Published in final edited form as:

Neuropharmacology. 2016 November ; 110(Pt A): 419–430. doi:10.1016/j.neuropharm.2016.07.028.

Inhibition of the prostaglandin E2 receptor EP2 prevents status epilepticus-induced deficits in the novel object recognition task in rats

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Abstract

Survivors of exposure to an organophosphorus nerve agent may develop a number of complications including long-term cognitive deficits (Miyaki et al., 2005; Nishiwaki et al., 2001). We recently demonstrated that inhibition of the prostaglandin E2 receptor, EP2, attenuates neuroinflammation and neurodegeneration caused by status epilepticus (SE) induced by the soman analog, diisopropylfluorophosphate (DFP), which manifest within hours to days of the initial insult. Here, we tested the hypothesis that DFP exposure leads to a loss of cognitive function in rats that is blocked by early, transient EP2 inhibition. Adult male Sprague-Dawley rats were administered vehicle or the competitive EP2 antagonist, TG6-10-1, (ip) at various times relative to DFP-induced SE. DFP administration resulted in prolonged seizure activity as demonstrated by cortical electroencephalography (EEG). A single intraperitoneal injection of TG6-10-1 or vehicle 1 h prior to DFP did not alter the development of seizures, the latency to SE or the duration of SE. Rats administered six injections of TG6-10-1 starting 90 min after the onset of DFP-induced SE could discriminate between a novel and familiar object 6–12 weeks after SE, unlike vehicle treated rats which showed no preference for the novel object. By contrast, behavioral changes in the light-dark box and open field assays were not affected by TG6-10-1. Delayed mortality after DFP was also unaffected by TG6-10-1. Thus, selective inhibition of the EP2 receptor may prevent SE-induced memory impairment in rats caused by exposure to a high dose of DFP.

Keywords

DFP; organophosphate; status epilepticus; hippocampus; EP2 receptor; novel object recognition; light-dark box preference test; open field; electroencephalography; seizure; epilepsy; anxiety

1. INTRODUCTION

Exposure to organophosphorus based nerve agents and pesticides poses a major public health concern. Organophosphorus agents bind and potentially inhibit acetylcholinesterase

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(AChE), leading to an irreversible structural change of the enzyme termed “aging”, accompanied by the rapid accumulation of acetylcholine (Colovic et al., 2013; Costa 2006), and the consequent clinical presentation of cholinergic symptoms following organophosphorus poisoning. Exposure to high levels of an organophosphorus agent often leads to seizures that can evolve into status epilepticus (SE, an unremitting seizure lasting longer than 5 min, or a series of seizures without intervening regain of consciousness). In the event of an exposure to high levels of organophosphorus agents the response time and treatment measures carried out by first responders is critical to minimize casualties, so it would be desirable to develop treatments that can mitigate long-term consequences. In animal models of organophosphorus agent exposure, survivors exhibit a number of consequences such as weight loss, neuroinflammation, neurodegeneration, long-term cognitive deficits and the development of spontaneous recurrent seizures (SRS) (Binukumar et al., 2011; Chen 2012; Gilat et al., 2005; Joosen et al., 2009; Pan et al., 2012; Raveh et al., 2003; Reddy & Kuruba, 2013; Rojas et al., 2015; Tattersall 2009; Tilson et al., 1990). Short-term inhibition of the EP2 receptor following SE in rats and mice induced by DFP or the muscarinic receptor agonist pilocarpine attenuates the consequences of status epilepticus that manifest within hours to days following the initial insult, including neuroinflammation, neuronal injury and breakdown of the blood-brain barrier (Jiang et al., 2013; Jiang et al., 2015; Rojas et al., 2015). Whether EP2 inhibition also ameliorates the long-term consequences of DFP-induced SE such as cognitive deficits has not been investigated and is the focus of the current study. Here we ask the questions: Does DFP-induced SE lead to long term cognitive deficits in rats? And, does EP2 inhibition by TG6-10-1 alter DFP-induced seizure characteristics or improve cognitive function after DFP? To address these questions we used a model of SE in adult rats involving exposure to a high dose of DFP. Following exposure to DFP rats were injected with either the EP2 receptor antagonist TG6-10-1 or its vehicle using two dosing paradigms. The rats were allowed to recover and were examined functionally 4 weeks and 6–12 weeks after the initial insult. The results shown here promote a better understanding of the pathophysiology of organophosphorus poisoning and further support therapies targeting the EP2 receptor to combat neuropathologies following exposure to organophosphorus based agents.

2. MATERIALS AND METHODS

2.1. Ethics Statement

All procedures concerning animals were approved by Emory University Institutional Animal Care and Use Committee and conformed to NIH guidelines.

2.2. Diisopropylfluorophosphate (DFP)-induced status epilepticus (SE)

Adult male Sprague–Dawley rats (200–240 g body weight) were purchased from Charles River Labs (Wilmington, MA, USA) and housed in standard plastic cages (2 rats/cage) in a temperature controlled room (22 ± 2 °C) on a 12 h r everse light–dark cycle. Food and water were provided *ad libitum*. On the day of DFP exposure the rats were weighed, placed individually into a plastic cage and moved into a ventilation hood. The rat DFP model of SE was the same as described previously (Rojas et al., 2015). Awake rats were injected subcutaneously (sc) with the reversible acetylcholinesterase inhibitor pyridostigmine

bromide (P1339, TCI America, Portland, OR) at 0.1 mg/kg in 0.9% saline. Twenty minutes later the rats were injected (sc) with the muscarinic receptor antagonist atropine methylbromide (A6883, Sigma, St. Louis, MO) at 20 mg/kg in 0.9% saline. Pyridostigmine bromide and atropine methylbromide, which are unable to cross the blood-brain barrier, were administered to rats prior to DFP to reduce peripheral toxicity and increase survival of the rats following DFP exposure without altering the development of seizures. Ten minutes after the atropine methylbromide injection, rats were injected with DFP (D0879, Sigma) at 9.5 mg/kg (ip) diluted in sterile distilled water. DFP was prepared fresh with thorough mixing within 5 minutes of administration. Control rats were treated similarly except they were given sterile water instead of DFP. Each rat received an injection volume based on weight (1 ml/kg).

2.3. Behavioral scoring of seizure activity

Organophosphorus-induced seizure behaviors began within 10 minutes of DFP exposure and consisted of distinct motor behaviors that included forelimb clonus, tail extension, and whole body clonic seizures. Rats presenting these behaviors with increasing seizure intensity, duration, and frequency after exposure to DFP were declared to be in status epilepticus, which is characterized by non-intermittent whole body clonic seizures that persist. Twenty percent of the rats injected with 9.5 mg/kg DFP (ip) experienced occasional seizures but did not enter status epilepticus. The seizure activity was scored and recorded every 5 minutes for 80–90 min using a modified Racine scale (Racine, 1972) (below). All rats monitored for at least 5 hours exhibited persistent non-intermittent seizure activity, which eventually waned. The rats were then placed in clean plastic cages with fresh bedding, soft food and water and allowed to recover. To maintain hydration, lactated Ringer's solution (2 ml, sc) was administered when the rats were placed into the new cages and then once daily until rats were able to eat and drink on their own.

2.4. Modified Racine scale for seizures after DFP exposure

<u>Behavioral Score</u>	<u>Observed Motor Behavior</u>
0 Normal Behavior:	walking, exploring, sniffing, grooming
1 Freeze Behavior:	immobile, staring, heightened startle, curled-up posture
2 Repetitive Behavior:	blinking, chewing, head bobbing, scratching, face washing, whisker twitching
3 Early Seizure Behavior:	myoclonic jerks, partial body clonus
4 Advanced Seizure Behavior:	whole body clonus
5 Status Epilepticus (SE):	repeated seizure activity (2 events in stages 3, 4 or 6 within a 5 minute window)
6 Intense Seizure Behavior:	repetitive jumping or bouncing, wild running, tonic seizures
7 Death	

2.5. Modified Irwin test

A modified Irwin test (Irwin, 1968) was performed to assess the health of rats prior to and after DFP-induced status epilepticus. The test comprised 12 parameters (ptosis, exophthalmos, lacrimation, body posture, bushy tail, tremors, running vs. walking, dragging body, hyper/hypoactive, aggression when handled, muscle tone when handled and

vocalization when handled) that can be measured simply by experimenter observation and gentle handling. Some parameters require agitating the rats by forcing them to move about their home cage. The test was given twice (once prior to DFP and again 24 hours after DFP exposure). Each parameter was scored on a three point scale (i.e., 0=normal, 1=mild to moderate impairment and 2=severe impairment) with a total score ranging from 0–24. A total score of 0 as the sum of all 12 parameters indicates a normal healthy rat. A total score ranging from 1–11 indicates a healthy animal that appears slightly impaired. A compromised animal would fail the modified Irwin test with a total score of 12.

2.6. Pharmacokinetics and administration of TG6-10-1

The EP2 receptor antagonist TG6-10-1 was synthesized in our laboratory as previously reported (Ganesh et al., 2014a; Ganesh et al., 2014b; Jiang et al., 2012). TG6-10-1 was greater than 97% pure as analyzed by nuclear magnetic resonance (NMR), liquid chromatography mass spectrometry (LC/MS) and elemental composition. Pharmacokinetic analysis and brain distribution of TG6-10-1 were performed in normal healthy adult rats at Sai Life Sciences Limited (India). A total of 9 male Sprague Dawley rats (8–12 weeks old) weighing between 200 to 250 g were used for these studies. Rats were administered a single intraperitoneal dose of TG6-10-1 (10 mg/kg) dissolved in a formulation solution consisting of 10% DMSO, 40% water and 50% polyethylene glycol. Blood samples were collected for bioanalysis from cannulated jugular veins under light isoflurane anesthesia from three rats each at 1, 2 and 4 hours after injection.

Plasma was harvested by centrifugation at 4000 rpm for 10 min at 4°C and stored frozen until analysis. A separate group of rats was used for each time point after TG6-10-1 dosing. After blood collection, the rats were euthanized and the brains were isolated. The brains were dipped three times in ice-cold phosphate-buffered saline, blotted and weighed. Brain samples were homogenized using ice-cold phosphate-buffered saline (pH 7.4) and homogenates were kept frozen until analysis.

For TG6-10-1 exposure, rats were assigned to a random number stream and received intraperitoneal administration of vehicle (10% DMSO, 40% water, 50% polyethylene glycol) or the EP2 receptor antagonist, TG6-10-1 (5 mg/kg), dissolved in the same vehicle, with the following protocol: i) one injection, 1 hr prior to DFP, ii) three injections (1.5, 6 and 21 hours after DFP-induced SE onset) or iii) six injections (1.5, 6, 21, 30, 45–47, and 52–55 hours after SE onset). The working concentration of TG6-10-1 was 2.5 mg/ml. Each rat received a volume of either vehicle or TG6-10-1 based on weight (2 ml/kg).

2.7. Electrode implantation and EEG recording

Adult male Sprague-Dawley rats (220–260 g body weight) were implanted with monopolar surface skull electrodes under general anesthesia [ketamine (80 mg/kg) and xylazine (5 mg/kg), ip]. The screw electrodes were made by attaching male miniature pin connectors (A-M Systems, Carlsborg, WA) to stainless steel screws with stainless steel wire (PlasticsOne, Roanoke, VA). Two screw electrodes were positioned through burr holes above the left and right parietal cortices at the following coordinates (relative to bregma): AP -3 mm, L $\pm$ 2 mm. The third surface skull electrode was positioned at the base of the frontal bone (AP +4

mm, $L \pm 2$ mm, relative to bregma) and served as the reference. The screw electrodes penetrated the skull but not the dura matter. The three screw electrodes were secured to the skull with Instant Tray Mix (Lang Dental, Wheeling, IL), which is a rapid self-curing dental acrylic resin that forms a hardened cap on the skull. Rats were then injected with yohimbine (8–9 mg/kg, sc) to reverse the effects of the anesthesia, meloxicam (1.5 mg/kg, sc) for pain management, and then were allowed to recover from the surgery for 7–10 days before experimentation. On the day of EEG recording wire leads with a female miniature pin (A-M Systems) were fastened to the screw electrodes on the skull of the rats. EEG signals were recorded using a Nicolet Endeavor CR system (Viasys Healthcare, Madison, WI) with a Tornado v32 amplifier (Natus Medical Inc, San Carlos, CA) and the NicoletOne recorder software (Viasys Healthcare). The EEG signals were bandpass filtered at 1–100 Hz and 20–70 Hz, and acquired at 500 Hz. Evaluation of acquired EEG was carried out by NicoletOne reader (Viasys Healthcare) and dCLAMP (Pediatric Epilepsy Research, Massachusetts General Hospital, Boston, MA). The EEG analysis software dCLAMP was used to identify spikes, defined as abrupt and sharp transients that had an amplitude $>2\times$ of a 10 minute baseline period prior to drug administration; spikes were verified visually and counted.

2.8. Light-dark exploration

Light-dark exploration testing was carried out to examine anxiety-related behavior in rats that were administered DFP and sham treated rats. The test room was the same room the rats were housed in, but a different isolated cubicle to isolate odor, light intensity and noise. Light-dark preference was examined in all surviving rats 4 weeks following DFP exposure using a covered plexiglass box (40 cm length X 40 cm width X 30 cm height). The box is divided evenly by a partition that contained an opening (12 cm height x 14 cm width) located at the center of the floor into a light side with clear walls/cover and a dark side with black walls/cover as represented schematically in Figure 4A. On the test day each animal was placed in the dark compartment facing away from the opening and allowed to freely explore the entire apparatus for 5 min. Rat behavior and movement were recorded with a sony handycam video camera (Sony, New York, NY) mounted directly above the apparatus and the videos were later analyzed by an observer blinded to the treatment groups. Each rat was tested only once. Time spent in the light and latency to enter the light (with all four paws) were used as measures of anxiety. The latency to the first head poke was defined as the elapsed time for the head of the rat to completely emerge through the opening between the light and dark compartments. The latency to the first head poke, the number of head pokes before the rat fully transitioned to the lit side, the number of full body crosses into the lit side and time spent in the lit side were all measured and compared between the two groups of rats. None of the rats experienced a generalized seizure in the light-dark box during testing.

2.9. Novel object recognition and open field tests

A novel object recognition (NOR) test was used to assess recognition memory in rats that had experienced SE 6–12 weeks earlier. The simple NOR testing paradigm used in the current study invokes minimal stress, which is an important consideration as the rats could develop seizures that may influence their performance during the task. Sham treated rats that received six injections of the vehicle were tested along with rats that received six injections

of TG6-10-1. The NOR test was performed as previously described (Bevins & Basheer, 2006) with a lapse of two hours between training and testing. NOR was carried out in a box similar to that used for light-dark exploration (40cm x 40cm x 30 cm). The test room was the same room the rats were housed in, but a different isolated cubicle to control for odor, light intensity and noise isolation. NOR testing consisted of three parts: habituation, training/object familiarization and novel object recognition testing (Figure 5A). Several pairs of appropriate objects were used for familiarization and testing. Adult male Sprague-Dawley rats that experienced SE 6–12 weeks earlier and sham treated controls were transported individually to the testing arena in their home cages for the three parts of the test. During the habituation session rats were placed in the empty arena and allowed to freely move about, and their distance traveled was analyzed. Total distance moved, time in the center, and rearing were measured during the pre-training phase. On the next day habituation was repeated for 5 minutes. Following the habituation the rats were briefly removed from the arena for approximately 5 minutes and then returned for object familiarization that was carried out once, however now the arena contained two identical objects 20 cm apart from each other and total time spent exploring the identical objects was recorded for 5 minutes. Novel object testing was performed 2 hours after object familiarization in the same manner except one of the objects was replaced by a novel object. The rats were allowed to freely move about the box and explore the objects for 5 minutes. Object exploration was later scored in its entirety from video recordings of the trial by an experimenter blinded to the treatment of the rats during testing. Object exploration was defined as orientation of the head towards the object with the nose within 1 cm of the object with behaviors including sniffing, touching and gnawing. Excluded from the total exploration time was any time spent with the object where a rat simply propped the forepaws onto the object with the nose pointing away from the object. Rats were evaluated for their ability to remember the familiar object by expressing a preference for exploring the novel object. Preference for the novel object was expressed as a discrimination index (DI, equation below), which compares the amount of time a rat spent exploring the novel object compared to the familiar object. Which object served as the novel object and the left/right position of the objects were changed between rats within each group. NOR scoring was automated using a TopScan system (Clever Sys, Reston, VA). Between each trial, the arena and the objects were cleaned with 30% isopropyl alcohol and then dried with paper towels to remove any trace odors. Only one rat (vehicle injected) experienced a generalized seizure in the box during testing and so was not included in the analysis. A trained investigator that was blind to the experimental treatment ran all of the behavioral tasks.

$$DI = \frac{(\text{time exploring novel object} - \text{time exploring familiar object})}{(\text{time exploring novel object} + \text{time exploring familiar object})}$$

2.10. Data analysis

Data are presented as means $\pm$ standard error. Statistical analysis was performed with GraphPad Prism version 4 (GraphPad software, San Diego, CA). Student's *t* test or one-way ANOVA (with Bonferroni or Dunnett's *posthoc* tests) of selected means were performed as appropriate to examine differences of chemical or behavioral effects. Fisher's exact test was

used to compare mortality rates and the percent of rats that entered status epilepticus. The differences were considered to be statistically significant if $p < .05$. A Grubb's test was performed in GraphPad to identify outliers, as explained in Table 2.

3. RESULTS

3.1. Pharmacokinetics of TG6-10-1 in rats and DFP-induced SE characteristics

The pharmacokinetic studies were carried out on normal rather than DFP-treated rats for logistical reasons. Administration of TG6-10-1 (10 mg/kg ip) dissolved in 10% DMSO, 50% PEG400 and 40% water in adult male rats revealed a brain to plasma concentration ratio in the range of 0.3–0.4 from 1 to 4 hours after injection, and an average plasma concentration of 138 ng/ml (308 nM) at 4 hours (Figure 1B, C and Table 1). The TG6-10-1 exposure profile found here with DMSO, PEG400 and water is similar to that in rats administered TG6-10-1 dissolved in N-methylpyrrolidone (NMP), solutol and saline (Rojas et al., 2015). Based on studies showing that SE degrades the blood-brain barrier (e.g., Jiang et al., 2013; Serrano et al., 2011; Van Vliet et al., 2014a; Van Vliet et al., 2014b; Van Vliet et al., 2015), it is possible that brain penetration of TG6-10-1 is even higher following DFP treatment in rats.

Adult male Sprague-Dawley rats were injected subcutaneously with pyridostigmine bromide and atropine methylbromide followed 10 min later by a single dose of DFP (9.5 mg/kg, ip) according to the protocol in Figure 1A. This dose of DFP produced status epilepticus in adult male rats that lasted at least 5 hours without pharmacological intervention. Rats were administered the EP2 receptor antagonist TG6-10-1 during and following status epilepticus according to the two dosing paradigms in Figure 1A. The exposure schedules for TG6-10-1 were informed by pharmacokinetics analysis and by previous experiments (Rojas et al., 2015). In the current study DFP was administered to 140 adult male Sprague-Dawley rats, of which 112 entered status epilepticus resulting in an 80% success rate. The other 20% of rats displayed episodic seizure like activity but failed to develop non-intermittent seizures, and were labeled “DFP-no-SE”. In total, 52 of 140 rats died either within minutes of DFP administration (apparently by respiratory cessation), or during the ensuing SE experience, or within 7 days after SE had waned (37% total mortality). A large fraction (73%) of the total mortality involved rats that died before or within 8 hours of the onset of status epilepticus. These results are consistent with those previously reported for this DFP SE rat model (Rojas et al., 2015).

3.2. EP2 inhibition does not alter DFP-induced status epilepticus

Recently, we demonstrated in rats that inhibition of EP2 by a single dose of TG6-10-1 administered 60 minutes prior to DFP did not alter the behavioral characteristics of status epilepticus, the temporal evolution of seizures towards entry into status epilepticus, or the ability to enter status epilepticus, suggesting that TG6-10-1 is not an acute anticonvulsant in the rat DFP model (Rojas et al., 2015). In the current study we observed a very similar temporal evolution of seizure activity following DFP exposure as previously reported by Rojas et al., 2015 (Figure 1D). The temporal evolution of behavioral seizure activity revealed a rapid advancement towards status epilepticus with seizures progressively

intensifying and status epilepticus being reached within 40–50 minutes after DFP exposure (Figure 1D). The latency to enter status epilepticus following DFP exposure was similar for all rats regardless of whether they were randomized to receive TG6-10-1, vehicle or nothing. The waning of status epilepticus as determined by behavioral seizure activity via visual observation for 6 hours did not appear to be different between treatment groups suggesting that rats injected with vehicle or TG6-10-1 may have experienced a similar intensity of SE.

To more specifically determine whether TG6-10-1 or the vehicle delays the development of SE or alters the intensity or duration of SE we made cortical electroencephalography (EEG) recordings prior to and during DFP exposure. Adult male rats were instrumented with bilateral cortical electrodes 7–10 days prior to EEG recording and DFP exposure. On the day of experiment rats were connected to the EEG instrument and their baseline brain activity was recorded for at least 30 minutes prior to DFP. All rats tested displayed normal cortical activity prior to any drug administration as determined by the low amplitude, frequency and shape of the waveforms as well as the absence of spikes. A subset of rats was exposed only to DFP and served as controls (Figure 2A). Additional subsets of rats were injected with a single intraperitoneal dose of vehicle or TG6-10-1 (5 mg/kg) (Figure 2B, C) 1 hour prior to DFP. EEG was recorded continuously for 24 hours. Exposure to DFP led to the appearance of seizures defined by the appearance of a sudden burst of large amplitude ($>2\times$ the baseline prior to drug treatment) and high frequency spikes. The onset of SE was determined by the appearance of the initial electrographic seizure that consisted of large amplitude spikes that persisted for more than 10 seconds followed by a rapid quieting of electrical activity. Recurring large amplitude, high frequency spikes persisted following the first EEG seizure for at least 5 hours without pharmacological intervention (Figure 2A–C). All rats received a single injection of DFP and the latency to SE onset was determined. There was no significant difference in the average latency to SE onset as determined by one-way ANOVA with Bonferroni *post hoc* test for the three groups tested (Figure 2D). The duration of SE was defined as the period in which spikes detected by dCLAMP persisted from the initial seizure to the time of the last detectable spike just prior to a minimal 2 hour period of no detectable spikes. In all rats the large amplitude and high frequency spikes detected by dCLAMP eventually waned during the 24 hour recording. Without pharmacological intervention SE lasted from 5 hours to ~12 hours (Figure 2E). Rats exposed to vehicle or TG6-10-1 one hour prior to DFP experienced SE lasting 9 ± 1 hours ($n=4$) (Figure 2B, E) and 8 ± 1 hours ($n=3$) (Figure 2C, E), respectively, whereas rats that received only DFP displayed an average duration of SE lasting 9 ± 1 hours ($n=5$) (Figure 2A, E). Also, there was no difference in the total number of spikes detected by dCLAMP during the entire 24 hour recording [1650 ± 456 spikes ($n=5$) for DFP-only rats, 1220 ± 571 spikes ($n=4$) for vehicle treated rats and 2020 ± 708 spikes ($n=3$) for TG6-10-1 treated rats (Figure 2F)] as determined by one-way ANOVA with Bonferroni *post hoc* test. Taken together, these data reinforce the conclusion that TG6-10-1 or its vehicle do not act like an acute anticonvulsant to prevent the development of seizures or SE, decrease the intensity of SE or shorten the duration of SE in this rodent organophosphorus model.

3.3. TG6-10-1 had no effect on the early functional decline and delayed mortality of rats exposed to DFP

A modified Irwin test consisting of a selective subset of normal behaviors was used as a functional assessment on all rats prior to any drug administration. All rats subjected to the modified Irwin test prior to any manipulation on day zero scored 0 (not shown), indicative of normal rat health and behavior and were deemed suitable for further experimentation. The modified Irwin test was repeated for all rats 24 hours after DFP induced SE. Rats given DFP that endured status epilepticus were randomized and administered TG6-10-1 (5 mg/kg, ip) or vehicle at various time points after DFP exposure. Consistent with our previous findings (Rojas et al., 2015), most rats that endured SE failed (score = 12) the modified Irwin test measured 24 hours after DFP exposure (Figure 3A, B), indicative of compromised health. None of the rats that failed the modified Irwin test showed signs of infection or injury from the multiple injections on day zero, but instead they displayed lethargy. No difference was detected in the 24 h modified Irwin test score of rats that were administered 3 injections of TG6-10-1 or the vehicle after DFP (14.5 ± 0.7 for vehicle, $n=11$; 14.1 ± 0.6 for TG6-10-1, $n=14$; $p=.6$, t test) (Figure 3A). Additionally, no difference was detected in the 24 h modified Irwin test score of rats that were being administered 6 injections of TG6-10-1 or the vehicle after DFP (14.5 ± 0.5 for vehicle, $n=22$; 13.9 ± 0.4 for TG6-10-1, $n=25$; $p=.6$, t test) (Figure 3B), suggesting that inhibition of the EP2 receptor does not alter sensorimotor function measured 24 hours after DFP-induced SE. Furthermore, the average modified Irwin test score for rats used in the EEG experiments administered TG6-10-1 prior to DFP was very similar to that of rats that received a single injection of vehicle (16.5 ± 0.6 for vehicle, $n = 4$; 17 ± 0.4 for TG6-10-1, $n = 4$; $p = .5$, t test) (not shown). Rats exposed to DFP that did not enter status epilepticus (DFP-no-SE) had an average 24 hour modified Irwin test score of 9.7 ± 1.5 ($n = 7$) (Figure 3A), indicative of moderate functional impairment. The DFP-no-SE rats recovered much earlier from the acute effects of DFP exposure compared to rats that endured SE.

Rats that survive the acute episode of status epilepticus elicited by DFP usually survive the next four days (Rojas et al., 2015). In the current study, to investigate delayed mortality we monitored rats that endured DFP-induced SE continuously 26 days for rats that received three injections of TG6-10-1 or vehicle and up to 742 days for rats that received six injections. Of rats that received 3 injections of TG6-10-1, 14 of 19 survived over the 26 day period after status epilepticus whereas 11 of 15 rats administered vehicle survived ($p = 1$, Fisher's exact test) (Figure 3C). Likewise, no difference was detected in mortality measured during the first 26 days for rats that received 6 injections of TG6-10-1 (23 of 27 rats survived) compared to rats that received 6 injections of the vehicle (21 of 23 rats survived) ($p = .7$, Fisher's exact test). Analysis of long term survival (to 742 days) of rats that had received six injections also revealed no difference in mortality between TG6-10-1 and vehicle treated rats (5 of 27 TG6-10-1 injected rats survived whereas 6 of 23 vehicle injected rats survived) ($p = .7$, Fisher's exact test) (Figure 3D). The median survival of untreated male Sprague Dawley rats is 595-703 days (Keenan et al., 1994; Liang et al., 2010), which is much longer than the 450 day median survival of our cohort of rats that had experienced DFP SE (Fig 3D). Overall, the 26 day survival of rats regardless of whether they were injected with TG6-10-1 or vehicle was high as the range of 7-day mortality was only 10–

25% in either subset. Rats that experience DFP-induced status epilepticus tend to lose body weight for the first two days and then usually start to regain weight the days following (Rojas et al., 2015). Here, we weighed the rats daily for 26 days after DFP-induced status epilepticus. All rats exposed to DFP lost a similar amount of weight on day 1 (~10% average) regardless of whether they had been injected with vehicle or TG6-10-1 (Figure 3E, F). Rats administered TG6-10-1 or the vehicle 90 minutes, 6 hours and then again 21 hours after DFP-induced status epilepticus did not differ in weight regain on day 4 (Figure 3E). In the current study rats that received 6 injections of TG6-10-1 did not show accelerated weight regain compared to vehicle injected rats contrary to the finding by Rojas et al., 2015 (Figure 3F).

3.4. Behavior in the light-dark exploration task is unaffected by EP2 inhibition

Experiments were performed to investigate the role of EP2 receptor activation in seizure induced long-term cognitive deficits. The initial behavioral analysis was conducted 4 weeks after SE in a light-dark preference apparatus (Figure 4A). We chose 4 weeks as an appropriate time to investigate the behavior of the rats for the following reasons: 1) by week 4 all rats had recovered from the SE experience as assessed by weight regain and the modified Irwin score, and 2) by week 4 we had not observed generalized behavioral seizures in a majority of rats (29 of 42, 69%). To determine whether the combination of pyridostigmine bromide, atropine methyl bromide and DFP affects anxiety in rats that do not experience SE we first performed light-dark preference testing on a cohort of DFP-no-SE rats that were administered 3 injections of TG6-10-1 or vehicle. The DFP-no-SE rats did not significantly differ from sham treated controls in their exploration of the box or their preference for the dark as determined by the head poke latency, number of head pokes before initial entry into the light compartment, latency to enter the light compartment, number of entries into the light compartment or time spent in the light (Figure 4).

Regardless of whether TG6-10-1 or vehicle was administered, rats that endured status epilepticus displayed a trend of a longer head poke latency (Figure 4B), smaller number of head pokes before entering the lit compartment (Figure 4C), shorter latency to enter the light compartment (Figure 4D), an increase in the number of entries into the light (Figure 4E) and increased time spent in the light (Figure 4F) compared to sham treated control rats (all values are in Table 2), although statistical significance was only attained for the number of head pokes (Figure 4C). Comparison of the rats that experienced SE revealed that rats administered TG6-10-1 behaved similarly as vehicle injected rats as shown in Figure 4 and Table 2, suggesting that TG6-10-1 does not play a role in anxiety behaviors. However, rats that endured DFP-induced status epilepticus spent significantly more time in the lit compartment of the light-dark box compared to rats that did not experience SE [36 ± 6 sec for DFP-SE (TG6-10-1 and vehicle combined, $n=41$) vs. 4 ± 2 sec for No-SE (controls and DFP-No-SE combined, $n=22$); $p=.0003$, t test], suggesting that DFP-induced SE may cause anxiolytic behavior. It should be noted that all rats regardless of treatment had a preference for the dark as most of their time in the apparatus was spent in the dark (Figure 4F).

3.5. Transient inhibition of EP2 after SE restores the ability to form memories

One long-term consequence of SE induced by an organophosphorus nerve agent (soman, sarin) in animals is the loss of the ability to form new memories (Chen 2012; Filliat et al., 1999; Gilat et al., 2005; Joosen et al., 2009). This cognitive impairment is also observed in humans following exposure to the nerve agent sarin (Miyaki et al., 2005; Nishiwaki et al., 2001). To determine if DFP-induced SE in rats results in memory impairment we performed novel object recognition (NOR) testing 6–12 weeks after the SE experience on rats that received 6 injections of TG6-10-1 or the vehicle over a 2.5 day period after SE onset. On the day prior to novel object recognition testing, all rats were subjected to a habituation trial in the NOR arena. This habituation trial was analyzed as an open field exploration test as the rats were allowed to freely move about the empty box and explore (Figure 5A). Video recording analysis revealed that the majority of rats traveled 6–10 m during the 5 minute recording period (Figure 5B). Rats administered TG6-10-1 traveled a similar distance (Figure 5B) and spent nearly the same amount of time in the center of the arena (an area defined as a 20 cm square in the very middle) (Figure 5C) as rats injected with vehicle (7.1 ± 0.70 m traveled and 157 ± 17 sec in the center for TG6-10-1, $n = 19$ vs. 6.9 ± 0.74 m traveled and 172 ± 14 sec in the center for vehicle, $n = 20$). The distance traveled was also similar for sham treated control rats (7.4 ± 0.35 m, $n = 12$). However, the sham treated control rats spent significantly less time in the center of the arena (74 ± 10 sec, $n = 12$) (Figure 5C) compared to the rats that experienced SE 6–12 weeks earlier. Taken together, the open field data in Figure 5C suggest that TG6-10-1 treatment did not alter anxiety behavior measured during the habituation trial.

To determine whether TG6-10-1 treatment rescues impaired memory following DFP-induced SE, we compared NOR performance among three experimental cohorts of rats. The experimental cohorts were as follows: 1) sham treated control rats injected with TG6-10-1 or vehicle, 2) DFP-SE rats that had received 6 injections of TG6-10-1 and 3) DFP-SE rats that had received 6 injections of vehicle. Rats from all three cohorts spent the same percent of time exploring both identical objects during the training phase (not shown). The data revealed that control rats spent more time exploring the novel object compared to the familiar object with an average discrimination index of 0.31 ± 0.1 ($n = 12$) ($p = .003$, 1-sample t test compared to zero) (Figure 5D), indicating that they remembered the familiar object from the object familiarization phase and thus had a preference for the novel object. In contrast, rats from the DFP-SE group treated with vehicle spent the same amount of time exploring both the novel and familiar objects with an average discrimination index of 0.0 ± 0.1 ($n = 19$) (ns, t test compared to zero) (Figure 5D), suggesting that these rats did not remember the familiar object. Rats from the DFP-SE group that were administered 6 doses of TG6-10-1 spent significantly more time exploring the novel object with an average discrimination index of 0.24 ± 0.1 ($n = 18$) ($p = .02$, t test compared to zero) (Figure 5D). The DFP-SE rats administered TG6-10-1 performed similar to the control group in the NOR task. The time from DFP exposure on day 0 to the NOR test day varied between the rats, and there was a moderate negative correlation for the discrimination index as a function of time for the vehicle treated rats ($p = .03$) compared to a nonsignificant ($p > .05$) positive correlation for TG6-10-1 treated rats (Figure S1). No correlation was detected in either group of rats for time in the center plotted as a function of time from day 0 (Figure S1),

suggesting that rats treated with vehicle (but not rats treated with the EP2 antagonist) trend towards more impaired memory with increasing time. Taken together, these data indicate that early, short-term EP2 inhibition after SE may prevent the long-term inability to form new memories caused by organophosphorus-induced SE.

4. DISCUSSION

Status epilepticus induced by nerve agents in animals and humans leads to the development of spontaneous recurrent seizures and long-term cognitive deficits (Chen 2012; de Araujo Furtado et al., 2012; Joosen et al., 2009; Miyaki et al., 2005; Nishiwaki et al., 2001). In animal studies investigating learning and memory, these long term cognitive deficits are associated with the early neurodegeneration and the neuronal plasticity that occurs in the brain after status epilepticus (Chen 2012; Filliat et al., 1999; Joosen et al., 2009; McDonough et al., 1987; Myhrer et al., 2005). We recently demonstrated that short-term EP2 inhibition by the EP2 receptor antagonist used here (TG6-10-1) reduces neurodegeneration, accelerates weight regain and improves the overall functional health of rats within 4 days following SE (Rojas et al., 2015). In the current study, we demonstrated that rats develop an inability to remember an object 1-4 months following status epilepticus induced by DFP.

Rats that survived status epilepticus tend to survive the duration of the experiment as there appeared to be little delayed mortality over 26 days and no significant difference in delayed mortality for rats administered TG6-10-1 compared to rats injected with the vehicle to 742 days following DFP-SE. In the current study we monitored surviving rats for almost 2 years. The high acute mortality observed is common following exposure to high levels of organophosphorus based compounds and is attributed to a multifaceted effect of these agents on the function of vital organ systems. Administration of a single dose of TG6-10-1 prior to DFP resulted in a similar latency to enter SE and temporal evolution of seizures as rats given the vehicle, suggesting that TG6-10-1 is not an acute anticonvulsant and does not prevent the development of seizures or status epilepticus. The results also show that TG6-10-1 did not terminate SE early in this rodent organophosphate model of SE. Although accelerated weight regain following TG6-10-1 treatment occurred in our recent study (Rojas et al., 2015), this finding was not confirmed here with a different TG6-10-1 dosing paradigm. The rats were allowed to recover from SE and cognitive functions were investigated.

Exploratory behaviors, anxiety and memory retention were investigated in all rats. A dose of DFP sufficient to produce SE caused a significant reduction in anxiety-related behavior in two assays (time in the center of the NOR arena or time in the lit compartment in the light-dark box), compared to rats that did not endure SE, regardless of whether rats had been administered TG6-10-1 or vehicle. The DFP-no-SE rats, by contrast, did not significantly differ from sham treated controls in any anxiety-like measure during light-dark exploration, suggesting that any alterations in behavior are likely produced by the SE experience rather than the chemical agents themselves. Although in the current study rats exposed to a high dose of DFP displayed reduced anxiety behavior, exposure of rats to an SE-producing dose of soman was reported to increase anxiety behavior (Prager et al., 2014; Prager et al., 2015), whereas exposure to sub-SE doses of other organophosphorus agents showed a trend

towards decreased anxiety behaviors (Schultz et al., 1990; Wright et al., 2010) or had no effect (Nieminen et al., 1991; Valvassori et al., 2007). Thus, the net effect of organophosphorus agents on anxiety-related behaviors could depend on the agent and its dose. Nevertheless, administration of TG6-10-1 did not affect anxiety behaviors after status epilepticus in either of the two assays, suggesting that EP2 may not be involved in regulating anxiety.

Novel object recognition testing revealed a loss of the ability to remember a familiar object in rats that had been administered vehicle following a SE experience induced by DFP; rats treated with TG6-10-1 performed similar to control rats that had not experienced SE. Whether this inability to form new memories correlates with early neuropathologies following SE remains uncertain in the current study. The memory deficit observed in rats that endured SE was not due to spontaneous generalized seizures that occurred during testing as only 1 rat (vehicle injected) displayed a generalized seizure during any part of the NOR test and this rat was not included in the data analysis. TG6-10-1 treated rats showed a preference for the novel object similar to that of the sham treated controls. Taken together, these results suggest that EP2 receptor activation promotes not only the early consequences of SE such as neurodegeneration, neuroinflammation and gliosis (Rojas et al., 2015), but also the development of long term cognitive deficits such as the ability to form new memories. The robust response observed in the NOR task encourages the further study of EP2 receptor inhibition after SE in other tests of learning and memory such as contextual fear conditioning, Morris water maze or object displacement recognition.

5. CONCLUSION

Inhibition of the EP2 receptor in rats exposed to DFP blocks long term memory impairment, but does not affect anxiety behaviors. One somewhat trivial explanation for the beneficial effect of TG6-10-1 in the DFP model, that the compound simply aborts status epilepticus, has been ruled out. We conclude that short-term treatment with an EP2 receptor inhibitor beginning > 90 min after DFP exposure produces long-term cognitive benefits. These studies give insight into therapeutic modalities for the inhibition of EP2 in cognitive pathologies. In the future it will be important to determine whether this beneficial effect of TG6-10-1 occurs following exposure to other organophosphorus based neurotoxins and whether TG6-10-1 treatment alters the development of spontaneous recurrent seizures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work is supported by NIH UO1 NS058158-08, R01 NS097776 (RD) and T32 DA15040 (AR). We thank Dr. Jason Schroeder of the Emory rodent behavioral core facility for help with light-dark box preference and novel object recognition experiments.

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ABBREVIATIONS

PGE2	prostaglandin E2
EP2	prostaglandin E2 receptor 2
SE	status epilepticus
Con	control
COX-2	cyclooxygenase 2
DFP	diisopropyl fluorophosphate
OP	organophosphorus compound
IP	intraperitoneal
SC	subcutaneous
Veh	vehicle
mpk	mg/kg
TG6-10-1	potent and selective EP2 receptor antagonist
EEG	electroencephalography
LDPT	light-dark box preference test
NOR	novel object recognition
PEG	polyethylene glycol
NMP	N-methylpyrrolidone
SRS	spontaneous recurrent seizure
AChE	acetylcholinesterase
DI	discrimination index
SD	Sprague-dawley

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HIGHLIGHTS

- We investigated cognitive impairment in rats caused by status epilepticus (SE).
- Deficits in a novel object recognition task developed 6–12 weeks after SE.
- Treatment with the EP2 antagonist TG6-10-1 after SE blocked the memory impairment.
- TG6-10-1 did not affect behavioral changes in the light-dark box and open field assays.
- TG6-10-1 is not an acute anticonvulsant as it did not alter acute seizure characteristics.

Figure 1. Pharmacokinetics of TG6-10-1 and temporal evolution of SE

A, Experimental paradigm of chemical administration in a rat model of DFP-induced status epilepticus. Following DFP, rats were injected with vehicle (40% water, 50% PEG400, 10% DMSO) or TG6-10-1 (5 mg/kg) dissolved in the vehicle multiple times beginning 90 min from the onset of SE (3 injections or 6 injections at times shown on the right after SE onset). **B**, plasma concentration of normal adult rats that received TG6-10-1 (10 mg/kg, ip) ($n = 3$ rats). **C**, the brain to plasma ratio in rats administered TG6-10-1 over the same time span ($n = 3$ rats for each time point). Open circles represents individual rats. **D**, the mean behavioral seizure scores of rats that received DFP followed by nothing ($n = 10$ rats), DFP followed by the vehicle ($n = 45$ rats) and DFP followed by TG6-10-1 ($n = 53$ rats) are plotted as a function of time (data from that received 3 injections and 6 injections were combined for this analysis as there was no difference in the groups). Also shown is the behavioral seizure activity of rats that did not enter status epilepticus ($n = 11$ rats). The dashed line indicates the behavioral seizure activity score at the onset of status epilepticus.

Figure 2. Characteristics of DFP induced SE

A, Cortical EEG activity was recorded prior, during and after SE induced by exposure to DFP for 24 hours. Shown is a representative EEG trace from the cortical recording of an adult male rat with large spikes that appear within 30 minutes after exposure to DFP. The duration of SE is denoted by the horizontal bar above the trace. **B**, representative EEG trace from the cortical recording of an adult male rat that was administered the vehicle (ip) 1 hour prior to DFP. **C**, representative EEG trace from the cortical recording of an adult male rat that was injected with a single dose of TG6-10-1 (5 mg/kg, ip) 1 hour prior to DFP. **D**, plot of the latency to the onset of status epilepticus comparing the three groups of rats [DFP-SE (n = 4), vehicle followed by DFP-SE (n = 4), TG6-10-1 followed by DFP-SE (n = 4)]. **E**, bar graph of the mean duration of SE for each group of rats tested [DFP-SE (n = 5), vehicle followed by DFP-SE (n = 4), TG6-10-1 followed by DFP-SE (n = 3)]. The open symbols represents each individual rat. The filled triangles denotes the traces shown in A, B and C. **F**, plot of the total number of spikes identified by dCLAMP during the 24 hour recording for the three groups of rats [DFP-SE (n = 5), vehicle followed by DFP-SE (n = 4), TG6-10-1 followed by DFP-SE (n = 3)]. Error bars represent the standard error of the mean. ns = $p > .05$, One-way ANOVA with Bonferroni *posthoc*.

Figure 3. Analysis of functional recovery and delayed mortality

Plot of the mean Irwin score for rats that received 3 injections (**A**) or 6 injections (**B**) of TG6-10-1 or vehicle following DFP ($p = .6$ for 3 injections; $p = .3$ for 6 injections, t test). Each open square represents an individual rat that received TG6-10-1 after DFP-SE [3 injections, $n = 14$; 6 injections, $n = 25$]. Open circles represent individual rats that received vehicle after DFP-SE [3 injections, $n = 11$; 6 injections, $n = 22$]. The up facing closed triangles in **A** represent individual rats that did not enter status epilepticus (DFP-no-SE, $n = 7$ rats) following DFP administration. The short horizontal bold lines denotes the average of the individual animals within the group. The long horizontal dashed line represents the cutoff for determining whether an animal was healthy or impaired. **C**, **D** survival plots of rats that experienced DFP-induced SE and were administered TG6-10-1 or vehicle. Rats that

received 3 injections are shown in panel C measured to day 26 after DFP-induced SE ($p = 1$, Fisher's exact test comparing treatment groups) and rats that received 6 injections measured to day 742 after DFP-induced SE are shown in panel D ($p = .7$, Fisher's exact test comparing treatment groups). The number in parentheses represents the total number of rats in each group. **E**, graph of the weight change for rats that received 3 injections of TG6-10-1 or vehicle ($p = .5$, t test, day 4). **F**, shown is the weight change for rats that received 6 injections of TG6-10-1 or vehicle ($p = .3$, t test, day 4). The small inserts show the weight change over 26 days. Rats that lost 30% or more of the original body weight on any day over the 4 day period was not included in the analysis.

Figure 4. Exploration and anxiety behavior 4 weeks following SE

A, schematic of light-dark exploration apparatus. Latency to the first head poke (**B**), the number of head pokes (**C**), latency to enter the light compartment (**D**), number of entries into the light compartment (**E**) and the time spent in the light compartment (**F**) are shown for the four groups of rats (sham treated controls, DFP-no-SE, DFP-SE followed by vehicle, DFP-SE followed by TG6-10-1). The bars show the mean of the group and the number in the white box within the bar represent the total number of rats in each group. The error bars represent the standard error of the mean. The “+/-” symbol next to TG6-10-1 denotes sham treated control rats and DFP-no-SE rats that received TG6-10-1 (n=6) or vehicle (n=6). These rats were combined into one group as they were not different in any measure. $p < .01$, one-way ANOVA with Bonferroni *posthoc*; ns = $p > .05$ by One-way ANOVA with

Bonferroni *posthoc*. Grubb's test identified one sham treated control rat as an outlier. This rat was removed during analysis in panels B and F reducing the total number of sham treated control rats from 12 to 11. One DFP-SE rat that was injected with vehicle was also identified as an outlier by the Grubb's test and removed from the analysis in panels B, C and E reducing the total number of rats in the DFP-SE followed by vehicle group from 20 to 19. Time in the light was not obtained for one DFP-no-SE rat reducing the number from 12 to 11.

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Figure 5. Novel object recognition memory in rats that experienced SE. A, schematic of novel object recognition testing consisting of three epochs carried out over two days. **B**, a graph of the mean total distanced traveled for each group (sham treated controls, DFP-SE followed by vehicle, DFP-SE followed by TG6-10-1) is shown. $ns = p > .05$, One-way ANOVA with Dunnett's *posthoc*. **C**, time spent in the center is shown for the three groups tested. $p < .01$, One-way ANOVA, Dunnett's *posthoc*. **D**, a discrimination index was used as a measure of memory retention. The individual groups were compared to zero by a 1-sample t test. The number in the white box within the bar represents the total number of rats in each group. The "+/-" symbol next to TG6-10-1 denotes sham treated control rats that received TG6-10-1 (n=6) or vehicle (n=6). These rats were combined into one group as they were not different in any measure. The horizontal dashed line at 0 indicates the point at which there is no discrimination between the novel and familiar objects. $ns = p > .05$. One DFP-SE rat that received vehicle experienced a spontaneous seizure in the arena during NOR testing and was not included in that analysis of the discrimination index reducing the

total number of rats from 20 to 19. One DFP-SE rat that received TG6-10-1 was deemed not fit to perform NOR testing and was not included in the analysis of the discrimination index reducing the total number in the group from 19 to 18.

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Table 1

Individual plasma pharmacokinetic parameters of TG6-10-1 in normal male SD rats.

Time (hr)	Brain (ng/mL)	Plasma (ng/mL)	Brain-to-Plasma ratio	Mean Brain-to- plasma ratio
1	44.9	435	0.31	0.32
	47.5	406	0.35	
	60.2	625	0.29	
2	24.1	278	0.26	0.31
	29.4	244	0.36	
	42.0	396	0.32	
4	15.8	146	0.32	0.39
	21.3	146	0.44	
	16.7	124	0.41	

Rats received a single intraperitoneal injection of TG6-10-1 (10 mg/kg).

Table 2

Individual exploratory and anxiety behaviors in control and DFP administered male SD rats after DFP-induced SE in a light-dark preference apparatus.

Treatment	Head Poke latency (sec)	# of Head Pokes	Latency to enter light (sec)	# of entries into light	Time in light (sec)
Control	27 ± 5 (11)	8 ± 1 (12)3	242 ± 27 (12)3	1 ± 0.5 (12)3	7 ± 4 (11)3
DFP-no-SE	34 ± 9 (12)	5 ± 1 (12)	271 ± 12 (12)	1 ± 0.3 (12)	0.4 ± 0.2 (11)
DFP-SE + Vehicle	63 ± 13 (19)3	4.5 ± 1 (19)3	195 ± 26 (20)3	1.2 ± 0.4 (19)3	35 ± 10 (20)3
DFP-SE + TG6-10-1	49 ± 8 (21)3	5.7 ± 0.5 (21)3	155 ± 24 (21)3	2 ± 0.4 (21)3	37 ± 7 (21)3

Rats were subjected to a single experience (300 seconds) in a light-dark preference box. The value in each block is the mean ± SEM. The number in parentheses represent the number of rats. Control and DFP-no-SE rats received TG6-10-1 (n=6) or vehicle (n=6). Grubb's test identified one sham treated control rat as an outlier. This rat was removed during analysis reducing the total number of sham treated control rats from 12 to 11 for head poke latency (outlier value = 149) and time in the light (outlier value = 85). Grubb's test also identified outliers in the group of rats administered vehicle after DFP-SE. One separate outlier was identified for head poke latency (outlier value = 300), another for the number of head pokes (outlier value = 15) and a third for the number of entries into the light (outlier value = 8). These outliers were removed from the analysis reducing the total number of rats in the DFP-SE followed by vehicle group from 20 to 19 for the above mentioned parameters. Time in the light was not obtained for one DFP-no-SE rat reducing the number from 12 to 11.