11-1-2018

Gene Expression Changes by Neuroprotectant Novel Antidotes to Organophosphates

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By

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A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Undergraduate Honors
in Biological Sciences
in the Department of Biological Sciences

Mississippi State, Mississippi

November 30, 2018

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my primary investigator, Dr. Janice Chambers. I am wholly indebted to her for her patience, generosity, and mentorship, and it has been an absolute privilege and true pleasure to work in her laboratory over the past three years.

I would also like to thank:

Mary Beth Dail, who provided endless guidance and encouragement and without whom this work would be impossible.

Dr. Eddie Meek, who trained me when I first arrived and has continued to be invaluable.

The late Dr. Howard Chambers, who designed the novel oximes at the heart of this project.

Dr. Seth Oppenheimer, for all of the opportunities I have received as a Provost Scholar. It's been a wonderful three and a half years, and I am beyond honored to be one of the first to graduate.

The Shackouls Honors College, for providing me with constant support, both financial and human, throughout my time at MSU.

This work was funded by the National Institutes of Health CounterACT program U01 NS083430, and by the Mississippi State University Office of Research and Economic Development Undergraduate Research Program.

My sincere thanks to the Center for Environmental Health Sciences, the College of Veterinary Medicine, and everyone from my laboratory, both past and present.

ABSTRACT

Recent assassinations and terrorist attacks demonstrate the need for a more effective antidote against nerve agents and other organophosphate (OP) compounds which inhibit the nervous system enzyme acetylcholinesterase (AChE). This study explored the changes in gene expression induced by novel phenoxyalkyl pyridinium oximes (U.S. patent 9,277,937) that have demonstrated the ability to cross the blood-brain barrier and attenuate brain damage in a rat model. More specifically, this study determined whether rats treated with high levels of a surrogate for the nerve agent sarin (nitrophenyl isopropyl methylphosphonate; NIMP) and rats treated with NIMP followed by a novel oxime displayed significant differences in their levels of messenger RNA.

First, RNA samples were drawn from brain regions susceptible to damage by seizure-inducing OP exposure levels (piriform cortex and hippocampus), harvested from rats treated with NIMP alone or NIMP followed by a novel oxime. The samples of rat brain RNA were converted to complementary DNA (cDNA) via reverse transcription. The resultant cDNA was used in quantitative polymerase chain reaction (qPCR) to examine expression levels of genes that reflect inflammation (*Ccl2*) and nerve growth and repair (*Ngfr*) in the piriform cortex, along with expression levels of genes involved in potential brain damage repair (*Bdnf*) and astrocyte damage (*Gfap*) in the hippocampus. Following these initial four genes, two additional immediate early genes were evaluated, also in the hippocampus: *Fos*, associated with signal transduction, and *Bcl2l1*, which may provide neuroprotection. Changes in the levels of gene expression were quantified and compared across the experimental animal groups, i.e., NIMP alone, NIMP plus novel oxime, novel oxime alone, and vehicle controls.

Bdnf, *Fos*, and *Bcl2l1* showed statistically significant transcriptional changes between

several of the NIMP only and oxime combination groups, indicating that novel oximes may be able to directly affect these genes' expression and thereby attenuate NIMP-related neural damage. While the other genes did not display statistical significance, average cycle threshold (Ct) values for NIMP, oxime, combination, and vehicle groups still demonstrated notable differences suggestive of oxime therapeutic efficacy. Ultimately, these comparisons should demonstrate whether novel oximes can remediate some of the damage from OP poisoning and stimulate tissue repair. The data produced also support the OP-induced neuropathological and behavioral changes, as well as the therapeutic effects following novel oxime treatment, observed in live rats.

INTRODUCTION

The toxicity of organophosphates (OPs), including nerve agents and some insecticides, stems from their inhibition of the enzyme acetylcholinesterase (AChE) in the central and peripheral nervous systems. The neurotransmitter acetylcholine (ACh) is present at neuromuscular junctions and throughout the central nervous system, making it instrumental in muscle contraction, respiratory function, functions of the autonomic nervous system, and higher brain functions such as cognition.

Normally, within milliseconds of release and subsequent binding to its receptors, ACh is hydrolyzed by AChE into choline and acetic acid, allowing for normal cholinergic function. Any delay in the degradation of ACh has an immediate effect as a result of the accumulation of ACh, which causes prolonged transmission and excitation. Organophosphates are some of the most potent AChE inhibitors, capable of persistent inhibition which can last days and may become irreversible if a time-dependent dealkylation process known as “aging” occurs. Inhibition occurs following binding of the OP to the AChE active site, as the result of phosphorylation of the serine at the active site (Satoh and Gupta, 2010).

Following exposure to an OP, the subsequent buildup of ACh in neuromuscular junctions and synapses leads to overstimulation of both muscarinic and nicotinic acetylcholine receptors. Overstimulation of muscarinic receptors prompts acute signs including salivation, urination, diarrhea, bradycardia, and broncho- and laryngospasm, while nicotinic effects include vomiting, muscular fasciculation, and paralysis of the respiratory muscles (Satoh and Gupta, 2010). High exposure may also prompt symptoms originating within the CNS, including generalized or partial seizures, impaired coordination, and respiratory failure due to inhibition of the brain stem’s respiratory center. OP-induced overstimulation of muscarinic ACh receptors within the brain has

been connected to the onset of seizures, and overstimulation of nicotinic receptors may lead to the propagation of excitatory postsynaptic potentials.

It has also been demonstrated that seizure-like activity prompts inflammatory responses in the brain, impacting neuronal excitation and epileptic processes. Proinflammatory cytokines (such as *Ccl2*) are produced during seizures, while activation of astrocytes and microglia contributes to damage following convulsions. Seizures also damage the blood-brain barrier (BBB), causing leakage which enables the entry of peripheral inflammatory molecules that prompt further injury (Satoh and Gupta, 2010). As a result, prolonged seizures have been implicated as one of the main contributors to permanent brain damage, manifest through behavioral alterations and cognitive deficits lasting years after exposure. Two particular brain regions, the piriform cortex and hippocampus, have been observed throughout the literature to experience tissue damage more strongly and more frequently following seizure-inducing levels of the nerve agent sarin (Spradling *et al.*, 2011).

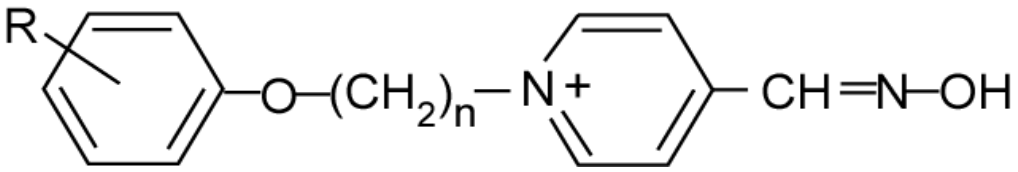
As evidenced by recent events, OP poisoning is a concern not only for members of the military but for civilian populations either accidentally or purposefully exposed to nerve agents and OP pesticides. The current treatment for OP poisoning involves atropine, which blocks peripheral muscarinic ACh receptors, and pralidoxime (2-PAM), an oxime which reactivates the inhibited AChE. The main deficiency of this therapy is 2-PAM's inability to cross the BBB to reactivate brain AChE as a result of the permanent positive charge contained within 2-PAM's pyridinium ring. Thus, while the current antidote can enable survival through the alleviation of peripheral effects, 2-PAM is unable to prevent permanent brain damage. Novel substituted phenoxyalkyl pyridinium oximes (U.S. patent 9,227,937) synthesized by this laboratory incorporate increased lipophilicity, allowing them to more easily cross the BBB, and have provided

convincing evidence of brain penetration and AChE reactivation *in vivo* using rats, although they are typically less effective reactivators (Chambers *et al.*, 2013).

Earlier *in vivo* survival experiments have shown that rats treated with a combination of a novel oxime alone or in combination with 2-PAM usually displayed lower levels of mortality than those treated with 2-PAM alone. Some of these novel oximes also showed a shorter time to cessation of seizure-like behavior than 2-PAM, strongly suggesting entry of the oximes into the brain (Chambers *et al.*, 2016). Additionally, the lead novel oxime showed a suppression of glial fibrillary acidic protein (*Gfap*) in histochemical studies that 2-PAM did not (Pringle *et al.*, 2018) and neuroprotection as indicated by NeuN nerve cell nuclear staining, while 2-PAM did not (unpublished). These histological data also strongly suggest that some of our novel oximes can enter the brain. Other studies conducted by this laboratory have also produced preliminary data on altered gene expression in the piriform cortex. These observed changes, some of which indicated inflammation and histological damage, followed the administration of high levels of NIMP in laboratory rats. One of the lead novel oximes showed some ability to reduce these changes in gene expression prompted by NIMP. Ultimately, the changes in gene expression discussed here will help to further substantiate the novel oximes' capacity for attenuating brain damage.

In place of actual nerve agents, this lab has developed non-volatile surrogates for use in experimentation. While the surrogates differ by leaving group, they phosphorylate AChE with the same chemical moiety as their respective nerve agents, making them highly relevant for both *in vitro* and *in vivo* use (Meek *et al.*, 2012). In this study, nitrophenyl isopropyl methylphosphonate (NIMP), a sarin surrogate, was employed. Messenger RNA (mRNA) was used as an indicator of gene expression because it codes for the production of new proteins and thus reflects the response of the tissue to chemical challenge.

Ultimately, the objective of this study was to evaluate any changes in gene expression prompted by the novel oximes. It was hoped that these changes would indicate the oximes' therapeutic properties, namely the ability of the novel oximes to up-regulate neuroprotective genes and/or down-regulate inflammatory or damage-reflective genes. The results of this study, therefore, serve to expand on previously observed changes induced by novel oximes and establish their potential in attenuating OP neural damage via changes in gene expression.



Oxime 1	n = 4	R = 4-Cl-
Oxime 20	n = 4	R = 4-Ph-CH_2-O-

FIGURE 1: Generic structure of novel substituted phenoxyalkyl pyridinium oximes, where n is the number of C's in the alkyl chain and R is the substitution on the phenoxy moiety.

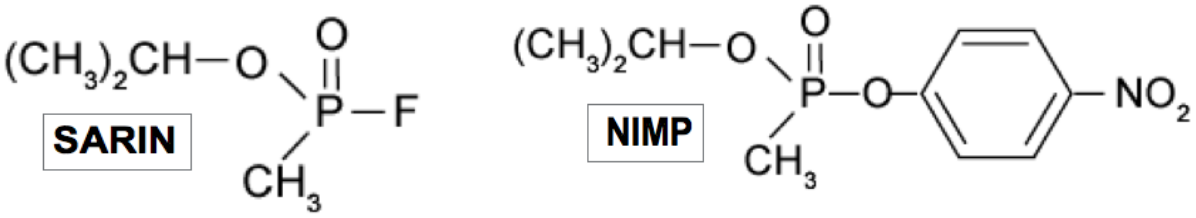


FIGURE 2: Structures of sarin and its surrogate, NIMP.

METHODS

Before the start of this study, adult male (250-300g) Sprague Dawley-derived rats were administered subcutaneously (SC) either a high sublethal dosage of the sarin surrogate NIMP (0.325mg/kg) or vehicle (DMSO). At 1-hour post-NIMP exposure, peak inhibition (about 80%) of brain AChE and signs of hypercholinergic toxicity, including seizure-like behavior, occur. At this point, rats were administered intramuscularly (IM) 146 μmoles/kg of a novel oxime or 2-PAM in Multisol (48.5% H_2O, 40% propylene glycol, 10% ethanol, 1.5% benzyl alcohol) or the vehicle alone (Multisol). This oxime dosage is equivalent to the human use of three auto-injectors, the method of delivery used by the military following an OP poisoning event. Three rats were collected for each treatment, representative of either an oxime alone, NIMP alone, a combination of oxime and NIMP, or the vehicles. Rats were euthanized 2 hours after oxime administration. The piriform cortex and hippocampus were quickly dissected, placed in RNAse-free tubes, snap frozen in liquid nitrogen, and stored at -80°C until used for total RNA extraction. Total RNA from both brain regions was purified using the RNeasy® Plus Mini Kit from Qiagen (Valencia, CA), following the manufacturer's instructions.

At the start of the present work, the frozen RNA samples were removed from long-term storage. The purity and quantity of the samples were determined by Abs 260/230 and 260/280 ratios using a Nanodrop ND-1000 spectrophotometer, in order to ensure that the RNA had not degraded, with a concentration of no less than 40 ng/μL. The RT2 First Strand Kit from Qiagen was used to reverse transcribe 0.5μg of total RNA into cDNA following the manufacturer's instructions. Each RNA sample was treated with a genomic DNA elimination mix prior to reverse transcription to eliminate any remaining genomic DNA. RE3 reverse transcriptase was primed in

an unbiased manner with random hexamers and oligo-dT primers. The plate was then spun in a Marathon tabletop centrifuge at roughly 1900 RPM for one minute.

PCR, a method commonly used in assessing genes, determines relative values for gene expression via fluorescence emissions. Once the mix's DNA polymerase synthesizes a new DNA strand, its exonuclease activity separates the fluorescent dye from a quencher dye. As PCR continues, more and more of the dye is released, intensifying fluorescence. The level of fluorescence, quantified by the PCR machine, is indicative of the amount of mRNA originally present in the tissue. More intense fluorescence, or an earlier break of the machine's cycle threshold (Ct), indicates higher initial mRNA levels. In measuring fluorescence against a threshold, the PCR machine takes into account the emission intensity of the reporter dyes (HEX and FAM) to the reference dye ROX. A more highly expressed gene produces fluorescence in a smaller number of cycles, reaching the threshold at an earlier cycle. Thus, more gene expression is indicated by a smaller Ct value, or the number of cycles required to cross the threshold.

Duplex qPCR was performed on the Stratagene MX3005 qPCR system with IDT PrimeTime® qPCR Assays. Assays contained 500 nm primers and 250 nm fluorogenic probes designed by IDT for both the genes of interest and RPLP1. The mRNA values were normalized using the housekeeping gene RPLP1 (ribosomal protein, large, P1), chosen because it had previously displayed the smallest changes between sample groups in arrays containing the same oximes and oxime combinations. RPLP1, notated by the marker dye HEX, was run in each well in addition to the gene of interest in order to provide a "housekeeping" or baseline comparison, as well as a positive control. Each duplex qPCR was performed in triplicate in a final reaction volume of 20µl, using PrimeTime Gene Expression Master Mix, which included addition of the reference dye ROX per the manufacturer's instructions. The cDNA was assayed at concentrations

empirically determined for each gene of interest, denoted by the marker dye FAM, and ranging from 36 to 75 ng. The cycling protocol was as follows: 95° C for 3 min, followed by 40 cycles of 15 s at 95° C, and then 1 min at 60° C. In all experiments, three wells without template were included as negative controls. Standard curves of both the housekeeping gene (RPLP1) and the gene of interest were used to calculate the PCR efficiency using dilutions of a template mixture from pure to 1:1000. Ct values for each well were calculated using the Stratagene MxPro qPCR software by manually defining the non-adaptive baseline and threshold per the manufacturer's instructions. The Ct values of each gene of interest were normalized to the Ct values of RPLP1 to correct for variations in pipetting. A total of 3 biological replicates and 9 technical replicates were collected for each treatment.

After normalization, mean Ct differences from each treatment and the vehicle controls were analyzed for outliers and normal distribution using the Kolmogorov-Smirnov test. After outlier removal, some of the groups were normally distributed, but unequal variances barred the use of an ANOVA. Instead, the non-parametric Kruskal-Wallis test was used to look for statistically significant differences among the groups at $P < 0.05$. When difference was found, the Mann-Whitney U test was used to determine which of the groups were statistically different at $P < 0.05$. The statistically different groups were then corrected for multiple comparisons using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995).

RESULTS

As defined in this study, a statistically significant change is one which produces a P value of $P < 0.05$, indicating a 90% confidence interval. Considering all six genes of interest, the following P values were determined:

- *Ccl2*: no significant changes (P = 0.26)
- *Ngfr*: no significant changes (P = 0.16)
- *Gfap*: no significant changes (P = 0.99)
- ***Bdnf*: significant changes (P = 0.000021)**
- ***Fos*: significant changes (P = 0.045)**
- ***Bcl2l1*: significant changes ($P = 1.0 \times 10^{-15}$)**

Bdnf

Of the initial four genes studied, *Bdnf* was the only gene to display statistically significant changes (P = 0.000021) among experimental groups. NIMP alone and Oxime 1 alone both repressed *Bdnf* expression to similar levels, displaying the greatest down-regulation of *Bdnf* and prompting no statistically significant differences compared to the control. However, when compared to that observed for the NIMP only group, *Bdnf* expression was significantly increased by 2-PAM and Oxime 20 alone as well as by the combination groups of NIMP plus 2-PAM and NIMP plus Oxime 1. These four groups also displayed statistically significant differences when compared to the vehicle control group, increasing expression of *Bdnf*. Oxime 20 alone and 2-PAM alone increased *Bdnf* expression to similar levels, with both groups displaying statistical significance when compared to Oxime 1 alone.

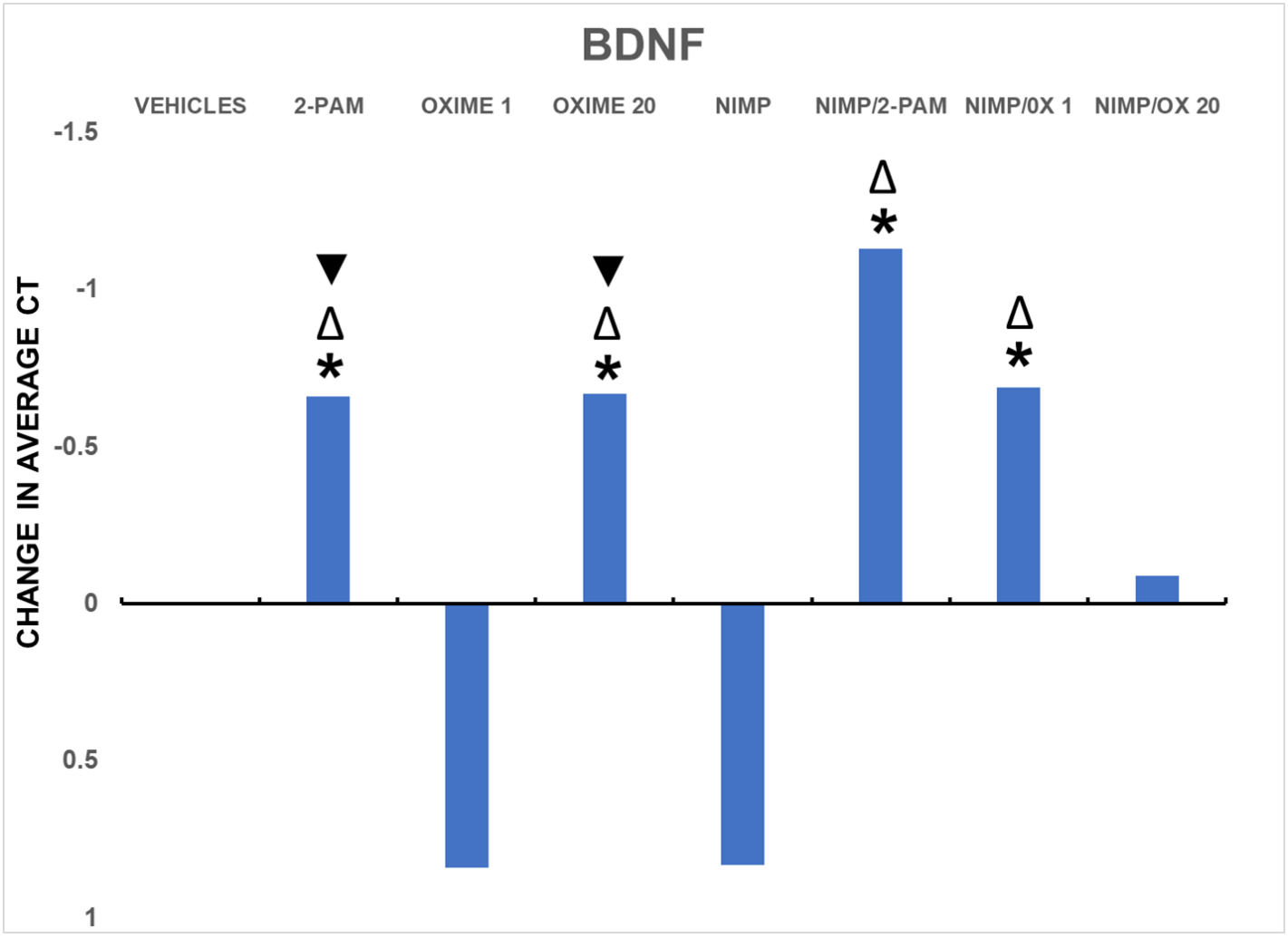


FIGURE 3: The vertical axis denotes changes in hippocampal *Bdnf* average Ct relative to the vehicle control value, which was set equal to zero. A value above the line reflects an increase in expression, and a value below the line reflects a decrease in expression. The * symbol indicates a statistically significant difference from NIMP, and the Δ symbol indicates a statistically significant difference from the vehicle group. The ▼ symbol indicates a statistically significant difference from Oxime 1. Statistical significance determined at $P < 0.05$.

Fos

The Ct values for *Fos* displayed statistical significance, with P = 0.045 across all experimental groups. The highest level of *Fos* expression was observed for the NIMP only group. Significant differences were observed between the 2-PAM and Oxime 20 only groups, with the 2-PAM expression levels approximating those of the vehicle control group. Oxime 20, conversely, significantly up-regulated *Fos*. Significant differences were also seen between the vehicle and NIMP plus 2-PAM groups, with the 2-PAM combination group down-regulating *Fos* to the greatest degree, lowering expression beyond even that of the control group.

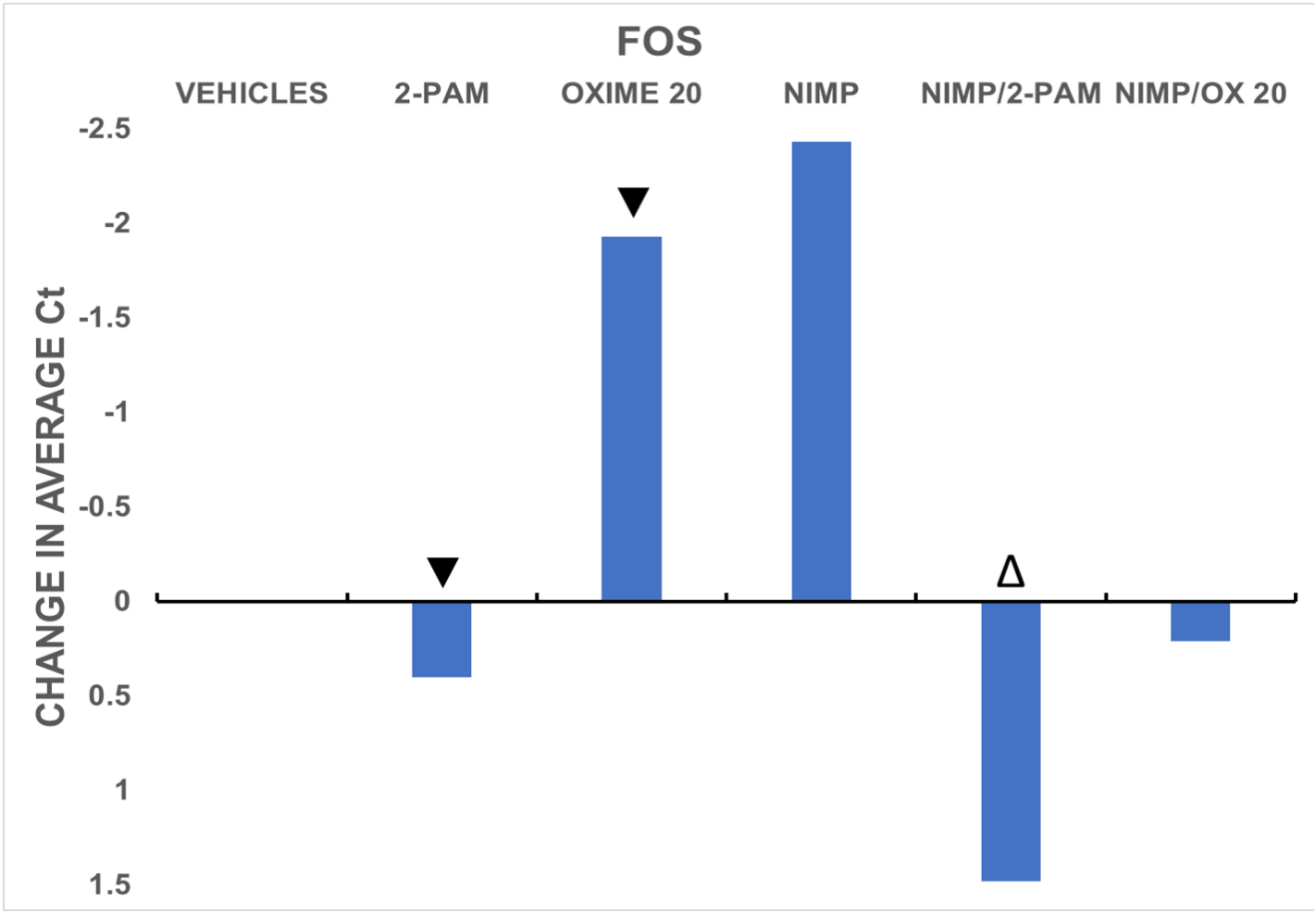


FIGURE 4: The vertical axis denotes changes in hippocampal *Fos* average Ct relative to the vehicle control value, which was set equal to zero. A value above the line reflects an increase in expression, and a value below the line reflects a decrease in expression. The Δ symbol indicates a statistically significant difference from the vehicle group. The ▼ symbol indicates a statistically significant difference between 2-PAM and Oxime 20. Statistical significance determined at $P < 0.05$.

Bcl2l1

The Ct values for *Bcl2l1* displayed marked statistical significance, with $P = 1.0 \times 10^{-15}$ across all experimental groups. The NIMP only group down-regulated *Bcl2l1*, prompting expression levels below those observed for the vehicle control group. Significant differences were observed between the NIMP only group and the vehicle, 2-PAM alone, and both combination groups. While either oxime alone was unable to increase *Bcl2l1* expression, both the NIMP plus 2-PAM and NIMP plus Oxime 20 combination groups prompted expression of *Bcl2l1* at levels which approximated those seen for the vehicle control group.

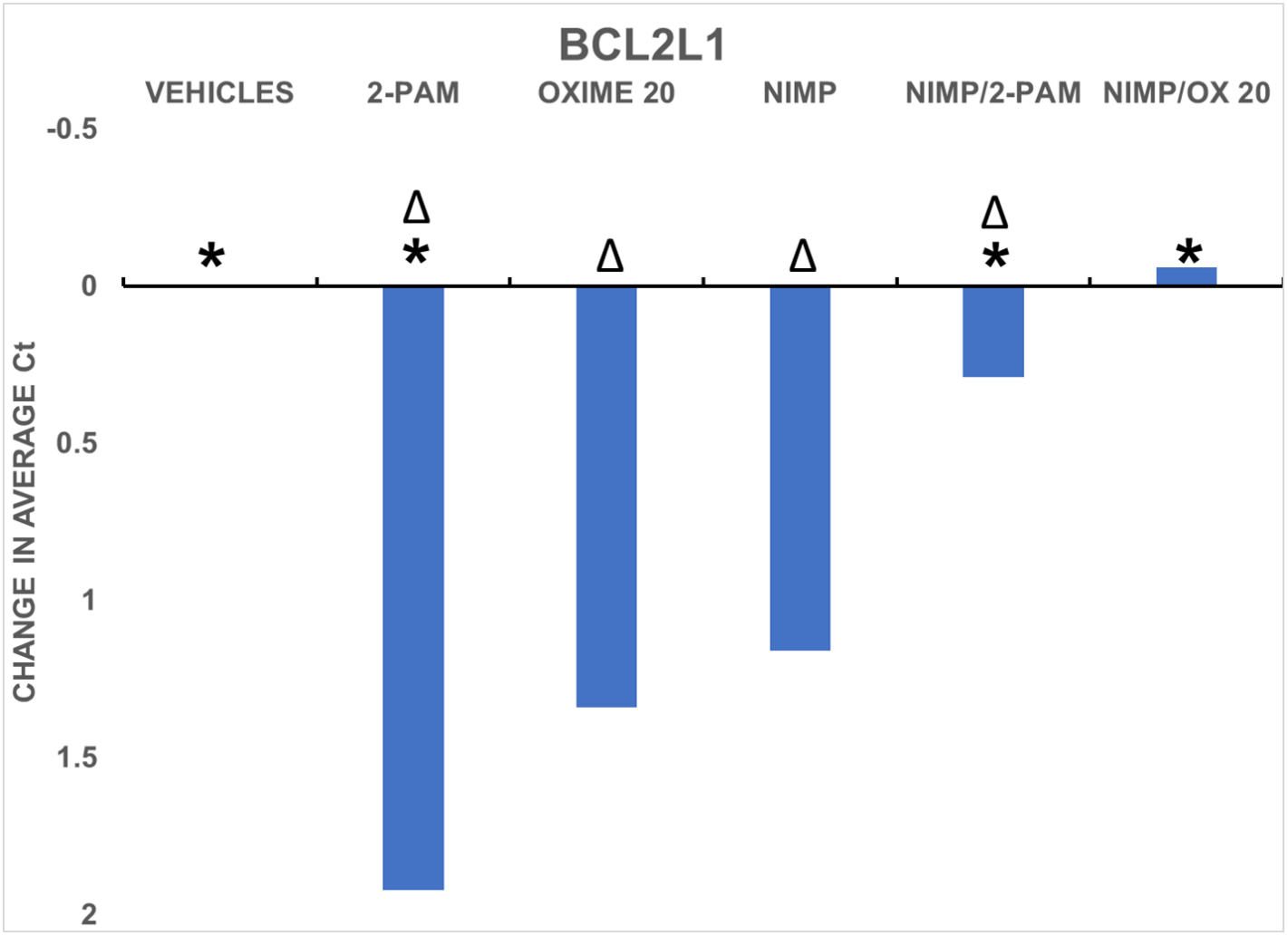


FIGURE 5: The vertical axis denotes changes in hippocampal *Bcl2l1* average Ct relative to the vehicle control value, which was set equal to zero. A value above the line reflects an increase in expression, and a value below the line reflects a decrease in expression. The * symbol indicates a statistically significant difference from NIMP, and the Δ symbol indicates a statistically significant difference from the vehicle group. Statistical significance determined at $P < 0.05$.

DISCUSSION

Ccl2 (C-C motif chemokine ligand 2; piriform cortex)

The gene *Ccl2*, a proinflammatory cytokine, has been associated in the literature with inflammation and immune function (Liu *et al.,* 2017). Since inflammation is one of the factors implicated in long-term brain damage, the novel oximes were expected to suppress the inflammatory response prompted by OP exposure. In previous work by this lab, *Ccl2* demonstrated a strong response with inflammatory PCR arrays (Dail, personal comm., 2018). Thus, in this study, *Ccl2* acted as a positive control, and was the first gene to be run using RNA from the piriform cortex (PC).

Both PC genes were only assayed with Oxime 1, leaving four experimental groups: NIMP; Oxime 1; NIMP plus Oxime 1; and vehicle control. While the results were not statistically significant, multiple trends may still be observed. As expected, the lowest level of *Ccl2* expression was demonstrated by the vehicle group. NIMP prompted the highest level of expression, indicating that NIMP plays a role in activating *Ccl2* and causing the resultant inflammation.

Oxime 1, when administered alone, trended toward a marked repression of *Ccl2* expression, indicating its ability to attenuate OP-induced inflammation. The combination of NIMP plus Oxime 1 elicited higher gene expression than that of Oxime 1 alone, but lower expression than that of the NIMP only group. These results suggest that Oxime 1 is able to decrease transcription of *Ccl2*. As *Ccl2* is a proinflammatory gene, Oxime 1 is thus acting therapeutically, demonstrating its potential to attenuate the factors involved in permanent brain damage.

Ngfr (nerve growth factor receptor; piriform cortex)

In the literature, the neurotrophin nerve growth factor (*Ngf*) is acknowledged as essential in the postnatal development of basal and striatal cholinergic neurons that project into the hippocampus and regulate cognitive functions such as learning and memory. Previous experiments have shown that exposure to the OP chlorpyrifos stimulates significant decreases in *Ngf* mRNA levels, indicating OP-induced damage as a precursor for inhibited neural development (Betancourt *et al.*, 2006).

As a potential limiting factor, it should be noted that *Ngfr* was one of multiple genes isolated from the original *Ngf* gene. Thus, it is possible that the chosen gene for this study, *Ngfr*, may not have been the best representative of *Ngf* functionality in relation to NIMP and oxime treatments.

Like *Ccl2*, *Ngfr* was only assayed with Oxime 1, leaving four experimental groups. While no significance was found, multiple trends were still established. Oxime 1 alone prompted the highest level of *Ngfr* expression, surpassing control levels. However, the combination of NIMP plus Oxime 1 displayed the lowest level of *Ngfr* expression, even falling below that observed for the NIMP group. Thus, it is possible that Oxime 1, in combination with NIMP, actually works to suppress *Ngfr* expression levels, suggesting that Oxime 1 is unable to attenuate NIMP damage by up-regulating *Ngfr*.

Gfap (glial fibrillary acidic protein; hippocampus)

In the literature, *Gfap* expression increases are associated with neural damage and neurodegeneration, as *Gfap* is up-regulated following neural injury and resultant cellular signaling in astrocytes (Benkovic *et al.,* 2006). Gene expression levels of *Gfap* were also observed to

increase following chlorpyrifos exposure in rats, suggesting heightened astrocyte activity as a response to OP poisoning (Betancourt *et al.*, 2006). Previous experiments performed by this laboratory have also demonstrated that, following challenge with an OP, administration of either no oxime therapy or 2-PAM resulted in increased *Gfap* expression in the piriform cortex and hippocampus. However, administration of novel Oxime 20 resulted in *Gfap* expression levels which were statistically indistinguishable from control levels, providing support for the oximes' ability to enter the brain and promote normalization (Pringle *et al.*, 2018).

In support of these findings, this project found that the NIMP group prompted *Gfap* expression higher than that observed for the vehicle control group, indicating that *Gfap* is activated by OP exposure. While no significance was found, some of the novel oxime groups still elicited demonstrable effects on *Gfap* expression. In suggesting their efficacy, two of the oximes given alone, 2-PAM and Oxime 1, were able to reduce *Gfap* expression levels, and the combination of NIMP plus Oxime 1 lowered *Gfap* expression below control levels. Thus, at least one novel oxime, Oxime 1, supports the desired trend toward suppression of *Gfap* levels. Following from previous findings, the results of this study suggest that Oxime 1, both alone and after NIMP exposure, may therefore have therapeutic attributes in its ability to down-regulate *Gfap* and thus prevent neural damage.

Bdnf (brain-derived neurotrophic factor; hippocampus)

In the literature, *Bdnf* has been associated with neural growth and repair. *Bdnf* mRNA levels have been reported to increase following chlorpyrifos and methyl parathion exposure in the cerebral cortex and hippocampus of juvenile rats, indicating that the proteins encoded by *Bdnf* possess some relation to neuroprotection (Betancourt *et al.*, 2007). *Bdnf* transcriptional increases

have also been shown to prompt neuronal growth and repair following subarachnoid hemorrhage (Lee *et al.,* 2016).

As a neuroprotective gene, *Bdnf* was predicted to display increased activation and associated protein production following the administration of novel oximes. In this study, as expected, expression of *Bdnf* was significantly increased for several of the oxime and combination groups, indicating that some novel oximes may be able to increase *Bdnf* transcription levels for neuroprotective purposes. As novel oximes have previously been shown to reduce observed seizure duration, it is possible they may achieve this attenuation through the reduction of NIMP's neurotoxic effects, primarily through their activation of *Bdnf*.

Within this study, it was observed that both 2-PAM and Oxime 20 alone were able to significantly up-regulate *Bdnf* when compared to the NIMP only group. The finding that Oxime 1 alone repressed *Bdnf* to approximately the same level as NIMP was countered by the observation that, when in combination with NIMP, Oxime 1 was able to significantly up-regulate *Bdnf* expression. These findings suggest that the oximes were able to provide some degree of neuroprotection, as *Bdnf* transcriptional increases correspond to neuronal growth and repair. Likewise, the other two combination groups (with 2-PAM and Oxime 20) managed to increase *Bdnf* transcription when compared to the NIMP only group, with the 2-PAM combination also demonstrating significance. While the Oxime 20 combination did not differ significantly from the NIMP group, its average Ct value closely approximated that of the vehicle control group, indicating that Oxime 20 may be able to normalize *Bdnf* transcription, returning gene expression levels to those observed in homeostasis.

These results demonstrate that 2-PAM and at least one of the novel oximes, Oxime 1, were able to up-regulate *Bdnf* following NIMP exposure to levels which differed significantly not only

from the NIMP group but from the baseline controls. Administered following NIMP exposure, Oxime 20 may also possess some normalization capacity regarding *Bdnf* expression. Thus, all three oximes were able to positively affect *Bdnf* expression, thereby demonstrating therapeutic capacity following NIMP exposure.

Fos (Fos proto-oncogene; hippocampus)

In the literature, mRNA levels for *Fos*, a marker of neuronal activation, have been reported to increase following chlorpyrifos and methyl parathion exposure in the cerebral cortex and hippocampus of juvenile rats, indicating that *Fos* may play a role in indicating neural damage (Betancourt *et al.*, 2007). *Fos* was also identified as an immediate early gene which encodes a nuclear protein involved in signal transduction, allowing it to play a role in long-term neuronal plasticity resulting from changes to the cellular or extracellular environment (Perez-Cadahia *et al.*, 2011). Novel environmental stimuli have also been reported to increase *Fos* expression levels within the hippocampus, indicating its role in neuronal excitation and changes related to neuroplasticity (VanElzakker *et al.*, 2008). Therefore, within this study, *Fos* was viewed as an indicator of OP-induced neurological damage.

The NIMP only group displayed the highest levels of *Fos* transcription, markedly increased over the vehicle control group, suggesting that *Fos* may play a role in indicating neural damage following OP exposure. Thus, down-regulation of *Fos* could be related to normalization processes following the administration of novel oximes.

Concerning the oxime only groups, *Fos* was significantly down-regulated by 2-PAM when compared to Oxime 20, indicating that 2-PAM may be able to outperform Oxime 20 in reversing any harmful effects of NIMP.

The NIMP plus Oxime 20 combination returned *Fos* levels to those observed for the control group, with no significant difference found between the two groups. This result suggests that Oxime 20 may be able to induce control levels of *Fos* expression following NIMP exposure and thus prompt a return to normalization, or homeostasis, following OP poisoning. In this regard, the Oxime 20 combination was observed to more closely approximate the control group than that of NIMP plus 2-PAM, which differed significantly from the vehicle group in lowering levels of *Fos* transcription.

Following from these results, it can be concluded that both 2-PAM and Oxime 20 may be able to play a role in neuroprotection through their normalization of factors related to NIMP-induced damage. These neurotoxic factors most likely prompt *Fos* expression as a type of alarm system; thus, it is suggested that, through their down-regulation of *Fos*, the oximes are working to reverse neural damage. This ability to dampen gene expression changes following NIMP exposure could prove key in Oxime 20's therapeutic potential, allowing it to attenuate OP-induced brain damage and reduce the possibility of permanent neural deficits.

Bcl2l1 (Bcl2-like 1; hippocampus)

In previous studies, *Bcl2l1* has been implicated as an immediate early gene pivotal to the inhibition of neuronal apoptosis, and potentially able to provide neuroprotection against ischemic brain injury. More specifically, the *Bcl* gene family was found to regulate neuronal development and provide neuronal protection against neurodegenerative factors (Park *et al.*, 2015). Up-regulation of *Bcl2l1* would therefore be associated with an increased potential for neuroprotection and should have been observed with oxime administration following NIMP exposure.

As observed within this study, the NIMP only group displayed statistically significant differences when compared to the vehicle control group, 2-PAM alone, and both oxime combination groups. Statistical significance in relation to the NIMP group was not found with Oxime 20 alone, whose average Ct value roughly approximated that of NIMP alone.

Thus, while the oximes administered alone were observed to down-regulate *Bcl2l1*, both of the combination groups, including that with Oxime 20, elicited similar expression of *Bcl2l1*. Gene expression values for the combination groups most closely approximated the values observed for the vehicle control group, with no significant differences found between the vehicle and the Oxime 20 combination. As both combination groups up-regulated *Bcl2l1* when compared to NIMP, producing values which neared those of the vehicle control group, it is suggested that novel oximes may exhibit some potential for normalization of *Bcl2l1* expression. Thus, the therapeutic potential of Oxime 20 may be related to its ability to increase *Bcl2l1* expression to control levels, allowing it to inhibit OP-induced apoptosis and provide neuroprotection following a poisoning event.

One of the main limitations of this study concerns the three-hour post-exposure window preceding sacrifice. Following NIMP exposure, a waiting period of one hour was undergone in order to achieve peak brain AChE inhibition as well as clear physical signs of hypercholinergic toxicity. Oximes were then administered, and the rats were euthanized two hours later. However, as different genes may be affected independently of peak AChE inhibition, this three-hour window may be too short of a timeframe to observe extensive changes in gene expression. As a result, these findings reflect gene expression at a given time point, which may or may not represent the oximes' peak response. Due to the different solubilities and rates of distribution and absorption of the tested

oximes, it is also possible that the peak effects of individual oximes might occur at different time points. Longer exposure windows, especially following administration of the oximes, could reveal more nuanced or significant changes to gene expression, as the phenomenon is influenced by a number of different factors unable to be fully accounted for here.

Other considerations include the OP used in this study. As NIMP is a nerve agent surrogate compound which is not being studied by many research groups, no other laboratories at this time have published work detailing its effects. It is important to note that NIMP may affect the chosen genes of interest differently than its respective nerve agent, sarin. NIMP also functions differently from other OP compounds that were not evaluated within this study, making these results NIMP-specific.

In addition, all six of the genes of interest were chosen based on previous literature, occasionally in relation to different OP compounds. It is possible that some of these genes were simply unresponsive to provocation with NIMP, or did not experience changes in their expression within the experimental timeframe used here. The last two genes, *Fos* and *Bcl2l1*, were chosen in light of this concern because of their identification as immediate early genes. It was expected that gene expression changes would occur more quickly, and thus allow for observation within the given window.

CONCLUSION

This study aimed to elucidate and quantify the changes in gene expression prompted by the administration of novel oximes. While not all genes of interest demonstrated statistically significant differences, a number of the observed Ct values indicated the potential for normalization of gene expression by the oximes in response to NIMP intoxication, or indicated trends in the direction of significance. In the case of two damage-related genes, *Ccl2* and *Gfap*, novel oxime combinations were shown to decrease expression when compared to the NIMP only group. These results demonstrated the novel oximes' potential for attenuation of inflammation and neural damage following OP exposure.

Novel oximes were also found to increase expression of the neuroprotective gene *Bdnf,* suggesting neuroprotection via brain cell growth and repair. The novel Oxime 1 combination was found to increase *Bdnf* expression above even control levels, indicating the potential for attenuation, while the Oxime 20 combination approximated the vehicle control. The immediate early genes *Fos* and *Bcl2l1* also demonstrated changes in gene expression consistent with normalization of gene transcription following OP exposure. For both genes, no significant differences were found between the Oxime 20 combination and the vehicle control, indicating that Oxime 20 may be able to induce a return to homeostatic gene expression levels following an OP poisoning event.

This study thus supports the potential of the novel oximes to attenuate neural damage after sublethal OP exposure, providing therapy via changes in gene expression. Their ability to up-regulate neuroprotective genes and down-regulate damage-reflective genes, therefore normalizing them to control levels, again suggests the novel oximes' capacity to enter the brain and provide neuroprotection not offered by the currently approved therapeutic. Ultimately, this study further

demonstrates the novel oximes' value in modern therapy, cementing them as promising alternatives to the current antidote and highlighting their potential in the endeavor to save not only lives, but brains.

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