Monoamine transporter inhibitors and norepinephrine reduce dopamine-dependent iron toxicity in cells derived from the substantia nigra

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Abstract

The role of dopamine in iron uptake into catecholaminergic neurons, and dopamine oxidation to aminochrome and its one-electron reduction in iron-mediated neurotoxicity, was studied in RCSN-3 cells, which express both tyrosine hydroxylase and monoamine transporters. The mean ± SD uptake of 100 μM $^{59}FeCl_3$ in RCSN-3 cells was 25 ± 4 pmol per min per mg, which increased to 28 ± 8 pmol per min per mg when complexed with dopamine (Fe(III)–dopamine). This uptake was inhibited by 2 μM nomifensine (43% $p < 0.05$), 100 μM imipramine (62% $p < 0.01$), 30 μM reboxetine (71% $p < 0.01$) and 2 mM dopamine (84% $p < 0.01$). The uptake of ^{59}Fe–dopamine complex was Na^+, Cl^- and temperature dependent. No toxic effects in RCSN-3 cells were observed when the cells were incubated with 100 μM $FeCl_3$ alone or complexed with dopamine. However, 100 μM Fe(III)–dopamine in the presence of 100 μM dicoumarol, an inhibitor of DT-diaphorase, induced toxicity (44% cell death; $p < 0.001$), which was inhibited by 2 μM nomifensine, 30 μM reboxetine and 2 mM norepinephrine. The neuroprotective action of norepinephrine can be explained by (1) its ability to form complexes with Fe^{3+}, (2) the uptake of Fe–norepinephrine complex via the norepinephrine transporter and (3) lack of toxicity of the Fe–norepinephrine complex even when DT-diaphorase is inhibited. These results support the proposed neuroprotective role of DT-diaphorase and norepinephrine.

Keywords: dopamine, iron, monoamine transporter, neuroprotection, neurotoxicity, norepinephrine.

Parkinson's disease is a neurodegenerative disorder characterized by the progressive loss of neurons that comprise the nigrostriatal dopaminergic system. Although it is generally accepted that free radicals are involved in the neurodegenerative process affecting the nigrostriatal system in patients with Parkinson's disease, the exact mechanism of neurodegeneration *in vivo* is still unknown (Alam *et al*. 1997; Facchinetti *et al*. 1998; Jenner 1998; Selley 1998; Ilic *et al*. 1999; Berg *et al*. 2001). One possible mechanism for free radical formation involves iron, as total iron levels in the substantia nigra (SN) of patients with Parkinson's disease are reportedly raised compared with those in age-matched controls (Sofic *et al*. 1988, 1991). Iron is indispensable for life, and it is an essential component of heme and non-heme

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Abbreviations used: COMT, catechol-*O*-methyltransferase; DAT, dopamine transporter; DMEM, Dulbecco's modified Eagle's medium; DMPO, 5,5-dimethylpyrroline-*N*-oxide; DT-diaphorase, NAD(P)H-quinone oxido-reductase (E.C.1.6.99.2); ESR, electron spin resonance; 5HTT, 5-hydroxytryptamine transporter; MAO, monoamine oxidase; NET, norepinephrine transporter; PBS, phosphate-buffered saline; SOD, superoxide dismutase; SN, substantia nigra; TBARS, thiobarbituric reactive substances; TH, tyrosine hydroxylase; VMAT, vesicular monoamine transporter.

proteins that play a vital role in a broad range of cellular functions. However, reduced iron(II) may catalyze formation of hydroxyl radicals in the presence of H_2O_2 via the Fenton reaction.

An alternative pathway for iron-dependent neurotoxicity involves its ability to oxidize dopamine to aminochrome. Indeed, iron(III) has been reported to form an intermediate 1 : 1 complex with dopamine, which decomposes releasing Fe(II) and oxidizing dopamine (Linert *et al*. 1996). Formation of Cu(II)–dopamine also generates aminochrome. (Paris *et al*. 2001). The formation of aminochrome seems to be a normal process as this compound is the precursor of neuromelanin, which is found in dopaminergic neurons located in the SN. However, aminochrome can be one-electron reduced by flavoenzymes to yield leukoaminochrome *o*-semiquinone radical, which is extremely reactive with oxygen and hence neurotoxic (Baez *et al*. 1995; Segura-Aguilar *et al*. 1998, 2001; ; Paris *et al*. 2001; Arriagada *et al*. 2004). Therefore, leukoaminochrome *o*-semiquinone radical has been proposed as one of the major sources for endogenous generation of reactive species involved in the degenerative process leading to Parkinson's disease (Graumann *et al*. 2002; Segura-Aguilar *et al*. 2001).

DT-diaphorase (NAD(P)H-quinone oxido-reductase, EC.1.6.99.2) has been reported to prevent one-electron reduction of aminochrome to leukoaminochrome *o*-semiquinone radical by reducing aminochrome with two electrons to leukoaminochrome (Segura-Aguilar and Lind 1989). DT-diaphorase provides protection against the toxic effects of aminochrome (Arriagada *et al*. 2004) and copper sulfate in RCSN-3 cells (Paris *et al*. 2001), and it prevents both 6-hydroxydopamine-like contralateral rotational behavior and the extensive loss of tyrosine hydroxylase (TH) staining in rats stereotaxically injected with the oxidizing agent manganese pyrophosphate complex into medial forebrain bundle and substantia nigra respectively (Segura-Aguilar *et al*. 2002; Diaz-Veliz *et al*. 2004).

In the present work, we investigated the possible role in iron neurotoxicity of (1) Fe–dopamine complex formation, (2) monoamine transporters in the uptake of Fe–dopamine complex into RCSN-3 cells, (3) dopamine oxidation to aminochrome and its one-electron reduction to reactive species, and (4) the possible protective role of DT-diaphorase and norepinephrine (Troadec *et al*. 2001).

Experimental procedures

Chemicals

Dopamine, nomifensine, norepinephrine, dicoumarol, $FeCl_3$, DMEM/Ham's F-12 nutrient mixture (1 : 1), 5,5-dimethylpyrroline-*N*-oxide (DMPO) and anti-rabbit dopamine transporter (DAT) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Calcein acetoxymethyl/(AM) and ethidium homodimer-1, were from Molecular Probes (Eugene, OR, USA). Rabbit anti-norepinephrine transporter (NET) and rabbit anti-5-hydroxytryptamine transporter (5HTT) were obtained from Chemicon International (Temecula, CA, USA). Reboxetine was from Pharmacia & Upjohn (Kalamazoo, MI, USA) and imipramine was from Laboratory Chile (Santiago, Chile).

Fe–dopamine complex formation

The formation of Fe–dopamine complex was performed in a ratio 1 : 1 at 100 μM in the cell culture medium before addition to the cells. The spectrum was obtained in phosphate-buffered saline (PBS), pH 7.4. Dopamine was determined by HLPC coupled to an electrochemical detector system, as reported previously (Bustamante *et al*. 2004). The concentration of dopamine and $FeCl_3$ was 0.1 μM in PBS pH 7.4.

Establishment of cell line (Raul Caviedes Substantia Nigra (RCSN)-3)

The SN of a 4-month-old Fisher 344 rat was carefully dissected, and the tissue was minced and suspended in 3 mL PBS containing 0.12% (w/v) trypsin (Sigma) and incubated for 30 min at 37°C. The trypsin reaction was stopped by adding an equal volume of plating medium, consisting of Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 nutrient mixture (1 : 1; Sigma) modified to contain 6 g/L glucose, 10% bovine serum, 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma). The suspension was centrifuged (1000 *g*) and the pellet resuspended in 2 mL plating medium. The tissue was dissociated by passages through a fire-polished Pasteur pipette, and the cells plated on to a collagen coated 60-mm culture dish at a density of 40 000 cells/cm^2. At the time of seeding, the plating medium was supplemented with 5% (v/v) medium conditioned by the UCHT1 rat thyroid cell line (Caviedes and Stanbury 1976), which reportedly induces transformation *in vitro* (Caviedes *et al*. 1993, 1994; Cárdenas *et al*. 1999; Allen *et al*. 2000). After 24 h, the initial plating medium was replaced by feeding medium consisting of DMEM/Ham's F-12 nutrient mixture (1 : 1) modified to contain 6 g/L glucose, 10% bovine serum, 2.5% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin (Sigma) and 10% UCHT1 conditioned medium. The cultures were kept in an incubator at 37°C with 100% humidity and an atmosphere of 5% CO_2, and were monitored routinely for appearance of transformation foci or morphological changes, which became evident after variable periods of time (7–8 months) and signaled the establishment of the RCSN cell line.

Cell culture conditions

The RCSN-3 cell line grows in monolayers, with a doubling time of 52 h, a plating efficiency of 21% and a saturation density of 56 000 cells/cm^2 when kept in normal growth media composed of DMEM/Ham's F-12 (1 : 1), 10% bovine serum, 2.5% fetal bovine serum and 40 mg/L gentamicin sulfate (Paris *et al*. 2001; Dagnino-Subiabre *et al*. 2000). The cultures were kept in an incubator at 37°C with 100% humidity and an atmosphere of 10% CO_2.

Cell death

For toxicity experiments, the cells were incubated with cell culture medium deprived of bovine serum and phenol red for 120 min. The concentration used for toxicity experiments was 100 μM

Fe–dopamine complex in the presence and absence of 100 μM dicoumarol. As controls, the cells were incubated with 100 μM dopamine, 100 μM $FeCl_3$, 100 μM dicoumarol or cell culture medium alone. The cells were visualized at 100 × magnification in a Nikon (Melville, N.Y., USA) Diaphot inverted microscope equipped with phase-contrast and fluorescence optics. The toxicity was measured by counting live and dead cells after staining with 0.5 μM calcein AM for 4 min at room temperature (20°C), followed by incubation with 5 μM ethidium heterodimer-1 for 45 min at 37°C. Calcein is a marker for live cells, and ethidium heterodimer-1 intercalates in the DNA of dead cells. The cells were counted in a phase-contrast microscope equipped with fluorescence optix, using the following filters: calcein AM, 450–490 nm (excitation) and 515 nm (emission); ethidium heterodimer-1, 450–495 nm (excitation) and 515–565 nm (emission). Apoptosis was measured by using Alexa fluor 488 annexin V/propidium iodide [Vybrant apoptosis assay kit #2 (Molecular Probes, Engene, OR, USA] with flow cytometry and Alexa fluor annexin V/ethidium heterodimer-1 with fluorescent microscopy.

Fe uptake

The uptake of 100 μM $FeCl_3$ (54 μCi $^{59}FeCl_3$) into RCSN-3 cells was measured under the same conditions used to study cell viability. ^{59}Fe–dopamine complex and ^{59}Fe-norepinephrine complex were formed by adding 100 μM dopamine or 100 μM norepinephrine to cell culture medium containing 100 μM $FeCl_3$ (54 μCi $^{59}FeCl_3$). The uptake of ^{59}Fe was assessed after incubation for 1 min at room temperature. At the end of the uptake period, the extracellular medium was removed and the cells were rapidly washed five times with 2 mL medium to remove residual ^{59}Fe tracer. Cell membranes were disrupted with 1 mL 1% Triton X-100 and, after 15 min incubation, 900 μL of the cell/Triton X-100 extract was removed and analyzed for ^{59}Fe tracer content by liquid scintillation counting. The remaining 100 μL was used for protein determination by the bicinconinic acid method. The results were expressed as nmoles per milligram protein per minute. ^{59}Fe–dopamine complex was also incubated in the presence of 2 μM nomifensine (an inhibitor of the DAT), 30 μM reboxetine (an inhibitor of the NET); 100 μM imipramine (an inhibitor of the 5HTT), 2 mM dopamine or 200 μM norepinephrine.

Immunofluorescence analysis by confocal microscopy

Coverslips containing control RCSN-3 cells grown to 50% confluence were washed twice with Dulbecco's PBS, pH 7.4. They were then fixed for 30 min with methanol at − 20°C. The cells were rinsed twice with PBS and blocked with 1.5% bovine serum albumin diluted in PBS for 40 min. The blocking solution was then aspirated and the cells were rinsed with PBS. The coverslips were incubated with the primary antibody (rabbit anti-DAT, 1 : 1000; rabbit anti-NET, 1 : 1000; rabbit anti-5HTT, 1 : 500) in PBS overnight. The primary antibody was aspirated, and the cells were washed three times with PBS. After washing, the cells were incubated for 1 h with secondary antibody (biotinylated anti-rabbit IgG [H + L]; Vector Laboratories, Burlingame, CA, USA) diluted 1 : 250 in PBS. The secondary antibody was removed and the cells were washed three times with PBS. The cells were incubated with Cy™-3-conjugated streptavidin 3 μg/mL (Jackson Inmunoresearch Laboratories, West Grove, PA, USA) for 1 h. The streptavidin solution was removed and cells were washed three times with PBS. Coverslips were mounted on to slides with fluorescent mounting medium (Dako, Carpinteria, CA, USA) and kept in the dark at 4°C. Confocal microscopy (Zeiss, Göttingen, Germany; model LSM-410 Axiovert-100) was used to study the cells. Sample illumination was carried out via a He–Ne laser with 543-nm excitation filter and emission filter over 560 nm. Z-resolution imaging allowed a thickness of 1.5 μm for the DAT, and 1.0 μm for the NET and 5HTT.

Spectroscopy

Electron spin resonance (ESR) spectra were recorded in X band (9.85 GHz) using a Bruker (Siberstreifen, Germany) ECS 106 spectrometer with a rectangular cavity and 500-KHz field modulation. Spectrometer conditions were microwave frequency 9.68 GHz, microwave power 20 mW, modulation amplitude 0.4 G, scan rate 0.83 G/s, time constant 0.25 s, number of scans 10. The hyperfine splitting constants were estimated to be accurate within 0.05 G. The cells were incubated for 5 min with 100 mM DMPO before the addition of 100 μM $FeCl_3$, 100 μM Fe–dopamine complex, 100 μM Fe–dopamine complex and 100 μM dicoumarol, 100 μM Fe–dopamine complex plus 100 μM dicoumarol in the presence of 2 μM nomifensine, or 100 μM Fe–dopamine complex plus 100 μM dicoumarol in the presence of 30 μM reboxetine, and incubation for 1 h. In order to compare the ESR spectra for cells incubated with Fe–dopamine complex in the presence and absence of dicoumarol we integrated the peaks to calculate the area and the statistical significance was assessed by Student's *t*-test. The spectra of 100 μM $FeCl_3$, 100 μM norepinephrine or 100 μM $FeCl_3$ plus 100 μM norepinephrine were obtained in water at room temperature in an Agilent (Palo Alto, CA, USA) 8453 spectrophotometer.

Determination of thiobarbituric reactive substances (TBARS)

The amount of aldehydic products generated by lipid peroxidation was quantified by the thiobarbituric acid reaction (Ohkawa *et al*. 1979).

Messencephalic cell cultures

Animals were treated in accordance with the Care and Use of Laboratory Animals, the European Directive no. 86/609, and the guidelines of the local institutional animal care and use committee. Cultures of post-mitotic dopaminergic neurons were prepared from the ventral mesencephalon of 15.5-embryonic-day Wistar rat embryos as described previously (Michel and Agid 1996). Cultures plated at a density of 1–1.5 × 10^5 cells/cm^2 were maintained in 500 μL chemically defined serum-free medium consisting of equal volumes of DMEM and Ham's F12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 1% penicillin and streptomycin, 1% glutamine, 1% N_2 and 2 μM MK-801. The survival of dopaminergic neurons was quantified by counting the number of cells immunoreactive for TH according to the method of Troadec *et al*. (2001). The cultures were fixed with a mixture of glutaraldehyde/formaldehyde (0.5 : 4%) in PBS for 1 min, and then with formaldehyde (4%) alone for another 20 min. Cells were then washed three times with PBS and processed for immunohistochemistry by incubating for 2 days at 4°C with an anti-TH antiserum (Pel-Freez, Rogers, AR, USA) diluted 1 : 250 in PBS containing 0.2% Triton X-100. Subsequent incubations were performed at room temperature with a

secondary biotinylated anti-mouse IgG (1 : 500 in PBS; Jackson Immunoresearch Laboratories) followed by amplification with an avidin biotinylated horseradish peroxidase preformed complex (Vectastain; Vector Laboratories). Finally, the cells were stained by incubation with a solution of diaminobenzidine (1 mg/mL) containing 0.006% hydrogen peroxide.

Data analysis

All data were expressed as mean ± SD values. Statistical significance was assessed using ANOVA for multiple comparisons, and Student's *t*-test for comparison between two groups.

Results

We have studied the possible role of Fe–dopamine complex formation and subsequent oxidation of dopamine to aminochrome (Linert *et al*. 1996) as a mechanism of iron toxicity in RCSN-3 cells. Incubation of 200 μM $FeCl_3$ with 200 μM dopamine resulted in formation of a new absorption maximum at 293 nm, suggesting the formation of Fe–dopamine complex (Fig. 1a). Under these conditions of Fe–dopamine complex formation (1 : 1), 90% of dopamine reacted with $FeCl_3$, as observed by HPLC-electrochemical detector (ED) (Fig. 1c). Incubation of RCSN-3 cells for 2 h with 100 μM $FeCl_3$ alone or 100 μM Fe–dopamine complex did not result in significant cell death (7.8 ± 4 and 15 ± 4%, respectively (Fig. 2). However, cell death increased significantly on incubation with 100 μM Fe–dopamine complex plus 100 μM

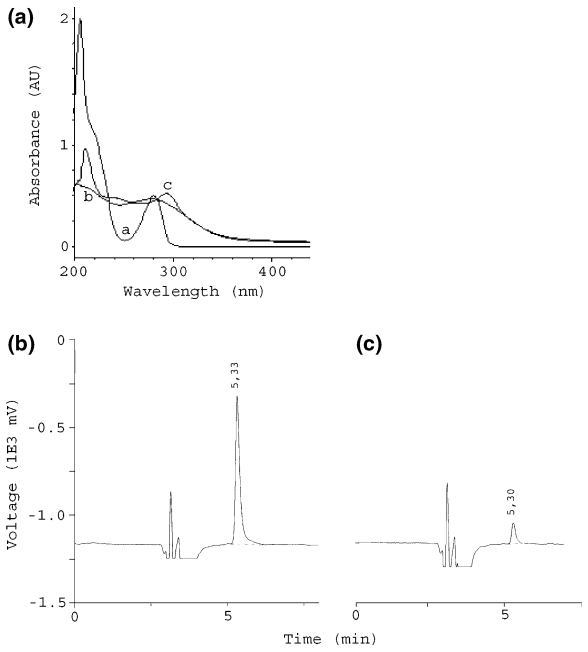

Fig. 1 Fe–dopamine complex formation. (a) Absorption spectra of 200 μM dopamine (a), 200 $FeCl_3$ (b) and 200 μM Fe–dopamine complex (c) in PBS, pH 7.4. Dopamine (signal at 5.3 min) determined by HPLC with an electrochemical detector in 0.1 μM dopamine (b) and 0.1 μM Fe–dopamine complex (c) in PBS, pH 7.4.

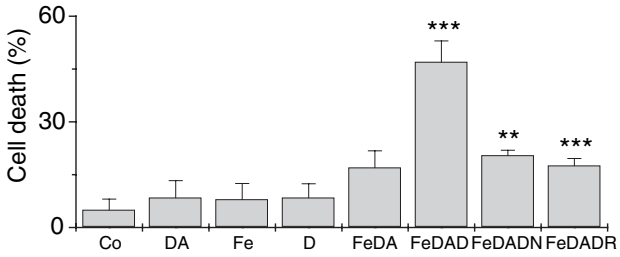

Fig. 2 Effect of Fe–dopamine complex plus dicoumarol on RCSN-3 cell death in the absence and presence of nomifensine and reboxetine. RCSN-3 cells were treated with 100 μM Fe–dopamine complex plus 100 μM dicoumarol (FeDAD) and in the presence of 2 μM nomifensine (FeDADN) or 30 μM reboxetine (FeDADR). As controls, the cells were incubated with cell culture medium (Co), 100 μM dopamine (DA), 100 μM $FeCl_3$ (Fe), 100 μM dicoumarol (D) or or 100 μM Fe–dopamine complex (FeDA). Values are mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$ (ANOVA for multiple comparisons and Student's *t*-test). The significance for FeDAD was obtained versus control and for FeDADN and FeDADR were obtained versus FeDAD.

of the DT-diaphorase inhibitor dicoumarol (44 ± 5% $p < 0.001$). No significant cell death was observed when the cells were first loaded with 100 μM $FeCl_3$, washed, then loaded with 100 μM dopamine and finally incubated for 2 h in the presence of 100 μM dicoumarol (data not shown). Interestingly, this cell death was inhibited by compounds that block the DAT (nomifensine 2 μM; 54% inhibition; $p < 0.01$) and the NET (reboxetine 30 μM; 62% inhibition; $p < 0.001$). The effect of inhibiting the 5HTT with 100 μM imipramine was inconclusive, owing to the direct toxic action of the compound on RCSN-3 cells (data not shown). No significant cell death was evident in RCSN-3 cells incubated solely with 100 μM dopamine (8.4 ± 4%) or 100 μM dicoumarol (8.3 ± 4%) (Fig. 2).

In order to determine whether the toxic effects of Fe–dopamine complex plus dicoumarol were restricted to cells expressing monoamine transporter, we used a neuronal cell line CNh derived from mouse cerebral cortex, which does not express such transporters. No toxic effects were observed in CNh cells exposed to 100 μM Fe–dopamine complex plus dicoumarol for 2 h (data not shown).

We also tested the effect of Fe–dopamine complex on dopaminergic neurons in mesencephalic cell cultures at lower concentrations and a longer exposure time (5 days). The cell culture treated with 10 μM Fe–dopamine complex together with 10 μM dicoumarol showed a 77% decrease (186 ± 16; $p < 0.001$) in the number of TH-positive cells compared with control cells (802 ± 42). Incubation with 10 μM Fe–dopamine complex induced a 51% decrease in TH-positive cells (396 ± 63; $p < 0.01$) but there was no significant effect of 10 μM $FeCl_3$ (599 ± 76), 10 μM dopamine (644 ± 52) or 10 μM dicoumarol (753 ± 95) on the number of TH-positive cells. It was not possible to test the role of Fe–dopamine

complex uptake via the DAT because of an intrinsically toxic effect of 2 μM nomifensine (data not shown).

In order to determine whether this cell death induced by 100 μM Fe–dopamine plus 100 μM dicoumarol involved an apoptotic process, we used annexin V/propidium iodide or annexin/ethidium heterodimer-1 with fluorescent microscopy, but no apoptosis was observed after 2 h. As a positive control, we used cells exposed to 100 μM menadione for 2 h (not shown).

The possibility that monoamine oxidase (MAO) or catechol-*O*-methyl transferase (COMT) may inhibit cell death mediated by the Fe–dopamine complex by preventing aminochrome formation as a consequence of dopamine metabolism catalyzed by MAO and/or COMT was also studied. L-Deprenyl, an inhibitor of MAO-B, 4-methylthioamphetamine, an inhibitor of MAO-A, and tocalpone, an inhibitor of COMT, were used to study the possible role of these enzymes in the observed Fe–dopamine complex-dependent toxicity. However, no increase in cell death was observed in the presence of MAO or COMT inhibitors, and a non-significant reduction in toxicity of cells treated with 100 μM Fe–dopamine complex together with 100 μM dicoumarol was observed in the presence of 1 μM 4-methylthioamphetamine (80 ± 30%), 1 μM L-deprenyl (91 ± 6%) or 1 μM tocalpone (92 ± 16%). The presence of MAO or COMT inhibitors did not change the inhibitory effect of reboxetine (30 μM), nomifensine (2 μM) or norepinephrine (200 μM) on cell death induced by 100 μM Fe–dopamine complex together with 100 μM dicoumarol (data not shown).

Because DAT and NET inhibitors decreased the cell death induced by Fe–dopamine complex plus dicoumarol, we studied the uptake of $^{59}FeCl_3$, alone or complexed with dopamine, in the absence and presence of monoamine transporter inhibitors. The uptake of 100 μM $FeCl_3$ (54 μCi $^{59}FeCl_3$) into RCSN cells was 25 ± 4 pmol per min per mg, which was not inhibited at 4°C (26 ± 6 pmol per min per mg). A non-significant increase in Fe uptake compared with that in the presence of $FeCl_3$ alone was observed when the cells were incubated with 100 μM Fe–dopamine complex (28 ± 8 pmol per min per mg). The uptake of 100 μM Fe–dopamine complex was inhibited by 2 μM nomifensine (43% $p < 0.05$), 100 μM imipramine (62% $p < 0.01$), 30 μM reboxetine (71% $p < 0.01$) and 2 mM dopamine (84% $p < 0.01$) (Fig. 3). Monoamine transporter inhibitors did not inhibit uptake of 100 μM $^{59}FeCl_3$ (data not shown). The uptake was found to occur with high affinity, in a concentration-dependent and saturable manner (Fig. 4a), with an estimated K_m of 0.46 ± 4 μM and V_{max} of 23 ± 4 pmol per min per mg using a Lineweaver–Burk plot. Na^-, Cl^- and temperature dependency of Fe–dopamine complex uptake was also studied. ^{59}Fe–dopamine complex uptake decreased to 61% of control values ($p < 0.001$) when the cells were incubated with buffer in which Cl^- was substituted by sodium gluconate, potassium gluconate and $Ca(NO_3)_2$.

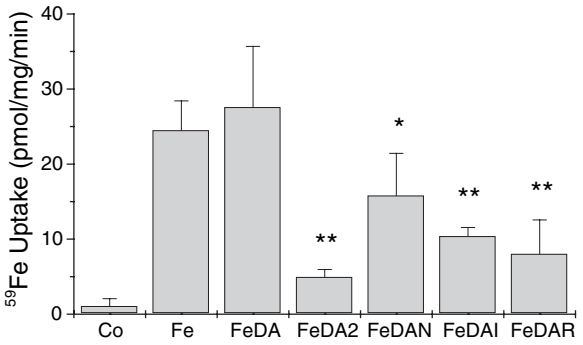

Fig. 3 Uptake of ^{59}Fe–dopamine complex into RCSN-3 cells in the absence and presence of inhibitors of monoamine transport. The uptake of ^{59}Fe–dopamine complex (FeDA) into RCSN-3 cells was measured in cells kept in cell culture medium without bovine serum. RCSN-3 cells were incubated with ^{59}Fe–dopamine complex in the presence of 2 mM dopamine (FeDA2), 2 μM nomifensine (FeDAN), 100 μM imipramine (FeDAI) or 30 μM reboxetine (FeDAR). As control the cells were incubated in the absence (Co) and presence of 100 μm $FeCl_3$ (Fe). The significance values were obtained by comparison with FeDA $^*p < 0.05$; $^{**}p < 0.01$ (ANOVA for multiple comparisons and student's *t*-test). Values are mean ± SD ($n = 3$). $^*p < 0.05$; $^{**}p < 0.01$ (ANOVA for multiple comparisons and Student's *t*-test).

Incubation of cells with buffer in which Na^+ was substituted by LiCl decreased the ^{59}Fe–dopamine uptake to 39% of the control values ($p < 0.001$) (Fig. 4b). The ^{59}Fe–dopamine complex uptake decreased to 49% at 4°C ($p < 0.05$) (Fig. 4c).

The inhibitory effect of monoamine transporter inhibitors on cell death and on the uptake of the Fe–dopamine complex, observed when the cells were treated with Fe–dopamine complex plus dicoumarol, suggests that RCSN-3 cells express these transporters. This was supported by the finding that RCSN-3 cells stained positive for the DAT, the NET and the 5HTT in immunohistochemical studies using specific antibodies (Figs 5–7 respectively). In order to determine whether these transporters are localized in the membrane of RCSN-3 cells we examined Z-sections 1 or 1.5 μm thick from the top to the bottom of the cell using confocal microcopy. In the Z-sections from 3.0 to 10.5 μm (Fig. 5b), 5 to 8 μm (Fig. 6b), and 3 to 8 μm (Fig. 7b) it was possible to ascertain that the DAT, NET and 5HTT respectively were mainly localized in the cell membrane.

The toxic effects of the Fe–dopamine complex plus dicoumarol on RCSN-3 cells suggest the intracellular formation of reactive species (superoxide radicals, hydrogen peroxide and hydroxyl radicals) and possible peroxidation of membranes. We therefore determined the formation of TBARS as an indication of lipid peroxidation (Fig. 8). A significant increase in TBARS was observed when the cells were incubated with Fe–dopamine complex plus dicoumarol (284 ± 13 pmol/mg; $p < 0.001$) compared with controls (139 ± 7 pmol/mg) or cells treated with 100 μM $FeCl_3$

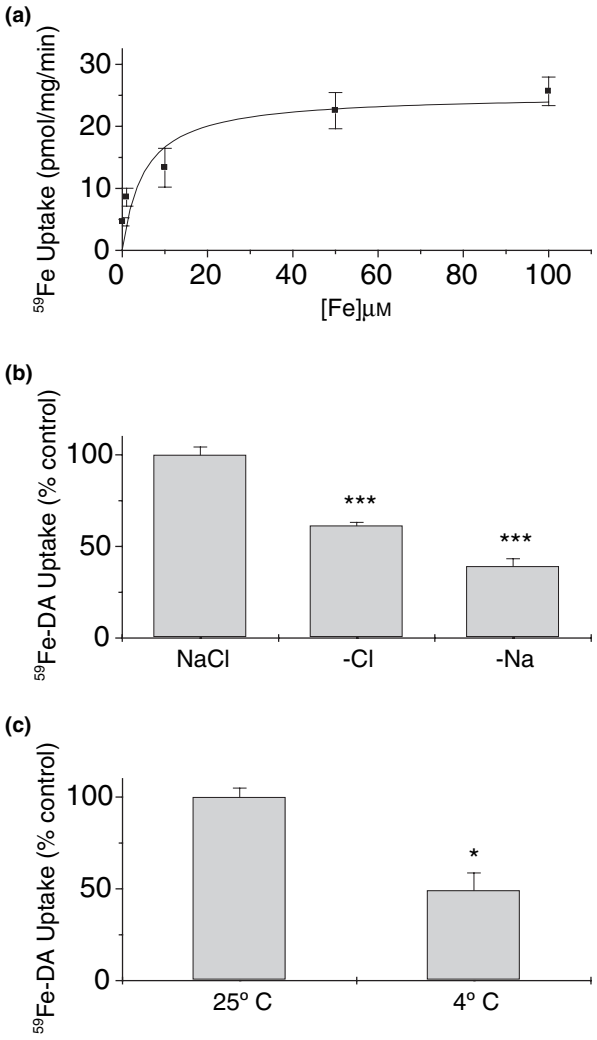

Fig. 4 Characterization of Fe–dopamine complex uptake into RCSN-3 cells. (a) Saturation of Fe–dopamine uptake into RCSN-3 cells was determined by incubating the cells with 100 μM dopamine, and with increasing concentrations of $FeCl_3$ for 1 min at room temperature. (b) Na^+, Cl^- dependence was determined by substituting Na^+ with LiCl, and Cl^- with sodium gluconate and $Ca(NO3)_2$. (c) Temperature dependence of Fe–dopamine uptake. Values are mean ± SD ($n = 3$). $^*p < 0.05$; $^{***}p < 0.001$ (ANOVA for multiple comparisons and Student's *t*-test).

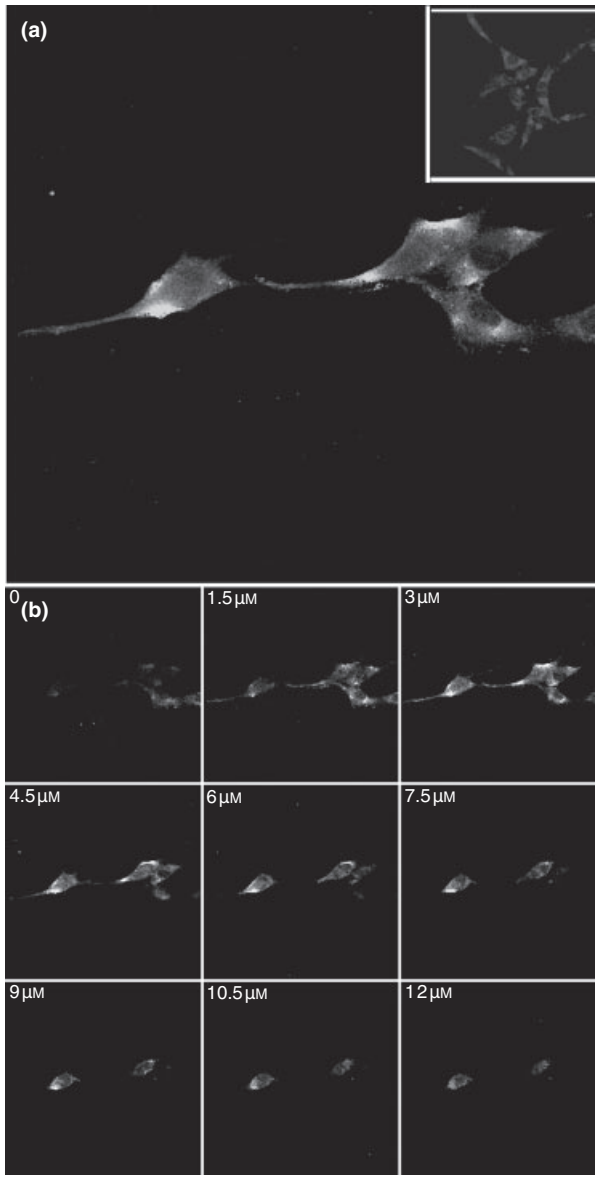

Fig. 5 Confocal microscopy images of fluorescence and Z-sections of RCSN-3 cells immunostained for the DAT. (a) Immunostained RCSN-3 cells visualized by confocal microscopy. Cells were incubated with anti-DAT antiserum, and Z-section images were integrated into one picture. The inset shows control conditions, in which RCSN-3 cells were incubated in the absence of anti-DAT antiserum. (b) Z-sections 1.5 μm thick from top to bottom of RCSN-3 cells incubated with anti-DAT antiserum.

(148 ± 6 pmol/mg). Interestingly, incubation of Fe–dopamine plus dicoumarol in the presence of 30 μM reboxetine or 2 μM nomifensine completely inhibited the formation of TBARS (140 ± 22 and 127 ± 39 respectively; both $p < 0.01$). No significant increase in TBARS was observed in cells treated with 100 μM dopamine, 100 μM dicoumarol, 2 μM nomifensine, 30 μM reboxetine or 100 μM Fe–dopamine complex (Fig. 8).

The formation of hydroxyl radicals during incubation of RCSN-3 cells for 1 h with 100 μM Fe–dopamine complex in the absence and presence of 100 μM dicoumarol was determined by using ESR with the spin-trapping agent DMPO. No ESR signal was observed in RCSN-3 cells treated with 100 μM $FeCl_3$ when the cells were incubated with DMPO (Fig. 9a). When the cells were incubated with 100 μM Fe–dopamine complex, a well resolved ESR spectrum appeared. The ESR signal intensity was consistent with the trapping of both the hydroxyl radical and an unknown

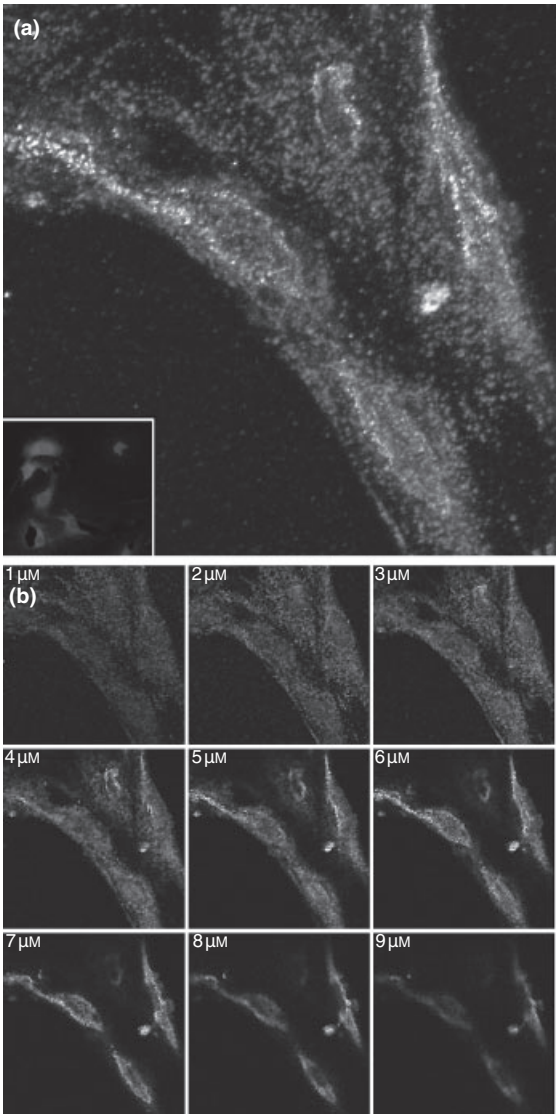

Fig. 6 Confocal microscopy images of fluorescence and Z-sections of RCSN-3 cells immunostained for the NET. (a) Immunostained RCSN-3 cells imaged using confocal microscopy. Cells were incubated with anti-NET antiserum, and Z-section images were integrated into one picture. The inset shows control conditions, in which RCSN-3 cells were incubated in the absence of anti-NET antiserum. (b) Z-sections 1.5 μm thick from top to bottom of RCSN-3 cells incubated with anti-NET antiserum.

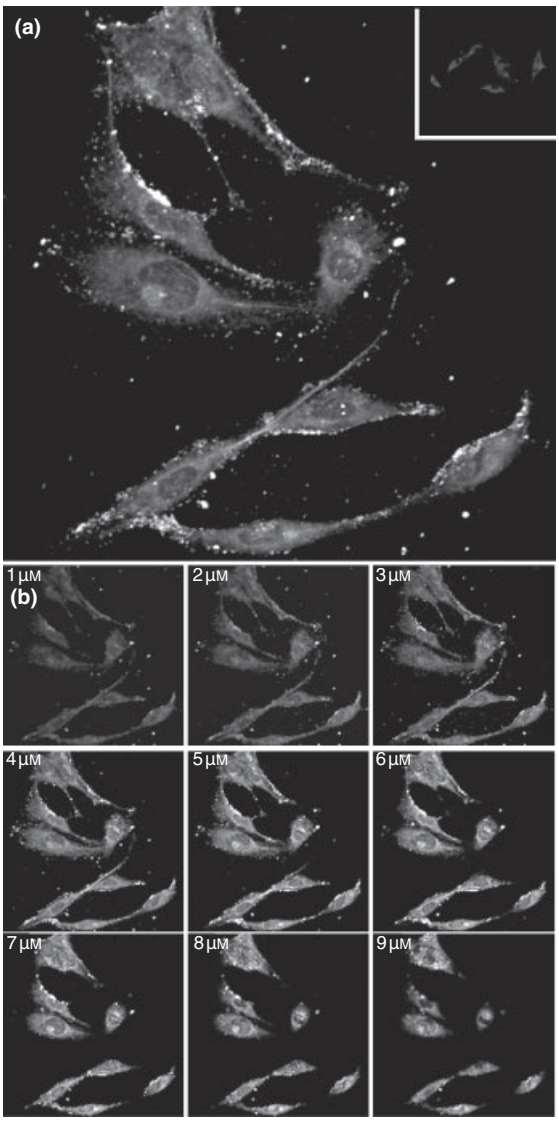

Fig. 7 Confocal microscopy images of fluorescence and Z-sections of RCSN-3 cells immunostained for the 5-HTT. (a) Immunostained RCSN-3 cells imaged using confocal microscopy. Cells were incubated with anti-5HTT antiserum and Z-section images were integrated into one picture. The inset shows control conditions, in which RCSN-3 cells were incubated in the absence of anti-5HTT antiserum. (b) Z-sections 1.5 μm thick from top to bottom of RCSN-3 cells incubated with anti-5HTT antiserum.

radical probably centered in C atom (DMPO-OH spin adduct $a_N = a_H = 14.78$ G; DMPO-Nitro 2 spin adduct $a_N = 16.20$ G, $a_H = 23.30$ G) (Fig. 9b). These hyperfine constants are in agreement with the splitting constants of other DMPO-OH adducts and carbon-centered radicals trapped by DMPO. A similar hyperfine pattern was observed when the cells were incubated with 100 μM Fe–dopamine complex together with 100 μM dicoumarol (Fig. 9c). However, the intensity of the signal attributed to the DMPO-OH adduct increased by 30% and that of the DMPO-C· adduct increased by 50% compared with the spectrum of the Fe–dopamine complex. No ESR signal was observed when RCSN-3 cells treated with 100 μM Fe–dopamine complex together with 100 μM dicoumarol in the presence of 2 μM nomifensine or 30 μM reboxetine (Figs 9e and d respectively) were incubated with DMPO.

Interestingly, the cell death observed in the presence of 100 μM Fe–dopamine complex plus 100 μM dicoumarol was

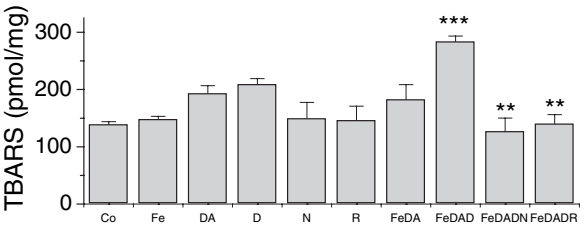

Fig. 8 Determination of TBARS during incubation of RCSN-3 cells with Fe–dopamine complex plus dicoumarol. Cells were incubated with 100 μM Fe–dopamine complex plus 100 μM dicoumarol (FeDAD) and in the presence of 2 μM nomifensine (FeDADN) or 30 μM reboxetine (FeDADR). As controls, cells were incubated with cell culture medium (Co), 100 μM $FeCl_3$ (Fe), 100 μM dopamine (DA), 100 μM dicoumarol (D) 2 μM nomifensine (N), 30 μM reboxetine (R) or 100 μM Fe–dopamine complex (FeDA). Values are mean ± SD. $^{**}p < 0.01$; $^{***}p < 0.001$ (ANOVA for multiple comparisons and Student's *t*-test).

inhibited by 200 μM norepinephrine (84% inhibition; $p < 0.001$) (Fig. 10). One possible explanation for the neuroprotective action of norepinephrine is that norepinephrine competes with dopamine to react with $FeCl_3$, thus forming a complex with Fe^{3+}. This is supported by the finding that the spectral peaks of 100 μM norepinephrine (192, 225 and 279 nm) were shifted to 204 and 281 nm when 100 μM norepinephrine was incubated with 100 μM $FeCl_3$ (Fig. 11a). The uptake of ^{59}Fe decreased by 40% when the cells were incubated with 100 μM Fe–norepinephrine complex (15 ± 4 nmol/mg/min) in comparison to the uptake of $FeCl_3$ alone (26 ± 5 nmol/mg/min) (Fig. 11b). The uptake mediated by Fe–norepinephrine complex was inhibited by 30 μM reboxetine (60% inhibition; $p < 0.05$) and 2 mM norepinephrine (72% inhibition; $p < 0.01$). Interestingly, no significant inhibitory effect of 2 μM nomifensine or 100 μM imipramine was observed (14 ± 1 and 14.1 ± 5 pmol/min/mg respectively; Fig. 11b). Finally, we studied the possible toxic effects of 100 μM Fe–norepinephrine in the absence and presence of 100 μM dicoumarol, but no such effects were observed (data not shown).

Discussion

Excess iron in the brain has been associated with several pathological conditions including Parkinson's disease; a significant increase in iron content has been observed in SN of both post-mortem parkinsonian brains and in live patients using imaging techniques. (Sofic *et al*. 1988; Dexter *et al*. 1989; Gorell *et al*. 1995; Berg *et al*. 2002b). Furthermore, the hereditary deficiency of ceruloplasmin in aceruloplasminemia is associated with basal ganglia degeneration and iron accumulation in the brain (Miyajima *et al*. 1996; Morita *et al*. 1995). In this regard, ceruloplasmin ferroxidase activity decreased by 40% in untreated patients with Parkinson's disease (Boll *et al*. 1999).

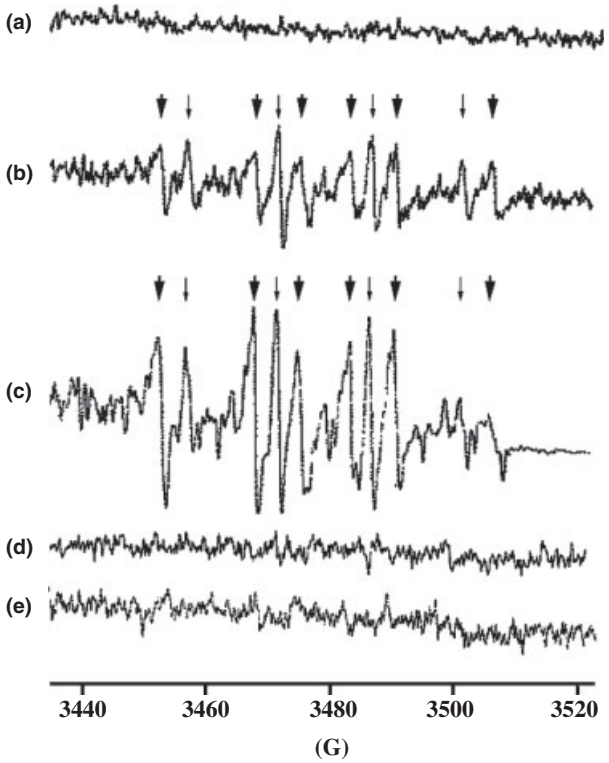

Fig. 9 Determination of hydroxyl radical-DMPO ESR signal during incubation of RCSN-3 cells. The formation of hydroxyl radicals was determined by using the spin-trapping agent DMPO when RCSN-3 cells were incubated with (a) 100 μM $FeCl_3$ and 100 mM DMPO, (b) 100 μM Fe–dopamine complex and 100 mM DMPO, (c) 100 μM Fe–dopamine complex plus 100 μM dicoumarol and 100 mM DMPO. Spectrometer conditions are described in Experimental Procedures. [DMPO-OH adduct (thin arrows): $a_N = a_H = 14.78$ G; DMPO-C3 unknown adduct (bold arrows): $a_N = 16.2$ G, $a_H = 23.30$ G]. (d) 100 μM Fe–dopamine complex plus 100 μM dicoumarol in the presence of 30 μM reboxetine and 100 mM DMPO; (e) 100 μM Fe–dopamine complex plus 100 μM dicoumarol in the presence of 2 μM nomifensine and 100 mM DMPO.

A pivotal role of iron in the pathogenesis of Parkinson's disease has been emphasized because of its ability to enhance the production of oxygen radicals and to accelerate neuronal degeneration (Sofic *et al*. 1988; Dexter *et al*. 1993; Shoham and Youdim 2000; Berg *et al*. 2002a; Andersen 2003; Gerlach *et al*. 2003). Until now, the neurotoxic action of iron has been thought to be restricted to its ability to catalyze the formation of hydroxyl radicals in the Fenton reaction. In this paper we propose a more specific neurotoxic action of iron in cells containing monoamine transporters (DAT, NET and 5HTT). However, the question is whether free iron is available in the extracellular space to form complexes with dopamine. It is possible that free iron exists in the extracellular space under conditions of iron overload in the brain. Iron uptake through the blood–brain barrier is carried out by receptor-mediated uptake of iron–transferrin complexes (for

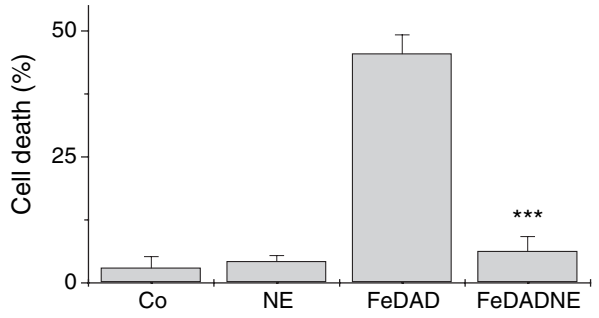

Fig. 10 Effect of norepinephrine on death of RCSN-3 cells induced by Fe–dopamine complex plus dicoumarol. The cells were incubated with 200 μM norepinephrine (NE), and 100 μM Fe-dopamine complex plus 100 μM dicoumarol in the absence (FeDAD) or presence (FeDADNE) of 200 μM norepinephrine. Control cells were incubated with cell culture medium (Co). The statistical significance of FeDADNE was compared to FeDAD. Values are mean ± SD. ***$p < 0.001$ (ANOVA for multiple comparisons and Student's *t*-test).

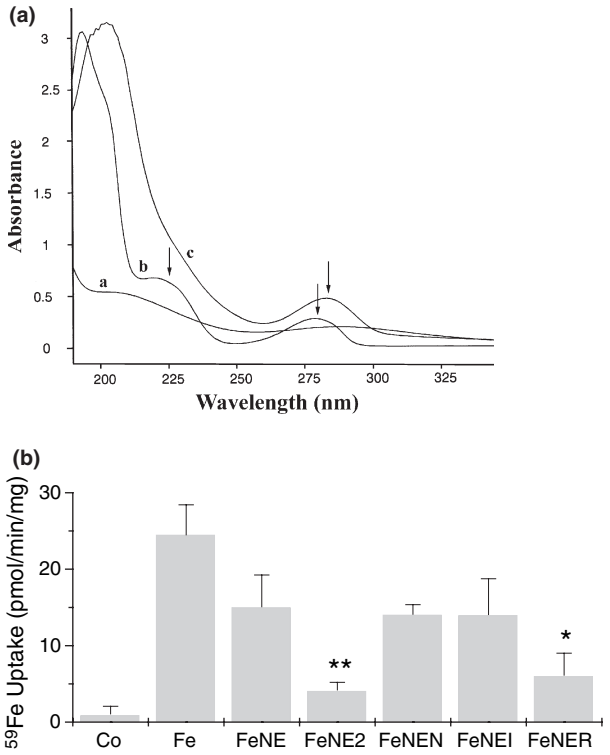

Fig. 11 Formation of Fe–norepinephrine complex and uptake into RCSN-3 cells. (a) The spectra of 100 μM $FeCl_3$ (a), 100 μM norepinephrine (b) and 100 μM $FeCl_3$ plus 100 μM norepinephrine (c) obtained in water. (b) Uptake of ^{59}Fe–norepinephrine complex (FeNE) into RCSN-3 cells was measured in cells kept in cell culture medium without bovine serum. RCSN-3 cells were incubated with ^{59}Fe–norepinephrine complex in the presence of 100 μM $FeCl_3$(Fe), control cells (Co), 2 mM norepinephrine (FeNE2), 2 μM nomifensine (FeNEN), 100 μM imipramine (FeNEI) or 30 μM reboxetine (FeNER). Values are mean ± SD ($n = 3$). **$p < 0.01$ (ANOVA for multiple comparisons and Student's *t*-test)

review see Moos and Morgan 2000). Iron is also bound to other plasma proteins such as ferritin and lactoferrin (Grau *et al*. 2001). However, there are studies showing that iron is transported across endothelial cells both bound and not bound to transferrin when the iron concentration exceeds the binding capacity of transferrin (Moos 2002; Burdo *et al*. 2003). Therefore, it seems plausible that extracellular free iron exists when iron-binding proteins are saturated under iron overload. In addition, reductants such as ascorbic acid, flavins ($FMNH_2$ and $FADH_2$), riboflavin, sulfide, thiols (L-cysteine and glutathione) and dopamine release iron from ferritin (Laulhere *et al*. 1996; Double *et al*. 1998; Cassanelli and Moulis 2001). Thiols also release iron from monoferric transferrin (Baldwin *et al*. 1990).

One important feature of the observed iron toxicity in RCSN-3 cells is its dependence on the uptake of the Fe–dopamine complex via the monoamine transporter, because the incubation of cells with $FeCl_3$ alone is not toxic, even though $FeCl_3$ alone is transported at a similar rate but independent of monoamine transporters. The uptake of $FeCl_3$ is probably mediated by the β_3-integrin and mobilferrin pathway, which has been reported in the intestine (Conrad and Umbreit 2002). There are several lines of evidence supporting the role of the monoamine transporter. (1) Fe–dopamine complex uptake into RCSN-3 cells was Na^+, Cl^- and temperature dependent, in agreement with previous studies showing the Na^+ and Cl^- dependence of the NET, 5HTT and DAT (Demchyshyn *et al*. 1994; Gu *et al*. 1994; Roubert *et al*. 2001). (2) Immunostaining specific for the DAT, NET and 5HTT in RCSN-3 cells supports the idea that these monoamine transporters are expressed in RCSN-3 cells. Examination of Z-sections from the top to the bottom of the cell using confocal microcopy showed that the DAT, NET and 5HTT are mainly localized in the cell membrane. (3) Inhibitors of monoamine transporters DAT (nomifensine), NET (reboxetine) and 5-HTT (imipramine) inhibited Fe–dopamine uptake into RCSN-3 cells. (4) Nomifensine and reboxetine inhibited Fe–dopamine complex toxicity in RCSN-3 cells independently of MAO or COMT metabolism of dopamine. (5) Nomifensine and reboxetine inhibited formation of TBARS induced by Fe–dopamine complex plus dicoumarol in RCSN-3 cells. (6) Nomifensine and reboxetine inhibited the formation of a DMPO-OH adduct ESR signal induced by Fe–dopamine complex plus dicoumarol in RCSN-3 cells. The rate of uptake of the ^{59}Fe–dopamine complex (28 ± 8 pmol per min per mg) was similar to that of [^{3}H]dopamine (2.3 pmol per mg per min) into RCSN-3 cells (Martinez-Alvarado *et al*. 2001) and the K_m was estimated at 0.46 μM, suggesting that the Fe–dopamine complex has a high affinity for the monoaminergic transporter. It therefore seems plausible that the Fe–dopamine complex may not only exert a biological effect by inducing toxicity when DT-diaphorase is inhibited, but may also have an impact on intracellular monoamine levels by competing with the reuptake of

monoamines. It is interesting to note that the formation of extracellular Fe–dopamine complex is required to obtain toxic effects in the presence of dicoumarol in RCSN-3 cells, because no significant cell death was observed in the presence of dicoumarol when the cells were first loaded with $FeCl_3$, washed, then loaded with 100 μM dopamine. A possible explanation for this lack of toxicity is that free ferric iron is chelated after intracellular uptake, preventing the formation of Fe–dopamine complex and thereby dopamine oxidation. Another interesting feature of Fe–dopamine complex uptake into RCSN-3 cells is the role of the NET, which is in agreement with a somewhat promiscuous role of this transporter in the uptake of dopamine (Yamamoto and Novotney 1998; Moron *et al*. 2002; Williams and Steketee 2004).

It is of interest to note that the incubation of RCSN-3 cells with Fe^{3+} alone did not induce formation of hydroxyl radicals or toxicity, despite the fact that the rate of Fe^{3+} uptake alone was similar to that of the Fe–dopamine complex. However, incubation of RCSN-3 cells with Fe–dopamine complex plus dicoumarol induced hydroxyl radical formation and toxicity. A possible explanation for the lack of toxicity of Fe alone probably resides in the fact that Fe uptake into the cells is followed by protein chelation, thus preventing the existence of free Fe^{2+}, and under normal conditions the amounts of H_2O_2 in the cell are insufficient to compete with protein chelation (Fig. 12). However, when Fe is complexed with dopamine after the uptake, Fe^{3+} is reduced to Fe^{2+} with concomitant oxidation of dopamine to aminochrome, which in the presence of dicoumarol is one-electron reduced to leukoaminochrome *o*-semiquinone radical. This radical is extremely reactive with oxygen (Segura-Aguilar *et al*. 1998) autoxidizing by reducing oxygen to superoxide radical, which can enzymatically or non-enzymatically dismutate to H_2O_2. This autoxidation continues until oxygen or NADH/NADPH is depleted, providing a permanent source of H_2O_2 to compete with protein chelation and also catalyzing the Fenton reaction to generate hydroxyl radicals. Another important feature during incubation of RCSN-3 cells with Fe–dopamine complex plus dicoumarol is the concomitant formation of a radical centered in a carbon. The identity of this radical is unknown, but it seems likely that the ESR spectra of this radical corresponds to leukoaminochrome *o*-semiquinone radical, with the radical delocalized into a carbon. The formation of hydroxyl radicals and C-center radicals when RCSN-3 cells were incubated with Fe–dopamine complex alone may be explained by the concentration of the Fe–dopamine complex achieved, which probably surpassed DT-diaphorase capacity to prevent one-electron reduction of aminochrome.

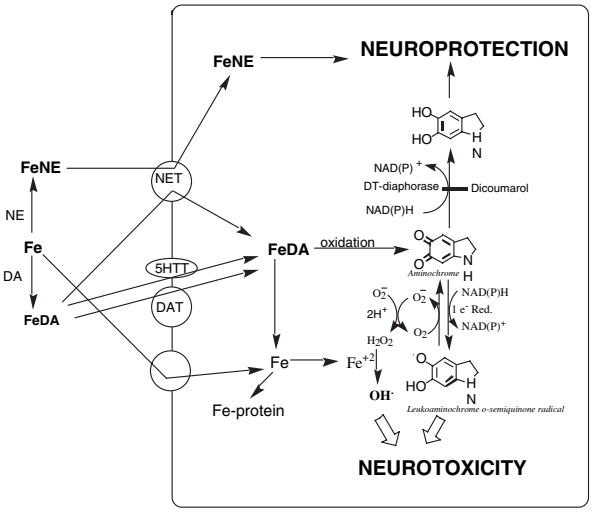

Fig. 12 Possible mechanism of Fe–dopamine complex toxicity in RCSN-3 cells. Fe^{3+} is able to form complexes with both dopamine (DA) and norepinephrine (NE). The uptake of Fe–dopamine complex (FeDA) is mediated by the DAT, NET and 5HTT. Complexed dopamine is oxidized to aminochrome and Fe^{3+} is reduced to free Fe^{2+}. Aminochrome then has two alternatives: (1) two-electron reduction by DT-diaphorase or (2) one-electron reduction of aminochrome to leukoaminochrome *o*-semiquinone radical when DT-diaphorase is inhibited by dicoumarol, which leads to neurotoxicity. Norepinephrine may compete with dopamine to form a complex with Fe (FeNE), which prevents neurotoxic effects as the uptake into RCSN-3 cells via the NET did not induce toxicity.

Inhibition of DT-diaphorase by dicoumarol plays an important role in the toxicity observed in RCSN-3 cells in the presence of Fe–dopamine complex. In contrast to all other flavoenzymes, which catalyze one-electron reduction of quinones, DT-diaphorase is a unique flavoenzyme as it catalyzes the two-electron reduction of quinones to hydroquinones (Iyanagi and Yamazaki 1970). DT-diaphorase constitutes 97% of the total quinone reductase activity in the rat SN, and is localized both in dopaminergic neurons and glia (Schultzberg *et al*. 1988). DT-diaphorase prevents one-electron reduction of aminochrome to leukoaminochrome *o*-semiquinone radical by reducing aminochrome with two electrons (Segura and Lind 1989; Baez *et al*. 1995; Segura-Aguilar *et al*. 1998; Paris *et al*. 2001; Graumann *et al*. 2002; Arriagada *et al*. 2004). One-electron reduction of aminochrome to leukoaminochrome *o*-semiquinone radical has been proposed to be one of the major mechanisms for endogenous generation of reactive species involved in the process leading to dopaminergic neurons degeneration (Baez *et al*. 1995; Segura-Aguilar *et al*. 1998, 2001; Paris *et al*. 2001; Arriagada *et al*. 2004). Inhibition of DT-diaphorase by dicoumarol induces (1) a 6-hydroxydopamine-like contralateral rotation and an extensive loss of TH staining in rats injected intracerebrally with the oxidizing agent manganese pyrophosphate complex into the medial forebrain bundle or the SN respectively (Segura-Aguilar *et al*. 2002; Diaz-Veliz *et al*. 2004), (2) toxicity in RCSN-3 cells exposed to Cu–dopamine complex (Paris *et al*. 2001), and (3) toxicity in RCSN-3 cells exposed to aminochrome formed by oxidizing dopamine with manganese pyrophosphate complex (Arriagada *et al*. 2004).

The results presented here also support the idea that DT-diaphorase plays a neuroprotective role in dopaminergic neurons by preventing one-electron reduction of aminochrome to leukoaminochrome *o*-semiquinone radical (Baez *et al*. 1995; Segura-Aguilar *et al*. 1998, 2001; Paris *et al*. 2001; Arriagada *et al*. 2004), as suggested by the increase in cell death observed when RCSN-3 cells were incubated with Fe–dopamine complex plus the DT-diaphorase inhibitor, dicoumarol.

Norepinephrine inhibited uptake and toxicity when RCSN-3 cells were incubated with Fe–dopamine complex plus dicoumarol in RCSN-3 cells. It seems plausible that the neuroprotective action of norepinephrine in this experimental model resulted from its ability to compete to form a complex with dopamine (Fig. 10) and the fact that Fe–norepinephrine complex was unable to induce toxicity in RCSN-3 cells, even though intracellular uptake of Fe–norepinephrine complex occurred through the NET and in the presence of dicoumarol. The neuroprotective effect of norepinephrine has also been observed at low concentrations (0.3–10 μM) in mesencephalic cultures, providing long-term survival and function of dopaminergic neurons (Troadec *et al*. 2001).

In conclusion, $FeCl_3$ alone did not induce toxicity in RCSN-3 cells despite the existence of effective uptake. However, Fe–dopamine complex formation and uptake through the monoaminergic transporter in the presence of dicoumarol induced toxicity in RCSN-3 cells. The uptake of Fe–dopamine complex was inhibited by nomifensine, reboxetine and imipramine, and was Na^+, Cl^- and temperature dependent. The toxicity induced by the Fe–dopamine complex in the presence of dicoumarol was inhibited by nomifensine, reboxetine and norepinephrine. Our results suggest that DT-diaphorase and norepinephrine prevent the observed toxic effects of the Fe–dopamine complex.

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