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PII: S0166-4328(19)31649-3

DOI: https://doi.org/10.1016/j.bbr.2020.112552

Reference: BBR 112552

To appear in: *Behavioural Brain Research*

Received Date: 12 November 2019

Revised Date: 30 January 2020

Accepted Date: 9 February 2020

Please cite this article as: Peris-Sampedro F, Guardia-Escote L, Basaure P, Cabré M, Colomina MT, Improvement of APOE4-dependent non-cognitive behavioural traits by postnatal cholinergic stimulation in female mice, *Behavioural Brain Research* (2020), doi: https://doi.org/10.1016/j.bbr.2020.112552

Improvement of APOE4-dependent non-cognitive behavioural traits by postnatal cholinergic stimulation in female mice

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Highlights

- Apolipoprotein E4 (apoE4) female mice display a spontaneous anxiety-like phenotype.
- ApoE4 female mice show repetitive behaviour.
- ApoE4 female mice exhibit a heightened motivation for high-fat diet (HFD).
- A postnatal exposure to chlorpyrifos (CPF) normalises the *APOE4*-dependent traits.
- A postnatal exposure to CPF fosters the over-consumption of HFD later in life.

Abstract

The apolipoprotein E (*APOE*) *ε4* allele hastens cognitive decline, but other non-cognitive behaviours, as well as underpinning interactions with the cholinergic system, have not been systematically addressed. Both C57BL/6 and humanised apoE4 female mice were transiently exposed to subclinical doses (0 or 1 mg/kg body weight) of the cholinesterase inhibitor chlorpyrifos (CPF), a widely-used pesticide, from

postnatal days 10 to 15. At 5 months of age, we assessed the impact of *APOE4* genotype, postnatal CPF exposure and *APOE4* x CPF interactions on anxiety (open field and light-dark tests), stereotypes (digging test) and neophobia (sucrose preference test), as well as on high-fat diet (HFD)-seeking and consumption (scheduled-feeding paradigm). We found that control *APOE4* female carriers displayed a robust anxiety-like phenotype, which was accompanied by exaggerated stereotypes and a subtle neophobic response to rewarding foods. In parallel, we observed an amplified "wanting" response for HFD in these mice, which did not entail enhanced "liking". Notably, postnatal CPF ameliorated the anxiety-like and the heightened HFD-seeking responses in adult apoE4 female mice, while caused them to gain weight steadily compared to control peers. In turn, an early-life transient exposure to CPF fostered the over-consumption of HFD during adulthood without affecting how much this reward was "wanted" or the total caloric intake. These data reveal a role for CPF towards fostering "unhealthy" dietary choices. We conclude that the *APOE4* genotype modulates non-cognitive behaviours and we provide support for an *APOE4*-dependent cholinergic dysfunction.

Keywords: Apolipoprotein E4; Anxiety; Cholinesterase inhibitor; Motivation; Chlorpyrifos

1. Introduction

Traditionally described as a part of an anchoring mechanism fostering lipid transport in the body, the apolipoprotein E (apoE) system only arose as a key regulator of neuronal processes few decades ago [1]. Among the three major *APOE* allelic variants that exist in humans (*ε2*, *ε3* and *ε4*), *ε4* represents the major genetic risk factor for developing Alzheimer's disease (AD), particularly in women [2, 3]. More recently, the role of the apoE system is being further challenged as many studies have highlighted its contribution in modulating cognitive processes in the absence of disease. Indeed, being carrier of the *ε4* allele has been associated not only with a faster cognitive decline in elderly non-demented subjects [4, 5], but also with impaired attention, working memory and spatial memory in middle-aged population [6, 7]. On the other hand, many studies in both rodents [8-10] and humans [11, 12] have described an enhanced anxiety-like phenotype in apoE4 subjects. For example, when subjected to either a zero plus maze or an elevated plus maze, apoE4 female mice displayed higher measures of anxiety compared to both apoE2 and apoE3 peers [9]. Meanwhile, other non-cognitive behaviours have not been systematically investigated in apoE transgenic mice. So far, it has been demonstrated that apoE4 female mice exhibit a deficient inhibition as revealed by increased perseveration and premature responding in the 5-choice serial reaction time task (5-CSRTT) [13, 14].

The hypothesis of a dysfunctional cholinergic neurotransmission to explain some of the cognitive deficits attributable to the *APOE4* genotype has steadily gained strength. Indeed, higher levels of acetylcholinesterase (AChE) [15], diminished release of acetylcholine (ACh) (partly caused by lower levels of vesicular acetylcholine transporter (VAChT)) [16], greater number of muscarinic receptors [17] or reduced activity of cholinergic neurons [18] have been proposed as potential factors contributing to their cognitive impairments.

Over the last years, our group has extensively investigated whether *APOE* polymorphisms confer different vulnerabilities upon exposure to toxic agents at both early-life [16, 17, 19] and adult stages [13, 20-22] in apoE-targeted replacement (apoE-TR) mice [23]. Particularly, we have focused on a widely-used pesticide belonging to the organophosphate (OP) family, namely chlorpyrifos (CPF). The toxicity of OP compounds relies on their ability to strongly inhibit both systemic and brain cholinesterases (ChE), thus triggering a cholinergic over-stimulation [24]. Nevertheless, non-cholinergic targets have been also described, and CPF is well-known to alter the proper signalling of other neurotransmitters like monoamines [25], dopamine (DA) [26] and serotonin [27]. Therefore, it is perhaps not surprising that broad-ranging behavioural disturbances emerge upon exposure to this toxic agent. While a worsening of cognitive skills has been recurrently described after exposure to CPF [20, 28, 29], the study of other complex behavioural domains linked to motivation or anxiety remain either understudied or controversial.

Our previous work has thus far underlined *APOE* x CPF interactions (at early-life stages) that, in part, endorse a cholinergic dysfunction in *APOE4* carriers, especially in female mice [16, 17, 19]. The present study was primarily designed to discover new potential interactions between the *APOE4* genotype and CPF, with a view to exploring behavioural domains, in female mice, hitherto overlooked. We reasoned that, because early-life exposures to CPF can cause long-lasting effects on components of the cholinergic system [17, 19] and given the hypothetical cholinergic hypofunction intrinsic to *APOE4* carriers, a non-symptomatic exposure to CPF during the postnatal period would normalise, at least in part, some of the behavioural deficits attributable to this genotype. Moreover, we sought (a) to determine whether an early-life exposure to CPF alters the motivation for a palatable food later in life, (b) to further support the effects of the *APOE4* genotype on anxiety, and (c) to investigate the engagement of the *APOE4* genotype on inhibitory control and motivation for food.

2. Material and methods

2.1 Animals and care

ApoE-TR mice were generated from a C57BL/6 background to express functional human apoE isoforms under the control of the endogenous murine promoter [23]. Both adult apoE-TR male and female mice, homozygous for the human *ε4* allele, were obtained from Taconic (Taconic Europe, Lille Skensved, Denmark). Following a 1-week quarantine period, female mice were mated with males of the same genotype for 5 days. Pregnant females were then individually housed, and the day of delivery designated as postnatal day (PND) 0. Each experimental group consisted of offspring from at least 4 different litters, and only female mice were used for this study. After weaning on PND 28, mice were group-housed in cages containing 2 to 5 counterparts and left undisturbed until the beginning of the experimental procedures (4 months later), from which they were individually housed. The same protocol was conducted in C57BL/6 female mice, so that they served as controls with similar genetic background (Charles River France, L'Arbresle, France). All the mice were housed under a 12-h light/dark cycle (lights off at 8 pm) in standard controlled conditions (22 ± 2°C and 50 ± 10% humidity) with free access to standard maintenance chow (SAFE A04 diet, Panlab, Barcelona, Spain; 3.34 kcal/g, where: 19.3% protein; 72.4% carbohydrate; 8.4% fat) and water, unless otherwise stated.

The use of animals and all the protocols were approved by the Animal Care and Use Committee of the Rovira i Virgili University (Tarragona, Spain) and were conducted in accordance with the Spanish Royal Decree 53/2013 on the protection of experimental animals, and the European Communities Council Directive (2010/63/EU).

2.2 Chemicals, treatment and experimental groups

CPF ([O,O-diethyl O-(3,5,6-trichloropyridin-2-yl) phosphorothioate], purity 99.5%; Sigma-Aldrich, Seelze, Germany) was dissolved in corn oil and adjusted to orally administer, with a micro-pipette, 1 mg/kg in 1 µL per g of body weight from PND 10 to PND 15. The control group received an equivalent volume of corn oil alone instead [16, 17, 19]. In previous studies, we have provided evidence indicating that 1 mg/kg/day of CPF produced a 50 % plasma ChE inhibition without effects in the central nervous system, thus remaining below the threshold for causing systemic toxicity [16]. We treated the pups within that window because it coincides with the highest peak in brain growth and development in rodents [30]. The four experimental groups were as follows: vehicle C57BL/6 (n=8), CPF-treated C57BL/6 (n=9), vehicle apoE4 (n=9) and CPF-treated apoE4 (n=9). All the mice were weighed regularly during the postnatal period (PND 4-28).

2.3 Behavioural assessment

Behavioural testing was carried out in 5-month-old female offspring. Animals were first allowed to acclimate to single housing for 2 weeks and handled regularly. The estrous cycle was monitored prior to behavioural testing. All behavioural procedures were undertaken during the light phase (10 am to 2 pm), and groups counterbalanced regarding the time of testing. Each behavioural test took place on alternate days. The experimenter was blind to the genotypes and treatments of the mice. To prevent the use of olfactory cues, we cleaned the behavioural equipment with 70 % ethanol between each session.

2.3.1 Estrous cycle

The estrous stage of the mice, which was assessed using a wet smear technique as previously described [31], was tracked prior to behavioural testing. The samples were examined under a microscope and assigned to a stage of the estrous cycle according to the most abundant cell type(s); (a) proestrus: mainly nucleated epithelial cells and few leucocytes; (b) estrus: only cornified cells; (c) metestrus: many leucocytes, cornified and nucleated epithelial cells; (d) diestrus: predominantly leucocytes. We classified the four groups into two subgroups: proestrus/estrus and metestrus/diestrus, according to progesterone levels [32, 33].

2.3.2 Open field test

Locomotor activity and behavioural responses to a novel environment were tested in an open field (OF) [28, 29]. The OF consisted of a dimly lit light-brown 60 x 60 cm wooden box, protected with 50 cm-high walls. The field was virtually divided into two zones: the periphery, an area up to 10 cm from the wall, and the central area. Each mouse was initially placed in the centre of the field and allowed to freely explore for 30 min. The following activity parameters were measured: total distance travelled, average velocity and time immobile. To study anxiety-like behaviour, we analysed activity in the central area (i.e., total distance in the centre/total distance ratio, time spent in the centre and frequency of visits to the centre). We also studied the habituation to the new environment by analysing changes in activity over six 5-min periods. The path and movements of the mice were recorded by a video camera (Sony CCD-IRIS), and then computerized by means of a video-tracking program (Etho-Vision©, Noldus Information Technologies, Wageningen, The Netherlands).

2.3.3 Digging test

A digging test was carried out to assess levels of repetitive behaviour [34]. Briefly, each mouse was individually placed in a clear plastic box (14 cm × 10 cm × 11 cm) filled with approximately 5-cm-deep wood chip bedding lightly tamped down to make a flat, even surface. The same bedding substrate was used for all the mice and flattened after each test. The latency to start digging, the total time spent digging and the number of digging bouts were manually recorded during the 5-min test.

2.3.4 Light-dark test

Anxiety-like behaviour was also tested in a light-dark test [35]. This paradigm, firstly described by Crawley and co-workers [36, 37], relies on the innate aversion of rodents to (bright) open areas and on their spontaneous drive to explore in response to a stimulating novel environment. On the other hand, behavioural inhibition may be partly accounting for the anxiety-like behaviour in this test, a trait that is commonly reflected by an increased risk assessment and a less risk-taking behaviour [38, 39]. The apparatus consisted of a ceiling-closed methacrylate cage (45 x 27 x 27 cm) divided into a dark chamber, which was not artificially lit, and a white chamber, brightly lit from above (~350 lux), which were connected by a centrally-positioned 7.5 x 7.5-cm opening. Each mouse was initially placed in the centre of the dark chamber and allowed to freely explore the entire apparatus for 5 min. The latency to enter the light chamber for the first time (as a measure of risk-taking behaviour; [38]), the time spent in the light chamber (as a measure of aversion; [40]), the total number of inter-compartmental transitions (as an index of activity and exploration; [40]), and the total number of aborted attempts to enter the aversive area (as a measure of risk assessment; [38, 39]) were scored. In any case, an entry/transition was counted when the four paws were in the zone, whereas an attempt was recorded when only the upper body crossed the boundary of the light chamber.

2.3.5 Sucrose preference testing

After familiarisation of the two-bottle setup (filled with water) over 3 days, mice were presented with two bottles (water vs 4% sucrose) for 4 h/day on 2 consecutive days. Data from the first day were used to assess preference for novelty. After the 5-week HFD scheduled-feeding (SF), sucrose preference (SP) was tested again for 4 h. To eliminate potential side preferences, the bottles were always side-switched 2 h after the test started, and the initial position of the sucrose bottle was alternated according to the position of the animal's cage in the rack. SP was calculated as follows: (mL of sucrose / total liquid intake) x 100.

2.4 Dietary intervention: scheduled-feeding paradigm

Both the dietary intervention and the binge-type feeding establishment protocols were updated from [41, 42]. After the behavioural assessment, we measured basal 24-h food intake and body weight for 1 week (i.e., baseline, BL). Following baseline measurements, all the mice underwent the same dietary manipulation with pelleted HFD (230 HF diet, SAFE, Augy, France; 5.32 kcal/g, where: 13.1% protein; 26.3% carbohydrate, mainly sucrose; 60.6% fat). First, during the habituation phase (i.e., HAB), mice were subjected to a 2-h scheduled access to HFD (late light phase, 3 to 5 pm) 5 days a week (Monday to Friday) for 5 weeks. HFD was always offered in addition to chow, because the presence of chow during the scheduled access to the palatable diet does not alter binge-type feeding establishment [42]. During the HAB phase, the mice had no access to HFD on Saturday and Sunday. After 5 weeks on the HFD SF paradigm, the mice were switched back to baseline, where only chow was available 24 h a day for 7 days (i.e., replacement phase, RP). After this 7-day period, mice were returned to the 2-h SF paradigm for 5 more days (i.e., refeeding phase, RF), after which SP was tested again. Body weight was measured 3 times per week. HFD intake was measured by weighing the amount given (which was always ~ 4g) and the amount left after the first 30 min and at the end of the 2-h SF. In order to assess the progression of the binge-type feeding establishment, we calculated the 30/120 min ratio. Arguably, data from the 2-h HFD consumption could be interpreted as a hedonic “liking” of the reward, whereas the 30/120 ratio mirrors the motivational “wanting” component of the reward. Chow intake was measured daily. In any case, we measured consumption of food by weight (g) and then converted it to energy (kcal).

2.5 Statistics

Data were processed using SPSS software 24 (IBM Corp., Armonk, New York, USA). We used repeated measures analyses of variance (ANOVA) analyses for comparing continuous variables, with both Genotype and Treatment as the between-subject factors and Time (minutes, days or weeks) as the within-subject factor. A three-way ANOVA (Cycle stage, Genotype and Treatment) test was conducted to assess whether the estrous cycle affected any behavioural parameter. Two-way ANOVA (Genotype and Treatment) analyses were used when comparing two or more groups. One-way ANOVA tests were used to follow up significances and interactions upon split data from both genotypes and/or treatments. We used a paired *t*-test to compare SP before and after the dietary intervention, and to compare the average 2-h HFD intake during the last HAB week and the RF phase. Statistical significance was set at $p < 0.05$, and the results are reported as mean values ± SEM.

3. Results

3.1 An early-life exposure to CPF triggers long-lasting weight gain in apoE4 female mice

Prior to the exposure period, there were no significant differences in body weight between groups (PND 4; vehicle C57BL/6: 2.08 ± 0.09 g, CPF-treated C57BL/6: 2.14 ± 0.11 g, vehicle apoE4: 2.20 ± 0.10 g and CPF-treated apoE4: 2.03 ± 0.06 g) (Fig. 1). However, differences in weight started to emerge early after exposure to CPF. Specifically, we found both a Time x Genotype and a Time x Treatment interaction ($F_{3, 34}$ = 3.353, p = 0.032; $F_{3, 34}$ = 3.237, p = 0.036; respectively) for the PND 4-28 period. Further analyses showed that CPF-treated apoE4 mice displayed already a higher body weight than their controls at PND 15 ($F_{1, 17}$ = 4.740, p = 0.045) that remained steady until PND 28 ($F_{1, 17}$ = 4.601, p = 0.048) (Fig. 1). Interestingly, these genotype-dependent differences persisted intact until and throughout adulthood (Time x Genotype: $F_{7, 34}$ = 3.970, p = 0.005; Genotype x Treatment: $F_{1, 34}$ = 4.506, p = 0.042); CPF-treated apoE4 mice weighed approximately 13 % more than their controls counterparts did throughout the experimental period (BL, HAB, RP and RF; Treatment: $F_{1, 17}$ = 6.037, p = 0.026) (Fig. 1).

Insert Figure 1

3.2 Behavioural assessment

3.2.1 The estrous cycle did not influence any behavioural outcome in the OF, digging or light-dark tests

Of the 35 females examined, 54.29 % were in the proestrus/estrus phase, while 45.71 % were in the metestrus/diestrus phase prior to starting the behavioural assessment. Moreover, these percentages were evenly distributed between groups (vehicle C57BL/6: 62.5 vs 37.5 %; CPF-treated C57BL/6: 44.44 vs 55.56 %; vehicle apoE4: 44.44 vs 55.56 %; and CPF-treated apoE4: 66.67 vs 33.33 %). Importantly, we did not find that the estrous cycle affected any of the behavioural parameters studied neither in the OF, nor in the digging or light-dark tests (data not shown). Therefore, we did not consider the stage of the estrous cycle for further analyses.

3.2.2 Neither postnatal CPF exposure nor APOE genotype affect exploratory behaviour or general motor activity during adulthood

The analysis of the changes in activity throughout the 30-min OF session reveals that all individuals habituated to the novel space ($F_{5, 34}$ = 7.343, p < 0.001) (Fig. 2a). However, we did not observe

significant differences between groups in the total distance travelled, average velocity or time spent immobile in the arena.

Insert Figure 2

3.2.3 An early-life exposure to CPF alleviates the anxiety-like phenotype inherent to APOE4 genotype during adulthood

As for anxiety measures in the OF, we detected a Genotype x Treatment interaction for both the time spent in the centre ($F_{1, 34} = 5.092$, $p = 0.031$) and the centre distance ratio ($F_{1, 34} = 3.826$, $p = 0.060$) (Fig. 2b, 2c). ApoE4 female mice postnatally treated with CPF tended to spend more time in the central area of the field compared to their controls ($F_{1, 17} = 3.292$, $p = 0.088$). However, we did not note any other relevant significances upon handling both genotypes separately.

The results from the digging test show that, regardless of the treatment, *APOE4* carriers spent 115 % more time digging (Genotype: $F_{1, 34} = 4.467$, $p = 0.043$) and performed 64 % more digging bouts (Genotype: $F_{1, 34} = 4.738$, $p = 0.037$) than did the C57BL/6 group (Fig. 2d, 2e), thus suggesting an exaggerated stereotypic behaviour.

Along the same lines, the results in the light-dark test point to a phenotype resembling anxiety in *APOE4* carriers (Genotype: time spent in the light chamber, $F_{1, 34} = 3.181$, $p = 0.084$; total number of transitions, $F_{1, 34} = 3.459$, $p = 0.072$; total number of attempted transitions, $F_{1, 34} = 6.049$, $p = 0.020$) (Fig. 2f-2i). Further analyses confirmed that, within the vehicle condition, apoE4 female mice spent 47 % less time in the light chamber ($F_{1, 16} = 7.114$, $p = 0.018$) (Fig. 2g), did 49 % less inter-compartmental transitions ($F_{1, 16} = 7.118$, $p = 0.018$) (Fig. 2h) and hesitated more to cross through the device opening (145 % increase in the number of attempted transitions) ($F_{1, 16} = 11.242$, $p = 0.004$) (Fig. 2i) compared to C57BL/6 peers. Interestingly, we pinpointed some Genotype x Treatment interactions, either significant or nearly-significant, for all the variables studied (latency to first enter the light chamber: $F_{1, 34} = 3.222$, $p = 0.082$; time spent in the light chamber: $F_{1, 34} = 6.062$, $p = 0.020$; total number of transitions: $F_{1, 34} = 4.270$, $p = 0.047$; and total number of attempted transitions: $F_{1, 34} = 4.156$, $p = 0.050$). Indeed, postnatal exposure to CPF caused adult apoE4 females to enter the light chamber a 55 % quicker ($F_{1, 17} = 5.450$, $p = 0.033$) (Fig. 2f), to spend 89 % more time in the light chamber ($F_{1, 17} = 8.807$, $p = 0.009$) (Fig. 2g), to do 85 % more inter-compartmental transitions ($F_{1, 17} = 7.270$, $p = 0.016$) (Fig. 2h) and to tend to reduce a 45 % the crossing attempts ($F_{1, 17} = 4.096$, $p = 0.060$) (Fig. 2i).

3.2.4 A previous experience with a HFD boosts sucrose intake in APOE4 carriers during adulthood

We found that *APOE* genotype tended to influence preference for novelty in the first SP test carried out before the dietary intervention (Genotype: $F_{1, 34} = 3.374$, $p = 0.076$) (Fig. 3a). Subsequent analyses indicated that, within the vehicle condition, apoE4 females drank 12 % less sugar basally than C57BL/6 controls did ($F_{1, 16} = 13.942$, $p = 0.002$) (Fig. 3a). Instead of catching up, vehicle apoE4 mice still displayed a lower preference for sucrose compared to C57BL/6 peers at the second test, yet the difference was not significant anymore (Fig. 3b). Notably, control apoE4 female mice exposed to a HFD SF paradigm during adulthood showed identical sugar intake to the other three experimental groups during the last SP test (Fig. 3c). Indeed, their SP score after the dietary manipulation was significantly higher from those of both the first (t [8] = -4.685, $p = 0.002$) and the second (t [8] = -2.942, $p = 0.019$) SP tests.

Insert Figure 3

3.3 Binge-type feeding establishment and food intake analysis

3.3.1 Acclimatisation to the SF paradigm

Daily chow intake was similar between groups prior to starting the dietary intervention (vehicle C57BL/6: 12.67 ± 0.43 kcal; CPF-treated C57BL/6: 13.11 ± 0.22 kcal; vehicle apoE4: 12.95 ± 0.33 kcal; and CPF-treated apoE4: 13.51 ± 0.51 kcal).

Mice took less than a week to adapt to the SF paradigm, although they showed a lower 2-h HFD intake and 30/120 ratio on Mondays compared to the rest of the week throughout the HAB phase. This effect, though, became less apparent as the HAB progressed (data not shown). Both the 2-h HFD intake and the 30/120 ratio increased rapidly over 3 days during the first week of HAB (Time: $F_{4, 34} = 156.980$, $p < 0.001$; $F_{4, 34} = 21.573$, $p < 0.001$, respectively) (Fig. 4a, 4b). Interestingly, the ratio during the first day of access to the SF paradigm was 37 % lower in *APOE4* carriers compared to C57BL/6 peers (Genotype: $F_{1, 34} = 5.429$, $p = 0.026$), pointing to a neophobic response to HFD (Fig. 4b). Overall, the mice compensated for greater HFD consumption by reducing their intake of chow gradually (Time: $F_{2, 34} = 141.274$, $p < 0.001$) (Fig. 4c).

After the RP phase, mice were returned to the 2-h SF paradigm for 5 more days during the RF phase. In general, the average 2-h HFD intake was 10 % higher during the RF phase compared to that of the last HAB week (6.73 ± 0.21 kcal vs 6.14 ± 0.21 kcal, respectively: t [34] = -4.264, $p < 0.001$). Interestingly, upon handling all groups separately, such difference was only significant in CPF-treated apoE4 (7.07 ±

0.45 kcal vs 6.17 ± 0.40 kcal, respectively: t [8] = -2.531, p = 0.035) and C57BL/6 mice (7.20 ± 0.46 kcal vs 6.70 ± 0.40 kcal, respectively: t [8] = -2.360, p = 0.046).

Insert Figure 4

3.3.2 An early-life exposure to CPF enhances HFD intake without affecting chow consumption after a 7-day withdrawal period

After the initial gradual increase during the first week of HAB, the 2-h HFD consumption plateaued during the HAB phase (Fig. 5a). Although we detected a Time x Treatment interaction already during that period ($F_{4, 34}$ = 3.240, p = 0.026), we did not notice any other remarkable effect upon handling both Genotype and Treatment factors separately.

On the first day of the RF phase, mice consumed less HFD compared to the last week of the HAB phase, an effect that was common to all groups (vehicle C57BL/6: 4.91 ± 0.46 kcal; CPF-treated C57BL/6: 5.38 ± 0.41 kcal; vehicle apoE4: 4.26 ± 0.26 kcal; and CPF-treated apoE4: 4.85 ± 0.48 kcal). Arguably, the animals needed an adaptation day after the RP phase, where they only had access to chow 24 h a day. For that reason, we excluded the first day of the RF phase from the analysis. However, we found a general effect of Treatment from the second RF day onwards ($F_{1, 34}$ = 4.252, p = 0.048) (Fig. 5b, 5c). Interestingly, mice postnatally treated with CPF, regardless of the genotype, consumed 13 % more HFD during the 2-h exposure compared to vehicle peers ($F_{1, 34}$ = 4.586, p = 0.040) (Fig. 5c). Importantly, daily total caloric intake remained similar between groups during the RF phase (vehicle C57BL/6: 12.76 ± 0.42 kcal; CPF-treated C57BL/6: 12.74 ± 0.36 kcal; vehicle apoE4: 12.37 ± 0.15 kcal; and CPF-treated apoE4: 13.26 ± 0.31 kcal).

Insert Figure 5

3.3.3 An early-life exposure to CPF normalises the amplified "wanting" response inherent to APOE4 genotype after a 7-day withdrawal period

In line with the HFD intake data, the 30/120 ratio values also plateaued during the HAB phase after its initial increase (Fig. 5d). From then on, all the groups showed similar scores throughout the HAB phase.

Following the same logic as above, we also excluded the first day of the RF phase from the 30/120 ratio analysis (30/120 ratio on the first day of the RF phase; vehicle C57BL/6: 0.74 ± 0.05; CPF-treated C57BL/6: 0.68 ± 0.03; vehicle apoE4: 0.72 ± 0.04; and CPF-treated apoE4: 0.73 ± 0.04).

We found a general effect of Genotype from the second RF day onwards ($F_{1, 34} = 5.509$, $p = 0.025$) (Fig. 5e, 5f). Strikingly, *APOE4* carriers exhibited higher 30/120 ratio scores overall than C57BL/6 counterparts did ($F_{1, 34} = 5.421$, $p = 0.026$) (Fig. 5f). Further analyses revealed that, within the vehicle condition, apoE4 female mice consumed 68 % of the total HFD intake in the first 30 min of exposure, and this result proved to be significantly different from C57BL/6 peers, who consumed 63 % instead ($F_{1, 16} = 6.148$, $p = 0.026$) (Fig. 5e).

4. Discussion

Here we primarily sought to address the impact of the *APOE4* genotype in non-cognitive behaviours so far uncharted, with a view to exploring interactions with the cholinergic system that could extend our insights on how the apoE system modulates behavioural attributes in the absence of disease. We found that adult apoE4 female mice displayed a robust anxiety-like phenotype, which was accompanied by complementary behavioural features including increased stereotypic behaviour and a neophobic response primarily to HFD. In addition, apoE4 mice exhibited a heightened incentive salience ("wanting" response) for a palatable food, here HFD, when switched back to the SF paradigm after a withdrawal week. Interestingly, a postnatal time-restricted (PND 10-15) exposure to the pesticide CPF normalised this *APOE4*-dependent phenotype, thus improving the anxiety-like and the excessive HFD-seeking responses, as well as caused apoE4 female mice to weight consistently more throughout adulthood. Finally, we found that a short postnatal exposure to CPF fostered the over-consumption of HFD later in life.

Our data showing an enhanced spontaneous anxiety-like behaviour in adult apoE4 female mice agree with those of previous studies [8-10] and further support a role for the *APOE* genotype in modulating anxiety. Remarkably, neither *APOE* genotype nor CPF influenced locomotor activity in the OF, fact that rules out the possibility that activity patterns may have misled the results on anxiety scores. The results herein also extent our insights on additional *APOE*-dependent behavioural traits that develop simultaneously to the anxiogenic response. Indeed, apoE4 female mice also displayed increased stereotypic behaviour and neophobia to HFD. Importantly, some of these responses were spontaneously elicited when the mice were exposed to unfamiliar environments (e.g., a clear plastic box with 5-cm deep bedding or the availability of a new food in the environment) and may be therefore linked to the animal's innate reaction with coping with a stressful situation. In recent decades, the non-

cognitive behavioural changes in AD, which are highly prevalent in diagnosed patients and even obvious at the pre-clinical stage, have been considered a core feature of the disease [43]. Despite the effort few authors have made to bridge the existent gap of knowledge [44-46], some of these behaviours, including apathy, stereotypes or neophobia, remain to be disregarded in mechanistic studies because of their heterogeneity and context specificity. Similarly, we were unable to find any study in rodents addressing the impact of the *APOE4* genotype on either stereotypic behaviour or neophobia upon rooting through the literature. Given the relevance of the *APOE4* genotype in the onset and progression of AD, we stress out the need for additional research assessing its impact on the AD-dependent affective symptomatology.

Both the dietary intervention and the binge-type feeding establishment protocols were updated from those used by Bake and co-workers [41, 42] and thus look very similar in essence. However, unlike these authors, we did all the food measurements manually, and hence we decided to limit the exposure to HFD to five days a week (Monday to Friday) during the HAB period. This protocol update entailed an "interrupted learning" during the five HAB weeks; the mice always showed a lower 2-h HFD intake and 30/120 ratio on Mondays compared to the rest of the week (even though this effect became less evident throughout the HAB phase). Arguably, the mice always used the first day of the week to recall the protocol, increasing their HFD intake thereupon. Hence, in hindsight, this could be regarded as a limitation of our study. When we analysed the data from the HAB phase (Fig. 5), we did include Mondays in the analysis since we calculated the mean of the corresponding week. After the RP phase, where the mice had access to chow only for an entire week, we presented again the HFD for 2 h on Monday (RF phase). To our surprise, mice consumed less HFD on the first RF day compared to the last week of the HAB phase, an effect that was common to all groups. We reason that mice used the first 2-h exposure (Monday) of the RF week as a recalling, and we interpret it as a trigger. Nevertheless, after excluding the first RF day from the analysis, the average 2-h HFD intake was 10 % higher during the RF phase compared to that of the last HAB week. It has been shown that introducing a period of HFD deprivation in rodents previously habituated to this diet can increase its consumption when subsequently reintroduced [47, 48]. This is relevant in terms of translational research, because it mimics an intermittent introduction of restricted caloric diets in humans. Our results from the refeeding phase sustain, on the one hand, that APOE4 carriers could be more sensitive to such rebound effect (because they exhibit an increased "wanting" response for HFD) and, on the other, that a postnatal CPF exposure could stand as a trigger for unhealthier dietary choices (because CPF-treated mice showed higher 2-h HFD intake compared to control peers).

Control apoE4 female mice exhibited an exacerbated motivation for a palatable food, which can be interpreted as an amplified "wanting" response [49]: they consumed more than two-thirds of their total HFD intake within the first 30 min of exposure to it when this was reintroduced after a withdrawal period. Although in a different setting, these data also accord with our previous observation of a deficient inhibitory control in apoE4 adult female mice. When subjected to a 5-CSRTT paradigm, apoE4 mice persevered more and did more premature responses than both apoE2 and apoE3 mice under baseline conditions [13, 14]. Of note, the increased "wanting" response for HFD was not coupled with a heightened "liking" of the rewarding food, because (control) apoE4 mice consumed similar calories from HFD compared to C57BL/6 peers. In this sense, the incentive-sensitisation theory underlines that the behavioural component of an addiction, whatever its nature, is triggered by an excessive amplification of "wanting" responses without necessarily affecting the extent to which the same reward is "liked" [49].

Most likely, the increased motivational response for HFD in (control) apoE4 mice hints the involvement of the brain mesocorticolimbic DA system, where dopaminergic neurons, mainly arising from the ventral tegmental area, project to both forebrain and cortical targets, such as the nucleus accumbens, striatum and prefrontal cortex [50]. This brain circuitry is involved in behavioural inhibition and impulsive action [51, 52], and rodent studies have demonstrated that blunted DA signalling in prefrontal cortical areas elicits an increase in motivation (involved in drug seeking) and/or impairs inhibitory control [14, 53]. In our recent study, adult apoE4 female mice, which displayed a heightened impulsive responding in the 5-CSRTT, presented lower levels of DA in the frontal cortex compared to apoE2 female mice [14]. Hence, a diminished DA content in cortical areas may account for the heightened "wanting" responding attributable to the *APOE4* genotype.

Arguably, the *APOE4*-dependent behavioural improvement observed in adult female mice upon postnatal exposure to CPF suggests that its administration during development may help redress a potential basal cholinergic deficit attributable to this genotype. We infer that an over-stimulation of the cholinergic system may underlie this functional enhancement. This hypothesis is supported by previous studies showing that adult apoE4 female mice particularly benefit from a limited postnatal exposure to CPF [17, 19]. To unravel the underlying mechanisms, the authors reported that apoE4 mice present basal differences in cholinergic components compared to wild-type peers (either apoE3 or C57BL/6 mice). These include lower levels of VAChT, greater number of muscarinic receptors and lower levels of alpha-7 nicotinic receptors in cortical areas, as well as diminished hippocampal ChE expression [17, 19]. Notwithstanding, the cholinergic system orchestrates an extensive amount of both cognitive and non-cognitive processes in a brain site-dependent manner, fact that emphasises its complexity

and heterogeneity. It seems therefore difficult to precisely identify candidate brain areas that may be relevant for the behavioural outcome herein described.

Still today, little attention has been paid to the contribution of developmental exposures to CPF to the global epidemic of obesity. Furthermore, the scarce existing literature thereon remains controversial. Indeed, while some authors have suggested that postnatal CPF exposure can trigger weight gain later in life [54], others have reported the opposite trend [55, 56]. In the current study, developmental CPF did not cause C57BL/6 mice to gain weight during adulthood. In the work by Lassiter and Brimijoin [54], pregnant rats were dosed daily by gavage with CPF at a dose of 2.5 mg/kg from gestational day 7 through the end of PND 21. Although both males and females were included in the study, the authors only found an increased body weight in male rats starting from PND 45. Arguably, (a) the use of different species (rats in their case vs mice in ours), (b) the choice of a specific developmental window (gestational and postnatal in their case vs only postnatal in ours), (c) the dose of CPF administered (2.5 mg/kg body weight in their case vs 1 mg/kg body weight in ours) or (d) the sex of the animals tested (significant effects were only found in males in their case, while we used females in our study) are all factors that may have contributed to such discrepancies. On the other hand, underlying mechanism through which this pesticide would enhance body weight exclusively in *APOE4* carriers remains uncertain to us and thus deserves further investigation.

Alternatively, it seems clear that a previous exposure with a palatable food, here HFD, may be enough to boost sucrose consumption in apoE4 control mice. In one study, a previous experience with a highly-salient sucrose solution abolished the intrinsic lower preference for glucose in rats, which resulted in a hyperphagic status regardless of the sugar consumed thereupon in the setting of a free-choice diet [57]. Presumably, a limitation of our study is that we did not include control groups (ideally for each genotype and treatment) that would have been kept on a regular chow diet during the SF paradigm, neither we performed additional behavioural tests after the dietary intervention. Although unlikely (because all the groups were kept under the same feeding paradigm, and thus the exposure to CPF arguably arises as the sole variable between groups), it would be difficult to completely rule out the possibility that the exposure to HFD could have complemented the (beneficial) effects of postnatal CPF in apoE4 mice, as reported for other OPs [58, 59].

Our results demonstrating that CPF did not affect anxiety or other related behaviours in wild-type mice fuels the controversy as to whether developmental exposures to OP pesticides contribute to the emergence of anxiety-like symptoms later in life. Indeed, while some studies in rodents have reported an anxiolytic or no effect for CPF [55, 60], others pointed to the opposite [61, 62]. It seems clear that

(a) the use of different species (rats or mice) and/or strains, (b) the choice of a specific developmental window (gestational, postnatal or a combination of both), (c) the dose of CPF administered or (d) the sex of the animals tested are all factors that may have contributed to such discrepancies.

An early-life transient exposure to CPF altered food choice during adulthood in a manner favouring more intake of an "unhealthier" palatable food, here HFD. Interestingly, our results reveal that this "liking" effect is diet-specific, because CPF-treated mice did not over-consume their regular chow. This suggests that CPF specifically turns on "liking" for a palatable food rather than enhancing total caloric intake. In their pioneering work, Slotkin and co-workers showed how a HFD exposure during adulthood reverses some of the effects of neonatal exposures to parathion, another pesticide belonging to the OP family [58, 59]. They argued that these developmental exposures to OP toxicants could ultimately unleash a "subconscious" preference for HFD later in life that could arguably ameliorate the toxicant-dependent effects [63]. To our knowledge, though, this hypothesis has never been empirically substantiated until now.

In summary, we provide evidence supporting a role for the *APOE4* genotype in anxiety and other non-cognitive behaviours, including stereotypes and neophobia to HFD in female mice. Certainly, the question arises as to whether these results would be applicable in male subjects too, and thus further studies are needed to address this issue. Interestingly, a non-toxic transient exposure to the pesticide CPF during the postnatal period redressed most of the behavioural outcomes described for the *APOE4* genotype. Specifically, it neutralised the anxiety-like and the heightened motivation for a rewarding food. Although we have not delved into the specific underlying mechanisms, we have made progress to endorse a hypothetical cholinergic dysfunction in *APOE4* carriers. Finally, our data indicate that a limited postnatal experience with the widely-used pesticide CPF is enough to redirect dietary choice behaviour in favour of "unhealthier" palatable foods later in life. This underscores the relevance of silent chronic exposures to environmental toxicants that may be relentlessly contributing to our poor dietary patterns in modern societies.

Disclosure

The authors declare they have no actual or potential competing financial interests.

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Acknowledgments

We thank Dr Celeste di Paolo, Esperanza Chernichero and Juan Valencia for their technical support with animal care. We thank Dr Tina Bake for insightful comments. This work was supported by the Ministry of the Economy and Competitiveness (MINECO, Spain) [grant numbers PSI2014-55785-C2-2-R, PSI2014- 55785-C2-1-R]; and the Commission for Universities and Research of the Ministry of Innovation, Universities and Enterprise of the Generalitat de Catalunya [grant number 2013 FI_B00170].

References

[1]. Mahley RW, Rall SC Jr. Apolipoprotein E: Far more than a lipid transport protein. Annu Rev Genomics Hum Genet 2000;1:507-37.

[2]. Raber J, Huang Y, Ashford JW. ApoE genotype accounts for the vast majority of AD risk and AD pathology. Neurobiol Aging 2004;25(5):641-50.

[3]. Ungar L, Altmann A, Greicius MD. Apolipoprotein E, gender, and Alzheimer's disease: An overlooked, but potent and promising interaction. Brain Imaging Behav 2014;8(2):262-73.

[4]. Berteau-Pavy F, Park B, Raber J. Effects of sex and APOE epsilon4 on object recognition and spatial navigation in the elderly. Neuroscience 2007;147(1):6-17.

[5]. Reitz C, Mayeux R. Endophenotypes in normal brain morphology and Alzheimer's disease: A review. Neuroscience 2009;164(1):174-90.

[6]. Bour A, Grootendorst J, Vogel E, Kelche C, Dodart JC, Bales K, Moreau PH, Sullivan PM, Mathis C. Middle-aged human apoE4 targeted-replacement mice show retention deficits on a wide range of spatial memory tasks. Behav Brain Res 2008;193(2):174-82.

[7]. Patel KT, Stevens MC, Pearlson GD, Winkler AM, Hawkins KA, Skudlarski P, Bauer LO. Default mode network activity and white matter integrity in healthy middle-aged ApoE4 carriers. Brain Imaging Behav 2013;7(1):60-7.

[8]. Reverte I, Klein AB, Ratner C, Domingo JL, Colomina MT. Behavioral phenotype and BDNF differences related to apoE isoforms and sex in young transgenic mice. Exp Neurol 2012;237(1):116-25.

[9]. Siegel JA, Haley GE, Raber J. Apolipoprotein E isoform-dependent effects on anxiety and cognition in female TR mice. Neurobiol Aging 2012;33(2):345-58.

[10]. Villasana LE, Weber S, Akinyeke T, Raber J. Genotype differences in anxiety and fear learning and memory of WT and ApoE4 mice associated with enhanced generation of hippocampal reactive oxygen species. J Neurochem 2016;138(6):896-908.

[11]. Michels A, Multhammer M, Zintl M, Mendoza MC, Klunemann HH. Association of apolipoprotein E epsilon4 (ApoE epsilon4) homozygosity with psychiatric behavioral symptoms. J Alzheimers Dis 2012;28(1):25-32.

[12]. Robertson J, Curley J, Kaye J, Quinn J, Pfankuch T, Raber J. ApoE isoforms and measures of anxiety in probable AD patients and Apoe-/- mice. Neurobiol Aging 2005;26(5):637-43.

[13]. Peris-Sampedro F, Reverte I, Basaure P, Cabré M, Domingo JL, Colomina MT. Apolipoprotein E (APOE) genotype and the pesticide chlorpyrifos modulate attention, motivation and impulsivity in female mice in the 5-choice serial reaction time task. Food Chem Toxicol 2016;92:224-35.

[14]. Reverte I, Peris-Sampedro F, Basaure P, Campa L, Suñol C, Moreno M, Domingo JL, Colomina MT. Attentional performance, impulsivity, and related neurotransmitter systems in apoE2, apoE3, and apoE4 female transgenic mice. Psychopharmacology 2016;233(2):295-308.

[15]. Eggers C, Herholz K, Kalbe E, Heiss WD. Cortical acetylcholine esterase activity and ApoE4-genotype in Alzheimer disease. Neurosci Lett 2006;408(1):46-50.

[16]. Basaure P, Guardia-Escote L, Cabré M, Peris-Sampedro F, Sánchez-Santed F, Domingo JL, Colomina MT. Postnatal chlorpyrifos exposure and apolipoprotein E (APOE) genotype differentially affect cholinergic expression and developmental parameters in transgenic mice. Food Chem Toxicol 2018;118:42-52.

[17]. Guardia-Escote L, Basaure P, Blanco J, Cabré M, Pérez-Fernández C, Sánchez-Santed F, Domingo JL, Colomina MT. Postnatal exposure to chlorpyrifos produces long-term effects on spatial memory and the cholinergic system in mice in a sex- and APOE genotype-dependent manner. Food Chem Toxicol 2018;122:1-10.

[18]. Salehi A, Dubelaar EJ, Mulder M, Swaab DF. Aggravated decrease in the activity of nucleus basalis neurons in Alzheimer's disease is apolipoprotein E-type dependent. Proc Natl Acad Sci U S A 1998;95(19):11445-9.

[19]. Basaure P, Guardia-Escote L, Cabré M, Peris-Sampedro F, Sánchez-Santed F, Domingo JL, Colomina MT. Learning, memory and the expression of cholinergic components in mice are modulated by the pesticide chlorpyrifos depending upon age at exposure and apolipoprotein E (APOE) genotype. Arch Toxicol 2019;93(3):693-707.

[20]. Peris-Sampedro F, Basaure P, Reverte I, Cabré M, Domingo JL, Colomina MT. Chronic exposure to chlorpyrifos triggered body weight increase and memory impairment depending on human apoE polymorphisms in a targeted replacement mouse model. Physiol Behav 2015;144:37-45.

[21]. Peris-Sampedro F, Cabré M, Basaure P, Reverte I, Domingo JL, Colomina MT. Adulthood dietary exposure to a common pesticide leads to an obese-like phenotype and a diabetic profile in apoE3 mice. Environ Res 2015;142:169-76.

[22]. Peris-Sampedro F, Blanco J, Cabré M, Basaure P, Guardia-Escote L, Domingo JL, Sánchez DJ, Colomina MT. New mechanistic insights on the metabolic-disruptor role of chlorpyrifos in apoE mice: a focus on insulin- and leptin-signalling pathways. Arch Toxicol 2018;92(5):1717-28.

[23]. Sullivan PM, Mezdour H, Aratani Y, Knouff C, Najib J, Reddick RL, Quarfordt SH, Maeda N. Targeted replacement of the mouse apolipoprotein E gene with the common human APOE3 allele enhances diet-induced hypercholesterolemia and atherosclerosis. J Biol Chem 1997;272(29):17972-80.

[24]. Eaton DL, Daroff RB, Autrup H, Bridges J, Buffler P, Costa LG, Coyle J, McKhann G, Mobley WC, Nadel L, Neubert D, Schulte-Hermann R, Spencer PS. Review of the toxicology of chlorpyrifos with an emphasis on human exposure and neurodevelopment. Crit Rev Toxicol 2008;38(2):1-125.

[25]. Moreno M, Cañadas F, Cardona D, Suñol C, Campa L, Sánchez-Amate MC, Flores P, Sánchez-Santed F. Long-term monoamine changes in the striatum and nucleus accumbens after acute chlorpyrifos exposure. Toxicol Lett 2008;176(2):162-7.

[26]. Torres-Altoro MI, Mathur BN, Drerup JM, Thomas R, Lovinger DM, O'Callaghan JP, Bibb JA. Organophosphates dysregulate dopamine signaling, glutamatergic neurotransmission, and induce neuronal injury markers in striatum. J Neurochem 2011;119(2):303-13.

[27]. Slotkin TA, Skavicus S, Levin ED, Seidler FJ. Prenatal nicotine changes the response to postnatal chlorpyrifos: Interactions targeting serotonergic synaptic function and cognition. Brain Res Bull 2015;111:84-96.

[28]. Basaure P, Peris-Sampedro F, Cabré M, Reverte I, Colomina MT. Two cholinesterase inhibitors trigger dissimilar effects on behavior and body weight in C57BL/6 mice: The case of chlorpyrifos and rivastigmine. Behav Brain Res 2017;318:1-11.

[29]. Peris-Sampedro F, Salazar JG, Cabré M, Reverte I, Domingo JL, Sánchez-Santed F, Colomina MT. Impaired retention in AβPP Swedish mice six months after oral exposure to chlorpyrifos. Food Chem Toxicol 2014;72:289-94.

[30]. Watson RE, Desesso JM, Hurtt ME, Cappon GD. Postnatal growth and morphological development of the brain: A species comparison. Birth Defects Res B Dev Reprod Toxicol 2006;77(5):471-84.

[31]. Caligioni CS. Assessing reproductive status/stages in mice. Curr Protoc Neurosci 2009;48(1): Appendix

[32]. Gangitano D, Salas R, Teng Y, Perez E, De Biasi M. Progesterone modulation of alpha5 nAChR subunits influences anxiety-related behavior during estrus cycle. Genes Brain Behav 2009;8(4):398-406.

[33]. Martini M, Pinto AX, Valverde O. Estrous cycle and sex affect cocaine-induced behavioural changes in CD1 mice. Psychopharmacology 2014;231(13):2647-59.

[34]. Langley EA, Krykbaeva M, Blusztajn JK, Mellott TJ. High maternal choline consumption during pregnancy and nursing alleviates deficits in social interaction and improves anxiety-like behaviors in the BTBR T+Itpr3tf/J mouse model of autism. Behav Brain Res 2015;278:210-20.

[35]. Heredia L, Torrente M, Colomina MT, Domingo JL. Assessing anxiety in C57BL/6J mice: A pharmacological characterization of the open-field and light/dark tests. J Pharmacol Toxicol Methods 2014;69(2):108-14.

[36]. Crawley J, Goodwin FK. Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. Pharmacol Biochem Behav 1980;13(2):167-70.

[37]. Crawley JN. Neuropharmacologic specificity of a simple animal model for the behavioral actions of benzodiazepines. Pharmacol Biochem Behav 1981;15(5):695-9.

[38]. Arrant AE, Schramm-Sapyta NL, Kuhn CM. Use of the light/dark test for anxiety in adult and adolescent male rats. Behav Brain Res 2013;256:119-27.

[39]. Kulesskaya N, Voikar V. Assessment of mouse anxiety-like behavior in the light-dark box and open-field arena: role of equipment and procedure. Physiol Behav 2014;133:30-8.

[40]. Bourin M, Hascoët M. The mouse light/dark box test. Eur J Pharmacol 2003;463(1-3):55-65.

[41]. Bake T, Murphy M, Morgan DG, Mercer JG. Large, binge-type meals of high fat diet change feeding behaviour and entrain food anticipatory activity in mice. Appetite 2014;77:60-71.

[42]. Bake T, Hellgren KT, Dickson SL. Acute ghrelin changes food preference from a high-fat diet to chow during binge-like eating in rodents. J Neuroendocrinol 2017;29(4).

[43]. Shin IS, Carter M, Masterman D, Fairbanks L, Cummings JL. Neuropsychiatric symptoms and quality of life in Alzheimer disease. Am J Geriatr Psychiatry 2005;13(6):469-74.

[44]. Baeta-Corral R, Giménez-Llort L. Bizarre behaviors and risk assessment in 3xTg-AD mice at early stages of the disease. Behav Brain Res 2014;258:97-105.

[45]. Torres-Lista V, Parrado-Fernández C, Alvarez-Montón I, Frontiñán-Rubio J, Durán-Prado M, Peinado JR, Johansson B, Alcaín FJ, Giménez-Llort L. Neophobia, NQO1 and SIRT1 as premorbid and prodromal indicators of AD in 3xTg-AD mice. Behav Brain Res 2014;271:140-6.

[46]. Torres-Lista V, López-Pousa S, Giménez-Llort L. Marble-burying is enhanced in 3xTg-AD mice, can be reversed by risperidone and it is modulable by handling. Behav Processes 2015;116:69-74.

[47]. Kreisler AD, Garcia MG, Spierling SR, Hui BE, Zorrilla EP. Extended vs. brief intermittent access to palatable food differently promote binge-like intake, rejection of less preferred food, and weight cycling in female rats. Physiol Behav 2017;177:305-16.

[48]. Kreisler AD, Mattock M, Zorrilla EP. The duration of intermittent access to preferred sucrose-rich food affects binge-like intake, fat accumulation, and fasting glucose in male rats. Appetite 2018;130:59-69.

[49]. Berridge KC, Robinson TE. Liking, wanting, and the incentive-sensitization theory of addiction. Am Psychol 2016;71(8):670-9.

[50]. Volkow ND, Wang GJ, Fowler JS, Tomasi D. Addiction circuitry in the human brain. Annu Rev Pharmacol Toxicol 2012;52:321-36.

[51]. Dalley JW, Everitt BJ, Robbins TW. Impulsivity, compulsivity, and top-down cognitive control. Neuron 2011;69(4):680-94.

[52]. Morein-Zamir S, Robbins TW. Fronto-striatal circuits in response-inhibition: Relevance to addiction. Brain Res 2015;1628(A):117-29.

[53]. Gutman AL, Ewald VA, Cosme CV, Worth WR, LaLumiere RT. The infralimbic and prelimbic cortices contribute to the inhibitory control of cocaine-seeking behavior during a discriminative stimulus task in rats. Addict Biol 2017;22(6):1719-30.

[54]. Lassiter TL, Brimijoin S. Rats gain excess weight after developmental exposure to the organophosphorothionate pesticide, chlorpyrifos. Neurotoxicol Teratol 2008;30(2):125-30.

[55]. Cole TB, Fisher JC, Burbacher TM, Costa LG, Furlong CE. Neurobehavioral assessment of mice following repeated postnatal exposure to chlorpyrifos-oxon. Neurotoxicol Teratol 2012;34(3):311-22.

[56]. De Felice A, Scattoni ML, Ricceri L, Calamandrei G. Prenatal exposure to a common organophosphate insecticide delays motor development in a mouse model of idiopathic autism. PLoS One 2015;10(3):e0121663.

[57]. Peris-Sampedro F, Mounib M, Schelé E, Edvardsson CE, Stoltenborg I, Adan RAH, Dickson SL. Impact of free-choice diets high in fat and different sugars on metabolic outcome and anxiety-like behavior in rats. Obesity 2019;27(3):409-19.

[58]. Lassiter TL, Ryde IT, Levin ED, Seidler FJ, Slotkin TA. Neonatal exposure to parathion alters lipid metabolism in adulthood: Interactions with dietary fat intake and implications for neurodevelopmental deficits. Brain Res Bull 2010;81(1):85-91.

[59]. Slotkin TA, Lassiter TL, Ryde IT, Wrench N, Levin ED, Seidler FJ. Consumption of a high-fat diet in adulthood ameliorates the effects of neonatal parathion exposure on acetylcholine systems in rat brain regions. Environ Health Perspect 2009;117(6):916-22.

[60]. Carr RL, Armstrong NH, Buchanan AT, Eells JB, Mohammed AN, Ross MK, Nail CA. Decreased anxiety in juvenile rats following exposure to low levels of chlorpyrifos during development. Neurotoxicology 2017;59:183-90.

[61]. Braquenier JB, Quertemont E, Tirelli E, Plumier JC. Anxiety in adult female mice following perinatal exposure to chlorpyrifos. Neurotoxicol Teratol 2010;32(2):234-9.

[62]. Venerosi A, Ricceri L, Rungi A, Sanghez V, Calamandrei G. Gestational exposure to the organophosphate chlorpyrifos alters social-emotional behaviour and impairs responsiveness to the serotonin transporter inhibitor fluvoxamine in mice. Psychopharmacology 2010;208(1):99-107.

[63]. Slotkin TA. Does early-life exposure to organophosphate insecticides lead to prediabetes and obesity? Reprod Toxicol 2011;31(3):297-301.

Figure 1. An early-life exposure to the cholinesterase inhibitor agent chlorpyrifos (CPF) increases body weight only in apolipoprotein E4 (apoE4) female mice. Body weight progression of (**a**) C57BL/6 and (**b**) apoE4 female mice over the postnatal period and throughout adulthood. Abbreviations: PND, postnatal day; BL, baseline; RP, replacement; RF, refeeding. Asterisks indicate significant differences between the two treatment conditions: * $p < 0.05$.

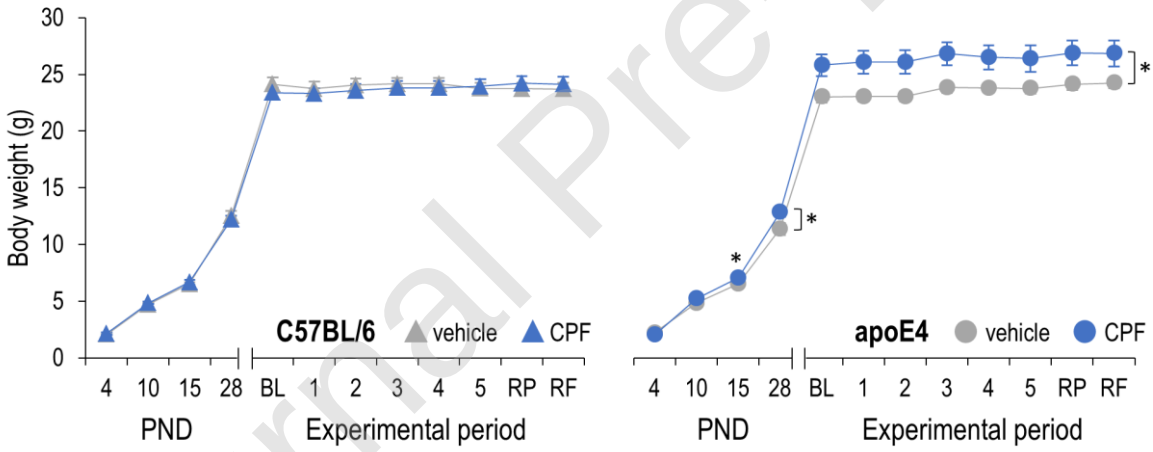

Figure 2. Effects of apolipoprotein E4 (*APOE4*) genotype, chlorpyrifos (CPF) treatment and underpinning interactions on locomotor activity and anxiety-like traits in female mice. (**a, b, c**) Parameters linked to locomotor activity and anxiety-like behaviour in both C57BL/6 and apoE4 female mice assessed in the open field. (**a**) Distance travelled in the arena throughout the 30-min session divided into 5-min periods, (**b**) total time spent in the centre and (**c**) total distance in the centre/total distance ratio. (**d, e**) Parameters linked to repetitive behaviour in all the groups assessed in the digging test. (**d**) Total time spent digging and (**e**) total number of digging bouts. (**f, g, h, i**) Parameters linked to anxiety-like behaviour in all the groups assessed in the light-dark test. (**f**) Latency to enter the light chamber for the first time (as a measure of risk-taking behaviour), (**g**) time spent in the light chamber

(as a measure of aversion), (**h**) the total number of inter-compartmental transitions (as a measure of activity and exploration) and (**i**) total number of attempted transitions (as a measure of risk assessment). Symbols indicate differences between: the two treatment conditions at $p < 0.1$ (†), $p < 0.05$ (*) or $p < 0.01$ (**); the two genotypes at $p < 0.05$ (Δ); apoE4 controls and C57BL/6 controls at $p < 0.05$ (◊) or $p < 0.01$ (◊◊).

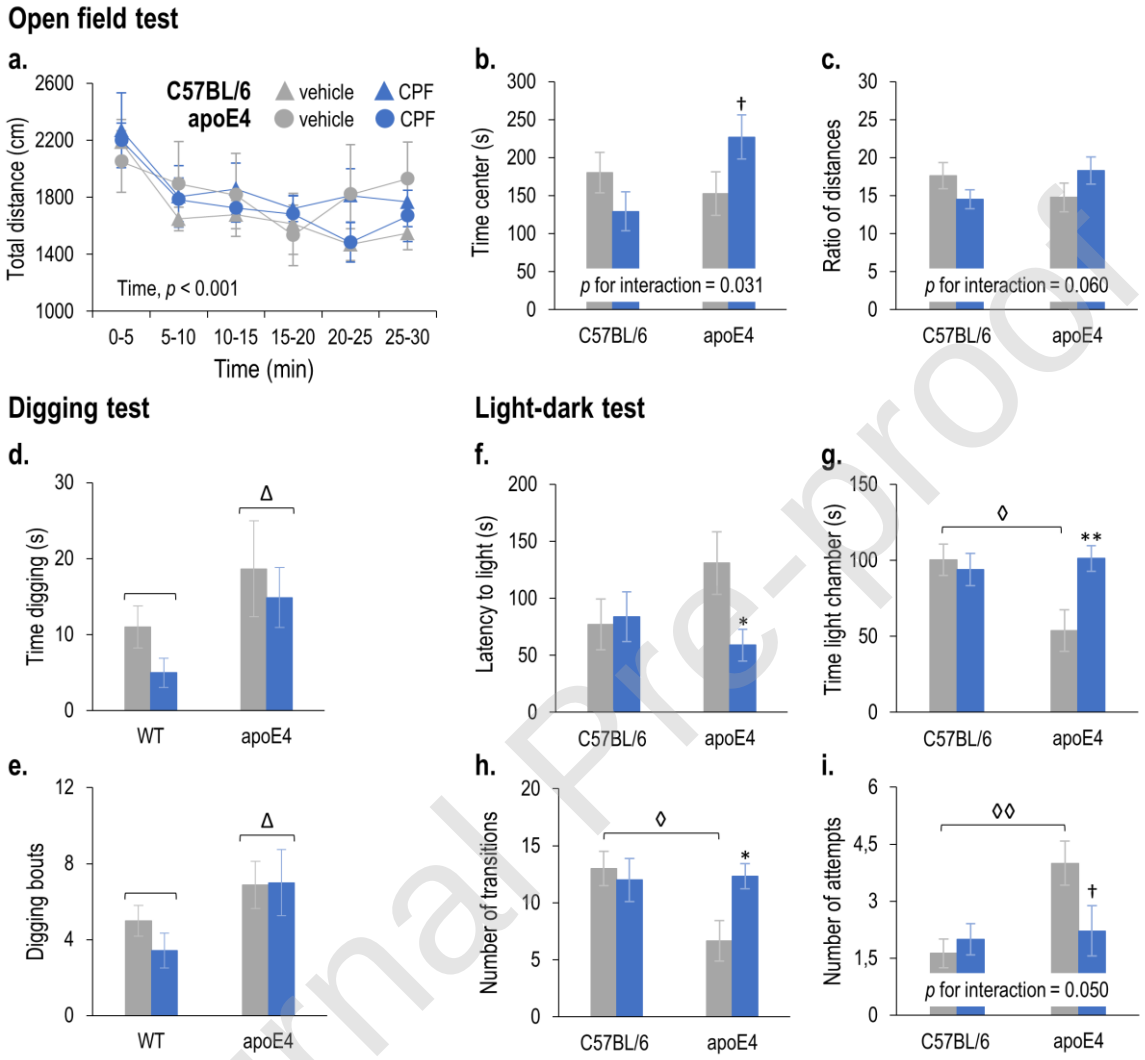

Figure 3. Effects of apolipoprotein E4 (*APOE4*) genotype, chlorpyrifos (CPF) treatment and underpinning interactions on sucrose preference (SP) in female mice. (**a**) Preference for novelty (in percentage of SP, day 1) and (**b**) preference for sucrose (in percentage of SP, day 2) in both C57BL/6 and apoE4 female mice assessed during the first SP test carried out before the dietary intervention with high-fat diet (HFD). (**c**) Preference for sucrose (in percentage of SP) in all the groups assessed in a SP test carried out after the dietary intervention with HFD. Symbols indicate differences between: apoE4 controls and C57BL/6 controls at $p < 0.01$ (◊◊); the three SP tests for vehicle apoE4 female mice at $p < 0.05$ (*) or $p < 0.01$ (**).

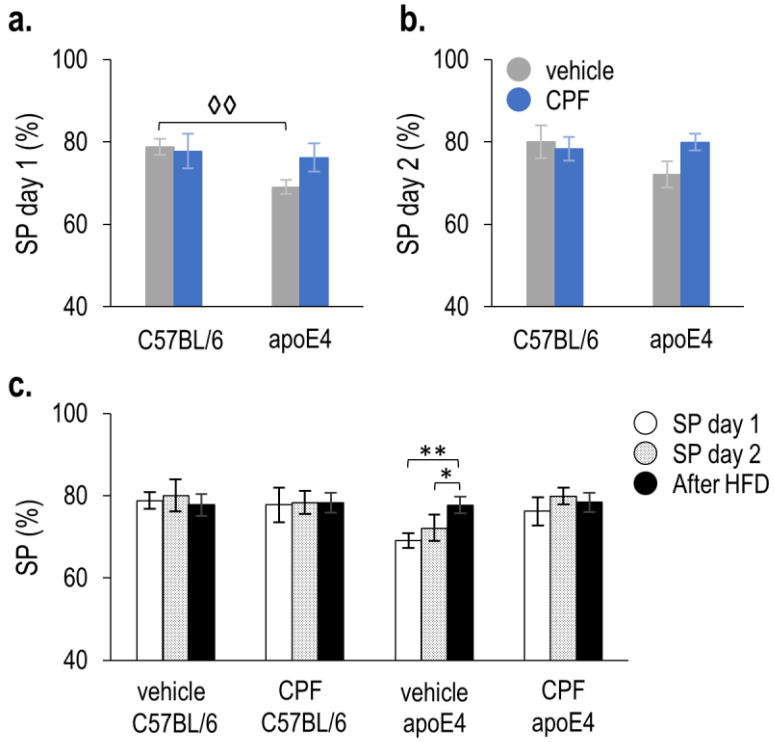

Figure 4. Acclimatisation to the scheduled-feeding paradigm over the first week of habituation. (**a**) Total 2-h high-fat diet (HFD) intake, (**b**) 30/120 ratio score and (**c**) 24-h chow intake in both C57BL/6 and apolipoprotein E4 (apoE4) female mice throughout the first week of habituation. Abbreviations: CPF, chlorpyrifos; D, day. The symbol indicates differences between the two genotypes at $p < 0.05$ (Δ).

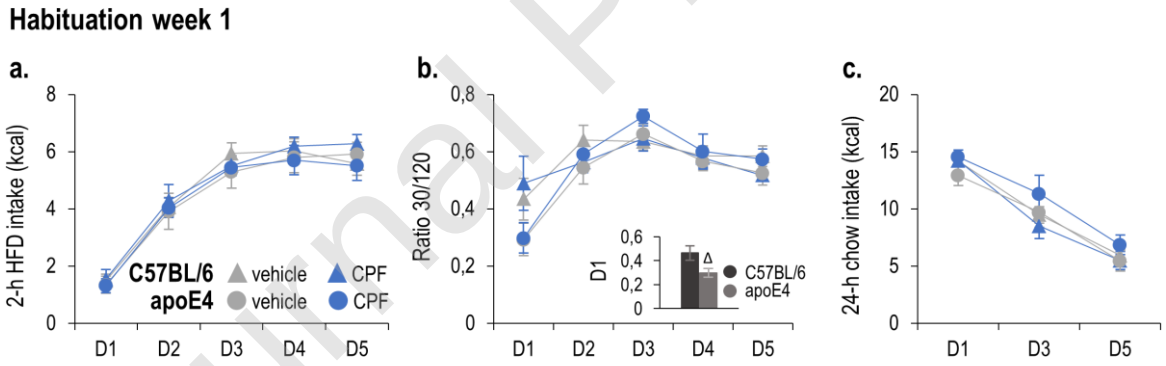

Figure 5. Effects of apolipoprotein E4 (*APOE4*) genotype, chlorpyrifos (CPF) treatment and underpinning interactions on high-fat diet (HFD) consumption and 30/120 ratio scores throughout the scheduled-feeding paradigm. Total 2-h HFD intake in both C57BL/6 and apoE4 female mice during both (**a**) the habituation (HAB) and (**b**) the refeeding (RF) phases. (**c**) Mean of the 2-h high-fat diet HFD intake during the entire RF phase for vehicle- and CPF-treated mice. The 30/120 ratio scores during both the (**d**) HAB and the (**e**) RF phases for all the groups. (**f**) Mean of the 30/120 ratio score for C57BL/6 and apoE4 mice. Symbols indicate differences between: the two treatment conditions at $p < 0.05$ (*); the two genotypes at $p < 0.05$ (Δ); apoE4 controls and C57BL/6 controls at $p < 0.05$ (◊).