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m-Trifluoromethyl-diphenyl diselenide attenuates pentylenetetrazole-induced seizures in mice by inhibiting GABA uptake in cerebral cortex slices

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Abstract:
The present study investigated the anticonvulsive effect of the disubstituted diaryl diselenides diphenyl diselenide $(PhSe)_2$, *m*-trifluoromethyl-diphenyl diselenide $(m\text{-}CF_3\text{-}C_6H_4Se)_2$, *p*-chloro-diphenyl diselenide $(p\text{-}Cl\text{-}C_6H_4Se)_2$ and *p*-methoxyl-diphenyl diselenide $(p\text{-}CH_3O\text{-}C_6H_4Se)_2$ on a chemical model of seizure induced by pentylenetetrazole (PTZ) in mice. $(PhSe)_2$, $(p\text{-}Cl\text{-}C_6H_4Se)_2$ and $(p\text{-}CH_3O\text{-}C_6H_4Se)_2$ did not abolish seizures induced by PTZ in mice. $(m\text{-}CF_3\text{-}C_6H_4Se)_2$ at the dose of 100 mg/kg significantly prolonged the latency of the onset of the first convulsive episode and reduced the number of animals that presented seizures. To investigate the possible mechanisms involved in the anticonvulsant effect of $(m\text{-}CF_3\text{-}C_6H_4Se)_2$, mice were submitted to different associations (all drugs in a sub-effective dose); aminooxyacetic acid hemihydrochloride (AOAA, a -aminobutyric acid (GABA)-T inhibitor), diazepam (a $GABA_A$ receptor agonist) or DL-2,4-diamino-*n*-butyric acid hydrochloride (DABA, an inhibitor of GABA uptake) were pre-administered together with $(m\text{-}CF_3\text{-}C_6H_4Se)_2$. $(m\text{-}CF_3\text{-}C_6H_4Se)_2$ + DABA abolished seizures induced by PTZ in mice. $(m\text{-}CF_3\text{-}C_6H_4Se)_2$ administered alone or with PTZ decreased the levels of GABA uptake in cerebral cortex slices. The present study demonstrates that $(m\text{-}CF_3\text{-}C_6H_4Se)_2$ exerts anticonvulsant action by decreasing the levels of GABA uptake.

Key words:
disubstituted diaryl diselenides, GABA, seizures, pentylenetetrazole, uptake, brain

Abbreviations:
AEDs – antiepileptic drugs, AOAA – aminooxyacetic acid hemihydrochloride, DABA – DL-2,4-diamino-*n*-butyric acid hydrochloride, GABA – γ-aminobutyric acid, $(m\text{-}CF_3\text{-}C_6H_4Se)_2$ – *m*-trifluoromethyl-diphenyl diselenide, $(p\text{-}CH_3O\text{-}C_6H_4Se)_2$ – *p*-methoxyl-diphenyl diselenide, $(p\text{-}Cl\text{-}C_6H_4Se)_2$ – *p*-chloro-diphenyl diselenide, $(PhSe)_2$ – diphenyl diselenide, PTZ – pentylenetetrazole)

Introduction

Among the different neurological disorders that affect the human condition, epilepsy has been largely studied during the last century [16, 27] and has become a dynamic research field in recent years [3]. Several hypotheses have been advanced to explain the cause of primary or idiopathic epilepsy, including alterations in several classic neurotransmitter systems such as glutamatergic and γ-aminobutyric acid (GABA)ergic neurotransmitter systems [6, 39].

GABA is recognized as the principal inhibitory neurotransmitter in the cerebral cortex [32], and compounds that potentiate GABAergic function may be useful as antiepileptic drugs (AEDs) [38]. GABAergic function in the central nervous system (CNS) may be potentiated with GABA receptor agonists [12] or

inhibitors of GABA catabolism [14]. These GABAergic agents display anticonvulsant activity in a variety of animal models and are currently being evaluated for anti-epileptic activity in humans [10, 21]. In addition, GABA function is potentiated by inhibition of GABA uptake from the synaptic cleft [37].

The current therapeutic treatment of epilepsy with modern AEDs is associated with side-effects, dose-related and chronic toxicity, and teratogenic effects. Furthermore, approximately 30% of patients continue to have seizures with the current AEDs therapy [18, 35].

Therefore, particular importance has been given to the design of new effective antiepileptic chemicals with high rates of response and remission and with fewer adverse effects [35]. In this regard, diphenyl diselenide $(PhSe)_2$, a selenium compound, displays neuroprotective activity [9, 28] and has been documented as a promising pharmacological agent against several experimental models such as depression, anxiety and oxidative stress [8, 22, 30, 31]. In addition, administration of *m*-trifluoromethyl-diphenyl diselenide (m-CF_3-$C_6H_4Se)_2$, a disubstituted diaryl diselenide, attenuates apomorphine-elicited stereotypy in mice [17].

Based on the chemistry and pharmacological properties of organoselenium compounds, the aim of this study was to examine whether administration of disubstituted diaryl diselenides exerts anticonvulsant activity in a pentylenetetrazole (PTZ)-induced seizure model in mice. The role of the GABAergic system in the protective effect of (m-CF_3-$C_6H_4Se)_2$ against PTZ-induced seizure was examined.

Materials and Methods

Chemicals

GABA, phenobarbital, diazepam, aminooxyacetic acid hemihydrochloride (AOAA) and DL-2,4-diamino-*n*-butyric acid hydrochloride (DABA) were purchased from Sigma (St. Louis, MO, USA). [^{3}H]GABA, specific activity 20 Ci/mmol, was purchased from Amersham International (Berkingshamshire, UK). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Diazepam was dissolved in a minimum amount of 1 M NaOH. This solution was adjusted to the appropriate volumes with 0.9% physiological saline solution. GABA, AOAA and DABA were dissolved in 0.9% physiological saline solution.

$(PhSe)_2$ and its disubstituted diaryl diselenides, (m-CF_3-$C_6H_4Se)_2$, (p-Cl-$C_6H_4Se)_2$ and (p-CH_3O-$C_6H_4Se)_2$, were prepared in our laboratory according to the method found in the literature [23]. Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed that $(PhSe)_2$ and its disubstituted diaryl diselenides presented analytical and spectroscopic data in full agreement with their assigned structures. The chemical purity of compounds (99.9%) was determined by GC/HPLC. The compounds were dissolved in canola oil.

Animals

Female (2–3 months old) Swiss albino mice (25–35 g) from our breeding colony were used. The animals were kept in a separate animal room, on a 12-h light/dark cycle, at a room temperature of 22 ± 2°C, with free access to food (Guabi, RS, Brazil) and water. The animals were treated according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil.

Drugs and treatment

Effect of disubstituted diaryl diselenides on PTZ-induced seizures

Mice were divided into 14 groups of 12–14 animals each. In 12 groups, mice were given $(PhSe)_2$, (m-CF_3-$C_6H_4Se)_2$, (p-Cl-$C_6H_4Se)_2$ and (p-CH_3O-$C_6H_4Se)_2$ at the doses of 25, 50 and 100 mg/kg by oral route (*po*) 30 min before the administration of PTZ (60 mg/kg, *ip*). The control group received canola oil (10 ml/kg) 30 min before administration of saline solution (*ip*). The PTZ group received canola oil 30 min before administration of PTZ (60 mg/kg).

Each animal was placed into an individual plastic cage for observation lasting 1 h. The onset of generalized seizures (tonic-clonic) was used as the endpoint. The generalized seizures (tonic-clonic) were characterized by full clonus of the body followed by rearing and falling [26, 36]. The time period before the onset of generalized convulsions was recorded.

Involvement of the GABA system in the anticonvulsive effect of (m-CF_3-$C_6H_4Se)_2$

Based on the results obtained, the involvement of the GABA system in the anticonvulsive effect of (m-CF_3-$C_6H_4Se)_2$ was investigated. In this regard, a sub-effective dose of (m-CF_3-$C_6H_4Se)_2$ (25 mg/kg, *po*) was co-administrated with a sub-effective dose of diazepam (0.1 mg/kg, *ip*, a $GABA_A$ receptor agonist), AOAA (4 mg/kg, *ip*, a GABA-transaminase inhibitor) and DABA (4 mg/kg, *ip*, an inhibitor of GABA uptake). The pre-treatment times prior to the injection of PTZ (60 mg/kg, *ip*) were: (m-CF_3-$C_6H_4Se)_2$ (30 min), diazepam (30 min), AOAA (20 min) and DABA (30 min) [1].

[^{3}H]GABA uptake by cerebral cortex slices

To verify the involvement of GABA uptake in the protective effect of (m-CF_3-$C_6H_4Se)_2$ against seizure induced by PTZ, [^{3}H]GABA uptake was carried out in slices of cortices from mice. The adequate [^{3}H]GABA concentration and incubation time for uptake assay were accomplished according to the method described by Schweigert et al. [33]. The animals were divided into four groups: control, (m-CF_3-$C_6H_4Se)_2$ 100 mg/kg, PTZ (60 mg/kg) and (m-CF_3-$C_6H_4Se)_2$ 100 mg/kg + PTZ 60 mg/kg (no convulsing mice).

The animals were euthanized after 1 h of drug administration; the brains were immediately removed and submerged in Hank's balanced salt solution (HBSS), pH 7.2. Parietal cerebral cortices were dissected and coronal slices (0.4 mm) were obtained from the parietal area using a Mc Illwain tissue chopper. Slices were transferred to multiwell dishes and washed with 1.0 ml HBSS. The same procedure was undertaken for the hippocampal GABA uptake assay. The uptake assay was performed by adding 50 µM [^{3}H]GABA in 300 µl HBSS, at 37°C. Incubation was terminated after 15 min by three ice-cold washes with 1 ml HBSS immediately followed by the addition of 0.5 M NaOH, which was kept overnight. Aliquots of lysate were taken to determine the intracellular content of [^{3}H]GABA through scintillation counting. Sodium-independent uptake was determined by using choline, and the resulting value was subtracted from the total uptake to obtain the sodium-dependent uptake. The experiments were done in duplicate.

Protein quantification

Protein concentration was measured by the method of Bradford [4], using bovine serum albumin as a standard.

Statistical analysis

Data are expressed as the means SEM. Statistical analysis was performed using a one-way analysis of variance (ANOVA), followed by the Duncan's multiple range test when appropriate. Values of $p < 0.05$ were considered statistically significant. Seizure incidence was statistically analyzed using the χ^2 method and Fisher's exact test.

Tab 1. Influence of pre-treatment with disubstituted diaryl diselenides on PTZ-induced seizures in mice

Groups	Appearance of seizures[a]	Latency[b] (min)
Control	0/11	ns
PTZ 60	12/12	3.00 ± 0.35
PTZ + $(PhSe)_2$ 25	12/12	4.21 ± 0.66
PTZ + $(PhSe)_2$ 50	9/12	3.67 ± 0.82
PTZ + $(PhSe)_2$ 100	4/12	5.46 ± 2.29
PTZ + (m-CF_3-$C_6H_4Se)_2$ 25	12/12	2.24 ± 1.45
PTZ + (m-CF_3-$C_6H_4Se)_2$ 50	6/12	5.07 ± 1.92
PTZ + (m-CF_3-$C_6H_4Se)_2$ 100	3/14*	8.05 ± 0.67**
PTZ + (p-Cl-$C_6H_4Se)_2$ 25	12/12	5.28 ± 0.93
PTZ + (p-Cl-$C_6H_4Se)_2$ 50	7/12	2.72 ± 0.40
PTZ + (p-Cl-$C_6H_4Se)_2$ 100	7/12	6.02 ± 1.67
PTZ + (p-CH_3O-$C_6H_4Se)_2$ 25	12/12	3.16 ± 0.28
PTZ + (p-CH_3O-$C_6H_4Se)_2$ 50	12/12	2.99 ± 0.50
PTZ + (p-CH_3O-$C_6H_4Se)_2$ 100	11/12	3.86 ± 0.77

[a] Number of animals that presented seizures/N of animals per group. [b] Time (min) prior to the appearance of the first seizure episode. "ns" animals that did not present seizure (in 60 min of observation). The doses of $(PhSe)_2$, (m-CF_3-$C_6H_4Se)_2$, (p-Cl-$C_6H_4Se)_2$, (p-CH_3O-$C_6H_4Se)_2$ and PTZ are presented in mg/kg. Data are reported as the mean ± SEM. * Denotes $p < 0.05$ as compared to the PTZ group (χ^2 method and Fischer's exact probability test), ** denotes $p < 0.05$ as compared to the PTZ group (one-way ANOVA/Duncan)

Results

Effect of disubstituted diaryl diselenides on PTZ-induced seizures

As shown in Table 1, pre-treatment with $(PhSe)_2$, $(p\text{-Cl-}C_6H_4Se)_2$ and $(p\text{-}CH_3O\text{-}C_6H_4Se)_2$ at the dose range of 25–100 mg/kg did not significantly alter the appearance of seizures induced by PTZ.

Pre-treatment with $(m\text{-}CF_3\text{-}C_6H_4Se)_2$ at the dose of 100 mg/kg significantly prolonged the latency of the onset of the first convulsive episode and reduced the number of animals that experienced convulsions induced by PTZ (Tab. 1).

Involvement of the GABA system in the anticonvulsive effect of $(m\text{-}CF_3\text{-}C_6H_4Se)_2$

Pre-treatment with diazepam + $(m\text{-}CF_3\text{-}C_6H_4Se)_2$, at sub-effective doses, did not significantly reduce the number of convulsing animals and did not increase the onset of the first seizure episode induced by PTZ (Tab. 2).

Pre-treatment with AOAA + $(m\text{-}CF_3\text{-}C_6H_4Se)_2$, at sub-effective doses, significantly prolonged the latency of the onset of the first convulsive episode but did not protect animals against seizures induced by PTZ (Tab. 2).

Pre-treatment with DABA + $(m\text{-}CF_3\text{-}C_6H_4Se)_2$, at sub-effective doses, abolished seizures induced by PTZ (Tab. 2).

Tab. 2. Influence of co-administration of DABA, AOAA and diazepam on $(m\text{-}CF_3\text{-}C_6H_4Se)_2$ in PTZ-induced seizures in mice

Groups	Appearance of seizures[a]	Latency[b] (min)
Control	0/10	ns
PTZ 60	10/10	2.89 ± 0.12
PTZ 60 + Diazepam 0.1	10/10	2.40 ± 0.29
PTZ 60 + AOAA 4	10/10	3.03 ± 0.34
PTZ 60 + DABA 4	10/10	3.65 ± 0.86
PTZ 60 + $(m\text{-}CF_3\text{-}C_6H_4Se)_2$ 25	12/12	2.67 ± 0.41
PTZ 60 + Diazepam 0.1 + $(m\text{-}CF_3\text{-}C_6H_4Se)_2$ 25	4/14	3.33 ± 0.93
PTZ 60 + AOAA 4 + $(m\text{-}CF_3\text{-}C_6H_4Se)_2$ 25	9/14	4.95 ± 1.21**
PTZ 60 + DABA 4 + $(m\text{-}CF_3\text{-}C_6H_4Se)_2$ 25	0/14*	ns

[a] Number of animals that presented seizures/N of animals per group. [b] Time (min) prior to the appearance of the first seizure episode. "ns" animals that did not present seizure (in 60 min of observation). The doses of $(m\text{-}CF_3\text{-}C_6H_4Se)_2$, diazepam, DABA (DL-24 diamino-n-butyric acid) and AOAA (aminooxyacetic acid) are presented in mg/kg. Data are reported as the mean ± SEM. * Denotes $p < 0.05$ as compared to the PTZ group (χ^2 method and Fischer's exact probability test), ** denotes $p < 0.05$ as compared to the PTZ group (one-way ANOVA/Duncan)

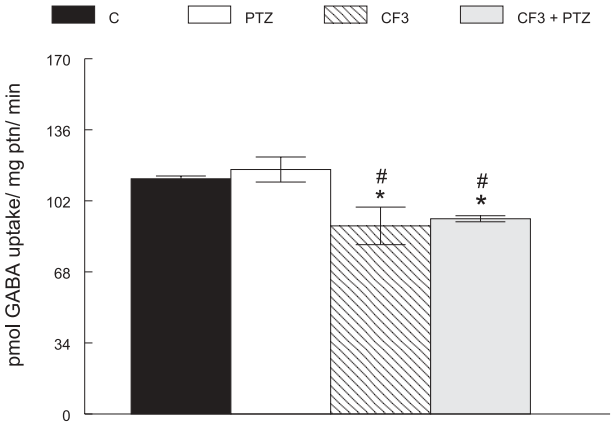

Fig. 1. Effect of $(m\text{-}CF_3\text{-}C_6H_4Se)_2$ on [^{3}H]GABA uptake levels by cerebral cortex slices. Data are reported as the mean ± SEM of two to four animals per group and GABA uptake levels are expressed as pmol of GABA uptake/mg protein/min. * Denotes $p < 0.05$ as compared to the PTZ group (one-way ANOVA/Duncan). Abbreviations: C – control, PTZ – pentylenetetrazole (60 mg/kg), CF_3 – $(m\text{-}CF_3\text{-}C_6H_4Se)_2$ (25 mg/kg) that presented seizure episodes; CF_3 + PTZ – $(m\text{-}CF_3\text{-}C_6H_4Se)_2$ (25 mg/kg) + pentylenetetrazole (60 mg/kg)

[^{3}H]GABA uptake by cerebral cortex slices

In cerebral cortex slices, the levels of [^{3}H]GABA uptake were not altered in mice treated with PTZ when compared to the control group. $(m\text{-}CF_3\text{-}C_6H_4Se)_2$ administered at the dose of 100 mg/kg significantly decreased [^{3}H]GABA uptake levels in cerebral cortices of mice when compared to the control and PTZ-treated groups. A significant decrease in the levels of [^{3}H]GABA uptake were found in $(m\text{-}CF_3\text{-}C_6H_4Se)_2$ + PTZ-treated group, animals which did not show seizure behavior, when compared to those of the control or PTZ-treated groups (Fig. 1).

Discussion

The present study demonstrates that $(m\text{-}CF_3\text{-}C_6H_4Se)_2$ at the dose of 100 mg/kg significantly protected against PTZ-induced seizures, with an increment in

onset of the first convulsive episode and a decrease in seizure incidence in mice. The GABAergic system, in particular GABA uptake, is involved in the anticonvulsive effect of (m-CF_3-$C_6H_4Se)_2$ in mice.

PTZ, a selective blocker of the chloride channel coupled to the $GABA_A$ receptor complex [34], is the most popular chemoconvulsant used for the evaluation of AEDs [25]. A sufficiently high dose of PTZ can produce a continuum of seizure activity that progresses from mild myoclonic erks to face and forelimb clonus without loss of righting reflex (which is known as minimal clonic seizure), to clonic seizures of limbs with loss of righting reflex, to full tonic extension of both forelimbs and hindlimbs (generalized tonic-clonic seizures) [15]. In this regard, data have revealed that PTZ-induced seizures are influenced by sex differences [11, 19]. For instance, Medina et al. [19] reported that female Swiss mice are more susceptible to seizures elicited by intraperitoneally injected PTZ than are their male counterparts. However, this sex difference is dose-dependent as it was demonstrated specifically with 50 and 60 mg/kg doses. The results of the present study are in accordance with these data since PTZ at the dose of 60 mg/kg induced generalized tonic-clonic seizures in 100% of the female mice tested.

Previous reports have demonstrated that disubstituted diaryl diselenides exhibit different pharmacological properties [17, 24]. In the current study, (m-CF_3-$C_6H_4Se)_2$ at the dose of 100 mg/kg abolished PTZ-induced seizures in mice, while $(PhSe)_2$, (p-Cl-$C_6H_4Se)_2$ and (p-CH_3O-$C_6H_4Se)_2$ were not effective. A possible explanation for this finding is that the electronic effects of m-CF_3 substituent bonded to the aromatic ring of diaryl diselenide. CF_3 is a strong electron withdrawing group, which causes the Se–Se bond of (m-CF_3-$C_6H_4Se)_2$ to be more susceptible to cleavage. However, (p-Cl-$C_6H_4Se)_2$ did not protect against PTZ-induced seizures even when it contained a substituted Cl, an electron withdrawing group. A possible explanation for this result is that Cl is a weak electron withdrawing group as compared to CF_3. The results obtained with $(PhSe)_2$ and (p-CH_3O-$C_6H_4Se)_2$ support the assertion that the electronic effects are, at least in part, related to the ability of diaryl diselenides to abolish PTZ seizures. In fact, $(PhSe)_2$ and (p-CH_3O-$C_6H_4Se)_2$, compounds without a substituent and with an electron-donating substituent bonded to the aromatic ring, did not demonstrate an anticonvulsant effect against seizures induced by PTZ.

Based on the anticonvulsant action of (m-CF_3-$C_6H_4Se)_2$ in PTZ-induced seizures in female mice, the involvement of the GABA system was examined. One of the principal therapeutic mechanisms of anticonvulsant drugs is the stimulation of receptors in the ionophore complex, which increases the chloride flux through chloride channels at $GABA_A$ receptors sites, enhancing GABAergic functions [20]. Central $GABA_A$ receptor synaptic function has been associated with epilepsy, and stimulation of $GABA_A$ receptors by GABA has been shown to overcome seizures [7]. The results presented here demonstrates that pre-treatment with a $GABA_A$ agonist diazepam plus (m-CF_3-$C_6H_4Se)_2$, both in sub-effective doses, neither abolishes convulsion nor alters the latency of the first seizure episode induced by PTZ. This result suggests that modulation of $GABA_A$ receptor in the benzodiazepinic site is not directly involved in the anticonvulsant action of (m-CF_3-$C_6H_4Se)_2$ in seizures induced by PTZ in mice.

It has been suggested that the reduction of GABA levels in the synaptic cleft increases predisposition to seizures, indicating that GABA modulates seizure susceptibility [29]. In this context, the animals were treated with AOAA + (m-CF_3-$C_6H_4Se)_2$. AOAA is a potent inhibitor of GABA-T, an enzyme that metabolizes GABA, accumulating GABA in the brain by preventing its breakdown. The results presented in this study showed that pre-treatment with AOAA + (m-CF_3-$C_6H_4Se)_2$, both at sub-effective doses, did not abolish convulsion but increased the latency of the first seizure episode induced by PTZ in mice. These results illustrate the contribution of GABA transaminase in the protective effect of (m-CF_3-$C_6H_4Se)_2$ against seizures induced by PTZ in mice.

However, a pharmacological approach to increasing GABAergic neurotransmission in the CNS is through inhibition of the uptake of GABA from the synaptic cleft. This mode of action potentiates endogenously released GABA, which may be a favorable mechanism as compared to the alternative approach of direct $GABA_A$ or $GABA_B$ receptor agonism [2]. In this study, pre-treatment with a potent and selective inhibitor of GABA uptake (DABA), along with (m-CF_3-$C_6H_4Se)_2$, resulted in the accumulation of GABA in the brain and abolished seizure episodes induced by PTZ in mice. These findings demonstrate the possible involvement of GABA uptake in the anticonvulsant action of (m-CF_3-$C_6H_4Se)_2$ in mice.

To provide evidence for the involvement of GABA uptake in the anticonvulsant action of (*m*-CF_3-$C_6H_4Se)_2$, we determined whether (*m*-CF_3-$C_6H_4Se)_2$ reduces GABA uptake by cortical slices. Figure 1 shows that both (*m*-CF_3-$C_6H_4Se)_2$- and (*m*-CF_3-$C_6H_4Se)_2$ with PTZ-treated groups significantly reduced GABA uptake levels. The reduction in GABA uptake levels in cerebral cortex slices could be associated with the anticonvulsant action of (*m*-CF_3-$C_6H_4Se)_2$ since the lower uptake of GABA increases extracellular GABA levels, leading to the observed decrease in the sensitivity to seizure episodes. For instance, it has been reported that an epilepsy-prone mice strain demonstrates a low concentration of GABA in the cortex [5]. Data have revealed that the utility of first-generation GABA uptake inhibitors is limited by their hydrophilic nature and subsequent inability to reach pharmacologically significant concentrations in the CNS [13]. The high lipophilicity of (*m*-CF_3-$C_6H_4Se)_2$ and its subsequent ability to cross the blood-brain barrier could add to the possible explanation of its anticonvulsant ability.

Taken together, the results of the present study show that (*m*-CF_3-$C_6H_4Se)_2$ exerts anticonvulsant action against PTZ-induced seizures in female mice. The precise mechanism through which (*m*-CF_3- $C_6H_4Se)_2$ exerts its action on PTZ-induced seizures in mice seems to involve, at least in part, GABA uptake.

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