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Research report

Long-term consequences of soman poisoning in mice Part 1. Neuropathology and neuronal regeneration in the amygdala

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ABSTRACT

To date, studies on soman-induced neuropathology mainly focused on the hippocampus, since this brain region is a well-delimited area with easily detectable pyramidal neurons. Moreover, the hippocampus is severely damaged after soman exposure leading to a substantial alteration of behavioral mnemonic processes. The neuropathology described in the hippocampus, however, and its behavioral consequences cannot be extrapolated to all other limbic damaged brain areas such as the amygdala.

Accordingly, in this inaugural paper, using hemalun–phloxin staining and NeuN immunohistochemistry, the number of damaged and residual healthy neurons was quantified in the amygdala in mice over a 90-day period after soman injection (1.2 LD_{50} of soman). On post-soman day 1, a moderate neuronal cell death (about 23% of the whole neurons) was evidenced. In parallel, a large quantity of degenerating neurons (about 36% of the whole neurons) occurred in this brain region and survived from post-soman day 1 to day 15. The death of these damaged neurons was initiated on post-soman day 30, and ended on post-soman day 90. Concomitantly, as quantified by NeuN immunohistochemistry, a clear neuronal regeneration was demonstrated in the amygdala of soman-poisoned mice between 60 and 90 days after neurotoxicant exposure.

In the companion paper (see part 2), the possible effects of both long-term neuropathology and delayed neuronal regeneration were evaluated on amygdala-driven emotional processes.

1. Introduction

Soman (pinacolyl methylphosphono–fluoridate) is a powerful irreversible cholinesterase inhibitor. The enzyme inhibition induces brain damage through synaptic acetylcholine accumulation and subsequent cerebral glutamate release leading to *N*-methyl-D-aspartate (NMDA) activation [28,36,30]. Most studies on soman-driven cerebral lesions reported early neuropathology development occurring within the first 48 h after neurotoxicant exposure. Brain damage was detected in all cortical areas, some subcortical limbic areas (claustrum, amygdala and hippocampus) and thalamic nuclei [31,44,37,11,2].

Lately, we investigated the evolution of cerebral lesions in the mouse hippocampal CA1 field, up to 3 months after soman exposure [14]. A massive quantity of degenerating neurons appeared in the CA1 field within the first day post-intoxication, but neuronal cell death remained restricted. These degenerating neurons survived for 1 month and eventually died between day 30 and day 90 after poisoning. Over the same period, delayed neuronal regeneration was evidenced in the hippocampal CA1 field of soman-poisoned mice [14]. Since the hippocampus is actively involved in spatial memory processes, we also performed cognitive behavioral testing (Morris water-maze and T-plus maze) on the same animal model to ascertain the influence of delayed neuronal cell death and regeneration over mnemonic performances [19]. One month after poisoning, spatial memory declined dramatically indicating that hippocampal degenerating neurons proved dysfunctional. Interestingly, however, after 3 months post-exposure, a slight recovery of the spatial memory was detected and may be due to the partial neuronal regeneration occurring in the CA1 field.

Nonetheless, the neuropathology described in the hippocampus may not be extrapolated to all other limbic damaged brain areas involved in behavioral processes, since regional differences exist in terms of neuropathological response intensity to soman-poisoning and treatment efficiency. For instance, early acute neuronal necrosis is more prevalent in the amygdala than in the hippocampus of

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soman-poisoned guinea pigs or rats [34,8]. Moreover, compared to the amygdala, the hippocampus is more easily protected by anti-NMDA drugs such as dizocilpine (MK-801) or thienylphencyclidine (TCP) [42,9].

Hence, the goal of the present study is to investigate the long-term neuropathology in the amygdala after soman exposure and to compare it with previously published data related to the hippocampus. For this purpose, the number of damaged eosinophilic- and residual healthy-neurons revealed by hemalun–phloxin (H&P) staining or neuronal nuclei antigen (NeuN) immunochemistry were quantified in the amygdala of soman-poisoned mice over a 3-month post-exposure period.

In the companion paper (see part 2), the long-term influence of soman intoxication on amygdala-driven emotional processes was also investigated using fear conditioning and fear reactivity behavioral testing.

2. Materials and methods

2.1. Soman exposure and brain sample collection

Nine-week-old adult male B6D2F1/j@rj mice (Janvier Laboratories, France) were housed in cages under standard conditions of temperature (24 °C) and humidity (50–60%), with a 12:12-h light/dark cycle and free access to water and standard laboratory chow. All the experiments in this study were reviewed and approved by the Institutional Animal Care and Research Advisory Committee in accordance with French law and the main international guidelines.

On day 0, soman (110 μg/kg in 200 μl of saline solution) – provided by the "Centre d'Etudes du Bouchet" (France) – was subcutaneously injected into the mice, followed 1 min later by an intraperitoneal injection of atropine methyl nitrate (MNA) at 5.0 mg/kg body weight (200 μl in saline). Only mice exhibiting well-characterized physical signs of convulsions were selected for the experiments, as previously described [12]. Indeed, according to numerous previous studies [29,9,2], it is commonly acknowledged that animals displaying convulsions for at least 2 h develop subsequent brain damage.

Pentobarbital (80 mg/kg) anaesthetized mice were sacrificed on days 1, 3, 8, 15, 30, 60 and 90 post-poisoning ($n = 8$ to 12 animals per experimental time from two distinct experiments) by intracardiac perfusion of saline with heparin (5 UI/ml) followed by a fixative solution of 4% formaldehyde and 3% acetic acid in saline. Brains were collected, post-fixed in 4% formaldehyde for 6 h and then processed for paraffin embedding. Coronal sections (6 μm thick) were serially cut with a microtome from −1.82 mm (hippocampus area) posterior to the bregma, as shown in the Franklin and Paxinos stereotaxic mouse brain atlas [20].

Similar conditions were applied to 9 control mice (intraperitoneal injections of MNA) but the soman injection was replaced by a subcutaneous injection of saline.

2.2. Hemalun–phloxin staining and immunochemistry

To detect eosinophilic and residual healthy cells, hemalun–phloxin (H&P) staining was performed on paraffin-embedded sections as previously described [33].

GFAP and NeuN were considered as specific markers for reactive astroglia and mature neurons, respectively. Immunochemistry procedures were previously detailed by Collombet et al. [14,16].

2.3. Quantification and statistical analysis

Quantitative evaluations of total, damaged and residual healthy cells were performed using a computerized image analysis system (SAMBA, Alcatel, France) linked to a CCD video camera-fitted microscope. For each mouse, cells were counted bilaterally in the basolateral amygdala (about 41,000 μm^2) from two non-adjacent sections and averaged. Then, for each experimental time, the mean value and its standard error (S.E.M.) were calculated for 8–12 animals (see above).

To appraise the evolution of cell density (total, degenerating or healthy cells) in poisoned mice over time, statistical comparisons were carried out with Dunnett's tests by comparing post-soman day 1 values (reference) with values from every other experimental time after intoxication. Significance was set at $p < 0.05$. All statistical comparisons were performed with Statistica 7.0 software (StatSoft, France).

3. Results

3.1. Long-term neuropathology in the amygdala of soman-poisoned mice

Using H&P staining, only intact healthy cells harboring white/violet large-rounded nuclei were detected in the amygdala of control mice (Fig. 1A). Following soman poisoning, H&P-stained eosinophilic cells appeared in the amygdala, as early as 1 day after neurotoxicant exposure (Fig. 1C). These eosinophilic cells exhibited red shrunken nuclei and emaciated dark pink cytoplasms. In addition, a massive cerebral edema was also revealed on post-soman day 1 and was characterized by the presence of numerous white spaces spread all over the amygdala area (Fig. 1C). Cerebral edema, however, was no longer present at later experimental times.

Density of total H&P-stained cells (including eosinophilic and healthy cells) was first determined. Total cell density reached 1300 ± 85 cells per mm^2 in the amygdala of control mice. One day after soman poisoning, only 1000 ± 70 total cells per mm^2 were measured in this cerebral region. This was significantly different from control value ($p < 0.05$). These results indicated that about 23% of H&P-stained cells died within the first day following soman exposure. Total cell densities at later experimental times were ranging from 1020 ± 80 to 1220 ± 60 cells per mm^2 and did not differ from the value determined on post-soman day 1.

To evaluate more precisely soman-induced neuropathology, the density of eosinophilic cells was also quantified in the amygdala. On post-soman day 1, the density of these degenerating cells raised to 470 ± 60 cells per mm^2 (Fig. 2A), representing 36% of total amygdalian H&P-stained cells. For later experimental times, eosinophilic cells were still detected, up to post-soman day 60. From post-soman day 3 to day 15, the quantity of these cells (ranging between 390 ± 30 and 430 ± 100 cells per mm^2) was similar to the one measured on post-soman day 1 (Fig. 2A). Thirty days after soman poisoning, a significant reduction of the eosinophilic cell density was initiated (210 ± 40 cells per mm^2; $p < 0.01$ compared with post-soman day 1) and was amplified at later experimental times (90 ± 20 cells per mm^2 on post-soman day 60; $p < 0.001$ compared with post-soman day 1). On post-soman day 90, most eosinophilic cells had almost disappeared and major tissue rearrangements were disclosed in the amygdala of soman-poisoned mice (Fig. 1E).

Altogether, the results clearly demonstrate the presence of two toxicity steps in the mouse amygdala after soman intoxication. The first step involves an acute toxicity occurring within the first 24 h following neurotoxicant exposure and leading to the appearance of numerous eosinophilic cells. Cell death was also observed with a loss of about 23% of H&P-stained cells. The second step, initiated on post-soman day 15 and lasting up to 3 months after poisoning, implies a delayed toxicity leading to the disappearance of almost all eosinophilic cells. Total cell density, however, remained unmodified over the 3-month period following soman injection despite the delayed loss of eosinophilic cells. This obviously implies cell regeneration in the amygdala to compensate this cell loss. Hence, the density of healthy H&P-stained cells was measured in the amygdala to validate such a cell regeneration hypothesis.

3.2. Delayed neuronal regeneration in the mouse amygdala following soman exposure

As mentioned above, the density of healthy H&P-stained cells in the amygdala of control mice amounted to 1300 ± 80 cells per mm^2 (Fig. 2B). One day after soman exposure, the density dramatically slumped to 540 ± 60 cells per mm^2 ($p < 0.001$; Fig. 2B), representing 41% of total amygdalian H&P-stained cells. This massive 59% drop in quantity of healthy cells accounted for the appearance of eosinophilic cells combined to the early cell death induced by the acute toxicity step. For later experimental times however, the density of healthy cells continuously increased over time. As shown in Fig. 2B, the density elevation became significant as early as post-soman day 8 (830 ± 40 cells per mm^2; $p < 0.05$ compared with

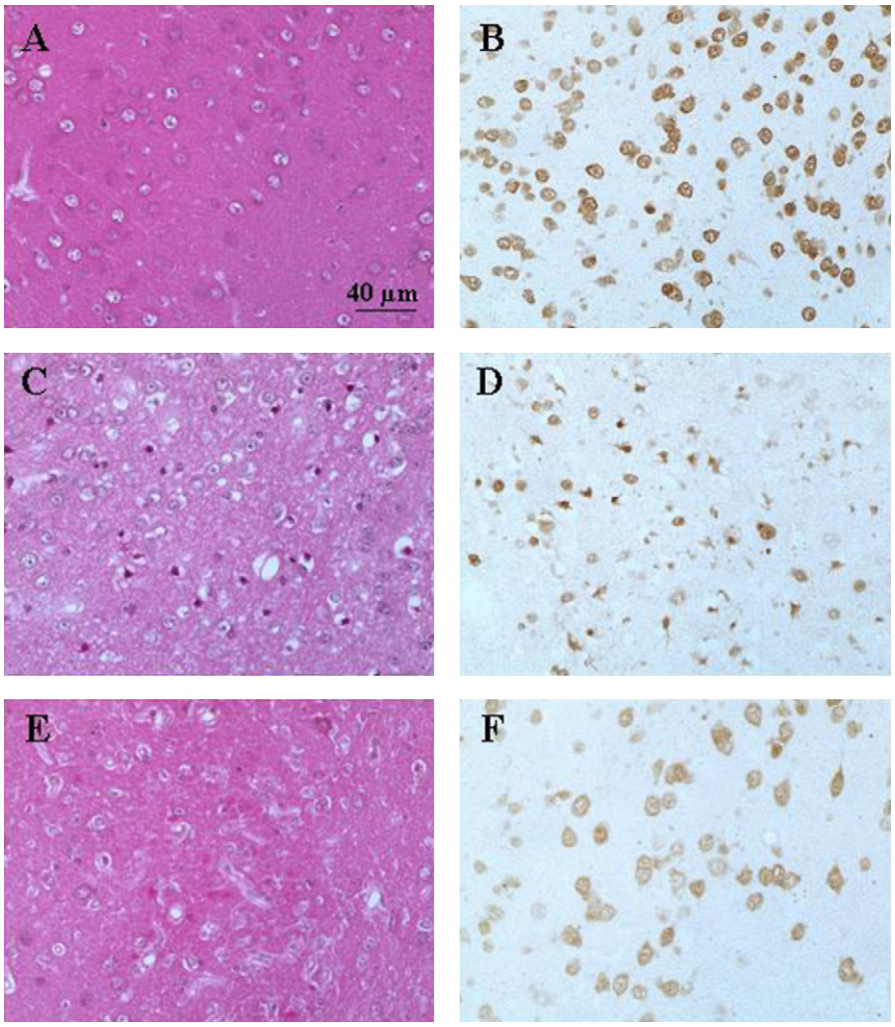

Fig. 1. Soman-induced neuropathology in the mouse amygdala. Brains collected from control or soman-poisoned mice were processed for hemalun–phloxin staining (A, C and E) or anti-NeuN immunochemistry (B, D and F). Cells were viewed in the amygdala under light microscope. For hemalun–phloxin staining, degenerating cells appeared in reddish fuchsia while healthy unaffected cells were stained in white and violet. NeuN-labeled neurons were stained in brown (DAB revelation). Histological comparisons were drawn between control (A and B) and exposed mice on post-soman day 1 (C and D) or day 90 (E and F). The same magnification was used for all the photographs (see scale bar on panel A). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

post-soman day 1) and reached 1020 ± 80 cells per mm^2 on post-soman day 90 ($p < 0.001$ compared with post-soman day 1).

Such data unequivocally reveal important delayed cell regeneration in the damaged amygdala of soman-poisoned mice. Yet, the nature of the cells involved in this regeneration event remained unknown. According to the nucleus size of quantified healthy H&P-stained cells, differentiated neurons or activated astrocytes could be potential candidates. Hence, to address the nature of newly integrated cells, anti-GFAP or anti-NeuN immunohistochemistry techniques were combined to H&P staining on same brain sections to reveal activated astrocytes or differentiated neurons, respectively.

As shown in Fig. 3A, all healthy H&P-stained cells harboring large-rounded nuclei expressed NeuN antigen in the amygdala of control mice. These cells may then be defined as intact neurons, since NeuN is a nuclear protein specific to differentiated neurons. One and three days after soman poisoning, two types of NeuN-labeled cells were revealed (Fig. 3B). Some cells (intact neurons) were similar to the healthy H&P-stained cells with large-rounded nuclei described in control mice. The other NeuN-labeled cells were eosinophilic H&P-stained cells with shrunken nuclei (damaged neurons). On post-soman day 8, there was only one cell type harboring NeuN immunoreactivity (Fig. 3C), namely: the healthy H&P-stained cells observed in control mice (intact neurons). However, two types of cells deprived of NeuN immunoreactivity were detected in the amygdala, 8 days after soman intoxication. The first type was eosinophilic H&P-stained cell with shrunken nucleus (damaged neurons lacking NeuN immunoreactivity). The second type consisted of healthy H&P-stained cell with large-rounded nucleus. The latter cell type could involve either degenerating neurons exhibiting a healthy neuron shape (large-rounded nucleus), or other sorts of activated cerebral cell such as astrocytes. To address the nature of these healthy H&P-stained cells with large-rounded nuclei lacking NeuN immunoreactivity, GFAP immunochemistry and H&P staining were combined on same brain sections (Fig. 3D). Several healthy H&P-stained cells with large-rounded nuclei harbored massive GFAP overexpression indicating that these cells were activated astrocytes (Fig. 3D).

To assess a potential delayed neuronal regeneration in the amygdala of soman-poisoned mice, only NeuN-labeled neurons with large-rounded nucleus (healthy neurons) were quantified, while NeuN-positive neurons with shrunken nucleus were ignored (degenerating neurons) in keeping with the data discussed above.

The density of NeuN-positive healthy neurons in the amygdala of control mice was 1290 ± 60 neurons per mm^2 (Figs. 1B and 2B),

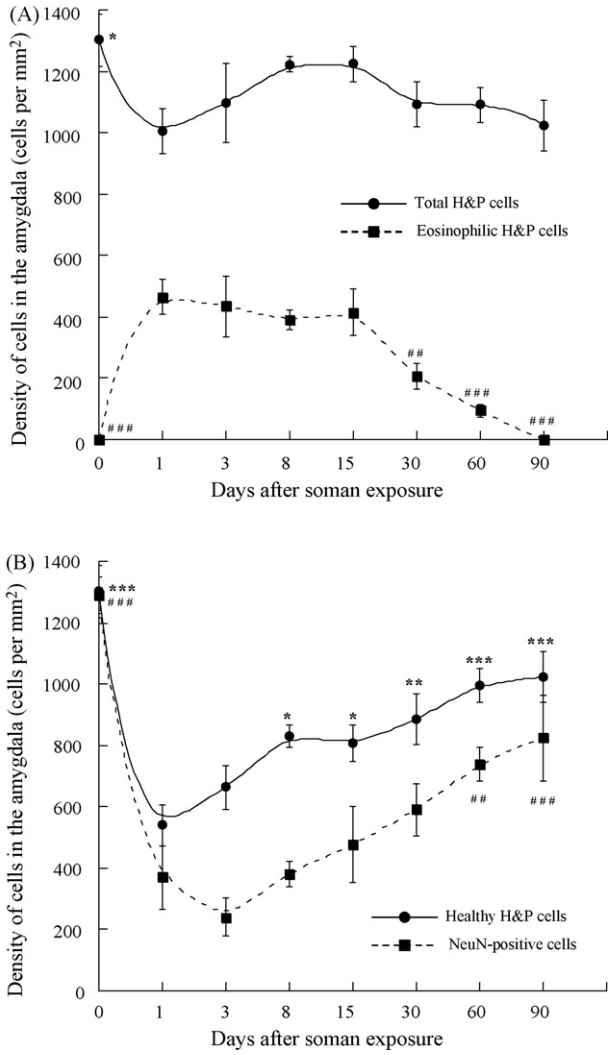

Fig. 2. Cell quantification in the mouse amygdala after soman exposure. Total, eosinophilic or healthy hemalun–phloxin-stained cells and NeuN-positive neurons were quantified in the amygdala of soman-poisoned mice on days 1, 3, 8, 15, 30, 60 and 90 after exposure or control animals. For each experimental group, the mean value for 8–12 mice and its standard error of the mean (S.E.M.) were calculated. Dunnett's *t*-tests were performed to compare cell densities between post-soman day 1 (reference) and other experimental times after intoxication or controls. Significance was set at $p < 0.05$. * or # for $p < 0.05$; ** or ## for $p < 0.01$ and *** or ### for $p < 0.001$.

a result which proves similar to the density found for healthy H&P-stained cells (1300 ± 80 cells per mm^2; Fig. 2B). Such a finding confirms that healthy H&P-stained cells are exclusively intact NeuN-positive neurons in the amygdala of control mice. On post-soman day 1, density of intact healthy neurons dramatically dropped to 370 ± 100 neurons per mm^2 ($p < 0.001$ compared to control mice; Figs. 1D and 2B) and was even lower on post-soman day 3 (240 ± 60 cells per mm^2; Fig. 2B). From post-soman day 8 onwards, a continuous and sustained amplification of the healthy neuron density was measured to reach 830 ± 140 neurons per mm^2 on post-soman day 90 ($p < 0.001$ compared to post-soman day 1; Figs. 1F and 2B). These data corroborate the suspected delayed neuronal regeneration occurring in the amygdala of soman-poisoned mice. The neuronal regeneration could be either due to an active neurogenesis process or to a reversion process with degenerating neurons reverting to healthy neurons.

Furthermore, at all tested experimental times after soman exposure, density of healthy H&P-stained cells proved higher than density of healthy NeuN-positive neurons (Fig. 2B). The density gap ranged between 170 and 450 cells per mm^2 with a maximum variation obtained on post-soman day 8. As mentioned above, such a gap may reflect the density of activated astrocytes appearing in the amygdala of soman-poisoned animals over the 90-day experimentation period.

4. Discussion

4.1. Long-term neuropathology in the amygdala

To date, most studies describing soman-induced neuropathology in rodent brain amygdala were restricted to the first days following neurotoxicant exposure. Indeed, investigations focused by and large on the effectiveness of new emergency treatments to impede *status epilepticus*, and to lessen subsequent early brain damage. The effectiveness of potent neuropharmacological treatments was usually evaluated at early experimental stages after nerve agent exposure, rarely exceeding 3 days post-intoxication. However, few studies reported qualitative long-term neuropathology in the amygdala of animal models after soman poisoning [31,24,37,2,11,16]. Globally, cellular damage was commonly characterized by the presence of H&P-stained eosinophilic neurons and was observed in all amygdaloid nuclei of rat or mice for experimental times ranging from 8 days to 3 months after soman exposure.

Our study hereby provides a first time ever-significant contribution, namely: a quantitative assessment of long-term neuropathology performed in the amygdala of soman-poisoned mice. Our results evidenced the occurrence of two different toxicity steps in the amygdalian neuropathological process related to soman exposure. An early and acute toxicity step took place within the first day following soman intoxication and was defined by the death of about 23% of total amygdalian neurons. In addition, 36% of total neurons were degenerating eosinophilic cells while the remainder (41% of amygdalian neurons) appeared to be healthy cells. The second toxicity step was initiated 15 days after soman poisoning and corresponded to the beginning of the death of eosinophilic neurons. The slow and delayed death of eosinophilic neurons continued up to 90 days after soman intoxication.

A slightly different two-step toxicity pattern was previously demonstrated in the hippocampal CA1 field of our animal model [14]. In the early and acute toxicity step, 20% of pyramidal neurons located in the hippocampal CA1 field died within the first 24 h after soman exposure, while 54% of other neurons adopted an eosinophilic feature. It clearly appears that the number of degenerating neurons is more substantial in the hippocampal CA1 field than in the amygdala even if the level of early neuronal cell death is equivalent in both cerebral structures. Conversely, the second toxicity step was initiated as early as post-soman day 15 in the amygdala while the beginning of this event was more delayed in the CA1 field since it started at least 1 month after soman poisoning [14]. These results seemed to refute the ones previously published by other authors with respect to soman-poisoned rats or guinea pigs [34,35,37,8]. In these qualitative studies, intoxicated animals (soman dose varying from 0.9 to 2.0 LD_{50}) subjected to various supportive treatments or pretreatments exhibited more severe cerebral damage in the amygdala than in the hippocampus. However, these authors estimated global brain injury including the presence of eosinophilic degenerating cells and tissue damage like cerebral edema. In our animal model, global tissue damage, mainly due to cerebral edema seems also to be more important in the amygdala than in the hippocampus. In a fully detailed discussion, Carpentier et al. [8] proposed several hypotheses to account for discrepancies in lesion severity between the amygdala and the hippocampus. Amongst these arguments, cytotoxic glutamate release and NMDA-receptor stimulation resulting from

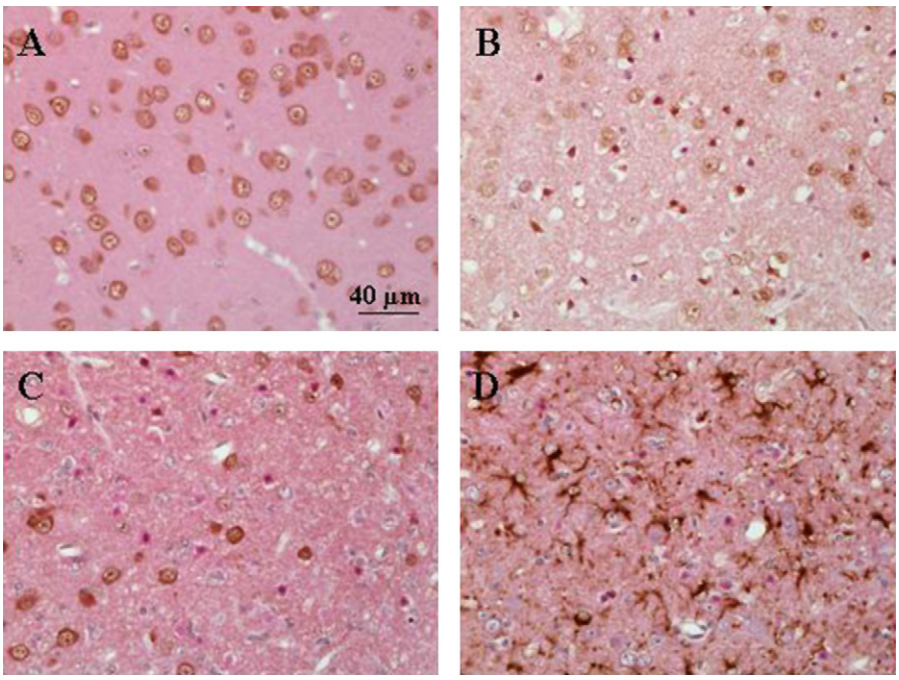

Fig. 3. Characterization of degenerating cells in the mice amygdala following soman exposure. To reveal the identity of degenerating cells, co-labeling of hemalun–phloxin staining with either anti-NeuN (A–C) or anti-GFAP (D) immunohistochemistry (DAB detection) was performed in brain sections from control (A) or intoxicated mice on post-soman day 1 (B) or day 8 (C and D). The same magnification was used for all the photographs (see scale bar on panel A).

soman-driven seizures are not comparable between both structures [25–27]. It is conceivable therefore, that subsequent cerebral lesions triggered by both physiological events could be different in the amygdala and hippocampus, respectively.

The nature of cell death involved in early and delayed toxicity stages remained questionable. Evidence for apoptotic events was noted in the amygdala but not in the hippocampus of soman-poisoned rats [18,3]. In a comprehensive ultrastructural study, Baille et al. [2] have investigated the morphology of damaged neurons in various brain structures, including the amygdala and hippocampal CA1 field of mice subjected to soman poisoning. In mice exhibiting long-lasting convulsions (more than 2 h of continuous convulsions) a large variety of distinct types of degenerating cells, including hybrid forms between apoptosis and necrosis were detected in brains but pure apoptosis remained scarce. [2]. However, 24 h after the administration of the convulsive dose of soman, the presence of rare autophagic- and apoptotic-like damaged cells was observed in the amygdala but not in the CA1 field, which partially confirmed Ballough's results [18,3]. In our earlier studies [14,16], we argued that neuronal cell death evidenced in the hippocampal CA1 field of our animal model during the early acute toxicity step might be a necrotic process rather than a pure apoptotic event. This was based on the fact that massive neuroinflammation process was demonstrated over the first days after soman poisoning in rodents [43,45,17]. Indeed, necrosis is always related to an inflammation response while pure apoptosis never triggers inflammation [46]. Furthermore, it is well known that neuronal damage resulting from excitotoxic insults is usually a necrotic process [21,40] and soman poisoning has typical excitotoxic origins [29,34]. Further, the early neuronal cell death (first 24 h after soman exposure) noticed either in the amygdala or hippocampal CA1 field could mainly be the result of a necrotic process.

The nature of the delayed neuronal cell death (post-soman day 15 onwards) in the amygdala remains unknown. We previously hypothesized that delayed death of hippocampal neurons could result from a programmed cell death pattern possibly including apoptosis. Such speculation was based on an *in vitro* study from Ankarcrona et al. [1] demonstrating that shortly after incubation with glutamate, a majority of cultured cerebellar granule neurons died by necrosis, whereas surviving neurons underwent apoptosis at a later stage after transient recovery of survival functions. A similar finding was evidenced in the brain of neonatal rats after intracerebroventricular administration of kainic acid [23]. As assessed by HSP-70 immunostaining and electron microscopy, early and acute necrotic neuronal cell death was evidenced in the hippocampal CA3 field, 1 and 7 days after kainic acid injection. Subsequently, progressive apoptotic neuronal loss extended to CA3 field up to 2 months after kainic acid administration as shown by electron microscopy and TUNEL-DNA analysis [23]. We hypothesized that an analogous process could occur in the brain of soman-poisoned mice to explain the two-step toxicity pattern shown in the CA1 field of the hippocampus [16]. This hypothesis would be also valid for current results obtained in the amygdala of soman-poisoned mice. Other types of delayed neurodegeneration, however, such as autophagy or neuronal cell death related to axonal deafferentation (Prof. Jean-Claude Martinou, University of Geneva, personal communication) may further account for the delayed neurotoxicity event visualized in either the hippocampal CA1 field or the amygdala in our animal model.

Autophagy is a caspase-independent cell death mainly involved in embryonic development and long-term neuropathology related to neurodegenerative diseases such as Parkinson, Alzheimer and Huntington diseases. Autophagic cell death is characterized by the sequestration of cytoplasmic proteins and organelles into double-membrane bound vacuoles originating from the endoplasmic reticulum (autophagosomes). A subsequent maturation of autophagosomes into acidified single-membrane bound autolysosomes implicates a fusion with preexisting lysosomes and the progressive cytoplasm destruction leads to cell death (for reviews see [10,47]). Interestingly, autophagic neuronal cell death was described in the hippocampus of kainic acid-intoxicated mice or in organotypic hippocampal cultures incubated with NMDA [6,41].

Since soman poisoning shares similar physiopathological processes with either kainic acid (AMPA receptor activation) or NMDA (NMDA receptor activation) challenging, it can be assumed that delayed autophagic neuronal cell death could occur in the hippocampus or amygdala after soman poisoning. To further substantiate this hypothesis, Baille et al. [2] have observed as mentioned above, the presence of sparse autophagic-like degenerating cells in the amygdala of soman-intoxicated mice, 24 h after neurotoxicant exposure.

In keeping with our earlier statement, deafferentation could also be responsible for a delayed neurodegeneration. To our knowledge, few studies described deafferentation-driven cell death in the rodent adult brain [7,22,5]. Deafferentation is usually defined as a mechanical destruction of specific afferent axons, which can trigger cell death in other specific cerebral target areas. As an example, fimbria-fornix transection produced delayed neuronal injury in the septum of rats [22] while bulbectomy causes degenerative changes and apoptotic cell death in the rat piriform cortex [7]. Massive axon degeneration was evidenced several days after soman exposure in various brain structures of mice, rats and cats, as assessed by electronic microscopy [39,2]. Deafferentation could therefore participate in the long-term neuropathology process observed in our animal model.

Our present results also give new qualitative insights into the soman-induced amygdalian neuropathology. Towards such an assessment, H&P staining was combined with either anti-GFAP (astrocytes) or anti-NeuN (neurons) immunohistochemistry on same brain sections to allow for a precise identification of either healthy or damaged H&P-stained cells. We demonstrated that healthy H&P-stained cells (large-rounded nuclei) harboring NeuN labeling, detected in control mice and in all tested experimental times after soman exposure, were intact neurons. From post-soman day 8 onwards, however, a new population of healthy H&P-stained deprived of NeuN immunoreactivity appeared in the amygdala. These cells were almost exclusively reactive astrocytes as evidenced by the presence of a positive GFAP labeling. This indicates that NeuN immunohistochemistry is a better option than H&P staining to quantify neurons, since reactive astrocytes could be mistaken for healthy neurons using H&P staining. Concerning degenerating eosinophilic cells (shrunken nuclei) revealed by H&P staining, 1 and 3 days after soman poisoning, all these cells were positively labeled by NeuN identifying them as degenerating neurons. None of eosinophilic H&P-stained cells were expressing GFAP excluding an astrocyte phenotype for these cells. From 8 days after intoxication onwards, eosinophilic cells were deprived of NeuN labeling indicating a loss of NeuN immunoreactivity in degenerating neurons at later experimental times. Interestingly, in the hippocampal CA1 field, degenerating neurons were deprived of NeuN immunoreactivity as early as 1 day after soman exposure. Shrunken eosinophilic neurons harboring NeuN labeling were never observed in the CA1 field at all tested experimental times [14]. These features confirmed the difference of damage susceptibility between the hippocampus and the amygdala. In an additional experiment based on western blot analysis, we demonstrated that NeuN immunoreactivity loss in hippocampal degenerating neurons was due to reduced NeuN antigenicity rather than a fall in protein expression level [15]. We hypothesized that masking NeuN antigenic sites by chaperone proteins may account for the decrease of NeuN immunoreactivity in damaged pyramidal neurons of the CA1 field. Obviously, a similar process could be invoked to justify the disappearance of NeuN labeling in degenerating amygdalian neurons.

4.2. Delayed neuronal regeneration in the amygdala

In the present study, an important neuronal regeneration has been clearly identified overtime in the amygdala, as assessed by quantification of NeuN-positively labeled neurons. Three months after poisoning, the number of new neurons appearing in the amygdala represents about 105% of the amount of amygdalian neurons quantified in poisoned mice, 24 h after soman exposure. Such a neuronal regeneration could be the result of a reparative neurogenesis process. However, the hypothesis of a reversion status of damaged neurons reverting to healthy ones to contribute to the amygdalian neuronal regeneration could not be totally discarded.

Neurogenesis results from the proliferation of neural stem cells located in the subgranular zone (SGZ) of the dentate gyrus and the subventricular zone (SVZ) of the brain, followed by a subsequent migration and engraftment of neural progenitors into other cerebral regions [32]. Neurogenesis in the amygdala of adult rats and primates has already been described [4,38]. According to Bernier et al. [4], newborn neurons detected in the amygdala and piriform cortex of healthy adult primates might have migrated from the SVZ that borders the lateral ventricles. In the specific research field of epilepsy, pentylenetetrazole (PTZ)-driven epileptic seizures induced an increase of neural progenitor proliferation in both SGZ and SVZ of adult rats and newborn neurons were subsequently encountered in the amygdala of these PTZ-injected animals [38].

In our animal model of soman-induced seizures, a massive and sustained expansion of neural progenitor proliferation was previously observed in both the SVZ and SGZ over the first month after neurotoxicant exposure [11]. Using BrdU/NeuN double labeling, newborn neurons were detected in either the hippocampal CA1 field and the amygdala of soman-poisoned mice, 1 month after soman administration [11,13]. Quantification of NeuN-positive neurons at various experimental times ranging from 1 to 90 days after soman exposure confirmed the presence of a neuronal regeneration in the hippocampal CA1 field of our animal model [14]. Three months after intoxication, newborn neurons represented about 20% of remaining pyramidal neurons found in CA1 field of poisoned mice, 1 day after neurotoxicant administration. Therefore, the present neuronal regeneration observed in the amygdala is five-fold higher than the one previously measured in the hippocampal CA1 field (105% versus 20% of newborn neurons, respectively). Altogether, these results would argue in favor of a reparative neurogenesis process occurring in the amygdala and hippocampal CA1 field of brain mice following soman poisoning.

5. Conclusions

In the present histological study, for the first time, a delayed neuronal cell death was evidenced in the amygdala of soman-poisoned mice. The development of the long-term neuropathology in this brain region differed from the one previously demonstrated in the hippocampal CA1 field of the same animal model [14] confirming thereby the suspected difference of damage susceptibility between the hippocampus and amygdala [8]. Concomitant to the delayed neuronal cell death, a massive neuronal regeneration was also observed in the amygdala of our intoxicated mice. Such a neuronal regeneration has already been described in the CA1 field of mice following soman exposure [14] but the regeneration intensity was five-fold lower than the one measured in the amygdala.

Interestingly, the long-term neuropathology and the delayed neuronal regeneration detected in the CA1 field had tremendous repercussions on mnemonic cognitive performances in our mouse model [19]. Indeed, soman poisoning induced a total annihilation of learning skills, 30 days after intoxication. However, 90 days after neurotoxicant exposure, a moderated improvement of water-maze mnemonic performances was evidenced in our animal model and was attributed to the low neuronal regeneration occurring in the hippocampal CA1 field [19]. However, long-term consequences of soman poisoning on amygdala-driven behavior have never been

studied. Therefore, in the companion paper (part 2: emotional behaviour), the effects of long-term neuropathology and delayed neuronal regeneration were evaluated on amygdala-driven emotional processes.

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