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DIFFERENTIAL REGULATION OF PROHORMONE CONVERTASE 1/3, PROHORMONE CONVERTASE 2 AND PHOSPHORYLATED CYCLIC-AMP-RESPONSE ELEMENT BINDING PROTEIN BY SHORT-TERM AND LONG-TERM MORPHINE TREATMENT: IMPLICATIONS FOR UNDERSTANDING THE "SWITCH" TO OPIATE ADDICTION

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Abstract—Drug addiction is a state of altered brain reward and self-regulation mediated by both neurotransmitter and hormonal systems. Although an organism's internal system attempts to maintain homeostasis when challenged by exogenous opiates and other drugs of abuse, it eventually fails, resulting in the transition from drug use to drug abuse. We propose that the attempted maintenance of hormonal homeostasis is achieved, in part, through alterations in levels of processing enzymes that control the ratio of active hormone to pro-hormone. Two pro-hormone convertases, PC1/3 and PC2 are believed to be responsible for the activation of many neurohormones and expression of these enzymes is dependent on the presence of a cyclic-AMP response element (CRE) in their promoters. Therefore, we studied the effects of short-term (24-h) and long-term (7-day) morphine treatment on the expression of hypothalamic PC1/3 and PC2 and levels of phosphorylated cyclic-AMP-response element binding protein (P-CREB). While short-term morphine exposure down-regulated, long-term morphine exposure up-regulated P-CREB, PC1/3 and PC2 protein levels in the rat hypothalamus as determined by Western blot analysis. Quantitative immunofluorescence studies confirmed these regulatory actions of morphine in the paraventricular and dorsomedial nucleus of the hypothalamus. Specific radioimmunoassays demonstrated that the increase in PC1/3 and PC2 levels following long-term morphine led to increased TRH biosynthesis as evidence by increased TRH/5.4 kDa C-terminal proTRH-derived peptide ratios in the median eminence. Promoter activity experiments in rat somatomammotrope GH3 cells containing the mu-opioid receptor demonstrated that the CRE(s) in the promoter of PC1/3 and PC2 is required for morphine-induced regulation of PC1/3 and PC2. Our data suggest that the regulation of the prohormone processing system by morphine may lead to alterations in the levels of multiple bioactive hormones and may be a compensatory mechanism whereby the organism tries to restore its homeostatic hormonal milieu. The down-regulation of PC1/3, PC2 and P-CREB by short-term morphine and up-regulation by long-term morphine treatment may be a signal mediating the switch from drug use to drug abuse.

Key words: post-translational processing, opiates, endorphin, TRH, hypothalamus, drug addiction.

As drug addiction is now viewed as a brain disease (Leshner, 1997), there has been an attempt to understand which neurochemical systems are altered by drug use. Abnormalities in neuroendocrine systems, such as corticotrophin-releasing hormone (CRH), pro-opiomelanocortin (POMC) and dynorphin have been reported in animals and humans exposed to drugs of abuse (Garcia de Yebenes and Pelletier, 1993; Spangler et al., 1996; Zhou et al., 1996; Rodriguez de Fonseca et al., 1997). For example, exogenous opiate administration down-regulates both brain POMC mRNA and plasma β-endorphin levels (Ho et al., 1980; Bronstein et al., 1990; Garcia de Yebenes and Pelletier, 1993). However, little is known about whether the changes in neurohormone(s) with drug intake are isolated or part of an altered hormonal environment and if these changes may account for some of the physiological effects of abused substances.

The precursors of opioid peptides, like most prohormones, are synthesized in an inactive form and are converted to the biologically active hormone by cleavage at paired basic residues. Two members of the family of pro-

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Abbreviations: AHiAL, anterolateral nucleus of the amygdala; ATF-1, activation transcription factor-1; CRE, cyclic AMP-response element; CREM, cAMP response element modulator; CRH, corticotrophin-releasing hormone; DMEM, Dulbecco's Minimum Essential Medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IEG, immediate early gene; IOD, integrated optical density; ME, median eminence; NIDA, National Institute of Drug Abuse; PaMP, paraventricular hypothalamic medial area; PaPo, posterior region of the hypothalamic nucleus; PBS, phosphate-buffered saline; PC, prohormone convertase; P-CREB, phosphorylated CREB; POMC, pro-opiomelanocortin; PVN, paraventricular nucleus.

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hormone convertases (PCs), PC1/3 and PC2, are located primarily in neuroendocrine tissues (Seidah et al., 1990, 1991; Hakes et al., 1991; Day et al., 1992) and mediate the processing of most prohormones (Seidah and Chretien, 1994). PC2 null mice lack proglucagon processing leading to hypoglycemia and also have impaired proinsulin processing (Furuta et al., 1997). POMC, proenkephalin and prodynorphin processing is blunted (Berman et al., 2000; Laurent et al., 2004). PC1/3 null mice are small due to impaired proGHRH processing and are hyperglycemic due to impaired proinsulin processing (Zhu et al., 2002a,b). The findings that the knockout mice have processing profiles that are expected from the known biochemistry of PC1/3 and PC2 support the notion that these two enzymes are necessary and sufficient for the cleavage of most prohormones/pro-neuropeptides at paired basic residues. Indirect experiments demonstrated that expression of PC1/3 is dependent on activation of the cyclic-AMP-response element binding protein (CREB)/cAMP system (Jansen et al., 1995; Lamas et al., 1997). Since these PCs have the potential to process a wide variety of prohormones, alterations in the activities of these enzymes in brain areas rich in neuropeptides would be expected to change the ratio of active hormone to inactive precursor for many hormonal systems.

Opiates and other drugs of abuse affect several intracellular messengers. The most well studied of these is CREB, a transcription factor responsive to cAMP, that along with the cAMP response element modulator (CREM) and activation transcription factor-1 (ATF-1), belong to the bZIP superfamily. CREB is activated by phosphorylation at serine-133 by cAMP-dependent protein kinase A (PKA) to become phosphorylated cyclic-AMP-response element binding protein (P-CREB). Changes in the phosphorylation of CREB and other cAMP-dependent transcription factors regulate transcription of genes that are dependent on activation of their cyclic AMP-response elements (CREs) (Guitart et al., 1992; Lane-Ladd et al., 1997). P-CREB is found in most tissues examined, with high levels in the brain (Martin and Kandel, 1996) and the pituitary (Sassone-Corsi, 1995) and is thought to be an important mediator in substance abuse (Blendy and Maldonado, 1998; Nestler, 2001; Chao and Nestler, 2004). In many brain regions such as the locus coeruleus, acutely administered morphine and other mu opioid receptor agonists bind to G_i protein-coupled mu opioid receptors, leading to inhibition of adenylate cyclase followed by a decrease in cAMP-dependent phosphorylation of CREB (Duman et al., 1988; Guitart et al., 1992). In contrast, in some brain regions, chronic opiate administration increases the level of cAMP by activating adenylate cyclase leading to an increase in the levels of P-CREB (Lane-Ladd et al., 1997; Nestler and Aghajanian, 1997). It has been proposed that CRE-mediated transcription serves as a functional marker for neuronal plasticity during opiate use (Shaw-Lutchman et al., 2002). The effect of exogenous opiates on the CREB system in regions involved in biosynthesis of opioids and other neuropeptides, such as the hypothalamus, has only briefly been examined (Shaw-Lutchman et al., 2002).

In humans, most drug users do not become drug-dependent. However other drug users become addicted to drugs of abuse. Koob and Le Moal (1997) postulated that the organism tries to maintain homeostasis when challenged by exogenous drugs such as opiates. However, it eventually fails to adapt to the environmental challenges, thereby the organism faces a new, albeit pathological, set point in which tolerance to the drug occurs. If drug intake ceases, withdrawal symptoms occur, leading to negative affects followed by craving, drug seeking behaviors and compulsive drug use (Leshner, 1997). Chronic drug exposure resulting in “the addicted brain” is likely to lead to an alteration of different brain genes compared with short-term drug exposure. The altered levels of these genes and proteins are likely to be multiple and the sum of changes in expression is likely to be the “switch” that moves the individual into a state of compulsive drug seeking and use (Leshner, 1997). We propose that this “switch to addiction” may be achieved, in part, by a series of gene products that are regulated by opiates via changes in intracellular transcription factors. One example of proteins affected differentially by short-term versus long-term opiate use may be the CRE-dependent processing enzymes that control the ratio of active hormones to prohormones. We tested the hypothesis that opioids alter PC levels and hence change the amount of active hormones via changes in CREB phosphorylation. The hypothalamus was chosen as it is a major site of neuropeptide biosynthesis and has an important role in addiction processes (DiLeone et al., 2003; de Lecea et al., 2006). Morphine, which acts primarily through the mu opioid receptors (Matthes et al., 1996), was chosen to study the effects of short-term and long-term morphine exposure on the levels of P-CREB and the PCs, PC1/3 and PC2.

EXPERIMENTAL PROCEDURES

Animals and treatments

All experimental protocols and animal procedures were approved by the Institutional Animal Care and Use Committee of The Charles Drew University of Medicine & Sciences–UCLA School of Medicine and performed in compliance with the NIH Guidelines for the Use of Animals in Research. The protocol used a minimum number of animals and minimized pain.

Adult male Sprague–Dawley rats (Harlan Teklad; Madison, WI, USA) weighing 200–230 g, were housed in a room with controlled light, temperature and humidity, and unrestricted access to food and water. To assess the effects of morphine on PC1/3, PC2 and P-CREB levels, rats were divided into four groups for both short-term and long-term studies; we used a 24-h morphine pellet regimen to mimic short-term opiate exposure and a 7-day morphine pellet regimen to mimic long-term opiate exposure. After rats were anesthetized with isoflurane (Attane, Minrad Inc., Bethlehem, PA, USA), pellets containing placebo or morphine [75 mg morphine pellets, matching placebo pellets, National Institute of Drug Abuse (NIDA, Bethesda, MD, USA)] were implanted s.c. between their scapulas and remained in place for 24 h. Rats were then killed to determine the effect of short-term morphine exposure on the expression of PC1/3, PC2 and levels of P-CREB (Boundy et al., 1998). To determine the effect of long-term morphine exposure on these parameters, rats were implanted with one placebo or morphine pellet as described above