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Research Report

5-Aminolevulinate and 4, 5-dioxovalerate ions decrease $GABA_A$ receptor density in neuronal cells, synaptosomes and rat brain

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Abbreviations:
ALA, 5-aminolevulinic acid
CSF, cerebrospinal fluid
DOVA, 4,5-dioxovaleric acid
NF, neurofilament
SA, succinylacetone
SAME, succinylacetone methyl ester
GABA, γ-aminobutyric acid
RA, retinoic acid

ABSTRACT

Porphyrias are heme-associated metabolic disorders such as intermittent acute porphyria (IAP) and lead poisoning, where 5-aminolevulinate (ALA) accumulates. Effects of ALA on the CNS have been explained by ALA binding to $GABA_A$ receptors, followed by receptor lesions from oxyradicals and 4, 5-dioxovalerate (DOVA) generated from metal-catalyzed ALA oxidation by oxygen. We have characterized the effects of ALA and DOVA on $GABA_A$ receptor density in synaptosomes and neurons in vitro and also in brains of rats treated with ALA or succinylacetone methyl ester (SAME), a tyrosine catabolite derivative able to induce ALA accumulation. Radiolabeling assays revealed that following exposure to DOVA the concentration of synaptosomal GABAergic sites decreased by approximately 50%. Pretreatment with DOVA resulted in less $GABA_A$ receptor density in P19 and WERI cells and altered cell morphology. Furthermore, exposure to DOVA also induced a 5-fold increase in WERI cell mortality rate. Treatment with ALA resulted in loss of neuronal morphology and decrease of $GABA_A$ density in P19 neuronal cells. ALA and SAME treatment diminished the density of GABAergic receptors in the habenular complex and the parabigeminal nucleus of rat brain as studied by immunohistochemical procedures. Our results strongly suggest that ALA- and DOVA-promoted damage to $GABA_A$ receptors may contribute to the neurological manifestations of AIP and plumbism.

1. Introduction

5-Aminolevulinic acid (ALA) is a heme precursor that accumulates in hereditary porphyrias such as acute intermittent porphyria (Hindmarsch, 1986), chemical porphyrias (e.g., lead poisoning) (Gurer and Ercal, 2000), and tyrosinosis (Berger et al., 1983). It has been proposed that the neuropsychiatric manifestations of these diseases, which include mood disorders, aggressiveness, hallucinations, convulsions and seizures (Sassa and Kappas, 1983), are associated with

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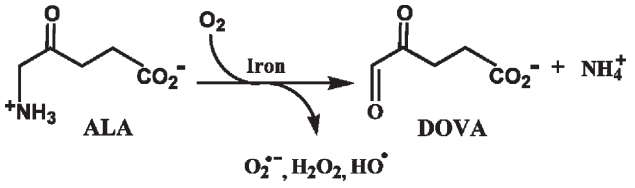
accumulation of ALA (Kappas et al., 1995) due to enzymatic deficiencies in the heme biosynthetic pathway (Sassa and Kappas, 1983). Although the mechanism through which ALA is transported to the brain is uncertain, there are reports that accumulation of ALA in the brain may occur through passive diffusion between the blood/brain barrier (Ennis et al., 2003). Moreover, recent evidence for the existence of specific active transport of peptides (PEPT2) in cortical synaptosomes has been shown (Fujitaa et al., 2004). It is very likely that ALA is transported through that system, as for ALA has been shown to be a substrate for almost identical transporters (Ocheltree et al., 2004). In the choroid plexus, PEPT2 transporter contributes to the clearance of ALA from the CSF into the blood, thus keeping ALA concentrations low in the CSF (Ocheltree et al., 2004). This finding suggests that the brain has regular mechanisms to avoid ALA accumulation by actively keeping low levels of that heme precursor through several peptide transporters, in particular PEPT2, within the CNS. To date, some previous studies have shown that ALA is produced in the brain (Kappas et al., 1995; DeMatteis et al., 1981) and that cerebral cortex can accumulate ALA (Juknat et al., 1995). Thus, one can speculate that ALA levels in the compartmentalized brain tissue may actually reach millimolar range (Kappas et al., 1995), although further clinical studies are needed to provide better evidence of the brain concentrations of ALA in health and disease. Nevertheless, ALA increase in the brain can be viewed as a pathological sign and underscores its likelihood of playing a central role to the development of the neuropsychiatric manifestations in porphyrias.

It has been shown that ALA accumulation induces convulsions (Emanuelli et al., 2000), for ALA toxicity also involves glutamatergic pathways, since ALA irreversibly inhibits glutamate uptake in astrocytes through inhibition of the GLT-1 glutamate transporter (Emanuelli et al., 2003). Moreover, ALA damages $GABA_A$ receptors (Demasi et al., 1996), and acts as a GABA antagonist (Pierach and Edwards, 1978), besides stimulating glutamate release (Brennan and Cantrill, 1979). This suggests that ALA may be involved in neuronal cell death due to calcium influx (Choi, 1994).

ALA was suggested to behave as a pro-oxidant because it undergoes an iron-catalyzed oxidation by oxygen (Monteiro et al., 1986) at physiological pH with production of reactive oxygen species and 4,5-dioxovaleric acid (DOVA) (Scheme 1). Previous reports indicate that ALA may disrupt $GABA_A$ receptor function through oxyradicals formed during ALA oxidation (Demasi et al., 1996). ALA may indeed play a significant role in the neuropsychiatric manifestations of these syndromes, considering that it promotes release of iron from ferritin (Oteiza et al., 1995), thus exacerbating oxidative injury.



Scheme 1 – Aerobic oxidation of ALA catalyzed by iron with generation of reactive oxygen species and DOVA.

Considering the reactivity of DOVA towards biomolecules (Di Mascio et al., 2000), and that ALA accumulation would lead to DOVA formation, we hypothesize direct toxicity of DOVA in these syndromes. The aim of this work was to determine whether DOVA affects $GABA_A$ receptor density and to further study ALA and DOVA neurotoxicity in vitro and in vivo. These studies employed ALA and DOVA to investigate their effects on $GABA_A$ receptor density on WERI human retinoblastoma and differentiated neuronal cell line P19. Succinylacetone methyl ester (SAME) and ALA-treated rats were used for the in vivo studies. Succinylacetone is a potent inhibitor of 5-aminolevulinate hydrolyase (K_i = 0.03 μmol/L, EC 4.2.1.24), as previously demonstrated (Berger et al., 1983), and hence is able to induce ALA accumulation and mimic the metabolic state observed in the aforementioned illnesses.

2. Results

2.1. ^{3}H-muscimol binding in synaptosomes

To identify effects of DOVA on $GABA_A$ receptors, a binding assay was performed with synaptosomes using the $GABA_A$ agonist ^{3}H-muscimol (Fig. 1). Preliminary experiments were conducted to find the range of ^{3}H-muscimol concentrations in which nonspecific binding was lowest (0 to 25 nM) (data not shown). The pretreatment of synaptosomes with 2 mM DOVA for 30 min at 4 °C resulted in a 50% decrease of GABAergic binding sites. As the synaptosomal preparation was thoroughly washed five times to completely remove DOVA, the observed decrease in the number of GABAergic binding sites is not likely to arise from competition of DOVA with ^{3}H-muscimol. In fact, the change in the number of binding sites was used by chemical damage to the receptor, resulting in loss of ligand binding. Moreover, after DOVA treatment the shape of the curve, (as its derivative) did not change, thus it can be concluded that the binding affinity or dissociation constant (K_d) of the receptor did not change (Bylund and Yamamura, 1988).

The Rosenthal's plot obtained in this assay was nonlinear, indicating that DOVA may interact with more than one GABAergic site or with different membrane sites (Bylund and Yamamura, 1988).

2.2. $GABA_A$ receptor immunolabeling of WERI and P19 cells

In an attempt to determine whether ALA and DOVA-driven toxicity in synaptosomes to GABAergic receptors could be reproduced in $GABA_A$ receptor-expressing cell cultures, we chose human retinoblastoma WERI cells as in vitro models for $GABA_A$ receptor expression (Chou et al., 1999), by evaluating cell viability and morphology after exposure to ALA and DOVA.

Following confirmation of $GABA_A$ receptor expression, WERI cells were treated with ALA or DOVA (1 and 10 mM). Exposure to 1 mM ALA did not alter GABAergic receptor density in WERI cells. Treatment of WERI cells with DOVA (1 and 10 mM) and ALA (10 mM) altered cell morphology (Fig. 2), as the cells ceased to grow in the grape-like aggregates observed in control samples. Images of WERI cells after immunocytochemical staining (Fig. 3E) showed that exposure

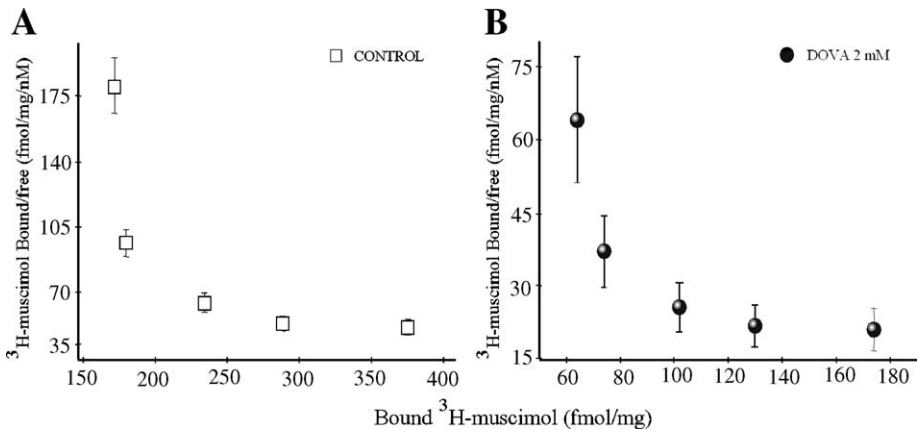


Fig. 1 – ^{3}H-muscimol binding in synaptosomes exposed to DOVA. Synaptosomes were incubated with (A) O.1 M phosphate buffer or (B) DOVA 2 mM at 4 °C in 10 mM Tris–HCl buffer, pH 7.35, for 30 min. Data are plotted as the mean ± SD of three independent experiments. Total binding was obtained for ^{3}H-muscimol (0 to 25 nM) and the nonspecific binding was evaluated in the presence of 1 mM GABA. Note that the values in the axis of the plot (B) (2 mM DOVA) are approximately half of those in plot (A) (phosphate buffer). This indicates that exposure to DOVA reduced the pool of GABAergic receptors by 48%.

to 10 mM DOVA resulted in a loss of $GABA_A$ receptor expression in the cell membrane, as well as possible receptor clustering (Fig. 3E). DOVA was also found to dramatically decrease cell viability (Fig. 4).

After observing an increase in mortality in WERI cells, we wanted to verify if the same toxicity would be observed in differentiated cells sharing fundamental neuronal properties. Hence, similar experiments were carried out with differentiated P19 cells, which express fully functional $GABA_A$ receptors (Lin et al., 1996). As DOVA 1 mM was already shown to increase WERI cell mortality (Fig. 4), P19 cells were not treated with ALA and DOVA concentrations higher than 1 mM. The success of neuronal differentiation of P19 cells was verified by the detection of neuron specific proteins NF 160 and NF 200 (data not shown) and neuronal morphology. Images were collected after P19-N cells at 12 DIV had been exposed to ALA

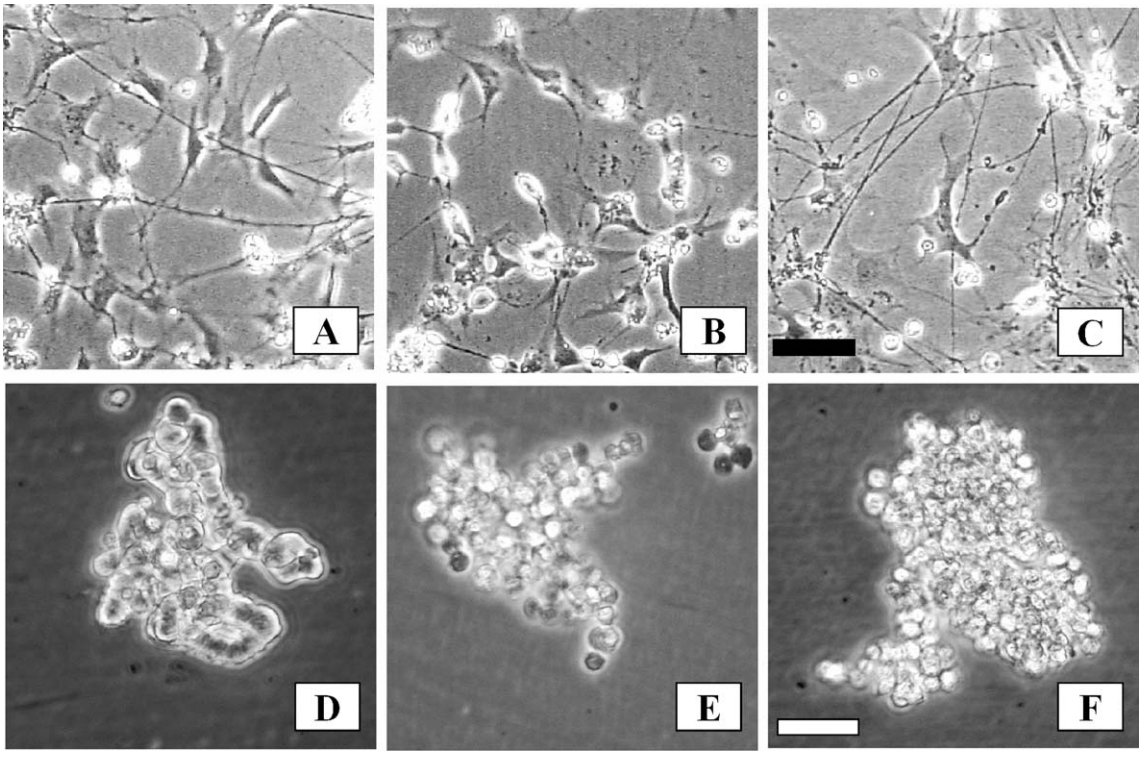


Fig. 2 – Digital images of P19-N and WERI cells exposed to ALA or DOVA. P19 EC cells (12 DIV) treated with (A) phosphate buffer, (B) 1 mM ALA and (C) 1 mM DOVA for 48 h. Morphological changes in WERI cells were obtained in DMEM medium supplemented with 10% FBS exposed to (D) phosphate buffer, (E) 10 mM ALA, and (F) 1 mM DOVA. All cells were grown at 37 °C with humidity and 5% CO2. The images are representative of 3 independent experiments. White scale bar = 25 μm, black scale bar = 50 μm.

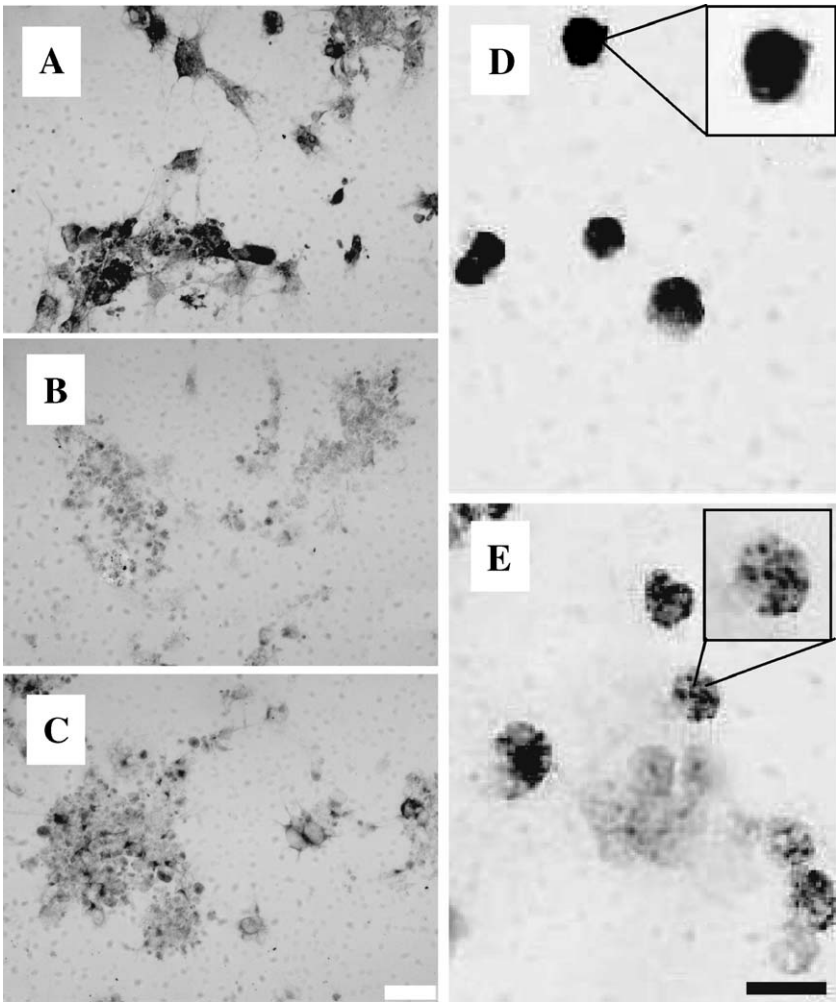


Fig. 3 – Immunocytochemical $GABA_A$ staining of WERI and P19-N cells at 14 DIV. P19 cells were exposed to: (A) phosphate buffer, (B) ALA 1 mM, or (C) DOVA 1 mM. WERI cells were incubated with: (D) phosphate buffer, (E) 10 mM DOVA. Cell cultures were treated with ALA or DOVA for 48 h. Inserts in panels D and E suggest possible clustering of $GABA_A$ receptors in WERI cells treated with 10 mM DOVA because these possess immunoreactive patches, instead of the homogeneous staining observed in control cells. All chemicals added to cell cultures were from fresh stock solutions. Images are representative of 3 experiments. White scale bar = 50 μm, black scale bar = 12 μm.

and DOVA at 1 mM concentrations for 48 h and $GABA_A$ receptor β subunit expression was detected by immunohistochemistry (Fig. 3). These data suggest that ALA and DOVA decrease $GABA_A$ receptor density and alter neuronal morphology. Images of differentiated P19 cells in culture bottles taken after treatment with 1 mM ALA or DOVA (Fig. 2) also show morphological modification such as a decrease in the density (40%) (Table 1). ALA and DOVA also induced, respectively a 4- and 3-fold decrease (Table 1) in the average length of cytoplasmic processes of P19 cells. These measurements are in agreement with the morphological changes apparent in the images of differentiated P19 cells (Fig. 2A, B and C), after exposure to ALA and DOVA 1 mM.

2.3. *Cell viability assays*

Exposure to ALA (10 mM) and DOVA (1 and 10 mM) altered WERI cell morphology, indicating damaged and possibly necrotic cells (Fig. 2). A Trypan Blue staining experiment was performed to assess WERI cell viability after treatment with ALA and DOVA (Fig. 4). The results show that exposure to 1 mM ALA, 1 mM and 10 mM DOVA for 48 h increased cell mortality 1.4-, 5.3- and 9.3-fold, in comparison with untreated control cells, respectively.

Identical experiments were conducted with P19 embryonic carcinoma cells undergoing neuronal differentiation to verify if the results observed with WERI cells were limited to this lineage. Control experiments were carried out with undifferentiated embryonal P19 carcinoma cells, in order to exclude ALA and DOVA toxicity to cells not expressing GABA receptors. Even though DOVA and ALA decrease $GABA_A$ receptor density in P19 neurons, an increase in cell mortality was not detected by using Trypan Blue staining, both in P19 neurons and P19 undifferentiated cells (Fig. 4). Basal mortality rates of P19 neurons (25%) similar to those observed in this experiment have been reported elsewhere (Ninomiya et al., 1997).

The main objective of the experiments conducted with these cell lines (WERI and P19) was to identify possible effects of ALA

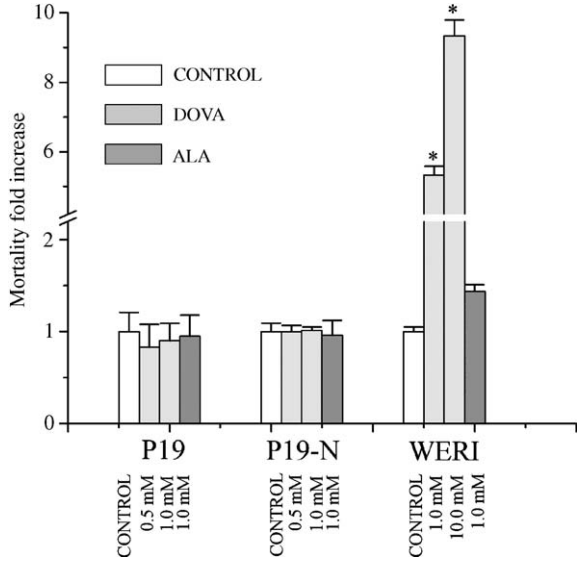


Fig. 4 – WERI and P19 cell viability after treatment with ALA and DOVA. WERI retinoblastoma and P19 cell mortality were determined by the Trypan Blue exclusion method, after exposure to 1 or 10 mM ALA and DOVA for 48 h. The results are the mean ± SD of at least three independent experiments, and were normalized according to the mortality rates of the control samples, which are 9, 26 and 30%, in WERI, P19 and P19-N cells, respectively. Similar basal mortality rates have been reported previously (Ninomiya et al., 1997) for P19-N cells. * Indicates a significant difference from controls at the $P < 0.001$ level.

and DOVA on cell viability and $GABA_A$ receptor distribution and expression. Therefore, $GABA_A$ receptor expression could not be quantified in these cells given their high intrinsic heterogeneity of their $GABA_A$ receptor expression immunoreactivity (Fig. 3A). Nevertheless, the observed effects of ALA and DOVA on $GABA_A$ receptors have been quantified and confirmed both in vitro with synaptosomes and later in vivo in rat brain.

2.4. *Rat treatment with SAME and ALA*

The effect of intraperitoneal injections of SAME and ALA into rats on $GABA_A$ receptor density in the CNS was assessed through immunohistochemistry of the receptors in the brains of rats. In these experiments animals treated with either ALA or SAME for 30 and 15 days. $GABA_A$ receptor density was found to be significantly decreased in different regions of the brain, such as the habenular complex and the parabigeminal nucleus (Figs. 5 and 6). This effect was not restricted to these regions, since it was also observed in other GABAergic nuclei, such as the supraoptic nucleus in the hypothalamus and the mesencephalic reticular formation (data not shown). Treatment for 30 days with ALA or SAME did not significantly increase these effects (results not shown). As increased mortality rates were observed in WERI, but not in P19 cells, our data do not suggest that ALA and its derivatives induce unspecific widespread cell death. Furthermore, the observed loss in $GABA_A$ receptor labeling in the brain is unlikely to be a result of great neuronal depletion by necrosis or apoptosis, but rather, a consequence of decreased availability of functional receptors by mechanisms yet not well understood.

Rats were not treated with DOVA because due to its high reactivity with proteins and thiols, it is unlikely that DOVA would reach the brain if injected intraperitoneally. Nevertheless, it has been reported that ALA accumulates in the CNS (Juknat et al., 1995), where it is expected to generate DOVA by oxidation and deamination, emphasizing the relevance of studying DOVA toxicity in vitro.

3. Discussion

Previous reports showed that ALA may induce iron-mediated oxidative stress in brain and cause damage to $GABA_A$ receptors, as suggested by the 2-fold increase in the dissociation constant of ^{3}H-muscimol binding to $GABA_A$ receptors in cortical synaptic membranes of rats treated with ALA (Demasi et al., 1996).

DOVA exhibits significant toxicity towards $GABA_A$ receptors. This hypothesis is supported by the both in the binding experiment with synaptosomes (Fig. 1) and in the immunocytochemical assays with P19 neurons and WERI cells (Fig. 3), where a decrease of GABAergic sites was observed. The observed decrease in GABAergic receptor density, which was induced by ALA in P19 neuron-like cells (Fig. 3) and in rat brain (Fig. 6), may lead to increased activity of excitatory pathways, which, in turn, are associated with high incidence of seizures and convulsions (Kappas et al., 1995; Obrenovitch and Urenjak, 1997), a common symptom in patients suffering from porphyrias (Kappas et al., 1995).

The results of the binding experiments of this work (Fig. 1) and of previous reports (Demasi et al., 1996) clearly show the differences between DOVA and ALA-originated damage to $GABA_A$ receptors in synaptosomes: while the former decreases the receptor pool, the latter increases the receptor-^{3}H-muscimol dissociation constant (Demasi et al., 1996). It is tempting to propose that ALA- and DOVA-driven damage to GABAergic pathways is likely to take place in these patients, since this effect was observed in vitro as well as in vivo in

Table 1 – Length and density of P19-N cell processes after treatment with 1 mM ALA or DOVA

Cell parameters	CONTROL	1 mM DOVA	1 mM ALA
Density (crossings/1 × 10^4 μm^2)	12.6 ± 1.4	4.1 ± 0.5	2.7 ± 0.4
Length (μm)	187.5 ± 20.9	117.8 ± 13.6	122.4 ± 11.3

Average length and density (number of crossings) of P19-N processes were measured with NIH-Image after exposure to 1 mM ALA or DOVA for 48 h. Results are representative of 5 independent experiments.

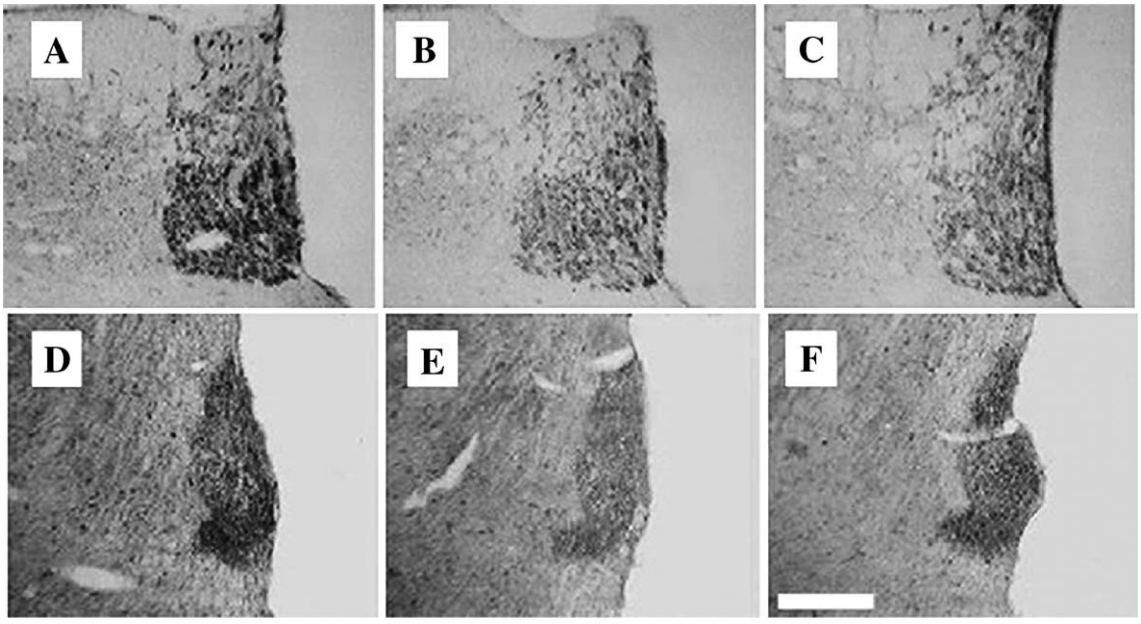


Fig. 5 – Effects of ALA and SAME on $GABA_A$ immunolabeling in rat brain. ALA and SAME (40 mg/kg) were injected intraperitoneally on alternate days for 15 days. Representative sections of the habenular complex of (A) phosphate buffer, (B) ALA, and (C) SAME treated rats are shown, as well as sections of the parabigeminal nucleus of rats treated with (D) phosphate buffer, (E) ALA, and (F) SAME. Representative images of three different animals for each condition are shown. Scale bar = 100 μm.

model systems used in this study (WERI and P19 cells, synaptosomes, and rat brain).

The results obtained from neurons from in vitro differentiation of embryonic carcinoma P19 cells are of particular interest, since ALA and DOVA toxicity to $GABA_A$ receptors during embryogenesis of the nervous system has never been studied. The P19 lineage is a well-established model for studying molecular mechanisms of neuronal differentiation in vitro (Martins et al., 2005). Moreover, these cells form excitable, functional synapses, and the sequence of expressions of its neurotransmitter receptors and differentiation markers closely resemble those observed in vivo (Lin et al., 1996). The hypothesis that ALA and DOVA interfere with GABAergic neurotransmission, and GABA mediated neurite outgrowth during development (Barbin et al., 1993) is strengthened by the altered aspect of P19-N cells after exposure to these drugs (Fig. 2), and by the measurement of the density and length of the processes of P19-N cells (Table 1), which reveal a 3- and 4-fold decrease in the average length of cytoplasmic processes after exposure to DOVA and ALA, respectively. However, further experiments are required to address if the ALA or DOVA disruption to $GABA_A$ receptor binding and expression, respectively, are involved in the impairment of neurite outgrowth and axonal development.

Although the mechanism of ALA transport and accumulation in the brain has not yet been resolved, some evidence suggests that there is ALA diffusion in the blood brain barrier (Ennis et al., 2003). Furthermore, the plasmatic concentration of ALA during crises in intermittent acute porphyria is 10 μM (40-fold higher than normal levels), as revealed previously (Minder, 1986). Consequently, it is expected, particularly during a crisis, that ALA concentration in the brain, which remains unknown, will be higher than in cerebrospinal fluid (10^{-5} to 10^{-7} M), as suggested elsewhere (Percy and Shanley, 1977). These findings support the idea that millimolar concentrations of ALA and DOVA may be adequate to assess their toxicity during acute exposure of neuronal cells to these drugs.

The fact that ALA did not alter P19 cell viability, even in concentrations as high as 1 mM (Fig. 4), does not necessarily imply that its pro-oxidant actions are irrelevant to its neurotoxicity, for it is possible that the decrease in GABAergic receptor

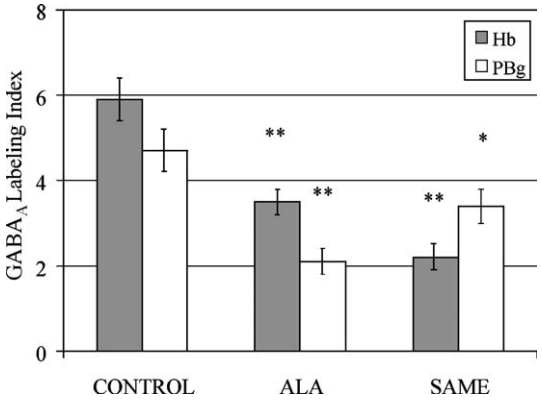


Fig. 6 – Optical density measurements of $GABA_A$ immunolabeling in brain of ALA and SAME treated rats. Measurements were made in the habenular complex (Hb) and the parabigeminal nucleus (PBg) of animals treated on alternate days with phosphate buffer, ALA or SAME for 15 days. Data shown are representative of three experiments; ** and * indicate a significant difference from controls at the $P < 0.01$ and $P < 0.05$ levels, respectively.

density observed in ALA-treated P19-N cells and rat brain involves oxidative damage to the receptors. This hypothesis is supported by previous reports findings (Demasi et al., 1996), according to which ALA-induced damage to $GABA_A$ receptors in synaptosomes may occur through an oxidative mechanism. DOVA treatment increased mortality rates in WERI, but not in P19 cells. These results may indicate that ALA and DOVA neurotoxicity occurs predominantly through specific pathways, and not through broad lipoperoxidation or random Schiff-base formation, for this would probably increase mortality rates in undifferentiated P19 cells as well.

The decrease observed in $GABA_A$ receptor availability in synaptosomes, cell cultures and rat brain may occur through several mechanisms, including direct damage of the receptor protein, inhibition and long-lasting receptor desensitization. Patch-clamp recordings are needed to elucidate the exact underlying mechanism.

Results of immunohistochemical assays in rat brain after ALA and SAME treatment (Fig. 5) indicate that GABA receptor density is generally decreased in GABAergic nuclei, i.e., in the habenular complex and the parabigeminal nucleus. The habenular complex is involved in many behavioral functions, connecting frontal, temporal and hypothalamic areas. These areas integrate and form the circuitries for numerous high order brain functions, such as planning, cognition, and visceral control. Thus, a lesion in the habenular complex can mostly contribute to the behavioral and emotional manifestations found in porphyric patients. The parabigeminal nucleus, on the other hand, has a role in visuomotor control, partly due to its projections to superior colliculus. Although eye movement disorders are not major complaints or findings in intermittent acute porphyria or lead poisoning, ocular involvement has been reported (Wolter et al., 1972; Baloh et al., 1980).

We hypothesize that functional impairments of brain structures by ALA or its derivatives may be related to neuropsychiatric and other symptoms observed in both inborn and acquired porphyrias. That SAME and ALA caused similar effects in rat brain (Fig. 6) suggests not only that the results observed were caused by ALA (and not by other heme precursors), but also that SAME treatment may be used as a porphyria model, as proposed elsewhere (Rocha et al., 2000). Intraperitoneal injections of ALA may not represent a good physiological model, as they probably induce accumulation of other heme precursors downstream from ALA in the heme's biosynthetic pathway. This makes it difficult to determine whether ALA or other heme precursors were responsible for the effects observed in this study. SAME treatment, on the other hand, is expected to induce only accumulation of ALA, for it is an inhibitor of 5-aminolevulinate hydrolyase.

Taken together, our results suggest that DOVA interacts with $GABA_A$ receptors and that ALA decreases GABAergic receptor density in vitro in neuronal cell lines and but also chronically in vivo in rat brains. The data presented here support the hypothesis that ALA and DOVA may play pivotal role in the neuropsychiatric manifestations of porphyrias, for the ALA- and DOVA-mediated $GABA_A$ receptor decrease in binding properties and membrane expression levels was observed in synaptosomes (Demasi et al., 1996), neuronal cell cultures and rat brains respectively.

4. Experimental procedures

4.1. *Materials*

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich Corporation, St. Louis, MO, USA). DOVA was prepared as described previously (Ihmels et al., 1991). ALA and DOVA stock solutions were freshly prepared and kept at –20 °C until use. All stock solutions and buffers were prepared with Milli Q Millipore deionized water.

4.2. *Synaptosomal preparation*

Rat brain cortex synaptosomes were prepared according to the Cotman's method (Cotman and Matthews, 1971). Male Wistar rats aged 45 to 60 days, fed with rat chow ad libitum, were sacrificed by cervical dislocation. Brains were removed and their cortexes isolated for the synaptosomal preparation. Protein concentrations were measured by the Bradford method (Sharma and Babitch, 1979), using a BIO RAD kit (BioRad, San Rafael, CA). Synaptosomes were resuspended in 0.32 M sucrose, 10 mM HEPES, pH 7.4, and stored at 4 °C for no longer than 2 days. Previous reports suggest that synaptosomes obtained through similar procedures retain a high density of post-synaptic $GABA_A$ receptors (Benavidez and Arce, 2002).

4.3. *Binding assays*

Synaptosomes in concentrations ranging from 0.3 to 0.8 mg of protein/mL were incubated in 10 mM Tris buffer at pH 7.4 at 37 °C for 30 min in the presence of 2 mM DOVA. Following incubation with DOVA, synaptosomes were washed (through centrifugation at 48,000 g and resuspension in the assay buffer) five times to assure complete removal of DOVA. After removal of DOVA, synaptosomes were incubated at 3 °C for 30 min with ^{3}H-muscimol (Amersham International, Buckinghamshire, UK) concentrations between 0 and 25 nM (0 to 71.3 nCi) in the presence or absence of 1 mM GABA (non-specific binding). Incubations were terminated by vacuum filtration of samples using GF/B Whatman filters (Whatman International, Maidstone, UK). Following filtration, the filters were rinsed twice with 3 mL of ice cold standard assay buffer, dried at 50 °C for 50 min, transferred to the scintillation liquid and counted. Radioactivity (dpm) was adjusted for counting efficiency and converted initially to curies (Ci) and then to mols of ^{3}H-muscimol (1 mmol of the ligand has an activity of 2.85 mCi). Data were plotted by the Scatchard method, and regression analyses (Bylund and Yamamura, 1988) performed to estimate the maximum density (B_{max}) and the dissociation binding constant (K_d) of receptors. ^{3}H-muscimol and GABA were diluted in standard assay buffer immediately before use.

4.4. *WERI human retinoblastoma cell culture*

Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and maintained in suspension. Cultures were kept at 37 °C and 5% CO_2. The cell viability of samples with a density of 5×10^5 cells/mL was measured by the Trypan Blue exclusion

method (Ignasi et al., 1999). The WERI cells were a generous gift from Prof. M.C. Sogayar (Department of Biochemistry, Universidade de São Paulo, Brazil).

4.5. *WERI cell immunocytochemistry*

Assays were performed as indicated below for P19 cell immunostaining. Goat anti- $GABA_A$ β subunit antibody (Santa Cruz Labs, Brazil) and conjugated anti-goat IgG antibody (Jackson Immunoresearch Laboratories, West Grove, PA) were used in 1:1000 and 1:200 dilutions, respectively.

4.6. *P19 cell culture and neuronal differentiation*

P19 embryonic carcinoma cells obtained from ATCC (CRL-1825; Rockville, MD) were differentiated to neuron-like cells (P19-N cells) by exposure to *all–trans* retinoic acid (RA). Cells were raised in Eagle's medium modified as previously reported (Martins et al., 2005). To initiate neuronal differentiation, cell cultures were induced to form embryonic bodies in bacterial-grade Petri dishes by the addition of 10^{-6} M RA at a density of 5×10^5 cells/mL in a defined medium (DMEM medium supplemented with 5 μg/mL insulin, 30 μg/mL transferrin, 20 μM ethanolamine and 30 nM sodium selenite). To prevent adhesion of the cell culture to plastic surfaces, the dishes were coated with 0.5% agarose prior to cell plating. Two days later, the embryonic bodies were plated in adherent culture flasks in DMEM medium with 10% fetal bovine serum for 48 h. Cells were then cultured in the defined medium, which was changed every 2 days. After 10 days, the cells were exposed to 50 μg/mL cytosine arabinoside to allow only post-mitotic cells to survive, thereby preventing the proliferation of glial cells. All the cells were cultured at 37 °C with humidity and 5% CO_2.

4.7. *Treatment of undifferentiated P19 cells with ALA and DOVA*

P19 cells, at a density of 10^5 cells/mL, were plated in adherent flasks and treated with phosphate buffer, in the presence of ALA (1.0 mM) or DOVA (0.2–1.0 mM) for 48 h days.

4.8. *Treatment of differentiated P19 cells (P19-N) with ALA and DOVA*

To determine the effect of ALA and DOVA on cell viability and morphology, cells were exposed for 2 days to 1 mM ALA or 1 mM DOVA, which had been administered to the cells 12 days after induction with retinoic acid. Control reactions were set up in the presence of phosphate buffer. On the 14th day after RA treatment, cell viability was determined by the Trypan Blue method (Ignasi et al., 1999). Randomly chosen areas of each cell culture flask were photographed after exposure to ALA or DOVA. Cell morphology was evaluated on an image analysis system based on NIH-Image (available on http://rsb.info.nih.gov/nih-image/Default.html).

4.9. *Immunocytochemistry of P19-N cells*

Cells at a density of 5×10^5 cells/mL were grown on glass coverslips, fixed in 2% paraformaldehyde for 10 min and dehydrated on a platform heated to 37 °C. Incubations were performed as described below for the immunohistochemistry procedure. A rabbit anti- $GABA_A$ β subunit (Alamone Labs, Jerusalem, Israel), mouse anti-NF160 and mouse anti-NF200 antibodies were used in 1:250, 1:500 and 1:500 dilutions, respectively. Goat anti-rabbit IgG and goat anti-mouse IgG were used in a 1:200 dilution. All the conjugated secondary antibodies, as well as anti-NF160 and anti-NF200 antibodies used here, were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Anti-$GABA_A$ β subunit antibodies were used as the target to explore the effects of ALA and DOVA on $GABA_A$ receptors. The ubiquitous β subunits have a pivotal role in the assembly of the pentameric receptor (Whiting et al., 1999). Moreover, they confer sensitivity for GABA (Pritchett et al., 1989) along with α subunits, and can have their function and intracellular trafficking modulated at the post-translational level by different phosphorylation states (Kumar et al., 2004).

4.10. *SAME preparation*

Succinylacetone (SA) was treated with diazomethane in ethyl ether as solvent. After evaporation of the ether, the SA methyl ester (SAME) was identified through its NMR spectra (data not shown). The ester was then dissolved in saline and the pH was adjusted to 7.4 before rat treatment. SAME, rather than SA, was injected because of its greater absorption and because it was used in a previous experimental porphyria study (Rocha et al., 2000). Diazomethane was kindly provided by Prof. Joao H. Lago (Dept. of Fundamental Chemistry, Universidade de São Paulo, Brazil).

4.11. *Rat treatment with ALA and SAME*

Male Wistar rats weighing 200–250 g were injected intraperitoneally with 0.5 mL saline, 40 mg/kg of ALA or SAME on alternate days for 15 or 30 days. The protocol was approved by the Bioethics Committee of the Chemistry Institute, Universidade de Sao Paulo, and is in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

4.12. *Immunohistochemistry*

Assays were performed according to a previously described protocol (Britto et al., 1992). Male Wistar rats aged 45 to 60 days were anesthetized and perfused intracardially with 0.1 M phosphate buffer, pH 7.35, followed by perfusion with 4% paraformaldehyde in the same buffer. Brains were removed and post-fixed overnight in 30% sucrose in phosphate buffer (0.1 M, pH 7.4). Frozen 30 μm brain coronal sections were collected in phosphate buffer and incubated overnight with a goat anti-$GABA_A$ β subunit antibody (1:500), which was purchased from Alamone Labs (Jerusalem, Israel), 0.3% polyoxyethylene isooctylcyclohexyl ether (TRITON X-100) and 5% normal goat serum. After washing with phosphate buffer, sections were incubated with a biotinylated goat anti-rabbit antibody (Vector Labs, Burlingame, CA, USA) at a 1:200 dilution for 1 h. Sections were rinsed with phosphate buffer and incubated with the avidin–biotin complex (Vectastain kit,

Vector Labs, Burlingame, CA, USA) for 1 h. Peroxidase activity was detected using H_2O_2 and diaminobenzidine tetrahydrochloride (Aldrich, Milwaukee, WI, USA) as a chromogen (Britto et al., 1992). Negative controls were obtained by repeating the above-described procedure in the absence of the primary antibody. Sections were mounted on gelatin-coated slides and coverslipped. Digital images were collected from an optic microscope and analyzed with Image J 1.33 (NIH/USA). Optical densities were measured in 8 representative fields of each of the parabigeminal nuclei and habenular complexes and compared with the optical densities of the neighboring background.

4.13. *Statistical analysis*

Data are reported as mean ± standard error of the mean (SEM) from at least three independent experiments, with data from each experiment being determined at least in triplicate. Statistical differences among treatment groups were evaluated by one-way analysis of variance (ANOVA), followed by Bonferroni's post hoc analysis. The level of significance was set at least at *P*.

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