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Effect of Manganese Treatment on the Levels of Neurotransmitters, Hormones, and Neuropeptides: Modulation by Stress

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Six weeks of daily intraperitoneal injection with manganese chloride (15 mg/kg body wt) reduced the normal weight gain of male Fischer-344 rats. This treatment depressed plasma testosterone and corticosterone levels, but prolactin levels were unaffected. The only significant changes in the levels of a variety of neuropeptides assayed in several regions were increases in the levels of hypothalamic substance P and pituitary neurotensin. Striatal serotonin, dopamine, and their metabolites were unchanged in manganese-exposed rats relative to saline-injected controls. However, the stress of injection combined with the effect of manganese appeared to significantly increase concentrations of striatal monoamines relative to uninjected controls.

INTRODUCTION

Manganese exposure is known to cause a series of neurological sequelae in a variety of species. In man, these signs are characterized by a biphasic response. Initially, a stage of psychiatric disturbance is seen, predominantly presenting as delusional thinking and a hyperactivity (Mena *et al.*, 1967). Subsequently, symptoms similar to Parkinson's disease appear, including akinesia, rigidity, and tremor (Cook *et al.*, 1974). These changes are paralleled by biochemical alterations of dopaminergic indices within the caudate nucleus (Cotzias *et al.*, 1974), and this has led to attempted treatment of manganism toxicity with L-DOPA (Mena *et al.*, 1970; Schunk, 1979). Rodents seem relatively resistant to manganese and after exposure to this metal do not clearly exhibit changes paralleling those seen in humans, guinea pigs, or monkeys (Bonilla, 1980; Shukla and Chandra, 1979; Neff *et al.*, 1969). However, behavioral dysfunction has been reported in rats (Roussel and Renaud, 1977) and mice (Chandra *et al.*, 1979b).

Because of the low potency of manganese treatment in producing motor dysfunction in rodents, the intent of the present work was to examine the possibility that those biochemical parameters of rodents that are susceptible to manganese may not be predominantly confined to the striatum and may involve molecular components not readily related to the dopamine system. In view of the potential vulnerability of the hypothalamo-pituitary axis to manganese (Deskin *et al.*, 1980)

and the vulnerability of neuropeptide levels to physiological change (Hong, *et al.*, 1984) emphasis was placed on endocrine and neuropeptide parameters. Since the dosing scheme involved repeated intraperitoneal injections of either manganese chloride or saline vehicle, an unhandled, uninjected control group was also studied. This gave us the opportunity to evaluate the effect of manganese treatment upon biological responses to the stress of the series of injections.

METHODS

Male rats of the Fischer-344 strain (Harlan Laboratories, Indianapolis, Ind.), aged 10 weeks, were dosed daily intraperitoneally with isotonic saline or manganese chloride (15 mg/kg body wt) for 6 weeks. A lower dose of manganese chloride (8 mg/kg) has previously been found to cause enzymatic changes in the rat brain (Shukla *et al.*, 1976). Another group of rats of the same age remained uninjected for this period. Twenty-four hours after the last injection, rats were decapitated and brain regions dissected (Glowinski and Iversen, 1966).

Estimation of dopamine and serotonin and their metabolites. The biogenic amine content of various brain regions was assayed by high performance liquid chromatography (HPLC) using the method of Wilson *et al.* (1983). In brief, tissue was homogenized in 19.3 vol of chilled 0.1 M HClO₄ containing 0.002 M sodium bisulfite. The homogenate was centrifuged (40,000g, 20 min) and the supernatant filtered through regenerated cellulose filter of 0.2- μ m pore size (Bio-Analytical Systems, Inc., West Lafayette, Ind.) prior to chromatography. The filtrate was used for automated analysis of serotonin, dopamine, and their acid metabolites by reversed phase HPLC using an electrochemical detection system. Compounds assayed were dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA).

Radioimmunoassay for neuropeptides. The contents of Met-Enkephalin (ME), β -endorphin (β -E), substance P (SP), and neurotensin (NT) were determined by previously described radioimmunoassays (Hong *et al.*, 1976, 1978; Fratta *et al.*, 1979; Govoni *et al.*, 1980).

Analysis of prolactin, luteinizing hormone, testosterone, and corticosterone levels. Blood was collected from rats at the time of decapitation and, after coagulation, was centrifuged at low speed (3000g, 10 min). Clear supernatant serum samples were stored at -70°C until assay. Prolactin and luteinizing hormone were measured using radioimmunoassay kits and reference standards supplied by the National Institute of Arthritis, Metabolism, and Digestive Diseases (Nemeroff *et al.*, 1977). Materials from commercial radioimmunoassay kits were used for determination of testosterone (Serono, Braintree, Mass.) and corticosterone (Corning Medical, Medford, Mass.). Corticosterone standard was purchased from Sigma Chemical Company (St. Louis, Mo.).

Protein determination. Protein was measured by the method of Lowry *et al.* (1951).

Statistical analysis. Differences between groups were assessed by Fisher's least significant difference test after a one-way analysis of variance (Keppel, 1973). The accepted level of significance in all cases was $P < 0.05$ using a two-tailed distribution.

RESULTS

Manganese treatment significantly reduced weight gain in rats by over 20% (Table 1), a result similar to that reported by Exon and Koller (1975) for Mn_3O_4 . The body weight of uninjected rats was significantly higher than in either injected group. The stressful nature of the injection series was also suggested by chronically elevated corticosterone and prolactin levels in saline-injected rats versus uninjected controls (Table 1). However, this corticosterone response to injection seemed blocked in manganese-injected rats. In addition, manganese treatment caused a reduction in circulating testosterone.

No significant changes in neuropeptide levels were found in the striatum or frontal cortex after manganese treatment (Table 2). However, the hypothalamic substance P level of exposed rats was significantly higher than in the other groups while other hypothalamic neuropeptide levels were unaffected by manganese. However, the stress of injection appeared to cause increases in the hypothalamic levels of Met-enkephalin, substance P, and β -endorphin (Table 2). Pituitary neurotensin levels were elevated by 50% in manganese-treated rats.

Striatal levels of DA, DOPAC, 5-HT, 5-HIAA, and HVA were determined (Table 3). Values obtained after manganese treatment were statistically indistinguishable from those of saline-injected controls. However, they were significantly greater than those of uninjected rats. In all cases, values for saline-injected rats were intermediate to corresponding values from the other two groups. The difference between injected and uninjected controls was significant only in the case of serotonin. Thus, both manganese and the injection procedure result in a trend toward elevated monoamine levels but only when these two parameters are considered together does this trend become significant. The magnitude of the amine level increases produced by the dual influence of manganese and injection stress was between 22 and 43%.

DISCUSSION

One of the salient features of this work is that manganese, when administered over a period of time in amounts sufficient to cause some physical debilitation in the rat as evidenced by weight loss, does not cause significant changes in striatal

TABLE 1
BODY WEIGHT AND LEVELS OF CIRCULATING HORMONES IN RATS EXPOSED TO $MnCl_2$ FOR 6 WEEKS

	$MnCl_2$ injected	Saline injected	Uninjected
Weight (g)	202 \pm 3 ^a	259 \pm 6	289 \pm 13 ^a
Hormone level (ng/ml serum)			
Corticosterone	181 \pm 28 ^a	352 \pm 65	152 \pm 43 ^a
Testosterone	1.4 \pm 0.2 ^a	3.4 \pm 0.5	4.6 \pm 0.6
Luteinizing hormone	9.5 \pm 1.6	13.5 \pm 1.6	37.9 \pm 11.4 ^a
Prolactin	24.5 \pm 12.4	25.5 \pm 2.3	11.1 \pm 3.3 ^a

Note. Each value represents a mean \pm SE ($n = 8-15$).

^a Value differs from that of saline-injected group ($P < 0.05$). Dosing details are given in the text.

TABLE 2
REGIONAL LEVELS OF NEUROPEPTIDES IN RATS EXPOSED TO $MnCl_2$ FOR 6 WEEKS

Neuropeptide	$MnCl_2$ injected	Saline injected	Uninjected
Hypothalamus			
β -Endorphin	4.30 \pm 0.20	4.40 \pm 0.30	3.10 \pm 0.30 ^a
Substance P	7.34 \pm 0.12 ^a	6.70 \pm 0.18	5.74 \pm 0.18 ^a
Neurotensin	1.76 \pm 0.04	1.67 \pm 0.07	1.49 \pm 0.06
Met-enkephalin	8.44 \pm 0.32	8.44 \pm 0.52	7.00 \pm 0.28 ^a
Striatum			
Substance P	3.90 \pm 0.10	3.60 \pm 0.10	3.40 \pm 0.10
Neurotensin	0.49 \pm 0.06	0.46 \pm 0.02	0.49 \pm 0.02
Met-enkephalin	20.86 \pm 0.62	19.66 \pm 0.69	18.90 \pm 0.69
Frontal cortex			
Substance P	0.23 \pm 0.02	0.24 \pm 0.01	0.23 \pm 0.07
Neurotensin	0.08 \pm 0.01	0.09 \pm 0.01	0.09 \pm 0.01
Met-enkephalin	1.02 \pm 0.03	1.02 \pm 0.02	0.98 \pm 0.02
Pituitary			
β -Endorphin	4.55 \pm 0.49	4.15 \pm 1.42	3.20 \pm 0.32
Neurotensin	1.21 \pm 0.10 ^a	0.81 \pm 0.07	0.73 \pm 0.05
Met-enkephalin	4.28 \pm 0.22	4.52 \pm 0.28	5.02 \pm 0.39

Note. Each value represents the mean (\pm SE) derived from 12–15 animals for $MnCl_2$ and saline groups and 6–8 animals in the unhandled group, given as ng/10 mg wet tissue except in the case of pituitary where β -endorphin levels were expressed as μ g/mg protein and neurotensin and met-enkephalin levels were expressed as ng/mg protein.

^a Value differs from that of saline-injected group ($P < 0.05$).

levels of dopamine and its metabolites. Pronounced biochemical changes are apparent in dosed animals but these are found neither within the caudate nucleus nor in any parameter related to dopaminergic function. The manganese levels of brain regions were not determined in this study. However, such values have been reported in a study using a similar dosing regiment (Shukla *et al.*, 1976). These authors administered a total of 840 mg $MnCl_2$ /kg body wt over 120 days while our overall dose was 960 mg $MnCl_2$ /kg in 56 days. The increase of cerebral man-

TABLE 3
LEVELS OF BIOGENIC AMINES AND THEIR ACID METABOLITES IN THE STRIATUM OF RAT EXPOSED TO $MnCl_2$ FOR 6 WEEKS

	$MnCl_2$ injected	Saline injected	Uninjected
DOPAC	1.38 \pm 0.14 ^a	1.13 \pm 0.08	0.96 \pm 0.08
Dopamine	16.30 \pm 1.30	13.60 \pm 1.10	13.40 \pm 1.10
5-HIAA	0.86 \pm 0.08 ^a	0.73 \pm 0.05	0.64 \pm 0.01
HVA	1.02 \pm 0.11 ^a	0.83 \pm 0.08	0.74 \pm 0.03
Serotonin	0.65 \pm 0.07 ^a	0.58 \pm 0.05 ^a	0.46 \pm 0.01

Note. Each value represents a mean (\pm SE) from 6–8 animals for the $MnCl_2$ and saline groups and four animals for the unhandled group given as μ g/g wet²wt.

^a Value differs from uninjected group ($P < 0.05$).

ganese content found by Shukla *et al.* (1976) was between 37 and 135% of control values. Thus, these rather high doses of manganese do little more than double normal endogenous manganese levels.

The selective increases of pituitary neurotensin and hypothalamic substance P suggest a predominantly hypothalamic–hypophyseal site of attack of manganese. It is possible that the depressed levels of circulating testosterone and corticosterone represent the consequences of the effects of manganese on the hypothalamo-pituitary axis. A lower level of corticosterone was also found in uninjected control rats (Table 1). The depressed level of corticosterone in manganese-exposed subjects relative to saline-injected controls suggests a failure of the usual response to the stress of repeated injection. However, the levels of circulating prolactin were very similar in manganese- and saline-injected rats, implying that this stress-related parameter reacted normally to the injections. Thus, the failure of the endocrine response was partial rather than absolute. The uninjected rats also appeared to be less stressed in that they were significantly heavier than other groups. The reduced body weight of manganese-treated rats is unlikely to solely account for the altered levels of hypothalamic peptides since we found that repeated injections of chlordecone, which caused a 25% reduction in body weight of rats, failed to change the levels of neuropeptides, such as β -E, SP, NT, and ME (manuscript in preparation). However, the possibility of an interaction between nutritional deprivation and response to manganese of experimental animals cannot be ruled out in the absence of data from pair-fed animals. The depressed levels of luteinizing hormone found in both groups of injected rats may be due to inhibitory regulation of this hormone, effected by increased levels of prolactin (Bardin, 1980). There may be a relation between lowered luteinizing hormone levels and the relatively higher hypothalamic Met-enkephalin or β -endorphin levels found in injected rats (Table 2), since opiates and endorphins increase and naloxone decreases serum luteinizing hormone levels in adult male rats (Bruni *et al.*, 1977).

The depression in testosterone levels of manganese-treated rats is reminiscent of a similar reduction following exposure to a variety of neurotoxic agents including monosodium glutamate (Nemeroff *et al.*, 1981), acrylamide (Ali *et al.*, 1983), lead (Braunstein *et al.*, 1978), and chlordecone (Uphouse and Hong, unpublished data). This cannot be related to the stress of injection since testosterone levels were not significantly altered by repeated injection of saline (Table 2). Although it was reported that the decrease in plasma testosterone level is a direct effect of manganese on the testis (Imam and Chandra, 1975), it is also possible that the lability of testosterone levels to manganese and other various neurotoxic agents may reflect a special vulnerability of the hypothalamo-hypophyseal-endocrine axis to these agents. This may be due to the pivotal role of the hypothalamus in that minor changes in this region are secondarily reflected by major metabolic and behavioral alterations. The mechanisms underlying altered endocrine levels may of course be quite varied, in spite of a common endpoint. The sensitivity of the pituitary may in part be related to the fact that the nerve terminals of the neural lobe and pars intermedia are partly outside the blood–brain barrier and appear to undergo a process of continuous degeneration and regen-

eration (Baumgarten *et al.*, 1972). Neurons are known to be subject to degenerative changes in the presence of a defective blood-brain barrier (Svenggaard *et al.*, 1975). Due to a relative ineffectiveness of the hypothalamic blood-brain barrier, highest concentration of manganese is found in the hypothalamus of manganese-treated rats, relative to other brain regions (Bonilla, 1980; Lai *et al.*, 1981). A special sensitivity of the rodent hypothalamus relative to the striatum was reported by Deskin *et al.* (1980). These authors found hypothalamic but not striatal tyrosine hydroxylase, dopamine, and monoamine oxidase to be modulated in manganese-treated rats. Such region specificity may be diminished when a higher dose of manganese is used (Bonilla, 1980). Reports of altered catecholamine levels in the striatum of manganese-treated rats generally have used a fluorimetric assay (Chandra and Shukla, 1981) and this assay method tends to be less precise than HPLC. Also, the above report employed oral administration of manganese and thus cannot be directly compared to our data based on intraperitoneal injection. The lack of effect of manganese alone on striatal dopamine and DOPAC levels is broadly in agreement with earlier data from this laboratory employing a shorter duration of exposure to this metal (Seth *et al.*, 1981). This latter study also found striatal levels of met-enkephalin and substance P to be unaltered by treatment but levels of 5-HIAA, the serotonin metabolite, were depressed in manganese-treated rats. Another report also indicates that dietary manganese may reduce cerebral serotonin levels (Kimura *et al.*, 1978), but we found no such changes in this study. The effect of manganese on the dopaminergic system may be biphasic. This is revealed by the clinical progression from schizophrenia-like states to Parkinsonism. Biochemically, the effect of a low dose of manganese upon synaptosomal dopamine uptake is opposite to the effect of a higher dose (Leung *et al.*, 1982; Lai *et al.*, 1982). This may account for the consensus that monoamine neurons are targets of manganese but lack of consensus as to the directionality of reported changes. The elevated levels of monoamines seen in the combined presence of injection stress and manganese relative to undisturbed controls is reminiscent of a report by Chandra *et al.* (1979a). These authors reported that immobilization stress altered levels of biogenic amines to a greater extent in manganese-treated rats. The interaction of exposure to toxic agents and over environmental parameters is a complicating factor in attempts to understand the biochemical injuries caused by a variety of neurotoxicants. The combination of adverse factors may serve to unmask changes that would otherwise be imperceptible.

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