

Neuroprotection for Nerve Agent-Induced Brain Damage by Blocking Delayed Calcium Overload: A Review

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ABSTRACT

Nerve agent-induced seizures often lead to irreversible brain damage. While seizures can be controlled if treated soon after onset, medical care for some battlefield casualties may be delayed beyond the therapeutic window of opportunity, i.e., after secondary pathways of neuronal injury have been activated and anticonvulsants can no longer stop seizure activity. Therefore, there is a need for adjunct drugs that can be administered one or more hours after nerve agent exposure to prevent or alleviate the seizure-related brain damage.

INTRODUCTION

Organophosphorus nerve agents are the principal chemical warfare agents known to produce brain injury. They exert their biological effects by inhibiting the enzyme acetylcholinesterase (AChE), thereby blocking hydrolysis of the neurotransmitter acetylcholine (ACh) resulting in greatly increased post synaptic ACh levels. This causes a spectrum of effects, including miosis, excess secretions, nausea, vomiting, and muscle fasciculations. At moderate to high doses, nerve agents also cause seizures and associated convulsions. If left untreated, seizures rapidly progress to *status epilepticus* (SE) and cause irreversible seizure-related brain damage (SRBD) (Solberg, 1997; Shih, 2003). U.S. military forces have adopted a regimen of pretreatment with the carbamate pyridostigmine bromide (PB) and post exposure treatment with atropine sulfate (AS) and the oxime pyridine-2-aldoxime methylchloride (2-PAM); this regimen greatly reduces morbidity and mortality. These drugs do not prevent nerve agent-induced seizures in all casualties, and the regimen now includes diazepam to terminate seizures and prevent SRBD (Shih, 2003).

Even with diazepam, the treatment regimen has limitations. The decision to include diazepam was based on animal data showing that it could terminate nerve agent-

induced seizures/convulsions and enhance survival when given in conjunction with carbamate pretreatment (e.g., PB), atropine (i.e., AS) and oxime (e.g., 2-PAM, HI-6) therapy (Lipp, 1972; Lipp, 1973; McDonough, 1989; McDonough, 1993; Shih, 1990). However, diazepam is unable to afford absolute protection against SRBD. Although neuropathology is reduced in diazepam-treated animals, the incidence and degree of protection afforded by diazepam is not complete (Clement, 1993; Hayward, 1990; McDonough, 1989; McDonough, 1995; Baze, 1993).

Battlefield nerve agent exposure levels are likely to be sufficient to induce seizures in troops that are not in full protective ensemble at the time of the attack (Fanzone, 2002). In such cases, the therapeutic window for arresting seizures with anticonvulsants and for preventing or reducing neuropathology is less than one hour following onset. By this time, seizures have progressed to SE and are refractory to anticonvulsant therapy; in addition, secondary pathways of neuronal injury have been activated (Lipp, 1972, 1973; Shih, 1990; Shih, 1991; Capacio, 1991; Philippens, 1992; Sparenborg, 1993; McDonough, 1993; Harris, 1994; Shih, 1999; Lallement, 2000; McDonough, 2000). The current regimen is thus unlikely to be effective in preventing SRBD if treatment is delayed for more than 1 hr following exposure. Prompt treatment of battlefield nerve agent casualties can be expected to be problematic. Confusion and high mobility of a battlefield scenario may cause delays in locating and evacuating casualties. Some casualties may not be received at the battalion aid station for one or more hours after nerve agent exposure. Thus, it is likely that the administration of anticonvulsants to some victims undergoing seizures may be delayed beyond the therapeutic window of opportunity to terminate seizures and prevent irreversible brain damage. It also is possible that some victims may undergo silent seizures, i.e. without convulsions (DeLorenzo, 1998). For these victims, treatment would almost certainly be delayed beyond the therapeutic window. If treatment is delayed, those soldiers who experience seizures and survive are more likely to develop irreversible brain damage and less likely to return to duty. In light of the above considerations, there is a clear need for drugs that will prevent or minimize brain damage when administered one or more hours after nerve agent exposure.

NEUROLOGICAL MECHANISMS OF INJURY

Nerve agent-induced SRBD is the result of a complex, multi-phasic response of individual neurons to numerous extra- and intracellular events. Following inhibition of AChE and accumulation of ACh at cholinergic synapses, seizures are triggered by hyperstimulation of cholinergic receptors on postsynaptic membranes (Lallement, 1992; McDonough, 1993; Tonduli, 1999). Subsequently, there is recruitment of, and excessive stimulation by, the glutamatergic neurotransmitter system. Glutamate is the major excitatory neurotransmitter in the brain and is responsible for sustaining soman-induced seizures and promoting the development of SE (Olney, 1983; Wade, 1987; Braitman, 1989; Sparenborg, 1992; Fosbraey, 1990; Solberg, 1997). Excessive stimulation of glutamate ionotropic receptors (see below) causes large pathological elevations in the concentrations of intracellular sodium and especially calcium, and prolonged depolarization of postsynaptic membranes. This initiates a deleterious cascade of

pathological processes, most of which center around a prolonged increase in intracellular free calcium or delayed calcium overload, and leads to excitotoxic cell death (Olney, 1983; Choi, 1988; Shih, 1993; Solberg, 1997). **[Figure 1]** provides a simplified overview of this cascade.

A transient elevation in intracellular free calcium is a ubiquitous signalling mechanism and regulator of intracellular processes ranging from cell growth and metabolism to cell death (Verkhatsky, 2003; Parekh, 2003; Carafoli, 2002). Increased cytosolic free calcium is also a critical neuronal mediator of learning and memory (Bliss, 1993). However, when normal homeostatic control of intracellular calcium is lost and a sustained elevation occurs, this delayed calcium overload triggers neuronal pathology by necrosis or apoptosis (a form of programmed cell death) (Randal, 1992; Orrenius, 1994; Orrenius, 1992; Nicotera, 2003; Nicholls, 2004). In neurons, the majority of calcium influx occurs through N-methyl-D-aspartate (NMDA) ionotropic glutamate receptors as well as through voltage-gated calcium channels (e.g., L-type). Calcium influx also occurs through alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid/kainate (AMPA/KA) receptors, another class of glutamate ionotropic receptors, but to a much lesser extent (for review, see Jayakar, 2004). Excessive stimulation of NMDA receptors is the first step in glutamate excitotoxicity (Olney, 1983; Choi, 1988). Release of intracellular stores is also responsible for increased cytosolic free calcium. The endoplasmic reticulum (ER) releases calcium following binding of the second messenger, inositol triphosphate (IP₃), to its ionotropic receptors. Calcium is also released from the ER *via* ryanodine receptors, i.e., ion-gated calcium channels that are responsive to calcium itself (Verkhatsky, 2003). The ER plays a critical role in normal calcium homeostasis. Excessive release or impaired uptake of calcium has been implicated in pathology resulting from calcium overload (Randal, 1992; Verkhatsky, 2003). Brain mitochondria are important for calcium buffering as cytosolic concentrations rise, and their ability to sequester calcium is ATP-dependent (Kulak, 2004). However, when calcium overload occurs, mitochondria undergo a permeability transition characterized by loss of mitochondrial transmembrane potential, curtailment of ATP synthesis, mitochondrial swelling, release of stored calcium, and neuronal death by necrosis (Duchen, 2000; Halestrap, 2002; Chang, 2002; Mattson, 2003).

It is widely acknowledged that the majority of soman-induced SRBD results from glutamate excitotoxicity and the delayed calcium overload that ensues (Olney, 1983; Braitman, 1989; Sparenborg, 1992; Solberg, 1997). When delayed calcium overload occurs in neurons, a pathological sequence is initiated that is characterized by activation of several potentially damaging enzymes. These include oxygenases, phospholipases, and nitric oxide synthase (NOS), which produce reactive oxygen species (ROS), i.e., superoxide radical, hydrogen peroxide, hydroxyl radical, nitric oxide, and peroxynitrite. ROS-induced neuronal injury includes direct damage to cell membranes, DNA and intracellular proteins, and it induces mitochondrial release of cytochrome c, and caspase activation (for review see Mattson, 2003). Release of cytochrome c, caspase activation, and DNA fragmentation are molecular hallmarks of apoptosis (Hou, 2002; Mattson, 2003; Nicholls, 2004). Cysteine proteases called calpains are also activated by sustained elevations in intracellular free calcium. Calpains degrade various intracellular proteins,

including those of the cytoskeleton, membrane channels, and metabolic enzymes, and cause neuronal death by necrosis (Hou, 2002; Mattson, 2003; Nicholls, 2004). It should be noted that necrosis produces localized inflammation, while apoptosis is not associated with inflammation. The culmination of these events may result in cell death hours or days after the initial insult (see for example, Orrenius, 1992; Orrenius, 1994; Nicotera, 2003).

CANDIDATE NEUROPROTECTANT COMPOUNDS

Much of our research on neuroprotective agents derives from the consensus that nerve agent-induced seizures lead to the development of glutamate-mediated excitotoxicity (Olney, 1983; Choi, 1987; Braitman, 1989; Lallement, 1991; Sparenborg, 1992; Solberg, 1997; Shih, 1997; McDonough, 1997) in which delayed calcium overload is the intracellular trigger of the final sequences leading to cell death (Olney, 1983; Choi, 1987; Randal, 1992; Verkhratsky, 2003; Nicholls, 2004). Classes of drugs that have been tested for their abilities to ameliorate nerve agent-induced SRBD by specifically mitigating delayed calcium overload [Table 1] include NMDA receptor antagonists, glycosphingolipids which reduce intracellular calcium by blocking the translocation of protein kinase C (PKC) (Manev, 1993; Tubaro, 1993; Otani, 2002; Monnet, 2003; Chaban, 2004); ryanodine receptor antagonists at the ER (Niebauer, 1999; Ballough, 2003; Krause, 2004); and poly(ADP-ribose) polymerase (PARP) inhibitors, that indirectly lower intracellular calcium by preventing ATP depletion. Increased ATP availability facilitates calcium efflux by the plasma membrane Ca²⁺ ATPase, calcium sequestration by the mitochondria, and indirectly enhances Na⁺/Ca²⁺ exchange by maintaining Na⁺/K⁺ ATPase functionality (Kulak, 2004).

There is an obvious need for a neuroprotectant drug that is capable of blocking delayed calcium overload resulting from nerve agent-induced seizures and is effective when administered 1-4 hours after exposure. Several candidate neuroprotectants have shown promise in animal models. When given in conjunction with PB, atropine methylnitrate (AMN) and 2-PAM, the non-competitive NMDA receptor antagonist dizocilpine (MK-801) was reported to reduce nerve agent-induced SRBD in the piriform cortex, amygdala, hippocampus, and thalamus (Sparenborg, 1992). These are among the most severely damaged brain regions in SRBD resulting from soman (Petras, 1981; Lemercier, 1983; McLeod, 1984; Pazdernik, 1985; Carpentier, 1990; Petras, 1994; Ballough, 1995; Ballough, 1998). In the Sparenborg study, MK-801 (0.5, 1.0 or 5 mg/kg, i.p.) reduced brain damage and diminished or arrested seizures, in guinea pigs, when administered as a pretreatment 30 min prior to soman, and the effects were dose-dependent. The anticonvulsant profile of MK-801 against soman-induced seizures was definitively characterized by Shih (Shih, 1990). He showed that the anticonvulsant effect of MK-801 is four times greater than that of diazepam, but at doses of 1 mg/kg or higher, MK-801 potentiated the lethal effects of soman. Concern was raised for the use of NMDA antagonists as treatments to counter neuropathology resulting from glutamate excitotoxicity when it was reported that MK-801 induces neuronal degeneration in the posterior cingulate, retrosplenial cortices, and other corticolimbic regions (Olney, 1989; Fix, 1993). The proposed mechanism by which this occurs is disinhibition of multiple converging excitatory pathways (Corso, 1997). Specifically, excessive blockage of glutamatergic pathways leads to excessive stimulation of cholinergic function (Olney,

1991). This is supported by the findings that neurotoxicity by MK-801 is augmented when cholinergic receptors (i.e., muscarinic) are activated (Wozniak, 1998).

Memantine is an uncompetitive NMDA receptor antagonist (Bormann, 1989) that has been tested for its anticonvulsant effects against soman-induced seizures. It has been suggested that memantine's pharmacokinetics make it a safer candidate than MK-801 (Chen, 1992). McLean (McLean, 1992) reported that memantine alone (18 mg/kg, sc) blocked the onset of soman-induced seizures and was able to terminate seizures when administered 15 minutes after soman injection. Their findings, however, are not in agreement with those of Shih (Shih, 1999), who report that memantine by itself is completely ineffective as an anticonvulsant against soman-induced seizures. The latter authors pointed to a need for electroencephalographic (EEG) monitoring when determining anticonvulsant efficacy, and suggested that the former workers may have mistaken diminished convulsive behavior as evidence of reduced seizure activity. Neither study addressed possible neuroprotective effects of memantine, i.e., reduced neuropathology independent of anticonvulsant activity. Irrespective of the above discrepancies, the neuroprotective benefit of memantine in other models of excitotoxicity is widely accepted (Parsons, 1999; Lipton, 2004). For example, in a rat model of stroke, memantine, given 2 hours after the ischemic event, reduced brain damage by approximately 50% (Chen, 1998). Memantine is well tolerated and does not produce neurotoxicity at therapeutic dosages. It was recently approved by the FDA for treatment of Alzheimer's disease.

Proof of concept that neuroprotection is possible following nerve agent-induced seizures and SE, irrespective of a drug's anticonvulsant activity, has been demonstrated using a nonpsychotropic derivative of tetrahydrocannabinol, dexanabinol (HU-211) (Filbert, 1999). HU-211 has been reported to inhibit NMDA receptors, act as an antioxidant and free radical scavenger, suppress nitrous oxide (NO) and tumor necrosis factor- α (TNF- α) generation and stabilize calcium levels (Shohami, 1993; Biegon, 1995; Lavie, 2001). HU-211 is generally well tolerated in humans (Darlington, 2003). When HU-211 (25 mg/kg, i.p.) was administered 5 minutes after onset of soman-induced seizures i.e., in conjunction with HI-6 and AMN pre- and post-treatment, respectively, temporal lobe lesion volume/necrosis (assessed at 28 hours after seizure onset) was reduced by 86%, compared with unprotected soman-positive controls [**Figure 2**]. Importantly, HU-211 had no effect on the strength or duration of seizure activity, as determined by quantitative EEG analysis. Significant neuroprotection was also observed when HU-211 administration was delayed 40 minutes after seizure onset. Neuroprotection by HU-211 was most evident in the piriform cortex and contiguous temporal lobe structures, e.g., amygdala, entorhinal, and perirhinal cortices, but did not extend to the thalamus. Co-administration of HU-211 and diazepam at 40 minutes after seizure onset did not augment the neuroprotection obtained with diazepam alone (data not shown). As regards the mechanisms of neuroprotection by these two drugs, it is important to differentiate between protection obtained by anticonvulsant effects vs. that produced by interfering with delayed calcium overload. In the above studies, HU-211 was protective despite the continued presence of undiminished seizures and SE, while diazepam attenuated (without stopping) seizure intensity and thereby reduced the initial

insult. The anticonvulsant action of diazepam, via agonistic modulation of GABA_A receptors, is well known and does not require elaboration. These mechanisms are non-overlapping and neuroprotective effects should be additive or synergistic.

Gacyclidine (GK-11) is another NMDA receptor antagonist that has shown considerable neuroprotective efficacy. When GK-11 (0.01 - 0.1 mg/kg, i.v.) was given to rats 10 minutes after soman exposure (in conjunction with PB pretreatment, and AS, 2-PAM and diazepam post-treatments, 1 minute after soman injections), it completely blocked SRBD when assessed 3 weeks after exposure (Lallement, 1997). In a more realistic battlefield scenario, GK-11 was administered 45 minutes after an 8-LD₅₀ soman exposure in nonhuman primates. Animals also received PB pretreatment, followed by AS, 2-PAM, and diazepam post-treatments (one minute after soman exposure) equivalent to a single autoinjector of each in man. When brain pathology was assessed 3 weeks after exposure, all three GK-11-treated primates showed little or no evidence of pathology in the frontal and entorhinal cortices, amygdala, caudate nucleus, hippocampus, thalamus, midbrain, pons, medulla, and cerebellum, compared with the only surviving soman-treated animal (1 of 3) that received AS, 2-PAM and diazepam but not GK-11 (Lallement *et al.*, 1998). In a study that approximates casualty management following a terrorist attack, soman-intoxicated primates (2.0 LD₅₀) did not receive PB pretreatment and received delayed AS, 2-PAM, and diazepam treatments (one man-equivalent of each, see above) 30 minutes post-exposure; this was followed by GK-11 (0.1 mg/kg, i.v.). In this study, the addition of GK-11 restored normal EEG activity and completely prevented neuropathology (assessed 5 weeks after exposure), compared with subjects that received AS/2-PAM/diazepam alone (Lallement, 1999). GK-11 has a binding affinity for NMDA receptors that is only one-tenth that of MK-801. In addition, it binds to non-NMDA receptors when interaction with NMDA receptors is prevented. For these reasons, it is considered substantially less neurotoxic than MK-801 (Hirbec, 2001). It is currently being evaluated in human clinical trials for a different neuroprotective indication (Hirbec, 2001; Lepeintre, 2004).

Perhaps the most promising neuroprotectant candidate to-date is ketamine. Ketamine is an FDA approved anesthetic that blocks neurotransmissions without depressing respiratory and circulatory functions. Its actions are mediated by low-affinity binding to NMDA receptor channels and prevention of calcium influx (for reviews, see MacDonald and Nowak, 1990; Werner *et al.*, 1997; Mion, 2003; Ivani, 2003). Ketamine is garnering considerable attention as a putative neuroprotectant against ischemic brain injury, damage resulting from seizures and SE, irrespective of etiology, and SRBD specifically resulting from nerve agent-induced seizures (Fujikawa, 1995; Werner, 1997; Mion, 2003; Van Rijckevorsel, 2005; Dorandeu, 2005). Fujikawa (1995) reported remarkable neuroprotection in 21 of 24 brain regions, in rats, when 100 mg/kg ketamine was administered (i.p.) 15 min after lithium-pilocarpine-induced SE onset. Similarly, 100 mg/kg ketamine (i.p.) prevented learning impairment in rats when administered immediately after lithium-pilocarpine-induced SE (Stewart and Persinger, 2001). Borris (2000) report that ketamine (58 mg/kg ED₅₀) can control prolonged SE, in rats, when administered 1 hr after onset. Cumulative evidence regarding the beneficial effects of ketamine following SE onset has led to its recommended use, in humans, when SE can

not be alleviated by conventional anticonvulsant therapy (Van Rijckevorsel, 2005). Based on its neuroprotective and anticonvulsant properties, Mion (2003) recommend ketamine as the anesthetic of choice for victims of nerve-agent exposure. Most recently, Dorandeu *et al.* (Dorandeu, 2005) reported that ketamine proved effective in stopping seizures, highly reducing SRBD, and improving guinea pig survival, when administered between 30 min and 2 hr following soman poisoning. Increasing dosages of ketamine (i.e., 10-60 mg/kg, i.m.) were required as post SE onset delay increased, and ketamine was always co-administered with atropine sulfate (2-10 mg/kg); in addition, guinea pigs also received pyridostigmine (26 mg/kg, i.m.) 30 prior to soman and atropine methyl nitrate (AMN, 4 mg/kg, i.m.) within 1 min following soman injection. Their study also provided compelling evidence of neuroprotection by ketamine at dosages that did not modify seizures (i.e., 2-10 mg/kg). These authors also suggested combining ketamine and benzodiazepine treatments when treatment is delayed 2 hr. Preliminary results by our laboratory corroborate the latter report of neuroprotection by ketamine following soman-induced SE. We observed greatly augmented neuroprotection by ketamine plus diazepam co-administration compared to diazepam alone. When soman-exposed (1.6 LD₅₀) rats were co-administered 20 mg/kg diazepam (i.m.) and 25 mg/kg ketamine (i.p.), 40 min post-seizure onset, mean cross-sectional area of temporal lobe necrosis was reduced $85.3 \pm 8.0\%$ %, compared soman-positive controls; diazepam alone reduced temporal lobe necrosis by only $40.0 \pm 20\%$. In these same animals, neuronal morphological assessments (via H&E) also indicated profound neuroprotection, in the piriform cortices of rats receiving combined diazepam and ketamine, compared to diazepam alone. Since 25 mg/kg ketamine is within the anesthetic range, it is likely that reduced seizure intensities contributed to the neuroprotection we observed. Taken together, the preponderance of evidence indicates that ketamine is a viable neuroprotectant candidate against nerve agent-induced SRBD.

In an effort to circumvent neurotoxicity associated with NMDA receptor antagonism and mitigate delayed calcium overload that has already become established, drugs that target events subsequent to calcium overload have been tested against soman-induced SRBD. Intracerebroventricular (i.c.v.) infusion in rats of GM1 monosialoganglioside (5 mg/kg/day, for 5 days prior to and 27 hours after soman exposure), in rats, markedly reduced cross-sectional areas of soman-induced temporal lobe necrosis, i.e., 85.9% lesion reduction in the piriform cortex and contiguous structures, compared with unprotected soman-positive controls (Ballough, 1998). In this study, all rats were pretreated with PB before soman exposure, and AMN and 2-PAM post-treatments. Considerable neuroprotection was also obtained with the water-soluble GM1 derivative WILD20. As an adjunct to HI-6 pretreatment and AMN post-treatment, WILD20 (2.5 mg/kg, ip) reduced volumetric temporal lobe necrosis by 75.2% (data not shown). Neuroprotection by these two compounds occurs although neither seizure intensity nor duration (assessed via EEG monitoring) was diminished.

The mechanism by which GM1 and WILD20 exert their neuroprotective effects involves inhibition of PKC translocation to the plasma membrane (Vacarino, 1987; Manev, 1993; Costa, 1994; Tubaro, 1993; Otani, 2002; Monnet, 2003; Chaban, 2004). It

has been shown that PKC activation and translocation enhance glutamate excitotoxicity (Wagey, 2001; Koponen, 2003). Furthermore, it has been reported that PKC's role in the excitotoxic process is to prolong NMDA receptor activation and possibly inhibit calcium extrusion mechanisms (Manev, 1993; Costa, 1994; Zhang, 1996). In addition, WILD20 is reported to reduce inflammatory response by its inhibitory effects on specific leukocytes, i.e., neutrophils (Tubaro, 1994). Despite our promising results with GM1 and WILD20, further studies have been discontinued due to concerns of possible contamination by prions associated with bovine spongiform encephalopathy, i.e., mad cow disease (Mattei, 2002).

Recent studies indicate that PARP inhibition is neuroprotective following neuropathological insults involving excitotoxicity, e.g., cerebral ischemia and traumatic brain injury (Eliasson, 1997; Mandir, 2000; Whalen, 2000; Abdelkarim, 2001; Kamanaka, 2004; Sharma, 2004; Nakajima, 2005; Ying, 2005). PARP is an abundant nuclear enzyme that is activated by ROS-induced DNA strand breaks (reviewed in Szabo, 1998; Ying, 2005). With moderate insults, it facilitates DNA repair by utilizing cellular nicotinamide adenine dinucleotide (NAD⁺) to form poly (ADP-ribose). Excessive PARP activation leads to NAD⁺ depletion, metabolic inhibition via glycolysis block, ATP insufficiency, and cell death by necrosis (Szabo, 1998; Abdelkarim, 2001; Virag, 2002). Neurons are especially vulnerable to metabolic insufficiency resulting from PARP over-activation, since glucose is normally the only metabolic substrate and the dependency on glycolysis is exceptionally high (Ying, 2005). In excitotoxic models, over-activation of PARP is closely linked to calcium-induced NOS activation which leads to the production of NO; the detrimental effects of NO are mostly mediated through peroxynitrite that forms when NO reacts with superoxide (Szabo, 1998; Park, 2004; Wang, 2004).

In 1999, Meier, reported reduced lesion volumes and increased survival in soman-exposed rats that received the PARP inhibitor benzamide (Meier, 1999). Further investigation of the neuroprotective efficacy of PARP inhibition warrants consideration. Subsequent studies might do well to include several new-generation PARP inhibitors that have shown increased efficacy, e.g., ONO-1924H, DR2313 and FR247304 (Kamanaka, 2004; Iwashita, 2004; Nakajima, 2005).

Another drug that has shown neuroprotective efficacy against soman-induced SRBD is dantrolene (Ballough, 2003). Dantrolene is a ryanodine receptor antagonist that prevents the release of calcium from the ER and is FDA-approved for use in malignant hyperthermia. While some neuroprotection is produced by diazepam alone (20 mg/kg, i.m., 40 min after seizure onset), this protection is significantly augmented in the dorsal and lateral cortices of rats by co-administration of 10 mg/kg, i.v., dantrolene (Ballough, 2003). Insolubility problems associated with dantrolene makes administration of a full dosage in a single injection difficult. To overcome solubility problems and achieve the desired 10 mg/kg dantrolene dosage, 4 separate i.v. injections were performed between 40 minutes and 8 hours after seizure onset, with a total injection volume approximating 1 mL per rat. A unique formulation of dantrolene (Lyotropic Therapeutics, Inc.) as a nano-crystal dispersion has been utilized to obviate solubility problems. With this formulation, it is possible to administer a much higher dosage of dantrolene in a much

lower injection volume. This is critical in as much as the concentration of dantrolene reaching the brain is lowered by liver enzymes when dantrolene is administered by i.p. injection. The nano-crystal formulation of dantrolene minimizes the effects of the liver enzymes.

The results with the nano crystal formulation of dantrolene corroborated and extended those of the previous study. Where the former study was unable to demonstrate significant protection in the piriform cortex.i.e., the most severely damaged region, 40 mg/kg, i.p. dantrolene (nano-crystal dispersion) plus diazepam (20 mg/kg, i.m.) reduced piriform cortical necrosis by an additional 15.6% more than that seen with diazepam alone (unpublished). In these experiments, all soman-exposed rats also received HI-6 (125 mg/kg, i.p., 30 min prior to soman) and AMN (2 mg/kg, i.m., < 1 min following soman) to protect against the peripheral effects of soman and ensure survival. Neuroprotection by dantrolene in the above experiments occurred without changes in seizure intensity or duration, i.e., dantrolene produced no discernable effects on the electrocorticographic (ECoG) profiles of soman-exposed rats. These findings are consistent with those of Niebauer and Gruenthal (Niebauer,1999) who examined the effects of dantrolene on hippocampal neuronal damage associated with 140 minutes of limbic SE in the rat. Administration of dantrolene within 15 minutes after onset of SE is associated with a significant reduction in the amount of neuronal injury in all hippocampal subregions. When dantrolene administration was delayed until 140 minutes after onset of SE, protection was seen in the CA3 pyramidal cell subregion only (Niebauer, 1999).

CONCLUSION

There is a need for adjunct therapy that is safe to use on victims in a far-forward situation and those for whom the therapeutic window of opportunity to arrest seizures has passed (i.e., > 40 minutes following nerve agent exposure). Ideally, therapy will be FDA approved and should improve neuronal survival and neurologic functioning, when administered with standard treatments, and should have demonstrated efficacy when given one or more hours after nerve agent exposure. Because of the many pathways leading to irreversible neuronal cell death in nerve agent-induced excitotoxicity, a "cocktail" mixture may be necessary. The present report identifies several neuroprotective strategies with proven efficacy in the alleviation of brain damage resulting from soman-induced seizures and SE. The mechanisms of action converge on the central theme of blocking the excitotoxic cascade and resultant delayed calcium overload. Of the neuroprotectant drugs discussed, three have received FDA approval for other indications, i.e., ketamine, memantine, and dantrolene, while several others are in clinical trials.

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Tables.

Table 1.
Potential Neuroprotective Compounds

Drug Class	Examples	Reference
NMDA antagonists	MK-801	Shih, 1990; Sparenborg, 1992.
	Memantine	McLean, 1992; Koplovitz, 1997; Parsons, 1999; Shih, 1999; Lipton, 2004.
	Dexanabinol (HU-211)	Biegon, 1995; Filbert, 1999.
	GK-11 Ketamine	Lallement, 1997; Lallement, 1999; Lepeintre, 2004.
PKC inhibitors	GM1 ganglioside	Manev, 1990; Ballough, 1998.
	Wild 20	Tubaro, 1993; present report.

PARP Inhibitors	Benzamide	Meier, 1999; Kamanaka, 2004; Nakajima, 2005.
Ryanodine receptor antagonists	Dantrolene	Niebauer, 1999; Ballough, 2003.

Notes for Table 1. None of the drugs in Table 1 block L-type calcium channels, and although some degree of neuroprotection by blocking L-type calcium channels could be expected, no studies have addressed this question.

Figures.
Figure 1.

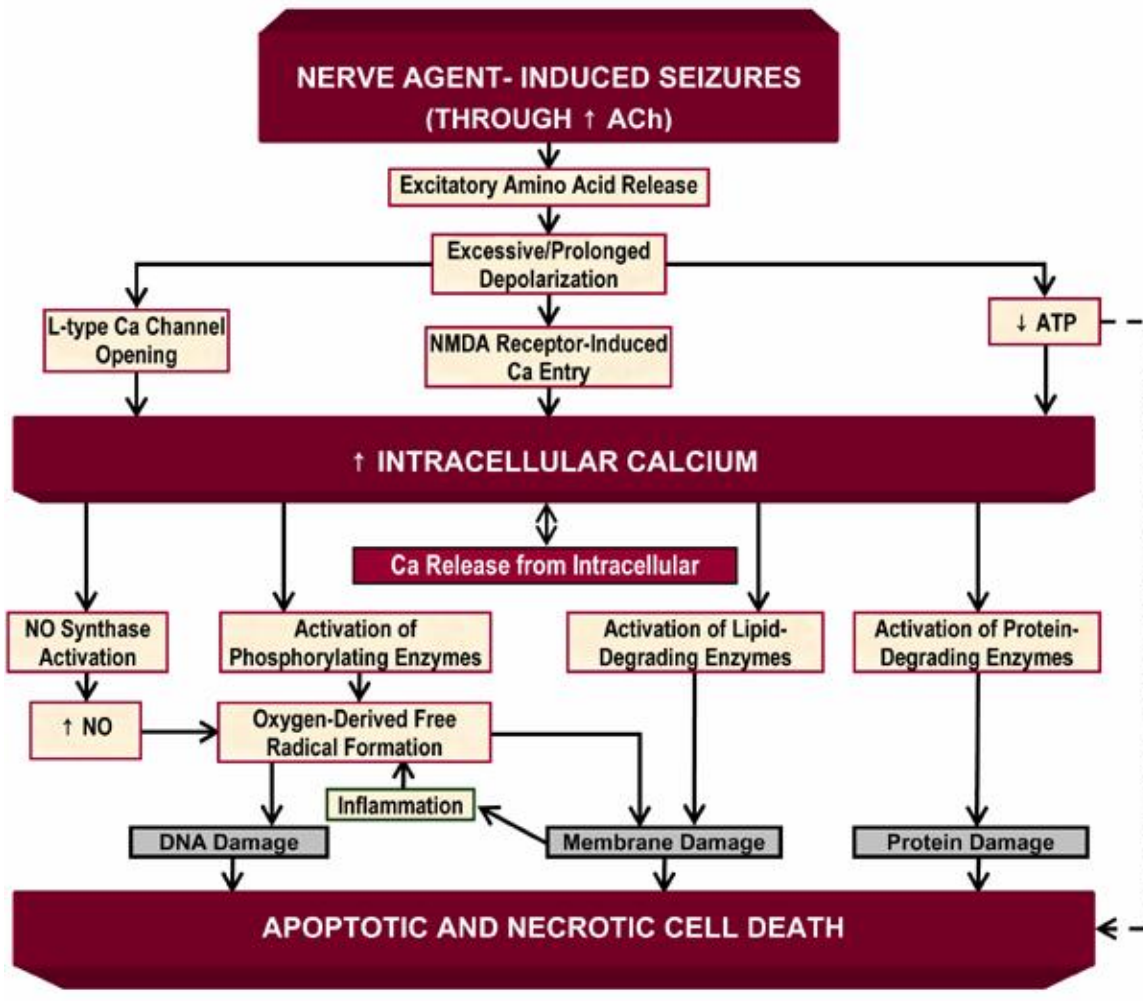
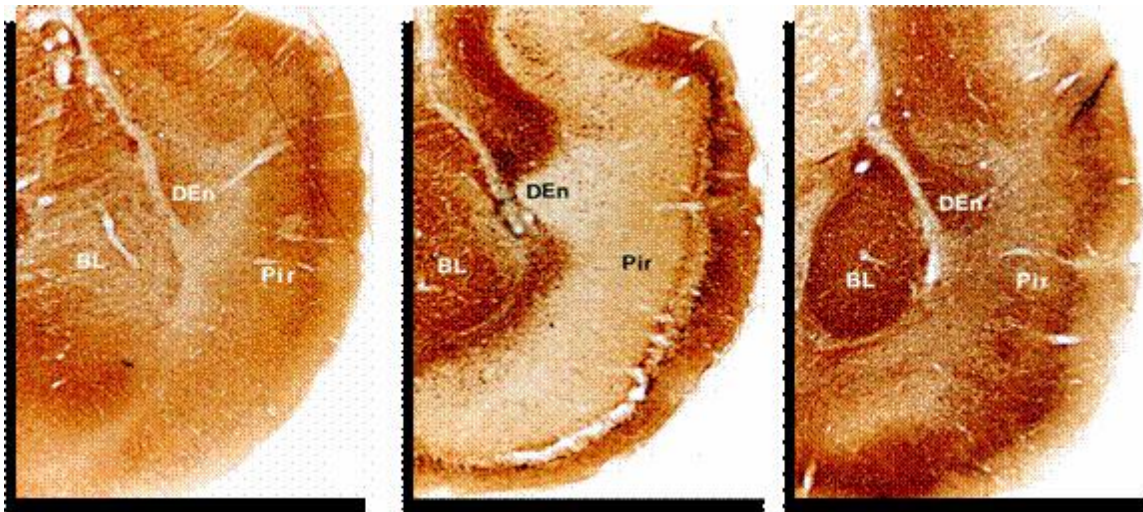


Figure 1. Proposed pathway of neuronal injury in nerve agent-induced brain damage. Various sites in this pathway have been targeted. MK-801, memantine, HU-211, GK-11 and ketamine are NMDA receptor antagonists. HU-211 also blocks free radical induced injury. GM1 ganglioside and Wild20 indirectly promote calcium extrusion by blocking PKC translocation (not indicated). Benzamide inhibits PAPR thereby increasing ATP availability. Dantrolene blocks calcium release from intracellular stores.

Figure 2.



Control

Soman

Soman + HU-211

Figure 2. HU-211 protects against soman-induced neurological damage. Microtubule-associated protein 2 (MAP2) staining is neuron-specific. MAP2-negative immunostaining indicates necrosis, except in areas of white matter. Den, dentate gyrus; Pir, piriform cortex; BL, basolateral amygdaloid nuclear group.