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PII: S0166-4328(16)30793-8
DOI: http://dx.doi.org/doi:10.1016/j.bbr.2016.10.014
Reference: BBR 10502

To appear in: *Behavioural Brain Research*

Received date: 13-7-2016
Revised date: 5-10-2016
Accepted date: 7-10-2016

Please cite this article as: Basaure Pia, Peris-Sampedro Fiona, Cabré Maria, Reverte Ingrid, Colomina Maria Teresa.Two cholinesterase inhibitors trigger dissimilar effects on behavior and body weight in C57BL/6 mice: the case of chlorpyrifos and rivastigmine.*Behavioural Brain Research* http://dx.doi.org/10.1016/j.bbr.2016.10.014

Two cholinesterase inhibitors trigger dissimilar effects on behavior and body weight in C57BL/6 mice: the case of chlorpyrifos and rivastigmine

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Abstract

Cholinesterases (ChE) are common targets of organophosphate (OP) pesticides and play a critical role in the pathology of some dementias. While chlorpyrifos (CPF) remains one of the most commonly used OPs in the world, numerous investigations have reported its neurotoxic potential and highlighted behavioral disturbances upon its administration. Rivastigmine currently serves to treat Alzheimer's disease, but it may induce cholinergic overstimulation in non-demented individuals. The present investigation aimed to compare the acute and delayed effects caused by both ChE inhibitors in adult C57BL/6 male mice. The animals were daily fed either a standard, a CPF- (5 mg/kg body weight) or a rivastigmine-supplemented diet (1 or 2 mg/kg body weight) for 8 weeks. After the treatment, we established an 8-week washout period to assess recovery. ChE enzyme activity, biomarkers, physical effects, and behavioral alterations were evaluated at different time points during the exposure and after the washout period. Both rivastigmine doses induced a time-dependent weight increase. CPF and rivastigmine inhibited brain acetylcholinesterase following an isoform-specific pattern. As for behavioral assessment, CPF negatively modulated learning strategies and impaired memory in a Barnes maze task at the end of the exposure. On the other hand, the low dose of rivastigmine improved memory recall at the end of the washout period in a Morris water maze. Indeed, our results endorse the positive effects of low doses of rivastigmine following a drug-free period in young mice. Therefore, doses and periodicity of treatment to improve cognition in elderly people upon rivastigmine administration should be revised.

Keywords: Cholinesterase inhibitor, chlorpyrifos, rivastigmine, learning, memory, acetylcholinesterase isoform

Abbreviations: ACH, Acetylcholine; ACHE, Acetylcholinesterase; AD, Alzheimer's disease; BM, Barnes maze; CHE, Cholinesterase; CPF, Chlorpyrifos, FDA, Food and Drug Administration; MWM, Morris water maze; OF, Open field; OP, Organophosphate; RIV, rivastigmine

1. Introduction

The central cholinergic system plays a critical role in numerous cognitive processes, including spatial learning and memory [1,2]. Likewise, such types of dementia as Alzheimer's disease (AD) and Parkinson disease have been associated with a cholinergic deficit [3]. One mechanism to increase the amount of synaptic acetylcholine (ACh) is the inhibition of acetylcholinesterase (AChE) [4], which justifies using cholinesterase (ChE) inhibitors to alleviate the symptoms of dementia. Specifically, rivastigmine is a carbamate that inhibits both AChE and plasma ChE for several hours [5] by pseudo-irreversibly binding to these enzymes [6]. Nowadays, it is used to treat both AD and Parkinson disease in their mild and moderate phases [5]. Traditionally, the drug has been prescribed in various formulations, including capsules, oral solution, and transdermal patch, being the latter the most clinically effective [7]. Among the beneficial effects exerted by this drug, a large volume of animal-based studies have reported that rivastigmine enhances memory [8-10].

On the other hand, organophosphate (OPs) compounds are a large class of chemicals that are extensively used worldwide as pesticides [11]. In recent decades, there has been increasing interest in relating the development of neurological disorders and cognitive deficits to exposure to pesticides. Nowadays, chlorpyrifos (CPF) is one of the most frequently used OPs to control crop damage by insects in agriculture [12]. Since CPF irreversibly binds to and inhibits both AChE and plasma ChE, cholinergic hyperstimulation occurs [13]. A huge number of studies involving experimental animals have indicated that when acutely administered CPF produces long-term behavioral alterations and neurological deficits, such as altered inhibitory control [14], spatial memory impairments [15], and changes in locomotor activity [16].

Currently, there is a growing body of literature that recognizes the importance of low-level pesticide exposures and their impact on the health of the general population. With this in view, low doses of CPF have been related to neurotoxic effects [17-19], deficits in spatial learning and inability to use newly acquired information [20-23], as well as metabolic

alterations in animal models [24-26]. Likewise, a number of researchers have indicated that dietary intake is the most common source of exposure for the general population [27,28].

Structural cholinergic elements and their relevance to cognitive functions is a continuing research concern. In this line, different isoforms of brain AChE are found in various synaptic compartments. The cytosolic G1 isoform is isolated from the salt-soluble fraction and can be readily detected in such brain areas as the cortex, hippocampus and amygdala [29]. The G4 isoform, though, is bound to the membrane and isolated from the detergent-soluble fraction [29]. Further, it has been recurrently reported that the G4 isoform is more abundant than G1 in healthy subjects [30-31]. In fact, Meshorer et al. [32] have revealed a long-term replacement of the G4 isoform by the G1 under stress conditions. A broader perspective has been adopted by Farchi et al. [33], who suggested that both G4 and G1 isoforms modulate cognitive performance differently.

By drawing on the concept of harmful effects, Pope et al. [34] mentioned that the mechanism of toxicity of ChE inhibitors is essentially the same as the mechanism for therapeutic uses. In parallel, a much debated question is whether ChE inhibitors can be prescribed to patients with mild cognitive impairment, but without a declared dementia [35]. According to Pope and collaborators [34], cholinergic overstimulation under normal conditions leads to an imbalance in ACh neurotransmission, which may ultimately trigger the emergence of cholinergic signs of toxicity. Up to now, no controlled studies have compared potential differences between two or more ChE inhibitors sharing the same primary target, but with different usages.

The present study sought to investigate the differences between CPF and rivastigmine, by evaluating the acute and delayed effects on physical and other general parameters, locomotor activity and spatial learning and memory, following a subchronic low dietary exposure in C57BL/6 adult male mice.

2. Materials and methods

2.1. Animals and care

Three-month-old C57BL/6 male mice (Charles River, Barcelona, Spain) were used for this study. The animals were housed in plastic cages containing 3-5 individuals and kept under a 12-h light-dark cycle (lights off at 8 pm) in an environmentally controlled room, held at 22ºC ± 2ºC and a relative humidity of 50 ± 10%. The mice were allowed free access to food and tap water and given a normal chow diet (Panlab, Barcelona, Spain) before the experiment started. The use of animals and the experimental 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/UE).

2.2. Chemical compounds

Rivastigmine tartrate ((*S*)-3-[1-(dimethylamino)ethyl]phenyl *N*-ethyl-*N*-methylcarbamate, purity 98%) was supplied by TCI Europe N.V. (Zwijndrecht, Belgium). CPF (O,O-diethyl O-(3,5,6-trichloropyridin-2-yl) phosphorothioate, purity 99.5%) was provided by Sigma-Aldrich (Seelze, Germany). Three diets were obtained by supplementing standard rodent chow with either CPF or rivastigmine. Previous to the treatment, basal food intake was recorded to determine the total amount of each chemical compound to be added to the standard diet (data not shown). Thus, the diet supplemented with CPF (37.5 mg CPF/kg chow) was intended to deliver a dose of 5 mg/kg body weight/day, which was expected to be below the range of acute signs of toxicity [36]. Likewise, two types of diet were manufactured to deliver either 1 (RIV1) or 2 (RIV2) mg/kg body weight/day rivastigmine (7.5 mg RIV1 and 15 mg RIV2/kg chow). According to the US Food and Drug Administration (FDA), the subsequent rivastigmine-equivalent dose in humans can be calculated using a conversion factor, which considers the body surface area and the body weight of both the human and the mouse [37]. Thus, the range of doses of the drug we used in this study (i.e., 1 and 2 mg/kg/day) corresponded to 0.08 and 0.16 mg/kg/day in humans,

respectively, and match those traditionally administered in transdermal patches (i.e., 4.6 mg/24 h and 9.5 mg/24 h, respectively) [7].

2.3. Treatment and experimental design

The experimental procedure is depicted in Figure 1. A total of 69 animals were weighed and divided into four experimental groups as follows: control (n=15), CPF (n=18), RIV1 (n=18), and RIV2 (n=18). On the basis of this classification, mice were daily provided with 4 g/mouse of either a standard, a CPF- (5 mg/kg body weight/day), or a rivastigmine-supplemented diet (1 or 2 mg/kg body weight/day) for 8 consecutive weeks. In order to ensure that the mice received the estimated dose, we monitored body weight and food consumption weekly to further calculate the real ingested doses, which were: 4.93 ± 0.22 mg/kg CPF, 0.97 ± 0.04 mg/kg RIV1, and 2.08 ± 0.08 mg/kg RIV2. Over the course of the whole study, the emergence of potential cholinergic signs was also monitored.

-- Insert Figure 1 about here –

2.4. Behavioral assessment

The total number of animals used for each behavioral task depended on when these tasks were carried out (Fig. 1). Notwithstanding, a minimum of n=10/group was guaranteed. 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).

2.4.1. Open field

Exploratory activity and behavioral responses to a novel environment were tested in an open field (OF) one month after the treatment began. The OF consisted of a light-brown wooden box of 60 × 60 cm and a 50 cm-high wall. The field was divided into two virtual zones: the periphery, an area up to 10 cm from the wall, and the central area, covering the

remaining arena space. At the beginning of the test, the animals were placed in the center of the arena and were allowed to move freely around it for 30 min. We determined the total distance travelled in the arena, the mean velocity, the frequency of visits to the central area, the total distance travelled in the center, and the total distance in the center/total distance as distance ratio. Habituation to the new environment was also studied by analyzing changes in activity over six 5-min periods.

2.4.2. Barnes maze

The potential acute effects of both ChE inhibitors on spatial learning and memory were assessed during the last week of exposure using a Barnes maze (BM). The apparatus consisted of a white methacrylate circular arena elevated 1 m above the floor, with 20 holes (4.5 cm diameter) equally distributed around the perimeter. Each hole was located 2.5 cm from the edge of the maze and assigned a number from 1 to 20. A dark escape box was placed beneath one of the holes (i.e., target hole). Bright and intense light was used to encourage the animals to escape into the dark box. During the training period, mice were subjected to a daily session of two trials with an inter-trial interval of 60 min for 5 consecutive days. Each trial started after the animal had been placed in the center of the maze. Then, the mice were allowed to move freely and the trial finished when they entered the escape box or after 180 s. If an animal did not find the target hole within this time, it was gently guided and placed into the escape box by the experimenter. In both cases, the mouse remained in the escape box for 30 s after the end of each trial before being returned to its holding cage. To avoid proximal cues and ensure hippocampus-dependent learning, the maze was rotated between trials, but the position of the escape box was maintained in a fixed position with respect to the external cues. We measured the total distance travelled in the arena, the escape latency to the target hole, and the average search velocity. The search strategies used by the mice to reach the escape box were also examined as previously evaluated by Peris-Sampedro and coworkers, [21] (Fig. 7A). The strategies were scored as “random” when the mouse displayed an arbitrary search with multiple crossings through the center of the maze; as “serial” when the animal turned around the edge of the maze, crossing at least three adjacent holes before entering the target hole; and as “spatial” if it

moved directly towards the target hole from the center of the maze. The apparatus and escape box were carefully cleaned between each trial to prevent the mice from using olfactory cues. The retention of the task was performed 24 h after the last training session, and consisted of a 120-s free exploration session without the escape box. During the probe trial, both the latency of escape to the target zone (i.e., target hole and its two adjacent holes) and the total time spent in this area were analyzed.

2.4.3. Morris water maze

The potential delayed effects of both ChE inhibitors on spatial learning and memory were assessed at the end of the 2-month washout period by means of a Morris water maze (MWM). The apparatus consisted of a circular tank (diameter: 1 m; height: 60 cm), virtually divided into four quadrants. An escape platform (10 cm diameter) was located 1 cm below the water surface in the target quadrant. Water was maintained at 23ºC (±1ºC) and rendered opaque by adding white tempera paint to ensure that the platform was camouflaged. In the course of the acquisition period, the animals performed two trials per day with a 60-min inter-trial interval for 10 consecutive days. During each trial, the mice were given 90 s to find the hidden platform, and were finally required to remain on it for 30 s. If an animal failed to find the platform within this time, it was guided and placed on it for 30 s by the experimenter. There were four different starting positions that were changed within each trial. To avoid proximal cues and to prevent non-spatial learning strategies, an internal mobile wall was added to the maze and rotated between trials. The latency to find the escape platform, the distance travelled and the swim velocity were measured. The retention of the task was evaluated by two probe trials, which consisted of a 60-s free swim without the escape platform. These probe trials were performed before the training session on acquisition day 10 and 72 h after the last training session. The total time spent in the target quadrant was recorded as a measure of unbiased recall for spatial memory.

2.5. Sacrifice and sampling

In order to follow-up the effects of both ChE inhibitors, biological samples of CPF- and rivastigmine-treated mice were obtained at three different time points: the end of the first week of exposure, during the last week of exposure and after a washout of 2 months. On the contrary, control samples were only collected at the end of exposure and after the washout (Fig. 1). Briefly, animals were deeply anesthetized with carbon dioxide before being euthanized. Blood was obtained by cardiac puncture and immediately centrifuged to obtain plasma, which was stored at -80ºC until analysis. After the blood draw, mice were rapidly decapitated and the whole brains were removed, dissected and stored at -80ºC until use.

2.5.1. Enzyme activity

Plasma ChE activity was determined as a measure of acute systemic effect [12] at the three aforementioned time points (see section *2.5*). The activity of G1 and G4 isoforms of the AChE in brain was also measured within these time points, according to Ferlemi et al. [38]. Briefly, brain samples were weighed and homogenized in cold in PBS (10% w/v) at pH 7.6 and then centrifuged at 15000 g for 20 min at 4°C. The supernatant (i.e., salt-soluble fraction) was removed for analysis and the pellet was resuspended with an equal volume of 1% Triton X-100 and centrifuged again at 15000 g for 20 min at 4°C. Finally, the remaining supernatant (i.e., detergent-soluble fraction) was kept for AChE assay. Several investigations have confirmed that the cytosolic G1 isoform of brain AChE is more abundant in the salt-soluble fraction of the homogenate, while the detergent-soluble fraction contains mainly the G4 isoform of the enzyme [38]. In both cases, ChE activity was determined spectrophotometrically using an update of the Ellman method [16,39]. Enzyme activity was expressed relative to protein concentration, which was assessed using the Bradford method [40]. Both ChE plasma and AChE brain activities of exposed animals were estimated on the basis of the activity value of the control mice and represented as a percentage.

2.5.2. General biochemistry

Plasma concentrations of albumin, total cholesterol and triglycerides were determined during the last week of exposure and after a washout of 2 months. All samples were analyzed with commercially available kits provided by QCA (Química Analítica Clínica S.A., QCA, Amposta, Spain). Every absorbance measurement was performed in triplicate according to the manufacturer's instructions at a constant temperature of 37ºC with a semiautomatic COBAS MIRA analyzer (Hoffman-La Roche & Co., Basel, Switzerland).

2.6. Statistical analysis

Data were analyzed with the SPSS Statistics software (version 20.0). Body weight and behavioral assessment were analyzed by a two-way analysis of variance (ANOVA) mixed design with one within-subject factor (time) and one between-subject factor (treatment). Further one-way ANOVAs (treatment) were used to analyze differences between groups. A post hoc Tukey test was used for multiple comparisons between groups. A paired *t*-test was used to analyze differences in the MWM retention task, to evaluate the search strategies in the BM task, and to determine potential changes in enzyme activity within the same treatment group. Statistical significance was set at $p<0.05$, and results are reported as mean values ± SE.

3. Results

3.1. Both RIV1 and RIV2 induce time-dependent weight increase.

The treatment led to gradual weight gain throughout the exposure period, as revealed by a treatment x time interaction [$F(24,52)=2.709$, $p<0.001$] (Fig. 2A). In addition, a tendency towards a major effect of the treatment was noted within this period [$F(3,52)=2.414$, $p=0.078$]. Post hoc analysis indicated that mice exposed to RIV1 generally weighed more than control mice ($p=0.065$), and that these differences were significant from the sixth week until the end of the exposure period (Fig. 2A). During washout, the treatment was also found to affect body weight [$F(3,38)=2.602$, $p=0.029$] (Fig. 2B). While the weight progression of RIV1-exposed mice levelled off across the recovery period, those animals

previously exposed to RIV2 showed greater body weights than their control peers (p=0.038). In order to get further insight into the weight status of the mice, we looked at their net weight gain over both periods. During exposure, the treatment was observed to have an overall effect [F(3,52)=4.170, p=0.010]: RIV1-exposed mice gained more weight than non-exposed mice (p=0.006) (Fig. 2C). Similarly, the treatment also affected body weight increase during washout, albeit at a slower rate [F(3,38)=4.197, p=0.012] (Fig. 2D). In line with these results, mice that were subjected to RIV1 exhibited the slowest rate of weight increase within this period (RIV1 *vs* control, p=0.027) (Fig. 2D).

-- Insert Figure 2 about here –

3.2. CPF triggers an acute but reversible systemic plasma ChE inhibition.

ChE activity was analyzed in plasma at the end of the first week of exposure, during the last week of exposure, and after a washout of 2 months (Fig. 3). Over the course of both the first and the last weeks of CPF exposure, plasma ChE activity dropped to 9.5% (t=-60.525, d.f.3, p<0.001) and 18.7% (t=-87.411, d.f.3, p<0.001) of the total activity of the control group, respectively. At the end of the CPF-free washout period, the plasma ChE activity was totally recovered. Neither dose of rivastigmine significantly inhibited plasma ChE.

-- Insert Figure 3 about here –

3.3. The inhibitory effect of both CPF and rivastigmine on brain AChE displays an isoform-specific pattern.

The brain G1 and G4 AChE isoforms were studied at the end of the first week of exposure, during the last week of exposure, and after a washout of 2 months (Fig. 4). Overall, the three treatment conditions decreased the G1 isoform activity relative to that inherent to the control group. Specifically, we observed a moderate G1 inhibition at the end of the first week of exposure to CPF (49.3%) (t=-7.205, d.f.3, p=0.006), RIV1 (41.3%) (t=-6.310, d.f.3, p=0.008), and RIV2 (64.8%) (t=-16.167, d.f.3, p=0.001). While CPF-fed mice

gradually recovered the G1 activity after the last week of exposure, both RIV1- (t=-6.056, d.f.3, p=0.009) and RIV2-treated mice (t=-7.217, d.f.3, p=0.005) continued to show a marked G1 inhibition within this period (i.e., 33.5 and 37.6%, respectively). In both cases, the G1 activity was thoroughly recovered at the end of washout, exceeding even the values inherent to the control group. On the other hand, CPF was the only agent that inhibited G4 throughout the experimental procedure (i.e., end of the first week of exposure, 77.4%: t=-15.578, d.f.3, p=0.001; last week of exposure, 30.3%: t=-4.253, d.f.3, p=0.024; and washout, 28.9%: t=-4.419, d.f.5, p=0.007). As expected, neither of the rivastigmine doses had any effect on G4 isoform activity.

-- Insert Figure 4 about here –

3.4. CPF reduces plasma albumin levels.

Plasma concentrations of biomarkers were analyzed during the last week of exposure, and after a washout of 2 months (Table 1). At the end of the exposure, the treatment affected albumin levels [F(3,15)=4.858, p=0.019]. A post hoc analysis confirmed that CPF reduced the plasma albumin concentrations relative to the control group (p=0.017). Nonetheless, albumin levels recovered during the washout period. Neither of the rivastigmine doses affected this parameter. On the other hand, no differences were observed for total cholesterol or triglyceride levels.

-- Insert Table 1 about here –

3.5. CPF and rivastigmine do not affect exploratory behavior or general motor activity after one month of treatment.

Exploratory behavior and general motor activity were evaluated one month after the treatment began by means of an OF. The results of the total distance travelled in the arena showed an effect for time factor, thereby indicating that all individuals habituated to the

novel space [F(5,52)=25.621, p<0.001] (Fig. 5). However, no significant differences in any of the parameters evaluated were noted between treatment groups.

-- Insert Figure 5 about here –

3.6. CPF negatively modulates learning strategies and produces acute memory impairments.

Spatial learning and memory were assessed in a BM during the last week of exposure. Throughout the acquisition period the performance of the mice improved overall: the total distance travelled in the arena [F(4,52)=37.753, p<0.001] (Fig. 6) and the escape latency to the target hole [F(4,52)=42.933, p<0.001] (data not shown) decreased over sessions. However, the treatment did not alter the total distance travelled, the escape latency to the target hole, or the search velocity.

-- Insert Figure 6 about here –

To ascertain whether there were differences in the use of search strategies, we compared the frequency of each strategy between the first and the last day of acquisition for each treatment group (Fig. 7). Control mice used the random strategy less frequently (t=2.739, d.f.12, p=0.018) (Fig. 7B) and the spatial strategy more frequently (t=-2.941, d.f.12, p=0.012) (Fig. 7D) in the last training session than in the first one. Likewise, the RIV1 group also performed the task better because by the end of the acquisition stage they were using the random strategy less (t=3.122, d.f.13, p=0.008) (Fig. 7B).

We also explored the differences between treatment conditions on the first and the last acquisition days (Fig. 7). The results revealed that choosing a random strategy during the last acquisition session was influenced by the treatment [F(3,52)=4.808, p=0.005]: CPF-exposed mice were the most regular users (CPF *vs* control: p=0.018, CPF *vs* RIV1: p=0.015, and CPF *vs* RIV2: p=0.021) (Fig.7B). No significant differences were observed when the first day of acquisition or the other search strategies were analyzed.

-- Insert Figure 7 about here –

The retention of the task was evaluated by a single probe trial performed 24 h after the last acquisition session (Fig. 8). The treatment was found to have a main effect on the latency to the target zone [$F(3,52)=5.137$, $p=0.004$]: CPF-exposed mice needed more time to reach this area than the other groups (CPF *vs* control: $p=0.023$, CPF *vs* RIV1: $p=0.015$, and CPF *vs* RIV2: $p=0.008$). The analysis of the total time spent in the target zone revealed no significant differences between treatment groups.

-- Insert Figure 8 about here –

3.7. RIV1 improves memory recall at the end of the washout period.

Spatial learning and memory were also assessed in a MWM after a washout of 2 months. An overall improvement in performance was observed throughout the acquisition period, manifested by a decrease in the escape latency [$F(9, 38)=12.893$, $p<0.001$] (data not shown) and the distance travelled to reach the platform [$F(9, 38)=34.540$, $p<0.001$] (Fig. 9A). We also noted changes in search velocity during acquisition [$F(9, 38)=7.920$, $p<0.001$] (Fig. 9B). The treatment affected the distance travelled [$F(3, 38)=2.966$, $p=0.045$], and tended to alter the search velocity [$F(3, 38)=2.513$, $p=0.074$]. We also found a treatment x time interaction for the distance travelled [$F(27, 38)=2.207$, $p=0.003$]. In general terms, mice that had been exposed to RIV2 swam a greater distance to get to the platform than those that had been fed CPF ($p=0.035$). Nevertheless, RIV2-treated mice tended to be faster than CPF-exposed mice ($p=0.080$), which suggests that differences in the distance travelled were a reflection of increased search velocity within the RIV2 group.

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Retention of the task was evaluated by two probe trials performed before the training session on acquisition day 10 and 72 h after the last training session (Fig. 10). To determine whether there were differences in task retention, we compared the time spent in the target

quadrant with the chance of being in any of the other three virtual quadrants of the maze. Despite the fact that all the groups swam in the target quadrant for more than 15 s during the first probe trial, none of them showed a marked preference for this area. However, 72 h later, only RIV1-exposed mice were able to remember the location of the platform (t=2.537, d.f.9, p=0.032).

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4. Discussion

The current study is the first to compare the neurobehavioral effects of subchronic dietary exposure to rivastigmine and CPF in healthy adult male mice. Although both of the ChE inhibitors increased body weight throughout the experimental period, only rivastigmine did so significantly. Thus, RIV1 and RIV2 triggered either a prompt or a delayed weight gain, respectively. Differences in the inhibition of both brain AChE isoforms were also observed among treatment groups. Specifically, whilst rivastigmine was a strong inhibitor of the G1 isoform, G4 was only inhibited by CPF. As expected, CPF induced a marked inhibition of plasma ChE with no signs of cholinergic toxicity, and a temporary decrease in plasma albumin levels. However, exposure to either RIV1 or RIV2 did not affect any of these parameters. The results concerning behavioral assessment suggested that both cholinergic agents induced opposite effects on learning and memory processes. In point of fact, CPF-fed mice were the most frequent users of the random strategy at the end of the acquisition period in the BM task, which could ultimately be the cause of their poor retention scores. Strikingly, however, the mice previously exposed to RIV1 enhanced memory recall in the MWM, as they were the only group that remembered the virtual location of the platform after 72 h.

Body weight was recorded weekly throughout the experimental procedure as a measure of the general condition of the mice. Although all the animals gained weight during the treatment period, the highest weights were inherent to RIV1-fed mice. A review of the literature shows that rivastigmine rarely causes weight gain. In fact, the expected effect of

its oral administration is weight loss as a consequence of various gastrointestinal side effects [41]. Nonetheless, a single case of weight gain, which was accompanied by an improvement in food intake, has been reported in a user of a rivastigmine-transdermal patch at a dose of 9 mg per day [42]. The authors suggested that administering rivastigmine dermally may lead to a better tolerability profile of the drug [42]. Indeed, several prescriber guides and literature reviews recommend that rivastigmine be dispensed during mealtimes, in liquid formulation or via transdermal patches [7,41,43-45]. Therefore, a possible explanation for the weight gain we observed is that rivastigmine may be absorbed slowly and continuously when dietary administered, thus avoiding the maximum concentration peak that would eventually cause acute side effects. Furthermore, the degree of peripheral inhibition of both AChE and plasma ChE is associated with the adverse gastrointestinal events characteristic of rivastigmine dosage [30,46,47]. Accordingly, the current results confirmed that RIV1 induced the lowest inhibition of plasma ChE. Along the same lines, RIV2 induced a notable plasma enzyme inhibition throughout the exposure period, despite not reaching the levels inherent to CPF. Intriguingly, the mice that had been exposed to RIV2 displayed greater body weights throughout the washout period. So, it is not so unreasonable to expect the body weight of RIV2-treated mice to begin to recover after the drug has been withdrawn. Despite the fact that CPF-exposed mice gained weight, the effect was not statistically significant. In a recent study, we described a weight gain in adult C57BL/6 male mice as from the eighth week of a dietary exposure to 2 mg/kg CPF [26]. One explanation for these discrepancies may lie in substantial differences between protocols. Indeed, our exposure schedule lasted for 8 weeks and the mice were younger than those used in our previous study. Moreover, the dose we used in the present work was higher and induced a marked plasma ChE inhibition, which might have caused gastrointestinal complaints. Another investigation performed in rats also found an increase in body weight after a chronic subcutaneous exposure to 5 mg/kg CPF [48]. Even though the dose they used was the same as ours, the animal model, the exposure duration, and the route of administration were different.

As expected, both cholinergic agents triggered a high inhibition of brain AChE with specific actions on each enzyme isoform. We found that rivastigmine exerted a potent

inhibitory effect on G1 isoform activity. Several authors have suggested that this drug preferably inhibits the G1 form of the enzyme rather than the G4 [41,47,49]. The current results for CPF match those reported by López-Granero et al. [20,50], which revealed a more robust inhibition of G4 after both a chronic dietary exposure to CPF and an acute subcutaneous injection of several OPs (i.e., CPF, diisopropylphosphorofluoridate, and parathion) [50]. We also noted that CPF induced a lasting inhibition of the G4, since the enzyme activity did not entirely recover even after a CPF-free washout of two months. In agreement with this, Middlemore-Risher et al. [51] noticed a full recovery of AChE activity in various brain regions except the striatum and basal forebrain after a 30-day CPF-free period. Taking into account that the concentration of the G4 isoform is greater in the striatum than in other brain areas [29,52], it is therefore likely that the residual inhibition we observed mainly corresponds to striatal G4 activity. It has also been suggested that the G4 isoform takes longer to recover its full activity because of the longer time required for membrane insertion of this form [50]. Overall, the current results are in line with those reported by Zhao and Tang [49]. Specifically, the authors compared the effects of five AChE inhibitors (i.e., huperzine A, tacrine, donepezil, physostigmine, and rivastigmine) on different molecular forms of AChE in several brain areas. They concluded that each drug inhibited AChE following an isoform- and region-specific pattern [49].

As far as behavioral endpoints are concerned, there has been little discussion about the association between brain AChE isoforms and cognitive processes. A number of researchers have suggested that G1 activity increases in response to chemical and physical stress, thereby protecting neurons from both internal and external damage [3,32]. Likewise, Das and collaborators [29] proposed G4 activity as a key regulator of both learning and memory. So, we speculate that the activity of both isoforms may be linked to the different behavioral responses noted in our experimental groups. Nevertheless, to date, a considerable amount of research has confirmed that CPF has numerous targets of action other than the cholinergic system [14,53,54]. Conversely, far too little attention has been paid to other potential targets for rivastigmine [55,56]. Therefore, further studies are needed to better correlate rivastigmine-dependent behavioral effects with other plausible neurotransmitter systems.

A considerable number of studies have stressed the importance of plasma albumin as a biomarker of CPF exposure [57-59]. In line with previous investigations, we found that repeated exposure to CPF decreased plasma albumin levels [25,60]. Nowadays, it is well-known that reduced albumin is synonymous with liver function failure. It has also been suggested that such hypoactivity may be related to changes in protein or free amino acid metabolism in the liver [25,60]. On the other hand, CPF is known to generally disturb lipid homeostasis [24-26]. However, we did not find altered total cholesterol or triglyceride levels after exposure to CPF, a fact that merits further investigation. It should be pointed out that neither rivastigmine dose altered albumin, total cholesterol or triglyceride levels. Likewise, these results deserve further investigation to determine whether the body weight gain we found in RIV1-treated mice could be linked to alterations in the regulation or the signaling pathways of energy-related hormones in specific brain regions.

Interestingly, neither exploratory behavior nor general motor activity were affected one month after the treatment period had begun. Although adulthood exposures to CPF have been reported to either increase or decrease activity [15,16,21], many investigations have found no differences at this level [23,61,62]. By the same token, Stahl [41] attested that, once in the body, rivastigmine requires at least 6 weeks before any behavioral change can be noticeable.

The adverse effects of CPF on spatial learning and memory have been widely addressed in both epidemiological [63-65] and empirical studies [15,20,21,66,67]. It should be noted that, once presumed that OP compounds act beyond their ability to inhibit ChEs, it can no longer be assumed that they will all act alike, since the different agents may diverge in their actions mediated by other mechanisms. For example, metrifonate and its metabolite dichlorvos exert a cognitive improvement by inhibiting acylpeptide hydrolase [68]. In the current study, we observed that CPF had a significant detrimental effect on spatial learning and memory when assessed at the end of the exposure period. CPF-treated animals displayed poor retention in the BM task, probably because they chose the wrong learning strategy during the acquisition period. Currently, increasingly more evidence points to a

disruptive role of CPF in attentional processes [14,39,50]. Bearing in mind that our protocol update conditioned attentional demand, as it was essential for the animals to rule out strategic searches and focus on a mapping strategy, the current results might be related to attentional deficits. Nevertheless, the present findings are in accord with our recent data [21], which showed that subchronic dietary exposure to 2 mg/kg CPF led to mild memory impairment in humanized apolipoprotein E3 adult male mice [21]. Further, Yan and collaborators [23] also found impaired spatial memory after a 4-week oral exposure to low doses of CPF in male rats. In contrast, rivastigmine-fed mice, together with the control group, were the most frequent users of both spatial and serial strategies, thereby implying they made a reflective choice. Nonetheless, although both groups improved their search strategy during the acquisition period, administering RIV1 did not entail any significant behavioral enhancement relative to their control peers. Although existing empirical research supports the ameliorative effect of this drug on several learning processes, the evidence is limited to animal models of memory impairment [10,56]. Therefore, it seems that the beneficial effect of administering the drug may depend on the intrinsic cholinergic status of an individual.

Additionally, the behavioral changes induced by rivastigmine may persist or develop long after the treatment, as was described in an epidemiological report by Gurevich et al. [69] in which elderly individuals, subjected to repeated escalating doses of rivastigmine, improved memory scores after a 4-week washout period. Corroborating this idea, the analysis of the retention period in the MWM demonstrated that RIV1-treated mice were the only ones who remembered the location of the platform. With these exceptions, there is very little empirical data on the delayed beneficial effect of rivastigmine. Indeed, to the best of our knowledge, this is the first study to have examined potential behavioral effects after a rivastigmine-withdrawal period in healthy adult mice. It ought to be outlined that RIV1-treated mice showed no learning or memory improvements during the exposure, but did at the end of the washout period. So, more investigations are needed to explore whether administering low doses of rivastigmine in an intermittent protocol may be more effective than continuous treatment.

One point worth stressing is that control animals did not reach the retention criterion during the last probe on the MWM task. The protocol update we performed for the MWM consisted of rotating the internal wall of the pool in order to avoid the use of intra-maze cues [15,70,71]. Therefore, the animals were required to discriminate between relevant and irrelevant stimuli, which involved a higher attentional demand to perform the spatial task.

In view of the results we obtained, some limitations need to be addressed. The first one lies in that both CPF and rivastigmine inhibit plasma ChE differently. Thus, the fact of achieving the same plasma enzyme inhibition would allow us to know the real contribution of both G1 and G4 to the results we found. Therefore, although the aim of the current research was to investigate similarities and differences between both agents at relevant doses for human exposure, we are aware of the need to perform further studies including additional doses and/or other OPs.

The second limitation refers to the subtle behavioral effects we reported upon exposure to both agents in both the short- and the long-term. In the short-term, the sharpest ChE inhibition exerted by CPF may explain the poorer retention and the maintenance of a random strategy over the learning process in the BM task. These effects may be attributable to perceptual processing or attentional deficits due to a cholinergic overstimulation (Kolisnyk et al., 2013). On the other hand, as previously discussed, it seems difficult to observe any improvements when using a cholinergic drug in the absence of cholinergic deficits. Therefore, the use of a mouse model with a deficient cholinergic system instead of a wild-type animal model would have meant finding more robust rivastigmine-related beneficial effects. In the long-term, RIV1 has been proved to significantly improve recall in the MWM task. One potential explanation for the absence of a dose-response effect upon rivastigmine exposure may be the action of this drug on other non-cholinergic targets. However, this result needs to be more closely examined in the future.

5. Conclusion

In summary, the present study reports differences in the pattern of effects of both ChE inhibitors. Overall, the results reinforce existing evidence on the detrimental effects of

dietary CPF on spatial learning and memory processes. At the same time, our findings endorse the beneficial delayed effects of low doses of rivastigmine in healthy adult male mice. Besides confirming that the drug enhances spatial memory, the current results may be able to contribute to the development of therapies for elderly people suffering from nutritional deficits. However, further studies need to make a closer examination of the metabolic or cognitive effects of rivastigmine when administered at low doses or intermittently in healthy subjects. Despite the relevance of these results, both ChE agents did not produce the same degree of enzyme inhibition. Consequently, the current study should be considered as a preliminary approach. Nevertheless, it is essential to underline that the two rivastigmine doses had different effects, thus underscoring the seriousness of customized patient therapy, especially for those with mild cognitive impairment or further diseases in which the role of the cholinergic system remains to be elucidated.

Disclosure statement

The authors declare that no conflict of interest has influenced the results presented in this article.

Acknowledgements

The authors would like to thank Amparo Aguilar, Esperanza Chernichero and Juan València (Faculty of Medicine and Health Sciences, Universitat Rovira i Virgili), for their technical support with animal care. Likewise, the authors want to thank Jordi Blanco, PhD; and Laia Guardia for their technical support. This research was supported by the Ministry of Economy and Competitiveness (MINECO, Spain) (grant number PSI2010-21743-C02-01), the Commission for Universities and Research of the Department of Innovation, Universities and Enterprise of the *Generalitat de Catalunya* (grant number 2014 FI_B 00075), and the European Social Fund.

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Figure captions

Figure 1. Experimental protocol and total number of animals per group used for behavioral (OF, open field; BM, Barnes maze; and MWM, Morris water maze), general biomarkers, and toxicity testing in every time point considered.

Figure 2. The body weight progression was recorded weekly throughout both the 8-week treatment period (A), and washout (B) in C57BL/6 male mice. Body weight increase during the treatment (C) and the washout periods (D) was also depicted for each treatment condition (CPF, chlorpyrifos 5 mg/kg; RIV1, rivastigmine 1 mg/kg; RIV2, rivastigmine 2 mg/kg). Asterisks (*) indicate differences between treated mice and their respective control group at $p<0.05$.

Figure 3. Plasma cholinesterase (ChE) activity throughout the whole experimental protocol for each treatment condition (CPF, chlorpyrifos 5 mg/kg; RIV1, rivastigmine 1 mg/kg; RIV2, rivastigmine 2 mg/kg). The dashed line represents the enzyme activity of the control group. Asterisks (*,**) indicate differences in ChE activity of treated C57BL/6 mice relative to their corresponding control group at $p<0.05$ and $p<0.005$, respectively.

Figure 4. Both G1 and G4 isoforms activities of brain acetylcholinesterase (AChE) during the whole experimental protocol for each treatment condition (CPF, chlorpyrifos 5 mg/kg; RIV1, rivastigmine 1 mg/kg; RIV2, rivastigmine 2 mg/kg). The dashed line represents the enzyme activity of the control group. Asterisks (*,**) indicate differences between treated C57BL/6 mice and their corresponding control counterparts at $p<0.05$ and $p<0.005$, respectively.

Figure 5. Exploratory activity and behavioral responses to a novel environment assessed in an open field one month after the treatment began for each group of treated mice (CPF, chlorpyrifos 5 mg/kg; RIV1, rivastigmine 1 mg/kg; RIV2, rivastigmine 2 mg/kg). Distance traveled in the arena over 30 min of time divided into six 5-min periods.

Figure 6. Acquisition of a spatial learning task assessed at the end of the treatment period in a Barnes maze for each group of treated mice (CPF, chlorpyrifos 5 mg/kg; RIV1, rivastigmine 1 mg/kg; RIV2, rivastigmine 2 mg/kg). Total distance traveled in the arena over the five acquisition days.

Figure 7. Acquisition of a spatial learning task assessed at the end of the treatment period in a Barnes maze for each group of treated mice (CPF, chlorpyrifos 5 mg/kg; RIV1, rivastigmine 1 mg/kg; RIV2, rivastigmine 2 mg/kg). Representative images of search strategies used in the Barnes maze (A): random (no distinguishable pattern), serial (consecutive visits to the holes) and spatial (direct path to the target hole). Comparison of the frequency of use of random (B), serial (C), and spatial (D) search strategies during the first and the fifth day of acquisition. Symbols indicate: changes between the first and fifth day of acquisition within the same treatment group (*) and differences between CPF-fed mice and the other groups of treatment during the fifth day of acquisition (#) at $p<0.05$.

Figure 8. Retention measured in a Barnes maze assessed at the end of the treatment period for each group of treated mice (CPF, chlorpyrifos 5 mg/kg; RIV1, rivastigmine 1 mg/kg; RIV2, rivastigmine 2 mg/kg). Latency to the target zone in the probe trial carried out 24 h after the last training session. The asterisk (*) indicates differences between CPF-exposed mice and the other groups of treatment at $p<0.05$.

Figure 9. Acquisition of a spatial learning task assessed two months after the end of the treatment period in a Morris water maze for each group of treated mice (CPF, chlorpyrifos 5 mg/kg; RIV1, rivastigmine 1 mg/kg; RIV2, rivastigmine 2 mg/kg). Total distance traveled to reach the platform over the ten acquisition days (A) and mean search velocity (B).

Figure 10. Retention measured in a Morris water maze assessed two months after the end of the treatment period for each group of treated mice (CPF, chlorpyrifos 5 mg/kg; RIV1, rivastigmine 1 mg/kg; RIV2, rivastigmine 2 mg/kg). Time spent in the target quadrant compared to the expected time spent in each quadrant by chance without any previous

learning (i.e., 15 s) for the probe trials performed before the training session on acquisition day 10 (10AD), and 72 h after the last training session. The asterisk (*) indicates a performance significantly different from the chance level at $p<0.05$.

Figure 1

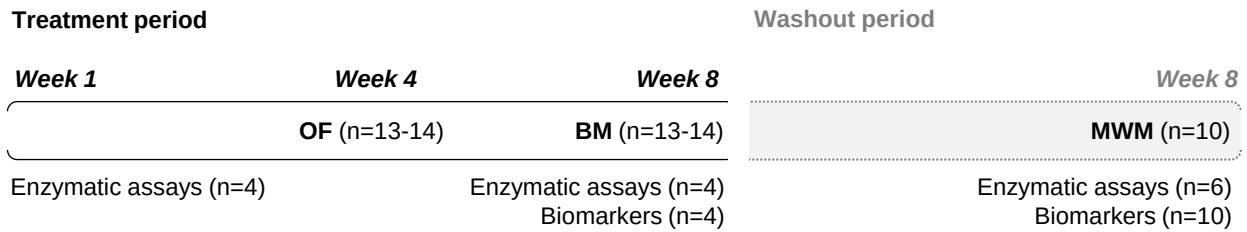
Treatment period
Washout period
Week 1
Week 4
Week 8
Week 8
OF (n=13-14)
BM (n=13-14)
MWM (n=10)
Enzymatic assays (n=4)
Enzymatic assays (n=4)
Biomarkers (n=4)
Enzymatic assays (n=6)
Biomarkers (n=10)

Figure 2

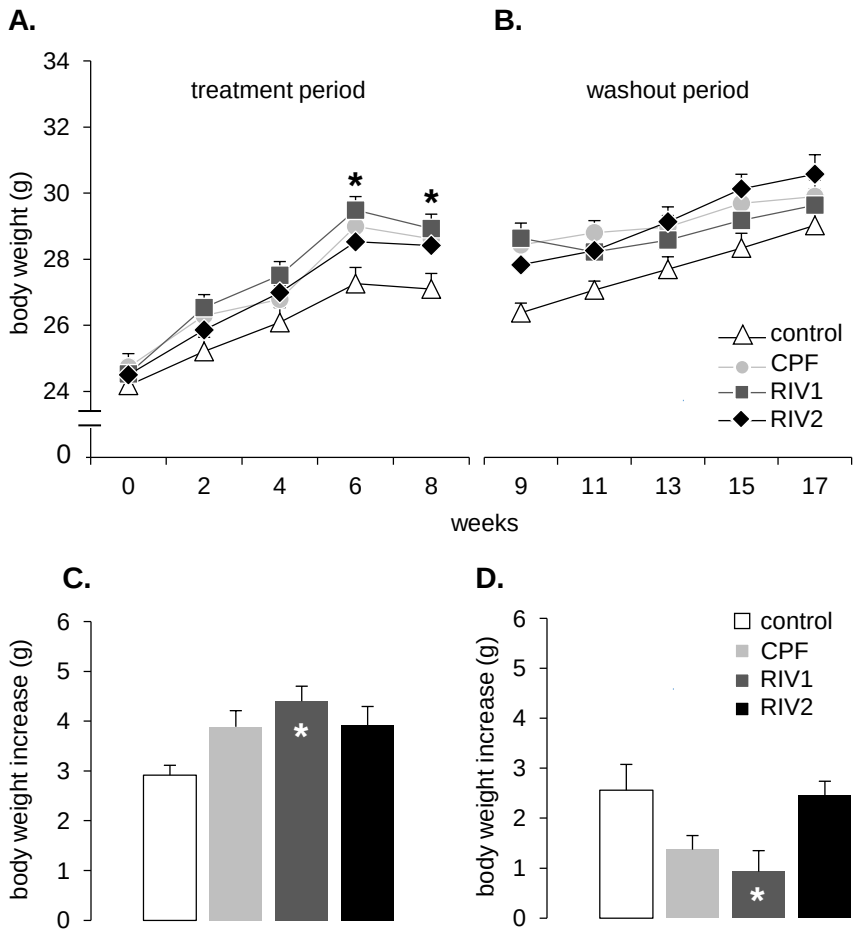
A.
B.
treatment period
washout period
body weight (g)
34
32
30
28
26
24
0
0
2
4
6
8
9
11
13
15
17
weeks
control
CPF
RIV1
RIV2
C.
D.
body weight increase (g)
6
5
4
3
2
1
0
control
CPF
RIV1
RIV2

Figure 3

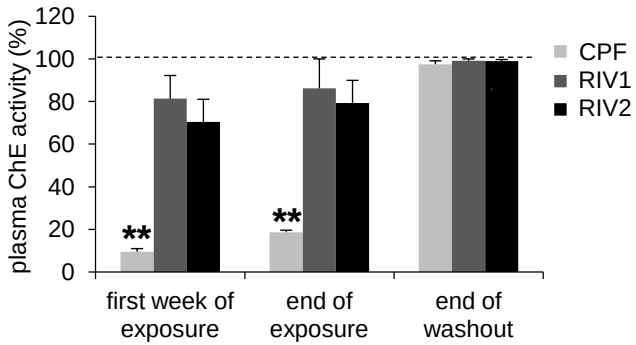
plasma ChE activity (%)
120
100
80
60
40
20
0
**
**
first week of exposure
end of exposure
end of washout
CPF
RIV1
RIV2

Figure 4

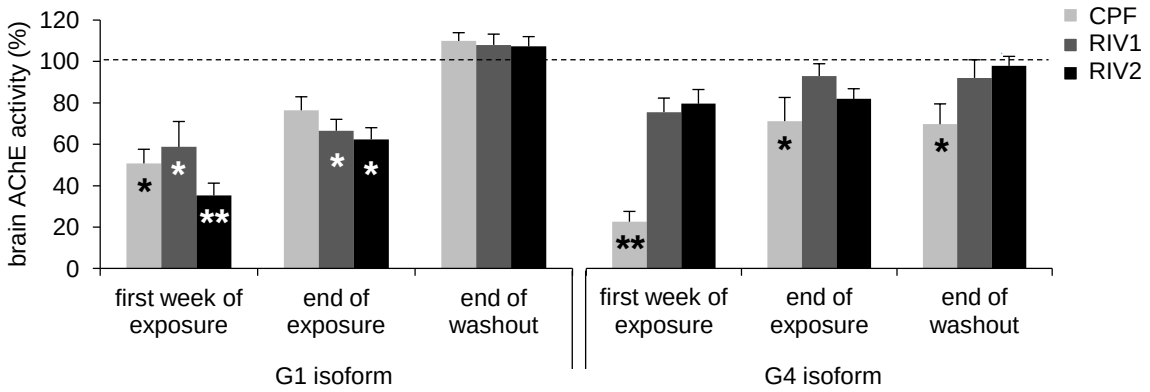
brain AChE activity (%)
120
100
80
60
40
20
0
CPF
RIV1
RIV2
*
*
**
*
*
**
*
*
first week of exposure
end of exposure
end of washout
first week of exposure
end of exposure
end of washout
G1 isoform
G4 isoform

Figure 5

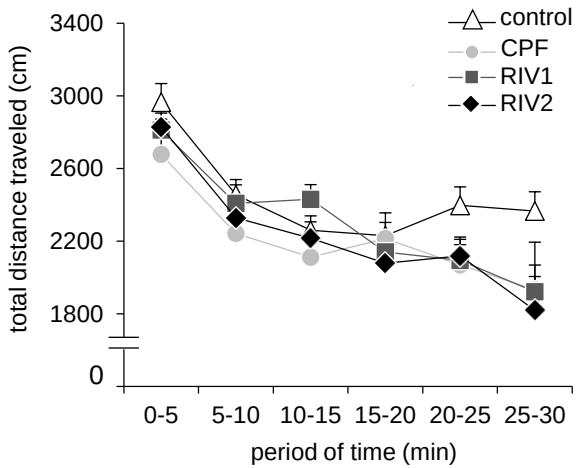
control
CPF
RIV1
RIV2
3400
3000
2600
2200
1800
0
total distance traveled (cm)
0-5
5-10
10-15
15-20
20-25
25-30
period of time (min)

Figure 6

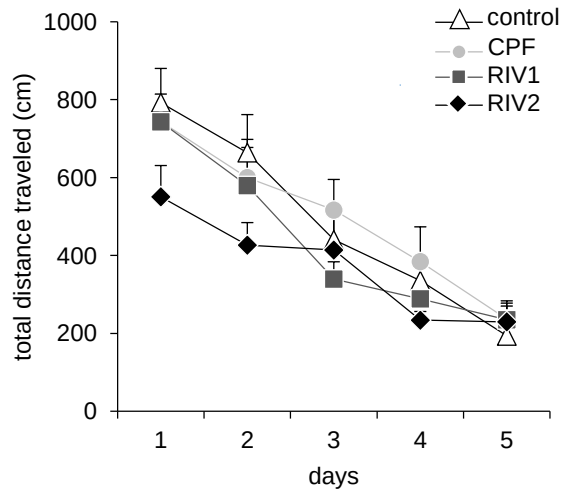
control
CPF
RIV1
RIV2
total distance traveled (cm)
1000
800
600
400
200
0
1
2
3
4
5
days

Figure 7

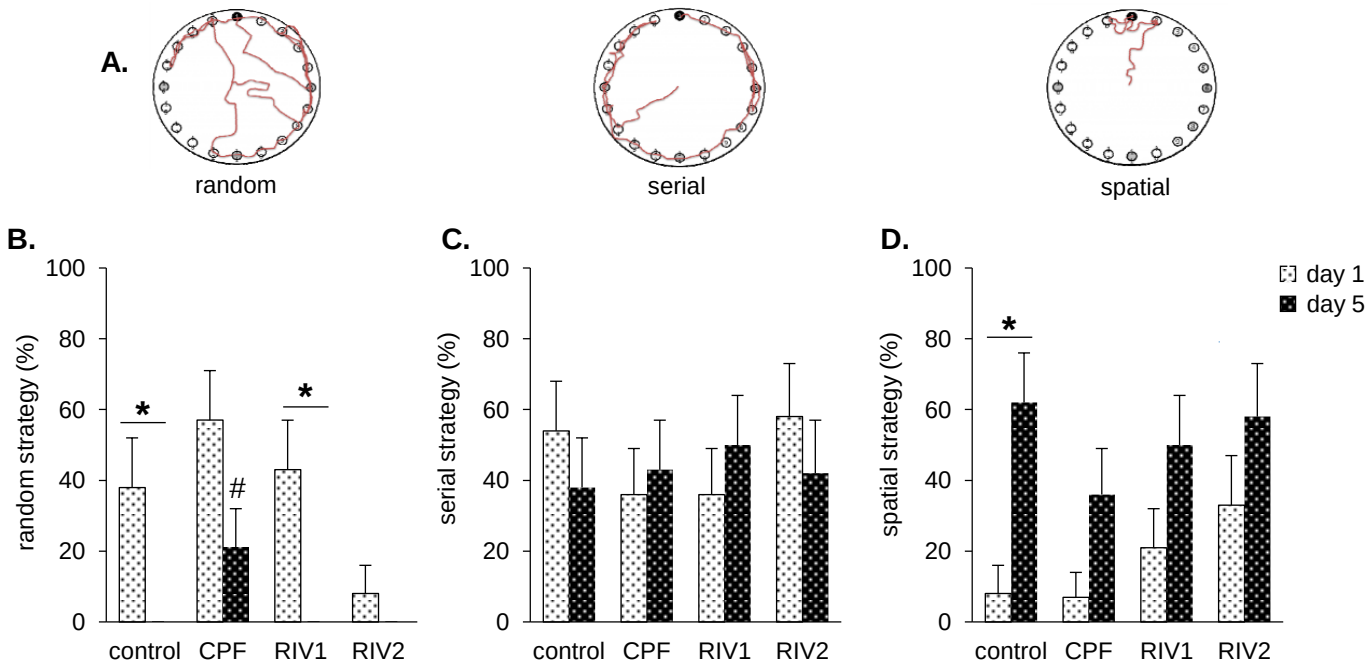
A.
random
serial
spatial
B.
random strategy (%)
100
80
60
40
20
0
*
#
*
control
CPF
RIV1
RIV2
C.
serial strategy (%)
100
80
60
40
20
0
control
CPF
RIV1
RIV2
D.
spatial strategy (%)
100
80
60
40
20
0
*
control
CPF
RIV1
RIV2
day 1
day 5

Figure 8

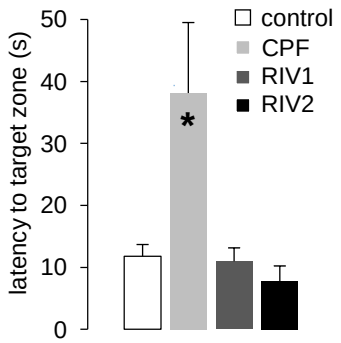

Figure 9

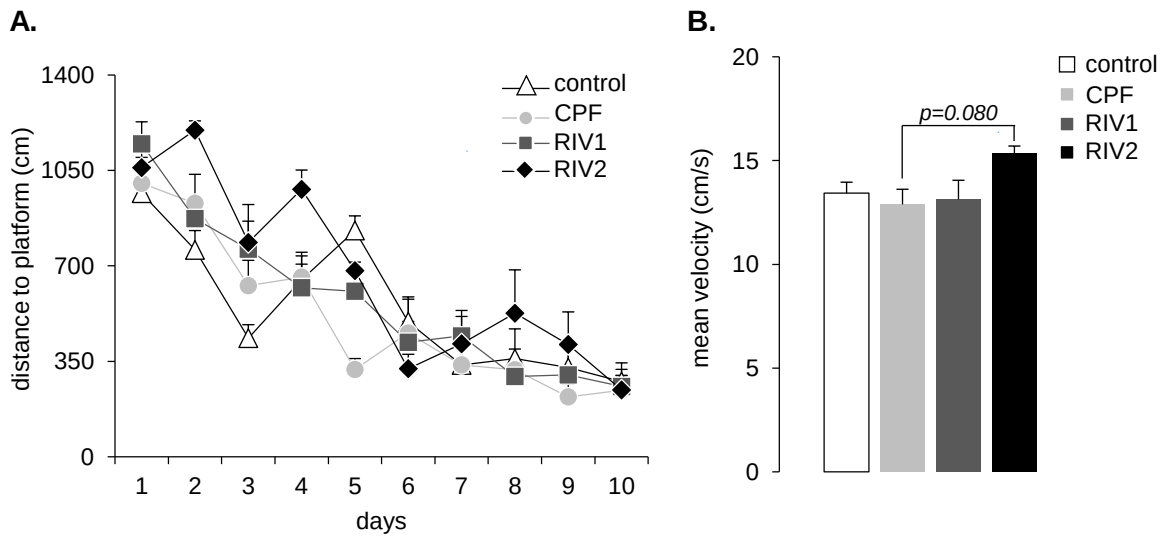
A.
1400
1050
700
350
0
distance to platform (cm)
1
2
3
4
5
6
7
8
9
10
days
control
CPF
RIV1
RIV2
B.
20
15
10
5
0
mean velocity (cm/s)
p=0.080
control
CPF
RIV1
RIV2

Figure 10

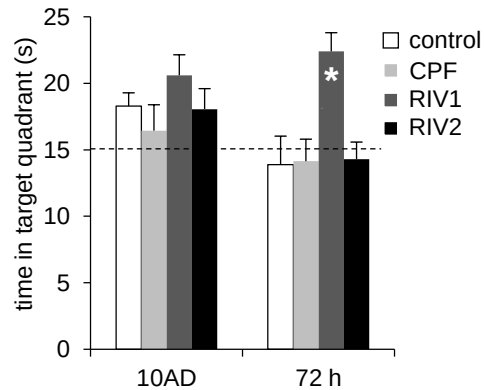
time in target quadrant (s)
25
20
15
10
5
0
10AD
72 h
control
CPF
RIV1
RIV2
*

Table 1. Plasma levels of biomarkers in C57BL/6 mice

	Control	Chlorpyrifos (5 mg/kg)		Rivastigmine (1 mg/kg)		Rivastigmine (2 mg/kg)	
		Treatment	Washout	Treatment	Washout	Treatment	Washout
Albumin (g/L)	35.60±2.2	**29.10±0.7***	35.95±0.48	32.30±1.10	35.24±0.24	30.30±0.32	36.40±0.21
Cholesterol (mmol/L)	3.34± 0.39	2.47± 0.09	3.56±0.20	2.69± 0.44	3.55±0.11	2.61± 0.07	3.69±0.09
Triglycerides (mmol/L)	1.20±0.08	1.02± 0.20	1.38±0.09	1.24± 0.28	1.20±0.08	1.04± 0.09	1.40±0.08

The asterisk (*) indicates differences between CPF-fed mice and the control group at $p<0.05$.