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Review

Neurobiology of organophosphate-induced seizures

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ABSTRACT

This review summarizes the efforts of our laboratories to develop a mechanism-based therapy for the treatment of organophosphate (OP) nerve agent-induced seizures. Organophosphate poisoning can occur during warfare and terrorist attacks and in the civilian sphere because of intentional or unintentional poisoning. Persons exposed to OPs experience seizures. We developed animal models of OP poisoning and then evaluated the effects of OP on excitatory α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated glutamatergic neurotransmission in the hippocampus using patch-clamp electrophysiology. Organophosphate agents enhance glutamatergic transmission by enhancing neurotransmitter release. M1 muscarinic receptors mediate this effect, at least in part. Muscarinic receptors exert this action by inhibiting specific KCNQ2/3 potassium channels, which mediate the M-current. Flupirtine, a drug that open channels, is effective against OP-induced seizures.

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1. Introduction

Organophosphate (OP) nerve agents, such as sarin, soman, and VX, act by inhibiting cholinesterase centrally and peripherally and enhancing cholinergic transmission. In experimental animals, all known nerve agents (cyclosarin, sarin, tabun, VX, and VR) produce convulsive seizures and status epilepticus (SE) within minutes of exposure [1]. Seizures occurred in humans exposed to nerve agent poisoning during the Iran–Iraq war and in the Tokyo subway attack, in which sarin was used [2,3]. More recent attacks in Syria and the UK using OP nerve agents re-emphasize the need for novel therapies [4]. We propose that the mechanism of OP-induced seizures is enhanced glutamate release from presynaptic terminals. Organophosphate causes the accumulation of acetylcholine in the synaptic terminals and the activation of M1 muscarinic receptors, which then inhibit KCNQ2/3-type potassium channels. Opening these M-type channels can terminate OP-induced seizures [5].

Nerve gases are a group of organophosphorus compounds that inhibit the acetylcholine-degrading enzyme cholinesterase. Humans exposed to these agent's experience signs of cholinergic toxicity, hypersecretion, fasciculations, seizures, coma, and death. Immediate therapy for nerve gas exposure consists of atropine and 2-pralidoxime (2-PAM). Atropine is a muscarinic receptor antagonist, and 2-PAM reactivates cholinesterase. This treatment controls the peripheral effects of cholinesterase inhibition but fails to protect against the systemic effect of nerve gas agents, seizures. Agents that readily cross the blood–brain barrier and are free of unwanted neurological effects are ideal prophylaxis for OP poisoning. A recent study has compared the efficacy and assessed the behavioral effects of galantamine and (−) huperzine against soman-induced seizures in male cynomolgus macaques and compared it with pyridostigmine [6]. These studies revealed a greater potency and minimal toxicity of huperzine as compared to galantamine and pyridostigmine [6]. Thus, agents that are superior in sequestering acetylcholinesterase may provide a better protection against OP exposure.

The Centers for Disease Control (CDC) currently recommends the use of 10 mg of diazepam given intravenously and slowly for nerve gas-induced seizures. This recommendation is derived from the recommendations of the US Army's chemical defense research institute and the current US Army doctrine on field treatment for nerve gas agent exposure.

The recommendation for using diazepam to treat nerve gas-induced seizures is based on a large body of work carried out by the chemical defense research community between 1972 and 89 [1]. Initial studies demonstrated that, when used in conjunction with atropine and oximes, diazepam and other benzodiazepines significantly reduce the mortality associated with OP nerve gas agent exposure. Other studies

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in the 1980s demonstrated that brain pathology associated with nerve gas exposure is primarily due to prolonged seizures and not the direct toxicity of the agent itself or hypoxic injury to neurons [7]. These studies demonstrated that nerve gas-induced neuropathology correlates well with seizure duration and that diazepam treatment significantly reduces the neuropathology associated with nerve gas exposure.

2. Development of OP induced seizure model for civilian labs

The OP nerve agents sarin, soman, and VX are restricted-use chemicals, and SE induced by these agents has been studied in defense labs. Therefore, acceptable surrogate OP agents must be used for civilian research to understand the mechanisms, pathophysiology, and treatment of OP-induced SE. Parathion and malathion are OP insecticides known to cause SE in humans [8]. The neuropathology associated with two OP agents available to civilian labs, paraoxon and diisopropyl fluorophosphate (DFP), have been studied, and in vitro models of poisoning have been developed [9–11]. However, these studies did not characterize the evolution of SE with electroencephalography (EEG). We characterized SE induced by paraoxon and DFP with EEG and behavior [12].

Animals were implanted with EEG recording electrodes and an intrahippocampal infusion cannula. Paraoxon was infused at a rate of either 0.5 or 1 μL/min in a total volume of 20 μL. Video and EEG monitoring began 10 min prior to paraoxon infusion and continued for 24 h after infusion completion. The infusion of paraoxon into the hippocampus caused electrographic seizures in a dose-dependent fashion. As the dose of paraoxon increased, the seizures became longer in duration and changed from intermittent to continuous. In animals with continuous seizures, there were brief periods of suppressed activity between seizures. Continuous electrographic seizures persisted for 4–18 h and constituted SE. These electrographic seizures were accompanied by behavioral seizures. The animals that experienced SE exhibited stop and stare behavior and thigmotactic movement, as well as wet-dog shaking for as long as 18 h. Paraoxon also caused SE after peripheral injection in animals that were protected from its peripheral effects by 2 PAM and scopolamine. Paraoxon-induced SE was responsive to diazepam given in early or late stages of SE [12]. This was unlike other cholinergic models of SE, in which benzodiazepine resistance is common [13].

We then characterized a DFP model of SE. Initial observations and published evidence were used to determine the DFP dose administered after treatment with atropine and 2-PAM. The behaviors consisted of a combination of chewing, head-bobbing, and generalized tonic–clonic seizures mixed with body tremors. In some animals, SE began with continuous electrographic seizure activity, while in others it progressed from periods of discrete seizures separated by normal EEG to seizures punctuated by suppression, after which the seizures merged. The SE caused by DFP was resistant to diazepam treatment when it was given at 30 min [12].

3. OP increases glutamatergic transmission

We tested whether cholinergic stimulation induced by the OP-mediated inhibition of cholinesterase increases glutamatergic transmission. We applied paraoxon to hippocampal slice preparations to study this effect. Whole-cell patch-clamp recordings of miniature excitatory postsynaptic currents (EPSCs) (mEPSCs), spontaneous EPSCs (sEPSCs), and evoked EPSCs (eEPSCs) were made from granule cells after the gamma-aminobutyric acid (GABA)-A receptor was blocked with the antagonist picrotoxin (50 μM) [14].

We investigated whether paraoxon can alter glutamatergic synaptic transmission by studying sEPSCs, mEPSCs, and eEPSCs recorded from dentate granule cells in sagittal hippocampal slices. After recording sEPSCs for 10 min, 3-μM paraoxon was bath-applied to a slice. The sEPSCs were more frequent and larger after the application of OP. This effect was dose-dependent, with higher doses causing a larger effect. We confirmed the effect of paraoxon by using ambenonium, a reversible high affinity cholinesterase inhibitor. It had a similar effect as that of paraoxon; it increased the frequency of sEPSCs. Since sEPSCs consist of action potential-dependent and action potential-independent events (mEPSCs), we tested whether paraoxon enhances the frequency and amplitude of mEPSCs. Paraoxon increased the frequency of mEPSCs but did not significantly change their sizes.

We tested whether glutamate release in response to a single action potential is increased by cholinesterase inhibition. Amplitude distribution histograms were used to analyze changes in presynaptic release mechanisms, and a new, larger peak appeared in the amplitude distribution histogram in response to paraoxon, suggesting increased presynaptic release. A minimally effective stimulus was applied to the perforant pathway by means of a glass electrode filled with artificial cerebrospinal fluid (ACSF), and evoked responses were recorded. Paraoxon reduced the failure rate and increased the amplitudes of the eEPSCs. These observations support the hypothesis that paraoxon acts at presynaptic sites and increases action potential-dependent release. To further test this, paired stimuli were delivered to evoke EPSCs. Paraoxon diminished the degree of paired-pulse facilitation.

To determine the type of cholinergic receptor that mediates the effect of paraoxon, we examined nicotinic and muscarinic agonists and antagonists. We first tested whether the nicotinic receptor agonist nicotine mimics the action of paraoxon. Nicotine (1 μM) increased the frequency of sEPSCs and their amplitudes. Increased sEPSC frequency caused by nicotine was completely reversed by 50-nM α-bungarotoxin. However, α-bungarotoxin did not prevent or reverse the increase in sEPSC frequency caused by the application of paraoxon.

We tested whether blocking muscarinic receptors selectively prevents the action of paraoxon. Atropine, a muscarinic antagonist, was applied after recording sEPSCs for 5 min. Paraoxon was then applied in the presence of atropine. Atropine did not alter the sEPSC frequency or amplitude. When paraoxon was applied in the presence of atropine, it did not enhance the sEPSC frequency or amplitude. Therefore, the muscarinic receptor antagonist atropine prevented paraoxon-induced increases in sEPSC frequency and amplitude. Although atropine blocked the action of paraoxon on sEPSCs, the muscarinic receptor agonist carbachol only partially mimicked the effects of paraoxon. We confirmed that muscarinic receptor blockade reduced release from presynaptic terminals. The muscarinic antagonist atropine reduced eEPSC amplitudes and increased the failure rate. Similarly, the application of paraoxon in the presence of atropine did not change the eEPSC amplitudes and did not increase the failure rate. Moreover, when atropine was applied after paraoxon, it reversed the effect of paraoxon on eEPSC amplitudes.

In summary, the cholinesterase inhibitor paraoxon enhances glutamatergic transmission in hippocampal granule cell synapses principally through presynaptic mechanisms. Furthermore, the effect of paraoxon on glutamatergic transmission is mediated in part by muscarinic receptors [14].

4. Mechanism of cholinergic enhancement of excitatory synaptic transmission

We investigated how muscarinic receptor activation modulates glutamatergic transmission. M-type potassium channels mediate the effects of muscarinic activation in the hippocampus, and it has been proposed that they modulate glutamatergic synaptic transmission [5]. Muscarinic acetylcholine receptors are widely expressed in the brain and are G protein-coupled receptors expressed throughout the central nervous system. The M1 subtype is the predominant muscarinic acetylcholine receptor (mAChR) in the cortex, hippocampus, striatum, and thalamus [15].

Previous studies have suggested that muscarinic receptor activation increases presynaptic glutamate release from the perforant path to dentate granule cells [14]. In the present study, we investigated the effect of the M1 muscarinic receptor agonist McN-A-343 on glutamate release

recorded from Schaffer collateral-CA1 synapses. The application of McN-A-343 (10 μM) to CA1 pyramidal neurons increased the mEPSC frequency, but the mEPSC amplitude did not change. The cumulative distribution of the interevent intervals shifted left with no change in the distribution of amplitudes. Thus, the activation of M1 receptors increased glutamate release from the presynaptic terminals in the absence of action potentials [5].

We tested whether M-channels regulate glutamate release from presynaptic terminals of Schaffer collaterals since M1 receptor activation inhibits M-channels [16,17]. The frequency of sEPSCs increased in response to M channel inhibition. XE991 (10 μM), an M-channel inhibitor, was applied to hippocampal slices, and it increased the frequency of sEPSCs but did not affect their amplitudes. The distribution of sEPSC interevent intervals shifted left in response to the inhibition of M-channels, but the inhibition of M-channels had no effect on the cumulative distribution of their amplitudes. Schaffer collateral-CA1 pyramidal neuron synapses are modulated by M-channels. Similar to an M1 agonist, an M1 inhibitor altered action potential-independent release from the presynaptic terminal. The effect of XE991 on release was studied after blocking action potentials. The frequency, but not amplitude, of mEPSCs was increased by XE991 (10 μM). There was a leftward shift in the cumulative distribution of the mEPSC interevent intervals. The frequency of mEPSCs continued to increase after 10 min of XE991 application and returned to baseline at 90 min. The effect of XE991 on mEPSC frequency was confirmed by applying linopirdine, another M channel antagonist, which also increased the frequency of mEPSCs but had no effect on their amplitude.

We tested whether opening M channels has the opposite effect and reduces release. Flupirtine (20 μM), an M-channel opener, decreased action potential-independent release. To confirm that M channels mediate the effect of the M1 muscarinic agonist, we incubated slices with XE991 for 25 min. After 5 min of baseline recordings in XE991-containing medium, McN-A-343 was applied, and it blocked the XE-991-induced increase in EPSC frequency. This result suggests that blocking M-channels prevents the enhancement of mEPSC frequency by McN-A-343. These results suggest that the inhibition of the M-channel current enhances the action potential-independent release of glutamate [5].

5. Calcium influx through voltage-gated calcium channels mediates the muscarinic effect

We investigated the role of calcium in the M-channel modulation of neurotransmitter release. The increase in mEPSC frequency caused by the inhibition of M-channels was not because of enhanced Ca^{2+} release from intracellular stores or calcium influx from the extracellular space. First, we investigated the role of intracellular stores by blocking release from these stores. Hippocampal slices were treated with 2.5-μM thapsigargin, and the effect of the M-channel blocker XE991 (10 μM) was preserved. It increased the mEPSC frequency with or without thapsigargin incubation. Release from intracellular stores does not contribute to the modulation of neurotransmitter release by M-channels. Therefore, we tested whether the effect of M-channel blockade on mEPSC frequency is modulated by extracellular Ca^{2+} by recording in calcium-free medium. The absence of calcium in ACSF eliminated M-channel modulation and release from Schaffer collaterals. Calcium-free medium also eliminated the effect of the muscarinic M1 agonist McN-A-343. The enhancement of mEPSCs was blocked in calcium-free medium; thus, the effect of M1 receptor activation on the frequency of mEPSCs is largely mediated by increased calcium influx from the extracellular space [5].

We propose that M-channel inhibition leads to the activation of presynaptic voltage-gated calcium channels. We studied the effect of blocking P/Q- and N-type calcium channels on mEPSC frequency when XE991 was applied. P/Q- and N-type channels are expressed in the presynaptic terminals of Schaffer collaterals [18,19]. Slices were incubated with either ω-agatoxin TK or ω-conotoxin GVIA for 15 min prior to data collection. Incubation with ω-agatoxin TK, which blocks P/Q-type calcium channels, prevented the XE991-mediated enhancement of mEPSC frequency in approximately 50% of the cells tested. ω-Conotoxin GVIA, which inhibits N-type channels, had a similar effect in half of the neurons. We simultaneously applied both the blockers, and the effect of XE 991 was no longer apparent. The inhibition of M-type channels causes calcium influx though P/Q- and N-type calcium channels into the presynaptic terminals [5].

6. McN-A-343 and XE991 depolarize and increase action potential firing in CA3 neurons

We tested the effect of M-channel inhibition on the membrane properties of CA3 pyramidal neurons using current-clamp recordings. McN-A-343 application depolarized CA3 neurons and increased the action potential firing frequency. The effect of McN-A-343 was mimicked by XE991. Furthermore, the inhibition of M-channels or the activation of M1 muscarinic receptors increases CA3 pyramidal neuron membrane input resistance [5].

In summary, glutamate release is potentiated by M1 muscarinic activation or M-channel inhibition. Ca^{2+} influx through P/Q- and N-type calcium channels appears to mediate this effect. These results suggest that M1 receptor activation inhibits M-type potassium channels and depolarizes CA3 neurons, leading to an influx of Ca^{2+} through presynaptic voltage-dependent calcium channels and increasing spontaneous glutamate release to CA1 neurons. This study demonstrates that M-channels modulate action potential-independent neurotransmitter release [5].

7. Flupirtine terminates OP-induced SE

We then tested whether flupirtine, a drug that opens M-channels, abolishes SE. Three SE models were employed for this study: a lithium/pilocarpine model, a continuous hippocampal stimulation (CHS) model, and DFP model. These models have been described previously [12,20,21]. Electroencephalographic activity was recorded continuously for at least 5 h following drug injection, and was inspected visually. Benzodiazepine refractory SE (established SE (ESE)) was considered abolished when the following two conditions were met: i) the EEG returned to normal baseline or showed arrhythmic spikes (<2 Hz), and ii) there was no recurrence of electrographic or behavioral seizures within 1 h. All drugs were administered intraperitoneally unless noted otherwise. We tested whether flupirtine alone or in combination with diazepam abolished ESE within 60 min of drug administration.

The initial evaluation of the effect of flupirtine treatment on ESE was performed in the lithium/pilocarpine model. Diazepam (10 mg/kg) or vehicle was administered 10 min after the onset of continuous electrographic seizures, which also corresponded with the first stage 5 behavioral seizures. Electrographic and behavioral seizures continued unabated after treatment with diazepam. SE was not abolished in any animal within 60 min [21,22]. Diazepam did not shorten the duration of seizures. Thus, 10 min after the onset of continuous electrographic seizure activity, the animals exhibited ESE. Based on a previous study [23], an initial dose of 50-mg/kg flupirtine (i.p.) was used to treat ESE. This dose did not abolish lithium/pilocarpine-induced ESE in any animal or reduce the duration of seizures. Higher doses (75 or 100 mg/kg) were ineffective and toxic. ESE generated by lithium/pilocarpine was not abrogated by flupirtine alone. However, ESE was abolished by a combination of diazepam and flupirtine. This combination abolished ESE rapidly in most animals and reduced the SE duration. Thus, combination treatment with flupirtine (50 mg/kg) and diazepam (10 mg/kg) can terminate lithium/pilocarpine-induced ESE within a narrow therapeutic window.

Flupirtine was tested in a continuous hippocampal stimulation (CHS) model, an electrical stimulation model of ESE [24]. Only 20% of the animals were treated with diazepam (10 mg/kg) 10 min after the onset of continuous electrographic seizure activity. We then tested the

effect of flupirtine (50 mg/kg, n = 5) on CHS-induced ESE. Flupirtine was ineffective in a majority of animals and did not reduce the mean seizure duration. A combination of diazepam and flupirtine abolished ESE within 1 h in 80% of the animals and reduced the mean SE duration. Thus, CHS-induced ESE can be effectively abolished by a combination of diazepam and flupirtine.

Flupirtine was most effective against DFP-induced SE. Seizures in response to DFP were variable, and only those animals that developed continuous electrographic seizures with behavioral signs were selected for further study. Flupirtine was administered 30 min after the onset of continuous electrographic seizure activity, and it reduced the seizure duration of DFP-induced ESE. When diazepam (10 mg/kg) was given in combination with flupirtine (50 mg/kg), it was highly effective in terminating DFP-induced SE. All animals quickly became seizure-free, and the mean SE duration was reduced. Thus, flupirtine in combination with diazepam is highly effective in terminating DFP-induced ESE. These studies show that a combination of a potassium channel opener and a benzodiazepine may be a candidate therapy for patients who develop ESE.

It is likely that flupirtine is effective in treating SE through its actions on potassium channels and GABA-A receptors, as it has long been reported to open potassium channels. Initial studies suggested that it opens an inwardly rectifying potassium channel. More recent studies have indicated that it preferentially opens Kv7.2/3 channels. Flupirtine also potentiates GABA-A receptors [25]. It is important to consider that the therapeutic range of this drug varies depending on its nociceptive action. Multiple actions of flupirtine have been reported in the last 30 years, and many of these occur at concentrations ten to one hundred times higher than the therapeutic range observed in vivo (from 1 to 10 μM).

Kv7.2/3 channels are critically involved in neuronal excitability and some forms of cholinergic excitation [26]. These channels are present at the axon initial segment and nodes of Ranvier, where they can modulate action potential generation and propagation [27,28]. These channels are inhibited by cholinergic muscarinic stimulation through the depletion of inositol diphosphate (IP2) [29,30]. The cloning and identification of the molecular constituents of M-channels has led to the development of a series of drugs that activate these channels. Three of these drugs, namely, flupirtine, retigabine, and BMS 204352, are well-investigated [31]. Retigabine was originally synthesized as a GABA-A receptor agonist and was later shown to open M-channels at lower therapeutic concentrations. Retigabine is an effective anticonvulsant in animal models of seizures, including the maximal electrical shock model, the kindling model, and the audiogenic seizure model, and in vitro seizures [32,33]. Retigabine was approved for clinical use as an add-on therapy for partial seizures. Retigabine has not been tested for the treatment of SE. Two other drugs are available to open potassium channels, namely, flupirtine and BMS-204352. Flupirtine is structurally similar to retigabine, whereas BMS-204352 (MaxiPost) is chemically distinct [31].

In summary, we developed models of OP poisoning. We investigated the effects of OP on excitatory AMPA receptor-mediated glutamatergic neurotransmission in the hippocampus using patch-clamp electrophysiology. Organophosphate enhances glutamatergic transmission by enhancing neurotransmitter release. M1 muscarinic receptors mediate this effect, at least in part. Muscarinic receptors exert this action by inhibiting specific KCNQ2/3 potassium channels, which mediate the M-current. Flupirtine, a drug that open channels, is effective against OP-induced seizures.

Conflict of interest

The authors have no conflicts of interest.

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