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Chronic opiate agonists down-regulate prodynorphin gene expression in rat brain

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The effects of long-term administration of opioid agonists on the regulation of prodynorphin gene expression in rat brain were investigated. Chronic intracerebroventricular treatment with the synthetic opioid agonist acting on the κ receptor, U-50,488H, and the classic μ agonist morphine markedly decreased prodynorphin mRNA levels in hypothalamus, hippocampus and striatum of tolerant rats. Levels of ir-Dynorphin A remained unchanged except in two cases. Chronic exposure to opiates thus appears to induce modifications of the endogenous opioid system, as regards gene expression regulation.

INTRODUCTION

Opiate tolerance may possibly be the result of an alteration in the function of brain circuits which utilize opioid peptides[1,2,7,19]. However, despite years of vigorous research in this field, the mechanisms underlying the development of tolerance to some of the effects of opiates are still not fully understood.

One possible mechanism involves changes at the opiate receptors, although conflicting results have been reported in vivo and in vitro[18,21,24,25]. Differences in opioid levels have also been reported, but none of these proposals convincingly explains the onset of tolerance (as well as dependence) after long-term opiate administration.

The possibility also exists that chronic exposure to opiates modifies the biosynthetic pattern of opioids in neuronal systems, since the gene expression of many neuropeptides can change in response to drug-induced alterations in neuronal function[16].

Recently evidence has appeared that morphine tolerance may depress the gene expression of the opioid precursors pro-opiomelanocortin (POMC) and pro-enkephalin in rat hypothalamus and striatum, respectively[17,28]. We ourselves have found that morphine tolerance may cause a decrease of prodynorphin mRNA levels in rat hippocampus and striatum[23]. The chronic opiate agonist, ethylketocyclazocine (EKC), also markedly reduced prodynorphin mRNA in rat hypothalamus and hippocampus[22]. However, other studies found no change in the endogenous opioid system of the hypothalamus of morphine-tolerant rats[14].

The aim of our study, therefore, was to investigate further the effects of chronic exposure to opiates on the neuronal gene expression of the opioid precursor prodynorphin in different rat brain areas. We employed the synthetic opiate agonist U-50,488H, which is highly selective for the κ opioid receptor[29]. We investigated chronic morphine effects as well in order to compare the κ-receptor agonist and a classic μ agonist opiate. At the same time we measured the content of the prodynorphin-derived peptides in those tissues in order to obtain information on posttranscriptional processing of the precursor.

We found that the synthetic κ-receptor agonist U-50, 488H, chronically administered, did down-regulate prodynorphin mRNA in the rat hypothalamus, hippocampus and striatum.

MATERIALS AND METHODS

Animals and treatment

Male Sprague–Dawley rats weighing 200–250 g were used. In each experiment 3 groups of 10 animals were used: controls, U-50, 488H-treated group and morphine-treated group. Each experiment was repeated four times. The following substances were used: *trans*(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl) cyclohexyl]-benzene acetamide methanesulfonate (U-50,488H, UpJohn Company, Kalamazoo, MI); morphine hydrochloride (Farmitalia-Carlo Erba, Milan, Italy). The intracerebroventricular (i.c.v.) route was used: U-50,488H (4 μg/μl) and morphine (1 μg/μl) were infused by mini-pumps (Alzet 2001, Alza Corporation, CA) at a constant rate of 1 μl/h for 7 days. Control rats received sterile artificial cerebrospinal fluid (CSF) 5 μl i.c.v. twice daily for seven days. For the i.c.v. experiment a cannula was permanently inserted into the lateral ventricle according to the method described elsewhere[4] and

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rats were allowed to recover for at least 5 days after surgery before beginning treatment.

Tissue processing

On day 7, the rats were killed, brains were rapidly removed, hypothalami, hippocampi and striata were dissected according to the method of Glowinsky and Iversen[13] and tissues were frozen on dry ice. Six of the 10 animals from each group were processed for total RNA extraction and the other four for peptide extraction.

Total RNA was prepared according to the method of Chirgwin et al[5]. RNA was extracted from pooled samples by homogenizing in five volumes of 4 M guanidinium thiocyanate/0.5% sodium *N*-laurylsarcosine/5mM 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 35,000 rpm at 20 °C. Pellets were resuspended in 10 mM Tris Cl (pH 7.4)/5 mM EDTA/1% SDS, extracted with a chloroform-1-butanol (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 11,000 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). The ratio $OD_{260}/OD_{280} > 1.8$ provided an estimate of the purity of the total RNA[15].

Probes

Blots were hybridized with two different cDNA probes[6]: (1) BgBa, the BglII to BamHI fragment (920 base pair) of the rat genomic DNA complementary to the prodynorphin mRNA, consisting of the 5'-translated region of the prodynorphin gene, encoding for all dynorphin; (2) BaBa, the BamHI to BamHI fragment (815 base pair) of the same genomic cDNA, consisting of the untranslated region of the prodynorphin gene. The cDNA fragments, inserted in pUC19 vectors, were kindly supplied by Drs. O. Civelli and J. Douglass[6]. BgBa was released by EcoRI and PstI digestion, and BaBa by BamHI digestion. Each fragment was labelled by either nick translation[20] or random priming[10] methods using α-[^{32}P]-dCTP to a specific activity of 7–9 × 10^5 cpm/ng.

A 30-base oligonucleotide recognizing α-tubulin mRNA (NEP 206, NEN Dupont, U.S.A.; spec. act. 1.27 × 10^9 dpm/μg) was used as internal standard to hybridize the same blots.

Northern blot analysis

Total RNA from each tissue (30 μg) was electrophoresed through 1% agarose gel containing 2.2 M formaldehyde at 75 V using 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 the filter was blotted, air-dried, baked at 80 °C for 2 h, and placed in a plastic bag which was sealed and stored at room temperature.

The blots were prehybridized overnight at 42 °C in a solution of 6 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate), 1 × Denhardt's solution (0.02% polyvinylpyrrolidone, Ficoll and BSA), 100 μg/ml denatured salmon sperm DNA, 0.1% SDS, 50% formamide, 10 mM Tris and 10% dextran sulfate. For the hybridization assay, each blot was prepared in duplicate and tested with the two ^{32}P-labelled cDNA probes. The probes were boiled for 10 min in the hybridization buffer (6 × SSC, 1 × Denhardt's solution, 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 × 10^6 cpm/ml; hybridization was carried out for 24 h at 42 °C.

After removal of the probe solution, blots were washed 3 times for 10 min at room temperature with a solution of 2 × SSC/0.1% SDS followed by 3 times for 10 at 65 °C with a solution of 0.1 × SSC/0.1% SDS, on a rocker. X-ray films (Amersham β-max) were exposed to the hybridized blot backed by an intensifying screen (Cronex DuPont) at −70 °C for 4 days.

Blots were hybridized serially with probes directed against prodynorphin and α-tubulin mRNA. Total RNA from treated animals was compared to RNA from control rats. Optical densities for autoradiographic bands produced by prodynorphin hybridization were determined using a Video Densitometer system (MDL 620). Hybridization values for samples from treated animals were expressed as percentages of controls (100%) for each experiment. Data were statistically analyzed by Student's *t*-test.

For the peptide content, tissues were obtained from four rats in each treatment group and used for dynorphin A detection. Radioimmunoassay was done using the 'Lucia' antiserum[12] kindly provided by Prof. B.M. Cox. Data were statistically analyzed by the Newman–Keuls test.

RESULTS

Under the conditions employed, we detected hybridization to prodynorphin mRNA (size ca. 2.4 kb) with both ^{32}P-labelled cDNA probes, giving the same results in each blot prepared in duplicate. Hybridization to prodynorphin mRNA in the Northern blot analysis of all tissues was reduced by U-50,488H and morphine, compared to control animals.

In the hypothalamus, chronic exposure to the selective κ opioid agonist U-50,488H markedly reduced prodynorphin mRNA, to 19.7 ± 4.8% (mean ± S.E.M.) of controls (mean of 4 independent experiments; $P < 0.01$); chronic morphine reduced prodynorphin mRNA to 50.5 ± 4.2% of control ($P < 0.01$). Fig. 1 refers to one single

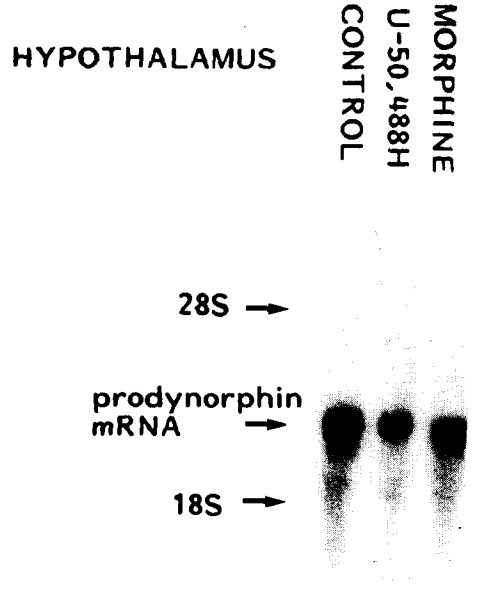

Fig. 1. Northern blot analysis of hybridization to prodynorphin mRNA in 30-μg samples of total hypothalamic RNA prepared from pools of 6 rats per group. Control: CSF, 5 μl i.c.v., twice daily for 7 days. U-50,488H and morphine: 4 μg/μl and 1 μg/μl, respectively, infused by minipumps at a constant rate of 1 μl/h for 7 days. Blots were hybridized and quantitated as described under Materials and Methods. A representative autoradiogram from one single experiment is shown.

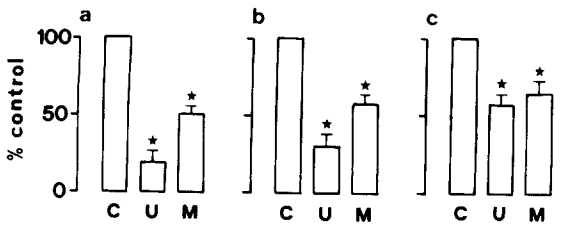

Fig. 2. Changes in prodynorphin mRNA levels in rat hypothalamus (panel a), hippocampus (panel b) and striatum (panel c). C, control rats; U, U-50,488H chronically treated rats; M, morphine chronically treated rats. Values are expressed as percentage of control (100%). Relative abundance of prodynorphin mRNA in autoradiogram from Northern blot analysis was quantitated by densitometer. Optical density values for autoradiographic bands were calculated as a fraction of the control value (always 100%) for each experiment and results are expressed as the mean from 4 separate samples, each pooled from 6 rats (* $P < 0.01$ vs control by t-test).

experiment and Fig. 2 (panel a) shows the mean values for all experiments, as histograms.

In the hippocampus, the hybridization of prodynorphin mRNA was significantly reduced, to 29.7 ± 6.9% (mean ± S.E.M.) of control values, by U-50,488H and to 58.7 ± 4.3% of control by morphine. Fig. 3 refers to a single experiment and Fig. 2 (panel b) shows the means of 4 replications.

In the striatum, chronic exposure to opioid agonists reduced the hybridization signal to 56.0 ± 4.5% of control after U-50,488H and to 63.2 ± 6.5% of control after morphine. Fig. 4 refers to a single experiment and Fig. 2 (panel c) shows the means of 4 replications.

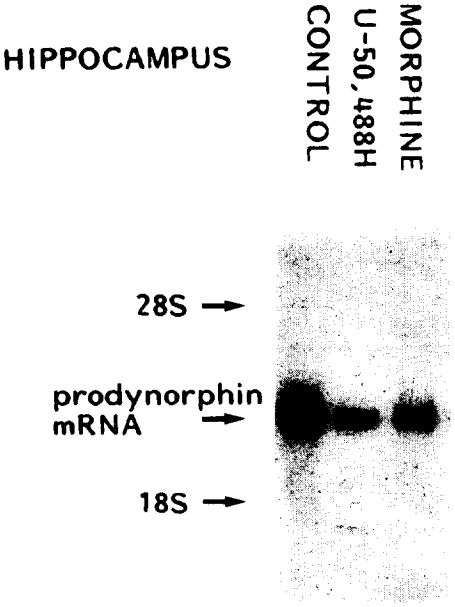

Fig. 3. Northern blot analysis of hybridization to prodynorphin mRNA in 30-μg samples of total hippocampal RNA prepared from pools of 6 rats per group. Control: CSF, 5 μl i.c.v., twice daily for 7 days. U-50,488H and morphine: 4 μg/μl and 1 μg/μl, respectively, infused by minipumps at a constant rate of 1 μl/h for 7 days. Blots were hybridized and quantitated as described under Materials and Methods. A representative autoradiogram from one single experiment is shown.

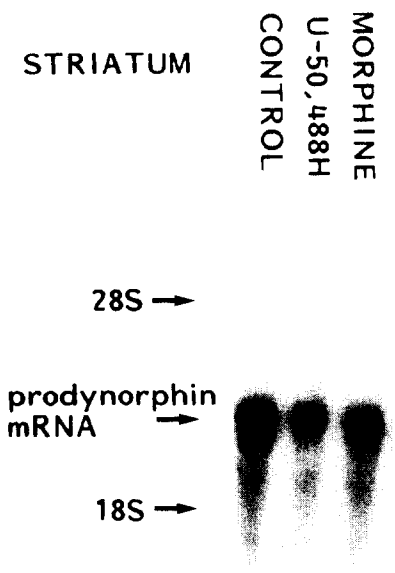

Fig. 4. Northern blot analysis of hybridization to prodynorphin mRNA in 30-μg samples of total striatal RNA prepared from pools of 6 rats per group. Control: CSF, 5 μl i.c.v., twice daily for 7 days. U-50,488H and morphine: 4 μg/μl and 1 μg/μl, respectively, infused by minipumps at a constant rate of 1 μl/h for 7 days. Blots were hybridized and quantitated as described under Materials and Methods. A representative autoradiogram from one single experiment is shown.

Although there may well be some inter-animal variability in the manifestation of the effect, the reduction was found in 4 independent replications of each experiment.

Many control experiments were made to validate these results. UV analysis of the RNA (electrophoresis of total RNA) showed no differences in the intensity of the 18S ribosomal bands for the control and the drug-treated rats, thus supporting the homogeneity of the total RNA concentrations. Hybridization to the structural protein α-tubulin mRNA was not significantly different for con-

TABLE I

Levels of ir-dyn A in tissues of rats chronically infused with U-50,488H and morphine

Values are mean ± S.E.M.

Group	*Hypothalamus*	*Hippocampus*	*Striatum*
	(ir-dyn A pmol/g tissue)		
Control	30.50 ± 3.47	7.85 ± 0.77	3.17 ± 0.43
U-50,488H	16.34 ± 3.23*	12.38 ± 1.92	3.55 ± 0.21
Morphine	32.70 ± 3.10	12.82 ± 1.87	6.79 ± 0.53*

*$P < 0.05$ vs control (Newman-Keuls test).

trol and treated rats; α-tubulin mRNA from U-50,488H-treated rats was 107 ± 5% of controls; α-tubulin mRNA from morphine-treated rats was 103 ± 6% (data not shown).

Except for two cases, no major changes were observed in peptide levels in these tissues after chronic exposure to opioid agonists: U-50,488H elicited a significant decrease of ir-dyn A in hypothalamus (16.34 ± 3.23 vs 30.50 ± 3.47 pmol/g tissue) and no changes in hippocampus and striatum; morphine induced an increase of ir-dyn A only in striatum (6.79 ± 0.53 vs 3.17 ± 0.43 pmol/g tissue), not in hypothalamus and hippocampus (Table I).

DISCUSSION

The present results indicate that chronic exposure to opioid agonists (necessary for the development of tolerance to their effects) clearly affects prodynorphin gene expression in the rat hypothalamus, hippocampus and striatum. Chronic U-50,488H significantly lowers prodynorphin mRNA. Both this synthetic selective κ agonist and the classic μ agonist morphine, as previously described[23], had the same decreasing effect on detectable prodynorphin mRNA, though the differences in the intensity of the action suggest that some degree of agonist selectivity may be involved in this effect.

Our data on prodynorphin follow the same pattern as findings on the other two opioid systems concerning chronic morphine. Mocchetti et al.[17] reported a 50% decrease in the POMC mRNA content in the hypothalamus of morphine-tolerant rats. A year later Uhl et al.[28] found a decrease of proenkephalin mRNA levels in striatum of chronically morphine-treated rats. Recently Tempel[26] found a 44% decrease on striatal proenkephalin mRNA after 4 days of postnatal morphine treatment in rats.

Only one contrasting result was reported, by Höllt et al.[14], who found no changes in mRNA coding for all 3 opioid peptide precursors in the hypothalamus of morphine-treated rats.

In the context of studies concerning the effects of chronic pharmacological treatments on biosynthesis of the endogenous opioid system, our data indicate that chronically administered opiates cause down-regulation of the prodynorphin system, besides the proenkephalin and POMC systems, already mentioned. Not only is this effect shown in the hypothalamus and in the striatum but, in our hands, also in the hippocampus.

We also explored the effects of chronic opiates on prodynorphin-derived peptide levels in the investigated areas. Except for two cases the opioid agonists employed did not alter the content of prodynorphin-related peptides in these tissues after chronic treatment: morphine raised ir-dyn A in the striatum and U-50,488H lowered ir-dyn A in the hypothalamus. The morphine-induced increase in dynorphin was also reported by Trujillo et al.[27], and it might reflect direct inhibition of striatal peptide release by morphine.

There are plenty of published reports of no correlation between the effect on mRNA and on the peptide, and no clear explanation has yet been offered. The neuronal level of a peptide is always based on a balance between several factors: biosynthesis of the precursor (in terms of transcription of DNA or mRNA), translation of precursor mRNA, processing of the precursor, packaging, transport, release and degradation of the biologically active peptides produced.

Our results indicate that chronic exposure to opioid receptor agonists leads to alterations in transcription of the prodynorphin mRNA, whose level drops, but at the same time it does not modify the peptide stores, probably because of a decrease in release or an increase in precursor processing. Rates of prodynorphin synthesis and release were probably down-regulated in parallel so that, as a consequence, the neuronal dynorphin store remained stable. Moreover, our data on ir-dyn A are consistent with other reports of no change in β-endorphin and proenkephalin-derived products after chronic treatment with morphine[17,28].

The search for a possible explanation of our data raises the question whether the down-regulation of the synthesis of prodynorphin mRNA is instrumental in opiate tolerance. The dynamics of the events do to some extent offer an explanation for the development of tolerance. The normal tone of the opioidergic system on receptor occupancy could be altered by continuous administration of exogenous opiate, so the synthesis and utilization of opioids could decrease because the receptors are completely occupied by the agonist. It seems of interest that all 3 opioid precursor mRNAs are affected by this mechanism in brain areas purportedly involved in the development of tolerance.

As regards the mechanisms underlying the development of tolerance, long-term administration of opioid agonists might cause alterations in the events following the signal transduction, thus inhibiting biosynthesis of the endogenous opioid system. Evidence has been provided of a signal transduction pathway linking membrane receptors to gene stimulation. Thus, the proenkephalin gene can be activated by cAMP[11,16], beside many other neuropeptide genes and one of the early genes, c-*fos* proto-oncogene, appears to be directly involved in the regulation of opioid gene expression[8,9,16].

Concerning receptor modifications occurring after chronic exposure to opioid agonists, a reduced coupling

efficiency of the opioid receptors to the associated G_i protein (desensitization)[30] and a down-regulation after further exposure[18] have been observed. This long-term uncoupling of opioid receptors from the appropriate transduction system might be responsible for the gene inactivation, which results in lower prodynorphin mRNA levels. These changes in opioid mRNA levels may be the direct effect of opioid receptor activity on the whole opioidergic system, including prodynorphin, POMC and proenkephalin[17,28], at least in rat hippocampus, hypothalamus and striatum. Focusing research on the second messenger pathway involved should elucidate the cellular mechanisms underlying the development of opiate tolerance.

In conclusion, our data indicate that chronic exposure to opioid receptor agonists (necessary for the development of tolerance) results in alterations of neuronal systems involving opioid peptides and their gene expression.

Further studies are in progress in our laboratory, utilizing opioid agonists and antagonists to clarify the interaction between the endogenous opioid system and the brain functions responsible for opiate tolerance.

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