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ORIGINAL ARTICLE

Organophosphates dysregulate dopamine signaling, glutamatergic neurotransmission, and induce neuronal injury markers in striatum

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Abstract

The neurological effects of organophosphate (OP) pesticides, commonly used on foods and in households, are an important public health concern. Furthermore, subclinical exposure to combinations of organophosphates is implicated in Gulf War illness. Here, we characterized the effects of the broadly used insecticide chlorpyrifos (CPF) on dopamine and glutamatergic neurotransmission effectors in corticostriatal motor/reward circuitry. CPF potentiated protein kinase A (PKA)-dependent phosphorylation of the striatal protein dopamine- and cAMP-regulated phosphoprotein of M_r 32 kDa (DARPP-32) and the glutamate receptor 1 (GluR1) subunit of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in mouse brain slices. It also increased GluR1 phosphorylation by PKA when administered systemically. This correlated with enhanced glutamate release from cortical projections in rat striatum. Similar effects were induced by the sarin congener, diisopropyl fluorophosphate, alone or in combination with the putative neuroprotectant, pyridostigmine bromide and the pesticide *N,N*-diethyl-meta-toluamide (DEET). This combination, meant to mimic the neurotoxicant exposure encountered by veterans of the 1991 Persian Gulf War, also induced hyperphosphorylation of the neurofibrillary tangle-associated protein tau. Diisopropyl fluorophosphate and pyrodostigmine bromide, alone or in combination, also increased the aberrant activity of the protein kinase, Cdk5, as indicated by conversion of its activating cofactor p35 to p25. Thus, consistent with recent findings in humans and animals, organophosphate exposure causes dysregulation in the motor/reward circuitry and invokes mechanisms associated with neurological disorders and neurodegeneration.

Keywords: chlorpyrifos, dopamine, Gulf War illness, insecticide, neurotoxicity, organophosphate.

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A wide variety of pesticides are known to have neurotoxic properties. For example, prenatal exposure to a class of compounds known as OP reduces child IQ (Bouchard *et al.*

Abbreviations used: ACh, acetylcholine; AChE, acetylcholinesterase; aCSF, artificial cerebral spinal fluid; ADHD, attention deficit hyperactivity disorder; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Cdk5, cyclin-dependent kinase 5; CPF, chlorpyrifos; CPO, chlorpyrifos oxon; DARPP-32 or D32, dopamine- and cAMP-regulated phosphoprotein of M_r 32 kDa; DEET, *N,N*-diethyl-meta-toluamide; DFP, diisopropyl fluorophosphate; DMSO, dimethylsulfoxide; GluR1, glutamate receptor 1; i.p., intraperitoneal; mAChR, muscarinic acetylcholine receptor; mEPSC, miniature excitatory postsynaptic current; MSN, medium spiny neuron; nAChR, nicotinic acetylcholine receptor; OP, organophosphates; PB, pyridostigmine bromide; PKA, protein kinase A; P-T205, anti-phospho-tau; s.c., subcutaneous.

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2011), impairs mental development (Engel *et al.* 2011), and causes cognitive deficits (Eaton *et al.* 2008; Bouchard *et al.* 2010; Rauh *et al.* 2011). Furthermore, exposure to cholinesterase-inhibiting compounds including OP, pesticides, pyridostigmine bromide (PB, an anti-nerve agent medication), and low-level sarin nerve gas is linked epidemiologically with a chronic multi-symptom illness in veterans from the Persian Gulf War (Staines 2005; Thomas *et al.* 2006; Haley *et al.* 2009). Symptoms include cognitive disturbances, muscle fatigue, fever, diarrhea, and insomnia.

OP nerve agents and insecticides disrupt cholinergic neurotransmission by inhibiting acetylcholinesterase (AChE) (Wiener and Hoffman 2004). Acetylcholine (ACh) is the major excitatory neurotransmitter at neuromuscular junctions of the peripheral nervous system and a widely distributed first messenger throughout the CNS. Termination of cholinergic transmission is completely dependent upon active AChE. Inactivation of AChE by OP increases nervous system levels of ACh, over-stimulating both nicotinic (nAChR) and muscarinic (mAChR) receptors (Felder *et al.* 2000). Acute high level exposure to OP induces seizures, respiratory failure, coma, and death (Wiener and Hoffman 2004). Low level exposures to CPF, the most well-known OP insecticide in agriculture and domestic use, alters the expression of genes involved in cell growth and differentiation, cAMP-related signaling, neurotransmitter synthesis and release, and receptors that target the actions of many neurotransmitters including ACh and dopamine (Auman *et al.* 2000; Slotkin and Seidler 2007; Eells and Brown 2009).

Dopaminergic pathways within the mesocorticolimbic circuitry mediate reward learning, motivation, and motor control. At the hub of this circuitry, striatal medium spiny neurons (MSNs) receive cholinergic input via α4β2 nAChRs and α7 mAChRs at dopaminergic and glutamatergic terminals, respectively (Nomikos *et al.* 2000; Wonnacott *et al.* 2000). Furthermore, nicotine exhibits differential dose-dependent actions on pre-synaptic nAChRs, stimulating dopamine release and activation of D1- and D2-dependent signaling pathways in the neostriatum (Hamada *et al.* 2004). Interestingly, OP exposure is linked to attention deficit hyperactivity disorder (ADHD) epidemiology, a disorder that targets corticostriatal circuitry (Bouchard *et al.* 2010; Drerup *et al.* 2010). Furthermore, single photon emission computed tomography revealed abnormal brain responses to cholinergic challenges in the basal ganglia of Gulf War veterans (Haley *et al.* 2009).

These findings suggest that the harmful effects of OP exposure are because of, at least in part, dysregulation of dopamine neurotransmission and neurotoxic effects on striatal neurons. Therefore, we assessed the effects of AChE-inhibiting agents on neostriatal dopamine signal transmission. The results revealed dysregulation of the D1 receptor/cAMP/PKA signaling pathway, potentiation of corticostriatal glutamatergic transmission, hyperphosphorylation of tau, and induction of aberrant activity of the neuronal protein kinase Cdk5. These data define novel molecular targets that may mediate OP neurotoxicity and provide an avenue for the development of therapeutic treatments following nerve agent exposure.

Experimental procedures

Materials

Adenosine deaminase, anti-phospho-tau (P-T205), CPF, diisopropyl fluorophosphate (DFP), DEET, NMDA, pyridostigmine bromide (PB), and R(+)-SCH-23390 hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phospho-DARPP-32 (P-T34), phospho-DARPP-32 (P-T75), and DARPP-32 antibodies were from Cell Signaling Technology Inc. (Beverly, MA, USA). Chlorpyrifos oxon (CPO) was from Chem Service Inc. (West Chester, PA, USA), GluR1 subunit of AMPA receptor antibody from Abcam (Cambrigde, MA, USA), anti-phospho-GluR1 (P-S845) from PhosphoSolutions (Aurora, CO, USA), mouse (monoclonal) anti-tau from Biosource (Camarillo, CA, USA), and P35 (C-19) sc-820 antibody from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Goat anti-mouse IgG and goat anti-rabbit IgG peroxidase conjugated secondary antibodies were from Thermo Fisher Scientific (Rockford, IL, USA). All other chemicals were purchased from Sigma-Aldrich.

Animals

All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center or the CDC-NIOSH. For experiments involving systemic injections with nerve agents, drugs were administered via subcutaneous (s.c.) or intraperitoneal (i.p.) route to adult male FVB or adult male or female C57BL/6 mice at the indicated dose levels. For studies of the systemic effects of CPF, female C57BL/6 mice were dosed daily for 7 days (30 mg/kg, s.c.). On the seventh day, the mice were killed 2 h following CPF treatment and striatal tissue was rapidly dissected for immunoblotting. Vehicle (Veh) treated mice received corn oil alone. CPF was dissolved in dimethylsulfoxide (DMSO) (for slice pharmacology experiments), and corn oil or peanut oil (for systemic injections). All experiments involving the use of DEET and DFP were performed at the CDC-NIOSH. In those experiments, for evaluation of phospho-Ser845 GluR1 and phospho-Thr205 tau, male FVB mice were dosed daily for 15 days with PB (2.5 mg/kg, s.c.) and DEET (5 mg/kg, s.c.). On the 15th day, a challenge dose of DFP (4 mg/kg, i.p.) was given and the mice were killed 2 h following DFP for immunoblot analysis. The mice were killed by focused microwave fixation. For evaluation of p25 levels, the dose of DEET was increased to 30 mg/kg (s.c.). Control mice received vehicle only. Mice that received DFP injections exhibited seizure-like behaviors and mortality was approximately 10% among all DFP treatment groups. For studies of the systemic effect of PB following a delay period from the time of exposure, male C57BL6 mice were dosed daily for 10 days (1 mg/kg, s.c.) and sacrificed 4 weeks after

the last treatment for immunoblotting. Pyridostigmine bromide, diisopropyl fluorophosphates, and *N,N*-diethyl-meta-toluamide were dissolved in 0.9% saline.

Acute slice pharmacology

Brains from male C57BL/6 mice (6–10 weeks old) were rapidly dissected and placed in ice-cold oxygenated Krebs buffer (124 mM NaCl, 4 mM KCl, 26 mM $NaHCO_3$, 10 mM D-glucose, 1.5 mM $CaCl_2$, 1.5 mM $MgSO_4$, 1.25 mM KH_2PO_4). Using a vibratome, coronal slices (350 μm) were prepared, and the striatum was microdissected and incubated at 30°C under 95% O_2/5% CO_2 for the indicated periods. Slices were separately treated with pharmacological reagents following a pre-incubation and recovery period in Kreb's buffer containing adenosine deaminase (10 μg/mL) to allow homeostasis to be achieved. For experiments involving the D1 antagonist SCH-23390, a 10 min pre-incubation with the compound was performed before treatments with CPO as specified in the experiment. Following drug treatment, slices were snap frozen in dry ice and stored at −80°C.

Quantitative immunoblotting

Protein phosphorylation levels were evaluated by quantitative immunoblotting with phosphorylation state-specific antibodies. Samples were transferred to dry ice, then removed, immediately sonicated in boiling lysis buffer (10 mM NaF in 1% sodium dodecyl sulfate), and boiled for 5 min. Protein concentrations were determined by bicinchoninic acid protein assay (Thermo Fisher Scientific). Equal amounts of total protein (100 μg) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by overnight transfer to nitrocellulose membranes. Membranes were blocked in 5% powdered skim milk dissolved in Tris-buffered saline-Tween, then probed with primary antibodies for P-T34 DARPP-32 (1 : 500), P-T75 DARPP-32 (1 : 1000), DARPP-32 (1 : 1000), P-S845 GluR1 (1 : 1000), GluR1 (1 : 1000), P-T205 tau (1 : 1000), tau (1 : 1000), and P35 (C-19) (1 : 500). Blots were washed several times and incubated with HRP-conjugated secondary antibodies (1 : 10,000). For detection of phosphorylation, blots were stripped and re-probed for total protein. Antibody was detected using enhanced chemiluminescence (GE Healthcare, Piscataway, NJ, USA). Multiple exposure times were evaluated for each blot to ensure linearity.

Electrophysiological recordings

Following anesthetization of male or female p14-p18 Sprague-Dawley rats, brains were removed and immediately immersed in ice-cold, oxygenated, modified artificial cerebral spinal fluid (aCSF) containing the following (in mM): sucrose, 194; NaCl, 30; KCl, 4.5; $MgCl_2$, 1; $NaHCO_3$, 26; NaH_2PO_4, 1.2; D-glucose, 10. Three hundred micrometer slices were generated using a vibratome (Leica Microsystems, GmbH, Wetzlar, Germany) and subsequently placed in continuously oxygenated aCSF containing the following (in mM): NaCl, 124, KCl, 4.5; $MgCl_2$, 1; CaCl, 2; $NaHCO_3$, 26; NaH_2PO_4, 1.2; D-glucose, 10. The aCSF osmolarity was 320 mOsm and the pH was 7.3. Slices were equilibrated at 33°C for 1 h and subsequently incubated at 25°C until hemisected slices were transferred to the recording chamber.

Whole-cell electrophysiological recordings of dorsolateral striatal MSN miniature excitatory post-synaptic currents (mEPSCs) were performed in 29–31°C aCSF containing picrotoxin (50 μM), tetrodotoxin (TTX) (1 μM), and 0.1% DMSO. Intracellular glass recording electrodes were pulled from borosilicate glass on a Flaming/Brown micropipette puller (Sutter Instrument Co., Novato, CA, USA) to a resistance of 2–5 mΩ. Recording electrodes were filled with a $CsMeSO_3$–based solution of 295–300 mOsm containing (in mM); 120 $CsMeSO_3$, 5 NaCl, 10 TEA-Cl, 10 HEPES, 5 QX-314, 1.1 EGTA, 0.3 Na-GTP, and 4 Mg-ATP. MSNs were voltage clamped at −60 mV using a Multiclamp 700 A amplifier (Molecular Devices, Sunnyvale, CA, USA) following rupture of the cell membrane and achievement of the whole-cell recording configuration. After a 3-min equilibration period, baseline mEPSC recordings were taken for 5 min, followed by a 10 min bath-application of CPO (100 μM, dissolved in DMSO) for a total of a 15 min recording session. The concentration of DMSO remained at 0.1% throughout the experiment. Recordings were filtered at 2 kHz and digitized at 10 kHz and were discarded if series resistance exceeded 25 MΩ or changed by more than 15%.

Data analysis

For quantitative immunoblotting analysis, immunoreactivity signals were captured by autoradiography and quantified with ImageJ software (NIH). The quantified effects were derived by normalization of the signals to total protein levels. Data are presented as mean ± SEM. Student's *t*-test or ANOVA was performed to compare data sets using GRAPHPAD Prism (GraphPad Software Inc., San Diego, CA, USA).

For electrophysiological analysis, baseline (0–5 min) and CPO (10–15 min) mEPSC data were analyzed using the Mini Analysis program version 6.0.7 (Synaptosoft, Decatur, GA, USA). Amplitude and area thresholds were manually set, and the accuracy of mEPSC detection was manually verified for all data sets. A paired *t*-test was used to compare data sets using GraphPad Prism. Data are expressed as mean ± SEM.

Results

Chlorpyrifos and pyridostigmine bromide enhance PKA-dependent phosphorylation of downstream effectors of dopamine neurotransmission in acute striatal slices

To explore the effect of nerve agents on dopamine neurotransmission, we evaluated the influence of the organophosphate, CPF, on the PKA-dependent phosphorylation state of two important downstream effectors of striatal dopamine neurotransmission, DARPP-32 and the GluR1 subunit of the AMPA receptor. Treatment of acutely prepared mouse striatal slices with the CPF active metabolite, CPO, caused a 1.9 ± 0.1-fold increase in phospho-Thr34 DARPP-32 (Fig. 1a). When PKA phosphorylates DARPP-32 at Thr34, it is converted into a potent inhibitor of protein phosphate 1, thereby allowing dopamine to control the phosphorylation state of numerous phosphoproteins that regulate striatal neuron function (Greengard *et al.* 1999). CPO did not alter the phosphorylation state of DARPP-32 at Thr75, a Cdk5 site that converts it into a PKA inhibitor (Bibb *et al.* 1999) (Fig. 1b). GluR1 is another important downstream effector of the D1 receptor/cAMP/PKA cascade. Phosphorylation at

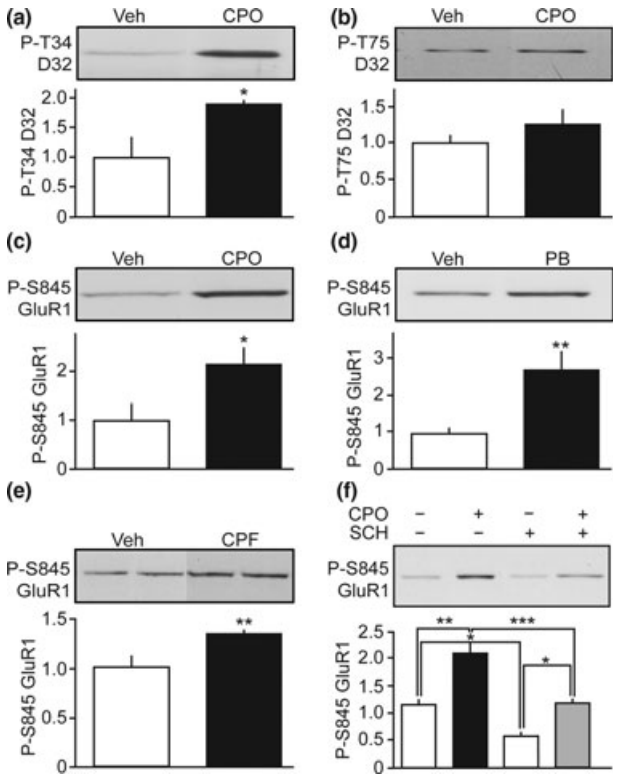

Fig. 1 CPF and PB activate PKA signaling in striatum. Effects of treatment of mouse striatal slices with CPO (100 μM, 60 min) on DARPP-32 (D32) phosphorylation by (a) PKA at Thr34 (P-T34) or (b) Cdk5 at Thr75 (P-T75) vs. vehicle (Veh) are shown as representative blots with quantification. The effects of treatment of CPO (c) or PB (d) (100 μM, 60 min) on GluR1 phosphorylation at Ser845 (P-S845) is shown. (e) The effect of systemic exposure to CPF on striatal P-S845 GluR1 is shown. (f) Effects of treatment of mouse striatal slices with CPO (100 μM, 60 min) on P-S845 GluR1 in the absence (−) and presence (+) of the D1 receptor antagonist SCH 23390 (SCH, 1 μM). Data represent means ± SEM normalized for total levels. *$p = 0.0298$, Student's *t*-test, for (a), $n = 3$. *$p = 0.0305$, Student's *t*-test, for (c), $n = 4$. **$p = 0.0100$, Student's *t*-test, for (d), $n = 4$–5. **$p = 0.0085$, Student's *t*-test, for (e), $n = 5$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ANOVA with Newman–Keuls *post hoc* test, for (f), $n = 3$–4.

Ser845 GluR1 is increased by D1 dopamine receptor agonists, and the phosphorylation of this site controls GluR1 trafficking, stability, and striatal neuron excitability (Song and Huganir 2002). Treatment of striatal slices with CPO enhanced PKA-dependent phosphorylation of Ser845 GluR1 by 2.1 ± 0.4-fold (Fig. 1c). Furthermore, treatment of striatal slices with the anti-nerve agent cholinesterase inhibitor PB revealed a 2.7 ± 0.5-fold enhancement in phospho-Ser845 GluR1 (Fig. 1d). Thus, CPO and PB regulate important downstream effectors of striatal dopamine neurotransmission.

Chlorpyrifos increases PKA signaling *in vivo*

To determine if OP exposure *in vivo* could activate striatal PKA, the effect of systemic administration CPO on PKA-dependent phosphorylation of GluR1 was assessed. Mice were treated with eight daily injections of 1 mg/kg or 2.5 mg/kg of CPO. These doses were empirically determined to cause minimal lethality. However, no significant effect on phospho-Ser845 GluR1 was detected under these conditions. As an alternative, mice were injected subcutaneously with 30 mg/kg of the less lethal CPO parent compound, CPF, for 7 days while control mice received only vehicle. Striatal tissue was acutely dissected 2 h after the last dose of nerve agent. Interestingly, repeated treatment with CPF resulted in a 1.36 ± 0.04-fold increase in striatal phospho-Ser845 GluR1 levels (Fig. 1e). These *in vivo* data were consistent with the effects observed in striatal slices indicating that OP exposure activated striatal D1 receptor/cAMP/PKA effectors.

Chlorpyrifos modulation of PKA signaling may involve the activation of D1 dopamine receptors

To understand the mechanism by which nerve agents could dysregulate dopamine neurotransmission, we evaluated the effects of CPO on phospho-Ser845 GluR1 levels in mouse striatal slices in the absence or presence of the D1 receptor antagonist, SCH 23390. SCH 23390 caused a reduction in the basal levels of phospho-Ser845 GluR1 to 50% (0.6 ± 0.1-fold) of control levels in untreated slices (1.2 ± 0.1-fold) (Fig. 1f). Furthermore, the increase in phospho-Ser845 GluR1 induced in the presence of the D1 receptor antagonist was 57.1% (1.2 ± 0.1-fold) of that induced by CPO treatment alone (2.1 ± 0.2-fold) (Fig. 1f). These findings suggest that at least a portion of the effect of the OP in phospho-Ser845 GluR1 was mediated by a pathway other than the activation of D1 receptors. These data also suggest that antagonists of D1 receptors may serve to counter some of the effects of nerve agents on dopamine signaling.

Chlorpyrifos augments corticostriatal glutamatergic transmission

To further define the pathophysiological effects of OP on striatal neuron function, the effects of CPO on corticostriatal glutamatergic neurotransmission was assessed by whole-cell patch clamp recording. For these neurophysiological analyses, TTX-insensitive mEPSCs from dorsolateral striatal MSNs were evaluated. Compared to baseline values, a 10 min bath-application of CPO did not affect mEPSC amplitude (baseline, 29.16 ± 1.32 pA vs. CPO, 28.81 ± 2.65 pA), but caused a significant decrease in the inter-event interval (increase in frequency) of mEPSC events (baseline, 0.68 ± 0.16 s vs. CPO, 0.51 ± 0.15 s) (Fig. 2a–d). CPO application did not change the holding current (Fig. 2e). These results suggest that CPO alters striatal neurotransmission by enhancing glutamate release from corticostriatal terminals in an action potential-independent manner.

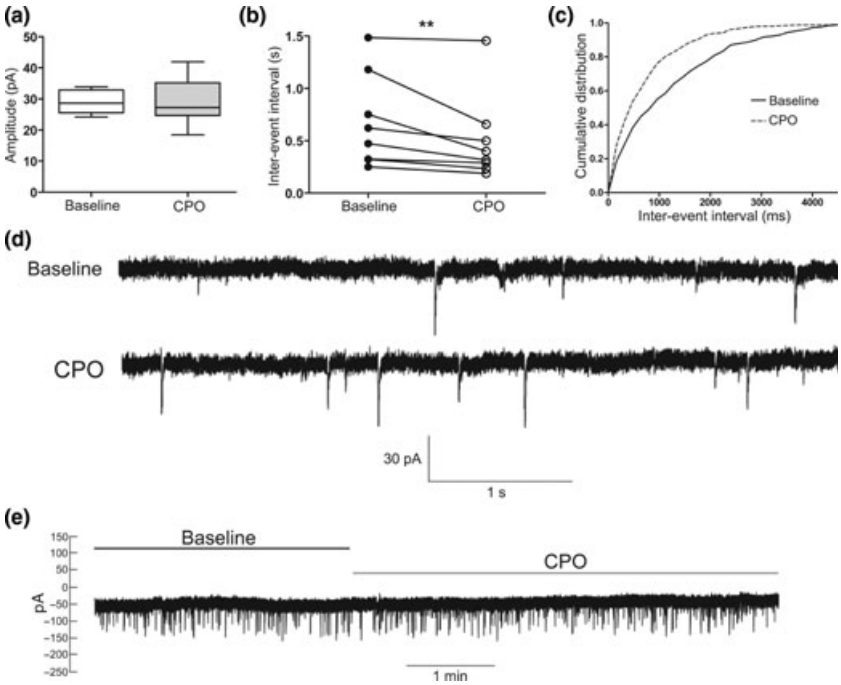

Fig. 2 CPF enhances corticostriatal glutamatergic transmission. (a) Box plots demonstrating that bath application of CPO (100 μM) did not significantly change TTX-insensitive mEPSC amplitude. (b) A decrease in mEPSC inter-event interval during CPO application was observed in all recorded MSNs. (c) The cumulative distribution of mEPSC inter-event interval data during baseline (solid line) and CPO (dashed line) conditions is shown. (d) Example of mEPSC traces during baseline (top) and CPO application (bottom). (e) The holding current was unchanged by application of CPO. $^{**}p = 0.0078$, Student's *t*-test, $n = 8$.

DFP alone or in combination with other neurotoxicants alters PKA signaling *in vivo*

Studies using CPO and CPF revealed effects of this OP on striatal dopamine signaling and excitability. During the 1991 Gulf War, exposure to CPF used as an insecticide likely occurred in combination with use of the insect repellant, DEET, and, potentially, exposure to low levels of sarin gas (Golomb 2008). In addition, the reversible OP cholinesterase inhibitor, PB, was used prophylactically as a neuroprotectant in the event of nerve agent exposure. To mimic these conditions, mice were treated with different combinations of agents of potential relevance to Gulf War Illnesses. One cohort of subjects received a single s.c. injection of 4 mg/kg of DFP, a sarin surrogate compound. Another group was given 2.5 mg/kg PB and 5 mg/kg of DEET daily for 15 days. For another group, to this regimen, a challenge dose of DFP was added on the final day. Quantitative immunoblot analysis was conducted on lysates from acutely dissected regions of the mesolimbic circuitry including striatum and hippocampus 2 h following the final treatment. Mice treated with DFP alone exhibited a trend toward enhanced phosphorylation of GluR1 at the Ser845 site ($p = 0.0586$). However, PB/DEET treatment with or without the DFP challenge had no detectible effect at this phosphorylation site in the striatum (Fig. 3a).

Although the vast majority of dopamine neurotransmission occurs in the striatum, dopamine neurons also innervate the hippocampus. Nerve agents have been suggested to cause deficits in hippocampal-dependent tasks designed to measure cognition. Therefore, we also assessed the effects of systemic administration of PB, DEET, and DFP on phospho-Ser845 GluR1 levels in the hippocampus (Fig. 3b). Immunoblot analysis from hippocampal lysates of mice treated with the same dose described above showed a 1.5 ± 0.1-fold increase in phospho-Ser845 GluR1 following DFP treatment. Exposure to PB and DEET alone did not induce changes in phosho-Ser845 GluR1 compared with vehicle-treated mice. However, a 1.7 ± 0.1-fold increase in PKA-dependent phosphorylation was induced by the combination of these agents with DFP. Together, these data suggest that PKA signaling is enhanced *in vivo* by DFP alone or in combined exposure to other neurotoxicant AChE inhibitors.

Systemic exposure to nerve agents causes hyperphosphorylation of tau

In addition to the dysregulation of PKA-dependent phosphorylation of downstream effectors of dopamine neurotransmission, the effects of OP on neurological function suggest more severe neuronal injury. The hyperphosphorylation of the neurofilament binding protein, tau, has been strongly

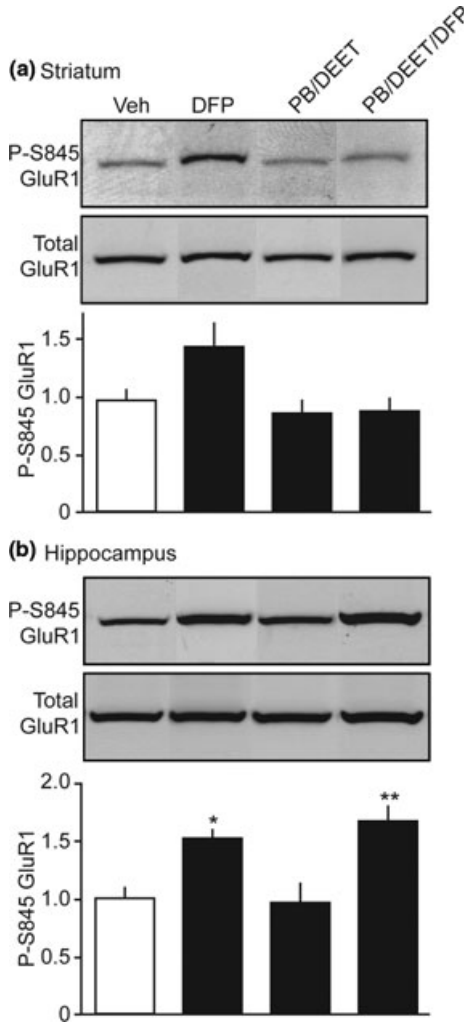

Fig. 3 Nerve agents exposure enhances PKA-dependent phosphorylation of GluR1 *in vivo*. Effects of systemic exposure to the different combinations of PB (15 days), DEET (15 days), and DFP (1 day, final day), indicated, and on P-S845 GluR1 levels in mouse striatum (a) and hippocampus (b) are shown. $*p < 0.05$, $**p < 0.01$, ANOVA with Newman–Keuls *post hoc* test, $n = 4–5$.

implicated in an array of neurological and neurodegenerative diseases (Baumann *et al.* 1993; Lopes and Agostinho 2011). Moreover, phosphorylation of tau at Thr205 by the protein kinase Cdk5 has been associated with loss of neuronal function and death (Baumann *et al.* 1993; Lopes and Agostinho 2011). Interestingly, immunoblot analysis from mice exposed to PB, DEET, and DFP revealed a dramatic 17.0 ± 1.9-fold increase in the levels of phosphorylation of the aberrant Cdk5 substrate Thr205 tau in striatal lysates (Fig. 4a). DFP exposure alone caused a 15.5 ± 2.0-fold increase at that site. Furthermore, hippocampal lysates from mice exposed to all three neurotoxicants showed a 21.5 ± 1.3-fold increase in phospho-Thr205 tau levels, whereas DFP-treated mice exhibited a 17.1 ± 1.1-fold enhancement compared with mice that received vehicle treatment alone (Fig. 4b). These marked effects suggest that toxicity induced upon DFP exposure, alone or in the presence of PB and DEET, induces neuropathological effects in striatum and hippocampus.

Systemic exposure to nerve agents dysregulates Cdk5

Cdk5 regulates dopamine neurotransmission (Bibb *et al.* 1999) and contributes to many other neuronal functions (Cheung *et al.* 2006). However, neuronal injury and loss of homeostasis can cause activation of the Ca^{2+}-dependent protease, calpain (Kusakawa *et al.* 2000; Lee *et al.* 2000). Cleavage of the Cdk5-activating neuronal cofactor p35 to p25 by calpain, results in hyper-activation and redirection of the kinase towards aberrant substrates including Thr205 tau. Cdk5 bound to p25 exhibits aberrant activity, contributes to neuronal death, and has been implicated in neurotoxicity and neurodegenerative diseases (Cruz *et al.* 2003; Lopes and Agostinho 2011). Furthermore, animal models of p25 over-expression exhibit behavioral deficits that correlate with dysregulation of dopamine signaling (Meyer *et al.* 2008).

To investigate the ability of nerve agents to induce aberrant Cdk5 activity, we examined p25 generation in different paradigms of AChE inhibitors exposure. Although CPO alone as a systemic (1.0 or 2.5 mg/kg, s.c. 7 days) didn't induce appreciable p25 generation, the effects of DFP alone or in combination with other OPs on tau hyperphosphorylation, suggested these, possibly more severe, treatments might have an effect on p25 generation. To investigate this possibility, mice were treated with the same dose regimen of PB, DEET, and DFP described above (i.e. Figs 3 and 4) and lysates from acutely dissected regions of the striatum and hippocampus were analyzed 2 h following DFP exposure. Surprisingly, these conditions did not result in a detectable increase in p25 production. However, striatal lysates from a cohort of mice that received a higher dose regimen of the insecticide DEET (30 mg/kg, vs. 5 mg/kg in the earlier experiments) in combined exposure with PB and DFP reveal a 13.4 ± 3.3-fold increase in p25 generation 2 h following the last exposure (Fig. 5a). These mice displayed no changes in phosphorylation at Thr75 DARPP-32 (Fig. 5a). Interestingly, 6 h after the last exposure, mice exhibited a 10.0 ± 1.5-fold increase in p25 generation that was accompanied by a reduction in phospho-Thr75 DARPP-32 to 60% (0.6 ± 0.1) of control levels in vehicle-treated mice (1.0 ± 0.1) (Fig. 5b). Similar effects were also observed in hippocampus with 12.8 ± 3.9 and 17.4 ± 1.1-fold increases at 2 and 6 h, respectively, after the final treatment (Fig. 5c and d). These data indicate that nerve agents induce aberrant Cdk5 activity and shift its specificity away from physiological substrates, most likely, toward aberrant substrates such as tau.

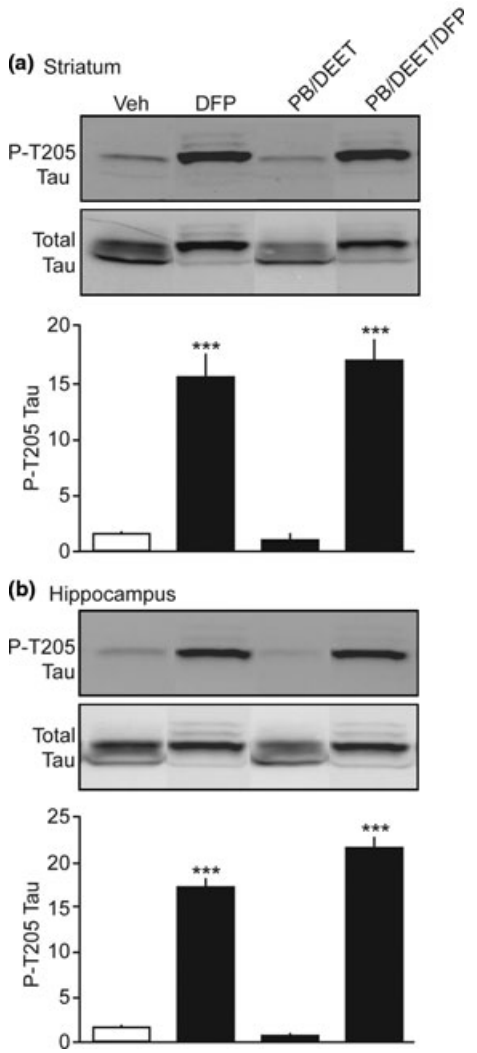

Fig. 4 Nerve agents exposure causes tau hyperphosphorylation *in vivo*. Effects of systemic exposure to different combinations of PB, DEET, and DFP, and on the phosphorylation state of Thr205 tau (P-T205 Tau) in mouse striatum (a) and hippocampus (b) are shown. ***$p < 0.001$, ANOVA with Newman–Keuls *post hoc* test, $n = 4$–5.

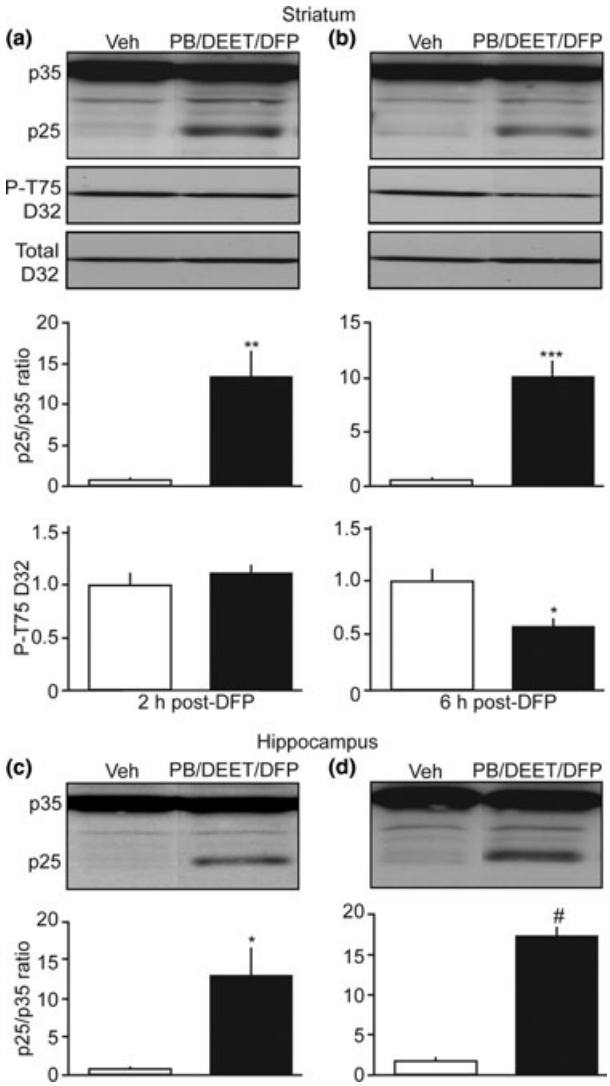

Fig. 5 Nerve agents exposure dysregulates Cdk5 activity *in vivo*. Effects of systemic exposure to PB, DEET, and DFP on p25 generation and Cdk5-dependent phosphorylation of P-T75 DARPP-32 in mouse striatum (a and b). Representative blots from tissue taken 2 or 6 h after treatments are shown above quantification. Effects of systemic exposure to PB, DEET, and DFP on p25 generation in hippocampus (c and d). *$p = 0.0108$, **$p = 0.0045$, ***$p = 0.0004$, Student's *t*-test, for (a), $n = 3$–4. *$p = 0.0102$, #$p < 0.0001$, Student's *t*-test, for (b), $n = 3$–4.

Systemic exposure to pyridostigmine bromide dysregulates Cdk5 activity following a delay period from time of exposure

Although neurotoxic exposure could be limited to self-administration of PB, the data indicated that PB in the presence of DEET had little initial neurotoxic effect. However, it has been suggested that the pathological effects to subclinical doses of OP such as PB may only manifest after a delay period of the last exposure (Kamel and Hoppin 2004). Therefore, we examined the effect of systemic administration of PB following a 4-week delay period after the last exposure. Mice were treated with 10 daily injections of 1 mg/kg PB and immunoblot analysis was conducted on lysates from acutely dissected regions following the delay period. Mice treated with PB exhibited a 1.6 ± 0.3-fold increase in p25 generation accompanied by a decrease in phospho-Thr75 DARPP-32 levels to 69% (0.69 ± 0.05) of control levels in vehicle-treated mice (1.0 ± 0.1) (Fig. 6a). Comparable effects were detected in hippocampus with 1.8 ± 0.2-fold increase following the delay treatment. The detection of high levels of p25 in these two brain regions and redirection of Cdk5 specificity support the hypothesis that this compound which was administered to Gulf War veterans during combat has the capacity to induce neuronal injury, even after considerable delay from the time of exposure to subclinical doses.

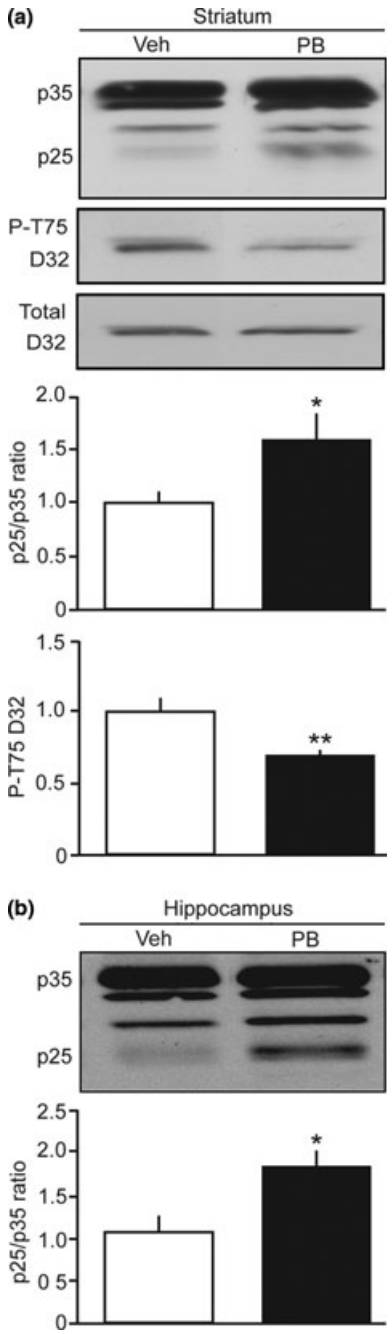

Fig. 6 Exposure to PB dysregulates Cdk5 activity *in vivo* after a delay period. (a) Effects of systemic exposure to PB following a 4-week delay period after the last treatment on p25 generation and P-T75 DARPP32 in mouse striatum. (b) Delayed effects of systemic exposure to PB on p25 generation in mouse hippocampus. *$p = 0.0297$, **$p = 0.0065$, Student's *t*-test, for (a), $n = 6$. *$p = 0.0106$, Student's *t*-test, for (b), $n = 6$.

Discussion

Nerve agents are thought to induce brain damage through hyperstimulation of cholinergic receptors (McDonough and Shih 1997). However, both glutamatergic and dopaminergic neurotransmitter systems mediate the biological and pathological effects of AChE inhibitors (McDonough and Shih 1997; Wonnacott 1997; Wonnacott *et al.* 2000). Furthermore, the neostriatum has been implicated as a target of the long-term neurotoxic effects of OP in clinical studies (Haley *et al.* 2000, 2009). To better understand the deleterious and pathogenic effects of these compounds, we examined the consequences of nerve agent exposure on dopamine signaling pathways, glutamate neurotransmission, and neuronal injury mechanisms in the neostriatum in rodent brain slices and whole animals *in vivo*.

We found that CPF and PB increased PKA-dependent phosphorylation of downstream effectors of striatal dopamine neurotransmission including the GluR1 subunit of the AMPA receptor and/or DARPP-32 in slices and *in vivo*. A previous study found that high concentrations of nicotine induced a D1 receptor-dependent activation of PKA and increased phosphorylation of Thr34 DARPP-32 through activation of the striatonigral pathway (Hamada *et al.* 2004). However, given that a potent D1 receptor antagonist did not block the activation of striatal PKA, it is possible that at least a portion of the effects of CPF are mediated by pathways, such as those modulated by mAChR, which do not directly involve D1 receptor activation. Accordingly, ACh released from striatal cholinergic interneurons interacts in a dynamic manner with dopamine signaling at multiple levels including pre-synaptic regulation of neurotransmitter release and post-synaptic effects (Threlfell and Cragg 2011). The degree to which CPF activates PKA through non-dopamine mediated mechanisms may warrant further study. Moreover, the deleterious effects of these agents may occur across a spectrum of biological and pathological mechanisms. At relatively low levels of exposure, effects may occur via AChR activation and subsequent dopamine release, whereas higher doses may induce dysregulation of Cdk5, removal of tonic inhibition of PKA, or direct covalent modifications because of organophosphate reactivity.

The effects of CPF on the phosphorylation state of downstream effectors of the D1 receptor/cAMP/PKA pathway suggest that nerve agents may exert some of their pathological effects via alterations in dopamine neurotransmission. CPF has been shown to increase dopamine turnover and to alter dopamine and other monoamine metabolite levels (Moreno *et al.* 2008; Eells and Brown 2009). Furthermore, CPF exposure during the gestational days of developing rats evoked long-term increases in serotonin and dopamine turnover (Aldridge *et al.* 2005). Some of the positive effects of CPF on PKA activity in slices and whole animal subjects (see Fig. 1) were not replicated when combined OP regimens, including higher DEET concentrations, were used (see Fig. 3a, for example). However, these more toxic exposures may have invoked mechanisms of neuronal injury associated with Cdk5/p25 that possibly occluded effects observed at lower doses.

Excessive glutamate release may constitute a principle cause of neuronal injury and death. ACh can modulate glutamate release through pre-synaptic α7 nACh receptors in striatal synaptosomes (Marchi *et al.* 2002). Furthermore, nicotine has been shown to enhance hippocampal synaptic transmission on pre-synaptic terminals containing α7 nAChRs (Gray *et al.* 1996). Consistent with these observations, CPF attenuated the inter-interval period between mEPSC events, presumably reflecting enhanced striatal glutamate release. Surprisingly, there are few other reports of the potentiation of striatal glutamate neurotransmission by AChE inhibitors. On the other hand, a study on the effects of nerve agent soman-induced seizures has shown that the levels of excitatory amino acids are attenuated following intoxication (Shih and McDonough 1997). AChE inhibitors such as donepezil, rivastigmine, and galantamine are used as clinical treatments to delay or slow the onset of cognitive deficiencies accompanying Alzheimer's disease pathology, and these compounds have been suggested to attenuate excitotoxicity in the hippocampus and cortex (Standridge 2004; Hansen *et al.* 2006). Furthermore, it has been suggested that the elicited neuroprotective effects of donepezil may involve down-regulation of NMDA receptors, thereby attenuating glutamate-induced Ca^{2+} increase following α7 nAChRs activation (Shen *et al.* 2010).

Nerve agents induce seizure-related brain damage through hyper-stimulation of cholinergic receptors. Subsequent excessive stimulation of the glutamatergic system triggers a prolonged intracellular increase in Ca^{2+}, causing the activation of secondary pathways responsible for neuronal injury (McDonough and Shih 1997). NDMA receptor antagonists such as memantine, a drug used clinically to treat Alzheimer's (Thomas and Grossberg 2009), have been recognized for their ability to reduce Ca^{2+} overload. However, controversy exists regarding their efficacy and resulting neurotoxic effects (Filbert *et al.* 2005). Current studies are focused on identifying secondary intracellular signaling pathways that could be targeted for the development of therapeutics that prevent or alleviate the symptoms following nerve agent exposure.

DFP alone or as part of a combination of neurotoxicants meant to mimic the exposure encountered by veterans of the 1991 Persian Gulf War caused hyper-phosphorylation of Thr205 tau. Tau functions to stabilize the cytoskeleton, and its hyper-phosphorylation can result in the formation of neurofibrillary tangles and neurodegeneration (Hanger *et al.* 2009). Phosphorylation at this site is Cdk5-dependent and it has been implicated as a biomarker for Alzheimer's pathology (Cruz *et al.* 2003; Cruz and Tsai 2004). Cdk5 plays a critical role in corticogenesis, synaptic plasticity, drug addiction, and cognition (Bibb 2003; Angelo *et al.* 2006; Cheung *et al.* 2006). Dysregulation of its activity may depend on prolonged NMDA receptor activation. The resulting intracellular Ca^{2+} influx could then cause calpain-dependent conversion of p35 to p25 (Lee *et al.* 2000). Unnecessary NMDA receptor activation because of excessive cholinergic stimulation is one of the most prominent effects of nerve agent exposure that ultimately leads to neuropathology (McDonough and Shih 1997).

PB/DEET/DFP exposure also resulted in p25 generation and induction of aberrant Cdk5 activity in both striatum and hippocampus. Interestingly, the high levels of p25 in striatum correlated with a decrease in phospho-Thr75 DARPP-32, possibly disinhibiting PKA activity. Similar effects were induced by PB after an extensive delay from the time of exposure. High levels of nicotine also reduce Cdk5-dependent phosphorylation of DARPP-32 at Thr75, possibly through a dopamine-release dependent pathway (Hamada *et al.* 2005). Moreover, induction of transgenic p25 overexpression reduced Thr75 DARPP-32, altered dopamine signaling, and shifted Cdk5 specificity with regard to physiological and aberrant substrates (Meyer *et al.* 2008). Nonetheless, striatal signaling may be differentially dysregulated depending on the profile of exposure.

Our data indicate that dysregulation of Cdk5 is a component of the long-term effects that arise from exposure and is consistent with at least two reports suggesting OP neurotoxicants dysregulate Cdk5 (Wang *et al.* 2006; Zhu *et al.* 2010). Thus, nerve agent-induced dysregulation of Cdk5 may dramatically affect striatal-dependent brain function, and may be relevant to subclinical neurotoxicity and disorders involving dopamine neurotransmission. For example, pregnant women exposed to pesticides such as CPO and DEET have a higher risk to manifest deficits in fetal neurodevelopment and altered birth outcomes (Berkowitz *et al.* 2004; Barr *et al.* 2010). Prenatal insecticide exposure reduces IQ and delays mental and psychomotor development (Rauh *et al.* 2006, 2011; Bouchard *et al.* 2011; Engel *et al.* 2011), and children with elevated urinary levels of OP metabolites had a higher ADHD prevalence (Bouchard *et al.* 2010). Furthermore, a recent study has shown that CPF oxon alters the firing activity of the locus coeruleus noradrenergic neurons, a brain region associated with anxiety and stress (Cao *et al.* 2011). Taken together, the present study is among the first to examine the mechanisms by which OP may alter brain function and cause long-term pathological effects. The secondary signaling targets identified here may contribute to the development of treatments to prevent or counter these neurotoxic effects.

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