Chronic Naltrexone Treatment Increases Expression of Preproenkephalin and Preprotachykinin mRNA in Discrete Brain Regions

Ann Tempel,¹ John A. Kessler,^{1,2} and R. Suzanne Zukin¹

Departments of ¹Neuroscience and ²Neurology, Albert Einstein College of Medicine, Bronx, New York 10461

Long-term blockade of brain opioid receptors by the opiate antagonist naltrexone increases methionine-enkephalin content in the striatum and nucleus accumbens (Tempel et al., 1984). To determine whether these changes in peptide levels reflect increased peptide synthesis, we examined preproenkephalin mRNA content in discrete brain regions of control (placebo-treated) and chronic naltrexone-treated animals by Northern analysis. Chronic naltrexone treatment (8 d) led to an approximately 12-fold increase in the striatal content of preproenkephalin mRNA relative to that of control animals. In contrast, no statistically significant change was observed in striatal mRNA for cyclophilin (1B15) or actin. Small increases in preproenkephalin mRNA content occurred in the hippocampus (+40%) and hypothalamus (+19%). No significant changes occurred in the frontal cortex. Increases in levels of the mRNA were seen as early as 24 hr after antagonist treatment. In contrast, changes in opioid receptor density required 3–4 d to reach half-maximal up-regulation after chronic antagonist treatment. Recent evidence has suggested that substance P is regulated by opioid peptides. To determine whether substance P synthesis is altered by chronic antagonist treatment, the mRNA corresponding to the precursor for substance P was examined using a probe for exon-7 of the preprotachykinin gene. Preprotachykinin mRNA content in the striatum was increased 6-fold after chronic antagonist treatment relative to that of control animals. Substance P content was increased 3-fold after chronic antagonist treatment. These data suggest that chronic blockade of brain opioid receptors leads to the increased synthesis of both enkephalin and substance P in the striatum and that these changes are relatively specific. The finding of opiate antagonist-induced increases in preprotachykinin mRNA and substance P levels supports the concept of a role for enkephalin in the regulation of substance P gene expression.

Antagonist-induced opioid supersensitivity and opioid receptor up-regulation are well characterized. Long-term *in vivo* administration of the opioid antagonist naloxone or naltrexone results in enhanced morphine-induced analgesia (Lahti and Collins, 1978; Tang and Collins, 1978; Tempel et al., 1985; Yoburn et al., 1985, 1989a) and enhanced effects of morphine on neurons of the locus coeruleus (Bardo et al., 1983) and the myenteric plexus (Schulz et al., 1979). This functional supersensitivity reflects both an increased number of μ and δ opioid receptors (Tempel et al., 1985; Danks et al., 1988; Yoburn et al., 1989b) and an increased coupling of receptor to the inhibitory guanyl nucleotide binding protein G_i (Zukin et al., 1982; Tempel et al., 1985). The changes in opioid receptor density vary heterogeneously throughout the brain; highest increases in μ receptor density occur in the nucleus accumbens, amygdala, striatum, layers I and III of the neocortex, and the periaqueductal grey region (Tempel et al., 1984).

In addition to these changes in opioid receptors, chronic administration of opioid antagonists produces changes in opioid peptide levels. Chronic naltrexone treatment increases methionine enkephalin-like immunoreactivity in the striatum and nucleus accumbens, structures that also show large increases in receptor number (Tempel et al., 1984). More moderate increases occur in the periaqueductal grey and hypothalamus. In contrast, no changes in opioid peptide levels are discernible in other brain areas, including the neocortex.

The present study was carried out to characterize the effects of opioid antagonist treatment on preproenkephalin (PPE) and preprotachykinin (PPT) mRNA levels. Our study indicates that long-term blockade of opioid receptors by naltrexone leads to large increases in the striatum of both PPE mRNA and PPT mRNA. These findings suggest that chronic blockade of opioid receptors leads to increased enkephalin synthesis and that the enkephalinergic system is capable of modulating PPT gene expression.

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Correspondence should be addressed to Ann Tempel, Department of Psychiatry, Hillside Hospital/Div. of LIJHC, 266th St. & 76th Ave., Glen Oaks, NY 11004. Copyright © 1990 Society for Neuroscience 0270-6474/90/030741-07\$02.00/0

Materials and Methods

Animals and drug delivery. Male Sprague-Dawley rats (200–250 gm) were used in all experiments and were maintained *ad libitum* on food and water. For chronic drug experiments, 7–10 rats were implanted with naltrexone pellets (two 30-mg pellets, NIDA) in the dorsal subcapsular space under anesthesia. Control animals were either implanted with placebo pellets or sham-operated. A separate group of animals served as nonimplanted controls. Animals were killed at the designated times after implantation.

Preparation of total cellular RNA. Rats were killed by decapitation, and brains were removed rapidly. Dissections were performed immediately on ice. Coordinates were determined by reference to the atlas of Paxinos and Watson (1986), as previously described (Tempel et al.,

Figure 1. *A*, Northern blot analysis of PPE mRNA from control (C) and chronic (8 d) naltrexone-treated (N) rats. A representative autoradiogram of a 48-hr exposure at -70°C is shown. *B*, Northern blot analysis of 1B15 mRNA from control and antagonist-treated rats. A representative autoradiogram of a 24-hr exposure at -70°C is shown. Total RNA was prepared from dissected rat brain as described under Materials and Methods. Ten micrograms of total RNA were loaded onto each lane. Northern blots were hybridized and quantitated as described under Materials and Methods. The PPE and 1B15 hybridization signals displayed are derived from the same lanes for each brain region shown. *C*, Alterations in levels of PPE mRNA in response to chronic naltrexone treatment. Ratio values of PPE to 1B15 mRNA (Table 1) from each brain region from control animals were compared to those of antagonist-treated animals to calculate changes in mRNA levels. Alterations are expressed as percentage of control values. Scale bars represent the mean $\pm$ SEM from 10 experiments (each experiment = 1 animal examined independently; $*p < 0.01$). STR., striatum; HIPP., hippocampus; HYPO., hypothalamus; F.C., frontal cortex.

1984). The following 5 regions were dissected from each brain: (1) frontal cortex, (2) hypothalamus, (3) striatum, (4) hippocampus, and (5) substantia nigra. Brain regions were immediately frozen in liquid nitrogen and stored at -70°C .

Cellular RNA was isolated by a modification of the chloroform-phenol method of Bingham and Zachar (1985). Tissue from a single brain region from a single rat was placed in 0.5 ml lysis solution of the following composition: 1 mM EDTA, 350 mM NaCl, 2% SDS, 7 M urea, 10 mM Tris-HCl (pH 8.0). Samples were homogenized with a hand-held glass homogenizer. Protease K was added to a final concentration of 10 $\mu\text{g}/\text{ml}$, and the entire lysis sample was incubated at 37°C for 30

min. Each sample was then extracted 2 times with an equal volume of a solution containing 50% phenol, 48% chloroform, and 2% isoamyl alcohol and once with one volume of a solution containing 96% chloroform and 4% isoamyl alcohol. Total nucleic acids were precipitated from the remaining aqueous layer by adding 0.1 vol of 3 M NaOAc, pH 5.0, and 2.5 vol of ethanol and storing at -20°C overnight. RNA was collected by centrifugation in a microcentrifuge at maximum speed (4°C for 15 min). Pellets were dried and resuspended in distilled H_2O , and the RNA content was determined by measurement of ultraviolet absorbance at 260 nm.

Northern gel and blot analysis. Denatured RNA samples (5–10 μg of

Table 1. Ratio of preproenkephalin mRNA and preprotachykinin mRNA to 1B15 mRNA in control rat brain regions

Brain region	Ratio PPE mRNA/ 1B15 mRNA	Ratio PPT mRNA/1B15 mRNA
Striatum	2.56	2.83
Hypothalamus	1.64	1.78
Hippocampus	0.24	0.26
Frontal cortex	0.21	0.18

Densitometric analysis of levels of PPE, PPT, and 1B15 mRNA was performed. The values obtained for PPE and PPT RNA were each divided by the value obtained for 1B15 mRNA to normalize for possible differences in the amount of mRNA loaded onto each lane of the gel.

total nucleic acids) were electrophoresed on a 1.5% agarose gel containing 16.6% formaldehyde. RNA samples were transferred to Gene Screen Plus (NEN) by overnight capillary blotting in $10 \times$ SSC ($1 \times$ SSC = 0.15 M sodium chloride, 0.015 M sodium citrate). The Northern blot was then vacuum baked at 80°C for 2 hr. A 32 P-labeled RNA probe (Yoshikawa and Sabol, 1986) synthesized by using phage SP6 RNA polymerase was used to detect PPE mRNA [1.5 kilobases (kb)]. For substance P hybridization (1.7 kb), a 32 P-labeled 460 bp EcoRI-PstI fragment of plasmid RSP p23 was derived from a PPT exon 7-derived rat genomic clone (Bonner et al., 1987). 1B15 mRNA (1.0 kb) was detected with a probe generously supplied by Dr. Jim Douglass (Danielson et al., 1988).

RNA blots were prehybridized for 5 hr at 65°C in 50% formamide, 1 M NaCl, 1% SDS, and 0.1 mg/ml denatured herring sperm DNA. Blots were hybridized at 65°C for at least 20 hr in prehybridization solution to which $1-4 \times 10^5$ dpm/ml of one of the probes had been added. Blots were then washed at 65°C with a solution of $0.1 \times$ SSC/0.01% SDS and exposed to Kodak XAR-5 film at -70°C for the appropriate time. The densities of bands corresponding to mRNA samples of interest were determined with a computer program for gel quantification using a Quantimet 970 image analysis system. Autoradiogram exposure times were chosen so that the optical densities of all bands were within the linear response range of the x-ray film as determined by the computer program. Blots were then prepared for subsequent hybridizations with additional cRNA probes. Blots were hybridized serially with probes directed against PPE, 1B15, PPT, and actin mRNAs.

Quantification of Northern blots. Equal amounts of RNA (as determined by measurement of optical density at 260 nm) were loaded onto each lane of the gels. In order to quantitate more accurately the amount of RNA actually present in each lane, Northern blots were rehybridized to the neutral probe, 1B15 (Schnitzer and Schachner, 1982; Sutcliffe et al., 1983) and were analyzed by densitometry. Normalization of PPE and PPT mRNA levels for the amount of RNA loaded was accomplished by taking the ratio of PPE or PPT mRNA to 1B15 mRNA for each sample.

Biochemical measurement. Protein contents were determined by the method of Lowry et al. (1951). Substance P was measured by radioimmunoassay as previously described (Kessler et al., 1981). Characterization of the antisera indicated less than 0.01% cross-reactivity with any other peptides tested.

Statistics. Statistical significance was determined using a 2-tailed paired Student's *t*-test.

Drugs. Naltrexone pellets were generously supplied by NIDA.

Results

Naltrexone-induced alterations of preproenkephalin mRNA levels

To determine whether PPE mRNA levels were altered after chronic naltrexone treatment, mRNA samples from brain regions of control and naltrexone-treated rats were examined by Northern blot analysis (Fig. 1, Table 1). Equal amounts of RNA (as determined by optical density at 260 nm) were loaded in each lane of the gels. Autoradiograms of representative blots from several separate experiments were analyzed by densitome-

Figure 2. Time course of naltrexone-induced alterations in striatal PPE message. mRNA was prepared and quantitated as described under Materials and Methods and the legend to Figure 1, A, B. Scale bars represent the mean $\pm$ SEM from 6 independent experiments (each experiment = 1 animal examined independently; **p* < 0.01).

try to compare mRNA levels quantitatively. Northern blot analysis of the PPE mRNA revealed a single band $\approx$ 1.5 kb for all conditions and brain regions; that for PPT mRNA was a single band at $\approx$ 1.7 kb. Normalization of PPE and PPT mRNA content for possible variation in the amount of RNA applied to the gel was accomplished by taking the ratio of the optical density reading for the band corresponding to PPE or PPT mRNA to that for 1B15 mRNA for each sample (Table 1), as described under Materials and Methods. Autoradiograms of representative Northern blots of 1B15 mRNA for various brain regions are shown in Figure 1B. Ratio values ranged from a high of 2.56 for striatum to a low of 0.21 for frontal cortex for PPE message. Thus, striatum expresses $\approx$ 12 times greater PPE message than that of hippocampus and frontal cortex. Ratios for PPT mRNA ranged from a high of 2.83 in striatum to a low of 0.18 in frontal cortex. Again, striatum expresses 16 times greater PPT message than frontal cortex and hippocampus. Moderate levels of both PPE and PPT message are expressed in the hypothalamus.

Ratio values from each brain region from drug-treated and control animals were compared to calculate changes in mRNA levels. Naltrexone treatment for 8 d produced a 12-fold increase in striatal PPE mRNA levels relative to control or saline-treated animals (Fig. 1, A, C). Although there was individual animal

Table 2. Substance P content in brain regions from control and chronic naltrexone-treated animals

Brain region	Substance P (pmol/mg protein)		
	Control	Treated	% Change
Striatum	0.08 (6)	0.26 (5)	+225% ^b
Hypothalamus	8.98 (6)	7.20 (5)	-20%
Substantia nigra	21.37 (4)	25.08 (3)	+17%
Hippocampus	ND ^a	ND	—
Frontal cortex	ND	ND	—

Rats were treated with either naltrexone or saline for 8 d. Brains from control and chronic naltrexone-treated rats were dissected into various brain regions, and radioimmunoassay was performed as described in Materials and Methods. Values are means from the number of animals indicated in parentheses.

^a ND, not detected.

^b Statistically significant difference (2-tailed *t*-test, *p* < 0.01).

Figure 3. *A*, Northern blot analysis of PPT mRNA from control (C) and chronic (8 d) naltrexone-treated (N) rats. A representative autoradiogram of a 72-hr exposure at -70°C is shown. Total RNA was prepared from dissected rat brain as described under Materials and Methods. Ten micrograms of total RNA were loaded onto each lane. Northern blots were hybridized and quantitated as described under Materials and Methods. *B*, Alterations in levels of PPT mRNA in response to chronic naltrexone treatment. Ratio values of PPT to 1B15 mRNA (Table 1) from each brain region from control animals were compared to those of naltrexone-treated animals to calculate changes in mRNA levels. Alterations are expressed as percentage of control values. Scale bars represent the mean $\pm$ SEM from 5 experiments (each experiment = 1 animal examined independently; $*p < 0.01$). STR., striatum; F.C., frontal cortex; HYPO., hypothalamus; HIPPO., hippocampus.

variability in the magnitude of the effect, increases in the range of 9–14-fold relative to control values were found in 10 independent replications (1 replication = 1 animal examined independently) of the experiment. No significant changes in the level of actin or 1B15 mRNA were apparent in the striatum following antagonist treatment (Fig. 1*B*). Small increases in PPE content also were observed in the hippocampus (+40%) and hypothalamus (+19%) of naltrexone-treated rats relative to that in the corresponding brain regions of control animals. No significant change was observed in PPE mRNA content in the frontal cortex. These results are summarized in Figure 1, *A*, *C*.

We next investigated the time course of the increase in PPE mRNA levels in striatal tissue. RNA was isolated from rats treated with naltrexone for 0, 1, 4, or 8 d. A 6-fold increase in striatal PPE mRNA content was observed as early as 24 hr after naltrexone treatment was initiated. A 12-fold increase in striatal PPE message was reached following 8 d of antagonist treatment (Fig. 2).

Naltrexone-induced alterations in preprotachykinin mRNA

Are other peptide systems affected after chronic opiate receptor blockade? To answer this question, Northern blots were rehybridized to a cRNA probe for PPT, the precursor of substance P. The results are illustrated in Figure 3. Chronic naltrexone treatment produced a 6-fold increase in striatal PPT mRNA level relative to that of control tissue. A 66% increase occurred in the frontal cortex. Conversely, PPT mRNA levels in the hippocampus and hypothalamus were slightly decreased (–75% and –58%, respectively) after naltrexone treatment. These data suggest that the opioid system is involved in the regulation of substance P synthesis.

mRNA changes are accompanied by alterations in the levels of the encoded neuropeptide

Previous studies have demonstrated that methionine-enkephalin content in the striatum is increased by long-term blockade

of opioid receptors (Tempel et al., 1984). To determine if substance P content also is altered, the content of peptide in brain regions from control and chronic naltrexone-treated rats was examined.

Chronic naltrexone treatment induced more than a 3-fold increase in striatal substance P levels relative to that of control tissue. This increase is consistent with the 5-fold increase in mRNA levels mentioned previously. No significant changes were observed in any other brain region examined. These results are summarized in Table 2.

Discussion

The present study examined the changes in peptides and peptide mRNA caused by long-term blockade of brain opioid receptors. Specifically, substance P content and PPE and PPT mRNA levels were examined in brain regions from rats treated chronically with the opiate antagonist naltrexone. The results of our study demonstrate that chronic blockade of opioid receptors leads to a stimulation of proenkephalin and protachykinin synthesis.

Several observations indicate that enkephalin biosynthesis in the striatum is regulated by opioid receptors. Naltrexone treatment stimulates methionine-enkephalin content in the striatum (Tempel et al., 1984). In the present study, chronic administration of the opioid antagonist naltrexone was shown to cause a rapid increase in PPE mRNA (6-fold increase in 24 hr), which remained elevated with prolonged treatment. Conversely, chronic administration of the opioid agonist morphine reduces striatal levels of PPE mRNA (Uhl et al., 1988). It is not clear whether these changes in opioid peptide content and mRNA levels occur as a result of a direct effect of opioid receptor activity on enkephalinergic neurons in the striatum, or whether indirect mechanisms involving one or more other neurons are involved. Interactions between the enkephalinergic and dopaminergic systems are well documented. For example, a significant number of opioid receptors in the striatum are located on the nigrostriatal dopaminergic terminals (Gardner et al., 1980; Murrin et al., 1980; Eghbali et al., 1987). Furthermore, levels of PPE mRNA and enkephalin in the striatum are regulated by this dopaminergic pathway. Chronic dopamine receptor blockade stimulates levels of striatal methionine-enkephalin and PPE mRNA (Hong et al., 1978a, b; Hanson et al., 1981; Sabol et al., 1983; Tang et al., 1983; Thal et al., 1983; Bannon et al., 1986). Thus, it is possible that modulation of dopamine release plays at least some role in the effects of opioid receptor activation or blockade on striatal enkephalin biosynthesis.

Chronic blockade of opioid receptors also increased levels of PPT mRNA and substance P in the striatum. The nature of the interaction between enkephalinergic and substance P systems has received much attention (for a review, see Cuello, 1983). Electrophysiological evidence that opioids inhibit the release of substance P from slices of the trigeminal nucleus (Jessell and Iversen, 1977) and from cultured sensory neurons (Mudge et al., 1979) led to the proposal that enkephalin released from local interneurons acts presynaptically via axoaxonic synapses on substance P-containing fibers. In support of this idea is the finding of a high density of opioid receptors on primary sensory fibers in the spinal cord (Lamotte et al., 1976; Atweh and Kuhar, 1977, 1983). However, ultrastructural data would indicate that axoaxonic synapses between enkephalin and substance P elements are unlikely (Hunt et al., 1980; Cuello et al., 1982). The direct simultaneous visualization of substance P- and enkepha-

lin-immunoreactive sites by electron microscopy reveals that most of these sites are well separated, although occasionally both may terminate on a common dendrite. Thus, the inhibition of substance P release by enkephalin could occur either postsynaptically or, more likely "parasynaptically," via diffusion of transmitter substance (Cuello, 1983). A similar model of parasynaptic interaction between the enkephalinergic and dopaminergic system in the striatum has been invoked by others (Herkenham and McLean, 1986). The concept of "parasynaptic interaction" has gained additional support from a recent study in which opioid receptors were localized at the ultrastructural level (Hamel and Beaudet, 1987). In opioid receptor-rich striatal patches, only 7% of μ receptors are located at synapses. Of the remaining sites, 46% are located at nonjunctional axodendritic contacts (lacking membrane specializations), and 18% are at nonjunctional axoaxonic contacts. Thus, it is likely that opioid peptides exert their modulatory actions via these nonsynaptic contacts by diffusion beyond the synapse. In the case of opioid receptor blockade by naltrexone, substance P neurons would be disinhibited, thereby increasing substance P release and/or turnover and necessitating increase in production of message and peptide in the striatum. Our findings of increased PPT mRNA and peptide in the striatum would support this hypothesis.

It is of interest that naltrexone stimulates the biosynthesis of two peptides that, in the striatum, are largely localized to different neuronal subpopulations (Penny et al., 1986; Gerfen and Young, 1988). This observation suggests that naltrexone acts on multiple types of neurons. Pharmacological blockade of dopamine receptors or destruction of dopaminergic cells by lesions of the striatonigral pathway decreases the content of substance P and of PPT mRNA in the basal ganglia (Hong et al., 1978a; Hanson et al., 1981; Oblin et al., 1984; Bannon et al., 1986). It is possible that opioid receptors on dopaminergic terminals participate in the regulation of striatal tachykinin metabolism. However, although naltrexone treatment results in similar increases in both PPE mRNA and PPT mRNA, dopamine receptor blockade decreases PPT mRNA while increasing PPE mRNA. Thus, the parallel increases in tachykinin and enkephalin biosynthesis cannot result solely from modulation of dopamine release in the striatum, and complex interactions among multiple neuronal populations must be postulated. Clearly, opioid receptors are involved in the regulation of several transmitter systems in the basal ganglia.

It is interesting that levels of substance P in the striatum increased in parallel with levels of PPE mRNA but that peptide levels in the substantia nigra were unchanged. Several explanations exist for this apparent discrepancy: (1) Almost all substance P in the substantia nigra is localized within nerve terminals of striatal neurons (Gerfen and Young, 1988). The lack of change in peptide levels in these terminals in the face of an apparent increase in perikaryal peptide synthesis suggests either that peptide release or turnover was elevated after naltrexone treatment or that not all PPT mRNA is translated. (2) It is well known that not every striatal cell body projects to the substantia nigra. A substantial proportion of the substance P-synthesizing striatal neurons project to the globus pallidus. Thus, enhanced activity in these neurons could account for some of the elevation in striatal PPT mRNA, without necessarily being reflected in enhanced release from striatonigral terminals. Therefore, the changes in peptide mRNAs after opiate antagonist treatment could be most pronounced in neurons of striatofugal pathways.

Activation of specific opioid receptors may differentially reg-

ulate the release of transmitter in specific brain areas. For example, μ opioids enhance the release of substance P from slices of rat spinal cord, whereas δ opioids reduce peptide release (Jessell and Iversen, 1977). The κ -specific opioid peptide dynorphin and some κ opioids have been shown to potently and selectively inhibit dopamine release from slices of rat striatum, whereas the δ -receptor agonist enkephalin [Leu⁵]enkephalin selectively inhibits acetylcholine release (Mulder et al., 1984). Therefore, delineation of the mechanisms mediating the effects of chronic opioid receptor blockade on striatal peptide biosynthesis will require identification of the specific opioid receptor subtypes that are localized in the appropriate neural circuits.

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