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BRAIN IRON IN THE FERROCENE-LOADED RAT: ITS CHELATION AND INFLUENCE ON DOPAMINE METABOLISM

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Abstract—After administration of the ferrocene derivative 3,5,5-trimethyl hexanoyl ferrocene to rats for 4 weeks various brain regions including substantia nigra, cerebellum and cerebral cortex showed up to 50% increase in iron content. Subsequent administration of one of the hydroxypyridones CP20, CP24 and CP94, or the siderophore desferrioxamine caused a significant decrease in the iron content of these various brain regions. Each of the hydroxypyridones and the siderophore influenced dopamine metabolism by causing significant variations in both homovanillic acid and dopamine turnover.

Key words: Parkinson's disease; iron-loaded brain; iron chelation

Iron is essential for normal brain function [1] although, in contrast to other organs, little is known of brain iron metabolism. Transferrin acts as the iron transporter, the iron-loaded transferrin being taken up by receptor-mediated endocytosis at the luminal membrane of the endothelial brain capillaries [2]. Iron dissociates from transferrin in endosomal compartments and is transcytosed by unknown mechanisms. The iron will complex with a transferrin-like molecule in the interstitial space of the brain from where it is transported to neurons and glia cells.

Alterations occur in iron metabolism in various neurodegenerative diseases. Excessive iron deposits are found in basal ganglia diseases, for example, in substantia nigra in Parkinson's disease [3] in substantia nigra, caudate nucleus and putamen in progressive supranuclear palsy [4] and the caudate nucleus in Huntington's disease [5]. In Alzheimer's disease iron accumulates around and within the senile plaques [6]. The cause and consequence of such site specific perturbations in iron homeostasis in these brain regions is unknown.

Considerable attention has been focused on the accumulation of iron in substantia nigra of parkinsonian patients which could induce oxidative stress [7]. The nature of the iron complex may be ferritin [8] or not [9] or alternatively iron complexed with neuromelanin [10], other proteins or smaller molecules. The explanation for the excessive iron is unclear but could be caused by changes in the density of transferrin receptor on substantia nigra or as a result of neuronal degradation.

The ability of iron chelators to displace iron from these iron storage complexes presents a new therapeutic approach for the treatment of parkinsonian patients. However, it is clear that for such treatment to be effective, the iron chelator should be able to cross the blood–brain barrier, chelate the excess iron in the specific regions of the brain and not adversely interfere with other essential systems which require iron for their metabolism, e.g. ribonuclease reductase, dopamine synthetase.

It has been difficult to engender an animal model which shows similar excessive and selective accumulation of iron to the brain regions found in various human neurodegenerative disorders. Administration of MPTP¶ produces a parkinsonian lesion in substantia nigra in experimental primates with a concomitant increase in iron [11]. In rats the blood–brain barrier will restrict the uptake of iron into the brain when it is bound to transferrin. However, our recent studies of rats supplemented with 3,5,5-trimethyl hexanoyl ferrocene derivative for 4 weeks, showed increased amounts of iron in many organs, heart, liver, spleen and pancreas [12]. This high content of tissue iron is caused by the ferrocene derivative being carried intact within the blood, not associated with transferrin, which rapidly permeates cellular membranes. Therefore, the blood–brain barrier should not present a major obstacle to this compound.

In these present studies, we have quantitated the

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¶ Abbreviations: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; CP24, 1,*n*-butyl-3-hydroxy-2-methyl pyridone-4-one; CP94, 1,2-diethyl-3-hydroxypyrid-4-one; CP20, 1,2-dimethyl-3-hydroxypyrid-4-one; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; EAAS, electrothermal atomic absorption spectroscopy; ICP, inductively coupled plasma; HPLC, high-performance liquid chromatography; DHBA, dihydroxybenzylamine; DFO, desferrioxamine; IRF, iron responsive factor; 6-OHDA, 6-hydroxy-dopamine; 5-HT, 5-hydroxytryptamine.

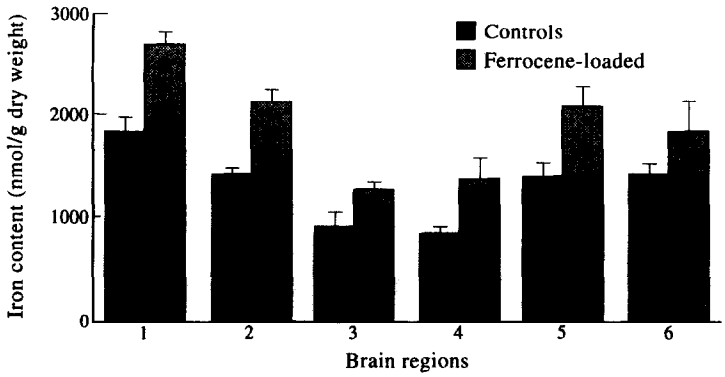

Fig. 1. Iron content of different regions of rat brain after supplementation with the ferrocene derivative 3,5,5-trimethyl hexanoyl ferrocene, compared to those areas in control rats. (1) Cerebellum, (2) cerebral cortex, (3) hippocampus, (4) brain stem, (5) striatum, (6) substantia nigra.

iron content of different brain regions after iron loading for 4 weeks, while assessing its possible adverse effect on dopamine content and turnover. Specific iron chelators such as desferrioxamine and various hydroxy pyridones have been administered to the rats after iron loading to ascertain their specificity for iron removal from brain regions and to assess whether such chelators might interfere with dopamine metabolism.

MATERIAL AND METHODS

Male Wistar rats (starting weight 75–100 g) in groups of six, were used in each of the following studies.

Two methods were compared in their ability to load rat brain with iron, firstly by feeding a diet supplemented with ferrocene (0.1 g/kg prepared in an iron-free diet) for 4 weeks or secondly by daily gavage of the ferrocene compound dissolved in 10% cremophore, 75 mg/kg, for 5 weeks.

For the iron chelation studies, either desferrioxamine (30 mg/kg) or one of the hydroxypyridones, CP24 (30 mg/kg), CP94 (100 mg/kg), was administered intraperitoneally three times per week for 2 weeks to the ferrocene-loaded rats.

Possible alterations in dopamine metabolism by the chelators were investigated by two approaches. Firstly, by studying the dose–response of one of the hydroxypyridones, CP94, on striatal dopamine, DOPAC, HVA and dopamine turnover in ferrocene-loaded rats. Secondly, by assessing dopamine metabolism at two and four weeks after intraperitoneal administration of desferrioxamine, CP20 or CP94 (50 mg/kg body weight) to rats which had been administered the ferrocene derivative for the previous four weeks.

Prior to killing the rats, blood was removed by cardiac puncture for estimation of haematological parameters, after which the animal was killed by cervical dislocation and the liver removed, for the estimation of iron content by EAAS. The brain was removed from the cranium, and specific areas dissected out for assay of iron by inductively coupled plasma emission spectroscopy, apart from the substantia nigra in which the iron content was assayed by EAAS.

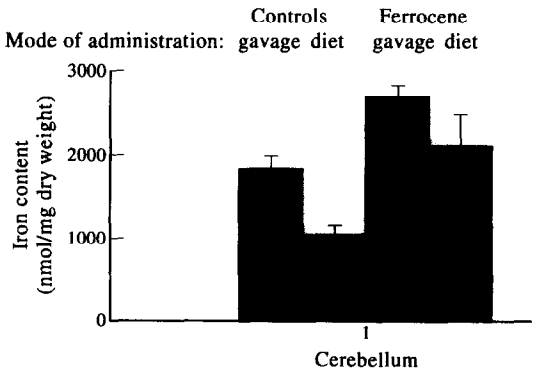

Fig. 2. Comparison of the efficiency of iron-loading with the ferrocene derivative given either in the diet or by gavage in the cerebellum.

To assess the iron status of the rats after loading with ferrocene, a small portion of the liver was homogenized in water, 10% (w/v), further diluted with water to the standard reference range for iron of 0.1–0.4 μg Fe/mL prior to analysis by EASS.

Iron was analysed in the various brain regions by ICP spectroscopy analysis. The brain samples were freeze-dried and solubilized by heating the sample in 200 μL sulphuric acid at 60°, followed by a dropwise addition of 80 μL, 30% (w/v) hydrogen peroxide, both to enhance tissue solubilization and to bleach the sample. The samples were cooled to room temperature, made up to 1 mL with water and analysed by an Applied Research Laboratory ICP spectrophotometer.

Dopamine and its metabolites, HVA and DOPAC, were assayed by HPLC by a modification of the

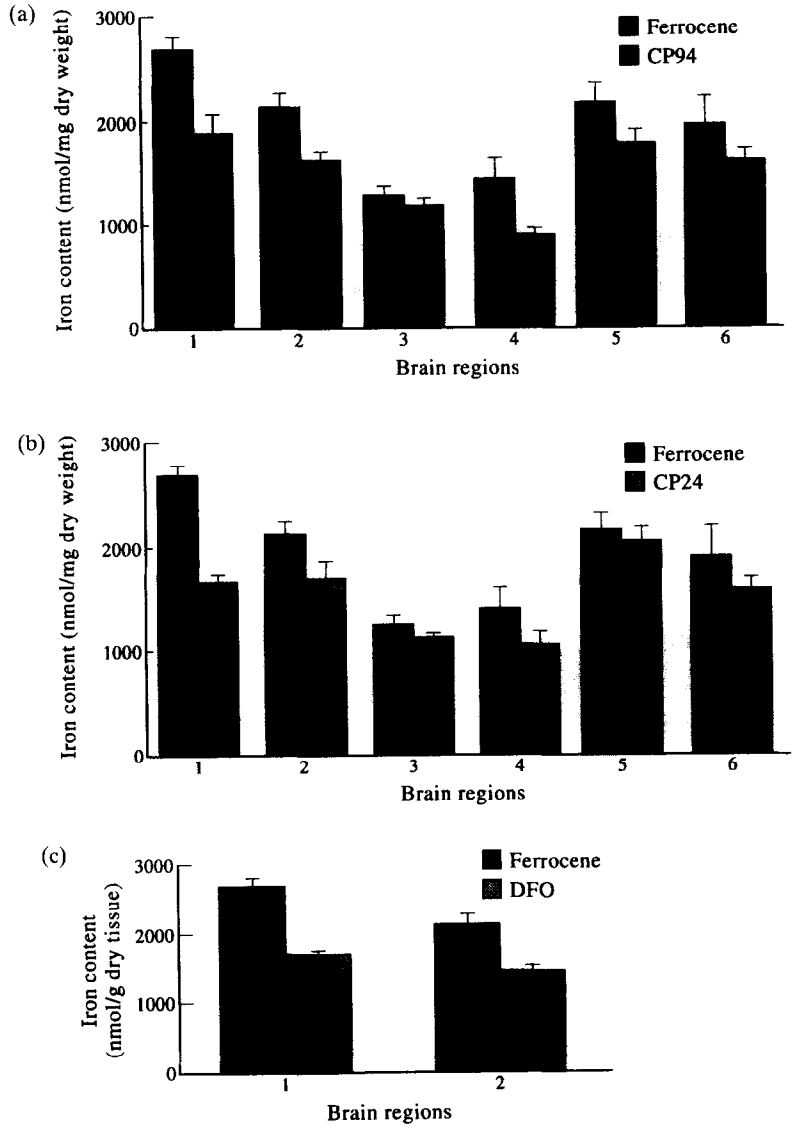

Fig. 3. Alteration in the iron content of the various brain regions after administration of (a) CP94 (100 mg/kg), (b) CP24 (30 mg/kg), and (c) DFO (30 mg/kg). (1) Cerebellum, (2) cerebral cortex, (3) hippocampus, (4) brain stem, (5) striatum, (6) substantia nigra.

method of Rose *et al.* [13]. The striated region of the brain was dissected on ice, weighed and sonicated in 10 vol. (w/v) 0.4 M perchloric acid containing 1 mM EDTA and 0.5% sodium metabisulphite by a Microson tissue disrupter. The resulting homogenate was added to a solution of the internal standard DHBA in a 9:1 (v/v) to give a final concentration of DHBA of 100 ng/mL. The homogenate was centrifuged at 15,000 rpm for 10 min at 4°. An aliquot of the supernatant was injected onto a Spherisorb ODS-2 reverse-phase column and chromatographic peaks detected by a BAS LC-4B amperometric detector with a thin-layer electrochemical cell fitted with a glassy carbon working electrode and Ag/AgCl reference electrode.

The results are presented as mean and standard deviation. Significance was assessed by paired Student's *t*-test.

RESULTS

The iron status, as assessed by liver iron content, was significantly increased in the rats administered the ferrocene derivative, 5243 ± 1240 and 3679 ± 486 μg/g tissue in the groups of rats either given the ferrocene derivative by gavage or in the diet, respectively, compared to the control values of 514 ± 238 and 599 ± 122 μg/g tissue. The haematological indices were unspectacular, transferrin saturation remaining unchanged with iron loading, while there were no consistent alterations in white cell counts.

Table 1. Striatal dopamine and its metabolites after chelation

	Dopamine	DOPAC	HVA	Turnover	5-HT	5HIAA
Ferrocene + 2 weeks Fe-free	11.4 ± 0.35	0.71 ± 0.03	1.17 ± 0.04	0.17 ± 0.01	0.68 ± 0.05	0.49 ± 0.02
Ferrocene + 4 weeks Fe-free	12.86 ± 0.66	0.84 ± 0.05	1.30 ± 0.06	0.17 ± 0.01	0.63 ± 0.04	0.66 ± 0.07
Ferrocene + 2 weeks CP94	11.25 ± 0.58	0.65 ± 0.03	0.69 ± 0.04	0.12 ± 0.01	0.54 ± 0.04	0.53 ± 0.03
Ferrocene + 4 weeks CP94	11.72 ± 0.74	0.66 ± 0.04	0.68 ± 0.1	0.11 ± 0.01	0.59 ± 0.03	0.55 ± 0.03
Ferrocene + 2 weeks DFO	9.57 ± 0.83	0.63 ± 0.06	0.64 ± 0.05	0.13 ± 0.01	0.67 ± 0.03	0.73 ± 0.03
Ferrocene + 4 weeks DFO	12.08 ± 0.66	0.81 ± 0.06	0.77 ± 0.05	0.13 ± 0.005	0.64 ± 0.06	0.62 ± 0.007
Ferrocene + 2 weeks CP20	13.01 ± 0.44	0.74 ± 0.06	0.72 ± 0.09	0.11 ± 0.01	0.60 ± 0.01	0.66 ± 0.02
Ferrocene + 4 weeks CP20	12.00 ± 0.96	0.61 ± 0.05	0.59 ± 0.04	0.10 ± 0.01	0.56 ± 0.01	0.59 ± 0.03

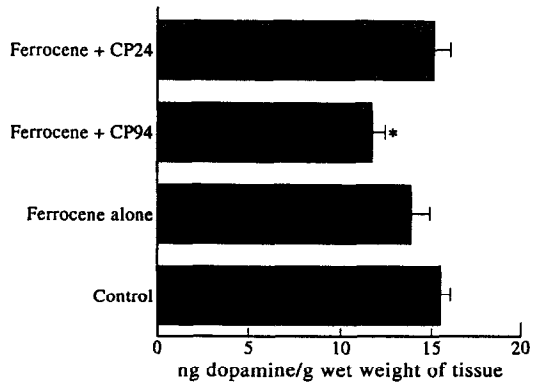

Fig. 4. Brain dopamine levels in rats administered the ferrocene derivative for 5 weeks and then receiving either CP94 (100 mg/kg), or CP24 (30 mg/kg) for 2 weeks, compared to ferrocene only.

In the rats loaded with ferrocene by gavage, each of the brain regions selected increased by a similar amount (between 50 and 55%), compared to the corresponding area in the control rat brains (Fig. 1), apart from the substantia nigra which showed a smaller increase of 30%. There was little difference in the extent of the iron loading whether the ferrocene was given by diet or gavage, the percentage increase from the control value being of a similar extent (Fig. 2).

Figure 3 shows the brain iron content of the different brain regions after 2 weeks administration of either CP94 (100 mg/kg; Fig. 3(a)) or CP24 (30 mg/kg; Fig. 3(b)) to the ferrocene-loaded rat. The iron content decreased in each of the brain regions examined in an order of magnitude, cerebellum and brain stem > substantia nigra and cerebral cortex > striatum and hippocampus. DFO was also effective in decreasing the iron content of the few regions assayed (Fig. 3(c)).

Figure 4 shows that striatal dopamine levels were not adversely affected by iron loading of the brain or its subsequent chelation with CP24 (30 mg/kg). However, administration of CP94, at a dose of 100 mg/kg for 2 weeks, significantly reduced striatal dopamine content. Figure 5 shows the effect of increasing IP doses of CP94 (12.5–100 mg/kg) on striatal dopamine metabolism in ferrocene-loaded rats. There was a gradual decrease in dopamine levels which reached significance at the highest dose, 100 mg/kg. Similarly the higher doses of CP94, 50 mg/kg and 100 mg/kg induced significant changes in DOPAC and dopamine turnover, while HVA was significantly altered only at the highest dose (Fig. 5(b)). Administration of one of the chelators, CP94, CP20 or DFO intraperitoneally, at doses of 50 mg/kg over a longer period, i.e. 4 weeks, did not significantly affect dopamine levels. However, dopamine turnover and DOPAC were significantly reduced at both 2 and 4 weeks after such therapy (Table 1).

DISCUSSION

It has proved difficult to increase experimentally the iron content of the rat brain. An earlier study by Taylor *et al.* [14] showed no change in brain iron content after administration of the carbonyl iron compound. The explanation for such a significant increase in the brains of the ferrocene rats in this present study, relates to the fact that the ferrocene derivative is carried in the blood, after crossing the intestinal mucosa directly, and does not associate with transferrin. It therefore readily crosses all cell membranes, possibly by endocytosis, and in particular the blood–brain barrier because of its lipophilic nature. The variable iron content of each region of the brain may reflect their lipid content. The form in which the iron is present in these brain regions is currently under investigation. Such a disruption in brain iron homeostasis might be predicted to alter iron regulatory proteins such as

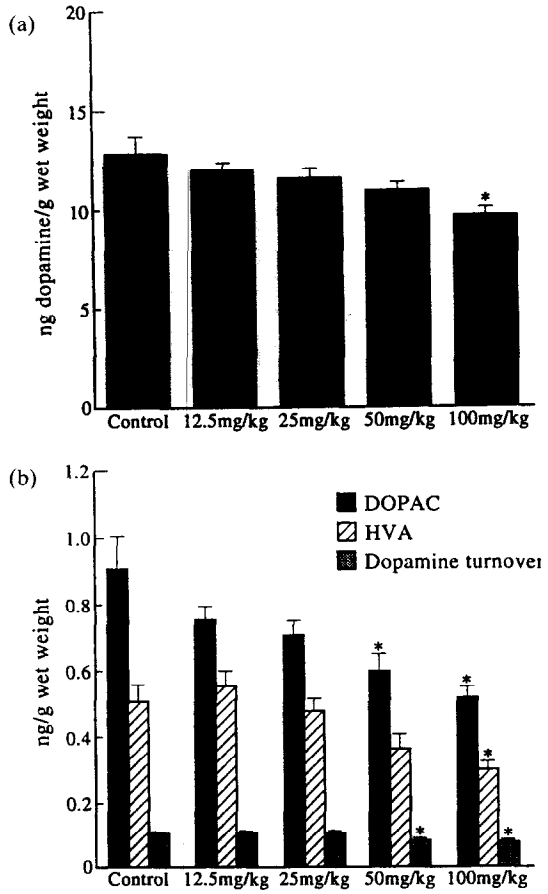

Fig. 5. Effect of CP94 administration (12.5–100 mg/kg) on (a) striatal dopamine levels, and (b) striatal DOPAC, HVA and dopamine turnover in ferrocene-loaded rats.

transferrin/transferrin receptors and ferritin. Taylor *et al.* [14] showed that there were alterations in the regulation of transferrin receptors in the brains of rats loaded or depleted with iron, as assessed by ^{125}I-transferrin uptake. However, we have been unable to show any consistent changes in the affinity of the cytosolic IRF for the iron responsive element with iron status, the IRF activity in the cytosol of whole brains from ferrocene rats being similar to that of controls by band shift assay. Interestingly, the cytosolic fraction from the brains of rats administered iron dextran did show alterations in their affinity for IRE [15].

The removal of the excess iron in the brains of parkinsonian patients might be beneficial by slowing the progressive disabling course of the illness. Initial studies showed that desferrioxamine prevented the 6-OHDA induced degeneration of nigrostriatal dopamine neurons [16] but brain iron content was not specifically assayed. In this present study we have clearly shown that the hydroxypyridones in addition to desferrioxamine are able to cross the blood–brain barrier and significantly reduce the brain iron content after only 2 weeks of administration. The chelator CP24 removed similar amounts of iron to that mobilized by CP94 even though the amount of the former drug administered was three-fold less, underlining the greater lipophilicity of CP24. Furthermore, there was regional specificity in the removal of iron by the chelators; for example, CP94 induced comparable decreases in the iron brain content of each region investigated, approx. 30%, apart from hippocampus where the diminution in iron was only 18%. There was a more variable response with CP24 where the decreases in brain iron content ranged from only 7% in the striatum to almost 50% in the cerebellum after chelation. The administration of CP24 or CP94 to non-iron-loaded rats has been shown to have little effect on brain iron content (Dexter, D., unpublished data).

Caution, however, must be exercised in the use of chelators to remove excess iron often seen in pathological conditions, since it is important that the excess iron chelated is principally that associated with the disease process while iron essential for normal cellular function is retained. Removal of iron from essential iron-containing enzymes such as tyrosine hydroxylase or ribonuclease reductase, key enzymes in dopamine synthesis and DNA synthesis, respectively, may cause other adverse effects to brain cellular function.

There were alterations in the levels of dopamine and its metabolites in the striatum two weeks after administration of each of the iron chelators, although these only reached significance at the higher doses of CP94. This may indicate that there is inhibition of dopamine metabolism directly caused by co-ordination of the iron from tyrosine hydroxylase to the chelator. An acute study where a single dose of CP20 was administered to rats (at doses between 1 and 100 mg/kg, i.p.), and compared to a similarly administered dose of desferrioxamine (100 mg/kg) caused marked alterations in HVA, DOPAC, dopamine and 5-HT within a few hours [17].

The beneficial use of iron chelation for the treatment of Parkinson's disease remains conjectural and will be dependent, in part, upon whether the increased iron stores within substantia nigra are actually exacerbating the tissue damage either by catalysing free radical generation or by interfering with dopamine metabolism. However, the alterations in dopamine metabolism reported in this communication, although minimal if low doses are administered, may preclude their therapeutic use.

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