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© Med Sci Monit, 2007; 13(9): BR194-199
**PMID:** 17767114



**Received:** 2007.02.13
**Accepted:** 2007.06.05
**Published:** 2007.09.03

# The effect of paraoxon on GABA uptake in rat cerebellar synaptosomes

**Authors' Contribution:**
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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**Source of support:** Departmental sources

## Summary

**Background:** The compounds used to treat organophosphate (OP) poisoning are not able to fully alleviate long-lasting effects. They are mainly used to antagonize the cholinergic effects of OPs; however, non-cholinergic effects such as interference with different neurotransmitter systems, especially GABA release and uptake, are now attracting more attention.

**Material/Methods:** Cerebellar synaptosomes were used to investigate any potential interaction between paraoxon and GABA uptake. The cerebella of 250- to 280-g Wistar rats were rapidly dissected out, homogenized, centrifuged, and incubated with 0.004 μM [$^3$H]GABA in the presence of different doses of paraoxon for 15 minutes at 37°C. At the end of the incubation period, the synaptosomes were layered in chambers of a superfusion system (UGO). To assay the amount of [$^3$H]GABA uptake, radioactivity was measured using a β-counter (Winspectrul).

**Results:** Mean GABA uptake was 111, 95, 71, 73, and 75 percent of the control values in the presence of paraoxon concentrations of 0.01, 0.1, 1, 10, and 100 μM, respectively. Accordingly, GABA uptake was significantly reduced at doses 1, 10, and 100 μM of paraoxon ($P$<0.05).

**Conclusions:** Paraoxon may interfere with GABA uptake by cerebellar synaptosomes at micromolar concentrations or higher.

**key words:** **organophosphate • Paraoxon • Cerebellum • synaptosome • GABA**

**Full-text PDF:** http://www.medscimonit.com/fulltxt.php?IDMAN=10130
**Word count:** 2421
**Tables:** –
**Figures:** 5
**References:** 33

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## BACKGROUND

The organophosphates (OPs) constitute a diverse group of chemical structures exhibiting a wide range of physicochemical properties. The toxic effects of OP compounds are classified into three categories: muscarinic, nicotinic, and central nervous system (CNS) effects [1]. The primary mechanism of OP action is irreversible inhibition of acetylcholinesterase and accumulation of acetylcholin (Ach) [2]. An excess of ACh stimulates seizure activity (convulsions) and, if uncontrolled, can lead to brain damage [3]. In spite of various anticonvulsant drugs on the market, convulsion is still a problem in poisoning management. It therefore seems that OPs may have other targets apart from cholinesterases. In fact, there is now a large body of evidence supporting the concept that OPs do interact directly with other molecular targets or cellular functions in the CNS, such as membrane channels [4], receptors [1], pump [5], and morphological changes in neurons [6], and even neurotransmitters [1].

A change in the levels of the GABA (gamma-aminobutyric acid) during nerve agent seizures has also been reported [1,7]. GABA is the major inhibitory neurotransmitter in the mammalian brain [8], where it plays a fundamental role in controlling neuronal excitability [9]. The terminating step in GABA transmission is its removal from the synaptic cleft. Many toxicants or drugs can directly or indirectly interfere with transmitter uptake and can therefore have profound effects on signaling. However, many transporters can also reverse, causing the release of neurotransmitter in a calcium-independent manner [10,11]. Richerson and Wu proposed that GABA transporter is the major determinant of the level of tonic inhibition and an important source of GABA release during seizures, therefore playing a much more dynamic role in the control of brain excitability than was previously recognized [12]. The behavioral consequences of changes in the "balance" between inhibition and excitation are often profound (e.g. following administration of convulsion or anesthetic drugs which are known to alter GABAergic neurotransmission).

When examining reports of OP intoxication, alterations in GABA concentration are controversial. Brain GABA levels are reported as being increased [1], decreased [7], or unchanged in rats following OP intoxication [13]. There are even fewer reports specifically on the effect of OPs on GABA uptake. We used paraoxon to investigate its possible mediation in GABA uptake in rat cerebellar synaptosomes.

## MATERIAL AND METHODS

### Animals

All experimental procedures involving animals were in accordance with the protocols established in the guidelines for animal care issued by the Baqiyatallah University of Medical Sciences and reviewed and authorized by the Ethics Committee of the university. Adult male Wistar rats (250–280 g) purchased locally were kept in a temperature-controlled room (22±1°C) with a 12-hour light/dark illumination cycle and free access to rat chow (Pars Dam Co. Iran) and water.

### Chemicals

γ-aminobutyric acid (GABA), triton X-100, and ATC (acetylthiocholine) were obtained from Sigma Chemical Co., Germany. [$^3$H]GABA (86 ci/mmol) was purchased from Amersham Bioscience, UK. Diethyl p–nitrophenyl phosphate (paraoxon), nipecotic acid, aminooxyacetic acid (AOAA), DTNB (5, 5'-dithiobis (2-nitrobenzoic acid)), and bovine serum albumin (BSA) were all purchased from Fluka, Switzerland. Other materials were from Merck, Germany.

### Preparation of synaptosomes

Rats were anesthetized with diethyl ether and sacrificed by decapitation. Dissection, homogenization, and all fractionations were conducted at 0–4°C and synaptosomes were prepared according to Maura's method [14]. For each experiment one rat cerebellum in three replicates was used. A total number of 73 rats was used in the experiments. In brief, the cerebellum was quickly removed and homogenized in 40 volumes of ice-cold medium A (0.32 M sucrose in 100 mM phosphate buffer, pH 7.4) in a glass potter homogenizer adjusted at 90 rotations/min and 25 up-and-down strokes. The homogenate was then centrifuged for 5 min at 1000 × *g*. The pellet ($P_1$: crude nuclear) was resuspended in an equal volume of medium A, filtered through a double gauze layer, and re-centrifuged for another 5 min. The pellet containing the synaptosomes was resuspended in medium B (125 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 1 mM $NaH_2PO_4$, 22 mM $NaHCO_3$, and 10 mM glucose. Medium B was aerated at room temperature for a minimum of 30 min with 5% $CO_2$ and 95% $O_2$, adjusting the pH of the solution to 7.2–7.4. All solutions were made fresh from stock solutions. Next we assayed the protein to normalize the samples.

### Electron microscopy

Electron micrographs were taken from a few samples for morphological verification of the synaptosomes, as described elsewhere [14]. Briefly, the suspension of giant synaptosomal fractions were fixed with 1% glutaraldehyde, then mixed with agarose and placed in a plastic mold. The synaptosome-containing agarose films were post-fixed in osmium tetroxide, suctioned by ultramicrotome, stained with uranyl acetate and lead citrate, and then examined with a Zeiss EM 900 electron microscope.

### Protein and Enzyme assay

Protein concentration was determined by Bradford's method using bovine serum albumin as the standard [15]. The changes in activity of the marker enzyme, lactate dehyrogenase (LDH), were measured before and after membrane disruption by triton X-100 according to Moss and Henderson [16].

### Uptake of GABA in synaptosomes

Uptake assays were performed according to the method of Mantz and coworkers [17]. The synaptosomal pellets were resuspended to a final protein concentration of 1 mg/ml in medium B. Aliquots of synaptosomes (0.5 ml, containing 0.5 mg protein) were added to Eppendorf tubes, preincubated for 20 min at 37°C in either the absence (control) or presence of various concentrations of paraoxon (0.01, 0.1, 1, 10, and

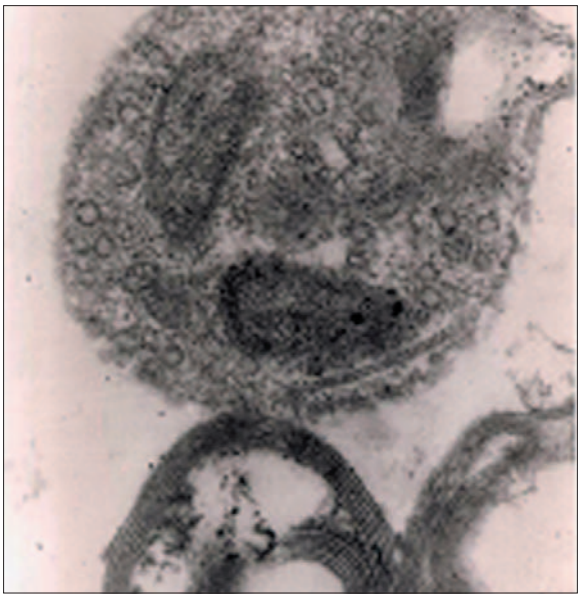

**Figure 1.** Electron micrograph of rat cerebellar giant synaptosomes. The synaptoplasm contains synaptic vesicles and two mitochondria. (Uranyl acetate-lead citrate staining, ×50000).

100 µM). Since the concentration of paraoxon in the stock solution was 1 mM, 100 µl of solution for 100 µM paraoxon was added. Attempts were made to minimize the volume change when using higher concentrations of paraoxon. Then aminooxyacetic acid (final concentration: 10 µM), a GABA transaminase inhibitor, was added to the incubation solution during the GABA uptake assay. Also, 0.5 ml of each synaptosomal preparation was added to the Eppendorf tube containing the GABA uptake inhibitor nipecotic acid (final concentration: 50 mM). [$^3$H]GABA uptake by the transporter was considered as the difference between the radioactivity measured in both the presence and absence of nipecotic acid. This concentration was in excess of the $IC_{50}$ values of the inhibitor.

### Incubation

Three microliters of tritiated GABA were added to a final concentration of 0.004 µM, then the synaptosomes were incubated in a bath shaker for 15 min (loading period) at 37°C. The duration of incubation was determined according to our experiment. After incubation, the reaction was stopped by addition of 2 ml of ice-cold medium B. This was followed by washing three times with ice-cold medium B after transfer to superfusion chambers. The synaptosomes were distributed on filters (0.65 µm pore, Millipore) placed at the bottom of a set of parallel superfusion chambers maintained at 37°C [18]. A peristaltic pump was connected to the bottom of the superfusion chambers and its flow adjusted to 0.5 ml/min/chamber. The filters containing the remnant of synaptosomes were completely covered by scintillated liquid. The presence of radioactivity in the various filtrates was quantified by β-counter (Winspectrul LSD).

### Statistical analysis

Eight rats were used for each concentration of paraoxon. To evaluate reproducibility, the experiments were carried

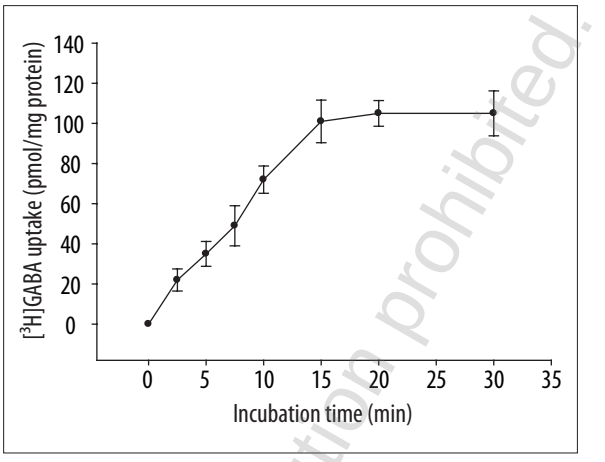


**Figure 2.** Time dependency of GABA uptake. Uptake reaches a maximum at 15 min.

out in replicates of three. Statistical comparisons were made by Student's *t*-test for independent samples (paired or unpaired, where appropriate). Multiple comparisons between animal groups were made by one-way analysis of variance (ANOVA) followed by the post hoc Newman-Keuls procedure. Values of $P$ with a probability of less than 0.05 were considered to indicate a statistically significant difference between the means. All statistical analyses were performed with the aid of the SPSS computer program. Unless otherwise indicated, experimental data are represented as mean ±*SEM*.

## Results

### Synaptosomal integrity

An electron micrograph of a synaptosome prepared from rat cerebellum showed that most of the synaptosomes were densely packed with synaptic vesicles containing well-preserved intra-synaptosomal mitochondria. We also used changes in occluded LDH activity as a marker for the integrity of the synaptosome (Figure 1). Disruption of particles with triton X-100 increased enzyme activity significantly and in such a way that the ratio of this activity relative to the samples obtained from the same intact synaptosomes was 88.38±2.37% ($P$<0.05, n=18). When expressed as a percentage of the total, occluded and free LDH activities were 88.38±2.37% and 11±62%, respectively ($P$<0.001, n=18).

### Time dependency of [$^3$H]GABA uptake

Synaptosomal accumulation of [$^3$H]GABA was time dependent and peaked at 15 min. [$^3$H]GABA uptakes were 22, 35, 49, 72, 101, 100, and 100 pmol/mg protein at 2.5, 5, 7.5, 10, 15, 20, and 30 min from the beginning of incubation, respectively. The increase was not significant until 15 minutes into the incubation time (Figure 2).

### Effect of paraoxon on GABA uptake

Paraoxon inhibited the cholinesterase activity of the synaptosomes in a concentration-dependent manner. The levels of [$^3$H] GABA uptake in the cerebellar synaptosomes were not significantly different in lower doses of paraox-

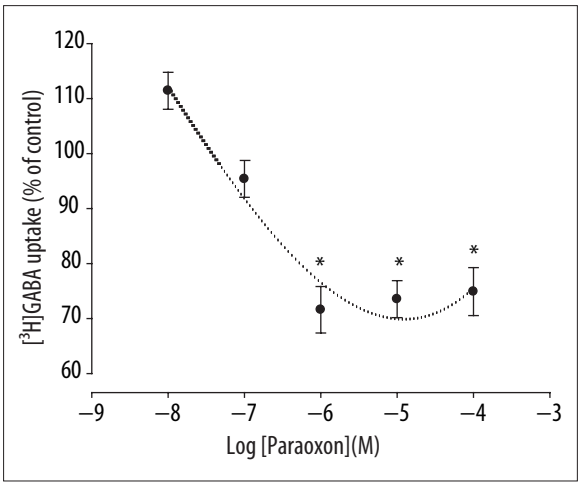


**Figure 3.** Effect of paraoxon on [3H]GABA uptake. Aliquots of synaptosomes were pre-exposed at 37°C to 0.01, 0.1, 1, 10, or 100 µM paraoxon for 20 minutes, then incubated for 15 min with the indicated concentrations of GABA plus 4 nM of [3H]GABA. Values are expressed as the percentage of the control uptake. Lower concentrations of paraoxon (0.01 or 1 µM) had no effect. [3H]GABA uptake in the control group was 55±3 pmol/mg protein. *$P$<0.05 compared with the control.

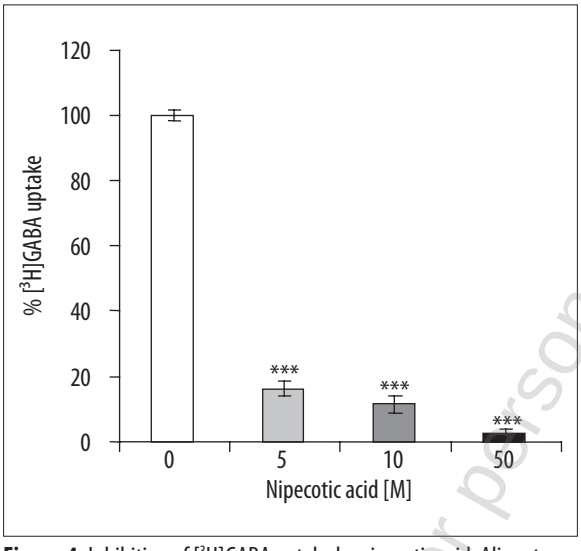


**Figure 4.** Inhibition of [3H]GABA uptake by nipecotic acid. Aliquots of synaptosomes were incubated for 15 min with [3H]GABA (50 nM) in the absence or presence of the indicated concentrations of inhibitors, added 5 min before. ***$P$<0.001 compared with the control.

on compared with those obtained at higher doses. At micromolar concentrations of paraoxon ($10^{-6}$–$10^{-4}$ M), GABA concentration was reduced to approximately 30–28% of the control level, this being statistically significant ($P$<0.05, Figure 3). Exposure of the cerebellar synaptosomes to a selective inhibitor of the GABA transporter, nipecotic acid, reduced the level of GABA uptake by more than 80% (n=6, Figure 4). This significant difference indicates that most of the GABA uptake into the synaptosomes occurred via transporters.

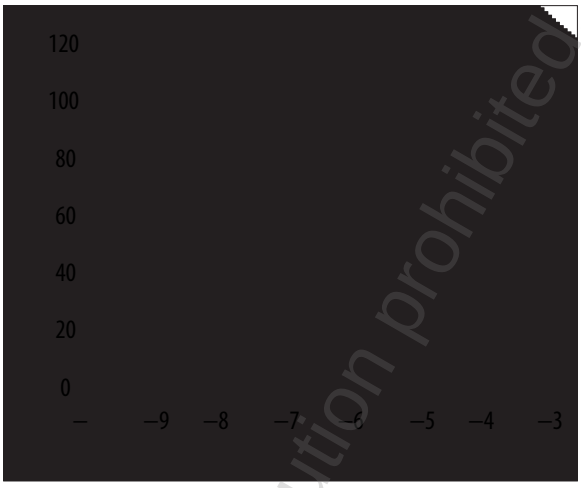


**Figure 5.** Synaptosomal cholinesterase inhibition by paraoxon. ***$P$<0.001 compare with the control.

**Cholinesterase activity**

Paraoxon inhibited cholinesterase activity of synaptosomes, and this inhibition was concentration dependent (n=6, Figure 5).

**LDH activity after and before**

In order to determine the effects of paraoxon itself and the incubation procedure on synaptosome integrity, we use LDH as a marker. Accordingly, synaptosomal membrane structure was unaffected (88% vs. 85%, n=6).

## Discussion

We found that paraoxon at a micromolar concentration interfered with GABA transmission and reduced its uptake by cerebellar synaptosomes in rats. To provide evidence to assure the accuracy of our results, we examined our synaptosomes morphologically and functionally. After examining the electron micrographs it is believed that the synaptosomes are indeed intact and well-prepared membrane-bound bodies that contain synaptic vesicles. The LDH assay and nipecotic acid tests [19] provided further support regarding the intactness of the membranes. Occluded LDH activity was 88.38% of the total, a value in good accordance with other reports [20,21]. Similar values of LDH activity before and after paraoxon exposure indicate that paraoxon did not disrupt membrane integrity, as reported by Cecchini [22]. Since aminooxyacetic acid prevents GABA metabolism, any detected tritium in the present study represents [3H]GABA uptake. It was also shown that the metabolic changes in [3H]-neurotransmitter content following incubation of the synaptosomes with [3H]GABA in the presence of this inhibitor were negligible [23].

Our results correlate well with studies by Szilagyi and colleagues (1993) reporting that high doses (1–2 mM) of tabun decreased the uptake of [3H]GABA [24]. Coudray-Lucas and associates (1984) investigated the effect of sublethal doses of paraoxon and soman on the metabolism of GABA and the total concentration in rat brain areas (hypothalamus, striatum, cerebellum) [13]. They, however, did not report any

data regarding GABA uptake. We decided to examine the effects of exposure to very low concentrations of paraoxon. Although the blood concentration of paraoxon during intoxication can temporarily reach the micromolar concentration range [25], it is quite possible that cell function in the target tissues may be altered by exposure to much lower concentrations of organophosphates. Other investigators have suggested that nerve agents may inhibit the metabolism of GABA in synaptosomal preparation [24]. It is also reported that in rat cerebral cortex synaptosomes, paraoxon inhibits GABA uptake at high doses [26].

It is important to note the $P_1$ synaptosome had impurity that may have affected our results, although the data from the experiments with nipecotic acid indicate that this should not be significant. We also observed that the optimum time for synaptosomal [$^3$H]GABA uptake was about 15 min, which is partially consistent with that reported by Sutch and coworkers (1999), who claimed [$^3$H]GABA uptake was maximum at 20 min [27]. We also observed that a 15-min incubation time does not disrupt synaptosomes by measuring the LDH activity of a few samples before and after incubation.

Looking back at our results, there is an apparent contradiction. Reduction in GABA uptake would apparently result in an increased GABA concentration at the synaptic level, which does not seem to favor convulsion or seizure. Therefore, the interpretation of our results needs a thorough survey in the literature to explain the complexity of this phenomenon.

Paraoxon is an OP causing convulsion, so it must somehow affect the balance between excitatory and inhibitory neurotransmitters in different brain regions in favor of excitation. To do so, paraoxon can theoretically increase the synaptic level of excitatory neurotransmitters (e.g. glutamate) or it may reduce GABA level. In synaptic terminals, neurotransmitter release is a complex phenomenon and is modulated at several putative sites, including the transporter as well as the release process itself [28]. GABA has a significant degree of extracellular freedom followingr elease, and one function of GABA transport is to remove GABA from the extrasynaptic space during both low- and high-frequency firing [29]; thus the GABA transporters regulate synaptic and extra-synaptic concentrations of GABA and, in this capacity, are partly responsible for the regulation of inhibitory neurotransmission in the nervous system [30]. GABA transporters may have other important functions as well, such as supplying a source of extracellular GABA via reversal of the GABA transporter and regulating paracrine GABA [29,31]. Under physiological conditions, a small increase in cellular GABA (5–10%) may be sufficient to enhance GABA transporter reversal with stimulation [31]. If uptake is damped by any means, in our case by paraoxon, the synaptic content of GABA decreases, favoring a reduction in GABA release. In other words, it is possible that inhibition of GABA transporter causes the decrease in GABA release mediated by transporters, contributing to the seizure activity by paraoxon. This would only occur if existing GABA diffuses out of its microenviroment, a phenomena reported by **Gregory** [29]. For the mechanism of the inhibitory effect of paraoxon on GABA uptake, it is interesting to consider that GABA transporter has Na dependency, and the direct toxic inhibitory effect of OPs on sodium-potassium ATPase [5,32,33] that leads to disruption of the concentrations of Na may interfere of GABA transporter.

## CONCLUSIONS

The present results suggest that paraoxon at high doses decreases [$^3$H]GABA uptake in synaptosomal preparations. Because transporter function will be positively correlated with neurotransmitter release, GABA transporters may play a role in OP-induced convulsion.

**Acknowledgements**

The authors would like to thank Baqiyatallah University for sponsoring the project The technical efforts of Mrs. Soleymani are also appreciated.

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