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MPMT-OX up-regulates GABAergic transmission and protects against seizure-like behavior in *Caenorhabditis elegans*

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ABSTRACT

The signal transmission in the nervous system operates through a sensitive balance between excitatory (E) inputs and inhibitory (I) responses. Imbalances in this system contribute to the development of pathologies such as seizures. In *Caenorhabditis elegans*, the locomotor circuit operates via the coordinated activity of cholinergic excitatory (E) and GABAergic inhibitory (I) transmission. Changes in E/I inputs can cause uncontrolled electrical discharges, mimicking the physiology of seizures. Molecules derived from 1,3,4-oxadiazole have been found to exhibit diverse biological activities, including anticonvulsant effect. In this work, we study the activity of the compound 2-[(4-methoxyphenylselenyl)methylthio]-5-phenyl-1,3,4-oxadiazole (MPMT-OX) in the GABAergic and cholinergic systems. We demonstrate that MPMT-OX reduced the locomotor activity of *C. elegans* with a normal balance between the E/I systems and increased the resistance to paralysis in worms exposed to pentylenetetrazol and aldicarb. MPMT-OX increased seizure resistance and assisted in the recovery of locomotor activity in worms with deletions in the genes *unc-46*, which regulates the transport of GABA into vesicles, and *unc-49*, which encodes the $GABA_A$ receptor. *C. elegans* with deletions in the *unc-25* and *unc-47* genes did not respond to treatment. Therefore, we suggest that the compound MPMT-OX upregulates GABAergic signaling in a manner dependent on the *unc-25* gene, which is responsible for GABA synthesis, and *unc-47*, which encodes the vesicular GABA transporter.

1. Introduction

The nervous system works based on the subtle balance of excitatory and inhibitory signals (Mccormick and Contreras, 2001). Several neurotransmitters are involved in this balance, such as γ-aminobutyric acid (GABA). GABA is the primary inhibitory neurotransmitter in the mammalian brain (Mody et al., 1994). Chlorine (Cl^-) influx through $GABA_A$ receptors results in neuronal hyperpolarization and inhibition. This response depends on the maintenance of a relatively low intracellular Cl^- concentration, resulting in a Cl^- reversal potential more negative than the cell's resting membrane potential (Farrant and Kaila, 2007). Acetylcholine (ACh) is a fast-acting, point-to-point neurotransmitter at the neuromuscular junction (NMJ) and in the autonomic ganglia. However, in the mammalian central nervous system, ACh acts by modulating neuronal excitability, altering the presynaptic release of neurotransmitters and coordinating the firing of groups of neurons (Rice and Cragg, 2004; Kawai et al., 2007; Zhang et al., 2007). The imbalance between excitatory (E) and inhibitory (I) signaling pathways is essential for seizures' occurrence. Seizures can be described as paroxysmal hypersynchronous transient electrical discharges in the brain that result from too much excitation or too little inhibition in the area in which the abnormal discharge starts (Scharfman, 2007; Berg et al., 2010).

Despite its simple nervous system, *Caenorhabditis elegans* has important conserved features of neuronal function at the level of ion channels, axon guidance cues, receptors, transporters, synaptic components, and neurotransmitters, i.e., ACh, dopamine, GABA, and serotonin (Bargmann, 1998). Previous studies have established *C. elegans* as a simple model for the study of seizure-like behavior (Williams et al., 2004; Risley et al., 2016). In adult worms, cholinergic motor neurons excite body wall muscles as well as GABAergic neurons that synapse onto opposing body wall muscles. Thus, when ACh excites and

Abbreviations: GABA, γ-aminobutyric acid; WT, wild-type; ACh, acetylcholine; PTZ, pentylenetetrazol; VGAT, vesicular GABA transporter; GAD, glutamic acid decarboxylase; AChE, acetylcholinesterase; NGM, nematode growth medium; GFP, green fluorescent protein; NMJ, neuromuscular junction; MPMT-OX, 2-[(4-methoxyphenylselenyl)methylthio]-5-phenyl-1,3,4-oxadiazole

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contracts one set of muscles, GABA is released onto the opposing muscles to inhibit and relax them (White et al., 1976), allowing adults to coordinate body bending (Mclntire et al., 1993a,b; Schuske et al., 2004). Due to the sensitivity of this nematode to changes in this E/I balance, some studies have used pentylenetetrazol (PTZ) to produce seizures in *C. elegans* (Williams et al., 2004). PTZ is a selective blocker of the $GABA_A$ receptor, which decreases the seizure threshold by altering the muscles' E/I input ratio (Huang et al., 2001). However, detecting the effects of PTZ requires seizure-sensitive strains, such as *unc-25* (Jin et al., 1999), *unc-47* (White et al., 1986), or *unc-49* (Schwartz, 1988) deletion mutants. Recently, it has been demonstrated that anticonvulsant drugs (retigabine, sodium valproate, and levetiracetam) promote the recovery of *unc-25* and *unc-49* mutants after induction of seizures (Risley et al., 2016). This response confirms the suitability of the experimental model *C. elegans* for the study of new molecules with pharmacological potential to modulate the GABAergic system and the diseases related to imbalance in this system.

In this sense, oxadiazole-derived molecules have attracted attention due to their neuroprotective activity and low toxicity in mammalian models (Liu et al., 2001; Jiang et al., 2015). Oxadiazole is a five-membered heterocyclic ring consisting of two carbons, two nitrogens, one oxygen, and two double bonds, with a general formula of $C_2H_2ON_2$. Several molecules derived from 1,2,3-oxadiazole have shown anticonvulsant activity in maximal electroshock seizure models (MES), subcutaneous pentylenetetrazol models (scPTZ), and subcutaneous strychnine models (scSTY), by positively modulating GABAergic signaling in mammals (Zarghi et al., 2005; Rajak et al., 2010; Mashayekh et al., 2014).

Considering the above information, the aim of this study was to investigate the effects of a new molecule, 2-[(4-methoxyphenylselenyl) methylthio]-5-phenyl-1,3,4-oxadiazole (MPMT-OX), on GABAergic signaling and seizure-like behavior induced by PTZ in *C. elegans*. In this study, we report that the MPMT-OX compound could acts to modulate the genes of the GABAergic pathway, resulting in an increase in inhibitory signaling and protection against seizure-like behavior.

2. Material and methods

2.1. Chemicals and reagents

Aldicarb, dimethyl sulfoxide (DMSO), muscimol, PTZ, bovine serum albumin (BSA), 5,5-dithiobis (2-nitrobenzoic acid) (DTNB), sodium azide, and acetylthiocholine iodide (ASChI) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The MPMT-OX compound (Fig. 1) was synthesized as described elsewhere (Sauer et al., 2016), and chemical purity (78%) was assessed by hydrogen and carbon nuclear magnetic resonance and gas chromatography.

2.2. C. elegans strains and their maintenance

The strains used in this study were obtained from the *C. elegans* Genetics Center (University of Minnesota, Minneapolis) and were maintained on nematode agar growth medium (NGM) plates seeded with *OP50 Escherichia coli* at 20 °C under standard conditions (Brenner, 1974). The *C. elegans* Bristol strain N2 was used as the control wild-type; the other strains are described in Table 1.

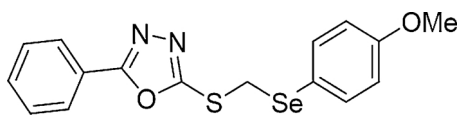

Fig. 1. Chemical structure of the 2-[(4-methoxyphenylselenyl)methylthio]-5-phenyl-1,3,4-oxadiazole (MPMT-OX).

2.3. Treatment and survival test

NGM agar plates for treatment were seeded with *E. coli OP50* and incubated overnight at 37 °C to allow bacterial growth. MPMT-OX was added to the agar surface at final concentrations of 0, 5, 15, and 50 μM. DMSO was added to control plates, and the final concentration never exceeded 0.5%.

Gravid adult hermaphrodite worms (WT or mutants) were lysated by bleach solution (1% NaOCl, 0.25 M NaOH), and the eggs obtained hatched after 16 h in M9 buffer to obtain all worms in stage L1 (Sulston and Hodgkin, 1988). The population of L1 larval-stage worms was transferred to NGM plates with DMSO or MPMT-OX and cultured until the 1-day adult stage, about 46 h. After exposure, WT and *unc-47* worms were analyzed for lethality. All other tests were performed after this period of exposure to the test compound.

2.4. PTZ-induced paralysis assay

Approximately 20 WT or *unc-30* worms were transferred from the treatment plates to new NGM plates seeded with *E. coli* and containing a final concentration of 5 mg PTZ/mL of agar, and the worms were assayed for paralysis every 15 min for 120 min. Paralysis was defined as the absence of movement when the worm was prodded three times with a platinum wire on the head and tail, and when pharyngeal pumping was absent (Calahorro and Ruiz-Rubio, 2013).

2.5. Aldicarb-induced paralysis assay

The resistance of WT worms to aldicarb was performed as described elsewhere (Nurrish et al., 1999) with minor modifications. Approximately 20 worms were transferred to NGM plates seeded with *E. coli* and containing 1 mM aldicarb and assayed for paralysis every 30 min for 2.5 h. Paralysis was defined by the absence of movement when prodded three times with a platinum wire on the head and tail.

2.6. Acetylcholinesterase (AChE) activity assay

AChE activity was measured in WT and mutant worms using the colorimetric method (Ellman et al., 1961) with adaptations (Cole et al., 2004). After exposure to treatments, 8000–10,000 adult worms were washed three times in M9 buffer and transferred to Eppendorf tubes. The samples were frozen and thawed three times using dry ice and sonicated 5 × 15 s at 30% amplitude with 10 s breaks on the ice and centrifuged for 30 min at 15,000 × *g* at 4 °C. The resulting supernatant was used to measure AChE activity. The proportions of reagents were adjusted for ELISA plates, where 40 μL of sample were mixed with 200 μL of 0.25 mM DTNB and 10 μL of 156 mM ASChI, and the plates were incubated at 30 °C for 5 min. The rate of change in absorbance was measured at 405 nm at 60 s intervals for 5 min in a spectrophotometer. Protein content was determined as described previously (Bradford, 1976).

2.7. Muscimol assay

Muscimol assays were performed as described previously (Dabbish and Raizen, 2011). Twenty worms were transferred to NGM plates seeded with *E. coli* containing 1 mM muscimol. After 1 h of exposure, worms were scored for the following categories: category 0: the worm moved away rapidly from the mechanical stimulus; category 1: the worm briefly contracted and relaxed and then moved away from the stimulus; category 2: the worm contracted and then relaxed while at the same time moving slightly away from the stimulus; category 3: the worm contracted and then relaxed but showed no moving away; category 4: the worm contracted incompletely and then relaxed and showed no moving away; and category 5: the worm showed flaccid paralysis and did not respond at all to the mechanical stimulus.

Table 1
List of the *C. elegans* strains.

Strain	Genotype	Transgene
CZ333	juIs1 [*unc-25p*::snb-1::GFP + lin-15(+)] IV	GFP expression in presynaptic terminals of GABAergic DD and VD motor neurons and RME neurons.
CB156	*unc-25* (e156) III	*unc-25* gene encodes the biosynthetic enzyme glutamic acid decarboxylase (GAD).
CB 307	*unc-47* (e307) III	*unc-47* (e307) III, *unc-47gene* encodes the vesicular GABA transporter VGAT.
BC 277	*unc-46* (e177) *dpy-11* (e224) V	*unc-46* gene regulates the transport of GABA into carrier vesicles.
CB 407	*unc-49* (e407) III	*unc-49* gene encodes the $GABA_A$ receptor that mediates body muscle inhibition during locomotion.
EW 49	*unc-30* (e191) IV; deIs12	*unc-30* gene regulates the expression of *unc-25* and *unc-47* in 19 D-type neurons.
RM2710	*snf-11*(ok156) V	snf-11 gene encodes plasma membrane transporter required for GABA re-uptake from the synaptic cleft.
CB 193	*unc-29(e193) I*	*unc-29* gene encodes non-a subunits of the nicotinic acetylcholine receptor (nAChR).

2.8. Seizure-like behavior assay

Seizure-like behavior assays were performed as previously described (Williams et al., 2004) with minor modification. About 20–30 mutant worms (*unc-25*, *unc-46*, *unc-47*, and *unc-49*) were transferred from the treatment plates to new NGM plates with *E. coli* OP50 and 5 mg PTZ/mL of agar and observed every 10 min for 1 h. Worms were scored positive for seizure-like behavior if they demonstrated contractions in the whole body or head-bobbing, posterior immobilization, and lack of pharyngeal pumping, an observation indicative of disrupted GABA neurotransmission (Williams et al., 2004).

2.9. Locomotion behavior assay

WT or mutant worms were transferred from the treatment plates to new food-free NGM plates and allowed to move freely. After 1 min of adaptation, worms were scored for the number of body bends in 1 min. A body bend was defined as a change in the direction of propagation of the part of the worm corresponding to the posterior bulb of the pharynx along the *y*-axis, assuming the worm was traveling along the *x*-axis (Tsalik and Hobert, 2003). The worms' locomotor capacity was evaluated in two situations: after exposure to MPMT-OX, and 1 h after the end of induction of seizure-like behavior with PTZ. After exposure to PTZ, the worms were transferred to new NGM plates containing only *E. coli* and recovered for 1 h. This time period was stipulated for the re-establishment of basic behaviors: body bends, pharyngeal pumping, and defecation.

2.10. Distribution of synaptic vesicles at the pre-synaptic terminals in GABAergic neurons

Neuronal trafficking of GABA transporter vesicles (VGAT) was measured in *juIs1 [unc-25p::snb-1::GFP + lin-15(+)] IV* transgenic worms. SNB-1::GFP worms were analyzed after exposure to MPMT-OX or DMSO and after 1 h of recovery from exposure to 5 mg PTZ/mL. The nematodes were washed from the exposure plates with M9 and placed on slides containing 3 mM sodium azide. The images were obtained under a confocal microscope (Olympus® FLUOVIEW FV101) at 60× magnification. Green fluorescent protein (GFP) is expressed in the presynaptic terminals of GABAergic DD and VD motor neurons as discrete fluorescent puncta. Gaps were defined as GFP failures of GABAergic motor neurons.

2.11. Digital videos of convulsions

Worms were exposed to 5 mg PTZ/mL of agar for 15 min and then examined under a stereomicroscope (OLYMPUS® CX21). We chose this time because approximately 50% of the worms presented convulsion-like behavior, as shown in Fig. 6. Seizures were recorded for 20 s using a digital video camera at 29 frames/s. The captured videos were saved using the ISCapture program.

2.12. Statistical analysis

Statistical analysis was performed using GraphPad (Version 6.0 for Macintosh OSX, GraphPad Software, San Diego, CA, USA). Significance was assessed by Student's *t*-test (for two groups). For more than two groups, we used one-way analysis of variance (ANOVA) or two-way ANOVA, followed by a suitable post hoc test. All assays were repeated at least three times, and $p < 0.05$ was considered statistically significant. We measured the mean fluorescence of each animal's puncta through the ImageJ program and transformed the values into the percentage of control group, which we submitted to the statistical tests in GraphPad.

3. Results

3.1. MPMT-OX changes locomotor activity and reduces paralysis

We first evaluated whether MPMT-OX caused toxicity in WT or deletion mutant worms (*unc-47*). In this assay, both strains showed similar tolerance to 5, 15, and 50 μM MPMT-OX during exposure of 46 h, without increasing mortality in relation to the DMSO control (data not shown). Fig. 2 shows the effect of MPMT-OX on locomotor activity in *C. elegans*. WT worms exposed to MPMT-OX showed a 23.2% and 20.8% decrease in the number of body bends at 15 and 50 μM, respectively, compared to the control group (Fig. 2A).

A decrease in locomotor activity has been related to a decrease in excitatory signaling (cholinergic) or an increase in inhibitory signaling (GABAergic) (Dabbish and Raizen, 2011; Stawicki et al., 2011). To determine if treatment with MPMT-OX altered the E/I ratio at the NMJ, we exposed the WT worms to PTZ. Fig. 2B shows that treatment with 15 μM MPMT-OX reduced the percentage of PTZ-paralyzed worms by 20% starting at 30 min, and that 50 μM reduced the number of paralyzed worms by approximately 20.5% only at 45 and 60 min of PTZ exposure.

3.2. MPMT-OX slows aldicarb-induced paralysis without acting on the cholinergic system

To investigate possible alterations in cholinergic signaling, worms treated with MPMT-OX were tested for resistance to paralysis induced by aldicarb. The rate at which worms become paralyzed reflects the balance of excitatory cholinergic and inhibitory GABAergic inputs to the muscle (Vashlishan et al., 2008). Treatment with 5 μM of MPMT-OX reduced the number of WT worms paralyzed after 90 min of exposure to aldicarb, while 15 μM of MPMT-OX reduced the number of WT worms paralyzed after 60 min compared to the control (Fig. 3A). The concentration of 15 μM was chosen for further analysis.

Resistance to aldicarb may be associated with a reduction in ACh levels at the neuromuscular synapse (Locke et al., 2006; Mahoney et al., 2006). To investigate this possibility, we measured AChE activity in WT and GABA deletion mutants *unc-25*, *unc-47*, *unc-49*, *unc-46*, and *unc-30*. WT and mutant worms showed no significant differences in AChE activity in response to treatment with 15 μM MPMT-OX compared to the

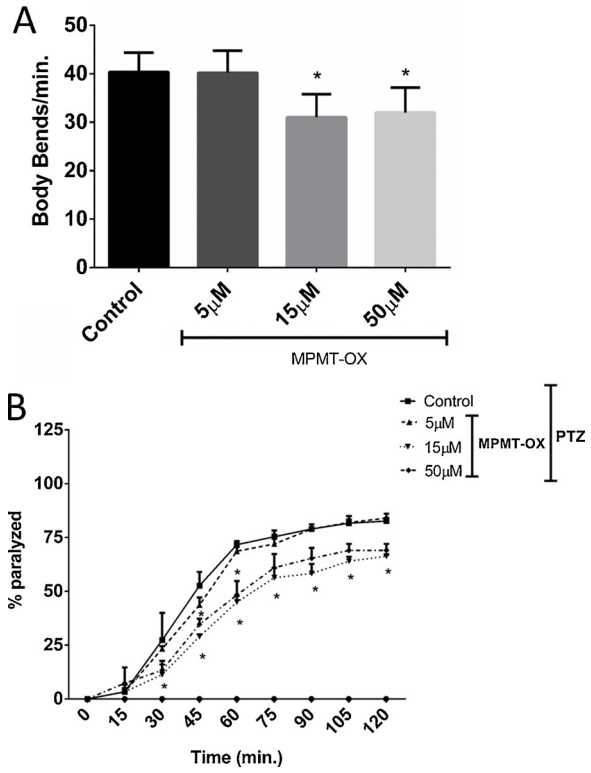

Fig. 2. Effect of MPMT-OX on locomotor activity in WT *C. elegans*. (A) Data are expressed as number of body bends from three independent assays with 10 worms in each group ($n = 30$). * indicates $p < 0.05$ compared to the control group by one-way ANOVA followed by the Tukey multiple comparison test. (B) PTZ-induced paralysis. Worms were placed on plates containing 5 mg/mL PTZ and assayed for body bends every 15 min for 2 h. Data represent the percentage of paralyzed animals from three independent assays with 20 worms in each group ($n = 60$). * indicates $p < 0.05$, 15 and 50 μM MPMT-OX versus control, by two-way ANOVA followed by the Tukey multiple comparison test. Data are represented as means ± SEM.

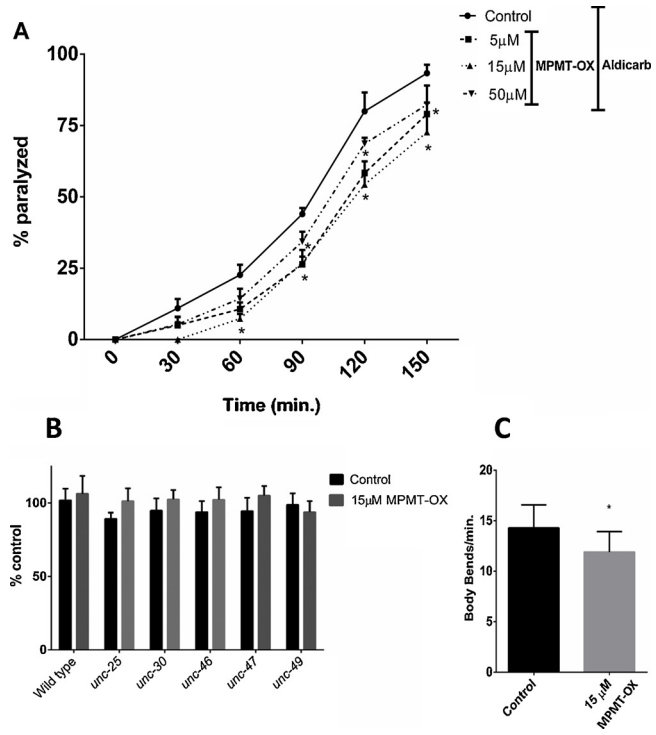

Fig. 3. Effect of MPMT-OX on cholinergic signaling. (A) Paralysis was induced with 1 mM aldicarb in WT. Locomotion was evaluated every 30 min for 150 min. Data represent the percentage of paralyzed animals from three independent assays with 20 worms in each group ($n = 60$). * indicates $p < 0.05$, 15 μM and 50 μM MPMT-OX versus control, with two-way ANOVA followed by the Tukey multiple comparison test. (B) AChE activity in WT and transgenic worms. Data are expressed as the percentage of control of each strain from four independent assays, $n = 4$. (C) Effect of MPMT-OX on locomotor activity of *C. elegans unc-29* knockout. Data are expressed as number of body bends performed by the worms from four independent assays with 10 worms in each group ($n = 40$). * indicates $p < 0.05$ by Student's *t*-test. Data are represented as means ± SEM.

control (Fig. 3B). To determine the participation of the ACh receptor in the effect of MPMT-OX, we evaluated the locomotor activity of worms with the deletion of the *unc-29* gene, responsible for encoding non-a subunits of nicotinic acetylcholine receptor (nAChR) (Fleming et al., 1997). *unc-29* deletion mutants exposed to MPMT-OX shows a reduced number of body bends of per minute compared to the control (Fig. 3C).

3.3. MPMT-OX acts on inhibitory signaling and alters the degree of relaxation of body muscle

We exposed WT worms to muscimol, which acts as a $GABA_A$-receptor agonist. *C. elegans* placed on agar containing GABA agonists showed a variety of responses that reflected the degree of body muscle relaxation (De La Cruz et al., 2003). Fig. 4A shows that MPMT-OX exposure reduced the number of worms with a normal response to the stimulus to 12.4%, (category 0: the worm moved away rapidly from the mechanical stimulus) compared to control worms. In contrast, the treatment caused a 17% increase in the number of worms in category 4 (the worm contracted incompletely and then relaxed and showed no moving away) and a 12% increase in category 5 (the worm showed flaccid paralysis and did not respond to the mechanical stimulus). To determine the effect of MPMT-OX on *C. elegans* with high levels of GABA in the neuromuscular synapse, we evaluated the locomotor activity of *snf-11* worms (Mullen et al., 2006). Treatment with MPMT-OX reduced the number of body bends per minute of *snf-11* worms by 15% compared to control (Fig. 4B).

3.4. MPMT-OX modulates locomotor activity of worms with deficiency in GABAergic signaling

The *C. elegans* mutant strains *unc-25*, *unc-30*, *unc-46*, *unc-47*, and *unc-49* have reduced GABA inhibitory activity, which compromises these animals' locomotor capacity, since body wall muscles operate on a greater proportion of excitatory inputs (Schuske et al., 2004, 2007). We found that the *C. elegans* GABA mutants *unc-25*, *unc-30*, *unc-46*, *unc-47*, and *unc-49* respectively showed decreased locomotion by 23%, 19%, 31%, 32%, and 45% compared to WT worms (Fig. 5A). This indicates that a reduction in inhibitory signaling contributes to a reduction in locomotion rate because the E/I balance is critical to the normal nematode locomotion rate. Treatment with MPMT-OX increased the number of body bends in *unc-30* (10.46%), *unc-46* (16.75%), and *unc-49* (15.6%) worms compared to the control group of each strain. *unc-25* and *unc-47* worms showed no significant change in their body bend rate compared to controls (Fig. 5B).

3.5. MPMT-OX increases GABAergic signaling and protects against seizure-like behavior

We tested whether MPMT-OX was able to alter susceptibility to PTZ-induced seizures in *C. elegans* GABAergic mutants (*unc-25*, *unc-46*, *unc-47*, and *unc-49*). Using digital video imaging, we captured the phenotypic effects of chemically induced seizures in *C. elegans* with deletion of *un-47* gene (supplementary material: video 1A, WT control (normal behavior); video 1B, *unc-47* (control behavior), and 1C, *unc-47*

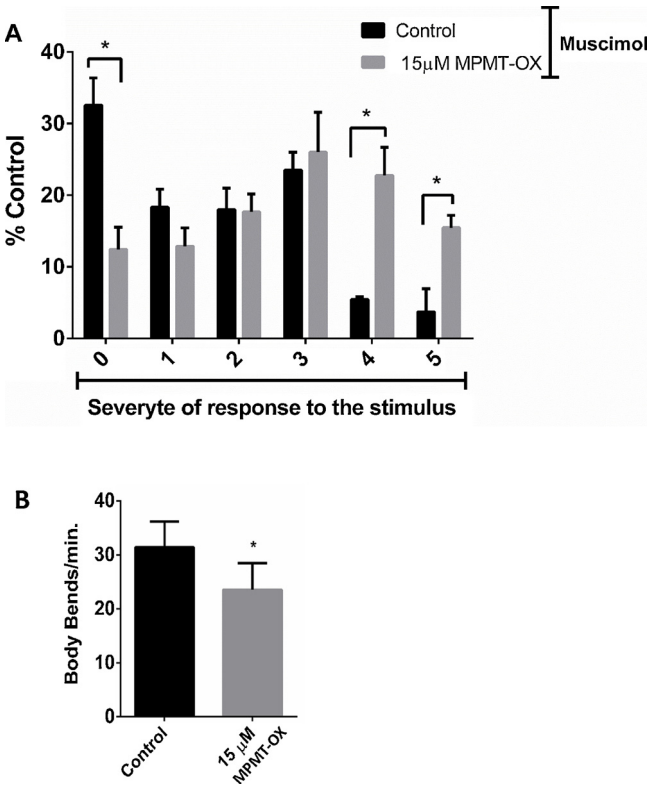

Fig. 4. Effect of MPMT-OX on GABAergic signaling. (A) Treatment increases WT worms' sensitivity to $GABA_A$ receptor agonists. Data are expressed as the fraction of worms that displayed each of five phenotypes after a 1-h exposure to 0.5 mM of muscimol, from three independent assays with 20 worms in each group ($n = 60$) (see Section 2 for full description of phenotypes). * indicates $p < 0.05$ by Student's *t*-test. (B) Data are expressed as number of body bends performed by the *snf-11* worms, from three independent assays with 10 worms in each group ($n = 30$). * indicates $p < 0.05$ by Student's *t*-test. Data are represented as means ± SEM.

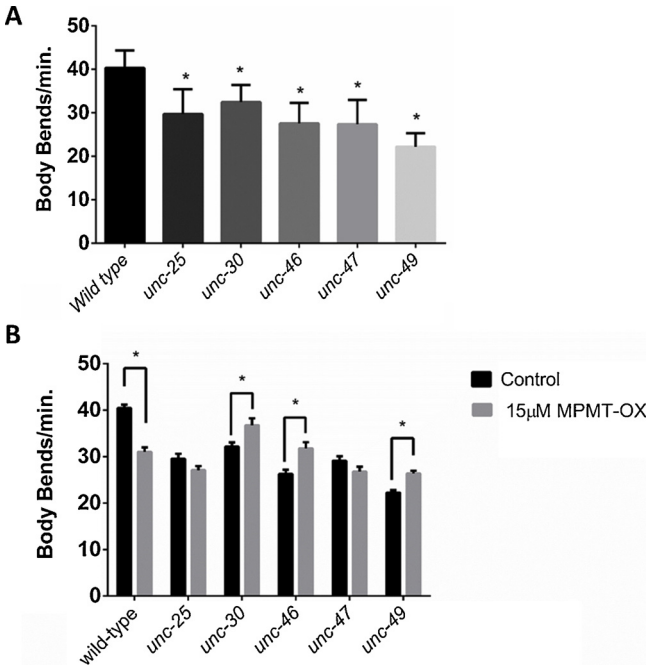

Fig. 5. Effect of MPMT-OX on locomotor activity in *C. elegans* mutants with reduced GABAergic signaling. (A) All mutant worms (*unc-25*, *unc-30*, *unc-46*, *unc-47*, and *unc-49*) showed a reduction in locomotor activity when compared to the wild-type strain. * indicates $p < 0.05$ with one-way ANOVA followed by the Tukey multiple comparison test. (B) Number of body bends of the WT and mutant worms after exposure to MPMT-OX. * indicates $p < 0.05$ by Student's *t*-test. (A, B) Data are expressed as mean body bends/min performed by the worms, from three independent assays with 10–15 worms in each group ($30 \leq n \leq 45$). Data are represented as means ± SEM.

(convulsion-like behavior)). Only animals with posterior paralysis, the absence of pharyngeal pumping and defecation, and head-bobbing movements or spasms all over the body were scored positive for seizure.

Treatment with MPMT-OX did not affect the susceptibility of *unc-25* (Fig. 6A) and *unc-47* worms (Fig. 6C) to PTZ-induced seizures. The number of *unc-46* worms treated with MPMT-OX that displayed seizure-like behavior was 25% lower than in the control group following 30 min of PTZ exposure (Fig. 6B). The number of treated *unc-49* worms that showed convulsion-like behavior was approximately 20% lower than that of control worms after 20 min of exposure to PTZ (Fig. 6D). *unc-30* worms did not convulse in the presence of PTZ, and therefore, we tested whether treatment with MPMT-OX could affect the susceptibility of these worms to PTZ-induced paralysis. Fig. 6E shows that the treatment reduced the number of paralyzed *unc-30* worms by 20–30% after 45 min of PTZ exposure compared to control.

3.6. MPMT-OX facilitates recovery of GABAergic signaling

Previous work has shown a reduction in GABA levels after seizures in mammals (Bradford, 1995; Treiman, 2001). In the present study, we observed that after one hour of PTZ exposure, worms were not able to coordinate body bends due to the drastic imbalance in E/I signaling.

The recovery of locomotor activity is indicative of the reestablishment of inhibitory signaling. Therefore, after 1 h of recovery, we evaluated the locomotion rate of these animals (Fig. 7A). All not treated worms of mutant strains *unc-25*, *unc-30* (Jin et al., 1994), *unc-46* (Schuske et al., 2007), *unc-47*, and *unc-49* showed a significant reduction in the number of body bends after exposure to PTZ. The unc-30, *unc-46*, and *unc-49* worms treated with MPMT-OX showed a significant increase in the number of body bends after the recovery interval. Again, the *unc-25* and *unc-47* mutant strains did not respond to treatment with MPMT-OX.

3.7. MPMT-OX acts pre-synaptically altering traffic of GABAergic vesicles

Fig. 8 (and supplementary material Fig. S1) shows the density of synapses in the GABAergic nerve cords in SNB-1::GFP worms treated with MPMT-OX, before and after exposure to PTZ. SNB-1::GFP worms have a WT phenotype, and exposure to PTZ caused a paralysis rate similar to that of WT worms (data not shown).

Control worms showed a small number of gaps in the GABAergic motor neurons (12.5%) (Fig. 8A (I) and B); approximate values have been previously reported (Williams et al., 2004). Worms treated with MPMT-OX showed similar numbers of gaps compared to control (Fig. 8A (II) and B); however, the fluorescence of SNB-1::GFP puncta increased significantly compared to the control (Fig. 8A (II) and C). Exposure only to PTZ yielded a 16% increase in the number of gaps (Fig. 8A (III) and B) and a reduction in the fluorescence of GFP puncta compared to control (Fig. 8A (III) and C). Worms that were treated with MPMT-OX before PTZ exposure showed higher fluorescence of GFP puncta (Fig. 8A (IV) and C) and reduction in the number of gaps compared to the worms not treated exposed to PTZ (Fig. 8A (IV) and B).

4. Discussion

In the present study, we investigated the effect of a new molecule derived from 1,3,4-oxadiazole on neurotransmission in *C. elegans*. The data presented here are in line with previous studies demonstrating the anticonvulsant activity of other 1,3,4-oxadiazole derivatives (Zarghi et al., 2008; Rajak et al., 2010; Siddiqui et al., 2014). We also observed

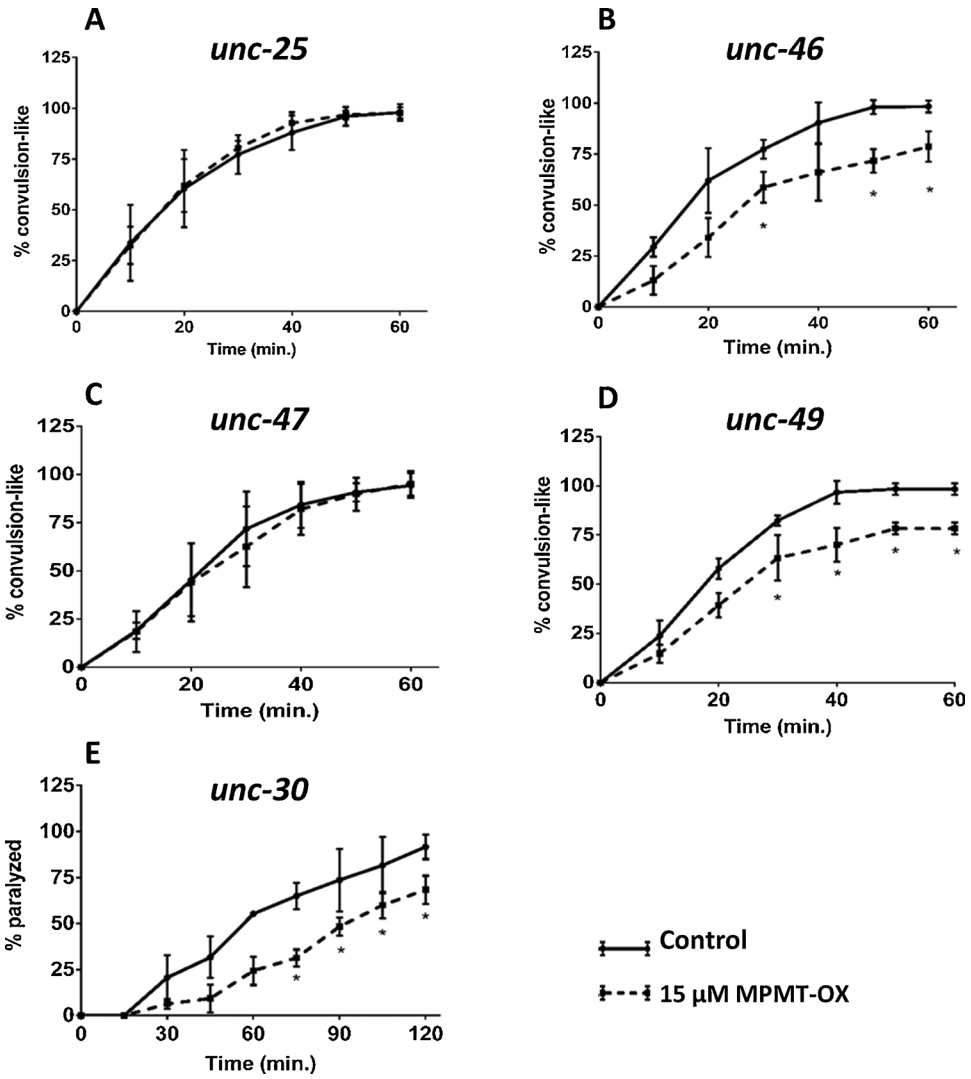

Fig. 6. MPMT-OX increases threshold for seizure-like behavior. (A–D) Data are expressed as the percentage of worms with seizure-like behavior after being exposed to 5 mg/mL PTZ for 1 h. (A) *unc-25*, four independent assays with 24 worms in each group ($n = 96$). (B) *unc-46*, three independent assays with 30 worms in each group ($n = 90$), $^*p < 0.05$ with two-way ANOVA followed by Bonferroni's multiple comparison test. (C) *unc-47*, four independent assays with 28 worms in each group ($n = 112$). (D) *unc-49*, three independent assays with 20 worms in each group ($n = 60$), $^*p < 0.05$ with two-way ANOVA followed by Bonferroni's multiple comparison test. (E) *unc-30*, paralysis was induced with 5 mg/mL PTZ. Mobility was evaluated every 15 min for 2 h. Data represent the mean percentage of paralyzed *unc-30* worms, from three independent assays with 23 worms in each group ($n = 70$). $^*p < 0.05$ with two-way ANOVA followed by Bonferroni's multiple comparison test. Data are represented as means ± SEM.

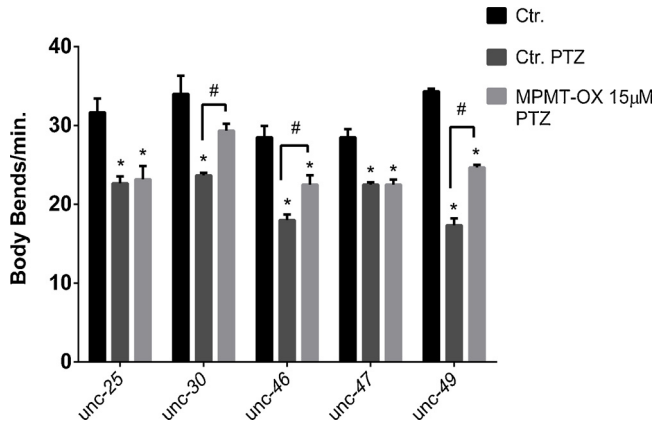

Fig. 7. MPMT-OX assists in locomotor recovery after seizure-like behavior. Data are expressed as number of body-bends/min of Ctr. (control worms before seizure), Ctr. PTZ (control after seizure), and MPMT-OX 15 µM PTZ (worms treated after seizure). The worms recovered for 1 hour after seizure induced by 5 mg/mL PTZ. Locomotion was evaluated in three independent assays with 10–15 worms in each group ($30 \leq n \leq 45$). * indicates $p < 0.05$ compared to Ctr., $\#p < 0.05$ versus Ctr. PTZ with two-way ANOVA followed by Bonferroni's multiple comparison test. Data are represented as means ± SEM.

that MPMT-OX showed no toxicity to the worms during the exposure period. This corroborates recent work which shows that oxadiazole derivatives did not cause toxicity in rodents at doses that exhibit anticonvulsant activity (Siddiqui et al., 2008; Bhat et al., 2010; Jain et al., 2011).

Genetic studies have shown that the neurotransmitters and ion channels that mediate synaptic transmission in mammals are highly conserved in *C. elegans* (Bargmann and Kaplan, 1998; Leung et al., 2008). In this regard, the NMJ of this nematode is a genetic model system appealing to the more complex and often inaccessible polynerved synapses of vertebrate central neurons.

C. elegans' locomotor activity is directly related to ACh and GABA neurotransmitters, and changes in their balance may reflect alterations in the worm's locomotor ability (Jospin et al., 2009). WT worms treated with MPMT-OX showed decreased locomotor activity. To determine if this behavioral change was related to imbalances in E/I signaling, we exposed MPMT-OX-treated worms to PTZ. The induction of paralysis with PTZ in WT worms is controversial, as previous work has reported that WT worms show normal behavior when exposed to up to 20 mg/mL PTZ (Williams et al., 2004). However, other studies have reported reduced locomotor activity and paralysis in WT worms (Calahorro and Ruiz-Rubio, 2013). We found that 5 mg/mL PTZ induced a high paralysis rate in WT worms and that MPMT-OX at concentrations of 15 and 50 µM MPMT-OX reduced these rates. These data may suggest that MPMT-OX increases inhibitory signaling, since it decreases the number

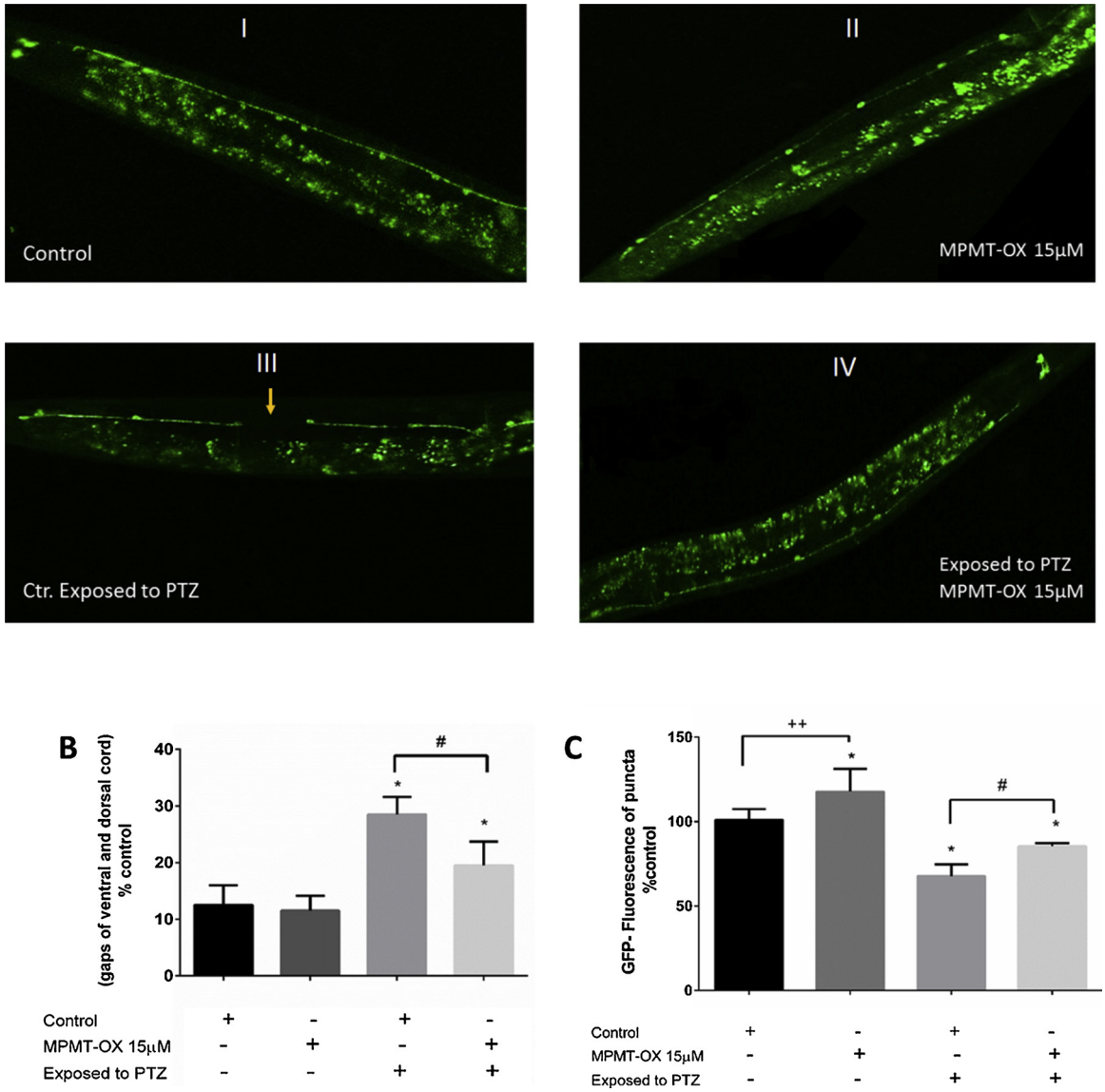

Fig. 8. Traffic of GABAergic vesicles before and after exposure to PTZ: (A) GABAergic presynaptic terminals are visualized in live adult animals with *Punc-25*-SNB-1::GFP (juIs1), and all images were obtained from ventral and dorsal cords before and after PTZ exposure. Arrows indicate gaps in nerve cord. (B) Quantification of gaps in GFP of the GABAergic nerve cords. (C) Quantification of SNB-1::GFP fluorescent puncta from images obtained from three independent assays with 10 animals in each group ($n = 30$). * indicates $p < 0.05$ compared to the Control group, #$p < 0.05$, MPMT-OX 15 μM-Exposed to PTZ group versus Control-Exposed to PTZ group, + +$p < 0.05$, MPMT-OX 15 μM group versus Control group, with two-way ANOVA followed by the Tukey multiple comparison test. Data are represented as means ± SEM. For more images, see Supplemental Information Fig. S1.

of body bends of worms with normal levels of ACh/GABA (Fig. 2A) and increases resistance to paralysis in worms with an inhibited GABA response (Fig. 2B).

The aldicarb assay has been previously used to assess alterations in cholinergic synaptic transmission at the NMJ (Mahoney et al., 2006). Aldicarb inhibits AChE, causing the accumulation of ACh in the synaptic cleft. This leads to increased excitatory input and eventually to hypercontractive paralysis. Worms treated with MPMT-OX were more resistant to aldicarb-induced paralysis (Fig. 3A). This can be attributed to changes in ACh levels at the neuromuscular synapse. We measured AChE activity in worms exposed to MPMT-OX, since the ability of some 1,3,4-oxadiazole derivatives to inhibit AChE activity has been previously described (Khan et al., 2013; Kamal et al., 2014). Our results indicate that MPMT-OX did not affect AChE activity in WT worms or GABA mutants (Fig. 3B).

The *unc-29* gene encodes a non-alpha subunit of the nicotinic ACh receptor (nAChR), which mediates fast actions of ACh at the NMJ and in the nervous system (Fleming et al., 1997). Worms with deletion of this subunit have a deficiency in cholinergic signaling, which is reflected in low locomotor activity compared to WT worms (compare Figs. 2A and 3C, reduction of 38%). The additional reduction in the locomotor activity of treated *unc-29* worms indicated a greater imbalance between (E) and (I), which tends to increase inhibitory signaling.

To investigate whether MPMT-OX may upregulate GABA, we exposed the WT worms to muscimol, a restricted conformational analog of GABA. Muscimol produced an inward current in the body wall muscle with a reversal potential of +20 mV, similar to the GABA response (Richmond and Jorgensen, 1999). The “elastic” phenotype, in which the animals contract and relax without displacement, is the more severe response to the tactile stimulus, indicating the loss of muscle tonus due to excessive inhibitory signaling in the NMJ (Dabbish and Raizen, 2011). The increased severity of the response observed in treated worms is an indication of the greater release of GABA in the synaptic

cleft (Fig. 4A).

snf-11 mutant worms treated with MPMT-OX had a reduction in locomotor activity (Fig. 4B). The *snf-11* gene encodes an electrogenic [+]/Cl[−]-coupled, high-affinity GABA transporter that is required in vivo for GABA uptake (Mclntire et al., 1993a,b; Schuske et al., 2004). Worms with *snf-11* gene knockout have increased basal levels of GABA in the cleft (Mullen et al., 2006). Therefore, the reduction in the locomotor activity of *snf-11* worms treated with MPMT-OX can be explained by the increase in GABA release in the synaptic cleft, in addition to the high levels of pre-existing GABA, leading to a reduction in the locomotor capacity of these animals.

Our data demonstrated that MPMT-OX acts by modulating GABAergic signaling, so we tested several mutants with defects in biosynthetic enzymes, transporters, and receptors. Defects in these proteins can lead to a specific imbalance of GABA neurotransmission and to diseases, such as epilepsy (Baulac et al., 2001). The *unc-25*, *unc-30*, *unc-46*, and *unc-47* genes encode proteins required in the presynaptic neuron, and the *unc-49* gene encodes postsynaptic GABA receptors (Schuske et al., 2004).

MPMT-OX-treated *unc-49* worms showed an increase in locomotor activity before (Fig. 5B) and after induction of seizure behavior (Fig. 7) and a reduction in the frequency of seizure-like behavior (Fig. 6D). *C. elegans unc-49* do not have $GABA_A$ receptors, but our data demonstrate that these animals respond to the possible increase in the release of the GABA in the synaptic cleft caused by MPMT-OX treatment. We believe that this is possible because *C. elegans* possess $GABA_B$ receptors, expressed by cholinergic motor neurons, which inhibit cholinergic neurons by feedback in response to GABA spillover (Schultheis et al., 2011). Thus, even in the absence of $GABA_A$, $GABA_B$ receptors may respond to the increased release of GABA in the synaptic cleft, inhibiting cholinergic motor neurons. As demonstrated in previous works, the null mutant for the $GABA_A$ receptor (*unc-49*) presented a convulsive behavior in the presence of PTZ, a chemical $GABA_A$ antagonist (Williams et al., 2004; Dabbish and Raizen, 2011). PTZ is known to induce convulsions through the suppression of GABAergic signaling; however, it also alters other neurotransmission systems in mammals, such as the adenosinergic system (Pagonopoulou and Angelatou, 1998) and glutamate/glutamine homeostasis (Eloqayli et al., 2003). Thus, we believe that imbalance in several neurotransmission pathways may contribute to the convulsion effect of PTZ in *unc-49*/$GABA_A$ worms.

The *unc-30* gene encodes a homeodomain transcription factor that regulates the expression of *unc-25*-GAD and *unc-47*-VGAT in 19 D-type neurons (Eastman et al., 1999). *unc-30* deletion worms showed a 10-fold reduction in *unc-25* mRNA in D-type neurons and normal levels of GAD and VGAT in RME, AVL, DVB, and RIS GABAergic neurons (Eastman et al., 1999). *unc-30* worms treated with MPMT-OX exhibited increased locomotor activity (Fig. 5B), greater resistance to PTZ-induced paralysis (Fig. 6E), and an increased locomotion rate (Fig. 7) after exposure to PTZ compared to controls. This indicates an increase in inhibitory signaling not dependent on the *unc-30* gene. We did not observe seizures in these animals exposed to PTZ, which corroborates previously reported data (Locke et al., 2009). We believe this occurred because these animals express WT levels of GABA in RME, AVL, DVB, and RIS neurons (Jin et al., 1994). Although these worms did not convulse, they did become paralyzed in the presence of PTZ at a higher rate than did WT worms (compare Figs. 2B and 6E), which corroborates the lower levels of GABA described in these animals.

The *unc-46* gene is required primarily at the synapse to localize VGAT to synaptic vesicles (Schuske et al., 2007). MPMT-OX treatment increased the locomotor activity of treated *unc-46* worms (Fig. 5B), reduced the number of worms with seizure-like behavior in the presence of PTZ (Fig. 6B), and improved locomotor capacity after seizure (Fig. 7). Previous work has demonstrated that the overexpression of *unc-47* (VGAT) in an *unc-46* mutant background partially rescues the defects in these animals (Schuske et al., 2007), therefore, these data may suggest the *unc-47* gene as one of the targets of the MPMT-OX compound.

unc-25 and *unc-47* worms treated with MPMT-OX showed no changes in locomotor activity (Fig. 6B) in the latency time up to the beginning of the convulsion-like behavior (Fig. 6A and C), and they did not recover locomotor activity after the seizures (Fig. 7A). These data suggest that the *unc-25* and *unc-47* genes are required for the effect of MPMT-OX. To verify this hypothesis, we used a method to observe the GABAergic synapses in vivo.

The *snb-1* gene encodes a neuronally expressed *C. elegans* homolog of the synaptic vesicle-associated membrane protein synaptobrevin (SNB-1), which is involved in vesicle docking and exocytosis (Nonet et al., 1998). The *unc-25* gene serves as a ubiquitous marker for the GABAergic system (Nonet, 1999). A transgenic strain carrying a fusion of GFP to the protein SNB-1 driven by the *unc-25* promoter allows the specific visualization of the GABAergic vesicle system.

Previous work has shown that differences in the fluorescence intensity of SNB-1::GFP puncta are correlated with changes in the number of synaptic vesicles that reach the synaptic terminals (Dittman and Kaplan, 2006; Bessa et al., 2013). In *C. elegans*, synapses occur *en passant*, that is, synaptic boutons are formed along the axon shaft (Jin, 2005). Therefore, our data (Fig. 8 and supplementary material Fig. S1) indicate that the increase in the fluorescence of SNB-1::GFP puncta, as well as the reduction of the number of gaps in the GABAergic motor neurons of worms treated with MPMT-OX and post-exposed to PTZ, is related to the greater recruitment of SNB-1 due to increased docking of GABAergic vesicles. Our behavioral experiments (locomotor activity of N2 worms and GABA mutants, response to muscimol, induction of seizure-like behavior) indicate an increase in inhibitory signaling. Therefore, we believe that the increase in the fluorescence of SNB-1::GFP puncta is not a mere accumulation of GABAergic vesicles in presynaptic neurons but an increase in the release of GABAergic vesicles in the synaptic cleft.

The coupling of neurotransmitter synthesis (*unc-25*) and packaging (*unc-47*) would provide a mechanism to ensure neurotransmitter function. Indeed, many neurotransmitter synthetases, although they are cytosolic proteins, are found predominantly in association with synaptic vesicles (Ueda et al., 1987; D'amelio et al., 1990; Erlander et al., 1991). In *C. elegans*, the expression of *unc-25* and *unc-47* is regulated by the same transcriptional factor in the type D neurons. It appears that an early duplication of certain promoter elements may have been maintained by selective pressure to coordinate the expression of two genes that function in the same process (Eastman et al., 1999).

Our data altogether lead us to believe that the MPMT-OX compound acts on the pre-synaptic GABAergic neurons, leading to an increase in inhibitory signaling. In nematodes with deletion of the *unc-47* gene, we did not observe any response to MPMT-OX treatment, even in the presence of *unc-25*. The same is true for nematodes with deletion of *unc-25* and the presence of *unc-47*. Thus, our experiments indicate that MPMT-OX's activity on GABAergic signaling in *C. elegans* is dependent on both the *unc-25* and *unc-47* genes.

Recently, several series of compounds derived from oxadiazole were synthesized and tested for their anticonvulsant activities. A new series of oxadiazole-based compounds exhibited better anticonvulsant activity and higher safety relative to the standard drug, phenobarbital (Mohammadi-Khanaposhtani et al., 2016). Another paper showed that a number of novel 1,3,4-oxadiazole derivatives of phthalimide exhibit anticonvulsant activity and lower neurotoxicity than the standard phenytoin drug (Bhat et al., 2010). In general, oxadiazole derivatives have anticonvulsive activity and low neurotoxicity in rodents, which corroborates the data presented in this study.

Some studies based on the structure-activity relationship (SAR) and molecular docking suggest that the anticonvulsive effect of oxadiazole derivatives is mediated by barbiturate receptors (Zarghi et al., 2008; Faizi et al., 2012; Perekhoda, 2014). In mammals, drugs barbiturates are GABAergic agonists that modulate $GABA_A$ receptors (Schumacher and McEwen, 1989). In *C. elegans*, $GABA_A$ receptors share significant

structural and pharmacological overlap with mammalian $GABA_A$ receptors (Bamber et al., 2003). However, in the present study we conclude that demonstrated that the effect of the MPMT-OX oxadiazole derivative does not depend on the *unc-49* gene encoding the $GABA_A$ receptor, but on the *unc-25* and *unc-47* genes responsible for the synthesis and transport of GABA, respectively.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.neuro.2019.08.001.

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