

**The M1 muscarinic receptor antagonist VU0255035 delays the development of status epilepticus after organophosphate exposure and prevents hyperexcitability in the basolateral amygdala**

Steven L. Miller, Vassiliki Aroniadou-Anderjaska, Volodymyr I. Pidoplichko, Taiza H. Figueiredo, James P. Apland, Jishnu K.S. Krishnan, Maria F. M. Braga

Department of Anatomy, Physiology and Genetics (S.L.M., V.A.-A., V.I.P., T.H.F., J.K.S.K., M.F.M.B.), Department of Psychiatry (V.A.-A., M.F.M.B.), and Program in Neuroscience (S.L.M., V.A.-A., M.F.M.B.), F. Edward Hébert School of Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD, 20814, USA

Neurotoxicology Branch, U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, Maryland, 21010, USA (J.P.A.)

[stevenmiller17@gmail.com](mailto:stevenmiller17@gmail.com), [vanderjaska@usuhs.edu](mailto:vanderjaska@usuhs.edu), [volodymyr.pidoplichko.ctr@usuhs.edu](mailto:volodymyr.pidoplichko.ctr@usuhs.edu), [taiza.figueiredo.ctr@usuhs.edu](mailto:taiza.figueiredo.ctr@usuhs.edu), [james.p.apland.civ@mail.mil](mailto:james.p.apland.civ@mail.mil), [jishnu.krishnan.ctr@usuhs.edu](mailto:jishnu.krishnan.ctr@usuhs.edu), [maria.braga@usuhs.edu](mailto:maria.braga@usuhs.edu)

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**Running title:** M1 receptor role in seizure generation by organophosphates

Corresponding author: Maria F.M. Braga, D.D.S., Ph.D, Department of Anatomy, Physiology, and Genetics, F. Edward Hébert School of Medicine, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814. Phone: (301) 295-3524 Fax: 301-295-3566 Email: [maria.braga@usuhs.edu](mailto:maria.braga@usuhs.edu)

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**List of non-standard abbreviations:**

BLA, basolateral amygdala

DMSO, Dimethyl sulfoxide

mAChRs, muscarinic acetylcholine receptors

M1, muscarinic receptor type 1

OP, organophosphate

2-PAM, pralidoxime chloride

VU0255035, [N-(3-oxo-3-(4-(pyridine-4-yl)piperazin-1-yl)propyl)-benzo[c][1,2,5]thiadiazole-4-sulfonamide]

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## Abstract

Exposure to organophosphorus toxins induces seizures that progress to status epilepticus (SE), which can cause brain damage or death. Seizures are generated by hyperstimulation of muscarinic receptors, subsequent to inhibition of acetylcholinesterase; this is followed by glutamatergic hyperactivity, which sustains and reinforces seizure activity. It has been unclear which muscarinic receptor subtypes are involved in seizure initiation and the development of SE in the early phases after exposure. Here, we show that pretreatment of rats with the selective M1 receptor antagonist VU0255035, significantly suppressed seizure severity and prevented the development of SE for about 40 min after exposure to paraoxon or soman, suggesting an important role of the M1 receptor in the early phases of seizure generation. In addition, in *in vitro* brain slices of the basolateral amygdala (BLA), a brain region that plays a key role in seizure initiation after nerve agent exposure, VU0255035 blocked the effects produced by bath application of paraoxon, namely, a brief barrage of spontaneous IPSCs, followed by a significant increase in the ratio of the total charge transferred by spontaneous EPSCs over that of the IPSCs. Furthermore, paraoxon enhanced the hyperpolarization-activated cation current  $I_h$  in BLA principal cells which could be one of the mechanisms underlying the increased glutamatergic activity an effect that was also blocked in the presence of VU0255035. Thus, selective M1 antagonists may be an efficacious pretreatment in contexts where there is risk for exposure to OPs, as they will delay the development of SE long enough for medical assistance to arrive.

## Introduction

There is ample evidence to suggest that the primary mechanism by which organophosphorus toxins (OPs) used as insecticides or in chemical warfare (nerve agents) induce prolonged, severe seizures (status epilepticus; SE) involves hyperstimulation of muscarinic acetylcholine receptors (mAChRs). Thus, phosphorylation of acetylcholinesterase by these toxins, inactivates the enzyme (Sirin et al., 2012), resulting in accumulation of acetylcholine at cholinergic synapses. Although both nicotinic acetylcholine receptors and mAChRs can be expected to be activated by the elevated acetylcholine, it is primarily the activation of mAChRs that elicits the generation of seizures. Evidence for that has been provided by studies showing that mAChR antagonists prevent the induction of seizures if administered before OP exposure (Ashani and Catravas, 1981; Capacio and Shih, 1991; McDonough et al., 1987; Skovira et al., 2010), and can arrest seizures if administered shortly after exposure (Anderson et al., 1997; McDonough et al., 1989), while nicotinic receptor antagonists do not suppress OP-induced seizures *in vivo* (Dekundy et al., 2003; Shih et al., 1991) or epileptiform activity *in vitro* (Harrison et al., 2004). Antagonists of mAChRs are ineffective in stopping seizures if injected at delayed time points after OP exposure, because after the initial phase of SE, glutamatergic mechanisms that have come into play are primarily responsible for reinforcing and sustaining seizures (McDonough and Shih, 1997; Weissman and Raveh, 2008). It has not been clear, however, which of the mAChR subtypes are involved in the initiation of seizures, leading to and sustaining SE in the early phases post-exposure.

There are five subtypes of mAChRs, M<sub>1</sub> to M<sub>5</sub>. They all are G-protein coupled; M<sub>2</sub> and M<sub>4</sub> are preferentially coupled to the Gi class of G proteins, while M<sub>1</sub>, M<sub>3</sub>, and M<sub>5</sub> are coupled to the Gq family (Kruse et al., 2014; Wess, 2003). Hamilton et al. (1997) and Bymaster et al.

(2003) have found that pilocarpine, a muscarinic agonist, induces SE in wild-type mice, but not in mice lacking the M<sub>1</sub> receptor, suggesting a key role for M<sub>1</sub> receptors in the induction of SE by pilocarpine. In addition, a highly selective M<sub>1</sub> receptor antagonist was developed recently, VU0255035 (Weaver et al., 2009), and was tested against seizures induced by pilocarpine; mice pre-treated with VU0255035 did not develop SE, at least for 40 min after injection of pilocarpine (Sheffler et al., 2009). Because pilocarpine and OPs share the primary mechanism of seizure induction (muscarinic receptor hyperstimulation), it has been suggested that findings in the pilocarpine model of SE may also apply to OP-induced SE (Tang et al., 2011; Tetz et al., 2006). Whether or not M<sub>1</sub> receptor activation, which is necessary for induction of seizures by pilocarpine, is also necessary for induction of seizures by OPs was recently tested in wild-type and M<sub>1</sub>-knockout mice (Kow et al., 2015) exposed to paraoxon (O,O-diethyl O-*p*-nitrophenyl phosphate), which is the active metabolite of the insecticide parathion (Garcia et al., 2003). No difference was found in paraoxon-induced seizure severity between the wild-type and the M<sub>1</sub>-knockout mice, leading to the conclusion that in contrast to the significant role that the M<sub>1</sub> receptor plays in pilocarpine-induced seizures, these receptors are not involved in the generation of seizures by paraoxon exposure (Kow et al., 2015).

Delineating the mechanisms that are involved in the generation of seizures after OP exposure can result in more targeted pretreatments and early-phase, post-exposure treatments that have minor or no side effects. Therefore, in order to shed more light into the role of the M<sub>1</sub> receptor in seizure generation by OPs, in the present study, we examined the effect of pretreatment with the selective M<sub>1</sub> antagonist VU0255035 on seizure development and severity by exposure of rats to soman or paraoxon. We further examined the effects of paraoxon on neuronal excitability parameters, in *in vitro* brain slices, and whether M<sub>1</sub> receptors are involved

in these effects. The *in vitro* studies were performed in the basolateral amygdala (BLA), a seizure-prone amygdala nucleus (Aroniadou-Anderjaska et al., 2008), because there is strong evidence to suggest that the BLA plays a key role in seizure generation after nerve agent exposure (McDonough et al., 1987; Prager et al., 2013).

## Materials and Methods

### Animals

Male, Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were housed in an environmentally controlled room (20–23°C, 12 hr light/dark cycle, lights on 06:00 A.M.), with food and water available *ad libitum*. The animal care and use programs at the Uniformed Services University of the Health Sciences and the U.S. Army Medical Research Institute of Chemical Defense are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All animal experiments were conducted following the Guide for the Care and Use of Laboratory Animals, by the Institute of Laboratory Animal Resources, the National Research Council USA, and were approved by the Institutional Animal Care and Use Committees of both of our institutions.

### Paraoxon and soman exposure

Ten-week-old male rats were administered 25 mg/kg (i.p.) of the selective M<sub>1</sub> receptor antagonist VU0255035 (Tocris Bioscience, Ellisville, MO) diluted at 25 mg/ml in DMSO (Sigma-Aldrich, St Louis, MO), or 1 ml/kg (i.p.) DMSO as the vehicle, 15 min before exposure to soman (pinacolyl methylphosphonofluoridate; obtained from the Edgewood Chemical Biological Center, Aberdeen Proving Ground, Edgewood, MD), or 30 min before exposure to

paraoxon (paraoxon-ethyl; Sigma-Aldrich). The concentration of VU0255035 that we used is within the range that has no significant effect on cognitive functions (Sheffler et al., 2009). For the soman experiments, animals were subcutaneously injected with  $1.8 \times LD_{50}$  soman (198  $\mu\text{g}/\text{kg}$ , diluted in cold physiological saline). For the paraoxon experiments, animals were subcutaneously injected with 4 mg/kg paraoxon; paraoxon solutions were prepared fresh by adding 10 ml stock solution (1.274 g/mL) to 3 ml of ice cold PBS in a glass vial and mixing thoroughly. Soman was used because of its high toxicity and the difficulty in counteracting soman-induced seizures, which implies that if a drug is effective in suppressing seizures induced by soman, it is likely to be effective also against other nerve agents. Paraoxon was used because it is highly potent, has similar effects to those of soman, and there is extensive literature on its use as a model of OP poisoning, including its use as a surrogate for chemical warfare nerve agents. The peripheral toxic cholinergic effects of soman and paraoxon were controlled by intramuscular injection of either 2 mg/kg atropine methylnitrate, or 2 mg/kg atropine sulfate along with 25 mg/kg pralidoxime (2-PAM; all 3 drugs from Sigma-Aldrich), administered within 1 min after injection of the OP. Seizures were monitored behaviorally in a blind fashion by two expert observers, and their severity was scored for 1 h, using a modified version of the Racine scale (Racine, 1972), as we have described previously (Figueiredo et al., 2011): Stage 0, no behavioral response; Stage 1, behavioral arrest; Stage 2, oral/facial movements, chewing, head nodding; Stage 3, unilateral/bilateral forelimb clonus without rearing, Straub tail, extended body posture; Stage 4, bilateral forelimb clonus plus rearing; Stage 5, rearing and falling; and Stage 6, full tonic-clonic seizures. Behavioral seizures above stage 3 indicate SE (Abdullah and Rafiqul Islam, 2012; Apland et al., 2014; Deshpante et al., 2014; Rossetti et al., 2012).

## Electrophysiology

Male rats, 25 to 30 days-old, were anesthetized with isoflurane prior to decapitation for preparation of brain slices. Coronal slices (400  $\mu\text{m}$  thick) containing the amygdala were cut as described previously (Aroniadou-Anderjaska et al., 2012). Recording solution (artificial cerebrospinal fluid, ACSF) consisted of the following (in mM): 125 NaCl, 2.5 KCl, 1.25  $\text{NaH}_2\text{PO}_4$ , 21  $\text{NaHCO}_3$ , 2  $\text{CaCl}_2$ , 1  $\text{MgCl}_2$ , and 11 D-glucose. The ACSF was saturated with 95%  $\text{O}_2$  / 5%  $\text{CO}_2$  to achieve a pH near 7.4, and the osmolarity was adjusted to 325 mOsm with D-glucose. The slice chamber (0.7 ml capacity) had continuously flowing ACSF ( $\sim 8$  ml/min) at a temperature of 31–32  $^\circ\text{C}$ . Tight-seal ( $>1$  G $\Omega$ ), whole-cell recordings from principal neurons in the BLA were performed as described previously (Pidoplichko et al., 2014). Neurons were visualized under infrared light using Nomarski optics of an upright microscope (Zeiss Axioskop 2, Thronwood, NY) through a 40x water immersion objective, equipped with a CCD-100 camera (Dage-MTI, Michigan City, IN). BLA principal cells were identified by their pyramidal shape and the presence of the hyperpolarization-activated cationic current ( $I_h$ ; Aroniadou-Anderjaska et al., 2012). Borosilicate glass patch electrodes had a resistance of 3.5–4.5 M $\Omega$  when filled with an internal solution of the following composition (in mM): 60  $\text{CsCH}_3\text{SO}_3$ , 60  $\text{KCH}_3\text{SO}_3$ , 10 EGTA, 10 HEPES, 5 KCl, 5 Mg-ATP, and 0.3  $\text{Na}_3\text{GTP}$ , pH 7.2, 290 mOsm. The 5 mM KCl allowed the simultaneous recordings of spontaneous inhibitory postsynaptic currents (sIPSCs) and excitatory postsynaptic currents (sEPSCs). Access resistance (15–24 M $\Omega$ ) was regularly monitored during recordings, and cells were rejected if the resistance changed by  $>15\%$  during the experiment. Ionic currents were amplified using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), and filtered (1 kHz) with a four-pole, low-pass Bessel filter; currents were digitally sampled (up to 2 kHz) using pClamp 10.2 software (Molecular Devices, Sunnyvale, CA), and



further analyzed using the Mini Analysis Program (Synptosoft, Fort Lee, NJ) and Origin (OriginLab, Northampton, MA). Drugs used for the *in vitro* experiments were paraoxon-ethyl, VU0255035, atropine sulfate, D-AP5 (N-methyl-D-aspartate receptor antagonist; Tocris Bioscience, Ellisville, MO), SCH50911 (GABA<sub>B</sub> receptor antagonist; Tocris Bioscience), and LY341495 (metabotropic glutamate group II/III receptor antagonist; Tocris Bioscience).

### Statistical Analysis

Seizure score differences between the OP-exposed and the vehicle groups were tested for statistical significance using independent-samples Student's *t*-test. *In vitro* electrophysiological results were analyzed using paired Student's *t*-tests. Differences were considered statistically significant when  $P < 0.05$ . Data are presented as Means  $\pm$  Standard Error of the Mean. Sample size "n" refers to the number of animals for the *in vivo* experiments and the number of recorded neurons for the whole-cell experiments.

### Results

#### Effects of VU0255035 pretreatment on seizure severity after exposure to soman

Eight rats were randomly divided into two groups: a group that was injected with VU0255035 (25 mg/kg, n = 4) and a group injected with the vehicle (DMSO; 1 ml/kg, n = 4), at 15 min before exposure to soman (198  $\mu$ g/kg). All rats developed seizures, and there was no statistically significant difference in seizure scores between the two groups at 5 min ( $0.75 \pm 0.75$  for the vehicle group and  $2.5 \pm 0.5$  for the VU0255035 group;  $p = 0.1$ ) or 10 min ( $3 \pm 0$  for the vehicle group and  $2.75 \pm 0.25$  for the VU0255035 group;  $p = 0.3$ ) after injection of soman. However, for the next 35 min, Racine scale scores for the VU0255035 group were significantly lower compared to the vehicle group (Fig. 1A). At 15 min post-soman injection, the seizure score

was  $5.25 \pm 0.75$  for the vehicle group and  $2.75 \pm 0.25$  for the VU0255035 group ( $p = 0.025$ ). At 20 min post-exposure, seizure scores were  $5.25 \pm 0.75$  for the vehicle group and  $3 \pm 0$  for the VU0255035 group ( $p = 0.025$ ). At 25 min, seizure scores were  $5.25 \pm 0.75$  for the vehicle group and  $2.75 \pm 0.25$  for the VU0255035 group ( $p = 0.020$ ). At 30 min, seizure scores were  $5.25 \pm 0.75$  for the vehicle group and  $2.5 \pm 0.3$  for the VU0255035 group ( $p = 0.015$ ). At 35 min after soman injection, seizure scores were  $5.25 \pm 0.75$  for the vehicle group and  $2.25 \pm 0.25$  for the VU0255035 group ( $p = 0.009$ ). At 40 min, seizure scores were  $5 \pm 1$  for the vehicle group and  $2.25 \pm 0.25$  for the VU0255035 group ( $p = 0.026$ ). At 45 min post-exposure, seizure scores were  $5 \pm 1$  for the vehicle group and  $2.25 \pm 0.25$  for the VU0255035 group ( $p = 0.026$ ). During the last 15 min of observation there were no significant differences in the Racine scale scores between vehicle and VU0255035 groups (Fig. 1A; at 50 min,  $5 \pm 1$  for the vehicle group and  $2.75 \pm 0.25$  for the VU0255035 group,  $p = 0.052$ ; at 55 min,  $5 \pm 1$  for the vehicle group and  $3 \pm 0.41$  for the VU0255035 group,  $p = 0.09$ ; and at 60 min,  $5 \pm 1$  for the vehicle group and  $3.25 \pm 0.25$  for the VU0255035 group,  $p = 0.10$ ). These results are summarized in Table 1.

### **Effects of VU0255035 pretreatment on seizure severity after exposure to paraoxon**

Next, we examined if pretreatment with VU0255035 also decreases seizure severity after exposure to paraoxon. Because behavioral seizure score did not differ between the VU0255035 group and the vehicle group during the first 10 min after soman exposure (Fig. 1A), with VU0255035 administered 15 min before soman injection, this time we administered VU0255035 30 min before paraoxon exposure, considering that perhaps 15 min were not sufficient for VU0255035 to take full effect. Eleven rats were randomly divided into two groups: a group that was injected with 25 mg/kg VU0255035 ( $n = 6$ ) and a group injected with the vehicle (DMSO; 1

ml/kg,  $n = 5$ ), at 30 min before exposure to paraoxon (4 mg/kg). All rats developed seizures. Again, the Racine score did not differ significantly between the two groups during the first 10 min after paraoxon injection (at 5 min,  $3.33 \pm 0.24$  for the vehicle group and  $3.4 \pm 0.21$  for the VU0255035 group,  $p = 0.8$ ; at 10 min,  $4.33 \pm 0.8$  for the vehicle group and  $3.8 \pm 0.33$  for the VU0255035 group,  $p = 0.5$ ). However, for the next 30 min, Racine scale scores for the VU0255035 group were significantly lower compared to the vehicle group (Fig. 1B). At 15 min post-paraoxon injection, the seizure score was  $4 \pm 0.37$  for the vehicle group and  $2.2 \pm 0.26$  for the VU0255035 group ( $p = 0.003$ ). At 20 min post-exposure, seizure scores were  $5 \pm 0.51$  for the vehicle group and  $2.4 \pm 0.37$  for the VU0255035 group ( $p = 0.002$ ). At 25 min, seizure scores were  $5.33 \pm 0.37$  for the vehicle group and  $2.2 \pm 0.33$  for the VU0255035 group ( $p = 0.0001$ ). At 30 min, seizure scores were  $4.67 \pm 0.51$  for the vehicle group and  $2.4 \pm 0.33$  for the VU0255035 group ( $p = 0.004$ ). At 35 min after paraoxon injection, seizure scores were  $4.83 \pm 0.58$  for the vehicle group and  $2.8 \pm 0.25$  for the VU0255035 group ( $p = 0.010$ ). At 40 min, seizure scores were  $4.33 \pm 0.51$  for the vehicle group and  $2.6 \pm 0.21$  for the VU0255035 group ( $p = 0.008$ ). During the last 20 min of observation, there were no significant differences in the Racine scale scores between vehicle and VU0255035 groups (Fig. 1B; at 45 min,  $4.67 \pm 0.51$  for the vehicle group and  $3.6 \pm 0.33$  for the VU0255035 group,  $p = 0.104$ ); at 50 min,  $4.83 \pm 0.2$  for the vehicle group and  $4.2 \pm 0.31$  for the VU0255035 group ( $p = 0.134$ ); at 55 min,  $5 \pm 0.24$  for the vehicle group and  $4.4 \pm 0.26$  for the VU0255035 group,  $p = 0.131$ ); and at 60 min,  $4.83 \pm 0.32$  for the vehicle group and  $5 \pm 0.17$  for the VU0255035 group,  $p = 0.635$ ). These results are summarized in Table 2.

## Effects of paraoxon on spontaneous synaptic activity in the BLA and the role of M<sub>1</sub> receptors

Whole-cell simultaneous recordings of sIPSCs and sEPSCs were obtained from principal BLA neurons, at  $V_h$  of -58 mV, in the presence of D-AP5 (50  $\mu$ M), SCH50911 (10  $\mu$ M), and LY341495 (3  $\mu$ M); under these conditions, outward currents are mediated by GABA<sub>A</sub> receptors, while inward currents are mediated by AMPA/kainate receptors (Pidoplichko et al., 2014). Bath application of paraoxon, at three different concentrations (0.1, 1, and 10  $\mu$ M) induced a transient barrage of large-amplitude sIPSCs with a duration of  $1.1 \pm 0.1$  min ( $n = 21$ ), which was followed by a lasting enhancement of preferentially sEPSCs (Fig. 2A). Paraoxon took effect (appearance of the barrage of sIPSCs) within 2 to 8 min after application, depending on the concentration (the lower concentration of paraoxon was slower to take effect). To quantify the relative increase of excitatory synaptic activity by paraoxon, we calculated the charge transferred by the excitatory and the inhibitory currents, during a 20 s time-window, in control conditions, and then again after paraoxon had taken effect; the charge transferred, in picoCoulombs, was calculated as the area delimited by the excitatory or inhibitory current and the baseline. The ratio of the charge transferred by sEPSCs to the charge transferred by sIPSCs was increased significantly in the presence of paraoxon. When 0.1  $\mu$ M paraoxon was applied, the sEPSC/sIPSC ratio was increased from  $0.6 \pm 0.1$  in control conditions to  $1.4 \pm 0.3$  at 17 min after paraoxon application ( $P < 0.05$ ,  $n = 7$ ). When 1  $\mu$ M paraoxon was applied, the sEPSC/sIPSC ratio was increased from  $0.6 \pm 0.1$  in control conditions to  $0.9 \pm 0.1$  at 9 min after paraoxon application ( $P < 0.05$ ,  $n = 4$ ). Finally, when 10  $\mu$ M paraoxon was applied, the ratio of sEPSC/sIPSC charge transfer was increased from  $0.8 \pm 0.1$  in control conditions to  $1.1 \pm 0.1$  at 6 min after paraoxon application ( $P < 0.01$ ,  $n = 10$ ; Fig. 2B).

To determine whether mAChRs were involved in mediating the effects of paraoxon, first, we pretreated the slices with atropine (1  $\mu$ M) for about 4 min, and then applied 10  $\mu$ M paraoxon, while recording from principal BLA neurons. The holding potential was again -58 mV, and D-AP5 (50  $\mu$ M), SCH50911 (10  $\mu$ M), and LY341495 (3  $\mu$ M) were present in the slice medium. Paraoxon, in the presence of atropine, had no significant effect on sIPSCs or sEPSCs (Fig. 3A, top traces). The total charge transferred by sIPSCs or sEPSCs during a 20 s time-window, 10 min after paraoxon application, was expressed as a percentage of the total charge transferred, during 20 s, in control conditions (in the presence of atropine). When 1  $\mu$ M atropine was present in the slice medium, the charge transferred by sIPSCs after 10 min in paraoxon was  $72 \pm 25\%$  of the control ( $n = 7$ ,  $P = 0.203$ ), while the charge transferred by sEPSCs was  $79 \pm 23\%$  of the control ( $n = 7$ ,  $P = 0.055$ ; Fig 3B, left bar graph). Next, we performed similar experiments in the presence of VU0255035, to determine whether the  $M_1$  receptor was involved in mediating the effects of paraoxon. In 10  $\mu$ M VU0255035, paraoxon had no significant effect on sIPSCs or sEPSCs (Fig. 3A, lower traces); the charge transferred by sIPSCs after 10 min in paraoxon was  $75 \pm 17\%$  of the VU0255035-containing control ( $n = 7$ ,  $P = 0.156$ ), while the charge transferred by sEPSCs was  $81 \pm 16\%$  of the VU0255035-containing control ( $n = 7$ ,  $P = 0.389$ ; Fig. 3B, middle bar graph). These results are in contrast to the effects of 10  $\mu$ M paraoxon in the absence of muscarinic receptor antagonists (Fig. 2A, third row of traces); under these conditions, the charge transferred by sIPSCs was increased by paraoxon to  $275 \pm 57\%$  of the control ( $n = 10$ ,  $P = 0.0001$ ), and the charge transferred by sEPSCs was increased by paraoxon to  $435 \pm 80\%$  of the control ( $n = 10$ ,  $P = 0.00002$ ; Fig. 3B, right bar graph). The difference between the increase in sIPSCs and the increase in sEPSCs was statistically significant ( $P < 0.001$ ).

### **Effects of paraoxon on the $I_h$ current**

Previous studies have shown that one of the mechanisms by which  $M_1$  receptor activation depolarizes pyramidal neurons in the hippocampus is the increase of the hyperpolarization-activated, mixed  $Na^+/K^+$  current ( $I_h$ ; Fisahn et al., 2002). The preferential increase of spontaneous glutamatergic activity by paraoxon, in the BLA, suggests that paraoxon may depolarize principal BLA neurons. To determine whether such an effect could be mediated via enhancement of the  $I_h$  current, in principal BLA neurons we evoked the  $I_h$  by voltage steps from a  $V_h$  of -70 mV to -110 mV, in 10 mV increments. Bath application of 10  $\mu$ M paraoxon had a consistent and clear enhancing effect on the  $I_h$  current ( $n = 4$ ; Fig. 4A). Addition of 10  $\mu$ M VU0255035 to the slice medium reversed the effect of paraoxon (Fig. 4B). Bath application of 10  $\mu$ M VU0255035 alone (in the absence of paraoxon) had no effect on the  $I_h$  (Fig. 4C). When 10  $\mu$ M paraoxon was added to the recording solution in the presence of 10  $\mu$ M VU0255035, there was no effect on the  $I_h$  (Fig. 4D). These results suggest that the enhancement of  $I_h$  by paraoxon was mediated by  $M_1$  receptor activation.

## Discussion

The present study demonstrated that pretreatment with the selective  $M_1$  receptor antagonist VU0255035 significantly suppresses behavioral seizures induced by either soman or paraoxon, for at least 40 min after exposure, preventing the development of SE during this time period. The *in vitro* experiments aiming to uncover some of the mechanisms involved in seizure generation by paraoxon and the involvement of  $M_1$  receptors, showed that paraoxon enhances both inhibitory and excitatory spontaneous activity recorded from BLA principal cells, with a lasting greater increase in sEPSCs, such that the ratio of the charge transferred by sEPSCs over that of sIPSCs is increased; these effects of paraoxon are blocked in the presence of VU0255035.

Lastly, we found that paraoxon increases the  $I_h$   $\text{Na}^+/\text{K}^+$  current in BLA principal cells, an effect that is also blocked by VU0255035. The depolarization that the increase of  $I_h$  can induce may be one of the mechanisms by which paraoxon increases spontaneous excitatory activity in the BLA.

Knowledge of the specific subtypes of mAChRs that are involved in the initiation of SE after exposure to an OP can lead to the development of pretreatments or early post-exposure treatments that do not have the side effects of non-selective mAChR antagonists. The role of  $M_1$  receptors, however, in seizure induction by OPs has been unclear. Previous studies have shown that pilocarpine, a muscarinic agonist, induces severe seizures in wild-type mice and in mice lacking the  $M_2$ ,  $M_3$ ,  $M_4$ , or  $M_5$  receptor (Bymaster et al., 2003), but not in mice lacking the  $M_1$  receptor (Bymaster et al., 2003; Hamilton et al., 1997). In consistency with these studies, pretreatment of wild-type mice with VU0255035 decreased the severity of seizures induced by pilocarpine; the effect was significant at 35 and 40 min after pilocarpine injection, resulting in seizure severity below the level of SE at least for the first 40 min post-injection (Sheffler et al., 2009). Since both pilocarpine and OPs are known to induce seizures by mAChR hyperstimulation, it is reasonable to suggest that  $M_1$  receptors may also be important in the induction of seizures by OPs, including nerve agents (Bhattacharjee et al., 2013). However, Kow et al. (2015) found no differences between wild-type and  $M_1$  knockout mice in seizure severity scores or proportion of animals displaying at least one tonic-clonic seizure after exposure to paraoxon; it was concluded that pilocarpine and paraoxon induce seizures by different mechanisms. The present study, however, in rats, shows that the  $M_1$  receptor plays a central role in the severity of seizures induced by paraoxon, at least during the first 40 min after the exposure. This discrepancy may imply species differences in the role of the  $M_1$  receptor in OP-induced seizures.

The data in the present study make it evident that during the first 10 min after OP injection, low intensity seizures were initiated despite the VU0255035 pretreatment. Behavioral seizures stage 2 to 3 and slightly higher  $\square$  which correspond to epileptic discharges on EEG (Abdullah and Rafiqul Islam, 2012)  $\square$  were observed during the first 10 min after soman or paraoxon injection in the VU0255035-treated rats as in the vehicle-treated rats, while the effects of VU0255035 pretreatment became significant after 10 min post-exposure. Pharmacokinetic studies have shown that VU0255035 has already reached high concentrations in the brain at 30 min after the injection (Sheffler et al., 2009). Therefore, the absence of a significant effect of the VU0255035 pretreatment during the first 10 min is probably not attributable to a delay of the drug reaching the brain. Other mAChR subtypes may be involved in the generation of seizure activity at this early phase, or an increase of glutamate may be the culprit, since extracellular glutamate has been found to increase in the amygdala within the first 10 min after exposure to soman (Lallement et al., 1991). During the next 30 min (after the initial 10 min period), the M<sub>1</sub> antagonist in the VU0255035-treated group prevented SE (stage 3 and higher), but seizures below stage 3 were ongoing; again, other mAChR subtypes and/or glutamatergic mechanisms may be involved in sustaining low-intensity seizures during this post-exposure period. After the 40 min post-exposure, the seizure-suppressing effects of VU0255035 pretreatment were no longer significant. Since VU0255035 concentrations in the brain remain relatively high for 2 h after injection (Sheffler et al., 2009), the reduction in the seizure-suppressing effect of VU0255035 after the 40 min period post-exposure may not be due to the clearance of the compound from the brain, but mainly reflect reduced significance of the M<sub>1</sub> receptors, as glutamatergic mechanisms take over the primary role in sustaining and reinforcing seizures (McDonough and Shih, 1997; Weissman and Raveh, 2008). Thus, M<sub>1</sub> receptors appear to be



important during a certain time period after OP exposure (after the first 10 min and before 1 h). This implies that if the effects of an M<sub>1</sub> antagonist or the consequences of knocking out the M<sub>1</sub> receptor (in mice) on seizure induction by pilocarpine or OPs are averaged over a period of time, instead of being evaluated every 5 min or during the 10 to 40 min post-exposure, a significant role of the receptor may be missed.

The *in vitro* experiments were performed in the BLA because, in addition to the importance of other brain regions in seizure generation by OPs (Myhrer, 2007; Myhrer et al., 2007, 2010), there is strong evidence to suggest that the BLA plays a key role (Aroniadou-Anderjaska et al., 2009). Thus, when McDonough et al. (1987) microinjected nerve agents into different brain regions, they observed that convulsions were elicited only when the nerve agent was injected into the BLA. Recently, Prager et al. (2013) found that in the small proportion of rats who do not develop SE when exposed to high doses of soman, the activity of acetylcholinesterase is reduced in the hippocampus and the piriform cortex to a similar extent as in the rats who develop SE, but to a significantly lesser extent in the BLA; this suggests a critical role of acetylcholinesterase reduction in the BLA for SE to be induced. The importance of the amygdala in nerve agent-induced seizure generation is also highlighted by the findings that the amygdala displays the earliest increase in extracellular glutamate after exposure to soman (Lallement et al., 1991), and generates ictal-like discharges after *in vitro* application of soman (Apland et al., 2009). It is to be expected, therefore, that when the BLA is exposed to an OP, neuronal excitability will increase, which will be reflected in an increase of spontaneous glutamatergic activity. Indeed, in the present study, paraoxon increased sEPSCs in the BLA significantly more than the increase of sIPSCs.

The *in vitro* effects of paraoxon were blocked in the presence of VU0255035, suggesting that both the increase in sIPSCs and that in sEPSCs were mediated via M<sub>1</sub> receptor activation. The cellular location of these receptors where paraoxon acted remains to be determined. Cholinergic synapses in the BLA are formed by the dense cholinergic afferents arising from the basal forebrain (Ben-Ari et al., 1977; Carlsen and Heimer, 1986; Muller et al., 2011; Nagai et al., 1982), but also by an intrinsic population of small cholinergic neurons (Carlsen and Heimer, 1986). Cholinergic nerve terminals synapse on both pyramidal cells and interneurons (Carlsen and Heimer, 1986; Muller et al., 2011; Nitecka and Frotscher, 1989), but the majority of the cholinergic input is directed onto pyramidal neurons (Muller et al., 2011). From the different types of mAChRs, both the M<sub>1</sub> and M<sub>2</sub> subtypes are present in the rat BLA (Cortes and Palacios, 1986; Mash and Potter, 1986; Mash et al., 1988; Spencer et al., 1986), as well as the M<sub>3</sub> and/or M<sub>4</sub> receptors (Smith et al., 1991), but the M<sub>1</sub> receptors predominate (Buckley et al., 1988). Immunocytochemistry along with electron microscopy have revealed that M<sub>1</sub> receptors in the rat BLA are located on somata, dendritic shafts, and, to a lesser extent, spines of pyramidal neurons, as well as on aspiny dendrites with morphological features typical of interneurons (Muller et al., 2013). In addition, M<sub>1</sub> immunoreactivity has been found on presynaptic terminals, most of which formed asymmetrical synapses with dendritic spines, but some of which formed symmetrical synapses with neuronal somata and dendritic shafts (Muller et al., 2013). It appears, therefore, that M<sub>1</sub> receptors in the rat BLA are in a position to affect the activity of both glutamatergic and GABAergic neurons, by postsynaptic or presynaptic mechanisms. The increase in GABAergic inhibitory activity by paraoxon could be due to M<sub>1</sub> receptor-mediated direct depolarization of GABAergic interneurons, facilitation of GABA release from GABAergic presynaptic terminals, or depolarization of glutamatergic neurons or terminals synapsing onto inhibitory interneurons;

presynaptic facilitation of GABA release may be the least likely of the three possibilities, as M<sub>1</sub> receptor activation inhibits GABA release in the rat lateral amygdala (Sugita et al., 1991). The increase in sEPSCs by paraoxon could be the result of M<sub>1</sub> receptor-mediated depolarization of BLA principal neurons via enhancement of the *I<sub>h</sub>* current, as shown previously in CA3 pyramidal neurons in the hippocampus (Fisahn et al., 2002), and in BLA principal neurons in the present study. In addition, M<sub>1</sub> receptors on glutamatergic terminals may facilitate glutamate release; this remains to be demonstrated in the BLA, but in granule cells of the hippocampus, the frequency and amplitude of sEPSCs are increased by application of paraoxon via a presynaptic mechanism, and this effect is blocked by pre-application of atropine (Kozhemyakin et al., 2010).

Up to 30 mg/kg of VU0255035 have been used in mice to block M<sub>1</sub> receptors, and no effect was observed on learning as determined by contextual fear conditioning (Sheffler et al., 2009). In M<sub>1</sub> knock-out mice, Miyakawa et al. (2001) found evidence of hyperactivity under stressful conditions and no significant cognitive impairments in the Morris water maze and in contextual fear conditioning, while Anagnostaras et al. (2003) found enhancements, deficits, or no effect in different types of memory. Taken together with the results presented here, it is suggested that selective M<sub>1</sub> antagonists may be an efficacious pretreatment in situations where there is risk for exposure to OPs, as the antagonist may produce only minimal or no significant side effects on cognitive function, while, upon exposure, it will delay the development of SE long enough for medical assistance to arrive.

### **Author Contributions**

Conceived research: Braga, Miller, Aroniadou-Anderjaska, Apland

Participated in research design: Braga, Miller, Aroniadou-Anderjaska, Apland

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Conducted experiments: Miller, Pidoplichko, Figueiredo, Aplan, Krishnan

Performed data analysis: Miller, Pidoplichko, Figueiredo

Drafted manuscript: Aroniadou-Anderjaska

Edited and revised manuscript: Aroniadou-Anderjaska, Aplan, Braga

Approved final manuscript: Miller, Pidoplichko, Figueiredo, Aplan, Krishnan, Aroniadou-Anderjaska, Braga

Other: Braga, Aroniadou-Anderjaska, Aplan acquired funding for the research.

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## Footnotes

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## Figure Legends

**Fig. 1.** Pretreatment with the selective  $M_1$  receptor antagonist VU0255035 reduces seizure severity after exposure to soman or paraoxon. **(A)** Administration of VU0255035 (25 mg/kg), 15 min before exposure to soman ( $1.8 \times LD_{50}$ ), significantly reduced seizure severity scores from 15 min to 45 min after soman injection ( $n = 4$  in each of the two groups;  $*p < 0.05$ ,  $**p < 0.01$ ). **(B)** Administration of VU0255035 (25 mg/kg), 30 min before exposure to paraoxon (4 mg/kg), significantly reduced seizure severity scores from 15 min to 40 min after paraoxon injection ( $n = 6$  in the VU0255035 group and  $n = 5$  in the vehicle group;  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ).

**Fig. 2.** Paraoxon enhances both spontaneous inhibitory currents (sIPSCs) and spontaneous excitatory currents (sEPSCs), with a significantly greater, lasting effect on the sEPSCs, in the BLA. Whole cell simultaneous recordings of sIPSCs and sEPSCs were obtained from BLA principal cells ( $V_h = -58$ ). **(A)** Representative examples of the effects of paraoxon at 0.1  $\mu$ M, 1  $\mu$ M, and 10  $\mu$ M (traces are from 3 different neurons). Outward currents (upward deflections) are GABAergic and inward currents (downward deflections) are glutamatergic. The number of min between successive traces in a row is the time lapse from the time point paraoxon was applied, and then the interval between the two successive recordings. **(B)** Group data of the effects of paraoxon on the ratio of the charge transferred by sEPSCs over the charge transferred by sIPSCs. The charge transferred was calculated during a 20 s time-window, in control conditions and after bath application of paraoxon. Left bar graph: at 17 min after application of 0.1  $\mu$ M paraoxon,  $n = 7$ . Middle bar graph: at 9 min after application of 1  $\mu$ M paraoxon,  $n = 4$ . Right bar graph: at 6 min after application of 10  $\mu$ M paraoxon,  $n = 10$ .  $*p < 0.05$ ,  $**p < 0.01$ .



**Fig. 3.** M<sub>1</sub> muscarinic receptors mediate the effects of paraoxon on sIPSCs and sEPSCs, in the BLA. Whole cell simultaneous recordings of sIPSCs and sEPSCs were obtained from BLA principal cells ( $V_h = -58$ ). **(A)** Representative traces showing that 10  $\mu$ M paraoxon had no significant effect when applied in the presence of atropine (1  $\mu$ M, top row of traces) or VU0255035 (10  $\mu$ M, second row of traces). **(B)** Effects of 10  $\mu$ M paraoxon on sIPSCs and sEPSCs under three conditions: pretreatment with atropine (left bar graph;  $n = 7$ ), pretreatment with VU0255035 (middle bar graph;  $n = 7$ ), and in the absence of muscarinic receptor antagonists (right bar graph;  $n = 10$ ). The total charge transferred by sIPSCs or sEPSCs during a 20 s time-window, 10 min after paraoxon application, was expressed as a percentage of the total charge transferred, during 20 s, in control conditions. Only when there was no atropine or VU0255035 in the slice medium, the total charge transferred by sIPSCs and sEPSCs was increased by paraoxon, and the difference between the increase in sIPSCs and the increase in sEPSCs was statistically significant ( $***P < 0.001$ ).

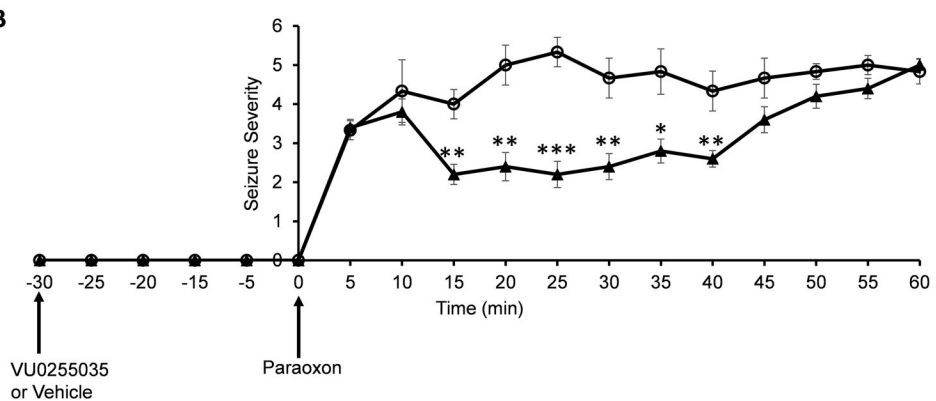
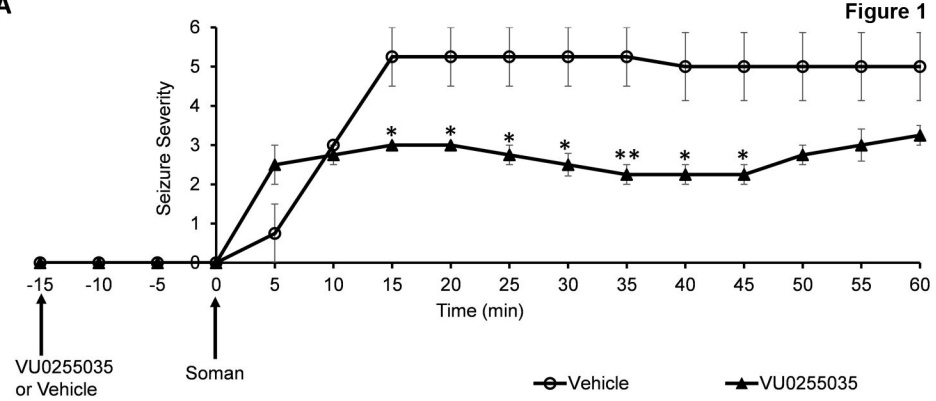
**Fig. 4.** Paraoxon enhances the hyperpolarization-activated cation current,  $I_h$ , in principal BLA neurons, via M<sub>1</sub> muscarinic receptor activation. **(A)** Bath application of 10  $\mu$ M paraoxon increased the  $I_h$  current elicited by hyperpolarizing steps (at 10 mV increments) from a  $V_h$  of -70 mV. The recordings shown are 3 min after application of paraoxon. **(B)** Addition of 10  $\mu$ M VU0255035 to the slice medium reversed the effects of paraoxon. **A** and **B** are from the same neuron. **(C)** Application of 10  $\mu$ M VU0255035 alone had no effect on the  $I_h$ . **(D)** Bath application of 10  $\mu$ M paraoxon in the presence of 10  $\mu$ M VU0255035 had no effect on the  $I_h$ . **C** and **D** are from the same neuron (different from **A** and **B**).

**Table 1: Seizure severity after exposure to soman, in VU0255035-pretreated compared to vehicle-pretreated rats.**

Time after soman injection (min)	Behavioral seizure score (vehicle group)	Behavioral seizure score (VU0255035 group)	<i>p</i> Value
5	0.75 ± 0.75	2.5 ± 0.5	0.1
10	3 ± 0	2.75 ± 0.25	0.3
15	5.25 ± 0.75	2.75 ± 0.25	0.025*
20	5.25 ± 0.75	3 ± 0	0.025*
25	5.25 ± 0.75	2.75 ± 0.25	0.02*
30	5.25 ± 0.75	2.5 ± 0.3	0.015*
35	5.25 ± 0.75	2.25 ± 0.25	0.009**
40	5 ± 1	2.25 ± 0.25	0.026*
45	5 ± 1	2.25 ± 0.25	0.026*
50	5 ± 1	2.75 ± 0.25	0.052
55	5 ± 1	3 ± 0.41	0.09
60	5 ± 1	3.25 ± 0.25	0.1

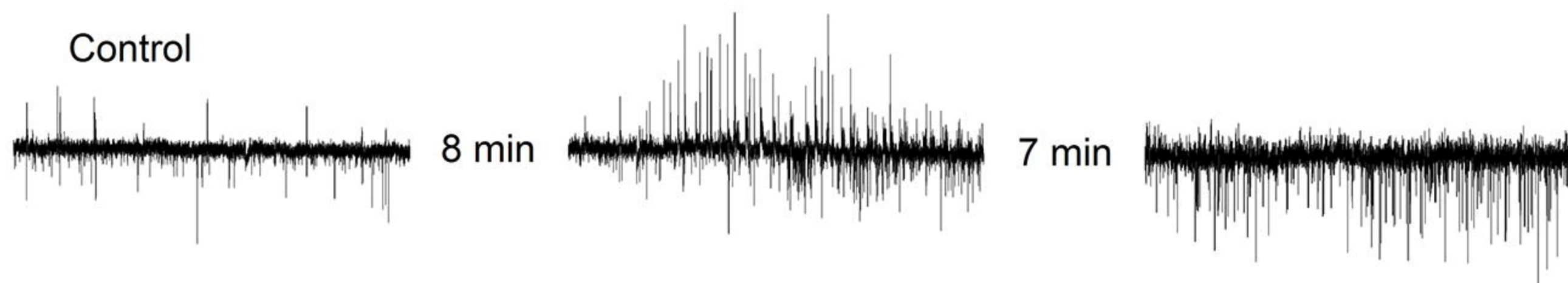
**Table 2: Seizure severity after exposure to paraoxon, in VU0255035-pretreated compared to vehicle-pretreated rats.**

Time after paraoxon injection (min)	Behavioral seizure score (vehicle group)	Behavioral seizure score (VU0255035 group)	<i>p</i> Value
5	3.33 ± 0.24	3.4 ± 0.21	0.8
10	4.33 ± 0.8	3.8 ± 0.33	0.5
15	4 ± 0.37	2.2 ± 0.26	0.003**
20	5 ± 0.51	2.4 ± 0.37	0.002**
25	5.33 ± 0.37	2.2 ± 0.33	0.0001***
30	4.67 ± 0.51	2.4 ± 0.33	0.004**
35	4.83 ± 0.58	2.8 ± 0.25	0.01*
40	4.33 ± 0.51	2.6 ± 0.21	0.008**
45	4.67 ± 0.51	3.6 ± 0.33	0.104
50	4.83 ± 0.2	4.2 ± 0.31	0.134
55	5 ± 0.24	4.4 ± 0.26	0.131
60	4.83 ± 0.32	5 ± 0.17	0.635

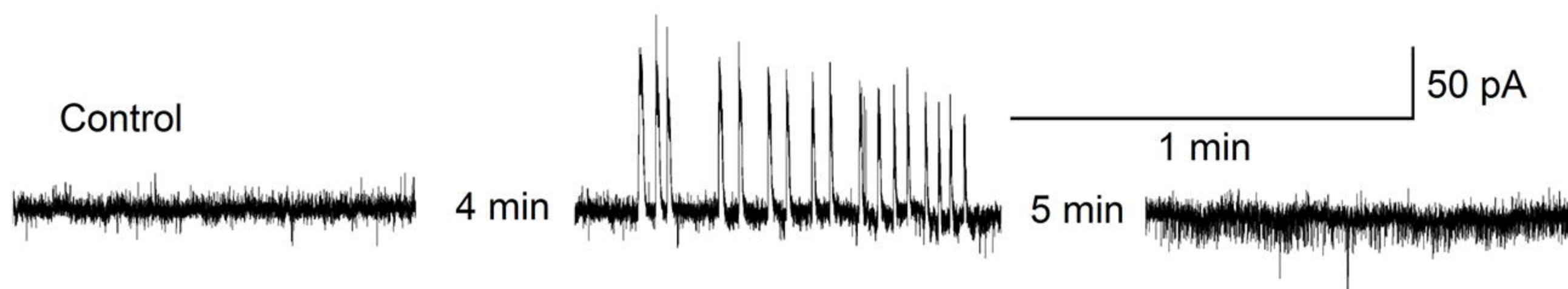
**Figure 1**

A

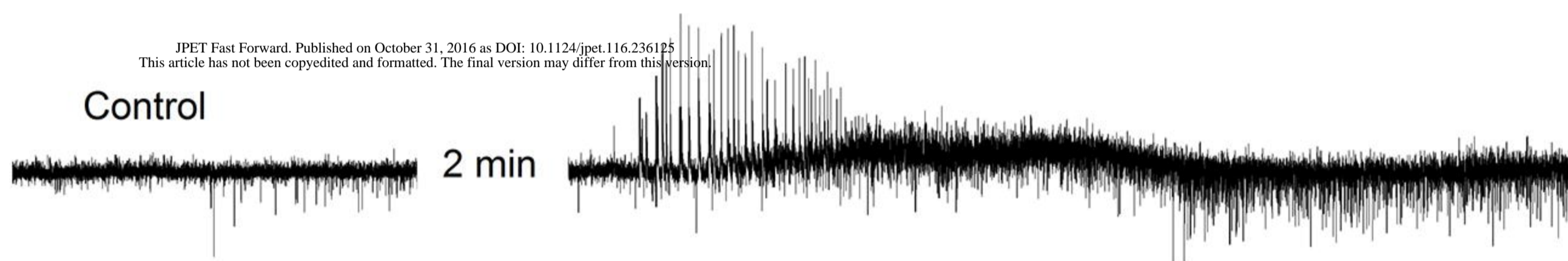
Paraoxon 0.1  $\mu$ M



Paraoxon 1  $\mu$ M



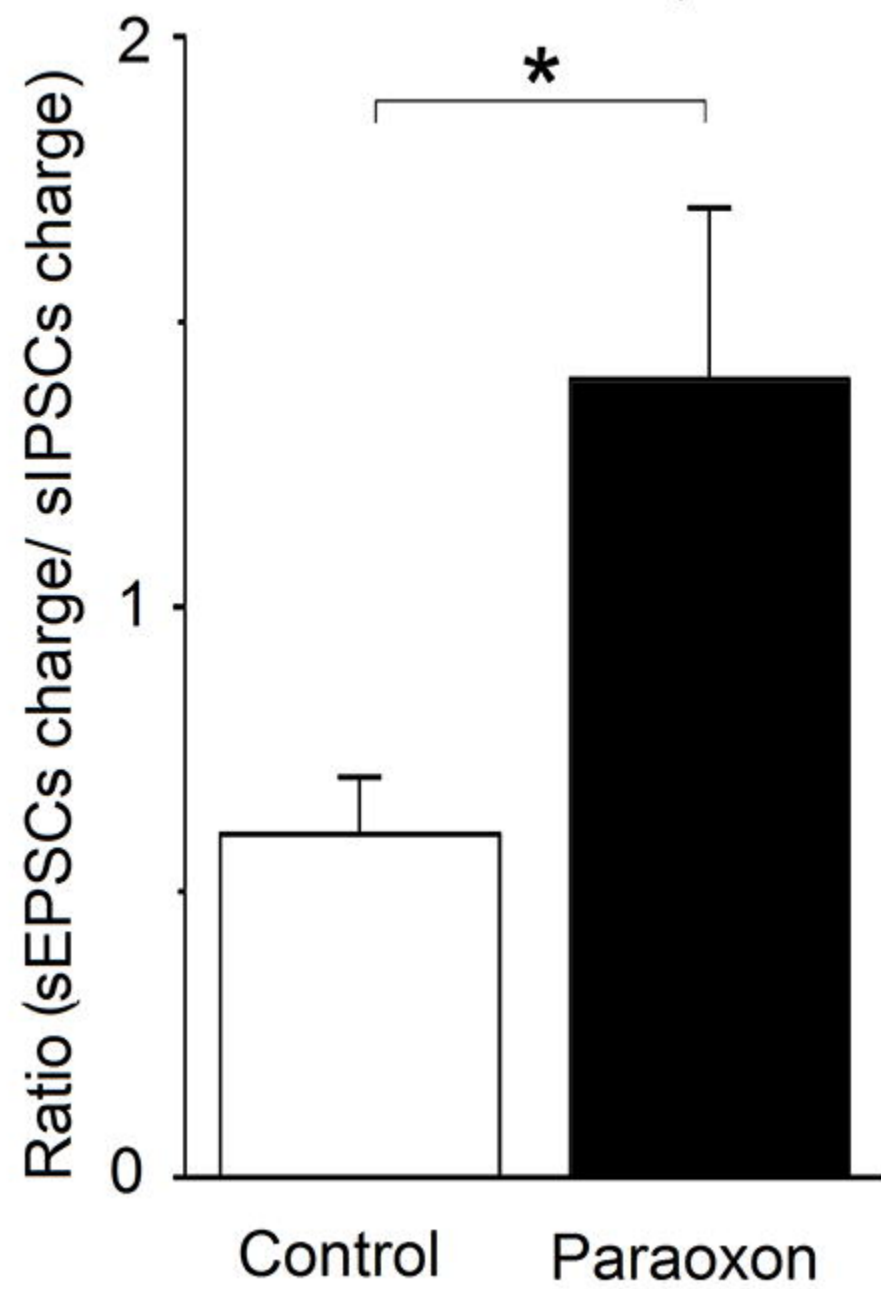
Paraoxon 10  $\mu$ M



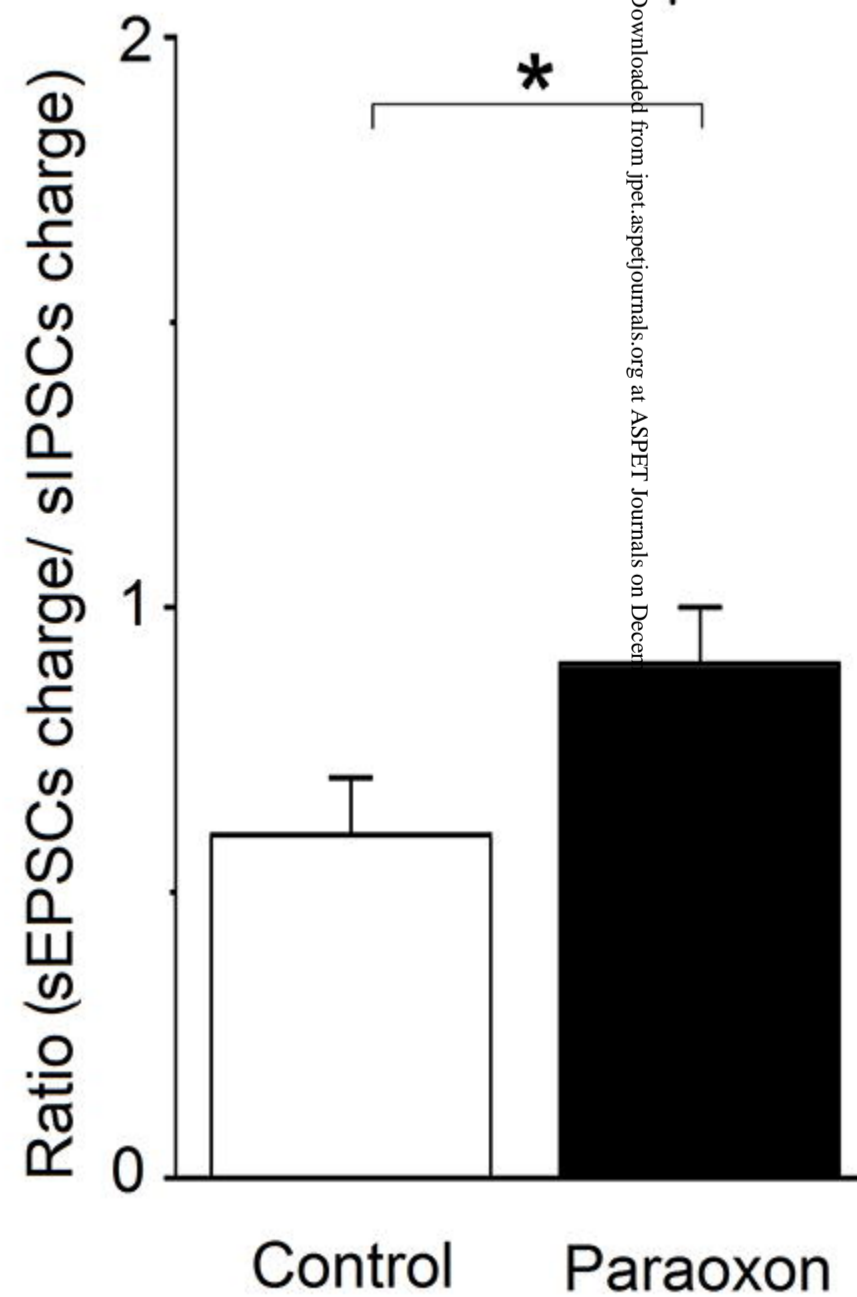
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B

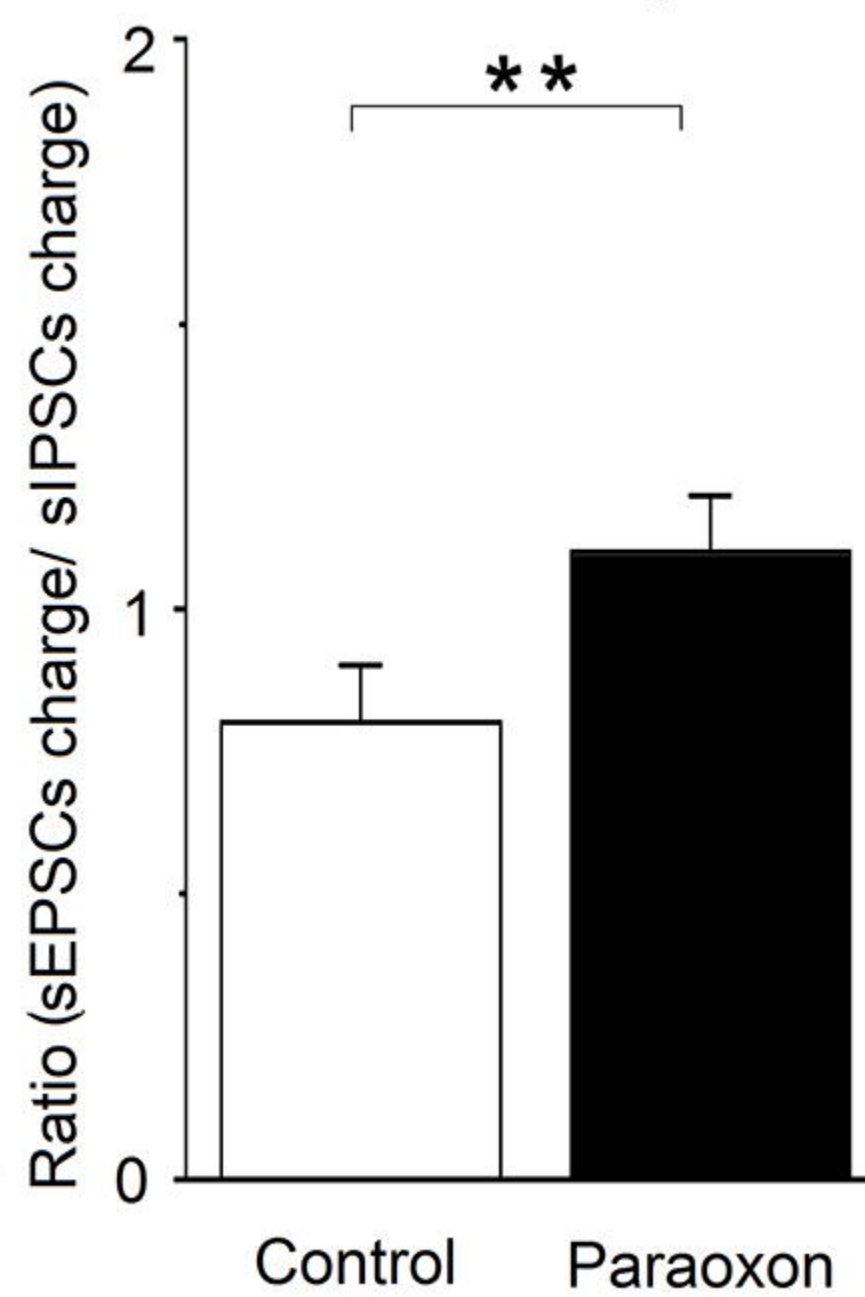
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Paraoxon 1  $\mu$ M

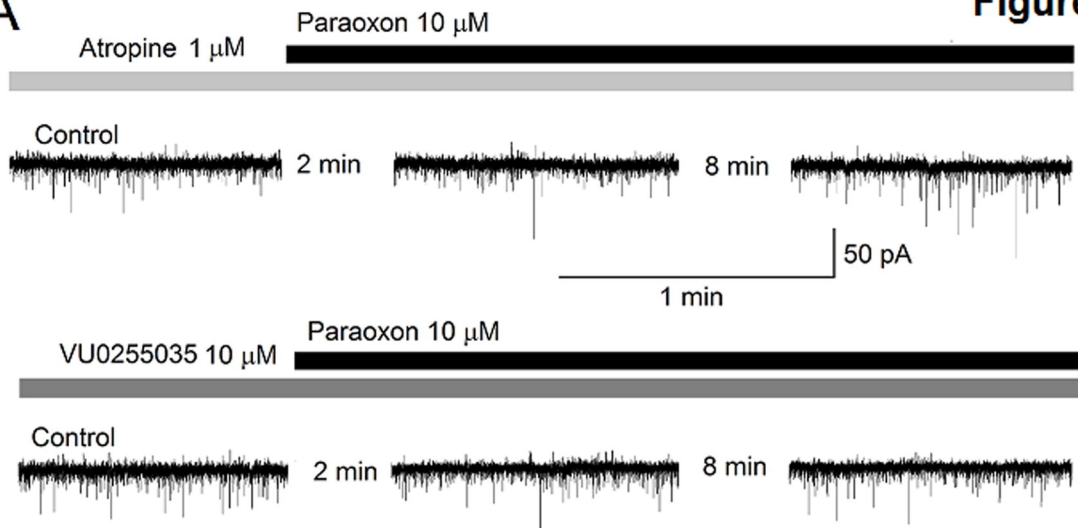


Paraoxon 10  $\mu$ M

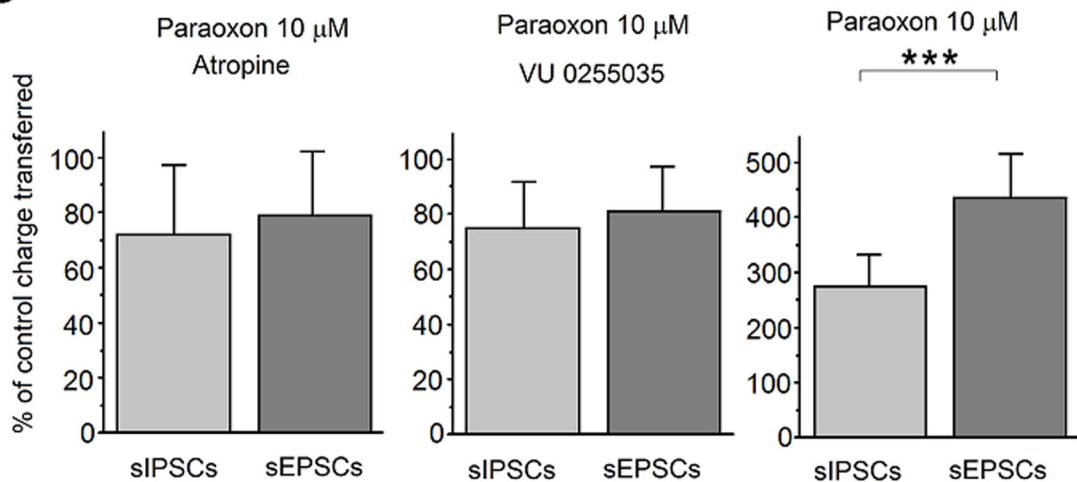


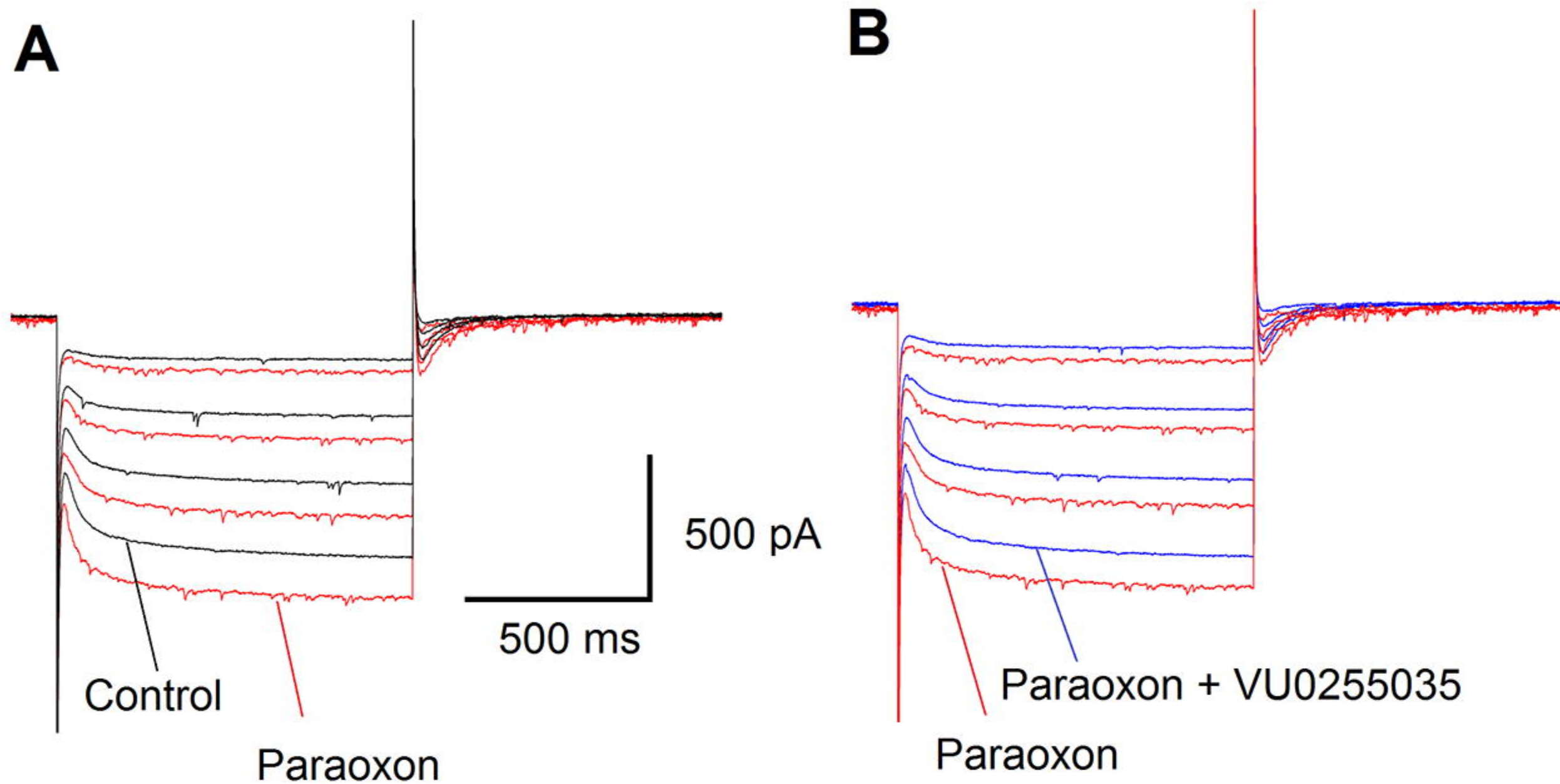
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