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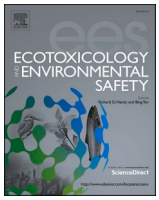

β-Hexachlorocyclohexane triggers neuroinflammatory activity, epigenetic histone post-translational modifications and cognitive dysfunction

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ABSTRACT

Persistent organic pollutants (POPs), which encompass pesticides and industrial chemicals widely utilized across the globe, pose a covert threat to human health. β-hexachlorocyclohexane (β-HCH) is an organochlorine pesticide with striking stability, still illegally dumped in many countries, and recognized as responsible for several pathogenetic mechanisms. This study represents a pioneering exploration into the neurotoxic effects induced by the exposure to β-HCH specifically targeting neuronal cells (N2a), microglia (BV-2), and C57BL/6 mice. As shown by western blot and qPCR analyses, the administration of β-HCH triggered a modulation of NF-κB, a key factor influencing both inflammation and pro-inflammatory cytokines expression. We demonstrated by proteomic and western blot techniques epigenetic modifications in H3 histone induced by β-HCH. Histone acetylation of H3K9 and H3K27 increased in N2a, and in the prefrontal cortex of C57BL/6 mice administered with β-HCH, whereas it decreased in BV-2 cells and in the hippocampus. We also observed a severe detrimental effect on recognition memory and spatial navigation by the Novel Object Recognition Test (NORT) and the Object Place Recognition Task (OPRT) behavioural tests. Cognitive impairment was linked to decreased expression of the genes BDNF and SNAP-25, which are mediators involved in synaptic function and activity. The obtained results expand our understanding of the harmful impact produced by β-HCH exposure by highlighting its implication in the pathogenesis of neurological diseases. These findings will support intervention programs to limit the risk induced by exposure to POPs. Regulatory agencies should block further illicit use, causing environmental hazards and endangering human and animal health.

1. Introduction

Synthetic pesticides brought a fundamental change in global food production and consumption practices. The availability of these chemical agents has enabled the cultivation of a wider variety of crops with higher yields, resulting in increased food supplies, dietary diversity, economic wealth, and demographic expansion. Pesticides have permeated the environment as a consequence of their massive application in agricultural and domestic purposes (Landrigan et al., 2020). It is noteworthy that the global pesticide market experienced a compounded annual growth rate (CAGR) of 8.9% from USD 78.16 billion in 2021 to USD 85.11 billion in 2022. Projections indicate that it is set to reach USD 105.39 billion by 2026, sustaining a compounded annual growth rate of 5.5%. This pesticide market includes insecticides, fungicides, herbicides, and other products (e.g., rodenticides, nematicides, fumigants, etc.), and their impact on public health as chemical interferents are and will continue to be devastating (*"Environmental and health impacts of pesticides and fertilizers and ways of minimizing them", published by UN Environment Programme in collaboration with the FAO and the WHO – June 2021*). Among these interferents are the Persistent Organic Pollutants

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(POPs) with a long half-life due to inadequate natural degradation processes, causing a continuous increase of the toxic agro-environmental burden (Breivik et al., 1999; Misra and Chaturvedi, 2007). POPs include synthetic compounds such as pesticides, polychlorinated biphenyls (PCBs) and dioxins and furans produced as a result of manufacturing procedures (Gavrilescu, 2005). Contaminated food intake represents the principal way of exposure to POPs, followed by inhaling polluted air during POP synthesis and usage (Bohlin et al., 2007).

The persistent “organochlorinated pesticides" (OCPs) included in POPs encompass a diverse group of organic compounds primarily comprised of carbon, hydrogen, and multiple chlorine atoms in each molecule (Guo et al., 2019; Mrema et al., 2013; Vaccher et al., 2020). One of these compounds is the β-isomer of hexachlorocyclohexane (β-HCH), which is a byproduct of the synthesis of lindane (γ-HCH) that was extensively utilized as an insecticide and pesticide from the 1940s to the 1990 s across various agricultural applications. β-HCH is distinguished by its high lipid solubility and long-lasting presence in the environment. Its widespread use and distribution constitute an environmental emergency in several countries, including Italy (Marra et al., 2023; Noi et al., 2021; Wang et al., 2018; Yang et al., 2023). Exposure to β-HCH can increase individual susceptibility to various health issues, including cancer and neurodegenerative diseases, once it contaminates human tissues (Varghese et al., 2021; Vellingiri et al., 2022). The EPA (United States Environmental Protection Agency) has classified β-HCH as "possibly carcinogenic to humans" (EPA, 2003) as assessed by several studies demonstrating the association of β-HCH with colorectal and breast cancer (Abolhassani et al., 2019; Mortazavi et al., 2019).

Pesticide exposure induces systemic inflammation associated with oxidative stress (Kumar et al., 2014; Singh et al., 2022; Tsitsimpikou et al., 2013; Zafiropoulos et al., 2014), although the precise molecular mechanism behind this phenomenon remains unclear (Sule et al., 2022). Systemic inflammation and oxidative stress are known to contribute significantly to pathological processes within the neuronal compartment, potentially leading to cognitive impairment (Felipo et al., 2012; Mossakowski et al., 2015) and motor dysfunction (Montoliu et al., 2015).

β-HCH triggered the production of ROS in dopaminergic neurons and microglial cells. Additionally, elevated levels of serum β-HCH were detected in Parkinson’s disease (PD) patients, suggesting a correlation between OCP exposure and neurodegenerative disorders (Richardson et al., 2011). OCPs were found in the substantia nigra and striata of post-mortem PD brains (Kanthasamy et al., 2005; Mao and Liu, 2008; Sharma et al., 2010). Several studies showed that fetal exposure to pesticides during pregnancy and, to a lesser extent, through breastfeeding negatively impacted neurodevelopment (Haddad et al., 2015; Savitz et al., 2014; Tan et al., 2009; Wang et al., 2021).

Epigenetic modifications should contribute to the development of pathological effects alongside the inflammatory response associated with pesticide exposure. These modifications encompass post-translational modification of histones (methylation, acetylation, PARylation, etc.) and change in DNA methylation. These modifications are critical in regulating numerous nuclear processes, such as DNA replication and repair, gene silencing, transcription and, cell cycle progression. They may have a significant role in influencing gene expression in response to environmental factors, leading to pathological changes. A recent study implies that environmental factors contribute to neurodegenerative diseases by inducing epigenetic changes and dysregulation of the epigenome (Ghosh and Saadat, 2023).

Despite several papers have explored the influence of β-HCH on the epigenome through studies on DNA methylation and miRNA expression (Giambo et al., 2021; Rusiecki et al., 2017; Van Cauwenbergh et al., 2020), limited research addressed its impact on histone post-translational modifications (PTM). This study investigated the relatively unexplored molecular aspects of β-HCH on the central nervous system (CNS) encompassing its neuroinflammatory potential and its effects on histone epigenetic changes and cognitive performance.

2. Materials and methods

2.1. β-hexachlorocyclohexane (β-HCH) preparation

β-HCH was obtained from Sigma chemicals (CAS No. 319–85–7, analytical grade). β-HCH was dissolved in DMSO at an initial concentration of 100 mM and then was added to the cellular medium to a final concentration of 10 μM. The animals were daily treated via an oral dose of β-HCH previously dissolved in groundnut oil at 5 mg/Kg.

2.2. Cell culture and treatment

N2a cells were grown in DMEM medium supplemented with 10% FBS, 2 mM L-Glutammine, 100 U/ml penicillin and 100 ug/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Mouse microglia cells (BV-2) were grown in DMEM-F12 medium supplemented with 10% FBS, 4 mM L-Glutammine, 100 U/ml penicillin and 100 ug/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2. Cells were treated with vehicle control (0.001% DMSO) or 10 μM β-HCH for 24 h or 48 h. The concentration of β-HCH used for this study was selected based on previous publications (Rubini et al., 2021a, 2021b).

2.3. Cell viability assays

To assess cell viability, the mitochondrial activity of cells treated with β-HCH either for 24 h and 48 h was evaluated by MTT assay, according to (Hansen et al., 1989) and (Morgan, 1998). The optical density (OD) of the soluble formazan crystals was measured at 570 nm, with a reference at 690 nm, using a microplate reader (Thermo Scientific Appliskan Multimode Microplate Reader) (Grieco et al., 2021). The cytotoxicity effect related to β-HCH exposure was also assessed by the trypan blue exclusion method (Grieco et al., 2021).

2.4. Animal care and treatment

Three-month-old C57BL/6 J male mice were obtained from Charles River (Calco, Lecco Italy) and housed in the animal facility. The present study followed the ARRIVE Guidelines (Percie du Sert et al., 2020) and complied with the General Management of Animal Care and Veterinary Drugs of the Italian Ministry of Health (D. L. 26/2014) and it was carried out according to European Communities Council Directive (86/609/CEE) for the use of animals in research.

Animals were housed with a light/dark cycle of 12 h at constant temperature and humidity in groups of 3–4 mice/cage in standard conditions with free access to food and water.

Four-months-old C57BL/6 J male mice were randomly assigned to placebo or β-HCH-group of treatment and daily administered with an oral dose (oral gavage) of 5 mg/Kg β-HCH (N=12) or 0 mg/Kg β-HCH (placebo) (N=11) for eight consecutive days in standard feeding conditions.

Mice were sacrificed after the end of treatment and conclusion of the cognitive assessment. Brains were removed (Latina et al., 2021) and selected areas such as hippocampus and prefrontal cortex dissected and immediately frozen as previously described (Latina et al., 2021).

2.5. Cognitive assessment

Recognition memory and spatial memory were assessed via the use of novel object recognition test (NORT) and object place recognition task (OPRT), respectively. As detailed in previous studies (Barbato et al., 2020; Latina et al., 2021), NORT and OPRT were carried out following a habituation phase of 5 min to the apparatus to reduce stress and to avoid a potential neophobic response. From a procedural point of view, for both tasks, mice are firstly placed into a squared arena, and either the

novel object (i.e., NORT) or the novel spatial location of a familiar object (i.e., OPRT) are changed randomly as previously detailed (Barbato et al., 2020; Coccurello et al., 2000; Latina et al., 2021)

The percentage of the discrimination index (DI) was calculated as follows: contact time with the novel object or time novel place (Tno)− contact time with the familiar one or time non-displaced object (Tfo) \total contact with objects (Tno+Tfo) × 100.

2.6. Total protein extraction

BV-2 and N2a cells, as well as frozen pre-frontal cortex and hippocampus from both hemispheres were homogenized in ice-cold RIPA buffer, (composed of 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1 mM EDTA) supplemented with proteases inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail (Sigma-Aldrich), for 1 h on ice. The resulting supernatants obtained after centrifugation at 4 °C for 20 min at 13,000 rpm were collected and used for western blot analysis. The protein concentration was determined using the Pierce BCA assay kit (Thermo Scientific, Waltham, MA, USA).

2.7. Nuclear and cytoplasmic protein fraction extraction

Cytoplasmic and nuclear proteins from BV-2 and N2a cells were extracted using ProteoJET™ Cytoplasmic and Nuclear Protein Extraction Kit" (Fermentas) according to the manufacturer's instructions. Briefly, cells were lysed by adding 10 volumes of cell lysis buffer, provided by the kit, containing protease inhibitors and 1 mM DTT to 1 vol of cells provided by the kit. Cells incubated on ice for 20 min were centrifuged at 500 g at 4°C for 10 min. The cytoplasmic and nuclear fractions were separated and utilized for different assays. The nuclear pellet was resuspended in 1/10 vol of the Nuclei lysis reagent containing 5 mM DTT. The solution was rocked for 30 min at 4°C and then the nuclear protein extract was collected after centrifugation at 20000 g 4°C for 10 min. Proteins in both lysates were quantified by Pierce bicinchoninic assay (BCA) kit (Thermo Scientific, Waltham, MA, USA) and stored at −80°C until use.

2.8. Histone protein extraction

Histone proteins from both cellular lines, mice pre-frontal cortex and hippocampus were extracted using the EpiQuik Total Histone Extraction Kit (EpiGentek, Farmingdale, NY), according to the manufacturer's instructions and quantified as (Napoletani et al., 2021).

2.9. Western blot analysis

Equal amounts of histone and endogenous protein extracts (2 μg and 20 μg for each sample, respectively) loaded onto the 4–20% Criterion™ TGX Stain-Free™ Protein Gel (BioRad) were resolved by SDS-PAGE electrophoresis and processed as (Grieco et al., 2021). The membranes were probed with following primary antibodies: Anti-Histone H3 (acetyl K9) (dil. 1:1000 - #ab32129- Abcam – Rabbit), Anti-Histone H3 (acetyl K27) (dil. 1:1000 - #39133 – Active Motif – Rabbit), Tri-Methyl-Histone H3 (Lys 9) (dil. 1:1000 - #ab8898 - Abcam – Rabbit), Tri-Methyl-Histone H3 (Lys 27) (dil. 1:1000 - #9733 - Cell Signaling – Rabbit), NF-κB p65 (dil. 1:1000 - #06418 - Merck Millipore - Rabbit), Phospho-NF-κB p65 (dil. 1:500 - #3033- Cell Signaling - Rabbit), HDAC1 (dil. 1:700 - #sc81598 - Santa Cruz - Mouse), HDAC3 (dil. 1:700 - #sc376957 - Santa Cruz - Mouse), H3 (dil. 1:20.000 - #ab1791 - Abcam - Rabbit), β-Actina (dil. 1:5000 - #a1978 - Sigma - Mouse), GAPDH (dil. 1:1000 - #sc32233 - Santa Cruz - Mouse). Densitometric analyses were performed by ImageLab software (Biorad) and normalized to a reference protein (H3, β-Actin and GAPDH). Three biological replicates were performed with three technical replicates.

2.10. Real-time quantitative PCR analysis

Total RNA was extracted from both cells and brain tissue using TRIzol, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA) and following the protocol (Grieco et al., 2021; Masoud et al., 2018). Results obtained using the primers shown in Table S1 were analyzed relative to RPS27a according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.11. Protein identification by MALDI-TOF mass spectrometry

Histon proteins were resolved by 4–20% SDS-PAGE gel electrophoresis. The histone H3 protein bands were excised, cut into small pieces, destained, reduced, and alkylated by iodoacetamide (Brisdelli et al., 2020) After alkylation, each gel slice was digested with Arg-C (Promega, Madison, WI, USA) in buffer (50 mM Tris-HCl, 5 mM CaCl2, 2 mM EDTA, 5 mM DTT) with an enzyme-substrate ratio of 1:100 for 3 h at 37°C. An aliquot of each peptide mixture, blended with α-Cyano-4-hydroxycinnamic acid matrix, was analyzed by a mass spectrometry platform ultrafleXtreme (Bruker, Bremen DE, Germany) MALDI ToFToF, provided with a smartbeam-II laser. MS-spectra were acquired in reflector and positive mode. Peptide mass fingerprinting was achieved by Flex Analysis and the protein identification by BioTools software against UniProt_mus musculus database, selecting ArgC as enzyme, up to 1 missed cleavage, carbamidomethylated cysteine residues (+57.021 Da) as fixed modification and oxidated methionine (+15.995 Da), mono- (+14.016 Da), di- (+28.031 Da), tri-methylated (+42.047 Da) and acetylated (+42.011 da) lysine residues as variable modifications. All *m/z* values were submitted to tandem mass experiments in LIFT mode. The peptide and relative post-translational modification (PTM) identifications were achieved by applying the same parameters for protein identifications by peptide mass fingerprinting (PMF). The presence of "diagnostic" ions at a low molecular weight of 98.097, 130.09 and 126.092 in MSMS spectra, allowed to discriminate among mono-, di- and/or tri-methylated and acetylated lysine residues respectively (Hseiky et al., 2021). This information was crucial to distinguish between acetylation and tri-methylation which share the same Δmass.

2.12. Statistical analysis

All experiments were conducted in triplicate and the results are presented as mean ± standard error of the mean (SEM). Statistical evaluations were carried out using a t-test in GraphPad Prism 8.0.1 (GraphPad Software, La Jolla, CA, USA). A significance threshold of $p \leq 0.05$ was applied to all analyses, indicating statistical significance.

3. Results

The present work investigates the neurotoxic effect induced by the organic pesticide β-HCH and its potential to evoke neuroinflammatory responses and epigenetic modifications of histone H3 in cellular models and C57BL/6 J mice. We also explored cognitive dysfunctions in mice following β-HCH administration.

3.1. Effect of β-HCH on cellular viability

Cell viability in BV-2 and N2a cells treated with β-HCH was assessed by MTT assay. The analyses were carried out at 24 h and 48 h using a β-HCH concentration of 10 μM. The data showed that treatment with β-HCH induces a moderate decrease of viability in BV-2 at 24 h (Fig. S1 a). No changes were observed in N2a at each time point (Fig. S1 b).

Based on these results and following the literature (Kumar et al., 2014; Rubini et al., 2021a), we decided to use a β-HCH concentration of 10 μM.

3.2. β-HCH exposure and NF-κB activation in different cellular models

Organochlorinated compounds participate in the inflammatory processes by modulating several transcription factors (Abdollahi et al., 2004). NF-κB is a transcription factor involved in multiple cellular processes such as cell growth, apoptosis, and immune response (Lawrence, 2009). To verify whether β-HCH can influence the expression of inflammatory agents in BV-2 and N2a cells, the activation of NF-κB was evaluated. Cytosolic and nuclear proteins obtained from BV-2 cells after treatment with or without 10 μM β-HCH, were analyzed by Western blot. A significant nuclear translocation of NF-κB was observed up to 1 h with a slight decrease in the following 24 h (Fig. 1a). The analysis of N2a cells cell lysates with anti-phospho-NF-κB antibody revealed that β-HCH induced NF-κB activation up to 6 (Fig. 1b). The temporal delay of NF-κB activation may reside in the functional difference of the two cell types.

3.3. β-HCH exposure and inflammatory cytokines in different cellular models and in C57BL/6 mice

We investigated whether the activation of NF-kB by β-HCH triggered the expression of pro-inflammatory cytokines genes. IL-1β, IL-6, TNF-α and TGF-β mRNA expression levels in BV-2 and N2a cells treated with or without 10 μM β-HCH for 24 h were analyzed by qPCR. Fig. 2a and b showed an increase of IL-1β in both cellular models treated with β-HCH, whereas IL-6 expression was higher in BV-2 compared with N2a. TNF-α and TGF-β do not appear to be modulated by β-HCH treatment.

To evaluate whether β-HCH may induce an *in vivo* inflammatory response, we analyzed the expression of inflammatory cytokines in the prefrontal cortex (PFC) and hippocampal regions from C57BL/6 mice after administration of 5 mg/kg/day β-HCH for eight consecutive days. Based on experiments reported in the literature on mice, we chose this dose which was the lowest that can still induce a pharmacological effect (Anand et al., 1998; Gilbert and Mack, 1995; Rivera et al., 1998; Wong

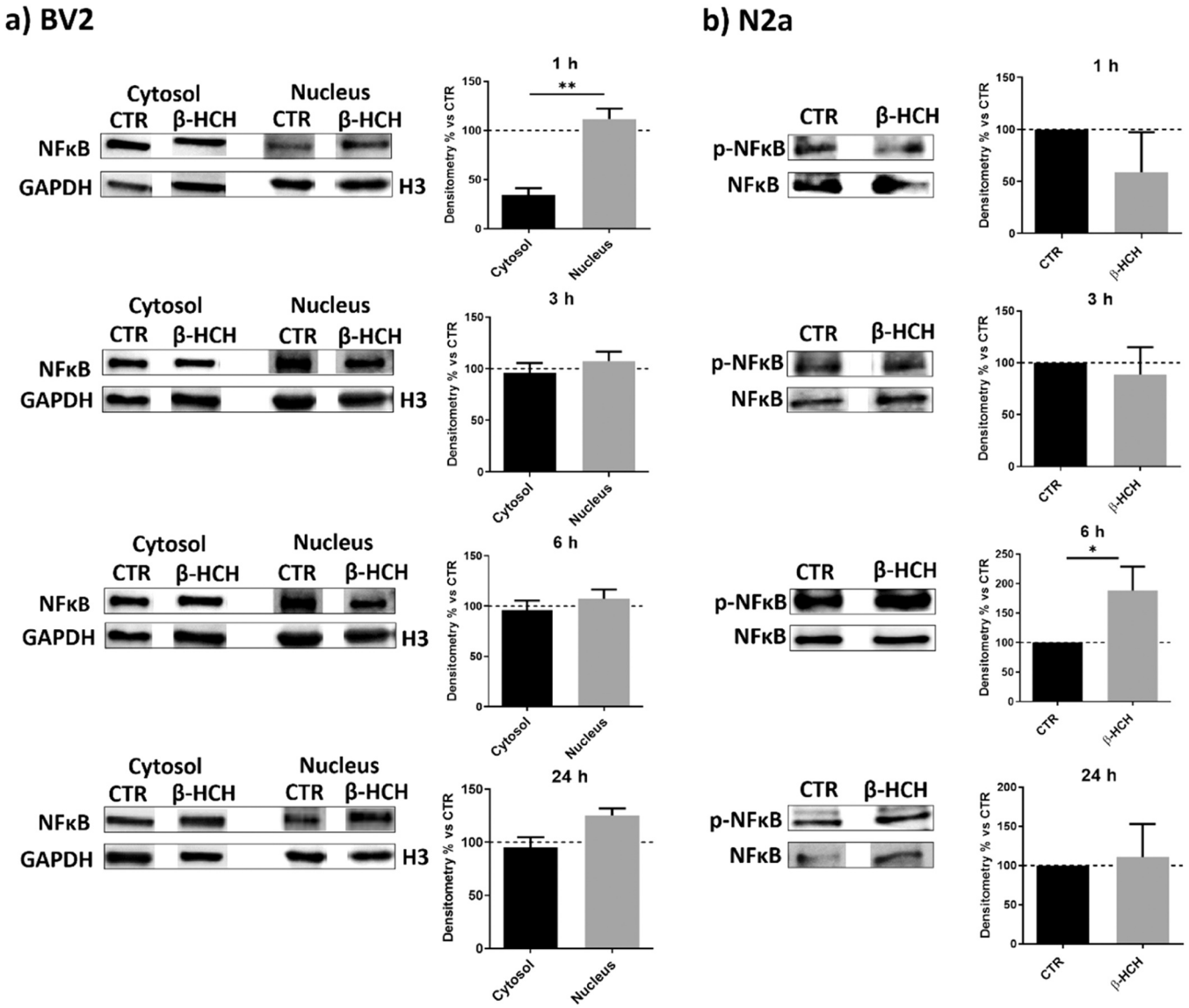

Fig. 1. - NF-κB activation was analyzed by Western blot. a) Cytoplasmic and nuclear fraction obtained from BV-2 cells treated with or without 10 μM β-HCH at 1 h, 3 h, 6 h and 24 h. Samples were subjected to 4–12% SDS-PAGE, blotted and the membrane was probed with NF-κB p65 specific antibody. NF-κB p65 signal was normalized on GAPDH for the cytoplasmic fraction and on histone H3 for the nuclear fraction. b) N2a cells treated as described above were immunoblotted with anti-phospho-NF-κB p65 Ser antibodies. As a control, the blotted membrane was probed with anti-NF-κB p65 antibodies and normalized against NF-κB p65 antibodies. Densitometric analyses were performed with ImageLab software (Biorad). Data are expressed as percentage versus cytosol or control (CTR). The values are shown as mean ± SEM from three independent experiments. Statistical evaluations were carried out using a t-test. * $p < 0.05$ *vs CTR*, ** $p < 0.01$ *vs Cytosol.*

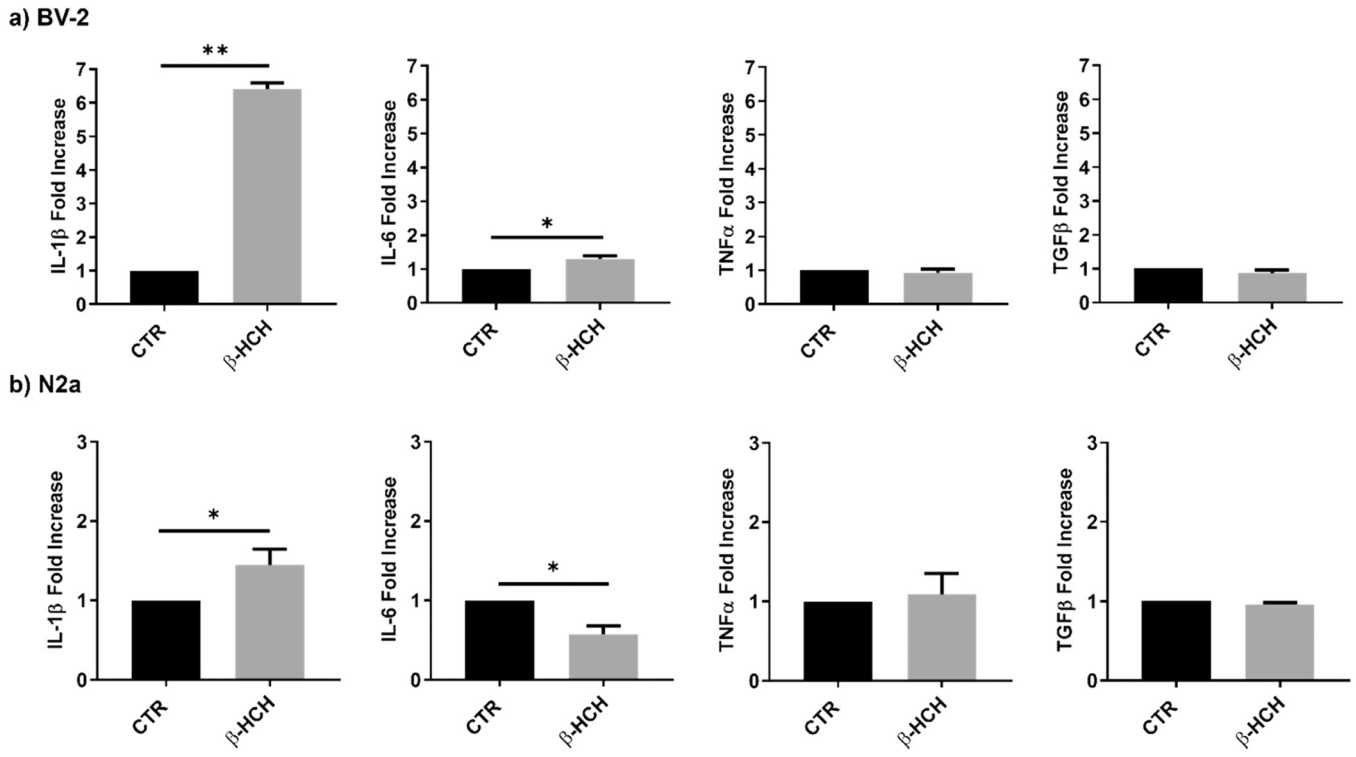

Fig. 2. - qPCR of IL-1β, IL-6, TNF-α, TGF- β in cellular models. mRNA extracted from a) BV-2 and b) N2a cells treated with or without 10 μM β-HCH for 24 h. The RPS27a gene was used for normalization. Expression profiles were determined using the 2−ΔΔCT method. The values are shown as mean ± SEM from three independent experiments. Statistical evaluations were carried out using a t-test. * $p < 0.05$ *vs CTR*, ** $p < 0.01$, *vs CTR*.

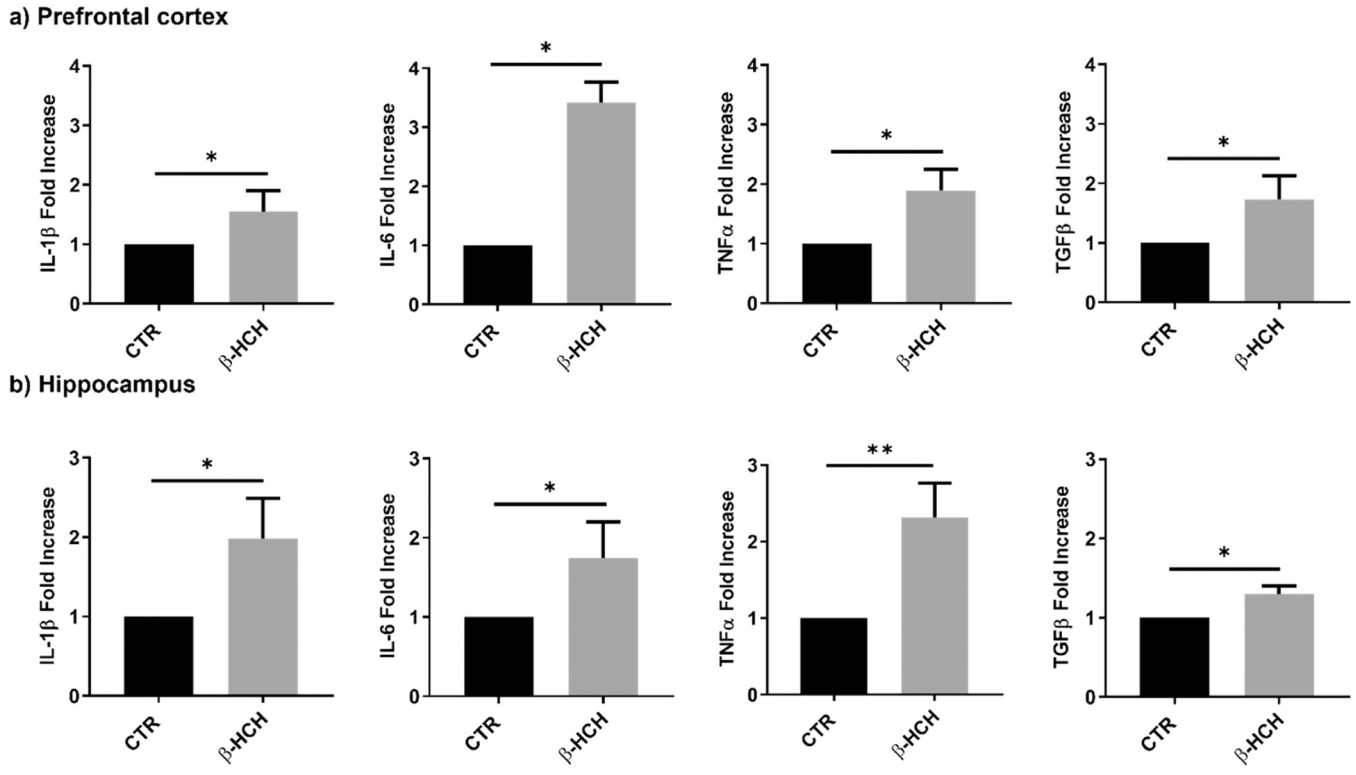

Fig. 3. - qPCR of IL-1β, IL-6, TNF-α, TGF-β in a mouse model. mRNA extracted from a) PFC and b) hippocampus regions of C57BL/6 mice (n.12) subjected to 5 mg/kg/day β-HCH or 0 mg/kg/day β-HCH (CTR) for 8 days. The RPS27a gene was used for normalization Expression profiles were determined using the 2−ΔΔCT method. The values are shown as mean ± SEM from three independent experiments. Statistical evaluations were carried out using a t-test. * $p < 0.05$ *vs CTR*, ** $p < 0.01$, *vs CTR*.

and Matsumura, 2007; Wu et al., 2000). A significative increase of IL-1β, IL-6, TNFα and TGF-β in the PFC and hippocampus was observed in C57BL/6 mice treated with β-HCH compared to mice treated with a placebo as reported in Fig. 3. These data suggested that β-HCH evoked a marked neuroinflammatory response *in vivo* (Fig. 3a, b).

3.4. Proteomic analyses of histone post-translational modifications

Epigenetics represents a relevant mechanistic link by which environmental signals could influence host gene expression by contributing to inflammation response alterations (Ho et al., 2012; Mukherjee et al., 2021). Classes of chemicals such as metals (arsenic, nickel, chromium, and methylmercury), air pollutants (particulate matter, benzene), and endocrine/reproductive disruptor toxicants (diethylstilbestrol, dioxin) contribute to epigenetic modifications (Baccarelli and Bollati, 2009; Collotta et al., 2013).

In this context, we investigated whether exposure to β-HCH of neural cells can induce an alteration of histone H3 PTMs.

MALDI-ToFToF analyses of the Arg-C fragments of histone H3 were performed after histone purification from BV-2 and N2a cells. Identification of methylation and acetylation PTMs on lysine residues were achieved. We found a similar pattern of PTMs in control BV-2 and N2a cells (Fig. 4).

In treated cells, we observed an increase in acetylation on H3K9 and H3K14 in N2a, whereas these lysine residues in BV-2 showed enrichment in methylated forms (Fig. 4).

3.5. Histone H3 acetylation and methylation assessment in cellular models and in C57BL/6 mice

Histone acetylation was associated with nucleosome remodelling and transcriptional activation, while histone methylation is associated with transcriptional silencing or increased gene expression (Hernández-Muñoz, 2010). In this scenario, we assessed the level of activating acetylation and repressing methylation in the two histone hot spots, H3K9 and H3K27. Western Blot experiments were performed on treated or untreated BV-2 and N2a cells with 10 μM β-HCH. Fig. 5a showed a decrease in acetylation of histones H3K9 and H3K27 in BV-2 cells treated with β-HCH as compared to control cells. Trimethylated histones H3K9 and H3K27 do not appear to be affected by treatment. In contrast, acetylated histones of N2a neuronal cells showed an opposite trend (Fig. 5b). We observed a higher level of the acetylation of histones H3K9 and H3K27 counterbalanced by a decrease in their methylation.

The levels of proteins associated with histone deacetylase (HDAC), which are enzymes responsible for eliminating acetyl functional groups from lysine residues on histone proteins, were also investigated. Our results showed an increase of HDAC1 and HDAC3 in β-HCH-treated BV-2 compared to control cells (Fig. S2a). On the contrary, treatment of N2a cells induced a significant decrease of both enzymes compared with the control (Fig.S2b).

3.6. Histone modifications in C57BL/6 mice after β-HCH exposure

To corroborate our finding on histone H3 post-translational modifications on neural cells, we utilized western blot analysis to investigate the β-HCH effect in epigenetic regulation in mice PFC and hippocampus. Histone fractions obtained from the two C57BL/6 mice brain regions, treated either with 5 mg/kg/day β-HCH or 0 mg/kg/day β-HCH for eight consecutive days, were probed with antibodies against H3K9 acetylated, H3K27 acetylated, H3K9me3, and H3K27me3. The results obtained from PFC samples (Fig. 6a) showed a significant increase in histone H3K9 and H3K27 acetylation, associated with a decrease in histone H3K9 and H3K27 methylations. A significant reduction in both acetylated and methylated H3K27 was observed in the hippocampus (Fig. 6b).

3.7. Cognitive performance in C57BL/6 mice after β-HCH exposure

The inflammatory processes and epigenetic changes observed in cellular and mouse models following β-HCH treatment may induce cognitive dysfunction (Mostafalou and Abdollahi, 2023; Nabi and Tabassum, 2022; Yi et al., 2022). In this frame, we evaluated the impact and the potential neurotoxic effects of β-HCH administration on cognitive performance in mice.

C57BL/6 (n. 12) mice were treated either with 5 mg/kg/day β-HCH or 0 mg/kg/day β-HCH for eight consecutive days. Cognitive performance was evaluated through the implementation of NORT and OPRT to assess recognition memory and spatial memory/spatial navigation, respectively. Results indicate the presence of an impairment in both the ability to discriminate a new object (ORT) and to orient the exploratory behaviour towards stimuli moved to a novel location (OPRT) in mice administered with β-HCH (Fig. 7a).

To further support the results indicating memory impairment results, we investigate the synaptic integrity of C57BL/6 mice treated with β-HCH. The gene expression of BDNF and SNAP-25, essential mediators of synaptic function and activity, were assessed by qPCR analyses. We focused on the PFC and hippocampus, two specific brain areas involved in cognitive and behavioural dysfunctions associated with neurodegenerative processes (Fathy et al., 2020; Mu and Gage, 2011; Olajide et al., 2021). In C57BL/6 mice that underwent β-HCH exposure, we observed a reduction in BDNF and SNAP-25 transcripts in both PFC and hippocampal regions (Fig. 7b).

4. Discussion

This study was solicited by the environmental alarm raised by the

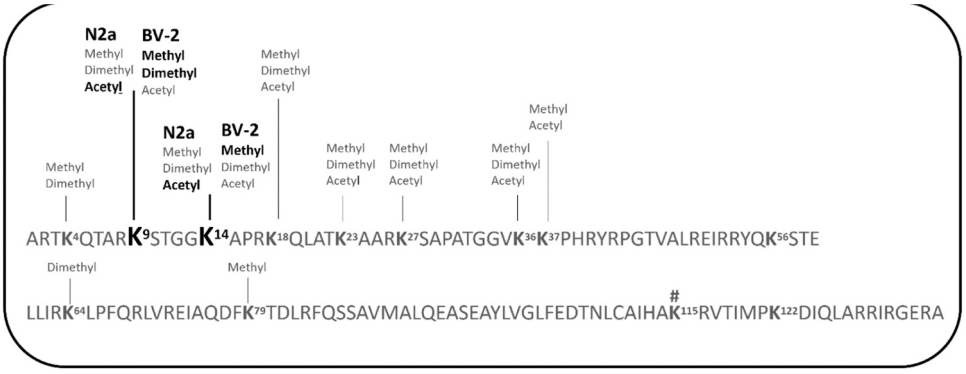

Fig. 4. - Histone H3 sequence. Lysine residues are numbered according to the position in the sequence and highlighted in bold. Identical PTMs were found in N2a and BV-2 cells and are reported above each lysine residue. The relative increase in PTM for K9 and K14 observed in treated cells is highlighted in bold. For K56 and K122 no PTM was observed. # not detected.

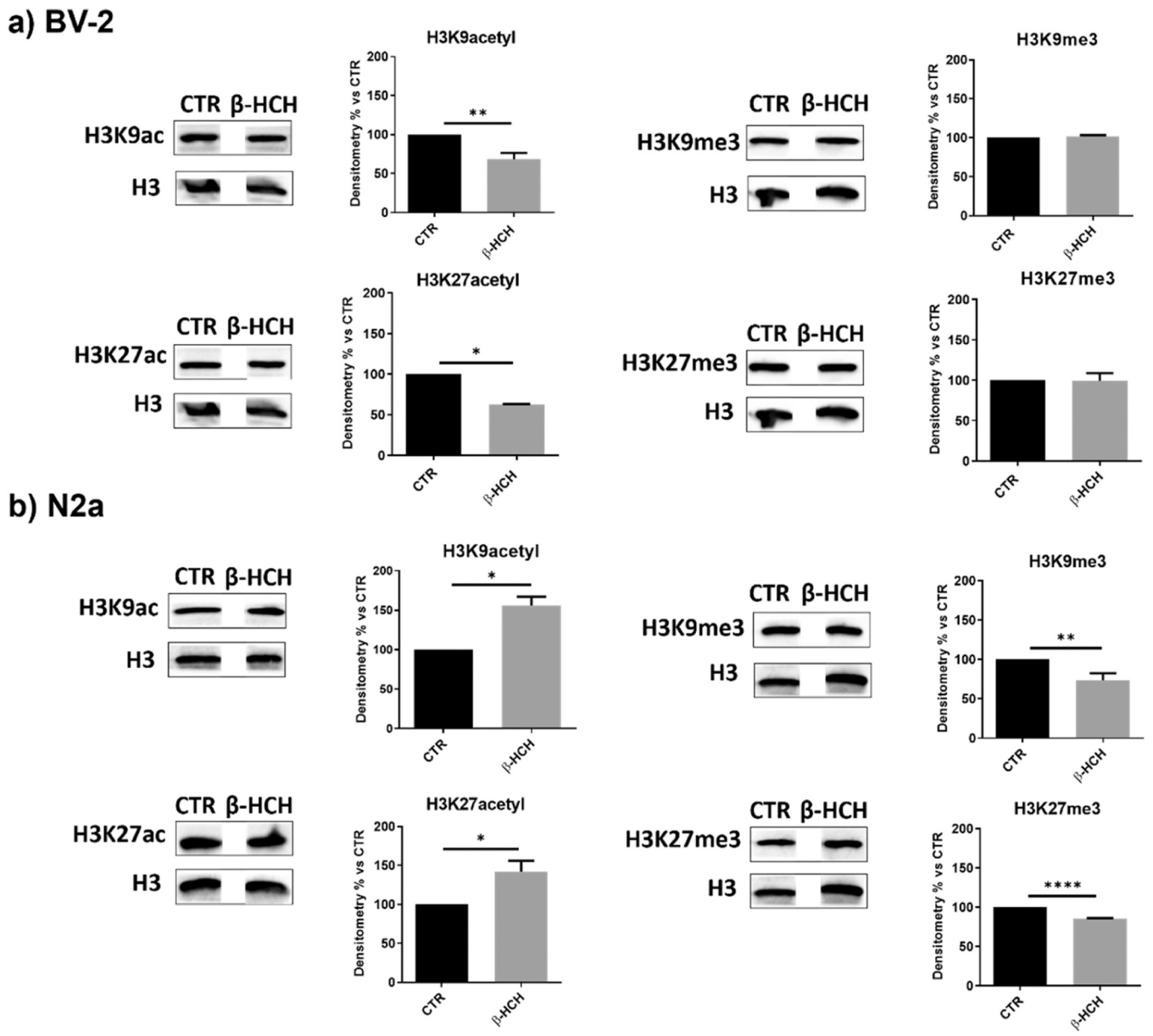

Fig. 5. – Evaluation of histone modifications. Histone proteins obtained from a) BV-2 cells and b) N2a treated with or without 10 μM β-HCH for 24 h were analyzed by western blot analysis, probed with anti-H3K9acetylated, anti-H3K27acetylated, anti-H3K9me3 and anti-H3K27me3. Densitometric analyses were normalized with histone H3. Data are reported as a percentage versus CTR. The values are shown as mean ± SEM from three independent experiments. Statistical evaluations were carried out using a t-test. * $p<0.05$ *vs CTR;* ** $p<0.01$ *vs CTR,* **** $p<0.0001$ *vs CTR.*

contamination from β-HCH, which has been documented in a vast area of Central Italy known as Valle del Sacco where this pesticide polluted the soil, the river water and downstream sediment (Porta et al., 2013). β-HCH can contaminate a variety of environmental matrices due to its persistence in soil and water, posing a threat to human health due to its bioaccumulation in animal tissues (Tsygankov et al., 2019).

Given that pesticides can induce severe chronic alteration of the CNS, including cognitive deficits and psychomotor dysfunction as well as mood disorders (e.g., depression), psychiatric morbidity, neurodegenerative diseases (such as AD and PD) and neurodevelopmental disorders (Kanthasamy et al., 2012; Kwok, 2010; Migliore and Coppedè, 2009), this study aimed at investigating the consequences of β-HCH exposure in neural cell models and mouse brains.

In both microglial and neuronal cells treated with β-HCH, we observed a significant increase in NF-κB activation, indicating the involvement of β-HCH in promoting an inflammatory landscape. These findings align with those observed in MCF-7 breast cancer cell lines exposed to β-HCH (Wong and Matsumura, 2007) as well as in human neuroblastoma SH-SY5Y cells treated with the OPC Zineb and Endosulfan (Jia and Misra, 2007). Moreover, the upregulation of pro-inflammatory cytokines such as IL-1β and IL-6 in BV-2 cells further confirmed the inflammatory response following exposure to β-HCH. Similar results were found in the PFC and hippocampus of mice treated with β-HCH. Levels of TNFα, IL-6 and IL-1β were increased in different neuropathological processes as well as in major depressive disorder (Das et al., 2021), and in the brains of patients with AD (von Bernhardi et al., 2010) or in the cerebrospinal fluid of patients with PD (Leal et al., 2013; Sterling et al., 2022). These inflammatory cytokines appear to act as mediators between the immune system and the CNS, as they can modulate synapse sensitivity and release of neurotransmitters, suggesting their involvement in the onset of cognitive disorders (Centonze, 2015). Specifically, the overexpression of pro-inflammatory molecules

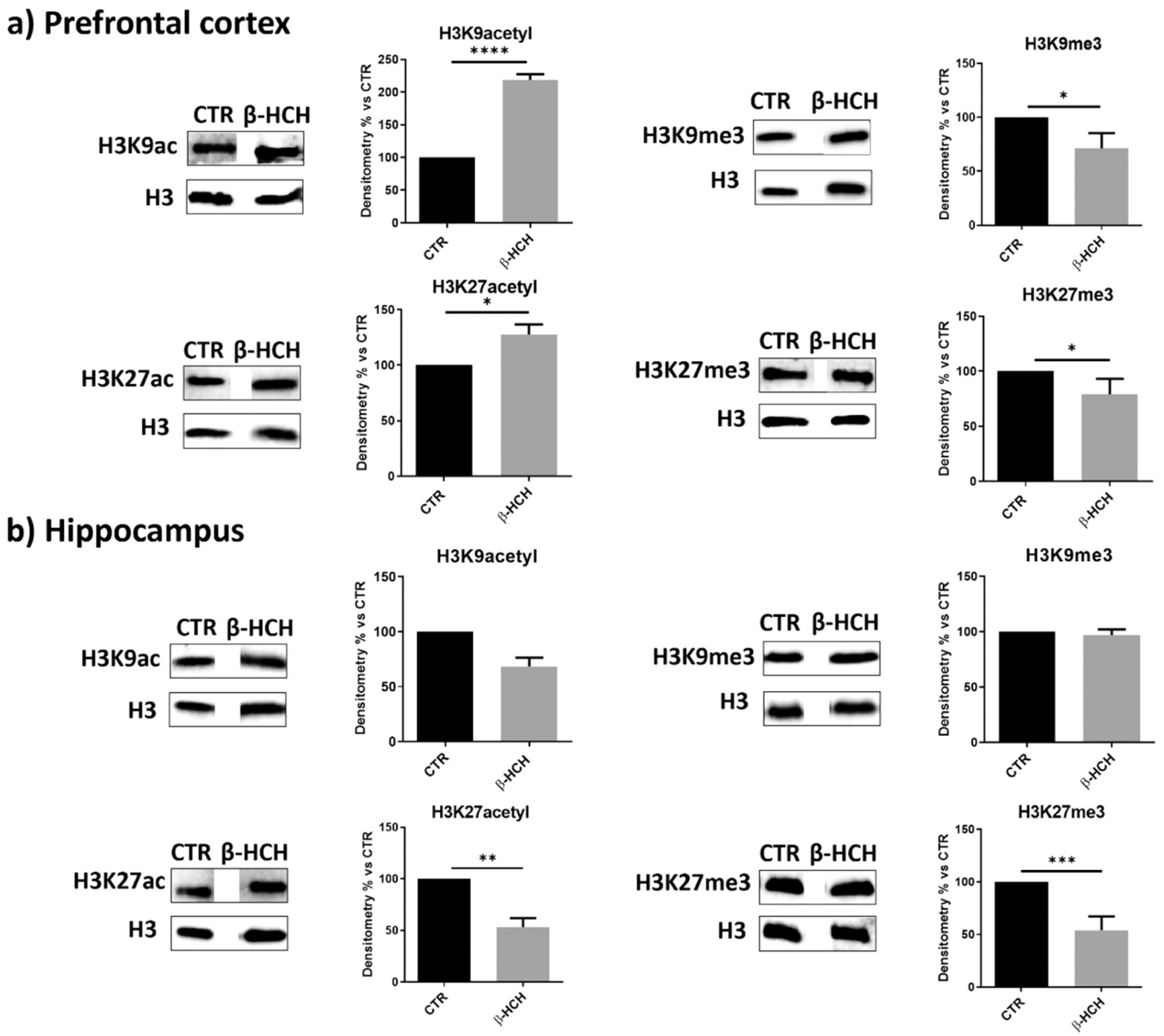

Fig. 6. – Evaluation of histone modifications. Histone proteins obtained from the PFC and hippocampus of C57BL/6 mice subjected to 5 mg/kg/day β-HCH or 0 mg/kg/day β-HCH (CTR) for eight days. a) PFC and b) Hippocampus from C57BL/6 mice were assessed by western blot analysis, probed with anti-H3K9acetylated, anti-H3K27acetylated, anti-H3K9me3 and anti-H3K27me3. Densitometric analyses were normalized on histone H3. The values are shown as mean ± SEM from three independent experiments. Statistical evaluations were carried out using a t-test. * $p<0.05$ *vs (CTR);* ** $p<0.01$ *vs CTR,* *** $P<0.001$ *vs CTR.*

such as TNFα, IL-1β, and IL-6 may represent “lethal triplets” for the mechanisms underlying neural plasticity, learning and memory processes. Several pieces of evidence support the role of TNF-α mediated signalling in synaptic plasticity and neural transmission (Beattie et al., 2002; Kaneko et al., 2008). Physiological levels of IL-1β are required for long-term potentiation (LTP), synaptic plasticity, and hippocampal-dependent memory (Lynch et al., 2007; Prieto et al., 2019). IL-6 promotes astrocytes and microglia activation, particularly in neurodegenerative diseases, and its increase during ageing is correlated with cognitive decline (Economos et al., 2013).

TGF-β is a modulatory cytokine whose expression can be induced by different traumatic events such as brain injury and neurodegenerative diseases (Buckwalter and Wyss-Coray, 2004). In AD, TGF-β may exert a protective role, although this effect can be counteracted by the increase of pro-inflammatory molecules, including TNF-α (Blasko et al., 2004).

A similar neuroinflammatory milieu could have occurred in our animal model where the β-HCH-induced inflammatory challenge may have abolished the neuroprotective potential of the slightly increased level of TGF-β expression observed in PFC and hippocampus, thus compromising the ability of TGF-β to promote microglia function and inhibit neurotoxicity (Eyüpoglu et al., 2003).

Considering, the relevance of epigenetic changes in the interplay among genes, the environment and behaviour (Grova et al., 2019), several scientific papers have analyzed the impact of exposure to environmental pollutants as a potential cause of short and long-term lowering in the expression of miRNAs, influencing levels of DNA methyltransferases, methyl-CpG binding protein 2, and other proteins implicated in histone modifications (Masoud et al., 2016).

Moreover, it is now well established that specific histone modifications generate a "histone code" that can alter the chromatin structure and lead to the modulation of gene transcription (Collotta et al., 2013; Park et al., 2022). The present study analyzed β-HCH-induced H3 histone modifications by mass spectrometry (MS) and western blot techniques. MS showed wide heterogeneity in PTMs. To better clarify how β-HCH

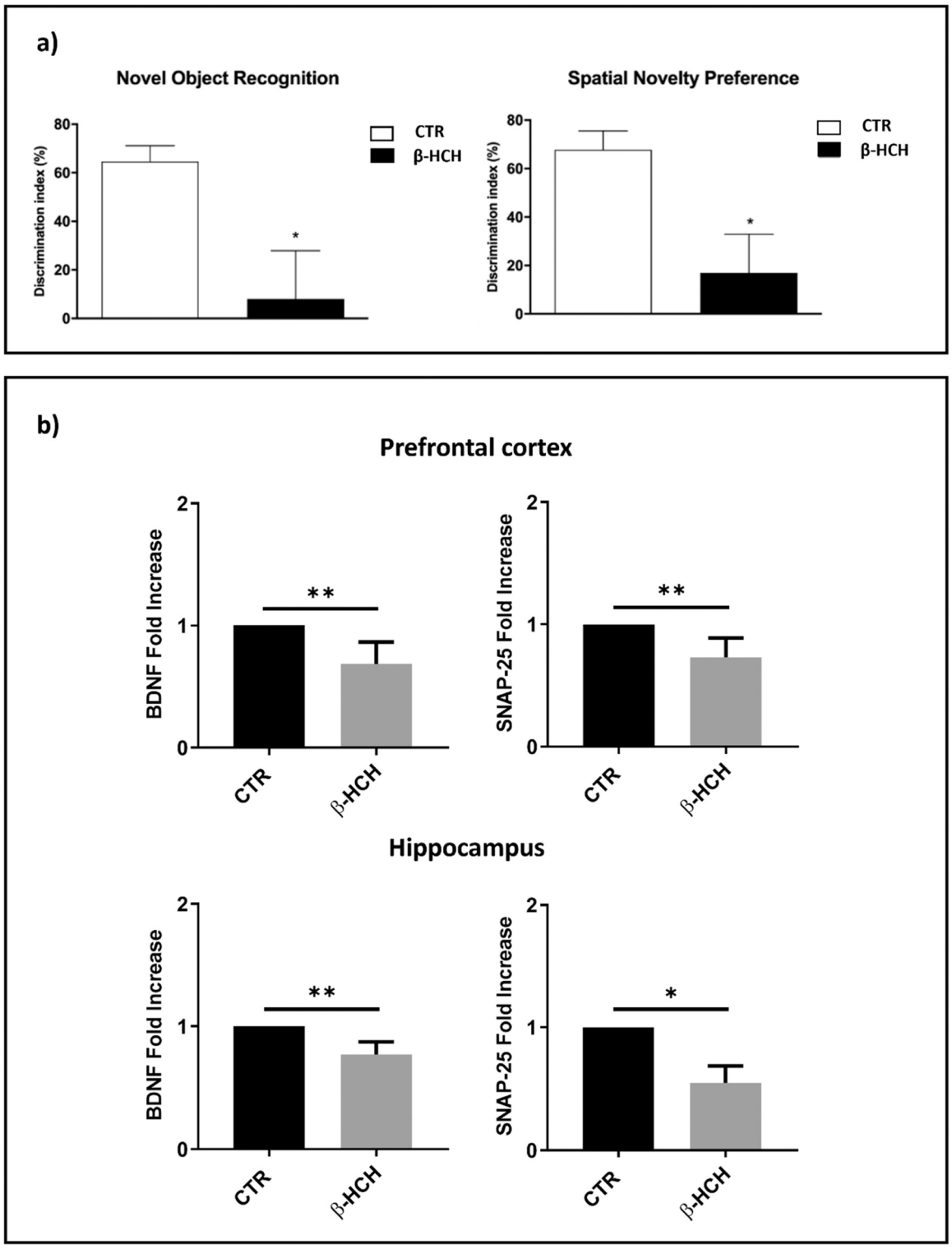

Fig. 7. – Cognitive assessment and BDNF and SNAP-25 expression. Panel a) C57BL/6 (N=23) mice were administered either with 5 mg/kg/day β-HCH (N=12) or with 0 mg/kg/day β-HCH (N=11) for eight consecutive days. Panel a) mice subjected to the NORT and to OPRT test. Panel b) The mRNA extracted from the a) PFC and b) hippocampus of C57BL/6 mice, treated either with 5 mg/kg/day β-HCH (N=12) or 0 mg/kg/day β-HCH (N=11) (CTR) for eight consecutive days, was analyzed by qPCR. The RPS27a gene was used for normalization. $2-\Delta CT$ method was employed to determine expression profiles. Data are reported as percent of mean ± SEM of values of individual treated mice versus untreated mice considered as control. Statistical evaluations were carried out using a t-test. *$p < 0.05$ vs CTR, ** $p < 0.01$ vs CTR.

impacts the epigenetic changes in H3 histone, we utilized western blot analyses to identify H3K9 and H3K27 acetylated, which are considered as marker of active chromatin configuration, along with the corresponding H3 methylated lysines, that lead to inactive euchromatin and constitutive heterochromatin (Creyghton et al., 2010; Karmodiya et al., 2012). Our results disclosed cell-type specific changes in histone PMTs associated with different expressions of HDAC enzymes in response to β-HCH treatment. The reduction in histone H3K9 and H3K27 acetylation observed in BV-2 cells correlated with increased HDAC1 and HDAC3 levels. In N2a cells, the increased acetylation in H3K9 and H3K27 was parallel to decreased deacetylase levels. The neuronal deregulation of HDAC activity, associated with aberrant cyclin p25/Cdk5 modulation and DNA damage accumulation, was reported as a pathological mechanism found in different neurodegenerative conditions, highlighting the importance of HDAC1 activity in preventing neurodegenerative processes and neuronal death (Kim et al., 2008). To further elucidate the epigenetic impact of β-HCH on histone modifications, we performed the same analyses in the PFC and hippocampus from C57BL/6 mice after β-HCH exposure. We observed a different epigenetic pattern in these two brain regions, underscoring the occurrence of regional-specific epigenetic alterations. These specific alterations across the two brain regions reflect structural and functional differences, and they are frequently associated with the progressive decline of cognitive functions and the onset of neurodegenerative disorders (Rizzardi et al., 2019). The decrease in H3K9 and H3K27 methylation, observed in PFC from C57BL/6 mice could be associated with learning contextual conditioning as fear conditioning in rodents (Gupta et al., 2010). Considering that histone acetylation has been linked to learning and memory processes (Gräff et al., 2012), the decrease in the acetylation of H3K9 and H3K27 found in the hippocampus can contribute to explain the impairment observed in the execution of hippocampal-dependent memory tasks such as NORT and OPRT, as described here. The inhibition of H3K9 methylation in the hippocampus improved memory decline and spatial learning in aged animals. (Snigdha et al., 2016). Increasing evidence highlights the role of epigenetic mechanisms in neuronal differentiation, maturation, neural plasticity and dynamic processes of memory and learning (Karpova et al., 2017; Lockett et al., 2010). Therefore, we analyzed the expression of BDNF, a key player in the synaptic plasticity required in learning, memory processes, neuroprotection, development, survival of neurons, and synaptic differentiation (Bathina and Das, 2015; Lu et al., 2014). We also evaluated the expression of SNAP-25 involved in synaptic transmission. A significant reduction in BDNF and SNAP-25 genes after β-HCH administration was found in both the PFC and hippocampus. In mice where microglia were depleted, a reduction in BDNF led to decreased cortical expression of specific glutamate receptors and impaired synaptic function, resulting in deficits in learning and mobility (Parkhurst et al., 2013). Reduced BDNF levels were reported both in the hippocampus and PFC from patients with bipolar disorder or major depressive disorder, showing a comorbid deficit in memory processing (Reinhart et al., 2015). In addition, the hippocampus of patients with PD and dementia with Lewy bodies showed a reduced expression of BDNF (Imamura et al., 2005; Parain et al., 1999). Moreover, SNAP-25 contributes to hippocampal-dependent memory consolidation in contextual spatial navigation and fear conditioning (Hou et al., 2004). Our data on BDNF and SNAP-25 decrease supported the analyses assessing a cognitive decline in C57BL/6 mice exposed to β-HCH.

5. Conclusion

The overall data from this study provide evidence that exposure to β-HCH triggers the activation of neuroinflammatory pathways that may be responsible for several epigenetic changes and the alterations of proteins involved in neural plasticity. The interplay of these effects may cause the cognitive impairment we observed in this study. These findings may expand our knowledge concerning the harmful potential of β-HCH exposure in neurodegenerative disease development. They also strongly suggest a rapid intervention to protect human health and serve as a further warning against the illegal and excessive use of pesticides for pests and disease carriers.

CRediT authorship contribution statement

Maddalena Grieco: Writing – review & editing, Validation, Investigation, Formal analysis. **Alessandra Giorgi:** Writing – original draft, Visualization, Software, Methodology, Investigation. **Anna Maggiore:** Writing – original draft, Investigation, Data curation. **Giacomo Giacovazzo:** Validation, Methodology, Formal analysis. **Maria d'Erme:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization. **Serena Ficchì:** Formal analysis, Data curation. **Giuseppina Mignogna:** Writing – review & editing, Writing – original draft, Supervision, Data curation, Conceptualization. **Luciana Mosca:** Writing – review & editing, Funding acquisition. **Roberto Coccurello:** Writing – review & editing, Writing – original draft, Supervision, Data curation, Conceptualization. **Bruno MARAS:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.116487.

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