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The Role of DARPP-32, an Intracellular Signaling Molecule, in the Actions of the Nerve Agent Sarin

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14. ABSTRACT

We investigated the role of DARPP-32 in mediating changes in phosphorylation after sarin exposure. Wild type mice and mice bearing a targeted disruption of the gene for DARPP-32 were exposed to sarin; levels of brain phosphoproteins were measured. Mice receiving 1.0 x LD_{50} dose of sarin displayed motor convulsions. No significant change in phosphorylation level of T75 DARPP-32 was observed in brains of wild type mice. Also, no significant changes in phosphorylation were observed in the brains of wild type or DARPP-32 knockout mice after 0.5 x LD_{50} sarin. At a sarin dose of 1.0 x LD_{50} a significant increase in CREB phosphorylation was observed. One difference was noted in brain phosphorylation between wild type and DARPP-32 knockout mice. The presence of DARPP-32 significantly affected the ability of sarin to alter phosphorylation of the AMPA receptor GluR1 at S831. An increase in phosphorylation measured in wild type mouse brain after sarin was significantly attenuated in the brain of DARPP-32 knockout mice, indicating that DARPP-32 may modulate the extent to which S831 responses are induced by sarin. The results of this study do not support the hypothesis that changes in phosphorylation levels of T75 DARPP-32 are necessary for phosphorylation changes observed in brain after a 1.0 x LD_{50} dose of sarin.

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ABSTRACT

Organophosphorus (OP) nerve agents exert acute effects by inhibiting the enzyme acetylcholinesterase in the central and peripheral nervous systems. Inhibition of acetylcholinesterase results in accumulation of acetylcholine and, which in turn causes overstimulation of nicotinic and muscarinic receptors. Previous study indicated that treatment of rats with the nerve agent sarin both at a convulsive dose (1.0 x LD_{50}) and a sub-convulsive threshold dose (0.5 x LD_{50}) resulted in a change in phosphorylation of the dual function protein kinase/protein phosphatase inhibitor DARPP-32 at the threonine residue 75 (T75). Moreover, sarin treatment also increased phosphorylation of the protein kinase ERK and the transcription factor CREB, and reduced phosphorylation of the cytoskeletal anchoring protein spinophilin and the glutamate receptor NR1, only at seizure-inducing doses of sarin. In the present study, we sought to investigate the possible role of DARPP-32 in mediating changes in phosphorylation after sarin exposure. Wild type mice and mice bearing a targeted disruption of the gene for DARPP-32 were exposed to sarin and levels of brain phosphoproteins were measured. As in our previous rat studies, all mice receiving 1.0 x LD_{50} dose of sarin displayed motor convulsions. However, in contrast to the previous study, no significant change in phosphorylation level of T75 DARPP-32 was observed in brains of wild type mice. In addition no significant changes in phosphorylation were observed in the brains of wild type or DARPP-32 knockout mice after 0.5 x LD_{50} sarin. At a sarin dose of 1.0 x LD_{50} a significant increase in CREB phosphorylation was observed, in agreement with previous observations in rat. One difference was noted in brain phosphorylation between wild type and DARPP-32 knockout mice. The presence of DARPP-32 significantly affected the ability of sarin to alter phosphorylation of the AMPA receptor GluR1 at S831. An increase in phosphorylation measured in wild type mouse brain after sarin was significantly attenuated in the brain of DARPP-32 knockout mice, indicating that DARPP-32 may modulate the extent to which S831 responses are induced by sarin. However, the results of this study do not support the hypothesis, derived from previous studies in rats, that changes in phosphorylation levels of T75 DARPP-32 are necessary for phosphorylation changes observed in brain after a 1.0 x LD_{50} dose of sarin.

INNOVATION

These studies are unique in that they serve to characterize *in vivo* the specific intracellular signaling pathways and neuronal proteins that are targeted by exposure to convulsive and sub-convulsive doses of sarin. The studies are innovative in that they utilize as tools for this analysis a strain of mice that individually has been genetically engineered to lack the gene coding for the dopamine signaling protein, DARPP-32. Since these mice consequently lack expression of these signaling proteins, they can be used to assess the unique roles of this molecule in mediating the biochemical and behavioral effects of sarin. This goal is made possible by the use of 1) a specially designed microwave to freeze alterations of phosphorylation state *in vivo* after nerve agent exposure and 2) by the use of phospho-specific antibodies that have been developed to specifically monitor changes in phosphorylation. These techniques have enabled us to measure the biochemical effects of sarin, focusing on the precise measurement of the state of phosphorylation of key neuronal proteins in the brain. These experiments provide a "snapshot" of how nerve agents alter the neurotransmitter signaling pathways in the brain.

TRANSITION OF RESEARCH

Results from this project provide the groundwork for additional studies that will characterize the changes in protein phosphorylation resulting from 1) acute exposure to other nerve agents, 2) seizures induced by nerve agents, and 3) low-level acute and chronic exposure to various nerve agents.

MILITARY RELEVANCE

These studies have major potential to lead to the development of novel pharmaceuticals and/or diagnostics that may have multiple applications in United States military operations. An understanding of the acute effects of nerve agents on brain function will aid in the development of protective agents with better central nervous system efficacy than drugs, such as diazepam, that are currently available. For instance, we anticipate that compounds will be developed that are more effective in negating convulsant effects of exposure to organophosphorus agents. An understanding of the long-term effects of nerve agent exposure as distinguished from the response to stress and fear present in combat deployments may lead to the development of diagnostics that are effective in distinguishing between, and perhaps treating, the ill effects of chemical agents and stress physiology. Such diagnostic agents would be useful in monitoring the health of military personnel in ways that are not possible at present.

INTRODUCTION

A well-characterized mediator of the biochemical, electrophysiological, transcriptional and behavioral effects of several major brain neurotransmitters is DARPP-32 (dopamine and cAMP regulated phospho-protein of molecular weight 32,000). DARPP-32 expression is highly enriched in prefrontal cortex and striatum. Activation of dopamine (DA) D1 receptors, due to the stimulation of protein kinase A (PKA), phosphorylates DARPP-32 at Thr-34 (T34) and thereby converts DARPP-32 into a potent inhibitor of protein phosphatase 1 (PP-1) (Hemmings et al., 1984a) (see Figure 1). This effect is antagonized by activation at DA D2 receptors, which results in (a) inhibition of PKA and (b) stimulation of the Ca^{+2}/calmodulin-dependent protein phosphatase signaling cascade, which dephosphorylates phospho-T34-DARPP-32 (Nishi et al., 1999). DARPP-32 is also phosphorylated at a single threonine residue (T75) by cyclin dependent kinase-5 (CDK5), a neuronally enriched and brain-specific member of the cyclin-dependent kinase family. When phosphorylated at T75, DARPP-32 is converted into a potent inhibitor of PKA (Bibb et al., 1999). Activation of D1 receptors also decreases phosphorylation at Thr-75(T75)-DARPP-32, which reduces inhibition of PKA and thereby facilitates transmission by means of the PKA/T34-DARPP-32/PP-1 signaling cascade (Bibb et al., 1999). The efficacy of this signaling cascade is also regulated by the phosphorylation state of DARPP-32 at Ser-102 (S102) and Ser-137 (S137). For example, S102 on DARPP-32 is phosphorylated by casein kinase II (CK2). In previously published experiments, increases in phosphorylation at site S102 increase the efficiency of phosphorylation of T34 by PKA but not by protein kinase G (Girault et al., 1989). DARPP-32 is also phosphorylated on amino acid S137 by casein kinase I (CK1). Increases in phosphorylation at this site decrease the rate of dephosphorylation by protein phosphatase 2B (PP-2B) at T34. The physiological effect of phosphorylation at S102 and S137 is to potentiate signaling through the dopamine/D1/PKA/DARPP-32/PP-1 pathway and to reduce signaling through the glutamate/Ca^{+2}/PP-2B/DARPP-32/PP-1 pathway. PP-1 controls the state of phosphorylation and activity of numerous physiologically important substrates including neurotransmitter receptors, voltage gated ion channels, ion pumps and transcription factors. As a result, neurotransmitters that increase or decrease the phosphorylation state of DARPP-32 either inhibit or activate, respectively, PP-1 and thereby increase or decrease the state of phosphorylation and activity of a large array of downstream physiological effectors (Greengard et al., 1999).

As shown in Figure 2 the DARPP-32/PP-1 cascade is responsive to a large number of neurotransmitters in addition to DA (Hemmings et al., 1984b; Walaas and Greengard, 1984). These include glutamate (Halpain et al., 1990), γ-amino-butyric acid (GABA) (Snyder et al., 1994), adenosine (Svenningsson et al., 1998), cholecystokinin (CCK) (Snyder et al., 1993), and others (Tsou et al., 1993). Direct proof for the role of the DARPP-32/PP-1 signaling cascade in mediating the actions of these various first messengers has come from both *in vitro* manipulations using intracellular injection of kinase and phosphatase molecules and from gene knockout experiments. The latter type of experiment using DARPP-32 knockout mice has shown that DARPP-32 is essential to the action of various neurotransmitters and that the DARPP-32/PP-1 cascade modulates the phosphorylation state of several important ligand and voltage gated ion channels. For example, the state of phosphorylation of the NMDA receptor subunit-1 (NR1) and the AMPA receptor subunit-1 (GluR1) has been shown to be controlled by the DARPP-32/PP-1 cascade (Snyder et al., 1998; Snyder et al., 2000). The ability of the DARPP-

32/PP-1 pathways to regulate the phosphorylation state of these receptors is critical to the control of receptor activity for both the NMDA-type receptor (Fienberg et al., 1998) and the AMPA-type receptor (Yan et al., 1999).

In addition to the direct inhibition of PP-1 activity mediated via binding to T34-phosphorylated DARPP-32, PP-1 activity can be regulated through the selective targeting of the phosphatase to membrane-bound neuronal targets through the actions of a variety of PP-1 targeting proteins (Greengard et al., 1999). One of these targeting proteins, spinophilin, targets PP-1 to regions of DARPP-32-containing neurons that contain high concentrations of NMDA and AMPA receptors (Ouimet et al., 1995). Phosphorylation of spinophilin at Ser-94 (S94) by PKA reduces association of the PP-1/spinophilin complex with membranes and would be expected to alter the ability of PP-1 to dephosphorylate membrane-bound receptors (Hsieh-Wilson et al., 2003). Mice genetically engineered to lack spinophilin have altered glutamate receptor activity and exhibit a resistance to seizures induced by glutamate agonists such as kainate (Feng et al., 2000).

In a previous collaboration, we documented novel changes in the phosphorylation states of selected striatal phosphoproteins that are associated with exposure of rats to the organophosphorus (OP) nerve agent sarin (Shih et al., 2004). Both seizure-inducing doses (i.e., 1.0 x LD_{50}) and sub-seizure threshold doses (i.e., 0.5 x LD_{50}) of the nerve agent were tested to determine whether certain of these markers are predictive of sarin exposure in non-symptomatic animals. Rats administered a 0.5 x LD_{50} dose (62.5 μg/kg, sc) of sarin displayed a significant and selective increase in the state of phosphorylation of DARPP-32 at T75 30 min after administration ($p=0.03$, t-test). Rats exposed to a 1.0 x LD_{50} dose (125 μg/kg, sc) of sarin displayed significant changes in phosphorylation of several sites. Three sites increased in phosphorylation level 15 min after sarin treatment: Ser-133 (S133) of cyclic AMP response element binding protein (CREB), Thr-183 (T183) of extracellular signal-regulated protein kinase (ERK), and T75 of DARPP-32. Thus, levels of T75-phosphorylated DARPP-32 were increased in both symptomatic and asymptomatic rats. The state of phosphorylation of several sites was reduced 30 min (but not 15 min) after 1.0 x LD_{50} sarin treatment: T34, S102, and S137 of DARPP-32, S94 of spinophilin, and Ser-897 (S897) of NR1. The reduction in phosphorylation at S897 of NR1 was not indicative of a generalized dephosphorylation of all glutamate receptors, since levels of phosphorylation of the AMPA receptor subunit GluR1 at Ser-845 (S845), a PKA site, and at S831, a substrate for PKC and the Ca_2^+/calmodulin-dependent protein kinase II (CaMKII), were not reduced by 1.0 x LD_{50} sarin treatment.

Taken together these data suggest that changes in T75 DARPP-32 may serve as a marker for asymptomatic nerve agent exposure. This increase in T75 DARPP-32 phosphorylation, an event that converts DARPP-32 into a potent PKA inhibitor, resulted in large reductions in phosphorylation at two sites phosphorylated by PKA, S94 of spinophilin and S897 of NR1. In the present study, we use DARPP-32 knockout mice to test the hypothesis that an inhibition of PKA activity, mediated by increased levels of phospho-T75 DARPP-32, is responsible for the reduced phosphorylation of S94 on spinophilin. Reduced phosphorylation of spinophilin at S94 would be expected to promote PP-1 association with membrane-bound receptors such as NR1 and promote dephosphorylation by PP-1 of NR1 at S897. Thus, future studies will utilize spinophilin knockout mice to test the hypothesis that regulation of the spinophilin

phosphorylation state, induced by sarin exposure, is responsible for the effect of NR1 phosphorylation.

Based on our previous results with sarin in rats (Shih et al., 2004), we anticipated that exposure of mice to convulsive doses of sarin would result in reduced phosphorylation of both the NR1 subunit of the NMDA receptor and spinophilin. We, furthermore, expected that these effects would occur as a result of the ability of sarin to increase phosphorylation of DARPP-32 at T75, converting DARPP-32 into a PKA inhibitor. We speculated that this results in a decrease in phosphorylation of spinophilin at S94, leading to an increased association of spinophilin (and PP-1) at NMDA-type glutamate receptors. This then would result in the observed decrease in NR1 phosphorylation at S897. Thus, we anticipated that sarin-induced reductions in NR1 and spinophilin phosphorylation will be absent in the striatum of DARPP-32 knockout mice.

This report summarizes the results of experiments designed to examine the changes in protein phosphorylation that occur in response to sub-convulsive and convulsive doses of sarin in normal animals and compare these changes with those occurring in the brains of animals that genetically lack DARPP-32.

MATERIALS AND METHODS

Animals: A total of 50 male C57Bl/6 mice, weighing 20-30g at the time of experiment, were used in this study. Twenty wild type C57Bl/6 mice were obtained from Jackson laboratories (Bar Harbor, ME) and were used in dose finding studies intended to establish a 1 x LD_{50} and a 0.5 x LD_{50} dose for sarin exposure in mice. Subsequently, fifteen wild type mice and fifteen mice bearing a disruption of the gene coding for DARPP-32 (knockout) were used for experiments (see Fienberg et al., 1998). All of these animals (n=30) were provided by the laboratory of Dr. Paul Greengard at The Rockefeller University (New York, NY) where they were bred and housed in the Rockefeller University Laboratory Animal Research Center (LARC). After shipment to the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) they were group-housed in temperature (21 ± 2^{o}C) and humidity (50 ± 10%) controlled animal quarters maintained on a 12-h light-dark full spectrum lighting cycle with lights on at 0600 h. Laboratory chow and water were freely available. Experiments were conducted at USAMRICD and brain samples shipped to Intracellular Therapeutics, Inc. (ITI) for processing. The research environment and protocol for animal experimentation were approved by the institutional animal care and use committee at USAMRICD. Animal facilities at USAMRICD are fully accredited by AAALAC International.

Materials: Saline (0.9% NaCl) injection, USP, was purchased from Cutter Labs Inc. (Berkeley, CA). Sarin, obtained from the U. S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD), was diluted in ice-cold saline prior to injection. Saline or sarin injection volume was 5 ml/kg subcutaneously (sc). BCA protein assay kits were purchased from Pierce Chemical Co. (Rockford, IL). Phospho-specific antibodies for DARPP-32 T75, DARPP-32 S137 and spinophilin S94 were kindly provided by Dr. Paul Greengard (Rockefeller University). Anti-phospho-T34 DARPP-32 antibodies were provided by Dr. Angus Nairn (Rockefeller University). Anti-phospho-S845 GluR1, phospho-S831 GluR1, phospho-S897 NR1, and phospho-S133 CREB antibodies were obtained from Upstate USA, Inc. (Charlottesville, VA).

Alexa-680 fluorescent labeled goat anti-mouse IgG was obtained from Molecular Probes (Eugene, OR). IR dye 800CW fluorescent tag labeled goat anti-rabbit IgG was purchased from Rockland Immunochemicals (Gilbertsville, PA). Blocking buffer for Western blotting was obtained from LiCor (Lincoln, NE).

Animal experimental procedures: A preliminary verification of the LD_{50} of sarin in the wild type mice was conducted by the "up and down" method (Dixon, 1965) using five doses (four animals per dose level) with 125 μg/kg as the middle dose at intervals of 0.05 $\log_{10}$ unit. In the main study, mice from each genotype (wild type and DARPP-32 knockout) were divided into 5 groups with 3 animals in each group. Group one was injected subcutaneously (sc) with the vehicle saline (5 ml/kg) to serve as controls. Group two was injected with sarin at dose of 1.0 x LD_{50} (LD_{50} = 130 μg/kg, sc) and euthanized 15 min after injection. Group three was injected with sarin at dose of 1.0 x LD_{50} and euthanized 30 min after injection. Group four was injected with sarin at dose of 0.5 x LD_{50} (62.5 μg/kg, sc) and euthanized 15 min after injection. Group five was injected with sarin at dose of 0.5 x LD_{50} and euthanized 30 min after injection. Animals were euthanized by a head-focused microwave device (3.0 kW, 2.45 MHz for 0.85 sec; Gerling-Moore Metabostat System, Gerling-Moore, Inc., Santa Clara, CA) to arrest alterations of phosphorylation state *in vivo* at specified times after injection (Guidotti et al., 1974). Cerebral cortex, striatum and hippocampus were dissected rapidly after microwave procedure, immediately flash frozen, and then stored at –80^{o} C until phosphoprotein analysis.

Sample Processing: Frozen tissue samples from microwave-irradiated animals were sonicated in 1% sodium dodecyl sulfate (SDS) and boiled for 10 min. Small aliquots of the homogenate were retained for protein determination by the bicinchoninic acid (BCA) protein assay method (Pierce Chemical Co., Rockford, IL). Equal amounts of protein were processed using 10% acrylamide gels as described by Nishi et al. (1997) and immunoblotted as described below. DARPP-32 phosphorylation sites were analyzed in wild type mice by the use of phospho-specific antibodies that have been developed to specifically monitor changes in phosphorylation by the procedure described by Czernik et al. (1991). Phosphorylation sites examined were the T34, T75, S102 and S137 of DARPP-32, S94 of spinophilin, S897 of the NMDA receptor NR1 subunit, and S831 and S845 of the AMPA receptor GluR1 subunit, S40 of tyrosine hydroxylase (TH), S133 of CREB, and T183 of ERK2 (see Table 1).

Immunoblotting for DARPP-32 phosphorylated at T34, T75 or S137: Aliquots (3 μl) of brain homogenates were used for protein determination. Equal amounts of protein (50 μg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (BioRad, Hercules, CA). The membranes were blocked in Tris-buffered saline (TBS)/Tween with LiCor Blocking Buffer (LiCor, Lincoln, NE) followed by incubation with antibodies against phospho[T34]-DARPP-32, phospho[T75]-DARPP-32, phospho[S137]-DARPP-32 or total DARPP-32. The membranes were then washed 4 times for 5 min each with TBS/Tween, and antibody binding was revealed using Alexa 680 labeled goat anti-mouse IgG (Molecular Probes, Eugene, OR) or IRdye 800CW labeled goat anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA). Antibody binding was detected and quantitated using a LiCor Odyssey infrared fluorescent detection system (LiCor, Lincoln, NE). Reagents for detecting the other phosphorylation sites of interest, including S133 CREB, T183

ERK, S897 NR1, S831 and S845 GluR1, and S94 spinophilin, have been previously described (Svenningsson et al., 2002; Pozzi et al., 2003).

Data Analysis: The state of phosphorylation of several neuronally enriched phosphoproteins was monitored and quantified in striatal and cortical samples from sarin-exposed mice and saline-treated control mice at 15 and 30 min after sarin or saline administration. Levels of phosphorylation at each site were quantified, averaged across all samples in each group, then expressed as a percent $\pm$ SEM of levels present in the saline-injected control mice. Since no significant differences were observed in levels of phosphorylation for any of the phospho-sites as a function of time (i.e., 15 vs. 30 min) these data were pooled for each dose level and genotype. Statistical analyses were then performed as two-factor (dose x genotype) ANOVA with a Newman-Keuls post-hoc test. A difference of $p<0.05$ was considered significant.

RESULTS

Toxicity: An initial experiment was conducted with 20 wild type mice to verify the subcutaneous (sc) LD_{50} dose of sarin in mice, which was determined to be 130 µg/kg. This LD_{50} dose was used for both wild type and DARPP-32 knockout mice in the subsequent experiment. Animals were injected with saline (0.5 ml/kg, sc), a 0.5 x LD_{50} (65 µg/kg, sc) or a 1.0 x LD_{50} (130 µg/kg, sc) dose of sarin. All mice administered with the 1.0 x LD_{50} dose showed signs of tonic and clonic motor convulsions. Two of the mice administered a 0.5 x LD_{50} dose of sarin displayed mild forms of convulsive activity (both were DARPP-32 knockout mice).

Biochemical Observations: Three brain regions, striatum, cortex, and hippocampus, were dissected from the brains of mice. These brain samples were transported on dry ice to ITI for analysis. Preliminary analysis has been performed on the striatal and cortical samples from sarin-treated mice and their saline-injected controls. The data are summarized in Tables 2 and 3 as well as in Figures 3 and 4. A summary of the statistics is provided in Table 4.

Wild type and DARPP-32 knockout mice administered sarin did not show significant differences between phosphorylation of any of the phosphorylation sites measured at 30 min, compared with 15 min, post-injection. Based on this observation, data for 15- and 30-min time points were pooled for mice treated with either a 0.5 x LD_{50} dose or a 1.0 x LD_{50} dose of sarin. These data were then subjected to further analysis to determine whether phosphorylation levels were significantly affected by sarin dose and/or mouse genotype.

This subsequent analysis of results from wild type and DARPP-32 knockout mice revealed that sarin induced a dose-dependent increase in the state of phosphorylation of the transcription factor CREB at S133 in the striatum (Two-Factor ANOVA, $p<0.0001$) (Table 4 and Figure 3). Thus, CREB phosphorylation increased in striatum of both wild type and DARPP-32 knockout mice in response to exposure of mice to 1.0 x LD_{50}, but not 0.5 x LD_{50} sarin. Analysis revealed a significant effect of mouse genotype on the phosphorylation of residue S831 of GluR1 in the cortex. Sarin exposure increased phospho-S831 levels in wild type, but not in DARPP-32 knockout mice (Two-factor ANOVA, $p=0.02$) (Table 4 and Figure 4). Thus, the presence of DARPP-32 selectively affected the regulation of GluR1 phosphorylation at S831 in response to sarin.

In contrast to mice exposed to a convulsive dose of sarin (i.e., 1.0 x LD_{50}), mice receiving a sub-convulsive threshold dose of sarin (i.e., 0.5 x LD_{50}) showed no significant changes in the state of phosphorylation of any of the sites measured in striatum or cortex.

Table 1. Phosphorylation sites examined and their functions

Phospho-Site Abbreviation	Identity/Description of Site
T75	Thr75 is a CDK5-dependent site on DARPP-32 controlling the PKA-inhibitor activity of DARPP-32.
S133	Ser133 on CREB is essential for regulating CREB function; phosphorylation increases CREB activity.
T183	Phosphorylation at Thr183 on ERK1 (44kDa form) and ERK2 (42kDa forms) is essential for increasing ERK activity.
T34	Thr34 is a PKA-dependent site on DARPP-32 that converts DARPP-32 into a PP-1 inhibitor.
S102	Ser102 is a Casein Kinase II-dependent site on DARPP-32 that enhances T34 phosphorylation.
S94	Ser94 is a PKA-dependent site on spinophilin controlling association of this PP-1 targeting protein with actin
S137	Ser137 is a Casein Kinase I site on DARPP-32 that also facilitates Thr34 phosphorylation.
S897	Ser897 is a PKA-dependent site on the NR1 subunit of the NMDA receptors that is involved in regulating NMDA receptor conductance.
S845	Ser845 is a PKA-dependent site on the GluR1 subunit of the AMPA receptors that enhances open-time probability of the receptor channel.
S40	Ser40 on Tyrosine Hydroxylase is a PKA-dependent site that, when phosphorylated, increases the enzymatic activity of TH, and increases the rate of dopamine synthesis.
S831	Ser831 is a PKC/CaMKII-dependent site on GluR1 that controls conductance through the AMPA receptor channel.

Footnote: AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CaMKII = the $Ca2^{+}$/calmodulin-dependent protein kinase II
CDK5 = Cyclin-dependent kinase 5
CREB = cyclic AMP response element binding protein
DARPP-32 = dopamine (DA) and cAMP-regulated phosphoprotein of molecular weight 32KDa
ERK = extracellular signal-regulated protein kinase
GluR1 = subunit 1 of AMPA-responsive type glutamate receptor
NMDA = N-methyl-D-aspartate
NR1 = N-methyl-D-aspartate receptor subunit 1
PKA = cAMP-dependent protein kinase
PKC = protein kinase C
PP-1 = protein phosphatase 1
TH = Tyrosine Hydroxylase

Table 2. Effect of a 0.5 x LD_{50} dose or a 1.0 x LD_{50} dose of sarin on the state of phosphorylation of several striatal phosphoproteins. Levels of phosphorylation at each site are quantified and expressed as a percent ± (SEM) of levels present in striatum of the saline-injected control mice. NS=Not significant, two-way ANOVA for effect of dose on wild type mice; NA=measurement not available.

Phospho-Site	*WILD TYPE MICE*			*DARPP-32 KNOCKOUT MICE*		
	Control Mean (SEM) %	**0.5 LD_{50} Mean (±SEM) % control**	**1.0 LD_{50} Mean (±SEM) % control**	**Control Mean (SEM) %**	**0.5 LD_{50} Mean (±SEM) % control**	**1.0 LD_{50} Mean (±SEM) % control**
T75	100 (3)	106 (7)	127 (13)	NA	NA	NA
S133	100(2)	96 (2)	143 (38)	100 (2)	108 (2)	131 (7)
T183	100 (9)	111 (18)	111 (25)	100 (6)	126 (8)	136 (22)
T34	100 (7)	101(20)	112 (9)	NA	NA	NA
S102	100 (3)	99 (2)	90 (8)	NA	NA	NA
S94	100 (8)	94 (21)	87 (16)	100 (14)	119 (9)	99 (24)
S137	100 (3)	102 (5)	101 (14)	NA	NA	NA
S897	100 (12)	91 (5)	98 (19)	100 (4)	98 (5)	76(11)
S845	100 (5)	95 (7)	120 (13)	100 (4)	101 (7)	117 (23)
S40	100 (2)	101 (2)	115 (7)	100 (3)	103 (4)	101 (7)
S831	100 (19)	99 (22)	141 (36)	100 (18)	116 (23)	100 (14)

Table 3. Effect of a 0.5 x LD_{50} dose or a 1.0 x LD_{50} dose of sarin on the state of phosphorylation of several cortical phosphoproteins. Levels of phosphorylation at each site are quantified and expressed as a percent ± (SEM) of levels present in cortex of the saline-injected control mice. NS=Not significant, two-way ANOVA for dose effect in wild type mice; NA=measurement not available

Phospho-Site	*WILD TYPE MICE*			*DARPP-32 KNOCKOUT MICE*		
	Control (SEM) %	**0.5 LD_{50} Mean (±SEM) % control**	**1.0 LD_{50} Mean (±SEM) % control**	**Control (SEM) %**	**0.5 LD_{50} Mean (±SEM) % control**	**1.0 LD_{50} Mean (±SEM) % control**
T75	100 (2)	58 (13)	65 (15)	NA	NA	NA
S133	100(4)	93 (7)	134 (24)	100 (6)	84 (10)	93 (15)
T183	100 (7)	123 (15)	123 (22)	100 (23)	116 (20)	118 (28)
T34	100 (10)	57 (14)	97 (30)	NA	NA	NA
S102	NA	NA	NA	NA	NA	NA
S94	100 (13)	101 (10)	105 (18)	100 (8)	121 (11)	114 (12)
S137	NA	NA	NA	NA	NA	NA
S897	100 (5)	101 (10)	127 (20)	100 (4)	87 (6)	87 (11)
S845	100 (3)	96 (11)	149 (33)	100 (4)	84 (16)	89 (16)
S40	NA	NA	NA	100 (3)	NA	NA
S831	100 (6)	118 (12)	160 (37)	100 (13)	74 (13)	74 (12)

Table 4. Statistical summary for the effects of sarin and DARPP-32 genotype on the phosphorylation of several phosphoproteins in mouse striatum and cortex. Two-factor ANOVA was performed to evaluate the effects of sarin dose (0, 0.5, or 1.0 x LD_{50} sarin) and genotype (wild type, DARPP-32 knockout) and the interaction of these two variables on change in protein phosphorylation. Significant effects ($p<0.05$) are highlighted in bold. See text for details.

STRIATUM

Phosphoprotein site	***pValue***		
	Interaction	***Dose***	***Genotype***
S133 CREB	0.31	**<0.0001**	0.90
T183 ERK	0.67	0.22	0.29
S94 Spinophilin	0.66	0.66	0.28
S897 NR1	0.46	0.51	0.59
S845 GluR1	0.92	0.14	0.91
S40 TH	0.19	0.18	0.27
S831 GluR1	0.47	0.65	0.66

CORTEX

Phosphoprotein site	***pValue***		
	Interaction	***Dose***	***Genotype***
S133 CREB	0.39	0.22	0.21
T183 ERK	0.98	0.66	0.78
S94 Spinophilin	0.81	0.76	0.42
S897 NR1	0.34	0.56	0.12
S845 GluR1	0.33	0.33	0.19
S831 GluR1	0.22	0.56	**0.02**

Figure 1. Phosphorylation sites on DARPP-32

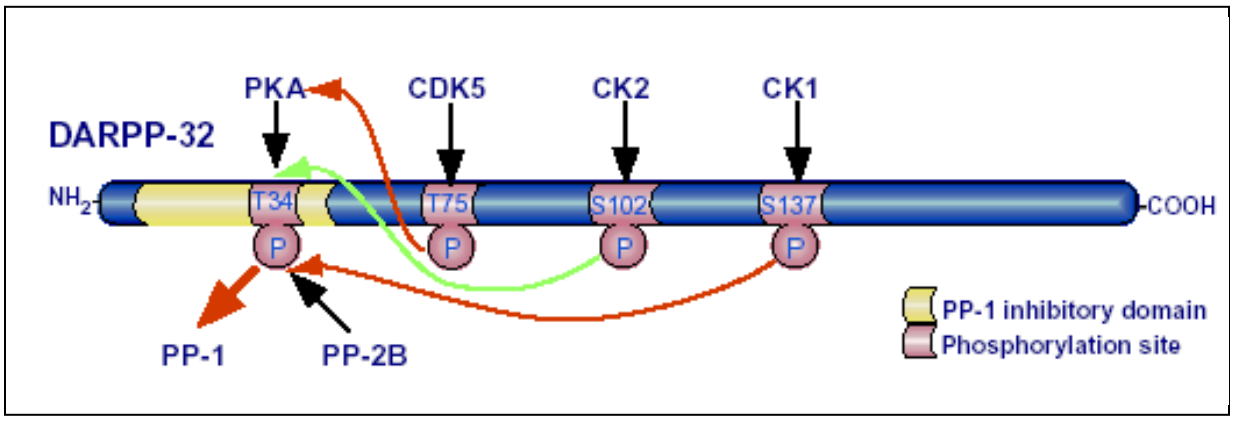

Figure 1. Diagram showing sites of phosphorylation of DARPP-32 and indicating the effect of each phosphorylation site on DARPP-32 function. Phosphorylation of DARPP-32 at T34 by PKA leads to inhibition of PP1 activity, whereas phosphorylation of DARPP-32 at T75 by CDK5 leads to inhibition of PKA activity (red, dark arrow). Phosphorylation of DARPP-32 at S102 by CK2 promotes phosphorylation at T34 by PKA (green, light arrow). Phosphorylation of DARPP-32 at S137 promotes phosphorylation at T34 (red, dark arrow) by reducing dephosphorylation at this site by PP-2B. See text for details.

Figure 2. Signaling pathways converging upon DARPP-32

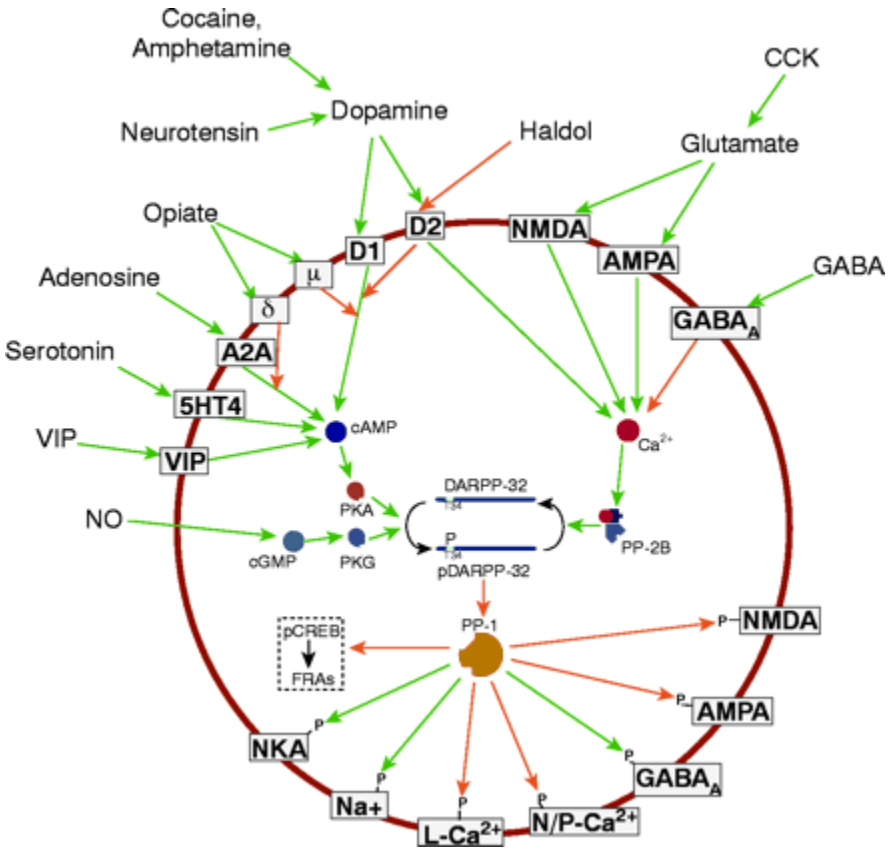

Figure 2. Diagram showing interactions of signaling pathways in nerve cells. Activation by dopamine of the D1 subclass of dopamine receptors stimulates phosphorylation of DARPP-32 at T34. This is achieved through a pathway involving the activation of adenylyl cyclase, the formation of cAMP, and the activation of PKA. Activation by dopamine of the D2 subclass of dopamine receptors causes the dephosphorylation of DARPP-32 through two synergistic mechanisms: D2 receptor activation (i) prevents the D1 receptor-induced increase in cyclic AMP formation and (ii) raises intracellular Ca^{2+}, which activates a Ca^{2+}-dependent protein phosphatase (PP-2B or calcineurin). Activated PP-2B dephosphorylates DARPP-32 at T34. Glutamate acts as both a fast-acting and slow-acting neurotransmitter receptor. Activation by glutamate of AMPA receptors causes a rapid response through influx of sodium ions, depolarization of the membrane, and firing of an action potential. Slow synaptic transmission, in response to glutamate, results in part from activation of the AMPA- and NMDA-type glutamate receptors, which increases intracellular Ca^{2+} and the activity of PP-2B, and causes the dephosphorylation of DARPP-32 at T34. All other neurotransmitters that have been shown to act directly to alter the physiology of dopaminoceptive neurons also alter the phosphorylation state of DARPP-32 at T34 through the indicated pathways. 5HT4, 5-hydroxytryptophan (serotonin) receptor 4; NKA, Na^+/K^+-ATPase; VIP, vasoactive intestinal peptide; L- and N/P-Ca^{2+}, L type and N/P type Ca^{2+} channels.

Figure 3.

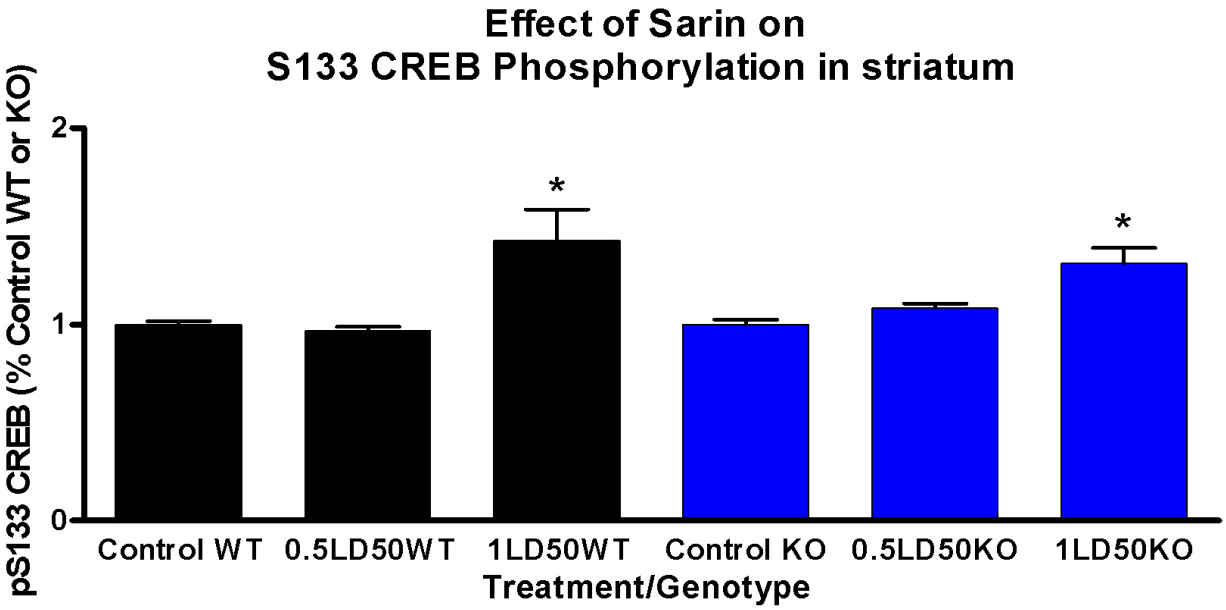

Figure 3. Bar graph depicting the effect of sarin dose (0.5 x LD_{50} or 1.0 x LD_{50}) on the phosphorylation of CREB at S133 in striatum from wild type (WT) and DARPP-32 knockout (KO) mice. Two-factor ANOVA, *$p<0.0001$.

Figure 4.

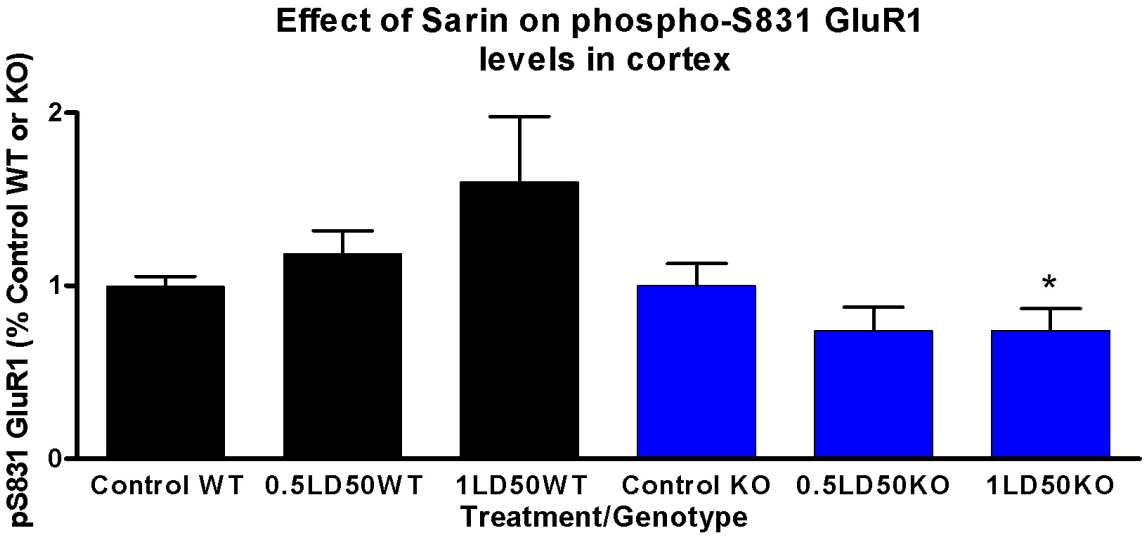

Figure 4. Bar graph depicting the effect of sarin dose (0.5 x LD_{50} or 1.0 x LD_{50}) on the phosphorylation of GluR1 at S831 in mouse cortex from wild type (WT) and DARPP-32 knockout (KO) mice. Two-factor ANOVA, *p=0.02.

SIGNIFICANCE OF FINDINGS

The results of the present study provide a preliminary evaluation of the role of a critical dopamine-responsive signaling protein, DARPP-32, in the biochemical effects of the nerve agent sarin. This study also serves as one of the first studies to evaluate the biochemical effects of sarin on protein phosphorylation specifically in the brains of mice rather than rats. The characterization of sarin biochemistry in mice may prove useful for the subsequent study of sarin effects in a wide range of gene knockout mouse models.

Consistent with our previous work in rats (Shih, 1981; Shih and McDonough, 1997; Scremin et al., 2003; Shih et al., 2004), mice receiving a high dose of sarin (i.e., 1.0 x LD_{50} dose) showed characteristic signs of convulsions. Mice treated with a 1.0 x LD_{50} dose of sarin displayed a significant increase in the state of phosphorylation of the transcription factor CREB in striatum. Since CREB is a key regulator of gene transcription in the brain, these data indicate that an early response to sarin exposure is the activation of signaling pathways that regulate gene expression. The fact that CREB phosphorylation is markedly increased in the brains of both rats and mice argues for this site as a commonly shared substrate for sarin effects in animals experiencing nerve agent-induced seizure activity. Interestingly, another recent study suggests that increased CREB phosphorylation is a significant effect of low-level exposure of brain neurons to the insecticide chlorpyrifos (Schuh et al., 2002). Thus, CREB activation may define a signaling pathway that is a common target for both organophosphate insecticides and nerve agents.

In contrast to our previous study in rats, a high dose of sarin did not significantly affect the phosphorylation state of DARPP-32 at T75 in mice. This site, when phosphorylated by the cyclin-dependent kinase 5 (CDK5), converts DARPP-32 into an inhibitor of PKA (see Figure 1), a major protein kinase. In addition, other changes in phosphorylation found in rats, including increases in phosphorylation of T183 ERK and decreases in phosphorylation of DARPP-32 at T34, spinophilin (at S94) and NR1 (at S897), were not observed in mouse brain after sarin treatment. One site of phosphorylation, S831 of GluR1, was differentially regulated in wild type compared with DARPP-32 knockout mice. Interestingly, S831 phosphorylation level was moderately increased in the striatum of rats exposed to a convulsive dose of sarin as well as in mouse striatum after this treatment. Since this site is an excellent substrate for NMDA-mediated phosphorylation via either protein kinase C (PKC) or CaMKII activity, it is likely that it is increased secondary to seizure activity involving massive NMDA receptor activation. The fact that deletion of DARPP-32 reduces this phosphorylation argues for the possibility that dysregulation of PP-1 activity, secondary to loss of DARPP-32, may interfere with the ability of NMDA to phosphorylate and activate this site during seizures and consequently to enhance NMDA activity in the striatum of knockout animals. Since the present studies involved no measures of neuronal damage due to seizure activity it is impossible to determine whether DARPP-32 knockout mice showed less neuronal damage as a result of sarin-induced seizures than did wild type mice. However, it would be of interest in future studies to determine whether the presence of signaling proteins such as DARPP-32 ameliorate the neuronal damage associated with nerve agent-related seizure activity.

In previous studies in rats, a dose of sarin (0.5 x LD_{50}) that was sub-threshold for inducing convulsions was nonetheless observed to induce a significant and selective increase in phospho(T75)-DARPP-32 levels in striatum, which was observed 30 min after sarin exposure. The appearance of increases in phospho(T75)-DARPP-32 levels at doses of sarin that are sub-threshold for convulsion suggests that changes in signaling pathways that control phosphorylation of this site (e.g., CDK5) may mediate brain responses to nerve agents. Since CDK5 has been associated with normal neuronal development and with the structural reorganization of neurons in response to drugs of abuse (Bibb et al., 2001; Norrholm et al., 2003), it may be an excellent candidate for mediating the subtle, long-lasting perceptual and motor deficits associated with low-level sarin exposure (Scremin et al., 2003). However, the present studies did not show a similar effect of sub-threshold sarin on T75 phosphorylation in mice. The basis for this difference between the two species may reflect differences between rats and mice in the binding of sarin to brain and peripheral cholinesterases. Thus, it is possible that mice more efficiently neutralize low-level doses of sarin, resulting in a lower brain exposure to the nerve agent, compared with rats. This potential difference in the distribution and binding of sarin in the two species should be further examined to test this hypothesis. In addition, the data from the present study is also affected by the rather small sample size that was available for analysis. It is possible then that when data are collected from larger groups of mice in subsequent experiments, some data trends noted in the present study may prove to be significant effects.

These studies demonstrate that phosphorylation of specific phosphoproteins is a sensitive indicator to monitor the effects of nerve agent exposure *in vivo*. Further work must be performed to determine whether some of the differences in brain phosphorylation response noted between rat and mouse are due to species differences in the response to nerve agent. Moreover, further studies will be necessary to better evaluate the role of DARPP-32 and other signaling proteins, including spinophilin, in the effects of sarin. Such studies may provide information to guide the search for small-molecule inhibitors to serve as novel antidotes to nerve agent exposure.

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ABBREVIATIONS

AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BCA = bicinchoninic acid
CaMKII- $Ca2^{+}$/calmodulin-dependent kinase II
cAMP= cyclic adenosine monophosphate
CCK = cholecystokinin
CDK5 = cyclin-dependent kinase 5
CK1 = casein kinase I
CK2 = casein kinase II
CREB = cyclic AMP response element binding protein
DA = dopamine
DARPP-32 = dopamine (DA) and cAMP-regulated phosphoprotein of molecular weight 32KDa
ERK = extracellular signal-regulated protein kinase
GABA = γ-aminobutyric acid
GluR1 = subunit 1 of AMPA-responsive type glutamate receptor
LD50 = median lethal dose or lethal dose 50%
NMDA = N-methyl-D-aspartate
NR1 = subunit 1 of N-methyl-D-aspartate-responsive type glutamate receptor
OP = organophosphorus compound
PKA = cAMP-dependent protein kinase
PKC = protein kinase C
PP-1 = protein phosphatase 1
PP-2B = protein phosphatase 2B
S40 = serine reside 40 on TH
S94 = serine residue 94 on spinophilin
S102 = serine residue 102 on DARPP-32
S133 = serine residue 133 on CREB
S137 = serine residue 137 on DARPP-32
S831 = serine residue 831 on the AMPA receptor GluR1 subunit
S845 = serine residue 845 on the AMPA receptor GluR1 subunit
S897 = serine residue 897 on the NMDA receptor NR1 subunit
SDS = sodium dodecyl sulfate
SDS-PAGE = SDS-polyacrylamide gel electrophoresis
T34 = threonine residue 34 on DARPP-32
T75 = threonine residue 75 on DARPP-32
T183 = threonine residue 183 on ERK
TBS = Tris-buffered saline
TH = Tyrosine Hydroxylase