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Morphine and Methadone Dependence in the Rat: Withdrawal and Brain Met-Enkephalin Levels

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PIERCE, T. L., G. K. L. TIONG AND J. E. OLLEY. *Morphine and methadone dependence in the rat: Withdrawal and brain met-enkephalin levels.* PHARMACOL BIOCHEM BEHAV **42**(1) 91–96, 1992.—Opioids were administered to female Long Evans rats in their drinking water. Maintenance doses of 0.8 and 0.4 mg/ml for morphine and methadone, respectively, were achieved using an ascending dosage schedule. Rats were decapitated 0, 20, or 60 min after naloxone (10 mg/kg, IP) or saline. Brain met-enkephalin-like immunoreactivity (ME-LI) was determined by radioimmunoassay (RIA). In morphine-drinking animals, ME-LI in all regions of the brain was unaltered following saline administration; however, 20 min after naloxone injection ME-LI had increased in the striatum, hypothalamus, midbrain, and pituitary. By 60 min, ME-LI was no longer elevated. In both methadone- and water-drinking rats, ME-LI did not deviate from normal. These elevated levels of ME-LI, 20 min after naloxone-precipitated withdrawal in morphine-dependent rats, coincided with the peak of behavioural signs in the precipitated withdrawal syndrome. The milder behavioural disturbances observed in the withdrawal of methadone-drinking rats were consistent with the unaltered ME-LI in these animals.

Met-enkephalin Methadone Methadone dependent Morphine Morphine dependent
Naloxone-precipitated withdrawal Rat

CHANGES in mechanisms relating to endogenous opioids have been implicated as responsible for the physical signs of the withdrawal syndrome precipitated by naloxone (19). Despite this, earlier studies on the direction of the change of endogenous opioid levels in morphine-tolerant or dependent rats has given rise to conflicting findings (7,18,19). Factors that may confuse the issue include the duration of opioid exposure, fluctuations between narcosis and withdrawal, stress at the time of brain sampling, and loss of met-enkephalin (ME) due to oxidation during extraction and assay (6,12). Studies on the effects of chronic methadone administration on endogenous opioid levels in the rat have not been carried out.

This study compared both morphine and methadone with respect to their influences on behavioural and biochemical events during physical dependence and withdrawal. To avoid alternating between states of narcosis and withdrawal, both opiates were administered in drinking water (1,9,15). The degree of dependence was estimated by naloxone-precipitated withdrawal (3,8,21). The time course of precipitated abstinence was investigated so behavioural aspects and changes in the endogenous opioid, ME, could be correlated during the withdrawal syndrome. A radioimmunoassay (RIA) that used an antiserum raised against oxidised ME (20) was used to obtain a more accurate indication of ME levels in the rat brain before, during, and after the behavioural effects of the syndrome.

METHOD

Animals

Virgin female Long Evans rats weighing initially 70–100 g were housed four per cage under controlled conditions of temperature (20 ± 2°C) and lighting (lights on 0600–1800 h). Pellet food (Clark King) and drinking fluid, with or without opiate was available ad lib. Fluid intake (assuming 1 g = 1 ml) per cage and weight of individual rats was recorded daily.

Assessment of Dependence (on Morphine and Methadone)

Naloxone HCl (10 mg/kg) or saline were injected intraperitoneally to precipitate a withdrawal syndrome (21) or act as a control, respectively. The degree of physical dependence was assessed by observing the severity of the precipitated withdrawal syndrome. Observations for 60 min recorded rearing, grooming, teeth-chattering, swallowing, ptosis, abnormal posture, diarrhoea, writhing, wet-dog shakes, and jumping. Any other behaviour during the observation period was also noted.

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TABLE 1
ASSESSMENT OF DEPENDENCE

Score	0–10 min	10–20 min	20–30 min	Weight Loss
0	Rearing	Rearing	Rearing	0–2 g
	Grooming	Grooming	Grooming	
1	Rearing	Rearing	Rearing	2–5 g
	Grooming	Grooming	Grooming	
	Swallowing	Swallowing	Swallowing	
	Teeth-chattering	Teeth-chattering	Teeth-chattering	
		Posture	Posture	
			Diarrhoea	
			Writhing	
2	Rearing	Swallowing	Swallowing	5–7 g
	Grooming	Teeth-chattering	Teeth-chattering	
	Swallowing	Posture	Posture	
	Teeth-chattering	Diarrhoea	Diarrhoea	
	Posture	Writhing	Writhing	
		Jumping	Ptosis	
3	Rearing	Swallowing	Swallowing	7–10 g
	Swallowing	Teeth-chattering	Teeth-chattering	
	Teeth-chattering	Posture	Posture	
	Posture	Diarrhoea	Diarrhoea	
	Diarrhoea	Writhing	Writhing	
	Writhing	Ptosis	Ptosis	
	Jumping			

Profile of naloxone-precipitated-withdrawal behaviours indicating the degree of opioid dependence. "Posture" indicates characteristic prostration.

Weight loss was recorded 1 h after naloxone administration. The degree of dependence was then scored (0–3) according to Table 1 using 0- to 30-min data only.

Measurement of ME-Like Immunoreactivity

After decapitation, brains were immediately removed and dissected over ice (10). Six regions (cortex, medulla oblongata, midbrain, striatum, hypothalamus, and pituitary) were separated and rapidly placed into ice-cold 1 M acetic acid (1 : 5 w/v). Tissues were incubated (15 min) in a shaking water bath (80–85°C), homogenised at 4°C, and centrifuged (Clements GS200) 780 × *g* (2000 rpm) for 15 min (4°C). The supernatant was stored in aliquots at −4°C until assay. ME-like immunoreactivity (ME-LI) content of the extracts was measured in duplicate by RIA using an antiserum specific for ME sulphoxide (20). Previous work has shown the antiserum to be specific for the sulphoxide derivative of the peptide. The only enkephalin fragments showing reactivity with the antiserum were Gly-Gly-Phe-Met (0.5%) and oxidised Gly-Gly-Phe-Met (12.5%). Unoxidised ME showed only 3.2% cross-reactivity in the RIA but both ME sulphoxide and ME oxidised with 1% hydrogen peroxide for 20 min were equipotent. Cross-reactivity with leu-enkephalin and the endorphins was less than 0.2%. High-performance liquid chromatography (HPLC) was also used to confirm that immunoreactive fractions eluted with characteristics of ME sulphoxide. Therefore, in this study, samples, tracer, and standard peptide were oxidised with 1% hydrogen peroxide 20 min before assay. Protein content in the pellet was also determined (14) to express ME content as per mg protein.

Experiment 1

An initial experiment was designed to determine if rats would accept morphine- and methadone-treated water and also assess the degree of dependence developed to various concentrations of these opioids. Morphine or methadone was administered via drinking water according to a procedure established for morphine (1). An initial concentration of 0.1 mg/ml morphine or methadone was increased every 48 h to reach a dose of 0.2, 0.4, or 0.8 mg/ml that was maintained for a further 4 days. Control rats drank tapwater.

Experiment 2

Using results obtained from Experiment 1, a single dose of morphine or methadone was chosen to investigate the effect of opiate dependence and withdrawal on regional brain ME levels in the rat.

Drinking water initially contained 0.1 mg/ml morphine or 0.05 mg/ml methadone, the concentration being increased every second day up to maintenance levels of 0.8 or 0.4 mg/ml for morphine (n = 24) or methadone (n = 24), respectively. Control animals drank tapwater (n = 24). These chronic treatments were continued for a further 4 days, giving a total opioid course of 14 days before the acute challenge with naloxone HCl (10 mg/kg, IP) or saline (IP). Animals were decapitated 0, 20, or 60 min after naloxone administration and ME-LI determined.

Drugs

Morphine hydrochloride (Macfarlan Smith), methadone hydrochloride (Burroughs Wellcome) and naloxone hydro-

chloride (Sigma) were calculated as base. ^{125}[I-ME] (Du Pont) and goat antirabbit antiserum (Physiology Dept., Monash University) were used for RIA. All other chemicals were of analytical grade.

Statistics

Weight gain and fluid intake of the treatment groups were analysed by analysis of variance (ANOVA) (Systat). ME-LI data analysis used a three-way ANOVA (3 × 2 × 3 factorial) consisting of three chronic treatment groups, two acute treatments, and three time intervals. Tukey's HSD test was used to determine significance. Assessment of dependence was analysed using Spearman's rank coefficient. Tukey's HSD test was also used to analyse the frequency of individual behaviours.

RESULTS

Naloxone-Precipitated Withdrawal Syndrome

The pattern of behaviour induced by acute administration of naloxone to rats drinking morphine showed a characteristic dose-dependent development. Normal behaviour of rearing and grooming receded, replaced by the bizarre withdrawal behaviours of teeth-chattering, swallowing, diarrhoea, and prostration. Abdominal writhing and jumping were only seen at the higher doses and wet-dog shakes and escape attempts were rarely observed. Behaviours evident in naloxone-treated methadone-drinking rats were characteristic of those seen in animals drinking the lower concentrations of morphine.

Analysis of the frequency of individual behaviours was attempted; however, it was complicated due to expression of the withdrawal syndrome varying considerably between animals. Thus, although statistical significance was reached between water-drinking or saline-treated controls and opioid-drinking rats at the higher doses (0.4 and 0.8 mg/ml) for behaviours such as grooming, swallowing, diarrhoea, writhing, and wet-dog shakes ($p < 0.05$), the variability of their display precluded differentiation between morphine- and methadone-exposed animals except at the 0.2-mg/ml dose of morphine and methadone, where the frequency of teeth-chattering, swallowing, and diarrhoea were found statistically significant ($p < 0.05$). The constellation of behaviours and their timing were more robust than any single indicator.

Observations for 60 min followed the time course that can be exemplified by swallowing (Fig. 1). Although absent initially, swallowing reached a peak 20 min after administration of naloxone, then became negligible by 40 min. Scoring the degree of dependence, therefore, utilised 0- to 30-min observations only. This led to the scoring system shown in Table 1, which has been used to produce the following data.

Dependence and Opioid Concentration in Drinking Water

Using an ascending dosage schedule, rats accepted all opioid solutions in their drinking water. Dependence on opioids (assessed using naloxone-precipitated withdrawal; Table 1) developed following introduction and 4 days of maintenance at doses of 0.2–0.8 mg/ml of morphine or methadone (Experiment 1). As shown in Fig. 2, dependence increased with the concentration of morphine consumed ($p < 0.01$) to a maximal score of 2.25 ± 0.02. The concentration-response curve for dependence following methadone treatment was shallow. The most severe mean dependence score was 1.4 ± 0.3; thus, although behaviour was significantly different from that of water-drinking rats at all dose levels there was no demonstrable relationship between the concentration of methadone consumed and the degree of dependence developed.

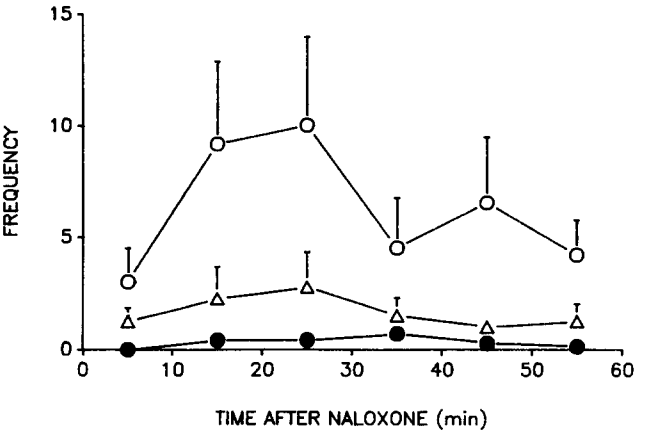

FIG. 1. Time course of naloxone-induced swallowing in rats drinking (●) water, (○) 0.8 mg/ml morphine, or (△) 0.4 mg/ml methadone. Each point represents the mean frequency ± SEM (n = 6–7) of swallowing in 10-min intervals following naloxone 10 mg/kg IP.

Fluid Intake and Weight Gain

The following data are from a second group of rats ingesting the highest tolerable concentrations of morphine (0.8 mg/ml) or methadone (0.4 mg/ml).

The average fluid intake of morphine- or methadone-drinking rats (49.09 ± 1.5 ml or 52.8 ± 1.3 ml, respectively) was less per cage than that of water-drinking (69.24 ± 1.4 ml) rats (Fig. 3). This reduction in fluid intake was not dose related for either opioid. There was significantly more day-to-day variation in fluid intake by opioid- than by water-drinking rats ($p < 0.05$). However, despite these fluctuations in drinking behaviour no animal showed any signs of withdrawal during the treatment period. Weight gain (Fig. 4) was not found

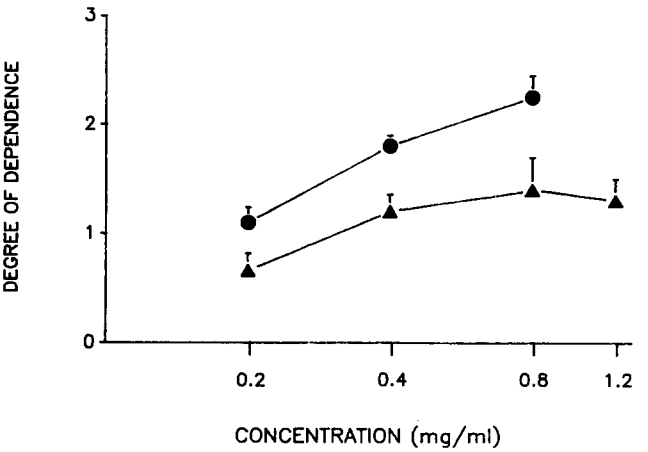

FIG. 2. Influence of opioid load on the degree of dependence of the rat. Degree of dependence was scored by precipitating withdrawal with 10 mg/kg naloxone IP (Table 1) following induction and maintenance for 4 days with (●) morphine or (▲) methadone in the drinking water. Each point represents the mean score ± SEM (n = 6–11).

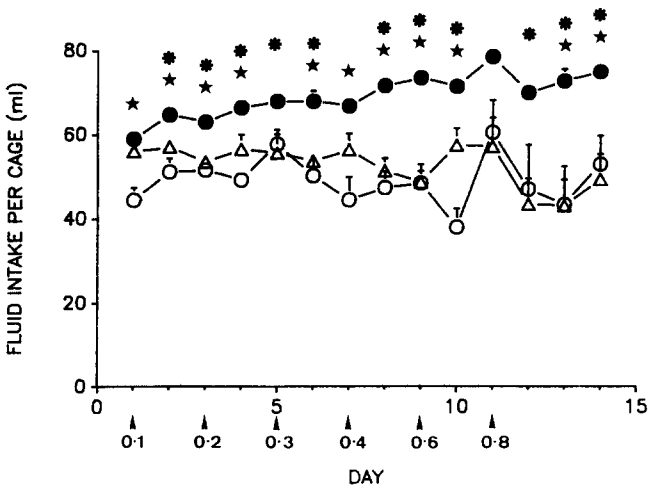

FIG. 3. Fluid intake (ml) of rats drinking (●) water, (○) 0.8 mg/ml morphine, or (△) 0.4 mg/ml methadone. Each point represents the average amount of fluid ± SEM consumed per cage of four rats (n = six cages). The concentration of opioid was increased every 48 h (indicated by arrows) from an initial concentration of 0.1 mg/ml morphine (as shown on graph) and 0.05 mg/ml methadone to doses of 0.8 mg/ml morphine or 0.4 mg/ml methadone and then was maintained for a further 4 days (Experiment 2). Significant differences ($p < 0.05$) from water-drinking animals are indicated for those drinking (★) morphine and (✱) methadone.

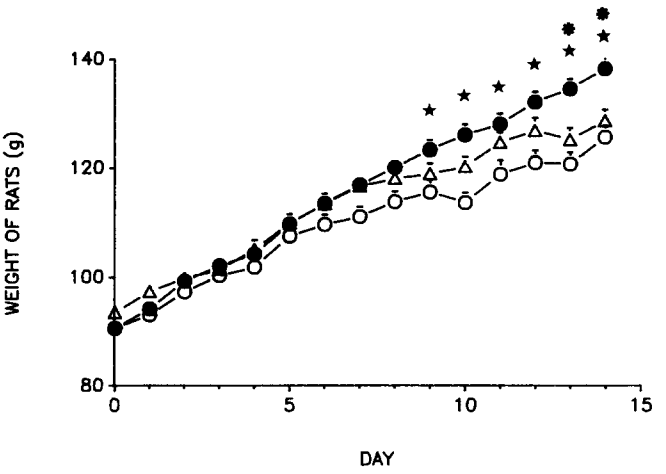

FIG. 4. Growth of rats drinking opioid solutions. Mean weight ± SEM (g) of rats drinking (●) water, (○) morphine, and (△) methadone. Significant differences from water-drinking rats are shown for those drinking (★) morphine and (✱) methadone.

significantly different until day 10. There was a mortality rate of 3.3% in the methadone-drinking rats at doses of 0.8 (2 rats) and 0.4 mg/ml (2 rats). All occurred on the first day of exposure to an increased opioid load. No morphine- or water-drinking animals died.

ME Content of Brain Regions

The intra-assay coefficient of variation for the RIA of ME was 7.81% (n = 6). Regional efficiency of the extraction of ME (n = 4) was as follows: cortex, 46.95 ± 4.03%; medulla, 65.28 ± 1.76%; midbrain, 51.82 ± 0.45%; striatum, 59.15 ± 2.36%; hypothalamus, 72.64 ± 1.82%; and pituitary, 76.04 ± 1.52%. ME content of each region was therefore corrected using these figures and was expressed per mg protein.

Levels of ME-LI (pmol/mg protein), corrected for extraction efficiency, are shown in Fig. 5. No changes in ME-LI were demonstrable in either the cortex or medulla oblongata and so have not been presented. Water-drinking rats, challenged acutely with either saline or naloxone, showed consistent levels of ME-LI at all times. Regional ME-LI for morphine-drinking animals was comparable to that of controls at all times following saline administration, but after acute naloxone treatment a significant elevation of ME-LI was found in the midbrain, striatum, hypothalamus, and pituitary at 20 but not at 0 or 60 min. However, in comparison to water-drinking rats the striatum was the only region to show a significant difference after naloxone at this time interval. Brains from methadone-drinking rats showed no significant disturbance of ME-LI in any region after either saline or naloxone administration.

DISCUSSION

The present study confirmed the results of other workers showing that rats can be induced to drink morphine (1,9,15) and also methadone (15) solutions. However, as has been previously reported (1,15) the volume of fluid consumed was less than that of water-drinking rats and showed a greater daily variation in intake. Taste aversion (5) could be a contributing factor to this occurrence. Nevertheless, rats were found to maintain their weight up until the latter part of the treatment regime and appeared healthy throughout. The sudden deaths of 3.3% of methadone-drinking animals indicated the doses chosen for the biochemical study were the maximum tolerable using this route of administration.

Administration of opioids in the drinking water had many advantages over parenteral methods. In addition to effectiveness and convenience, fluctuations between narcosis and withdrawal were negligible due to continuous access to the opioid solution; second, minimal stress was imposed on animals, allowing the study of biochemical and neurochemical effects unhampered by this factor. However, the limitations of this method were the amount of opioid animals would accept or could tolerate.

In the rat, dependence on morphine has been widely documented (1,3,8,15,21), with animals in this study displaying a characteristic pattern of behavioural signs indicative of opioid withdrawal following naloxone administration. Bizarre behaviours appeared and normal behaviours were reduced during withdrawal with maximal changes occurring at approximately 20 min. In accordance with earlier studies (15), morphine-treated rats displayed more extreme behavioural signs of opioid dependence during naloxone-precipitated withdrawal compared to methadone-maintained animals. This contrasts with many other aspects of morphine and methadone activity. Methadone has been found to be of equal or greater potency to morphine in vitro as a μ-receptor agonist (16), intravenously with respect to its euphoriant effects in nondependent postaddict volunteers (11), in cancer patients for pain relief (4), and in small animals as an antinociceptive agent (2). Ling et al. (13) postulated that the high concentrations necessary to produce physical dependence to methadone cannot be attained by oral dosing due to its low therapeutic index, whereas following administration to rats of high concentrations by prolonged intravenous infusion methadone can induce a

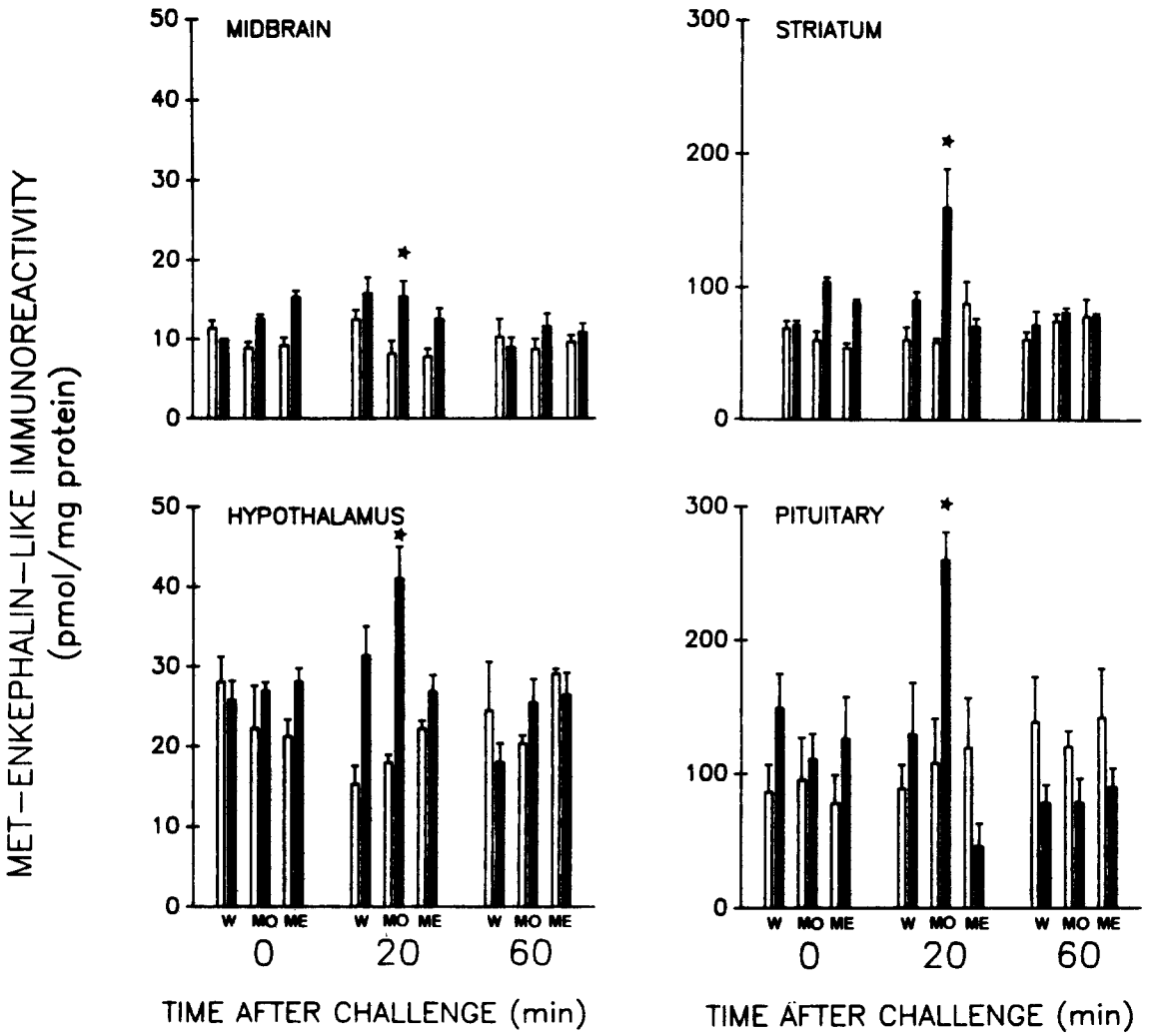

FIG. 5. ME-LI in specific brain regions of (W) water-, (MO) 0.8 mg/ml morphine-, and (ME) 0.4 mg/ml methadone-drinking rats decapitated 0, 20, or 60 min after (□) saline or (■) naloxone (10 mg/kg, IP) challenge. Each bar represents the ME-LI of specific brain regions corrected for extraction efficiency (n = 4). (★) Significant differences ($p < 0.05$) between saline and naloxone challenges.

morphine-like withdrawal syndrome; however, this means of administration is not comparable with methadone use in man.

Enkephalin content within the brain varies significantly between individual regions. It has been found (22) that of the two enkephalins, met- and leu-enkephalin, the former is present in higher concentrations throughout the brain. Furthermore, the striatum was found to contain the highest concentration of this endogenous opioid, which may be a contributing factor to it being the area most affected in this study. Levels of ME-LI in the brain were constant following prolonged exposure to the exogenous opioids, there being no significant variations in any brain region of either control or opioid-dependent rats after saline challenge. This suggests that the endogenous opioid system is able to reestablish a new equilibrium to accommodate the exogenously applied opioid. Such an occurrence is in accordance with earlier work using pellet implantation (18), where chronic morphine administration was found to elicit a transient decline in ME levels after 3 days of exposure but levels returned to normal after 5 days.

However, 20 min after naloxone administration to morphine- but not water- or methadone-drinking rats elevated levels of ME-LI were recorded and these coincided with the peak in behavioural signs of naloxone-precipitated withdrawal. This syndrome was most intense in the morphine-dependent rats, leading to the possibility of a link between the observed behavioural and biochemical events, where the visible signs of opiate withdrawal could be a reflection of gross disturbances of transmitter balance reflected by the marked elevation of peptide levels seen in the midbrain, hypothalamus, striatum, and pituitary. The elevated levels of peptide may indicate increased synthesis or processing, reduced release of transmitter, decreased degradation, or a combination of effects that resulted in an overall increase. To elucidate the possible underlying changes in these dynamic processes, further experiments would be required involving hybridisation of mRNA to assess synthesis and trypsin and carboxypeptidase treatment of extracts to clarify prohormone processing, release, and assessment of the metabolising enzymes. Because

of the rapid response and duration of the syndrome, we would postulate that at least one factor, acute, reduced release of ME-LI, was occurring but the mechanism is unclear.

Other neuropeptides, in particular substance P and calcitonin gene-related peptide, have also been shown to be elevated during naloxone-precipitated withdrawal in opiate-dependent rats (17). Regional changes were characteristic for each peptide yet followed a similar time course to that shown here for ME in morphine-dependent rats. This possibly indicates increased inhibition of neuronal transmitter release on a more generalised basis during withdrawal, the regional patterns varying with the neuronal topography. There were no discernible fluctuations of neuropeptide levels in methadone-treated animals, which indicates a difference from morphine in the biochemical and behavioural effects that is not explicable in the current understanding of their pharmacology in the rat.

The underlying neural mechanisms controlling levels of these peptides are not known, but in view of the temporal similarities a disturbance of striatal/brain stem function could be linked with the bizarre behaviours evident during the withdrawal syndrome. The less intense withdrawal response and lack of overt biochemical disturbance in methadone- as opposed to morphine-dependent rats requires further study. Possible factors involved could be the differences in kinetics at the neuronal level such that the capacity to maintain homeostasis of transmitters is not exceeded in withdrawing methadone-dependent rats. Alternatively, there may be qualitative differences between the changes induced during chronic exposure to morphine and methadone.

In conclusion, this study has demonstrated the potential for administering opioids in drinking water. Subchronic morphine treatment increases endogenous ME-LI levels in midbrain, hypothalamus, striatum, and pituitary of rats withdrawing from morphine but there was no comparable disturbance in methadone-treated animals. Finally, these observations correlate with the intensity and time course of the behavioural signs of withdrawal.

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