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Contemporary Issues in Toxicology

Nerve agent intoxication: Recent neuropathophysiological findings and subsequent impact on medical management prospects

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ABSTRACT

This manuscript provides a survey of research findings catered to the development of effective countermeasures against nerve agent poisoning over the past decade. New neuropathophysiological distinctive features as regards organophosphate (OP) intoxication are presented. Such leading neuropathophysiological features include recent data on nerve agent-induced neuropathology, related peripheral or central nervous system inflammation and subsequent angiogenesis process. Hence, leading countermeasures against OP exposure are down-listed in terms of pre-treatment, protection or decontamination and emergency treatments. The final chapter focuses on the description of the self-repair attempt encountered in lesioned rodent brains, up to 3 months after soman poisoning. Indeed, an increased proliferation of neuronal progenitors was recently observed in injured brains of mice subjected to soman exposure. Subsequently, the latter experienced a neuronal regeneration in damaged brain regions such as the hippocampus and amygdala. The positive effect of a cytokine treatment on the neuronal regeneration and subsequent cognitive behavioral recovery are also discussed in this review. For the first time, brain cell therapy and neuronal regeneration are considered as a valuable contribution towards delayed treatment against OP intoxication. To date, efficient delayed treatment was lacking in the therapeutic resources administered to patients contaminated by nerve agents.

Contents

Introduction

For ages, warriors have resorted to non-conventional weapons to cause major incapacitating injuries among their foes. As a rule, the choice for any non-conventional weapon rested upon its additional

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harmful potential over the enemies' psychological condition. Amongst massive destruction weapons such as biological, chemical or nuclear weapons, chemical weapons were the easiest to develop and hence they were the first to be used. As early as the third century, Byzantines invented an incendiary and toxic paste made of tar, sulphurs, saltpeter and antimony oxysulfide to intoxicate besieged enemies. The first large-scale use for chemical weapons undoubtedly occurred during the First World War. Between 1915 and 1917, tons of chlorine and sulfur mustard were spread on the battlefield. Thousands of soldiers were made useless and these massive attacks caused a wave of panic through the targeted troops and had a drastic fall-out effect on the fighters' morale. The period between the late 1930's and the end of the Second World War was favorable to the discovery of powerful cholinesterase inhibitors: organophosphate (OP)-base nerve agents such as tabun, sarin and soman (Meyer, 2001; Renaudeau, 2005). During the Iran–Iraq war (1980–1988), chemical weapons (mustard gas, tabun and lewisite) were used and caused the death of several thousands of people (Kadivar and Adams, 1991; Meyer, 2001; Renaudeau, 2005).

Terrorist threats remain another cause for concern in terms of chemical agent use. Terrorist attacks by the sect "Aum Shinrikyo" resorted to sarin in Matsumoto,Japan (June 1994) and in the Tokyo subway (March 1995). In the first attack (Matsumoto), 600 people were intoxicated and 7 of them died. In the second attack (Tokyo subway), 12 deaths were numbered out of 5500 poisoned individuals (Morita et al., 1995; Suzuki et al., 1995). Despite atropine sulfate and benzodiazepine treatments, about 10% of moderately/severely intoxicated victims suffered from neuropathy and ataxia between 3 days and 3 months after poisoning. Furthermore, six severely affected people were resuscitated from cardiopulmonary arrest or coma with generalized convulsions. Five of them recovered completely while one victim remained in a vegetative state due to anoxic encephalopathy with EEG abnormalities persisting for up to 5 years post-intoxication. A long-term follow-up study performed on victims from both terrorist attacks highlighted that about 8% of the victims presented post traumatic stress disorder (PTSD) up to 5 years after intoxication (Yanagisawa et al., 2006).

This review will focus on the pathophysiological effects of OP (mainly soman) and on the already existing or currently developed countermeasures against nerve agent poisoning.

Pathophysiological effects of nerve agents

OP such as soman, tabun, sarin, cyklosarin or VX are more or less powerful irreversible inhibitors of blood and cerebral cholinesterases. Usually, the time course of OP intoxication depends on the nature of the nerve agent, the route of exposure and the level of contamination. Exposure to nerve agents results in a sequence of toxic signs evolving in a few minutes according to the following order: hypersecretions and fasciculations, tremor, convulsions, coma and finally death by cardiac arrest (heart paralysis) and respiratory distress (diaphragm paralysis and suffocation with hypersecreted mucus). These toxic signs are directly related to the physiological way of actions of nerve agents (Bajgar, 2004; Lallement et al., 1998; McDonough and Shih, 1997). Indeed, cholinesterase inhibition prevents acetylcholine (ACh) hydrolysis leading to the accumulation of this neurotransmitter in the brain synaptic areas (Fig. 1). A few minutes after nerve agent poisoning, the cholinergic hyperactivity triggers peripheral or central muscarinic and nicotinic signs. Central signs (either muscarinic or nicotinic responses) are characterized by anxiety, headache, epileptic seizures with loss of consciousness, and inhibition of the medullary respiratory center. Peripheral muscarinic signs are mainly observed in exocrine glands with hypersecretion processes such as rhinorrhea, bronchorrhea, sweating, lacrimation and salivation. Peripheral nicotinic signs include fasciculation, tachycardia, hypertension, pallor and convulsions with a possible evolution to the paralysis of skeletal muscles (Fig. 1) (Bajgar, 2004; Lallement et al., 1998; McDonough and Shih, 1997).

In addition, the augmented ACh level rapidly produces a massive glutamate release in the brain. This glutamatergic response induces a prompt activation of AMPA receptors (within the first 10 min after OP poisoning), followed by a delayed stimulation of NMDA receptors (between 30 and 45 min after intoxication). NMDA receptor activation is combined with an intracellular accumulation of Ca^{2+} and by an overproduction of free radical compounds (Fig. 1). All these events (AMPA and NMDA receptor stimulations; intracellular accumulation of Ca^{2+}) are involved in the early propagation of seizure activity and in the maintenance of epileptic seizure that initiate a biochemical process responsible for neuronal cell death (Bajgar, 2004; Lallement et al., 1998; McDonough and Shih, 1997).

Neuropathology following nerve-agent exposure

Nerve agent-induced neuropathology has been widely studied over the first 3 days following intoxication of rodents subjected or not to various supportive treatments. Most of these studies were carried out in soman-poisoned animals since conventional emergency treatments against OP intoxication are only partially efficient compared to soman. As assessed by cresyl-violet or hemalum-phloxin (H&P) stainings performed on brain sections, significant neuronal cell death was demonstrated within the first 24–48 h after soman exposure in all cortical regions, some subcortical limbic areas (hippocampus, amygdala and claustrum) and several thalamic nuclei (Apland et al., 2010; Lemercier et al., 1983; McDonough et al., 1998; Tryphonas and Clement, 1995). Neuronal cell death was combined with the presence of degenerating neurons and edema in damaged cerebral areas (Figs. 2 and 3). The severity and location of brain lesions seemed to be correlated with the intensity and the duration of seizure activity (Carpentier et al., 2001). In a detailed immunohistochemistry and ultrastructural study, Baille et al. (2005) have examined the morphology of degenerating neurons in various brain structures (hippocampus, amygdala, piriform cortex and thalamus) of mice, 8 h, 24 h and 7 days after soman exposure. A total of 11 ultrastructurally different types of degenerating neurons were identified including various hybrid forms ranging between apoptosis and necrosis. Pure apoptosis remained however scarce (Baille et al., 2005). Further, neuronal cell death occurring during the early acute toxicity step might be a necrotic process rather than a pure apoptotic event, with a rapid elimination of dead cells by macrophages infiltrated in damaged rodent brains. Indeed, two major findings tend to substantiate such a hypothesis. First, an important neuro-inflammation process was evidenced over the first days after soman exposure in rodents (Dhote et al., 2007; Svensson et al., 2001; Williams et al., 2003) and necrosis is always related to a massive inflammatory response, whereas pure apoptosis does not produce any inflammation (Yakovlev and Faden, 2004). Second, neuronal damage resulting from soman poisoning has excitotoxic origins (Lallement et al., 1993) and it is widely acknowledged that neuronal cell death due to excitotoxic insult (kainate and glutamate challenging) is generally a necrotic process (Fujikawa et al., 2000; Puig and Ferrer, 2002).

Only a few studies focused on qualitative or quantitative long-term evolution of neuropathology following soman exposure in rodents (Collombet et al., 2005b, 2006a, 2008; Kadar et al., 1992; Lemercier et al., 1983; McDonough et al., 1998). A steady elevated number of H&P-stained eosinophilic neurons (degenerating neurons) was detected in subcortical limbic regions (mainly in the hippocampus and amygdala) of rodent brains several weeks after soman poisoning (Fig. 3). These eosinophilic neurons also exhibited a loss of NeuN immunoreactivity due to a reduced NeuN antigenicity rather than a decrease in protein expression level (Collombet et al., 2006b). Masking of NeuN antigenic sites by chaperone proteins could account for the abated NeuN immunoreactivity observed in damaged neurons. After a 1-month post-exposure survival period, a slow death of degenerating neurons was initiated leading to a complete disappearance of these cells in both the amygdala and hippocampus, 3 months

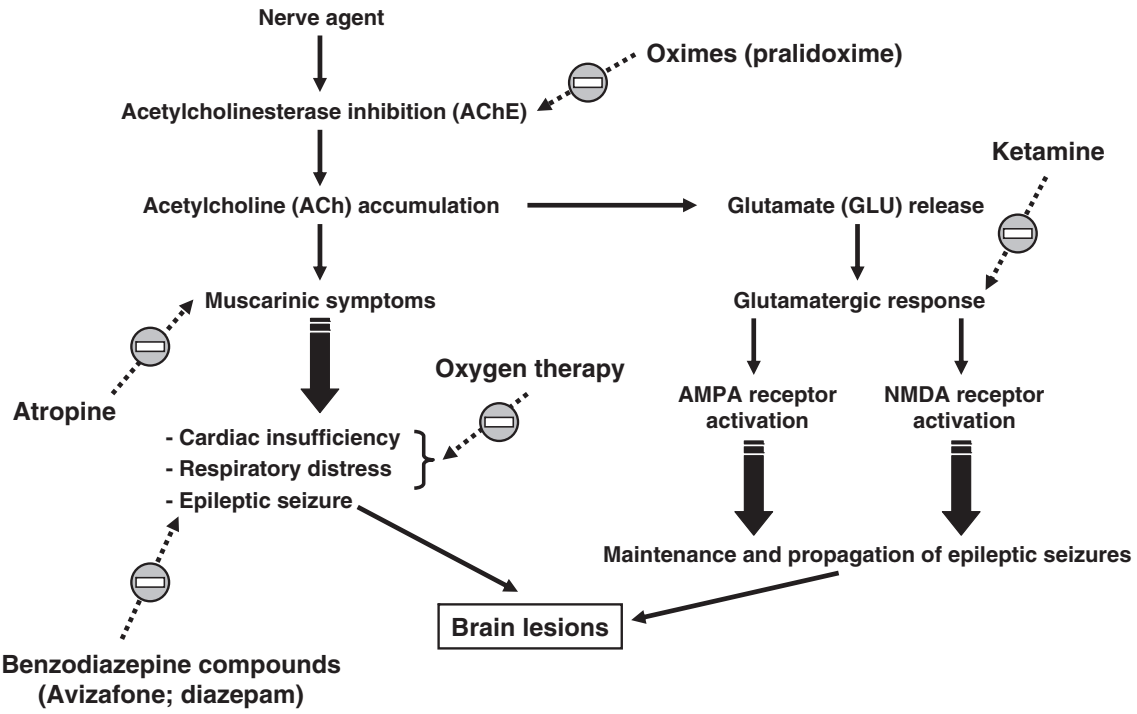

Fig. 1. Pathophysiological effects of nerve agent intoxication and mode of actions of emergency treatments. Inhibition of acetylcholinesterase (AChE) by nerve agents induces a rapid acetylcholine (Ach) accumulation followed, roughly 30 minutes later, by an important glutamate release. Ach overload leads to muscarinic symptoms responsible for epileptic seizures, cardiac insufficiency and respiratory distress. Glutamate accumulation induces an excitotoxic response (activation of AMPA and NMDA receptors) participating in the development of brain lesions (maintenance and propagation of epileptic seizures). Existing emergency treatments (oximes, atropine, benzodiazepine compounds and oxygen therapy) and their mode of actions in the pathophysiological cascade are indicated with dashed arrows with stop signs. Ketamine, a potential treatment currently under pharmacological testing, should be able to block the glutamatergic response.

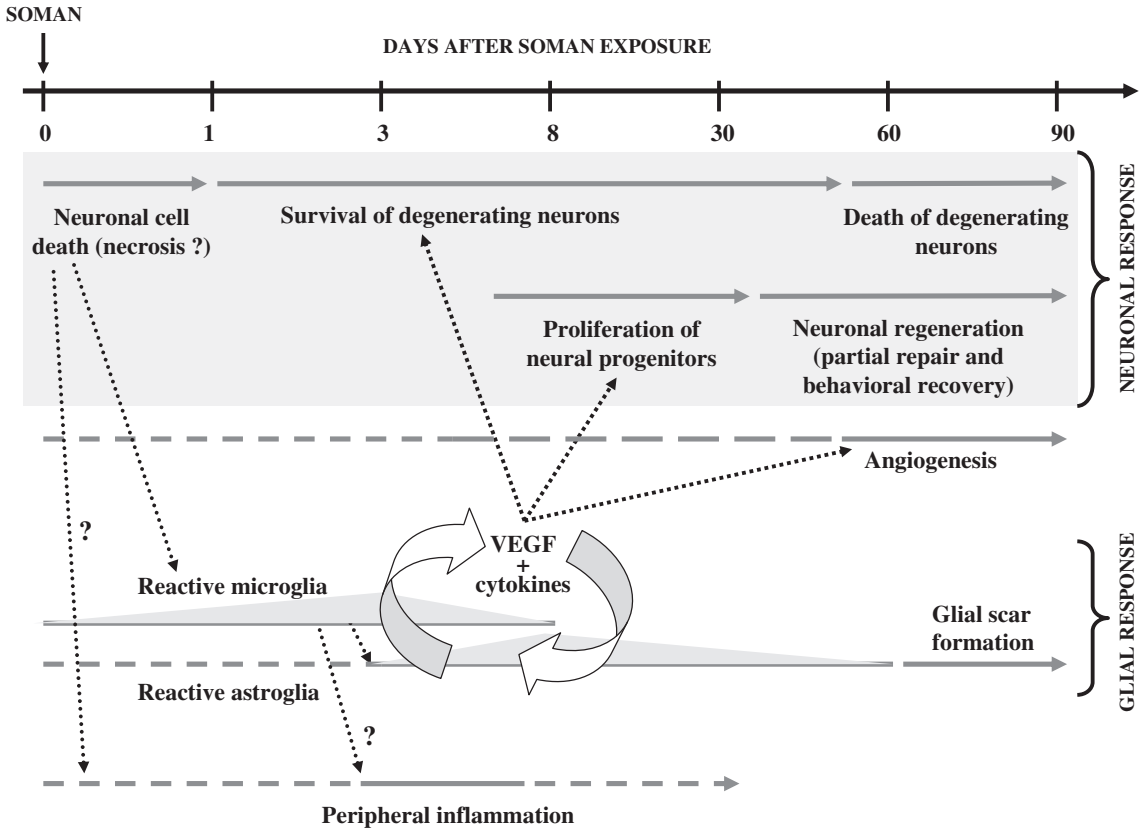

Fig. 2. Speculative overview of neuropathological and self-reparative events induced by soman poisoning. This figure summarizes the results obtained by the military research over the last decade, in terms of long-term soman-induced neuropathological and self-reparative mechanisms. Details are summarized in the conclusions of this review.
This figure is modified from Collombet et al. (2007).

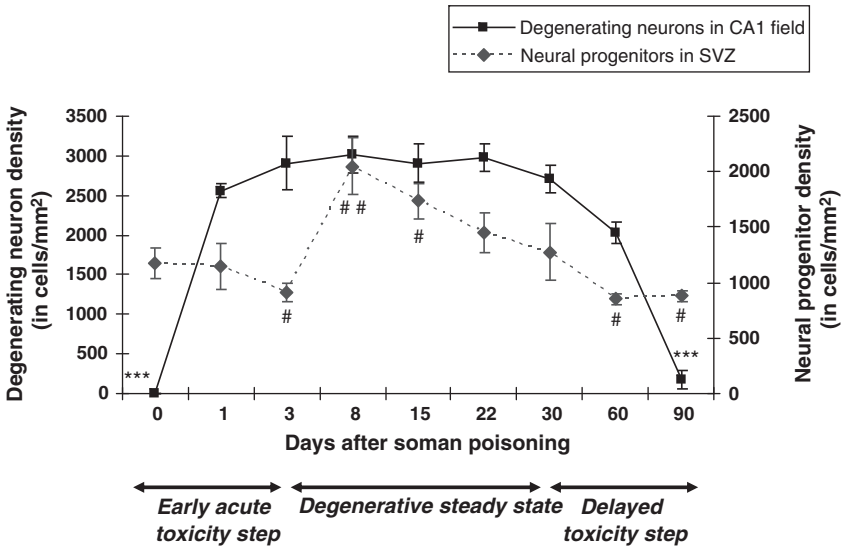

Fig. 3. Soman-induced neuropathology and its impact on neural progenitor proliferation in mice. Eosinophilic hemalum-phloxin stained neurons (degenerating neurons) or BrdU (5-bromo-2′-deoxyuridine)-labeled neural progenitors were quantified in hippocampal CA1 field or subventricular zone (SVZ) of mouse brain sections, respectively. Brains were collected from control mice (day 0) or soman-poisoned mice on post-soman days 1, 3, 8, 15, 22, 30, 60 or 90. For each experimental time, the values given represent the mean number of cells per mm^2 and the standard error of the mean (SEM) for 7–12 animals. Dunnett's t-tests were performed to compare degenerating neuron density between post-soman day 1 (reference) and other experimental times after intoxication or controls. Significance was set at $p<0.05$ ($^{***}p<0.001$). Neural progenitor quantification between control (day 0; reference) mice and poisoned mice at different experimental times was compared using Dunnett's t-tests. Significance was set at $p<0.05$ ($^{\#}p<0.05$ and $^{\#\#}p<0.01$). This figure is modified from Collombet et al. (2005b, 2006a).

after soman poisoning (Figs. 2 and 3) (Collombet et al., 2006a, 2008). Delayed neuronal cell death subsequent to the long-lasting presence of degenerating neurons has been already demonstrated in rodents' hippocampus after kainate injections (Hopkins et al., 2000) or global and focal brain ischemia (Fukuda et al., 1993; Onodera et al., 1993; Wang et al., 2004). The cause for long-term survival of degenerating neurons and the nature of the delayed neuronal cell death remains unknown. However, numerous cytokines, neurotrophic and growth factors synthesized during the neuro-inflammation process have disclosed neuroprotective effects (Brockington et al., 2004; Gora-Kupilas and Josko, 2005; Greenberg and Jin, 2005; Liberto et al., 2004) which may explain the long-term survival of degenerating neurons in injured cerebral regions (Fig. 2). The delayed death following soman exposure could result from a programmed cell death pattern possibly including apoptosis. Nevertheless, other types of delayed neurodegeneration such as autophagy or neuronal cell death induced by axonal deafferentation may not be wholly discarded.

Autophagic cell death is encountered in embryonic development and long-tem degenerative diseases such as Alzheimer, Huntington or Parkinson diseases. Autophagy is singled out by the sequestration of organelles and cytoplasmic proteins into double-membrane bound vacuoles called autophagosomes and originating from endoplasmic reticulum. The progressive annihilation of the cytoplasm triggers cell death (Chu, 2006; Yuan et al., 2003). Interestingly, Baille et al.(2005) have described the presence of a few autophagic-like degenerating neurons in mouse brains, but early after soman poisoning (24 h post-exposure). Furthermore, autophagic neuronal cell death was observed in the hippocampus of mice intoxicated with kainic acid, a neurotoxicant exhibiting similar pathophysiological processes in common with soman (Shacka et al., 2007).

Deafferentation is defined as a mechanical or physiological destruction of afferent axons which can lead to a delayed neuronal cell death in other brain target regions (Borsello et al., 2000; Capurso et al., 1997; Ginsberg et al., 1999). As assessed by electron microscopy, an important axon degeneration was evidenced in different brain areas of cats, mice and rats several days after soman intoxication (Baille et al., 2005; Petras, 1994). Hence, deafferentation could be involved in the soman-induced delayed neuronal cell death phase.

As a conclusion, a three-step neuropathological event would be induced after soman poisoning in rodents (Figs. 2 and 3). The first step involves a well-described early acute toxicity leading to the death of numerous neurons and occurring within the first 24 h following nerve agent challenging. This neuronal cell death pairs up with the appearance of an important number of degenerating eosinophilic neurons deprived of NeuN immunoreactivity. These degenerating neurons survived over a period of time ranging between 2 weeks and 2 months after soman exposure, depending on the specific wounded brain region under scrutiny. This second step can be defined as a degenerative steady state. Eventually the third step involves a delayed toxicity initiated by the slow death of these degenerating neurons. This step, lasting up to 3 months post-exposure, leads to the complete disappearance of degenerating neurons (Figs. 2 and 3).

Interestingly, these different toxicity steps were clearly evidenced in clinical (weight loss) and motor (rotarod) parameters measured in soman-poisoned mice, up to 3 months after nerve agent exposure (Filliat et al., 2007). From post-soman days 1 to 13—a period covering the early acute toxicity step—mice exhibited a drastic weight loss and a blatant motor performance slump. After a partial and transient recovery, a second reduction of weight and rotarod performances were visualized over a period extending from 30 to 45 days after poisoning and onwards, a time lapse matching the delayed toxicity step (Filliat et al., 2007).

Peripheral and neuro-inflammation after nerve-agent exposure

Many brain lesions resulting from chemical (kainic acid, MK-801, glutamate, pilocarpine) and mechanical (brain trauma) injuries, ischemia or epilepsy induce central and peripheral inflammatory response with glial cell activation followed by subsequent glial scar formation (gliosis) as part of the reparative tissue process (Chen and Swanson, 2003; Fawcett and Asher, 1999; Kempermann and Neumann, 2003; Liberto et al., 2004; Nakamura, 2002; Silver and Miller, 2004; Stoll et al., 2002). To sum up, metabolic deregulation and cell death resulting from brain lesions rapidly activate microglial and astroglial cells which both participate in the neuro-inflammation response. Reactive glial cells exhibit prominent perinuclear cytoplasm and hypertrophic thickened processes as compared to resting glia. In the first days following brain

injury, invading macrophages, microglial and astroglial cells secrete pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (Il-1β) and interleukin-6 (Il-6). However, microglia synthesizes a much higher quantity of these cytokines than astrocytes or macrophages. Depending on experimental conditions, the role of TNF-α, IL1-β and IL-6 remains controversial since these cytokines are mentioned either as neuroprotective or neurodegenerative factors. Reactive astroglia also secrete various growth and neurotrophic factors such as ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), basic fibroblast growth factor (FGF-2), nerve growth factor (NGF) and vascular endothelial growth factor (VEGF). All of these factors promote survival of degenerating neurons in various *in vitro* and *in vivo* brain damage models. Usually, activation of astroglial cells requires more time than microglial cell activation. Indeed, reactive microglia is detected a few days only following brain injury whereas reactive astroglia can be spotted several months after injury. Both reactive astroglial and microglial cells replicate and participate in the glial scar formation which is a cytokine-dependent mechanism mainly contributing to fill loss tissue areas following the neuropathology events (neuronal cell death, demyelination or axonal degeneration).

Following soman poisoning, a massive activation of microglia and astroglia was observed (Baille-Le Crom et al., 1995; Collombet et al., 2005b, 2007; Zimmer et al., 1997). Using *in situ* hybridization method, Baille-Le Crom et al. (1995) demonstrated an increase in GFAP (glial fibrillary acidic protein) mRNA expression in astrocytes of rat hippocampus and dentate gyrus from 6 h to 24 h after soman exposure. Such a finding implied a rapid astroglial cell activation since GFAP is a specific astrocyte cytoskeletal intermediate filament well-known to be over-expressed after brain injury. These results were further completed and substantiated by Zimmer et al. (1997). As assessed by immunohistochemistry techniques, both astroglia (GFAP staining) and microglia (OX-42 staining) were rapidly activated in the brain of soman-intoxicated rats. Indeed, reactive microglial cells were detected between 1 h and 4 h after nerve agent exposure in the amygdala, hippocampus, piriform cortex and thalamus of rats. Within the first 24 h following soman exposure, microglia activation was reduced in all these brain regions except in the piriform cortex. A similar time-course pattern was observed with reactive astrocytes. Indeed, between 4 h and 8 h after soman administration, intense GFAP immunostaining was shown in the amygdala, hippocampus, entorhinal cortex and piriform cortex. However, GFAP staining strongly decreased in all these cerebral regions, 24 h after soman intoxication (Zimmer et al., 1997). According to the authors, the rapid but short-term astroglia activation could be due to fast changes in the physical structure of GFAP filaments favoring the epitope exposure to the anti-GFAP antibody, rather than newly synthesized GFAP proteins.

In a long-term study, reactive microglia was detected as early as 24 h following soman administration in damaged mouse brain regions (amygdala, hippocampus, thalamus, lateral septum, cerebral cortex) as well as in undamaged cerebral areas (hypothalamus and striatum) (Collombet et al., 2005b). The peak for microglia activation and cell proliferation was reached on post-soman day 3 either in lesioned or unlesioned brain regions (Fig. 2). On post-soman day 8, some activated microglial cells were still present in damaged cerebral areas. In unaffected brain regions, reactive microglia remained undetectable. For later experimental times, up to 3 months after soman exposure, resting microglia only was observed regardless the considered damaged or undamaged brain region (Collombet et al., 2005b). Comparatively, the first signs of astroglial cell activation appeared on post-soman day 3 and the peak in terms of GFAP staining intensity and astrocyte proliferation was obtained on post-soman day 8 (Fig. 2). Reactive astroglial cells were viewed in damaged brain regions only (i.e. amygdala, cerebral cortex, hippocampus, lateral septum and thalamus). Further, astroglia activation decreased over time, up to 3 months after soman poisoning but remained detectable in some discrete regions of lesioned brains such as the amygdala and the hippocampus where an astrogliotic scar was present (Collombet et al., 2005b, 2007).

Altogether these results clearly pointed out two steps of astroglial cell activation. The first one, extending over several hours after nerve agent exposure, is characterized by morphological changes of astrocytes (hypertrophic activation) including cell swelling and possibly cytoskeletal changes increasing exposure of GFAP antigenic sites. The second one was disclosed a few days after soman intoxication and presented typical signs of gliosis (hyperplasic activation with astrocyte proliferation) resulting in the formation of a glial scar with over-expression of GFAP protein. Interestingly, both steps of astrocyte activation had already been reported by Dusart et al. (1991) in rats subjected to kainic acid poisoning (neurotoxicant sharing similar pathophysiological process with soman). In addition, the same research team noted that microglia activation preceded the astrogliosis in kainic acid-intoxicated rats (Marty et al., 1991) in keeping with our own findings. Hence microgliosis could possibly trigger astrogliosis via the secretion of cytokines such as IL-1β (Fig. 2) (Liberto et al., 2004). Indeed, in a mouse model of brain injury, IL-1β is secreted by activated microglia within 15 min after corticectomy (Herx et al., 2000) and this secreted Il-1β is required to promote activation of astrocytes *in vivo* (Herx and Yong, 2001).

Cytokine expression following nerve agent exposure in rodents has already been under scrutiny (Chapman et al., 2006; Dhote et al., 2007; Johnson and Kan, 2010; Svensson et al., 2001; Williams et al., 2003). Il-1β, IL-6 and TNF-α mRNAs or proteins are over-expressed in lesioned rat brain areas such as the cortex, hippocampus and thalamus, between 30 min and 6 h after soman poisoning (Svensson et al., 2001; Williams et al., 2003). In a complementary long-term experiment, Dhote et al. (2007) analyzed the time course of changes in mRNA levels of IL-1β, TNF-α and IL-6 in mice brains, up to 7 days after soman intoxication. A massive increase of the 3 mRNA levels was shown in the cerebral cortex and hippocampus between 6 h and 2 days after soman exposure. The level of Il-1β mRNA was still significantly elevated on post-soman day 7 in both the cerebral cortex and hippocampus (Dhote et al., 2007). Using bead multiplex immunoassays, Johnson and Kan (2010) evidenced a significant concentration increase in Il-1α, Il-1β, Il-6 and TNF-α in injured brain regions of rats (piriform cortex, hippocampus and thalamus), 12 h after soman administration. Interestingly, neurotoxic cytokines Il-1α and Il-1β were primarily expressed by activated microglia, whereas potentially neuroprotective cytokine Il-6 was expressed by neurons and hypertrophic astrocytes as assessed by immunohistochemistry (Johnson and Kan, 2010). Almost similar results were obtained in rats subjected to sarin poisoning and treated with midazolam 30 min after the nerve agent exposure (Chapman et al., 2006). Indeed, the synthesis of TNF-α, IL-1β and IL-6 proteins was raised in the cerebral cortex and hippocampus between 2 h and 2 days following sarin-induced seizures. Interestingly, a second increase in levels of these inflammatory markers was revealed in the rat brains 30 days after sarin exposure (Chapman et al., 2006). All these results clearly indicate the presence of an important neuro-inflammatory process in rodent brains following nerve agent exposure. In the case of soman poisoning, the neuro-inflammation was combined with a long-term peripheral inflammation. Indeed, from post-soman day 4 to post-soman day 22, the number of monocytes and granulocytes in the blood circulation had considerably increased (Collombet et al., 2005a). Phagocytic events involving activated monocytes (invading macrophages) and granulocytes are required in damaged brain regions to remove accumulated cell debris, as suggested by the macrophage invasion already observed in wounded brain regions 1, 2 and 7 days after soman exposure (Baille et al., 2005; Lallement et al., 1993). The extensive need for activated monocytes and granulocytes in the brain could contribute to further cell quantity increase in blood (Fig. 2).

Hypothetical relationship between neuropathology and inflammation

As mentioned above, the early acute toxicity step possibly triggers the neuro-inflammation process and the related long-term activation of glial cells due to the massive neuronal cell death occurring during the

first 24 h after soman administration (Fig. 2). Cytokines, neurotrophic and growth factors produced by activated microglia and astroglia may impact the balance between neuroprotection and neurodegeneration in soman-damaged rodent brains. Even if the neuroprotective or neurodegenerative role of IL-1β, TNF-α and IL-6 remains controversial, other astrocyte-secreted cytokines such as BDNF, CNTF, FGF-2, NGF or VEGF are well-defined factors promoting *in vitro* and *in vivo* survival of degenerating neurons after various types of brain injuries. Therefore, these secreted cytokines could promote the long-term survival of degenerating neurons in lesioned brain regions. This argument is further substantiated by the fact that the presence of degenerating neurons (between 1 day and 1 month after soman administration) was shown over the same period of time than the one covering the massive activation of astroglial cells. Hence, we may legitimately hypothesize that the death of degenerating neurons occurs when the glia activation decreased (extending from 1 month following intoxication and beyond) and then, the amount of secreted cytokines does not suffice to ensure survival of degenerating neurons (Fig. 2).

Angiogenesis

Angiogenesis is characterized by the formation of new blood vessels either by sprouting or splitting of pre-existing vessels. Angiogenesis participates in the reparative process taking place after various cerebral injuries (brain tumors; epileptic seizures; trauma and ischemia) and its role is to provide nutrients and oxygen to enhance the reparation process (Brockington et al., 2004; Harrigan, 2003; Merrill and Oldfield, 2005). Under pathological conditions, several angiogenic factors such as angiopoietin-1, ephrins, integrins and VEGF are mainly involved in the proliferation and migration of endothelial cells responsible for the initiation step of the neo-vascularization event (Harrigan, 2003; Merrill and Oldfield, 2005). Among these factors, VEGF plays a key role in the development of angiogenesis. Indeed, VEGF is rapidly secreted by reactive glia (both microglia and astroglia) and neurons via other factors produced by glial cells such as EGF (epidermal growth factor), FGF-2, IL-1β, IL-6 and TNF-α (Croll et al., 2004; Harrigan, 2003; Salhia et al., 2000). Interestingly, conversely, VEGF participates in the *in vivo* activation and proliferation of both microglial and astroglial cells (Krum and Khaibullina, 2003; Krum et al., 2002; Tran et al., 2005; Zand et al., 2005) maintaining the up-regulation of IL-1β, IL-6 and TNF-α pro-inflammatory cytokines responsible for VEGF secretion. This reciprocal regulation process between VEGF and other pro-inflammatory cytokines in glial cells remains a genuine "vicious circle" (Fig. 2).

Following soman administration in mice, noticeable VEGF overexpression was detected in the lesioned hippocampus and amygdala on post-soman day 3 (Collombet et al., 2007). This is not surprising according to the "vicious circle" hypothesis developed above and the previous studies mentioning an up-regulation of IL-1β, IL-6 and TNF-α pro-inflammatory cytokines in rodents' wounded brain regions during the first 7 days following soman exposure (Dhote et al., 2007; Johnson and Kan, 2010; Svensson et al., 2001; Williams et al., 2003). As assessed by quantitative anti-claudin-5 immunochemistry allowing detection of highly differentiated blood vessels, angiogenesis was evidenced 3 months after soman exposure (Fig. 2) in damaged brain areas (hippocampus and amygdala). Neo-vascularization was not present in unlesioned cerebral regions such as the hypothalamus (Collombet et al., 2007).

Existing countermeasures against nerve agent poisoning in France

As shown in Fig. 4, the evolution of nerve agent intoxication can be broken down into 3 different phases: (i) contamination, (ii) early clinical signs onset (i.e. toxicity signs including epileptic seizures) and (iii) delayed clinical signs onset (i.e. development of brain lesions). In ideal terms, research programs catered to developing countermeasures intended to fend off nerve agent poisoning aimed at pharmacological compounds or protective systems able to provide beneficial effects potentially encompassing the 3 phases referenced herein. To date, existing countermeasures against chemical weapons, whether in France or worldwide mainly focus on the initial 2 phases (i.e. contamination and early clinical signs onset) and involve namely, pre-treatments, protection and decontamination or emergency treatments (Fig. 4).

Pre-treatment

Pre-treatments will be given to military staff suspected to encounter an elevated risk towards nerve agent exposure (dismantling of chemical weapon stocks; emergency assistance staff after a terrorist attack; risk of nerve agent spreading over a battlefield). Pre-treatments are expected to protect contaminated people long enough (i.e. prior to the onset of early clinical signs of intoxication, if possible) prior to enhanced emergency treatment implementation.

In France, pyridostigmine bromide or Mestinon®—the drug used to treat myasthenia gravis—is the market-approved pre-treatment against organophosphate exposure (Fig. 4). This carbamate compound is a reversible inhibitor of cholinesterases. As such, it prevents any easy access from nerve agents to the catalytic sites of these enzymes. Enzyme protection provided by the pyridostigmine bromide is only partial and temporary. Then, in case of OP contamination of pre-treated military staff, an emergency treatment will be necessary anyway. In addition, pyridostigmine bromide does not cross the blood–brain barrier (BBB). Hence, it does not protect the central nervous system from epileptic seizures.

Detoxifying enzymes, also called bioscavengers, stand as valuable contenders for the development of the next generation of pre-treatment for OP poisoning (Fig. 4). If a contamination risk is suspected, bioscavengers will be intravenously injected to relevant military staff. After contamination and subsequent penetration in the blood circulation, nerve agents will be neutralized by detoxifying enzymes. Major research programs conducted in this area consist in modifying by directed mutagenesis, natural enzymes found in humans so as to upgrade their capacity to either entrap nerve agent molecules (stoichiometric bioscavengers) or to neutralize them (catalytic bioscavengers). Investigations also aim at increasing enzyme half-time or reducing immune response induced by bioscavengers when injected in human blood circulation (Masson et al., 2008; Rochu et al., 2007). Studied detoxifying enzymes are cholinesterases, the natural targets of OP, and paraoxonase. Recently, a recombinant human butyrylcholinesterase was produced in the milk of transgenic mice and goat, making this enzyme easy to purify. This recombinant enzyme exhibited normal *in vitro* affinity for standard nerve agents such as soman, sarin, tabun or VX (Huang et al., 2007).

Protection and decontamination

Inhalation, absorption through the skin and oral route are the three possible pathways for OP penetration in the human body. Gas mask and protective clothes including butyl gloves, carbonaceous over-garments and over-boots are the main countermeasures within the French army to prevent OP penetration. However, in case of nerve agent exposure, a decontaminating glove containing Fuller's earth is made available to the military staff so as to absorb OP droplets. Unfortunately, there are no protective resources for civilians except their own clothes which are very ineffective against massive nerve agent attack (terrorist attack) since these clothes can be easily impregnated with neurotoxicants. As a matter of interest, flour can be a substitute for the Fuller's earth glove for the emergency decontamination of the civilian population. As regards both military staff and civilians, the recommended delayed decontamination method is a shower with soap or with 0.5% to 0.8% hypochlorite solution prepared in water (diluted bleach). Decontamination is a crucial step within the intoxication countermeasure process insofar as it allows elimination of OP which has not penetrated through the skin of contaminated people. Thus, self-contamination of exposed people or accidental contamination of emergency assistance staff can be avoided.

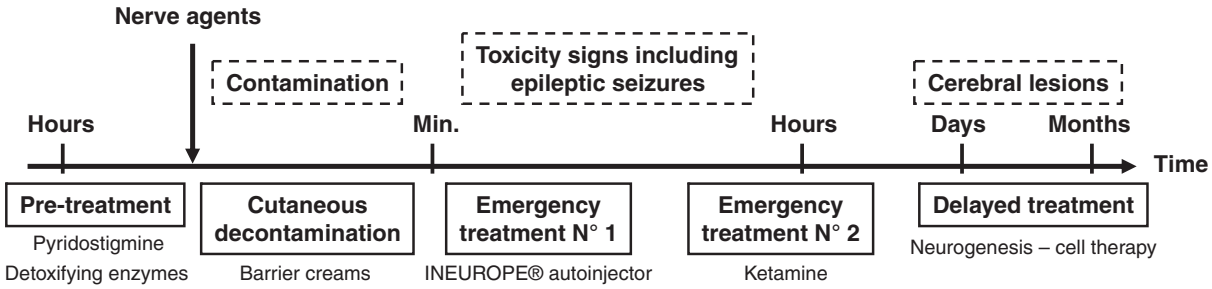

Fig. 4. Existing and potential countermeasures against nerve agent intoxication. Time-course evolution of nerve agent intoxication includes 3 different phases: the contamination, the onset of toxicity signs and the development of brain lesions. Countermeasures developed by the French military research mainly focused on four different therapeutic aspects covering the full time-course of the nerve agent poisoning: pre-treatment (pyridostigmine; detoxifying enzymes), cutaneous decontamination (barrier creams), emergency treatment (INEUROPE® autoinjector; ketamine) and delayed treatment (neurogenesis and cell therapy).

Future protection systems are orientated towards skin lotions or creams able to prevent neurotoxicant penetration through the skin, or even better, able to inactivate nerve agents (Fig. 4). Some passive protective barrier creams are currently used in different armies such as SERPACWA (USA), AG-7 (UB) and IB-1 (Israel). In addition, RSDL (reactive skin decontaminant lotion—Canada and USA) is a decontaminating solution which readily sequesters and destroys chemical warfare agents when applied on the skin of exposed people. In France, a new hydro-alcoholic gel called A16 (patent pending), exhibits a better protection against VX contamination compared to AG-7 or SERPACWA (Millerioux et al., 2009). In the future, detoxifying enzymes or chemical compounds could be incorporated in this gel in order to inactivate nerve agents.

Emergency treatments

After OP exposure, emergency treatments will be promptly administered to contaminated military staff at the onset of the first clinical signs to prevent the respiratory distress and the development of epileptic seizures. Since nerve agent-induced neuronal damage originates from epileptic seizures, all available emergency treatments take seizure prevention into consideration.

As an example, French military troops have access to the INEUROPE® autoinjector (Fig. 4). This two compartment wet/dry system autoinjector contains 3 pharmacological compounds: 350 mg pralidoxime (contrathion®), 2 mg atropine and 20 mg avizafone (Lallement et al., 2007). Pralidoxime is an oxime acting as a reactivator of OP-inhibited cholinesterases (Fig. 1). Atropine is a muscarinic receptor antagonist. Hence, it blocks the muscarinic response due to the hypercholinergic event triggered by the nerve agent poisoning (Fig. 1). Avizafone, a diazepam pro-drug, is a powerful anticonvulsant able to terminate OP-induced epileptic seizures (Fig. 1). However, this autoinjector is efficient only if the contained antidotes are delivered in the first 20 min following nerve agent exposure.

Once treated with the autoinjector, OP-intoxicated military staff will be rushed to a first-aid emergency unit to receive oxime, atropine and benzodiazepine infusions.

If necessary, artificial breathing can be provided to nerve agent-poisoned soldiers. It can vary from a simple oxygen mask in a first-aid post on the battlefield to controlled breathing lasting between several hours to several days in the hospital (Fig. 1).

In France, the main research on emergency treatment aims at testing on soman-poisoned rodent models, new pharmacological molecules potentially more efficient than the ones present in the INEUROPE® autoinjector or new molecules able to stop prolonged epileptic seizures. For example, the oxime contained in the INEUROPE® autoinjector cannot reactivate cholinesterases inhibited by soman, tabun or cyklosarin. The oxime HI-6 would display enhanced enzymatic reactivation properties as compared to pralidoxime (Dorandeu et al., 2007a). Another molecule, ketamine (a NMDA receptor antagonist), is a general anesthetic used to stop refractory epileptic seizures in hospitalized patients (Figs. 1 and 4). Studies are carried out to evaluate the effect of ketamine on different animal models after soman intoxication (Dorandeu et al., 2007b).

Delayed treatment and cell therapy

As mentioned above, emergency treatment (i.e. oxime, atropine and benzodiazepine mix) must be administered to rodent models in the first 20 min following soman exposure to prevent either respiratory distress-induced death or brain lesion development. In case of nerve agent spreading over a battlefield or terrorist attack, the delivery of the emergency treatment to contaminated humans would probably not be performed in time to ensure optimal treatment efficiency due to the obvious lack of appropriate first-aid assistance. Such a delay would in all likelihood lead to the development of damage in the brain of some surviving exposed people. This fear was unfortunately confirmed by the terrorist attack in Tokyo's subway with sarin gas. Indeed, some victims exhibited signs of neuropathy and ataxia within several months to several years after exposure (Yanagisawa et al., 2006). An obvious therapeutic approach would be to prevent or at least to modulate OP-induced neuronal cell death to protect victims against brain lesions. However, very little has been published to date on a potentially efficient neuronal cell death inhibitor after nerve agent poisoning in animal models. In the absence of an adequate emergency treatment, there is an extensive need therefore for adequate availability of a delayed treatment to repair nerve agent-induced brain lesions. Brain cell therapy and neurogenesis could provide valuable approaches in terms of delayed treatment to repair cerebral damage triggered by neurotoxicant intoxication.

Cell therapy

Cerebral cell therapy can be defined as a replacement of dead cell by the transplantation of cultured progenitor cells in lesioned brains. These engrafted cultured progenitor cells can originate from neural stem cells (brain-derived stem cells) but also from non-neural stem cells (such as bone marrow or umbilical cord stem cells).

Neural stem or precursor cells were isolated from adult or embryonic rodent brains, amplified by culture and then, transplanted in animal models of spinal cord injuries, stroke (ischemia induced by middle cerebral artery occlusion) or neurodegenerative diseases (Parkinson's disease, multiple or amyotrophic lateral sclerosis, Alzheimer's and Huntington's diseases). After engraftment in lesioned brain or spinal cord, neural progenitors differentiated into either mature neurons or glial cells. This cell replacement led to partial or total functional recovery in the animal models (Einstein and Ben-Hur, 2008; Lindvall and Kokaia, 2006; Okano and Sawamoto, 2008).

Bone marrow stromal cells (BMSCs), also called mesenchymal stem cells (MSCs) have the ability to differentiate both *in vitro* and *in vivo* into non-mesodermal mature cells such as neurons and astrocytes. For this

reason, wild-type or genetically-engineered MSCs were injected either by intracerebral or intravenous routes to different rodent models of ischemia, traumatic brain injuries or neurodegenerative diseases (Li and Chopp, 2009; Phinney and Isakova, 2005). After migration and engraftment in damaged brain areas, a subsequent differentiation of MSCs into mature neurons and fibroblasts was reported, promoting repair and regeneration of lesioned cerebral tissue. MSC engraftments generated noticeable therapeutic benefits in terms of functional recovery (Li and Chopp, 2009; Phinney and Isakova, 2005).

We recently injected by stereotaxic procedure, BrdU or Qtracker nanocrystal-labeled human MSCs in the damaged hippocampus of soman-poisoned mice (MSCs intracerebral injections performed on post-soman day 7). Engrafted MSCs were detected on post-soman days 21 only in the injection site but not in other lesioned hippocampal areas such as CA1 field, indicating thereby the absence of any injected MSCs migration [unpublished data]. It can be hypothesized that chemotactic cytokines (i.e. proteins involved in MSC migration process) generated by the damage provoked by the injection needle certainly attracted MSCs with enhanced efficiency compared to chemotactic cytokines resulting from soman-induced lesions. Interestingly, the absence of any graft rejection was noticed in our experiment despite the xenograft procedure we applied (graft of human MSCs into mouse brains). This phenomenon is probably due to the poorly immunogenic status of injected human MSCs (Sotiropoulou and Papamichail, 2007; Uccelli et al., 2006). However, MSCs transplantation into OP-lesioned brains through intracerebral injection route stands out as the least adequate alternative for delayed treatment since the amount of engrafted MSCs remained restricted (limitation of injected volumes) and the intracerebral injection procedure is an additional cause for further brain injury.

Neurogenesis

Neurogenesis is a natural regenerative process occurring in mammalian adult brains and is based on the continuous *in vivo* proliferation of endogenous neural stem cells (NSCs). NSCs are located in the subgranular zone (SGZ) of the dentate gyrus and in the subventricular zone (SVZ). In unlesioned mammalian adult brains, proliferating NSCs give birth to neural progenitors also called neuroblasts. SGZ-derived neural progenitors migrate a short distance mainly into the dentate gyrus granule cell layer and, to a lesser extent, into other regions of the hippocampus. After neuronal differentiation, SGZ-derived newborn neurons functionally integrate the existing brain circuitry and participate into hippocampal-dependent cognitive learning and memory processes (Zhao et al., 2008). In rodent, SVZ-originating neural progenitors migrate through the path known as the rostral migratory stream to the olfactory bulb. In the olfactory bulb, SVZ derived-neuroblasts differentiate into neurons and might be involved in odor memory and olfactory learning mechanisms (Zhao et al., 2008). Most of both SVZ and SGZ-derived newborn neurons die within the first month after engraftment and subsequent neuronal differentiation. Interestingly, enriched environments (e.g. cages with a variety of objects such as boxes, chains, ladders, running wheels, etc.) and enriched odor exposure increase the survival of newly generated neurons in the hippocampus and olfactory bulb, respectively (Zhao et al., 2008). In addition, an enriched environment also enhances the proliferation rate of neural progenitors in undamaged rodent brains and heightens learning and memory skills in these animals (Nithianantharajah and Hannan, 2006).

Several molecules and proteins are known to increase neurogenesis in the intact brain of wild-type rodents. Growth factors or neurotrophic factors such as CNTF, BDNF, EGF, FGF-2, IGF-1, TGFα and VEGF (Bauer et al., 2007; Hagg, 2005; Parent, 2007; Zhao et al., 2008) promotes *in vivo* proliferation of neural stem cells in either SVZ or SGZ. Some of these factors are also involved in the long-term progenitor survival (VEGF), neuroblast migration (CNTF) (Bauer et al., 2007) or neuroblast differentiation into neurons (BDNF, IGF-1) (Bauer et al., 2007; Parent, 2007). Erythropoietin (EPO), an erythropoiesis stimulator, exhibits neuroprotective functions but also enhances the neurogenesis process in various animal models of brain injuries including stroke, cerebral ischemia and traumatic brain injury (Liu et al., 2008). As another example, prolactin, an hormone secreted during reproductive states, induces neurogenesis in the maternal brain (Bridges and Grattan, 2003). Serotonin and antidepressant treatments (fluoxetine, rolipram, agomelatine) also promote the proliferation of neural progenitors in the dentate gyrus. Contrariwise, stress and related secreted glucocorticoid hormones (corticosterone; cortisol, etc.) reduce the proliferation of SGZ progenitor cells (Paizanis et al., 2007; Zhao et al., 2008).

Various damage occurrences in the mammalian adult brain, including global or focal ischemia, stroke, traumatic injury, electroconvulsive shocks and kainate or pilocarpine-induced *status epilepticus*, stimulate neurogenesis (Burns et al., 2009; Parent, 2007; Zhao et al., 2008). In most of these studies, brain lesions expanded the population of neural progenitors in both the SVZ and SGZ. Some newly engrafted neurons were also detected in lesioned cerebral areas such as the hippocampal CA1 and CA3 fields, cortices and striatum, depending on the nature of brain injury. A subsequent differentiation of these engrafted neuroblasts into neurons or astrocytes was generally observed (Burns et al., 2009; Parent, 2007; Zhao et al., 2008). The stimulation of neurogenesis after brain lesions could be explained by 2 different types of mechanisms involving endogenous signal production: factors synthesized by apoptotic dying cells and cytokines produced during the inflammation process (Burns et al., 2009). Even though little is known about factors synthesized by apoptotic dying cells, there is, however some sizeable amount of information available to date as regards the inflammation process. As mentioned above, pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 are secreted by both activated microglial and astroglial cells but microglia synthesizes much higher quantities of these cytokines than astrocytes. Il-6 has a detrimental effect by reducing hippocampal neurogenesis. The role of TNF-α and IL-1β is more controversial since these two pro-inflammatory cytokines have shown either negative or positive influence on the proliferation of NSC (Ekdahl et al., 2009; Whitney et al., 2009). During the inflammation process, activated astroglial cells also secrete various neurotrophic and growth factors such as VEGF, BDNF, CNTF and FGF-2. All these cytokines were described as stimulators of neurogenesis by increasing neural progenitor proliferation in both the SVZ and SGZ (Brockington et al., 2004; Hagg, 2005).

The long-term effect of soman poisoning on neurogenesis was recently studied in mice using BrdU labeling and immunohistochemistry (Fig. 3) (Collombet et al., 2005b, 2006a, 2008). BrdU, a nucleotide derivative, is only incorporated in the DNA of replicating cells and can be detected with immunochemistry technique. The validation of this method to detect neural progenitors has been widely substantiated (Taupin, 2007).

A transient reduction of neural progenitor proliferation was measured on post-soman day 1 or day 3 in the SGZ or SVZ, respectively (Fig. 3) (Collombet et al., 2005b). This decrease in NSC cell division may rely either on a detrimental effect of over-expressed pro-inflammatory cytokines or on a decline of ATP stocks preventing cell proliferation. Indeed, a massive microglial activation was detected in the brain of soman-intoxicated mice 1 and 3 days after neurotoxicant exposure and, as mentioned above, activated microglia synthesizes huge amounts of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) known to inhibit the proliferation of neural precursors. Concerning the hypothesis of the low bioenergetic status, a 50% reduction of *in vivo* ATP stocks was shown in the brain of rats shortly after soman exposure (Miller and Medina, 1986). This lack of ATP may impede neural progenitor proliferation.

After the transient decrease phase, a long-term increase in neural progenitor replication was observed over a 1-month period in both the SVZ and SGZ with a peak at 3 or 7 days after soman poisoning in the SGZ or SVZ, respectively (Fig. 3) (Collombet et al., 2005b). Interestingly, this 1-month period of NSC division heightened level coincides with the

long-term duration of astroglial cell activation present in the brain of soman-intoxicated mice (Fig. 2). Indeed, activated astroglia produce numerous growth factors or cytokines (VEGF, BDNF, CNTF and FGF-2) enhancing neural progenitor proliferation. As a result, findings display an expanded neuroblast population over the first month after soman exposure.

Two and 3 months following soman poisoning, a sizeable drop in neural precursor proliferation was evidenced in both the SVZ and SGZ of our animal model (Fig. 3) (Collombet et al., 2005b). This time-course matches with the delayed neuronal cell death observed after soman intoxication in mice (second toxicity step initiated 1 month after soman exposure as previously mentioned in this review). It can be assumed therefore that some unknown endogenous signals or factors produced by dying neurons could be detrimental to the replication of NSC.

In an additional experiment, the destiny of BrdU-labeled replicating neuroblasts was analyzed. As assessed by BrdU/NeuN double labeling observed under light microscope, some cells positively labeled with BrdU also expressed NeuN proteins (Collombet et al., 2005b). These differentiated cells are located in SVZ and SGZ neurogenic niches and in soman-damaged brain regions such as the hippocampal CA1 field and amygdala as well (Collombet et al., 2005b). More recently, fluorescence microscopy was performed to substantiate the previously published results obtained by light microscopy (unpublished data). In addition, mature healthy neurons expressing NeuN were numbered in a time-course experiment covering a 3-month period after soman exposure in mice (Collombet et al., 2006a, 2008). Degenerating neurons are excluded from this numbering since they are deprived of NeuN immunoreactivity (Collombet et al., 2006b). A neuronal regeneration was clearly demonstrated in both the hippocampal CA1 field and amygdala of soman-exposed mice (Figs. 2 and 5). Three months after intoxication, the neuronal regeneration observed in the amygdala is five-fold higher than the one measured in the hippocampus (Collombet et al., 2006a, 2008).

Cognitive impairment and delayed recovery after nerve agent poisoning

The impact of neuronal regeneration on functional and cognitive processes was investigated. T-maze and Morris water maze tests especially address spatial mnemonic processes which are functionally supported by the hippocampal formation (O'Keefe and Nadel, 1978). On the other hand, the amygdala is greatly involved in the fear conditioning response (Phelps and LeDoux, 2005). One month after intoxication, poisoned mice exhibited a dramatic performance slump in mnemonic cognitive tasks (T-maze and Morris water maze test) as well as an abnormal elevated anxiety profile (experiments of auditory and contextual conditioned fear or elevated-plus maze) (Coubard et al., 2008; Filliat et al., 2007; Mamczarz et al., 2010). Our results also clearly demonstrated that a rapid development of brain lesions in the hippocampus is a prerequisite to observe a delayed alteration of hippocampal-driven spatial memory in mice (Filliat et al., 2007). Such data was confirmed and completed by Pernot et al. (2009). Indeed, intrahippocampal injection of a low soman dose (1 nmol) in mice promoted delayed epileptogenesis in the absence of brain damage and failed to alter spatial memory scores of animal models subjected to Morris water maze experiments (Pernot et al., 2009). In our study, 3 months after poisoning, a very faint increase in spatial memory skills was observed in intoxicated mice as assessed by the Morris water maze test (Filliat et al., 2007). In parallel, T-maze abnormal exploratory behavior resulting from soman intoxication entirely recovered 3 months after nerve agent exposure (Coubard et al., 2008). At the same time, poisoned mice displayed a full recovery of anxiety and emotional behavior profiles (Coubard et al., 2008). The entire recovery of fear conditioning but not of spatial memory can be explained by the level of neuronal regeneration in the amygdala as compared to the one measured in the hippocampus. As mentioned above, the neuronal regeneration in the amygdala is five-fold higher than the one quantified in the hippocampal CA1 field (Collombet et al., 2006a, 2008). It can be assumed therefore that the magnitude of cell regeneration within the amygdala is elevated enough to allow a recovery of normal fear conditioning response. Conversely, the low level of cell regeneration into the hippocampus does not contribute any enhancement of spatial memory skills in soman-poisoned mice. In addition, the implementation of efficient spatial memory processes during the brain repair procedure may require more complex neuronal interconnections and more distributed neural networks than the ones necessary for the fear-conditioning task as previously stated by Coubard et al. (2008).

The presence of newborn neurons in soman-damaged brain areas could be the consequence of an active neurogenesis process involving the migration of BrdU-labeled neuroblasts (generated in SVZ or SGZ) to sites of injury followed by a subsequent differentiation of these cells into mature neurons expressing NeuN. In most studies describing brain damage repair, neurogenesis is validated by the presence of BrdU/NeuN double-labeled cells in lesioned cerebral regions. In the specific field of epilepsy, neurogenesis occurring in lesioned brain regions has already been reported, thus providing further legitimacy to our results obtained in soman-intoxicated mice. For example, the

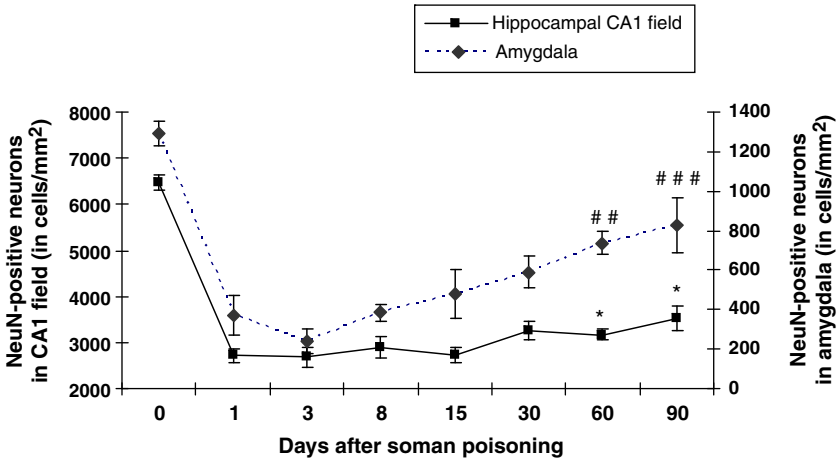

Fig. 5. Quantification of NeuN-positive neurons in mouse hippocampus and amygdala after soman exposure. NeuN-positive neurons (healthy mature neurons) were quantified in hippocampal CA1 field and amygdala of soman-poisoned mice on days 1, 3, 8, 15, 30, 60 and 90 after exposure or control animals. For each experimental group, the mean cell density value for 8–12 mice and its standard error of the mean (SEM) were calculated. Dunnett's t-tests were performed to compare neuron densities between post-soman day 1 (reference) and other experimental times after intoxication or controls. Significance was set at $p<0.05$ ($^{*}p<0.05$; $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$).
This figure is modified from Collombet et al. (2006a, 2008).

presence of newborn neurons was revealed in the amygdala of rats exhibiting pentylenetetrazole-driven epileptic seizures (Park et al., 2006). Neurogenesis was also found in the hippocampal CA3 field of adult or neonatal rats subjected to kainic acid- or pilocarpine-induced *status epilepticus* (Dong et al., 2003; Scharfman et al., 2000). However, an alternative hypothesis to neurogenesis can be put forward to account for the neuronal regeneration exhibited in soman-lesioned brain areas. Indeed, a possible reversion status of degenerating neurons reverting to healthy ones must also be taken in account. Nevertheless, the BrdU/NeuN double labeling immunohistochemistry method performed to detect neuronal regeneration does not suffice to differentiate between a neurogenesis process or a reverting status of degenerating neurons to account for the presence of newborn neurons in injured brain areas. Indeed, a DNA repair can occur in the nucleus of degenerating neurons by the time of BrdU injection and, then, these repairing cells will certainly incorporate BrdU in their nuclear DNA. If these BrdU-labeled degenerating neurons revert to healthy neurons, it can be hypothesized that these cells will also regain the NeuN immunoreactivity transiently lost after soman poisoning. Hence, reverted degenerating neurons would be detected as BrdU/NeuN double-labeled cells and could be mistaken with real newborn neurons similarly labeled and ensuing from the active neurogenesis process.

Since cytokines such as CNTF, BDNF, EGF, FGF-2, IGF-1, TGFα and VEGF are well known to promote neurogenesis (Bauer et al., 2007; Hagg, 2005; Parent, 2007; Zhao et al., 2008), some of these growth factors were subcutaneously injected to soman-exposed mice in order to evaluate their effect on neuronal regeneration and functional recovery. A cocktail of EGF and FGF-2 (dose of 40 μg/kg/day for both cytokines) was administered for 8 days to the mouse model of soman intoxication. As shown on post-soman day 9, the cytokine treatment did not modify the rate of proliferation of BrdU-labeled neural progenitor in either SVZ or SGZ but seemed to foster the migration of these cells to adjacent brain regions (e.g. hilus of the dentate gyrus, septum). At a later experimental time (post-soman day 34), mobilized neuroblasts were detected in damaged brain regions such as the hippocampal CA1 field and amygdala. In terms of differentiation, engrafted neural progenitors appeared to preferentially differentiate into mature NeuN-expressing neurons in the hippocampus while the astrocyte phenotype was favored in the amygdala (Collombet et al., 2005c). This result was confirmed with the quantification of NeuN-expressing neurons in the hippocampal CA1 field and amygdala of mice intoxicated with soman and treated or not with the EGF and FGF-2 cocktail (Collombet et al., 2011). One and 3 months after poisoning, the cytokine treatment increased by 15% the neuronal regeneration measured in the hippocampal CA1 field of poisoned mice. Contrariwise, the cytokine treatment did not modify the neuronal regeneration observed in the amygdala of exposed mice, possibly confirming the orientated differentiation of neuroblasts towards an astrocyte phenotype (Collombet et al., 2011). Interestingly, despite a positive effect on the hippocampal neuronal regeneration, the cytokine treatment bears no influence on the hippocampal-driven spatial memory skill (Morris water maze and T-maze experiments) which dramatically plummeted after soman exposure. As an additional hypothesis, we are entitled to assume that despite the quantity of newborn/recovered neurons detected in the CA1 field, the threshold of functional mature neurons with complex neuronal interconnections necessary to observe an improvement of spatial memory hasn't been reached as yet. It is noteworthy that in other pathologies, slightly different results were obtained. For example, in focal middle cerebral artery occlusion (MCAO) ischemia performed in mice, combined infusion of EGF and FGF-2 into cerebral ventricles induced massive proliferation of neural progenitors and subsequent migration of these cells into the CA1 field and temporal cortex. Furthermore, the authors (Nakatomi et al., 2002) evidenced that most of these mobilized neural progenitors differentiate into neurons after they achieved their migration into the CA1 field and temporal cortex. The cytokine treatment and the related increased neurogenesis greatly contributed to the improvement of ischemic neurological deficits as assessed by Morris water maze experiments (Nakatomi et al., 2002). As suggested by the authors, newborn neurons promoted by cytokine therapy were functional and correctly integrated into the hippocampal neural circuitry. For reasons that remain unknown as yet, it does not seem to be the case with soman-poisoned mice.

On the other hand, the cytokine treatment accelerates the complete recovery of a normal anxiety profile (tests of auditory and contextual conditioned fear) in soman-intoxicated mice. Indeed, normal anxiety behavior is recovered only 1 month after poisoning in cytokine-treated mice while a 3-month period is necessary to regain a normal emotional behavior in the absence of cytokine treatment (Collombet et al., 2011). The accelerated emotional behavior recovery after cytokine treatment cannot be explained by a positive effect of this treatment on the neurogenesis process occurring in the amygdala since the level of the neuronal regeneration in this brain region remained similar in soman-exposed mice subjected or not to cytokines. Amygdala is a central structure in the circuitry involved in fear conditioning but other regions such as the hippocampus, thalamus, septum and cortices are also involved in emotional learning and memory (Phelps and LeDoux, 2005). Thus, it can be hypothesized that the cytokine treatment-driven enhancement of the neuronal regeneration in one or more of these brain areas could be effective enough to accelerate the total recovery of the emotional behavior. Then, the increased hippocampal neurogenesis evidenced in soman-exposed mice treated by EGF and FGF-2 cytokines could be held accountable for the rapid normalization of the anxiety profile (Collombet et al., 2005c, 2011). The involvement of FGF-2 related-hippocampal neurogenesis in the regulation of mood and emotions has recently been investigated (Perez et al., 2009), confirming the latter hypothesis. In this study, two genetically different groups of rats presenting either low or high anxiety profiles were cross-examined to assess anxiety behavior (elevated plus maze and light–dark box anxiety tests), expression of endogenous hippocampal FGF-2 mRNA and hippocampal neurogenesis. High-anxiety driven rats significantly exhibited reduced levels of hippocampal FGF-2 mRNA and decreased hippocampal newborn cells as compared to low-anxiety rats. After a 3-week FGF-2 treatment (intraperitoneal injection of a daily 5 μg/kg dose), high-anxiety rats displayed a reduction of their anxiety status combined with an increase in newborn hippocampal cell survival, including astrocytes and neurons (Perez et al., 2009). According to the authors, hippocampal FGF-2 has a central role in modulating anxiety behavior probably via hippocampal neurogenesis. A similar mechanism could occur in soman-poisoned mice treated with FGF-2 and EGF and could explain a swift recovery of normal anxiety profile in keeping with increased hippocampal neuronal regeneration. An alternative hypothesis to the enhancement of neuronal regeneration by cytokine treatment can be put forward to explain the restoration of a standard mouse emotional behavior after soman poisoning. Indeed, a direct anxiolytic effect of injected growth factor can be proposed as an alternative explanation. A recent experiment corroborates such a hypothesis. A single administration of a 20 μg/kg FGF-2 dose in rats triggers long-term extinction of fear and attenuates reinstatement-induced recovery of learned fear (Graham and Richardson, 2009).

In conclusion, the use of a cytokine treatment seems to be a breakthrough therapeutic approach to repair brain lesions induced by nerve agent poisoning. With the administration of a cocktail containing FGF-2 and EGF, we clearly demonstrated that injected cytokines enhanced the neuronal regeneration occurring spontaneously in specific soman-injured cerebral regions. Furthermore, such neuronal regeneration seemed to participate in the partial or total recovery of behavioral functions altered by soman intoxication. However, the capability to increase neuronal regeneration or engraftment of newborn neurons in a lesioned brain area cannot be considered as a leading therapeutic contribution if the regenerated neurons are not adequately integrated in the brain network circuitry responsible for complex mnemonic and social behaviors. Then, more sophisticated treatments consisting of a

combination of various drugs providing specific but different roles in the brain therapy strategy could be developed. For example, cytokines such as BDNF or NGF, fostering axonal growth and extended axonal connections, neuronal survival or differentiation could be incorporated in the treatment. EPO is likely to stand as a valuable contender since such a molecule showed both neurogenesis stimulation and neuroprotective roles in rodents with traumatic brain injury, cerebral ischemia and stroke (Liu et al., 2008). At last, housing environment for animal models in upgraded conditions provides a further valuable therapeutic resource catered to repairing brain lesions (Will et al., 2004). Indeed, an enriched environment interacts at different levels within the cerebral repair process. Such instances reported herein provide substantial evidence to the effect that an enriched environment increases neural progenitor proliferation and neurogenesis in the SVZ in rat models with MCAO stroke. Using limb placement test on a rotating pole, a functional improvement is demonstrated in enriched rats but this cannot be related to an increased replacement of mature neurons in the infarct zone (Komitova et al., 2005). The authors argue that functional recovery in enriched animals could be related to an unknown neuronal plasticity or re-organization. Secretion of trophic factors by immature neuronal cells found in the infarct area could also exert some effects on the recovery process. In a sophisticated experiment, Rampon et al. (2000) examined the effects of enriched environmental conditions on 3 types of non-spatial memory in knockout mice lacking NMDA receptor-1 in a CA1 hippocampal field (Rampon et al., 2000). The enrichment enhances learning and memory skills in mutant mice and further increases synapse density in the CA1 field. According to the authors, it remains to be determined whether an enrichment-induced rise in CA1 synaptic density has any functional role in the concomitant behavioral improvement (Rampon et al., 2000). If an enriched environment participates in the axonal connections and plasticity to recreate a functional brain network circuitry then, such an approach would hence contribute a further potent therapeutic strategy catered to repairing brain damage.

Conclusions

Over the past decade, military research based on countermeasures aimed at preventing the effects of nerve agent poisoning mainly focused on four different therapeutic areas encompassing the comprehensive time-course of a neurotoxicant contamination: pre-treatment, cutaneous decontamination, emergency treatment and delayed treatment (Fig. 4). The research performed in the field of delayed treatment generated valuable results leading the way to a speculative cascade of long-term neuropathological mechanisms induced by nerve agents such as soman, in rodent models (Fig. 2). To summarize, a massive inflammatory response is rapidly initiated by the early acute neuronal cell death occurring within 24 h of soman exposure. Indeed, this neuronal cell death, possibly a necrotic process, triggers the activation of both microglial (peak on post-soman day 3) and astroglial cells (peak on post-soman day 8). This phenomenon pairs up with an important peripheral inflammation evidenced in rodent blood. The activation of astroglia is a long-term process, lasting up to 3 months after soman exposure, leading to the formation of a glial scar in some discrete highly damaged brain regions (mainly in amygdala and hippocampus). To date, it is widely acknowledged that activated glial cells secrete various growth factors and pro-inflammatory cytokines, including BDNF, FGF-2, NGF, VEGF, TNF-α, IL-6 and IL-1β. The secretion of some of these cytokines was observed in soman-poisoned rodents. Extensive conducted research has demonstrated the key role of glial-secreted factors to promote angiogenesis (VEGF), to increase the proliferation level of neural progenitors (VEGF, BDNF and FGF-2) and to interact in the balance between the survival and the death of neurons (IL-1β, IL-6, TNF-α, NGF, FGF-2 and BDNF). Interestingly, the early acute neuronal cell death revealed after soman poisoning combined with the appearance of degenerating neurons in lesioned cerebral regions. Degenerating neurons survived at least 1 month after soman intoxication before dying (phase of delayed neuronal cell death). Cytokines secreted by activated glia could afford for the long-term survival of these degenerating neurons. In addition, our data clearly indicates that an angiogenic process and a heightened proliferation of neural progenitors followed by a neuronal regeneration in injured brain areas was detected after soman exposure in mice. Both angiogenesis and neuronal regeneration certainly constitutes an attempt at physiological self-repair promoted by *in vivo* secreted cytokines. The consequence of spontaneous neuronal regeneration on behavioral recovery was also investigated in soman-intoxicated mice. A complete recovery of emotional behavior was assessed over time while spatial memory skills were only partially recovered 3 months after soman exposure.

A cytokine treatment (cocktail of EGF and FGF-2) was administered to soman-poisoned mice and the effect of this treatment was scrutinized in terms of neuronal regeneration and behavioral recovery. With the cytokine treatment, neuronal regeneration was enhanced in the hippocampal CA1 field but remained unchanged in the amygdala. The enhanced neuronal regeneration in the hippocampus had no effect on the spatial memory skill (a memory process related to the hippocampus functioning) despite a full recovery of the exploratory behavior. The weak magnitude of the neuronal regeneration or an incomplete integration of newborn neurons in the lesioned hippocampus could prevent spatial memory recovery. Surprisingly, the complete recovery of emotional behavior (mainly mediated by the amygdala) was speeded up by the cytokine treatment. A direct anxiolytic effect of injected cytokines could account for the accelerated normalization of the emotional behavior.

To conclude, cell therapy and neurogenesis boosted by a cytokine treatment seem to be a promising therapeutic approach to repair soman-induced brain lesions. However, the neuronal regeneration in damaged cerebral areas must be accompanied by a correct incorporation of newborn neurons in the brain network circuitry. Enriched environment and/or administration of additional cytokines (BDNF or NGF) could favor a correct axonal growth and extended connections (axonal plasticity) to boost functional recovery after nerve agent exposure.

Conflict of interest statement

I declare no conflict of interest.

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