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Identification of MARPP-58, A Morphine- and Cyclic AMP-Regulated Phosphoprotein of 58 kDa, as Tyrosine Hydroxylase: Evidence for Regulation of its Expression by Chronic Morphine in the Rat Locus Coeruleus

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Previously, we have identified a number of morphine- and cyclic AMP-regulated phosphoproteins (MARPPs) in the rat locus coeruleus (LC) and other brain regions. We now show that one of these phosphoproteins, a 58 kDa protein designated MARPP-58, is tyrosine hydroxylase. First, MARPP-58 comigrates with immunolabeled, immunoprecipitated, and purified tyrosine hydroxylase on 1- and 2-dimensional electrophoresis. Second, MARPP-58, immunoprecipitated tyrosine hydroxylase, and purified tyrosine hydroxylase yield identical 1-dimensional phosphopeptide maps. Third, MARPP-58 exhibits a regional and subcellular distribution in brain consistent with tyrosine hydroxylase.

Identification of MARPP-58 as tyrosine hydroxylase made it possible to determine whether increases in MARPP-58 phosphorylation induced by chronic morphine in the LC reported previously are associated with alterations in enzyme activity and expression in this brain region. We show that chronic treatment of rats with morphine increases levels of tyrosine hydroxylase activity, immunoreactivity, and mRNA in the LC. Induction of the enzyme by chronic morphine was blocked by concomitant treatment of rats with the opiate receptor antagonist naltrexone, indicating that morphine produces this effect through the activation of opiate receptors. Consistent with previous observations that the chronic morphine-induced change in MARPP-58 phosphorylation is specific to the LC, changes observed in enzyme activity, immunoreactivity, and mRNA were not observed in a number of other brain regions studied.

The results indicate that chronic morphine regulates the expression of tyrosine hydroxylase specifically in the LC and suggest that such regulation reflects long-term adaptations of LC neurons to chronic morphine at the level of gene expression.

In a previous study, we identified a number of morphine- and cyclic AMP-regulated phosphoproteins (MARPPs) in the locus coeruleus (LC) and other regions of rat brain (Guitart and Nestler, 1989). Among these MARPPs was a 58 kDa protein, with an isoelectric point of about 5.5, designated MARPP-58. The state of phosphorylation of MARPP-58 was decreased by acute morphine and stimulated by acute forskolin and cyclic AMP analogs, in isolated, intact LC nuclei *ex vivo*. The protein was also shown to be an endogenous substrate for cyclic AMP-dependent protein kinase in broken cell preparations of this brain region. In addition, based on interpretations of protein phosphorylation data, it appeared that chronic morphine increased total levels of MARPP-58 in the LC. MARPP-58 was also present at high levels in the neostriatum but was not regulated by chronic morphine in that brain region, and the protein was barely detectable in the other brain regions studied, which included the frontal cortex and dorsal raphe (Guitart and Nestler, 1989).

Based on the molecular weight, isoelectric point, and regional distribution of MARPP-58, we considered the possibility that the protein represents tyrosine hydroxylase. Tyrosine hydroxylase is the rate-limiting enzyme in the biosynthesis of the catecholamine neurotransmitters and is localized in brain to catecholamine nuclei and their projection areas. Activity of the enzyme has been shown to be under the acute regulatory control of nerve impulses and neurotransmitters in adrenal medulla and a number of regions of the peripheral and central nervous systems. Such regulation appears to be mediated in large part through phosphorylation of the enzyme (for review, see Nestler and Greengard, 1984, 1989; Goldstein and Greene, 1987; Zigmond et al., 1989). Tyrosine hydroxylase is now known to be phosphorylated by at least 3 types of protein kinases, including cyclic AMP-dependent protein kinase, calcium/calmodulin-dependent protein kinase II, and protein kinase C, and has also been reported to be phosphorylated by second-messenger-independent protein kinases. These various protein kinases phosphorylate tyrosine hydroxylase on some distinct and some shared serine residues, and phosphorylation of the enzyme by each of

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these protein kinases appears to result in its activation (see Goldstein and Greene, 1987; Griffith and Schulman, 1988; Haycock et al., 1988; Waymire et al., 1988; Zigmond et al., 1989). More long-term regulation of tyrosine hydroxylase, achieved apparently at the level of its expression, has also been demonstrated in response to a variety of pharmacological and environmental stimuli. Thus, chronic stress, chronic synaptic activation, and chronic treatment with reserpine (and similar agents) have been shown to increase levels of enzyme activity, and more recently levels of enzyme immunoreactivity and messenger RNA, in cultured pheochromocytoma cells, adrenal medulla, peripheral sympathetic neurons, and specific regions of the CNS, including the LC (see Discussion). Such acute and chronic regulation of tyrosine hydroxylase is thought to play a critical role in modulating the functional activity of adrenal chromaffin cells and catecholaminergic neuronal systems.

We report here that MARPP-58 is indeed tyrosine hydroxylase based on comigration of the proteins on 1- and 2-dimensional electrophoresis and on immunochemical and peptide mapping criteria. We show further that chronic morphine-induced increases in tyrosine hydroxylase (MARPP-58) phosphorylation in the LC are associated with increases in levels of enzyme activity, immunoreactivity, and mRNA specifically in this brain region. Such regulation of tyrosine hydroxylase expression presumably contributes to the functional adaptations that occur in LC neurons in response to chronic morphine treatment.

Materials and Methods

Chronic in vivo *morphine treatment.* Male Sprague-Dawley rats (initial weight 150–200 gm) were used for this study. One morphine pellet (containing 75 mg of morphine base; National Institute on Drug Abuse) was implanted subcutaneously in rats under light halothane anesthesia daily for 5 consecutive days, and animals were used on day 6. Such treatment provides continuous exposure to morphine and induces states of profound tolerance and dependence in the rats based on behavioral criteria, as well as tolerance and dependence in individual LC neurons based on electrophysiological criteria (see Blasig et al., 1973; Aghajanian, 1978; Rasmussen et al., 1990). Control animals underwent either identical surgery with implantation of placebo pellets or no treatment; no differences were observed between these 2 conditions. In some experiments, rats were treated concomitantly with chronic morphine (as above) plus naltrexone (Dupont) by daily subcutaneous and intraperitoneal injections, conditions shown to block the development of tolerance and dependence in the rats and in individual LC neurons (Nestler et al., 1989a). For some immunoblot experiments, rats received acute morphine treatment by a single subcutaneous injection of morphine sulfate (20 mg/kg; National Institute on Drug Abuse), with rats used 30 min later.

Back-phosphorylation assays. LC nuclei were excised from 0.75-mm-thick coronal cross-sections of brain by obtaining 15 gauge punches with a syringe needle as described (Nestler and Tallman, 1988). Other brain regions were isolated by gross dissection. Brain regions were homogenized (10 mg of wet weight/ml) in 10 mM Tris, 1 mM DTT, 1 mM EDTA, and centrifuged at 150,000 × *g* in a Beckman airfuge for 10 min at 4°C. Resulting supernatants, designated the soluble fraction, were acidified to contain (final concentrations): 20 mM citric acid/0.01% nonidet P-40 (Sigma) with a final pH of 2.8–3.0. Resulting pellets, designated the particulate fraction, were resuspended in the original buffer and acidified as above. The acidified soluble and particulate fractions were then centrifuged at 10,000 × *g* for 15 min at 4°C in a Savant microfuge, and the supernatants of each fraction, referred to as "soluble or particulate acid extracts," were neutralized with 200 mM Na_2HPO_4 (200 μl/ml).

Cyclic AMP-dependent protein phosphorylation was studied by "indirect back-phosphorylation" (Forn and Greengard, 1978; Nestler and Greengard, 1980) with modifications exactly as described by Guitart and Nestler (1989): Briefly, duplicate aliquots (containing 10–20 μg protein) of neutralized acid extracts of LC or other brain regions were back-phosphorylated with purified cyclic AMP-dependent protein kinase (Sigma) and [γ-^{32}P]ATP (30 Ci/mmol; New England Nuclear). Phosphorylation reactions were terminated by the addition of 40 mg crystalline urea plus 7 μl "IEF-stop solution" [final concentrations: 2% nonidet P-40, 2% ampholines (ratio of pH 3–10 to pH 5–7 of 1:1; Biorad), 5% 2-mercaptoethanol]. For experiments involving 1-dimensional electrophoresis, reactions were terminated by the addition of "SDS-stop solution" (final concentrations: 50 mM Tris pH 6.7, 2% SDS, 4% glycerol, 2% 2-mercaptoethanol, and bromophenol blue as a marker). In some experiments, 50–200 ng tyrosine hydroxylase purified from pheochromocytoma cells (Haycock, 1989) was subjected to back-phosphorylation as described above for brain extracts.

It should be pointed out that back-phosphorylation provides a measure of the amount of the dephosphorylated form of individual proteins. In indirect back-phosphorylation, tissue is homogenized at physiological pH, which allows the dephosphorylation of most known phosphoproteins by endogenous protein phosphatases, such that alterations in levels of indirect back-phosphorylation of a given protein tend to reflect equivalent alterations in its total amount (see Nestler and Greengard, 1984; Guitart and Nestler, 1989). Although such interpretations of back-phosphorylation data must be viewed with caution, the number of phosphoproteins whose total amount can be assessed accurately by indirect back-phosphorylation is steadily growing (see Guitart and Nestler, 1989). Nevertheless, interpretations of back-phosphorylation data should be confirmed, whenever possible, directly by immunochemical methods, as carried out in the present study (e.g., compare Figs. 1 and 4).

One- and two-dimensional electrophoresis. Back-phosphorylated samples were subjected to isoelectric focusing in the first dimension in tube gels according to published procedures (see Guitart and Nestler, 1989). The resulting tube gels were then layered across standard SDS–polyacrylamide slab gels, with 7.5% acrylamide/0.3% bisacrylamide in the resolving gels, and then subjected to electrophoresis in the second dimension. Isoelectric focusing resolved proteins with isoelectric points between 4.5 and 7.5 based on isoelectric point standards (BDH, London) and on the pH of the tube gels measured directly with a pH meter after homogenizing 0.5 cm sections of the tube gels in water. In some experiments, tissue samples were analyzed by 1-dimensional SDS–polyacrylamide gel electrophoresis with 7.5% acrylamide/0.3% bisacrylamide in the resolving gels. Resulting 1- and 2-dimensional gels were dried and phosphoproteins detected by autoradiography. ^{32}P incorporation into individual proteins was quantitated by liquid scintillation counting of excised bands. Levels of protein phosphorylation were normalized per punch of LC nuclei, as they contained equivalent amounts of protein, determined by the method of Lowry et al. (1951). Protein phosphorylation in other brain regions was normalized per mg protein.

Immunoprecipitation of tyrosine hydroxylase. In some experiments, tyrosine hydroxylase was immunoprecipitated from back-phosphorylated extracts of LC using an affinity-purified rabbit polyclonal antiserum prepared against enzyme purified from pheochromocytoma cells (Haycock, 1989). Immunoprecipitation was carried out exactly as described for Synapsin I by use of protein A-bearing *Staphylococcus aureus* cells (Nestler and Greengard, 1980). For 1-dimensional electrophoresis, resulting immunoprecipitates were resuspended in 100 μl of SDS-stop solution, boiled for 2 min, and subjected to electrophoresis as described above. For 2-dimensional electrophoresis, immunoprecipitates were resuspended in 20 μl of 2% SDS, boiled for 2 min, and added to a final volume of 80 μl urea and IEF-stop solution. The samples were subjected to electrophoresis as described above, except that 4% Nonidet P-40 was used in the IEF-stop solution. Tyrosine hydroxylase precipitated by this method was found to be specific for the antibody used, as immunoprecipitation reactions carried out in the presence of nonimmune rabbit serum resulted in no detectable precipitation of the enzyme.

Immunolabeling of tyrosine hydroxylase. In some experiments, LC or other brain regions were homogenized (10 mg/ml) in 2% SDS, and SDS-stop solution was added to aliquots of the homogenates (containing 25–75 μg protein). The samples were then subjected to 1-dimensional SDS–polyacrylamide gel electrophoresis with 7.5% acrylamide/0.3% bisacrylamide in the resolving gels. In other experiments, aliquots of soluble fractions of LC (containing 25–75 μg protein) were subjected to 2-dimensional electrophoresis as described above. Proteins in resulting 1- and 2-dimensional gels were transferred electrophoretically to nitrocellulose papers, which were then immunolabeled for tyrosine hydroxylase exactly as described (Nestler et al., 1989b) except that 0.5% non-fat dry milk was included in all immunoblotting buffers. A commercially avail-

able rabbit polyclonal antiserum (1:250; Eugene Tech) and ^{125}I-labeled goat anti-rabbit IgG (500 cpm/μl; New England Nuclear) were used in these experiments. Resulting blots were dried and autoradiographed with the use of intensifying screens (Dupont). Levels of immunolabeling were quantitated by densitometry or by counting excised bands in a gamma counter.

Under the immunoblotting conditions used, levels of tyrosine hydroxylase immunoreactivity were linear over a 3-fold range of tissue concentration. In some experiments, levels of tyrosine hydroxylase immunoreactivity in brain samples were compared to immunoreactivity levels for purified enzyme included in the same gels. These experiments demonstrated that mixtures of brain samples and purified enzyme led to additive levels of immunoreactivity, and enabled quantitation of the absolute concentration of the enzyme in the brain regions studied.

One-dimensional peptide mapping. Identity between MARPP-58 and tyrosine hydroxylase was studied by 1-dimensional peptide mapping analysis as described (see Guitart and Nestler, 1989). Briefly, bands of MARPP-58 and of immunoprecipitated or purified tyrosine hydroxylase were excised from dried 1- or 2-dimensional gels and swelled in 20 mM Tris, pH 6.7, 0.1% SDS for 10 min at room temperature. The gel pieces were then loaded onto standard 1-dimensional SDS–polyacrylamide gels except that the stacking gels were 4 cm long and the resolving gels contained 15% acrylamide/0.6% bisacrylamide. *S. aureus* V-8 protease (1–2.5 μg/lane; Miles) was then loaded on top of the bands and the gels were electrophoresed at 60 V overnight. Resulting gels were dried and autoradiographed.

Tyrosine hydroxylase activity assays. Soluble tyrosine hydroxylase activity was assayed by the coupled decarboxylase assay of Waymire et al. (1971) as modified by Kapatos and Zigmond (1979). Briefly, brain regions isolated from decapitated rats were frozen on dry ice and stored for up to 1 week before further analysis. Frozen tissue was homogenized in 50 mM Tris, pH 6.0, 25 mM NaF, and centrifuged at 40,000 × *g* for 30 min. Homogenization volumes for the different brain regions analyzed were 100 μl for LC pairs and 50× and 3× weight wet, respectively, for neostriatum and cerebellum. Triplicate aliquots (containing 2–50 μg protein) of resulting supernatants were incubated at 37°C for 10–30 min in 0.2 M Tris-acetate buffer at different pHs in the presence of: L-[1-^{14}C]tyrosine (75 μM; specific activity 50–55 Ci/mmol), 0.3 or 3 mM 6-methyl-5,6,7,8-tetrahydropterin-HCl ($6MPH_4$), catalase, dihydropteridine reductase (partially purified from rat liver), and NADH. The L-[1-^{14}C]DOPA generated by the assay was decarboxylated by addition of an excess of L-aromatic amino acid decarboxylase (partially purified from hog kidney) in the presence of (final concentrations) 0.1 M Tris-acetate, pH 6.8, pyridoxal-5′-phosphate, and 3-iodo-L-tyrosine. Finally, $^{14}CO_2$ was trapped in tissue solubilizer and quantitated by liquid scintillation. For LC, tyrosine hydroxylase activity was expressed as pmol $^{14}CO_2$ generated/min/LC pair. Since LC pairs have been found to contain approximately 100 μg protein, this number was used to express enzyme activity in pmol/min/mg protein. For other brain regions, enzyme activity was expressed as pmol/min/mg protein based on protein measurements.

Previous studies have shown that pH and $6MPH_4$ concentration influence tyrosine hydroxylase activity and, in the present study, enzyme activity was assayed under different conditions in order to assess both the degree of activation and total amount of the enzyme. Under basal conditions, the optimal pH for tyrosine hydroxylase activity in the LC is 6.2, while that for the activated enzyme is shifted to a more basic pH (Acheson and Zigmond, 1981). Therefore, in order to improve the detection of changes in the degree of enzyme activation, assays were performed both at pH 6.2 and 6.8. Optimal pHs used for the neostriatum and cerebellum were 5.7 and 6.2, respectively. Activation of the enzyme in the present assay system is generally expressed as an increase in affinity of the enzyme for $6MPH_4$, the enzyme's pterin cofactor. Therefore, tyrosine hydroxylase activity was measured in the LC in the presence of subsaturating concentrations of $6MPH_4$ (0.3 mM) to determine the degree of enzyme activation and in the presence of saturating concentrations of $6MPH_4$ (3 mM) to determine changes in maximal enzyme activity. Similar concentrations of $6MPH_4$ were used for the other brain regions studied.

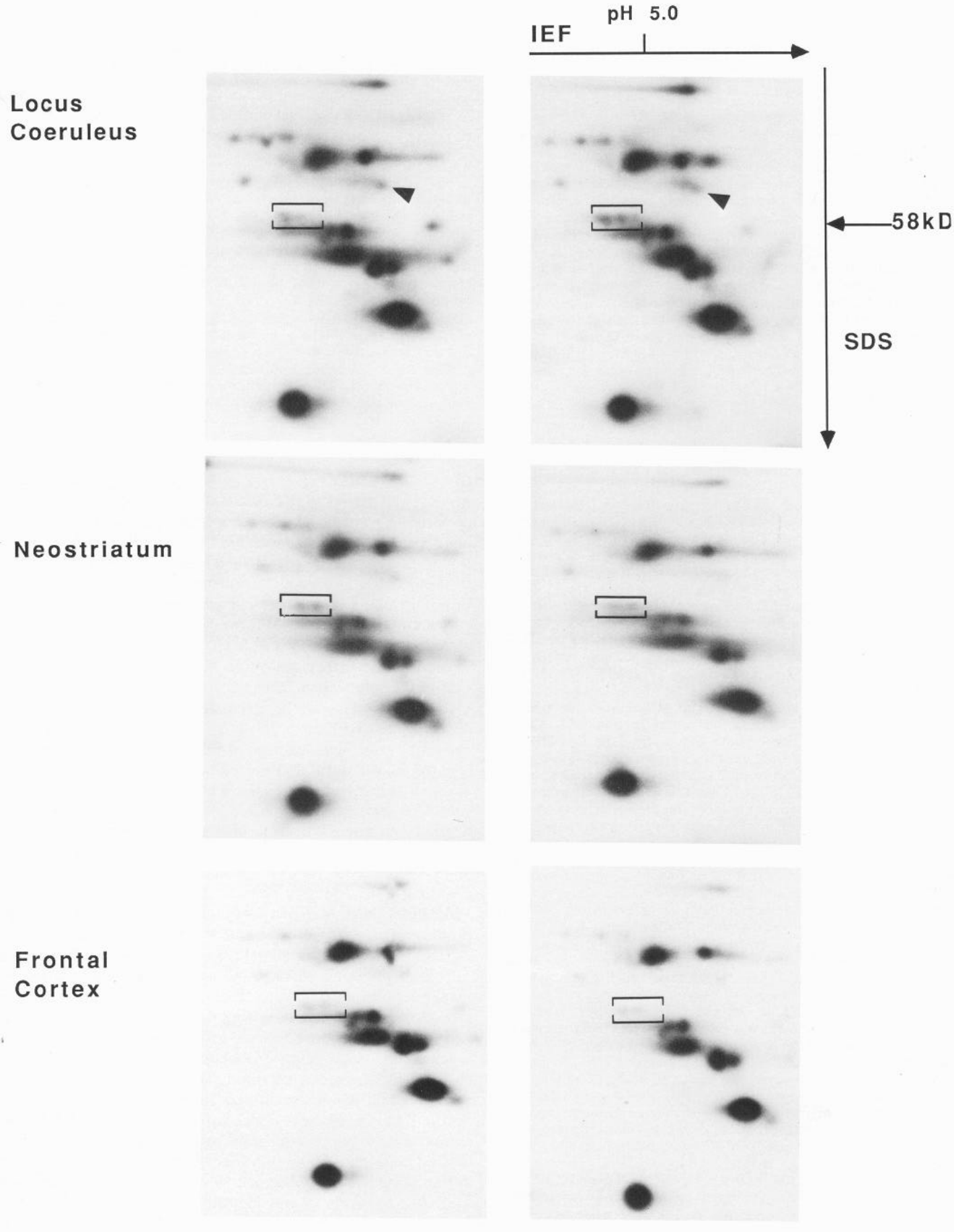
Northern blotting of tyrosine hydroxylase mRNA. Levels of mRNA for tyrosine hydroxylase were quantitated by Northern (hybridization) blot analysis. Total mRNA was extracted from LC using published procedures (Faucon-Biguet et al., 1986; Hayward et al., 1990). Briefly, LC and substantia nigra were isolated from control and morphine-treated rats and frozen at −70°C until further use. LC from 4–5 rats or substantia nigra from 2 rats were pooled and homogenized in 200 μl of 0.2 M Tris, pH 8.8, 25 mM EDTA, 0.1 M LiCl, 1% SDS, 5 μg/ml dextran T40 (Pharmacia). To the homogenates was added 400 μl phenol : chloroform : isoamyl alcohol (25:24:1) and the samples were rehomogenized and centrifuged at 10,000 × *g* for 10 min. The aqueous phase was again extracted with 2 volumes of the phenol : chloroform : isoamyl alcohol mixture and RNA in the final supernatants was precipitated by addition of 2 volumes of ethanol and by cooling the samples at −20°C for 60 min. RNA was recovered by centrifuging the samples in a microfuge, washing the pellets in 70% ethanol, and resuspending the final pellets in 50–100 μl of water. Total RNA generated from the samples (~5 μg) was then subjected to Northern blot analysis exactly as described previously (Berger and Kimmel, 1987; Saito et al., 1989) using a cDNA clone for bovine adrenal medulla tyrosine hydroxylase (D'Mello et al., 1988; provided by Dr. B. Kaplan, University of Pittsburgh). The probe was labeled with ^{32}P by a multiprimer method (Amersham); 1–2 × 10^7 cpm were used per blot. All blots were stripped of tyrosine hydroxylase probe by boiling in water for 5 min and were then rehybridized with a cDNA clone for 18S ribosomal RNA (provided by I. Wool, University of Chicago), also ^{32}P-labeled by the multiprimer method. Quantitation of tyrosine hydroxylase and 18S ribosomal RNA hybridization was carried out by analyzing resulting Northern blots with the Betascope 603 (Betagen); although such quantitation cannot be related to absolute values of mRNA, it is valid and reproducible when data obtained from a single experiment and on the same blot are being analyzed.

Results

Chronic morphine regulation of MARPP-58 phosphorylation. MARPP-58 was identified originally as an acidic 58 kDa phosphoprotein whose back-phosphorylation, i.e., degree of phosphorylation *in vitro,* was increased by chronic morphine in the rat LC (Guitart and Nestler, 1989). MARPP-58 was also present in the neostriatum but not regulated by chronic morphine in this brain region, and the protein was barely detectable in frontal cortex and dorsal raphe. In these earlier studies, crude homogenates of the brain regions were analyzed, making it impossible to determine whether morphine regulation of MARPP-58 occurs in the soluble or particulate fractions of the LC.

In the present study, therefore, we addressed this question by analyzing MARPP-58 back-phosphorylation in crude soluble and crude particulate fractions of the LC and other brain regions isolated from control and morphine-treated rats. As shown in Figure 1, we found that analysis of soluble fractions revealed chronic morphine regulation of MARPP-58 similar to that demonstrated previously for crude homogenates. Thus, chronic morphine increased back-phosphorylation levels of MARPP-58 by 36 ± 7% in the LC (percent change from control ± SEM, N = 6 animals). In contrast, chronic morphine had no effect on MARPP-58 back-phosphorylation in the neostriatum, and the protein was only barely detectable (and showed no morphine regulation) in the frontal cortex (Fig. 1). In contrast to analysis of soluble fractions, MARPP-58 was not detectable in particulate fractions of LC isolated from control or morphine-treated rats (data not shown), indicating that MARPP-58 is predominantly a soluble protein.

Regional distribution of MARPP-58. In earlier studies, MARPP-58 showed a dramatic variation in its levels among the four brain regions examined. In the current study, a more detailed analysis of the regional distribution of MARPP-58 was carried out. A number of brain regions, and some peripheral tissues, were analyzed by indirect back-phosphorylation and 2-dimensional electrophoresis, as described in Materials and Methods. MARPP-58 was identified by autoradiography and its level quantitated by counting excised gel bands by liquid scintillation. Highest levels of MARPP-58 were found in the ventral tegmentum, substantia nigra, neostriatum, nucleus ac-


pH 5.0
IEF
Locus Coeruleus
58kD
SDS
Neostriatum
Frontal Cortex
control
morphine

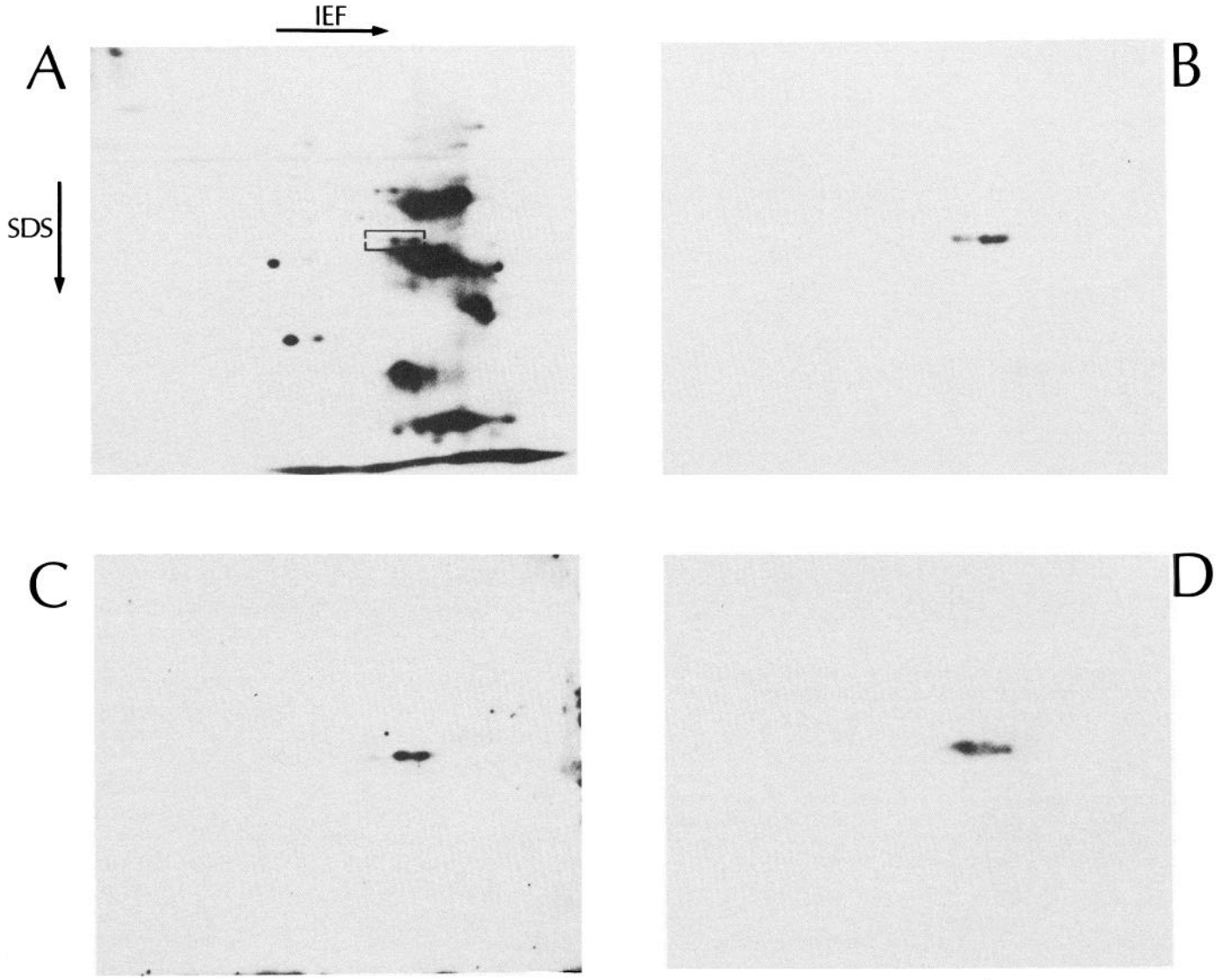


Figure 2. Autoradiograms of 2-dimensional gels showing the comigration of MARPP-58 and tyrosine hydroxylase in the rat LC. *A,* Aliquots of soluble fractions of LC were subjected to back-phosphorylation with purified cyclic AMP-dependent protein kinase and [γ-^{32}P]ATP and to 2-dimensional electrophoresis as described in Materials and Methods. *B,* Aliquots of LC soluble fractions were back-phosphorylated as above, after which tyrosine hydroxylase was immunoprecipitated from the extracts using an affinity-purified rabbit polyclonal antiserum as described in Materials and Methods. Resulting immunoprecipitates were then subjected to 2-dimensional electrophoresis. *C,* Aliquots of LC soluble fractions were subjected to back-phosphorylation as above but with nonradioactively-labeled ATP and subjected to 2-dimensional electrophoresis. Proteins in resulting gels were transferred electrophoretically to nitrocellulose papers, which were then immunolabeled for tyrosine hydroxylase using a commercially available rabbit polyclonal antiserum and ^{125}I-labeled goat anti-rabbit IgG as described in Materials and Methods. *D,* 0.1 μg tyrosine hydroxylase purified from pheochromocytoma cells was back-phosphorylated (as in *A* above) and subjected to 2-dimensional electrophoresis. Autoradiograms from resulting 2-dimensional gels are shown in their entirety. The brackets in gel *A* identify the position of MARPP-58. The figure shows that MARPP-58 comigrates with immunoprecipitated, immunolabeled, and purified tyrosine hydroxylase.

cumbens, ventral pallidum, superior cervical ganglion, and adrenal medulla, with very low levels found in cerebral cortex, cerebellum, hippocampus, hypothalamus, dorsal raphe, and whole pons (gels not shown). Levels of MARPP-58 in the LC were intermediate between those in these 2 groups. In contrast, MARPP-58 was not detectable in liver or kidney. This regional distribution of MARPP-58 parallels very closely the reported levels of tyrosine hydroxylase in these various tissues and brain regions (see Labatut et al., 1988; Weissmann et al., 1989; Haycock, 1989).

Comigration of MARPP-58 and tyrosine hydroxylase on 2-dimensional electrophoresis. The molecular weight (58 kDa),

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Figure 1. Autoradiograms of 2-dimensional gels showing chronic morphine regulation of MARPP-58 in soluble fractions of LC and other regions of rat brain. LC and other brain regions were isolated from control and morphine-treated rats, and aliquots of soluble fractions were subjected to back-phosphorylation and 2-dimensional electrophoresis as described in Materials and Methods. Portions of autoradiograms obtained from resulting gels are shown. *Brackets* indicate the position of MARPP-58. *Arrows* indicate the position of another morphine-regulated phosphoprotein, MRPP-62, present in the LC at much higher levels than in the neostriatum or frontal cortex (Guitart and Nestler, 1989).

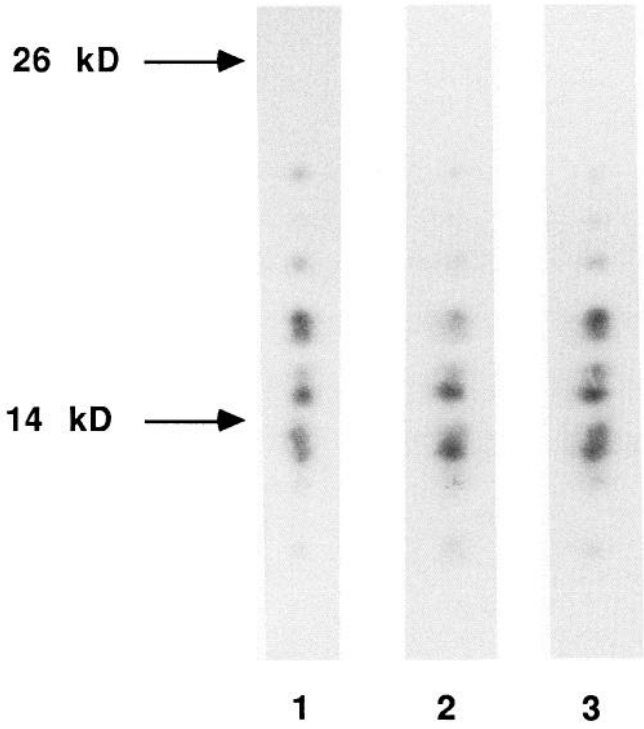

Figure 3. Autoradiograms showing phosphopeptide maps of MARPP-58 and tyrosine hydroxylase. Bands of MARPP-58/tyrosine hydroxylase were excised from 2-dimensional gels such as those shown in Figure 2. *Lane 1,* MARPP-58 from back-phosphorylated LC soluble fractions (i.e., gel A in Fig. 2). *Lane 2,* Tyrosine hydroxylase immunoprecipitated from back-phosphorylated LC soluble fractions (i.e., gel B in Fig. 2). *Lane 3,* Purified tyrosine hydroxylase (i.e., gel D in Fig. 2). The excised bands were then subjected to 1-dimensional peptide mapping analysis using *Staphylococcus aureus* V-8 protease (2.5 μg/lane), as described in Materials and Methods, and to autoradiography.

isoelectric point (~5.5), subcellular localization, and regional distribution of MARPP-58 raised the possibility that it represents tyrosine hydroxylase, known to be an acidic ~60 kDa protein localized to catecholaminergic neurons in the nervous system. The identity of MARPP-58 as tyrosine hydroxylase was tested directly by determining whether the 2 proteins comigrate on 1- and 2-dimensional electrophoresis. It was found that MARPP-58 in back-phosphorylated soluble fractions of the LC comigrates precisely on 1-dimensional gels (not shown) and 2-dimensional gels (Fig. 2, gels A) with (1) ^{32}P-labeled-tyrosine hydroxylase immunoprecipitated from back-phosphorylated soluble fractions of the LC (gel B); (2) immunolabeled tyrosine hydroxylase in crude LC homogenates (gel C) and LC soluble fractions (not shown); and (3) purified tyrosine hydroxylase ^{32}P-labeled by back-phosphorylation (gel D). Precise comigration was confirmed in 2 ways. First, with gels A, B, and D, protein standards were added to the original samples and resulting 2-dimensional gels were stained with Coomassie blue to enable exact alignment of the gels. Second, aliquots of LC soluble fractions that had been back-phosphorylated with [γ-^{32}P]ATP were also immunolabeled for tyrosine hydroxylase; this confirmed that the position of MARPP-58 corresponded exactly to that of the immunolabeled protein (data not shown).

Peptide mapping analysis of MARPP-58 and tyrosine hydroxylase. Further evidence for the identity between MARPP-58 and tyrosine hydroxylase was obtained by subjecting the proteins to 1-dimensional peptide mapping analysis with *S. aureus* V-8 protease, as described in Materials and Methods. Individual protein bands excised from 2-dimensional gels such as those shown in Figure 2 were used in this experiment. As shown in Figure 3, identical phosphopeptide fragments were generated from: MARPP-58 from back-phosphorylated soluble fractions of LC, tyrosine hydroxylase immunoprecipitated from back-phosphorylated LC soluble fractions, and back-phosphorylated, purified tyrosine hydroxylase. Such identity of the phosphopeptide maps of these proteins was observed for several concentrations of the protease (data not shown).

Table 1. Chronic morphine regulation of tyrosine hydroxylase activity in the rat locus coeruleus and neostriatum

	Tyrosine hydroxylase activity (pmol/min/mg protein)	
	Control	Chronic morphine
Locus coeruleus		
Saturating conditions	310 ± 21 (8)	446 ± 24 (8)[a]
Subsaturating conditions	116 ± 10 (8)	171 ± 12 (8)[a]
Neostriatum	2742 ± 139 (7)	2783 ± 184 (7)

Brain regions were isolated from control and chronic morphine-treated rats and assayed for tyrosine hydroxylase activity under maximal and submaximal conditions as described in Materials and Methods. Results of enzyme assays under both conditions are shown for the LC, whereas only those under saturating conditions are shown for the neostriatum. Enzyme activity is expressed as pmol of CO_2 generated/min/mg protein.

[a] $p < 0.05$ by 2-tailed *t*-test.

Chronic morphine regulation of tyrosine hydroxylase activity. Identification of MARPP-58 as tyrosine hydroxylase made it possible to determine whether increases in back-phosphorylation levels of the protein in the LC are associated with increases in enzyme activity in this brain region. It was found that chronic morphine treatment increased levels of maximal tyrosine hydroxylase activity in the LC, but not in the neostriatum (Table 1). Interestingly, the increase in tyrosine hydroxylase activity observed in the LC was approximately the same when the enzyme was assayed under maximal conditions (44% increase) and submaximal conditions (47% increase) (Table 1), suggesting that no apparent activation of tyrosine hydroxylase had occurred in response to chronic morphine. In addition, we found no change in tyrosine hydroxylase activity in the cerebellum, a projection area of the LC (data not shown). These results are consistent with earlier findings that increases in tyrosine hydroxylase levels in the LC in response to a variety of stimuli may not be associated with equivalent increases in LC projection areas (e.g., see Zigmond, 1980; Acheson and Zigmond, 1981).

Chronic morphine regulation of tyrosine hydroxylase immunoreactivity and mRNA. Next, we studied whether the increased levels of tyrosine hydroxylase phosphorylation and activity in the LC in response to chronic morphine treatment are associated with increased expression of the enzyme. As shown in Figure 4, it was found that chronic morphine increased levels of tyrosine hydroxylase immunoreactivity by about 50% in the LC, an effect not observed in the neostriatum or substantia nigra. This effect of chronic morphine was blocked by concomitant administration of the opiate receptor antagonist naltrexone (102 ± 12% of control ± SEM, $N = 6$), indicating that chronic morphine regulation of the enzyme is mediated through opiate receptors. In contrast to chronic morphine, acute morphine failed

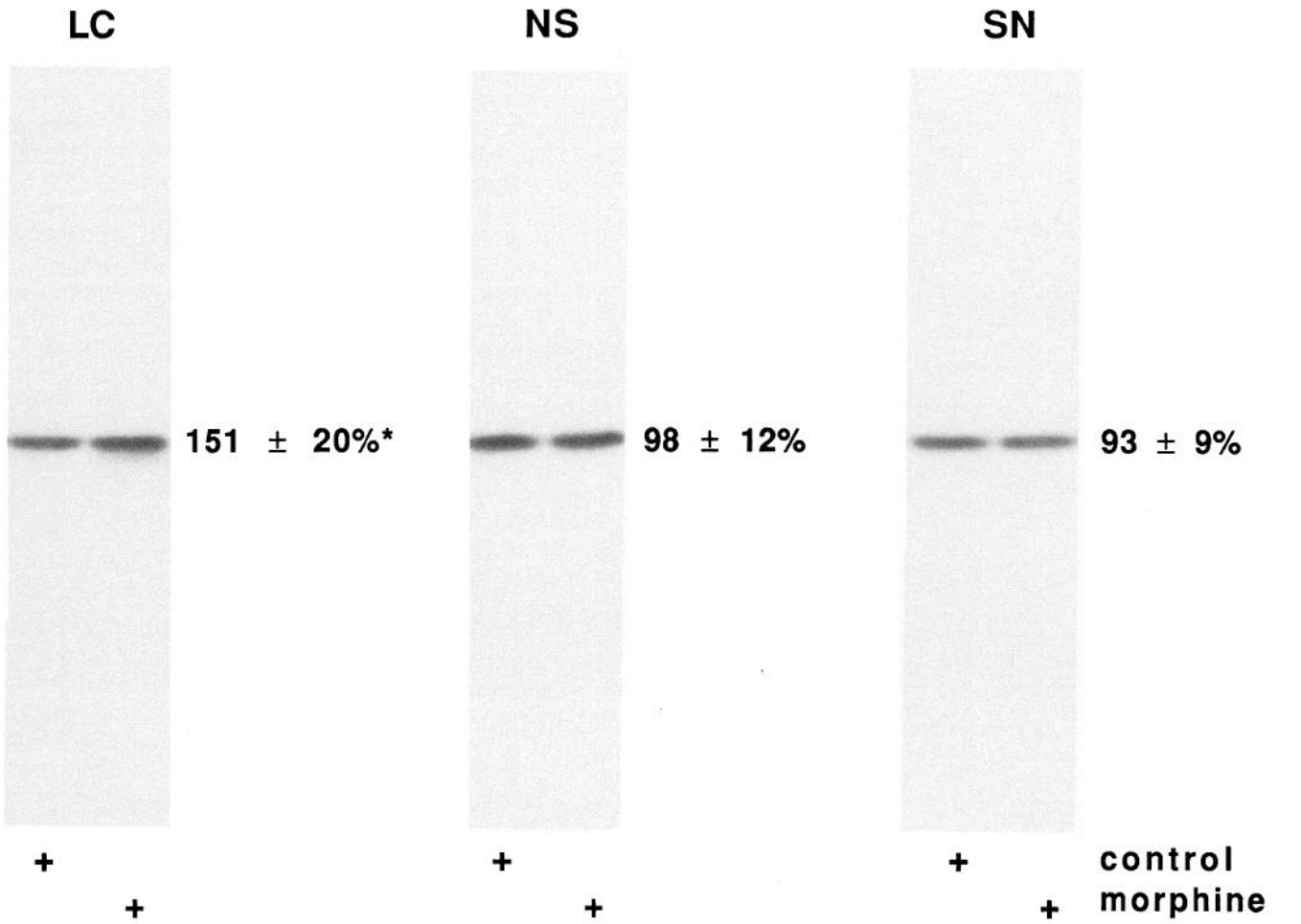

Figure 4. Autoradiograms showing chronic morphine regulation of tyrosine hydroxylase immunoreactivity in the LC and other regions of rat brain. Brain regions were isolated from control and morphine-treated rats, and aliquots of crude homogenates [containing 75 μg of protein for LC and 35 μg of protein for neostriatum (*NS*) and substantia nigra (*SN*)] were subjected to 1-dimensional electrophoresis. Proteins in resulting gels were transferred electrophoretically to nitrocellulose papers, which were then immunolabeled for tyrosine hydroxylase using a commercially available rabbit polyclonal antiserum and ^{125}I-labeled goat anti-rabbit IgG as described in Materials and Methods. Autoradiograms were obtained from resulting blots. Levels of tyrosine hydroxylase immunoreactivity were quantitated by densitometry or by counting excised bands in a gamma counter and are expressed as a percentage of control ± SEM [$N = 6$ (LC and NS) or 3 (SN)]. Based on immunolabeling of purified tyrosine hydroxylase, levels of the enzyme (μg/mg protein) in brain regions from control rats were: LC, 0.6; NS, 2.4; and SN, 1.7. The value determined for the concentration of the enzyme in the neostriatum agrees well with that found previously (Haycock, 1989). *$p < 0.05$ by χ^2 test.

to alter levels of tyrosine hydroxylase immunoreactivity (data not shown).

Analysis of tyrosine hydroxylase mRNA by Northern blotting revealed a predominant mRNA species of about 1.8 kb as reported previously (D'Mello et al., 1988). As shown in Figure 5, it was found that chronic treatment of rats with morphine dramatically increased levels of tyrosine hydroxylase mRNA in the LC. In contrast, no increase in mRNA levels of the enzyme was observed in the substantia nigra, another catecholaminergic nucleus in brain.

Discussion

We have identified previously a number of morphine- and cyclic AMP-regulated phosphoproteins (MARPPs) in the LC and other regions of rat brain (Guitart and Nestler, 1989). Among these proteins was an acidic 58 kDa protein designated MARPP-58. Back-phosphorylation levels of MARPP-58 were shown to be regulated in the LC by acute and chronic morphine treatment. In addition, MARPP-58 appeared to be a physiological substrate for cyclic AMP-dependent protein kinase, inasmuch as forskolin and cyclic AMP analogs increased the phosphorylation state of MARPP-58 in intact LC nuclei *ex vivo* and cyclic AMP increased the endogenous phosphorylation of the protein in broken cell preparations of this brain region. MARPP-58 showed a region-specific distribution in brain. It was present at high levels in neostriatum, but was not regulated by chronic morphine in that region, and it was barely detectable in frontal cortex and dorsal raphe.

Based on the molecular weight, isoelectric point, and regional distribution of MARPP-58, we considered the possibility that it represents tyrosine hydroxylase. In the present report, we demonstrate the identity between the 2 proteins. First, MARPP-58 in crude LC extracts comigrated on 2-dimensional electrophoresis with (1) tyrosine hydroxylase immunoprecipitated from such extracts, (2) tyrosine hydroxylase immunolabeled in such extracts, and (3) phosphorylated, purified tyrosine hydroxylase. Second, MARPP-58 and immunoprecipitated and purified tyrosine hydroxylase yielded identical phosphopeptide fragments

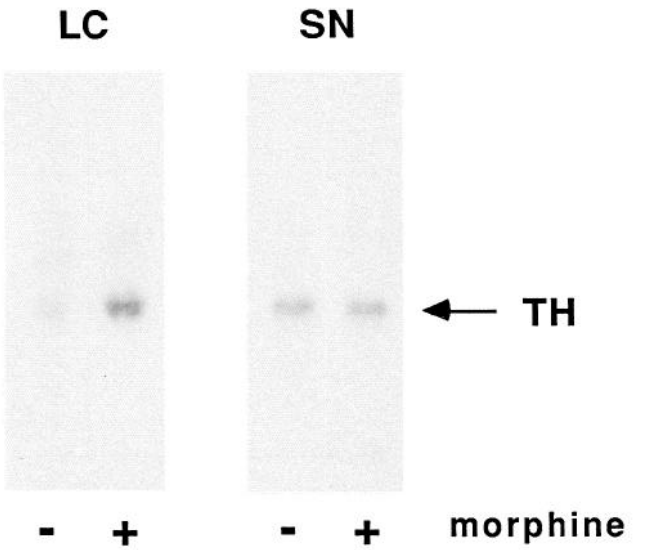

Figure 5. Autoradiograms showing chronic morphine regulation of tyrosine hydroxylase mRNA in the LC and substantia nigra. Total RNA (~5 μg) was extracted from brain regions isolated from control and morphine-treated rats and subjected to electrophoresis in 1% agarose gels. RNA was transferred to nitrocellulose papers, which were then analyzed by Northern blotting using a ^{32}P-labeled cDNA probe for tyrosine hydroxylase. The same blots were reprobed for 18S ribosomal RNA, as described in Materials and Methods, which indicated that each of the lanes shown contains comparable levels of RNA. This figure is representative of results obtained from 3 separate experiments, with each determination made on LC and substantia nigra (*SN*) pooled from 4–5 and 2 rats, respectively.

upon 1-dimensional peptide mapping analysis. Third, chronic morphine regulation of MARPP-58 phosphorylation was reproduced in the present study in soluble fractions of the LC, indicating that MARPP-58, like tyrosine hydroxylase, is predominantly a soluble protein. Finally, a more detailed study of the regional distribution of MARPP-58 was consistent with the known distribution of tyrosine hydroxylase.

In previous studies, we demonstrated that the state of phosphorylation of MARPP-58 is increased acutely in intact LC *ex vivo* by forskolin (which increases neuronal levels of cyclic AMP through activation of adenylate cyclase) and by cyclic AMP analogs, and is decreased by acute exposure to morphine (Guitart and Nestler, 1989). Acute morphine regulation of MARPP-58 was shown to be achieved through the activation of specific opiate receptors, since such regulation is blocked by concomitant exposure to naloxone, an opiate receptor antagonist. Such morphine-induced decreases in the phosphorylation state of the enzyme would be expected to decrease its catalytic activity (see introductory remarks). The results raise the possibility that such decreases in tyrosine hydroxylase activity contribute to the acute actions of morphine in the nervous system. In addition, as the LC contains high levels of endogenous opioids (Simantov et al., 1977; Zamir et al., 1985) that regulate LC neuronal activity *in vivo* (e.g., see Aghajanian, 1978; Abercrombie and Jacobs, 1988), identification of MARPP-58 as tyrosine hydroxylase provides the first direct evidence that the state of phosphorylation of the enzyme may be under the regulatory control of neurotransmitters in the CNS, as has been observed in adrenal medulla, peripheral noradrenergic (sympathetic) neurons, and cultured cells (see Goldstein and Greene, 1987; Waymire et al., 1988; Nestler and Greengard, 1989; Zigmond et al., 1989).

Back-phosphorylation data obtained in previous studies had suggested that, in contrast to the actions of acute morphine, chronic morphine increased the total amount of MARPP-58 in the LC (see Guitart and Nestler, 1989). Identification of MARPP-58 as tyrosine hydroxylase enabled direct confirmation of this hypothesis. We found that chronic treatment of rats with morphine increased levels of tyrosine hydroxylase immunoreactivity and maximal levels of tyrosine hydroxylase catalytic activity in the LC, effects not observed in the other brain regions studied. Chronic morphine also increased levels of tyrosine hydroxylase mRNA in the LC as determined by Northern blotting. These results indicate that chronic morphine regulation of tyrosine hydroxylase occurs at a pretranslational level. One possibility is that chronic morphine increases levels of tyrosine hydroxylase protein and mRNA at the level of gene expression, that is, by increasing the transcription of the tyrosine hydroxylase gene. Another possibility, not incompatible with the first, is that chronic morphine regulates tyrosine hydroxylase by altering the turnover of enzyme mRNA and/or protein.

Previously, chronic morphine had been shown to produce a small (~20%) increase in tyrosine hydroxylase activity in whole cross-sections of rat anterior pons (DiStefano and Brown, 1985). Results of the present study demonstrate that this increase in enzyme activity is associated with increases in enzyme protein and mRNA, and demonstrates that a considerably larger increase (~50%) occurs within the LC. The effect of chronic morphine on tyrosine hydroxylase is not due to nonspecific stress associated with drug treatment, which by itself can induce tyrosine hydroxylase in the LC (Richard et al., 1988). Sham-operated animals showed no increase in enzyme levels and the effect of chronic morphine was blocked completely by concomitant treatment of rats with the opiate receptor antagonist naltrexone.

In addition to an induction of tyrosine hydroxylase in the LC, it was of interest to determine whether chronic morphine altered the state of activation (i.e., state of phosphorylation) of the enzyme. Previous back-phosphorylation experiments suggested that a similar fraction of the total enzyme was phosphorylated in LC from control and morphine-treated rats (Guitart and Nestler, 1989). However, these results must be viewed with caution, since tyrosine hydroxylase is known to be phosphorylated by at least three protein kinases (see introductory remarks), and the back-phosphorylation experiments examined phosphorylation of the enzyme by cyclic AMP-dependent protein kinase only. In the present study, we addressed this issue further by measuring tyrosine hydroxylase catalytic activity under maximal and submaximal conditions, which reflect, respectively, the total amount of the enzyme and the degree of enzyme activation (see Materials and Methods). Similar increases in enzyme activity in response to chronic morphine were observed in the LC under both conditions, suggesting that chronic morphine increased the amount of the enzyme without a change in its degree of activation. Since phosphorylation of tyrosine hydroxylase by cyclic AMP-dependent or calcium/calmodulin-dependent protein kinases or by protein kinase C appears to result in an increase in its degree of activation (see Goldstein and Greene, 1987; Zigmond et al., 1989), the results support the view that the ratio of phosphorylated to dephosphorylated tyrosine hydroxylase, at the phosphorylation sites for each of these protein kinases, is not altered in the LC by chronic morphine. Clearly, the va-

lidity of this interpretation must be studied directly in future studies by use of these other protein kinases in phosphorylation experiments.

One of the central questions raised by this study is: By what mechanism does chronic morphine lead to an induction of tyrosine hydroxylase in the LC? Regulation of tyrosine hydroxylase expression has been studied extensively in adrenal medulla and peripheral sympathetic neurons, where the enzyme is known to be induced at a pre-translational level by drugs that lead to increased synaptic activation of the cells, as well as by chronic stress and glucocorticoids (Thoenen, 1970; Reis et al., 1974; Guidotti and Costa, 1977; Edgar and Thoenen, 1978; Acheson and Zigmond, 1981; Fluharty et al., 1984; Tank et al., 1985; Faucon-Biguet et al., 1986; Richard et al., 1988; Schalling et al., 1989). Similar regulation may occur in the central nervous system. For example, tyrosine hydroxylase expression, reflected in levels of enzyme activity, immunoreactivity, and mRNA, is increased in central noradrenergic nuclei, including the LC, by reserpine (Reis et al., 1974; Faucon-Biguet et al., 1986; Berod et al., 1987; Labatut et al., 1988), which depletes stores of catecholamine neurotransmitters, by 6-hydroxydopamine (Acheson and Zigmond, 1981), which destroys noradrenergic nerve terminals, and by chronic stress (Zigmond et al., 1974; Richard et al., 1988), but the mechanisms involved remain unclear.

One current view is that tyrosine hydroxylase induction in the CNS is regulated to match the functional activity of the catecholaminergic neurons, similar to adrenal chromaffin cells and sympathetic neurons, where increased synaptic activation of the cells induces the enzyme at least in part through increased levels of cellular cyclic AMP (see Guidotti and Costa, 1977; Zigmond, 1980). Recent evidence, obtained from experiments with pheochromocytoma cells, confirms that cyclic AMP regulation of tyrosine hydroxylase expression occurs at the level of gene transcription (Lewis et al., 1987). Although there is no direct evidence that synaptic activity regulates tyrosine hydroxylase expression in central neurons, indirect evidence supports this possibility. Thus, chronic stress activates LC neurons (Abercrombie and Jacobs, 1987) and increases tyrosine hydroxylase levels (see above), whereas chronic antidepressant treatments depress LC neurons (Huang et al., 1979; Blier and De Montigny, 1985) and decrease tyrosine hydroxylase levels (McMahon et al., 1989), and the effects of chronic stress and antidepressants on the enzyme have been shown to occur at the protein and mRNA levels.

However, regulation of the enzyme by chronic morphine does not fit readily with this scheme. Under the treatment conditions used in the present study, morphine is known to initially inhibit LC neuronal activity (Aghajanian, 1978). LC firing rates then return gradually toward control levels over the next 5 d, but remain slightly inhibited at the time the animals are killed (Aghajanian, 1978; Rasmussen et al., 1990). Upon the removal of the opiate, LC firing rates increase several-fold above control levels. An increase in the excitability of LC neurons appears to contribute to this withdrawal activation (see below and Nestler, 1990). Although these assessments of LC neuronal activity are based on extracellular recordings from anesthetized rats, 2 recent studies have indicated that similar changes occur in awake, behaving rats. First, acute systemic morphine has been shown to decrease LC firing rates in unanesthetized rats (Valentino and Wehby, 1988). This is in marked contrast to the known excitatory actions of acute systemic morphine on LC neurons in awake cats (Abercrombie and Jacobs, 1987, 1988). Second, we have recently examined regulation of the nuclear proto-oncogene c-*fos* in the LC in response to morphine treatments in awake, unanesthetized rats (Hayward et al., 1990). It has been proposed that expression of c-*fos* can be used as a molecular marker of neuronal activity (Sagar et al., 1988). This study demonstrated decreased c-*fos* expression (i.e., presumably decreased neuronal activity) in response to acute and chronic morphine, and increased c-*fos* expression (i.e., presumably increased neuronal activity) in response to opiate withdrawal. Therefore, the increase in tyrosine hydroxylase induction observed in response to chronic morphine in the LC occurs during a period of time when LC neurons are not firing at increased rates. The results raise the novel idea that the induction of tyrosine hydroxylase in the LC in response to chronic morphine does not reflect the actual activity of the neurons per se, but reflects instead an increase in the intrinsic excitability of the neurons, that is, an increase in their responsiveness to afferent synaptic inputs.

Concomitant with tyrosine hydroxylase induction, we have also shown that chronic morphine increases levels of adenylate cyclase (Duman et al., 1988), certain G-proteins ($G_{i\alpha}$ and $G_{o\alpha}$; Nestler et al., 1989a), cyclic AMP-dependent protein kinase (Nestler and Tallman, 1988), and a number of phosphoprotein substrates for the protein kinase (MARPPs; Guitart and Nestler, 1989) in the LC. Since cyclic AMP is known to be excitatory in LC neurons (Wang and Aghajanian, 1987; North et al., 1987), this up-regulation of the cyclic AMP system could contribute to the increased intrinsic excitability of these neurons. Many of these morphine-induced changes appear to involve increases in protein levels, raising the possibility that they are achieved by morphine regulation of gene expression. It is possible that the mechanism underlying the up-regulation of the cyclic AMP system also regulates tyrosine hydroxylase expression. For example, inhibition of the cyclic AMP system, with decreased levels of cyclic AMP and of activated cyclic AMP-dependent protein kinase, induced by acute morphine (see Duman et al., 1988; Beitner et al., 1989) may trigger the subsequent induction of tyrosine hydroxylase and of the various protein constituents of the cyclic AMP system. Alternatively, it may be the up-regulated cyclic AMP system, with increased levels of adenylate cyclase and cyclic AMP-dependent protein kinase, that triggers the induction of tyrosine hydroxylase. This scheme would fit with what is known about tyrosine hydroxylase induction in peripheral tissues and cultured cells (see above). It is also possible that decreases in the phosphorylation of tyrosine hydroxylase per se, induced by acute morphine in the LC (Guitart and Nestler, 1989), which would be expected to decrease activity of the enzyme, signals the neurons to increase their synthesis of new enzyme. In any of these schemes, the induction of tyrosine hydroxylase, like the up-regulation of the cyclic AMP system, would represent a homeostatic mechanism by which LC neurons overcome persistent opiate inhibition of the cells. Further work is needed to clarify the mechanisms underlying the chronic morphine regulation of tyrosine hydroxylase in the LC. Such studies will improve our understanding, at the molecular level, of the processes by which acute and chronic morphine regulates the functional activity of its target neurons and leads to addictive phenomena.

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