2016

Repeated low-dose organophosphate DFP exposure leads to the development of depression and cognitive impairment in a rat model of Gulf War Illness

Laxmikant Deshpande
Virginia Commonwealth University, laxmikant.deshpande@vcuhealth.org

Kristin Phillips

Repeated low-dose organophosphate DFP exposure leads to the development of depression and cognitive impairment in a rat model of Gulf War Illness

Kristin F. Phillips and Laxmikant S. Deshpande*

Department of Neurology, Virginia Commonwealth University, Richmond, VA 23298, USA

To whom correspondence should be addressed:
*Laxmikant S. Deshpande, Ph.D.
Virginia Commonwealth University
School of Medicine
PO Box 980599
Richmond, VA 23298
Phone: 804-828-3392
Fax: 804-828-6432
E-mail: Laxmikant.Deshpande@vcuhealth.org

Abstract:

Approximately 175,000 to 250,000 of the returning veterans from the 1991 Persian Gulf War exhibit chronic multi-symptom illnesses that includes neurologic co-morbidities such as depression, anxiety and cognitive impairments. Amongst a host of causative factors, exposure to low levels of the nerve agent Sarin has been strongly implicated for expression of Gulf War Illness (GWI). Nerve agents similar to pesticides are organophosphate (OP) compounds. There is evidence from civilian population that exposure to OPs such as in agricultural workers and nerve agents such as the survivors and first-responders of the Tokyo subway Sarin gas attack suffer from chronic neurological problems similar to GWI symptoms. Given this unique chemical profile, OPs are ideal to study the effects of nerve agents and develop models of GWI in civilian laboratories. In this study, we used repeated low-dose exposure to OP agent diisopropyl fluorophosphate (DFP) over a 5-day period to approximate the duration and level of Sarin exposure during the Persian Gulf War. We tested the rats at 3-months post DFP exposure. Using a battery of behavioral assays, we observed the presence of symptoms of chronic depression, anxiety and memory problems as characterized by increased immobility time in the Forced Swim Test, anhedonia in the Sucrose Preference Test, anxiety in the Elevated Plus Maze, and spatial memory impairments in the Object Location Test, respectively. Chronic low dose DFP exposure was also associated with hippocampal neuronal damage as characterized by the presence of Fluoro-Jade staining. Given that OP exposure is considered a leading cause of GWI related morbidities, this animal model will be ideally suited to study underlying molecular mechanisms for the expression of GWI neurological symptoms and identify drugs for the effective treatment of GWIs.

Keywords: Gulf War Illness, Organophosphate, DFP, depression, anxiety, memory impairments

Introduction

About 25-35% of the deployed soldier population from 1991 Gulf War suffer from a constellation of inexplicable symptoms referred to as Gulf War Illness (GWI). According to the Institute of Medicine's report, GWI also known as chronic multi-symptom illness is defined as the presence of a spectrum of chronic symptoms experienced for 6 months or longer in at least two of six categories: development of fatigue, mood and cognitive changes, musculoskeletal changes, gastrointestinal symptoms, respiratory difficulty, and neurologic abnormalities including major co-morbidities such as depression and anxiety (Institute of Medicine: Board on the Health of Select Populations, 2013). There are several confounding factors attributed to development of GWI, including exposure to depleted uranium from tanks and body armor, prophylactic use of pyridostigmine bromide (PB) tablets, heavy use of insect repellants such as DEET and permethrin, smoke from oil-well fires, and dust particulate matter among others (Friedl et al., 2009; Steele et al., 2012; Wolfe et al., 2002). Interestingly, GWI symptoms have not been reported in veterans returning from other military conflicts suggesting that deployment-related stress is not a major factor in the expression of these multi-symptom illnesses (Haley, 1997). Newly assembled epidemiological, meteorological and intelligence data now indicate soldiers were exposed to organophosphate (OP) nerve agents Sarin and Cyclosarin from fallout released from demolitions of the ammunition dump at Khamisiyah, Iraq (Couzin, 2004; Haley and Tuite, 2013; Special Assistant to the Secretary of Defense for Gulf War Illnesses, 2001; Tuite and Haley, 2013). After reviewing all the available data, the Research Advisory Committee on Gulf War Veterans' Illnesses has strongly implicated exposure to OPs as one of the leading cause for GWI (Couzin, 2004; U.S. Department of Veterans Affairs, 2008, White et al., 2015). Animal studies using various combinations of GW agents particularly combinations of PB with

insecticides have reported expression of GWI related symptoms in rodents (Hattiangady et al., 2014; Ojo et al., 2014; Parihar et al., 2013; Zakirova et al., 2015). However, the consequences of a repeated low-level OP agent exposure on the development of neurological morbidities as observed in GWI have not been clearly documented (White et al., 2015).

Diisopropyl fluorophosphate (DFP) is an OP compound that is used in civilian laboratories as a surrogate nerve gas agent (Deshpande et al., 2010; Li et al., 2011b; O'Callaghan et al., 2015; Terry et al., 2012). Similar to Sarin, DFP is also an irreversible inhibitor of the enzyme acetylcholinesterase (AChE). Significant inhibition of AChE such as observed during lethal OP exposures leads to rapid buildup of the neurotransmitter acetylcholine at the synapses precipitating a "cholinergic crisis" as characterized by miosis, salivation, bradycardia, seizures and ultimately death, if left untreated (Bajgar, 2004). There is also evidence from the civilian population that repeated exposure to insecticides (Rosenstock et al., 1991; Savage et al., 1988; Steenland et al., 1994; Wesseling et al., 2002) or exposure to nerve agents (Brown and Brix, 1998) as seen in the survivors and first responders of the Tokyo subway and Matsumoto Sarin gas attacks in Japan (Hood, 2001; Nishiwaki et al., 2001) can lead to the development of chronic neurological morbidities. Animal studies have also reported neuropsychiatric morbidities following high levels of OP exposures or chronic exposures to insecticides (Abdel-Rahman et al., 2004a; Deshpande et al., 2014b; Henderson et al., 2002; Johnson et al., 2009). For example, stress and combined exposure to low doses of PB and insecticides permethrin and DEET have been reported to produce chronic neuropathology and neurobehavioral deficits (Abdel-Rahman et al., 2004b; Hattiangady et al., 2014). Studies have also shown that repeated administration of low-dose chlorpyrifos in rats produced chronic memory impairments in the radial-arm maze task (Terry et al., 2012). Repeated exposure to DFP (0.5 mg/kg every other day for 30-days) in rats

has also been reported to produce cognitive deficits in the water-maze test (Terry et al., 2011) and persistent impairments of inhibitory response control in a 5-Choice Serial Reaction Time Task (Terry et al., 2014). In an attempt to develop an OP based rodent model of GWI, we used Sarin surrogate DFP to mimic a low-level OP exposure corresponding to the 4-5 day period when soldiers were exposed to nerve agents during the 1991 Persian Gulf War. Behavioral assays for determination of neurological morbidities were then carried out 3-months following DFP exposure.

Materials and methods

Animals

All animal use procedures were in strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by Virginia Commonwealth University's Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing ~300 g and 8 weeks of age were used in this study. Animals were housed two per cage at 20-22^{o} C with a 12 hour light-dark cycle (lights on 0600-01800 h) and given free access to food and water.

Chemicals

All the chemicals were obtained from Sigma Aldrich Company (St. Louis, MO, USA) unless otherwise noted.

DFP exposure

DFP (catalog # D0879) was prepared fresh daily by dissolving in ice-cold phosphate buffered saline just before the exposure. Rats were injected with DFP (400 μg/kg, s.c.) once-

daily for 5-days, while control rats received DFP vehicle injections for the same period. Animal health including weight measurements were assessed every day during the exposure and for the next seven days following the end of chronic DFP injections.

Assessment of depression and memory

All behavioral assays were carried out 3-months following DFP exposure. Testing was carried out in a quiet, dimly lit room between 0800 to 1400 hrs. Behavioral testing moved from the least stressful to most stressful tasks. Thus, rats were first subjected to sucrose preference test followed by object location test, then elevated plus maze and finally forced swim test. No two tests were carried out on the same day. These tests are described below.

Forced Swim Test (FST)

Porsolt's modified FST was used to assess behavioral despair (Castagne et al., 2011; Deshpande et al., 2014b; Overstreet, 2012). Briefly, animals were forced to swim by being placed in a glass cylindrical chamber (46cm H x 30cm D) filled with water (30 cm height, 25°C). Two swimming sessions were carried out with an initial 15 min 'pre-test' followed by a 5 min 'test' after 24 h. Swimming sessions were recorded for off-line analysis. Active (swimming, climbing, diving) and passive (immobility) behavior was evaluated by 2 reviewers blinded to the treatment conditions. Immobility (primary outcome) was defined as the period during which the animal floats in the water making only those movements necessary to keep its head above water. The tank was emptied and thoroughly cleaned for every rat to be tested in a session.

Sucrose Preference Test (SPT)

This test measures hedonia (pleasure-seeking) or lack of it (anhedonia) by monitoring a rat's preference to sucrose-laced water (Deshpande et al., 2014b; Overstreet, 2012). Briefly, rats

were habituated to having two bottles in the cage lid for three days. The bottles were fitted with ball-bearing sipper tubes that prevented fluids from leaking. Following this acclimation, rats had the free choice of either drinking the 1% sucrose solution or plain water for a period of 2 days. Water and sucrose solution intake was measured daily, and the positions of two bottles were switched daily to reduce any confounding effects produced by a side bias. Sucrose preference was calculated as a percentage of the volume of sucrose intake over the total volume of fluid intake and averaged over the 2 days of testing. Reviewers were blinded to treatment conditions. A spill-cage without rat was also employed using bottles and sipper tubes from the same batch as test cages. Measurement errors were ± 2 ml.

Elevated Plus Maze (EPM)

This test assesses anxiety by taking into account the innate behavior of rats to prefer dark enclosed spaces over bright open spaces (Deshpande et al., 2014b; Walf and Frye, 2007). The maze (Med Associates Inc., St. Albans, VT) was made of black polyvinyl chloride and consisted of four arms, 50 cm long x 10 cm wide, connected by a central square, 10 x 10 cm: two open without walls and two closed by 31-cm-high walls. All arms were attached to sturdy metal legs; the maze was elevated 55 cm above the floor level and was set in a dimly lit room. A video camera was suspended above the maze to record the rat movements for analysis. A video-tracking system (Noldus Ethovision XT 11) was used to automatically collect behavioral data. The procedure consisted of placing the rats at the junction of the open and closed arms, the center of the maze, facing the open arm opposite to where the experimenter was. The video-tracking system was started after the animal was placed in the maze so that the behavior of each animal was consistently recorded for 5 min. At the end of the 5 min test session, the rat was removed from the plus maze and returned to its home cage. The maze was cleaned with 70%

ethanol and air-dried to remove any scent traces and allowed to dry completely before introducing the next animal in the arena. Time spent and entries made in the various arms of EPM were calculated.

Object Location Test (OLT)

This test assess place recognition memory (spatial memory) by calculating the preference of the rat to explore an object that has been moved to a new location. Briefly, rats were placed in black Perspex box 90 x 60 x 50 cm in a dimly illuminated and quiet animal behavior testing room. Rats were habituated individually, by allowing them to explore the empty box for 10-min per session for 2 days. The arena was cleaned with a 70% ethanol solution and dried completely in between each subject so as to eliminate any potential odor cues left by previous subjects. On the third day, in the sample phase, two identical objects were placed in opposite corners of the box, 20 cm from the wall. A rat was allowed to explore for 3-min, and then it was removed from the box and returned to its home cage. In the choice phase (1-h later), one of the object was moved to a novel location, and the rat was allowed to explore for 2-min. Objects were similar in size and emotionally neutral. A video-tracking system (Noldus Ethovision XT 11) was used to automatically collect behavioral data. Direct contacts included any contact with mouth, nose or paw and did not include contacts that were accidental (backing or bumping into the object). Also, standing, sitting or leaning on the object was not scored as object interaction. A rat is considered to be exploring an object when its nose is within 2 cm of the object. Time spent exploring the object at novel location versus the object remaining in the familiar location was calculated for each group. A place discrimination index was calculated as the percentage of time spent with the object at novel location/the total time spent in exploring both the objects. (Barker and Warburton, 2011; Deshpande et al., 2014b; Hattiangady et al., 2014).

Fluoro-Jade staining

Rats were sacrificed 48-h following the last injection of DFP exposure regimen for Fluoro-Jade staining. Briefly, deep anesthesia was induced in rats with ketamine/xylazine (75mg/kg/7.5mg/kg i.p.) mixture. Anesthetized animals were flushed transcardially with saline and perfused with 4% paraformaldehyde in a 100 mM sodium phosphate buffer (pH 7.4). Fixed brains were removed and post-fixed in 4% paraformaldehyde/phosphate buffer overnight, cryoprotected in 30% sucrose/phosphate buffer (pH 7.4) (48 h), flash frozen in isopentane and stored at -80°C until used for sectioning. Coronal sections (40 μm) were cut on a cryostat (Leica Microsystems, Wetzlar, Germany) and mounted onto microscope slides (Trubond 380; Tru Scientific LLC, Bellingham, WA). Slides were dried in a desiccant chamber at 55°C for 30 min prior to staining. Slides were first incubated in a solution of 1% NaOH in 80% ethanol for 5 minutes followed by hydration in a 70% ethanol and then ddH_2O for 2 minutes each. Slides were then incubated in a 0.06% $KMnO_4$ solution for 10 min followed by washing in ddH_2O for 2 min. Slides were then stained in a 0.0004% Fluoro-Jade C (FJC) solution in 0.1% acetic acid for 20 min (Deshpande et al., 2014a; Li et al., 2011b). Stained slides underwent 3x washes in ddH_2O for 2 min each and then dried in a desiccant chamber at 55°C for 30 min. Stained slides were then cleared with xylene for 5 min and cover slipped with DPX mounting agent. Stained sections were evaluated with a fluorescent IX-70 inverted microscope with a 20X (UApo 340, 0.7 n.a., water) objective (Olympus America, Center Valley, PA) and excitation/emission filters for visualization of FITC. Greyscale digital images (1324x1024, 16-bit, 1x1 binning) of FJC staining for hippocampus were acquired with a Hamamatsu ORCA-ER camera (Hamamatsu Photonics, Japan).

Data analysis

Data were analyzed and graphs plotted using the SigmaPlot 12.5 software (SPSS Inc, Chicago, IL). All the data that passed the normality test was further subjected to t-test. A value of $p<0.05$ was considered significant for all data analyses. Analysis of digital images to count FJC positive cell staining was carried out with ImageJ (U. S. National Institutes of Health, Bethesda, MD) by thresholding for specific stain and obtaining positive cell counts using the particle analysis component (size range in pixel: 25-1000). All parameters for digital acquisition and analysis of staining remained constant throughout. Representative digital images were processed with Adobe Photoshop (Adobe Systems Inc., San Jose, CA).

Results

DFP exposure

Rats were injected with DFP (400 μg/kg, s.c.) for a 5-day period which corresponds to the approximate time period for OP nerve gas exposure during the First Gulf War. No signs of cholinergic crisis were observed for the first 4-days of exposures. Few rats displayed lacrimation and mild tremors on the fifth day of exposure but these symptoms were resolved without any intervention by the end of the day. No significant differences were observed in the weight gain dynamics between the controls and DFP-exposed rats post-exposure period. A transient loss in weight in the DFP-exposed rats was observed on the 5^{th} day of DFP injection. This brief loss in the weight lasted for couple of days and the DFP-exposed rats quickly regained their pre-exposure weights and maintained weight-gain trajectory similar to the control rats (Fig. 1). No significant weight differences were observed between the control and DFP-exposed rats at 3-months post-exposure when the behavioral tests were conducted (Table 1). There were no visual signs of pain and discomfort such as hunched posture, poor grooming, porphyrin around eyes or

nose in DFP exposed rats. DFP exposure (400 μg/kg, s.c.) for 1-day did not exhibit any behavioral abnormalities in the various screening tests at 3-months post exposure (data not shown).

Performance on FST

The FST was an effective test in evaluating the presence of a despair-like state in the chronic DFP exposed rats. DFP rats subjected to the modified FST exhibited increased immobility time (79.7 ± 11.5 s) that was significantly higher compared to the immobility time (37.7 ± 6.5 s) in age matched control rats ($p<0.01$, n= 15, Fig. 2).

Performance on SPT

Chronic DFP exposed rats also displayed absence of preferential sucrose consumption on SPT. DFP rats consumed 53.2 ±4.8 % sucrose-laced water and 46.8 ± 5.2% of non-sweetened water. In contrast, age-matched control rats overwhelmingly preferred sucrose water (74.1 ± 4.1 %) over non-sweetened water (25.9 ± 5.2%). This indicates presence of anhedonia in the low-dose DFP rats ($p<0.01$, n= 15, Fig. 3A). No differences were found between total fluid consumption and fluid consumed on right vs. left-side amongst the two groups ($p>0.5$, n= 15, Fig. 3B).

Performance on EPM

Chronic DFP exposed rats displayed significant anxiety-like behavior compared to age-matched control rats. DFP rats spent significantly less time (9.4 ± 2.2 % vs. 29.3 ± 3.7%) and made significantly less entries (12.2 ± 4.8% vs. 37.1 ± 4.5%) in the open-arm of EPM compared to age-matched control rats ($p<0.05$, **n= 15**, Figs. 4A, B). To investigate whether these differences in open-arm behavior were not due to global differences in exploratory or locomotor

activity, we also measured the distance travelled and total arm entries. No significant differences were observed in these two parameters between the rats in the two groups ($p>0.5$, **n= 15**, Figs. 4C, D).

Performance on OLT

In the test phase of OLT, age-matched control rats spent more time exploring the object at the new location (B) versus the object at the old place (A) (70.87% vs 29.13%, respectively, Fig. 4A), indicating that these rats remembered the earlier location. In contrast, chronic DFP exposed rats showed little preference when the object was at novel location (B) and spent almost equal time exploring object at both locations (48.7% vs 51.3%, respectively, Fig. 5A), indicating that these rats displayed spatial memory impairments. Calculating the place discrimination index revealed impaired place recognition memory in the DFP exposed rats ($p<0.01$, n= 15, Fig. 5B). No significant differences were observed between distance travelled and mean velocity amongst the two groups ($p>0.5$, n= 15, Fig. 5C, D).

Neuronal injury associated with DFP exposure

To assess neuronal injury brain sections from animals injected with DFP or vehicle were labeled with FJC (Deshpande et al., 2014a; Li et al., 2011b). Across all brain regions examined, there was negligible FJC labeling in brain sections obtained from vehicle controls. In contrast, all the DFP exposed rats exhibited hippocampal damage as characterized by presence of FJC-positive staining in the polymorphic layer and along the hilus/granule cell border of the dentate gyrus (Fig. 6). Quantitative analysis revealed presence of 2.1 ± 0.22 FJC positive cells/ 100 μM^2 area in DFP exposed rats (n= 7 rats).

Discussion

After the end of 1991 Gulf War the returning military soldiers started reporting health problems including psychiatric impairments that could not be adequately explained by existing medical knowledge (U.S. Department of Veterans Affairs, 2008). Following detailed factor-based analysis (Haley et al., 1997) and exhaustive studies by Center for Disease Control (Fukuda et al., 1998) and Institute of Medicine a cluster of symptoms were identified in Persian Gulf War veterans which included musculoskeletal, gastrointestinal and central nervous system deficits to constitute GWI syndrome or Chronic Multi-symptom Illnesses (Institute of Medicine: Board on the Health of Select Populations, 2013). Studies on the causative factors revealed a number of possible agents that were present during the Iraqi theater (U.S. Department of Veterans Affairs, 2008). Chief among them was the detonation of bunkers that housed Iraqi chemical weapon rockets. Destruction of these bunkers generated a toxic plume of nerve gas and exposed the troops in the surrounding area to low-levels of nerve agents (Directorate for Deployment Health Support, Khamisiyah ammunition point case narrative, 2002). Taking into account the satellite data, meteorological conditions at that time, dispersal characteristics of nerve agents, and intelligence information (Tuite and Haley, 2013) it is estimated that this low-level exposure lasted for a 4-day period from March 10-13'1991. While there are no correct estimates available for levels of nerve gas exposure (United States General Accounting Office, 2004) , it is believed that there were no troops in the vicinity of "first-noticeable effect" zone. The majority of plume exposed soldiers were in "low-level hazard" zone (Directorate for Deployment Health Support, Khamisiyah ammunition point case narrative, 2002). In an attempt to develop an OP-based rodent model of GWI neurological morbidities, we used DFP dose that was less than $1/5^{th}$ the LD_{50} estimates (Misik et al., 2015) to mimic low-level GW nerve agent exposures over a 5-day

period. These exposures did not produce any symptoms of overt cholinergic stimulation, when tested 3-months following the end of OP exposure period these rats exhibited significant psychiatric impairments including chronic depression-like symptoms, anxiety, and cognitive deficits similar to those reported by GW veterans (Black et al., 2004; Blore et al., 2015; Odegard et al., 2013; Institute of Medicine: Board on the Health of Select Populations, 2013). While the inhalational DFP exposure would have been an ideal route of exposure, this route has unique dose administration challenges (Wong et al., 2013). In an actual exposure scenario, affected casualties would come into contact with varying concentrations of toxicant for varying periods of time. The exposure dose will also vary depending upon the exposure methodology such as whole-body, nose-only, lung-only, etc. This makes modelling difficult since the exposure dose is not a toxicokinetically relevant dose (Wong et al., 2013). Under such scenario, other exposure routes provide a greater degree of dose control. Amongst these routes, we have previously tested dose, solvent, and route of administration conditions for DFP exposure (Deshpande et al., 2010). Based on these studies, we found the subcutaneous route provided a stable, controlled response for DFP exposure.

Depression is a complex psychological phenomenon and as such is difficult to analyze using a single test (Overstreet, 2012). For identifying depressive symptoms we used the Forced Swim Test (FST) that models despair along with the Sucrose Preference Test (SPT) that signifies anhedonia. Anxiety was tested using Elevated Plus Maze (EPM) paradigm. DFP exposed rats showed significantly higher immobility time in FST, did not show higher preference towards sweetened water in SPT and preferred the dark-closed arms in EPM. Taken together, the helplessness, despair, anxiety, and lack of feeling pleasure were reflective of the symptoms of depression-like state in DFP exposed rats.

Similar to depression, assessment of memory is also a complex behavioral task. We assessed hippocampus-dependent spatial memory using the OLT paradigm. Compared to the water-maze test, OLT is relatively stress-free and reliably predicts place-recognition memory functioning. We observed that DFP exposed rats had difficulties in OLT and they spend less time with the object in a novel place than the object in the familiar place. Age-matched control rats spent more time in exploring the moved object to the new location than when the object that remained in the same position. Histological analysis revealed neuronal damage within the hippocampus characterized by FJC-positive staining in the polymorphic layer and along the hilus/granule cell border of the dentate gyrus, possibly underlying the spatial memory impairments. Indeed, the hippocampus is essential in memory functioning (Battaglia et al., 2011) and plays a major role in pathophysiology of depression (Campbell and Macqueen, 2004). Studies have shown hippocampal dysfunction in Gulf War veterans using both imaging and neuropsychological testing (Chao et al., 2011; Chao et al., 2010; Menon et al., 2004; Odegard et al., 2013). Chronic hippocampal perfusion dysfunction (Li et al., 2011a) and smaller hippocampal volume (Apfel et al., 2011) has been observed in GW veterans. Reduced gray matter, white matter and hippocampal subfields have also been reported in GW veterans suspected with Sarin and Cyclosarin exposure (Chao et al., 2011; Chao et al., 2010; Chao et al., 2015). Animal models of GWI have also demonstrated hippocampal neuronal loss, reduced neurogenesis, inflammation, and reduced synaptic transmission underlying the expression of anxiety, depression, mood and memory deficits (Abdel-Rahman et al., 2004a; Abdullah et al., 2012; Hattiangady et al., 2014; O'Callaghan et al., 2015; Parihar et al., 2013; Speed et al., 2012; Torres-Altoro et al., 2011). It will be interesting to investigate if other critical brain areas such as amygdala, thalamus, and piriform cortex demonstrate neuronal damage in this animal model.

Using GWI associated chemicals and conditions (PB, permethrin, DEET, stress), Hattiangady and colleagues showed the presence of neurological deficits and neuronal damage similar to the findings reported here (Hattiangady et al., 2014). PB which was used as prophylactic against nerve-agent during the First Gulf War does not normally cross the blood-brain barrier. However, in the presence of stress and insecticides, the blood-brain barrier is compromised and PB can enter the brain (Abdel-Rahman et al., 2002). PB is a reversible inhibitor of AChE, while permethrin and DEET are known sodium channel activators. Application of these agents in combination with PB will cause cholinergic activation, neuronal excitation and downstream glutamatergic stimulation leading to neuropathology and neurotoxicity in GWI rodents (Abou-Donia et al., 1996, Hattiangady et al., 2014). In this study, we sought to establish if OP exposure by itself could lead to the development of GWI-related psychiatric deficits. We hypothesize that the low-grade, sustained cholinergic stimulation following DFP-induced AChE inhibition would recruit the glutamatergic system, activating the signaling mechanisms which would lead to excitotoxic neuronal damage and precipitate the behavioral deficits observed in our study. Indeed, we have previously reported that DFP intoxication produced N-methyl-D-aspartate (NMDA) receptor-mediated elevations in hippocampal neuronal calcium levels that lasted for weeks after the initial OP exposure (Deshpande et al., 2010). These sustained hippocampal calcium elevations could underlie neuronal toxicity and some of the long-term plasticity changes associated with OP exposure (Deshpande et al., 2014a; Deshpande et al., 2014b). Future studies will explore hippocampal calcium dynamics in this GWI model. The work by Hattiangady and colleagues also found reduced neurogenesis in their non-OP GWI rodent model (Hattiangady et al., 2014). It will be very interesting to investigate if such a response is also observed in our OP-based GWI model

system. Thus, while these two studies used different agents to induce GWI related symptoms, the underlying mechanism for neuronal damage likely remains the same and may explain for the similar outcomes.

In conclusion, our study demonstrates that repeated low-dose exposure to OP DFP produces neuronal damage and exhibits chronic behavioral and cognitive morbidities similar to those commonly reported in GW veterans. Given that OP exposure is considered a leading cause of GWI related morbidities, this animal model will be ideally suited to study underlying molecular mechanisms for the expression of GWI neurological symptoms and identify drugs for the effective treatment of GWIs.

Acknowledgements

This work was funded by Congressionally Directed Medical Research Program (CDMRP), specifically, the Gulf War Illness Research Program (GWIRP), grant award (W81XWH-14-1-0478) to Dr. Laxmikant S. Deshpande. Authors would like to thank Ms. Beverly Huang and Alaina Hudson for their help during the experiments.

References

Abdel-Rahman A, Abou-Donia S, El-Masry E, Shetty A and Abou-Donia M (2004a) Stress and combined exposure to low doses of pyridostigmine bromide, DEET, and permethrin produce neurochemical and neuropathological alterations in cerebral cortex, hippocampus, and cerebellum. *J Toxicol Environ Health A* **67**:163-192.

Abdel-Rahman A, Dechkovskaia AM, Goldstein LB, Bullman SH, Khan W, El-Masry EM and Abou-Donia MB (2004b) Neurological deficits induced by malathion, DEET, and permethrin, alone or in combination in adult rats. *J Toxicol Environ Health A* **67**:331-356.

Abdel-Rahman A, Shetty AK and Abou-Donia MB (2002) Disruption of the blood-brain barrier and neuronal cell death in cingulate cortex, dentate gyrus, thalamus, and hypothalamus in a rat model of Gulf-War syndrome. *Neurobiol Dis* **10**:306-326.

Abdullah L, Evans JE, Bishop A, Reed JM, Crynen G, Phillips J, Pelot R, Mullan MA, Ferro A, Mullan CM, Mullan MJ, Ait-Ghezala G and Crawford FC (2012) Lipidomic profiling of phosphocholine-containing brain lipids in mice with sensorimotor deficits and anxiety-like features after exposure to Gulf War agents. *Neuromolecular Med* **14**:349-361.

Abou-Donia MB, Wilmarth KR, Abdel-Rahman AA, Jensen KF, Oehme FW and Kurt TL (1996) Increased neurotoxicity following concurrent exposure to pyridostigmine bromide, DEET, and chlorpyrifos. *Fundam Appl Toxicol* **34**:201-222.

Apfel BA, Ross J, Hlavin J, Meyerhoff DJ, Metzler TJ, Marmar CR, Weiner MW, Schuff N and Neylan TC (2011) Hippocampal volume differences in Gulf War veterans with current versus lifetime posttraumatic stress disorder symptoms. *Biological psychiatry* **69**:541-548.

Bajgar J (2004) Organophosphates/nerve agent poisoning: mechanism of action, diagnosis, prophylaxis, and treatment. *Adv Clin Chem* **38**:151-216.

Barker GRI and Warburton EC (2011) When Is the Hippocampus Involved in Recognition Memory? *J Neurosci* **31**:10721-10731.

Battaglia FP, Benchenane K, Sirota A, Pennartz CM and Wiener SI (2011) The hippocampus: hub of brain network communication for memory. *Trends Cogn Sci* **15**:310-318.

Black DW, Carney CP, Peloso PM, Woolson RF, Schwartz DA, Voelker MD, Barrett DH and Doebbeling BN (2004) Gulf War veterans with anxiety: prevalence, comorbidity, and risk factors. *Epidemiology* **15**:135-142.

Blore JD, Sim MR, Forbes AB, Creamer MC and Kelsall HL (2015) Depression in Gulf War veterans: a systematic review and meta-analysis. *Psychological medicine* **45**:1565-1580.

Brown MA and Brix KA (1998) Review of health consequences from high-, intermediate- and low-level exposure to organophosphorus nerve agents. *J Appl Toxicol* **18**:393-408.

Campbell S and Macqueen G (2004) The role of the hippocampus in the pathophysiology of major depression. *J Psychiatry Neurosci* **29**:417-426.

Castagne V, Moser P, Roux S and Porsolt RD (2011) Rodent models of depression: forced swim and tail suspension behavioral despair tests in rats and mice. *Curr Protoc Neurosci* **Chapter 8**:Unit 8 10A.

Chao LL, Abadjian L, Hlavin J, Meyerhoff DJ and Weiner MW (2011) Effects of low-level sarin and cyclosarin exposure and Gulf War Illness on brain structure and function: a study at 4T. *Neurotoxicology* **32**:814-822.

Chao LL, Rothlind JC, Cardenas VA, Meyerhoff DJ and Weiner MW (2010) Effects of low-level exposure to sarin and cyclosarin during the 1991 Gulf War on brain function and brain structure in US veterans. *Neurotoxicology* **31**:493-501.

Chao LL, Zhang Y and Buckley S (2015) Effects of low-level sarin and cyclosarin exposure on white matter integrity in Gulf War Veterans. *Neurotoxicology* **48**:239-248.

Couzin J (2004) VA Advisers Link Gulf War Illnesses to Neurotoxins. *Science* **306**:26-27.

Deshpande LS, Carter DS, Blair RE and DeLorenzo RJ (2010) Development of a prolonged calcium plateau in hippocampal neurons in rats surviving status epilepticus induced by the organophosphate diisopropylfluorophosphate. *Toxicol Sci* **116**:623-631.

Deshpande LS, Carter DS, Phillips KF, Blair RE and DeLorenzo RJ (2014a) Development of status epilepticus, sustained calcium elevations and neuronal injury in a rat survival model of lethal paraoxon intoxication. *Neurotoxicology* **44**:17-26.

Deshpande LS, Phillips K, Huang B and DeLorenzo RJ (2014b) Chronic behavioral and cognitive deficits in a rat survival model of paraoxon toxicity. *Neurotoxicology* **44**:352-357.

Directorate for Deployment Health Support of the Special Assistant to the Under Secretary of Defense (Personnel and Readiness) for Gulf War Illness Medical Readiness and Military Deployments US demolition operations at the Khamisiyah ammunition point (case narrative) (2002) http://www.gulflink.osd.mil/khamisiyah_tech/.

Friedl KE, Grate SJ and Proctor SP (2009) Neuropsychological issues in military deployments: lessons observed in the DoD Gulf War Illnesses Research Program. *Mil Med* **174**:335-346.

Fukuda K, Nisenbaum R, Stewart G, Thompson WW, Robin L, Washko RM, Noah DL, Barrett DH, Randall B, Herwaldt BL, Mawle AC and Reeves WC (1998) Chronic multisymptom illness affecting Air Force veterans of the Gulf War. *JAMA* **280**:981-988.

Haley RW (1997) Is Gulf War syndrome due to stress? The evidence reexamined. *American journal of epidemiology* **146**:695-703.

Haley RW, Kurt TL and Hom J (1997) Is there a Gulf War Syndrome? Searching for syndromes by factor analysis of symptoms. *JAMA* **277**:215-222.

Haley RW and Tuite JJ (2013) Epidemiologic evidence of health effects from long-distance transit of chemical weapons fallout from bombing early in the 1991 Persian Gulf War. *Neuroepidemiology* **40**:178-189.

Hattiangady B, Mishra V, Kodali M, Shuai B, Rao X and Shetty AK (2014) Object location and object recognition memory impairments, motivation deficits and depression in a model of Gulf War illness. *Frontiers in behavioral neuroscience* **8**:78.

Henderson RF, Barr EB, Blackwell WB, Clark CR, Conn CA, Kalra R, March TH, Sopori ML, Tesfaigzi Y, Menache MG and Mash DC (2002) Response of rats to low levels of sarin. *Toxicol Appl Pharmacol* **184**:67-76.

Hood E (2001) The Tokyo attacks in retrospect: sarin leads to memory loss. *Environ Health Perspect* **109**:A542.

Johnson FO, Chambers JE, Nail CA, Givaruangsawat S and Carr RL (2009) Developmental chlorpyrifos and methyl parathion exposure alters radial-arm maze performance in juvenile and adult rats. *Toxicol Sci* **109**:132-142.

Li X, Spence JS, Buhner DM, Hart J, Jr., Cullum CM, Biggs MM, Hester AL, Odegard TN, Carmack PS, Briggs RW and Haley RW (2011a) Hippocampal dysfunction in Gulf War veterans: investigation with ASL perfusion MR imaging and physostigmine challenge. *Radiology* **261**:218-225.

Li Y, Lein PJ, Liu C, Bruun DA, Tewolde T, Ford G and Ford BD (2011b) Spatiotemporal pattern of neuronal injury induced by DFP in rats: a model for delayed neuronal cell death following acute OP intoxication. *Toxicol Appl Pharmacol* **253**:261-269.

Menon PM, Nasrallah HA, Reeves RR and Ali JA (2004) Hippocampal dysfunction in Gulf War Syndrome. A proton MR spectroscopy study. *Brain Res* **1009**:189-194.

Misik J, Pavlikova R, Cabal J and Kuca K (2015) Acute toxicity of some nerve agents and pesticides in rats. *Drug and chemical toxicology* **38**:32-36.

Nishiwaki Y, Maekawa K, Ogawa Y, Asukai N, Minami M, Omae K and Sarin Health Effects Study G (2001) Effects of sarin on the nervous system in rescue team staff members and police officers 3 years after the Tokyo subway sarin attack. *Environ Health Perspect* **109**:1169-1173.

O'Callaghan JP, Kelly KA, Locker AR, Miller DB and Lasley SM (2015) Corticosterone primes the neuroinflammatory response to DFP in mice: potential animal model of Gulf War Illness. *J Neurochem* **133**:708-721.

Odegard TN, Cooper CM, Farris EA, Arduengo J, Bartlett J and Haley R (2013) Memory impairment exhibited by veterans with Gulf War Illness. *Neurocase* **19**:316-327.

Ojo JO, Abdullah L, Evans J, Reed JM, Montague H, Mullan MJ and Crawford FC (2014) Exposure to an organophosphate pesticide, individually or in combination with other Gulf War agents, impairs synaptic integrity and neuronal differentiation, and is accompanied by subtle microvascular injury in a mouse model of Gulf War agent exposure. *Neuropathology* **34**:109-127.

Overstreet DH (2012) Modeling depression in animal models. *Methods Mol Biol* **829**:125-144.

Parihar VK, Hattiangady B, Shuai B and Shetty AK (2013) Mood and Memory Deficits in a Model of Gulf War Illness Are Linked with Reduced Neurogenesis, Partial Neuron Loss, and Mild Inflammation in the Hippocampus. *Neuropsychopharmacology*.

Institute of Medicine:Board on the Health of Select Populations (2013) Gulf War and Health: Treatment of Chronic Multisymptom Illness. http://www.iom.edu/Reports/2013/Gulf-War-and-HealthTreatment-for-Chronic-Multisymptom-Illness.aspx

Rosenstock L, Keifer M, Daniell WE, McConnell R and Claypoole K (1991) Chronic central nervous system effects of acute organophosphate pesticide intoxication. *The Lancet* **338**:223-227.

Savage EP, Keefe TJ, Mounce LM, Heaton RK, Lewis JA and Burcar PJ (1988) Chronic neurological sequelae of acute organophosphate pesticide poisoning. *Arch Environ Health* **43**:38-45.

Special Assistant to the Secretary of Defense for Gulf War Illnesses, Medical Readiness and Military Deployments (2001) Case narrative, chemical warfare release at Muhammadiyat ammunition storage site. http://wwwgulflinkosdmil/muhammadiyat.

Speed HE, Blaiss CA, Kim A, Haws ME, Melvin NR, Jennings M, Eisch AJ and Powell CM (2012) Delayed reduction of hippocampal synaptic transmission and spines following exposure to repeated subclinical doses of organophosphorus pesticide in adult mice. *Toxicol Sci* **125**:196-208.

Steele L, Sastre A, Gerkovich MM and Cook MR (2012) Complex factors in the etiology of Gulf War illness: wartime exposures and risk factors in veteran subgroups. *Environ Health Perspect* **120**:112-118.

Steenland K, Jenkins B, Ames RG, O'Malley M, Chrislip D and Russo J (1994) Chronic neurological sequelae to organophosphate pesticide poisoning. *Am J Public Health* **84**:731-736.

Terry AV, Jr., Beck WD, Warner S, Vandenhuerk L and Callahan PM (2012) Chronic impairments in spatial learning and memory in rats previously exposed to chlorpyrfos or diisopropylfluorophosphate. *Neurotoxicol Teratol* **34**:1-8.

Terry AV, Jr., Buccafusco JJ, Gearhart DA, Beck WD, Middlemore-Risher ML, Truan JN, Schwarz GM, Xu M, Bartlett MG, Kutiyanawala A and Pillai A (2011) Repeated, intermittent exposures to diisopropylfluorophosphate in rats: protracted effects on cholinergic markers, nerve growth factor-related proteins, and cognitive function. *Neuroscience* **176**:237-253.

Terry AV, Jr., Callahan PM, Beck WD, Vandenhuerk L, Sinha S, Bouchard K, Schade R and Waller JL (2014) Repeated exposures to diisopropylfluorophosphate result in impairments of sustained attention and persistent alterations of inhibitory response control in rats. *Neurotoxicol Teratol* **44**:18-29.

Torres-Altoro MI, Mathur BN, Drerup JM, Thomas R, Lovinger DM, O'Callaghan JP and Bibb JA (2011) Organophosphates dysregulate dopamine signaling, glutamatergic neurotransmission, and induce neuronal injury markers in striatum. *J Neurochem* **119**:303-313.

Tuite JJ and Haley RW (2013) Meteorological and intelligence evidence of long-distance transit of chemical weapons fallout from bombing early in the 1991 Persian Gulf War. *Neuroepidemiology* **40**:160-177.

U.S. Department of Veterans Affairs, Research Advisory Committee on Gulf War Veterans' Illnesses (2008) Gulf War illness and the health of Gulf War veterans, scientific findings and recommendations.

United States General Accounting Office (2004) DOD's conclusions about U.S. troops' exposure cannot be adequately supported. http://www.gao.gov/products/GAO-04-159

Walf AA and Frye CA (2007) The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat Protocols* **2**:322-328.

Wesseling C, Keifer M, Ahlbom A, McConnell R, Moon JD, Rosenstock L and Hogstedt C (2002) Long-term neurobehavioral effects of mild poisonings with organophosphate and n-methyl carbamate pesticides among banana workers. *Int J Occup Environ Health* **8**:27-34.

White RF, Steele L, O'Callaghan JP, Sullivan K, Binns JH, Golomb BA, Bloom FE, Bunker JA, Crawford F, Graves JC, Hardie A, Klimas N, Knox M, Meggs WJ, Melling J, Philbert MA and Grashow R (2015) Recent research on Gulf War illness and other health problems in veterans of the 1991 Gulf War: Effects of toxicant exposures during deployment. *Cortex (in-press)* doi:10.1016/j.cortex.2015.08.022.

Wolfe J, Proctor SP, Erickson DJ and Hu H (2002) Risk factors for multisymptom illness in US Army veterans of the Gulf War. *J Occup Environ Med* **44**:271-281.

Wong B, Perkins MW, Santos MD, Rodriguez AM, Murphy G and Sciuto AM (2013) Development of a model for nerve agent inhalation in conscious rats. *Toxicology mechanisms and methods* **23**:537-547.

Zakirova Z, Tweed M, Crynen G, Reed J, Abdullah L, Nissanka N, Mullan M, Mullan MJ, Mathura V, Crawford F and Ait-Ghezala G (2015) Gulf War Agent Exposure Causes Impairment of Long-Term Memory Formation and Neuropathological Changes in a Mouse Model of Gulf War Illness. *PLoS One* **10**.

Table 1 Body weight changes during the study

Experimental Group	Weight before OP exposure (g)	Weight 12-weeks post OP exposure (g)
Control	301.5 ± 7.5	516.4 ± 16.5
DFP exposed	304.4 ± 6.5	521.6 ± 18.5

Data presented as mean ± SD (n= 15 rats, p=0.3, *t*-test)

Figure Legends

Figure 1. Body weight changes before, during, and after DFP exposure. A transient loss in the weights of the DFP-exposed rats was observed on the 5th day of DFP injection, which lasted for couple of days. No significant differences were observed in the weight gain dynamics between the controls and DFP-exposed rats in the post-exposure period. Data expressed as mean ± SEM, *p<0.05, t-test, n= 15 rats.

Figure 2. Increased immobility time in DFP exposed rats during FST. The immobility time in DFP exposed rats was significantly higher compared to age matched control rats. Data expressed as mean ± SEM, *p<0.05, t-test, n= 15 rats.

Figure 3. Loss of sucrose consumption preference in DFP exposed rats on SPT. Control rats overwhelmingly consumed sucrose water over regular water, whereas DFP exposed rats did not exhibit any such preference indicating anhedonia-like condition. Data expressed as mean ± SEM, *p<0.05, t-test, n= 15 rats.

Figure 4. Increased anxiety in DFP exposed rats on EPM test. DFP exposed rats displayed significantly lower open arm time (A) and open arm entries (B) compared to age-matched control rats. No differences were observed in the distance travelled (C) and total arm entries (D) between the two-groups. Data expressed as mean ± SEM, *p<0.05, t-test, n= 15 rats.

Figure 5. Impaired spatial memory in DFP exposed rats on OL test. (A, B) DFP exposed rats showed no preference for when the object was moved to new location-B indicative of impaired spatial memory that was significantly lower compared to the time spent by age matched control rats at the new location. No significant differences were observed in the distance travelled (C)

and mean velocity (D) during the test session between the two groups. Data expressed as mean ± SEM, *p<0.05, t-test, n= 15 rats.

Figure 6. Low dose DFP induced neuronal injury. A representative photomicrographs of Fluoro-Jade C (FJC) staining in the dentate gyrus-hilus region from (A) control and (B) DFP exposed rat. Scale bar, 200 μm.

Fig. 1

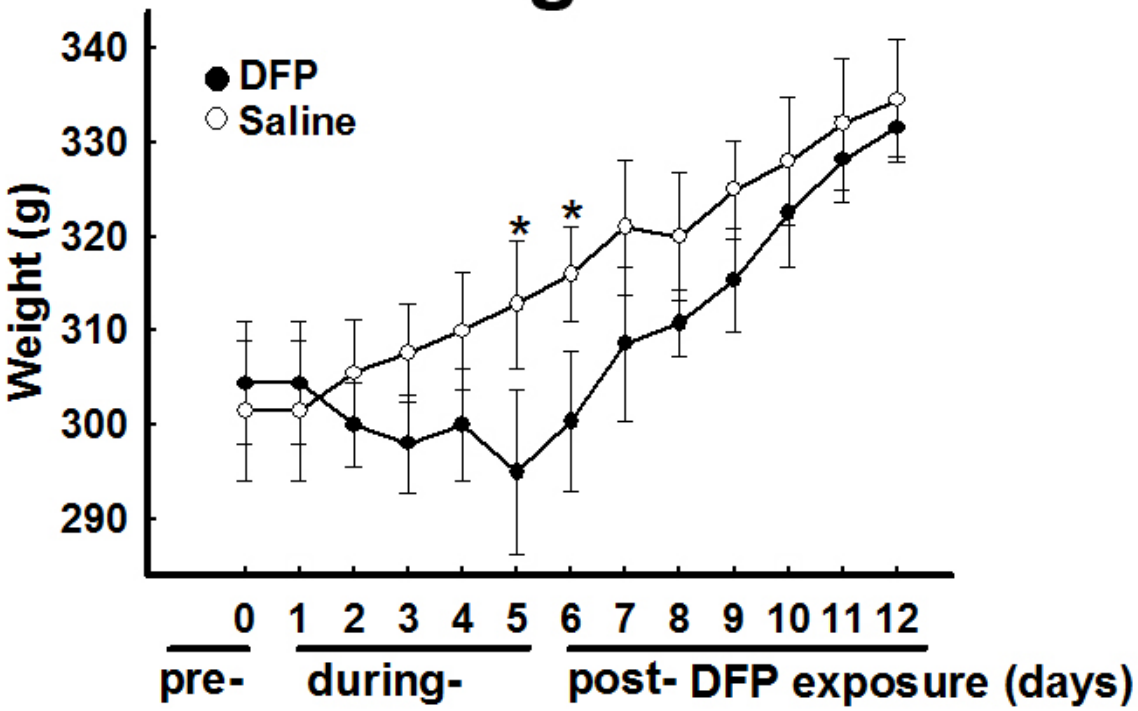
340
330
320
310
300
290
Weight (g)
DFP
Saline
*
*
0 1 2 3 4 5 6 7 8 9 10 11 12
pre-
during-
post- DFP exposure (days)

Fig. 2

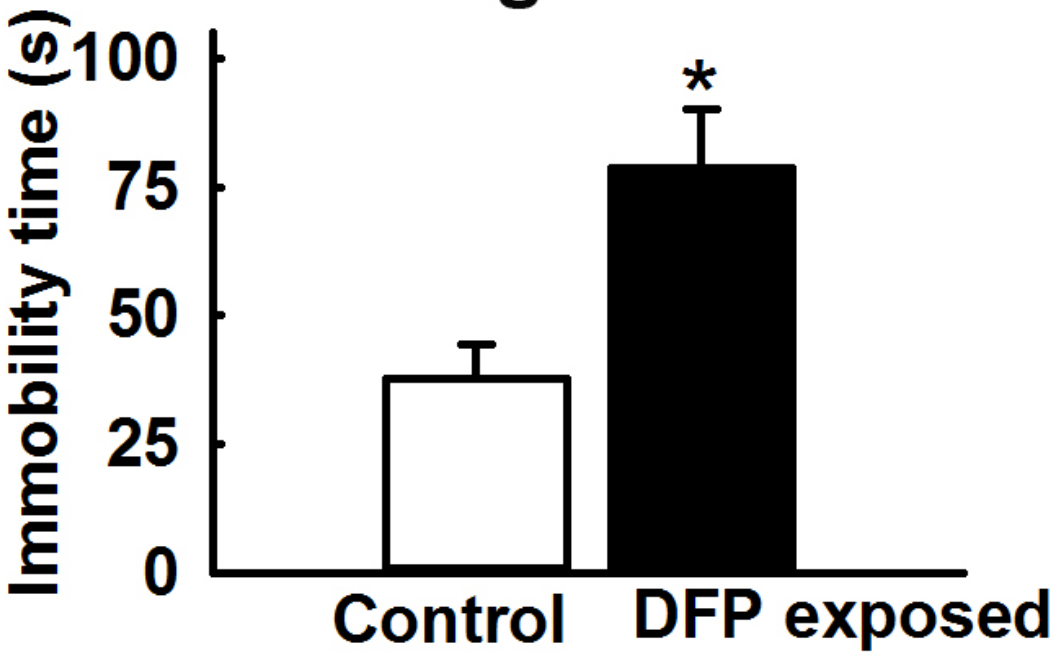

Fig. 3

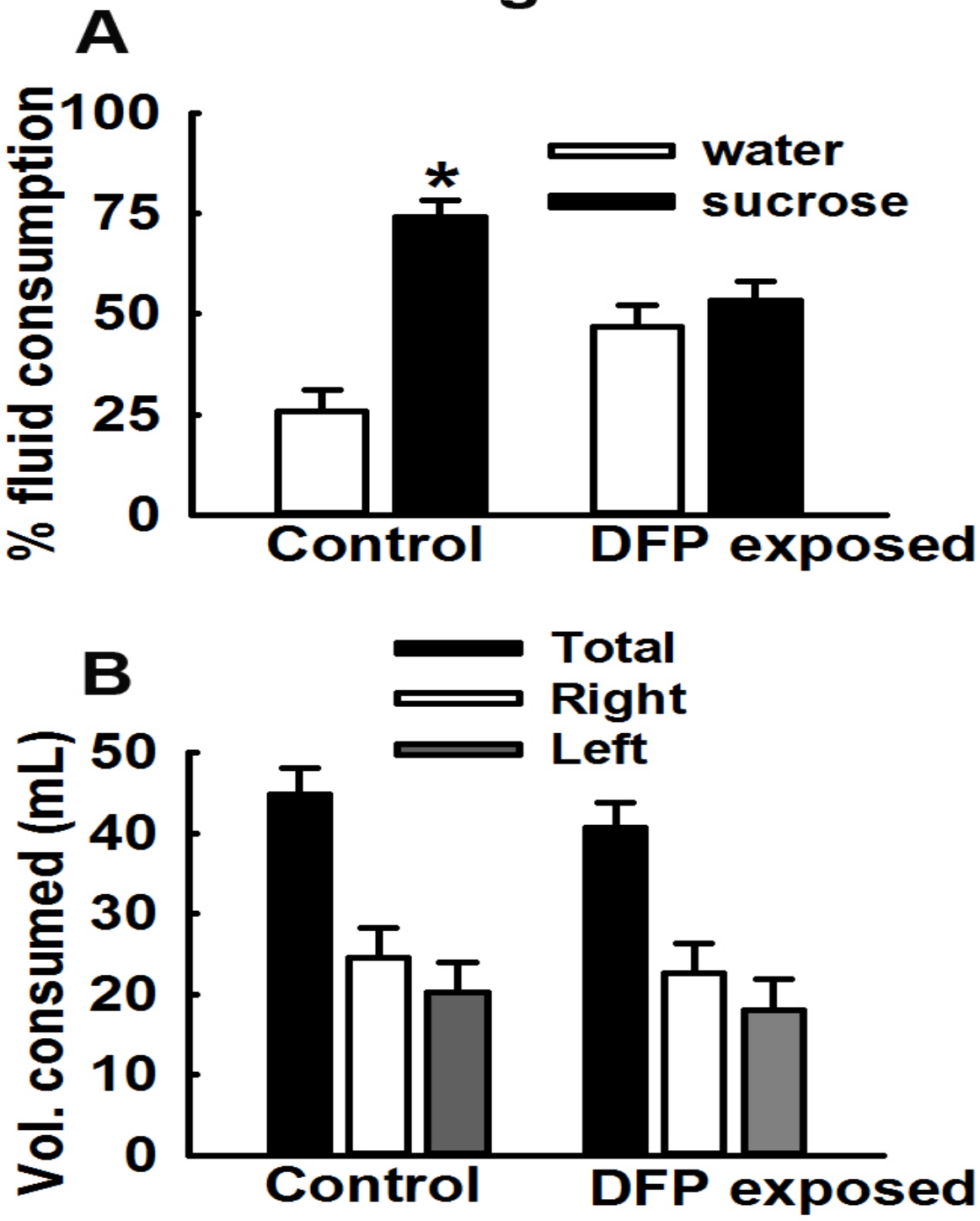
A
% fluid consumption
100
75
50
25
0
*
water
sucrose
Control
DFP exposed
B
Vol. consumed (mL)
50
40
30
20
10
0
Total
Right
Left
Control
DFP exposed

Fig. 4

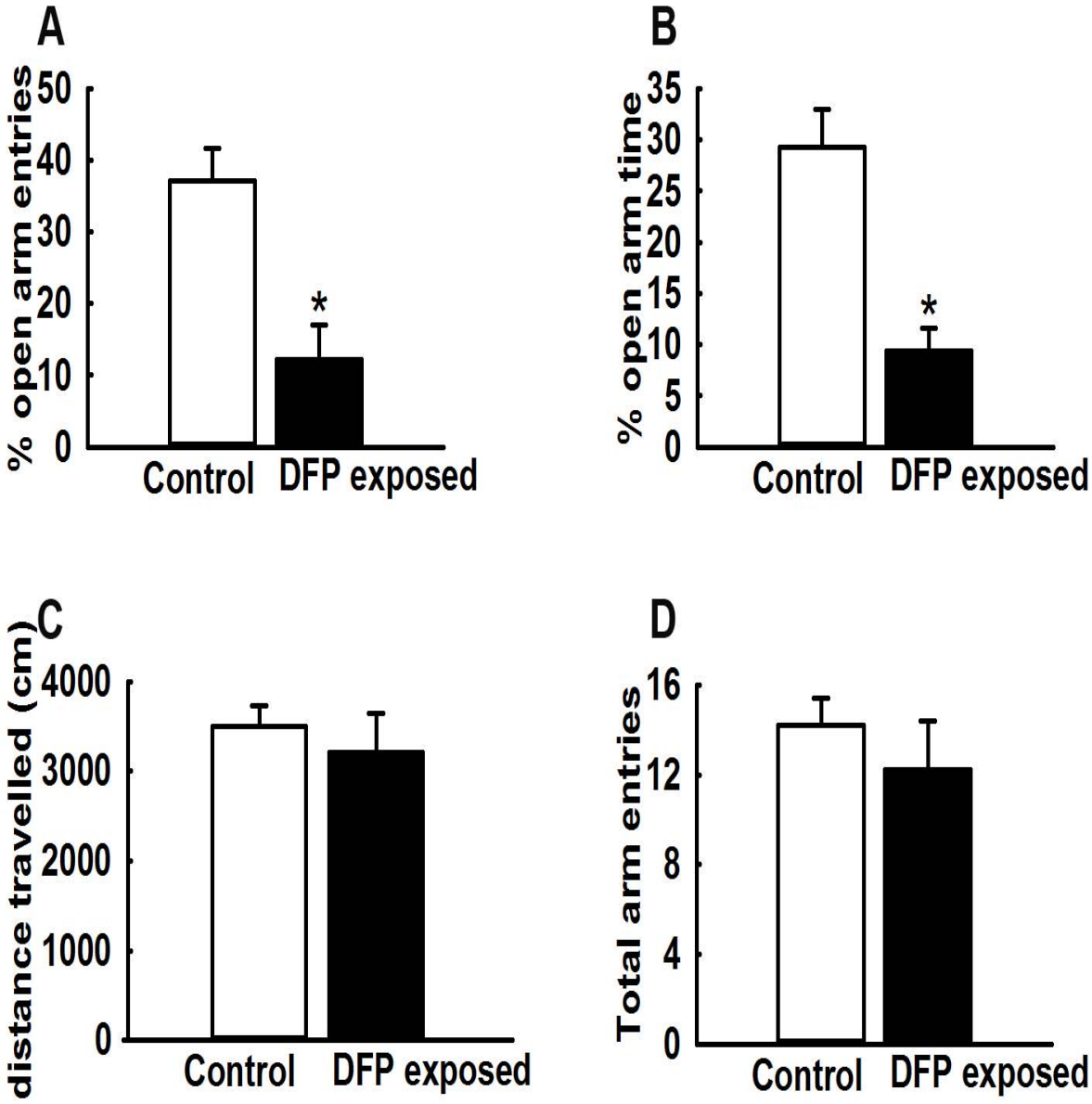
A
% open arm entries
50
40
30
20
10
0
*
Control
DFP exposed
B
% open arm time
35
30
25
20
15
10
5
0
*
Control
DFP exposed
C
distance travelled (cm)
4000
3000
2000
1000
0
Control
DFP exposed
D
Total arm entries
16
12
8
4
0
Control
DFP exposed

Fig. 5

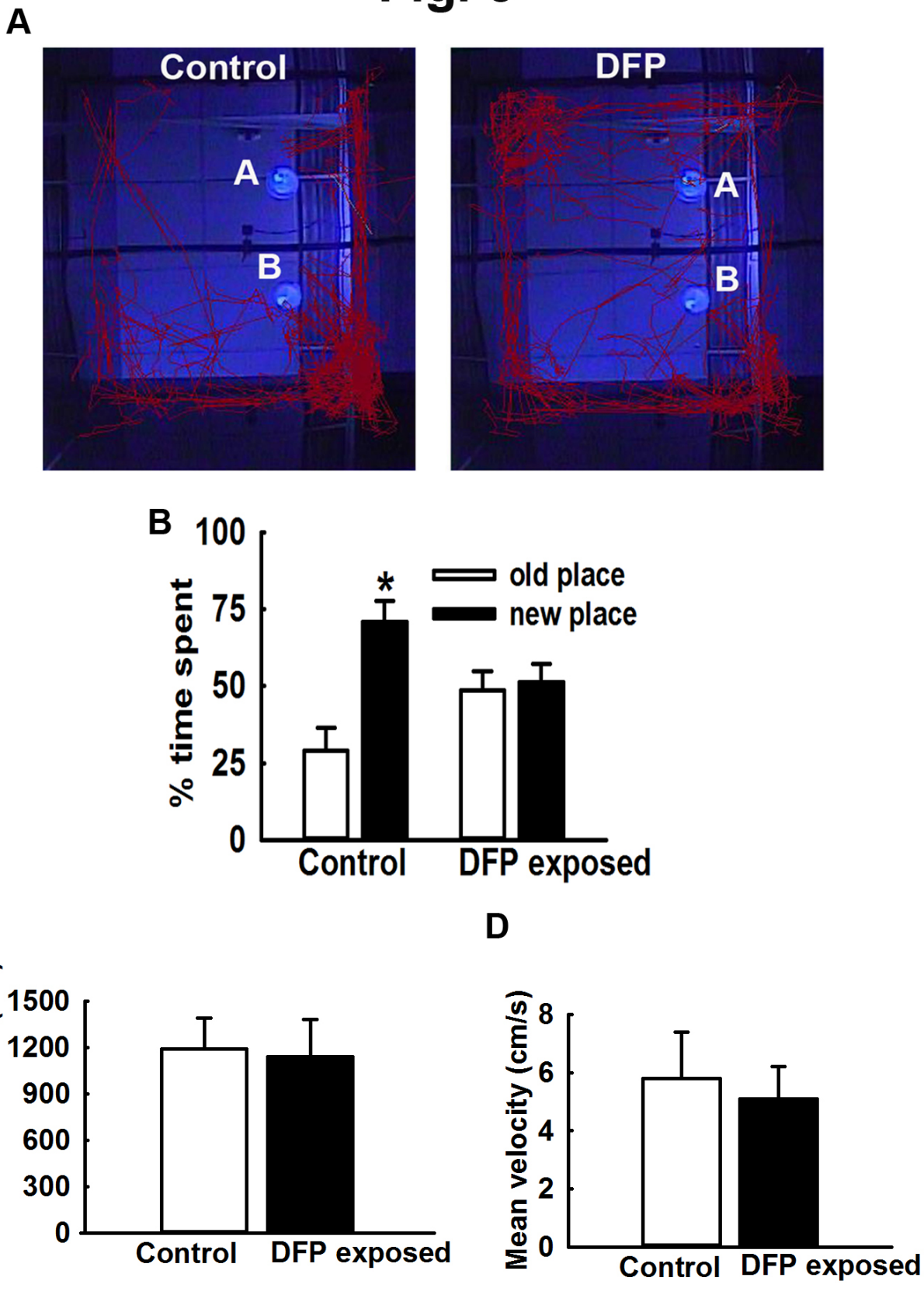
A
Control
A
B
DFP
A
B
B
100
75
50
25
0
% time spent
*
old place
new place
Control
DFP exposed
C
distance travelled (cm)
1500
1200
900
600
300
0
Control
DFP exposed
D
Mean velocity (cm/s)
8
6
4
2
0
Control
DFP exposed

Fig. 6