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Heptafluoroisobutyronitrile (C$_4$F$_7$N), a gas used for insulating and arc quenching in electrical switchgear, is neurotoxic in the mouse brain

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Abstract

Fluoronitrile gas (C_4F_7N, CAS number 42532-60-5) is one of the most promising candidates as insulating and/or breaking medium in high and medium voltage electrical equipment. Besides its promising properties, C_4F_7N gas is however not devoid of acute toxicity when used pure or in gas mixtures. The toxicity was not extensively analyzed and reported. The aim of the present study was to analyze in mice the consequences of a single exposure to C_4F_7N gas, at different concentrations and different timepoints after exposure. Male and female Swiss mice were exposed to breathable air or C_4F_7N gas, at 800 ppmv or 1,500 ppmv, for 4 h on day 0. Behavioral tests (spontaneous alternation in the Y-maze and object recognition) were performed on days 1, 7 and 14 to assess memory alterations. The animals were then sacrificed and their brains dissected for biochemical analyses or fixed with paraformaldehyde for histology and immunohistochemistry. Results showed behavioral impairments and memory deficits, with impairments of alternation at days 1 and 7 and object recognition at day 14. Histological alterations of pyramidal neuronal layer in the hippocampus, neuroinflammatory astroglial reaction, and microglial alterations were observed. Moreover, the biochemical analyses showed a reductive stress with decreased lipid peroxidation and release of cytochrome c, leading to apoptosis with increases in caspase-9 cleavage and γ-H2AX/H2AX ratio. Finally, electrophysiological analyses using a multi-electrode array allowed the measure of the extracellular activity of pyramidal neurons in the CA2 area and revealed that exposure to the gas not only prevented the induction of long-term potentiation but even provoked an epileptoid-like activity in some neurons suggesting major alterations of synaptic plasticity. This study therefore showed that an acute exposure of mice to C_4F_7N gas provoked, particularly in female animals, memory alterations and brain toxicity characterized by a reductive stress, microglial toxicity, loss of synaptic plasticity and apoptosis. Its use in industrial installations must be done with extreme caution.

Introduction

Sulfur hexafluoride (SF_6) gas has been widely used since the fifties in high and medium voltage electrical equipment as insulating and/or breaking medium without any issue regarding toxicity or flammability, particularly. Despite its interesting properties, the gas contributes to the greenhouse effect. Its global warming potential (GWP) is 23,500, meaning that 1 Kg of SF_6 has a similar effect than 23.5 Tons of CO_2 when it is released in the atmosphere. For this reason, many studies were launched about origin natural gases (air, CO_2, N_2, O_2…) and fluorinated gases which can be used alone or in mix with natural origin gases. Some fluorinated alternative candidates were identified [Berouad & Haddad, 2017; Kosse et al., 2017; Xiao et al., 2018] to replace SF_6, including fluoroketone ($C_5F_{10}O$), fluoronitrile (C_4F_7N), trifluoroiodomethane (CF_3I), or hydrofluoroolefins like 1,3,3,3-tetrafluoropropene (HFO-1234ze, $C_3H_2F_4$).

2,3,3,3-Tetrafluoro-2-(trifluoromethyl)-propanenitrile (fluoronitrile, C_4F_7N, CAS number 42532-60-5), despite its high GWP of 2,300, is one of the most promising candidates to replace SF_6 in electrical equipments. In medium voltage, when the pressure inside apparatus in lower than 1.5 bar abs, C_4F_7N mixed with air or CO_2 is better than SF_6 for dielectric properties and close to SF_6 for arc quenching. In high voltage, when the pressure inside apparatus in higher than 6 bar abs, C_4F_7N mixed with CO_2 and O_2 enables to have comparable dielectric and arc quenching performances to SF_6.

Whatever the electrical equipment, medium or high voltage, the partial pressure of fluoronitrile inside the equipment is the same. This value depends on the minimum operating temperature of the equipment. For instance, the partial pressure of C_4F_7N inside a switch having a minimum operating temperature of -25°C is 0.475 bar at 20°C.

The acute toxicity of C_4F_7N gas, pure or in gas mixtures, after breaking (presence of many by-products) was studied by many authors. The LC_{50} (4 h) value depends on the animal species, rats or mice, and on the volume of gas inside the switch. In case of breaking

in a small volume of gas, the LC_{50} (4 h) value of by-products can be lower than 0.02% for mice [Preve et al., 2019] in presence of perfluoroisobutene (PFIB).

The aim of the present study was therefore to analyze the toxicity induced in mice after a single exposure to C_4F_7N gas, at different concentrations (800 ppmv and 1,500 ppmv), between 24 h and 2 weeks after exposure. Male and female Swiss mice were exposed to breathable air (control) or C_4F_7N gas for 4 h on day 0. Behavioral tests, namely spontaneous alternation in the Y-maze and the novel object test, were performed on days 1, 7 and 14 to assess alteration of memory. The animals were then sacrificed and their brains dissected for biochemical analyses or fixed with paraformaldehyde for histology and immunohistochemistry. The biochemical analyses were done in the hippocampus or cortex, structures involved in memory, by colorimetric tests, western blotting and commercial ELISA kits. Markers of different aspects of toxicity were quantified: lipid peroxidation and release to the cytosol of mitochondrial cytochrome c for oxidative stress; pro-inflammatory cytokines, IL-6 and TNFα, and markers of astroglial (GFAP) or microglial (Iba-1) reactions for neuroinflammation; level of expression of the anti- or pro-apoptotic proteins Bax and Bcl-2, the pro- and cleaved forms of caspase-9, and H2AX and γ-H2AX. Morphological analyzes in the hippocampus focused on cell loss in the pyramidal neuron layers and neuroinflammation using GFAP or Iba-1 immunostaining for the astroglial or microglial reaction, respectively. Finally, electrophysiological analyses using a multi-electrode array allowed the measure of the extracellular activity of pyramidal neurons in the CA2 area and particularly their ability to establish long-term potentiation as well as synaptic facilitation. Data showed that an acute exposure to C_4F_7N gas indeed provoked in mice memory alterations and brain toxicity characterized by reductive stress, microglial toxicity, loss of synaptic plasticity and apoptosis/parthanatos.

Material and Methods

Animals

The male and female Swiss CD-1 mice (RjOrl: SWISS) originated from Janvier (Le Genest-Saint-Isle, France). They were 7 weeks old from the start of the experiments. The animals were housed in groups of 6 individuals in plastic cages, with free access to food and water, in a regulated environment (23 ± 1°C, 40-60% humidity, 12 h/12 h light/night cycle.The animal testing procedures complied with the European Union directive 2010/63 and the ARRIVE directives [Kilkenny et al., 2010]. They have been authorized by the National Ethic Committee (Paris).

Gas exposure

Poisoning with 2,3,3,3-tetrafluoro-2-(trifluoromethyl)-propanenitrile gas (C_4F_7N) (PM Invest, supplied by Schneider) was carried out by S3F Chimie company (Grabels, France), which prepared tanks of C_4F_7N gas mixture in breathing air, installed at the animal center of the Faculty of Pharmacy of Montpellier. These mixtures were made using a vacuum manifold fitted with manometers allowing additions by pressure. The bottles were then heated to 65°C in an oven for 48 h to speed up the homogenization of the contents. The different concentrations of C_4F_7N in breathing air prepared for this study were 800 ppmv and 1,500 ppmv. The two mixtures were assayed by FTIR (Fourier Transformed InfraRed) spectroscopy before and after exposure in order to precisely quantify the concentrations of inhaled gas, that were, respectively, in the 827 ppmv and 1,531-1,555 ppmv ranges (see Supplementary Table 1). Male and female Swiss mice 7-10 weeks old were exposed to breathable air (control) or C_4F_7N gas on day 0 per group of 6 animals. The experiments were carried out on several batches for each condition to reach n = 12 or 24 per treatment group. On day 1, 7 and 14, animal weight was monitored.

Spontaneous alternation test in the Y-maze

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The maze was in gray PVC. Each arm was 40 cm long, 13 cm high, 3 cm wide at the bottom, 10 cm wide at the top and converged at an equal angle. Each mouse was placed at the end of one arm and was allowed to move freely through the maze for an 8 min session. The series of arm entries, including possible returns in the same arm, were visually recorded. An alternation was defined as the successive entry into three different arms. The maximum number of possible alternations therefore corresponded to the total number of entries in the arms minus two and the percentage of alternations was calculated as: real alternations / maximum number of possible alternations x 100. The parameters analyzed are the percentage of alternation (memory index) and the total number of arm entries (exploration index) [Maurice et al., 1994, 1996; Meunier et al., 2006, 2013; Rodriguez Cruz et al., 2017; Couly et al., 2021]. The *a priori* exclusion criteria were: number of arm entries <10 or alternation percentage <20% or > 90%. Data from animals not meeting these criteria were excluded from the calculations (attrition). The spontaneous alternation test was performed on days 1, 7 and 14.

Novel object test

Recognition memory was analyzed using the novel object test, according to previously published protocols [Rodriguez Cruz et al., 2017; Couly et al., 2021]. The mice were placed individually in a square arena of 50 x 50 cm^2. The test was performed in 3 sessions with an inter-session time interval of 1 h. In session 1 (open-field exploration), the animals were allowed to acclimatize to the arena for 10 min. In session 2, two identical objects were placed at ¼ and ¾ of a diagonal of the arena. The activity of the mouse and the position of the nose were recorded for 10 min (Nosetrack® software, Viewpoint, Lissieu, France). The number of contacts with the objects and the duration of the contacts were measured. In session 3, the object in position 2 was replaced with a new object of different color, shape and texture. Mouse activity was recorded for 10 min and analyzed. A preferential exploration index was calculated as the ratio of either the number or duration of contacts with the object in position 2 over the total number or duration of contacts with the two objects. Animals showing no

contact with an object or less than 10 contacts with objects, during session 2 or 3, were excluded from the calculations. The test of the new object was carried out on Day 1, 7 and 14.

Measurement of lipid peroxidation

Female mice were sacrificed by decapitation on the indicated days after the injections and the brains were quickly removed, the cortex was dissected, weighed, frozen in liquid nitrogen and stored at -80°C until analysis. After thawing, the cortex was homogenized in cold methanol (1/10 w/v), centrifuged at 1,000g for 5 min and the supernatant was collected. The homogenate was added to a solution containing 0.25 mM $FeSO_4$, 25 mM H_2SO_4, 0.1 mM orange xylenol and incubated for 30 min at room temperature. The absorbance was measured at 580 nm ($A_{580}1$), and 10 µl of 1 mM cumene hydroperoxide (CHP) was added to the sample and incubated for 30 min at room temperature, to determine the maximum level of oxidation. Absorbance was measured at 580nm ($A_{580}2$). The level of lipid peroxidation was determined in CHP equivalents according to: $CHP_{eq} = A_{580}1 / A_{580}2$ x [CHP (nmol)] x dilution, calculated as CHP_{eq} per weight of wet tissue, and presented as a percentage of the value of the control group.

Release of cytochrome c from the mitochondria to the cytosol

Female mice were sacrificed on the indicated days after the injections and the cortex was quickly dissected on ice and kept at -80°C until use. The cortex was homogenized with a piston grinder in ice-cold homogenization buffer (250 µM sucrose, 10 mM HEPES, pH 7.4), including a cocktail of protease inhibitor and phosphatase (Roche Diagnostics, Meylan, France) in a final volume of 500 µl. The homogenates were centrifuged at 600 g for 5 min and the supernatant was collected and centrifuged again at 10,300 g for 20 min. The supernatant, corresponding to the cytosolic fraction, and the pellet, corresponding to the crude mitochondrial fraction, were separated. The mitochondrial fraction was resuspended in 100 µl of ice cold isolation buffer (250 mM mannitol, 5 mM HEPES, 0.5 mM EGTA, pH 7.4).

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The proteins, 30 µg per lane, were resolved on a 12% SDS-polyacrylamid gel and transferred to a polyvinylidene fluoride (PVDF) membrane (GE Healthcare, Orsay, France). After 1 h of blocking in 5% skimmed milk powder in phosphate buffered saline (PBS) pH 7.5 containing 0.1% Tween-20 (PBS-T), the membranes were incubated overnight at 4°C with the primary antibodies: mouse anti-cytochrome c (dilution 1/1,000; ab13575, Abcam) or anti-oxphos subunit I of mouse complex IV (Oxphos, 1/1,000; ab14705, Abcam). After 3 washes of 10 min, the membranes were incubated for 1 h at room temperature with the corresponding secondary antibody, conjugated with goat anti-mouse IgG peroxidase (1/2000; ab6789, Abcam). Immunoreactive bands were visualized with ECL chemiluminescence reagent (Millipore, Molsheim, France) using an Odyssey Fc fluorescent imaging system (Li-Cor, Eurobio, Courtaboeuf, France). The intensity of the peroxidase activity was quantified using the Odyssey Fc software (Li-Cor).

Toxicity marker analyses using ELISA kits

The contents of TNF-α, interleukin-6 (IL-6), Iba-1, Bax and Bcl-2 were analyzed by ELISA tests. The commercial kits used are detailed in the Supplementary Table 2. For n = 6 animals per group, both hippocampi were used. The tissue was homogenized after thawing in 500 µl of RIPA buffer and sonicated on ice for 2 times 10 s. After centrifugation (10,000 *g*, 5 min, 4°C), the supernatants were then aliquoted and stored at -80°C and used within one month for ELISA assays according to the manufacturer's instructions. For each test, the absorbance was read at 450 nm and the sample concentration was calculated using the standard curve. The results are expressed in ng of marker per mg of protein and in % of the control value.

Protein concentration measurement

Protein quantification was performed with a protein assay kit (Pierce BCA, ThermoScientific) to assess extraction performance and allow normalization. Standard solutions were prepared by serial dilution from a stock solution of 2 mg/ml bovine serum albumin in MilliQ water. Twenty-five µl of each standard was added to a 96-well plate. The samples (8.4 µl) were

diluted in the plate 1/6 in 41.6 µl of bidistilled water. Then, serial dilutions(1/12, 1/24, 1/48) were made for each sample. The working reagent was added, 200 µl/well (reagent A + reagent B according to a 50/1 ratio), then incubation at 37°C for 30 min. Absorbance was measured at 562 nm.Total protein concentrations were then calculated from standard curve dilutions and served to normalize western blot results.

Measurement of Caspase-9 and histone γ-H2AX / H2AX of the cortex

Following the determination of the protein contents of the cortex of female mice, 30 µg per route, were resolved on a 12% SDS-polyacrylamid gel and transferred onto a membrane made of PVDF. After 1 h blocking in 5% skimmed milk powder in PBS-T, the membranes were incubated overnight at 4°C with the primary antibodies: rabbit anti-caspase-9 (dilution 1/1000; # 9506, Cell Signaling), rabbit anti-histone H2AX (1/5,000; ab11175, Abcam) and anti-γ-H2AX (phospho S139) from rabbit (1/5,000; ab11174, Abcam). After 3 washes of 10 min, the membranes were incubated for 1 h at room temperature with the corresponding secondary antibody,conjugated with goat anti-rabbit IgG peroxidase (1/2,000; ab6721, Abcam). Immunoreactive bands were visualized with ECL chemiluminescence reagent (Millipore, Molsheim, France) using an Odyssey Fc fluorescent imaging system (Li-Cor, Eurobio, Courtaboeuf, France). The intensity of the peroxidase activity was quantified using the Odyssey Fc software (Li-Cor).

Brain fixation and slicing

On day 15, 6 mice of each condition were anesthetized intraperitoneally with 200 µl of a premix of ketamine (80 mg/kg) and xylazine (10 mg/kg) and infused transcardiac with 50 ml of saline followed by 50 ml of Antigenfix (Diapath). The samples were stored for 48 h after fixation in the antigenfix solution, at +4°C. The brains were immersed in 30% sucrose PBS and sliced within one month.

Each brain was cut into an area comprising the cortex, the nucleus basalis magnocellularis and the hippocampal formation, between Bregma +1.80 and Bregma -2.80

according to the stereotaxic coordinates of the mouse brain [Paxinos & Franklin, 2004]. Serial frozen coronal sections (25 μm thick) were cut with a freezing microtome (Microm HM 450, Thermo Fisher), collected in a 24-well plate and stored in a cryoprotectant at -20°C. The slices were then made on slides, each containing three coronal sections of a mouse.

Quantification of viable neurons in CA1-3 areas of the hippocampus

Sections were stained with 0.2% cresyl violet reagent (Sigma-Aldrich), then dehydrated with graduated ethanol, treated with xylene and mounted with Mountex medium (BDH Laboratory Supplies). After mounting, the slides were stored at room temperature for 24 h of drying. Examination of the CA1 area was performed using scanned slices using a Nanozoomer virtual microscopy system (Hamamatsu, Massy, France). The measurement of the thickness of CA1, CA2, CA3 and the number of pyramidal cells were analyzed using a 20x objective with the cell count macro of ImageJ v1.46 (NIH) software. The data were expressed as the average number of viable cells per mm², 4-6 hippocampi for each mouse and 4-6 mice per condition, according to the method previously reported [Villard et al., 2009; Rodriguez Cruz et al., 2017; Maurice et al., 2019].

Immunohistochemical labeling of microglia and astrocytes

For immunohistochemical labeling, slices in a 24-well plate were incubated overnight at +4°C with polyclonal rabbit anti-Iba-1 (1/250, 019-19741, Wako) and l mouse monoclonal anti-GFAP (1/400, G3893, Sigma-Aldrich). Then, the slices were incubated for 1 h at room temperature with secondary anti-rabbit Cy3 (1:/400) and anti-mouse secondary antibodies 488 (1/1,000). The slices were incubated for 5 min with 10 μg/ml DAPI and rinsed with PBS. Finally, the slices were mounted with ProLong (ThermoFischer). Images of each slice were taken with a confocal microscope (Leica SPE).

Functional analysis of neurotransmission by electrophysiology

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Experiments were carried out on freshly prepared hippocampi (250 µm) obtained from female mice aged 10 weeks. After decapitation, the brains were quickly dissected and placed in ice-cold cutting buffer containing 2.5mM KCl, 25mM $NaHCO_3$, 1.2mM NaH_2PO_4, 0.5mM $CaCl_2$, 7mM $MgSO_4$, 11mM glucose and 234mM sucrose (infused with O_2/CO_2, 95%/5%). The slices were then prepared using a vibratome (Microm HM 650 V) and kept at 25°C for at least 1 h before electrophysiological recording in KREBS buffer (124 mM NaCl, 2.2 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, 2 mM $MgSO_4$, 10 mM glucose and 10 mM HEPES). This latter buffer was used for the recordings.

For electrophysiological recordings, the slices were transferred to an MEA (MEA60; Multi Channel Systems, Reutlingen, Germany) continuously perfused with the extracellular medium described above (flow rate 1-2 ml/min) and maintained at 32°C. The MEA consists of 60 extracellular electrodes. The inter-electrode distance was 200 µm. Each individual electrode in the array can be used either to record or to stimulate. A nylon mesh was positioned over the wafer to achieve satisfactory electrical contact between the surface of the wafer and the electrode array. Biphasic stimulation (-300 µA to 300 µA) was delivered by an external stimulator (STG-1004; Multi Channel Systems) in the Schaffer collateral pathway and recordings were made in the CA1 area of the hippocampus. Basal neurotransmission was demonstrated by stimulations spaced 15 s apart. In order to achieve a long-term potentiation (LTP) process, a protocol of 100 Hz for 1 s was applied to the wafer. In addition, to evaluate the facilitation of the slice, a paired-pulse protocol was applied. This consisted of two stimuli with an intensity ranging from -300 µA to + 300 µA, separated by an interval ranging from 25 ms to 500 ms. Postsynaptic field excitation potentials (fEPSP) were then recorded by all remaining electrodes in the array at the same time. The signals were recorded and analyzed (MC Rack; Multi Channel Systems).

Statistical analyses

Statistical analyses were performed using Prism v9.1 (GraphPad Software, San Diego, CA, USA). Parametric data were analyzed by one-way or two-way analysis of

variance (ANOVA, F value), followed by a Dunnett's test or by a Student's t-test (detailled in the Supplementary Table 3). One-sample analyses were performed by one-column t-test $vs.$ the baseline value (0% or 50%). The levels of statistical significance considered were: $p<0.05$, $p<0.01$ and $p<0.001$.

Results

Effects of an exposure to C_4F_7N on the behavior of male and female Swiss mice

Male and female mice were exposed to a concentration of 1,500 ppmv of C_4F_7N gas, or breathable air, during 4 h on day 0. A lower (800 ppmv) concentration was added during the course of the study as female seemed to be more sensitive to the gas. The mouse weights were easured on day 0 and before each behavioral analysis session, *i.e.*, on days 1, 7 and 14. Control male or female animals regularly gained weight during the 2 weeks (Fig. 1a,b). C_4F_7N gas-exposed animals loosed 7% to 10% of their initial weight 24 h after exposure, whatever the gender and dose (Fig. 1a,b). Female exposed to the lower concentration regained then weight (Fig. 1b), but not male or female exposed to the higher concentration (Fig. 1a,b). This observation suggested long term impacts of gas exposure on the general condition of the animals.

Animals were first tested for spontaneous alternation in the Y-maze. The alternation percentage is considered as a rapid, predictive test measuring spatial working memory [Sarter et al., 1988; Maurice et al., 1994]. The total number of arms entered is an index of exploration. We also analyzed attrition, since some mice deceased after exposure to the gas and some were not able to perform the test, performing less than 10 arm entries during the 8-min session or showing alternation percentages <20% or >90%. The alternation percentage decreased after gas exposure at 1,500 ppmv and appeared non-significantly different from the random choice value (50%), on day 1 in male mice (Fig. 2a) and on day 7 for female mice (Fig. 2b). The exploratory behavior was altered 24 h after gas exposure, in male and female mice and whatever the concentration in female (Fig. 2c,d). At longer timepoint, mice showed similar number of arm entries as control animals. Noteworthily, the highest gas concentration led to a considerable attrition, for both gender at day 14. Indeed, deceased mice or mice unable to correctly perform the task represented until 50-60% of the groups (Fig. 2e, f).

Mice were then submitted to an object recognition test. The procedure includes 3 sessions. In session 1, mice are placed individually in an empty square arena and their mobility analyzed during 10 min for distance traveled, immobility, walking speed and presence in the center (thigmotaxis) (Fig. 3). One hour after this open-field session, 2 similar object were placed in the arena and the mouse interactions with each object were analyzed during 10 min, by videotracking in terms of number and duration of contacts (Suppl. Fig. 1). After 1 h, 1 familiar object were replaced by a novel one and mouse exploration analyzed (Fig. 4).

During the first, open-field, session, we observed that the mouse mobility was significantly decreased, mainly at day 14, in male and female mice (Fig. 3a,b), with significant decreases in walking speed as comapred with control groups, at different timepoints (Fig. 3c,d). Immobility profiles were unchanged in male gas exposed mice as compared to controls (Fig. 3e) but decreased in female mice exposed to 1,500 ppmv at day 7, as compared to controls (Fig. 3f). Control mice tended to increase their presence in the center of the arena with time (Fig. 3g,h). Such trend was not observed for gas exposed mice and significant decreases appeared at day 14 for males (Fig. 3g) and females at the lowest concentration of gas (Fig. 3h), suggesting an increased anxiety status. During session 2, male gas-exposed mice showed a significant alteration of object exploration 24 h after exposure (Suppl. Fig. 1a). Female responses and balanced exploration of each object was unaltered. During session 3, the decrease in the number of contact was still observed for male gas-exposed mice (Fig. 4a) and an increase was noted for female exposed to 800 ppmv on day 7 (Fig. 4b). All groups but one showed a significant preferential exploration of the novel object, when data were analyzed in terms of contact (Fig. 4c,d) or duration of contacts (Fig. 4e,f). Indeed, female mice exposed to the highest concentration showed a progressive decrease of the scores with no statistical difference from 50% at day 14 (Fig. 4d,f).

These observations thus revealed general impact on body weight, mobility, anxiety, spatial working memory and recognition memory in gas-exposed mice between 24 h to 14 days after exposure.

Neurotoxicity induced by exposure to C_4F_7N in Swiss mice

We then analyzed several parameters of neurotoxicity in the brain of mice exposed to 1,500 ppmv of C_4F_7N gas. First, the pyramidal neuronal layer of the hippocampus, a key structure for memory, was analyzed. The number of viable cells and the layer thickness were determined in the CA1, CA2 and CA3 areas for male (Fig. 5a-c) and female mice (Fig. 5d-f). In males, results showed a 6% decrease in cell number in CA1 (Fig. 5b) and decreases of 28% and 23% of the thickness of CA1 and CA2, respectively (Fig. 5c). In females, significant reduction in the number of viable cells were noted in all CA1-3 areas (-32%, -28%, -19%, respectively; Fig. 5e). Cell layer thickness was slightly increased in CA1, CA3 and significantly in CA2 (Fig. 5f). These alterations were important, more marked in females, and suggested an important toxicity with putatively major impact on functional hippocampal circuitry.

The oxidative status was analyzed using several markers. First, lipid peroxidation, an indirect but stable indicator of the oxidative status of brain tissue, was analyzed in cortex extracts from female mice exposed to 1,500 ppmv of C_4F_7N. A very significant 64% diminution of lipid perxidation was measured, suggesting no oxidative stress but rather a major reduction status of the brain tissue.

Alteration of mitochondrial integrity can be measured by the levels of cytochrome c, a cofactor for several enzymes of the mitochondrial respiratory chain, that is, in pathological condition, no longer stabilized at the inner membrane of the mitochondria and released into the cytosol. After subcellular fractionation to separate the cytosolic and mitochondrial fractions, cytochrome c was assayed by western blot (Fig. 6b). OXPHOS labeling was performed in parallel to confirm the quality of the subcellular separation, this marker being selective and specific for mitochondria (Fig. 6b). Exposure to C_4F_7N gas only moderately

affected cytochrome c expression levels in cytosolic (Fig. 6c) and mitochondrial fractions (Fig. 6d) in the hippocampus of mice exposed to 1,500 ppmv, but the cytosol / mitochondria ratio increased significantly by 33% (Fig. 6e), which indicated a significant impairment of mitochondrial integrity.

The expression level of caspase-9, a cellular protease which activation signal originates in the mitochondria, were measured by western blot in the mouse hippocampus, for its precursor, *i.e.*non-cleaved form, and active, cleaved form (Fig. 6b).Exposure to C_4F_7N gas did not affect pro-caspase-9 levels (Fig. 6f), but very significantly increases the cleaved form (+92%; Fig. 6g) and therefore the cleaved/pro ratio, which increase was close to reach statistical significance (+79%; Fig. 6h). Both parameters denoted a major impact of the gas-exposure on mitochondrial integrity in brain tissue.

Neuroinflammation was first measured in female mice using immunofluorescence analyses of microglial and astroglial reactions in brain coronal sections particularly comprising the hippocampus and cortex. The astroglial reaction was measured using a GFAP immunostaining (Fig. 7) and the microglial reaction was visualized using an Iba-1 immunostaining (Fig. 8). Cell density was counted directly in three sub-regions of the hippocampus: the stratum radiatum (Rad), the molecular layer (Mol) and the polymorphic layer of the dendate gyrus (PoDG). One region was analyzed in the cortex, the lateral parietal associative cortex (LPtA). Exposure of mice to C_4F_7N gas induced significant, concentration-dependent increases in GFAP immunostaining in PoDG (Fig. 7a,b), Mol (Fig. 7c,d) and Rad (Fig. 7e,f), indicative of a marked astrogliosis reaction and therefore of neuroinflammation. However, the gas-exposure provoked, on the contrary, concentration-dependent decreases in the number of microglial cells: -21% in PoDG (Fig. 8a,b), -19% in Mol (Fig. 8c,d) and -16% in Rad (Fig. 8e,f) and -29% in the cortex (Suppl. Fig. 2). This alteration signed a specific alteration of microglia.

In order to confirm neuroinflammation, ELISA analyses of different cytokines were performed in hippocampus extract of mice exposed to 1,500 ppmv of C_4F_7N (Fig. 9a-c). Gas-exposure resulted in a non-significant decrease in the levels of interleukin-6 in the

hippocampus by 18% (Fig. 9a), but in a very significant decrease in TNF-α levels of 56% (Fig. 9b). The cytokine is massively released by the microglial cells. Coherently, tissue levels of the microglial marker Iba-1 were also significantly reduced by 53% (Fig. 9c). These observations confirmed the immunofluorescence analysis and indicated a major toxicity of the gas-exposure affecting specifically microglial cells.

Neurotoxicity could also involve apoptosis and DNA double strand breaks. We analyzed the levels of pro- and anti-apoptotic proteins Bax and Bcl-2. The gas-exposure did not change the tissue levels in Bax (Fig. 9d) nor Bcl-2 (Fig. 9e). The Bax/Bcl-2 ratio was therefore unchanged (Fig. 9f), suggesting no apoptotic component.

H2Ax is one of several genes encoding histone H2A. H2Ax contributes to the formation of nucleosomes and, therefore, to the structure of DNA. It is phosphorylated at the level of serine 139, then called γ-H2Ax, in reaction to double-strand breaks in DNA. It is therefore a marker of cell death by parthanatos. Exposure to C_4F_7N gas only moderately affected the expression levels of H2Ax (Fig. 9g,h) or its phosphorylated form (Fig. 9g,i) in the hippocampus of mice exposed to 1,500 ppmv, but the γ-H2Ax/H2Ax ratio was significantly increased by 61% (Fig. 9j), which indicated a noticeable increase in the activity of the DNA repair process and therefore an increase in double-stranded lesions.

Functional impact of the exposure to C_4F_7N gas on hippocampal plasticity in Swiss mice
A neuron generates and propagates an electrical signal, then transmitted to neighbour cell through a synapse, releasing neurotransmitters and triggering an amplified reaction of the signal. The intensity of the signal varies with neuronal activity, sustaining cellular plasticity. This phenomenon occurs particularly during learning and memory formation. It translates memorization at the cellular level. It is measured directly by electrophysiological analyzes of the activity of neurons on slices of the brain. Using a multi-electrode assay, we measured paire-pulse facilitation and long-term potentiation (LTP) in hippocampus slices from control and gas-exposed female mice (Fig. 10). We observed that the paire-pulse facilitation, measured in control animals, was absent in gas-exposed mice (Fig. 10a). Analysis of the

impact of inter-pulse time intervals (Fig. 10b) showed a bell-shaped curve with a maximum effect at 100 ms for controls and an absence of effect for gas-exposed mice. Analysis of the LTP (Fig. 10c,d) showed a complete absence of plasticity in gas-exposed animals. Moreover, the recording of these facilitation and PLT processes led to the observation of electrical hyperactivity in brain slices of 1,500 ppmv C_4F_7N mice (Fig. 10e). Gas-exposure gas therefore provoked a major alteration in the functional activity of neurotransmission by hypersensitizing synaptic cell communication to an "epileptoid" type activity.

Discussion

In the present study, we report that acute (4 h) exposure of Swiss mice to C_4F_7N gas, a standard laboratory animal model, at two concentrations (800 and 1,500 ppmv) provoked physical and behavioral impairments and neurological toxicity. The selected gas concentrations were expectedly far beyond the claimed LC_{50} in rats (15,000-20,000 ppmv) [Li et al., 2019]. However, a recent report by Zhang et al. [2020] determined much lower LC_{50} values of 1,175 ppmv in male and 1,380 ppmv in female BALB/c mice after a 4 h exposure, which is much closer to the concentrations used in the present study. However, BALB/c is an immunodeficient inbred line and LC_{50} values are likely much lower than values that can be expected in the vigourus outbred Swiss line used in our study, for which data are not available. Interestingly, both male and female mice were examined and sex-related differences in the intensity of the alterations were observed, female animals appearing more sensitive to C_4F_7N gas intoxication. This is opposite to the observation of Zhang et al. [2020] who concluded that female mice were more tolerant to C_4F_7N gas than the males by measuring a slightly higher LC_{50} value [Zhang et al., 2020].

As soon as 24 h after exposure, mice lost 5-10% of body weight and did not recover at day 7 or 14 after exposure at the high dose tested. Mice showed hypolocomotion at day 1, evidenced in the open-field test and during Y-maze exploration and decreased walking speed in the open-field particularly at day 14. Moreover, a very high percentage of attrition was observed at day 14, with intoxicated animals dying, showing a redhibitory weight loss (>20% of their initial weight at day 0), or unable to explore sufficiently the Y-maze (*i.e.*, not reaching the exclusion criterion of a minimum of 10 arm entries during the 8-min session). These observations denoted a global impairment of the general status of the animals that appeared quickly after gas exposure and lasted for at least 2 weeks, leading to the important level of attrition observed.

The neurological alterations culminated as learning and memory impairments and alterrations of the anxiety response. Indeed, male and female mice showed decreased

alternation percentage in the Y-maze, at day 1 for males and days 1 and 7 for females. Females, but not males, also showed recognition memory deficits in the novel object test at day 14. Moreover, the presence in the center of the open-field, the most anxiogenic place of the arena [Kelly, 1993], was significantly decreased for both male and female mice particularly at days 1 and 14. These observations identified neurologic alterations affecting cognitive functions of the animals and led us to explore at the biochemical and morphological levels the toxicity likely affecting the brain structures and fonctionality.

The hippocampus is a brain structure of major importance in memory [Howland & Wang, 2008; Mancini et al., 2022] and anxiety [Ghasemi et al., 2021; Park et al., 2022]. We first examined the number of viable neurons in hippocampal layers by staining sections of the brain with the viable cell dye cresyl violet. The morphological examination showed that exposure to C_4F_7N gas produced, particularly in female mice, damages to the pyramidal neuronal layers of the hippocampus, with approximately 30% decreases in the number of cells in the CA1 and CA2 areas and a 40% increase in the width of the CA2 area. These alterations were therefore very significant and more marked in female than in male mice. They suggested that a major excitotoxicity occured during gas exposure with expectedly major functional damages to the hippocampal connections.

Functional analyses using an electrophysiological approach has indeed shown, in female mice 14 days after exposure to C_4F_7N gas at 1,500 ppmv, an inability of the hippocampal neuronal network to induce long-term potentiation or even paired-pulse facilitation in the analyzed slices. This meant that neurotransmission was very disturbed in this structure considered as is a reference structure for its complex neuroanatomical organization and its major role in learning and memory [Hayashi, 2022; Mancini et al., 2022]. Moreover, the recording of electrophysiological responses allowed the observation of a clear electrical hyperactivity of the slices. Exposure to C_4F_7N gas produced a major alteration in the functional activity of neurotransmission by hypersensitizing synaptic cell communication resulting in "epileptoid-like" activity. The extra population spikes generated were very close to what could observed with the application of very high concentration of choline, for instance

[Motin, 2011], and are likely to result from NMDA receptor over-activation, thus confirming an important excitotoxic process after C_4F_7N gas exposure.

The biochemical examination of different markers in the hippocampus or cortex of the mice first showed several interesting features. First, the levels of lipid peroxidation was reduced suggesting marked reductive stress. Indeed, exposure to gas produced a very significant 64% decrease in membrane lipid peroxidation levels in the cortex of mice. Reductive stress is the counterpart oxidative stress and it occurs when physiological conditions shift towards reducing status the redox balance of major biological redox couples [Perez-Torres et al., 2017]. Such conditions could happen, for instance, when an overexpression and overactivity of the antioxidant enzymatic systems is observed, resulting in complete depletion of reactive oxidative species and related cellular signaling. Among the consequences, modifications of protein and membrane lipids, alteration of mitochondrial function and decreased cellular metabolism are observed [Perez-Torres et al., 2017]. ~~Among the pathological conditions leading to reductive stress is inflammation and~~ we particularly observed in the brains of the mice exposed to C_4F_7N, mitochondrial damage together with alterations in astroglial and microglial cells and reactivity. Mitochondrial damage was shown by increased level of cytochrome c, the cofactor for cytochrome oxidase, aka, complex IV of the mitochondrial respiratory chain, in the cytosol as compared to the mitochondria. In relation with the release of cytochrome c into the cytsol, an activation of th caspase-9 was induced as the level of its cleaved form increased, as well as the ratio between its cleaved form over the pro-enzyme level. Caspase-9 is a signaling cysteine-aspartic protease involved in apoptosis and cytokine signalling [Kuida, 2000]. It is directly cleaved from the pro-enzyme of caspase-9 into the active dimer form upon activation of apaf-1 (apoptosome) that is due to the release of cytochrome c from mitochondria, and thus generates apoptotic signals. Interestingly, exposure to C_4F_7N gas did not appreciably affect the levels of pro- or anti-apoptotic proteins of the Bax family, suggesting that the toxic processes revealed by the analyses of cellular markers proceded downstream from these markers. Indeed, a strong reductive stress is likely to markedly alter the mitochondrial homeostasis, sufficiently to

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trigger a prtapoptotic signal resulting in apoptosis, DNA breaks, as shown by the significant increase in γ-H2Ax/H2Ax levels ratio, and cell loss, as observed in the hippocampus. The toxic cascade is schematized in Fig. 11a.

One open question arising from our observations is the paradoxical effect of C_4F_7N gas inducing reductive stress although fluorine and fluorine-based gas are expected to be oxidant, fluorine being the most electronegative among halogens. Analyses aiming at determining how C_4F_7N gas diffuses and interacts with biological tissues are therefore needed to determine the pathways responsible for the reductive stress observed here after gas exposure. Such analyses were beyond the scope of the present study but deserves precise studies to fully address the toxicity induces by C_4F_7N gas.

As previously stated, reductive stress can result from inflammation. Inflammation in the brain, so-called neuroinflammation, relies the reactivity on different types of glial cells, astroglia and microglia, the brain resident macrophages. A contribution from peripheral cells is also involved as neuroinflammation rhymes with permeation/alteration of the blood-brain barrier. reactive astrocytes or microglia release numerous cytokines and inflammatory factors sustaining the global inflammatory status of the tissue. We analyzed specific glial cells markers using a morphological immunofluorescent approach, GFAP being a marker of astrocytes and Iba-1 a marker of microglia, in three areas of the hippocampal formation. All three analyses performed in the *stratum radiatum*, the *stratum moleculare* or the polymorphic layer of the dendate gyrus led to convergent observations showing that astroglial reactivity dose-dependently and significantly increased with the concentration of C_4F_7N gas while, on the contrary, microglial expression dose-dependently and significantly decreased. The decrease in Iba-1 expression was quantitavely confirmed using an ELISA assay in tissue homogenates. Interestingly, the level of interleukin-6, a cytokine released by both reactive astroglia and microglia, was unchanged in the tissue, but the level of TNFα, a cytokine mainly released by microglia, was significantly decreased by 56%. The alteration of microglia was therefore shown coherently morphologically and biochemically using Iba-1 and TNFα.

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We concluded that exposure to C_4F_7N gas induced a strong neuroinflammation but also selectively damaged microglia. The cellular impact of the gas exposure is summarized in Fig. 11b. Microglia proliferation, differentiation, and survival are regulated by different signals. For instance, as colony-stimulating factor 1 (CSF1) has been shown to play such a role, CSF1 inhibitors are currently proposed as pharmacological tools to mitigate microglia dynamics in inflammatory pathological conditions where those cells support the disease progression [Barca et al., 2021]. However, although attenuating microglial reactivity could offer a therapeutic interest in neuroinflammatory conditions, microglia alterations in physiological conditions is detrimental as these cells play a major role in maintaining the brain tissue homeostasis.

In summary, we reported major neurological alterations induced in mice by an acute (4 h) exposure to C_4F_7N gas, at 8000 or 1,500 ppmv concentration. Alterations in the general status of the animals including lethality, in the activity, memory and anxiety responses were noted at delays up to 2 weeks after exposure. The gas exposure impacted the brain tissue integrity and physiology, with defects in neurotransmission including epileptoid forms of activity, induction of neuroinflammation, reductive stress and mitochondria-driven apoptosis (Fig. 11a), leading to cellular damages in neurons and microglia (Fig. 11b).

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Legends for the figures

Figure 1. Effect of a single exposure to C_4F_7N gas on weight change in male (a) and female (b) mice. The animals were exposed during 4 h to either breathable air (control) or two doses of gas: 800 ppmv (females only) or 1,500 ppmv. They were weighed before exposure (day 0) and then on day 1, 7 and 14. Data are calculated in variation vs. day 0 and expressed as mean ± SEM. ^{oo} $p<0.01$, ^{ooo} $p<0.001$ *vs.* 0; one-column$t$-test. *** $p<0.001$ *vs.* control group on the same day; Student's t-test.

Figure 2. Effect of a single exposure to C_4F_7N gas on spontaneous alternation performances of (a,c,e) male and (b,d,f) female mice, in the Y-maze test (a,b) alternation performance; (c,d) number of entries in the arms; (e,f) percentage of attrition. The animals were exposed for 4 h on day 0, either to breathable air for the control animals or to C_4F_7N gas, 800 ppmv (females only) or 1,500 ppmv. The data show the mean ± SEM in (a-d). ^{o} $p<0.05$, ^{oo} $p<0.01$, ^{ooo} $p<0.001$ *vs.* 50% level in (a,b) or *vs.* 0 in (e,f); one-column$t$-test. ** $p<0.01$ vs. control group; Dunnett's test in (c,d).

Figure 3. Effect of a single exposure to different doses of C_4F_7N gas on open-field exploration. (a,c,e,g) Male and (b,d,f,h) female mice were free to explore, for 10 min on days 1, 7 and 14, a squared empty arena: (a,b) distance traveled; (c,d) walking speed; (e,f) duration of inactivity; (g,h) time in the center of the arena. The animals were exposed for 4 h on day 0 to either breathable air (control) or to C_4F_7N gas, 800 ppmv or 1,500 ppmv. Data show the mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* control, Dunnett's test.

Figure 4. Effect of a single exposure to C_4F_7N gas on recognition memory: session 3 of the novel object test. (a,c,e) Male and (b,d,f) female mice mice interacted with a familiar and a novel object: (a,b) number of contacts with the objects and (c,d) preference for the novel

object calculated in number of contacts or (e,f) in duration of contact. The data shows the mean ± SEM. * p<0.05, ** p<0.01 *vs.* control on the same day; Dunnett's test in (a, b).^{o} *p*<0.05, ^{oo}*p*<0.01, ^{ooo}*p*<0.001 *vs.* 50%; one-column*t*-test in (c-f).

Figure 5. Effects of a single exposure to C_4F_7N on cell loss in the CA1, CA2 and CA3 layers of hippocampal pyramidal neurons in (a-c) male and (d-f) female mice, by cresyl violet staining. (a,d) Typical histological images and quantification of (b,e) the number of viable cells and (c,f) cell layer thickness. 3-6 slices were counted per animal. * *p*<0.05, ** *p*<0.01, *** *p*<0.001 *vs.* control group; one-column *t*-test.

Figure 6. Effects of a single exposure to C_4F_7N on oxidative status in the cortex extracts of female mice: (a) lipid peroxidation; (b-e) release of cytochrome c into the cytosol, measured by Western blot; (b,f-h) of expression of pro-caspase-9 and of its cleaved form measured by Western blot. (b) Typical blots; (c) quantification of cytochrome c levels in the cytosolic fraction; (d) mitochondrial fraction; (e) cytosol/mitochondria ratio; (f) quantification of pro-caspase-9 expression levels; (g) quantifying the levels of expression of cleaved caspase-9; (h) c-caspase-9 / pro-caspase-9 ratio.The analyses were carried out 15 days after a single exposure to breathable gas (control) and to a dose of 1,500 ppmv for C_4F_7N. * *p*<0.05, ** *p*<0.01, Student's *t*-test.

Figure 7. Effects of a single exposure to C_4F_7N gas (control, 800 ppmv, 1,500 ppmv) on the astroglial reaction in the female mouse hippocampus by immunohistofluorescent labeling of the GFAP protein: (a,b) polymorphic layer (PoDG), (c,d) molecular layer (Mol) and (e,f) stratum radiatum (Rad). (a,c,e) Typical immunofluorescence images (blue: DAPI, green: GFAP) and (b,d,f) quantifications. Coronal sections 25 µm thick were labeled with the antibodies and the hippocampal areas were analyzed. Scale = 50 µm. * *p*<0.05, ** *p*< 0.01, *** *p*<0.001, Dunnett's test.

Figure 8. Effects of a single exposure to C_4F_7N gas (control, 800 ppmv, 1,500 ppmv) on the microglial reaction in the mouse hippocampus by immunohistofluorescent labeling of the Iba-1 protein: (a,b) polymorphic layer (PoDG), (c,d) molecular layer (Mol) and (e,f) stratum radiatum (Rad). (a,c,e) Typical immunofluorescence images (blue: DAPI, red: Iba-1) and (b,d,f) quantifications. Coronal sections 25 μm thick were labeled with the antibodies and the hippocampal areas were analyzed. Scale = 50 μm. * $p<0.05$, ** $p< 0.01$, *** $p<0.001$, Dunnett's test.

Figure 9. Effects of a single exposure to C_4F_7N gas (control, 800 ppmv, 1,500 ppmv) on: (a-c) markers of neuroinflammation IL-6, TNF-α and Iba-1;(d-f) markers of apoptosis Bax, Bcl-2 and the Bax/Bcl-2 ratio; (g-j) the expression levels of the histone variant H2Ax measured by ELISA in the hippocampus (a-f) or by western blot in the cortex (g-j) of female mice. (g) Typical blots for H2Ax; and γ-H2Ax; (h-j) quantifications of the expression levels of H2Ax, γ-H2Ax and γ-H2Ax/H2Ax ratio. The analyses were carried out 15 days after a single exposure to breathable gas (control) or to the dose of 1,500 ppmv for C_4F_7N. * $p<0.05$, ** $p<0.01$ *vs.* control value; Student's *t*-test.

Figure 10. Effects of a single exposure to C_4F_7N gas (1,500 ppmv) on synaptic plasticity of female mouse brain slices. (a) The facilitation process was elicited by two stimulations with a time interval ranging from 25 to 500 ms (control mice: N = 3; n = 5 and C_4F_7N mice 1,500 ppm: N = 2; n = 2). (b) The long-term potentiation process (LTP) was induced by stimulation of 100 Hz for 1 s on the brain slices of control mice and C_4F_7N 1,500 ppmv (control mice: N = 3; n = 3 and C_4F_7N mice 1,500 ppm: N = 2; n = 2) (c) These facilitation and LTP processes led to electrical hyperactivity of the brain slices of C_4F_7N mice 1,500 ppmv. Coronal sections 250 μm thick were recorded. *** $p< 0.001$ *vs.* control values; two-way ANOVA.

Figure 11. Summary of (a) the toxicity observed in the mouse brains after exposure to C_4F_7N gas; and (b) the impact on brain cells. In (a), the toxic signal is likely downstream from Bax/Bcl-2 signaling, impacting mitochondria homeostasis through reductive stress. The cytochrome c release into the cytosol activate the cleavage of caspase-9 and downstream effectors inducing apoptosis, DNA double-strand lesions and cell death.