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REVIEW

Recent advances in cholinergic mechanisms as reactions to toxicity, stress, and neuroimmune insults

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Abstract

This review presents recent studies of the chemical and molecular regulators of acetylcholine (ACh) signaling and the complexity of the small molecule and RNA regulators of those mechanisms that control cholinergic functioning in health and disease. The underlying structural, neurochemical, and transcriptomic concepts, including basic and translational research and clinical studies, shed new light on how these processes inter-change under acute states, age, sex, and COVID-19 infection; all of which modulate ACh-mediated processes and inflammation in women and men and under diverse stresses. The aspect of organophosphorus (OP) compound toxicity is discussed based on the view that despite numerous studies, acetylcholinesterase (AChE) is still a vulnerable target in OP poisoning because of a lack of efficient treatment and the limitations of oxime-assisted reactivation of inhibited AChE. The over-arching purpose of this review is thus to discuss mechanisms of cholinergic signaling dysfunction caused by OP pesticides, OP nerve agents, and anti-cholinergic medications; and to highlight new therapeutic strategies to combat both the acute and chronic effects of these chemicals on the cholinergic and neuroimmune systems. Furthermore, OP toxicity was examined in view of cholinesterase inhibition and beyond in order to highlight improved small molecules and RNA therapeutic strategies and assess their predicted pitfalls to reverse the acute toxicity and long-term deleterious effects of OPs.

KEYWORDS
acetylcholine, inflammation, microRNA, neurotoxicity, transfer RNA fragments

INTRODUCTION

Acetylcholine (ACh) was the very first neurotransmitter discovered, but the full scope of its brain and body impact, as well as the corresponding mechanisms involved, are not yet fully understood. Discussions on issues related to cholinergic mechanisms often focus on the proteins triggering and/or terminating such mechanisms or the genes encoding for them, including their alternative splicing products and the non-coding RNAs regulating them. We aimed to briefly cover and connect both of these topics following information presented at the 17th International Symposium on Cholinergic Mechanisms held in Dubrovnik, Croatia from 8 to 12 May 2022. The

Abbreviations: 2PAM, pralidoxime; ACh, acetylcholine; AChE, acetylcholinesterase; BBB, blood–brain barrier; BChE, butyrylcholinesterase; CNS, central nervous system; HI-6, asoxime; mAChR, muscarinic acetylcholine receptor; miRs, microRNAs; NA, nerve agent; nAChR, nicotinic acetylcholine receptor; NeuN, neuronal nuclear antigen; OP, organophosphorus compound; tRFs, transfer RNA fragments.

This Review is part of the special issue "17th Symposium on Cholinergic Mechanisms (ISCM2022)".

symposium was attended by neurochemistry specialists, including basic, translational, and clinical investigators, of all ages and varied scientific disciplines from around the world. This diverse assortment of talent allowed for an open discussion and novel insights on the processes controlling ACh signaling, the cellular and tissue structures involved, and the recent clinical big data reports demonstrating the long-term impact of anti-cholinergic medications as risk factors for dementia in the elderly.

Specifically, recent advances have offered an opportunity to design novel innovative steps toward an integrative approach to address the multi-leveled impact of cholinergic systems, which spans both multiple aspects of basic fundamental science and the progress in clinical and personalized medicine. Considering such a broad picture of cholinergic mechanisms, in this review we focused on reactions that are crucial for neurotransmission and degradation of ACh, but also important for toxicity in case of organophosphate poisoning, as well as on the regulation of balanced cholinergic signaling. Any change in the balance between these processes, either through cholinesterase inhibitors or RNA regulators including microRNAs and transfer RNA fragments, may lead to a pronounced imbalance accompanied by immediate and long-term changes in those biological roles controlled by cholinergic signaling. Furthermore, OP toxicity was examined in view of cholinesterase inhibition and beyond, in order to highlight therapeutic strategies and pitfalls to reverse the acute toxicity and long-term deleterious effects of OPs.

INHIBITION OF CHOLINESTERASES WITH OLD AND NEW ORGANOPHOSPHATES

The term "cholinesterases" (ChEs) refer to two structurally similar enzymes: acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8). Although both ChEs share the catalytic mechanism of hydrolysis of choline esters, their roles in live organisms are quite different. AChE terminates nerve impulse transmission in neuronal synapses by hydrolyzing the neurotransmitter acetylcholine (ACh). BChE also performs such hydrolysis, but at a slower pace and in distinct tissues, and is generally considered as having no natural physiological function. However, a growing body of evidence indicates that BChE plays a central role in the development of the symptomatology of Alzheimer's disease (Maxwell et al., 2022). In addition, BChE is engaged in the metabolism of many xenobiotics and serves as a backup for AChE, as well as in the protection of synaptic AChE from man-made and naturally occurring poisons (Čadež & Kovarik, 2021).

Triesters of phosphoric acid, diesters of phosphonic acid as well as phosphoramidic acid esters are commonly known as organophosphorus (OP) inhibitors of AChE (Taylor, 2017). OPs form a stable covalent bond with the nucleophilic serine of the AChE catalytic triad, thus acting as hemi-substrates of ChE (Aldridge & Reiner, 1972). Extremely slow rates of dephosphorylation are measured in time-intervals of hours and days, rendering OP inhibitory and AChE unavailable for its physiological function (Aldridge & Reiner, 1972). Although some of the most toxic substances made by man are OPs, such as the nerve agent (NA) poison sarin, VX, and Novichok, other compounds belonging to this class are frequently used as pesticides (Figure 1). General susceptibility to hydrolytic degradation allows non-volatile OP pesticides to convert to non-toxic products within days of their application in the field. Nerve agent warfare OPs, on the other hand, are both more stable (VX, tabun, and Novichoks) and much more volatile (sarin, soman) (Taylor, 2017), which makes them extremely hazardous. Furthermore, some OP pesticides such as parathion, chlorpyrifos, or malathion are applied in their low toxicity phosphorothioate (P=S) forms, which are bio-transformed to their active phosphate (P=O) analogs (Kwong, 2002).

Phosphorothioates are more lipophilic than the phosphate versions of these compounds and are stored extensively in fat. Therefore, the appearance of toxic symptoms after exposure to phosphorothioate pesticides is delayed. Most patients become symptomatic within 12 h of exposure if the causative agents are not fat-soluble organophosphates. For more lipophilic OPs, elimination is slow because of extensive fat storage, and may take days (Kwong, 2002). In the case of OP pesticide poisoning, an early treatment following the acute cholinergic phase is required to avoid health problems such as muscle paralysis, which can occur 1–4 days after OP poisoning, or OP-induced delayed neuropathy (OPIDN), which affects long axons in the spinal cord and peripheral nerves after a latent period of 10–14 days. Both intermediate syndrome and OPIDN have not been reported for NA poisoning. Nevertheless, both pesticide and nerve agent OPs further owe their exquisite toxicity to their uncharged chemical nature, which allows them to quickly traverse biological membranes (such as skin or alveoli), enter the blood, and penetrate both the peripheral and central nervous systems, reaching target AChE (Taylor, 2017; Zorbaz & Kovarik, 2021).

Inadequate or malicious handling of otherwise safe OP pesticides may result in exposure to still reactive, toxic compounds. OP intoxication is a global issue, and the OPCW (Organization for the Prohibition of Chemical Weapons) has recently warned about the potential for a further escalation of this threat and the pending need for effective antidotes (Timperley et al., 2019). Intoxication by OPs is the cause of over 200 000 deaths per year worldwide. Most fatalities are because of human exposure to OP pesticides (Dawson et al., 2010) and, more recently, also to exposure to nerve agent OPs. Sarin fatally wounded hundreds of innocent civilians in the 2017 Syrian conflict (Dolgin, 2013). VX was used in the 2017 terrorist assassination of dissident Kim Jong Nam at the Kuala Lumpur airport, and Sergei and Julia Skripal were insidiously poisoned in 2018 in the United Kingdom (Stone, 2018), as was Alexey Navalny in 2020 in Russia (Stone, 2020), with the new A-type NAs, Novichoks (Mirzayanov, 2008).

REVERSAL OF OP TOXICITY BY OXIMES

The toxic effects of OP poisoning are the consequence of the inhibition of AChE in the central nervous system (CNS) leading to an

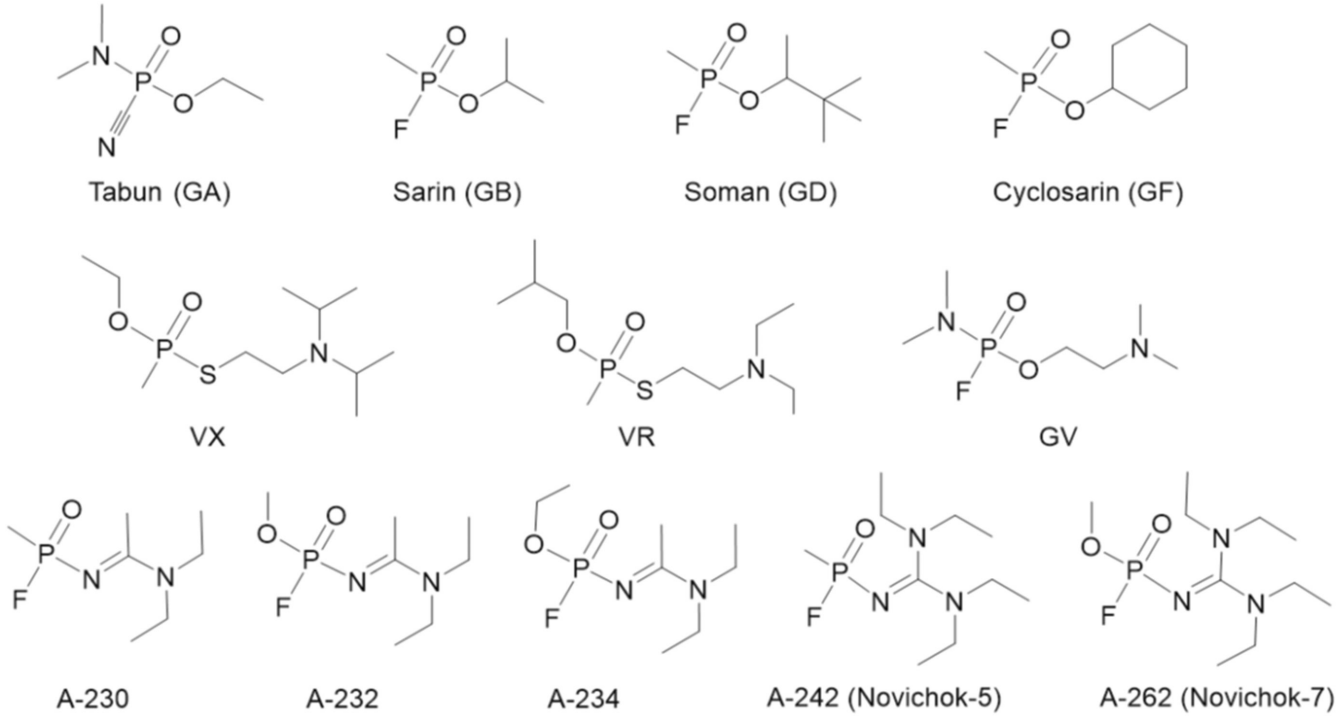

FIGURE 1 Representatives of the G-series (tabun, sarin, soman, and cyclosarin), V-series (VX, VR,), IVA agents (GV), and Novichok series of nerve agents (A-230, A-232, A-234, A-242, and A-262).

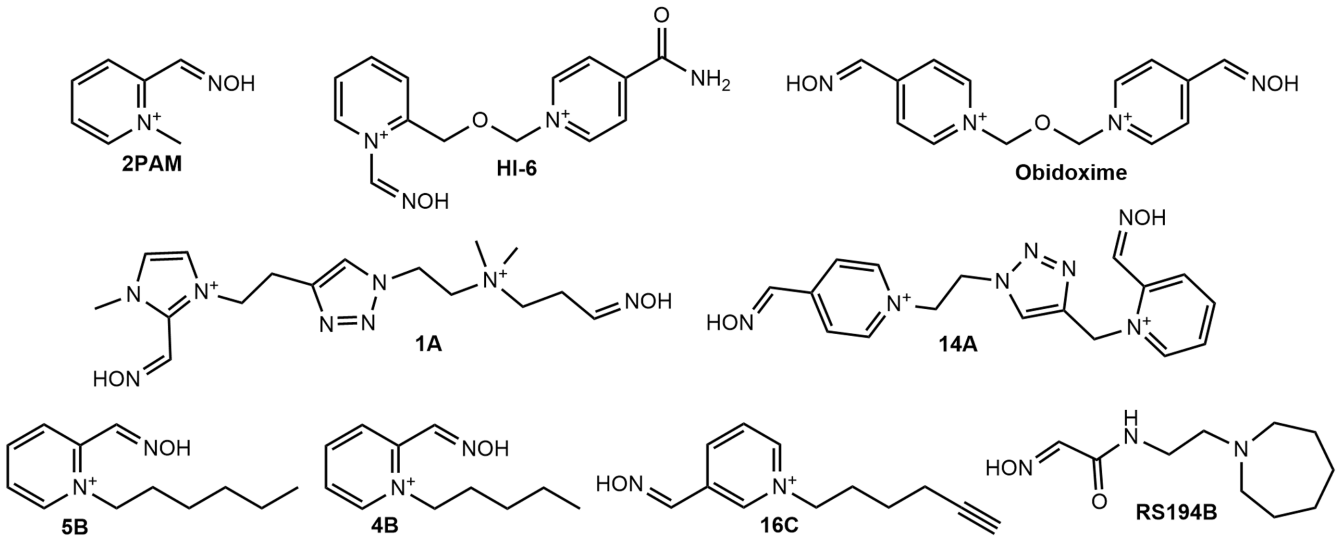

FIGURE 2 Chemical structures of the selected reactivators: standard pyridinium aldoxime reactivators of phosphorylated acetylcholinesterase (2PAM, HI-6, and obidoxime), triazole-annulated click oximes (1A and 14A), 2PAM analogs (4B, 5B, and 16C), and zwitterionic oxime RS194B.

accumulation of the neurotransmitter ACh at synapses and neuromuscular junctions. Therefore, the standard treatment consists of the use of atropine, a competitive ACh antagonist, to reverse the biochemical abnormalities at the synapses resulting from excess ACh, and the use of nucleophilic oxime reactivators of covalently inhibited AChE (Figure 2) (Kovarik Maček & Hrvat, 2020). All oximes in clinical use, such as pralidoxime (2PAM), asoxime (HI-6), and obidoxime are cationic pyridinium derivatives that can access OP-inhibited AChE in blood, organs, skeletal muscles, and the peripheral nervous system (PNS), but barely in the CNS. These drugs are highly valued for treating NA intoxications, but their clinical efficacy depends on the OP that caused the toxicity, which limits their applicability (Worek et al., 2004). Specifically, nearly all severe cases of OP poisoning now result from self-poisoning with large volumes of less potent (WHO hazard class Ib and II) insecticides and co-formulated solvents, but some casualty treatments have

shown that pralidoxime may be associated with harm, including increased mortality (Eddleston, 2022). The reason for this is that standard oximes do not form complementary interactions with the AChE active site residues and therefore show limited efficiency in reactivation (Kovarik et al., 2004; Mercey et al., 2012). Hence, oximes should not be used routinely for the care of OP insecticide-poisoned patients (Eddleston, 2022). The problem lies in the fact that the oxime-assisted reactivation of OP-inhibited AChE implies intertwined processes that depend not only on oxime structure, but also on the structure and characteristics of the OP compound and of the OP-AChE conjugate (Kovarik et al., 2004; Kovarik & Maček Hrvat, 2020; Mercey et al., 2012; Zorbaz et al., 2020).

Recently, the reactivation of OP-inhibited AChE was shown to depend on a fine fit of the oxime not only being close to the phosphorylated catalytic serine, but also acquiring an angle of 180 degrees, which is essential to ensure a thermodynamically favorable nucleophilic attack from the side of the oxime group (Bennion et al., 2021). The advantage of the technology available today in terms of enzyme 3D structure determination and introduction of specific mutations can assist in designing new, more efficient antidotes for treatment of OP-poisoned patients. Using such knowledge can shed new light on the amino acid residues implicated in the reactivation reaction, while providing appropriate conditions for creating stoichiometric or catalytic scavengers as a new therapeutic approach to OP intoxication (Kovarik & Maček Hrvat, 2020; Masson & Nachon, 2017).

Apart from these advances, recent events involving A-series agents have highlighted the urgency of achieving a better understanding of their toxic profile, particularly for antidotes capable of reactivating inhibited AChE, in order to develop more efficient medical countermeasures (Lewis, 2018; Steindl et al., 2021). Since obidoxime was inefficient in treating patient (Steindl et al., 2021), it became apparent that the vulnerability to this class of NAs demands urgent research and global efforts aimed at overcoming the lack of knowledge about their properties, deleterious effects, and plausible countermeasures (Bolt & Hengstler, 2022; Imrit et al., 2020; Jeong & Choi, 2019; Nepovimova & Kuca, 2018; Santos et al., 2022).

The crucial approach of treatment is to reduce or eliminate longer term health effects arising from NA exposure and acute cholinergic crisis (Timperley et al., 2019). Only rapidly administered adequate antidotal treatment consisting of a reactivator of NA-inhibited AChE, an anti-cholinergic drug (preferably atropine), and an anticonvulsive drug (preferably a benzodiazepine) can stop the overstimulation of peripheral and central cholinergic receptors and subsequent clinical signs and symptoms. Therefore, atropine has remained the first drug of choice against symptoms of NA poisoning, because muscarinic effects are the most life-threatening. Although atropine does not cross the blood–brain barrier (BBB) readily, it has beneficial effects in the CNS and PNS, against central apnea, over-secretion, and even convulsions and cardiac toxicity (Kovarik & Maček Hrvat, 2020; Timperley et al., 2019).

STRUCTURAL-MECHANISTIC INSIGHTS INTO THE REACTIVATION OF PHOSPHORYLATED AChE

The cholinergic system and AChE activity are severely harmed by the neurotoxicity of OPs. Correspondingly, the search for novel and enhanced oxime antidotes relies on X-ray structures of native and OP-conjugated AChE, in particular those of human AChE/BChE that are used as valuable drug discovery templates. A recent set of six PDB-deposited X-ray structures of A-230-, A-232-, and A-234-conjugated AChE in the absence and presence of HI-6 (Available online: https://www.rcsb.org/structure/6NTL) showed that the large size of these agents fills the active center gorge of AChE more tightly than any other covalent or reversible ligand (Figure 3) (Luedtke et al., 2021). However, A-234, the largest of the three Novichoks, did not promote any distortions in AChE, although its large ethoxy substituent gets inserted into the acyl pocket and simultaneously fills the choline-binding site with a large phosphoramide substituent. One consequence of these interactions is the stabilization of the conjugates that renders them more resistant toward nucleophilic reactivation with oximes. In addition, a recent study on pre-reactivation kinetics, supplemented with structural data, showed that efficient reactivation is linked to longer residence time, and revealed that the organophosphates dictate both the initial binding and the structural prerequisites for the reactivation reaction (Lindgren et al., 2022).

The above studies indicate that reactivating nucleophiles possess better prospects of accessing the conjugated phosphorus when they approach from the direction of the peripheral site and align with the axis of the active center gorge channel with their extended

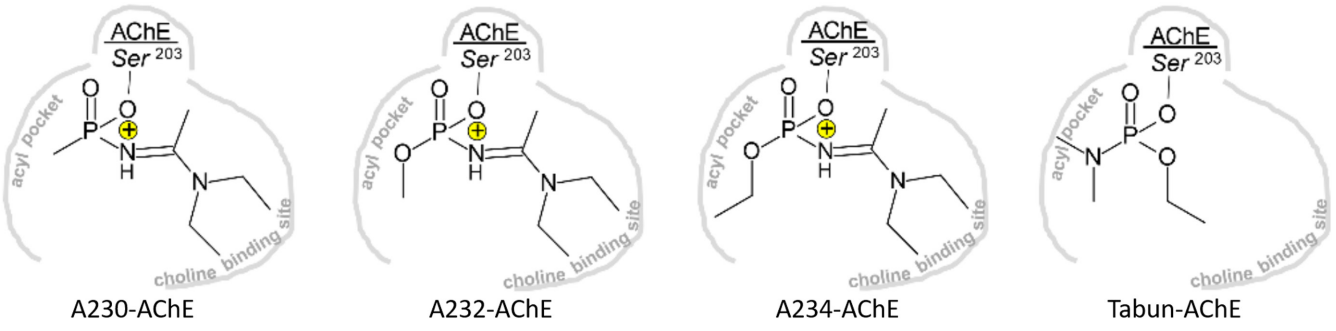

FIGURE 3 Schematic representation of OP-AChE conjugates formed by OP inferred from respective X-ray structures (adapted from Luedtke et al., 2021).

nucleophilic aldoxime (Luedtke et al., 2021). In the case of oximes that rely on stabilizations (or flexibility), their substituted pyridinium rings appear misaligned for nucleophilic attack, as was observed for 2PAM and HI-6 in the respective X-ray structures (PDB IDs 6NTN, 6NTM, 6NTG, 6CQW, 6WVO, 6WUY, and 5HF9). Reactive nucleophilic aldoxime groups are attached to their pyridinium rings in orientations that are non-effectively reactivated. More recent studies generated the centrally active zwitterionic monoxime RS194B (Radić et al., 2012; Sit et al., 2018) or zwitterionic LG-bisoximes (Gorecki et al., 2020) that approach to the conjugated phosphorus immediately from the direction of the peripheral site and seem to better comply with this structure-based requirement (Luedtke et al., 2021).

OXIME REACTIVATORS OF PHOSPHOAMIDATED AChE

A-agents are phosphoroamidates and structural analogs of the NA tabun, which is resistant to oxime reactivation when conjugated with the catalytic serine. Among the standard oximes, TMB-4 showed some potential in reactivating the tabun-AChE conjugate, but the dose required for in vivo application was too toxic (Čalić et al., 2006; Kovarik et al., 2009). So far, pyridinium K-oxime studies have shown that only pyridinium oximes with the *para*-positioned oxime group like K203, K048, K074, and K075 have sufficient potency, superior to TMB-4, to restore AChE activity after tabun inhibition (Čalić et al., 2006, 2008; Katalinić et al., 2018; Kovarik, Čalić, et al., 2007; Kovarik, Radić, et al., 2007; Kovarik et al., 2009).

A more recent study with a triazole-containing oxime library designed by double Nobel laurate Karl Barry Sharpless pointed out several oximes that were better reactivators of the tabun-AChE conjugate than obidoxime and 2PAM (Kovarik et al., 2019). The library of over 100 oximes, mainly those encompassing a triazole ring synthesized using the copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) to form a linking 1,4-triazole, was screened for oxime-dependent reactivation kinetics of tabun-, VX-, sarin-, cyclosarin-, and paraoxon-inhibited human AChE, and several novel oximes were particularly efficient reactivators in tabun inhibition (Figure 4). Out of a substantial number of 1,2,3-triazole-linked *N*-methylpyridinium and 2-methylimidazolium oximes, compounds were discovered with significantly improved in vitro reactivating efficacies for tabun-inhibited AChE compared to standard bis-pyridinium oximes. In initial in vivo studies, antidotal efficacy for three leads was established in tabun-exposed mice. Although several of the tabun antidote leads showed reasonable potency in reactivating the other tested OP-AChE conjugates, a universally superior oxime against various organophosphates was not identified.

Multidimensional and hydrogen bonding constraints in the AChE active center gorge conjugated with methylphosphonates, phosphorates, and phosphoramidates, mitigate against the likelihood of finding a single reactivator that would be the most efficient for NAs and their congeners. Nevertheless, the above findings offered a valuable and comprehensive platform for the further development of antidotes and scavengers against tabun and related phosphoramidate exposures, such as the Novichok series of compounds (Kovarik et al., 2019). Correspondingly, the same library of oximes and oxime RS194B was used for reactivation of AChE and BChE inhibited by fenamiphos and methamidofos (Čadež et al., 2021), which showed that for both insecticides, oximes 14A, 1A, and RS194B were the most effective reactivators of AChE and BChE, which can establish catalytic decomposition of OP in the circulation (Zandona et al., 2020) or pseudo-catalytic bio-scavenging in combination with an efficient reactivator of BChE (Kovarik, Čalić, et al., 2007; Kovarik, Radić, et al., 2007; Kovarik & Maček Hrvat, 2020). It is worth mentioning that administration of plasma BChE enabled the successful recovery of patients poisoned by A-agents (Steindl et al., 2021).

NEUROPROTECTION BY CNS-ACTIVE OXIMES

Acute OP exposure promotes neuroinflammation, which is considered to lead to the development of long-term consequences

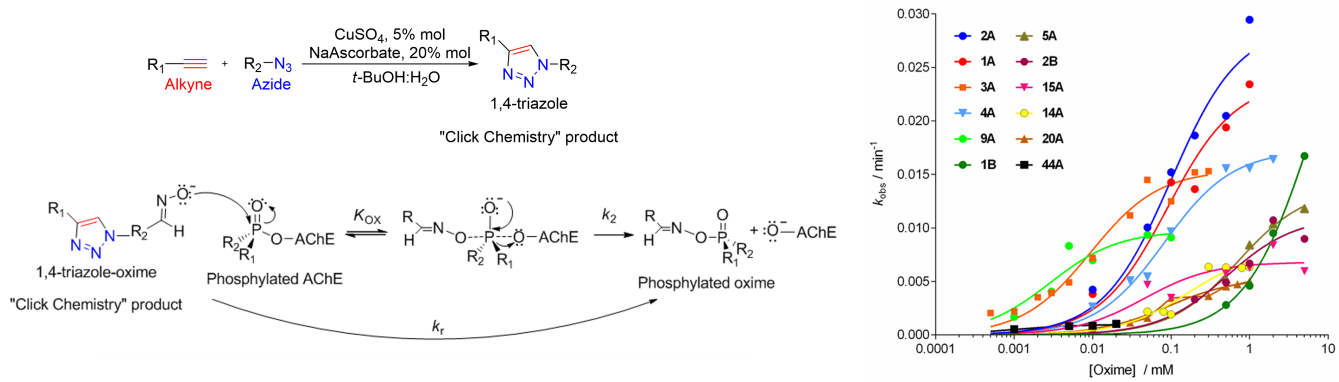

FIGURE 4 General scheme of copper(I)-catalyzed azide–alkyne cycloaddition employed to synthesize an oxime library tested for the antidotal activity against tabun and kinetics of reactivation by selected oximes. Non-linear regression fit was used for determination of reactivation constants given at the general scheme of oxime-assisted reactivation of phosphorylated AChE (adapted from Kovarik et al., 2019).

following the release of pro-inflammatory cytokine, inflammatory gene expression, and gliosis, with gliosis being the most consistently observed of these neuroinflammatory responses (Chen, 2012; Collombet, 2011; Collombet et al., 2005; de Araujo Furtado et al., 2012; Flannery et al., 2016; Guignet & Lein, 2019; Johnson et al., 2011; Spradling et al., 2011). So far, no studies have addressed the neurotoxicity of A-agents.

Recently, a study of neurotoxicity tested the hypothesis that treatment with uncharged, but ionizable oximes that cross the BBB and reactivate OP-inhibited synaptic AChE could protect the brain of mice exposed to a NA (Sit et al., 2018). The comparison of levels of the IBA-1 protein as a measure of microglial response, and the GFAP protein as a measure of astrogliosis, and neuronal cell viability detected following NeuN immunoreactivity, in the brain of sarin-exposed mice with and without treatment by the CNS-active oxime RS194B and 2PAM revealed that RS194B (but not 2PAM) attenuated the symptoms of poisoning quickly and acted neuro-protectively, as shown by employing lower IBA-1-levels compared to sarin-poisoned mice, and maintained GFAP at control levels. NeuN was significantly lower in mice exposed to sarin and those treated with oxime 2PAM. In mice treated with RS194B, the NeuN protein level was similar to that of the control group, which confirmed a high rate of diffusion across the BBB and the neuroprotective effect of the RS oxime.

CHOLINERGIC-ASSOCIATED EFFECTS OBSERVED FROM THE ASPECT OF OP TOXICITY

There are strong indications that both AChE and BChE play non-catalytic roles in the nervous system and that their inhibition could possibly affect neurodevelopment, where they have a "morphogenic" role in vertebrate systems (Layer, 1990; Pope & Brimijoin, 2018) as well as neurodegeneration. Synaptic toxicity, which is strongly associated with neurodegenerative diseases, was lately investigated based on a new angle, in relation to acute OP poisoning when hippocampal slice culture exposed to paraoxon showed a progressive reduction of pre- and postsynaptic biomarkers (Farizatto et al., 2019). If confirmed in an in vivo model, these observations could indicate a novel long-lasting mechanism underlying the delayed neurologic dysfunction after acute OP exposure.

It is worth mentioning that the long-term low dose inhibition of AChE by OPs and other contributing factors, such as physiological stress and other chemical exposures, in the absence of an acute cholinergic crisis may have also played a role in facilitating the development of a chronic neuroimmune disorder known as Gulf War Sickness. The myriad of cellular and molecular effects demonstrated in the brain as a result of these exposures have been hypothesized to be the consequences of phosphorylation of neuroinflammatory signaling mediators (Michalovicz et al., 2020) and involved transcriptional changes (Kaufer et al., 1998). In the case of a munition dump destruction during the Gulf War, it was found that it had contained sarin and cyclosarin. The US Army soldiers that were on the site were found to have exhibited less proficient neurobehavioral functioning on tasks involving fine psychomotor dexterity and visuospatial abilities 4–5 years after exposure (Heaton et al., 2007; Proctor et al., 2006). Furthermore, long-term cognitive impairments as a result of exposure to low-doses of sarin have been documented both in the Gulf War victims and in the first responders of the Tokyo subway attack (Miyaki et al., 2005; Nishiwaki et al., 2001), as well in active-duty soldiers who have been to Iraq (Loh et al., 2010.).

Neurological diseases may also derive from the disruption of the balance between oxidant and antioxidant components in neurons that is caused by OPs, which have a potent pro-oxidant activity that disturbs the mitochondrial function in neurons (Kumar, 2016). Overproduction of free radicals consequently leads to impaired mitochondrial function through the oxidation of proteins, DNA, and lipid content (Mostafalou et al., 2012). Mitochondrial impairment induces oxidative stress which is strongly associated with excessive cholinergic and glutamatergic activity, leading to cell dysfunction and finally, cell death (Aroniadou-Anderjaska et al., 2020).

One other aspect of OP exposure is that OPs exert immune side effects through mechanisms unrelated to AChE inhibition, but rather because of their binding to cholinergic receptors, thus modulating their expression (Mehrani & Golmanesh, 2008; Smulders et al., 2004; Trailović et al., 2017). AChE inhibition combined with the inhibition of nicotinic receptors (nAChRs) could explain the consequent blockade of the anti-inflammatory metabolic pathways. Moreover, overactivation of nAChRs and muscarinic receptors (mAChRs) leads to an increased calcium influx, which generates mitochondrial stress that promotes ROS production (Gorlach et al., 2015; Toledo-Ibarra et al., 2021). As a response to OP stimuli, immune cells can release a variety of inflammation mediators, activating pro- and anti-inflammatory processes and regulating intracellular pathways (Costa et al., 2020). OPs interfere with cell signaling pathways changing cytokine production, surface marker expression, and cell activation. TLR, IL-1R, IL-6R, and TNFR receptors get activated by pro-inflammatory cytokines and affect the intracellular signaling pathways like MAPK, NF-kB, JAK, or STAT, in return modulating the inflammatory genes, such as IL-1, TNF-α, IL-6, interferons, transforming growth factor (TGF), and chemokines (Farkhondeh et al., 2020; Fioranelli et al., 2021; Kianpour et al., 2021; Mitra et al., 2019; Proskocil et al., 2019).

Exposure to OPs can also lead to neurobehavioral deficits and affect the transcription of genes associated with learning, memory, and synaptic plasticity (Eckel-Mahan et al., 2008; Lonze & Ginty, 2002; Moshitzky et al., 2020; Verma et al., 2009). Yet, some studies suggest that chronic, unlike acute OP exposure, may trigger cholinergic anti-inflammatory pathways through the down-regulation of cholinergic receptors, which leads to suppressed T-cell activity and predisposition to cancer (Banks & Lein, 2012; Mitra et al., 2019; Tarkowski et al., 2004). In OP-induced neuroinflammation, microglia regulate the production of pro-inflammatory cytokines, causing damage to neurons and provoking neurodegenerative alterations. With this in mind, a beneficial impact of focusing on the therapies that will inactivate microglia and inhibit the inflammatory response was proposed

(Andrew & Lein, 2021; Deshpande et al., 2016; Fang et al., 2022; Karami-Mohajeri & Abdollahi, 2013; Naughton & Terry Jr, 2018; Ojha & Srivastava, 2012).

At the longer term, ACh accumulation because of OP-mediated AChE inhibition leads to the overstimulation of nicotinic or muscarinic receptors, consequent activation of the glutamatergic system and the development of seizures. OP-induced seizures and the blockade of the cholinergic anti-inflammatory response may lead to neuroinflammation and brain damage (Borovikova et al., 2000). A recently investigated approach for preventing this deleterious outcome is to block cholinergic and glutamatergic cascades. Promising results have been reported for tezampanel, an antagonist of GluK1 kainate and AMPA receptors, especially in combination with caramiphen, an anti-cholinergic and anti-glutamatergic agent (Aroniadou-Anderjaska et al., 2020). Recently, attention was drawn to OP compounds other than pesticides or NA like flame retardants. Zhong et al. (2020, 2021) showed that neonatal exposure to TDCPP, a phosphorus-based flame retardant common in consumer goods and baby products, induces neuronal damage through microglia-mediated inflammation, and that exposure to another flame retardant, TPHP causes abnormal learning and memory behaviors by disturbing synaptogenesis and neurotransmission.

REGULATORY RNAs AND THEIR FUNCTIONING IN THE CHOLINERGIC SYSTEM

At the upstream molecular level, systemic reactions to cholinergic signaling such as those caused by the cholinesterase inhibitors described above depend on both coding and non-coding RNAs. First and foremost, brain reactions to many small molecule agents that penetrate the BBB induce changes in the levels and activities of small regulatory RNAs, depend on the integrity of the BBB, which tends to be impaired under mental stress. For example, pyridostigmine, a carbamate AChE inhibitor and the most used one for several indications, penetrates into the brain and induces a transcriptional response, indicating a multi-leveled reaction to the combined impact of stress and cholinergic blockade (Friedman et al., 1996). This response is further seen in the hippocampus, and involves both choline acetyl transferase, leading to acetylcholine synthesis, and the muscarinic receptor (Kaufer et al., 1998). However, the complex impact of non-coding RNA controllers on brain-to-body functioning is largely unresolved, although microRNAs (miRs) and transfer RNA fragments (tRFs) rapidly acquire wide recognition as global controllers of regulatory processes (Figure 5).

Notably, both families of small non-coding RNAs targeting the cholinergic pathway transcripts (CholinomiRs and CholinotRFs) can operate by forming hybridization links with short, 6 or 7 nucleotide long sequences in messenger RNA chains carrying sequences complementary to miRNAs. This hybridization attenuates the capacity of those target mRNAs to get translated and may initiate their nucleolytic destruction (Bartel, 2009). However, such short

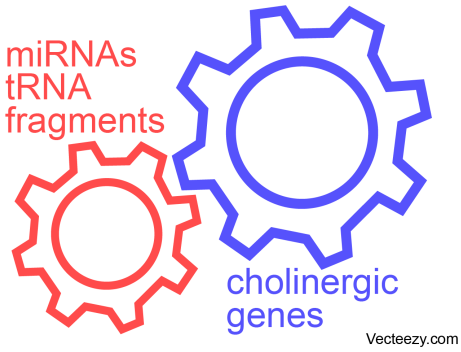

FIGURE 5 We have learned that cholinergic genes, presented here as the larger circle, may be regulated by small non-coding RNAs including microRNAs (miRs) and transfer RNA (tRNA) fragments (tRFs) that are represented as the smaller circle interacting with the larger one. The stripes in the drawing surrounding the larger circle represent the components constituting the nuclear membrane. Over the years, research from many laboratories including ours shed new light on the cholinergic mechanisms and their dependence on small regulatory RNAs. Hence, these studies should expand to the realm of employing these regulatory RNAs and their functioning for improving the therapeutics of malfunctioning cholinergic processes.

6 or 7 nucleotide long regions may occur in numerous mRNA transcripts; therefore, each CholinomiR may target numerous cholinergic mRNAs, which often contribute to this pathway's activities; reciprocally, each cholinergic mRNA may be targeted by numerous competing CholinomiRs or CholinotRFs (Lobentanzer et al., 2019; Winek et al., 2020). While relatively short tRFs (up to about 30 nucleotides in length) share their activities with miRs and can suppress the expression of target mRNA transcripts carrying complementary motif sequences, longer tRFs primarily interact with particular RNA-binding proteins. Whenever those short non-coding RNAs interact with mRNA transcripts belonging to the cholinergic family (e.g., ACh and choline receptors, synthesizing enzymes, etc.), they are part of the miR/tRF family that contributes to regulating the cholinergic system, and are defined as "CholinomiRs" or "CholinotRFs" (Shulman et al., 2023; Winek et al., 2020). Notably, these interactions are by definition the most complex and context dependent, since the repertoire of both the small non-coding RNAs and their so-called cholinergic targets are consistently subject to regulatory processes. Taken together, the cumulative impact of small non-coding RNAs could indicate a potential role for miRNA therapeutics against chronic cholinergic-related diseases, as distinct from acute OP poisoning. For example, using antisense oligonucleotides blocking the impact of specific miRs may limit the long-term inflammation damages which were observed in engineered mice with impaired functioning of miR-132 (Shaked et al., 2009). Detailed lists of CholinomiRs and CholinotRFs may be found in recent papers (Lobentanzer et al., 2019; Shulman et al., 2023; Winek et al., 2020).

When focusing on ACh-related pathways, one needs to combine advanced computational neuroscience with RNA sequencing technologies, transgenic engineering, and microscopy analysis tools

to investigate controller RNA functions in the healthy and diseased brain and body, which consistently communicate with each other via the vagus nerve. Furthermore, changes in miR genes may merely involve alterations of single nucleotides in the genome; consequently, evolutionary changes dictate genomic alterations, such that a significant fraction of the CholinomiRs are primate-specific silencers of multiple genes that compete with each other on suppressing their targets (Barbash et al., 2014). This, in turn has led to altered cholinergic brain-to-body regulation of anxiety and inflammation (Soreq, 2015). An example are Israeli human volunteers who contributed data on their stress responses and showed consistent cholinergic-associated pulse increases under fear of terror that coincided with changes in their blood cholinesterase activities along four successive years (Shenhar-Tsarfaty et al., 2015); in contradiction, a massive CholinomiRs decline occurs in Alzheimer's brains (Barbash et al., 2017) and CholinotRF levels decline in the nucleus accumbens of females living with Alzheimer's disease in association with their dementia symptoms (Shulman et al., 2023).

Importantly, altered levels of miRs that are directed at those mRNAs that were classified as "cholinergic" transcripts ("CholinomiRs"; namely, those miRs whose targeted mRNAs encode proteins associated with cholinergic pathways) are further accompanied by changes in the levels of long non-coding RNAs. Considering that non-coding RNAs mount up to 98% of our genome, these changes reflect a global impact of altered cholinergic transmission which extends to responses to therapeutics such as the routinely administered Statins (Simchovitz et al., 2020). Additionally, circular RNA transcripts may cause interventions with miRs by "sponging" their activities, for example in Parkinson's disease brains (Hanan et al., 2020; Simchovitz et al., 2020). At earlier ages, personal humiliation of newborn mice by a unilateral plucking of their whiskers leads to long-term changes in the mammalian cortical network of cholinergic interneurons, whose dendrite trees and location in specific cortical layers are altered while keeping the total length of their cumulative dendrites unaltered and presenting massively changed levels of particular dendrite regulatory transcripts (Yayon et al., 2023).

Taken together, this complex picture of multi-leveled changes in cholinergic features calls for assessing the links of regulatory RNA functioning with cholinergic target genes. In this context, an engineered strain of mice, with ablated 3′-terminal domain changing the recognition of the AChE-targeted miR-132 revealed a largely altered phenotype of elevated anxiety, hyper-activated locomotion, and increased inflammation (Shaked et al., 2009), which demonstrated a massive reaction to this alteration of the 3′-terminal non-translated domain interacting with a CholinomiR in the AChE transcript. However, CholinomiRs may fail to provide a rapid response under acute conditions, especially in nucleated blood cells that rapidly divide. Moreover, replacing these agents with other CholinomiRs would require transcription, processing, and transport to their sites of action, which may take long and be unsatisfactory under acute disease states. In such cases, for example in nucleated blood cells of patients following ischemic stroke, whose protection from pneumonia demands rapid changes in their immune response, CholinomiR levels decline while CholinotRFs accumulate (Winek et al., 2020).

In yet another example, CholinomiR-132 operates to regulate liver fattening (Hanin et al., 2018). Another CholinomiR, miR-211, targets a brain-abundant muscarinic receptor to prevent epileptic seizures if available at relatively high levels (Bekenstein et al., 2017). Intriguingly, identifying CholinomiR profiles in brain tissues discovered significant differences between men and women living with schizophrenia and bipolar disorder (Lobentanzer et al., 2019; Simchovitz-Gesher & Soreq, 2020), and mild social humiliation by a unilateral plucking of whiskers caused persistently modified structure of cholinergic interneurons in young mice (Yayon et al., 2023). Taken together, these studies could indicate a potential role for miRNA therapeutics against chronic cases, as opposed to acute OP poisoning. Moreover, this massive body of work leads to planning of molecular neuroscience-driven prevention and/or intervention protocols with diseases involving impaired ACh signaling, possibly via RNA therapeutics, which is no longer merely a dream. Several main topics come to mind when discussing such regulatory processes, as is briefly listed below for selected issues.

miRs AND TRFs MEDIATE THE REGULATION OF NEUROIMMUNE CHOLINERGIC SIGNALING

The work of Drs Kawashima and Tracey taught us over two decades that cholinergic signals may block or minimize the inflammatory pathway through the action of several key proteins (Borovikova et al., 2000; Fujii et al., 2017). However, those proteins as well, like any other protein, are subject to upstream control by RNA regulators. Supporting this view, miR-132 which carries a short sequence motif complementary to a 3′-region of AChE mRNA and suppresses its translatability controls neuroimmune signaling from brain to body; namely, it may regulate both neuronal stress responses and intestinal reactions (Barbash et al., 2014; Shaked et al., 2009). Moreover, other small RNAs may share such activities, such that tRFs carrying parallel sequence motifs may likewise affect the cholinergic pathway. Consequently, a shift from such CholinomiRs to CholinotRFs targeted to cholinergic transcripts in nucleated blood cells exerts a pronounced impact of stroke-induced neuroimmune changes (Winek et al., 2020); and parallel studies have aimed to pursue the impact of loss of CholinomiR controllers on hepatic hyperlipidemia (Hanin et al., 2018). Figure 6 shows schematically the alternate roles of miRNAs and tRFs in controlling stressful insults affecting the cholinergic signaling pathway. Briefly, this drawing presents acute situations as "SOS" ones and highlights the fact that the production of tRFs from either the nuclear or the mitochondrial genome merely requires a nuclease breakdown (shown as a hammer), unlike miRs which are produced by a longer process of transcription and subsequent transport.

CholinomiRs DECLINE MEDIATES THE LONG-LASTING SUPPRESSION OF STRESS REACTIONS

Over evolutionary times, the DNA of all organisms got modified thanks to the insertion of single nucleotide mutations or polymorphisms as well as because of viral-induced changes in the genome. Therefore, all humans carry individual copies of DNA. Because miRNAs are very small, every nucleotide modulation may create a new miRNA, meaning that numerous miRNAs have been altered along evolutionary time. Consequently, numerous miRNAs are primate specific, limiting the value of murine models of disease (Barbash et al., 2014). For example, the BChE gene includes a 3′-terminal region complementary to miR-298, which emerged in primates (Figure 7). Changes in the levels of miR-298 should therefore be studied in primates alone, limiting the value of murine models.

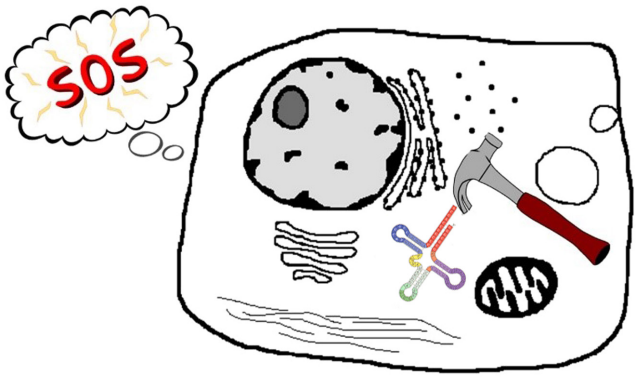

FIGURE 6 MiR activities depend on transcription, processing, and transport events and may take relatively long times, which hampers their prospects to handle the organismal responses to acute situations. As they may not suffice for controlling standard stressful insults, tRFs may take this regulatory role under acute situations. Production of tRFs merely requires a nuclease breakdown of the tRNA molecule from which they are derived. This is a relatively short process, and hence tRFs are essential when acute rapid response is required. The rapid production of tRFs by a single cut of pre-existing tRNA molecules in nucleated blood cells (e.g., by the nuclease angiogenin, presented in the figure as a hammer, and the blood levels of which are elevated in ischemic stroke patients) ensures efficient production of tRFs at times of urgent need.

In principle, miRNAs may exert their activities either when their levels increase (e.g., like miR-132 in intestinal tissues from Crone's disease patients) (Shenhar-Tsarfaty et al., 2016); or alternatively, when their levels decline. A relevant example is that of CholinomiR miR-211, which may attenuate epileptic seizures if its levels are high enough but the levels of which are too low to enable that role in Alzheimer's disease brains, such that a significant fraction of Alzheimer's disease patients suffer from epileptic seizures (Bekenstein et al., 2017). Intriguingly, altered levels of the miR-211 homologous miR-204 associate with stereotypic behavior in AChE-overexpressing mice (Moshitzky et al., 2020); likewise, a mutated miR-608-binding site in the AChE gene causes psychological arousal, elevated brain AChE, blood pressure, and inflammation (Hanin et al., 2014; Lin et al., 2016). Importantly, however, miRNA profiles in cortical brain tissues from men and women are not similar, such that the AChE-targeting miR-125b differs sex dependently in individuals living with schizophrenia (Lobentanzer et al., 2019). Additionally, men and women show distinct reactions to numerous therapeutics (Simchovitz-Gesher & Soreq, 2020). All of these phenomena may potentially be caused by the altered impact of

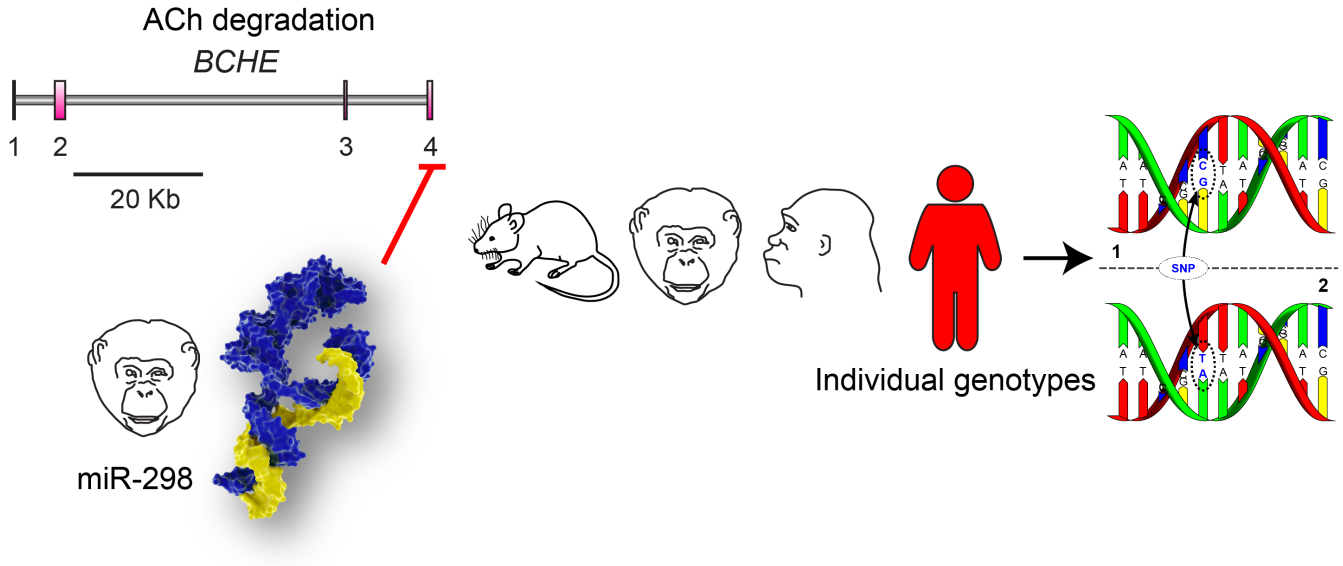

FIGURE 7 Primate-specific miRNAs evolved to modulate cholinergic signaling. Shown here is a scheme of the BChE mRNA transcript encoding butyrylcholinesterase (BChE), which includes a primate-specific recognition element to miR-298 in exon 4 (see Barbash et al., 2014). Evolutionary processes including single nucleotide mutations as well as single nucleotide polymorphisms (SNPs) alter the genotypes of all living organisms, leading to individual genotypes that may include changes in small genes such as miRNAs.

cholinergic signaling, for example in male and female patients with Alzheimer's disease where the decline of mitochondrially originated CholinotRFs in the nucleus accumbens of women patients reflects their accelerated cognitive decline compared to men living with Alzheimer's disease (Shulman et al., 2023). Figure 8 shows schematically the hierarchy of such stress insults as related to imbalanced cholinergic signaling.

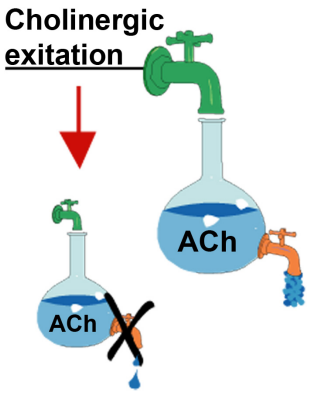

FIGURE 8 Cholinergic imbalance is susceptible to small RNAs-mediated regulatory changes under acute stressors. Balanced cholinergic signaling like any balanced process depends on two inverse events: on the one hand, it initiates under ACh production and on the other hand, it may be stopped by its degradation, which is schematically reflected as scales. As reported in our review, any change in the balance between these processes may alter ACh production, shown schematically as cross signs. Such blockade may operate via small molecule cholinesterase inhibitors (see above) or by non-coding RNA regulators thereof, including miRNAs and tRFs. In both cases, this blockade would lead to a pronounced imbalance accompanied by immediate and long-lasting changes in the biological roles of the corresponding regulator molecules, and will be subject to control by cholinergic signaling.

CholinomiRs-ASSOCIATED CHANGES IN STROKE, CARDIAC, AND INFLAMMATORY DISEASE

Collaborative studies by both basic research and clinical experts observed serum AChE changes that predict recovery and survival from ischemic stroke (Ben Assayag et al., 2010). Subsequent tests found a "changing of the guards" phenomenon in stroke patients' nucleated blood cells, where CholinomiRs are exchanged by cholino-targeted transfer tRfs (Winek et al., 2020). Inversely, inflamed intestinal biopsies from patients with Crone's disease showed drastic miR-132 increases, which indicated reduced AChE levels and up-regulated cholinergic signaling (Maharshak et al., 2013). Also, cardiac disease patients with lower AChE levels in blood samples were found to be under intensified risk of non-survival (Arbel et al., 2015). Furthermore, research volunteers presenting stress-response elevation of the C-reactive protein reflecting elevated inflammatory reactions showed consistent annual increases in their pulse levels (by about eight beats more per minute) over 4 successive years, reflecting increased risk of death because of all causes (Shenhar-Tsarfaty et al., 2015). The multi-organ features of cholinergic signaling are hence relevant for numerous organs, systems, and processes and are consistently subjected to regulation by miRNAs and tRFs (CholinomiRs, CholinotRFs).

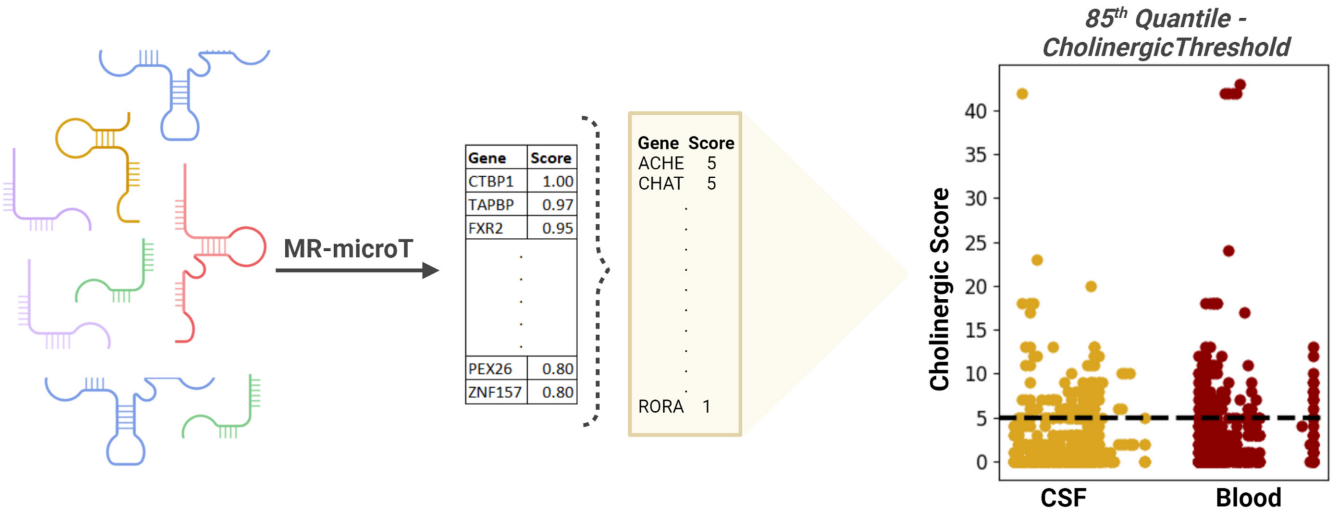

FIGURE 9 CholinotRFs target many mRNAs in the nucleus accumbens of women with Alzheimer's disease. The cholinergic potential of each tRF found in blood or CSF was set according to the number of predicted targets it had among a list of 94 known cholinergic genes weighted according to their direct effect on the cholinergic system. The cholinergic score of each tRF was set as the number of cholinergic genes multiplied by their weights, and the cholinergic threshold was set as the 85th quantile of the cholinergic scores in the CSF and blood independently. Importantly, depletion of mitochondrial genome-originated CholinotRFs reflected cognitive decline, rather than the pathology of women patients living with Alzheimer's disease (adapted from Shulman et al., 2023).

CHOLINERGIC-MEDIATED RNA METABOLISM IMPAIRMENTS ARE PROMINENT IN NEURODEGENERATING BRAIN AND BLOOD SAMPLES

Compelling evidence indicates that inhibiting the catalytic activity of enzyme modulators of cholinergic signals induces massive functional alterations, in particular under aging. Recent studies from the United States, mainland Europe, and the United Kingdom all show elevated risk of dementia in patients subjected to long-term treatment with anti-cholinergic medications (e.g., blockers of muscarinic receptors because of incontinence; Coupland et al., 2019). At the neurochemistry level, such medications also alter the levels of RNA regulators of cholinergic signaling (Winek et al., 2021). Furthermore, RNA metabolism-related brain damages were observed in Alzheimer's disease brains in cholinergic neurons and in Parkinson's disease brains from human donors and cholinergic-deprived mice (Barbash et al., 2017; Berson et al., 2012; Hanan et al., 2020; Paldor et al., 2022; Shulman et al., 2023). Altogether, combining these numerous lines of studies strongly suggests rapid and pronounced responsive reactions at the level of small RNA regulators to those cholinergic signaling changes that are intertwined with modulations of the protein targets of those regulatory RNAs. As shown in Figure 9, CholinotRFs target many mRNAs in the nucleus accumbens of women with Alzheimer's disease.

IMPLICATING CHOLINERGIC MECHANISMS TO STRESS-RELATED DAILY LIFE

The first part of our review mainly relates to either the immediate responses to acute poisoning with anti-cholinesterases or to treatment with small molecule agents aimed to save the lives of acutely poisoned individuals. However, the cholinergic system is also impaired under neurodegeneration and psychiatric diseases (Barbash et al., 2017; Moshitzky et al., 2020), and its fine tuning may also impact the daily lives of stress-exposed individuals. While the stress-afflicted daily life in 2023 Israel is seemingly tolerable, its long-term stress-related impact notably increases the risk of diverse disease. Supporting this notion, a collaborative Big-Data study with clinical experts combined machine learning with patient serum tests (Shenhar-Tsarfaty et al., 2015) linked anxiety and metabolism regulating miRNAs that affect both inflammation and metabolic disease (Meydan et al., 2016; Shaked et al., 2009). Yet more recently, mapped cortical cholinergic interneurons in the brain of early-stressed mice revealed altered profile of their stress-induced controller transcripts, which led to altered maps of the dendritic branches and cortical layer positions of cortical cholinergic interneurons (Yayon et al., 2023). Cholinergic-regulating small RNAs are hence causally involved in controlling the cholinergic balance at both the basic and translational biomedical neuroscience aspects and across the complete scope of mild and acute stressors.

AUTHOR CONTRIBUTIONS

ZK and HS contributed to conception, design of this work, and drafted a significant portion of the manuscript. All authors contributed to revise the manuscript. All authors read and approved the present version of manuscript to be published. All co-authors contributed to the writing and style of this mini-review.

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CONFLICT OF INTEREST STATEMENT

HS and ZK are co-editors of this special issue. HS is an editor for the Journal of Neurochemistry. The authors declare no other potential conflict of interest.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/jnc.15887.

DATA AVAILABILITY STATEMENT

Data sharing is not relevant since no new data were generated or analyzed for this Review article.

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