). (B) N-6 LCPUFA arachidonic acid (AA) (sensitization: ns, diet: $P < 0.0001$, interaction: ns). (C) N-6 LCPUFA docosatetraenoic acid (DTA) (sensitization: ns, diet: $P < 0.0001$, interaction: ns). Two-way ANOVA followed by Bonferroni's multiple comparisons test was conducted and data are presented as mean ± S.E.M. $^{****}P < 0.0001$, ns: not significant, $n = 11$ per group.

control mice (9.218 ± 1.278 nmol/g, $P < 0.05$, Fig. 6A). Decreased levels of DA were not present in allergic mice when they were fed the n-3 LCPUFA-enriched diet (8.989 ± 2.154 nmol/g). Furthermore, levels of DA metabolites DOPAC (0.6450 ± 0.1071 nmol/g, Fig. 6B), 3-MT (0.2800 ± 0.08567 nmol/g, Fig. 6C) and HVA (0.8700 ± 0.07421 nmol/g, Fig. 6D) were all reduced in food allergic mice fed the control diet compared to control mice (DOPAC: 1.439 ± 0.2618 nmol/g; 3-MT: 0.8688 ± 0.1750 nmol/g; HVA: 1.393 ± 0.1623 nmol/g, $P < 0.05$). The n-3 LCPUFA-enriched diet was able to prevent this reduction in metabolite levels in the PFC of allergic mice (DOPAC: 1.267 ± 0.3585 nmol/g; 3-MT: 0.7286 ± 0.2018 nmol/g; HVA: 1.640 ± 0.2739 nmol/g). The turnover rate of DA, assessed by calculating the ratio between metabolites and DA ((DOPAC+3-MT + HVA)/DA), was not altered between groups (data not shown). In contrast to DA, levels of 5-HT and 5-HIAA were not altered in the PFC of allergic mice (5-HT: 3.268 ± 0.3141 nmol/g; 5-HIAA: 2.188 ± 0.1127 nmol/g) compared to control mice (5-HT: 3.294 ± 0.1937 nmol/g; 5-HIAA: 2.639 ± 0.1073 nmol/g, Fig. 6E and F). However, when fed the n-3 LCPUFA-enriched diet, increased levels of 5-HIAA were observed in allergic mice (3.146 ± 0.4454 nmol/g, Fig. 6F).

4. Discussion

The present study demonstrates that dietary supplementation of n-3 LCPUFA prevents food allergy-induced abnormalities in social behaviour and associated dampening of the dopaminergic system in the PFC of whey-sensitized mice. We suggest that this effect of n-3 LCPUFA on social behaviour may be mediated by a reduced allergic response, decreased intestinal 5-HT signalling to afferent neuronal fibres, or directly by incorporation of DHA into neuronal membranes in the brain. Locomotor activity in the open field was not different between groups, suggesting that impaired social behaviour was not affected by altered mobility and food allergic mice did not show any overt signs of sickness.

It was previously shown that n-3 LCPUFA supplementation prevented allergic sensitization and reduced the allergic skin response in female C3H/HeOuJ mice (van den Elsen et al., 2013; van den Elsen et al., 2014). In contrast, we used male mice in the present experiment, because a male preponderance is observed in ASD patients (Fombonne, 2005). The n-3 LCPUFA-enriched diet was able to reduce the allergic skin response in whey-sensitized male mice. However, n-3 LCPUFA supplementation did not completely abolish the allergic skin response and it lowered neither mucosal mast cell degranulation nor production of whey-specific immunoglobulins. This suggests that the effects of n-3 LCPUFA observed at the behavioural level were not mediated by these humoral factors. However, we cannot exclude effects of n-3 LCPUFA on allergy-induced *de novo* synthesis of arachidonic acid metabolites or cytokines in mast cells. Systemic cytokine levels were measured in this study, but no differences between groups were observed (data not shown). Tissue content of DHA is known to be higher in females than males and is dependent on sex hormones (Childs et al., 2008), which may explain the gender differences observed in this study in males compared to the previous study in females.

A pro-inflammatory environment in the intestine decreases serotonin transporter activity on epithelial cells, both *in vitro* (Mossner et al., 2001; Foley et al., 2007; Mendoza et al., 2009) and *in vivo* (Wheatcroft et al., 2005; Gershon, 2005; Linden et al., 2005), resulting in less 5-HT reuptake and metabolism, consequently reducing 5-HIAA levels. Depressed levels of 5-HIAA were observed in intestinal biopsies of patients with coeliac disease (Coleman et al., 2006) and IBS (Dunlop et al., 2005). In line with these observations, we showed that 5-HIAA levels were reduced in the intestine of allergic mice. The reduction in 5-HIAA levels was prevented when allergic mice were fed the n-3 LCPUFA-enriched diet. This indicates that n-3 LCPUFA supplementation restored

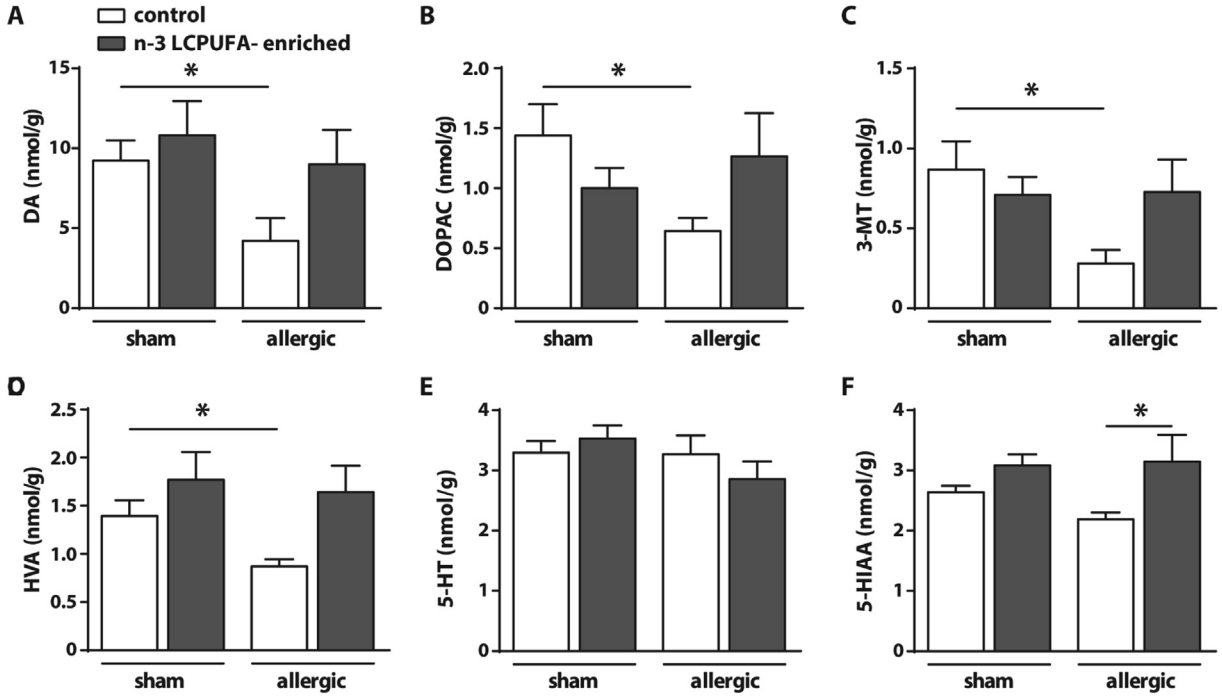

Fig. 6. Dopamine (DA), serotonin (5-HT) and metabolite levels in prefrontal cortex (PFC) of whey-sensitized allergic mice and sham-sensitized control mice and the effect of n-3 LCPUFA supplementation. (A) Levels of DA (sensitization: $P < 0.05$, diet: ns, interaction: ns). Levels of (B) 3,4-dihydroxyphenylacetic acid (DOPAC), (C) 3-methoxytyramine (3-MT), and (D) homovanillic acid (HVA) (all three metabolites: sensitization: ns, diet: ns, interaction: $P < 0.05$). (E) Levels of 5-HT (sensitization: ns, diet: ns, interaction: ns) and 5-HIAA (sensitization: ns, diet: $P < 0.05$, interaction: ns). Two-way ANOVA followed by Bonferroni's multiple comparisons test was conducted and data are presented as mean ± S.E.M. $^*P < 0.05$, ns: not significant, $n = 8$ per group.

intestinal 5-HT metabolism, while mast cell degranulation remained unaffected. Food allergy-induced signalling to the brain is suggested to be mediated by 5-HT binding to receptors on vagal afferents in the intestine (Mazda et al., 2004; Castex et al., 1995). Therefore, the beneficial effects of n-3 LCPUFA supplementation on food allergy-induced behavioural deficits may be the result of restored 5-HT metabolism. To our knowledge, the effect of LCPUFAs on intestinal 5-HT has not been investigated and could provide an interesting avenue for future research on the treatment of inflammatory and allergic diseases of the intestinal tract and activation of the gut–brain axis.

We speculate that the improvements in social behaviour may also be mediated by increased incorporation of DHA into the brain. Supplementation with N-3 LCPUFA enhanced n-3 DHA content in the brain, mainly at the expense of n-6 AA and DTA. LCPUFA, in particular AA and DHA (Palsdottir et al., 2012), are incorporated in the neuronal cell membrane to maintain normal brain function (Schuchardt et al., 2010; Bourre et al., 1991; Uauy and Dangour, 2006). Brain fatty acid content is dependent on nutritional intake and it was shown that suboptimal n-3 LCPUFA intake during gestation and postnatal development caused neurodevelopmental deficits in preclinical (Chen and Su, 2013; Bhatia et al., 2011) and clinical studies (Birch et al., 2000; Willatts et al., 1998; Drover et al., 2011). Therapeutic effects of n-3 LCPUFA have been postulated in several neurodevelopmental (Amminger et al., 2007; Bloch and Qawasmi, 2011) and neurodegenerative disorders (Karr et al., 2011). Regarding ASD, conflicting results exist on the efficacy of n-3 LCPUFA supplementation on behaviour and large well-controlled randomized trials are required (James et al., 2011).

LCPUFA in the brain act on physiological functions via several mechanisms. LCPUFA are incorporated in membrane-based phospholipids of neural tissue modifying membrane integrity and fluidity (Stubbs and Smith, 1984; Suzuki et al., 1998). Functioning of transmembrane proteins, such as receptors and transporters, is affected by membrane fluidity (Schuchardt et al., 2010; Chalon, 2006). Monoaminergic neurotransmission, in particular mesocortical dopamine, was reported to alter upon changes in nutritional composition of fatty acids (Delion et al., 1994). Chronic n-3 LCPUFA deficiency in rats reduced levels of DA (Delion et al., 1994), the number of vesicular monoamine transporter-2 binding sites (Kodas et al., 2002), and the number of storage vesicles in DA terminals in the PF C (Zimmer et al., 2000). This suggests that n-3 LCPUFA depletion reduces the prefrontal DA neurotransmission. Moreover, a diet high in n-3 LCPUFA increased prefrontal DA levels of rats compared to a diet high in n-6 LCPUFA (Chalon et al., 1998). This is in line with our observation that supplementation with n-3 LCPUFA normalized decreased DA and metabolite levels in PFC of whey-sensitized allergic mice. It was previously shown that reduced activity of the prefrontal dopaminergic system during repeated social stress was mediated by increased levels of prostaglandin E2 (PGE2) (Tanaka et al., 2012), a pro-inflammatory metabolite of n-6 AA. In order to investigate whether normalization of allergy-induced deficits in social behaviour are mediated by PGE2, we measured PGE2 levels in whole brain homogenates obtained from the current study. Although levels of PGE2 were somewhat elevated in brain homogenates of allergic mice, no significant differences were observed between groups in a two-way ANOVA (data not shown). In addition, PGE2 levels tended to inversely correlate with brain content of DHA ($P = 0.08$, $r^2 = 0.15$, data not shown). It is possible that measuring PGE2 in the prefrontal cortex would lead to significant increase in PGE2 levels in allergic mice and correlation with DHA, but this would require further investigation. As the dopaminergic system in the PFC regulates social behaviour (Young et al., 2011), n-3 LCPUFA supplementation may beneficially affect social behaviour in allergic mice directly via its incorporation into neuronal membranes, affecting dopaminergic neurotransmission in the PFC. Because the mesocortical DA system is thought to be involved in aberrant social behaviour (Tanaka et al., 2012) and in the pathophysiology of ASD (Ernst et al., 1997), dietary n-3 LCPUFA supplementation could be of relevance in the management of abnormal social behaviour of patients with ASD and co-morbid food allergic symptoms.

In summary, we demonstrate that dietary fish oil, high in n-3 DHA, prevented food allergy-induced deficits in social behaviour of mice and restored dopamine and metabolite levels in the PFC of whey-sensitized allergic mice. Together, we suggest that n-3 LCPUFA supplementation may have beneficial effects on social behaviour of food allergic mice, either indirectly by reducing the allergic response and decreasing intestinal 5-HT signalling, or directly by incorporation of DHA into neuronal membranes in the brain improving prefrontal dopaminergic neurotransmission.

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