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Role of nitric oxide synthase on brain GABA transaminase activity and GABA levels

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In an attempt to clarify the controversial role of nitric oxide (NO) in seizures, the effects of NO on brain GABA transaminase (GABA-T) activity and GABA levels were investigated. To this aim, the effects of the substrate (L-arginine) and inhibitors (Nω-nitro-L-arginine methyl ester, 7-nitroindazole) of NO synthase (NOS) on GABA-T activity and GABA levels *in vitro* and *ex vivo* were analyzed. *In vitro* NO diminished GABA-T activity and increased GABA. *Ex vivo* NO modified GABA-T activity and GABA levels biphasically. Inhibition of endothelial and neuronal NOS (eNOS and nNOS) had opposite effects on GABA-T activity and GABA levels, even during seizures induced by pentylenetetrazole. Different effects of NO on GABA-T activity and on GABA levels, depending on the NOS isoform involved, may explain its contradictory role in seizures, the endothelial NOS acting as an anticonvulsant and the neuronal NOS as a proconvulsant. nNOS inhibitors may represent a new generation of antiepileptics.

Keywords: nitric oxide, GABA-transaminase, GABA, seizures

Nitric oxide (NO) is a freely diffusible gas synthesized from the oxidation of L-arginine by one of the isoforms of the enzyme NO synthase (NOS): neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). In the brain, NO acts both as a second messenger and as a neurotransmitter/neuromodulator, and influences several physiological functions (1, 2) such as: interneuronal communication, synaptic plasticity, memory, intracellular signal transmission and mediator release (3). Also, it has been proposed that NO can induce pathologies that can lead to different neurological disorders such as ischemia, stroke and epileptiform seizures (2).

The role of NO in epileptogenesis has been examined in a number of *ex vivo* and *in vitro* studies; however, the results are still contradictory. In the present state of knowledge,

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the only reasonable conclusion is that NO behaves as a neuromodulador with dual – pro-convulsive or anticonvulsive – action (4).

The main intracellular effect of NO in the central nervous system (CNS) is the activation of soluble guanylate cyclase, leading to the synthesis of cyclic guanosine monophosphate (cGMP) (5). As an example of the importance of the NO-cGMP pathway, its involvement in the neuroprotective effect of hesperidin in mice could be mentioned (6). However, NO also evokes the release of neurotransmitters including acetylcholine, catecholamines and neuroactive amino acids such as gamma aminobutyric acid (GABA) (7).

Seizures may appear when the balance between excitatory and inhibitory impulses in the brain is perturbed. GABA is considered to be the main inhibitory neurotransmitter of the CNS that counterbalances neuronal excitation. One of the strategies to prevent and/or control convulsions is to increase GABA brain levels through inhibition of GABA transaminase (GABA-T, EC 2.6.1.19), the enzyme responsible for its degradation. Based on this principle, anticonvulsant drugs such as amino oxyacetic acid (AAOA) and antiepileptic drugs such as valproic acid (8), vigabatrin (9) and ethanolamine-*O*-sulfate (10) were designed.

Stimulation of glutamate receptors, mainly of the *N*-methyl-D-aspartate (NMDA) type, is a consequence of the increase in cGMP caused by NO. It has been reported that NMDA receptors may contribute to both anti- and pro-convulsant effects of D-penicillamine (11). NMDA receptor activation induces release of GABA from the cerebral cortical and striatal neurons (12–14). In addition, it has been reported that the increase in NO concentration is associated with the release of GABA in cerebral cortex (7), hippocampus (15) and striatum (16–18); however, the mechanisms still remain unclear. As an example, it is not clear why moderate and high increases of NO levels had opposite effects on GABA-T activity (18).

Considering the foregoing and the widespread role of NO in the nervous system, we conducted an *in vitro* and *ex vivo* study in order to explore the possible factors influencing the effect of NO on GABA-T activity. To this aim, the effects of promotion of NO synthesis induced by L-arginine (L-Arg) on GABA-T activity and GABA levels in whole mouse brain were investigated. The effect of NO synthesis inhibition on the described parameters was also studied using *Nω*-nitro-L-arginine-methyl ester (L-NAME), an unspecific inhibitor of NOS. Since nNOS is distributed in several sensory pathways (19, 20), 7-nitroindazole (7-NI), a specific inhibitor of nNOS that administrated in doses up to 80 mg kg^{-1} was reported to have no effect on the mean arterial pressure (21), was also included in the study. Effect of the mentioned drugs on described parameters was also studied during seizures induced by pentylenetetrazole (PTZ).

EXPERIMENTAL

Chemicals

L-Arg, L-NAME, 7-NI, AAOA, GABase [a mixture of GABA-T and succinic semialdehyde dehydrogenase (SSDH, EC 1.2.1.16) from *Pseudomonas fluorescens*] and PTZ were purchased from Sigma Chemical Co. (USA). Standard analytical grade laboratory reagents were obtained from Merck (Germany) or Sigma-Aldrich Chemical Co. (USA).

Animals

Experiments were conducted in compliance with the Helsinki Guide for the Care and Use of Laboratory Animals, adopted and promulgated by the EU Directive 2010/63/EU for animal experiments and approved by the institutional committee of ethics of E.N.C.B. (Escuela Nacional de Ciencias Biológicas, Ciudad de México, México).

CD1 male albino mice with the mean body mass of 25 g (aged 5–6 weeks) were used in the experiments. Animals were maintained under a 12/12 h light/dark cycle, fed *ad libitum* a stock laboratory diet (%, *m*/*m*: 49.8 carbohydrates, 23.5 protein, 3.7 fats, minerals and added vitamins and amino acids) and had free access to drinking water.

Brain GABA-T activity determination

Tissue processing. – After appropriate treatment, animals were sacrificed and whole wet brains were removed. Homogenates were prepared in 5 % Triton X-100 solution (25 %, *m*/*V*) in a tissue homogenizer (Glas-Col, USA). After centrifugation at 12,500 rpm for 45 min, GABA-T activity was determined in the supernatants.

Enzymatic activity evaluation. – GABA-T activity quantification was performed according to the method of Jung *et al.* (22) using 6 μmol L^{-1} GABA plus 5 μmol L^{-1} 2-oxoglutarate as substrate. Enzymatic activity was proportional to the formation of NADH from NAD, which was recorded as the increase in absorbance at 340 nm. Specific GABA-T activity was calculated by subtracting the blank values (measured in the absence of substrate) from the total activity. The anticonvulsant AAOA was employed as a control in all cases.

In vitro *effect of NO on brain GABA-T activity*

To the supernatants obtained from mouse brain homogenates, different concentrations of L-Arg (10^{-5}–10^{-3} mol L^{-1}), L-NAME (10^{-4}–10^{-3} mol L^{-1}), 7-NI (10^{-5}–10^{-3} mol L^{-1}) or AAOA (10^{-3} mol L^{-1}) were added. Several minutes afterwards (10, 15, 20 or 30 min), GABA-T activity was determined. Enzymatic activity was compared to that of homogenates from animals that did not receive any treatment.

Ex vivo *effect of NO on GABA-T activity*

Groups of 5 animals each received different doses of L-Arg (30–250 mg kg^{-1}), L-NAME (10–200 mg kg^{-1}), 7-NI (10–50 mg kg^{-1}) or AAOA (20–50 mg kg^{-1}) *via i.p.* Animals were sacrificed one hour after administration, brains were quickly removed and GABA-T activity was determined.

In a different set of experiments, L-Arg (50 mg kg^{-1}), L-NAME (50 and 100 mg kg^{-1}), 7-NI (10 mg kg^{-1}) or AAOA (40 mg kg^{-1}) were *i.p.* administrated to groups of 5 animals each. After 1 hour of exposure to the respective agent, animals received PTZ (95 mg kg^{-1}). Twenty minutes later, animals were sacrificed and GABA-T activity was determined. Similar experiments were performed at the same time under the same conditions, but without PTZ administration.

In all cases, enzymatic activity was compared to that of animals that received only saline solution.

Determination of brain GABA concentration

Tissue processing. – Animals were sacrificed and whole brains were removed. Homogenates in 80 % ethanol were obtained with a Glas-Col tissue homogenizer. Homogenates were centrifuged (3,500 rpm/5 min), pellets were washed in 75 % ethanol and centrifuged at 5000 rpm for 5 min. Supernatants were brought together and chloroform was added. After vigorous shaking and centrifugation at 3,000 rpm for 20 min, the aqueous phase was extracted and concentrated. Aliquots of this sample were used for GABA quantification.

Enzymatic quantification of GABA. – GABA quantification was performed with GABase according to the manufacturer's instructions (Sigma Chemical Co). Briefly, in this method GABA-T converts GABA to succinate semialdehyde (SSA). SSA is then converted to succinate in a reaction catalyzed by SSDH, using $NAD(P)^+$ as a cofactor. Stoichiometric reduction of the cofactor, resulting in the increment of the absorbance at 340 nm, reflects the levels of GABA in the reaction mixture. Reaction medium was composed of pyrophosphate buffer pH = 8.6, NADP 0.004 mol L^{-1}, pH = 7, GABA solution and alpha ketoglutaric acid 0.02 mol L^{-1}, pH = 7.9. AAOA was used as a control in all experiments.

Ex vivo *effect of NO on GABA brain levels*

Different doses of L-Arg (30–250 mg kg^{-1}), L-NAME (10–200 mg kg^{-1}), 7-NI (10–50 mg kg^{-1}) or AAOA (20–50 mg kg^{-1}) were *i.p.* administrated to groups of 5 animals each. Animals were sacrificed after 1 hour, the brains were quickly removed and GABA levels were quantified.

In all cases, GABA concentration was compared with the GABA concentration in animals of the control group, which received only saline solution.

Statistical analysis

All results were normalized against the control and expressed as the mean ± SEM (standard error of the mean) of at least five determinations ($n > 5$). GABA-T activity was compared between groups using the one-way analysis of variance (ANOVA). *F*-value was

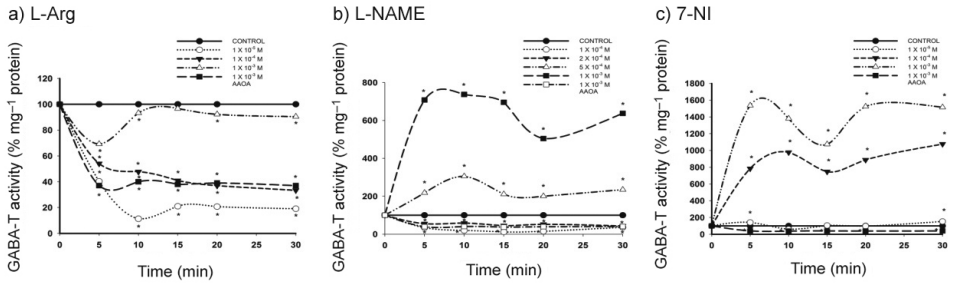

Fig. 1. *In vitro* influence of some nitric oxide synthase (NOS) effectors on mouse brain gamma amino butyric acid transaminase (GABA-T) activity. a) L-arginine (L-Arg), b) Nω-nitro-L-arginine methyl ester (L-NAME), c) 7-nitroindazole (7-NI) and amino-oxyacetic acid (AAOA). Results are mean ± SEM ($n > 4$). *Significantly different compared to the control: $p < 0.05$.

calculated and, if and where indicated, followed by Tukey's multiple comparison test. Graph Pad Prism version 5.0 software (GraphPad Software, Inc, La Jolla, CA, USA) was used, and $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The aim of this work was to investigate the effect of NO on GABA-T activity and brain GABA levels in order to find out why moderate and high increases in NO levels had opposite effects on GABA-T activity, as reported by Paul *et al*. (18). NO levels were modified by influencing the NOS activity with its natural substrate (L-Arg) and with unspecific (L-NAME) and neuronal NOS specific (7-NI) inhibitors of its activity, and the resulting changes in GABA-T activity and GABA levels were analyzed *in vitro* as well as *ex vivo*. AAOA, a traditional GABA-T inhibitor, was used as a positive control.

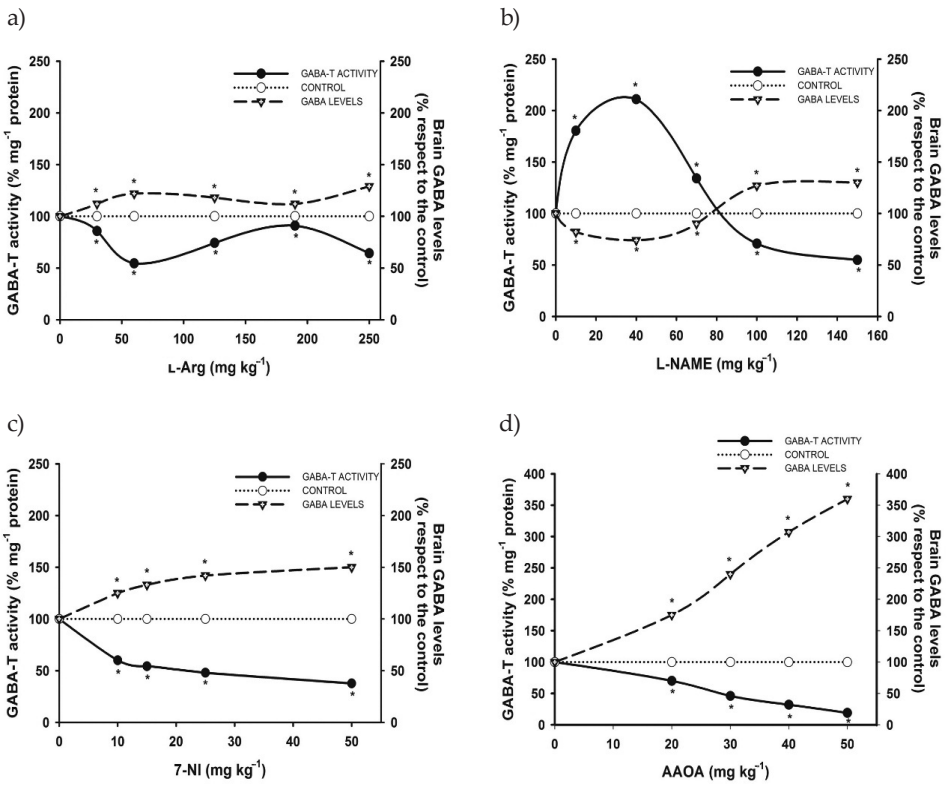

Fig. 2. *Ex vivo* influence of some nitric oxide synthase (NOS) effectors on mouse brain gamma amino butyric acid transaminase (GABA-T) activity and brain GABA levels. a) L-arginine (L-Arg, 30–250 mg kg^{-1}), b) Nω-nitro-L-arginine methyl ester (L-NAME, 10–200 mg kg^{-1}), c) 7-nitroindazole (7-NI, 10–50 mg kg^{-1}) or d) amino-oxyacetic acid (AAOA, 20–50 mg kg^{-1}). Results are mean ± SEM ($n > 4$). *Significantly different compared to the control: $p < 0.05$.

L-Arg, 10^{-3} mol L^{-1}, significantly decreased GABA-T activity in a dose dependent manner *in vitro*, reaching an activity of only 10 % compared to the control (Fig. 1a). L-NAME increased GABA-T activity in a dose response manner up to 700 % compared to the control (Fig. 1b). The described *in vitro* variations of GABA-T activity induced by changes in NO levels indicate a clear correlation between these parameters, and are in accord with the reports of Jayakumar *et al*. (17) and Paul *et al*. (18, 23).

Considering only the results for L-NAME, it could be presumed that an *in vitro* induced decrease in NO levels caused an increase in GABA-T activity. However, since L-NAME is a non-specific NOS inhibitor with an affinity for eNOS of 63–75 % and only of 7 % for nNOS, its high concentration would be necessary to inhibit the latter NOS isoform. In other words, the role of nNOS is difficult to determine using L-NAME. Thus, a more selective inhibitor of this NO isoform, 7-NI, was used.

Similarly as L-NAME, 7-NI enhanced GABA-T activity but by a much bigger amount, reaching 1.500 % of activity at 10^{-4} mol L^{-1} compared to the control group (Fig. 1c). An about 15-fold rise of enzymatic activity compared to the control suggests the important role of nNOS in the modulation of GABA-T activity. Considering that high concentrations of L-NAME had effects similar to low 7-NI concentrations, the effects of high concentrations of L-NAME on GABA-T activity could be attributed to the inhibition of nNOS.

Fig. 2 represents *ex vivo* effects of the different NOS effectors. It could be presumed that L-Arg decreased GABA-T activity at two different stages, represented by two different picks. First, NO levels decreased by 40–50 % at a dose of 60 mg kg^{-1}, returning slowly to the control levels at a dose of 190 mg kg^{-1}. In a second peak or stage, GABA-T activity dropped once again by 40–50 % at a dose of 250 mg kg^{-1} (Fig. 2a). Therefore, it could be taken that the diminished GABA-T activity generated by NO is observed not only *in vitro* but also *ex vivo,* but showing two stages, which will be called biphasic. This biphasic shape may represent the effect of NO generated by different NOS isoforms, implying different effects of NO on GABA-T activity, which could depend on whether it is generated by eNOS or nNOS (location dependent).

The former hypothesis seems to be enforced by the effects of different doses of L-NAME (Fig. 2b), which also shows two peaks, reaching an increase of 225 % compared to the control (40 mg kg^{-1}), whereas higher doses decreased the activity, reaching a 50 % activity compared to the control.

As regards the effects of 7-NI *ex vivo,* it decreased GABA-T in a dose dependent manner, reaching about 40 % of enzymatic activity compared to the control and showing only one peak (Fig. 2c). Considering the specific inhibition of 7-NI on nNOS and that its inhibition on GABA-T activity did not show a biphasic effect, along with the fact that 7-NI decreased enzymatic activity by a similar magnitude to that observed at the higher tested dose of L-NAME, it is proposed that the increase of GABA-T activity may be attributed to the inhibition of eNOS, whereas the diminution of the enzymatic activity could be considered as a result of nNOS inhibition.

Overall, *ex vivo* results suggest that endothelial and neuronal NO have opposite effects on GABA-T activity and their effect as an activator or inhibitor of the enzyme may be related to their anticonvulsant or proconvulsant properties. Therefore, NO brain levels may constitute a new mechanism for GABA-T activity regulation, modulating different pathways and suggesting a possible dependence on location (in the glia or in neurons). This hypothesis, however, needs further investigation.

In vitro results for 7-NI are opposite to those observed *in vivo*, since it caused an increase but also a decrease of GABA-T activity, resp. *In vitro* experiments were designed to study the direct effect of drugs on GABA-T activity without the influence of any other factor, whereas *in vivo* studies showed the effect on enzymatic activity as a result of integral effects of drugs on the different body systems. Thus, the contrast between *in vitro* and *in vivo* effects of 7-NI is considered to be the result of its effect not only on the enzyme but also on other systems that may influence GABA-T activity. Since brain GABA-T is the subject of this study, the effects of NO on the nervous system, which may have repercussions on GABA-T activity, are of particular interest. For example, it has been reported that NO evokes the release of several neurotransmitters, including GABA (7, 24).

Considering the forgoing and the role of GABA-T on the regulation of GABA brain levels, L-Arg, L-NAME and 7-NI effects on brain GABA levels were also investigated. L-Arg increased brain GABA levels, showing also two different peaks (Fig. 2a). This means that an increase in NO levels induced by L-Arg induced, in turn, an increase of brain GABA levels, in a biphasic process corresponding to the two stages of the effect of NO on GABA-T activity. This is in accord with the findings of several authors (7, 15, 16). The fact that sodium nitroprusside, another NO donor, also increased brain GABA levels (18) supports the idea that NO could modulate the GABA-ergic system.

The effect of L-NAME on brain GABA levels show also two peaks, diminishes with the dose up to 75 mg kg^{-1} but rises with 100 mg kg^{-1} or higher (Fig. 2b). This is in agreement with the biphasic effect of L-NAME on GABA-T activity and with the biphasic modulation of GABA release by NO from rat hippocampus reported by Getting *et al.* (25).

7-NI increased brain GABA levels in a dose dependent manner (Fig. 2c) to a value similar to the highest dose of L-NAME (Fig. 2c), but showed only one peak. The described increase in GABA levels may explain the enhanced anticonvulsant effects of conventional antiepileptic drugs such as carbamazepine, phenobarbital, phenytoin and valproate induced by 7-NI, obtained in maximal electroshock-induced seizures in a mouse model (26).

Altogether, the described effects on brain GABA levels confirm that NO promotes the release of GABA and suggest that this happens through the changes in GABA-T activity. Besides, considering the results for L-NAME and 7-NI and their affinity for eNOS and nNOS, resp., it could be said that neuronal and endothelial NO have opposite effects not only on GABA-T activity but also on brain GABA levels.

Opposite effects of NO, depending on whether eNOS or nNOS is involved, may explain its anticonvulsant but also proconvulsant properties, as reported in different studies (4). Considering that NO as well as GABA-T activity are related to seizures, the possible repercussions of the changes of NO levels on GABA-T activity during convulsions were also studied, investigating the effect of L-Arg, L-NAME and 7-NI on GABA-T activity during seizures induced by PTZ.

PTZ did not modify GABA-T activity by itself but the decrease of GABA-T activity caused by 50 mg kg^{-1} of L-Arg as well as the increase produced by 50 mg kg^{-1} of L-NAME were partially counteracted during the seizures induced by PTZ. L-NAME 150 mg kg^{-1} and 7-NI 10 mg kg^{-1} decreased enzymatic activity by a similar value (about 60 %), the effect that was not modified by PTZ (Fig. 3). The increase in enzymatic activity induced by a low dose of L-NAME and the decrease caused by a high dose of the same drug indicate that also during seizures induced by PTZ, L-NAME had different effects on GABA-T activity and, in turn, on brain GABA levels, which seems to be related to the NOS isoform involved.

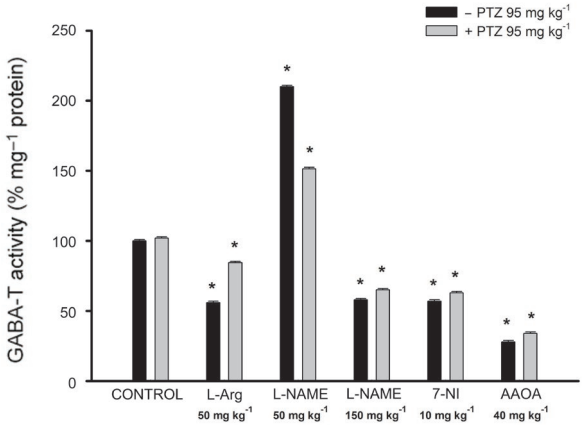

Fig. 3. *Ex vivo* influence of some nitric oxide synthase (NOS) effectors on mouse brain gamma amino butyric acid transaminase (GABA-T) activity during seizures induced by pentylenetetrazole (PTZ). Animals received L-arginine (L-Arg, 50 mg kg^{-1}), Nω-nitro-L-arginine methyl ester (L-NAME, 50 and 150 mg kg^{-1}), 7-nitroindazole (7-NI, 10 mg kg^{-1}) or amino-oxyacetic acid (AAOA, 40 mg kg^{-1}). Seizures were induced by PTZ (95 mg kg^{-1}). Results are mean ± SEM ($n > 4$). *Significantly different from the group that did not receive PTZ: $p < 0.05$.

The primary target of PTZ is to weaken the inhibitory action of GABA, acting as a $GABA_A$ receptor antagonist. However, seizures induced by this chemoconvulsant are also a result of the activation of glutamatergic synaptic transmission, and NMDA receptors are mainly involved in the genesis of clonic seizures (27). The results reported herein suggest that NO generated by nNOS may be the activator of NMDA receptors that participate in this process, since during seizures induced by PTZ inhibition of nNOS, caused by high doses of L-NAME as well as 7-NI, it decreased GABA-T activity leading to an increase of brain GABA levels. This conclusion is not surprising considering that it has been reported

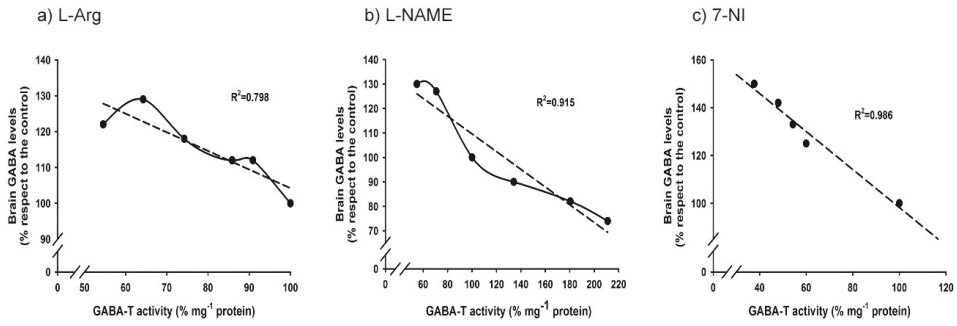

Fig. 4. Correlation between brain gamma amino butyric acid transaminase (GABA-T) activity and brain GABA levels under the influence of L-arginine (L-Arg), Nω-nitro-L-arginine methyl ester (L-NAME) and 7-nitroindazole (7-NI).

that NMDA receptors/NO pathway contribute to the anticonvulsant effect of different drugs such as D-penicillamine (11), dextrometrophan (28) and lithium (29).

Once established that NO influences the GABA-T activity modulating brain GABA levels, the correlations between GABA-T activity and GABA levels in the presence of L-Arg, L-NAME and 7-NI were explored. Fig. 4 shows, as expected, that GABA-T activity and brain GABA levels are inversely related, but only in the case of 7-NI (Fig. 4c) the correlation is linear with $R^2 = 0.986$. These results suggest that NO generated by nNOS had a direct influence on GABA-T activity and thereby on brain GABA levels.

CONCLUSIONS

Contradictory reports on the role of NO on the seizure mechanism may be explained by the NOS isoform involved. The results confer anticonvulsant properties on endothelial NO but proconvulsant on neuronal NOS. This attribution of convulsant properties to neuronal NO is in agreement with the finding that the anticonvulsant effect of dextrometrophan is mediated by a decline in nNOS activity (28). Proconvulsant or anticonvulsant properties of NO may be explained by the opposite effects of endothelial and neuronal NO on GABA-T activity, and hence on GABA levels.

More work is necessary to assess exactly the role of neuronal NO and its particular action site, since it has different effects on the brain. For example, it has been reported that nNOS- derived excess NO in the glutamatergic pathway plays a key role in the failure of the blood-brain-barrier during seizures induced by PTZ (30).

Understanding how to modulate brain GABA-T activity through NO brain levels represents a novel strategy for controlling seizures, and hence for the development of new antiepileptic drugs (AEDS), which is especially important in the cases refractory to the treatment with traditional AEDS, either administered alone or in combination.

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Acronyms, abbreviations, symbols. – AAOA – amino oxyacetic acid, AEDS – antiepileptic drugs, L-Arg – L-arginine, cGMP – cyclic guanosine monophosphate, CNS – central nervous system, GABA – gamma aminobutyric acid, GABA-T – GABA transaminase, NAD(P) – nicotinamide adenine dinucleotide(phosphate), L-NAME – *N*-nitro-L-arginine-methyl ester, 7-NI – 7-nitroindazole, NMDA – *N*-methyl-D-aspartate (NMDA), NO – nitric oxide, NOS – NO synthase (eNOS – endothelial NOS, iNOS – inducible NOS, nNOS – neuronal NOS), PTZ – pentylenetetrazole, SSA – succinate semialdehyde, SSDH – succinic semialdehyde dehydrogenase.

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