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Short communication

# Steady-state methadone in rats does not change mRNA levels of corticotropin-releasing factor, its pituitary receptor or proopiomelanocortin

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**Abstract**

Male Fischer rats received either methadone (a long-acting opioid agonist, 10 mg/kg/day) or saline (24 μl/day) subcutaneously by osmotic minipumps for 7 days. Chronic steady-state methadone administration did not alter (a) corticotropin-releasing factor (CRF) mRNA in the hypothalamus, (b) proopiomelanocortin (POMC) and CRF type 1 receptor (CRF-R1) mRNAs in the anterior lobe and neurointermediate/posterior lobe of the pituitary, or (c) circulating levels of corticosterone. No change was found in levels of either POMC mRNA in the hypothalamus and amygdala, or CRF mRNA in the frontal cortex, olfactory bulb and amygdala. These results demonstrate that neither the activity of the hypothalamic-pituitary-adrenal axis, nor the β-endorphin and CRF systems in the brain, are altered by steady-state occupancy of opioid receptors with the long-acting opioid agonist methadone.

*Keywords:* Methadone; POMC (proopiomelanocortin); CRF (corticotropin-releasing factor); CRF type 1 receptor; Solution hybridization; Corticosterone

## 1. Introduction

Corticotropin-releasing factor (CRF) of the hypothalamic paraventricular nucleus is the principal factor promoting proopiomelanocortin (POMC) gene expression and adrenocorticotropic hormone (ACTH)/β-endorphin secretion in rat anterior pituitary. CRF initiates its biological effects through specific CRF receptors which are encoded by two distinct genes: corticotropin-releasing factor type 1 receptor (CRF-R1) and type 2 receptor (CRF-R2). Potter et al. (1994) have reported that CRF-R1 mRNA is localized in the anterior lobe and intermediate lobe of rat pituitary, and that CRF-R1 mRNA expression in the anterior pituitary is associated with ACTH-positive cells. We have recently shown that CRF-R1 mRNA, and in addition to POMC mRNA, in the anterior pituitary is subject to negative feedback control by glucocorticoids (Zhou et al., 1996a). Therefore, glucocorticoids or stress may influence anterior lobe ACTH/β-endorphin release in vivo by several ways, including alterations of CRF gene expression and release, changing CRF receptor responsiveness in the anterior lobe of the pituitary to CRF input, by direct modulation of POMC gene expression, and by affecting the releasable pool of ACTH/β-endorphin.

There is evidence that opioids are important in the control of the hypothalamic-pituitary-adrenal axis. In rats, acute (1 or 2 day) administration of morphine results in increased ACTH or corticosterone secretion, while animals treated on a chronic basis with morphine (3 days or more) show attenuation of the morphine induced ACTH or corticosterone response, with the development of tolerance by 5 days of morphine treatment (Buckingham and Cooper, 1984; Ignar and Kuhn, 1990). In man, opioids exhibit an inhibitory effect on the hypothalamic-pituitary-adrenal axis (McDonald et al., 1959). For instance, basal levels of ACTH and cortisol are significantly disrupted in active heroin addicts: suppression of ACTH and cortisol and abnormal diurnal rhythms of these hormones are found (Kreek, 1973). In contrast, based on many clinical observations, basal levels and the diurnal rhythm of ACTH, β-endorphin and cortisol have been shown to become normalized in moderate to high dose, long-term, methadone

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(a long-acting exogenous opioid)-maintained patients compared to those of healthy volunteer subjects (Kreek, 1973; Kreek et al., 1983, 1984).

In the present study, the effects of chronic steady-state methadone administration by subcutaneous (s.c.) pump infusion on components of the hypothalamic-pituitary-adrenal axis were examined in an animal model to determine if there are modulations of: (1) CRF gene expression in the hypothalamus, (2) CRF-R1 and POMC gene expressions in the anterior pituitary (reflected by their respective mRNA levels) and (3) adrenal gland activity measured by circulating corticosterone levels. The pharmacokinetic profile of methadone differs across species: the half-life of methadone in rats is 70–90 min; in man, it is 24 h (Kreek, 1979). In this study, therefore, we used the steady-state administration of methadone (10 mg/kg per day) achieved by osmotic minipumps to more closely model the blood methadone levels in patients in maintenance pharmacotherapy.

## 2. Materials and methods

Male Fischer rats (190–220 g, Charles River Laboratories, Kingston, NY, USA) were housed individually in a stress-minimized facility with free access to food and water, under a 12/12 h light/dark cycle (lights on from 09:00 h to 21:00 h). After 7 days of adjustment to our facility, osmotic minipumps (Alzet, Model 2001) were implanted subcutaneously in the nape of the neck of the animals under methohexital sodium (Brevital) anesthesia. The minipumps were filled with saline or methadone, 100 mg/ml dissolved in saline. The contents were delivered at a constant rate of 1 μl/h resulting in a dose of methadone of 10 mg/kg per day. 7 days after the minipumps were implanted, rats were lightly anesthetized by brief (15 s) exposure to $CO_2$ and killed by decapitation at 15:00 h. Brains were rapidly removed, and regions to be studied were dissected on ice, homogenized in guanidinium thiocyanate buffer and extracted with acidic phenol and chloroform.

A 760-bp fragment from the rat CRF cDNA (a kind gift from Dr. R.C. Thompson at The University of Michigan) or a 538 base pair fragment from the rat POMC cDNA (a kind gift from Dr. J.L. Roberts at The Mount Sinai Medical Center, New York, NY, USA) was cloned into the polylinker region of the pSP64 plasmid (Promega, Madison, WI, USA) in both the sense and antisense orientations. A portion of the human 18S rRNA gene was inserted into the plasmid pS/E (a pSP65 derivative; a kind gift from Drs. T. Nilsen and P. Maroney at Case Western University, Cleveland, OH, USA). $^{32}$P-labeled cRNA antisense probes and unlabeled RNA sense standards were synthesized using a SP6 transcription system. $^{32}$P-labeled CRF-R1 antisense probe was synthesized using the SP6 polymerase from the pcDNA plasmid, which contains a long 2.5-kb fragment from the rat CRF-R1 cDNA (a kind gift from Dr. W. Vale at The Salk Institute), and its RNA sense standard was synthesized from the opposite orientation using the T7 polymerase from the same pcDNA plasmid. A denaturing agarose gel was used to insure that a single full-length transcript had been synthesized from each plasmid.

The solution hybridization RNase protection-trichloroacetic acid precipitation protocol for POMC, CRF or CRF-R1 has recently been described in detail and documentation of the protected fragment by gel electrophoresis included in these reports (Zhou et al., 1996a,b). A set of sense RNA calibration standards determined with concentration by optical absorbance at 260 nm was used to relate values obtained from experimental samples (brain or pituitary RNA) to specific RNA levels. The optimal hybridization conditions were: 10% formamide and 75°C for CRF-R1, 50% formamide and 75°C for POMC, and no formamide and 75°C for CRF. A new standard curve was generated each time experimental samples were analyzed and all RNA extracts of a particular tissue were assayed for each mRNA as a group on a single day. Total cellular RNA concentrations were measured by hybridization of diluted extracts to a $^{32}$P-labeled probe complementary to 18S rRNA at 75°C.

At the time of decapitation of each rat, trunk blood was collected in tubes placed on ice, spun in a refrigerated centrifuge, and plasma was separated and stored at −20°C for corticosterone measurement by radioimmunoassay (RIA), using a rat corticosterone $^{125}$I kit (ICN Biomedicals, Costa Mesa, CA, USA). All corticosterone values were determined in duplicate in a single assay. The plasma methadone levels were determined by gas liquid chromatography (Borg et al., 1995) at SmithKline Beecham (New York, NY, USA).

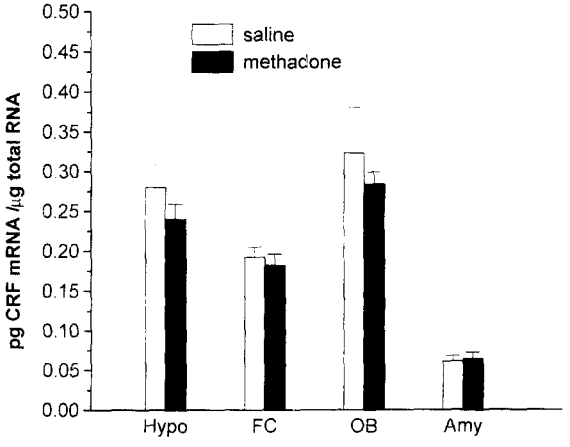


Fig. 1. Effects of treatment with methadone (10 mg/kg per day) or saline (24 μl/rat per day) subcutaneously by osmotic minipumps for 7 days on the corticotropin-releasing factor (CRF) mRNA levels in the brain of the rat. □ = saline, $n = 5–6$; ■ = methadone, $n = 6–7$. Hypo, hypothalamus; OB, olfactory bulb; FC, frontal cortex; Amy, amygdala. Data presented in graphs are the mean ± S.E.M.

Data in graphs are presented as the mean ± S.E.M. An unpaired two-tailed *t*-test between saline control and methadone groups was used to evaluate the statistical significance of differences.

## 3. Results

Chronic methadone administration by pump infusion had no effect on the CRF mRNA levels in the hypothalamus ($F(1,9) = 1.13$, $P = 0.32$) (Fig. 1). CRF in the hypothalamus initiates its neuroendocrine effects of stimulating POMC gene expression through the CRF-R1 receptors in the anterior pituitary. The sensitive solution hybridization assay allowed us to measure both CRF-R1 and POMC mRNAs from the same anterior pituitary extract in each animal. No significant difference was found between the methadone and saline groups in the CRF-R1 mRNA levels in both the anterior lobe ($F(1,11) = 0.19$, $P = 0.67$) and neurointermediate/posterior lobe ($F(1,11) = 0.98$, $P = 0.34$) of the pituitary (Fig. 2A). Chronic administration of methadone also had no significant effect on POMC mRNA in either the anterior lobe ($F(1,11) = 2.91$, $P = 0.12$) or neurointermediate/posterior lobe ($F(1,11) = 0.68$, $P = 0.43$) of the pituitary (Fig. 2B).

The mean plasma methadone level measured on day 7 was 123 ± 7.1 ng/ml (mean ± S.E.M., $n = 6$) in the methadone-treated rats. There were no significant differences in plasma corticosterone levels between the saline (50 ± 13 ng/ml, $n = 5$) and methadone (54 ± 5 ng/ml, $n = 7$) groups ($F(1,10) = 0.10$, $P = 0.76$).

In the central nervous system, the POMC mRNA is present in the hypothalamus (POMC cells in the arcuate nucleus of the medio-basal hypothalamus), and in the amygdala (Civelli et al., 1982; Zhou et al., 1996a). Methadone did not produce any significant change on the POMC mRNA levels in either the hypothalamus ($F(1,11) = 0.06$, $P > 0.8$) or amygdala ($F(1,11) = 0.27$, $P = 0.62$) (Fig. 2B). CRF mRNA levels were also unaltered in the frontal cortex ($F(1,11) = 0.30$, $P = 0.59$), amygdala ($F(1,11) = 0.15$, $P = 0.70$) and olfactory bulb ($F(1,11) = 0.53$, $P = 0.48$) (Fig. 1).

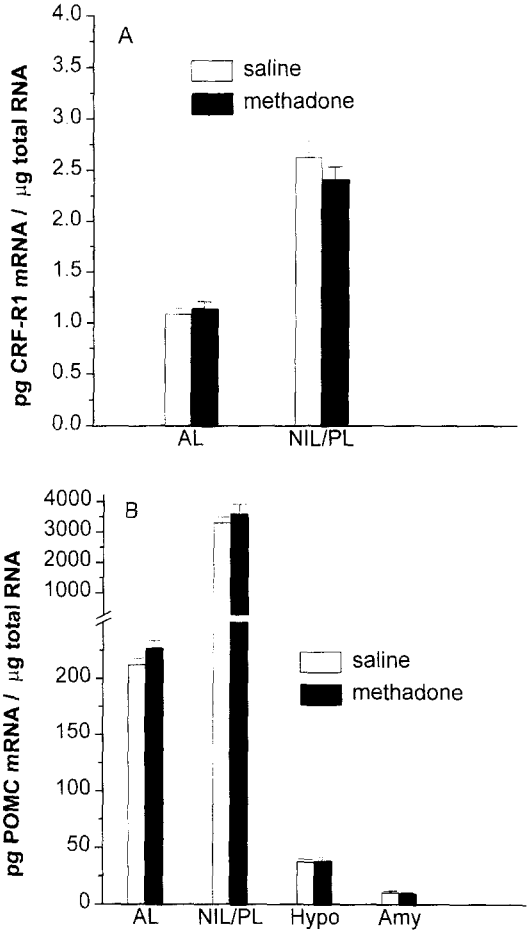


Fig. 2. Effects of treatment with methadone (10 mg/kg per day) or saline (24 µl/rat per day) subcutaneously by osmotic minipumps for 7 days on the CRF-R1 mRNA levels in the pituitary (A) and the proopiomelanocortin (POMC) mRNA levels in the brain and pituitary (B) of the rat. □ = saline, $n = 6$; ■ = methadone, $n = 7$. AL, anterior lobe of the pituitary; NIL/PL, neurointermediate lobe/posterior lobe of the pituitary; Hypo, hypothalamus; Amy, amygdala. Data presented in graphs are the mean ± S.E.M.

## 4. Discussion

In this study, we found that 7 day administration of steady-state methadone did not produce significant changes in any component of the hypothalamic-pituitary-adrenal axis studied: CRF mRNA levels in the hypothalamus, CRF-R1 and POMC mRNA levels in the anterior pituitary, or circulating corticosterone levels.

Despite the complex action of opioids and opiates on the hypothalamic-pituitary-adrenal axis in rats, tolerance (or even suppression) develops to the initial stimulatory effect on the hypothalamic-pituitary activity following long-term treatment with opiates such as morphine in vivo (Buckingham and Cooper, 1984). It has been shown that inhibitory modulation of hypothalamic-pituitary activity by opioids or opiates in vivo appears to occur principally through direct inhibition of CRF release from the hypothalamus (Plotsky, 1986). However, there is also evidence that the ACTH inhibition by opioids is mediated partially via CRF-independent mechanisms. Lamberts et al. (1983) have shown a direct inhibition of ACTH release from the rat pituitary in vitro by the [Met$^5$]enkephalin derivative [D-Ala$^2$,Me Phe$^4$,Met$^5$(*O*)-ol]enkephalin (FK 33824) (but not by β-endorphin), which is not naloxone reversible. These results suggest that opiates and opioids play an important role in hypothalamic-pituitary control of ACTH secretion in rats.

In man, the evidence from published reports is consistent with an inhibitory opioid effect on hypothalamic-pitui-

tary-adrenal function. Based on clinical research in the treatment of heroin addiction, our group and others have found that stress hormone levels of the hypothalamic-pituitary-adrenal axis are profoundly disrupted in active heroin addicts. These disruptions include suppression and abnormal circadian variation of circulating ACTH and cortisol levels, indicating that the long-term administration of short-acting opiates as is the case in heroin addiction, seems to persistently inhibit pituitary-adrenal activity (Kreek, 1973). When opiates are acutely withdrawn, there is an activation of the hypothalamic-pituitary-adrenal activity with increases of both ACTH and cortisol secretion (Kreek, 1973). In contrast, in methadone-maintained patients receiving moderate to high daily doses of methadone (60–120 mg/day) the basal levels and the diurnal rhythms of ACTH and cortisol are normalized and are comparable to those in healthy volunteer subjects (Kreek, 1973; Kreek et al., 1983, 1984), along with the reduction in drug hunger and the prevention of opiate withdrawal symptoms (Dole et al., 1966). In those studies, it has also been shown that the responsivity of hypothalamic-pituitary-adrenal axis is normalized in steady-state chronic methadone-maintained patients: their hypothalamic-pituitary-adrenal axis responses to metyrapone-induced stress appear to be no different from that of healthy volunteer subjects (Kreek, 1973; Kreek et al., 1984). In the current study the functional integrity of the hypothalamic-pituitary-adrenal response to a provocative stimulus in the steady-state methadone-treated rat was not determined.

It has previously been demonstrated that a single daily s.c. administration of methadone (2.5 mg/kg) causes multiple opiate effects, e.g., analgesic effect (Ziring et al., 1981). The steady-state administration of methadone (10 mg/kg per day) by osmotic pumps in this study achieved a mean plasma level of 123 ng/ml with a range from 100–150 ng/ml, which is comparable to levels achieved and sustained at 24 h after last administration during chronic methadone maintenance treatment in man on daily oral doses of 60–120 mg/day, yielding a range of plasma levels of 74–732 ng/ml (Borg et al., 1995). Generally accepted methadone plasma levels for analgesic effect in human is 100–400 ng/ml (SmithKline Beecham Clinical Laboratories Report, New York, NY, USA). We did not find any effect of steady-state methadone treatment on the mRNA levels of hypothalamic CRF and POMC, anterior pituitary CRF-R1 and POMC, or circulating corticosterone levels in the rat model. This demonstrates that steady-state occupancy of $\mu$-opioid receptors with methadone does not have any significant effect on the rat CRF, CRF-R1 or POMC mRNA levels. It is possible that there is an initial alteration of gene expression by an acute administration of an opioid agonist like morphine or methadone. Thus, even if the gene expression had been changed at some earlier time points, it was normalized during chronic steady-state methadone maintenance. Our results support the hypothesis (Kreek, 1973) that there is no disruption of the hypothalamic-pituitary-adrenal activity during steady-state administration of the exogenous opioid methadone.

One earlier study showed that 72 h, but not 24 h, of morphine treatment with a 75 mg s.c. pellet, led to significantly increased $\beta$-endorphin immunoreactivity in rat hypothalamus (Bronstein et al., 1990). Most previous studies on the effects of opioid agonists on the brain and pituitary $\beta$-endorphin content have consistently reported that chronic treatment with opiates or opioids did not change the immunoreactive-$\beta$-endorphin content in the hypothalamus, thalamus, midbrain, amygdala or pituitary of the rat, and that there was no effect on POMC precursor processing in the rat brain. Consistent with an earlier study from our group that chronic (36 day) methadone administration did not alter concentrations of immunoreactive $\beta$-endorphin in the rat amygdala and hypothalamus (Ragavan et al., 1983), no changes in POMC mRNA levels were found in these two regions. However, chronic (36 day) naltrexone administration significantly reduced levels of immunoreactive $\beta$-endorphin in both the amygdala and hypothalamus (Ragavan et al., 1983). In this study, as in our previous two studies (Zhou et al., 1996a,b), measurable levels of POMC mRNA were found in the amygdala, consistent with an early report of Civelli et al. (1982) using Northern blot assay. It is not known whether there are POMC mRNA producing cell bodies in the amygdala or whether this POMC mRNA arrives by axonal transport, possibly from the hypothalamus. In a study from another laboratory, it was reported that POMC mRNA in the hypothalamus tended to decrease after 7 days of morphine treatment by pellets, although significant alterations were noted in only one of two experiments (POMC mRNA was not measured in the pituitary) (Bronstein et al., 1990). Using chronic morphine treatment (60 mg/kg, 12 days) by pump infusion, Lightman and Young (1988) reported that both POMC mRNA in the anterior pituitary and pars intermedia, and CRF mRNA in parvocellular and magnocellular regions of the hypothalamus, measured by in situ hybridization, were unaltered.

In the present study, we used the sensitive and quantitative solution hybridization-RNase protection-trichloroacetic acid precipitation assay to accurately measure mRNA levels. For instance, the sensitivity of POMC mRNA assay was 5 pg (the rat hypothalamus and amygdala contain 1000–1200 pg and 400–500 pg POMC mRNA respectively, absolute but not relative amounts); intra-assay (precision) and inter-assay (reproducibility) coefficients of variation were 3.9% and 5.6% respectively; pituitary POMC mRNA levels for all experimental animals were determined in duplicate in a single assay. The present studies clearly show that chronic methadone administration by steady-state pump infusion has no effect on CRF, CRF-R1 or POMC gene expressions in the rat brain and pituitary, supporting the hypothesis that steady-state methadone administration does not alter the CRF and $\beta$-endorphin systems.

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