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Morphine treatment selectively regulates expression of rat pituitary POMC and the prohormone convertases PC1/3 and PC2

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ABSTRACT

The prohormone convertases, PC1/3 and PC2 are thought to be responsible for the activation of many prohormones through processing including the endogenous opioid peptides. We propose that maintenance of hormonal homeostasis can be achieved, in part, via alterations in levels of these enzymes that control the ratio of active hormone to prohormone. In order to test the hypothesis that exogenous opioids regulate the endogenous opioid system and the enzymes responsible for their biosynthesis, we studied the effect of short-term morphine or naltrexone treatment on pituitary PC1/3 and PC2 as well as on the level of pro-opiomelanocortin (POMC), the precursor gene for the biosynthesis of the endogenous opioid peptide, β-endorphin. Using ribonuclease protection assays, we observed that morphine down-regulated and naltrexone up-regulated rat pituitary PC1/3 and PC2 mRNA. Immunofluorescence and Western blot analysis confirmed that the protein levels changed in parallel with the changes in mRNA levels and were accompanied by changes in the levels of phosphorylated cyclic-AMP response element binding protein. We propose that the alterations of the prohormone processing system may be a compensatory mechanism in response to an exogenous opioid ligand whereby the organism tries to restore its homeostatic hormonal milieu following exposure to the opioid, possibly by regulating the levels of multiple endogenous opioid peptides and other neuropeptides in concert.

1. Introduction

Drug addiction is now viewed as a brain disease [22], rather than merely a social problem, as previously thought. Consequently, there has been an attempt to understand which neurochemical systems are altered by drug use. Although much research has focused on opioid use altering various neurotransmitter systems, other evidence has found abnormalities in neuroendocrine systems, such as corticotrophin-releasing hormone (CRH), pro-opiomelanocortin (POMC) and dynorphin in animals and humans exposed to drugs of abuse [14,37,47,52]. Although several studies have shown that exogenous opioid administration down-regulates both brain POMC mRNA and plasma β-endorphin levels [4,14,18], other studies using different opioid paradigms have been unable to reproduce this finding [53,54]. However, little is known if the changes in neurohormone(s) with drug intake are isolated or is a part of an altered hormonal environment, which may account for some of the pharmacological effects of abused substances.

Most prohormones, including precursors of opioid peptides, are synthesized in an inactive form and are converted to the biologically active hormone by cleavage at paired basic residues. PC1/3[3] and PC2, two members of the family of prohormone convertases (PCs), are located primarily in neuroendocrine tissues [8,16,43,44] and can activate many neurohormones [42]. PC1/3

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[3] At the 6th Gordon Research Conference on Proprotein Processing, Trafficking and Secretion (2004), the leading researchers agreed to use the terminology, PC1/3 to describe the identical PC1 and PC3 prohormone convertase.

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null mice are small due to impaired proGHRH processing and are hyperglycemic due to impaired proinsulin processing [55,56]. PC2 null mice have absent proglucagon processing leading to hypoglycemia and also have impaired proinsulin processing [13]. POMC, proenkephalin and prodynorphin processing is blunted [1,21]. The PCs have been shown to be regulated by several factors, including by dopamine receptor agonists/antagonists [2,3,8], CRH [3], glucose [41], suckling [33], glucocorticoids [8] and thyroid hormone [8]. Our laboratories have extensively studied the regulation of the PCs by cytokines [25], hyperglycemia [31,32], thyroid status [10,23,26,45,46] and fasting/feeding [39]. Opioid withdrawal was associated with increased PC2 protein levels in the midbrain periaqueductal gray matter [34] and we recently demonstrated that short-term morphine exposure down-regulated hypothalamic PC1/3 and PC2 protein levels, while long-term morphine exposure up-regulated expression of these proteins [11].

Indirect experiments demonstrated that expression of PC1/3 is dependent on activation of the cyclic AMP response element binding protein (CREB)/cAMP system [19,20]. Given that these PCs have the potential to process a wide variety of prohormones, alterations in the levels of these enzymes in areas rich in neuropeptides would be expected to change the ratio of active hormone to inactive precursor for many hormonal systems.

Morphine, a plant alkaloid, as well as the endogenous opioid peptide, β-endorphin, bind with high affinity to the mu-opioid receptor [50]. Furthermore, mu-opioid receptor expression has been detected in the pituitary [5,6], an endocrine gland that is the site of hormonal biosynthesis, including the bioactive peptides adrenocorticotropic hormone (ACTH) and β-endorphin from the POMC precursor. Thus, we tested the hypothesis that activation of the opioid receptor by its respective agonist as well as the blockade of the receptor by the opioid antagonist naltrexone, can alter the levels of PC1, PC2, the POMC precursor and potentially alter the amount of active hormones in the pituitary. We also studied the effect of short-term opioid exposure on the level of phosphorylated CREB (P-CREB) as this transcription factor regulates PC expression [19,20].

2. Materials and methods

2.1. Animals

Male Sprague-Dawley (275–300 g) rats, housed in a room with controlled light, temperature and humidity and unrestricted access to food and water. All experimental protocols and animal procedures were performed in compliance with the NIH Guidelines for the Use of Animals in Research and approved by the Institutional Animal Care and Use Committees of Charles Drew University of Medicine & Sciences and Cedars-Sinai Medical Center.

2.2. Animal procedures

Rats were implanted with either a 75 mg morphine or placebo pellet [National Institute on Drug Abuse (NIDA)], or two naltrexone (60 mg total, NIDA) or placebo pellets (NIDA) in the nape of the neck under methoxyflurane anesthesia. Morphine pellets have been used for more than 40 years to study the effects of chronic opioids [51]. We have recently submitted a manuscript comparing the regulation of P-CREB in mouse brain regions by different paradigms of morphine administration and found that morphine pellets have a much more robust effect on P-CREB levels than daily morphine injections (Ren et al., submitted). Six to eight animals per group were used. Previous studies using rat hypothalamus [11] have shown that the stress of surgery/pellet implantation did not affect PC1/3 or PC2 mRNA levels compared to untreated rats (data not shown). Rats were euthanized by cervical decapitation 6 h after implantation for ribonuclease protection assay (RPA) or 24 h later for measurement of ACTH and β-endorphin. Western blot analysis or perfused with paraformaldehyde and used for immunohistochemical studies, as preliminary data demonstrated these times showed optimal regulation by morphine. Pellet implantation and euthanasia occurred at the same time between 1300 and 1500. For RPAs, the whole pituitary was removed from animal, frozen in liquid nitrogen and then stored at −80 °C until analysis. For Western blot analysis, the anterior and neurointermediate lobes of the pituitary were carefully dissected under a dissection microscope, rapidly frozen in liquid nitrogen and then stored at −80 °C.

2.3. In vitro transcription of riboprobes

Rat POMC (exon 3) and furin cDNA fragments subcloned into pBluescript SK-vector (Stratagene, La Jolla, CA) were obtained from Dr. Malcolm Low (University of Oregon) and Dr. Richard Mains (University of Connecticut), respectively. Rat PC1/3 and PC2 cDNA fragments subcloned into pBluescript II SK-vector (Stratagene) were obtained from Dr. Richard Mains. The cDNAs were linearized with the following restriction enzymes and transcribed with T7 polymerase (PC1/3 used T3 polymerase) to yield the following fragments: rPOMC-Ehe1-141 nucleotides (nt) (287–427 of rat POMC); rPC1/3-PstI-306 (nt) (243–548 of rat PC1/3); rPC2-Eag1-361 (nt) (353–713 of rat PC2); rfurin-BalI-179 (nt) (263–441 of rat furin). For the PC1/3 probe, the cDNA was treated with Klenow after linearization to remove the 3′ overhang. After linearization, the cDNA fragment was purified by phenol/chloroform extraction and ethanol precipitation.

In vitro transcription was carried out according to the protocol described in Riboprobe in vitro transcription systems' manual (Promega, Madison, WI). Linearized cDNA (1 μg) was transcribed with 20 units of either T3 or T7 RNA polymerase and ^{32}P-UTP. All probes were gel purified on TBE mini-gels (Novex, San Diego, CA), stored at −20 °C and used within 3 days.

2.4. Ribonuclease protection assay

RPA was used due to its ability to accurately quantify low levels of mRNA. Tissues were homogenized in Trizol reagent (Gibco BRL, Gaithersburg, MD, USA) to prepare mRNA prior to use. Quality of mRNA was checked on a 1% agarose/TBE gel. The tissue POMC, PC1/3, and PC2 and furin mRNA levels were measured by RPA according to the protocol described in the RPAIITM Kit (Ambion, Austin, TX). For each ^{32}P-labeled probe, a control tube with yeast tRNA instead of tissue RNA and a probe only tube were used. Samples were hybridized at 55 °C for 16 h and then treated with 1:100 RNase A/RNase T1 mixture for 30 min at 37 °C and electrophoresed on a 6% denaturing polyacrylamide gel at 60 W for 2 h. Following electrophoresis, the gel were dried and exposed to X-ray film at −80 °C. The POMC, PC1/3, and PC2 and furin mRNA levels were quantified on an AlphaImager 2000 densitometry (Alpha Innotech Corporation, San Leandro, CA) using β-actin as an internal control. The intensity of the signal was found to be linear with the amount of RNA.

2.5. Tissue preparation for immunohistochemistry

Rats (n = 4/group) were anesthetized with an intraperitoneal injection of ketamine (40 mg/kg, Sigma Chemical Co., St. Louis, MO, USA) and xylazine (5 mg/kg, Phoenix Scientific Inc, St. Joseph, MO, USA) and then perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in PBS. Brains were carefully removed and post-fixed in 4% paraformaldehyde overnight at