Dopamine D$_2$ Receptor Expression Is Altered by Changes in Cellular Iron Levels in PC12 Cells and Rat Brain Tissue[1,2]

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Abstract

Iron deficiency anemia in early life alters the development and functioning of the dopamine neurotransmitter system, but data regarding the specific effects of brain iron loss on dopamine D$_2$ receptor regulation are lacking. Cell culture and animal models were employed in this study to determine whether D$_2$ receptor expression is altered when cellular iron levels are depleted. Endogenous D$_2$ receptor-expressing PC12 cells exposed to increasing concentrations of the iron chelator desferrioxamine (25–100 μmol/L) exhibited dose-dependent decreases in total D$_2$ receptor protein concentrations (20–65%), but there were minimal effects on D$_2$ receptor mRNA levels. When iron-deficient cells were repleted with ferric ammonium citrate for 24 h, D$_2$ receptor protein densities were similar to control. Dietary iron deficiency for 6 wk in weanling rats also reduced regional iron concentrations by nearly 50% in the ventral midbrain and caudate but did not affect D$_2$ receptor mRNA levels in the ventral midbrain. Iron deficiency significantly reduced membrane D$_2$ receptor protein levels by >70% in caudate, whereas cytosolic concentrations showed only 25% losses. D$_2$ receptor protein densities and regional iron concentrations were restored within 2 wk of dietary iron repletion. These results support the concept that D$_2$ receptor gene expression is not significantly changed by iron deficiency, whereas dopamine receptor trafficking is affected and is likely related to known dopamine system alterations in iron deficiency.   J. Nutr. 138: 2487–2494, 2008.

Introduction

Neural functioning and behavioral development are affected by iron deficiency in early life (1), which results in lethargy, reduced cognitive performance, decreased attention as well as abnormalities in auditory evoke potentials responses, spontaneous motor activity, and sleep patterns that may not improve after iron therapy (1–6). Deficits in iron metabolism in the central nervous system have also been linked to Restless Legs Syndrome (RLS).[5] Individuals diagnosed with RLS display diurnal-based deficiencies in motor control and the severity of symptoms increases during the evening hours. Support for the role of iron in RLS comes from a study in which infusion of iron dextran improved RLS symptoms and elevated substantia nigra and prefrontal cortex iron to normal levels (7). RLS is also linked to changes in the dopamine neurotransmitter system, because

enhancement of dopamine activity reduces RLS symptoms (8,9), whereas dopamine receptor antagonists exacerbate these symptoms (10,11).

Rodent and cell culture model examinations indicate that the dopamine system is particularly sensitive to dietary iron deficiency. In vivo microdialysis studies using postweaning iron-deficient rats and mice show that extracellular dopamine concentrations are consistently elevated in the striatum compared with controls (12–14). Postweaning iron-deficient animal models also have deficits in intracellular dopamine concentrations, but the amount of dopamine loss varies by brain region, illustrating the heterogenous effect of iron deficiency on the brain (15–17). Moreover, recent evidence shows that the response of iron-deficient rats to striatal infusion of 3,4-dihyroxy-L-phenylalanine, the precursor of dopamine, is blunted and delayed compared with control diet-fed rats (18). The expression and functioning of the dopamine transporter, the main site of dopamine reuptake into the cell, is also altered in striatal synaptosomes from iron-deficient rats (19) in an iron-chelated cell culture model (20) and in vivo (18). Together, these data provide evidence that decreased reuptake of dopamine by the dopamine transporter is associated with elevated extracellular striatal dopamine levels when iron is depleted. The exploration of the dopamine system after iron deficiency also includes studies that examine dopamine D$_1$ and

D_2 receptor densities (16,19,21). These studies consistently show decreased D_2 receptor levels, but not necessarily D_1 receptor levels, in striatum.

Through pharmacological manipulations in mice and rats, it has been hypothesized that a signal transduction pathway links the D_2 receptor and the dopamine transporter. Specifically, administration of the D_2 agonist quinpirole results in an enhancement of dopamine transport velocity in striatal synaptosomes (22), whereas the D_2 receptor antagonist raclopride decreases dopamine transport (23). Recent evidence suggests that this specific pathway is altered in iron deficiency (18). Using in vivo microdialysis, these authors showed that infusion of quinpirole into striatum increases dopamine transport velocity in rats fed control diet, whereas the D_2 receptor agonist did not affect uptake in iron-deficient rats. These effects are consistent with a reduced D_2 receptor levels (16,19,21) and possible alterations in the signal transduction pathway(s). To date, the mechanism(s) of effect of neuronal iron deficiency on dopamine receptor metabolism or gene expression remains unexplored.

In the current report, we examined the effect of iron depletion on D_2 receptor protein and gene expression levels in a cell culture model of iron deficiency and in ventral midbrain (substantia nigra and ventral tegmentum) and caudate putamen from iron-deficient rats. These studies will provide insights into the potential mechanisms by which iron influences D_2 receptor functioning and expression, thereby leading to alterations in dopamine neurotransmission.

Materials and Methods

Pheochromocytoma cell culture. PC12 cells, a pheochromocytoma cell line, were grown on Collagen IV Cellware (BD Biosciences) and maintained in RPMI 1640 medium (Gibco) supplemented with 10 mmol/L HEPES, 4.5 g/L glucose, 10% equine serum (HyClone), 5% defined fetal bovine serum (HyClone), 2 mmol/L glutamine (Sigma-Aldrich), and 100 kU/L penicillin-streptomycin (HyClone) at 37°C in a water-saturated atmosphere containing 7% CO_2. For all cell culture experiments, cells were treated with 25, 50, or 100 μmol/L desferrioxamine (DFO) for 24 h or 50 μmol/L DFO for 24 h followed by 24-h treatment with 50 mg/L ferrous ammonium citrate (FAC) for repletion studies.

Western blot analysis. All conditions for cell treatments, cell collection, and western blotting have been previously described (20,24) and were uniform over all experiments. Membranes were probed with either a monoclonal rabbit anti-D_2 receptor amino-terminal antibody (Research Diagnostics) or a mouse anti-transferrin receptor (TfR) monoclonal antibody (Zymed Laboratories) in blocking solution (1:1000) for 12 h at 4°C. Membranes were also probed for actin immunoreactivity (rabbit anti-actin antibody, 1:500, Santa Cruz Biotechnology) using assay conditions similar to those described above. Protein band densities were quantified by scanning exposed film (Epson Perfection 636U digital scanner, Epson America) into Adobe Photoshop (version 7.0) and band integrative densities were determined with NIH Image software. To enable comparisons of different blots with variable exposure times, results from experimental groups were normalized to the mean of control lanes from the same gel. Data are expressed as percent of control values.

PC12 mRNA expression. For cell RNA analysis, PC12 cells were washed 3 times with ice-cold PBS (9.1 mmol/L Na_2HPO_4, 1.7 mmol/L NaH_2PO_4, 150 mmol/L NaCl, pH 7.4) and exposed to 25, 50, and 100 μmol/L DFO for 24 h. The cells were harvested in PBS using vigorous trituration and collected by centrifugation. Total RNA was isolated using a RNeasy Mini kit (Qiagen), quantified spectrophotometrically, and examined for integrity by electrophoresis (1.25% agarose-0.67 mol/L formaldehyde gel) using ethidium bromide staining. We used quantitative real-time PCR analysis to determine D_2 receptor mRNA expression.

Triplicate aliquots of RNA were analyzed using the Perkin Elmer/Applied Biosystems Division 7700 Sequence Detector. The sequences for the primers were as follows: D_2 receptor forward primer, 5′-CTT CAC ATG GCT GGG CTA TGT-3′, reverse primer, 5′-GCG GAA CTC GAT GTT GAA GG- 3′; TfR forward primer, 5′-CAT CTC CAT CTG ACC CTC AC-3′, reverse primer, 5′-GTC TTT GGC TTC TGG TCT CA-3′; divalent metal transporter 1 (DMT1) + iron responsive element forward primer, 5′-GGA GGG ATT CCT GAA CCT AA-3′, reverse primer, 5′-AGG TGA GGA TAG GGA TGA GG-3′; ferritin H forward primer, 5′-ACT GAT GAA GCT GCA GAA CC-3′, reverse primer, 5′-GTG GGG ATC ATT CTT GTC AG-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward primer, 5′- GGA GTC TAC TGG CGT CTT CA-3′, reverse primer, 5′-ATG CCA AAG TTG TCA TGG AT-3′) and β-actin (forward primer, 5′-GAA GTA CCC CAT TGA ACA CG-3′, reverse primer, 5′- GAG GCA TAC AGG GAC AAC AC-3′) were used as controls. The primers were designed using the Perkin Elmer/Applied Biosciences Primer Express software based on mRNA sequences reported in the National Center for Biotechnology Information public database. Each value for mRNA was normalized relative to GAPDH expression.

Animals, dietary treatments, and iron assessment. Male 21-d-old Sprague-Dawley rats (Harlan Sprague Dawley) were randomly divided into 3 dietary treatment groups as follows: control (50 μg/g diet, 6 wk), iron deficient (3 μg/g diet, 6 wk), and iron repleted (3 μg/g diet for 4 wk followed by 50 μg/g diet for 2 wk). The diets were prepared according to the AIN-93G diet (24) with cornstarch as the sole source of carbohydrate and ferric citrate as the source of iron. Mean dietary iron concentrations were 48 ± 2 μg/g for the control rats and 3.6 ± 0.9 μg/g for the iron-deficient diets, respectively, as determined using atomic absorption spectrophotometry (Perkin Elmer AAnalyst 600, Perkin Elmer). All animals were housed under controlled environmental conditions with a 12-h-light:12-h-dark cycle (lights on 0600–1800; 25 ± 1°C) and consumed food and distilled water ad libitum. Hematology and liver and spleen non-heme iron concentrations were measured according to methods previously described (17,25). The Pennsylvania State University Institutional Animal Care and Use Committee approved all animal procedures used in this study.

Brain D_2 receptor protein and mRNA expression and iron determinations. For D_2 receptor protein determinations in brain tissue, rats were killed by decapitation between 0900 and 1100 and brains were dissected for ventral midbrain (substantia nigra and ventral tegmentum) and caudate putamen. We chose these 2 regions because the ventral midbrain contains dopamine cell bodies and the caudate is the terminal field of the nigrostriatal dopamine system. Brain regions were homogenized on ice in TBS (10 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, pH 7.4) supplemented with protease inhibitors (Roche Diagnostics). We performed protein extraction, SDS-PAGE, and immunoblotting on brain region homogenates according to the methods described above. Total iron concentrations from brain region homogenates were determined according to our standard laboratory method using ultra-pure nitric acid (Mallinckrot Baker) digestion and analysis with atomic absorption spectrophotometry (26). For tissue RNA analysis, ventral midbrain tissue was rapidly dissected, immediately placed in RNA*later* (Ambion), and stored at −20°C. RNA was isolated using the ToTALLY RNA kit (Ambion) according to the manufacturer's instructions, quantified spectrophotometrically, and examined for integrity by electrophoresis using ethidium bromide staining. Quantitative real-time PCR analysis, as described above, was used to determine mRNA expression in brain tissue.

Membrane-cytosol fractionation of brain homogenates. Caudate from each rat was weighed and homogenized in 10 volumes cold TBS (10 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, pH 7.4) supplemented with protease inhibitors (Roche Diagnostics). A 250-μL aliquot was centrifuged at 500 × g for 2 min at 4°C to clear cell debris. The pellets were discarded and the supernatants centrifuged at 20,000 × g for 30 min at 4°C. The supernatant (cytosolic fraction) was decanted and immediately stored on ice. The pellet (membrane fraction) was resuspended in 750 μL TBS and centrifuged at 20,000 × g for 30 min at

4°C and repeated 3 times. Membrane fractions were then solubilized in 200 μL of cold radio immunoprecipitation assay buffer (25 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% TritonX-100, 1% sodium deoxycholate, and 0.1% SDS) supplemented with protease inhibitors for 60 min at 4°C with constant shaking. Solubilized extracts were centrifuged (16,000 × g; 10 min, 4°C) and protein content of the supernatants was assessed using the BCA protein assay (Pierce) with bovine serum albumin as the standard. SDS-PAGE and immunoblotting of each fraction were performed according to the methods described above.

Data analysis. Data in graphs are represented as means ± SEM. Groups were compared by single-factor ANOVA (SAS). In the presence of significant *F*-values, individual comparisons between means were made using a Tukey's test (for multiple comparisons) or a Dunnett's test (for comparison of experimental groups to control group). Regression analyses were performed to analyze relationships between cellular iron status and D_2 receptor protein and mRNA levels. Significance was set at $P < 0.05$.

Results

Total D_2 receptor protein concentrations in PC12 cells are reduced with iron chelation. We utilized PC12 cell culture models to examine the effects of iron chelation on D_2 receptor expression. Exposure of PC12 cells to DFO for 24 h resulted in a concentration-dependent decrease in D_2 receptor protein densities (**Fig. 1A,B**). D_2 receptor densities were 81 ± 6.3% ($P = 0.07$), 53 ± 4.0% ($P < 0.05$), and 31 ± 5.2% ($P < 0.05$) of controls with DFO treatments of 25, 50, and 100 μmol/L, respectively. To monitor the response of PC12 cells to chelation of the labile iron pool, we measured TfR protein concentrations and mRNA levels of several iron-related proteins as biomarkers for cellular iron deficiency. There were dramatic, dose-dependent increases in TfR band intensities in DFO-treated cells, indicating that the severity of iron chelation is enhanced as the cells are exposed to increasing concentrations of DFO (Fig. 1A,B). TfR protein levels were higher than control at all doses of DFO ($P < 0.05$). Real-time PCR analyses showed a 31% elevation in TfR mRNA levels, a 37% reduction in ferritin H mRNA levels, and a 27% reduction in DMT1 (+IRE) within 24 h of 200 μmol/L DFO treatment ($P < 0.05$ for all; data not shown). Further experiments measuring the labile iron pool with Phen-Green GSK fluorescence showed that DFO treatment for 24 h reduced the chelatable iron pool by >20% ($P < 0.05$, data not shown).

Repletion of iron-chelated PC12 cells (50 μmol/L DFO, 24 h) with FAC (50 mg/L) caused D_2 receptor protein levels to return to control levels within 24 h of being repleted (Fig. 1C,D). Restorations in intracellular iron concentrations after FAC were demonstrated by the return of TfR densities to control levels. D_2 receptor protein concentrations did not change in control cells supplemented with FAC (50 mg/L, 24 h), indicating that iron did not affect D_2 receptor protein expression levels (Fig. 1C,D). In addition, iron supplementation did not change TfR levels. The doses of DFO and FAC used in these experiments did not affect total cellular protein concentrations and overall cell viability (trypan blue exclusion assays).

Moderate changes in PC12 cell D_2 receptor gene expression after iron chelation. The endogenous expression of the D_2 receptor protein in our PC12 cell culture model allowed us to examine whether DFO-induced reductions in the D_2 receptor may be the result of alterations in the synthesis of the protein. Real-time PCR analysis of mRNA extracted from PC12 cells exposed to increasing concentrations of DFO showed D_2 re-
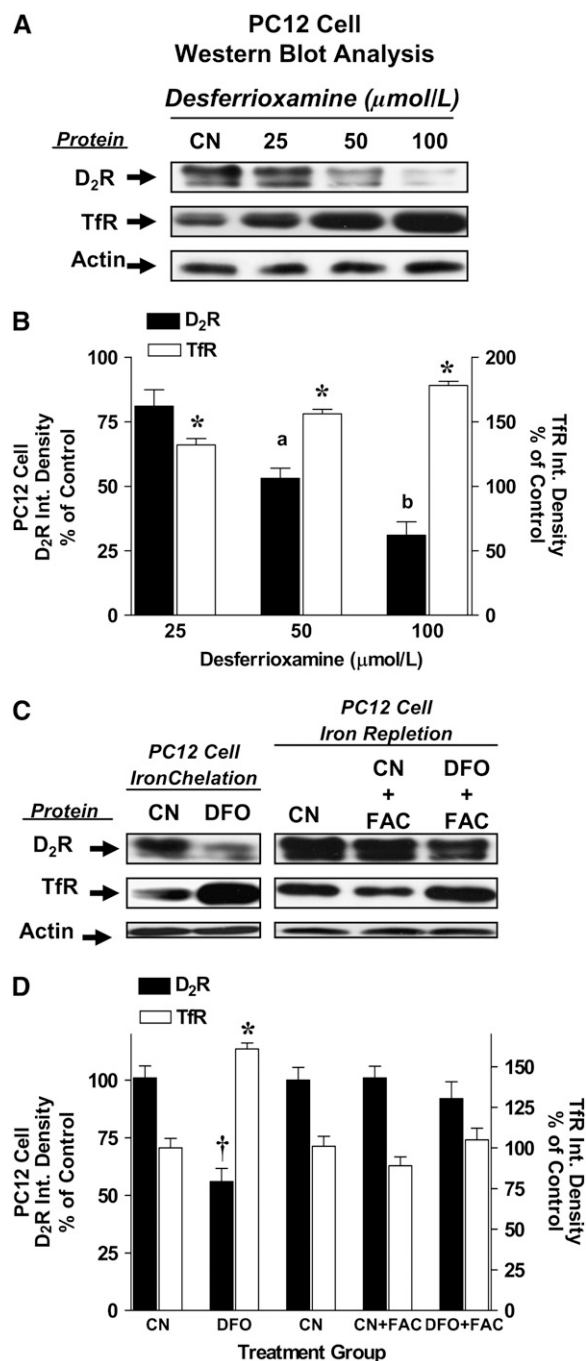

FIGURE 1 Dopamine D_2 receptor and TfR protein concentrations in iron-chelated and iron-repleted PC12 cells. (*A*) Representative ECL photograph of immunoblotted D_2 receptor, TfR, and actin in PC12 cells exposed to 0, 25, 50, and 100 μmol/L DFO for 24 h. CN, Vehicle control. (*B*) Integrated densities for D_2 receptor and TfR after 24 h DFO exposure. Data are mean percent of CN ± SEM, $n = 3$. *Different from CN, $P < 0.001$. Means without a common letter differ, $P < 0.05$. (*C*) Representative ECL photographs of immunoblotted D_2 receptor, TfR, and actin from vehicle- or DFO-treated PC12 cells exposed to FAC for 24 h. Control cells were treated identically excluding the addition of DFO and FAC. (*D*) Integrated densities for D_2 receptor and TfR after iron repletion. Data are percent of CN means ± SEM, $n = 3$. †,*Different from CN, $P < 0.05$.

ceptor mRNA expression levels were 106 ± 8%, 95 ± 8%, and 87 ± 7% of control after a 24 h treatment with 25, 50, and 100 μmol/L DFO, respectively (data not shown). Treatment with

200 μmol/L DFO for 24 h reduced D$_2$ receptor mRNA levels to 59 $\pm$ 7% of control ($P < 0.05$), but D$_1$ receptor mRNA expression did not change (data not shown). To monitor the effects of iron chelation on overall PC12 gene expression, the mRNA levels of GAPDH, β-actin, and Thy-1, an iron-responsive glycopeptide important to synaptic efficacy, were also measured in cell culture model. DFO (25–100 μmol/L) did not affect the expression levels of GAPDH or β-actin. Thy-1 mRNA levels, however, decreased by 38% in PC12 cells exposed to 200 μmol/L DFO for 24 h ($P < 0.05$).

Iron deprivation and repletion in 65-d-old rats. Male rats fed an iron-deficient diet from weaning (postnatal d 21) developed anemia with significantly lower hemoglobin, hematocrit, and serum iron levels than control diet-fed rats ($P < 0.05$ for all). Cellular iron stores in the liver and the spleen were depleted in iron-deficient rats ($P < 0.05$). Rats in the iron-repleted group were fed an iron-deficient diet for 4 wk followed by a control diet for 2 wk. After 2 wk of feeding a control diet, hemoglobin, hematocrit, plasma iron, liver iron, and spleen iron levels did not differ from rats fed the control diet for 6 wk (**Table 1**).

Iron levels but not D2 receptor mRNA expression are altered in ventral midbrain in iron-deficient rats. Dietary iron deficiency for 6 wk resulted in large reductions in ventral midbrain iron concentrations ($P < 0.05$; **Fig. 2A**). D$_2$ receptor mRNA levels tended to be reduced slightly ($P = 0.07$; Fig. 2B). In iron-replete rats, ventral midbrain iron levels were restored to control levels, and D$_2$ receptor protein and mRNA levels were unchanged (Fig. 2A,B). Although D$_2$ receptor mRNA levels in iron-deficient rats did not differ from controls, regression analysis of individual rat data revealed a relationship between ventral midbrain D$_2$ receptor mRNA and ventral midbrain iron concentrations through the influence of a small number of observations in the control and iron repletion groups ($r^2 = 0.412$; $P < 0.05$). Dietary treatment and regional iron changes did not affect β-actin and GAPDH mRNA levels, suggesting that losses in iron concentrations did not globally alter the transcriptional activity within the ventral midbrain (data not shown).

D2 receptor protein levels are reduced in the ventral midbrain in iron-deficient rats. The effects of dietary iron depletion on D$_2$ receptor protein expression patterns were examined in the ventral midbrain, which contains dopamine neuron cell bodies. In iron-deficient rats with 65% reductions in ventral midbrain iron, ventral midbrain D$_2$ receptor protein densities were
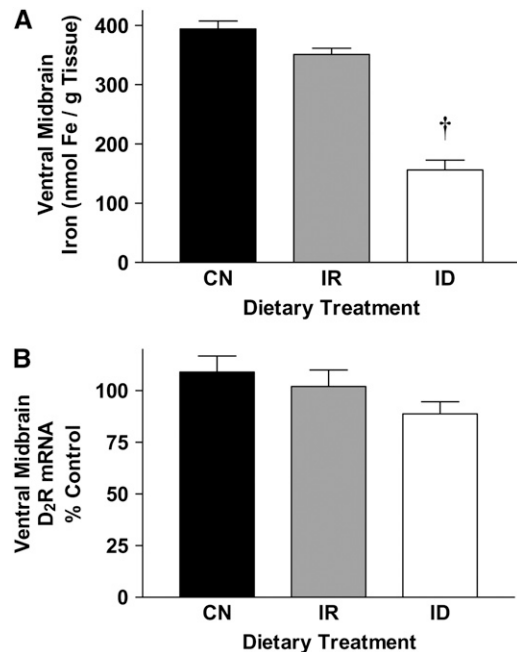

FIGURE 2 Iron and D$_2$ receptor mRNA levels in the ventral midbrain from control (CN), iron-repleted (IR), and iron-deficient (ID) rats. (*A*) Iron concentration in ventral midbrain from CN, IR, and ID rats. Values are means $\pm$ SEM, $n = 6$. †Different from CN, $P < 0.05$. (*B*) Real-time-PCR analysis of ventral midbrain D$_2$ receptor mRNA levels in CN, IR, and ID rats. Values are percent of CN means $\pm$ SEM, $n = 6$.

also significantly lower than rats fed the control diet (**Fig. 3A,B**; $P < 0.05$). Cellular responses to regional iron losses were demonstrated by the large elevations in TfR protein levels in the ventral midbrain of iron-deficient rats ($P < 0.05$; Fig. 3A,B). In iron-repleted rats (Fig. 2A), both D$_2$ receptor and TfR protein densities were similar to control diet-fed rats (Fig. 3A,B). D$_2$ receptor protein densities and total iron content in the ventral midbrain were correlated ($r^2 = 0.519$; $P < 0.05$).

Iron and D2 receptor protein levels are reduced in the caudate from iron-deficient rats. Iron and D$_2$ receptor protein densities were measured in the caudate putamen to demonstrate the impact of an iron-deficient diet on D$_2$ receptor expression patterns in one of the terminal fields of the dopamine system (**Fig. 4**). Iron concentrations and D$_2$ receptor protein densities in the caudate putamen were both reduced by ~50% in rats fed an iron-deficient diet (Fig. 4A–C; $P < 0.05$ for both). Reductions in caudate iron levels were accompanied by large elevations in caudate TfR densities in iron-deficient rats ($P < 0.05$; Fig. 4B,C). Iron, D$_2$ receptor, and TfR levels in iron-replete rats were similar to levels found in rats fed the control diet (Fig. 4A–C). Regression analysis of individual rat data revealed a direct linear relationship between D$_2$ receptor protein and iron levels in the caudate putamen ($r^2 = 0.584$; $P < 0.05$).

D2 receptor densities in caudate putamen membrane fractions are reduced with iron deficiency. Subcellular fractionation studies were conducted to examine the effects of iron deficiency on D$_2$ receptor partitioning between the membrane and intracellular (cytosolic) spaces. D$_2$ receptor levels in iron-deficient rats were significantly lower in both crude cytosolic and membrane fractions from the caudate putamen. The losses of D$_2$ receptor in iron-deficient rats were far greater in the crude
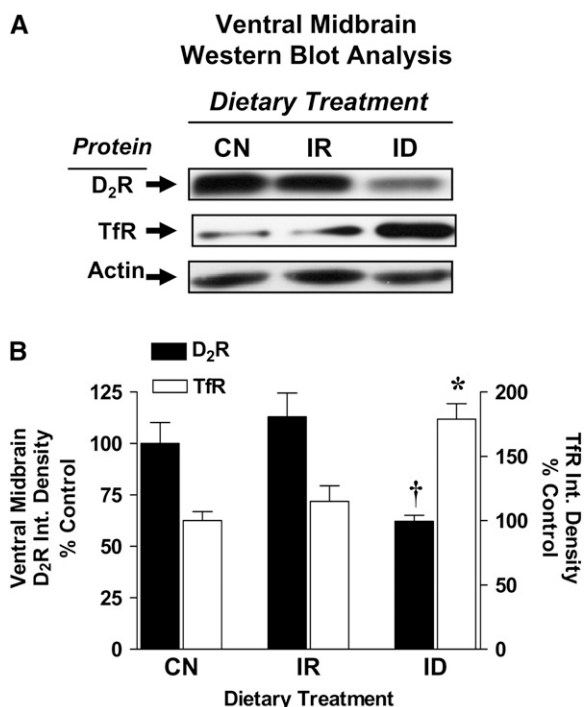
TABLE 1 Hematology and tissue non-heme iron measurements from control, iron-deficient, and iron-repleted rats[1]

	Control	Iron deficient	Repleted
Body weight, g	273 $\pm$ 3	197 $\pm$ 5*	259 $\pm$ 5*
Hemoglobin, g/L	145 $\pm$ 3	49 $\pm$ 2*	142 $\pm$ 3
Hematocrit, %	0.45 $\pm$ 0.01	0.20 $\pm$ 0.005*	0.45 $\pm$ 0.005
Serum iron, μmol/L	33 $\pm$ 8	12 $\pm$ 4*	29 $\pm$ 3
TIBC,[2] μmol/L	83 $\pm$ 7	133 $\pm$ 15*	79 $\pm$ 12
Tf saturation,[3] %	40 $\pm$ 7	9.2 $\pm$ 4*	37 $\pm$ 10
Liver iron, μmol/g	5.8 $\pm$ 0.2	1.3 $\pm$ 0.4*	7.4 $\pm$ 1
Spleen iron, μmol/g	12 $\pm$ 1	2.8 $\pm$ 0.2*	14 $\pm$ 1

[1] Values are means $\pm$ SEM, $n = 6$. *Different from control, $P < 0.05$.
[2] TIBC, total iron-binding capacity.
[3] Tf, Transferrin protein.

FIGURE 3 D_2 receptor protein levels in ventral mibrain from control (CN), iron-repleted (IR), and iron-deficient (ID) rats. (A) Representative ECL photographs of immunoblotted D_2 receptor, TfR, and actin in ventral midbrain from CN, IR, and ID rats. (B) Integrated densities for D_2 receptor and TfR after iron depletion and iron repletion. Data are mean percent of CN ± SEM, $n = 6$. [†],*Different from CN, $P < 0.05$.

FIGURE 4 Iron content and D_2 receptor protein levels in caudate-putamen from control (CN), iron-repleted (IR), and iron-deficient (ID) rats. (A) Mean caudate-putamen iron concentrations in CN, IR, and ID rats. Data are means ± SEM, $n = 6$. [†]Different from CN. (B) Representative ECL photographs of immunoblotted D_2 receptor, TfR, and actin in CN, IR, and ID rats. (C) Mean integrated densities for caudate D_2 receptor and TfR in CN, IR, and ID rats. Data are mean percent of CN ± SEM, $n = 6$. [†],*Different from CN, $P < 0.05$.

membrane fraction ($P < 0.05$; **Fig. 5A,B**) compared with the cytosolic fraction ($P < 0.05$; Fig. 5A,B). In iron-repleted rats, D_2 receptor densities did not differ from control preparations in either subcellular fraction (Fig. 5A,B). Large elevations in caudate putamen TfR densities were detected in both cytosolic and membrane fractions prepared from iron-deficient rats (Fig. 5A,C). Although cytosolic TfR densities normalized to control levels in iron-repleted rats (Fig. 5A,C), TfR densities remained elevated in caudate putamen membrane fractions (168 ± 11.0% of control; $P < 0.05$; Fig. 5A,C). Membrane Thy-1 densities were lower in iron-deficient rats ($P < 0.05$) and remained altered in iron-repleted rats ($P < 0.05$; Fig. 5A,D). Thy-1 immunoreactivity was not detected in any of the cytosolic fractions examined in these experiments, consistent with its location on the cell membrane.

Discussion

The present experiments were designed to determine the influence of cellular iron deficiency on D_2 receptor expression patterns in cell culture and in dopamine-containing regions of the brain. Abnormalities in D_2 receptor expression in iron deficiency have strong implications regarding human functioning, as a number of aspects of learning, decision making, addiction, and movement can all be altered either directly, or indirectly, when this dopamine receptor subtype loses functionality [for recent review see (27)]. Deficits in brain iron and dopamine signaling are also described in RLS, where patients show reduced symptoms after enhancement of dopamine activity (8,9) and an exacerbation in symptoms in response to dopamine receptor antagonists (10,11).
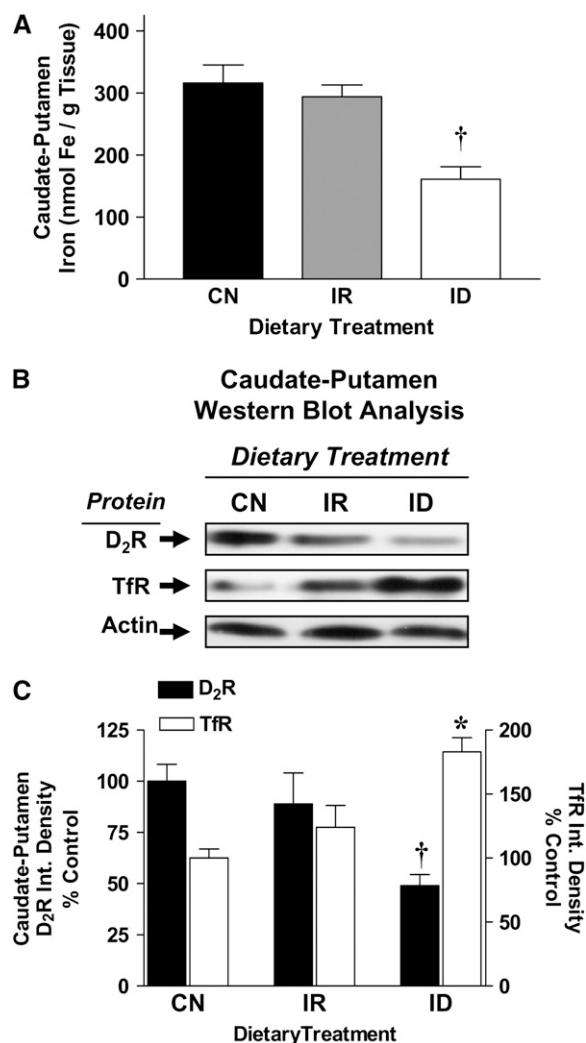
To examine the relationship between iron depletion and the D_2 receptor at the cellular level, we produced an iron deficiency in PC12 cells. PC12 cells were used to model iron deficiency, because they express the dopamine transporter, D_2 receptors, D_1 receptors, and many of the signal transduction components responsible for regulation of the dopamine system. The dopamine transporter expressed in these cells is also responsive to iron chelation in a similar fashion as this transporter is affected in the iron-deficient brain. The cell culture studies presented here revealed that 24-h DFO treatment decreased the cellular levels of the D_2 receptor in a dose-dependent manner without affecting expression of housekeeping proteins or changing cell viability. There were the appropriate changes in iron TfR protein expression and transferrin, ferritin H, and DMT1 gene expression to demonstrate that these cells were iron deficient. In fact, fluorescence assays showed that DFO reduces the iron pool by >20%. Addition of ferric ammonium citrate to iron-chelated cells caused D_2 receptor levels to return to normal levels, again dem-

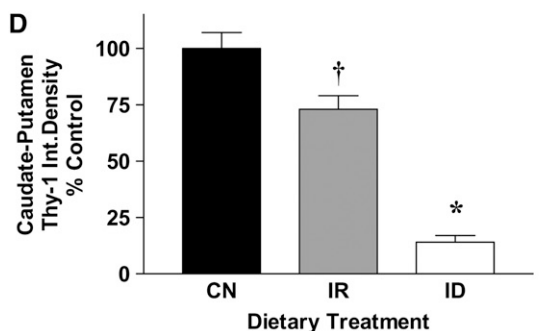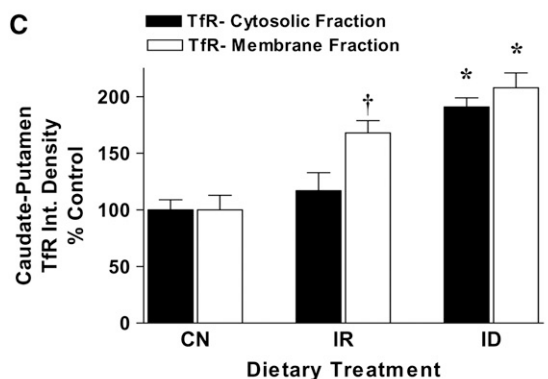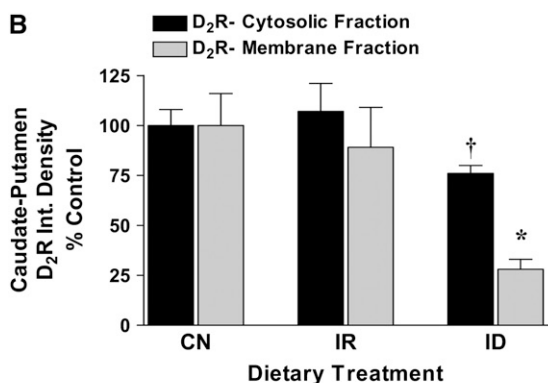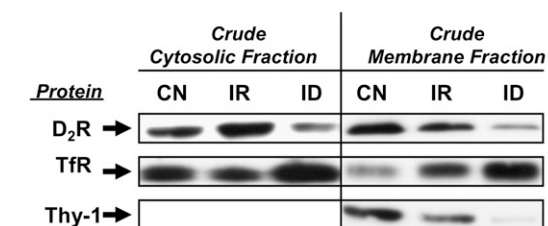**A**   **Caudate-Putamen Subcellular Fractionation Western Blot Analysis**

FIGURE 5 The distribution of D_2 receptor, TfR, and Thy-1 proteins in the caudate putamen from control (CN), iron-repleted (IR), and iron-deficient (ID) rats. (*A*) Representative ECL photographs of immunoblotted membrane and cytosolic fractions of D_2 receptor, TfR, and Thy-1 proteins in CN, IR, and ID animals. (*B*) Mean integrated densities for membrane and cytosolic D_2 receptor from CN, IR, and ID rats. Data are mean percent of CN $\pm$ SEM, $n = 6$. †Different from CN, $P < 0.05$; *$P < 0.01$. (*C*) Mean integrated densities for membrane and cytosolic TfR from CN, IR, and ID rats. Data are percent of CN means $\pm$ SEM, $n = 6$. †Different from CN, $P < 0.05$; *$P < 0.01$. (*D*) Mean integrated densities for membrane and cytosolic Thy-1 from CN, IR, and ID rats. Data are mean percent of CN $\pm$ SEM, $n = 6$. †Different from CN, $P < 0.05$; * Different from CN, $P < 0.01$.

onstrating that these changes in the D_2 receptor were a result of iron chelation. Because DFO chelates several other metals, including copper, cobalt, and zinc, these metals could also play a role in changing D_2 receptor levels. We believe that this is not the case, because DFO has a much higher affinity for iron with binding constants of 10^{31} for ferric iron, 10^{14} for copper (28), 10^{11} for cobalt (28), and 10^{11} for zinc (28–30).

Whereas D_2 receptor protein levels were robustly reduced in iron-chelated PC12 cells and the ventral midbrain of iron-deficient rats, mRNA levels were only modestly affected. At the highest concentration of DFO (100 μmol/L), D_2 receptor mRNA levels were reduced by 13% and protein levels decreased by 69%. Similarly, D_2 receptor mRNA and protein levels were reduced by 11% and 65%, respectively, in ventral midbrain. These data suggest that the decrease in D_2 receptor protein levels in iron-chelated PC12 cells and in the iron-deficient ventral midbrain may not be due to a decrease in transcription. The D_2 receptor gene is transcribed from a TATA-less promoter that has an initiator-like sequence and several putative Sp1/Sp3 and Ap1 transcriptional binding sites (31). Previous work shows that iron chelation alters the levels of these transcription factors but apparently this did not significantly influence mRNA levels in the current paradigm (28,32). Microarray analysis of DFO-treated PC12 cells also did not reveal any effect of iron chelation on these transcription factors (J. Beard and E. Unger, unpublished data).

In the caudate of iron-deficient rats, D_2 receptor protein expression is significantly reduced. We explored the possibility that there was an increase in rate of degradation of this protein due to changes in trafficking (33). Previous studies support the hypothesis that cellular iron deprivation alters the turnover of monoamine-related proteins (20,34). In those studies, iron chelation in neuroblastoma or PC12 cell cultures enhanced the rate of degradation of the dopamine transporter and norepinephrine transporter due to increased movement of the transporters to the lysosomal vesicle pool. Similar, but not identical, regulation of D_2 receptor functionality occurs through trafficking mediated by dopamine receptor multiplex proteins (35). Clardy et al. (36) recently showed that a number of these dopamine receptor-associated proteins are altered by iron deficiency in early life. Our own real-time PCR data also demonstrate that the mRNA of D_2 receptor multiplex proteins, including glycogen synthase kinase 3β, β arrestin 2, protein phosphatase 2A, and regulator of G protein signaling 2 are changed in PC12 cells treated with DFO (J. Beard, unpublished data). We did not explore the direct role of these regulatory proteins in the movement of the D_2 receptor from the cell membrane, although iron deficiency clearly altered membrane D_2 receptor levels far more than cytosolic levels (37,38). In addition, the important synaptic gylcopeptide, Thy-1, is dramatically decreased in striatum of iron-deficient rats, in PC12 cells treated with DFO, and in autopsy brains from RLS patients who have evidence of brain iron deficiency (39). The loss of Thy-1, or dopamine- and cyclic-AMP-regulated phosphoproteins, could affect D_2 receptor recycling on the membrane and thus decrease the half-life of the receptor (35).

The influence of iron status on dopamine is not restricted to just alterations with the dopamine transporter and the D_2 receptor, because there are associated changes in intracellular and extracellular dopamine concentrations in iron-deficient rodents (15,40,41), tyrosine hydroxylase activity in cell culture and rodent models (15,41), and phosphorylation of related proteins through protein kinase C (PKC)-mediated pathways in cells (42). D_2 receptor trafficking is increased with activation of PKC (43). PKC isoforms are known to be sensitive to changes in

cellular iron status, because iron-chelated neuro2A cells have time-dependent alterations in the PKC isoforms α, $\beta 1$, δ, and ε that may reflect a direct effect of iron on PKC or an indirect effect through signal transduction pathways (20). This recent report also demonstrates that application of the PKC inhibitor staurosporine to PC12 cells prevents the reduction in dopamine uptake after iron chelation, providing further support for a possible PKC involvement in D_2 receptor trafficking. Future studies are planned to explore the specific effects of PKC activation on downstream signaling deficits that result from iron chelation.

Overall, the decline in D_2 receptor protein levels in the current study is consistent with previous reports of reductions in D_2 receptor levels in the iron-deficient state (16,19,21). In this report, we did not investigate changes in the presynaptic compared with the postsynaptic D_2 receptors in iron-deficient rats. Presynaptic D_2 auto-receptors regulate reuptake of dopamine through signal transduction pathway(s). Pharmacological studies have revealed that administration of the D_2 agonist quinpirole enhances dopamine transport velocity in striatal synaptosomes (22), whereas the D_2 receptor antagonist raclopride decreases dopamine transport (23). Recent evidence also shows that infusion of quinpirole into striatum increases dopamine transport velocity in rats fed a control diet, whereas the D_2 receptor agonist did not affect uptake in iron-deficient rats. Together, these data suggest that the D_2 receptor, the signal transduction pathway(s), and the dopamine transporter is altered in iron deficiency (18). To date, the mechanism(s) of effect of neuronal iron deficiency on pre- vs. postsynaptic dopamine receptor metabolism or gene expression remains unexplored.

The importance of these observations to nutrition is based on the estimates that as many as 30–50% of the world's infants and children, and > 50% of reproductive-age women, have iron deficiency (44). Changes in brain iron status are related to a number of deficits in brain functioning, of which dopamine metabolism is only one (3). In summary, the results of this study show that in both a cell culture model and a rodent model of iron deficiency, the D_2 receptor is affected by iron status. Whether changes in the D_2 receptor are a direct effect of iron loss or a result of changes in dopamine signaling (possibly related to dopamine transporter deficiencies, altered PKC signaling, etc.) has yet to be elucidated. The persistence of these effects from a developmental perspective has already been demonstrated in rodent models (45,46). The efficacy of iron treatment in RLS to improve brain iron content and dopamine agonist treatment to alter dopamine metabolism is consistent with a relationship between brain iron status and clinical symptoms (7). Future studies will focus on the availability of iron to neurons and whether transients of iron flux in the brain are important to neural functioning.

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