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Neuroprotection against diisopropylfluorophosphate in acute hippocampal slices

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Abstract

Diisopropylfluorophosphate (DFP) is an irreversible inhibitor of acetylcholine esterase (AChE) and a surrogate of the organophosphorus (OP) nerve agent sarin. The neurotoxicity of DFP was assessed as a reduction of population spike (PS) area elicited by synaptic stimulation in acute hippocampal slices. Two classical antidotes, atropine, and pralidoxime, and two novel antidotes, 4R-cembranotriene-diol (4R) and a caspase 9 inhibitor, were tested. Atropine, pralidoxime, and 4R significantly protected when applied 30 min after DFP. The caspase inhibitor was neuroprotective when applied 5–10 min before or after DFP, suggesting that early synaptic apoptosis is responsible for the loss of PSs. It is likely that apoptosis starts at the synapses and, if antidotes are not applied, descends to the cell bodies, causing death. The acute slice is a reliable tool for mechanistic studies, and the assessment of neurotoxicity and neuroprotection with PS areas is, in general, pharmacologically congruent with in vivo results and predicts the effect of drugs in vivo. 4R was first found to be neuroprotective in slices and later we demonstrated that 4R is neuroprotective in vivo. The mechanism of neurotoxicity of OPs is not well understood, and there is a need for novel antidotes that could be discovered using acute slices.

Keywords

Muscarinic overstimulation; DFP neurotoxicity; Population spikes; Hippocampal CA1 area; 4R-cembranotriene-diol; Synaptic apoptosis

Introduction

The frequency of civilian poisonings and the threat of terrorist attacks prompted the development of strategies for protection against neurotoxic compounds. Among the most likely compounds to be involved in civilian accidents or to be used by terrorists are the organophosphorus (OP) irreversible acetylcholine esterase (AChE) inhibitors. The inhibition

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of AChE causes the accumulation of acetylcholine (ACh), resulting in muscarinic and nicotinic receptors overstimulation. The peripheral muscarinic symptoms include increased mucosal secretions, bronchial constriction, and if the dose is high enough suffocation. In the brain, the accumulated ACh triggers a cascade leading to enhanced glutamate release [1], excitotoxicity, and neuronal damage or death. Current emergency therapy for acute OP poisoning consists of administration of atropine, an oxime, and a benzodiazepine. This therapy has not changed substantially in five decades, and although it is relatively effective in reducing mortality [2], survivors are often afflicted with delayed brain damage and years of neurological problems [3].

The sequence of events following AChE inhibition, which leads to glutamatergic seizures causing permanent neuropathological damage is only poorly understood, signifying the need for a more suitable model for studying these events [4]. In vitro models are often more suitable for addressing functional assays that are difficult to perform in vivo. Here we use the acute hippocampal slice to study the mechanism of diisopropylfluorophosphate (DFP) neurotoxicity and its reversal by the classical antidotes atropine and pralidoxime. Two novel antidotes, the 4R-cembratrienediol [5] and the cell-permeable caspase 9 inhibitor LEHD-CHO, were tested.

The acute hippocampal slice has been used for more than three decades to study the mechanism of neuroprotection and injury caused by oxygen and glucose deprivation, excitotoxic amino acids, and lately OPs. The slices preserve most of the pharmacological, anatomical, and other characteristics of the native hippocampus, including the plasticity to acquire long-term potentiation. Stimulation of the Schaffer collaterals and commissural afferents synaptically elicits PSs from CA1 pyramidal neurons. The PS is the sum of axon potentials of a population of neurons and is directly proportional to the number of functionally active pyramidal neurons [6]. The decrease or loss of PSs has proven to be a measure of neuronal injury, which can be prevented or reversed by various antidotes or neuroprotective compounds. The loss of PSs is an early event that precedes neuronal death. In most experimental settings, it is problematic to determine the PSs and neuronal death simultaneously. Small et al. have shown a correlation between the loss of PS and neuronal death at increasing times of oxygen and glucose deprivation [7]. The same group described that a fluorescence live/dead confocal viability assay was consistent with the PSs assessment of neuronal damage after several pharmacological neuroprotective treatments [8]. PS have been successfully used to study the mechanism of neurotoxicity and neuroprotection [7, 9-13].

Remarkably, the neuroprotective effect observed in vitro has been repeatedly confirmed in vivo proving the usefulness of this method [14-18].

Previously, we determined the neuroprotective properties of 4R against NMDA in vitro [9, 13, 19]; now we report that 4R has in vitro neuroprotective activity against DFP. These in vitro results prompted us to confirm the neuroprotective activity of 4R in vivo [14, 15].

Although DFP is not a chemical warfare nerve agent, it has the main neurotoxic properties of nerve agents. It irreversibly inhibits AChE, causing accumulation of ACh and

hyperphysiological stimulation of muscarinic receptors. DFP is a safer and less volatile surrogate of sarin, a chemical war nerve agent [20].

Materials and Methods

Common laboratory chemicals, dimethylsulfoxide, and diisopropylfluorophosphate were obtained from Sigma-Aldrich (St. Louis, MO). The cell-permeable caspase 9 inhibitor II LEHD-CHO was obtained from Calbiochem, San Diego, CA. The cembranoid (1S,2E,4R,6R,7E,11E)-cembra-2,7,11-triene-4,6-diol (4R) was prepared by K. El Sayed (School of Pharmacy, University of Louisiana, Monroe, LA).

Male Sprague Dawley rats (120–200 g) from our colony were used for the preparation of hippocampal slices.

Slice Preparation and Electrophysiological Recordings

The methods used were described [9]. For dissection and incubation, a standard artificial cerebrospinal fluid (ACSF) saturated with 95% O₂, 5% CO₂ was used, containing: 125 mM NaCl, 3.3 mM KCl, 1.25 mM NaH₂PO₄, 2 mM MgSO₄, 2 mM CaCl₂, 25 mM NaHCO₃, and 10 mM glucose. Hippocampi were dissected over ice, and 400-μm transverse slices were cut with a manual slicer and transferred to an incubation chamber. The chamber was a temperature-controlled bath surrounding an acrylic plate with three lanes. The slices were kept in the lanes at the interface between ACSF and warm and humidified 95% O₂, 5% CO₂ at 34±1 °C.

A bipolar electrode placed in the stratum radiatum was used to stimulate the Schaffer collaterals incoming fibers with a constant current for 0.2 ms. The resulting population spike (PS) was recorded in the stratum pyramidale with a glass electrode (impedance of 1–5 MΩ) filled with 2 M NaCl. Slices from the hippocampi of two rats were distributed among the three lanes and PSs were recorded from 7 slices per lane. When the slices had recovered their electrophysiological activity one hour after dissection, the minimum stimulus needed to elicit a threshold PS was determined for each slice. Each slice was then stimulated at twice the strength required to elicit the threshold PS. This initial response was recorded as PS area (ms × mV) and compared with the final response elicited by the same stimulus strength recorded from the same site after the experimental treatment was finished. Throughout this work, the standard noxious stimulus was a 10 min application of 100 μM DFP in the presence of standard O₂ and glucose. The percentage of the initial response remaining at the end of the experiment was used as a measure of electrophysiological recovery. DFP controls are slices exposed to DFP and superfused with ACSF for 90 min before recording the final PSs. The time necessary to record the PSs from 7 slices in each lane was 30–45 min.

Fig. 1 shows the standard timeline for recording the initial, the final PSs, and the application of DFP and of the antidote in all experiments unless otherwise stated.

AChE activity determination

AChE was determined using the method of Ellman [21]. Slices were treated as those used for PS recording. After 2 hours in the incubation chamber, the slices were superfused for 10

min with either ACSF or 0.5, 10, 50, or 100 μ M DFP. DFP was then washed out with ACSF for 90 min, and the slices were frozen on dry ice, and stored for AChE determination. Frozen slices were homogenized using an electronic homogenizer in cold sodium phosphate buffer (0.1 M, pH 8.0) plus 1% Triton X-100 in the proportion of 1 ml buffer/0.1 g of tissue. Homogenized samples were centrifuged at $13,400 \times g$ for 1 min and the supernatant used to perform the AChE assay. An aliquot of 10 μ l of supernatant was added to 180 μ l of 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) solution (0.3 mM DTNB, 0.6 mM NaHCO_3 , 97 μ M tetra isopropyl pyro phosphoramidate). After 5 min, 10 μ l of acetylthiocholine iodide (from a 40 mM stock solution) was added, and the absorbance was read at 405 nm with 16 kinetic cycles. AChE activity was measured in triplicate wells. The color changes were read in a VersaMax microplate reader (Molecular Devices) at 405 nm with 16 kinetic cycles using a minimal kinetic interval. Enzyme activity was normalized to protein concentration, which was determined using the Bradford reagent [22]. Data are expressed as μ mol substrate transformed/min/mg of protein using the following formula: activity (OD/min sample – (OD/min blank) * 0.2 (total volume, ml) / 0.014 (extinction coefficient) * 0.01 (sample volume, ml). The specific activity was expressed as μ M/min/mg of protein.

Data acquisition and statistical analysis

The field potentials were acquired and the PS area (msec $\times$ mV) analyzed with the Labman program (a gift from Dr. T. J. Teyler, WWAMI Medical Education Program, University of Idaho, Moscow, ID). Curve fitting and statistical analysis were done with Sigma Plot, version 12.5 (Systat Software, Inc.). One-way analysis of variance was used whenever the data were normally distributed. In some experiments, a large proportion of slices treated with DFP had zero recoveries, and the data failed the normality test. In these cases, non-parametric Kruskal-Wallis one-way analysis of variance on ranks was used. The post hoc test used is indicated for each experiment. The data is presented as mean $\pm$ S.E.M.

Results

DFP inhibited AChE and reduced the PS areas recorded from the CA1 region of the acute hippocampal slices

The primary reason for DFP neurotoxicity is inhibition of AChE [23]. To compare the effect of DFP on AChE inhibition and on the decrease of PS areas, we measured both under the same conditions, as shown in Fig. 1. The inhibition of AChE by DFP followed a logistic equation, with an IC_{50} of 0.8 μ M DFP (Fig. 2).

DFP decreased the PS areas in a dose- and time-dependent manner. Representative recordings were done before and after exposure to various concentrations of DFP are shown in Fig. 3A. The PS areas of ACSF controls displayed <10% rundown (Fig. 3A; a). The plot showing the reduction of PSs by DFP is shown in Fig. 3B, the line represents the equation: $f = \text{min} + (\text{max} - \text{min}) / (1 + (\text{DFP conc.} / \text{IC}_{50}) ^ {(-\text{Hill coefficient})})$; where min = 13 ± 12 ; max = 96 ± 14 ; IC_{50} = 39 ± 24 μ M; and Hill coefficient = -1.2 ± 0.7 . The decrease in PS area produced by DFP did not cause multiple PSs or other alterations of the waveforms. The effect of DFP was dependent on the length of exposure (Fig. 3C). When 100 μ M was

applied for 10 min, the effect of DFP on the PS areas was unchanged for at least 3 h (Fig. 3D).

4R protected the PS areas from DFP toxicity within a 30-min window of opportunity

Applying the conditions shown in Fig. 1, 4R ameliorated the neurotoxic effect of DFP. However, when applied to slices not exposed to DFP, 4R did not alter either the area or the shape of the PS (Fig. 4A, B). ACSF and DFP controls showed the expected final PS areas.

The neuroprotection by 4R was durable, and only after 3 hours of washing with ACSF was there a non-significant decrease attributable to rundown of the slices (Fig. 4C). This result together with the data in Fig. 3D proves that PS areas are a stable, predictable parameter suitable for neurotoxicity and neuroprotection studies.

Atropine and pralidoxime protected the PSs against DFP toxicity

In clinical practice, atropine and pralidoxime are routinely applied antidotes for OP poisoning. Both of these classical antidotes were tested to compare them with 4R. Atropine alone, applied 30 min after DFP, significantly protected the PSs. The simultaneous application of atropine and 4R did not significantly increase neuroprotection. In the absence of DFP, 1 μ M atropine did not alter the PSs (Fig. 5A).

The interaction between the neuroprotective effect of 4R and atropine was further studied by comparing the dose responses of 4R alone with 4R in the presence of 1 μ M atropine. Fig. 5B shows that from 0.5–10 μ M 4R there was no significant increase in neuroprotection conferred by the addition of 1 μ M atropine. However, at 0.05 μ M 4R, 1 μ M atropine increased the recovery of PS areas above the level of 4R alone (Student's t-test, $p < 0.01$) but only up to the neuroprotection by atropine alone (open triangles). We conclude that the neuroprotective effects of 4R and atropine were not additive.

Pralidoxime, a reactivator of AChE, is a classic antidote of OP inhibitors of AChE. The neuroprotection by 2–100 μ M pralidoxime did not show a clear dose dependence in the range tested, and its neuroprotective activity was equivalent to 10 μ M 4R (Fig. 6).

DFP-induced decrease in PS area is prevented by inhibition of caspase 9

We tested whether apoptosis is involved in decreasing the PS areas by DFP. For that purpose, the cell-permeable, reversible caspase 9 inhibitor II LEHD-CHO was applied for 1 hour at different intervals relative to DFP (Fig. 7).

When this inhibitor was applied for 1 hour and stopped for 5 min before application of DFP, the neuroprotection of the PSs was robust and significant (Fig. 7, $p < 0.001$). Most likely, the inhibitor penetrated the slices and was present when DFP was applied and still present when DFP was washed out with ACSF. Application of the inhibitor 5 min after DFP was significantly less neuroprotective, presumably because there was 5 min for DFP to trigger the activation of caspases. Application of the caspase inhibitor 30 min before or 30 min after DFP was ineffective in protecting the slices. These results suggest that DFP triggers apoptosis within a narrow time window by an apoptotic cascade that involves activation of caspase 9.

Discussion

The objective of this work was to explore the potential of the acute in vitro hippocampal slice to study the mechanism of neurotoxicity of nerve agents and their antidotes. In emergencies, antidotes must be applied after the poisoning; therefore, we applied the antidotes 30 min after DFP and assessed the neurotoxicity or neuroprotection 90 min after DFP. In each experiment, the field potentials of 21 slices were manually recorded, making it impossible to record all slices simultaneously. The variability of the time of recording did not introduce measurable distortion of the data, as was shown by the effect of time on the results.

Under our conditions, DFP inhibited AChE with an IC_{50} of 0.78 μ M but decreased the PS areas with an IC_{50} of 39 μ M. This divergence illustrates that the steps that link AChE inhibition and neurotoxicity are not directly related. Interestingly, the neuroprotection by 4R was dose-dependent, reaching a plateau of about 80% recovery of PS areas at 0.2 μ M 4R with an EC_{50} of 68 nM. It is reasonable to conclude that 4R applied 30 min after DFP protected the PS areas against DFP by interfering with the steps necessary for the neurotoxic effect to be manifested as decreased PS areas.

Previously, we reported that 4R protected the PSs against NMDA excitotoxicity by an anti-apoptotic nicotinic mechanism that involves activation of Akt/PKB and inactivation of GSK-3 β [19, 24]. Since at least part of the neurotoxicity of OPs is glutamatergic excitotoxicity [1, 25], it is likely that 4R protects against DFP by the same mechanism as it does against externally applied NMDA.

The supraphysiological activation of muscarinic receptors is the main link in the chain of events leading to peripheral toxicity and the neurotoxicity of DFP and other irreversible AChE inhibitors. Atropine, a competitive antagonist of muscarinic receptors, is the antidote administered to patients poisoned with OPs insecticides or nerve agents [26]. In our conditions, atropine applied 30 min after DFP was significantly neuroprotective. The neuroprotection by atropine and by 4R were not significantly additive perhaps because they act at a different step of the same chain of events leading to neurotoxicity. Atropine, per se, did not cause any untoward effects even at 50 μ M (data not shown). Atropine per se does not protect the brain against injury caused by OPs. However, it increases the neuroprotection of other antidotes [27-29]. The result that atropine alone protected against DFP was unexpected since we reported that atropine did not protect acute hippocampal slices against paraoxon [9]. In vivo atropine alone increased the toxicity of paraoxon to guinea pigs [30] paralleling our in vitro lack of efficacy of atropine.

Pralidoxime was as neuroprotective as atropine and 4R. A controversial issue about pralidoxime is whether it protects the brain. The quaternary structure of pralidoxime and other AChE reactivators limit their penetration through the intact BBB. However, there are proofs of central reactivation of AChE [31] and it was demonstrated that pralidoxime penetrates the blood-brain barrier [32]. The accepted clinical application and mechanism of the action of oximes, including pralidoxime, is a reactivation of AChE inhibited by OPs before the aging of the OP-AChE complex is completed. However, the effect of pralidoxime

is still an open question because of its low efficacy [33] and the wide range of pharmacological activities. In cardiac myocytes, 10 mM pralidoxime inhibits the Na⁺/Ca²⁺ exchanger (NCX) by a mechanism that does not involve dephosphorylation of serine residues [34]. NCX has been reported to be involved in neuroprotection, but its role is marred by contradictory reports [35, 36]. The clinical use of oximes is controversial because of the reported negative balance between benefits and harmful effects [37, 38].

OPs are known to induce apoptosis [39, 40]. To test the hypothesis that the decrease of PS areas by DFP is mediated by apoptosis, a cell-permeable, reversible caspase 9 inhibitor was used. Superfusion with the inhibitor for 1 hour and ending 5 min before DFP provided complete protection, but application of the inhibitor for 1 hour starting 5 min after DFP only provided modest protection. However, washing out the inhibitor for 30 min before DFP or applying it 30 min after DFP removed the protection, proving that the inhibitor must be present during DFP application or shortly thereafter. Remarkably, the time course of the effect of the caspase 9 inhibitor differs from the other antidotes, suggesting that DFP triggers caspase-dependent apoptosis during the first 5–10 min. Although this inhibitor is selective for caspase 9, it may possibly inhibit caspases 4 and 5. These results support the conclusion that the initial stage of DFP neurotoxicity is mediated by a caspase-dependent process that can be stopped by antidotes.

The proposed time course of 5–10 min for apoptosis in acute slices is different from what is observed in vivo. DFP treatment in vivo demonstrated apoptotic cells in the hippocampus, cortex, amygdala, and thalamus, starting at 4 h and persisting until 72 h after DFP treatment [41]. This apparent contradiction can be explained by the fact that, in vivo, only the final stages of apoptosis in neuronal cell bodies were observed. In slices, the early stages of apoptosis in the synapses that fail to sustain synaptic transmission, are manifested as a decrease in PS area. This interpretation is in agreement with the notion that neuronal apoptosis starts at the synapses, and only if the noxious stimulus persists or is strong enough it propagate to the nucleus [42, 43].

In conclusion, the results obtained with acute hippocampal slices show that the pharmacological profile of DFP neurotoxicity and the antidotal effect of atropine and pralidoxime are consistent with in vivo findings. As mentioned in the introduction, the neurotoxicity and neuroprotection observed in slices is a good predictor of the result in vivo. In the present case, the neuroprotection by 4R, first observed in slices [32], was reproduced in vivo [14]. A puzzling finding is the very early onset of the caspase-dependent neurotoxic effect, which is most likely apoptosis. Such short-onset effects could not be observed in vivo. The harmful consequences of AChE inhibition are not conclusively understood, and this in vitro model could shed light on these processes and aid in identifying targets for novel antidotes.

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References

1. Weissman BA, Raveh L. Therapy against organophosphate poisoning: the importance of anticholinergic drugs with antiglutamatergic properties. *Toxicology and applied pharmacology*. 2008; 232(2):351–8. [PubMed: 18680758]
2. Newmark J. Therapy for nerve agent poisoning. *Arch Neurol*. 2004; 61(5):649–52. [PubMed: 15148139]
3. Miyaki K, Nishiwaki Y, Maekawa K, Ogawa Y, Asukai N, Yoshimura K, Etoh N, Matsumoto Y, Kikuchi Y, Kumagai N, Omae K. Effects of sarin on the nervous system of subway workers seven years after the Tokyo subway sarin attack. *J Occup Health*. 2005; 47(4):299–304. [PubMed: 16096354]
4. Crawford, SM.; Compton, JR.; Tetz, LM.; Ratcliffe, RH.; Steele, KH.; Gordon, RK.; Nambiar, MP. Development Of A Rat Diisopropylfluorophosphate-Induced Seizure/Status Epilepticus Model For Screening Of Neuroprotectants Following Exposure To Chemical Warfare Agents.. Paper presented at the Scientific Conference on Chemical and Biological Defense Research; Hunt Valley, MD, A976944.. 2004. p. 1-15.
5. Ferchmin PA, Pagan OR, Ulrich H, Szeto AC, Hann RM, Eterovic VA. Actions of octocoral and tobacco cembranoids on nicotinic receptors. *Toxicon*. 2009; 54(8):1174–82. [PubMed: 19281835]
6. Andersen P, Bliss TV, Skrede KK. Unit analysis of hippocampal population spikes. *Exp Brain Res*. 1971; 13(2):208–21. [PubMed: 5123965]
7. Small DL, Monette R, Chakravarthy B, Durkin J, Barbe G, Mealing G, Morley P, Buchan AM. Mechanisms of 1S,3R-ACPD-induced neuroprotection in rat hippocampal slices subjected to oxygen and glucose deprivation. *Neuropharmacology*. 1996; 35(8):1037–48. [PubMed: 9121606]
8. Small DL, Monette R, Buchan AM, Morley P. Identification of calcium channels involved in neuronal injury in rat hippocampal slices subjected to oxygen and glucose deprivation. *Brain Res*. 1997; 753(2):209–18. [PubMed: 9125405]
9. Eterovic VA, Pérez D, Martins AH, Cuadrado BL, Carrasco M, Ferchmin P. A Cembranoid Protects Acute Hippocampal Slices Against Paraoxon Neurotoxicity. *Toxicology in Vitro*. 2011; 25:1468–74. [PubMed: 21569834]
10. Ferchmin PA, Perez D, Biello M. Spermine is neuroprotective against anoxia and N-methyl-D-aspartate in hippocampal slices. *Brain Res*. 2000; 859(2):273–9. [PubMed: 10719074]
11. Schurr A, Payne RS, Heine MF, Rigor BM. Hypoxia, excitotoxicity, and neuroprotection in the hippocampal slice preparation. *J Neurosci Methods*. 1995; 59(1):129–138. [PubMed: 7475243]
12. Wang T, Raley-Susman KM, Wang J, Chambers G, Cottrell JE, Kass IS. Thiopental attenuates hypoxic changes of electrophysiology, biochemistry, and morphology in rat hippocampal slice CA1 pyramidal cells. *Stroke*. 1999; 30(11):2400–7. [PubMed: 10548677]
13. Martins AH, Alves JM, Perez D, Carrasco M, Torres-Rivera W, Eterovic VA, Ferchmin PA, Ulrich H. Kinin-B2 receptor mediated neuroprotection after NMDA excitotoxicity is reversed in the presence of kinin-B1 receptor agonists. *PLoS One*. 2012; 7(2):e30755. [PubMed: 22348022]
14. Ferchmin PA, Andino M, Reyes Salaman R, Alves J, Velez-Roman J, Cuadrado B, Carrasco M, Torres-Rivera W, Segarra A, Martins AH, Lee JE, Eterovic VA. 4R-cembranoid protects against diisopropylfluorophosphate-mediated neurodegeneration. *Neurotoxicology*. 2014; 44:80–90. [PubMed: 24928201]
15. Martins AH, Hu J, Xu Z, Mu C, Alvarez P, Ford BD, El Sayed K, Eterovic VA, Ferchmin PA, Hao J. Neuroprotective activity of (1S,2E,4R,6R,-7E,11E)-2,7,11-cembratriene-4,6-diol (4R) in vitro and in vivo in rodent models of brain ischemia. *Neuroscience*. 2015; 291:250–9. [PubMed: 25677097]
16. Wallis RA, Panizzon KL. Felbamate neuroprotection against CA1 traumatic neuronal injury. *Eur J Pharmacol*. 1995; 294(2-3):475–82. [PubMed: 8750708]
17. Wallis RA, Panizzon KL, Fairchild MD, Wasterlain CG. Protective effects of felbamate against hypoxia in the rat hippocampal slice. *Stroke*. 1992; 23(4):547–51. [PubMed: 1561687]
18. Wasterlain CG, Adams LM, Wichmann JK, Sofia RD. Felbamate protects CA1 neurons from apoptosis in a gerbil model of global ischemia. *Stroke*. 1996; 27(7):1236–40. [PubMed: 8685935]

19. Ferchmin PA, Hao J, Perez D, Penzo M, Maldonado HM, Gonzalez MT, Rodriguez AD, de Vellis J. Tobacco cembranoids protect the function of acute hippocampal slices against NMDA by a mechanism mediated by $\alpha 4 \beta 2$ nicotinic receptors. *J Neurosci Res*. 2005; 82(5):631–41. [PubMed: 16247800]
20. Deshpande LS, Carter DS, Blair RE, DeLorenzo RJ. Development of a prolonged calcium plateau in hippocampal neurons in rats surviving status epilepticus induced by the organophosphate diisopropylfluorophosphate. *Toxicol Sci*. 2010; 116(2):623–31. [PubMed: 20498005]
21. Ellman GL, Courtney KD, Andres V Jr. Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol*. 1961; 7:88–95. [PubMed: 13726518]
22. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976; 72:248–54. [PubMed: 942051]
23. Maxwell DM, Brecht KM, Koplovitz I, Sweeney RE. Acetylcholinesterase inhibition: does it explain the toxicity of organophosphorus compounds? *Arch Toxicol*. 2006; 80(11):756–60. [PubMed: 16770629]
24. Ferchmin PA, Perez D, Eterovic VA, de Vellis J. Nicotinic receptors differentially regulate N-methyl-D-aspartate damage in acute hippocampal slices. *The Journal of pharmacology and experimental therapeutics*. 2003; 305(3):1071–8. [PubMed: 12649299]
25. Zaja-Milatovic S, Gupta RC, Aschner M, Milatovic D. Protection of DFP-induced oxidative damage and neurodegeneration by antioxidants and NMDA receptor antagonist. *Toxicology and applied pharmacology*. 2009; 240(2):124–31. [PubMed: 19615394]
26. Robenshtok E, Luria S, Tashma Z, Hourvitz A. Adverse reaction to atropine and the treatment of organophosphate intoxication. *Isr Med Assoc J*. 2002; 4(7):535–9. [PubMed: 12120467]
27. Kassa J, Koupilova M. Neuroprotective effects of currently used antidotes in soman-poisoned rats. *Pharmacol Biochem Behav*. 2000; 67(3):663–8. [PubMed: 11164099]
28. Kassa J, Krejeova G. Neuroprotective effects of currently used antidotes in tabun-poisoned rats. *Pharmacol Toxicol*. 2003; 92(6):258–64. [PubMed: 12787257]
29. Shih TM, Rowland TC, McDonough JH. Anticonvulsants for nerve agent-induced seizures: The influence of the therapeutic dose of atropine. *J Pharmacol Exp Ther*. 2007; 320(1):154–61. [PubMed: 17015638]
30. Albuquerque EX, Pereira EF, Aracava Y, Fawcett WP, Oliveira M, Randall WR, Hamilton TA, Kan RK, Romano JA Jr. Adler M. Effective countermeasure against poisoning by organophosphorus insecticides and nerve agents. *Proc Natl Acad Sci U S A*. 2006; 103(35):13220–5. [PubMed: 16914529]
31. Bajgar J, Fusek J, Kuca K, Bartosova L, Jun D. Treatment of organophosphate intoxication using cholinesterase reactivators: facts and fiction. *Mini Rev Med Chem*. 2007; 7(5):461–6. [PubMed: 17504181]
32. Ferchmin, P.; P.D.; Martins, AH.; Cuadrado, BL.; Carrasco, M.; Eterovic, VA. *Neuroscience 2009, S.f. Neuroscience. Society for Neuroscience; Washington, DC 20005: 2009. A cembranoid protects acute hippocampal slices against neurotoxic organophosphates.*
33. Kuca K, Hrabina M, Soukup O, Tobin G, Karasova J, Pohanka M. Pralidoxime--the gold standard of acetylcholinesterase reactivators--reactivation in vitro efficacy. *Bratisl Lek Listy*. 2010; 111(9):502–4. [PubMed: 21180265]
34. Watanabe Y, Iwamoto T, Matsuoka I, Ohkubo S, Ono T, Watano T, Shigekawa M, Kimura J. Inhibitory effect of 2,3-butanedione monoxime (BDM) on $\text{Na}^{+}/\text{Ca}^{2+}$ exchange current in guinea-pig cardiac ventricular myocytes. *Br J Pharmacol*. 2001; 132(6):1317–25. [PubMed: 11250883]
35. Muthuraman A, Jaggi AS, Singh N, Singh D. Ameliorative effects of amiloride and pralidoxime in chronic constriction injury and vincristine induced painful neuropathy in rats. *Eur J Pharmacol*. 2008; 587(1-3):104–11. [PubMed: 18486127]
36. Annunziato L, Pignataro G, Di Renzo GF. Pharmacology of brain $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger: from molecular biology to therapeutic perspectives. *Pharmacol Rev*. 2004; 56(4):633–54. [PubMed: 15602012]

37. Buckley NA, Eddleston M, Li Y, Bevan M, Robertson J. Oximes for acute organophosphate pesticide poisoning. *Cochrane Database Syst Rev*. 2011; 2:CD005085. [PubMed: 21328273]
38. Eddleston M, Eyer P, Worek F, Juszczak E, Alder N, Mohamed F, Senarathna L, Hittarage A, Azher S, Jeganathan K, Jayamanne S, von Meyer L, Dawson AH, Sheriff MH, Buckley NA. Pralidoxime in acute organophosphorus insecticide poisoning--a randomised controlled trial. *PLoS Med*. 2009; 6(6):e1000104. [PubMed: 19564902]
39. Wu X, Tian F, Okagaki P, Marini AM. Inhibition of N-methyl-D-aspartate receptors increases paraoxon-induced apoptosis in cultured neurons. *Toxicology and applied pharmacology*. 2005; 208(1):57–67. [PubMed: 16164961]
40. Te JA, Spradling-Reeves KD, Dillman JF 3rd, Wallqvist A. Neuroprotective mechanisms activated in non-seizing rats exposed to sarin. *Brain Res*. 2015; 1618:136–48. [PubMed: 26049129]
41. Li Y, Lein PJ, Liu C, Bruun DA, Tewolde T, Ford G, Ford BD. Spatiotemporal pattern of neuronal injury induced by DFP in rats: A model for delayed neuronal cell death following acute OP intoxication. *Toxicology and applied pharmacology*. 2011; 253(3):261–9. [PubMed: 21513723]
42. Mattson MP, Keller JN, Begley JG. Evidence for synaptic apoptosis. *Exp Neurol*. 1998; 153(1): 35–48. [PubMed: 9743565]
43. Guo ZH, Mattson MP. Neurotrophic factors protect cortical synaptic terminals against amyloid and oxidative stress-induced impairment of glucose transport, glutamate transport and mitochondrial function. *Cereb Cortex*. 2000; 10(1):50–7. [PubMed: 10639395]

Figure 1.

Timeline of recording the initial PSs, the application of DFP, ACSF, the antidote, and recording of the final PSs. This timeline was applied to all experiments unless otherwise stated.

Figure 2.

DFP inhibits the activity of acetylcholinesterase (AChE). Slices were treated as those used for PS recording. There were three or more slices for each DFP concentration. The line represents the equation: $f = \text{min} + (\text{max} - \text{min}) / (1 + (\text{DFP conc} / \text{IC}_{50})^{\text{Hill coefficient}})$; where $\text{min} = 0.55 \pm 0.02$; $\text{max} = 23.44 \pm 0.02$; $\text{IC}_{50} = 0.78 \pm 0.01 \mu\text{M}$; and Hill coefficient = -1.7 ± 0.04 .

Figure 3.

DFP decreases the PS areas in a dose- and time-dependent manner. **A)** PSs recorded before and after treatment with 0, 10, 100, 500, 700, and 1000 μ M DFP. **B)** Mean $\pm$ S.E.M. of the remaining PS after DFP exposure. The number of slices (in parentheses) per DFP concentration was 0 (28), 10 (7), 50 (6), 100 (28), 300 (14), 500 (14), 700 (7), and 1000 μ M (7). **C)** The effect of DFP on PS areas was time dependent. DFP was applied for 1 (21), 5 (7), 10 (28), 30 (7), or 60 min (21) (number of slices). The decrease in PS area after 10, 30, and 60 min of DFP were significantly different from the PS area after 1 and 5 min of exposure (* $p < 0.05$; PSs ANOVA on ranks followed by Dunn's test). The line is a single exponential decay (best-fit parameter $\pm$ S.E.M.). **D)** After application of DFP, the slices were washed with ACSF before recording the final PSs (n=7 for each group).

Figure 4.

4R applied 30 min after DFP significantly protected the PSs. **4A)** Pairs of representative recordings before and after the corresponding treatments. a) PSs from slices superfused with ACSF. b) DFP controls. c) Slices exposed to 100 μ M DFP, 30 min ACSF, followed by 10 μ M 4R for 1 hour. d) 4R controls not exposed to DFP treated with 10 μ M 4R for 1 hour. **4B)** Treatments are as in Fig 4A. A significant difference from DFP controls is shown (* $p < 0.05$, Kruskal-Wallis ANOVA on ranks followed by Dunn's test). The slices per group were 14, 21, 21, and 7. **4C)** The neuroprotection by 4R is stable for 3 hours. Slices were treated as in 4Ac followed by 1, 2, or 3 hours of ACSF before recording the final PSs ($n = 21$ in each group).

Figure 5.

Neuroprotection by atropine and 4R **A)** Atropine is neuroprotective, alone and in the presence of 4R. The gray bars show the effect of atropine applied either alone or together with 4R after DFP. The last two bars are controls superfused for 1 hour with ACSF or 1 μM atropine. All groups were significantly different from DFP controls (* $p < 0.05$; Kruskal-Wallis ANOVA on ranks followed by Dunn's test). **B)** Concentration-neuroprotection curves of 4R (filled circles) and of 4R in the presence of 1 μM atropine (open squares). The concentrations of 4R were 0 (14), 0.05 (28), 0.075 (14), 0.1 (14), 0.2 (7), 0.5 (28), 2 (28), and 10 μM (68); number of slices in parentheses. For the dose-neuroprotection curve of 4R in the presence of 1 μM atropine the following 4R concentrations were tested 0.05 (14), 0.5 (7), and 10 (21) μM 4R. At zero 4R concentration, two groups of slices were tested: DFP controls (filled circles; $n = 109$) and ACSF controls superfused with ACSF for 3 hours (triangles; $n = 14$). The line represents a four-parameter logistic curve: $f = \min + (\max - \min) / (1 + (4R \text{ concentration} / EC_{50})^{-\text{Hill coefficient}})$; where $\min = 29.6 \pm 2.6$, the % PS recovery in the absence of 4R; $\max = 84.1 \pm 5.8$, the % PS recovery at 4R saturation; $EC_{50} = 0.068 \pm 0.034 \mu\text{M}$; and Hill coefficient = 0.7 ± 0.4 .

Figure 6.

Pralidoxime was neuroprotective in vitro. Slices exposed to DFP were superfused with 2, 10, or 100 μ M pralidoxime or 10 μ M 4R. All conditions were significantly different from the DFP control (* p <0.001; ANOVA followed by Bonferroni test). The number of slices per condition was 49, 14, 28, 21, and 14.

Figure 7.

A caspase 9 inhibitor II protected the PS from DFP. LEHD-CHO (5 μ M) was superfused for 1 hour at four different times relative to DFP application. The first bar is the DFP control. In the second bar, LEHD-CHO superfused for 1 hour up to 5 min before DFP provided complete protection. In the third bar, LEHD-CHO applied for 1 hour starting 5 min after DFP provided only modest protection. For the fourth and fifth bars, the interval between DFP and the inhibitor was 30 min. The final PSs were recorded 90 min after DFP. The significance of the difference from the DFP control was (***) $p < 0.001$; (*) $p < 0.05$, ANOVA followed by Bonferroni post hoc test). There were 5 slices per each group treated with LEHD-CHO and 21 slices in the DFP control group.