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Long-term exposure to opioid antagonists up-regulates prodynorphin gene expression in rat brain

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Abstract

We investigated the effect of long-term administration of opioid antagonists on the regulation of prodynorphin gene expression in rat brain. Intracerebroventricular (i.c.v.) injections for seven days of nor-binaltorphimine (nor-BNI), the highly selective κ opioid antagonist, naloxone and its longer acting analog naltrexone, both relatively selective antagonists for the μ opioid receptor, markedly raised prodynorphin mRNA levels in rat hypothalamus, hippocampus and striatum. Peptides, namely immunoreactive-dynorphin A (ir-dyn A), were unaffected after chronic treatment with all antagonists, in the same tissues. These results, taken together with our previous observations, suggest that chronic opioid antagonists, acting on κ and μ opioid receptors, clearly up-regulate prodynorphin gene expression in discrete rat brain regions, activating its biosynthesis. Moreover, our data support the hypothesis that the endogenous opioid system plays a role in the mechanisms underlying the development of opiate tolerance.

Keywords: Opioid; Gene expression; Prodynorphin mRNA; Chronic treatment; Rat brain; Nor-binaltorphimine; Naloxone; Naltrexone

1. Introduction

Chronic treatment with different opioid agonists causes a down-regulation of the gene expression of the endogenous opioid system in some areas of the rat brain [7,16,22–25,34]. Morphine, the classic μ opioid receptor agonist, ethylketocyclazocine and the synthetic Upjohn compound U-50,488H, selective agonists for the κ opioid receptor, markedly inhibited opioid gene expression, lowering mRNA levels in rat hypothalamus, hippocampus and striatum [23], when chronically administered for seven days.

Chronic treatment with opiate agonists acting at μ and δ opioid receptors has also been shown to induce a down-regulation of opioid binding sites, lowering both the affinity and numbers of receptors available [7,19,29,31,35]. However, chronic treatment with opiate antagonists reportedly increases the number of opioid receptors in rat brain [7,15,17,30,37], indicating that opioid binding sites are up-regulated in these conditions [11].

Therefore, in this context, cellular changes in response to chronic opioid treatments migth be related to the development of opiate tolerance [1,8,14,33]. The agonist-induced down-regulation and the antagonist-induced up-regulation of opioid receptors, with the exception reported by Rothman et al. [26], might be the result of adaptative responses by the cell to the increased level of receptor activation in vivo during chronic morphine or to the decreased level of receptor activation after chronic naloxone or naltrexone [7]. All these molecular changes may play important roles in the development of opiate tolerance.

In the light of these findings [7,16,23,34], the aim of our study, therefore, was to investigate the effects of chronic exposure to opioid antagonists on the regulation of gene expression of the opioid precursor prodynorphin [3,27] in the rat brain, which we had already found was reduced during the development of tolerance, following opioid administration [22–25].

Nor-binaltorphimine (nor-BNI) [28], a highly selective antagonist for the κ opioid receptor, and naloxone

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and its longer acting analog naltrexone, relatively selective antagonists for the μ opioid receptor [20], were injected intracerebroventricularly (i.c.v.) to the rat for seven days. Prodynorphin mRNA levels were then measured in the rat hypothalamus, hippocampus and striatum. Prodynorphin mRNA levels were clearly upregulated by all opioid antagonists. At the same time, immunoreactive dynorphin A (ir-dyn A) content was measured in the same tissues: no changes were observed after chronic treatment with all antagonists.

2. Materials and methods

2.1. Animals and treatment

Male Sprague–Dawley rats (Charles River, Como, Italy) weighing 200–250 g were used. Four groups of twelve animals were used in each experiment: controls, nor-BNI-treated group, naloxone-treated group, naltrexone-treated group. Each experiment was repeated four times. The following substances were given i.c.v.: nor-BNI (1 μg/μl), naloxone (3 μg/μl) and naltrexone (1 μg/μl) infused by mini-pumps (Alzet 2001, Alza Corp., CA) at a constant rate of 1 μl/h for seven days. Control rats received sterile artificial cerebrospinal fluid (CSF), 1 μl/h i.c.v. for seven days, by mini-pumps. For i.c.v. injections a cannula was permanently inserted into the lateral ventricle according to the method described elsewhere [2] and rats were allowed to recover for at least five days after surgery before beginning treatment.

2.2. Tissue processing

On treatment day 7, the rats were killed, their brains were rapidly removed and hypothalami, hippocampi and striata were dissected according to the method of Glowinsky and Iversen [12] and tissues were frozen on dry-ice. Six of 12 rats from each group were processed for total RNA extraction and the other 6 for peptide extraction.

Total RNA was prepared according to the method of Chirgwin et al. [5], RNA was extracted from pooled samples (2–3 rats) by homogenizing in five 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 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 OD/ml = 25 μg RNA/ml). A ratio of OD_{260} to OD_{280} of more than 1.8 provided an estimate of the purity of the total RNA.

For peptide extraction samples were weighed, homogenized in 10 ml/g of 0.1 M acetic acid at 95°C and held at this temperature for 10 min. Homogenates were then centrifuged at 10,000 × *g* at 4°C for 20 min, and the supernatants were removed and stored at −24°C until assay for immunoreactive dynorphin by an already described radioimmunoassay [10].

2.3. Probes

Blots were hybridized with the probe BgBa, the *Bgl*II to *Bam*HI 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 prodynorphin.

The cDNA fragment, inserted in pUC19 vector, was kindly supplied by Drs. O. Civelli and J. Douglass [6]. BgBa was released by *Eco*RI and *Pst*I digestion, labelled by either nick translation or random priming methods using [α-^{32}P]-dCTP to a specific activity of $7–9 \times 10^5$ cpm/ng.

A cDNA recognizing β-actin mRNA (clone pHFβA-1, containing the full-length cDNA insert for human cytoplasmatic β-actin) was used as internal standard to hybridize the same blots [13].

2.4. 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 morpholinopropane sulfonic 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.

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 $1–2 \times 10^6$ cpm/ml; hybridization was carried out for 24 h at 42°C.

After removal of the probe solution, blots were washed three times for 10 min at room temperature

with a solution of 2 × SSC/0.1% SDS followed by three times for 10 min 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 twice with probes directed against prodynorphin and β-actin mRNA. For β-actin mRNA hybridization, blots were prehybridized and hybridized overnight at 65°C in a solution of 4 × SSC, 50 mM NaH_2PO_4, 5 × Denhardt's solution and 10% dextran sulfate. Blots were washed three times for ten min at 65°C with a solution of 0.5 × SSC, 0.1% SDS on a rocker and then exposed to X-ray films at −70°C for 24 h.

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). Briefly, background densities from areas of film away from the lanes were subtracted from observed values, which were obtained from non-saturated autoradiographic exposures in which standardizing lanes revealed a linear relationship between the amount of prodynorphin mRNA and hybridization signal. Two autoradiograms for each blot were scanned. Multiple exposures to film and the presence of standardizing lanes allowed us to avoid saturation of the X-ray film. Hybridization values for samples from treated animals were expressed as percentages of controls (100%) for each experiment. Data were statistically analyzed by Dunnett's test.

2.5. Radioimmunoassay

For the opioid peptide ir-dyn A content, tissues were obtained from six rats in each treatment group and analyzed for dynorphin A detection. Dynorphin A(1–13) (Peninsula Labs.-Europe, Merseyside, UK) was iodinated by the chloramine-T method and purified by micro-column Sep-Pak C18 reversed phase chromatography. Antiserum employed was 'Lucia' (kindly supplied by Prof. B.M. Cox), raised against dynorphin A(1–13) and displaying full cross-reactivity to dynorphin A(1–17), partial cross-reactivity to the COOH-extended form (e.g. dynorphin A(1–32)) and not recognizing dynorphin A(1–8), dynorphin B or any other opioid peptide [10]. In the light of these properties, the total immunoreactivity detected by the antiserum is referred to as ir-dyn A. It was used at the appropriate dilution to give about 30% binding of [^{125}I]dynorphin A(1–13) added (≈ 5,000 cpm). In a typical assay, the IC_{50} was 10 fmol/assay tube (intra- and interassay coefficents of variation were 5% and 7%).

Radioimmunoassays were carried out in 0.15 M sodium phosphate buffer (pH 7.4), with incubation for 18–24 h at 4°C. The reaction was terminated by the addition of 1.0 ml of buffer containing 3.0% charcoal and 0.3% dextran. Bound peptide was separated by centrifugation at 5,000 × g at 4°C and 1 ml samples of the supernatants were counted for 1 min on a gamma-counter. Data were statistically analyzed by ANOVA followed by the Newman-Keuls test.

3. Results

Under the conditions employed we detected hybridization to prodynorphin mRNA (size ca. 2.3–2.4 kb) with our probe. Northern blot analysis revealed a marked increase in prodynorphin mRNA levels after chronic exposure to nor-BNI, naloxone and naltrexone in the tissues, compared to control animals.

In the hypothalamus, the κ opioid antagonists nor-BNI, administered i.c.v. for seven days, raised prodynorphin mRNA levels to 158 ± 12% (mean ± S.E.M.) of controls (mean of four independent experiments; $P < 0.01$); the μ opioid antagonists naloxone and naltrexone chronically injected i.c.v. for seven days significantly raised prodynorphin mRNA levels to 170 ± 7% and 199 ± 15% (mean ± S.E.M.) of controls (mean of four independent experiments; $P < 0.01$). Fig. 1 refers to one single experiment and Fig. 2 shows the mean values for all experiments, as histograms.

In the hippocampus, hybridization to prodynorphin mRNA was significantly increased after chronic nor-BNI (to 180 ± 20% of controls, $P < 0.01$), naloxone (to 200 ± 24% of controls, $P < 0.01$) and naltrexone (to 155 ± 11% of controls, $P < 0.01$). Fig. 1 refers to a single experiment, and Fig. 2 shows the means of four independent experiments.

In the striatum, the κ opioid antagonist nor-BNI, administered i.c.v. for seven days, raised prodynorphin mRNA levels to 145 ± 9% of controls ($P < 0.01$); chronic exposure to the μ antagonists naloxone and naltrexone raised the hybridization signal to prodynorphin mRNA to 197 ± 13% and 180 ± 17% of controls ($P < 0.01$). Fig. 1 refers to a single Northern blot analysis and Fig. 2 presents the mean values of four replications.

Prodynorphin mRNA biosynthesis was stimulated in all replications of each experiment and the difference from controls was significant.

Internal control experiments were made to confirm and validate these data. First of all, UV analysis of total RNA (ribosomal 18S and 28S bands) after electrophoresis showed no differences in the intensity of single bands for control and drug-treated rats, supporting the homogeneity of total RNA loaded concentrations.

Each blot was then serially hybridized with the

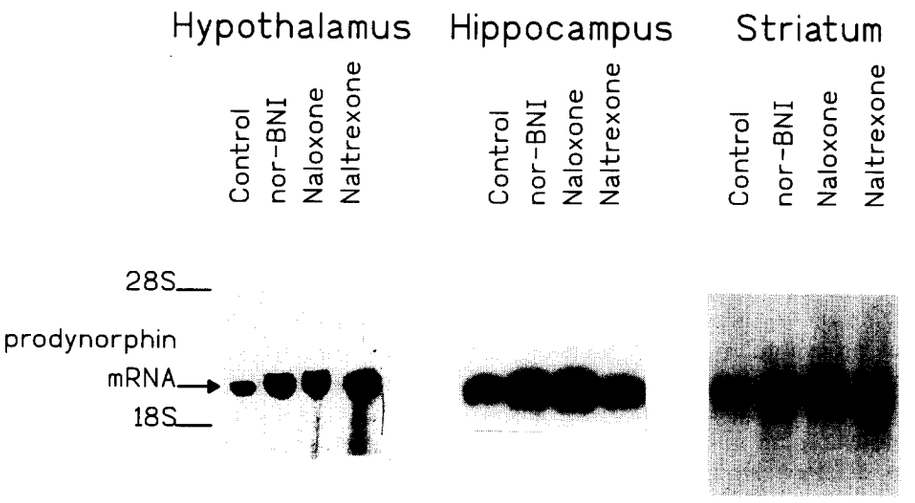

Fig. 1. Northern blot analysis of hybridization to prodynorphin mRNA in 30 μg samples of total hypothalamic, hippocampal and striatal RNA prepared from pools of 2–3 rats for group. Control: CSF, 1 μl/h i.c.v., for 7 days. Nor-BNI: 1 μg/μl, naloxone: 3 μg/μl, naltrexone: 1 μg/μl, infused i.c.v. by mini-pumps at a constant rate of 1 μl/h for seven days. Blots were hybridized and quantitated as described in section 2. Representative autoradiograms from single experiments are shown.

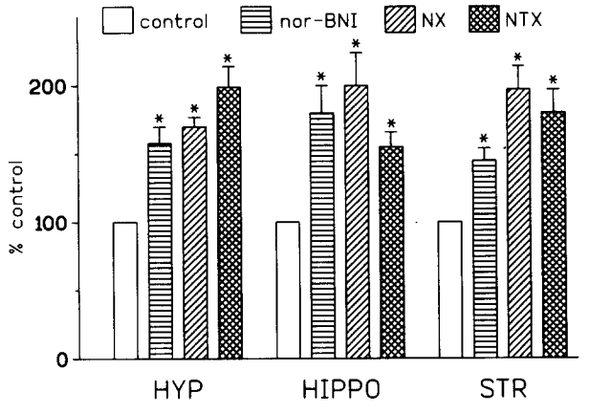

Fig. 2. Changes in prodynorphin mRNA levels in rat hypothalamus, hippocampus and striatum. Control rats; nor-BNI, norbinaltorphimine; NX, naloxone; NTX, naltrexone chronically treated rats. Values are expressed as percentage of control (100%). Relative abundance of prodynorphin mRNA in autoradiogram from Northern analysis was quantitated by densitometer. Optical density values for autoradiographic bands were calculated and results are expressed as the mean from four separate samples, each pooled from 2–3 rats (* $P < 0.01$ vs. control by Dunnett's test).

cDNA probe recognizing β-actin mRNA. No differences were reported between controls and treated animals in all tissues; β-actin mRNA levels from nor-BNI, naloxone and naltrexone treated rats were 98 ± 7%, 103 ± 6%, 107 ± 7% of controls, respectively. Fig. 3 shows a representative Northern blot analysis of β-actin mRNA in the striatum of controls, nor-BNI, naloxone and naltrexone treated rats.

Table 1
Levels of ir-Dyn A in tissues of rats, chronically treated with nor-BNI, naloxone and naltrexone

Treatment	Hypothalamus (ir-Dyn A pmol/g tissues)	Hippocampus	Striatum
Control	30.52 ± 3.47	7.85 ± 0.77	3.17 ± 0.43
Nor-BNI	28.10 ± 2.59	7.10 ± 1.39	3.95 ± 0.35
Naloxone	25.02 ± 3.80	11.64 ± 1.95	4.80 ± 0.55
Naltrexone	25.37 ± 4.58	9.48 ± 0.95	3.24 ± 0.44

Values are mean ± S.E.M. * $P < 0.05$ vs. Control (Newman-Keuls test).

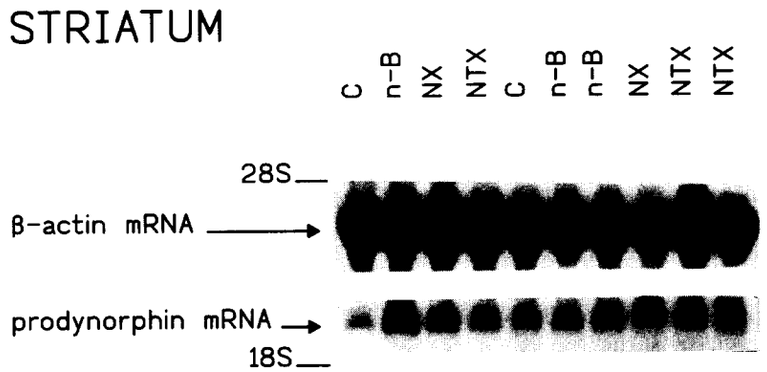

Fig. 3. Northern blot analysis of hybridization to prodynorphin and β-actin mRNA levels in rat striatum. C, control rats; n-B, norbinaltorphimine; NX, naloxone; NTX, naltrexone chronically treated rats. Blots were hybridized and quantitated as described in section 2. A representative autoradiogram from one single experiment is shown.

Concerning the peptide content, no changes were observed in ir-dyn A levels after chronic exposure to nor-BNI, naloxone and naltrexone in rat hypothalamus, hippocampus or striatum (Table 1).

4. Discussion

These results indicate that chronic exposure to opioid antagonists acting on κ and μ opioid receptors clearly up-regulates prodynorphin gene expression in the rat hypothalamus, hippocampus and striatum. The highly selective κ opioid antagonist nor-BNI significantly increased prodynorphin mRNA biosynthesis in these brain regions, as after chronic administration of the μ opioid antagonists naloxone and naltrexone.

Our data on prodynorphin mRNA are in accordance with Tempel et al. [32], concerning an increase of mRNA for another opioid precursor, proenkephalin, in rat striatum and hippocampus after naltrexone for seven days.

Taken together, these results appear to confirm that the whole of the endogenous opioidergic system is affected by chronic blockade of the μ opioid receptor; moreover, this means the prodynorphin system is affected by this chronic blockade besides that of the κ receptor, which is specific for this system [3].

Recent reports that receptor density is also up-regulated after chronic antagonist treatment well matches our results on opioid precursor mRNA levels [8,11,17,30,37]. Chronic occupancy of the opioid receptors by antagonists may possibly induce this up-regulation, since the sites available are silent [7]. Thus, our results suggest there is an increase in endogenous opioid gene expression, since no agonist interacts with opioid receptors occupied by the antagonists.

Chronic i.c.v. treatment with the opioid antagonists nor-BNI, naloxone and naltrexone, changed the levels of mRNA, but did not appear to modify ir-dynorphin A peptide levels in the rat hypothalamus, hippocampus and striatum. Thus, the peptide dynorphin A, derived from the precursor prodynorphin, is normal when prodynorphin mRNA content is up-regulated. The lack of correlation between the effects on the peptide and on mRNA seems typical of the neurochemical pattern of endogenous opioids, but no explanations have yet been offered.

As we suggested in an earlier work, and as happens with chronic treatment with agonists [22–24], the chronic antagonists affect opioid gene expression regulation and opioid receptor density [7]. If, at the same time, release is increased or precursor processing is inhibited, the neuronal balance of the dynorphin store does not appear to be affected [7,23].

Overall, findings to date show that chronic opioid agonists, acting on κ and μ opioid receptors, down-regulate prodynorphin gene expression in rat brain. The data presented in this study indicate that occupancy of these opioid receptors by opioid antagonists up-regulates the dynorphinergic system in the same rat brain regions, thus supporting the suggestion that chronic antagonists counteract an endogenous opioid inhibitory tone, with the opposite effects to agonists [7,21].

The present findings might, however, indicate that prolonged exposure to opioid antagonists acting on μ and κ receptors causes a lasting lack of activation of intracellular pathways, somehow resulting in marked stimulation of the endogenous opioid gene expression. Research on the second messenger pathway involved will help clarify the underlying cell mechanisms and could cast useful light on the neuronal plasticity in which the endogenous opioid system participates in the rat CNS [18].

Like the results of research into tolerance, these findings might help find answers to questions whether the modulation of opioid gene expression regulation by opioid agonists and antagonists is perhaps instrumental in the development of opiate tolerance [7,8,14,23]. Lastly, the recent cloning of the opioid receptors [4,9,36] will contribute to our understanding of the interaction of chemical structures with specific protein receptor domains.

Investigations on other molecules interacting with κ and μ opioid receptors and, above all, with the other opioid receptor, δ, should help clarify the neuronal pathways linking the opioid receptors, the post-transductional effector systems and the opioid gene activation or repression.

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