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The neurotoxin diethyl dithiophosphate impairs glutamate transport in cultured Bergmann glia cells

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ABSTRACT

Glutamate, the main excitatory neurotransmitter in the vertebrate Central Nervous System, is involved in almost every aspect of brain physiology, and its signaling properties are severely affected in most neurodegenerative diseases. This neurotransmitter has to be efficiently removed from the synaptic cleft in order to prevent an over-stimulation of glutamate receptors that leads to neuronal death. Specific sodium-dependent membrane transporters, highly enriched in glial cells, elicit the clearance of glutamate. Once internalized, it is metabolized to glutamine by the glia-enriched enzyme Glutamine synthetase. Accumulated glutamine is released into the extracellular space for its uptake into pre-synaptic neurons and its conversion to glutamate that is packed into synaptic vesicles completing the glutamate/glutamine cycle. Diverse chemical compounds, like organophosphates, directly affect brain chemistry by altering levels of neurotransmitters in the synaptic cleft. Organophosphate compounds are widely used as pesticides, and all living organisms are continuously exposed to these substances, either in a direct or indirect manner. Its metabolites, like the diethyl dithiophosphate, are capable of causing brain damage through diverse mechanisms including perturbation of neuronal-glial cell interactions and have been associated with attention-deficit disorders and other mental illness.

In order to characterize the neurotoxic mechanisms of diethyl dithiophosphate, we took advantage of the well characterized model of chick cerebellar Bergmann glia cultures. A significant impairment of [^{3}H] D-Aspartate transport was found upon exposure to the metabolite. These results indicate that glia cells are targets of neurotoxic substances such as pesticides and that these cells might be critically involved in the associated neuronal death.

1. Introduction

Glutamate (Glu), the main excitatory neurotransmitter in the vertebrate Central Nervous System (CNS) participates in cognitive, sensory and motor circuits. This neurotransmitter is considered a potent endotoxin in the CNS (Choi, 1988). Glu exerts its actions through the activation of specific membrane receptors and transporters expressed both in neurons and glial cells. In astrocytes, exposure to this amino acid increases $[Ca^{2+}]_i$ (Glaum et al., 1990), inhibits cyclic adenosine monophosphate (cAMP) formation (Prezeau et al., 1994), and leads to the accumulation of Reactive Oxygen Species (ROS) (Mawatari et al., 1996). The proper function of glutamatergic synaptic transmission requires a highly efficient removal of this amino acid *via* its uptake through a family of sodium-dependent high affinity excitatory amino acid transporters (EAATs) highly expressed in glial cells (Danbolt, 2001).

Several studies have suggested that these transporters, particularly those enriched in glial cells, play a critical role in the prevention of excitotoxic injuries attributed to an over-stimulation of neuronal Glu receptors (Danbolt, 1994). Glial Glu transporters comprise EAAT-1, also known as glutamate/aspartate transporter (GLAST), and EAAT-2, first named glutamate transporter 1 (GLT-1) (Lehre et al., 1995; Rothstein et al., 1994). Within the cerebellum, Bergmann glial cells (BGC) completely surround the most abundant glutamatergic synapses in the entire CNS, the one established between the axons of the granule cells (parallel fibers) and Purkinje cells (Mugnaini, 1969). BGC are responsible for more than 80% of the total Glu uptake activity in the cerebellar molecular layer, and express exclusively EAAT-1/GLAST (Ruiz and Ortega, 1995).

Recent evidences have suggested that GLAST is involved in Glu

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Abbreviations

AChE	Acetylcholinesterase
BGC	Bergmann Glia Cells
cAMP	Cyclic Adenosine Monophosphate; CNS Central Nervous System
DAPs	Dialkyl phosphates
DEDTP	Diethyl dithiophosphate
DEP	Diethyl phosphate
DETP	Diethyl thiophosphate
DMDTP	Dimethyl dithiophosphate
DMP	Dimethyl phosphate
DMTP	Dimethyl thiophosphate
EAATs	Excitatory Amino Acid Transporters
GLAST	Glutamate Aspartate Transporter
Glu	Glutamate
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OP	Organophosphate compounds
ROS	Reactive Oxygen Species
SOCS3	Suppressor of cytokine signaling 3
STAT5	Signal transducer and activator of transcription 5

signaling transactions. In fact, Glu regulates its own uptake process in a receptor-independent manner (González and Ortega, 2000). It has also been reported that GLAST is coupled to the Na^+/K^+ ATPase (Gegelashvili et al., 2007; Rose et al., 2009). Moreover, nitric oxide (NO) regulates GLAST through the activation of guanylate cyclase and the signaling cascade includes NO/cGMP/PKG and Ca^{2+} influx through the Na^+/Ca^{2+} exchanger and might be related to the plasma membrane Glu transporters turnover (Balderas et al., 2014).

Organophosphorus or organophosphates (OPs) are esters of phosphoric acid and its derivatives. These compounds are widely used worldwide as pesticides due to their low residual power in the environment. Some commercial uses of OPs include plague control (Marselos and Vainio, 1991), industrial disinfection of hospitals and schools, or fumigants for flower production and maintenance of golf courts or football pitches (Duggan et al., 2003). Due their widespread use, it has been proposed that all living organisms are continually exposed to these compounds. In fact, epidemiologic studies have revealed linkage between OPs exposure and a higher risk of cancer development and attention-deficit disorder, suggesting a deleterious effect of these compounds in the CNS (Brown et al., 1990; Marks et al., 2010; Waddell et al., 2001). Several authors have suggested that adverse short-term effects of these chemicals are mainly associated to CNS damage (Gupta et al., 2001). The toxicity of OPs depends of diverse factors, such as their chemical structure, metabolism in target organism, dose, and degree of decomposition, among others. It is important to mention that the primary mechanism of OP toxicity is the inhibition of acetylcholinesterase (AChE) (Edwards and Tchounwou, 2005; Nolan et al., 1984). OPs such as Disulfothon, Ethion, Phorate, Phosalone and Terbufos, generate metabolites called dialkyl phosphates (DAPs) like diethyl dithiophosphate (DEDTP), diethyl thiophosphate (DETP), diethyl phosphate (DEP), dimethyl dithiophosphate (DMDTP), dimethyl thiophosphate (DMTP) and dimethyl phosphate (DMP) (Duggan et al., 2003). These metabolites are formed once the OPs are absorbed by organisms like plants, insects or mammals. Subsequent to their absorption, many biotransformation reactions take place to facilitate either activation or a detoxification processes (Jokanovic, 2001). Above all, the lipophilic metabolite DEDTP is absorbed through a respiratory, gastrointestinal or dermal route (Vale, 1998), and once in the organism it is distributed rapidly through the bloodstream reaching different systems including the CNS, since due to its hydrophobicity it can permeate the blood brain barrier (BBB) (Gupta et al., 1999). Thus far, no detailed DEDEPT information is available about its toxicokinectic properties, and its metabolism is far from being established. It has been suggested that DEDPT is metabolized by secondary desulfurization reactions that generate the DETP and DEP products, with half-lives of 60 and 15 h, respectively (Vasilić et al., 1993). *In vitro* studies reported by Esquivel-Sentíes et al. (2010), demonstrated that in human CD4 + T lymphocytes, DEDTP increased suppressor of cytokine signaling 3 (SOCS3) phosphorylation and the de-phosphorylation of signal transducer, and activator of transcription 5 (STAT 5). A significant increase in the phosphorylation pattern of several members of the mitogen-activated protein kinase family (MAPK) was also present (Esquivel-Sentíes et al., 2010). The fact that OPs exposure has been associated to cognitive disorders, suggest an effect on glutamatergic transmission that might be glia-mediated. In this context, in the present contribution we explored the effect of DEDTP exposure in GLAST function. Our results demonstrate a dis-regulation of the Glu uptake process that might be linked to the reported CNS damage. These results favor the notion of a critical involvement of glial cells OPs neurotoxicity.

2. Methods

2.1. Animals

Chick embryos (10 day-old) were obtained from Avimex, S.A de C.V. (Mexico City) and maintained at 37 °C until used. All experiments were performed according to International Guidelines on the Ethical Use of Animals and had the specific approval of the Animal Ethics Committee of Cinvestav-Mexico. All efforts were made to reduce the number of embryos used and their suffering.

2.2. Materials

Tissue culture reagents were obtained from Life Technologies (Carlsbad, CA, USA). D-Aspartic acid, L-Glutamate acid, dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), choline chloride, ethylenediaminetetraacetic acid (EDTA) and DEDTP were acquired from Sigma-Aldrich (St. Louis, MO, USA). [^{3}H]-D-aspartate (Specific activity16.5 Ci/mmol, 1 mCi/mL) was obtained from Perkin Elmer (Boston, MA, USA). DEDTP (99.9% purity) was dissolved in 100% of DMSO.

2.3. Cell culture and stimulation protocol

Primary cultures of cerebellar Bergmann glial cells were prepared from 14-day-old chick embryos as previously described (Ortega et al., 1991). Cells were plated in 24-well plastic culture dishes in Opti-MEM medium containing 2.5% fetal bovine serum, 2 mM glutamine, and gentamicin (50 mg/mL) at 37 °C under standard conditions (5% CO_2 and 95% humidity) and used on the 4th–7th day after culture.

2.4. MTT assay

Cell viability was evaluated with the MTT assay, which determines the ability of healthy cells to produce formazan after the cleavage of the tetrazolium ring of MTT (Denizot and Lang, 1986). Briefly, BGC were grown in ELISA plates and treated as follows: Control (with vehicle only), Glu 1 mM, or DEDTP-treated cells (0.1, 1, 5, and 10 μM). All wells were washed and incubated with MTT (0.5 mg/mL) for 3 h at 37 °C. The MTT medium was discarded and the cells were incubated in DMSO to dissolve the formazan aggregates. The intensity of the MTT products was determined at 570 nm with an ELISA microplate reader (Epoch, Biotek). Cell viability was calculated as: Cell Viability (%) = average OD of treated wells/average OD of control wells.

Experiments were performed in triplicate. Statistical analysis was done comparing against non-stimulated cells *via* One-way ANOVA test and Dunnett's multiple comparison test.

2.5. [^{3}H]-D-Aspartate uptake

Confluent BGC monolayers seeded in 24-well plates were washed three times to remove all non-adhering cells with 0.25 mL aliquots of solution A (25 mM HEPES-Tris, 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 33.3 mM glucose, and 1 mM NaH_2PO_4 at pH 7.4) as described previously (González and Ortega, 2000; Ruiz and Ortega, 1995). To prepare a Na^+-free solution, NaCl was replaced by choline chloride and for a Ca^{2+}-free solution, $CaCl_2$ was omitted and 500 µM EDTA was added. The stimuli were included at the indicated time periods and added simultaneously with the substrate, followed by the addition of solution A containing 0.4 µCi/mL [^{3}H]-D-Asp. The uptake was stopped by rapid aspiration of the radioactive medium and each well was rinsed with ice-cold solution A. The monolayers were solubilized with 0.1 M NaOH for 2 h at room temperature. The radioactivity associated to the solubilized suspension was determined in a Perkin Elmer B291001 scintillation counter. Assays were done by quadruplicate, and statistical data analysis was performed using a One-way ANOVA with a Dunnett's multiple comparison test.

2.6. Statistical analysis

Data are expressed as the mean (average) ± standard error (SEM). A one- or two-way analysis of variance (ANOVA) was performed to determine significant differences between conditions. When one-way ANOVA indicated significance (at the 0.05 level), a Dunnett's multiple comparison test was used to determine which conditions were significantly different; for two-way ANOVA a Bonferroni's posttest was applied. A non-linear regression test for saturation experiments was performed to calculate K_M and V_{max}. Statistical analyses were performed using the GraphPad Prism Software (La Jolla California USA).

3. Results

3.1. Cell viability

Prior to evaluate any DEDTP effect on Glu transport, it was imperative to evaluate the toxicity of this compound in our culture system. Exposure of the cultured cells to DEDTP had no significant negative effects on cell viability at any of the concentrations used (0.1, 1, 5 and 10 µM), not even after a 48 h treatment (Fig. 1C). These results indicate that cells not only are surviving to the tested DEDTP concentrations, but also that upon the presence of the OPs metabolite these cells increase their proliferation rate after 12 and 24 h (Fig. 1A, and B respectively), and their cell viability did not decrease significantly at any time. Similar results have been reported in human hepatoma HepG2 cells stimulated with Dimefox, a model OPs compound, that increased cell proliferation at concentrations between 0.01 and 100 µg/mL (Hreljac et al., 2008).

3.2. DEDTP interferes with [^{3}H-D-aspartate] uptake in BGC

The effect of the exposure to DEDTP in Glu transport activity was evaluated through the use of a [^{3}H]-D-aspartate uptake assay (Ruiz and Ortega, 1995). Of the various conditions tested (Fig. 2), it is evident that 0.1 µM DEDTP is the most effective concentration in GLAST modulation. An effect is evident after a 12 h treatment, where a 20% decrease in the uptake activity is present (Fig. 2A), effect that is reverted and even increased after 24 h (Fig. 2B), just to diminish to approximately 60% after 48 h (Fig. 2C). Note also that after 48 h, a 10 µM DEDTP concentration is also capable to reduce the uptake activity.

Since these results were obtained using a fixed 24 nM [^{3}H]-D-Asp concentration, we decided to establish GLAST kinetic parameters upon a 0.1 µM DEDTP concentration for 12 and 24 h (Fig. 3A, and B, respectively). After DEDTP stimulation, alterations in GLAST activity could occur through changes in K_M, which refers to affinity of

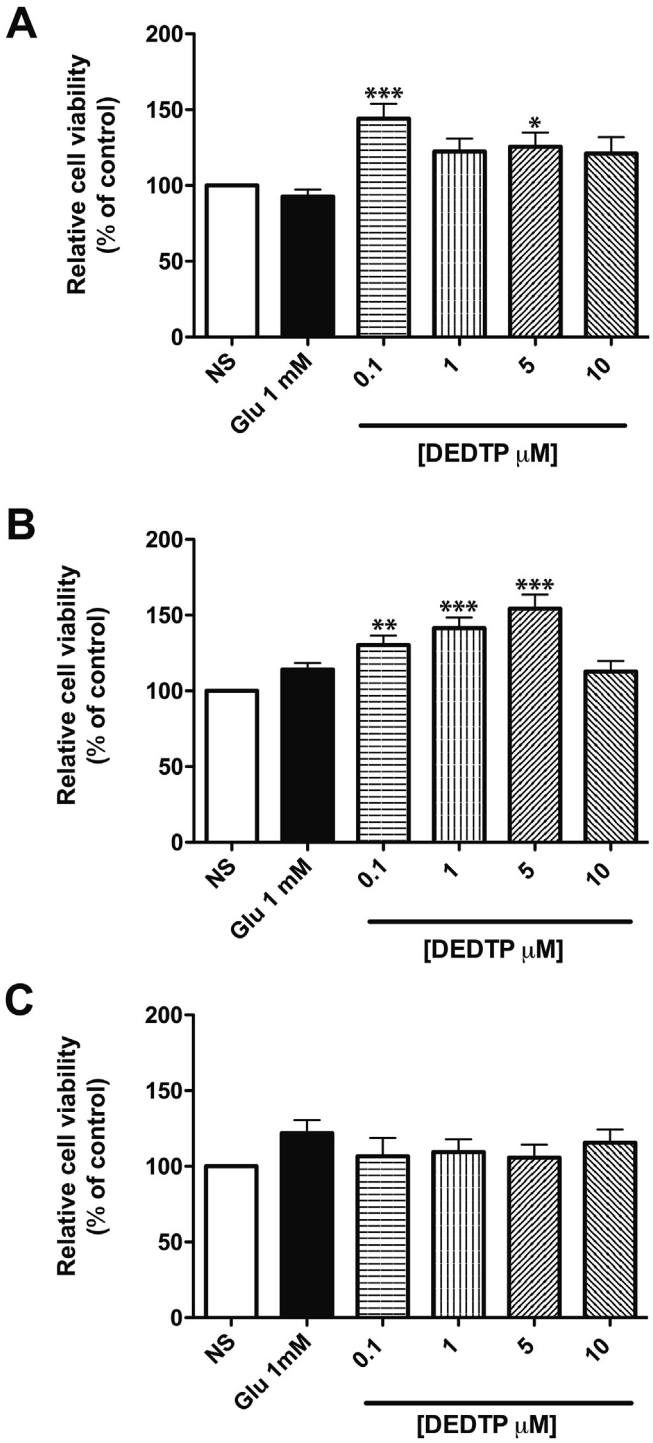

Fig. 1. Cell viability. Bergmann glia cells were treated with vehicle (non-stimulated, NS), 1 mM Glu for 30 min, or increasing DEDTP concentrations (0.1, 1, 5 and 10 µM) for different time periods: 12 h (**A**), 24 h (**B**) or 48 h (**C**). Thereafter, the cells were treated with the MTT reagent. The optical density of the samples was determined to be 570 nm in a plate reader and reported as a percentage of the control (cells not stimulated with DEDTP). Data are expressed as mean ± SEM from at least three independent experiments, each tested in quadruplicate. One-way ANOVA with a Dunnett's multiple comparison test were performed to analyze the data (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

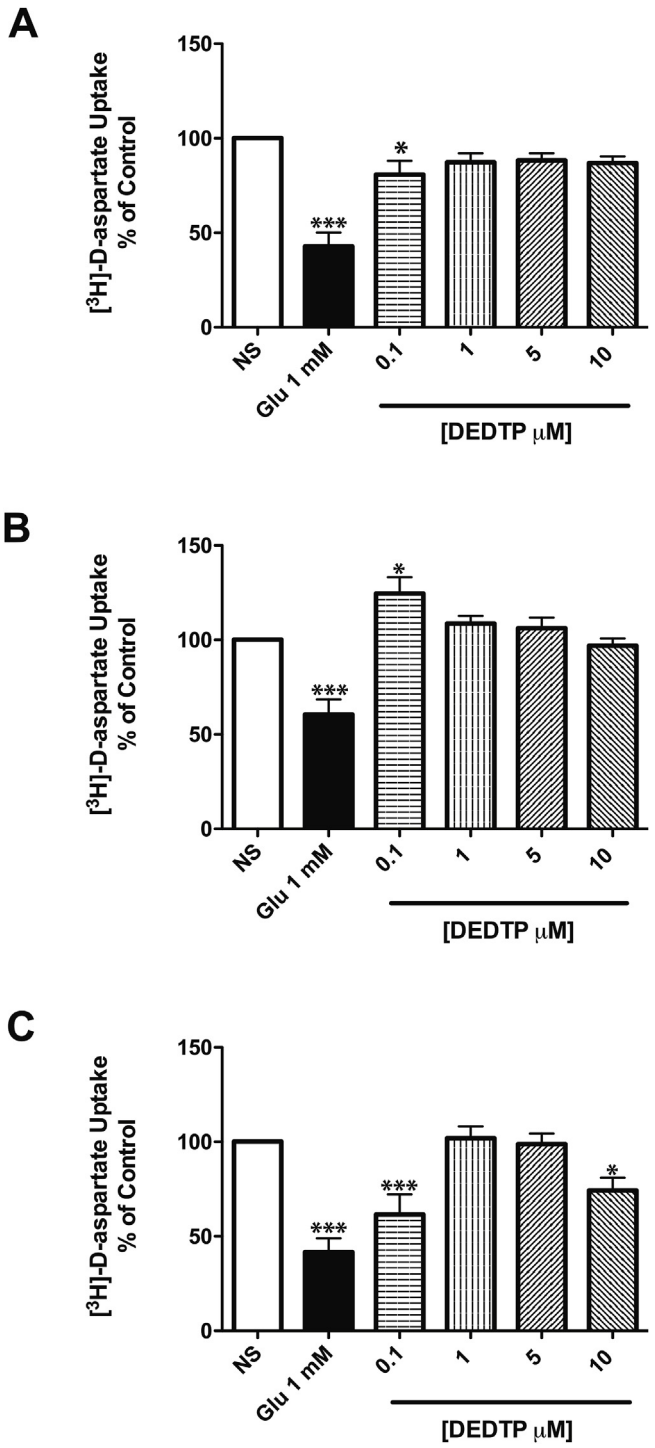

Fig. 2. DEDTP regulates [^{3}H]-D-Aspartate uptake activity. Confluent Bergmann glia cells were stimulated with vehicle (non-stimulated, NS), 1 mM Glu for 30 min, or increasing DEDTP concentrations (0.1, 1, 5 and 10 μM) for different time periods: 12 h (**A**), 24 h (**B**) or 48 h (**C**). The [^{3}H]-D-Aspartate uptake assay was performed for 30 min. Data are expressed as mean ± SEM from at least three independent experiments, each tested in quadruplicate. Statistical analysis was performed using a One-way ANOVA with a Dunnett's multiple comparison test; ***p < 0.001, **p < 0.01, *p < 0.05.

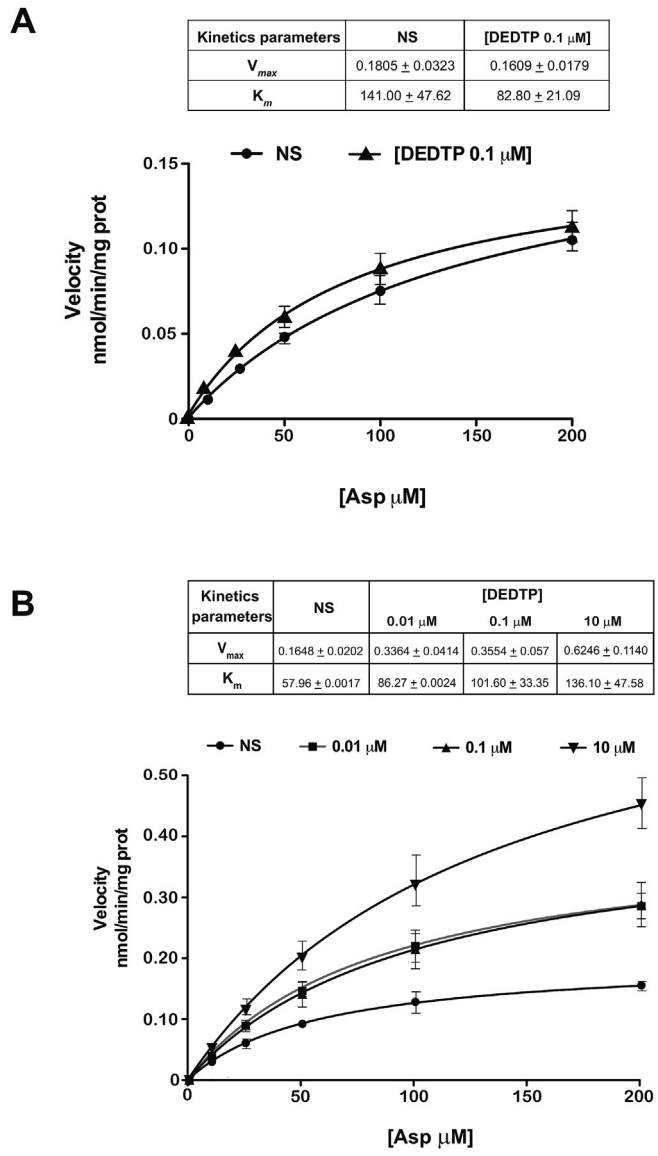

Fig. 3. DEDTP modifies GLAST kinetic parameters. Confluent Bergmann glia cells monolayers were exposed to vehicle (non-stimulated, NS) or 0.1 μM DEDTP for 12 h (**A**) or 24 h (**B**). The [^{3}H]-D-Asp uptake assay was performed with increasing non-labeled D-Asp concentrations (0, 10, 25, 50, 100 and 200 μM) for 30 min. Data are expressed as mean ± SEM from at least three independent experiments, each assayed in quadruplicate. Transport kinetics V_{max} (nmol/min*mg) and K_M (μM) were determined by non-linear regression using the GraphPad Prism Software (La Jolla California USA).

transporters, and/or in V_{Max}, which denotes changes in the rate of GLAST transport. A slight increase in V_{Max} was present after a 12 h DEDTP treatment, suggesting a plausible increase in plasma membrane GLAST transporters probably due to a direct interaction between DEDTP and these transporters, as has been reported for distinct compounds (Fumagalli et al., 2008; Susarla and Robinson, 2003). When the kinetic parameters were determined after a 48 h exposure, a significant increase in V_{Max} was detected for all the DEDTP concentrations used (Fig. 3B).

In order to gain insight into the complex effects of DEDTP on GLAST-mediated transport, we decided to explore if DEDTP would modify the well-characterized Glu-dependent decrease in [^{3}H]-D-Asp uptake activity (González and Ortega, 2000). For this purpose, we pre-incubated Bergmann glia cultures with 0.1 μM DEDTP for 12 (Fig. 4A), 24 (Fig. 4B), and 48 h (Fig. 4C), and on these cultures assayed the reported Glu decrease in [^{3}H]-D-aspartate uptake. Although a non-linear response is present upon DEDTP as a function of time, it is quite clear that the dialkyl compound triggers distinct signaling pathways that modify the Glu response depending on the time of exposure. After 12 h (Fig. 4A) it significantly decreases [^{3}H]-D-Asp transport, while after

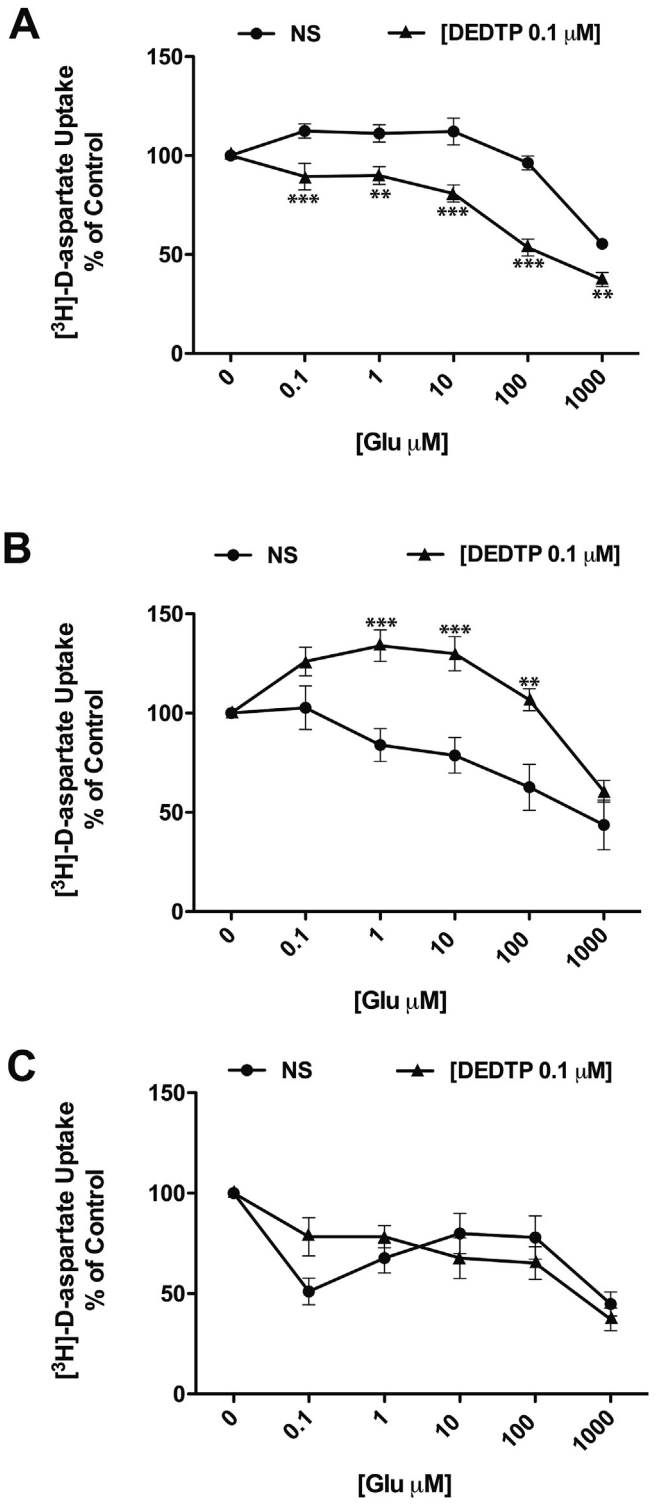

Fig. 4. DEDTP and Glu regulate GLAST activity. Confluent Bergmann glia monolayers were pre-incubated with 0.1 μM DEDTP for 12 h (**A**), 24 h (**B**) or 48 h (**C**). Thirty minutes before finishing the incubation time with DEDTP, different Glu concentrations were added (0.1, 1, 10, 100 and 1000 μM). The [^{3}H]-D-Asp uptake assay was performed for 30 min. Data are expressed as mean ± SEM from at least three independent experiments, each tested in quadruplicate. Statistical analysis was performed using a Two-way ANOVA and Bonferroni's post-hoc test; ***p < 0.001, **p < 0.01, *p < 0.05. Non-stimulated cells (NS) were pre-incubated with only vehicle (negative control), glutamate was not added to the cells before the uptake assays.

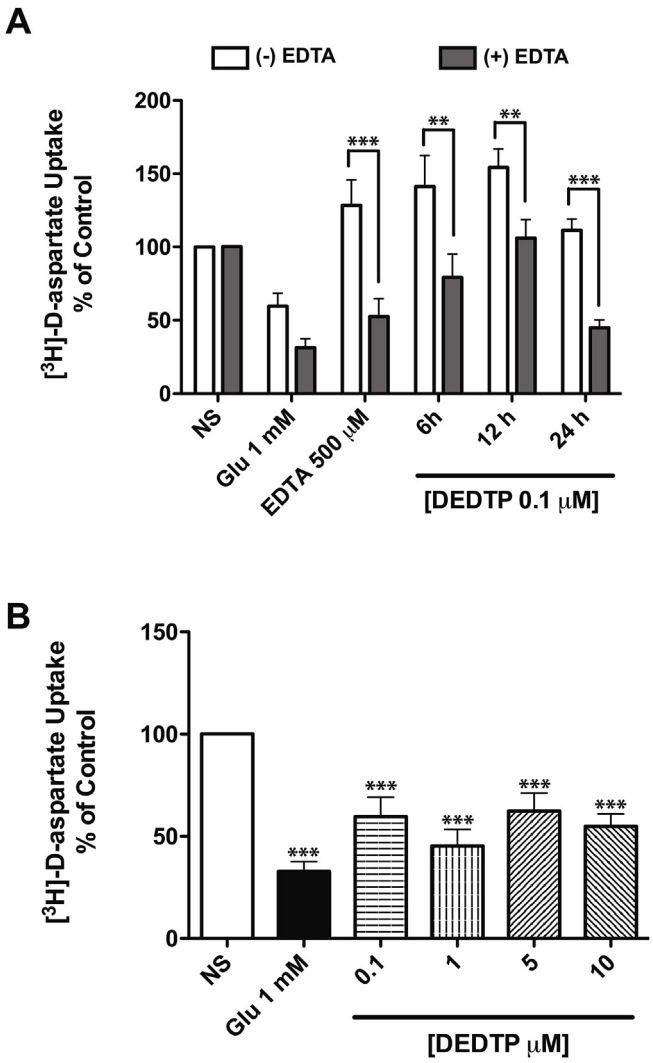

Fig. 5. Ca^{2+} dependence of the DEDTP effect in Bergmann glia cultures. **A:** Confluent monolayers were treated for 6, 12 and 24 h with a fixed 0.1 μM DEDTP concentration. The media was removed and the cells exposed for 30 min to 1 mM Glu, 500 μM EDTA and 0.1 μM DEDTP as indicated in the graph. [^{3}H] D-Asp uptake was started by the addition of the radioactive solution A containing EDTA and DEDPT as indicated. The uptake was stopped after 30 min. NS = Non-stimulated cells. **B:** Cells were treated for 12 h with different DEDTP concentrations (0.1, 1, 5 and 10 μM) in Na^+ free assay buffer. The [^{3}H]-D-Asp uptake assay was performed for 30 min. Data are expressed as mean ± SEM from at least three independent experiments, each tested in quadruplicate. **A,** statistical analysis was performed using a Two-way ANOVA and Bonferroni's post-hoc test, ***p < 0.001, **p < 0.01, *p < 0.05; **B,** data statistical analysis was achieved using One-way ANOVA and Dunnett's multiple comparison test, ***p < 0.001, **p < 0.01, *p < 0.05.

24 h (Fig. 4B) it augments the uptake. These results are favor the notion of a different and probably deteriorated uptake activity that without any doubt affects proper glutamatergic transmission.

3.3. Role of Ca^{2+} influx on the DEDTP response

As a preliminary step into the characterization of the signaling cascades involved in DEDTP toxic effects in BGC, we decided to explore the involvement of Ca^{2+} ions, given the importance of this second messenger in the signaling transactions triggered by Glu in these cells (reviewed in Martínez-Lozada and Ortega, 2015). As depicted in Fig. 5A, the removal of Ca^{2+} from the media prevents the DEDTP effect. BGC pre-treated with the Ca^{2+} chelator, display a significant decrease

[^{3}H]-D-Asp uptake after 6, 12 and 24 h compared to the cells treated in complete medium most probably due to the reported GLAST-dependent Ca^{2+} influx, through the Na^+/Ca^{2+} exchanger (Fairman et al., 1995; Kirischuk et al., 1999; Martínez-Lozada et al., 2013). As expected, when the [^{3}H]-D-Asp uptake is carried out in a Na^+-free medium, no increase in the transporter activity is present (Fig. 5B). These results suggest that the exposure of BGC to this toxic activates a biochemical mechanism that is linked to a relevant Ca^{2+} influx, opening the possibility of a diverse molecular mechanism of action of DEDTP in the cerebellum.

4. Discussion

It has been traditionally assumed that the neurotoxic effects of OPs are restricted to the well-characterized the inhibition of acetylcholinesterase (AChE). Nevertheless, the defined cholinergic pathways in the brain cannot explain the damage to other neurotransmitter systems that is reflected in the reported attention-deficient disorder (Yu et al., 2016). It has been reported that Bis(isopropyl methyl)phosphate (BIMP) and Bis(pinacolyl methyl)phosphonate (BPMP), two synthetic OPs derived from sarin (isopropyl methylphosphonofluoridate) and soman (pinacolyl methylphosphonofluoridate), can induce extracellular regulated kinase (ERK) and N-terminal c-Jun kinase (JNK) phosphorylation *via* the Ca^{2+}/diacylglicerol-dependent protein kinase (PKC) phosphorylation in a phospholipase C γ (PLC-γ) dependent manner in rat brain (Niijima et al., 1999). A study by Dam et al. (2003) characterized an augmentation of p53 mRNA levels in the rat cerebellum during the peak of granular cells neurogenesis after OPs (chlorpyrifos) exposure. Chlorpyrifos also induces phospho-ERK and phospho-p38 MAPK mediated apoptosis in mouse cortical neurons (Caughlan et al., 2004).

The sources of exposure to DEDTP are diverse due to its wide commercial use as a fuel additive, for the extraction of trace elements, as bath and metal cleaners, for drains, rust removers, batteries, lubricants, in metal refining, plumbing, fertilizers production, chemical reagents, petroleum additives, flame retardants, and initiators in the polymerization of artificial nails (Ferreira et al., 2007; Hamawand et al., 2013; Marklund et al., 2005; Ramos et al., 2012; Wexler, 1995). Non-occupationally exposed population is in chronic and close contact with DEDTP, as demonstrated by several studies that report elevated urine DAP levels (Barr et al., 2004; Griffith and Duncan, 1985). For example, in Italy DEDTP levels have been reported in 0.02 μg/g (Aprea et al., 1996), in Germany a concentration of 0.02–0.09 μg/g of DEDTP was found in the urine of children and adults (Heudorf and Angerer, 2001), and in Israel 0.02 μg/g DEDTP was reported in urinary samples from urban and rural population (Berman et al., 2013). Limited information about the tissue distribution of OPs compounds is available, and particularly in humans, we could only find a single report of a Japan case study where 0.0081 mg of chlorpyrifos-methyl was reported in brain tissue (Moriya et al., 1999). In the present work, DEDTP concentrations used were from 0.00186 mg (0.01 μM) to 1.86 mg (10 μM) and evaluated for a plausible BGC cytotoxicity. In fact the concentrations used are similar those reported in the cerebrospinal fluid of 46.5 kg male individual who ingested a dichlorvos, a OP insecticide (Moriya et al., 1999). Occupational risk of Brazilian agricultures has been evaluated too according to the physico-chemical characteristics of thirty nine OPs insecticides, and the distribution of these compounds in eight tissues was calculated; the highest concentrations were found in the adipose tissue contrast to the kidney, that had the lowest (Paraíba et al., 2009). It has also been demonstrated that in occupationally exposed women to pesticides during the first months of pregnancy, a negative impact on the embryonic neurodevelopment is present, resulting in decreased language function, long-term memory, manual motor speed, and autism risk (Andersen et al., 2012; Philippat et al., 2018). Besides, increased DAP levels are associated to hyperactivity and attention deficit disorders (Bouchard et al., 2010; Jokanović and Kosanović, 2010), behavioral changes (Salvi et al., 2003), impairment of memory and learning (Tan et al., 2009; Voorhees et al., 2017), dystonia and resting tremor (Kamel and Hoppin, 2004), seizures (Tattersall, 2009), among others. In mammals, the neuronal damage by OPs compounds have been related to an increased BBB permeability in a dose-response relationship, due to easy access in some regions of the brain like the cerebral cortex and the hippocampus (Abdel-Rahman et al., 2002; Sinha et al., 2004). BBB is essential for CNS osmolality and normal function, and exposure to higher doses of dialkyl compounds increase its permeability, probably as a consequence of the inhibition of acetyl cholinesterase. Interestingly, changes in BBB permeability are likely to be both age and species specific (Sinha et al., 2004; Sinha and Shukla, 2003). Therefore, the extrapolation to humans of the results present in animal models is difficult, even if mechanisms of action operate similar fashion.

In this context, we decided to explore the possibility that DEDTP could affect the major excitatory system of the CNS. Moreover, we challenged the notion of a neurotoxic that could affect glial cells. We took the advantage that in glutamatergic synapses, the removal of the transmitter is carried out mostly in the glial compartment (Danbolt, 2001). The proper recycling of Glu depends on its biotransformation to glutamine and its release to the vicinity of the presynaptic terminal that uptakes it up to metabolizes it back to glutamate that is packed in

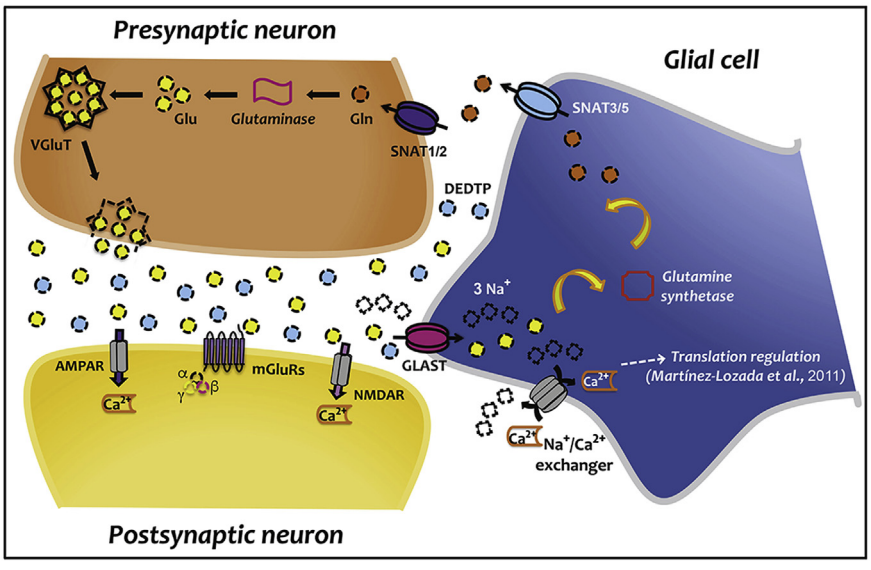

Fig. 6. Current model of the DEDTP effects in cultured Bergmann glial cells. Through the activation of glutamatergic receptors and transporters expressed in glial cells, cells sense synaptic activity and regulate their protein repertoire *via* transcriptional and translational control, also the localization of specific proteins in a given cell compartment is regulated. In the presynaptic neuron, glutamine (Gln) is converted to glutamate by *glutaminase* and packaged into synaptic vesicles (VGluT). After its release into the extracellular space, glutamate binds to its ionotropic (NMDAR and AMPAR) and metabotropic receptors (mGluRs) in the membranes of postsynaptic neurons and surrounding glial cells. Glutamate is cleared from the synaptic space by GLAST. Glutamate uptake leads to Na^+ influx, which activates the Na^+/Ca^{2+} exchanger increasing intracellular Ca^{2+} levels, resulting in the GLAST translational regulation. Within the glial cell, Glu is converted to Gln by *Glutamine synthetase* and the Gln is subsequently released by SNAT3/5, and taken up by neurons through SNAT1/2 to complete the Glu-Gln cycle. DEDTP disrupts glutamate recycling and by these means eventually glutamatergic transmission.

synaptic vesicles in what has been known as the *glutamate/glutamine* shuttle (Shank and Campbell, 1984). Disruption of this shuttle compromises glutamatergic transmission, scenario common to several neurodegenerative diseases (Murphy-Royal et al., 2017).

A detailed analysis of our results shown herein cannot allow us to report that the exposure of cultured Bergmann glia cells to DEDTP results in a dose-dependent modulation of GLAST function. A non-linear behavior was present throughout the different aspects of the transporter activity that we analyzed. Therefore, we decided to focus in a known and characterized function of Bergmann glia: Glu turnover; since these cells completely wrap the synapses established between the parallel fibers and the Purkinje cells (PF-PC synapse) in the cerebellar molecular layer (Somogyi et al., 1989). Taking into consideration that this synapse represents a point of convergence of millions of signals, Glu turnover and metabolism has to be tightly regulated (Zhou and Danbolt, 2014). If a neurotoxin would be deleterious to the cerebellar circuit then it might be possible that it would disrupt Glu-dependent GLAST regulation, as it has been reported for others toxins (Razafimanjato et al., 2011). The result presented in Fig. 4 demonstrate that indeed this is the case.

The lack of dose-dependency of cholinesterase inhibition by DAPs has been reported and has hampered its use as a marker of exposure to OPs (Sudakin and Stone, 2011); however, this does not demonstrate that it is safe for neurons. DEDTP is likely capable to disrupt plasma membrane protein complexes, such as the one formed by GLAST with other transporters like the sodium-dependent neutral amino acid transporter 3 (SNAT 3) (Martínez-Lozada et al., 2013), the Na^+/K^+ ATPase (Robinson and Jackson, 2016), and the cystine/glutamate antiporter (Suárez-Pozos et al., 2017) leading to an impairment of GLAST activity and most possibly glutamatergic transmission.

Work currently in progress in our laboratory is aimed to the characterization of the effect of DEDTP on GLAST protein-protein interactions and into a plausible regulation of its gene expression. A summary of our interpretation of DEDTP is presented in Fig. 6.

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Conflicts of interest

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in this manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuint.2018.06.004.

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