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Aluminium-induced impairment of Ca^{2+} modulatory action on GABA transport in brain cortex nerve terminals

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Abstract

The γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in vertebrate CNS. At GABAergic synapses, a high-affinity transporter exists, which is responsible for GABA reuptake and release during neurotransmission. GABA transporter activity depends on the phosphorylation/dephosphorylation state, being modulated by Ca^{2+}/calmodulin-dependent protein phosphatase 2B (calcineurin). Aluminium is known to interfere with the Ca^{2+}/calmodulin signalling pathway. In this work, we investigate the action of aluminium on GABA translocation mediated by the high-affinity transporter, using synaptic plasma membrane (SPM) vesicles and synaptosomes isolated from brain cortex. Aluminium completely relieved Ca^{2+} downregulation of GABA transporter, when mediating uptake or release. Accordingly, aluminium inhibited Ca^{2+}/calmodulin-dependent calcineurin activity present in SPM, in a concentration-dependent manner. The deleterious action of aluminium on the modulation of GABA transport was ascertained by comparative analysis of the aluminium effect on GABA uptake and release, under conditions favouring SPM dephosphorylation (presence of intracellular micromolar Ca^{2+}) or phosphorylation (absence of Ca^{2+} and/or presence of W-7, a selective calmodulin antagonist). In conclusion, aluminium-induced relief of Ca^{2+} modulatory action on GABA transporter may contribute significantly to modify GABAergic signalling during neurotoxic events in response to aluminium exposure.

Keywords: Aluminium; GABA; Calcineurin; Ca^{2+}/CaM signalling; Brain cortex

1. Introduction

The blooming industrialized society increases aluminium bioavailability as a result of continued acidification of the environment. Aluminium salts are used in the pharmaceutical industry, and they are generally used as flocculants in treating drinking water. Nowadays, aluminium is recognised as a neurotoxic agent [1,2]. During the last 30 years, the emergence of extensive evidence on aluminium poisoning has demonstrated the adverse affect of aluminium in inducing memory impairment, personality changes and dementia in humans [3–6]. Additionally, bioavailable aluminium seems to be associated with neurological deterioration during aging [7].

The mechanisms by which aluminium exerts its neurotoxic action remain poorly understood. Current hypotheses suggest the aluminium-induced impairment of particular chemically coded neuronal populations [8–10]. However, the explanation for the development of aluminium deleterious effects seems to be founded in the imbalance of ubiquitous cellular processes [2,11–20]. Over the past three decades, aluminium has been shown to interfere with the main steps of the neurotransmission event (synthesis, storage, release, postsynaptic reception and inactivation of neurotransmitters), which takes place at the synapse. Aluminium could modify the homeostasis of neurotransmitters in presynaptic nerve terminals in several ways: (i) by chelating neurotransmitter molecules [8,10,21,22]; (ii) by modifying the activity of enzymes responsible for transmitter synthesis and degradation [23–28]; and (iii) by compromising carrier-mediated neurotransmitter uptake [21,29–31]. Although, no direct effects of aluminium on neurosecretion machinery have previously been reported, the putative deleterious action of aluminium on Ca^{2+}-dependent, depolarisation-evoked neurotransmitter exocytosis has been postulated [22,32–35]. Conversely, several investigators provided unequivocal evidence of

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aluminium action on neurotransmitter postsynaptic receptors [15,36–38].

Previous research highlighted the hypothesis that aluminium may exert its toxic properties by interacting with calmodulin (CaM), a ubiquitous regulatory protein in Ca^{2+} signalling pathways of all eukaryotic cells, that plays a pivotal role in the regulation of stimulus-secretion coupling in nerve cells [39–42]. Several investigators clearly demonstrated that aluminium bound to calmodulin leads to the loss of calmodulin ability to interact with target proteins, producing impairment of its regulatory role [18,43–45]. In fact, aluminium changes the conformation of calmodulin [18,45–52], thus preventing the Ca^{2+}-induced transition between conformational states that are associated with the regulatory function of this protein. The protein phosphatase 2B (calcineurin), a key enzyme involved in the regulation of neurotransmission, is dependent on the presence of Ca^{2+} and calmodulin (Ca^{2+}/CaM) in order to modulate the activity of multiple proteins at the synapse [39–42,53–55].

Over the past few years, we have been reporting the involvement of calcineurin on the regulation of carrier-mediated translocation of GABA, the major inhibitory neurotransmitter in the adult mammalian CNS. Ca^{2+}/CaM-dependent calcineurin activity seems to be responsible for the downregulation of GABA transporter, either when mediating its release or reuptake from the synaptic cleft [56–60]. In the present study, we investigate the effect of aluminium on the carrier-mediated GABA transport across the synaptic plasma membrane (SPM) and on its regulation by Ca^{2+}/CaM-dependent calcineurin activation. We found that aluminium inhibits Ca^{2+}/CaM-dependent calcineurin activity and completely reverts the Ca^{2+}-induced inhibition of both GABA uptake and release by SPM vesicles. Finally, we verified whether aluminium-induced relief of Ca^{2+} modulatory action on GABA transporter occurs under more physiological conditions, by using a preparation of intact nerve terminals (synaptosomes).

2. Materials and methods

2.1. Reagents

The 4-amino-*n*-[2,3-^{3}H]butyric acid ([^{3}H]GABA), with a specific activity of 81.0 Ci/mmol, was purchased from Amersham. RII phosphopeptide was obtained from Biomol. W-7 and ionomycin were supplied by Calbiochem–Novabiochem. Malachite green, cyclosporin A, aminooxyacetic acid (AOAA) and $AlCl_3$ were obtained from Sigma–Aldrich. Aluminium trials were performed by adding aliquots (~4 μl) of $AlCl_3$ from freshly made stock solutions to reaction media, immediately before the beginning of the incubation period. The filters used for retention of subcellular fractions were obtained from Whatman.

2.2. Isolation of synaptic plasma membrane vesicles and synaptosomes

The SPM vesicles were isolated from sheep brain cortex, as previously described [61]. After homogenisation of the brain cortical grey matter, the homogenate was centrifuged at 900×*g* during 10 min, and the resulting supernatant was centrifuged at 10 000×*g* during 20 min. After lysis of the pellet material (crude synaptosomal fraction) in hypotonic alkaline medium, followed by successive centrifugations at 8000×*g* (10 min) and 35 000×*g* (30 min), the resuspended pellet was centrifuged through a discontinuous Dextran T500 gradient for 2 h at 23 000×*g*. The collected bands, containing the SPM, were diluted and centrifuged at 35 000×*g* during 30 min. Then, the pelleted vesicles were resuspended (5 mg protein/ml) and loaded with K^+ by incubating 30 min, at 30 °C, in a medium containing 0.1 mM $MgSO_4$ and 150 mM MES-potassium salt at pH 6.5. After the incubation period, SPM vesicles were centrifuged 30 min at 35 000×*g* and the pellet was resuspended in the same buffer to give a final concentration of about 20 mg protein/ml. Finally, the preparation was divided into several aliquots, which were frozen in liquid nitrogen and stored at −70 °C. When required, the SPM vesicles were thawed at room temperature.

The synaptosomes were prepared according to the method of Hajós [62]. Male Wistar rats were sacrificed by decapitation and the brains were removed. The brain cortices were rapidly dissected, homogenized in 9 volumes of ice-cold 0.32 M sucrose buffered with 10 mM Hepes–Tris (pH 7.4) and centrifuged at 1500×*g* for 10 min. The supernatant was centrifuged at 9000×*g* for 20 min and the pellet was resuspended in the homogenisation solution. The crude synaptosomal fraction was layered over 0.8 M sucrose and centrifuged at 9000×*g* for 30 min. Then, the synaptosomal enriched fraction, collected from the 0.8 M sucrose layer, was 2-fold diluted and centrifuged at 20 000×*g* for 30 min. The final pellet was resuspended in 0.32 M sucrose and 10 mM Hepes–Tris (pH 7.4) at a final concentration of about 8 mg protein/ml. All procedures were performed at 0–4 °C.

The protein content of both subcellular fractions was measured according to the method developed by Gornall et al. [63], using bovine serum albumin as a standard.

2.3. Measurement of protein phosphatase 2B (calcineurin) activity of synaptic plasma membrane

The measurement of calcineurin activity was performed by using RII phosphopeptide as specific substrate. The utilization of RII phosphopeptide was performed with modification of the method described by Martin et al. [64]. SPM vesicles (0.5 mg protein/ml) were incubated, at 30 °C, in 170 μl of 50 μM EGTA and 10 mM Hepes–Na (pH 7.4). After 2 min, the reactions were initiated by

adding 5 μM RII phosphopeptide and, at various time intervals, they were stopped with 30 μl of 2.5 M H_2SO_4. Subsequently, the malachite green reagent (800 μl) was added, and the suspensions were mixed and allowed to sit for 15 min. The absorbance changes were measured at 660 nm in a spectrophotometer (Perkin-Elmer Lambda 14P). The malachite green reagent was prepared as described by Harder et al. [65].

2.4. Measurement of [^{3}H]GABA uptake by synaptic plasma membrane vesicles and synaptosomes

The uptake of [^{3}H]GABA by SPM vesicles and synaptosomes was measured isotopically as described elsewhere [61,66]. The [^{3}H]GABA uptake reactions were carried out at 30 °C in the presence of 0.5 μM GABA supplemented with [^{3}H]GABA (0.25 μCi/ml). The reactions were initiated by adding SPM vesicles (final concentration 0.5 mg protein/ml) to the reaction media or by the addition of [^{3}H]GABA to media containing synaptosomes (final concentration 0.5 mg protein/ml). Unless otherwise indicated in the legends of the respective figures, uptake reactions were performed in media containing 150 mM NaCl, 10 mM Hepes–Na (pH 7.4), 50 μM EGTA and 2 μM ionomycin (SPM vesicle medium), or containing 128 mM NaCl, 1.2 mM $MgCl_2$, 5 mM KCl, 10 mM glucose, 10 μM AOAA, 10 mM Hepes–Na (pH 7.4), 100 μM EGTA and 2 μM ionomycin (synaptosome medium). The reactions were stopped by rapid filtration of 500-μl aliquots through glass-fiber filters (Whatman GF/B), prewashed with 5 ml of 150 mM NaCl or 320 mM sucrose, 10 mM Tris–HCl (pH 7.4) and 100 μM EGTA for SPM vesicle and synaptosomal preparations, respectively. The filters were then washed with 10 ml of the same medium and they were plunged in scintillation cocktail (Universol™ ES) for further radioactivity measurement by liquid scintillation spectrometry. The values for [^{3}H]GABA uptake were expressed as pmol/mg protein per min after subtraction of blank values obtained by filtering aliquots of reaction medium containing 0.5 μM GABA supplemented with [^{3}H]GABA (0.25 μCi/ml).

2.5. Measurement of [^{3}H]GABA release by synaptic plasma membrane vesicles

The release of [^{3}H]GABA from SPM vesicles was followed by the superfusion technique previously described [61]. The SPM vesicles were loaded with [^{3}H]GABA during 2 min in the presence of 0.5 μM GABA as described above, except that ionomycin was absent. Using a peristaltic pump, the loaded SPM vesicles were spread on glass microfiber filters (Whatman GF/B) mounted in Millipore metal 13 mm Swinnex filter holders. The filters were continuously washed with a medium containing 150 mM NaCl, 50 μM EGTA and 10 mM Hepes–Na (pH 7.4), at a flow rate of 0.31 ml/min. After 8 min of superfusion, the medium was quickly substituted for K^+ depolarisation medium (150 mM KCl, 50 μM EGTA and 10 mM Hepes–K at pH 7.4). Fractions were collected every minute directly into scintillation vials. The radioactivity was measured as described above for the uptake assays. The values for [^{3}H]GABA release by SPM vesicles were expressed as dpm/fraction or as arbitrary units (a.u.) that were calculated using the following equation:

$$\text{GABA released (a.u.)} = \int_a^x [({}^3H_x - {}^3H_b)/{}^3H_b]\, dt$$

where ^3H_x corresponds to the dpm counted in fraction x and ^3H_b corresponds to the extrapolated basal dpm level in the fraction $x(a)$.

GABA release values were calculated by using Origin™ computer program, and J_{max} (maximal translocation velocity) corresponds to the slope of the resulting curves.

2.6. Treatment of the data

All results were treated statistically with Origin™ computer program. Results are presented as means±S.E.M. of the number of experiments indicated in the figures. Statistical significance was determined by means of an unpaired two-tailed Student's *t*-test or by analysis of variance (ANOVA) followed by Bonferroni post-hoc test. A P value <0.05 was considered significant.

3. Results

3.1. Aluminium effect on Ca^{2+}/calmodulin-dependent protein phosphatase 2B (calcineurin) activity

Calcineurin, the most important Ca^{2+}/CaM-dependent protein phosphatase activity involved in the regulation of presynaptic events during neurotransmission [41,67], was shown to be widely distributed in various organelles within neurons (from the soma to dendrites), including the plasma membrane [41,42].

In previous reports we have demonstrated that SPM vesicles exhibit Ca^{2+}/CaM-dependent calcineurin activity, reaching maximal levels by raising free Ca^{2+} concentrations up to few micromolar [58]. Therefore, we investigated whether aluminium could interfere with the Ca^{2+}/CaM-dependent activity of calcineurin. Fig. 1 shows the activity of this enzyme in the presence of increasing $AlCl_3$ concentrations, in the absence or in the presence of Ca^{2+} ions. Under Ca^{2+} concentration (10 μM free Ca^{2+}) that assures maximal activation of calcineurin (~10 nmol P_i/mg protein per 50 min), aluminium affected significantly P_i production, in a dose-dependent manner. In fact, Ca^{2+}/CaM-dependent calcineurin activity was completely abolished in the presence of 300 μM $AlCl_3$. It is notewor-

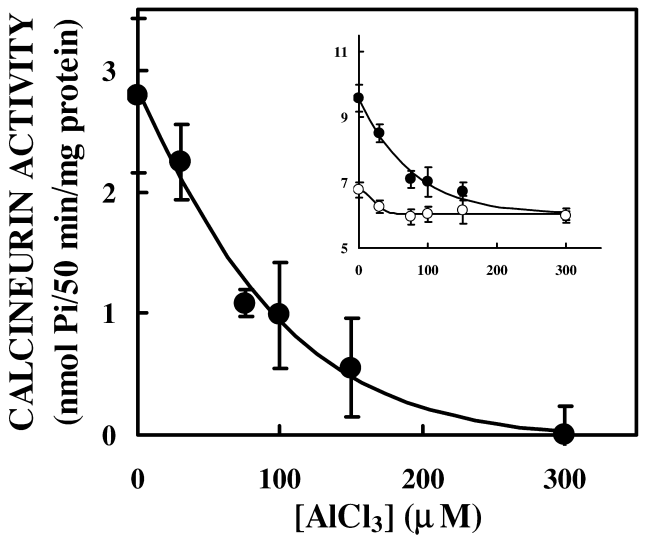

Fig. 1. Calcineurin activity as a function of aluminium concentration. The synaptic plasma membrane (SPM) vesicles (0.5 mg protein/ml) were incubated in a medium containing 10 mM Hepes–Na (pH 7.4), 50 μM EGTA and 5 μM RII phosphopeptide as specific calcineurin substrate. The reactions were carried out for 50 min in the presence of increasing $AlCl_3$ concentrations (0–300 μM). The reactions were stopped with 30 μl of 2.5 M H_2SO_4, and inorganic phosphate (P_i) was quantified as described in Section 2. The Ca^{2+}/CaM-dependent calcineurin activity was determined from the amount of P_i produced in the presence of 10 μM free Ca^{2+} by subtracting the amount of P_i produced in the absence of added $CaCl_2$. The insert represents the calcineurin activity in the absence (○–○) or in the presence of 10 μM free Ca^{2+} (•–•) as a function of $AlCl_3$ concentration. Vertical bars denote S.E.M. of the mean value of at least six separate determinations.

thy that even when calcineurin activation could be attributed to contaminating levels of Ca^{2+} in the nanomolar range (~7 nmol P_i/mg protein per 50 min), aluminium was able to decrease P_i production down to ~6 nmol P_i/mg protein per 50 min, which corresponds to values obtained in the presence of cyclosporin A, a selective calcineurin inhibitor (~6 nmol P_i/mg protein per 50 min).

These results clearly show that Ca^{2+}/CaM-dependent calcineurin activity of SPM is inhibited by aluminium, in a concentration-dependent manner, and maximal inhibition is reached in the presence of 300 μM $AlCl_3$. Therefore, further experiments, addressing aluminium effect on GABA transport in brain cortex nerve terminals, were performed in the presence of 300 μM $AlCl_3$, since total inhibition of Ca/CaM-dependent calcineurin activity is required to clearly demonstrate the effect of aluminium-induced impairment of Ca^{2+} modulatory action on GABA transport.

3.2. Aluminium effect on Ca^{2+}/calmodulin regulation of carrier-mediated GABA uptake and release by SPM vesicles

Intravesicular Ca^{2+}, at micromolar concentrations, was previously found to decrease, via stimulation of Ca^{2+}/CaM-dependent calcineurin activity, maximal uptake of GABA and its maximal release rate by ~50 and ~65%, respectively [56,57,59].

Fig. 2 shows the time course of GABA uptake mediated by its high-affinity Na^+-dependent GABA transport system, in the absence and in the presence of 300 μM $AlCl_3$. As it can be observed, aluminium produced a statistical non-significant decrease of the initial rate of accumulation and of the maximal uptake of [^{3}H]GABA (after 2–3 min incubation). However, a 30% reduction of the amount of [^{3}H]GABA retained by SPM vesicles could be observed when incubation period was extended up to 8 min. This effect should be the result of aluminium-induced alterations of membrane physical properties rather than a direct action on the GABA transport system, since GABA uptake by SPM vesicles is driven by artificially imposed ionic gradients [58,68].

The first evidence that aluminium jeopardizes the Ca^{2+} modulatory action on GABA transport derives from the observation that Ca^{2+}-induced inhibition of GABA uptake by SPM vesicles was totally relieved by 300 μM $AlCl_3$ (Fig. 3). As we can see, the addition of Ca^{2+} to the reaction medium in the presence of ionomycin, a specific Ca^{2+} ionophore, reduced the amount of [^{3}H]GABA accumulated by SPM vesicles from 141 to 79 pmol/mg protein. This reduction is in accordance with the previously reported effect of intravesicular Ca^{2+} on GABA uptake [56,57,59]. In contrast, Ca^{2+}-induced decrease of GABA uptake did not occur when 300 μM $AlCl_3$ was present in uptake medium, corresponding to a total prevention of the intravesicular Ca^{2+} effect by aluminium.

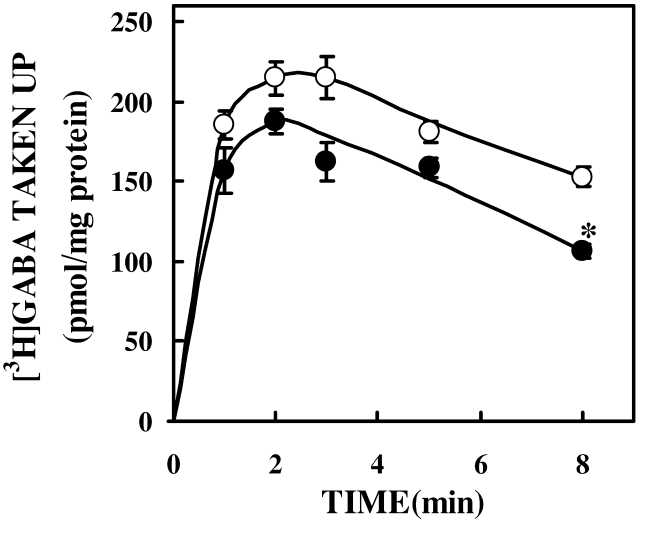

Fig. 2. Effect of aluminium on [^{3}H]GABA uptake by SPM vesicles. SPM vesicles (0.5 mg protein/ml) were incubated in a medium containing 150 mM NaCl, 50 μM EGTA, 10 mM Hepes–Na (pH 7.4) and 0.5 μM [^{3}H]GABA, in the absence (○–○) or in the presence of 300 μM $AlCl_3$ (•–•). The reactions were stopped by filtering 0.5 ml aliquots and the filters radioactivity was measured as described in Section 2. Vertical bars denote S.E.M. of at least six independent experiments. *$P<0.01$, statistically significant from control value.

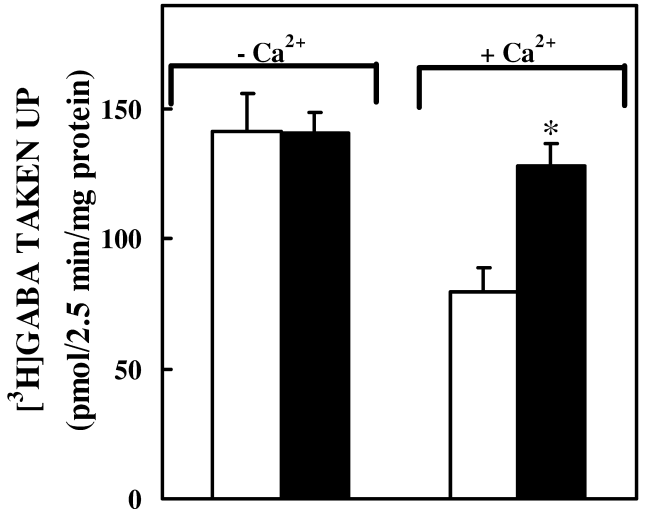

Fig. 3. Aluminium relieves the Ca^{2+}-induced [^{3}H]GABA uptake inhibition in SPM vesicles. Experimental conditions were the same as described in the legend of Fig. 2, except that the assays were performed for 2.5 min in the presence of 2 μM ionomycin (a specific Ca^{2+} ionophore). The reactions were carried out in the absence or in the presence of 10 μM free Ca^{2+}. Control condition (absence of $AlCl_3$), white bar; presence of 300 μM $AlCl_3$, black bar. Vertical bars denote S.E.M. of four independent experiments. *$P<0.02$, statistically significant from control value.

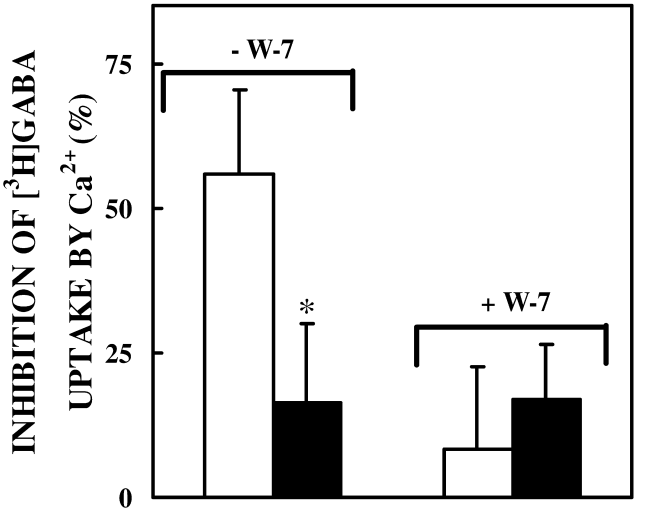

Fig. 4. Aluminium relieves the Ca^{2+} inhibitory effect on [^{3}H]GABA uptake by SPM vesicles: sensitivity to W-7 (calmodulin antagonist). Experimental conditions were the same as described in the legend of Fig. 2. The reactions were carried out for 2.5 min in the absence and in the presence of 10 μM free Ca^{2+}, under various experimental conditions (reaction medium supplemented with 2 μM ionomycin, 2 μM ionomycin plus 300 μM $AlCl_3$, 2 μM ionomycin plus 10 μM W-7 or 2 μM ionomycin plus 300 μM $AlCl_3$ and 10 μM W-7). Bars show the percentage of inhibition by Ca^{2+} in the absence (white bar) or in the presence of 300 μM $AlCl_3$ (black bar). Vertical bars denote S.E.M. of four independent experiments. *$P<0.01$, statistically significant from control value.

As previously reported, Ca^{2+}-induced inhibition of GABA uptake by SPM vesicles can be relieved by W-7, a specific calmodulin antagonist, and this is also observed in our experimental conditions (Fig. 4). In fact, the inhibitory effect of Ca^{2+} on GABA uptake (56%) was found to be almost completely prevented by either aluminium (Figs. 3 and 4) or W-7 (Fig. 4). Furthermore, concomitant presence of 300 μM $AlCl_3$ and 10 μM W-7 during GABA uptake reaction did not show any additive effects. These results are in line with those shown in Fig. 1, where total inhibition of Ca^{2+}/CaM-dependent calcineurin activity by 300 μM $AlCl_3$ was observed.

Fig. 5 shows the effect of aluminium on K^+ depolarisation-induced [^{3}H]GABA release from SPM vesicles, which corresponds to neurotransmitter translocation in the backward direction by the GABA transport system [57,58,69]. SPM vesicles were actively loaded with [^{3}H]GABA during 2 min, and then were superfused with 150 mM NaCl, 50 μM EGTA and 10 mM Hepes–Na (pH 7.4) during 8 min, to remove the non-accumulated [^{3}H]GABA. Fig. 5 represents a typical experiment, showing the late 4 min of NaCl washout of non-accumulated [^{3}H]GABA, followed by induction of carrier-mediated [^{3}H]GABA release due to substitution of the superfusion medium by similar medium, where Na^+ was replaced by K^+. A massive GABA efflux from the vesicles occurred during 4 min. The time course of K^+ depolarisation-induced release of [^{3}H]GABA from SPM vesicles can be plotted in a cumulative curve of arbitrary units (a.u.) as shown in the insert of Fig. 5. It can be seen that [^{3}H]GABA release triggered by K^+ depolarisation was slightly increased when SPM vesicles were superfused with depolarisation medium containing 300 μM $AlCl_3$. It should not be ruled out that aluminium could neutralize membrane-surface charges of sialic acid molecules and cause rigidification of SPM [70–74], which may contribute in improving the translocation capacity of the high-affinity GABA transporter [75,76]. However, the statistical non-significant aluminium stimulatory effect (19%) is probably attributed to the enhancement of GABA translocation rate by its transport system, due to aluminium inhibition of Ca^{2+}/CaM-dependent calcineurin activity in presence of residual Ca^{2+} in the nanomolar range (Fig. 1).

Aluminium-induced relief of the Ca^{2+} inhibitory effect on [^{3}H]GABA release is clearly shown in Fig. 6. [^{3}H]GABA released from SPM vesicles in response to K^+ depolarisation in the presence of Ca^{2+} was 1.2 a.u. (Fig. 6A), representing a 63% decrease when compared to K^+ depolarisation-induced release of [^{3}H]GABA in the absence of Ca^{2+} (3.2 a.u.). Conversely, it was observed that in presence of 300 μM $AlCl_3$ (Fig. 6B), the maximal translocation velocity (J_{max}) of [^{3}H]GABA release in response to K^+ depolarisation of SPM reached maximal values (1.1 a.u./min) even when 10 μM Ca^{2+} was added to the superfusion medium. Actually, when 300 μM $AlCl_3$ was introduced in K^+ depolarisation media, both J_{max} and total amount of [^{3}H]GABA released from SPM vesicles reached maximal values, which correspond to a 3-fold increase of the above mentioned parameter values when

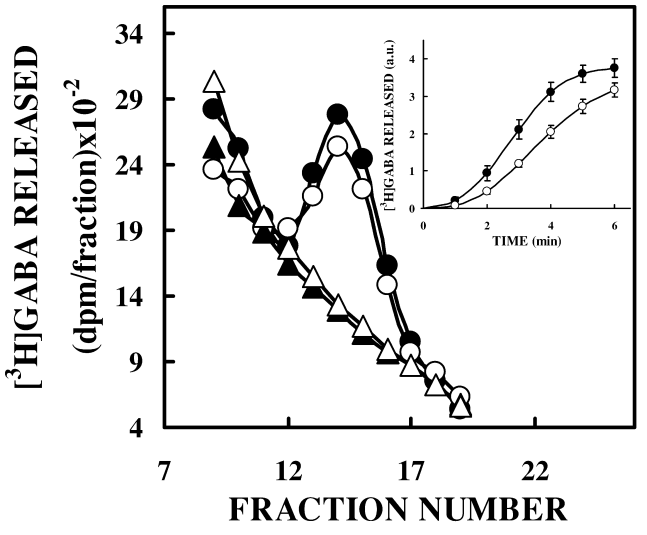

Fig. 5. Effect of aluminium on [^{3}H]GABA release by SPM vesicles. SPM vesicles (0.5 mg protein/ml), actively loaded with [^{3}H]GABA (incubation at 30 °C, during 2 min, with [^{3}H]GABA 0.5 μM, 150 mM NaCl, 50 μM EGTA and 10 mM Hepes–Na, at pH 7.4), were superfused during 8 min with 150 mM NaCl, 50 μM EGTA and 10 mM Hepes–Na (pH 7.4), as described in the text. Then, [^{3}H]GABA release from SPM vesicles was induced by replacement of the superfusion medium with different superfusion media buffered with 10 mM Hepes–Na (pH 7.4) and containing 50 μM EGTA plus 150 mM KCl (○–○), plus 150 mM KCl and 300 μM $AlCl_3$ (•–•), plus 150 mM NaCl (△–△) or plus 150 mM NaCl and 300 μM $AlCl_3$ (▲–▲). The results are expressed in dpm/fraction and correspond to a representative experiment. Insert shows the K^+ depolarisation-induced release of [^{3}H]GABA (superfusion with media containing 150 mM KCl) in the absence (○–○) or in the presence of 300 μM $AlCl_3$ (•–•). Values represent the integrated area of the released [^{3}H]GABA as arbitrary units per minute, defined in Section 2. Vertical bars denote S.E.M. of at least six independent experiments.

compared to those obtained in response to K^+ depolarisation in the presence of 10 μM Ca^{2+} and absence of aluminium. These results suggest an increased transport efficiency due to aluminium impairment of intravesicular Ca^{2+}-induced reduction of GABA transport across SPM, shown to act by decreasing J_{max} (Fig. 6A and [59]).

In order to investigate the involvement of calmodulin in aluminium relief of Ca^{2+}-induced inhibition of GABA release in response to K^+ depolarisation in SPM, we assessed the effect of the calmodulin antagonist, W-7. Fig. 7 shows that W-7 did not significantly modify either J_{max} or total amount of GABA released observed when 300 μM $AlCl_3$ was presented in superfusion media. Either the presence of aluminium, or aluminium and W-7, allowed achieving release parameters (J_{max} and total GABA released) values close to those obtained in control experiments (K^+ depolarisation in the absence of Ca^{2+}).

The results indicate that aluminium stimulation of GABA uptake and K^+ depolarisation-induced release by SPM vesicles occurs, when intravesicular Ca^{2+} concentrations are in micromolar range, and the underlying mechanism seems to involve inhibition of the Ca^{2+}/CaM-dependent calcineurin activity.

3.3. Aluminium effect on GABA uptake by synaptosomes

The study of aluminium effect on GABA transport greatly benefited from SPM vesicle preparation, since ionic gradients were artificially imposed, constituting the sole driving force for GABA transport. If SPM vesicle preparation facilitated identifying the underlying mechanism by which aluminium interferes with this transport system, it appears quite relevant to bring to light whether the disruption of Ca^{2+} modulatory action on GABA transport by aluminium can occur under more physiological conditions. Thereby, aluminium effect on GABA uptake by synaptosomes was studied, since this subcellular fraction consists of intact nerve terminals.

Fig. 8 shows that when synaptosomes were incubated in the presence of 300 μM $AlCl_3$, the amount of [^{3}H]GABA taken up during 15 min was reduced from 321 to 198 pmol/mg protein. This observation, aluminium-induced decrease (38%) of [^{3}H]GABA uptake by synaptosomes, is in contrast to the lack of effect of aluminium on [^{3}H]GABA uptake by SPM vesicles (Fig. 2), but it is in agreement with previous reports by other investigators [2,21,29–31,77–80]. In fact, it was hypothesised that (Na^+/K^+)-ATPase inhibition by aluminium could account for the reduced accumulation of neurotransmitters in the presence of aluminium, by compromising the magnitude of the Na^+ gradient and of membrane polarization state to which GABA transport is dependent on [68,81]. In this context, the observed aluminium effect on [^{3}H]GABA uptake by synaptosomes was compared with that of ouabain, a selective inhibitor of the (Na^+/K^+)-ATPase (Fig. 8). The presence of 50 μM ouabain, which inhibits the (Na^+/K^+)-ATPase by ~70% (data not shown), in the incubation medium produced a decrease (69%) of the total amount of [^{3}H]GABA taken up by synaptosomes. Furthermore, in the presence of both 300 μM $AlCl_3$ and 50 μM ouabain, the extension of reduction of [^{3}H]GABA uptake remained almost unaltered, which seems to reflect a restrained inhibitory action of aluminium on the GABA uptake by synaptosomes via the inhibition of the (Na^+/K^+)-ATPase activity.

Fig. 9 shows the effect of 300 μM $AlCl_3$ on [^{3}H]GABA uptake by synaptosomes under conditions, which favour Ca^{2+} modulatory action on the GABA transport system. It is clear that 10 μM free Ca^{2+}, in the presence of ionomycin, notably reduced the amount of [^{3}H]GABA taken up by synaptosomes from 278 to 227 pmol/mg protein, which is in accordance with the downregulatory action of intravesicular Ca^{2+} previously observed when SPM vesicles were used (Fig. 3). Accordingly, when 300 μM $AlCl_3$ was present in the incubation medium, aluminium relieved Ca^{2+} modulatory action on [^{3}H]GABA transport by synaptosomes (increasing the amount of [^{3}H]GABA taken up during 15 min from 227 to 262 pmol/mg protein) (Fig. 9).

The above results suggest that the modulation of GABA

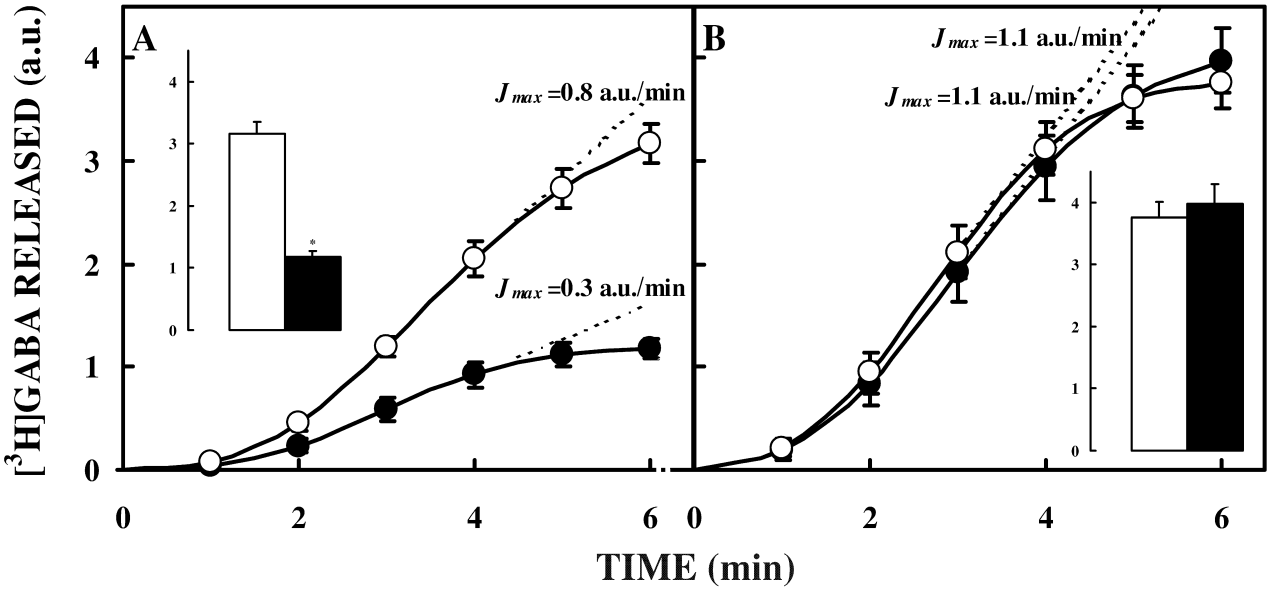

Fig. 6. Inhibition of K^+ depolarisation-induced [^{3}H]GABA release by Ca^{2+} from SPM vesicles (A) and relieve by aluminum (B). The assays were performed as described in the legend of Fig. 5. The release of [^{3}H]GABA from SPM vesicles was performed in the absence (A) or in the presence of 300 μM $AlCl_3$ (B), and was induced by superfusion with K^+ depolarisation medium (150 mM KCl, 50 μM EGTA and 10 mM Hepes–Na, at pH 7.4) in the absence (○–○) or in the presence of 10 μM free Ca^{2+} (•–•). The maximal velocities of [^{3}H]GABA release are represented by J_{max}. Values represent the integrated area of the released [^{3}H]GABA as arbitrary units per min. Inserted histograms represent the total amount of [^{3}H]GABA released under the different experimental conditions. Vertical bars denote S.E.M. of at least six independent experiments. *$P<0.01$, statistically significant from control value.

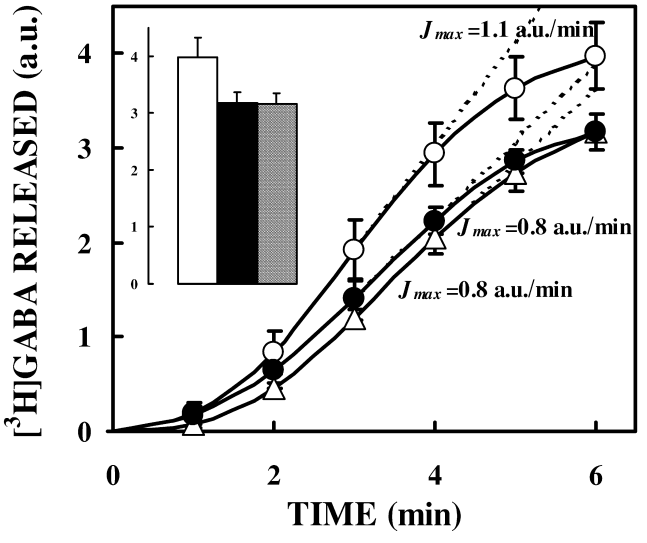

Fig. 7. Aluminium relieves the Ca^{2+} inhibitory effect on K^+ depolarisation-induced [^{3}H]GABA release from SPM vesicles: sensitivity to W-7 (calmodulin antagonist). The assays were performed as described in the legend of Fig. 5. The release of [^{3}H]GABA from SPM vesicles was induced by superfusion with K^+ depolarisation medium (150 mM KCl, 50 μM EGTA and 10 mM Hepes–Na, at pH 7.4) containing 10 μM free Ca^{2+}, 300 μM $AlCl_3$ in the absence (○–○) or in the presence of 10 μM W-7 (•–•). K^+ depolarisation-induced release (△–△) is plotted as reference values. The maximal velocities of [^{3}H]GABA release are represented by J_{max}. Values represent the integrated area of the released [^{3}H]GABA as arbitrary units per min. Inserted histograms represent the total amount of [^{3}H]GABA released under the different experimental conditions (absence of W-7, white bar; presence of W-7, black bar; K^+ depolarisation, grey bar). Vertical bars denote S.E.M. of at least six independent experiments.

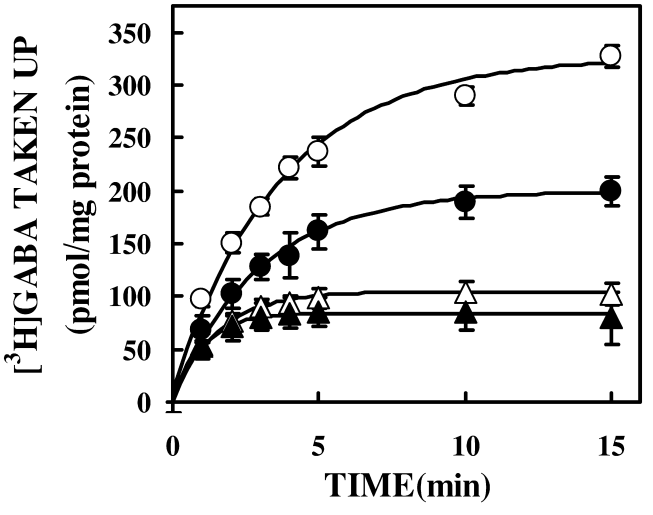

Fig. 8. Effect of aluminium on [^{3}H]GABA uptake by synaptosomes. Synaptosomes (0.5 mg protein/ml) were equilibrated during 2 min, at 30 °C, in incubation medium containing 128 mM NaCl, 1.2 mM $MgCl_2$, 5 mM KCl, 10 mM glucose, 10 μM AOAA, 100 μM EGTA and 10 mM Hepes–Na (pH 7.4), in the absence (○–○) or in the presence of 300 μM $AlCl_3$ (•–•), 50 μM ouabain (△–△) or 300 μM $AlCl_3$ and 50 μM ouabain (▲–▲). The reactions were started by adding [^{3}H]GABA (final concentration 0.5 μM), and were stopped by filtering 0.5 ml aliquots as described in Section 2. Vertical bars denote S.E.M. of at least five independent experiments.

transport system by Ca^{2+}/CaM-dependent calcineurin activation occurs in intact nerve terminals, and aluminium seems to interfere with this modulatory mechanism even

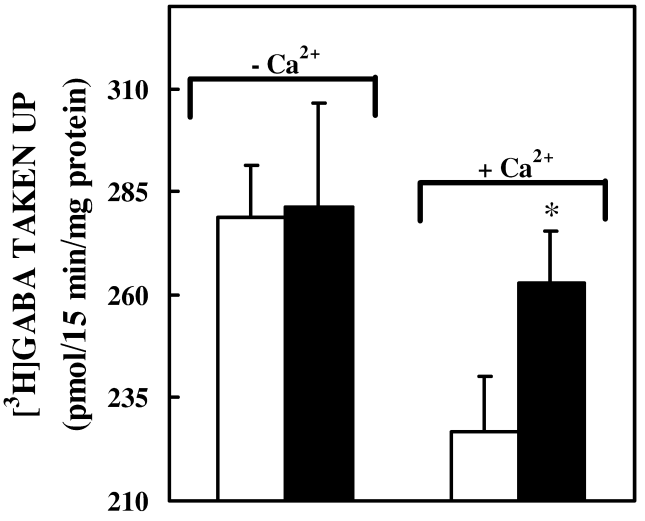

Fig. 9. Aluminium relives the Ca^{2+}-induced [^{3}H]GABA uptake inhibition in synaptosomes. Synaptosomes (0.5 mg protein/ml) were equilibrated as described in the legend of Fig. 8, except that the assays were performed for 15 min in the presence of 2 μM ionomycin. Bars show the amount of GABA taken up during 15 min in the absence or in the presence of 10 μM free Ca^{2+}. Control condition (absence of $AlCl_3$), white bar; presence of 300 μM $AlCl_3$, black bar. Vertical bars denote S.E.M. of at least nine independent experiments. *$P<0.01$, statistically significant from control value.

under conditions of partial disruption of ionic gradients across SPM.

4. Discussion

In accordance to the knowledge of the authors, this work represents the first experimental evidence that links the well-established effects of aluminium on calmodulin function [18,39–41,44–54] with the effect of aluminium on GABA transport in brain cortex nerve terminals. We have demonstrated that aluminium relieves the modulatory action of Ca^{2+} on GABA transporter, operating either in forward (uptake) or backward (release) directions (Figs. 3, 6 and 9), which involves Ca^{2+}/CaM/calcineurin signalling pathway (Figs. 1, 4 and 7). The modulation of cellular activities represents one of the major mechanisms responsible for coupling signals to cellular responses, which is foremost important in neurotransmission.

Calcineurin is one of the most abundant proteins in the brain, accounting for over 1% of total protein [82,83]. The regulation of neurotransmitter release by calcineurin was highlighted in glutamatergic and GABAergic transmission. Both uptake and release of these neurotransmitters are reduced upon activation of calcineurin [84,85]. Extrapolation of aluminium effects observed in in vitro experiments to in vivo conditions is difficult because of chemical speciation, bioavailability and relative toxicity of the distinct forms of aluminium. The average aluminium levels in human brain cortex ranges from 0.14 to 0.22 mg/kg [86], and aluminium concentrations in brain are significantly increased during aluminium intoxication, dementing diseases and metabolic encephalopathies [5,78,87,88]. Interestingly, Srabanti and Chaudhuri [89] showed that oral administration of $AlCl_3$ (50 mg/kg per day during 40 days) to rats resulted in a 70% decrease in calcineurin activity. This observation seems to support the physiological relevance of the results reported in this work (Fig. 1), suggesting that the relief of Ca^{2+} modulatory action on GABA transport by aluminium may occur during in vivo aluminum exposure.

Aluminium was reported to inhibit 40–50% the uptake of GABA, glycine, glutamate, choline, dopamine, noradrenaline and 5-hydroxytryptamine by synaptosomal fractions isolated from distinct brain regions [2,21,29–31,77–80]. The previously reported inhibitory effects of aluminium were mainly attributed to decreased (Na^+/K^+)-ATPase activity [29], rather than to direct interaction with neurotransmitter transporters. In fact, the transport systems responsible for the uptake of those neurotransmitters are driven by the Na^+ electrochemical gradient maintained by the (Na^+/K^+)-ATPase [60,61,68,81,90–92]. In the present work, the effect of aluminium on the [^{3}H]GABA uptake was studied by using SPM vesicles (Figs. 2–4) and synaptosomes (Figs. 8 and 9) isolated from brain cortex. As mentioned earlier, these presynaptic membrane fractions are especially different with respect to the process underlying formation of the Na^+ electrochemical gradient across plasma membrane. Ionic gradients are imposed artificially to SPM vesicles, whereas (Na^+/K^+)-ATPase is the main enzyme activity responsible for generation and maintenance of the Na^+ electrochemical gradient in synaptosomes. Accordingly, we observed that aluminium did not inhibit the uptake of GABA when the Na^+ electrochemical gradient was imposed artificially (SPM vesicles) whereas aluminium produced a ~40% reduction of the amount of GABA taken up by synaptosomes. These results are in agreement with the findings of Wong [30], which showed a 50% inhibition of GABA uptake by 316 μM $AlCl_3$. Moreover, no alterations in the amount of GABA taken up by synaptosomes during the addition of aluminium were observed, when the (Na^+/K^+)-ATPase activity was already partially inhibited by ouabain, a selective inhibitor of the (Na^+/K^+)-ATPase (Fig. 8).

In addition to the effect of aluminium on neurotransmitter transport due to inhibition of (Na^+/K^+)-ATPase, the results reported suggest that aluminium can possibly prevent the modulation of GABA transport by Ca^{2+} (Figs. 3, 4, 6, 7 and 9).

GABAergic transmission relies on the occurrence of a transient of GABA at the synaptic cleft, rising from submicromolar range to millimolar range and back down to

<1 μM in about 1 ms [93,94]. The high-affinity GABA transporter present at SPM is responsible for the shaping of GABAergic tone, not only because it mediates reuptake of GABA back to presynaptic terminals [95–97], but also because it mediates the release of this neurotransmitter to the synaptic cleft in response to depolarisation of SPM [57,60,91,98–102]. Recently, it has been reported that carrier-mediated release of GABA can occur in response to slight increases of K^+ concentrations (few millimolar) in the extracellular space [60,91,99], especially under conditions that favour a simultaneous reduction of the magnitude of Na^+ electrochemical gradient [60,81,91]. It seems that under resting conditions the high-affinity GABA transporter operates near its equilibrium, and the instantaneous direction of fluxes will depend mainly on GABA and ionic gradients across SPM, as well as on changes in membrane potential. On the contrary, when an action potential reaches the presynaptic terminal, GABA is released by both exocytosis and reversal of the carrier-mediated GABA transport, thereby assuring massive release of GABA into the synaptic cleft, necessary for GABAergic transmission [94]. Meanwhile, activation of calcineurin by the micromolar Ca^{2+} transient occurs, which, in turn, will decrease carrier-mediated GABA translocation across SPM (Figs. 3, 6 and 9, and Refs. [56,57,59]). The experimental evidence presented in this work clearly demonstrates that aluminium influences the tight spatio-temporal regulation of GABA translocation across SPM, by disrupting Ca^{2+}/CaM-dependent calcineurin signalling pathway, since aluminium inhibited Ca^{2+}/CaM-dependent calcineurin activity (Fig. 1) and relieved the Ca^{2+} modulatory action on the GABA translocation system, either when it mediates active uptake or K^+ depolarisation-induced release of GABA (Figs. 3, 6 and 9).

There are many reports relating GABAergic system dysfunction to neurological disorders and aluminium intoxication [25,99,102]. Epilepsy is perhaps the most evident one, because it involves failure of normal GABAergic transmission which results in seizure episodes [25,99,102]. Aluminium accumulation in GABAergic cell cultures was found to occur [9], and patients suffering from dialysis encephalopathy had their GABA contents reduced by 40% [25]. Recently, GABA uptake and carrier-mediated release were found to be impaired in patients suffering from human temporal lobe epilepsy [99,102], and high aluminium content in brain tissues seems to be related to epilepsy, dialysis encephalopathy, along with other neurological diseases, like Parkinson's and Alzheimer's diseases [2,7,78,103,104].

GABA transporters, and neurotransmitter transporters in general, are not passive players in regulating neurotransmission [92,105,106]. Neurotransmitter transporters share energetic resources and other common features, such as structure and functional similarities [92]. The findings reported in this study signal the need for further investigation of the effects of aluminium on other neurotransmitter transport systems.

5. Notation

AOAA, aminooxyacetic acid; CaM, calmodulin; GABA, γ-aminobutyric acid; J_{max}, maximal translocation velocity; Mes, 2[*N*-morpholino]ethanesulfonic acid; SPM, synaptic plasma membrane; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide hydrochloride

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