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MORPHINE DEPENDENCE AND ENZYME ACTIVITY IN THE HYPOTHALAMUS*

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Abstract Tolerance to and physical dependence on morphine were produced in rats by the implantation of morphine pellets. When both reached peak levels, the rats were sacrificed and malic dehydrogenase, lactic dehydrogenase and glucose-6-phosphate dehydrogenase were measured by sensitive microchemical methods in eight hypothalamic nuclei and in the cortex, cerebellum and liver. In the medial portion of the ventromedial nucleus of the hypothalamus, glucose-6-phosphate dehydrogenase was 20 per cent lower in the morphine-dependent animals than in the controls. This was the only significant change ($P < 0{\cdot}01$) detected for any of the three dehydrogenases in any of the regions or organs examined. The data indicate that chronic morphine administration does not produce a generalized change in the activity of major metabolic pathways in either the brain or liver. The regionally selective effect on glucose-6-phosphate dehydrogenase activity may reflect an involvement of the ventromedial hypothalamus in at least some aspects of the development of tolerance and physical dependence on narcotics.

Previous investigations of the effects of morphine, administered *in vivo* in pharmacologic dose ranges, on brain enzymic activity have indicated little or no influence of the narcotic on brain metabolic activity.[1] Also the addition of morphine, *in vitro*, to homogenates of rat cerebral hemispheres has no effect on a number of enzymes including glucose-6-phosphate dehydrogenase (G-6-PDH) and lactic dehydrogenase (LDH) even at very high drug concentrations.[2,3] In other studies, purified enzyme preparations of cytochrome C reductase alone or in combination with malic dehydrogenase (MDH) were inhibited by morphine, but the minimal effective concentration of the drug was 1×10^{-3} M.[4] This level is approximately 1000-fold greater than estimates of morphine levels in the brains of rats after subcutaneous injection.[5]

The major limitation of the foregoing investigations is that important regionally selective effects of morphine on cerebral enzymic activity are obscured by studying homogenates of whole brain. Moreover, it appears that morphine may act only at very discrete *loci* within the brain in producing its pharmacologic effects. For example, micro-injections of morphine into discrete areas of the hypothalamus result in dose-dependent analgesia and hypothermia, which are the principal effects of systemic morphine administration.[6,7] Discrete sites of the analgesic action of morphine have been localized in the central grey matter surrounding the third ventricle[7] and in structures near the ventricular wall surrounding the fourth ventricle and the aqueduct.[8] Because of these observations, it seems clear that enzymic studies conducted on a whole brain level are likely to obscure potentially important effects at a regional or micro-regional level in brain.

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The present study employing quantitative histochemical techniques was undertaken to examine the effect of chronic morphine administration on a number of brain enzymes. We have examined the effects of chronic morphine treatment on three enzymes representative of major metabolic pathways in brain: the citric acid cycle (MDH), glycolysis (LDH) and the pentose phosphate pathway (G-6-PDH). One enzyme from each metabolic pathway was studied, since levels of the enzymes within a metabolic group are relatively proportional[9,10] and changes in the level of one step may reflect an over-all alteration of the pathway. In the present study, rats were implanted with morphine pellets and their brains were analyzed when both tolerance and physical dependence reached peak levels.[11]

MATERIALS AND METHODS

Animals and treatment. Male Sprague–Dawley rats (165–190 g) were used in these studies. Animals were implanted subcutaneously with either 75 mg morphine pellets or placebo (lactose) pellets, according to procedures more fully described elsewhere.[11] Three days after pellet implantation, the point at which tolerance and physical dependence reach their peak levels, the animals were decapitated and their brains removed. Tissue blocks including hypothalamus, temporal lobes, cerebellum and liver were dissected out within 2 min and immediately frozen in liquid freon cooled by liquid nitrogen. Tissues were stored at −80° until microtomed at −20° in a cryostat.

Quantitative histochemistry. The procedures for obtaining unthawed, unfixed, unstained and histologically defined tissues have been described in detail elsewhere.[12] From 10 μm thick, freeze dried, coronal sections, small samples were dissected by hand at 25–50× magnification from 10 histologically discrete regions of brain and also from the periportal area of liver. The regions of brain studied included layer III of cerebral cortex, the internal granular layer of cerebellum (excluding Purkinje cells), and eight specific hypothalamic nuclei.[13] In the case of the ventromedial nucleus of

TABLE 1. CONDITIONS AND VOLUMES FOR ASSAY OF THE THREE ENZYMES*

Conditions	Enzymes		
	G-6-PDH	LDH	MDH
Assay conditions			
pH	9·3	7·6	8·6
Buffer	AMP_2 (100)	Tris (100)	Tris (100)
Substrate	G-6-P (1·0)	Pyruvate (1·0)	Oxaloacetate (2·5)
Pyridine nucleotide	NADP (0·14)	NADH (1·0)	NADH (2·0)
Nicotinamide		(20)	(18)
Bovine serum albumin (%)	0·05	0·05	0·05
Volumes			
During incubation (μl)	2	5	5
During reading (ml)	0·11	1·01	1·01
Fluorescence measurement			
Exciting light (nm)†	365	365	365
Emitted light (nm)‡	460–480	460–480	460–480

* Numbers in parentheses are concentrations during incubation in m-moles/l. Enzymic ir all enzymes was at 37° for 15 min (LDH and MDH) or 30 min (G-6-PDH).

† Obtained with Corning glass filter No. 5860.

‡ Obtained with Corning glass filters Nos. 5562, 4308 and 3387, in combination.

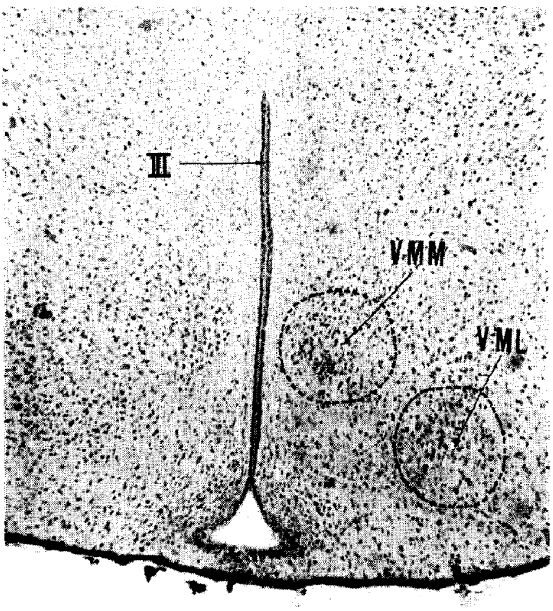

FIG. 1. Frozen section of rat brain hypothalamus (× 35) at level of ventromedial nucleus. Section was air-dried and then stained by the Richardson technique.[15] III, third ventricle; VMM, ventromedial nucleus, *pars medialis*; VML, ventromedial nucleus, *pars lateralis*.

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the hypothalamus, samples were obtained from both the medial and lateral portions as depicted in Fig. 1. The dry weight of the dissected samples ranged from 10 to 30 ng as determined on a quartz-fiber "fish-pole" balance.[14]

Chemical methods. The measurement of MDH, LDH and G-6-PDH was based on the formation of a fluorescent product of the oxidized pyridine nucleotide in a strongly alkaline solution.[16] Incubation conditions and volumes are summarized in Table 1. Assays were performed without knowledge of which animals were morphine dependent.

Statistical methods. Means and standard errors were calculated from 14 to 21 values pooled from the two animals in each group and therefore include both biologic and measurement variability. Prior work indicates that inter-animal variation is minimal so that repeated determinations on a small number of animals seem to have general validity according to Lowry *et al.*[17] The probability levels were determined by the two-tailed *t*-test from the standard error of the difference of means.[18]

RESULTS

The activities of G-6-PDH, MDH and LDH in the eight hypothalamic nuclei examined, and in the cortex, cerebellum and liver are shown in Table 2 for the morphine-dependent and control rats. As shown in this Table, in the medial ventromedial nucleus of the hypothalamus, G-6-PDH activity was 20 per cent lower in the morphine-dependent animals than in the controls (1·32 M/kg/hr vs 1·66 M/kg/hr, $P < 0.001$). This is the only change detected which is significantly different at the 0·01 level or less for any of the three dehydrogenases in any of the regions or organs assayed. In the lateral ventromedial nucleus and in the six other hypothalamic nuclei assayed, there were small variations in G-6-PDH activity (3–13 per cent), but the only difference significant at the 0·05 level is the 13 per cent decrease in the dorsomedial hypothalamus (1·59 M/kg/hr vs 1·82 M/kg per hr, $0.01 < P < 0.05$). In cortex, cerebellum and liver, G-6-PDH activity was not affected by morphine dependence (intergroup variation less than 3 per cent). The variation between morphine-dependent and control rats for MDH and LDH activities was less than 8 per cent in each region assayed, and none of these differences are significant at the 0·01 level. Some differences of small magnitude were significant at the 0·05 level: thus there was a 6 per cent increase in MDH activity in the anterior nuclei of morphine-dependent animals compared to controls (375 vs 354 M/kg per hr, $0.025 < P < 0.05$), a 6 per cent decrease in LDH activity in the lateral ventromedial nucleus of morphine-dependent rats compared to controls (69·4 vs 73·7 M/kg per hr, $0.01 < P < 0.02$), and a 3 per cent decrease in LDH activity in the paraventricular nucleus of morphine-dependent rats compared to controls (66·5 vs 68·8 M/kg per hr, $0.025 < P < 0.05$).

DISCUSSION

The most striking finding from this study is that the only change in morphine-dependent rats which is significant at the 0·01 level or less is the reduction in the level of one specific enzyme, G-6-PDH, in one discrete brain region, the medial ventromedial nucleus of the hypothalamus. There were few other differences significant at the 0·05 level and these changes represented variations of only 3–13

TABLE 2. ACTIVITY OF THREE DEHYDROGENASES IN MORPHINE-DEPENDENT AND CONTROL RATS*

Region assayed	Enzymes					
	G-6-PDH		LDH		MDH	
	Morphine-dependent	Control	Morphine-dependent	Control	Morphine-dependent	Control
Hypothalamus						
Anterior	1·28 ± 0·07	1·24 ± 0·12	69·4 ± 2·1	71·0 ± 2·9	375 ± 9†	354 ± 5
Supraoptic	2·03 ± 0·16	2·34 ± 0·14	61·8 ± 1·2	65·1 ± 2·0	351 ± 11	379 ± 10
Suprachiasmatic	2·12 ± 0·19	2·22 ± 0·20	63·4 ± 2·6	63·2 ± 2·2	407 ± 14	417 ± 10
Paraventricular	2·04 ± 0·10	2·22 ± 0·05	69·4 ± 1·2†	73·7 ± 1·3	348 ± 5	361 ± 6
Medial ventromedial	1·32 ± 0·03‡	1·66 ± 0·07	69·3 ± 0·8	69·8 ± 0·8	355 ± 6	356 ± 6
Lateral ventromedial	1·33 ± 0·04	1·49 ± 0·08	66·5 ± 0·8†	68·8 ± 0·6	359 ± 6	361 ± 7
Dorsomedial	1·59 ± 0·05†	1·82 ± 0·09	69·5 ± 0·6	69·1 ± 0·6	398 ± 8	404 ± 7
Arcuate	2·28 ± 0·08	2·53 ± 0·08	69·6 ± 0·7	69·7 ± 0·8	385 ± 9	396 ± 13
Cortex (layer III)	0·92 ± 0·03	0·90 ± 0·03	79·5 ± 1·8	81·2 ± 2·8	365 ± 3	371 ± 4
Cerebellum (granular layer)	0·91 ± 0·09	0·89 ± 0·05	72·4 ± 1·4	69·6 ± 1·2	378 ± 10	366 ± 5
Liver (periportal area)	0·89 ± 0·04	0·92 ± 0·09	111·0 ± 1·0	111·0 ± 1·1	255 ± 9	255 ± 5

* Activity level is expressed as moles of substrate oxidized kg (dry wt) hr. Results are presented as mean ± S. E. M.; each mean is based on 14–21 determinations in two animals.

† Difference between morphine-dependent rats and controls significant at $P < 0{\cdot}05$.

‡ Difference between morphine-dependent rats and controls significant at $P < 0{\cdot}001$.

per cent of control activity and are of doubtful physiological significance. Our data indicate that chronic morphine administration does not produce a generalized change in the activity of enzymes representative of three major metabolic pathways in either brain or liver. This result emphasizes the possibility that the biochemical events which result in morphine tolerance and dependence may well be both regionally and metabolically selective.

The impact of diminished G-6-PDH activity in the ventromedial hypothalamus (VMH) may be evaluated in terms of the known physiology of that region. Intravenous glucose administration increases the unit activity of single neurons in the VMH but not other hypothalamic or cortical neurons; the rate of discharge is correlated with the arterial venous glucose difference rather than with the blood glucose level *per se*.[19] It has been shown that stereotaxic injection of G-6-P into the VMH results in increased electrical activity recorded from a monopolar cannula implanted in the VMH.[20] The fact that G-6-PDH is the specific enzyme that oxidizes G-6-P suggests the possibility that the regionally selective change in G-6-PDH activity reflects an involvement of the VMH in some aspects of the development of dependence on narcotics. Thus, the decreased oxidation of G-6-P may lead to its local accumulation and thereby increase the activity of the G-6-P sensitive neurons in the VMH.

The hypothesis that morphine dependence may involve, at least in part, functional activation of the VMH is supported by other work on hypothalamic lesions in morphine-dependent rats. Kerr and Pozuelo[21] reported that the development of physical dependence on morphine was suppressed or markedly reduced by bilateral lesions which destroyed a major part of each ventromedial nucleus. Definitive conclusions cannot be drawn at this time, however, since other work indicates specific regions both within and outside the hypothalamus may be important in mediating some of the effects of morphine.[7,8,22,23] The inter-relationships between changes in different regions require further study. In addition, studies of G-6-P levels and control point enzymes, such as hexokinase, phosphofructokinase and transketolase, will be of particular interest.

In view of the highly selective metabolic and regional effects of morphine found in this study, further investigations utilizing quantitative histochemical techniques seem warranted to clarify the critical *loci* involved in the pharmacologic effects of morphine as well as in the development of tolerance and physical dependence.

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