Acta Pharm. **73** (2023) 59–74
https://doi.org/10.2478/acph-2023-0004

Original research paper

The opposite effect of convulsant drugs on neuronal and endothelial nitric oxide synthase – A possible explanation for the dual proconvulsive/anticonvulsive action of nitric oxide

LOURDES A. VEGA RASGADO[1,*]
EVA RAMÓN-GALLEGOS[2]
LORENA RODRÍGUEZ-PÁEZ[3]
VERÓNICA ALCÁNTARA-FARFÁN[3]

[1] *Laboratorio de Neuroquímica*
Departamento de Bioquímica
Escuela nacional de Ciencias Biológicas
Instituto Politécnico Nacional
Carpio y Plan de Ayala S/N, Colonia
Casco de Santo Tomás, C.P. 11340 Ciudad de México, México

[2] *Laboratorio de Citopatología Ambiental*
Departamento de Morfología
Escuela nacional de Ciencias Biológicas
Instituto Politécnico Nacional
Calle Wilfrido Massieu esquina Cerrada Manuel Stampa, Colonia Zacatenco, C.P. 07738 Ciudad de México, México

[3] *Laboratorio de Bioquímica Farmacológica*
Departamento de Bioquímica
Escuela nacional de Ciencias Biológicas
Instituto Politécnico Nacional
Carpio y Plan de Ayala S/N, Colonia
Casco de Santo Tomás, C.P. 11340 Ciudad de México, México

Accepted August 21, 2022
Published online August 21, 2022

ABSTRACT

Nitric oxide (NO) participates in processes such as endothelium-dependent vasodilation and neurotransmission/neuromodulation. The role of NO in epilepsy is controversial, attributing it to anticonvulsant but also proconvulsant properties. Clarification of this dual effect of NO might lead to the development of new antiepileptic drugs. Previous results in our laboratory indicated that this contradictory role of NO in seizures could depend on the nitric oxide synthase (NOS) isoform involved, which could play opposite roles in epileptogenesis, one of them being proconvulsant but the other anticonvulsant. The effect of convulsant drugs on neuronal NO (nNO) and endothelial NO (eNO) levels was investigated. Considering the distribution of neuronal and endothelial NOS in neurons and astrocytes, resp., primary cultures of neurons and astrocytes were used as a study model. The effects of convulsant drugs pentylenetetrazole, thiosemicarbazide, 4-aminopyridine and bicuculline on NO levels were studied, using a spectrophotometric method. Their effects on NO levels in neurons and astrocytes depend on the concentration and time of treatment. These convulsant drugs caused an increase in nNO, but a decrease in eNO was proportional to the duration of treatment in both cases. Apparently, nNO possesses convulsant properties mediated by its effect on the glutamatergic and GABAergic systems, probably through $GABA_A$ receptors. Anticonvulsant properties of eNO may be the consequence of its effect on endothelial vasodilation and its capability to induce angiogenesis. Described effects last as seizures do. Considering the limitations of these kinds of studies and the unexplored influence of inducible NO, further investigations are required.

Keywords: epilepsy, nitric oxide, seizures, endothelial nitric oxide, neuronal nitric oxide

* Correspondence; e-mail: lourdes_vega_rasgado@hotmail.com
[a] These authors contribute equally to the work.

Nitric oxide (NO) is a free radical synthesized from the oxidation of L-arginine by NO synthase (NOS). There is a growing amount of evidence that NO represents an important cellular messenger which plays a significant role in a variety of neurobiological processes. Specifically, in the central nervous system (CNS) NO participation has been recognized in different processes such as endothelium-dependent vasodilation, neurotransmission and the host-defense mechanisms (1–6).

NO influences several physiological functions such as: interneuronal communication, synaptic plasticity, memory, intracellular signal transmission and mediator release. Nevertheless, alterations of the NO system could be implicated in a wide variety of neurological diseases such as ischemia, stroke and epileptiform seizures.

Epilepsy is the most common neurological disorder that affects approximately 50 million people worldwide. It is characterized mainly by recurrent seizures accompanied by a loss of consciousness (7). NO regulates excitatory and inhibitory neurotransmission in both physiological and pathological conditions (8).

NO levels are regulated by two constitutive isoforms of the NOS: neuronal NOS (nNOS) and endothelial NOS (eNOS). But NO effects are conditioned not only by keeping their concentration between certain limits, as can be observed in the following examples. It is considered that in brain ischemia/reperfusion injury as well as in degeneration processes affecting the CNS, NO levels rise rapidly due to hyperactivity of nNOS (9, 10). However, in rats with epilepsy induced by *N*-methyl-D-aspartate (NMDA) injection the treatment with methylene blue, an nNOS inhibitor, increased the symptoms of epilepsy. In contrast, when the precursor of NO, L-arginine, was administered in the same experimental epilepsy model it reduced the symptoms of epilepsy (8). Also, in D,L-homocysteine-thiolactone-induced seizures model L-arginine provided protection and the NOS inhibitor *N(G)*-nitro-L-arginine methyl ester (L-NAME) treatment potentiated the incidence of seizures (11). Besides, in pathological conditions such as inflammation, NO levels produced by inducible NO synthase (iNOS) are temporally extremely high (12).

Thus, there is controversy over the role of NO signalling in the development of epilepsy, since available data show a lack of effect, a decrease or increase in susceptibility to seizures following administration of NOS inhibitors (13–18). Previous results in our laboratory about the effect of NO inhibitors on GABA (gamma amino butyric acid) transaminase activity suggest the possibility that this contradictory role of NO in seizures could depend on the NOS isoform involved (19). It means that NOS isoforms could play opposite roles in seizure generation; one of them being proconvulsant, but the other anticonvulsant.

Some differences between nNOS and eNOS are: (*i*) nNOS is cytosolic but eNOS is membrane-bound, (*ii*) nNOS is mainly soluble but eNOS is particulate, (*iii*) nNOS responds to glutamate receptor stimulation, but eNOS to acetylcholine receptor stimulation, (*iv*) nNOS induces NO release by neuronal dendrites but eNOS by vascular endothelium, (*v*) nNOS physiological function is neuronal communication but eNOS function is vasodilation and pain, (*vi*) nNOS can be found in neuronal tissue, skeletal and smooth muscles, whereas eNOS is located mainly in the endothelium, cardiac myocytes and astrocytes.

For many years astrocytes were regarded as the “glue” that bound neuronal elements together, providing mere structural support for the brain, but now it is considered that astrocytes play a pivotal role in brain homeostasis. Astrocytes have been shown to be involved in important processes such as: brain inflammation, oxidative stress, energy supply and metabolism, support of synaptic function and plasticity, the extracellular balance of

neurotransmitters, extracellular water and ion homeostasis, blood-brain barrier (BBB) maintenance and regulation of blood flow (20–33). In the past two decades, astrocytes have been increasingly acknowledged as key players in the etiology and pathogenesis of epilepsy. Nowadays astrocytes are considered key homeostatic regulators in the CNS and play important roles in the pathophysiology of epilepsy (34).

The clarification of the dual proconvulsant/anticonvulsant effect of NO might have implications for clinical medicine and could lead to new therapeutic opportunities, including the development of new antiepileptic drugs that may help the 30 % of patients who do not respond to the treatment with traditional antiepileptic drugs. Though the development of such new antiepileptics is a great challenge, the ubiquity of NO in the CNS implies that drugs designed to modify the biological activity of NO may have distinct effects.

The therapeutic challenge of the design of such antiepileptics would require the manipulation of the NO pathway selectively to succeed. The possibility of an opposite effect of NO produced by nNOS to that generated by eNOS, as well as the different locations of neuronal and endothelial NOS isoforms, represents an opportunity to design an antiepileptic drug with an innovative mechanism. Such selectivity also would avoid side effects, which is especially relevant for NO.

Because of the former, the potential of the opposite role of nNOS and eNOS in the generation of seizures is investigated here. With this aim, the effect of convulsant drugs on NO levels was studied. Considering the preferential distribution of nNOS and eNOS, resp., primary cultures of neurons and astrocytes were used as a study model.

EXPERIMENTAL

Animals

All experiments were performed according to the rules for the protection of animals used in research and other scientific purposes accordingly in the Declaration of Helsinki involving experimental animals, and were approved by the institutional ethical committee of Escuela nacional de Ciencias Biológicas, Instituto Politécnico Nacional (Ciudad de México, México).

Albino Wistar rats fed ad libitum on a stock laboratory diet (in %, *m*/*m*: 49.8 carbohydrates, 23.5 protein, 3.7 fat, 5.5 minerals, added vitamins and amino acids) were used for the experiments. The animals were maintained in a 12-h light-dark cycle. Females with a mean weight of 250 g were caged with males overnight (3 females and 1 male by cage) and conception was considered to occur at 01:00; this was verified the following morning by the presence of spermatozoa in the vaginal smears.

Reagents

Dulbecco's modified eagle's medium (DMEM), penicillin, streptomycin, poly-L-lysine, cytosine arabinoside, bovine serum albumin (BSA, fatty acid-free and dialyzed before use), pentylenetetrazole (PTZ), thiosemicarbazide (TS), 4-aminopyridine (4 AP), bicuculline (BIC), *N*-1-napthylethylenediamine (NED) and sulfanilamide were purchased from Sigma-Aldrich Chemical Co. (USA). Fetal calf serum (FCS) was obtained from Serva Boehringer Ingelheim (Germany).

Neurons and astrocytes cultures

Primary cultures were obtained as previously described by Tabernero *et al*. (35). For neuron cultures, rat fetuses at 17.5 days of gestation were delivered by rapid hysterectomy after cervical dislocation of the mother. Postnatal 1-day newborn rats were used to prepare astrocytes in culture. Animals were decapitated and their brains immediately excised. After removing the meninges and blood vessels, the forebrains were placed in Earle's balanced solution (EBS) containing 20 μg mL^{-1} DNase and 0.3 % (*m*/*V*) BSA. The tissue was minced, washed, centrifuged at 500×*g* for 4 min and incubated in 0.025 % trypsin (type III) and 60 μg mL^{-1} DNase I for 15 min at 37 °C. Trypsinization was terminated by the addition of DMEM containing 10 % FCS. The tissue was then dissociated by gently passing it eight times through a siliconized Pasteur pipette, and the supernatant cell suspension was recovered. This operation was repeated, and the resulting cell suspension was centrifuged. The cells were then resuspended in DMEM containing 10 % FCS and plated on Petri dishes coated with 10 μg mL^{-1} poly-lysine at a density of 10^5 cells cm^{-2}. Cells were maintained at 37 °C and 5 % CO_2. One and five days after plating for neurons and astrocytes respectively, 10 μmol L^{-1} cytosine arabinoside was added, to avoid glial cell proliferation on neuronal cultures and neuronal proliferation on astrocyte cultures. Under such conditions, culture purity was around 98 %. During the whole experiment, cellular morphology was reviewed under a microscope. After 7 and 21 days of culture, neurons and astrocytes were completely differentiated.

Treatment by convulsant drugs on neurons and astrocytes in primary culture and NO quantification

NO levels were determined as nitrites using a spectrophotometric method based on the Griess reaction. Briefly, a solution of 1 % sulfanilamide in 5 % phosphoric acid was added to the sample. After 5–10 min incubated at room temperature protected from light, a solution of 0.1 % NED in water was added and the mixture was incubated again for 5–10 min protected from light. The absorbance of the purple/magenta colour of the azo compound formed was measured within 30 min between 520–550 nm. A nitrite standard reference curve was prepared for each assay for accurate quantitation of nitrite levels in experimental samples.

One week after planting cultured neurons in DMEM supplemented with FCS 10 % (*V*/*V*), cells received 10, 25, 50, 75 and 100 μmol L^{-1} of different convulsant drugs: PTZ, 4 AP, TS or BIC. Each concentration indicated above of all convulsants acted for three specific times (24, 48 and 72 hours).

In a different set of experiments, astrocytes were cultured in DMEM with 10 % of FCS (*V*/*V*) for 21 days. Then, the cells were exposed to the same concentrations of PTZ, 4 AP, TS or BIC and specific times of treatment employed in neurons.

Once the corresponding treatments were completed NO levels were quantified in both cells, neurons and astrocytes, using the spectrophotometric method described above. Every sample was analyzed 4 times and the experiments were repeated 5 times. The resulting data were analyzed to study the influence of concentration and time of treatment with the convulsant drugs on NO level. NO level is compared with the control group, which consists of neurons or astrocytes that did not receive any convulsant drug but were treated exactly under the same conditions.

Statistical analysis

All results are expressed as the mean ± standard error of the mean (SEM) of at least five determinations, which were analyzed 4 times for each sample. NO levels, expressed as nitrite concentration, were compared between groups using independent factorial analysis of variance (ANOVA) or repeated-measures ANOVA. For *post hoc* analysis, Newman-Keuls multiple comparison test was employed. Graph Pad Prism version 5.0 software (GraphPad Software, Inc, La Jolla, CA, USA) was used. Statistical significance was indicated as $p < 0.05$.

RESULTS AND DISCUSSION

Influence of convulsant drugs concentration on nitric oxide levels in neurons and astrocytes in primary culture

Pentylenetetrazole (PTZ). – Results presented in Fig. 1 indicate that, after 24 hours of treatment in neurons, PTZ at low concentrations increased NO levels. The maximum was reached at 25 μmol L^{-1} followed by a decrease until 60 μmol L^{-1} and increased again from this point until 100 μmol L^{-1} (the highest concentration studied). After 48 hours of treatment, PTZ showed a similar biphasic tendency on a higher scale, but after 72 hours of treatment biphasic behaviour disappeared. The concentration of 10 μmol L^{-1} decreased slightly NO levels, but 25, 50 and 75 μmol L^{-1} increased them. Then, the highest concentration tested (100 μmol L^{-1}) significantly diminished NO levels.

PTZ in astrocytes (Fig. 2) had an opposite effect to that described in neurons, causing a decrease of NO levels at all concentrations tested after 24, 48 and 72 hours of treatment in an inversely proportional manner.

The primary action of PTZ is its role as a $GABA_A$ receptor antagonist (36). PTZ suppresses the function of inhibitory synapses, leading to increased neuronal activity. This regulation causes generalized seizures in animals (37). However, according to some reports the seizures induced by this chemoconvulsant are also a result of the activation of glutamatergic synaptic transmission. NMDA receptors are mainly involved in the genesis of clonic seizures induced by PTZ (38) and our results indicate that neuronal NO could be the mechanism through which PTZ causes the activation of NMDA receptors. It seems to be in coincidence with the consideration that nNOS contribute to kindling epilepsy induced by PTZ (39). On the other hand, the decreased NO levels in astrocytes induced by PTZ indicate a possible association between seizures and diminished levels of NO generated by eNOS.

Thiosemicarbazide (TS). – The effect of TS in neurons can also be seen in Fig. 1. The influence of TS on NO levels after 24 hours of treatment can be described as a biphasic curve, increasing at TS concentrations ≤ 10 μmol L^{-1} and then diminishing till 50 μmol L^{-1}, and then increasing slightly again at higher concentrations. In neurons, after 48 h TS concentrations ≤ 25 μmol L^{-1} caused a decrease in NO levels, but afterwards they increased until the control levels; after 72 hours of treatment, TS decreased NO levels in neurons, except for the concentration of 10 μmol L^{-1}.

Instead of increasing NO levels as observed in neurons, after 24 and 48 hours of treatment TS produced a reduction in the NO level in astrocytes, which was inversely propor-

tional to the concentration of the convulsant (Fig. 2). Seventy-two-hours-treatment with TS also caused a decrease inversely proportional to its concentration, but only at concentrations < 50 μmol L^{-1}. This is clearly seen from linear regression analysis which showed a much better correlation (R = –0.9929) for concentrations < 50 μmol L^{-1} instead of all concentrations (R = –0.8734).

Considering the described mechanism of action of PTZ on $GABA_A$ receptors, and the fact that TS also induces seizures by modifying GABA levels, diminishing its synthesis by inhibiting glutamic acid decarboxylase activity (GAD), it might be suggested that changes

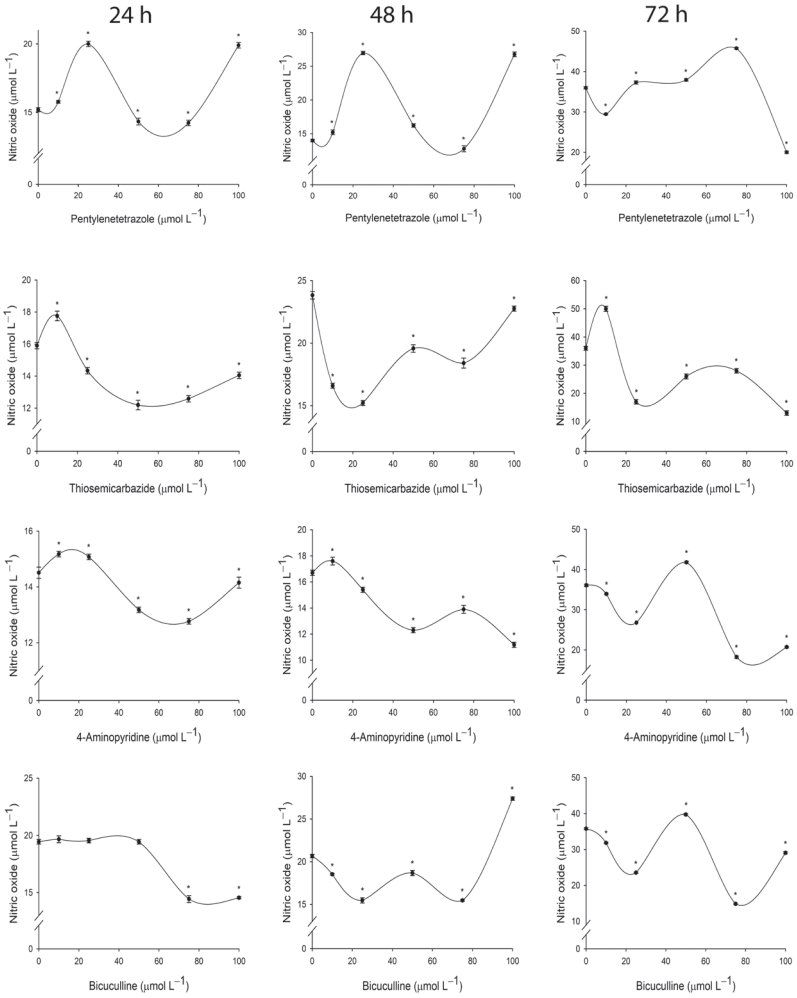

Fig. 1. Concentration-dependent effect of convulsant drugs on nitric oxide (NO) levels (expressed as nitrites, mean ± SEM, n = 5), in neurons in primary cultures. * Statistically significant difference as compared with control: $p \leq 0.05$ (*post-hoc* analysis Newman-Keuls test).

induced in the GABAergic system are related to an increase in neuronal NO levels. However, the decrease of NO levels in astrocytes reinforces the idea of a relationship between seizures and low levels of eNO. Besides, it must be considered that because of its structure, which includes sulfur and hydrazine groups, TS may act also act as an antioxidant.

4-Aminopyridine (4 AP). – Although on a different scale, 4 AP effects in neurons (as observed in Fig. 1), are very similar to that caused by TS. At all times of treatment studied a biphasic shape was observed. After 24 hours of treatment, the graphic shows a peak between 10 and 25 μmol L^{-1} as well as a through at 75 μmol L^{-1}. After 48 hours of treatment, 2 peaks

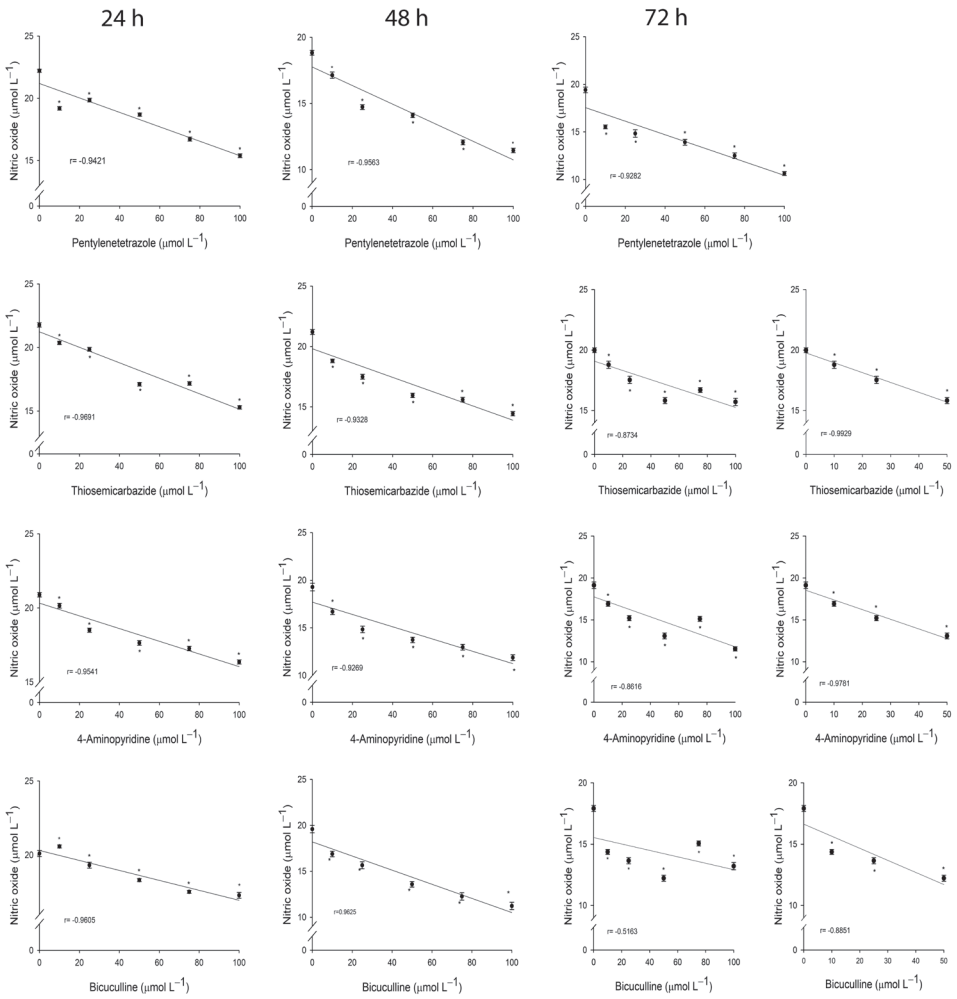

Fig. 2. Concentration-dependent effect of convulsant drugs on nitric oxide (NO) levels (expressed as nitrites, mean ± SEM, $n = 5$), in astrocytes in primary cultures. * Statistically significant difference as compared with control: $p \leq 0.05$ (*post-hoc* analysis Newman-Keuls test).

at the concentrations of 10 and 75 μmol L^{-1}, as well as 2 troughs at 50 and 100 μmol L^{-1} can be seen. After 72 hours of treatment, only one peak at a concentration of 50 μmol L^{-1} and 2 troughs at 25 and 75 μmol L^{-1} can be seen in the graphic.

In Fig. 2 an inverse proportionality was seen between the NO level and 4 AP in astrocytes, except for 75 and 100 μmol L^{-1} after 72 hours of treatment, as indicated by the change of the correlation parameter (from –0.8616 to –0.9781).

4 AP is a convulsant drug that blocks potassium channels, making the action potentials of neurons last longer (40, 41). This effect facilitates the non-specific release of neurotransmitters, including glutamate (Glu), which causes seizures. However, 4 AP at low doses was found to produce epileptiform activity without affecting Glu levels (42, 43), suggesting that other mechanisms, which do not implicate Glu, could be involved in generation of seizures in this model.

Considering that is particularly at low concentrations that 4 AP increased NO level in neurons, NO could be proposed as a possible mechanism by which this convulsant drug induces seizures at low doses. But NO would not exert its action through Glu receptors at such low doses, since Glu levels are not increased. During the 4 AP applications, one type of synchronous field potential discharges reported during this convulsant application is the "slow"-GABA-mediated interictal-like events. Thus, low doses of 4 AP could generate seizures by an effect on the GABAergic system mediated by NO. That would reaffirm the proposed idea of a correlation between effects on the GABAergic system of convulsant drugs and increased neuronal NO levels.

Another type of synchronous field potential discharges caused by a 4 AP application are described as "fast" interictal-like events, that are mainly mediated by Glu receptors. This could be the result of the action of neuronal NO on Glu receptors at high doses, especially considering its short half-life which would produce a very fast response. Hence, the biphasic shape of the effect of 4AP in NO level in neurons may represent the effect of this convulsant on the GABAergic system at low doses and on the glutamatergic system at high doses.

The decreased levels of NO in astrocytes caused by 4 AP might indicate that another effect of this convulsant would be to diminish endothelial NO. This possible mechanism of action of 4 AP would support the idea of the relation between low endothelial NO levels and seizures.

Bicuculline (BIC). – According to Fig. 1, BIC showed a different effect on NO levels in neurons. After 24 hours of treatment, it remained as the control value group until the concentration of 50 μmol L^{-1}, then NO levels fell abruptly. The addition of BIC to neurons after 48 hours of treatment decreased NO levels, showing two troughs at 25 and 80 μmol L^{-1}, to reach higher levels than observed in the control group at 100 μmol L^{-1}. About 72 hours of treatment, the corresponding graph at 50 μmol L^{-1} shows a peak with NO levels higher than the observed in the control, and 2 troughs at 25 and 75 μmol L^{-1}.

In astrocytes, BIC decreased NO level at all tested concentrations and once again an inversely proportional relationship between these parameters was observed, except for the concentrations 75 and 100 μmol L^{-1} after 72 hours of treatment. The former was demonstrated by the correlation parameter which indicated no strong inverse proportionality (R = –0.5163) unless concentrations < 50 μmol L^{-1} were considered.

The convulsant alkaloid BIC was continuously investigated for more than 50 years after its first publication in 1970 (44). It is considered a $GABA_A$ receptor antagonist, but BIC has other different actions such as on acetylcholine receptors (45). It must be highlighted that, unlike the other convulsant drugs, a concentration of 100 μmol L^{-1} and 24 hours of treatment are required to produce an increase in neuronal NO. It is considered that NO acts on the GABAergic system through $GABA_A$ receptors. Hence, in the presence of an antagonist of $GABA_A$ receptors, such as BIC, NO could not exert its action. Thus, it is considered that this result for BIC also supports the idea of the direct relation between neuronal NO levels and GABAergic system. On the other hand, the diminished endothelial NO levels caused by BIC once again speak on behalf of the relation between low endothelial NO levels and seizures.

Influence of times of treatment of convulsant drugs on NO levels in neurons and astrocytes in primary culture

The effect of different times of treatment with convulsant drugs on NO levels was also investigated, in neurons as well as in astrocytes. This is especially relevant since the damage induced by NO as a free radical and as a neuromodulator/neurotransmitter, particularly in neurons, is closely related to the lasting of its effects.

Pentylenetetrazole (PTZ). – Effects of time of treatment with PTZ in neurons are given in Fig. 3. It can be seen that NO levels increased with treatment duration. This might indicate that, despite its short half-life, neurons continue generating NO during the seizures. However, that is not the case for high concentrations of PTZ, such as 100 μmol L^{-1} and 72 hours of treatment. In astrocytes, PTZ caused the opposite effect, decreasing the NO level in all cases as the time of treatment was longer (Fig. 3). Hence, the proposed effect of NO in endothelial cells lasts as convulsions last.

Thiosemicarbazide (TS). – TS also induces an increase of NO levels in neurons, which is directly proportional to the time of treatment, as can be observed in Fig. 3 (except for 72 h at 100 μmol L^{-1}). Because of the TS mechanism of action, these results may suggest a possible relation between NO and GAD activity, the enzyme that catalyzes the formation of GABA from Glu. GAD is a key enzyme that ensures the balance between the concentration of Glu and GABA, necessary for the proper regulation of neuronal excitability.

In astrocytes, TS diminished the level of NO with time (Fig. 3).

4-Aminopyridine (4 AP) and bicuculline (BIC). – The time-dependent effects of 4 AP and BIC treatments were similar to that described for other convulsant drugs. That is an increase in NO level in neurons, but a decrease in astrocytes, depending on the duration of treatment (Fig. 3).

Such results support the proposal of long-lasting increases in NO levels in neurons as a new mechanism of action for all convulsants tested. Besides, all convulsant drugs tested decrease NO levels in astrocytes as long as convulsions last. This suggests that such diminished NO levels in astrocytes could be associated with these drugs' convulsant properties.

Summary

For the sake of comparison, a compilation of the effects of different times of treatment with convulsant drugs on the NO level in neurons as well as in astrocytes is presented in

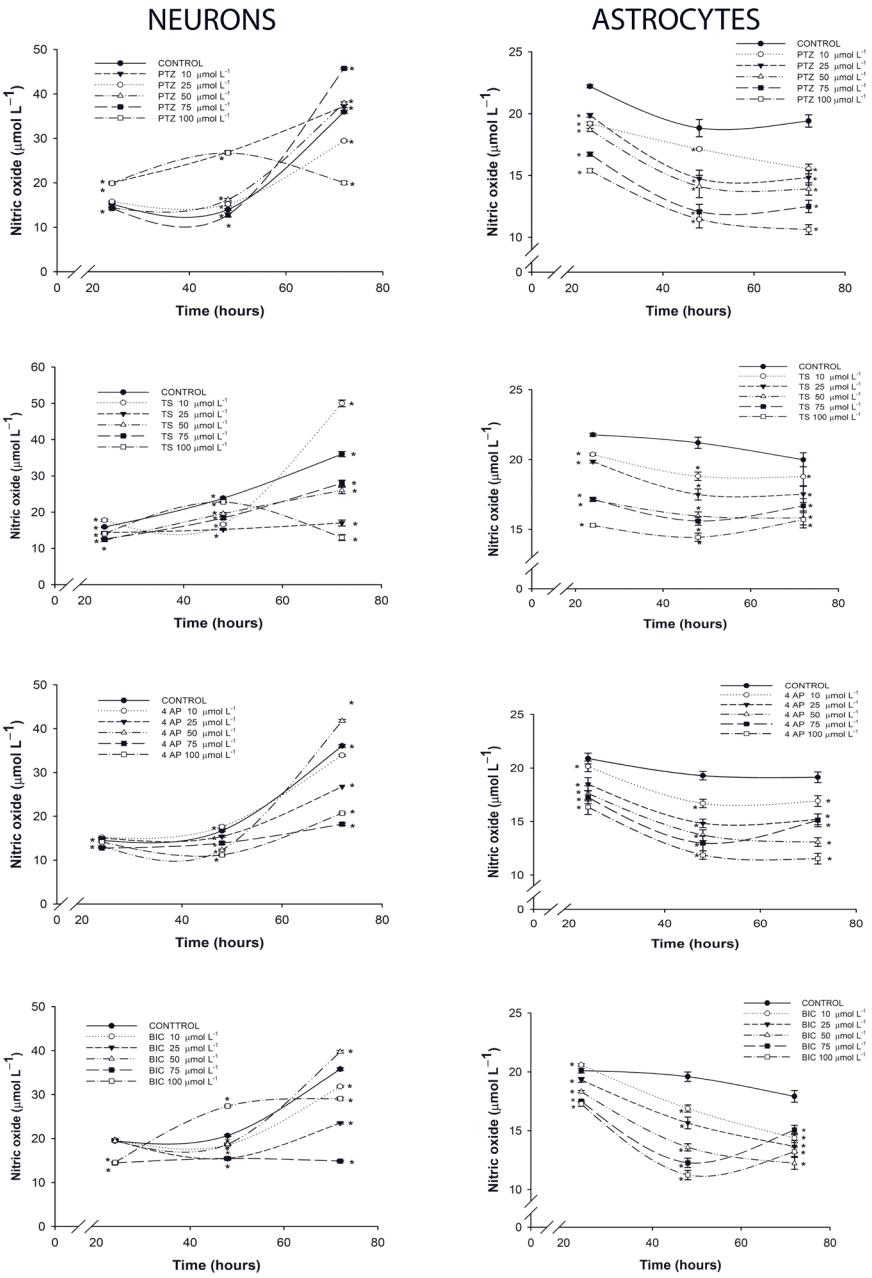

Fig. 3. Time-dependent effect of convulsant drugs on nitric oxide (NO) levels (expressed as nitrites, mean ± SEM, $n = 5$) in neurons and astrocytes in primary cultures. * Statistically significant difference as compared with control: $p \leq 0.05$ (*post-hoc* analysis Newman-Keuls test).

Fig. 4. In this figure, induction of seizures by tested convulsants seem to be associated with enhanced levels of neuronal NO, which are influenced by the concentration and the time of treatment used. In other words, such convulsant drugs would act as neuronal NO generators, which implies that NO generated by neurons could exert convulsant properties. This could be explained, by remembering that the main intracellular action of NO is the activation of soluble guanylate cyclase (cGMP) in the CNS (46–49). An increase in cGMP stimulates Glu receptors, mainly of the NMDA type. Thus, the result of the high levels of NO might be a release of Glu (the main excitatory neurotransmitter of the CNS), caused by the overstimulation of NMDA receptors. Hence, NO potentiates and facilitates glutamatergic neurotransmission, therefore seizure generation. However, NO also acts on inhibitory GABAergic synaptic transmission, which is reinforced considering the action mechanism of convulsant drugs tested. Due to PTZ and BIC results, it is also proposed that NO effects on the GABAergic system go through $GABA_A$ receptors.

NO induces GABA release, which would have an anticonvulsant effect. This paradoxical situation may represent indirect evidence of $GABA_A$ receptors' participation in neuronal NO in the generation of seizures. It was found that $GABA_A$ receptor signalling can be epileptogenic (50), and it is required for the generation of interictal-like events in human brain slices (51). This is particularly in immature neurons, and it is attributed to a slow GABAergic inhibition maturation and a developmentally increased NMDA and AMPA receptor-mediated excitability (52). In addition to excitation predominance, $GABA_A$ receptor activation leads to membrane depolarization and excitation, because of the high intracellular chloride concentration (53). In rats, changes in the chloride gradient and thereby the switch to $GABA_A$-mediated inhibition occur during the first 2 postnatal weeks (54). Hence, in cultured neurons prepared from fetal rats, which is the case in this study, enhanced GABAergic synaptic transmission leads to increased hyperexcitability. It is proposed that neuronal NO could be the mechanism of $GABA_A$ receptors stimulation under such conditions.

The decrease of endothelial NO caused by convulsant drugs indicates that, in contrast to the exercised effects of neuronal NO, it might exert anticonvulsant properties. There is

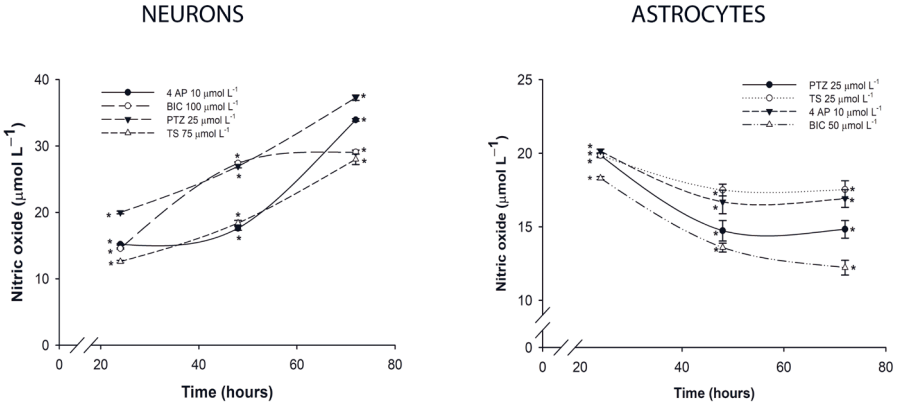

Fig. 4. Comparation of convulsant drugs' effect on nitric oxide (NO) levels (expressed as nitrites, mean ± SEM, $n = 5$) in neurons and astrocytes in primary cultures at different times of treatment. * Statistically significant difference as compared with control: $p \leq 0.05$ (*post-hoc* analysis Newman-Keuls test).

a coincidence in the effects of all convulsant drugs tested observed in the shape and magnitude of NO levels in astrocytes presented graphically. These facts suggest that this effect is caused through a common mechanism, which would be the lower relaxation of the endothelial cells caused for the decreased endothelial NO levels. If during the seizures endothelial NO levels diminish, this might cause a decrease in local cerebral flow with consequently diminished oxygen levels. All this might explain the generation of seizures when endothelial NO levels are decreased. Protective effects have been attributed to eNOS, mediated through maintenance of vascular homeostasis and promoting angiogenesis (55-57). Therefore, alterations in vascular homeostasis and hindered angiogenesis could also contribute to the convulsant effect of the lower levels of endothelial NO caused by the drugs tested.

It should be considered that the former interpretations are subjected to the inherent limitations of this kind of study. For example, it is true that nNOS and eNOS predominate in neurons and astrocytes, resp., but it does not mean that only one NOS isoform is to be considered. It has been reported that eNOS protein localization is not restricted to astrocytes (58, 59), but it can also be found in neurons. Specifically, eNOS is present in the dendritic spines of neurons in primary cultures (60). In addition, under pathological conditions, NO levels produced by iNOS should also be considered. In certain conditions, such as inflammation, NO levels produced by iNOS are temporally high. Thus, this factor should not be ruled out.

CONCLUSIONS

The dual convulsant/anticonvulsant effect of NO might be attributed, at least partially, to the NOS isoform implicated. The effects of convulsant drugs on the NO level depend on their concentration and time of treatment. This is valid for neurons as well as for astrocytes.

NO has several biological activities. Thus, as NO levels increase, different effects could be exercised. The increase of neuronal NO induced by low concentrations of convulsant drugs tested seems to be related to the generation of seizures. In addition, results indicate that the decrease of endothelial NO induced by the convulsant tested could also participate in epileptogenesis. As a free radical, high NO levels may cause different deleterious effects that may lead to neuronal death, depending on how long such a condition lasts. Thus, the fact that NO effects on neurons and astrocytes last as seizures do is especially relevant.

Apparently, convulsant properties of neuronal NO seem to be mediated not only by its effect on the glutamatergic system, but also on the GABAergic system probably through $GABA_A$ receptors. In contrast, endothelial NO appears to present anticonvulsant properties. These seem to be a direct consequence of NO's effect on endothelial cells, which make NO an important regulator of vascular homeostasis, and its capability to induce angiogenesis. Described effects last as seizures do.

All this requires further investigations considering the limitations of these kinds of studies and the unexplored influence of inducible NO. For example, it would be advisable to perform studies to investigate the possible correlation between brain GAD activity and NO and the specific role of inducible NO isoform.

Acronyms, abbreviations, symbols. – 4 AP – 4-aminopyridine, BIC – bicuculline, BSA – bovine serum albumin, cGMP – soluble guanylate cyclase, DMEM – Dulbecco's modified eagle's medium, EBS – Earle's balanced solution, eNO – endothelial nitric oxide, FCS – fetal calf serum, GABA – gamma amino butyric acid, GAD – glutamic acid decarboxylase, Glu – glutamate, iNOS – inducible NO synthase, L-NAME – *N(G)*-nitro-L-arginine methyl ester, NED – *N*-1-napthylethylenediamine, NMDA – *N*-methyl-*D*-aspartate, nNO – neuronal nitric oxide, NOS – nitric oxide synthase, PTZ – pentylenetetrazole, TS – thiosemicarbazide, nNOS – neuronal NO synthase, eNOS – endothelial NO synthase

Funds. – This work was supported by the Section of Postgraduate Studies and Research, Academic Secretary of National Polytechnic Institute, Mexico.

Author's contributions. – Lourdes Vega Rasgado: Design of experiments, experimental work, interpretations of results; Eva Ramón Gallegos: Design of experiments, interpretation of results; Lorena Rodríguez-Páez: Interpretation of results, redaction, bibliographic review; Verónica Alcántara Farfán: Design of experiments, experimental work.

Conflict of interest. – Authors declare do not have any conflict of interest.

REFERENCES

1. A. J. Duncan and S. J. Heales, Nitric oxide and neurological disorders, *Mol. Aspects Med.* **26**(1–2) (2005) 67–96; https://doi.org/10.1016/j.mam.2004.09.004
2. R. Stojanovic, Z. Todorovic, Z. Nesic, S. Vuckovic, N. Cerovac-Cosic and M. Prostran, NG-nitro-L-arginine methyl ester-induced potentiaton of the effect of aminophylline on rat diaphragm: the role of extracellular calcium, *J. Pharmacol. Sci.* **96**(4) (2004) 493–498; https://doi.org/ 10.1254/jphs.sce04001x
3. K. Yamamoto, H. Takei, Y. Koyanagi, N. Koshikawa and M. Kobayashi, Presynaptic cell type-dependent regulation of GABAergic synaptic transmission by nitric oxide in rat insular cortex, *Neuroscience* **284** (2015) 65–77; https://doi.org/10.1016/j.neuroscience.2014.09.062
4. L. Yassin, S. Radtke-Schuller, H. Asraf, B. Grothe, M. Hershfinkel, I. D. Forsythe and C. Kopp-Scheinpflug, Nitric oxide signaling modulates synaptic inhibition in the superior paraolivary nucleus (SPN) via cGMP-dependent suppression of KCC2, *Front. Neural Circuits* **8** (2014) Article ID 65 (12 pages); https://doi.org/10.3389/fncir.2014.00065
5. K. M. Boje, Nitric oxide neurotoxicity in neurodegenerative diseases, *Front. Biosci.* **9** (2004) 763–776; https://doi.org/10.2741/1268
6. O. Akyol, S. S. Zoroglu, F. Armutcu, S. Sahin and A. Gurel, Nitric oxide as a physiopathological factor in neuropsychiatric disorders, *In Vivo* **18**(3) (2004) 377–390.
7. O. Devinsky, A. Vezzani, T. J. O'Brien, N. Jette, I. E. Scheffer, M. de Curtis and P. Perucca, Epilepsy, *Nat. Rev. Dis. Primers* **4** (2018) Article ID 18024; https://doi.org/10.1038/nrdp.2018.24
8. G. Ferraro and P. Sardo, Nitric oxide and brain hyperexcitability, *In Vivo* **18**(3) (2004) 357–366.
9. K. Tieu, H. Ischiropoulos and S. Przedborski, Nitric oxide and reactive oxygen species in Parkinson's disease, *IUBMB Life* **55**(6) (2003) 329–335; https://doi.org/ 10.1080/1521654032000114320
10. M. Hsu, B. Srinivas, J. Kumar, R. Subramanian and J. Andersen, Glutathione depletion resulting in selective mitochondrial complex I inhibition in dopaminergic cells is via an NO-mediated pathway not involving peroxynitrite: implications for Parkinson's disease, *J. Neurochem.* **92**(5) (2005) 1091–1103; https://doi.org/10.1111/j.1471-4159.2004.02929.x
11. D. Hrncic, A. Rasic-Markovic, D. Krstic, D. Macut, D. Djuric and O. Stanojlovic, The role of nitric oxide in homocysteine thiolactone-induced seizures in adult rats, *Cell. Mol. Neurobiol.* **30**(2) (2010) 219–231; https://doi.org/ 10.1007/s10571-009-9444-9
12. S. H. Snyder, Janus faces of nitric oxide, *Nature* **364**(6438) (1993) 577; https://doi.org/10.1038/364577a0

13. G. Rondouin, M. Lerner-Natoli, O. Manzoni, M. Lafon-Cazal and J. Bockaert, A nitric oxide (NO) synthase inhibitor accelerates amygdala kindling, *Neuroreport* **3**(9) (1992) 805–808; https://doi.org/10.1097/00001756-199209000-00021
14. A. Becker, G. Grecksch and H. Schroder, N^{ω}-nitro-L-arginine methyl ester interferes with pentylenetetrazol-induced kindling and has no effect on changes in glutamate binding, *Brain Res.* **688**(1–2) (1995) 230–232; https://doi.org/10.1016/0006-8993(95)00565-8
15. P. Tutka, P. Klonowski, J. Dzieciuch, Z. Kleinrok and S. J. Czuczwar, NG-nitro-L-arginine differentially affects glutamate- or kainate-induced seizures, *Neuroreport* **7**(10) (1996) 1605–1608; https://doi.org/10.1097/00001756-199607080-00015
16. E. Przegalinski, L. Baran and J. Siwanowicz, The role of nitric oxide in chemically- and electrically-induced seizures in mice, *Neurosci. Lett.* **217**(2–3) (1996) 145–148.
17. M. S. Starr and B. S. Starr, Do NMDA receptor-mediated changes in motor behaviour involve nitric oxide? *Eur. J. Pharmacol.* **272**(2–3) (1995) 211–217; https://doi.org/ 10.1016/0014-2999(94)00644-m
18. E. M. Urbanska, E. Drelewska, K. K. Borowicz, P. Blaszczak, Z. Kleinrok and S. J. Czuczwar NG-nitro-L-arginine, a nitric oxide synthase inhibitor, and seizure susceptibility in four seizure models in mice, *J. Neural Transm.* (Vienna) **103**(10) (1996) 1145–1152; https://doi.org/10.1007/BF01271199
19. L. A. Vega, G. Ceballos and F. Vega-Diaz, Role of nitric oxide synthase on brain GABA transaminase activity and GABA levels, *Acta Pharm.* **68**(3) (2018) 349–359; https://doi.org/10.2478/acph-2018-0022
20. O. Devinsky, A. Vezzani, S. Najjar, N. C. De Lanerolle and M. A. Rogawski, Glia and epilepsy: excitability and inflammation, *Trends Neurosci.* **36**(3) (2013) 174–184; https://doi.org/ 10.1016/j.tins.2012.11.008
21. Y. Chen, C. Qin, J. Huang, X. Tang, C. Liu, K. Huang, J. Xu, G. Guo, A. Tong and L. Zhou, The role of astrocytes in oxidative stress of central nervous system: A mixed blessing, *Cell. Prolif.* **53**(3) (2020) e12781; https://doi.org/ 10.1111/cpr.12781
22. A. Falkowska, I. Gutowska, M. Goschorska, P. Nowacki, D. Chlubek and I. Baranowska-Bosiacka, Energy metabolism of the brain, including the cooperation between astrocytes and neurons, especially in the context of glycogen metabolism, *Int. J. Mol. Sci.* **16**(11) (2015) 25959–25981; https://doi.org/10.3390/ijms161125939
23. D. Boison and C. Steinhauser, Epilepsy and astrocyte energy metabolism, *Glia* **66**(6) (2018) 1235–1243; https://doi.org/ 10.1002/glia.23247
24. M. De Pitta, N. Brunel and A. Volterra, Astrocytes: Orchestrating synaptic plasticity?, *Neuroscience* **323** (2016) 43–61; https://doi.org/10.1016/j.neuroscience.2015.04.001
25. S. M. Q. Hussaini and M. H. Jang, New roles for old glue: astrocyte function in synaptic plasticity and neurological disorders, *Int. Neurourol. J.* **22**(Suppl. 3) (2018) S106–S114; https://doi.org/ 10.5213/inj.1836214.107
26. A. Volterra and J. Meldolesi, Astrocytes, from brain glue to communication elements: the revolution continues, *Nat. Rev. Neurosci.* **6**(8) (2005) 626–640; https://doi.org/ 10.1038/nrn1722
27. L. K. Bak, A. Schousboe and H. S. Waagepetersen, The glutamate/GABA-glutamine cycle: aspects of transport, neurotransmitter homeostasis and ammonia transfer, *J. Neurochem.* **98**(3) (2006) 641–653; https://doi.org/ 10.1111/j.1471-4159.2006.03913.x
28. N. N. Haj-Yasein, V. Jensen, I. Ostby, S. W. Omholt, J. Voipio, K. Kaila, O. P. Ottersen, O. Hvalby and E. A. Nagelhus, Aquaporin-4 regulates extracellular space volume dynamics during high-frequency synaptic stimulation: a gene deletion study in mouse hippocampus, *Glia* **60**(6) (2012) 867–874; https://doi.org/ 10.1002/glia.22319
29. F. Vasile, E. Dossi and N. Rouach, Human astrocytes: structure and functions in the healthy brain, *Brain Struct. Funct.* **222**(5) (2017) 2017–2029; https://doi.org/ 10.1007/s00429-017-1383-5
30. N. J. Abbott, L. Ronnback and E. Hansson, Astrocyte-endothelial interactions at the blood-brain barrier, *Nat. Rev. Neurosci.* **7**(1) (2006) 41–53; https://doi.org/ 10.1038/nrn1824

31. Y. Serlin, I. Shelef, B. Knyazer and A. Friedman, Anatomy and physiology of the blood-brain barrier, *Semin. Cell Dev. Biol.* **38** (2015) 2–6; https://doi.org/10.1016/j.semcdb.2015.01.002
32. G. R. Gordon, S. J. Mulligan and B. A. MacVicar, Astrocyte control of the cerebrovasculature, *Glia* **55**(12) (2007) 1214–1221; https://doi.org/ 10.1002/glia.20543
33. C. Iadecola and M. Nedergaard, Glial regulation of the cerebral microvasculature, *Nat. Neurosci.* **10**(11) (2007) 1369–1376; https://doi.org/10.1038/nn2003
34. S. Robel and H. Sontheimer, Glia as drivers of abnormal neuronal activity, *Nat. Neurosci.* **19**(1) (2016) 28–33; https://doi.org/10.1038/nn.4184
35. A. Tabernero, J. P. Bolanos and J. M. Medina, Lipogenesis from lactate in rat neurons and astrocytes in primary culture, *Biochem. J.* **294**(3) (1993) 635–638; https://doi.org/10.1042/bj2940635
36. R. F. Squires, E. Saederup, J. N. Crawley, P. Skolnick and S. M. Paul, Convulsant potencies of tetrazoles are highly correlated with actions on GABA/benzodiazepine/picrotoxin receptor complexes in brain, *Life Sci.* **35**(14) (1984) 1439–1444; https://doi.org/10.1016/0024-3205(84)90159-0
37. A. Tourov, R. Ferri, S. Del Gracco, M. Elia, S. A. Musumeci and M. C. Stefanini, Spike morphology in PTZ-induced generalized and cobalt-induced partial experimental epilepsy, *Funct. Neurol.* **11**(5) (1996) 237–245.
38. N. Y. Lukomskaya, N. I. Rukoyatkina, L. V. Gorbunova, V. E. Gmiro and L. G. Magazanik, Studies of the roles of NMDA and AMPA glutamate receptors in the mechanism of corasole convulsions in mice, *Neurosci. Behav. Physiol.* **34**(8) (2004) 783–789; https://doi.org/10.1023/b:neab.0000038128.02725.7e
39. X. Zhu, J. Dong, B. Han, R. Huang, A. Zhang, Z. Xia, H. Chang, J. Chao and H. Yao, Neuronal nitric oxide synthase contributes to PTZ kindling epilepsy-induced hippocampal endoplasmic reticulum stress and oxidative damage, *Front. Cell. Neurosci.* **11** (2017) Article ID 377 (16 pages); https://doi.org/10.3389/fncel.2017.00377
40. A. Morales-Villagran and R. Tapia, Preferential stimulation of glutamate release by 4-aminopyridine in rat striatum in vivo, *Neurochem. Int.* **28**(1) (1996) 35–40; https://doi.org/ 10.1016/0197-0186(95)00064-f
41. L. Medina-Ceja, A. Morales-Villagran and R. Tapia, Action of 4-aminopyridine on extracellular amino acids in hippocampus and entorhinal cortex: a dual microdialysis and electroencephalographic study in awake rats, *Brain Res. Bull.* **53**(3) (2000) 255–262; https://doi.org/ 10.1016/s0361-9230(00)00336-1
42. F. Pena and R. Tapia, Relationships among seizures, extracellular amino acid changes, and neurodegeneration induced by 4-aminopyridine in rat hippocampus: a microdialysis and electroencephalographic study, *J. Neurochem.* **72**(5) (1999) 2006–2014; https://doi.org/ 10.1046/j.1471-4159.1999.0722006.x
43. F. Pena and R. Tapia, Seizures and neurodegeneration induced by 4-aminopyridine in rat hippocampus in vivo: role of glutamate- and GABA-mediated neurotransmission and of ion channels, *Neuroscience* **101**(3) (2000) 547–561; https://doi.org/10.1016/s0306-4522(00)00400-0
44. D. R. Curtis, A. W. Duggan, D. Felix and G. A. Johnston, GABA, bicuculline and central inhibition, *Nature* **226**(5252) (1970) 1222–1224; https://doi.org/10.1038/2261222a0
45. V. Seutin and S. W. Johnson, Recent advances in the pharmacology of quaternary salts of bicuculline, *Trends Pharmacol. Sci.* **20**(7) (1999) 268–270; https://doi.org/10.1016/s0165-6147(99)01334-6
46. W. P. Arnold, C. K. Mittal, S. Katsuki and F. Murad, Nitric oxide activates guanylate cyclase and increases guanosine 3′:5′-cyclic monophosphate levels in various tissue preparations, *Proc. Natl. Acad. Sci. USA* **74**(8) (1977) 3203–3207; https://doi.org/ 10.1073/pnas.74.8.3203
47. J. Garthwaite, Glutamate, nitric oxide and cell-cell signalling in the nervous system, *Trends Neurosci.* **14**(2) (1991) 60–67; https://doi.org/ 10.1016/0166-2236(91)90022-m
48. J. Garthwaite, Nitric oxide signalling in the nervous system, *Semin. Neurosci.* **5** (1993) 171–180.

49. J. Garthwaite, S. L. Charles and R. Chess-Williams, Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain, *Nature* **336**(6197) (1988) 385–388; https://doi.org/10.1038/336385a0
50. A. Klaassen, J. Glykys, J. Maguire, C. Labarca, I. Mody and J. Boulter, Seizures and enhanced cortical GABAergic inhibition in two mouse models of human autosomal dominant nocturnal frontal lobe epilepsy, *Proc. Natl. Acad. Sci. USA* **103**(50) (2006) 19152–19157; https://doi.org/10.1073/pnas.0608215103
51. I. Cohen, V. Navarro, S. Clemenceau, M. Baulac and R. Miles, On the origin of interictal activity in human temporal lobe epilepsy in vitro, *Science* **298**(5597) (2002) 1418–1421; https://doi.org/10.1126/science.1076510
52. F. Jensen, Developmental factors regulating susceptibility to perinatal brain injury and seizures, *Curr. Opin. Pediatr.* **18**(6) (2006) 628–633; https://doi.org/10.1097/MOP.Ob013e328010c536
53. Y. Ben-Ari, C. Rovira, J.L. Gaiarsa, R. Corradetti, O. Robain and E. Cherubini, GABAergic mechanisms in the CA3 hippocampal region during early postnatal life, *Prog. Brain Res.* **83** (1990) 313–321; https://doi.org/10.1016/s0079-6123(08)61259-5
54. S. Zanelli, M. Naylor and J. Kapur, Nitric oxide alters GABAergic synaptic transmission in cultured hippocampal neurons, *Brain Res.* **1297** (2009) 23–31; https://doi.org/10.1016/j.brainres.2009.08.044
55. Z. Huang, P. L. Huang, J. Ma, W. Meng, C. Ayata, M. C. Fishman, and M. A. Moskowitz, Enlarged infarcts in endothelial nitric oxide synthase knockout mice are attenuated by nitro-L-arginine, *J. Cereb. Blood Flow Metab.* **16**(5) (1996) 981–987; https://doi.org/10.1097/00004647-199609000-00023
56. E. Morikawa, M. A. Moskowitz, Z. Huang, T. Yoshida, K. Irikura and T. Dalkara, L-arginine infusion promotes nitric oxide-dependent vasodilation, increases regional cerebral blood flow, and reduces infarction volume in the rat, *Stroke* **25**(2) (1994) 429–435; https://doi.org/10.1161/01.str.25.2.429
57. M. Endres, U. Laufs, Z. Huang, T. Nakamura, P. Huang, M. A. Moskowitz and J. K. Liao, Stroke protection by 3-hydroxy-3-methylglutaryl (HMG)-CoA reducta se inhibitors mediated by endothelial nitric oxide synthase, *Proc. Natl. Acad. Sci. USA* **95**(15) (1998) 8880–8885; https://doi.org/10.1073/pnas.95.15.8880
58. K. Iwase, K. Miyanaka, A. Shimizu, A. Nagasaki, T. Gotoh, M. Mori and M. Takiguchi, Induction of endothelial nitric-oxide synthase in rat brain astrocytes by systemic lipopolysaccharide treatment, *J. Biol. Chem.* **275**(16) (2000) 11929–11933; https://doi.org/10.1074/jbc.275.16.11929
59. J. L. Dinerman, T. M. Dawson, M. J. Schell, A. Snowman and S. H. Snyder, Endothelial nitric oxide synthase localized to hippocampal pyramidal cells: implications for synaptic plasticity, *Proc. Natl. Acad. Sci. USA* **91**(10) (1994) 4214–4218; https://doi.org/ 10.1073/pnas.91.10.4214
60. A. Caviedes, M. Varas-Godoy, C. Lafourcade, S. Sandoval, J. Bravo-Alegria, T. Kaehne, A. Massmann, J. P. Figueroa, F. Nualart and U. Wyneken, Endothelial nitric oxide synthase is present in dendritic spines of neurons in primary cultures, *Front. Cell. Neurosci.* **11** (2017) Article ID 180; https://doi.org/10.3389/fncel.2017.00180