Development of neuropathology following soman poisoning and medical countermeasures

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Abstract

Nerve agent-induced seizures can cause varying degrees of neuropathology depending on level of poisoning and duration of seizing. The intention of this review was to validate a novel approach for establishing effective treatment regimens against soman poisoning. Identification of seizure controlling sites in the forebrain of rats poisoned by soman was made by means of lesions, and the anticonvulsive properties of a number of relevant drugs were tested by microinfusions into the identified areas. By using these procedures, procyclidine emerged as the most potent drug. Its potency was confirmed in systemic studies and is further enhanced when combined with levetiracetam. Acute treatment with a combination of HI-6, levetiracetam and procyclidine (procyclidine regimen) can effectively manage supralethal poisoning by any of the classical nerve agents. Extended treatment with the procyclidine regimen is able to terminate residual "silent", local epileptiform activity in the severely poisoned rats. Evident advantages are seen when the same regimen exerts both powerful anticonvulsant and neuroprotectant efficacies. According to the results presented, the new strategy for establishing therapies against soman-induced seizures appears to be valid.

Key words: Nerve agents, seizures, neuropathology, countermeasures

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1. Introduction and historical background

Intoxication by the organophosphorus (OP) nerve agent soman, an irreversible acetylcholinesterase (AChE) inhibitor, causes a progression of toxic signs including miosis, hypersalivation, respiratory distress, tremor, seizures/convulsions, coma, and death. In survivors, soman can evoke sustained seizure activity resulting in neuropathology in vulnerable brain areas like the piriform cortex, amygdala, and hippocampus. A large body of evidence relates brain damage to secondary massive release of glutamate, a neurotransmitter with excitotoxic potential (Carpentier et al., 2010).

It has been hypothesized that several neurotransmitter systems probably become involved sequentially in the initiation and maintenance of seizures elicited by nerve agents (McDonough and Shih, 1997). The progression of events can be divided into 3 phases. An early cholinergic phase lasting from the time of exposure to about 5 min after onset of seizures is dominated by high cholinergic activity followed by a transitional phase of cholinergic and glutamatergic hyperactivity and finally a predominantly glutamatergic phase after about 40 min (McDonough and Shih, 1997).

In a majority of nerve agent studies, soman has been used in animal models to evaluate potential anticonvulsant drugs. The reason for this is that a higher dose of anticonvulsants is required to terminate seizures induced by soman than by other classical nerve agents (tabun, sarin, cyclosarin, VX). This finding suggests that drugs effective against soman will most likely also be effective against other nerve agents (Shih and McDonough, 2000). In the latter study, it was shown that soman, tabun, sarin, cyclosarin, VX, and VR in most cases can evoke seizure activity when given at toxic doses (2 x LD_{50}) in guinea pigs.

Brain damage caused by nerve agent-evoked seizures is most likely associated with glutamatergic excitotoxicity. About 20 min after onset of seizures the first signs of

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neuropathology are usually detectable (Lallement et al., 1994; McDonough et al., 1995). Seizures lasting beyond 40 min are increasingly difficult to terminate (Carpentier et al., 2001b; Lallement et al., 1999). Convulsive activity lasting for only 45 min can result in large variations in brain damage (from none to about 10 %), and these variations correlate positively with impairments in cognitive tasks (Myhrer et al., 2005). Hence, it is very important to control soman-induced seizures at an early stage (< 20 min) to prevent long-term cognitive deficits.

A number of armed forces have based their therapy against nerve agent intoxication on an oxime (obidoxime, 2-PAM, HI-6), an anticholinergic (atropine), and a $GABA_A$ agent (diazepam, avizafone) combined with carbamate (pyridostigmine) pretreatment (Aas, 2003). However, such treatment regimens can reduce immediate lethality, but they do not attenuate the occurrence of nerve agent-induced seizure activity and subsequent brain damage, unless atropine is given early and at a high dose (McDonough and Shih, 1997). Thus, there is an urgent need to search for novel strategies able to save lives and prevent or terminate nerve agent-induced seizures.

The purpose of the present study was to validate a novel strategy for establishing therapies that effectively can terminate seizure activity and prevent or reduce the development of neuropathology in soman poisoned rats. Ideas have been derived from epilepsy research in which seizure controlling sites in the rat brain have been mapped for screening of antiepileptics. Through studies from our laboratory, target areas for soman have been identified by use of selective lesions, and effects of microinfusion of relevant anticonvulsants into the specified areas have been tested to map critical pharmacological receptors. Furthermore, promising drugs from microinfusion experiments have been tested by systemic administration, and anticonvulsant and neuroprotectant efficacies of some drug combinations have been examined. This work is designed as an extensive analysis of factors

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crucial for the evolvement of neuropathology and differs from other recent nerve agent reviews like the one of de Araujo Furtado et al. (2012) and documents focusing on clinical treatment procedures (CDC; Center for Disease Control, EPA; Environmental Protection Agency, OSHO; Occupational Safety and Health Organization, ATSDR; Agency for Toxic Substances and Disease Registry, OPCW; Organization for Prohibition of Chemical Weapons). The term seizure is only used when EEG recordings have been performed in the study cited. Otherwise, the term convulsion is used. The term epilepsy refers to the use in preclinical and clinical epilepsy research.

Morphological changes in the brains of guinea pigs and monkeys after exposure to VX were initially described in 2 technical reports from Canada in the early 1960s. In these unpublished reports, the brain lesions were attributed to secondary effects of hypoxia presumably caused by the poisoning (McDonough and Shih, 1997). Subsequent studies have predominantly focused on soman. The first published study on the subject appeared in 1981. Rats received various doses of soman and the brain tissue was analyzed with 5 different staining techniques. Neuropathology was most evidently seen in the rats that convulsed (Petras, 1981). In an extension of his first study, Petras (1994) concluded that convulsant doses of soman produce bilateral brain damage in rats, cats, and monkeys.

Since the early 1980s, 3 hypotheses have been raised for the mechanism of nerve agent-induced neuropathology. The first is the excitotoxic hypothesis that associates sustained seizure activity with the primary cause for neuronal damage. A second hypothesis ascribes the neuropathology to hypoxia/anoxia/ischemia and emphasizes systemic factors (oxygenation, blood flow) as responsible for the initiation of neuropathology. The third hypothesis maintains that nerve agent causes direct toxic action on the brain neurons (McDonough and Shih, 1997). There is, however, little experimental evidence for the latter hypothesis. Hippocampal neurons exposed *in vitro* only to soman for 15-20 min show no

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signs of reduced cell viability after 24 h. On the other hand, exposure to glutamate alone for 30 min produces neuronal death in almost 80% of the cells at 24 h (Deshpande et al., 1995). Likewise, direct microinfusion of soman or VX into various brain sites fails to cause neuropathology unless such injections trigger sustained seizures (McDonough et al., 1987; Myhrer et al., 2010a).

It has been reported that there are minimal changes in pO_2 in blood or brain during sustained seizure activity induced by soman (McDonough and Shih, 1997). Actually, Carpentier et al. (1990) have demonstrated increased oxygenation in the brains of rats during the first hour after a convulsant dose of soman. In rats, soman-induced seizures cause a marked hypertensive response, large increases in cerebral regional blood flow, and large increases in regional glucose utilization (McDonough and Shih, 1997). It has been hypothesized that nerve agent-induced neurotoxicity may produce disruption of the microvasculature, for instance by hypertension, and/or a relative hypoxia/ischemia due to mismatches between cerebral metabolic demands during seizure activity and blood flow and/or glucose availability. Marked leakage in the blood-brain barrier (BBB) occurs during soman-induced seizures, most prominently during the first hour when blood pressure increases are most pronounced (McDonough and Shih, 1997).

Since nerve agents are well-known for causing respiratory distress, the development of neuropathology may reasonably be attributed to hypoxia/anoxia/ischemia. The latter factors as well as sustained seizure activity can produce neuropathology through a similar final common pathway. At the light microscopy level, the resultant morphological changes would appear similar, especially when viewed late after the insult when neurodegenerative changes are well advanced (McDonough and Shih, 1997). It is, however, apparent that neuropathology can be induced by nerve agents without concomitant impairment of respiratory function. Microinfusion of soman into the perirhinal or posterior piriform

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cortices evokes seizure activity in 75% of the rats, and brain damage is seen in all of them. In the non-convulsing animals, no neuropathology is detected (Myhrer et al., 2010a). Hence, it is hard to believe that the focal administration of soman could affect the respiratory organs by way of the blood stream. Furthermore, it takes up to 14 x LD_{50} of systemically administered soman to block diaphragmatic responses in anesthetized cats kept alive by electrical stimulation of the respiratory center (Rickett et al., 1986). By endotracheal aerosolization of nerve agent the situation is different for the respiratory organs. Soman-induced acute respiratory toxicity in anesthetized guinea pigs causes lung histopathology that is reduced by endotracheal aerosolization of atropine sulfate that ensures high level of survival even in absence of AChE reactivating oxime or any pretreatment (Perkins et al., 2011). Since the authors previously have shown that methyl atropine that does not cross the BBB, also protects against soman intoxication, they suggest that the protection must involve peripheral effects or toxic effects of soman on the respiratory system (Aas et al., 1988; Walday et al., 1993) and may not be due to the effects on the central respiratory disturbance (Perkins et al., 2011). Although the potential contributions of hypoxia/anoxia/ischemia to the development of neuropathology following nerve agent exposure cannot be entirely ruled out, the majority of experimental evidence favors the excitotoxic hypothesis (McDonough and Shih, 1997). During systemic administration of convulsant doses of nerve agent, seizure activity and respiratory distress may both be induced by declined AChE-activity in the blood. However, during focal administration of nerve agent in the brain, respiratory impairment is most likely initiated by central nervous mechanisms.

2. Cholinergic systems and target areas for nerve agent-induced seizures

When AChE is inhibited by nerve agent, the buildup of acetylcholine (ACh) can theoretically activate all cholinergic receptors in the organism. In the brain, cholinergic

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innervation is seen throughout the entire organ. Three major projection systems have been identified in the rat brain (Fig. 1). One system sends axons from the nucleus basalis magnocellularis to the whole cortical mantle (Wenk et al., 1980). Another system complex in the septal area consisting of the medial septal nucleus, diagonal band nucleus and preoptic magnucellular area projects to the hippocampal region, amygdala, and piriform, insular, cingular, and entorhinal cortices (Woolf et al., 1984). A third system arising in the dorso-lateral tegmental nucleus of the brainstem innervates the medial septal nucleus and the vertical and horizontal limbs of the diagonal band area (Woolf and Butcher, 1986) as well as the ventral respiratory group (Ellenberger and Feldman, 1990; Kubin and Fenik, 2004). The ventral respiratory group also receives cholinergic input from the pedunculopontine tegmental nucleus (Kubin and Fenik, 2004). Additionally, cholinergic interneurons are located in the striatum and nucleus accumbens (Rang and Dale, 1991). The innervated areas are provided with both muscarinic and nicotinic receptors. The highest densities of muscarinic M_1 and M_2 receptors in the rat brain are seen in limbic structures and neocortical areas (Spencer et al., 1986). The nicotinic receptors have a different distribution. They are highly concentrated in interpenduncular nucleus, medial habenula, and thalamic areas related to sensory function, and they have very low density in the hippocampus (London et al., 1985). Muscarinic receptors seem to be the responsive ones involved in evoking seizures, whereas nicotinic receptors are probably not involved in this process, since mecamylamine has no anticonvulsant efficacy (McDonough and Shih, 1997). Among cholinergic projection systems endowed with muscarinic receptors, some systems appear to play a more active role than others in inducing seizures by exposure to soman. The muscarinic receptors in the piriform cortex, perirhinal cortex, entorhinal cortex, septal area, and hippocampal region may play a pivotal role in seizure induction (Denoyer et al., 1992; Lallement et al., 1992; Myhrer et al., 2010a; Zimmer et al., 1998).

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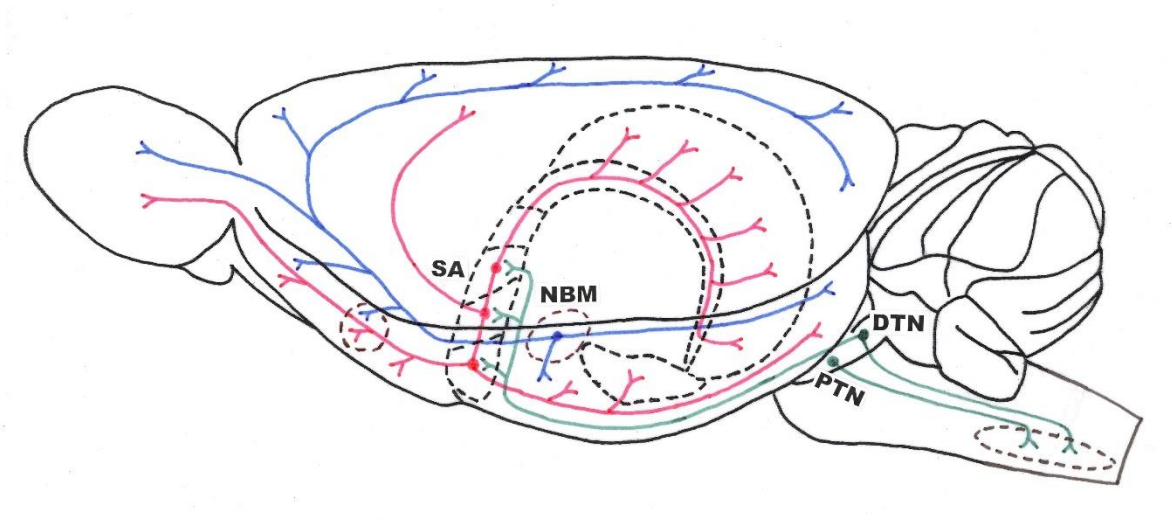

Fig. 1. Lateral view of the rat brain showing simplified version of cholinergic projection systems. Neurons in the nucleus basalis magnocellularis (NBM) send axons to the entire cortical mantle and olfactory bulb(blue). Neurons in the septal area (SA) send axons to the hippocampus and cingular, piriform, and enthorhinal cortices in addition to the olfactory bulb and amygdala (red). Neurons in the dorsolateral tegmental nucleus (DTN) send axons to the medial septal and diagonal band nuclei and to the ventral respiratory group in the brainstem that also receives input from the pedunculopontine tegmental nucleus(PTN) (green). From Myhrer (2007). For more labels of brain structures see Fig. 2.

Within the rat brain, there are control mechanisms with capacities to attenuate all aspects of convulsive activity. The substantia nigra (SN) pars reticulata and the area tempestas (AT) have been identified as two critical substrates for the control of experimentally induced seizures (Fig. 2) (Gale 1988). Microinfusions of pharmaceuticals into the anterior substantia nigra as well as the subthalamic nucleus have been shown to assure anticonvulsant effects (Dybdal and Gale, 2000; Gernert and Löscher 2001). The area tempestas (located in the deep prepiriform cortex) has been defined morphologically and termed the pre-endopiriform nucleus (Ekstrand et al., 2001). Infusion of the $GABA_A$ agonist, muscimol, into the substantia nigra has been demonstrated to attenuate generalized convulsive seizures induced by several mechanisms (Gale 1988). Infusion of muscimol into the area tempestas prevents the appearance of seizures on subsequent microinfusions in the area tempestas of cholinergic agonist (carbachol), glutamatergic agonist (kainic acid), or GABAergic antagonist (bicuculline) (Piredda and Gale 1985). The mediodorsal thalamus

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(MDT) has been identified as an area with abilities to modulate limbic seizures (Bertram et al., 1998). In rats with fully kindled seizures (low-intensity electrical stimulation) from the amygdala, microinfusion of muscimol into the mediodorsal thalamus results in reduced afterdischarge in the hippocampal CA1, whereas infusion of glutamate, AMPA, or NMDA results in prolongations of the hippocampal afterdischarge (Bertram et al., 2008). The mediodorsal thalamus appears to be a critical site in the initiation and spread of seizures that are driven from the medial temporal lobe, but it is not a trigger site for seizure activity in itself (Bertram, 2014).

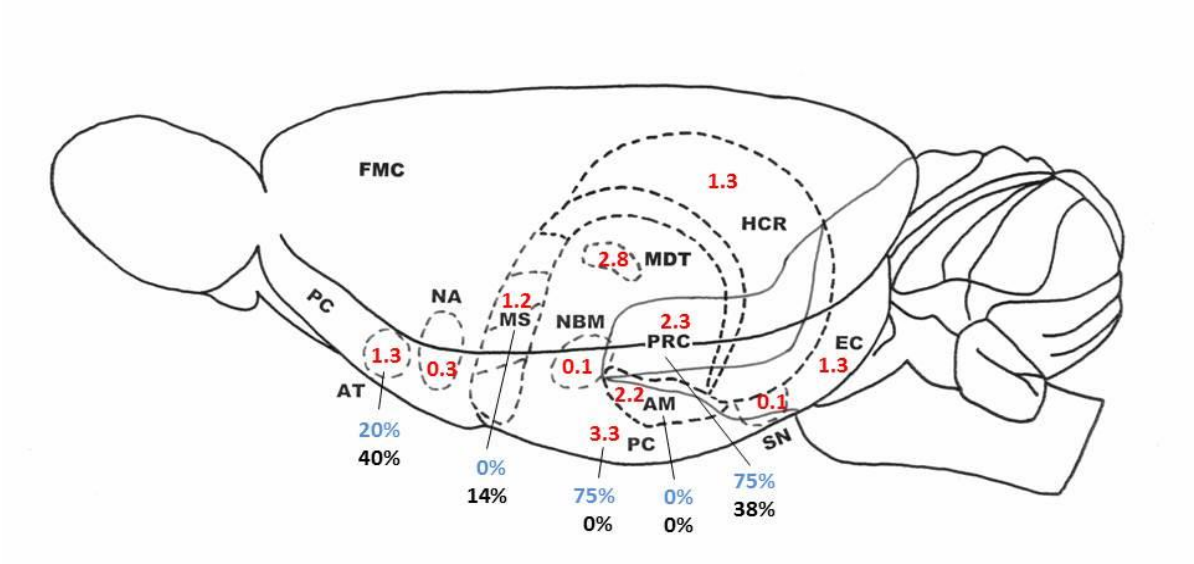

Fig. 2. Lateral view of the rat brain showing potential neuronal target areas for nerve agents. Anticonvulsant efficacy against soman poisoning was obtained by lesions in the area tempestas (AT), medial septum (MS), piriform cortex (PC), or perirhinal cortex (PRC). Lack of anticonvulsant effect followed damage to the nucleus accumbens (NA), nucleus basalis magnocellularis (NBM), hippocampal region (HCR), amygdala (AM), substantia nigra (SN) or entorhinal cortex (EC). MDT is the mediodorsal thalamus and FMC is the frontal motor cortex. The

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number in each structure (in red) represents the mean soman-induced neuropathology score derived from Table 1 in the study of McDonough et al. (1998). The other numbers, in blue and black, are data from the research performed in our own laboratory. The percentages presented below the figure (numbers in blue) denote the share of rats that developed convulsions upon microinfusion of soman into the structure linked with line (see text). The bold numbers (in black) beneath denote the percentages of rats with aspiration lesion in the same structure that did not respond with convulsions to systemic soman poisoning (complete protection). NBM corresponds to the ventral pallidum and preoptic nucleus in Table 1 of McDonough et al. (1998).

Areas critical for generation and/or control of seizures can be identified by means of various methods. Nerve agents or related chemoconvulsants can be microinfused into relevant structures for generation of seizures. Alternatively, anticonvulsants can be microinfused into corresponding structures in animals exposed to a convulsant dose of nerve agent. However, for the initial screening of target areas selective lesion of relevant brain structures may be the most pertinent choice. If selective damage to an area ensures anticonvulsant effects against nerve agent intoxication, the area affected may serve as a trigger site for seizures or may make up an important link in the propagation of seizure activity. The use of aspiration lesions causes complete disruption of seizure propagation, because passing fibers are also affected. The constellation of receptor types in the damaged region may provide clues for designing drugs with powerful anticonvulsant properties. Primary structures are those containing large assemblies of cholinergic neurons and areas demonstrated to have control capabilities in experimental epilepsy. Among structures giving rise to cholinergic projections, aspiration lesion of the medial septum (MS) causes prevention or increased latency to onset of convulsions, whereas damage to the nucleus basalis magnocellularis (NBM) or nucleus accumbens (NA) does not have anticonvulsant effects in rats exposed to a convulsant dose of soman (Fig. 2) (Myhrer et al., 2007). Rats with aspiration lesion of the seizure controlling substrate area tempestas in the anterior

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piriform cortex (PC) display marked anticonvulsant impact, whereas such effect is not seen when the substantia nigra is destroyed (Myhrer et al., 2007). Similar lesions made in the perirhinal cortex (PRC) or posterior piriform cortex (PC) produce anticonvulsant efficacy against soman intoxication, but anticonvulsant impact is not achieved when lesions are made in the entorhinal cortex (EC), hippocampal region (HCR), or amygdala (AM) (Myhrer et al., 2008a). Potential effects of mediodorsal thalamic (MDT) lesions against seizures initiated by nerve agents or other chemoconvulsants do not seem to have been studied. Such lesions have apparently been confined to studies of emotional and cognitive functions (Vertes, 2006).

Information has been collected to give an overview of how seizures can be generated in the brain areas (not mediodorsal thalamus) identified as target areas for nerve agents. The results presented are from both nerve agent research and epilepsy research. In the reported studies, 100% of the rats responded with seizures, except for the percentages described for the studies of nerve agents. In the area tempestas, microinfusion of soman induces seizures in 20% of the animals, whereas preconvulsive reactions are observed in the others (Myhrer et al, 2008b). The medial septum appears to be the least sensitive structure, since only preconvulsive responses are obtained by soman infusions (Myhrer et al., 2009). Repeated injections of VX cause hyperactivity (McDonough et al., 1987). A single infusion of soman results in wet dog shakes along with frenetic jumping bouts (Myhrer et al., 2009). In the perirhinal and piriform cortices, seizures are obtained in 75% of the animals after soman injections (Myhrer et al., 2010a). The glutamatergic agonist (kainic acid), cholinergic agonist (carbachol), and GABAergic antagonist (bicuculline) can generate seizures when infused into the area tempestas (Pirreda and Gale, 1985). In the medial septum, seizures are evoked by affecting opioid receptors (naloxone infusions) involved in the regulation of cholinergic activity (Mizuno and Kimura, 1996). The potent glutamatergic agonist, kainic

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acid, induces seizures in the perirhinal cortex (Imamura et al., 1998), whereas corresponding reaction is triggered by the GABAergic antagonist, bicuculline, in the piriform cortex (Giorgi et al., 2003). The kindling technique generates seizures in all 4 brain areas (Baxter et al., 1991; Ebert and Löscher, 1995; McIntyre et al., 1993). The perirhinal cortex is known to kindle faster than any other structure, and this area has direct projections to the frontal motor cortex in rats (McIntyre and Kelly, 2000). According to the results presented, there exists substantial evidence in support of the notion that the area tempestas, medial septum, perirhinal cortex, and piriform cortex represent particularly seizurogenic brain regions.

3. Distribution of soman-induced neuropathology and areas critical for initiation and/or control of seizures

In the search for relief measures against nerve agent-evoked neuropathology, it appears appropriate to use differential criteria for the identification of brain structures with potential role as target areas for nerve agent and areas with seizure controlling properties. In recent years, it has become a routine to present neuropathological evaluation of the efficacy of medical treatment for nerve agent poisoning. Ideally, large parts of the brain should be examined, but since this would require meticulous and time-consuming procedures most studies choose to present some representative index areas only. In 1 single study, however, a comprehensive description of neuropathology has been performed in which 34 brain structures have been analyzed (McDonough et al., 1998). Rats, instrumented to record cortical EEG activity, were pretreated with HI-6 (125 mg/kg) and then challenged with soman (1.6 x LD_{50}). All animals developed continuous epileptiform seizures that lasted in excess of 4 h. Groups with various survival times were perfused 1, 3, 10 or 30 days after exposure. Sections of the paraffin-embedded brains were stained with hematoxylin and eosin. Fifteen coronal forebrain sections (5 micron) separated by about 1 mm were evaluated

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in each animal from +5.2 to -8.8 anterior – posterior levels (A-P ±) with bregma as reference. The atlas of Paxinos and Watson (1986) was used to delineate the brain areas that were scored with the following system: 0=none; 1= minimal, 1-10%; 2=mild, 11-25%; 3=moderate, 26-45%; 4=severe, >45%. The results showed that the severity of lesions in some brain areas were evaluated as less severe at longer survival times than when judged 1 day following exposure. Hence, the mean scores across all survival time groups yielded a mean score for the structure for each anterior – posterior level. The results are presented in Table 1 in McDonough et al (1998). All cortical areas sustained damage, with the piriform and perirhinal cortices exhibiting the most severe changes. Subcortical areas such as amygdala, amygdala-piriform transition zone, and thalamic nuclei were the most consistently and severely damaged in all animals regardless of survival time. We have made a mean score of the areas presented in Table 1 of McDonough et al (1998) corresponding to the brain structures outlined in Fig. 2, and transferred the scores to the latter figure. In addition, the percentages presented below Fig. 2 (numbers in blue) denote the share of rats that developed convulsions upon microinfusion of soman into the structure linked with line. The bold numbers (in black) beneath denote the percentages of rats with aspiration lesion in the same structure that did not respond with convulsions to systemic soman poisoning (complete protection). The percentage for the amygdala was derived from the study of McDonough et al (1987). In the latter study, bilateral microsyringe injections of soman into the amygdala fail to evoke convulsions and brain pathology. However, if the rats were pretreated with lithium chloride (to enhance ACh turnover and release) (McDonough et al., 1987), or when carbachol was coadministered, soman injections evoke repetitive clonic convulsions and neuropathology (McDonough et al., 1987).

Potential brain sites in which seizure activity begins after nerve agent poisoning have been a topic of discussion for a long time. In this respect, a study has been undertaken to

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map a number of brain sites in rats, and 11 limbic forebrain areas show varying degree of sensitivity to the seizurogenic effects of directly infused VX (McDonough and Shih, 1997). The basolateral amygdala, amygdala-piriform cortex transition zone, and a restricted portion of the piriform cortex (A-P + 1.2) are the most sensitive areas. Two other levels of the piriform cortex (A-P + 3.2, + 2.2) and the entorhinal cortex are intermediately sensitive. The least sensitive areas are the dorsal hippocampus, central amygdala nucleus, and another area of the piriform cortex (A-P + 0.2). Virtually all animals that developed seizure activity displayed some degree of neuropathology, much of which occurred in structures quite distant from the injection site. Comparable infusions of VX into 13 other brain sites did not cause seizure or neuropathology (McDonough and Shih, 1997). These findings imply that there is a hierarchy of sites within the rat brain that are sensitive to the seizurogenic effects of nerve agent. All the sensitive sites, with the exception of the dorsal hippocampus, lie within the ventrolateral aspects of the forebrain and would be classified as cholinoceptive since most contain high concentrations of AChE (McDonough and Shih, 1997). However, some structures typically classified as cholinergic (e.g. septum, ventral pallidum, substantia innominata) do not respond with convulsant effect after infusion of VX (McDonough et al., 1987).

The findings outlined above are reconcilable with the results presented in Fig. 2. The structures most sensitive to microinfusion of soman are localized in the ventrolateral parts of the forebrain. A relationship is seen between the severity of neuropathology and the sensitivity to focal administration of soman. The posterior piriform cortex and the perirhinal cortex have high neuropathology scores and display convulsant reactions to infusion of soman. The area tempestas and medial septum have relatively moderate neuropathology scores and respond predominantly with preconvulsive or proconvulsive activity to soman infusions. Two measures express the anticonvulsant impact of the lesions described in this

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section. One is mean latency to onset of seizures and the other one is the percentage of nonconvulsing rats (complete protection). The latter percentages have been inserted in Fig. 2. As can be seen, the most efficacious anticonvulsant reactions are obtained by lesions in the area tempestas and perirhinal cortex, whereas the response rate is weaker after medial septal lesions and non-existing after posterior piriform cortical lesions. The most discrepant percentage of convulsant effect of soman infusion versus anticonvulsant effect of lesion is seen for the posterior piriform cortex. In view of the results from preclinical epilepsy research in which the amygdala commonly is used as a kindling site, the lack of response to both soman infusion and lesion in the amygdala may appear somewhat unexpected. Though, microinfusion of atropine into the amygdala does not result in anticonvulsant action against soman poisoning (Myhrer and Enger, unpublished data). The neuropathology score is also high for the mediodorsal thalamus from which seizures cannot be triggered, only modified (Bertram et al., 2008). It should be noted, however, that the neuropathology score given for the mediodorsal thalamus is the general score for the entire thalamus, since no differentiation among the various nuclei was made in the study of McDonough et al (1998). However, a specific score of 2.4 for the mediodorsal thalamus has been given in the study of Moffett et al (2011). Collectively, the posterior piriform cortex and the perirhinal cortex seem to fulfil most adequately the criteria set in terms of severity of neuropathology, percentage of responding to soman infusion with full-blown clonic-tonic convulsions, and the percentage of nonconvulsing rats in response to lesions. Of these 2 structures, the perirhinal cortex emerges as the most responsive one, since lesion of the posterior piriform cortex causes convulsions in all rats (although, significantly longer latencies to seizure onset than in sham operated control rats). This means that the perirhinal cortex and the posterior piriform cortex probably serve as target areas for nerve agents and probably will serve as strategic sites for microinfusion of anticonvulsant drugs as well. Support for these

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assumptions is found in the neurophysiological principles of the convulsive "machinery" evolved in experimental epilepsy (McIntyre and Gilby, 2008).

In epilepsy research, great emphasis has been put on identification of seizure controlling brain sites. It is assumed that the ability of a systemically administered drug to confer seizure protection depends on the drug's relative impact on the defined action sites (Gale, 1988). Epileptiform activity does not spread randomly throughout the brain, but the discharges seem to be generated and propagated by specific anatomical pathways (Gale, 1988; Löscher and Ebert, 1996). In epilepsy models, epileptiform activity is usually recruited in structures localized in the ventrolateral forebrain of rats (McIntyre, 2006). If not terminated, such partial epileptiform activity propagates to the motor cortex by way of the perirhinal cortex and clonic convulsions will occur (McIntyre, 2006). If the activity reaches a sufficiently high intensity level, electrical discharges spread further to the brainstem by way of the basal ganglia, and generalized seizures accompanied by tonic-clonic convulsions are seen (Browning and Nelson, 1986). The tonic extension of hind limbs likely reflects the level of seizure activity involving both the forebrain and brainstem (Swinyard, 1973). Nerve agent-generated seizures appear to be complex partial seizures evolving to secondarily generalized seizures accompanied by tonic-clonic convulsions (Myhrer, 2007). As an example, tonic-clonic convulsions in rats can be evoked by unilateral microinfusion of VX into the amygdala (McDonough et al., 1987).

4. Neurodegenerating processes and potential mechanisms

A major issue in nerve agent poisoning is the time-dependent development of the seizure-induced sequences that lead to neuropathology. Early termination of the seizures (< 10 min), during the initial cholinergic activity, results in no neuropathology when the level of nerve agent poisoning is 1-2 x LD_{50}. Twenty min of seizure activity is the minimal

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amount of time needed for the first observable signs of neuronal damage in some rats. However, the detrimental processes increase rapidly after this time with 40 min of seizing being sufficient to cause moderate lesions in most subjects (Lallement et al., 1994; McDonough et al., 1995).

The time course of degeneration after soman-induced seizures has been examined in the basolateral amygdala (Prager et al., 2014). Rats were exposed to a soman dose of 1.4 x LD_{50} and seized after about 8 min. Twenty min following soman they received atropine sulfate (2 mg/kg) to increase survival rate, and the brains were harvested at 1, 7, 14, or 30 days. AChE-activity was very low up to 7 days in the prelimbic cortex, piriform cortex, and hippocampus, but not in the basolateral amygdala. Extensive neuronal death was observed by 24 h and gradually decreased at 7, 14, and 30 days in the basolateral amygdala. Of the total neuropathology seen after 30 days, 50% occurred within 24 h. Within 7 days, 76% of the total neuropathology had evolved. The initial neuronal death was probably the result of excitotoxicity-elicited necrosis, and slower death may result from simultaneous influences and competition of survival- and death-inducing factors (Prager et al., 2014). The degeneration course described above may be transferable to other brain structures.

Epileptogenesis relies heavily on synaptic plasticity (Vismer et al., 2015). It has been demonstrated in epilepsy research that repeated seizure activity incrementally produces an indiscriminate and widespread induction of long-term potentiation (LTP; an important form of synaptic plasticity for learning and memory) (Reid and Steward, 1997). The involvement of LTP means that an initial epileptic episode can facilitate future attacks and also indicates that nerve agent-evoked seizures can be self-sustaining. Increased insights into the mechanism underlying the convulsive “machinery” of temporal lobe seizures has been considered as a key issue in achieving more efficacious antiepileptic drugs (Gale, 1988; Löscher and Ebert, 1996). A similar approach will probably benefit nerve agent research as

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well, because seizure activity accompanied by tonic-clonic convulsions follow common propagation pathways regardless of how the epileptiform activity is initiated (Myhrer and Aas, 2014). Also the brain areas affected by neuropathology in experimental epilepsy and nerve agent research are similar. Countermeasures with optimal anticonvulsant efficacy, however, must be specific for the nature of the medium used to trigger seizures.

Although early reports indirectly suggested that the circuits of the piriform cortex might act as a critical conduit for limbic seizure discharges to access motor systems, later studies more strongly implicate the perirhinal cortex in this process (McIntyre and Plant, 1993). To determine the involvement of glutamate-sensitive NMDA receptors in the development of burst responses, amygdala-piriform slices from control and kindled rats were exposed to perfusion medium that was magnesium (Mg^{2+}) free. Exposure of slices to zero Mg^{2+} would serve to augment the activity of the NMDA channel. Contrary to expectations, the perirhinal cortex has a greater propensity to develop and sustain epileptiform events than the amygdala and piriform cortex (McIntyre and Plant, 1993). Consistent with the in vitro findings, the perirhinal cortex distinguishes itself on several critical measures during in vivo assessment of epileptogenicity. The extremely brief latencies observed with perirhinal cortical kindling suggest a close anatomical relation between the perirhinal cortex and the motor systems. Anterograde and retrograde tracing studies of perirhinal and insular areas show that the perirhinal cortex heavily innervates the rat frontal cortex, particularly, the region that contains many neurons that project directly to the spinal cord (McIntyre et al., 1996). The potential importance of cortical structures to convulsive seizure expression is seen further in their varied rates of kindling. The structures with the fastest kindling (number of stimulations to first stage-5 convulsion) in the limbic system are, in descending order with neuropathology scores from Fig. 1 in brackets, the perirhinal cortex (2.3), piriform cortex (3.3), amygdala (2.2), entorhinal cortex (1.3), ventral

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hippocampus (1,3), and dorsal hippocampus (1.3). These findings suggest that the perirhinal cortex may be provided with the projection that relays limbic seizures to frontal cortical areas to drive brainstem/spinal cord convulsive expression (McIntyre and Gilby, 2008). Hence, in order to prevent seizures or to terminate ongoing seizures the perirhinal cortex will probably be the most strategic site to interfere with pharmacologically.

It has been a prevalent opinion that nerve agent-induced neuropathology is intimately associated with seizure activity and tonic-clonic convulsions or other overt signs of intoxication. Only 2 studies have previously reported the development of brain pathology in animals totally devoid of clinical signs of nerve agent poisoning. In the study of Hymowitz et al (1990), rats were trained in an operant task and repeatedly administered soman doses that were varied (mean dose of soman 56 µg/kg a day for 5-17 days) to elicit disruption of behavioral performance (80% of baseline) without causing clinical signs of intoxication. The minimum recovery time between exposures was 5 weeks. Histological examinations showed a marked increase in glial cells. In the study of Kadar et al (1992), rats were exposed to 0.5 x LD_{50} of soman (50 µg/kg) and displayed no clinical signs of intoxication. Three months following exposure histopathological examination showed hippocampal degeneration. In 3 more recent studies, a similar incongruence between toxic signs of soman poisoning and the development of neuropathology has been encountered (to follow below).

A comparison of the anticonvulsant and life preserving capacities between 2 types of therapies consisting of HI-6 (125 mg/kg)/ atropine (100 mg/kg)/ avizafone (15 mg/kg) (termed the atropine regimen) and HI-6 (125 mg/kg)/ scopolamine (1 mg/kg)/ physostigmine (0.1 mg/kg) (termed the physostigmine regimen) against various doses of soman (2, 3, 4 x LD_{50}) has been performed (Myhrer et al., 2013a). The results show that each regimen administered 2 times (1 and 5 min after exposure) effectively prevents or terminates epileptiform activity within 10 min. However, the regimens differ markedly in their life

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saving properties with the physostigmine regimen ranking highest. Since seizures were absent or only lasted for 1-6 min in some rats, the finding of morphological changes emerged as a surprise. No relationship was found between neuropathology and seizures/nonseizures. Fluorescent staining (Fluoro-Jade B) was more often observed in rats that received the physostigmine regimen than the atropine regimen (survival time 7 days). Because the latter regimen contains avizafone, some neuroprotection might have been obtained, even if avizafone has previously been reported to have less neuroprotectant potency than diazepam (Clement and Broxup, 1993). However, an intriguing feature of the neuropathology was that it nearly always occurred in the left hemisphere in the rats treated with the physostigmine regimen (Myhrer et al., 2013a). Also when given as a prophylactic treatment 20 min before a soman dose of 1.3 x LD_{50} the physostigmine regimen provides complete protection against seizures, incapacitation, and other toxic signs, but marked bilateral neuropathology occurs in the index areas of the piriform cortex and amygdala (Myhrer et al., 2013c). When rats exposed to 4 or 5 x LD_{50} are treated with the physostigmine regimen 1 and 5 min after exposure, higher neuropathology scores were observed in the left piriform cortex compared with the right side (survival time 7 days). In these animals, no overt toxic signs were observed and normal EEG recordings were made 1, 2, and 6 days following intoxication. Rats challenged with 4 or 5 x LD_{50} of soman and treated with HI-6 (125 mg/kg), levetiracetam (50 mg/kg), and procyclidine (20 mg/kg) 1 and 5 min after poisoning display negligible neuropathology in both hemispheres (Myhrer et al., 2013c).

Hemispheric lateralization of many functions has been found in the rat brain (Bianki, 1981; Goldstein et al., 2002; Klur et al., 2009; Martinez and Sarter, 2004). The left frontal/piriform cortex in rats of our Wistar strain has higher glutamatergic activity than in the right hemisphere (Myhrer et al., 1992). The neuropathology in the left piriform cortex or

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amygdala seen in rats that survived for 7 days in the studies cited above may be related to glutamatergic overstimulation in response to the high levels of soman poisoning (2, 3, 4, or 5 x LD_{50}). It has been demonstrated in hippocampal slice culture that after subtoxic soman (150 nM) treatments for 7 days the tissue becomes vulnerable to brief episodes of glutamate receptor overstimulation by AMPA that normally results in little or no excitotoxic damage (Munirathinam and Bahr, 2004). The predominantly unilateral nature of the neuronal injury suggests that local excitotoxic processes unrelated to global seizure activity can be induced by nerve agents. As previously mentioned, neuropathology has been reported following soman intoxication without concomitant overt toxic signs in 2 previous studies (Hymowitz et al., 1990; Kadar et al., 1992). Collectively, these findings of asymptomatic development of neuronal damage in response to nerve agent imply that further treatment can be needed after termination of recordable epileptiform activity, because a "silent" lesion process may still go on.

An even more conspicuous development of neuropathology without concomitant overt toxic signs has been observed after supralethal poisoning by various nerve agents. The treatment regimen consisting of HI-6 (125 mg/kg), levetiracetam (50 mg/kg), and procyclidine (20 mg/kg) (termed the triple regimen) has previously been shown to protect against soman poisoning in rats, since it has capacities to function as both prophylactic and therapeutic measure (Myhrer et al., 2013b). In a subsequent study (Myhrer et al., 2015), it was examined whether the triple regimen may have antidotal efficacy against intoxication by other classical nerve agents than soman. The treatment was given 1 and 5 min after exposure to a supralethal dose of nerve agents, and the results show that the triple regimen successfully prevented or terminated seizures and preserved the lives of rats exposed to 5 x LD_{50} of soman, sarin, cyclosarin, or VX, but solely 3 x LD_{50} of tabun was managed by this regimen. To meet the particular antidotal requirements of tabun, the triple regimen was

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reinforced with obidoxime (36 mg/kg) and was made to a quadruple regimen that effectively treated rats intoxicated by 5 x LD_{50} of tabun. The rats recovered very well and the majority gained pre-exposure body weight within 7 days. Even if pharmacological treatments given 1 and 5 min after soman poisoning prevented or terminated epileptiform activity within 10 min in all rats, evident neuropathology was discovered in the index areas piriform cortex and amygdala, but less evident in the hippocampal CA1 field. Fluorescent staining (Fluoro Jade B) was seen in all animals in 2 or 3 of the index areas. The neuronal injury was unrelated to whether the rats convulsed or not. Both the sarin and cyclosarin groups had more extensive neuropathology than the soman and VX groups. Hemispheric differences in terms of higher levels of neuronal damage in the left versus the right side occurred. This was seen in both the piriform cortex and amygdala in 1 of the 2 tabun groups, and in the amygdala only in the cyclosarin and VX groups. A peculiar lesion pattern was occasionally observed in the hippocampal CA1 layer. Half of the CA1 ending in the subiculum or the CA3 was demolished, whereas the other half was intact. This phenomenon was seen bilaterally in 1 rat in the tabun 5 x LD_{50} group, in the left side of 2 rats in the cyclosarin group, and in the right side of 1 rat in the VX group. The most convincing evidence that neuropathology can occur without the induction of seizures/convulsions was seen in the VX group. When rats challenged with 5 x LD_{50} of VX remained untreated, the mean latency to seizure onset is 6.1 min. Thus, the triple regimen administered 1 and 5 min after exposure was able to prevent onset of seizures, but still ample neuronal injuries were detected (Myhrer et al., 2015). It has previously been maintained that the initial signs of neuronal damage are detectable about 20 min after onset of soman-induced global seizure activity that is accompanied by tonic-clonic convulsions (Lallement et al. 1994; McDonough et al. 1995). From the results of Myhrer et al. (2015), it is apparent that the development of neuropathology can follow an alternative route when the degree of nerve agent intoxication is very high. The severe poisoning might

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have induced a long-lasting local, "silent" glutamatergic excitotoxic process. Even if no EEG abnormalities were recorded by the use of superficial cortical recordings, depth electrodes in critical structures (e.g., piriform cortex, perirhinal cortex) might have revealed focal epileptiform activity. In a recent study, we injected rats with 5 x LD_{50} of sarin and treated them 1 and 5 min after exposure with our procyclidine regimen (HI-6 125 mg/kg, levetiracetam 50 mg/kg, procyclidine 20 mg/kg). On the following days, the rats received 1 extra treatment a day with the procyclidine regimen for 3 or 6 consecutive days. At day 7, the brains were harvested, and sections stained with Fluoro Jade B. The neuroprotectant efficacy was impressive regardless of 3 or 6 treatments. Negligible neuropathology occurred in the left perirhinal cortex and/or the left basolateral amygdala, and none was seen in the hippocampal CA1, or pathology was absent in all 3 index areas (Myhrer, Enger, and Mariussen, unpublished data). Supralethal intoxication by sarin (5 x LD_{50}) without extra treatment causes extensive brain damage (Myhrer et al., 2015). The prolonged treatment with the procyclidine regimen that yields pronounced NMDA antagonism has likely been able to terminate the local epileptiform activity.

In human epilepsy, it has been assumed by neurologists performing deep recordings from various temporal lobe structures in patients that focal seizures restricted to the hippocampus are without clinical symptoms, and that it is only when focal hippocampal seizures recruit other structures, like the parahippocampal cortices and amygdala, that clinical symptoms become evident. Presumably, individuals could have many clinically silent hippocampal seizures over a period of days or years without recruitment of neighboring cortical structures (McIntyre and Gilby, 2008). The importance of the parahippocampal structures (including the piriform, perirhinal, and entorhinal areas) in the recruitment and manifestation of convulsive seizures as well as the sequential activation of the structures has been described in experimental animal models (Biagini et al., 2013; De

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Salvo et al., 2010; Shih et al., 2007). Of particular interest in the present context is evidence that silent, focal seizures can occur in the rat brain.

Focal protracted seizures can be provoked by using kindling of the amygdala resulting in relatively steady-state seizures well suited for neuroanatomical examination of metabolic activity (McIntyre and Gilby, 2008). Mapping with the ^{14}C-2-deoxyglucose (2-DG) has been applied for studies of seizure spread and correlations between seizure substrates and behavioral manifestations, as seizure activity greatly increases glucose utilization within activated areas (Handforth and Ackerman, 1995). Sustained amygdaloid kindling usually culminates in one of several forms of status epilepticus: Immobile seizures, ambulatory/exploratory seizures (preconvulsive), masticatory seizures (proconvulsive), or generalized seizures (convulsive). During immobile status epilepticus, the rats were sitting quietly with no gross movement. Metabolic 2-DG studies indicate considerable discrete activity in the ipsilateral amygdala/hippocampal transition area, perirhinal cortex, basolateral amygdala, and anterior limbic field. Loss of neurons was seen in the ipsilateral amygdala/hippocampal transition zone only (Handforth and Ackerman, 1995; McIntyre et al., 1991, 2006). If the status epilepticus is more severe, it manifests as ambulatory/exploring seizures, where the rats are constantly moving. The 2-DG recording 1 h after ambulatory onset shows a broadening of neuronal involvement from the immobile situation to additionally include all aspect of the piriform cortex, extending unilaterally from the entorhinal cortex posteriorly into the olfactory bulbs anteriorly and the ipsilateral septum. Other additionally activated structures include the midline thalamus with bilateral activation of reunions and mediodorsal thalamus. Following the spontaneous termination of this form of status epilepticus, total neuronal loss is seen within the ipsilateral piriform cortex and basolateral amygdala, in addition to extensive damage to the midline thalamus, but with variable lesions in the perirhinal and entorhinal cortices. In about half the rats,

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hippocampal CA1 damage was observed bilaterally (McIntyre et al., 2006). Hence, focal seizure activity may occur in several brain areas resulting in neuronal pathology, while no overt behavioral signs of epileptiform activity can be seen. It has been shown in rats that recurrent and spontaneous epileptiform electrocorticography events (0.8-2 sec) are undetectable by scalp EEG (D'Ambrosio et al., 2009).

The morphological characteristics of excitotoxic injury in vivo are consistent with a necrotic type of death. However, some neuronal subpopulations may die via apoptosis following the activation of an endogenous cell death program. Necrosis and apoptosis are 2 distinct forms of cell death that have markedly different impact on the surrounding tissue. The same neuronal population can undergo either an immediate, necrotic death or a slow, apoptotic death after excitotoxic damage. Necrosis is associated with loss of mitochondrial function and produces localized inflammation, which exacerbates damage. Apoptosis ensues in neurons that recover their mitochondrial function and energy levels (Ankarcrona et al, 1995). Neuronal injury resulting from soman-induced seizures exhibits a large variety of hybrid forms between necrosis and apoptosis, but the majority show more necrotic features (Baille et al., 2005).

It has recently been argued that direct actions of nerve agents, independent of seizures, may also contribute to delayed neuronal death (Pereira et al., 2014). Nerve agents can trigger mechanisms that eventually lead to neurotoxicity that develops either in absence of an overt acute phase of OP poisoning or long after the acute phase of OP poisoning has resolved. Toxicogenomic studies have reported that expression of genes linked to neuroprotective and neurodegenerative pathways is altered time-dependently after exposure of rats to either a subconvulsant or a convulsant dose of sarin or soman (Damodaran et al., 2006; RamaRao et al., 2011; Spradling et al., 2011). Direct interactions of nerve agents with molecular targets other than AChE have also been proposed to play a key role in the development of delayed

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OP neurotoxicity. Sarin and soman can decrease neuronal viability by disrupting the axonal transport of nutrients from the cell body to the axon terminals of neurons (Grigoryan et al., 2008). Furthermore, it has been suggested that metabolic inhibition, as seen in awake, soman-intoxicated mice, may lower seizure threshold and contribute to soman-related neurodegeneration and lethality (Nguyen et al., 2007).

5. Neuronal damage and behavioral consequences

As seen from Fig. 2 in the present study and Table 1 in McDonough et al. (1998), sustained seizure activity induced by soman can lead to widespread neuronal injuries. The neuronal condition created can be expected to cause a number of behavioral malfunctions. According to the severe damage produced in the posterior piriform cortex, perirhinal cortex, and amygdala, functions governed by these structures might be particularly affected. In some studies of rodents, attempts have been made to relate various degrees of brain damage to variations in performance impairment following exposure to nerve agent. In the studies to be referred, soman was used in all of them and rats were used in all but 2 studies in which mice served as subjects. Only studies presenting histological results are included.

In 4 studies, operant training has been used to assess potential behavioral impairment after exposure to soman. In the first study (McDonough et al., 1986), rats pretrained to lever press for milk reinforcement on a continuous reinforcement (CRF) schedule were injected with 110 μg/kg (s.c.) of soman or saline. Subjects exposed to soman experienced moderate to severe acute symptoms of intoxication. After a recovery period of 3 weeks, all surviving rats were retrained on CRF, then given 45 training sessions (each daily session 30 min) on a differential reinforcement of low rate (DRL) 20 sec schedule, followed by 10 sessions of extinction. The intoxicated rats showed no improvement over sessions in the number of reinforcement earned on the DRL schedule because of an ineffective response pattern. There

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were no differences between the groups in the number of total DRL responses or terminal extinction responses. Neuropathology was most evident in the dorsal thalamic areas, piriform cortex, and amygdala. Significant correlations were found among the severity of acute intoxication scores, degree of neuropathology, and deficits in DRL performance. It was concluded that exposure to a relatively high dose of soman can result in neuronal damage and persistent decrements in performance of certain operant tasks (McDonough et al., 1986). In the second study, rats were trained on an operant alternation task with a time-out between response periods following recovery from administration of soman (s.c.) doses of 75, 85, or 95 µg/kg. Training sessions continued until the rats attained criterion or 100 training sessions. All saline animals attained criterion, while only 60%, 33%, and 33% of the rats given 75, 85, or 95 µg/kg respectively reached the criterion. Variable degrees of neuropathology were found in the hippocampus, thalamus, amygdala, and piriform cortex. Significant correlations among the severity of neuronal injury and both the number of sessions required to terminal performance level of the alternation task and the number of sessions required to achieve the criterion performance were found across all soman doses (Modrow and Jaax, 1989). In the third study, rats were trained in an operant task and repeatedly administered soman in an average of 56 µg/kg (s.c.) a day for 5-17 days to suppress the performance level to 80% of the baseline. Soman attenuated response rates in both components of a multiple fixed interval 50-sec fixed-ratio 25 schedule of reinforcement, although all animals revealed marked tolerance to repeated soman administration. The most salient neuronal damage was glial cell proliferation in layer 4 and the deeper parts of layer 3 of the cerebral cortex, whereas the hippocampus was modestly affected (Hymowitz et al., 1990).

The forth study in which no treatment was given against the soman poisoning (a single dose of 50 or 85 µg/kg, s.c.) open field and a 14 choice point multiple T-maze (Stone maze)

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were used to measure behavioral changes. The rats with the most severe neuropathology displayed increased locomotor activity in the open field and impaired learning in the Stone maze, while those with minimal pathology had normal scores. Even after extensive handling during testing in the Stone maze (nearly 4 months) several high dose rats continued to react aggressively. Neuronal injury was observed in the hippocampus, amygdala, thalamus, and piriform cortex (Raffaele et al., 1987).

In 3 studies, the performance of rodents exposed to soman was examined in the water maze. In the first study, rats were pretreated with HI-6 (125 mg/kg) 30 min before injection of soman (1 x LD_{50}, s.c.). Only rats that convulsed displayed a learning deficit and neuropathology. A correlation between the extent of hippocampal CA1 damage and the degree of attenuated spatial learning was found. When rats pretreated with HI-6 (30 min before soman) were given a triple therapy at doses not totally preventing convulsions (atropine 2 mg/kg, TCP 0.5 mg/kg, NBQX 10 mg/kg) immediately after exposure, reduced impairment of spatial learning parameters was seen, despite lack of effect on the convulsions of the animals. There was no effect of treatment on neuropathology in CA1 (Filliat et al., 1999). In the second study using the water maze, it was shown in a preliminary experiment that mice challenged with 1.2 x LD_{50} of soman and treated with 5 mg/kg of atropine methyl nitrate (not crossing the BBB) 1 min after exposure could after 3 days be divided into either high weight loss (HWL) group or a low weight loss (LWL) group. A positive correlation was seen between hippocampal CA1 degeneration and high loss of body weight. One month after intoxication, it was seen in another set of animals that LWL mice displayed water maze performance like controls, whereas HWL mice did not exhibit any spatial learning. Three months after poisoning, the LWL group showed a mild deficit in the water maze, whereas the learning skills slightly recovered in the HWL group. The latter finding of behavioral recovery might be related to the observation of neurogenesis and

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sprouting in the hippocampal formation of the poisoned mice (Filliat et al., 2007). In the third study with water maze, beneficial neuronal effects of cytokine treatment was tested in mice injected with soman (1.2 x LD_{50}) and treated with 5 mg/kg methyl nitrate atropine 1 min after exposure. Only convulsing mice were included in the study, and cytokine was administered daily during post-soman days 1 to 8. The animals were tested 30 and 90 days after soman in water maze in addition to spontaneous alternation in T-maze, elevated plus maze, auditory and contextual fear conditioning. Cytokine treatment enhanced neuronal regeneration in the hippocampal CA1 area, but not in the amygdala. Soman poisoning caused memory impairment (water maze, T-maze), as well as increased anxiety or fear-like responses (plus maze, fear conditioning). No recovery of memory performance over time was produced in soman-intoxicated mice whether treated or not with cytokines (Collombet et al., 2011).

Avoidance procedures have been employed in 2 studies. In the first one, 2 groups of rats were trained during 6 days to acquire a two-way active avoidance task (shuttle-box). On the 6^{th} day, the rats were intoxicated by 1 x LD_{50} of soman (100 µg/kg, s.c.). Immediately after, half of the animals in each group received the High AS/DZ treatment (40 mg/kg of atropine sulfate plus 2.5 mg/kg of diazepam) and the other half the Low AS/DZ treatment (2 mg/kg of atropine plus 0.625 mg/kg of diazepam). After a recovery period of 6 days, a second 6 days period of relearning the avoidance response begun. During the 3 first training sessions, the Low AS/DZ group displayed markedly declined performance. No such decline was seen among the High AS/DZ-treated rats. In the Low AS/DZ group, neuropathology was only observed in the rats that had convulsed, whereas in the High AS/DZ group neither convulsions nor brain injuries were observed. Morphological changes were predominantly seen in the hippocampus and piriform cortex (Philippens et al., 1992). In the second study, a passive avoidance task (step-through) was used for rats implanted with osmotic minipumps

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containing physostigmine optimized to obtain 30-35% of blood AChE activity for 3 days and procyclidine (blood concentration of 50-100 ng/ml). Subsequently the rats received a soman dose of 1.3 x LD_{50} (160 µg/kg) along with control rats that had received HI-6 (125 mg/kg) 30 min prior to soman. Passive avoidance learning started 1 day after challenge with soman. In the control rats that received HI-6 only, soman induced profound convulsions, 30% mortality, incapacitation, learning and memory deficits, and neuropathology in the hippocampus, piriform cortex, amygdala, thalamus, and entorhinal cortex. The prophylactic treatment provided full protection against lethality, convulsions, incapacitation, learning and memory dysfunctions, and neuropathology (Choi et al., 2004).

In a single study, auditory and contextual fear conditioning has been used. Rats were exposed to soman (1.0 or 1.2 x LD_{50}) or saline followed by injections of HI-6 (93.6 mg/kg), atropine sulfate (2 mg/kg), and diazepam (10 mg/kg). All rats displayed signs of poisoning, and seizures were evident in 10/17 in the 1.0 x LD_{50} group and 12/14 in the 1.2 x LD_{50} group. Fear conditioning was performed on post-exposure day 8 followed by measuring of freezing to contextual and auditory conditioned stimuli on post-exposure day 9 and 10, respectively. Both forms of conditioning were severely impaired in both soman groups. Both groups also spent less time freezing than controls when returned to the context in which conditioning occurred. Neuronal fiber degeneration was present in the piriform cortex, thalamus, and amygdala in the rats that convulsed in response to soman regardless of dose (Moffett et al., 2011).

In the last study to be reported, behavioral changes were examined in a novelty test and a visual discrimination task. The rats were exposed to 1 x LD_{50} of soman (80 µg/kg, s.c.) and during 30-40 min following onset of convulsions they were treated with procyclidine (6 mg/kg), diazepam (10 mg/kg), and pentobarbital (30 mg/kg) (injections 5 min apart) that effectively terminated the seizure activity 45 min after onset. Non-convulsing control rats

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received saline or the above treatment regimen. Convulsing rats with neuropathology displayed impaired ability to recognize novelty, intact acquisition of the discrimination task, but markedly reduced retention of the same task. The group with convulsing rats without neuropathology performed like normal control rats. Significant correlations between neuropathology scores and behavioral measures were found for the animals that convulsed. Neuronal damage varied from none at all to 30% injury in the index areas of the hippocampus, amygdala, and piriform cortex (Myhrer et al., 2005).

In compliance with the assumption made in the introduction to this section, the results reported above show that soman-induced brain lesions can impede a number of behavioral functions. In all but one study (Hymowitz et al., 1990), overt signs of seizure activity were related to the development of brain damage. The most evident neuropathology is seen in the posterior piriform cortex, perirhinal cortex, and amygdala (Fig. 2). Results from studies of behavioral neuroscience show that the posterior part of the extensive piriform cortex is involved in olfactory perception encoding odor quality (e.g. fruity, spicy) (Kadohisa and Wilson, 2006). The perirhinal cortex is involved in cognitive processes, most notably detection of novelty (Albasser et al., 2015). The amygdala is involved in fear conditioning and fear extinction (Kochli et al., 2015; Sotres-Bayon et al., 2004). Many aspects of conditioned fear depend on the integrity of both the amygdala and the perirhinal cortex (Otto et al., 2000).

A general feature of the results presented after soman-induced brain injuries is cognitive impairment and/or increased fear or anxiety-like responses. Various performances in operant chambers are severely attenuated after brain damage caused by exposure to soman. Also spatial functions are markedly affected in the Stone-maze and water maze. Learning and memory deficits are seen in both two-way active avoidance and passive avoidance, procedures which encompass cognition as well as fear. The latter elements were clearly

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affected in auditory and contextual fear conditioning. Impaired novelty detection and impaired retention of a discrimination task are also prominent features after soman-induced brain injuries.

Nerve agent-evoked neuronal pathology will probably affect the same areas of the brain regardless of the type of nerve agent producing the damage, and the regions affected will be the same even if the index areas chosen can differ across studies. The degree of neuronal damage will probably be related to the duration of seizing and/or level of poisoning. Furthermore, the degree of neuropathology will depend on whether treatment has been given or not. The degree of pathology also seems to have differential potency to affect behavior, since clear correlations between neuronal damage and behavioral impairment have been demonstrated in several of the studies cited above. In a more recent study (Schultz et al., 2014), it was shown that rats exposed to 1.2 x LD_{50} of soman with standard therapy (atropine sulfate 2 mg/kg, HI-6 93.6 mg/kg, diazepam 10 mg/kg, im) display prolonged seizure activity, body weight loss, and behavioral deficits to include impairment in vestibulomotor function, locomotor activity, spatial memory acquisition in the water maze, and cued fear conditioning, as well as increased startle response and decreased prepulse inhibition. Caramiphen treatment (20, 100 mg/kg, im) as adjunct to standard therapy dose-dependently reduces seizure activity, attenuates deficits in spatial memory and fear conditioning, and protects against the development of severe neuronal degeneration.

Injuries in the brain after soman-elicited seizure activity are more widespread than those outlined in Fig. 2 and closer to the real situation as outlined in Table 1 in McDonough et al. (1998). In fluorescent brain sections (Fluoro-Jade B) neuropathology can be seen throughout the entire cortical mantle in addition to numerous subcortical structures (Myhrer et al., 2003). Hence, behavioral changes measured after soman-induced damage cannot be related to lesion of a single or several structures, because complex interactions in effects will result

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from the widespread injuries caused by nerve agents. If effects of nerve agent-induced brain damage and effects of selective lesion of a specific structure had been examined in the same task with relatively high cognitive loading, behavioral differences would probably have emerged from using the 2 different lesion procedures.

A relationship has been found between changes in EEG pattern and occurrence of neuropathology in animals both during nerve agent-induced seizures and after seizure termination. Power spectra, obtained from EEG 6 days after soman-intoxication of rats, revealed significant differences in the delta, theta, and beta frequencies (Philippens et al., 1992). In a later study of rats, it was shown that correlation analysis unveiled strong predictive relationship between the amount of delta frequency (0-3.5 Hz) activity on the day following exposure to soman and the degree of subsequent neuronal damage (McDonough et al., 1998). Increased delta activity is considered as an indicator of ongoing degenerative processes. A similar finding is also made in guinea pigs, but not in monkeys (Carpentier et al. 2001a). On the other hand, delta activity appears to decrease in monkeys during seizures induced by soman (Lallement et al., 1998). When rats or guinea pigs are effectively treated to prevent or reduce soman-induced seizure activity, delta waves are normal or barely affected (Carpentier et al., 2001a). In the above studies, superficial, cortical electrodes were used for EEG recordings. Similar EEG instrumentation was used in the study in which rats exposed to 5 x LD_{50} of VX displayed no signs of seizure activity (but evident neuropathology) following successful acute treatment with the triple procyclidine regimen (Myhrer et al. 2015). Although, spectral analysis was not performed in the latter study, change in delta waves would hardly be expected to be recorded since global epileptiform activity was absent. For recording local, silent epileptiform activity, depth electrodes in specified structures like the perirhinal and piriform cortices may be required.

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Conventionally, neuropathology is expected to be seen following nerve agent-elicited seizures lasting 20 min or more (Lallement et al., 1994; McDonough et al., 1995). The underlying process is believed to be glutamate-driven excitotoxicity leading to degeneration of neurons predominantly in the ventrolateral part of the brain as seen for rats (McDonough et al., 1998). These sequences are associated with global epileptiform activity accompanied by full-blown tonic-clonic convulsions. Results from more recent research, however, suggest that there are alternative avenues leading to corresponding neuronal damage. As outlined in Section 5, unilateral neuropathology is found when seizures are absent or only last for 1-6 min in rats effectively treated after supralethal (3 and 4 x LD_{50}) intoxication by soman (Myhrer et al., 2013a). Also prophylactic treatment (physostigmine regimen) given before a convulsant dose of soman (1.3 x LD_{50}) provides complete protection against seizures and other toxic signs, but marked bilateral neuronal injuries are seen (Myhrer et al., 2013c). Clear evidence that marked neuropathology can develop without concomitant recordable seizure activity is demonstrated when rats exposed to 5 x LD_{50} of VX and treated soon after poisoning display no clear toxic signs (Myhrer et al., 2015).

Nerve agent-induced neuronal lesions without seizures or other overt toxic signs may be related to local, silent epileptiform activity and/or direct toxic action of nerve agent on neurons as suggested by the third hypothesis dealt with in Section 1. Sustained amygdaloid kindling can trigger silent seizures during immobility or active exploring resulting in neuronal damage in the amygdala/hippocampal transition zone, piriform cortex, basolateral amygdala, perirhinal cortex, and entorhinal cortex and in about half the rats, hippocampal CA1 damage is observed (McIntyre et al., 2006). These areas are identical to those injured by soman-elicited seizures (Fig. 2, Table 1 in McDonough et al., 1998). In some studies with supralethal levels of intoxication, nerve agent-triggered neuropathology is predominantly seen in the left hemisphere (Myhrer et al., 2013a, 2013c, 2015). The latter finding may be

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associated with the observation that the left frontal/piriform cortex in our Wistar strain has higher glutamatergic activity than in the right hemisphere (Myhrer et al., 1992). Additionally, it has been found hemispheric lateralization of both the striatal glutamatergic receptors and the cortical presynaptic glutamatergic system, probably related to limbic function, with predominance on the left side in Sprague Dawley rats (Capper-Loup et al., 2009). Hence, the findings of higher glutamatergic activity in the left versus the right hemisphere give further support to the notion that local, silent glutamate-mediated excitotoxic activity results in neuronal damage in rats exposed to supralethal poisoning of nerve agent without displaying overt toxic signs after adequate therapy.

It may be questioned whether AChE inhibition leading to glutamatergic excitotoxicity is the only cause of brain damage after supralethal poisoning (5 x LD_{50}) without concomitant recordable seizures and overt convulsions. Petras (1981, 1994) raised the possibility that direct toxic action of the nerve agent on brain neurons may take place. This issue has recently received renewed attention. Subconvulsant doses of sarin or soman can result in neurodegeneration in absence of overt acute phase of OP poisoning or long after the acute phase of intoxication has terminated (Damodaran et al., 2006; RamaRao et al., 2011; Spradling et al., 2011). It has also been proposed direct action of nerve agents on molecular targets other than AChE (Grigoryan et al., 2008). In accordance with the latter notion, evidence for non-AChE targets of VX has been demonstrated in AChE knockout mice (Duysen et al., 2001). It was a surprise that AChE knockout mice can live up to 80 days, although they have low body weight, poor grip strength, and their tremor suggests the presence of excess acetylcholine. Mice with 0, 50, or 100% AChE activity were treated (s.c.) with VX. The LD_{50} for VX was 10 to 12 µg/kg in AChE-/-, 17 µg/kg in AChE-/+, and 24 µg/kg in AChE+/+ mice. The same cholinergic signs of toxicity including convulsions were present in AChE-/- mice as in wild-type mice, even though AChE-/- mice have no AChE

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whose inhibition could lead to cholinergic signs. Wilde type mice, but not AChE-/- mice, were protected by treatment with atropine. OP ester toxicity in wild-type mice is probably due to inhibition or binding to several proteins, only one of which is AChE (Duysen et al., 2001). Silent epileptiform activity and accompanying neuropathology in rats poisoned by supralethal level of nerve agents may therefore be caused by several additional factors other than inhibition of AChE. Some mechanisms can play in concert with AChE inhibition when the degree of intoxication is particularly severe, and the detoxification processes will be expected to be long-lasting.

When guinea pigs are exposed to 2 x LD_{50} of tabun, sarin, soman, cyclosarin, VX, or VR each of the 6 nerve agents is capable of inducing seizure activity. Histological analyses showed that there are no differences in the appearance of damage produced by one nerve agent versus another within a given structure and there was no difference between agents in the brain areas affected (Shih et al., 2003). When rats are exposed to 5 x LD_{50} of corresponding agents except VR and effectively treated with procyclidine regimens, sarin and cyclosarin produce more extensive injuries than soman and VX (Myhrer et al., 2015). Such differentiation between nerve agents' potency to induce neuropathology has, to our knowledge, not been shown before, and was probably seen because the efficacious therapies ensured survival of supralethal poisoning. This intriguing finding of differences in lesion capability among nerve agents is not readily accounted for.

By way of summary, it has been maintained in a number studies that only nerve agent intoxication resulting in seizures lasting more than 20 min can cause neuronal damage. This "axiom" has been challenged by some novel results showing that both unilateral and bilateral lesions can occur even if the animals did not seize at all or only seized for a brief time (< 20 min). These instances take place when rats are exposed to supralethal doses of nerve agent and treatment starts 1 min after exposure. Evident neuropathology can also

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develop without the occurrence of overt toxic signs after prophylactic treatment of rats challenged with 1.3 x LD_{50} of soman. A potential explanation of the latter findings of neuropathology may be associated with local, silent glutamate-driven epileptiform activity in brain areas identical to those damaged by silent kindling seizures in experimental epilepsy. In light of the results presented in this review, it cannot be precluded that several mechanisms other than inhibition of AChE can trigger epileptiform activity leading to damage of neurons in individuals exposed to nerve agent. The number of mechanisms involved may be related to the level of nerve agent intoxication.

6. **Microinfusion of drugs, determination of critical pharmacological receptors, and systemic utility**

Brain structures critical for control of soman-induced seizures have been identified by selective lesions. Pharmacological blocking of neuronal activity in the same structures (the area tempestas, medial septum, perirhinal cortex, posterior piriform cortex) would be expected to exert powerful anticonvulsant efficacy. In order to determine what receptors are important for efficacious anticonvulsant impact, pharmacological agents with potential antiseizure properties have been microinfused (1 µl/min) into the various target areas of rats subsequently exposed to a convulsant dose of soman. The results are summarized in Table 1. The muscarinic receptors appear to be critical in most brain regions, but there are distinct differences among other receptor types. In the area tempestas, cholinergic, and not glutamatergic antagonism is likely the active property of the antiparkinson agents, caramiphen and procyclidine, since the NMDA antagonist ketamine and MK-801 do not have anticonvulsant effects (Myhrer et al., 2008b). The $GABA_A$ modulators muscimol ethanol and propofol produce anticonvulsant effects, whereas diazepam and pentobarbital do not (Myhrer et al., 2006). In the medial septum, only muscarinic receptors seem to be the critical ones, because atropine, scopolamine, and procyclidine are effective, whereas

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ketamine and muscimol do not produce anticonvulsant effects (Myhrer et al., 2009). In the perirhinal cortex, both muscarinic and glutamatergic receptors have to be blocked simultaneously as by procyclidine in order to achieve anticonvulsant effect. Neither scopolamine alone nor ketamine alone causes anticonvulsant effects (Myhrer et al., 2010a). However, the scopolamine dose of 1 µg/µl in the latter study was too low, because it has been shown that the lowest dose of scopolamine yielding anticonvulsant impact in the perirhinal cortex in response to soman is 4.59 µg/µl (Skovira et al., 2012). The positive effect of NBQX suggests that AMPA receptors are critical for anticonvulsant impact in the perirhinal cortex (Myhrer et al., 2010a). The central roles of NMDA and AMPA receptors in this area suggest that there is an early increase of glutamatergic activity in the perirhinal cortex. This view receives further support from the findings that modulation of metabotropic glutamate receptors (mGluRs) in the perirhinal cortex also has anticonvulsant effects. Injection of MPEP (antagonizing mGluR5) or DCG-IV (agonistic effect on both mGluR2 and 3) results in marked anticonvulsant efficacy (Myhrer et al., 2010b). In a recent study, double infusions of anticonvulsants were used to reach a larger area of the perirhinal cortex. With such technique, MPEP protects 67 percent of the rats from evolving soman-induced seizures, whereas the percentage for DCG-IV is 83 (Myhrer et al., 2013b). In the posterior piriform cortex, muscarinic and $GABA_A$ receptors seem to be the critical ones. This constellation of receptors has similarity to area tempestas located in the anterior piriform cortex, and might indicate a general feature of the piriform cortex. In the mediodorsal thalamus, NMDA receptors emerge as the sensitive ones, since infusion of MK-801 (0.21-0.61 µg) results in anticonvulsant action against seizures triggered by soman, whereas scopolamine (3.33-12.24 µg) does not (Skovira et al., 2012). According to the present results, efficient anticonvulsant pretreatment has to be based on pharmacological agents affecting a number of subreceptors. Also post-exposure treatment will probably profit by

using similar agents. Determination of critical subreceptors in the identified target areas is crucial for analyses of potential mechanisms involved in the development of neuropathology in response to convulsant doses of nerve agents

Procyclidine emerged as the most potent anticonvulsant drug tested in the microinfusion studies (Myhrer, 2010). Enhancement of procyclidine's excellent anticonvulsant efficacy in the seizure controlling sites of the forebrain would make up a novel and interesting approach. A drug with such enhancing properties has been developed in preclinical epilepsy research. Levetiracetam (Keppra®) is an antiepileptic drug that strongly enhances the anticonvulsant effects of compounds affecting either glutamatergic or GABAergic neurotransmission (Kaminski et al., 2009). It has also been reported that levetiracetam may both reduce release of acetylcholine and reduce postsynaptic responsiveness in cholinergic synapses (Oliveira et al., 2005). Levetiracetam (50 mg/kg) combined with either procyclidine (20 mg/kg) or caramiphen (20 mg/kg) terminate seizure activity, but the survival rate is considerably higher for levetiracetam and procyclidine than levetiracetam and caramiphen at the tested doses. Both therapies can also save the lives of rats that were about to die 5-10 min after seizure onset. Prophylactic use of the combination therapies 20 min before exposure to soman prevent onset of seizures more effectively when levetiracetam is combined with procyclidine than caramiphen (Myhrer et al., 2011). These results show that the information achieved from microinfusion studies can be very valuable for systemic application in combination with other relevant drugs.

We have carried out a study in which effects of a physostigmine regimen (HI-6, scopolamine, physostigmine) were compared with those obtained by our procyclidine regimen (HI-6, levetiracetam, procyclidine) against various doses of soman at different times after intoxication. The prophylactic potencies of the regimens were also examined (Myhrer et al., 2013c). The results show that both regimens administered 2 times (1 and 5

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min after exposure to 3, 4, or 5 x LD_{50} of soman) effectively prevent or terminate epileptiform activity within 10 min. Both regimens also protect against death, and the rats recovered well. However, when the regimens were administered 10 and 14 min after a soman dose of 1.6 x LD_{50}, only the procyclidine regimen terminates seizure activity and protects all rats against death. When rats pretreated with pyridostigmine received treatment 20 and 24 min after a soman dose of 1.3 x LD_{50}, only the procyclidine regimen successfully stops seizures and prevents death among all rats. When the regimens were given as prophylactic treatment 20 min before a soman dose of 1.3 x LD_{50}, both the physostigmine and procyclidine regimens prevent convulsions, but only the procyclidine regimen yields neuroprotection (Myhrer et al., 2013c). In a recent study, it was examined whether the triple regimen may have antidotal efficacy against intoxication by other classical nerve agents than soman (Myhrer et al., 2015). The treatment was given 1 and 5 min after exposure to a supralethal dose of nerve agents, and the results showed that the triple regimen successfully prevent or terminate seizures and preserve the lives of rats exposed to 5 x LD_{50} of soman, sarin, cyclosarin, or VX, but solely 3 x LD_{50} of tabun was managed by this regimen. To meet the particular antidotal requirements of tabun, the triple regimen was reinforced with obidoxime and was made to a quadruple regimen that effectively treated rats intoxicated by 5 x LD_{50} of tabun. The rats recovered very well and the majority gained pre-exposure body weight within 7 days. Neuropathology was seen in all groups regardless of whether the rats seized or not. The most extensive damage was produced by sarin and cyclosarin.

Not all combinations derived from microinfusion studies will work equally well. For example, the potency of the metabotropic glutamate modulators DCG-IV and MPEP is very powerful in the perirhinal cortex (Myhrer et al., 2013b). However, when administered systemically together with procyclidine and HI-6, both combinations exert effective anticonvulsant impact, but they do not reach the same high level of protection as the

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procyclidine regimen (Myhrer et al., 2013d).The multifunctional properties of procyclidine (antiglutamatergic and anticholinergic) seem to profit from the enhancing effects of levetiracetam, whereas the single function of DCG-IV and MPEP (antiglutamatergic) does not result in increased anticonvulsant capacity when combined with levetiracetam and HI-6 (Myhrer et al., 2013d). The metabotropic glutamate modulators have only been tested in the perirhinal cortex. Hence, it is not known whether they are able to affect multiple action sites. It is also possible that some drugs found to be effective with microinfusions may have more moderate influence during systemic testing.

7. Treatment strategies

7.1. Neuroprotectants

Development of neuropathology is associated with nerve agent-induced epileptiform activity leading to glutamatergic excitotoxic activity. Large pathological elevation in the concentration of intracellular sodium and calcium are produced by excessive stimulation of ionotropic glutamate receptors and prolonged depolarization of postsynaptic membranes. In neurons, the majority of glutamate-induced calcium influx occurs through NMDA receptors as well as AMPA and kainate receptors, although to a lesser degree in the latter 2 types of receptors. Excessive stimulation of NMDA receptors represents the first step in glutamate excitotoxicity (Ballough et al., 2008). On the basis of this situation, the most efficacious countermeasures against nerve agent poisoning would be ionotropic glutamatergic antagonists blocking extracellular calcium influx or pharmacological compounds enhancing GABAergic activity that can attenuate glutamatergic activity.

A number of anticonvulsant drugs have neuroprotective properties (e.g., ionotropic antagonists), but some neuroprotectants (e.g. immunomodulators, receptor active drugs like neuropeptides) do likely not have anticonvulsive properties. Neuroprotection in this respect may be defined as chemical intervention to prevent or reduce neurodegenerative processes

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in the CNS. Assumptions about a drug's neuroprotectant potency against nerve agent poisoning can generally only be determined from the use of the same drug in treatment regimens against nerve agent poisoning. Determination of pure neuroprotectant efficacy is likely not feasible, even when used solely in survivors after treatment with other drugs. Remnant effects of the latter drugs may confound the results, because neuroprotectants are usually given short time after or along with the anticonvulsant regimen. Some life-preserving measure has to be administered.

In the initial phase of response to soman, elevated regional cholinergic activity subsequently induces extremely high global neuronal activity leading to seizures accompanied by convulsions. Gradually, structures dominated by excessive glutamatergic activity probably take over the excitatory drive of sustained seizures. Studies based on microdialysis procedures have reported an immediate increase in extracellular glutamate concentrations in the septum, piriform cortex, hippocampal region, and amygdala following the start of soman-triggered seizures (Lallement et al., 1991, 1992; Wade et al., 1987). This shift of neurochemical seizure generator is not readily understood. A similar phenomenon is also seen for the induction of seizures by pilocarpine or carbacol. In the latter case, anticholinergics can prevent seizures, but termination of ongoing seizures is achieved by antiglutamatergic drugs (Cavalheiro et al., 2006).

Ideally, countermeasures against nerve agent poisoning should be designed to have their primary efficacy in the seizure controlling brain sites (the area tempestas, medial septum, perirhinal cortex, posterior piriform cortex). For the choice of effective anticonvulsant drugs with neuroprotective properties it is important to know the general constellation of receptors in the respective seizure controlling areas and the neuronal connectivity of these critical areas.

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The area tempestas receives cholinergic input from the diagonal band nucleus of the septal area (Woolf et al., 1984) and is provided with both GABAergic and glutamatergic intrinsic connections, whereas the output is glutamatergic (Doherty et al., 2000; Gale, 1988; Halonen et al., 1994). In their pioneer work, Piredda and Gale (1985) showed that the area tempestas is provided with GABA receptors responding with antiseizure effect to microinfusion of muscimol. Furthermore, the brain site is also endowed with muscarinic receptors responding with antiseizure effect to atropine, and AMPA receptors responding to NBQX, but no response from NMDA receptors (Fornai et al., 2005). Demonstration of the presence of receptors unrelated to management of seizures does not seem to exist for the area tempestas.

The medial septal area receives cholinergic input from the dorsolateral tegmental nucleus, and neurons in the medial septum send cholinergic projections to the hippocampal formation and anterior cingular region (Woolf et al., 1984). In addition to muscarinic receptors responding to acetylcholine or atropine (Dutta and Ghosh, 2016), the medial septum also contains GABA receptors responding to microinfusion of bicuculline (Ang et al., 2015) and NMDA receptors responding to the antagonist D-AP7 or NMDA (Khakpai et al., 2016).

Both the perirhinal cortex and posterior piriform cortex receive cholinergic inputs from the septal diagonal band nucleus (Woolf et al., 1984). However, the cholinergic input to the posterior piriform cortex is far more extensive than the input to the perirhinal cortex, although the input to the latter structure is more evident than the dorsally located neocortical areas, as reflected in the AChE stained plates in the atlas of Paxinos and Watson (1986). A critical link from the area tempestas is represented by glutamatergic projections to the perirhinal cortex and posterior piriform cortex (Halonen et al., 1994). The posterior piriform cortical connections with the perirhinal cortex (Burwell and Amaral, 1998) are likely

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glutamatergic, since glutamate is used as neurotransmitter by cortico-cortical pathways (Fonnum, 1984). Furthermore, the perirhinal cortex has direct projections to the frontal motor cortex (McIntyre et al., 1996) that most likely are glutamatergic (Myhrer, 2007).

The perirhinal cortex possesses NMDA receptors responding to intracerebral infusion of AP-7 and AMPA receptors responding to NBQX (Malkova et al., 2015). Muscarinic receptors responding to the antagonist pirenzepine are localized in this brain area (Bartko et al., 2014) as well as GABA receptors responding to infusion of bicuculline (Kim et al., 2014). The posterior piriform cortex contains NMDA receptors responding to application of MK-801 (Yang et al., 2007) and muscarinic receptors responding to carbachol (Saar et al., 2007). GABA receptors in this area are found to respond to application of the antagonist 4AP (Panuccio et al., 2012). According to the results presented above, glutamatergic, GABAergic as well as cholinergic receptors are present in the 4 seizure controlling sites identified in Section 2. In addition, the metabotropic glutamate receptors consisting of Group I (mGluR1, mGluR5), Group II (mGluR2, mGluR3), and Group III (mGluR4, mGluR6, mGluR7, mGluR8) are all present in the perirhinal cortex of the rat (McCafferey et al., 1999).

Among the brain areas with seizure controlling abilities against nerve agent-induced epileptiform activity, the area tempestas and perirhinal cortex appear to be the most potent ones (Table 1). This differentiation is probably associated with how the respective structures are linked to other critical structures involved in triggering and/or propagating seizure activity. The medial septum has its major projection to the hippocampus which is one of the slowest kindling sites and not critical for either septal or amygdaloid kindling (Racine et al., 1988). Furthermore, activation of the hippocampus is not necessary for induction of focal seizures in the amygdala or piriform cortex kindling (Ebert and Löscher, 1995). The posterior piriform cortex is provided with a neurochemical pathway from the area tempestas

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that is weaker than the pathway from the area tempestas to the perirhinal cortex (Tortorella et al. 1997). Moreover, the posterior piriform cortex has a relatively small projection to the perirhinal cortex (Burwell and Amaral, 1998). Thus, the projection from the area tempestas to the perirhinal cortex makes up a very strategic propagation pathway of seizure activity to the frontal motor cortex.

Seizure activity lasting beyond 40 min is gradually more difficult to terminate (Carpentier et al., 2001b; Lallement et al., 1999). The refractory nature of sustained seizures represents a great challenge in treatment of nerve agent poisoned victims long time after exposure. Sustained seizures are associated with both brain damage and death (Shih et al., 2003). Seizure activity lasting 30 min or more (status epilepticus) has been shown to result in externalization of NMDA and AMPA receptors along with internalization of $GABA_A$ receptors. These alterations of the receptors' functional properties cause pharmacoresistance in sufferers of epilepsy and most likely in individuals exposed to a high dose of nerve agent. Despite the appearance of novel anticonvulsants, status epilepticus is still associated with significant morbidity and mortality (Wasterlain et al., 2013).

It has been shown in hippocampal slices that after repeated seizures in NMDA synapses, subunits are mobilized to the synaptic membrane and assemble into additional receptors. They may move initially to the perisynaptic area and then laterally to the synaptic area. As a result of this trafficking, the number of functional NMDA receptors per synapse increases (Niquet et al., 2016; Wasterlain et al., 2013). A similar process is also seen for AMPA receptors when AMPA-mediated glutamatergic transmission is strengthened during sustained seizure activity (Rajasekaran et al., 2013). After repeated seizures, the synaptic membrane of $GABA_A$ receptors forms clathrin-coated pits, which internalize as clathrin-coated vesicles, inactivating the receptors because they are no longer within reach of the neurotransmitter (Niquet et al., 2016). By this process, the antiseizure potency of

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benzodiazepines can decrease 20-fold in 30 min (Kapur and Macdonald, 1997). These maladaptive changes due to receptor trafficking will require correcting by a dual or triple therapy.

Extensive neuronal damage is seen in the index areas piriform cortex and amygdala, but modest damage occurs in CA1 after exposure to 5 x LD_{50} of any of the classical nerve agents. Even if onset of seizure activity was prevented or seizures were terminated within 10 min after exposure, ample neuronal injury was observed after treatment with the triple or quadruple regimens (Myhrer et al., 2015). Continued treatment with levetiracetam and procyclidine for several days after poisoning will likely reduce or prevent brain damage, as described in Section 4 (Myhrer, Enger, and Mariussen, unpublished data).

In instances of insult, the CNS generally responds with inflammatory reactions. After nerve agent-induced seizures in mice development of inflammation is detected in their brains. In order to ensure a reasonable number of survivors mice were pretreated with HI-6 (50 mg/kg) and then after 5 min exposed to a convulsant dose of soman (1.6 x LD_{50}). An important and highly significant increase of RT-PCR inflammatory gene expression was recorded in the whole neocortex with peak response 6-24 h after intoxication. In the hippocampus, the gene up-regulation reached a peak response after 24-48 h. The inflammatory reaction was still seen after 7 days (Dhote et al., 2007). In a subsequent study of the same group (Dhote et al., 2012), it was shown that treatment with ketamine in various doses combined with atropine sulfate (10 mg/kg) is neuroprotective and reduces neuroinflammation as well as heightened levels of microglia and astroglia in mice exposed to a convulsant dose of soman (1.6 x LD_{50}). The mice were pretreated with HI-6 (50 mg/kg) 5 min prior to poisoning and convulsed about 4 min after exposure. Treatment that started 30 min after soman was given as 25 mg/kg ketamine along with atropine 6 times every 30 min or 100 mg/kg of ketamine 3 times every 1 h. Both forms of treatment terminated or

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reduced convulsive activity, protected neurons in selected areas of the brain, and reduced inflammatory reactions. However, the overdosage of ketamine by both therapies killed a number of animals. It was concluded that antagonists of NMDA receptors are currently among the few compounds able to arrest seizures and provide neuroprotection even during refractory status epilepticus (Dhote et al., 2012) (Table 2).

Chemical substances other than recognized anticonvulsants have been shown to exert neuroprotectant impact against nerve agent-induced neuronal injuries. Alpha-linolenic acid (ALA or LIN) is an omega-3 fatty acid that is found in vegetable products and has no known side effects. LIN is neuroprotective against kainic acid-induced brain damage. Additionally, LIN exerts anti-depressant and anti-inflammatory activities and enhances synaptic plasticity in vivo. These properties make LIN a potential candidate against nerve agent-induced neuropathology (Pan et al., 2012). In the latter study, rats were pretreated with HI-6 (125 mg/kg) 30 min prior to soman exposure (1.6 x LD_{50}), received atropine methyl nitrate (2 mg/kg) 1 min later and received diazepam (10 mg/kg) after 40 min of seizing. A single dose of LIN (500 nmol/kg) was administered systemically either 3 days prior to soman injection or 30 min after exposure. Both pretreatment and posttreatment regimens provided marked protection of neurons in a similar way in the hippocampus, prefrontal cortex, amygdala, and piriform cortex. Subcutaneous injection of LIN shows greater neuroprotectant efficacy than intravenous injection in a brain region-specific manner. It was suggested that LIN may attenuate glutamate-mediated excitotoxicity and modulate convulsive activity (Pan et al., 2012). In a subsequent study of the same group (Pan et al., 2015), identical experimental paradigm and regimen of countermeasures were used, but LIN (500 nmol/kg, i.v.) was administered intravenously 30 min, 3 days and 7 days after challenge with soman (1.6 x LD_{50}). It was hypothesized that 3 sequential injections of LIN would provide long-lasting neuroprotection against soman-induced neuropathology, because such LIN regimen has

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been shown to increase the levels of brain-derived neurotrophic factor (BDNF) in the cortex and hippocampus and is associated with increased synaptogenesis, neurogenesis, and synaptic function (Blondeau et al., 2009). The results from the study of Pan et al. (2015) showed that the dosing schedule significantly reduces soman-induced neuronal degeneration (survival 21 days) in the cingulate cortex, amygdala, hippocampus, and piriform cortex. Furthermore, it was shown that nerve agent-induced impairment in rotarod test, forced swim test, and passive avoidance is markedly mitigated by the LIN treatment. It was suggested that BDNF is involved in the mechanisms underlying protection of neurons and preservation of behavioral functions (Pan et al., 2015).

BDNF is the most widely expressed and well characterized member of the neurotophin family in the mammalian brain. It is translated as a precursor protein which consists of an N-terminal prodomain and a C-terminal mature domain, and its effects are tightly regulated. This neuroprotective protein is likely mediated via activation of its postsynaptic binding site TrkB. BDNF can exert functions in a highly localized manner and also at distance by anterograde or retrograde transport. Modest changes in BDNF levels affect the development and regulation of neural circuits and brain functions. After brain injury or exposure to soman neuronal systems responds differently. Intravenous administration of LIN results in the formation of lipid rafts, increased kappaB levels and enhanced levels of BDNF mRNA and protein in the hippocampus. Release of mature BDNF into the extracellular space results in reduced release of glutamate and NMDA receptor-mediated excitotoxicity (Piermartiri et al., 2015a, 2015b). In rats intoxicated by soman, the increase in mature BDNF-label cells is principally localized to non-neuronal cells in the hippocampus. In sharp contrast, a marked increase in the number of NeuN-positive neurons is seen to follow administration of injection of LIN in soman poisoned rats (Piermartiri et al., 2015a).

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In a recent study, BDNF levels have been recorded in rats exposed to soman and treated with LIN (Piermartiri et al., 2015a). HI-6 (125 mg/kg) was given 30 min before a soman dose of 1.6 x LD_{50}. Then followed atropine methyl nitrate (2 mg/kg) 1 min later, and the animals were allowed to seize for 40 min before they received diazepam (10 mg/kg). LIN (500 nmol/kg) was administered intravenously at 30 min, 3 and 7 days after soman. The results showed that LIN treatment increases the endogenous expression of mature BDNF, activates Akt (a serine/threonine kinase) and the mammalian target of rapamycin complex 1 (mTORC1), increases neurogenesis in the hippocampal dental granular zone, alleviates passive avoidance performance and increases animal survival. While soman injection also enhances levels of BDNF, this enhancement does not activate downstream signaling pathways (Akt, mTOR) or neurogenesis (Piermartiri et al., 2015a).

Regulation of microglial response by adaptive immunity has previously been shown to reduce the consequences of acute insult to the CNS. In a study using the same experimental design and dosing schedule of countermeasures as the one above (Piermartiri et al., 2015a), rats were treated with a single injection of the immunomodulator poly-YE (1 mg/rat s.c.) 24 h after soman (180 µg/kg, s.c.) exposure (Finkelstein et al., 2012). Neuronal count revealed that poly-YE-treated rats had significantly more surviving neurons than control rats in the piriform cortex, but not in the amygdala, thalamus, and hippocampus. Furthermore, a significant restoration of the impaired performance in the Barnes maze was not achieved by treatment with poly-YE. It is suggested that immunomodulatory treatment may represent a supplementary approach against consequences of organophosphate-induced brain damage (Finkelstein et al., 2012).

N-acetyl-aspartyl-glutamate (NAAG) is the most abundant neuropeptide in the mammalian brain. In a variety of animal models of brain injury, administration of NAAG-related compounds, or inhibitors of glutamate carboxypetidases (GCPs; the enzymes that

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hydrolyze NAAG) have been shown to be neuroprotective. In the study of Guo et al. (2015), NAAG was used alone or in combination with 2-PMPA (2-phosphonomethyl pentanedioic acid; an inhibitor of GCP enzymes). When using the same experimental design and dosing schedule of countermeasures as in the study of Piermartiri et al. (2015a) cited above, NAAG (10 or 50 mg/kg i.p.) and /or 2-PMPA (50 mg/kg i.p.) were given 1 min after injection of soman. Single or combined administration of NAAG compounds and 2-PMPA significantly reduced the number of Fluoro-Jade-positive neurons in a number of brain areas among them the entorhinal and piriform cortices and amygdala. It is concluded that NAAG compounds and GCP enzyme inhibitors may be effective in brain regions known to be particularly susceptible to nerve agent exposure (Guo et al., 2015).

Over the last years, it has become apparent that new neurons can spontaneously be formed in the brain of various adult animal species including man. This process of neurogenesis is seen in the subgranular zone of the dentate gyrus and in the subventricular zone (Ryu et al., 2016). Cytokines, including neurotrophic factors (e.g. BDNF) and growth factors have been shown to enhance spontaneous neurogenesis triggered by cerebral injury. In a nerve agent study, soman-poisoned (1.2 x LD_{50}, s.c.) mice received atropine methyl nitrate (0.5 mg/kg) 1 min after exposure and seized for about 2 h. On the next day and daily over 8 days, the animals were treated with cytokines (a mixture of 40 µg/kg fibroblast growth factor-2 and epidermal growth factor) and the brains were harvested at 9, 30, and 90 days following poisoning. The results showed that the cytokine treatment did not exhibit any neuroprotectant effects after soman exposure. Cytokine treatment enhanced neuronal regeneration in the hippocampus, but not in the amygdala. Moreover, a spontaneous neuronal regeneration occurred in both the hippocampal CA1 and basolateral amygdala. (Collombet et al., 2011).

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7.2. Anticonvulsants

Treatment regimens for nerve agent poisoning should ideally possess neuroprotective properties. With this idea in mind, it will not be advisable to screen among any relevant candidate compound which may make up thousands. Clear-cut specifications should be worked out for what subreceptors in what sites of the brain that preferentially should be affected. To achieve these specifications several experimental procedures are necessary to follow: Screening brain sites for involvement in seizure generation by selective lesions; microinfusion of nerve agent to map target areas; microinfusion of anticonvulsants to screen critical receptors in seizure controlling structures; testing systemic utility of promising anticonvulsants.

By following the above procedures, we have examined the anticonvulsant potency of 18 pharmacological agents (Table 1). Based on data from microinfusions an anticonvulsant impact factor has been expressed as percentage of positive effects for drugs tested in at least 3 of the 4 seizure controlling brain sites identified (area tempestas, medial septum, perirhinal cortex, piriform cortex). Scopolamine (75%) and procyclidine (75%) rank on top followed by muscimol (50%), NBQX (33%), caramiphen (33%), and ketamine (0%). According to these results, efficient anticonvulsant pretreatment has to be based on pharmacological agents affecting plural brain areas and plural subreceptors. This design is also well suited for measuring neuroprotectant efficacy, because all subjects are without any neuronal damage at the start of the experiment. Conversely, when post-exposure treatment is carried out, some degree of neuropathology has usually to be produced before measurement of neuroprotection can start, or alternatively insufficient anticonvulsant treatment is given along with the neuroprotectant. Table 3A shows the neuroprotectant impact of 2 therapies involving scopolamine or procyclidine, respectively. The physostigmine/scopolamine regimen causes pronounced soman-induced neuropathology, whereas the procyclidine

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regimen protects virtually completely against neuronal damage. No relationship between life-saving capacity and neuroprotectant impact was seen for the physostigmine regimen. The development of neuropathology is likely linked to lack of glutamatergic antagonism and/or GABAergic agonism of the physostigmine regimen. In Table 3B showing the results for 4 therapies, some relationship between life-preserving capability and neuroprotectant action can be seen. The neuroprotectant efficacy of procyclidine is conspicuous, because by lowering the dose from 20 mg/kg to 6 mg/kg evident neuropathology emerges. Only the regimen with the highest dose of procyclidine saved all rats that displayed no overt toxic signs apart from a brief period of incapacitation in 2 rats. Procyclidine's NMDA antagonism is supposed to be enhanced by levetiracetam, but not by the metabotropic glutamate modulators MPEP and DCG-IV.

During post-exposure treatment of nerve agent poisoning, neuroprotecting compounds are either integrated part of the therapy or are given as an adjunct to a standardized regimen (HI-6, atropine, and diazepam). Some novel therapies have been based on caramiphen or the AMPA/kainate antagonist LY293558. Caramiphen has for many years been studied by Israeli investigators (Raveh et al, 2014) and has been carried on by US groups. One min after exposure to soman (1.2 x LD_{50}) rats were given HI-6 (93.6 mg/kg), atropine sulfate (2 mg/kg) with or without physostigmine (0.2 mg/kg). At 10, 20, or 30 min after seizure onset the animals received caramiphen (20 mg/kg), diazepam (10 mg/kg), or both. The combination of caramiphen and diazepam reduced duration of seizures and reduced neuropathology compared to diazepam alone, when treatment was delayed 20-30 min after seizure onset. Caramiphen by itself was not effective at the later treatment times, since seizure activity lasted for a long time (up to 72 h), and neuropathology was marked in the amygdala and piriform cortex (Schultz et al., 2012). The latter authors suggested that a higher dose of caramiphen (100 mg/kg) as used by Figueiredo et al. (2011a) would be more

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effective. In the latter study, HI-6 (125 mg/kg) was given 30 min before a soman dose of 1.4 x LD_{50} followed by atropine sulfate (2 mg/kg) 1 min later and the rats seized after about 10 min. Caramiphen (100 mg/kg) was administered 30 or 60 min after soman exposure, but required 1-4.5 h for complete cessation of seizures. Neuronal damage was attenuated in the amygdala, piriform cortex, hippocampus, and entorhinal cortex. It appears that the effectiveness of caramiphen is reduced with time after exposure, and some concern is expressed that the dose required might be toxic (Figueiredo et al., 2011a).

In our laboratory, we have compared the anticonvulsant and neuroprotectant impact of caramiphen (20 mg/kg) and procyclidine (20 mg/kg) when they are combined with levetiracetam (50 mg/kg). The rats were given HI-6 (125 mg/kg) 20 min before they were challenged with soman (1.6 x LD_{50}). Latency to seizure was 5-8 min for both groups and the animals were allowed to seize for 40 min before they were treated with levetiracetam combined with either caramiphen or procyclidine. Latency to seizure termination was 12-17 min for both groups. However, all rats in the caramiphen group died within 2 days, whereas all rats treated with procyclidine survived. After having seized for more than 50 min, marked neuropathology was seen in the piriform cortex and amygdala of the procyclidine group (Myhrer et al., 2011).

LY293558 is a GluK1R/AMPA receptor antagonist which has been shown to have promising anticonvulsant and neuroprotectant properties. In one study, rats received HI-6 (125 mg/kg) 30 min prior to soman (1.4 x LD_{50}) followed by atropine sulfate (2 mg/kg) 1 min later. Seizures were triggered after 5-15 min and treatment with LY293558 (50 mg/kg) was given 1 or 1.5 h after soman exposure. In rats instrumented for EEG recording, seizure activity was terminated after 20-30 min at 1 h, and survival rate was 67%. Epileptiform activity, however, recurred in most rats, and the total duration of the seizure activity during the initial 24 h was 1.78 h for the LY293558- treated rats. Also later treatment stopped

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seizures. LY293558 prevented or reduced damage in the amygdala, piriform cortex, hippocampus, and entorhinal cortex (Figueiredo et al., 2011b). In a subsequent study of LY293558, rats were poisoned by a soman dose of 1.2 x LD_{50} which resulted in seizures after about 9.5 min. Twenty min following exposure (about 10 min of seizing), HI-6 (125 mg/kg), atropine sulfate (2 mg/kg), and LY 293558 (15 mg/kg) were administered, and the mean time to seizure termination was 36.9 min. The total duration of seizures during the initial period was about 2 h, because in some animals the epileptiform activity returned at varying time points after cessation of the first seizures. After a survival time of 7 days, histological examination showed reduced neuropathology in the amygdala, piriform cortex, hippocampus, and entorhinal cortex in the treated rats (Apland et al., 2013).

The therapies based on procyclidine, caramiphen, or LY293558 have in common that they yield pronounced glutamatergic antagonism. Excessive stimulation of NMDA receptors, although to a lesser degree AMPA/kainate receptors, makes up the first step in glutamate-driven excitotoxicity (Ballough et al., 2008). Hence, the above regimens are apparently well provided with neuroprotective and anticonvulsive properties. This combination is very important, because the total duration of epileptiform activity will influence the extent of neuropathology.

LY293558 has been suggested used as an anesthetic (Lee et al., 2006; Von Bergen et al., 2002). By acknowledging the sedative effect, the dose of LY293558 was reduced from 50 mg/kg to 15 mg/kg, because with the highest dose the rats became deeply sedated and died from respiratory depression within the next 4 h (Apland et al., 2013). The anticonvulsant efficacy of the procyclidine regimen (HI-6, levetiracetam, procyclidine) can be exemplified by comparing it with effects of the LY293558 regimen. Twenty min following soman exposure (after about 10 min of seizing), LY293558 along with atropine and HI-6 was administered, and the mean time to seizure termination is 36.9 min (Apland et

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al., 2013). When the procyclidine regimen was administered 20 min following onset of soman-induced seizures, the mean time to seizure termination is 12.7 min (Myhrer et al., 2013d). This time difference in terminating seizure activity between the above studies will probably be decisive for the development of neuropathology.

The anticonvulsant potency turned out to be higher for procyclidine than caramiphen as judged from the microinfusion results presented in Table 1. Both drugs were effective in the area tempestas in which muscarinic receptors appear to be critical. In the perirhinal cortex, however, where glutamatergic receptors are responsive, procyclidine produces anticonvulsant action, whereas caramiphen does not. This difference is probably associated with the degree of NMDA antagonism among the drugs. Procyclidine inhibits the phencyclidine site at the NMDA receptor very potently (Reynolds and Miller, 1988) in a concentration-dependent manner (Myhrer et al., 2004), whereas caramiphen appears to bind to the Zn^{2+} site at the NMDA receptor (Raveh et al., 1999). Procyclidine and caramiphen differ in their capabilities to antagonize a lethal dose of NMDA in mice. Procyclidine at a dose of 30 mg/kg protects 80 % of NMDA-injected mice, whereas caramiphen doses of 10-80 mg/kg protect only 11- 40 % of the mice (Raveh et al., 1999). Among other pharmacological agents with anticonvulsive properties, it has been shown that they antagonize NMDA lethality in mice in the following ranked order: procyclidine, ketamine, benactyzine, pentobarbital, biperiden, trihexyphenidyl (McDonough and Shih, 1995). Caramiphen was not included in the latter study.

Pharmacoresistance occurs frequently during sustained seizure activity. Two distinct mechanisms seem to be activated. One is the externalization of NMDA and AMPA receptors. The other one is the transport of antiepileptics by drug efflux transporters such as P-glycoprotein (Pgp) at the BBB that can extrude antiepileptics from their intended site of action (Luna-Tortós et al., 2008). The constellation of the procyclidine regimen may

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contribute to protect against effects of the externalization process, because the decreased release of glutamate caused by levetiracetam makes the NMDA antagonism by procyclidine particularly effective, and levetiracetam also exerts GABAmimetic effects (Myhrer and Aas, 2014). An additional important aspect is that levetiracetam has been shown to be a Pgp substrate that can bind to the Pgp transporter and inhibit efflux of other antiepileptics (Luna-Tortós et al., 2008). It has been demonstrated that Pgp inhibition can increase the efficacy of nerve agent treatment. Tariquidar, a non-competitive, specific Pgp inhibitor, enhances the levels of HI-6 in the brain of rats about 2-fold during the first hour when administered as pretreatment or post soman (2 x LD_{50}) exposure. With the post-treatment paradigm, the efficacy of both atropine and HI-6 is elevated with the presence of tariquidar (Joosen et al., 2011, 2016). The caramiphen and LY293558 regimens do apparently not benefit from means correcting pharmacoresistance. The meticulous long-term work with the development of the procyclidine regimen appears to pay off and lead to results that are important for consideration in the development of future medical countermeasures.

7.3. Human aspects

While there is a growing body of knowledge about the effects of nerve agents and countermeasures on animals, corresponding data for humans are relatively scant. Even if nerve agents have been used in several recent conflicts (e.g., Iraq-Iran, Syria), systematic studies of reactions of the victims only seem to exist for the terror attacks in Japan (Yanagisawa et al., 2006). Members of a cult named "Aum Supreme Truth" attacked citizens with sarin in 1994 in Matsumoto and in 1995 in Tokyo, Japan. The cult manufactured sarin and VX and biological weapons to fight its societal enemies, especially the police and other governmental agencies. The target in Matsumoto was the district court and in Tokyo the subway and National Police Agency.

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The first attack using sarin occurred in summer at midnight in a residential area. Nearly 600 people were poisoned with 7 deaths and 56 hospitalized victims. In the second attack, sarin was released in several subway cars on different lines leading to the government office center in Tokyo during a Monday morning rush hour. Twelve deaths and more than 5000 were exposed (Yanagisawa et al., 2006). The clinical conditions of the injured victims, responses to various therapies, physical and psychological sequelae were recorded in both incidents with follow up to 10 years. Because these were the first incidents with mass casualties caused by sarin, it was important to describe the various aspects of human toxicity.

Oximes (pralidoxime, 2-PAM), atropine sulfate, diazepam, and ample intravenous fluid infusions were effective treatments for epileptic convulsions, agitation, and loss of body fluid. Severely affected subjects required intubations and mechanical ventilation. The main site of sarin action in critically ill victims was on the CNS leading to convulsions and coma. In both the Matsumoto and Tokyo attacks, plasma cholinesterase (ChE) served as useful index of sarin exposure. In 92% of hospitalized patients, plasma ChE levels returned to normal on the following day (Okumura et al., 2015). In 6 victims, however, with serum ChE below 29% of the lowest normal level were resuscitated from cardiopulmonary arrest or coma with generalized convulsions. Five recovered completely, but 1 remained in a vegetative state due to anoxic brain damage. EEG abnormalities lasted for up to 5 years. At 5 years, partial post-traumatic stress disorder was suspected in 6 subjects. In addition, a considerable number of people who had been in the area of sarin had psychological complaints (Yanagisawa et al., 2006).

For experimental purposes in animals, the doses of sarin or soman are usually between 1 and 2 x LD_{50}. In a study with more moderate use of nerve agent, rats received a single injection of sarin (0.5 x LD_{50}) that resulted in a significant decline in plasma ChE

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levels. Plasma ChE was inhibited up to 73% of control by 2.5 h and gradually recovered to 56% inhibition level on 1st, 38% on 3rd, 32% on 5th, and 23% on 7th day post-exposure (Chaubey et al., 2016). The restoration of AChE in brain tissue can take 7-14 days in rats exposed to a soman dose of 1.4 x LD_{50} (Prager et al., 2014). Thus, results from animal research are probably only comparable with the symptoms found in the most severely poisoned sarin victims. Changes in EEG patterns and anxiety disorders may reflect long-term impact on the CNS. Electrophysiological experiments with amygdaloid slices from young rats exposed to a convulsant dose of soman (brains harvested 24 h or 14 days after exposure) reveal reduced GABAergic inhibition in the basolateral amygdala which may relate to increased anxiety in in vivo studies (Prager et al., 2014). Likewise in humans, commercial pesticide (organophosphate compounds) sprayers show elevated anxiety and lower plasma cholinesterase activity than control subjects (Levin et al., 1976). Furthermore, there is an apparent correspondence between findings of increased fear/freezing in animals and elevated anxiety in humans following exposure to AChE inhibitors (Myhrer and Aas, 2016).

It has been suggested that delta activity may serve as an early indicator of soman-elicited brain damage. The monitoring of delta waves during the 24-72 h period that follows soman exposure may potentially be a useful tool to follow "online" the progression of neuropathology and to control the neuroprotective action of medication. Since the method is non-invasive in man and the animal results have partly been found in primates, the utility of spectral analysis as a prognostic means in human OP poisoning ought to be considered (Carpentier et al., 2001a).

Development of nerve agent-induced neuropathology without concomitant toxic signs has been demonstrated in several animal studies (Hymowitz et al., 1990; Kadar et al., 1992; Myhrer et al., 2015). It is suggested that local, silent epileptiform activity may be the

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underlying mechanism (Section 4). Similar findings are also made in human epilepsy (McIntyre and Gilby, 2008). After successful medical termination of nerve agent-triggered convulsions in humans, further treatment with neuroprotectants might be necessary depending on level of poisoning. Extracranial EEG is likely not able to unveil local, silent seizure activity. A potential option can be use of imaging techniques in scanning potential local increase of neuronal activity.

Soman-triggered seizure activity lasting for only 45 min is sufficient to produce marked neuropathology and severe cognitive deficits in rats (Myhrer et al., 2005). If humans exposed to nerve agent are inflicted with neuropathology corresponding to that seen in Fig. 2, the future prospects will be rather grim. For this reason, it will be very important to reduce or terminate epileptiform activity as soon as possible and to follow up with search for potential residual degenerating processes. It may come as an unexpected finding that the most frequently used nerve agent, sarin, has the highest potency among the classical nerve agents to make damage upon the CNS in animals as shown in a study by Myhrer and coworkers (2015).

8. Concluding comments

The introduction of a novel strategy for establishing therapies against soman poisoning appears successful according to recent results presented in this review. Identification of critical pharmacological receptors in seizure controlling brain sites in rats intoxicated by soman opens a new avenue in nerve agent research. It is assumed that the ability of a systemically administered drug to yield seizure protection depends on the drug's relative impact on the defined action sites (Gale, 1988).

The 3-phase model has been suggested to serve as a guide for future development of treatment regimens for nerve agent intoxication (McDonough and Shih, 1997). However, the

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criteria derived from this model are rather simple; viz. cholinergic and glutamatergic antagonism along with GABAergic agonism. No clear-cut specifications are worked out for what subreceptors in what sites of the brain that preferentially should be affected. Without such specifications further pharmacological research on anticonvulsants against nerve agents may take the form of a seemingly endless series of trial and error. The vast number of candidate compounds to choose among can as an example be seen in preclinical epilepsy research. The Antiepileptic Drug Development Program funded by the National Institute of Health (USA) has screened over 31.000 compounds of which 13 are approved for use as antiepileptic drugs (Crepeau and Treiman, 2010). In nerve agent research, specified abilities of drugs with anticonvulsive and neuroprotective potency can probably radically shorten the list of candidates not yet tested with conventional pharmacological approach. In a future perspective, research identifying receptor subtypes, receptor subunit composition, or unique cellular markers may provide the possibility of specifically targeting control sites. Such an approach may also provide potential benefits to novel drug synthesis for protection against nerve agent-induced seizures and neuropathology. It will also be of urgent interest to investigate the regions identified as seizure controlling with in vitro techniques to achieve deeper insights into mechanisms of relevance for evolvement of improved therapies.

Future treatment regimens for humans should ideally fulfil the requirements for serving both anticonvulsive and neuroprotective functions. With such combined capacities, the same therapy can be used for both acute poisoning and follow-up treatment to prevent development of neuropathology. Rapid and efficacious treatment of nerve agent victims should be able to save their lives by preventing or terminating epileptiform activity, relieve sufferings from somatic symptoms, and avoid brain injuries.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure legends

Fig. 1 Lateral view of the rat brain showing simplified version of cholinergic projection systems. Neurons in the nucleus basalis magnocellularis (NBM) send axons to the entire cortical mantle and olfactory bulb (blue). Neurons in the septal area (SA) send axons to the hippocampus and cingular, piriform, and enthorhinal cortices in addition to the olfactory bulb and amygdala (red). Neurons in the dorsolateral tegmental nucleus (DTN) send axons to the medial septal and diagonal band nuclei and to the ventral respiratory group in the brainstem that also receives input from the pedunculopontine tegmental nucleus (PTN) (green). From Myhrer (2007). For more labels of brain structures see Fig. 2.

Fig. 2 Lateral view of the rat brain showing potential neuronal target areas for nerve agents. Anticonvulsant efficacy against soman poisoning was obtained by lesions in the area tempestas (AT), medial septum (MS), piriform cortex (PC), or perirhinal cortex (PRC). Lack of anticonvulsant effect followed damage to the nucleus accumbens (NA), nucleus basalis magnocellularis (NBM), hippocampal region (HCR), amygdala (AM), substantia nigra (SN) or entorhinal cortex (EC). MDT is the mediodorsal thalamus and FMC is the frontal motor cortex. The number in each structure (in red) represents the mean soman-induced neuropathology score derived from Table 1 in the study of McDonough et al (1998). The percentages presented below the figure (numbers in blue) denote the share of rats that developed convulsions upon microinfusion of soman into the structure linked with line (see text). The bold numbers (in black) beneath denote the percentages of rats with aspiration lesion in the same structure that did not respond with convulsions to

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systemic soman poisoning (complete protection). NBM corresponds to the ventral pallidum and preoptic nucleus in Table 1 of McDonough et al (1998).

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Table 1

Effects of microinfusion (1 µl) of drugs (µg unless specified) against soman-induced seizures with percentage of nonconvulsing rats in each group. Anticonvulsant efficacy is based on latency (min) to seizure onset or prevention of seizures. A criterion in min was set for nonconvulsing rats so that data from both categories of results could be combined.

Brain	Anticonvulsant effect relative to saline infusion				No effect relative to saline infusion				
region	Drug	Dose	Receptor	Percent	Drug	Dose	Receptor	Percent	Study
Area	Atropine	100	M1-M5	62	Benactyzine	0.60/2	M1-M2+NMDA	0	Myhrer et
tempestas	Scopolamine	1	M1-M5	71	Biperiden	0.11/1	M1-M2+NMDA	13	al., 2006,
	Caramiphen	10	M1	13	Trihexyphenidyl	0.12/1	M1-M4+NMDA	0	2008b
	Procyclidine	6	M1-M4	25	Ketamine	50	NMDA	0	
	Muscimol	120 ng	$GABA_A$	75	MK-801	1	NMDA	0	
	Ethanol	0.47 µmol	$GABA_A$	50	NBQX	40	AMPA+KA	0	
	Propofol	20	$GABA_A$	62	Diazepam	5	$GABA_A$	14	
					Pentobarbital	50/100	$GABA_A$	0	
Medial	Atropine	100	M1-M5	0	Ketamine	50	NMDA	0	Myhrer et
septum	Scopolamine	1	M1-M5	0	Muscimol	120 ng	$GABA_A$	0	al., 2009

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	Procyclidine	6	M1-M4	0					
Perirhinal cortex	Procyclidine	6	M1-M4+NMDA	0	Scopolamine	1	M1-M5	0	Myhrer et al., 2010a, 2010b
	NBQX	40	AMPA+KA	0	Ketamine	50	NMDA	0	
	MPEP	0.1	mGluR5	25	Caramiphen	10	M1+NMDA	0	
	DCG-IV	1	mGluR2/3	25	Muscimol	120 ng	$GABA_A$	0	
	MPEP *	0.1	mGluR5	67					Myhrer et al., 2013b
	DCG-IV *	1	mGluR2/3	83					
Posterior piriform cortex	Scopolamine	1	M1-M5	0	Procyclidine	6	M1-M4+NMDA	0	Myhrer et al., 2010a
	Muscimol	120 ng	$GABA_A$	0	Caramiphen	10	M1+NMDA	0	
					NBQX	40	AMPA+KA	0	
					Ketamine	50	NMDA	0	

* Double infusions (reaching a larger area of the perirhinal cortex than single infusion). The percentage of nonconvulsing rats was 0 in some groups with anticonvulsant effect, but the latency to seizure onset was significantly higher than for the saline group. Infusion of biperiden (N=8) or diazepam (N=7) into the area tempestas prevented convulsions in 1 rat in each group, but the total score for these groups was not significantly different from the saline-treated group. From Myhrer and Aas (2014).

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Table 2

Agents with neuroprotective properties and putative mechanisms

Neuroprotective agents	Mode of action	Study
Alpha-linolic acid (omega-3 fatty acid)	Increased brain derived neurotrophic factor Increased neurogenesis in hippocampus	Piermartiri et al. (2015b) Piermartiri et al. (2015a)
Brain derived neurotrophic factor (BDNF)	Reduced release of glutamate	Piermartiri et al. (2015b)
Poly-YE immunomodulator	Increased BDNF Reduced microglia activation	Finkelstein et al. (2012)
Neuropeptide *N*-acetyl-aspartyl-glutamate	Inhibited GCP enzyme protection of nerve cells	Guo et al. (2015)
Cytokines	Increased neurogenesis in fascia dentate and subventricular zone	Collombet (2011)
Ketamine	NMDA antagonism	Dhote et al. (2012)
Procyclidine	NMDA antagonism	Myhrer et al. (2015)
Levetiracetam	Reduced release of glutamate GABAmimetic	Myhrer et al. (2015)
LY293558	AMPA antagonism	Apland et al. (2013)
Caramiphen	NMDA antagonism	Figueiredo et al. (2011a)

Table 3

A. Median (range) neuropathology scores of rats that lived for 7 days after exposure to a soman dose of 1.3 x LD_{50}. The rats were treated with 2 medical therapies 20 min before challenge with soman.

Group	Dose mg/kg	N	Neuropathology score		
			Piriform cortex	Hippocampal CA1	Amygdala
HI-6	125				
Scopolamine	1	6	2.8 (1 – 3)	0.0 (0 – 0)	1.0 (0 – 3)
Physostigmine	0.1				
HI-6	125				
Procyclidine	20	6	0.0** (0 – 1)	0.0 (0 – 0)	0.0* (0 – 0.5)
Levetiracetam	50				

Significantly different from the HI-6, scopolamine, physostigmine group: ***P*=0.0022, **P*=0.026. Study: Myhrer et al. (2013c).

B. Median (range) neuropathology scores of rats that lived for 2-7 days after exposure to a soman dose of 1.3 x LD_{50}. The rats were treated with 4 medical therapies 20 minutes before challenge with soman.

Group	Dose mg/kg	N	Neuropathology score			
			Piriform cortex	Hippocampal CA1	Amygdala	Total
HI-6	125					
Procyclidine	20	2/6	1.0 (0-2)	0.5 (0-1)	0.8 (0-1.5)	2.3 (1.5-3)
MPEP	30					
HI-6	125					
Procyclidine	20	5/6	1.0 (0-4)	0.0 (0-1)	1.5 (0-3)	3.0 (0.5-7)
DCG-IV	4					
HI-6	125					
Procyclidine	20	6/6	0.0 (0-1)	0.0 (0-0)	0.0 (0-0.5)	0.0[a] (0-1)
Levetiracetam	50					
HI-6	125					
Procyclidine	6	5/6	1.5 (0-4)	0.5 (0-3.5)	1.0 (0-2)	3.0 (0.5-7)
Levetiracetam	50					

[a] Significantly different from the HI-6, procyclidine, DCG-IV and HI-6, procyclidine (6 mg/kg), levetiracetam groups with one-way Kruskal-Wallis ANOVA and Dunn's multiple comparison test ($P<0.05$). N ratio indicates that some rats died before 7 days. Study: Myhrer et al. (2013d).

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Abbreviations

ACh – Acetylcholine

A-P – Anterior-posterior

AChE – Acetylcholinesterase

AD – Alzheimer's disease

Akt – Serine/threonine kinase

ALA – Aplpha linoleic acid

AM – Amygdala

AMPA – α-Amino- 3-hydroxy-5-methyl-4-isoxazolepropionic acid

AP-7 - 2-amino-7-phosphonoheptanoic acid

AS - Atropine sulfate

AT – Area tempestas

BBB – Blood brain barrier

BDNF – Brain-derived neurotropic factor

ChE - Cholinesterase

CNS – Central nervous system

CRF – Continuous reinforcement

DCG-IV - (1R, 2R)-3-[(1S)-1-amino-2-hydroxy-2-oxoethyl]cyclopropane-1,2-dicarboxylic acid

DG – Deoxyglucose

DRL – Differential reinforcement of low rate

DZ - Diazepam

EC – Entorhinal cortex

EEG – Electroencephalography

GABA – Gamma-aminobutyric acid

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GCP – Glutamate carboxypeptidase

GluK1 – Glutamate Kainate 1 receptor with the K1 subunit

HCR – Hippocampal region

HI-6 – 1-(2- hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxapropane dichloride

HWL - High weight loss

LD – Lethal dose

LIN – Linoleic acid

LTP – Long term potentiation

LWL – Low weight loss

LY293558 - AMPA/GluK1 receptor antagonist

MDT – Mediodorsal thalamus

mGluRs – Metabotropic glutamate receptors

MK – 801 - (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine

MPEP - 2-methyl-6-(phenylethynyl)pyridine hydrochloride

MS – Medial septum

mTORC1 – Mammalian target of rapamycin complex 1

mTOR - Mammalian target of rapamycin

NA – Nucleus accumbens

NAAG – N-acetyl-aspartyl-glutamate

NBM – Nucleus basalis magnocellularis

NBQX - 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline -7-sulfonamide

NMDA – N-metyl-D-aspartate

OP – Organophosphorus

PAM – Pralidoxime

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PC – Piriform cortex

PgP – P-glycoprotein

PMPA – Phosphonometyl pentanedioic acid

PRC – Perirhinal cortex

RT-PCR - Real time polymerase chain reaction

SN – Substantia nigra

TrkB – Tyrosine kinase receptor B

VR - N,N-diethyl-2-(methyl-(2-methylpropoxy)phosphoryl)sulfanylethanamine

VX - Ethyl ({2-[bis(propan-2-yl)amino]ethyl}sulfanyl)(methyl)phosphinate

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