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MORPHINE AFFECTS PRODYNORPHIN GENE EXPRESSION IN SOME AREAS OF RAT BRAIN

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Summary. - *The effect of a chronic morphine treatment on prodynorphin gene expression has been studied. Morphine has been intraperitoneally administered twice daily for seven days into rats. RNAs from hippocampus and striatum have been extracted and analyzed with probes complementary to the prodynorphin mRNA. A marked reduction in mRNA levels was detected in hippocampus, following chronic morphine treatment; in striatum results showed either a slight decrease or no substantial changes in mRNA levels. These results indicate that the chronic morphine is able to induce modifications in the homeostasis of the endogenous opioid gene expression, at least in some areas of the rat brain. In addition, our data support the hypothesis that a tolerance to opiates might involve alterations in functions of brain pathways which utilize the opioid peptidergic system.*

Riassunto (La morfina modifica l'espressione genica della prodinorfina in alcune aree del cervello di ratto). - *È stato indagato l'effetto di un trattamento cronico con morfina sull'espressione genica del peptide precursore oppioide, prodinorfina. La morfina è stata somministrata (10 mg/kg) per via intraperitoneale nel ratto, due volte al giorno per sette giorni. Gli RNA estratti dall'ippocampo e dallo striato sono stati analizzati con sonde di cDNA complementari al mRNA della prodinorfina. Nell'ippocampo si è evidenziata una marcata diminuzione dei livelli di mRNA per la prodinorfina in seguito a trattamento con morfina; nello striato invece si è osservato un leggero decremento o variazioni non statisticamente significative dei livelli di mRNA. Questi risultati indicano che un trattamento cronico con un oppiaceo, la morfina, può indurre modificazioni nell'omeostasi dei sistemi oppioidi endogeni, in particolare a livello della loro espressione genica, almeno in alcune aree cerebrali del ratto. Questi dati, inoltre, avvalorano l'ipotesi che il fenomeno della tolleranza agli oppiacei possa coinvolgere alterazioni nei circuiti neuronali che utilizzano i sistemi peptidergici oppioidi.*

Introduction

It is well known that chronic opiate administration results in the development of a tolerance to their effects.

It has been proposed that tolerance to opiates may be explained with alterations in functions of brain circuits which utilize opioid peptides [1].

One possible mechanism of tolerance could involve changes at the level of opiate receptors, although this issue is still unclear since conflicting results have been shown either *in vivo* or *in vitro* [2-4].

Differences in opioid levels have also been described, none of them, however, convincingly explains the occurrence of tolerance (as well as dependence) after long-term opiate administration.

The possibility also exists that a chronic exposure to opiates induces a modification of the biosynthetic pattern of opioids in neuronal systems since expression of several neuropeptide genes may change in response to drug-induced alterations in neuronal function. The aim of our study was to examine the influence of a chronic opiate treatment on the gene expression of the opioid peptide dynorphin, a purported endogenous ligand for the k opioid receptor [5].

A clear-cut tolerance to antinociceptive and motor effects of this opioid peptide develops after chronic intrathecal infusion [6].

So far, we have ascertained the influence of the chronic administration of morphine on the gene expression of the precursor of the opioid peptide, prodynorphin, in some areas of rat CNS: prodynorphin mRNAs were analyzed in striatum and

hippocampus of morphine-treated rats and compared to the values of control animals.

These areas are surely involved in morphine actions and, moreover, they have been described to be particularly enriched in dynorphin-related peptides [7-9].

Materials and methods

Male Sprague-Dawley rats weighing 200-250 g were used. For each experiment animals were divided into two groups: control rats receiving saline 0.2 ml/100 g i.p. twice daily for seven days; morphine-treated rats receiving morphine hydrochloride 10 mg/kg i.p. twice daily for seven days.

On days 1, 3 and 5 rats were tested for the nociceptive threshold 30' after the treatment with morphine, by means of the tail flick test. On day 6 all rats receiving morphine showed a tolerance to the antinociceptive effect of the opiate. On day 7 rats were sacrificed, brains were rapidly removed, striata and hippocampi dissected and frozen on dry ice.

RNA was analyzed from three different experiments and tissues from 10 animals were pooled for each one.

Total striatal and hippocampal RNA were prepared according to the method of Chirgwin *et al.* [10].

Briefly, RNA was extracted from pooled samples by homogenizing in 5 volumes of 4 M guanidinium thiocyanate/0.5% sodium N-laurylsarcosine/5 mM sodium citrate/0.1 M 2-mercaptoethanol, and the total RNA was isolated by centrifugation through a dense cesium chloride cushion (5.7 M CsCl / 0.1 M EDTA, pH 7.5) for 20 h at 35000 rpm at 20 °C. Pellets were resuspended in 10 mM Tris.HCl (pH 7.4)/5 mM EDTA/ 1% SDS, extracted with a chloroform-1-buthanol (4:1) mixture; RNA was precipitated with ice-cold 95% EtOH overnight at -20 °C and pelleted again by centrifugation in an Eppendorf microfuge at 11000 rpm at 4 °C for 15 min. Pellets were resuspended in distilled water and the RNA content was quantitated by measurement of absorbance at 260 nm (1 O.D./ml = 25 µg RNA/ml).

Blots were hybridized with two different cDNA probes: 1) BgBa, the 920 base pair fragment of the rat genomic DNA complementary to the prodynorphin mRNA, consisting of the 5'-translated region of the prodynorphin gene, encoding for all dynorphins; 2) BaBa, the 815 base pair fragment of the same genomic cDNA, consisting of the 3'-untranslated region of the prodynorphin gene.

The cDNA fragments, inserted in pUC13 vectors, were kindly supplied by Drs Civelli and Douglass [11].

Each fragment was labelled with α-[^{32}P]dCTP by nick translation to a specific activity of 7-9 x 10^5 cpm/ng.

30 µg of each total RNA sample were electrophored through a 1% agarose gel containing 2.2 M formaldehyde at 100 V using a 0.04 M morpholinopropanesulfonic acid (MOPS, pH 7.0) buffer containing 10 mM sodium acetate and 1 mM EDTA.

RNA was transferred to nitrocellulose overnight and after blotting the filter was air-dried, baked at 80 °C for 2 h, placed into a sealed plastic bag and stored at room temperature.

The blots were prehybridized overnight at 42 °C in a solution of 6XSSC (1XSSC = 0.15 M NaCl, 0.015 M sodium citrate), 1X Denhardt's solution (0.02% polyvinylpyrrolidone, Ficoll and BSA), 100 µg/ml denaturated salmon sperm DNA, 0.1% SDS, 50% formamide, 10 mM Tris and 10% dextran sulfate.

For hybridization assays, each blot was prepared in duplicate and tested with two different probes, (^{32}P)-BgBa and (^{32}P)-BaBa; the probes were boiled for 10 min in the hybridization buffer (6XSSC, 1X Denhardt's, 50% formamide, 100 µg/ml denatured salmon sperm DNA, 10 mM Tris and 10% dextran sulfate) and added to the sealed bags at the concentration of 1-2 x 10^6 cpm/ml; the hybridization was carried out for 20 h at 42 °C. Upon removal of the probe solution, blots were washed three times for ten min at room temperature with a solution of 2XSSC/0.1% SDS followed by three times for ten min at 65 °C with a solution of 0.1XSSC/0.1% SDS, on a rocker.

X-rays films (Amersham β-max) were exposed to the hybridized blots backed by an intensifying screen (Dupont Cronex) at -70 °C for 4 days.

Total RNAs from hippocampus and striatum of morphine-treated rats were compared to control animals.

U.V. analysis of the RNAs showed no differences in intensity of the 18S ribosomal bands between the control and the drug-treated rats, therefore supporting the homogeneity of the total RNA concentrations.

Results

Under the adopted conditions we were able to detect hybridization signal as single band corresponding to the prodynorphin mRNA (size ca. 2.9 kb, Fig. 1).

As regards hippocampus, prodynorphin mRNA levels were markedly reduced (approximately 2 fold) following chronic treatment with morphine, in comparison with control rats; this result was observed in all of the three sets of experiments (Fig. 2).

As regards striatum, in the first and in the second experiment, a slight decrease was found in prodynorphin mRNA levels after chronic morphine treatment; on the contrary, the third experiment showed no substantial change in striatal prodynorphin mRNA density of morphine-treated rats compared to controls.

Similar results were obtained with both probes, except for a better resolution and a higher signal detected with the [^{32}P]-BaBa probe.

Discussion

These results indicate that the chronic treatment with the opioid agonist morphine cause a marked and constant reduction in hippocampal prodynorphin mRNA, whereas changes are still unclear in striatum.

Thus the possibility exists that gene expression of the opioid peptide dynorphin may be modified in response to chronic treatment with an opiate, at least at hippocampal level.

The mRNA changes might cause time-dependent alterations in the levels of the encoded peptides.

Changes in prodynorphin mRNA levels can parallel alterations in the rate of synthesis and release of dynorphin-related peptides if a balance exists between rates of peptide synthesis and release.

However, it is not possible to exclude a selective impact of morphine at other levels, such as on the translation of prodynorphin system, the processing, transport and degradation of the precursor [12-14].

Concerning data obtained in striatum, the ambiguous result could depend on the biological variability of the animals, together with the fact that in each experiment we pooled 10 animals for each tissue, giving an average in the measurement of the mRNAs levels.

Our data on prodynorphin are however in agreement with very recent results concerning the effect of chronic morphine on gene expression of another opioid precursor, proenkephalin, in rat CNS [13]; moreover, differential expression of the opioid precursors at striatal level has been reported [15, 16].

In conclusion, our data support the hypothesis that a chronic exposure to opiates may produce alterations in function of neuronal systems involving opioid peptides, namely their gene expression, at least as regards dynorphin in rat hippocampus.

However, further studies are necessary to increase our understanding on interactions between opioid gene expression and brain functions in the development of opiate tolerance.

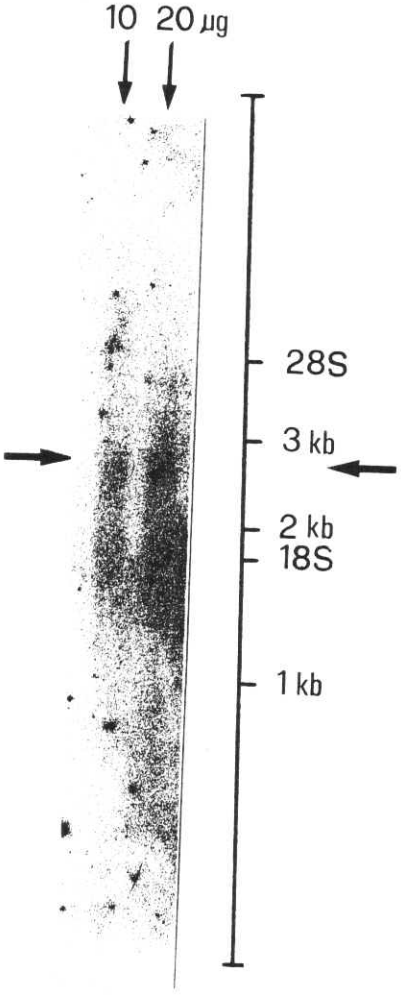

Fig.1 - Northern blot analysis of rat hippocampal total mRNA (10 and 20 µg). Hybridization to prodynorphin mRNA (approximately 2.9 kb size) by means of a ^{32}P-BgBa, a 920 bp fragment of the rat genomic cDNA (pUC19) complementary to prodynorphin mRNA. A kilobase ladder served as a molecular size marker.

Fig. 2. - Northern analysis of hybridization to prodynorphin mRNA in 30 µg samples of total hippocampal RNA, prepared from pools of rats treated with morphine (lane 2), compared with controls (lane 1). Hybridization carried out with the ^{32}P-BgBa probe.

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REFERENCES

1. ADLER, M.W. 1986. Mechanisms of opioid tolerance and dependence. In: *Problems of drug dependence 1986*. L.S. Harris (Ed.). NIDA Research Monograph 76, NIDA, Washington. pp. 63-68.

2. ROBSON, L.E., PATERSON, S.J. & KOSTERLITZ, H.W. 1983. Opiate receptors. *Handb. Psychopharmacol.* **17**: 13-19.

3. ROTHMAN, R.B., DANKS, J.A., JACOBSON, A.E., BURKE, T.R., RICE, K.C., TORTELLA, F.C. & HOLADAY, J.W. 1986. Morphine tolerance increases μ-noncompetitive δ binding sites. *Eur. J. Pharmacol.* **124**: 113-119.

4. PUTTFARKEN, P., WERLING, L.L. & COX, B.M. 1988. Effects of chronic morphine exposure on opioid inhibition of adenylyl cyclase in 7315c cell membranes: an useful model for the study of tolerance at μ opioid receptors. *Mol. Pharmacol.* **33**: 520-527.

5. CHAVKIN, C., JAMES, I.F. & GOLDSTEIN, A. 1982. Dynorphin is a specific endogenous ligand of the kappa opioid receptor. *Science* **215**: 413-415.

6. SPAMPINATO, S. & CANDELETTI, S. 1985. Characterization of dynorphin-A induced antinociception at spinal level. *Eur. J. Pharmacol.* **110**: 21-30.

7. AKIL, H., WATSON, S.J., YOUNG, E., LEWIS, M.E., KHACHATURIAN, H. & WALKER, J.M. 1984. Endogenous opioids: biology and function. *Annu. Rev. Neurosci.* **7**: 223-255.

8. COLLIER, H.O.J. 1980. Cellular site of opiate dependence. *Nature* **283**: 625-629.

9. SCHWARTZ, J.P. & COSTA, E. 1986. Hybridization approaches to the study of neuropeptides. *Annu. Rev. Neurosci.* **9**: 277-304.

10. CHIRGWIN, J.M., PRZYBYLA, A.E., MACDONALD, R.J. & RUTTER, W.J. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**: 5294-5299.

11. CIVELLI, O., DOUGLASS, J., GOLDSTEIN, A. & HERBERT, E. 1985. Sequence and expression of the rat prodynorphin gene. *Proc. Natl. Acad. Sci. USA* **82**: 4291-4295.

12. HABENER, J.F. 1981. Principles of peptide hormone biosynthesis. In: *Neurosecretion and brain peptides*. J.B. Martin, S. Reichlin & K.L. Bick (Eds). Raven Press, New York. pp. 21-34.

13. UHL, G.R., RYAN, J.P. & SCHWARTZ, J.P. 1988. Morphine alters preproenkephalin gene expression. *Brain Res.* **459**: 391-397.

14. ROMUALDI, P., LESA, G. & FERRI, S. 1989. An opiate chronic treatment affects prodynorphin gene expression. *Pharmacol. Res.* **21**: 477-478.

15. LIGHTMAN, S.L. & YOUNG, W.S. 1987. Changes in hypothalamic preproenkephalin A mRNA following stress and opiate withdrawal. *Nature* **328**: 643-645.

16. UHL, G.R., NAVIA, B. & DOUGLASS, J. 1989. Differential expression of preproenkefalin and preprodynorphin mRNAs in striatal neurons: high levels of preproenkephalin expression depend of cerebral cortical efferents. *J. Neurosci.* (in press).