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Impacts of Perigestational Exposure to Chlorpyrifos And High-Fat Diet on Ileum Contractility in Male Rats at Early Adulthood

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Abstract

Background: The perinatal period is characterized by strong plasticity and higher individual sensitivity to environmental factors through fetal programming. An association of junk food and early exposure to pesticide residues can be involved in metabolic disruption and increased pathologies including obesity.

Aims: This study established the effects of perigestational exposure to a widely used organophosphate pesticide, Chlorpyrifos (CPF), and high-fat diet (HFD) on the contractility of ileal smooth muscle in adult rats.

Methods: Four groups of four female rats were exposed during 4 months before and later during gestation and lactation periods to CPF (1 mg/kg bw/day vs. vehicle) with or without HFD. After being sacrificed at the age of 60 postnatal days (PND60), ileal smooth muscle strips were used for *in vitro* contractility measurements. Other ileal segments were used for AChE activity assessment, gene expression measurement, and histological analysis.

Results: At PND60, CPF exposure increased the ileal longitudinal and circular muscles contractility. The circular muscle thickness was also increased. CPF exposure was associated with greater expression of the tachykinin (substance P) and the muscarinic M2 acetylcholine receptor mRNA but lower expression of calmodulin mRNA in the ileum. The expression of tachykinin NK1 receptor mRNA was increased in HFD group compared to controls. The exposure to either CPF and/or HFD induced a decrease in the ileal AChE activity.

Conclusions: Despite the lack of direct exposure post-weaning, early life exposure to CPF and /or HFD programs the ileal homeostasis and increases the risk of altered gut contractile function at early adulthood.

Keywords: Chlorpyrifos; High-Fat Diet; Maternal Exposure; Ileum; Contractility

KeyPoints:

- Perigestational exposure to CPF increased ileal muscle contractility at adulthood.
- The altered ileal contraction involves cholinergic and non-cholinergic mechanisms.
- Offspring from HFD-fed mothers showed elevated NK1 receptor expression.

Introduction

The environment experienced by the developing offspring during the perinatal period is influenced by maternal health and dietary supply. According to the Developmental Origin of Health and Disease concept (DOHaD), nutritional environment perturbations during intrauterine life plays a pivotal role in programming the risks for adverse health outcomes at adulthood, promoting irreversible long-term effects on development [29,32]. As high-fat diet (HFD) intake is common among pregnant women in western countries, recent concerns have been raised about unbalanced maternal diet before and during pregnancy due to the well-recognized risk factors on the mothers and consequently on their infants. In particular, the gastrointestinal (GI) tract has been attracting increasing research in recent years given that the components of ingested food represent the major sources of chemical agents in contact with the digestive tract. Emergent evidence from animal and human studies suggests the ability of western-style diet depicted as the high intake of saturated fats in the contribution of endotoxemia, characterized by increased lipopolysaccharide (LPS) in blood, through changes in GI function associated with low-grade systemic inflammation [9,17]. Indeed, saturated food lipids act as pro-inflammatory molecules in the digestive tract, altering the intestinal epithelial barrier and the microbiota equilibrium [33]. The consumption of hypercaloric diet in rats has been shown to reduce the rat intestinal contractility due to modified architecture of the intestinal smooth muscle [48]. In this context, maternal HFD during gestation and lactation has been shown to inhibit intestinal development and disrupt the intestinal mucosal barrier [56]. However, the impact of continuous maternal HFD intake on the GI muscle activity in the offspring at adulthood has not been examined yet.

The gut represents the actor of nutrients absorption from ingested food but consequently the main point for the uptake of orally delivered chemicals and food toxicants, through internalization and subsequent interaction with other body organs and systems [20]. A growing body of evidence suggests the ability of maternal, fetal, and early-life postnatal exposure to environmental factors, in particular food contaminants, to affect the gut homeostasis settlement. Such exposure during a sensitive window of development is capable to induce changes in gut functions in the offspring through later life stages [42]. Among the well-known environmental contaminants, Chlorpyrifos (CPF) has been widely used for decades as an organophosphate insecticide in the world. Its residues are frequently detected in food and drinking water [5]. The primary toxic mechanism of CPF is inhibiting acetylcholinesterase (AChE) at the central and peripheral nervous system and neuromuscular junctions leading to overstimulation of cholinergic synapses [14, 50]. CPF exposure is associated with altered programming of fetal metabolism and long-term consequences on health [44, 45]. Pre- and postnatal exposure to CPF has been shown to increase the intestinal permeability [27] and metabolic effects [41] in the offspring at adulthood. Although the exposure to CPF from gestation and throughout lactation till early adulthood showed a weaker *in vitro* contractility of the longitudinal ileal smooth muscle, the impact of long term perigestational exposure to CPF at low levels, prior and throughout pregnancy till the end of lactation, in the progeny at early adulthood has not been elucidated [12].

Accordingly, among the risk factors associated with the perinatal period, most of these studies have only focused on a single exposure stimulus illustrating the maternal and early-life exposure to either nutritional or to an environmental contaminant. As pregnant women can be subjected to multiple adverse stimuli simultaneously during life, and knowing that the development of the gut function is mediated through several phases from gestation through weaning, our experimental protocol has focused on the long-term maternal exposure to different dietary risks including the exposure to a commonly used pesticide (CPF) and a western- diet nutritional style (HFD) in the progeny. In particular, the present study investigated the consequences of perigestational exposure to CPF and/or HFD, starting from 4 months before gestation till the end of lactation periods in young adult offspring maintained on a standard diet without CPF after weaning on the contractile response of the ileum, an essential smooth muscle for homeostasis maintenance and proper nutrients absorption. We further sought to identify several mediators involved in the alteration of the ileum function.

Material and Methods

Chlorpyrifos preparation

Chlorpyrifos (O, O-diethyl-O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate; 99.8% pure) was purchased from LGC Standards (Molsheim, France). Chlorpyrifos was dissolved in rapeseed oil (MP Biomedicals, Illkirch, France) as vehicle at 1 mg/ml and administered by gavage at 1 mg/kg body weight/day to the rats in the CPF group. The chosen dose corresponds to the oral no-observed-adverse-effect-level (NOAEL) for inhibition of brain cholinesterase activity in rats [11]. This dose has been used in our previous experiments [15].

Experimental Design

The study protocol was approved by the nationally accredited Regional Directorate for Health, Animal and Environ-

ment Protection (Amiens, France) and the French Ministry of Research (reference number APAFIS#8207-2016121322563594 v2). All animals were treated according to the European Communities Council's guidelines (2010/63/EU).

16 female Wistar rats (Janvier Labs, Le Genest Saint Isle, France,) aged 7 weeks on arrival were housed in cages under constant conditions in a controlled-air- temperature room (23°C), with a 12 hours light/dark cycle. The rats were housed in NexGen Max cage system with 81 in^2/523 cm^2 floor area mounted on EcoFlow rack system (Allentown Inc, Bussy Saint Georges, France). After 1 week of acclimation period, the female rats, with an average body weight of 225 ± 4.9 g, were assigned randomly to 4 groups and housed 2 per cage (n=4/group). The females were fed either standard chow diet (Serlab3436, 3.1 kcal/g consisting of 4.5% Crude fat with soybean oil as main component of fat) or high-fat diet HFD (Research Diets no. D12492, SSNIFF Spezialdiäten GmbH, 5.24 kcal/g, with 60% kcal from fat consisting of 54.4% lard and 5.6% soybean oil). The females were gavaged daily with organic rapeseed oil (as a vehicle) for control (oil, standard diet) and HFD (oil, HFD) groups or CPF (1 mg/kg/day in organic rapeseed oil) for CPF (CPF, standard diet) and CPF+HFD (CPF, HFD) groups during 4 consecutive months according to our recently developed protocol [15]. The HFD exposure protocol was based on the rat model developed by Lecoutre et al. [35], where female rats fed the same HFD before and during gestation and lactation have shown to induce metabolic alterations including mild glucose intolerance and hyperinsulinemia in the offspring at adulthood. In our model, the standard chow diet was used as a control diet based on a study demonstrated by Almeida-Suhett et al. [3] showing similar effects on phenotypic, metabolic, and behavioral outcomes in mice receiving standard chow diet and purified low-fat diet feeding during 16 to 18 weeks. At the end of these 4 months, the females were mated with the male rats (Janvier Labs, two females per male). Once the pregnancy was recognized (smear and presence of spermatozoa), the females were isolated in individual cages. During gestation, the dams were kept under the same conditions as before gestation until the end of the lactation period. The average litter size was 9 pups per litter without significant changes between groups. The male pups represented 52% of the total obtained offspring from all groups. To avoid the interference of hormonal changes in females during the estrous cycle [35, 46], the effects of perigestational exposure to CPF and/or HFD were investigated only in male offspring. At postnatal day (PND) 21, the weanlings were separated from their mothers and the male rats were categorized into 4 groups as below:

Control group: Maternal exposure to standard diet with vehicle (oil) (n=10)

HFD group: Maternal exposure to HF diet with vehicle (oil) (n=10)

CPF group: Maternal exposure to standard diet with CPF (n=10)

CPF+HFD group: Maternal exposure to both HF diet and CPF (n=7)

Then after, rats in all groups received only standard diet without CPF until the age of 2 months postnatal (n=7-10/group). At PND60, these offspring were then euthanized with sodium pentobarbital EXAGON (Axience, France) injection (1 $ml.kg^{-1}$; 200 $mg.ml^{-1}$ solution). The ileum was rapidly dissected and directly placed in Krebs Henseleit solution (Sigma Aldrich, Saint Quentin Fallavier, France). The abdomen was then opened and segments of ileum of around 1 cm in length that are 5 cm proximal to the ileocecal valve were dissected and used for *in vitro* contractility assessment as previously described by Darwiche et al. [12]. Other parts were used for AChE activity measurement, gene expression, and histological analysis.

Measurement of isolated ileum contractility

Longitudinal and circular ileum muscle strips were suspended under 1 g of tension in a 15 mL organ bath containing Krebs Henseleit solution at pH 7.4 (Sigma Aldrich, Saint Quentin Fallavier, France) oxygenated with 95% O_2 + 5% CO_2 and maintained at 37 °C. The strips were then left to equilibrate in the bath for 30 min and the Krebs Henseleit solution was replaced every 15 min. The electric field stimulation EFS (100 V; 30 ms) was performed by an electric stimulator (model 2100, A-M Systems; Phymep, Paris, France) using two rectangular platinum electrodes placed 2 cm apart, parallel to the muscle strips, as previously described by Darwiche and colleagues [12]. EFS induces ACh release from the myenteric plexus, promoting in turn muscle contraction [49]. The ileal strips were attached to a force transducer (model UF1; Pioden Controls Ltd, Newport, Isle of Wight, UK; precision ±10 mg), connected in turn to an isometric amplifier (HAZAX20208-01, Bionic Instruments; Phymep). A computerized data logger (MP100A-CE; Biopac Systems, Santa Barbara, CA, USA) was used to record the generated forces. The amplitude of contraction was analyzed with the peak analysis module in LabChart 7 software (AD Instruments, Oxford, UK) and normalized against the cross-section area (CSA, in cm2) according to the following formula: $CSA = M/(L \times \rho)$, where M is the mass (g), L is the length (cm) and σ is the muscle density (ρ= 1.056 $g.cm^{-3}$). Values were reported in g/cm^2.

Acetylcholinesterase activity

The Acetylcholinesterase activity in the ileum was measured according to a modified Ellman method [16]. The ileum samples were homogenized using a hand-held homogenizer in mammalian cell lysis buffer 5X (ab179835; Abcam, Cambridge, UK). Next, the ileum homogenates were centrifuged and 1 µL of protease inhibitor (Abcam) was then added to the supernatant. Before measuring the AChE activity, the supernatant was then diluted (1:10) and incubated with 10-5 M of butyrylcholinesterase inhibitor, tetra isopropyl pyrophosphoramide iso-OMPA (Sigma Aldrich, Saint Quentin Fallavier, France) for 15 minutes in order to obtain only AChE activity. The colorimetric assay kit (ab138871; Abcam) was used to measure the AChE activity according to the manufacturer's instructions. Briefly, 50 µl of the reaction mixture containing assay buffer, 20X 5,5'-dithio-bis-(2-nitrobenzoic acid): (DTNB), and acetylthiocholine were added to 96-well plate containing 50 µl of the supernatant and incubated for 10 min. Then, the absorbance was measured with a microplate reader (ELx808; Biotek, Winooski, VT, USA) at 412 nm during 15 minutes. The ileum protein content was quantified according to Bradford assay using Bovine Serum Albumin (BSA) as standard (Bio-Rad, Hercules, CA, USA). The AChE activity was normalized for the protein content, and the data were presented in µmol/min/mg protein.

Table 1: The sequences of primers used in real-time PCR

Gene		Primer sequence
GAPDH	Forward	5′-GGTGCTGAGTATGTCGTGGAGT-3′
	Reverse	5′-ATTGCTGACAATCTTGAGGGAG-3′
β-actin	Forward	5′-ACGTCGACATCCGCAAAGACCTC-3′
	Reverse	5′-TGATCTCCTTCTGCATCCGGTCA-3′
UBC	Forward	5′-TCGTACCTTTCTCACCACAGTATCTAG-3′
	Reverse	5′-GAAAACTAAGACACCTCCCCATCA-3′
HPRT1	Forward	5′-CTCATGGACTGATTATGGACAGGAC-3′
	Reverse	5′-GCAGGTCAGCAAAGAACTTATAGCC-3′
M2 AChR	Forward	5′-CACGAAACCTCTGACCTACCC-3′
	Reverse	5′-ACAGTCCTCACCCCTAGGATG-3′
M3 AChR	Forward	5′-GCTCCATCCTCAACTCTACCA-3′
	Reverse	5′-TTCTCTCCACATCCAGAGTCC-3′
Substance P (SP)	Forward	5′-TGTTTGCAGAGGAAATCGGTG-3′
	Reverse	5′- GAACTGCTGAGGCTTGGGTC-3′
rat NK1R	Forward	5′- GGTACTACGGCCTCTTCTATTGC-3′
	Reverse	5′- CAGGAAGTAGATCAGTACAGTCACG-3′
Calmodulin	Forward	5′- GAATGGCACCATTGACTTCC-3′
	Reverse	5′- GTAGCCATTGCCATCCTTGT-3′

Total RNA was extracted from the ileum samples using FastGene® RNA Basic Kit (Nippon Genetics Europe GmbH, Germany) according to the supplier's instructions. After quantification of the total RNA concentration using NanoDrop 1000 spectrophotometer (Thermo Scientific), 1 µg of RNA was used for cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Courtaboeuf, France). For gene expression measurement with quantitative real-time PCR (qRT-PCR), primers were purchased from Invitrogen (Life Technologies, Saint Aubin, France), and SYBR™ Green Master Mix (Applied Biosystems™) was used according to the manufacturer's instructions and PCRs were run on an ABI Prism 7900HT Real-time PCR System (Applied Biosystems™). The oligonucleotide sequences of the primers are presented in Table 1.

PCR for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), hypoxanthine guanine phosphoribosyl transferase (HPRT), Ubiquitin C (UBC) and β-actin was realized in the same conditions and the 4 genes were tested as robust housekeeping genes. Expression stability was determined by RefFinder [55] using the BestKeeper program [40], Normfinder program [4], Genorm program [52], and the comparative delta-Ct method [43] (Table 2). Consequently, the housekeeping gene coding for GAPDH was used as an endogenous control.

Table 2: The Expression stability for the candidate housekeeping genes determined by RefFinder

		Best Keeper	Norm Finder	Genorm	Delta CT
HOUSEKEEPING GENES	GAPDH	1.008	2.475	3.016	4.24
	β-actin	2.427	2.887	3.016	4.403
	HPRT	2.819	2.542	3.7	4.397
	UBC	3.098	4.998	4.67	5.64

PCR was performed under the following conditions: denaturation for 1min at 95 °C, annealing temperature was 56°C for calmodulin and 60°C for other primers, and elongation for 2 min at 72 °C. All PCR reactions were carried out in duplicate. The results were reported in arbitrary units using a $2^{-\Delta\Delta Ct}$ calculation, relative to the controls according to the following equation ($\Delta\Delta Ct = \Delta Ct$ exposed – mean ΔCt control). The expression level of each gene was expressed relative to GAPDH.

Histological analysis

Segments of ileum were fixed with Carnoy solution, embedded in paraffin blocks and cut to 5 µm thick-sections. Next, the sections were deparaffinized with xylene and further hydrated with successive baths of alcohol. The fixed sections were then stained with Mayer's hematoxylin/ eosin for histomorphometric analysis. The histological sections were observed under a light microscope (ZEISS, Imager.D2) attached with a camera and the digital images were acquired using Zen software (Carl Zeiss AG, Oberkochen, Germa-

ny). Then the Image J® software (U. S. National Institutes of Health, Bethesda, Maryland, USA) was used to measure the thicknesses of both longitudinal and circular smooth muscle layers.

Statistical analysis

Statistical analysis was performed with GraphPad Prism™ software (version 5.0; GraphPad Software Inc., San Diego, CA, USA). According to a Kolmogorov-Smirnov test of normality, all data were normally distributed. A two-way analysis of variance (ANOVA) was used to study the main effects of CPF (control and HFD groups vs. CPF and CPF+HFD groups) or diet (control and CPF groups vs. HFD and CPF+HFD groups) and the interaction between CPF and diet exposure. In case of significant CPF-diet interaction, unpaired t-test (post hoc analysis) was then applied to compare between different groups. The threshold for statistical significance was set to $p \leq 0.05$. Indicative results ($p<0.1$) are represented when needed.

Results

Body weight changes in the offspring by maternal exposure to CPF and/ or HFD according to postnatal age

The mean body weight of the male offspring was studied at different developmental stages. The male pups were not weighed at the PND1 to reduce early-life maternal separation stressor. At PND3 (3 days after birth) and PND21 (weaning), the mean body weight was significantly higher in HFD fed groups compared to standard diet fed groups ($p<0.01$). The increase in the body weight was still observed at early adulthood (PND60) ($p = 0.0577$). Besides, the mean body weight was lower in CPF groups as compared to the other groups not exposed to CPF ($p<0.05$) at PND60 (Figure 1).

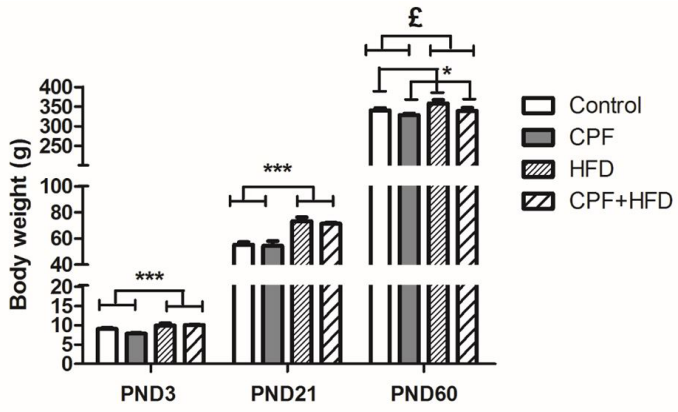

Figure 1: Effects of perigestational exposure to CPF and HFD on the body weight of male rat offspring at birth (PND3), as juveniles (PND21), and as young adults (PND60). Data are quoted as means ± SEM (n=7-10/group). Effect of diet (HFD and CPF+HFD groups vs. control and CPF groups) ***: $p<0.01$, £: $p<0.1$. Effect of CPF (CPF and CPF+HFD groups vs. control and HFD groups) *: $p<0.05$

Effects of CPF and HFD on the contractility of the ileum in male rats at PND60

The effects of perigestational exposure to CPF and/ or HFD and their interaction on the contractility of the ileum induced by electrical field stimulation were analyzed at PND60 (Figure 2). A main effect of CPF was observed. Compared to non-CPF groups, maternal CPF exposure was associated with a significant increase in the amplitude of contraction in the longitudinal muscle ($p = 0.0018$) and circular muscle ($p = 0.0223$) compared to controls. Despite a non-significant diet-CPF interaction for both muscles, the peak contractions of ileal longitudinal smooth muscle were higher in CPF group ($p<0.05$) and in CPF+HFD group ($p<0.1$) by 123% and by 149% respectively compared to the control group. For the circular muscle, the peak contraction was significantly higher in CPF+HFD group by 110% compared to the control group ($p<0.01$).

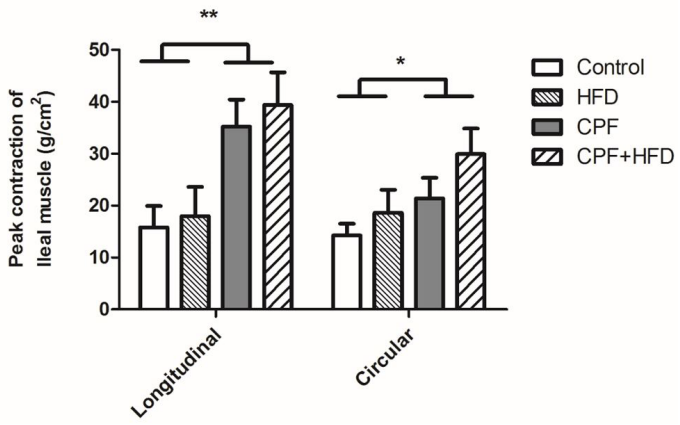

Figure 2: Effects of perigestational exposure to CPF and HFD on the *in vitro* peak contraction of ileal longitudinal and circular smooth muscles in male adult rats expressed as a function of cross-sectional area (g/cm2). Data are depicted as means ± SEM (n=7-10/group). Effect of CPF exposure (CPF and CPF+HFD groups vs. control and HFD groups) *: $p<0.05$; **: $p<0.01$

The activity of Acetylcholinesterase enzyme in the ileum

The perigestational exposure to HFD and CPF was associated with significant interaction for AChE activity in the ileum ($p = 0.05$). A main effect of CPF ($p = 0.0198$) and HFD ($p = 0.0080$) was also shown. AChE activity levels were significantly lower in HFD group ($p<0.01$) and CPF group ($p<0.01$) by 26% and 24% respectively compared to controls. A significant decrease by 29% was also observed in CPF+HFD group ($p<0.01$ vs control) (Figure 3).

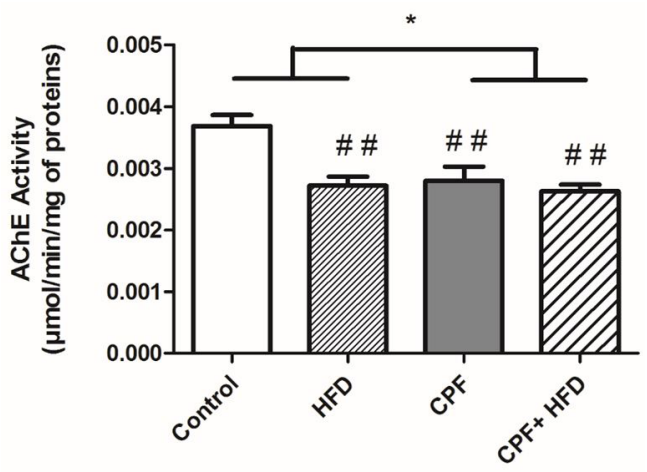

Figure 3: Effects of perigestational exposure to CPF and HFD on AChE activity in the ileum of male rats at PND60. Data are expressed as the mean ± SEM (n=7-10/group). Effect of CPF (CPF and CPF+HFD groups vs. control and HFD groups) *: p<0.05. For a significant CPF x diet interaction, significant differences according to unpaired t-test: # #: p<0.01 vs. control

Gene Expression analysis of muscarinic AChRs (M2 and M3 AChR), tachykinin (substance P) and neurokinin 1 receptor (NK1R), and calmodulin in the ileum

The effects of HFD and CPF exposure on the ileal gene expression were quantified by real-time PCR (Figure 4). A main effect of CPF was shown for M2 AChR mRNA. Indeed CPF exposure was associated with a significant increase in the M2 AChR mRNA compared to non-CPF groups (p = 0.0004). Even though the diet-CPF interaction for both M2 & M3 AChR mRNA was not significant (Figure 4A and B), the ileal expression of M2 AChR mRNA was significantly 1.5- and 1.45- fold higher in CPF and CPF+HFD groups, respectively, compared to controls (p<0.001 for CPF and p<0.01 for CPF+HFD) (Figure 4A). The ileal expression of M3 AChR mRNA was significantly higher by 1.9 fold in CPF group compared to controls (p<0.05) (Figure 4B).

Then, the effects of CPF and HFD exposure on the gene expression of SP and its preferred receptor (NK1R) were studied in the ileum. Despite a non-significant CPF-diet interaction for the expression of SP, a main effect for CPF exposure was observed. SP mRNA levels were significantly increased with maternal CPF exposure compared to non-CPF groups (p<0.05) (Figure 4C). However, the interaction between CPF and diet tends toward significance for NK1R mRNA expression (p = 0.0953). The ileal expression of NK1R mRNA was significantly higher by 1.5 fold in HFD compared to controls (p<0.05) (Figure 4D).

Although a non-significant CPF-diet interaction for the expression of calmodulin, a calcium (Ca2+)-binding protein, a main effect for CPF exposure was reported. Calmodulin mRNA levels were significantly decreased with maternal CPF exposure compared to non-CPF groups (p<0.05) (Figure 4E).

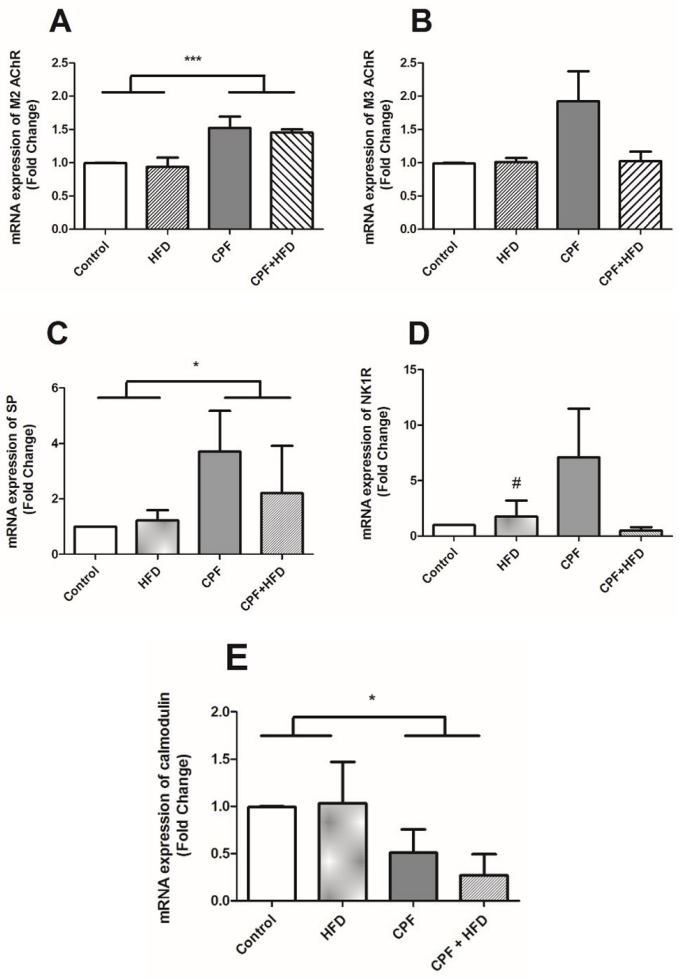

Figure 4: Gene expression analysis in the ileum of rats at PND60 quantified by real-time PCR. (A) M2 AChR mRNA levels, (B) M3 AChR mRNA levels, (C) SP mRNA coding for tachykinin (substance P), (D) NK1R mRNA coding for neurokinin 1 receptor, and (E) Calmodulin mRNA levels. Data are quoted as the mean ± SEM expressed relative to the control gene expression according to 2−ΔΔ CT method (n=7-10/group). Effect of CPF (CPF and CPF+HFD groups vs. control and HFD groups) *: p<0.05, ***: p<0.001. For a significant CPF x diet interaction, significant differences according to unpaired t-test: #: p<0.05 vs. control

Thickness of longitudinal and circular muscle layers in the ileum

The effects of HFD and CPF exposure on the thickness of longitudinal and circular muscles in the ileum are shown in Figure 5. Despite a non-significant CPF-diet interaction for both muscle layers, maternal CPF exposure was associated with a significant increase in the thickness of the circular ileal muscle compared to non-CPF groups (p = 0.0476). However, no significant changes were observed for the longitudinal muscle layer.

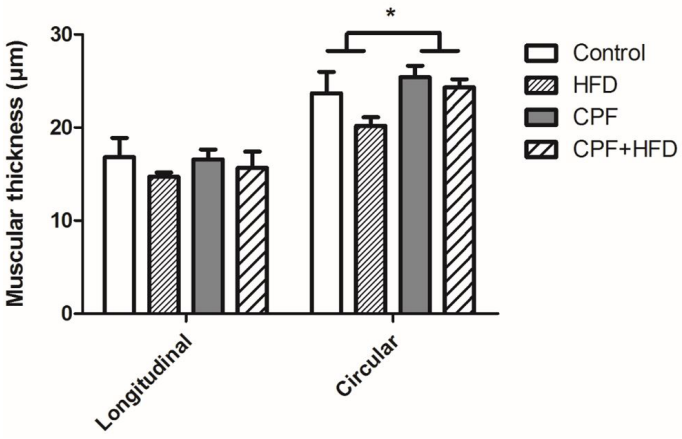

Figure 5: Effects of perigestational exposure to CPF and HFD on the thickness of the longitudinal and circular ileal smooth muscle layers in male rats at PND60. Data are expressed as the mean ± SEM (n=7-10/group). Effect of CPF (CPF and CPF+HFD groups vs. control and HFD groups) *: $p<0.05$

Discussion

Neural development during the prenatal and early postnatal environment is highly affected by the maternal nutrient provision, a prominent candidate for fetal programming. Knowing that the susceptibility of pregnant women to environmental factors is distinctly increased, in this study, we investigated the consequences of simultaneous maternal exposure to two major alimentary factors: OPs residues and HFD. Although recent studies on animal models have demonstrated the effects of HFD consumption on the metabolic disruptions induced by early-life OPs exposure [1, 34, 44], no studies have examined the impacts of continuous long-term maternal CPF and/ or HFD exposures starting from 4 months before gestation till the end of lactation on the GI smooth muscle function in the progeny at early adulthood.

In our study, only rats exposed during the perigestational period to HFD with or without CPF showed higher body weight at birth. Maternal HFD exposure assessed in animal models have reported contrasting results with some showing decreased body weight [10, 23, 26] while others reported unchanged body weight at birth [21, 51]. These contradictory results could be attributed to the duration of maternal HFD consumption and the fatty acid composition of the HFD used among these studies. Since HFD can be transformed into the milk [13], our rats reported an increase in the body weight at the end of lactation (PND21). Consistent with Lecoutre et al. [35], the elevated body weight was still observed at early adulthood (PND60).

On the other side, CPF exposure was associated with a decrease in body weight at adulthood (PND60) in agreement with our previously published work [12]. Since the effect was only observed at early adulthood suggests a prolonged effect of CPF toxicity through fetal programming.

The contractile activity of the longitudinal and circular muscle layers plays a functional role in propelling the luminal contents of the intestine [24]. Our results showed that the perigestational exposure to CPF was associated with higher amplitude of contraction for both longitudinal and circular muscles of the ileum. By contrast, in our previous experiments with a different exposure model [12], we have recently shown a weaker contraction of ileal longitudinal smooth muscle in rats exposed pre- and postnatally, throughout development, to a higher dose of CPF (5 mg/kg/day) at PND60. Noting that in the study of Darwiche et al. [12], the rats continue to be exposed to CPF after weaning until PND60. However, our rats stopped to receive either CPF or HFD after weaning. This could explain the difference between the results presented in both studies. On the other side, the amplitude of contraction for both muscles was not affected by HFD exposure. These findings are in agreement with Patten et al. [39] reporting no changes in the maximal contraction of the ileum with increasing levels of dietary saturated fats supplementation. In another work, Fu et al. [18] reported a stronger contraction/ relaxation of ileum in rats fed HFD for six weeks. The differences observed can be attributed to the species of rats used in the experiments (SD vs Wistar), the age of the animals and the duration of exposure to HFD, and another crucial element, the composition of HFD in terms of saturated fat.

It has been suggested that the architectural changes of the intestinal smooth muscle could affect the contractile response of the ileum [7, 48]. In our study, the increase in the amplitude of contraction of the ileum observed with CPF exposure could be attributed to the increased thickness of the circular ileal smooth muscle. Indeed, the hypertrophic growth of the circular musculature leads to increased hyperresponsiveness to contractile mediators and thus increasing the efficiency of contraction [7]. Unlike the circular muscle, the hypertrophy of longitudinal muscle develops a greater sensitivity to the relaxing factors reducing its contractile efficiency. This might explain the unchanged thickness of the longitudinal muscle despite its increased contractile activity.

Consistent with Darwiche et al. [12], ileal AChE activity was significantly decreased in the CPF group compared to controls despite that our rats were not directly exposed to CPF. In fact, CPF which is known to cross the placental barrier [2], elicits its toxicity through inhibition of AChE enzyme at cholinergic synapses [50]. Then, the accumulation of acetylcholine can overstimulate the postsynaptic cholinergic receptors [14], accounting at least in part,

to the observed increase in the ileal EFS-induced contractions. Indeed, the muscarinic acetylcholine receptors (mAChR) within the muscle myenteric circuits play an essential role in controlling intestinal mediated muscle contraction through the direct effect of ACh on muscle reflexes [22]. M2 AChR activation mediates adenylate cyclase inhibition, whereas M3 AChR leads to phosphoinositide hydrolysis, resulting in the mobilization of Ca2+ and consequent muscle contraction [19]. It has been demonstrated that cholinergic hyperstimulation can result in the desensitization of the muscarinic receptors in the ileum and brain [37]. In our study, CPF exposure is associated with a significant increase in the expression of M2 AChR mRNA without significant changes in M3 AChR mRNA expression. For instance, Chlorpyrifos oxon (CPO), the active metabolite of CPF, can bind directly to muscarinic receptors, in particular the M2 AChR, inhibiting the muscarinic agonist binding to cardiac [25] and striatum [8] muscarinic receptors. Since excess ACh may lead to desensitization of mAChRs, then the increase in the expression of M2 AChR observed in our study could represent an adaptive response to compensate for the possible decreased function of M2 AChRs [36].

On the other side, the perigestational exposure to HFD was associated with a significant decrease in the AChE activity in the offspring at PND60 suggesting prolonged cholinergic activation through inhibiting ACh degradation without modifications in the expression of M2 AChR. Although our rats did not directly ingest HFD after weaning, the increased levels of circulating energy molecules, such as free fatty acids, in rats consuming HFD has been shown to decrease the AChE activity in several brain areas [28, 38]. Interestingly, the decrease in the AChE activity in the CPF+HFD group was similar to both CPF and HFD groups, speculating that the indirect co-exposure to both CPF and HFD did not exacerbate the effect observed with the exposure to each factor separately. These findings corroborate our previous results showing a similar effect of decreased AChE activity in the diaphragm among the groups exposed to CPF and /or HFD [15].

Non-cholinergic excitatory neurotransmissions are mediated by tachykinins located in the central and peripheral nervous systems [47]. Besides the effects on cholinergic transmission, in our model, the perigestational CPF exposure induced a significant increase in the expression of the tachykinin Substance P (SP) without modifications in the expression of its preferred receptor the neurokinin 1 receptor (NK1R). Since the stimulation of the myenteric neurons induces the release of SP which can activate, in turn, other neurons and promote non-cholinergic intestinal smooth muscle contraction [6], then increased expression of SP can lead in turn to the increased amplitude of contraction of ileal muscles.

However, the expression of NK1R was significantly elevated in the HFD group compared to controls without significant changes in the expression of SP. A growing body of evidence suggests the significance of SP and NK1R interactions in the response of adipose tissue to HFD and weight gain. Indeed, administration of NK-1R antagonist has been shown to reduce weight gain and prevent fat accumulation in mice fed HFD for two weeks and promote weight loss in Diet-Induced Obesity (DIO) mice model [31]. Moreover, reduced weight gain has been reported in mice genetically deficient in NK1R in response to HFD feeding, suggesting the role of NK1R in the development of obesity [30]. Consequently, the increased expression of NK1R could alter the energy balance and induce the increase in body weight observed in our rats following the perigestational exposure to HFD.

Knowing that Ca^{2+} plays a pivotal role in regulating smooth muscle contractility [53], the expression of calmodulin, a Ca^{2+} binding protein, has been studied in ileum samples. In response to contractile stimuli and subsequent increased cytoplasmic Ca^{2+}, calmodulin activates the cross-bridge cycling resulting in smooth muscle contraction [54]. In our model, CPF exposure induced a decrease in the expression of calmodulin compared to groups that were not exposed throughout the perigestational period to CPF. This finding suggests an adaptive response to the increased cholinergic and non-cholinergic stimulation of the ileum. To confirm our results, the protein expression of calmodulin could be further assessed.

Conclusions

Therefore, the present study aimed to inspect the consequences of maternal exposure to CPF and HFD on the underlying contractility changes of the GI muscle in the progeny. In accordance with the DOHaD concept, the perigestational exposure to CPF is associated with increased ileal muscle contractility at adulthood via cholinergic and non-cholinergic mechanisms. Although maternal HFD did not affect ileal muscle contractility, the offspring were characterized by elevated NK1 receptor expression and reduced AChE activity levels. Despite the lack of direct exposure post-weaning, early life exposure to CPF and /or HFD programs the ileal homeostasis and increases the risk of altered gut contractile function at early adulthood. Such abnormal gut motility pattern can lead to irritable bowel syndrome (IBS) symptoms including abdominal pain, distention, diarrhea, and constipation. Irregular motor activity of the small intestine can be associated with pain; then it could be promising to study the perception of pain in the gut of the animals. Further studies are needed to assess current outcome indicators of irregular GI motor activity during development due to early life disturbances.

Author contributions

The contribution of each author are as follows: conception or design of the study (Hiba El Khayat El Sabbouri, Véronique Bach, Jérôme Gay-Quéheillard); methodology and investigation (Hiba El Khayat El Sabbouri, Marion Guibourdenche, Walaa Darwiche, Narimane Djekkoun, Jérôme Gay-Quéheillard), acquisition, analysis, or interpretation of data (Hiba El Khayat El Sabbouri, Jérôme Gay-Quéheillard); drafting or revising work critically for important intellectual content (Hiba El Khayat El Sabbouri, Jérôme Gay-Quéheillard) supervision (Wissam H. Joumaa, Véronique Bach, Wiam Ramadan, Jérôme Gay-Quéheillard) . All authors have approved the final version of the manuscript.

The authors declare that all data were generated in-house and that no paper mill was used.

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Conflict of Interest

The authors have no relevant financial or non-financial interests to disclose.

Statement on welfare of animals

The study protocol was approved by the nationally accredited Regional Directorate for Health, Animal and Environment Protection (Amiens, France) and the French Ministry of Research (reference number APAFIS#8207-2016121322563594 v2). All animals were treated according to the European Communities Council's guidelines (2010/63/EU).

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