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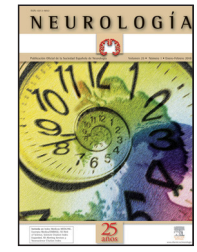

ORIGINAL ARTICLE

Understanding the pathophysiology of epilepsy in an animal model: pentylenetetrazole induces activation but not death of neurons of the medial extended amygdala

G. Pereno* and C. Beltramino

Cátedra de Neurología y Psicología, Facultad de Psicología, Universidad Nacional de Córdoba, Argentina

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KEYWORDS
Medial extended amygdala;
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Abstract
Introduction: Since middle of the 20th century the importance of amygdala in epilepsy it has suggested, although the basic mechanisms of this participation are still unknown. This ignorance increases when the different subdivisions of amygdala are considered, especially the medial amygdala. In this work we assess the involvement of the medial extended amygdala in an animal model of epilepsy and the consequences of its application in this brain structure.
Material and methods: Forty eight adult Wistar male rats were used, of which 24 of them received i.p. injections of pentylenetetrazole, and 24 (controls) were injected with saline. After 2, 6, 12 and 24 h survival, animals were fixed; the brains were sectioned serially and stained for fos (immunochemistry) and for neuronal death with the A-Cu-Ag technique. Data were analysed using two-way ANOVA followed by the Fisher post hoc test.
Results: Very few or no fos-immunoreactive neurons were seen in control animals. In experimental animals, fos was rapidly induced in structures of medial extended amygdala with peak levels at 2 h. Marked fos immunoreactivity persisted up to 12 h followed by a gradual return to baseline at 24 h. However, status epilepticus did not induced neuronal death.
Conclusions: These results show involvement of medial extended amygdala in epileptic mechanisms with an inhibitory component. However, neuronal death is not a consequence of status epilepticus-induced by pentylentetrazole.

*Author for correspondence.
E-mail: gpereno@psyche.unc.edu.ar (G. Pereno).

PALABRAS CLAVE
Amígdala extendida medial;
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Pentilentetrazol

Descifrando la fisiopatología de la epilepsia en un modelo animal: el pentilentetrazol induce la activación pero no la muerte de las neuronas de la amígdala extendida medial

Resumen
Introducción: Desde mitad del siglo xx se ha apuntado a la importancia de la amígdala en la epilepsia, aunque los mecanismos básicos de esta participación en su mayoría son aún desconocidos. Esta ignorancia es aún mayor cuando se tienen en cuenta las distintas subdivisiones de la amígdala, especialmente sus partes mediales. En este trabajo evaluamos la participación de la amígdala extendida medial en un modelo animal de epilepsia, así como las consecuencias que tiene el epileptógeno en esta estructura cerebral.
Material y métodos: Se utilizaron ratas adultas Wistar machos (n = 48); 24 animales recibieron inyecciones intraperitoneales de pentilentetrazol y 24, de salina. Luego de 2, 6, 12 y 24 h de sobrevida, los animales se fijaron, y sus cerebros se cortaron seriadamente y se procesaron para fos (inmunoquímica) y muerte neuronal con la técnica A-Cu-Ag. Los datos se analizaron con un ANOVA de 2 vías seguido de un test post-hoc (LSD de Fisher).
Resultados: Muy poca activación fos se halla en animales controles. En animales experimentales, fos fue rápidamente inducida en la amígdala extendida medial a las 2 h. Esta activación fue sostenida hasta las 12 h y retornó a valores basales a las 24 h. Sin embargo, el estado epiléptico no produjo muerte neuronal.
Conclusiones: Se demuestra así una participación de la amígdala extendida medial en mecanismos epilépticos en los cuales subyace un componente inhibitorio. Sin embargo, el estado epiléptico inducido no produce muerte neuronal en esta estructura.

Introduction

Knowledge of the cellular and molecular mechanisms underlying the different types of epilepsy is still limited. Currently, the factors that transform a normal region of the brain into an epileptic one are unknown, so studying animals with epilepsy is very useful for understanding the pathophysiology of this group of diseases1.

The most commonly used animal model in studying epilepsy is the model of status epilepticus (SE). Different convulsive agents are used to provoke SE, such as kainic acid[2], pilocarpine[3] and pentylenetetrazole (PTZ)[4] to induce an acute phase of SE characterised by unremitting tonic-clonic crises. The basic problem in experimental epileptology is to determine the conditions that affect the balance between neuronal excitation and inhibition in each case.

Furthermore, the anatomical structure called the medial extended amygdala (MeXAM) developed by our laboratory[5] is an area of the brain that has not been studied in depth in the field of experimental epilepsy. It consists of two major subdivisions, one that is the central amygdala and another that includes the medial amygdala and the interstitial nucleus of the medial stria terminalis (INSTm), known as the medial division. The MeXAM system is involved in pheromone signal processing, with effects on the endocrine system and the emotional and reproductive aspects of behaviour[6,7].

Numerous reports involve the amygdaloid complex in diseases such as epilepsy[8], Alzheimer's disease[9], depression[10] and anxiety[11]. Specifically in the field of epilepsy, interest in the amygdala as an epilepsy-related structure emerged in the decade of the fifties, when significant neuronal damage was found in patients who experienced SE. Animal models have shown that the amygdala is likely to be damaged in status epilepticus and that this causes histopathological changes[12,13]. However, very little is known about the details of temporal changes and distribution of these MeXAM changes.

One method of evaluating these changes is through the study of early gene expression[14]. Since its discovery as an early transcription gene, c-fos has been used as a tool for functional and anatomical mapping because it identifies neural cells and circuits that are activated in response to different stimuli[14]. In this study, we attempted to reveal the activation pattern with the expression of the c-fos gene, the fos protein, after inducing SE with PTZ. Similarly, we conducted a study to reveal possible neuronal death as an effect of the application of the epileptogenic agent. The agent PTZ has been used

frequently in animals as a model of generalised epilepsy; binding to the picrotoxin site for the $GABA_A$ receptor, PTZ damages chloride channel activity and thus blocks GABA-mediated inhibition[15].

Material and methods

Scope of the study

This study was carried out at the Laboratory of Limbic System Neurobiology at the Instituto de Investigaciones Médicas Mercedes y Martín Ferreyra.

Study period

The experimental work started in February 2006 and ended after 12 months, in March 2007.

Animals

We used 48 adult male Wistar rats (200-250 g), isolated in ad-hoc housing 48 h before the experiment in general conditions for animal facilities. All procedures were performed in accordance with Royal Decree 1201/2005 on the protection of experimental animals.

Induction of status epilepticus with PTZ

The animals (n=6) were injected with intraperitoneal PTZ diluted in 0.9% saline solution according to the scheme of Pineau et al.16. This study considered only the animals which reached state 4. In parallel, control animals received saline solution in the same manner.

Fixation of the brain

At 2, 6, 12 and 24 h after inducing SE, the animals were anaesthetised with chloral hydrate at 30%, then infused with 100 ml of washing solution and finally 300 ml of 4% paraformaldehyde. The brain was then placed in sucrose solution at 30%; 48 h later it was cut in 40 µm slices on a freezing microtome and collected serially in the same fixative solution.

Immunochemical technique

For fos protein detection, the brain sections were washed in buffer (PO4, 0.01 mol) and the internal peroxidase was inhibited. The sections were washed and the non-specific bindings were blocked and then incubated in normal horse serum at 5% Later, the sections were incubated in primary antibody (fos: 1/5,000, sc-52, Santa Cruz) and then incubated for 2 h in secondary biotinylated antibody (1/200, Vector) and 2 h with avidin-biotin-peroxidase complex (Vector); the reaction was developed with diaminobenzidine as the chromogen, adding cobalt chloride (black reaction product). The sections were mounted and coverslipped with DPX.

Amino-cupric-silver technique

The method of impregnation with A-Cu-Ag17 was selected because it is usually applied in the laboratory and is the latest, improved version of copper-silver methods.

Quantitative cell analysis

The sections were analysed with an Axioplan microscope, with an adapted Leica camera. The quantitative cell analysis was performed using Scion Image software (Scion Corporation, 2000, NIH Image, USA) in coronal sections increased by x20.

We selected the following MeXAM structures[18] for analysis: medial amygdaloid nucleus (Me) in the planes relative to bregma: —1.80 and —2.56 for the anterodorsal Me (MeAD), —2.30 for the anteroventral Me (MeAV), —3.14 and —3.60 for the posterodorsal Me (MePD) and —3.14 for the posteroventral Me (MePV); the INSTm in two planes relative to bregma: —0.30 for the anterior (INSTa) and ventral (INSTv) INST and —0.80 for the posterior (INSTp).

Statistical analysis

The data were analysed using a two-factor analysis of variance (ANOVA), followed by Fisher's *post-hoc* least significant difference (LSD) test. The values were expressed as the average of each group±standard error, and $p < 0.05$ was considered as the limit of statistical significance.

Results

Expression of fos after PTZ injection

In all control animals injected with saline solution, few, scattered nuclei of cells, activated in the areas evaluated here, were observed.

In experimental animals, the highest fos protein expression was found in those who survived 2 h after injection, where the differences with the control animals were statistically significant for all the areas where quantitative cell analysis of fos (+) nuclei was carried out (tables 1 and 2). The MeAD (table 1) and the INSTp (table 2) were the areas where it was possible to observe the greatest activation compared with controls. Likewise, the MePD (fig. 1) and the INSTa (fig. 2) were both heavily marked for fos protein. In all areas under evaluation, there were numerous cell nuclei stained with fos antibody. Three points stand out especially: the differentiation of the three columns in the INSTp (5), a considerable amount of activated cells in all Me divisions in the region near the accessory olfactory tract and the optic tract (fig. 1), and the INSTv (fig. 2) below the anterior edge.

After a survival of 6 h, although marking decreased, the differences remained significant, as was also true in the animals sacrificed 12 h after PTZ injection. Once again, the previously mentioned areas were the ones with the greatest activation (figs. 1 and 2; tables 1 and 2.) Finally, in animals

Table 1 Quantitative analysis of immunoreactive nuclei for fos in medial amygdaloid nucleus subdivisions after pentylenetetrazole (PTZ)-induced status epilepticus (SE)

Structure	Survival after PTZ-induced SE				
	Control	2 h	6 h	12 h	24 h
MeAV	6 ± 1.02	27.6 ± 2.43[a]	17.3 ± 1.66[a]	8.6 ± 2.44	5.3 ± 1.18
MeAD —1.80	16.1 ± 1.03	158.6 ± 7.71[a]	97.6 ± 5.5[a]	53.8 ± 3.01[a]	20.83 ± 1.86
MeAD —2.56	19 ± 1.2	202.5 ± 4.09[a]	102 ± 5.43[a]	49.16 ± 2.25[a]	23.8 ± 2.04
MePV	14.5 ± 1.49	111.5 ± 4.21[a]	98.33 ± 3.56[a]	48.3 ± 1.28[a]	22 ± 1.26[b]
MePD —3.14	16.1 ± 1.03	165.8 ± 7.03[a]	102.6 ± 4.86[a]	57 ± 2.08[a]	20 ± 1.96
MePD —3.60	19 ± 1.2	192 ± 4.86[a]	100.1 ± 4.36[a]	47.8 ± 3.32[a]	23.3 ± 2.33

MeAD: anterodorsal medial amygdaloid nucleus; MeAV: anteroventral medial amygdaloid nucleus; MePD: posterodorsal medial amygdaloid nucleus; MePV: posteroventral medial amygdaloid nucleus.
We used a two-factor ANOVA followed by Fisher's *post-hoc* LSD test. The data are expressed as average ± standard error of the average.
[a]$p<0.001$.
[b]$p<0.01$.

Table 2 Quantitative analysis of the immunoreactive nuclei for fos in medial INST subdivisions after pentylenetetrazole (PTZ)-induced status epilepticus (SE)

Structure	Survival after PTZ-induced SE				
	Control	2 h	6 h	12 h	24 h
INSTa	13.6 ± 1.59	167.6 ± 6.4*	126.5 ± 9.68*	50.1 ± 5.32*	16.5 ± 1.46
INSTp	18.3 ± 1.08	207.5 ± 8.42*	132.6 ± 5.68*	55.5 ± 3.97*	21.5 ± 2.87
INSTv	12.1 ± 1.48	84.16 ± 3.51*	62.5 ± 2.26*	45 ± 2.5*	11.16 ± 0.91

INST: interstitial nucleus of the stria terminalis; INSTa: interstitial nucleus of the anterior stria terminalis; INSTp: interstitial nucleus of the posterior stria terminalis; INSTv: interstitial nucleus of the ventral stria terminalis.
We used a two-factor ANOVA followed by Fisher's *post-hoc* LSD test. The data are expressed as average±standard error of the average.
*$p<0.001$.

Figure 1 Photographs of brain sections (?2.5) showing the activation in the posterodorsal and posteroventral medial amygdaloid nuclei at different survivals after pentylenetetrazole injection. Bar scale: 500 μm.

Figure 2 Photographs of brain sections (× 2.5) showing the activation in the interstitial nucleus of the anterior and ventral stria terminalis at different survivals after pentylenetetrazole injection. Bar scale: 500 μm.

whose survival reached 24 h, it could be observed that the differences between control and experimental animals were not significant, as activation reached baseline values comparable to those of controls (tables 1 and 2).

The ANOVA performed revealed what was stated previously, that is, that the activation peak was found at 2 h and marking then gradually decreased in animals with longer survival (figs. 3 and 4). The fact that the differences between the various experimental groups were significant for most of the structures in the quantitative cell analysis (tables 1 and 2) is noteworthy.

Neurodegeneration after PTZ injection

Under the paradigm of this model, it was not possible to find neuronal death in the MeXAM (fig. 5). While animals were used with survival up to 24 h, animals with survival rates of up to 30 days after PTZ injection were also used and no signs of neuronal death were found in any of them. However, it should be noted that it was possible to find signs of axon terminal neurodegeneration in the CA3 area in the hippocampus of animals with 24-h survival (results not shown). This feature was not observed in other brain structures.

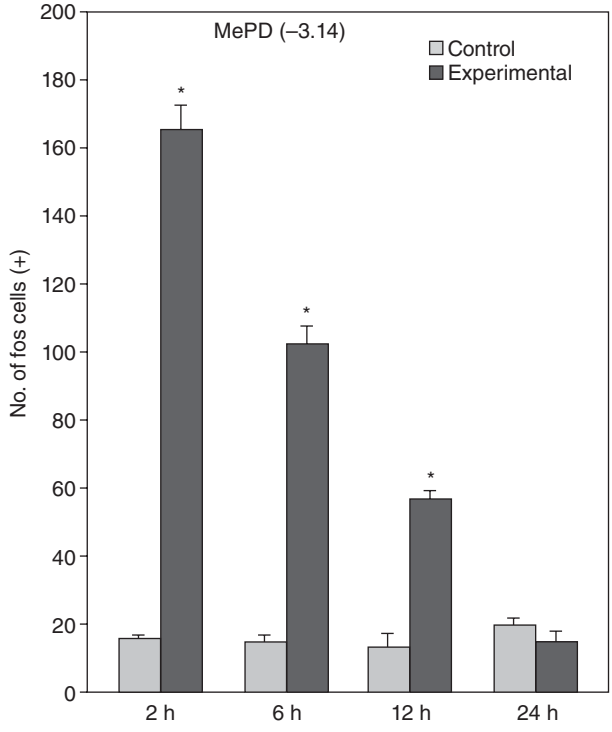

Figure 3 Quantitative cell analysis of fos+ nuclei of animals with survivals of 2, 6, 12 and 24 h after pentylenetetrazole application and subsequent two-factor ANOVA in the posterodorsal medial amygdaloid nucleus (MePD), whose factors are the "treatment" and the various "survivals". The result of the study of the factors is as follows: for treatment: $F_{(1.40)}$=1,111; p<0.001; for survival: $F_{(3.40)}$=220.3; p <0.001; interaction: $F_{(3.40)}$=209.4; p<0.001. The results are expressed as average±standard error (*p<0.001).

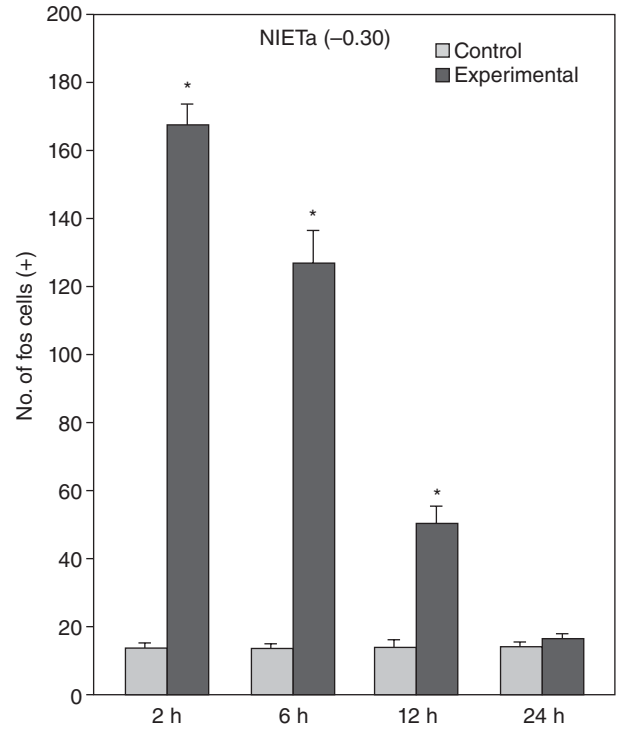

Figure 4 Quantitative cell analysis of fos+ nuclei in animals with survivals of 2, 6, 12 and 24 h after pentylenetetrazole application and subsequent two-factor ANOVA in the interstitial nucleus of the anterior stria terminalis (INSTa), whose factors are the "treatment" and the various "survivals". The result of the study of the factors is as follows: for treatment: $F_{(1.40)}$=640.46; p<0.001; for survival: $F_{(3.40)}$=130.34; p <0.001; interaction: $F_{(3.40)}$=132.90; p<0.001. The results are expressed as average±standard error (* p<0.001).

Figure 5 Photographs of brain sections (× 2.5) showing in A the posterodorsal and posteroventral medial amygdaloid nucleus, and in B the interstitial nucleus of the anterior and ventral stria terminalis of animals with survival 24 h after status epilepticus and which were processed with the A-Cu-Ag technique.

Discussion

GABAergic neurotransmission and epilepsy

Several findings demonstrate that the GABAergic system is involved in various types of epilepsies. For example, Muñoz

et al.[19], when analyzing the brains of epileptic patients, found a reduction in $GABA_B$ receptor 1a-b immunostaining in the granular cell layer of the hippocampus dentate gyrus. Other authors found the two receptor $GABA_B$ subtypes (1 and 2) overexpressed in the hippocampus of patients with temporal lobe epilepsy, which was interpreted as a compensating mechanism[20]. The values of the $GABA_A$ receptor were also altered in the brains of epileptic patients and animal models[21].

GABA is thus implicated in epilepsy, status epilepticus and other epileptic syndromes. Presynaptic and postsynaptic alterations, such as changes in receptor subunit composition and receptor structure or genetic modifications of receptors, could affect susceptibility to an epileptic seizure.

However, epilepsy is not a GABAergic syndrome. Several findings led to the idea that seizures would respond to a simple model in which inhibition and excitation would act as brake and accelerator, respectively. This scheme is nevertheless an oversimplified model. There is evidence that GABAergic neurotransmission can be excitatory in baseline conditions not only in a developing brain[22], but also in an epileptic brain[23].

In summary, an increase in the number of GABAergic synapses has been found in human epileptic brains and in animal models. These have been interpreted as a compensatory mechanism, but they could also be explained as being secondary to GABAergic function changes (from inhibition to excitation) and being, partially, the origins of system hyperexcitability[1].

Expression of early genes and epilepsy

Previous studies have used fos protein to assess the involvement of different neuronal populations in epilepsy models using PTZ[24,25]. Here we show, after the quantitative analysis of fos-activated neuron nuclei, that expression is maximal at 2- and 4-h survival, being consistent with acute expression, although there is evidence of sustained expression until 12 h after the SE. After 12 h, immunostaining decreases considerably; that is why, in this study, it was decided not to sacrifice animals surviving over 24 h after injection.

Taking fos protein expression as a tool for functional and anatomical mapping, we show the ability of the MeXAM to react by becoming active through the expression of early genes (such as c-fos) and its participation in epileptic mechanisms. This lets us state that the MeXAM is involved in epileptic processes involving the participation of GABAergic mechanisms.

The fact that fos expression was evident at 24 h pointed to the hypothesis that these neurons might be suffering from some form of neurodegenerative process. To this end, parallel sections of brains were processed with the A-Cu-Ag technique. Despite using different PTZ doses (from mild seizures to severe SE), no degeneration was found in these brains. This shows that, while the MeXAM is involved in this animal model, the neurons were able to recover from the stress represented by PTZ-induced SE.

The involvement of the medial extended amygdala in epileptic mechanisms

The amygdaloid complex has been little studied in the field of epilepsy, despite the fact that its involvement has been demonstrated, particularly that of the temporal lobe[26]. There are basic research studies that have addressed the problem of epilepsy using different animal models. They emphasise the role of the hippocampus and of some amygdaloid nuclei of the basolateral complex, leaving aside other amygdaloid complexes. Given the above, the question arises: is the MeXAM really involved in epilepsy? The results of our study show that the MeXAM clearly participates in epileptic mechanisms.

Although the use of early gene products, such as c-fos, as a marker of neural activity revealed that other brain areas were also activated, there are two important points to consider:

1. The animal model used here corresponded to secondary generalised epilepsy and it therefore also activated other brain areas.
2. In these areas, fos expression decreased rapidly (at about 4 h), whereas fos expression was maintained significantly, until 12 h of survival, in MeXAM structures.

The results obtained here demonstrate that the MeXAM is involved in epileptic mechanisms. However, unlike what happens with other animal models such as kainic acid[27] or pilocarpine[28], no neuronal death was found in the MeXAM. It has been reported that systemic PTZ injection induces neurodegeneration in the neurons of the hippocampus and the amygdala[29]; however, the neurodegeneration found in these studies was apoptotic, which cannot be detected with the technique used in this study. In addition, our study considers the amygdala as a unitary complex; it does not discriminate the different subdivisions. Likewise, it has also been demonstrated that PTZ-induced SE brings about oxidative stress in rat brains, with consequent neuronal death[30]. The technique used here is specifically for detecting neuronal death from necrosis and the death of neurons induced by oxidative stress very probably involves an apoptotic component[31].

Other studies[16] have shown that PTZ-induced status epilepticus in immature rats causes a period of strong activation characterised by a sharp increase in metabolic rate; this has the effect, at maturity, of permanent changes in cell membrane permeability, allowing acid fuchsin to enter. However, these cells never enter the stage of DNA fragmentation and are able to recover.

The contradictory results might be due to using different techniques for detecting neuronal death, or to considering the amygdaloid complex as a whole without taking its various subdivisions into account. Our findings demonstrate that the GABAergic antagonist, PTZ, does not cause neuronal death in the MeXAM, which shows that epilepsies in which the underlying cause is mostly an inhibitory component are unlikely to result in the death of its neurons.

Clinical projections and research practices

While this study has a distinctly basic orientation, it enables important conclusions to be drawn. First, this research provides a basis for understanding the complexity of the amygdaloid complex. This is a brain structure that, despite its small size, has great complexity, both in its functions and its anatomy, in terms of its connections with other structures.

This study is the starting point for further basic and clinical research. In the basic aspect, it is important for the development of new antiepileptic drugs, GABAergic antagonists or agonists, which interfere with the epileptogenic process. In the clinical aspect, it establishes a correlation between the present research with resistant temporal lobe epilepsy and the choice of different drugs for its treatment.

Detailed knowledge of the anatomy of the amygdala will facilitate, in the near future, selective surgery of temporal lobe epilepsy. Of course, all of the preceding points lie within the framework of basic research efforts, in constant interaction with clinical research, aimed at offering new initiatives that provide a deeper understanding of this disease.

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Conflict of interests

The authors declare no conflict of interests.

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