200400746A

厚生労働科学研究費補助金

こころの健康科学研究事業

パーキンソン病 PARK7 の原因遺伝子 DJ-1 の機能と創薬応用

平成１６年度　総括研究報告書

主任研究者　有賀寛芳

平成１７年 (2005) 年４月

目次

厚生労働科学研究費補助金（こころの健康科学研究事業）
総括研究報告書

パーキンソン病 PARK7 の原因遺伝子 DJ-1 の機能と創薬応用

主任研究者　有賀寛芳　北海道大学大学院薬学研究科教授

研究要旨

本研究室で新規癌遺伝子として単離同定した DJ-1 は一昨年度家族性パーキンソン病 PARK7 の原因遺伝子として同定され、現在まで１１箇所の DJ-1 遺伝子変異がパーキンソン病患者で報告されている。DJ-1 の機能解析と DJ-1 が関与するパーキンソン病発症機構の解明を目的として本研究がスタートした。DJ-1 は細胞増殖、受精、癌、不妊、パーキンソン病と多機能を有するが、本来の機能は転写調節、抗酸化ストレス、プロテアーゼ、更にミトコンドリア complex 1 の制御因子であることが明らかとし、これらのいずれかの欠損もパーキンソン病発症原因となる。また、弧発性パーキンソン病患者脳では不活性型と考えられる異常酸化型の DJ-1 が観察された。更に、パーキンソン病モデルラットの DJ-1 タンパク質を注射すると劇的にドーパミン神経細胞死の阻止とそれに伴う行動異常が改善されることから、DJ-1 はパーキンソン病の創薬ターゲットになることが示唆された。

A. 研究目的

申請者が新規癌遺伝子として1997年に単離し研究をしてきたDJ-1は家族性パーキンソン病(PARK7)の原因遺伝子であることが報告され本研究プログラムが始まった。現在までにパーキンソン病患者で１１箇所のDJ-1遺伝子変異（欠損、点突然変異）が報告されている。パーキンソン病は酸化ストレス、それに伴う異常タンパク質の凝集、ミトコンドリアのcomplex 1の機能阻害が原因と考えられているが詳細な分子機構は明らかでなかった。我々は以前及び本プログラムによって、DJ-1は転写調節、抗酸化ストレス、プロテアーゼの３つの機能を有し、その機能破綻はパーキンソン病などの脳神経変性疾患、男性不妊、細胞癌化の原因となることを明らかとした。具体的には、DJ-1は酸化ストレスを誘発する活性酸素により３つのシステインが自己酸化されることで活性酸素を消去し、酸化ストレスによる細胞死を防御する、DJ-1はプロテアーゼとして家族性パーキンソン病(PARK2)の遺伝子産物であるParkinのユビキチン化基質でありパーキンソン病患者で凝集体形成するPael受容体、家族性アミロードシス(FAP)の原因タンパク質であるトランスサイレチン(TTR)を分解する、更に転写因子としてストレス、シャペロン、アポトーシス関連遺伝子の転写調節を行うことを明らかとした。一方、**孤発性パーキンソン病患者脳においては、活性型である還元型DJ-1の欠如と異常なフォームの酸化型**DJ-1が存在を見出し、同時にパーキンソン病患者に見られる**DJ-1変異体は抗酸化ストレス能の消失、**現弱を示したことにより、DJ-1機能欠損との関連を明らかにした。更に、DJ-1はミトコンドリアcomplex 1サブユニットNDUFA4と結合し、DJ-1ノックダウン細胞ではcomplex 1の酵素活性が著しく低下していることにより、complex 1の正の制御因子であることも明らかとした。要約すると、DJ-1は種々のストレス、とりわけ酸化ストレスによって発現誘導され、酸化ストレスが起こった細胞内部位に局在変動し、種々のシャペロンと複合体形成し、活性酸素除去と、酸化ストレスによって変性したタンパク質の分解に預かる。この機能には106番目のシステインの酸化が必須である。更に、ドーパミン合成のキー酵素であるチロシンヒドロキシラーゼとDJ-1が結合することが明らかになりつつあり、ドーパミン作動性神経の直接な制御因子としてのDJ-1も考えられ始めた。

一方、6-OHDA投与パーキンソン病モデルマウスにDJ-1タンパク質を直接注入すると、ドーパミンニューロン死とそれに伴う行動異常が劇的に阻止されることを明らかとした。これらは、DJ-1がパーキンソン病治療薬となる可能性を示唆した。

A. 研究方法

１．DJ-1の抗酸化ストレス機能

大腸菌で発現後精製したDJ-1と過酸化水素を反応させ、スコルポチン反応で過酸化水素量を定量した。また、マウスNIH3T3細胞に野生型及びパーキンソン病に見られる各種変異体DJ-1を定常的に発現している細胞株を構築し、過酸化水素、6-ヒドロキシドパミンなどを細胞に加えた場合の細胞内活性酸素量をDCFH添加後、フローサイトメーターで定量した。

２．酸化ストレス誘導細胞死に対するDJ-1の抵抗性

上術した各種細胞株、及びDJ-1遺伝子をターゲットとしたsiRNAを導入DJ-1ノックダウン細胞に過酸化水素を1-2 時間作用させ、生細胞数をMTT法で定量した。

３．DJ-1のプロテアーゼ活性

精製DJ-1とウサギ網状赤血球抽出液で合成した各種のタンパク質を反応させ、反応物をSDS-電気泳動で展開させた後、フルオログラフィーでそれらのタンパク質の分解を検討した。また、パエル受容体とDJ-1 cDNAを293T細胞にtransfection後、抗パエル受容体抗体でその分解を検討した。

４．DJ-1のSUMO-1化

H1299 細胞抽出液を抗 DJ-1 抗体で免疫沈降させ、沈降物を抗 SUMO-1 抗体で Western blotting を行った。パーキンソン病に見られる L166P 変異体を H1299 細胞に transfection し、同様に解析した。

B. 研究結果

１．DJ-1の抗酸化ストレス機能

大腸菌で産生後精製した組み換えDJ-1と過酸化

水素をin vitroで反応させたところ、DJ-1のシステインが自己酸化されることで、DJ-11分子あたり最低6分子の過酸化水素を消去すること、また、マウスNIH3T3, ヒト神経細胞SH-SY5Y細胞に過酸化水素と投与したところ、細胞内でin vitro同様に過酸化水素が消去されることを明らかにした。次に、siRNAによるDJ-1ノックダウンSH-SY5Y細胞では過酸化水素、MPP+、6-ヒドロキシドーパミンが誘導する細胞死により感受性が高まることが明らかとなった。更に、L166P, C106S, DJ-1のSUMO-1修飾されないK130Rなどの種々のDJ-1変異体を導入したNIH3T3細胞株を作成し、過酸化水素の消去能、過酸化水素が誘導する細胞死に対する感受性を検討したところ、いずれもDJ-1機能の消失、現弱が見られた(Taira et al. EMBO Rep. 2004)。次に、パーキンソン病患者で見られるDJ-1変異体M26I, R98Q, D149Aで同様な解析を行ったところ、L166Pほどではないが抗酸化ストレス機能の低下が見られた(Takahashi-Niki et al. BBRC, 2004)。細胞に種々の濃度の過酸化水素を投与し、酸化されるDJ-1のシステインを質量分析法で解析したところ、C106→C53→C46の順でSO3Hの形で酸化されることが明らかとなった(Kinumi et al. BBRC, 2004)。同時に、DJ-1の機能発揮には後述のK130へのSUMO-1かとともに、C106が酸化されることが必須であることが明らかとなった(Takahashi-Niki et al. BBRC, 2004)。

一方、DJ-1のヒト脳での発現を免疫染色、イムノブロット法で解析し、ほとんどの組織でDJ-1が強く発現し、パーキンソン病患者では一部Lewy bodyにも局在していた。更に、弧発性及びR98Q変異を有するパーキンソン病患者脳のDJ-1を2次元電気泳動で検討したところ、健常人に見られる非酸化型DJ-1の消失と健常人と異なった酸化型DJ-1の存在が明らかとなった(Bandopadhyay et al. Brain, 2004)。

2．DJ-1のSUMO-1化と機能調節

ヒトH1299細胞のDJ-1をイムノブロット法で解析したところ、DJ-1はSUMO-1修飾されていることが明らかとなった。DJ-1には16個のリジンが存在するのでこれら全てをアルギニンに変化した変異DJ-1を作成しH1299細胞でのSUMO-1化の有無を解析したところ、種間で高度に保存されている130番目のリジン(K130)がSUMO-1化サイトであることが明らかとなった。そこでこれらの変異体を使いDJ-1の抗酸化、細胞増殖、細胞癌化能に対する機能を解析したところ、K130へのSUMO-1化が全ての機能に必須であった。更に、L166P変異体はすべてのリジンが、あるいはK130が多重にSUMO-1化されており、これによりミトコンドリアへの局在変動、不溶化が起こり、抗アポトーシス機能の消失が見られた(Shinbo et al. Cee Death. Diff. revised)。ハンチントン病の封入体構成タンパク質もSUMO-1化されることが最近報告されており、神経変性疾患関連タンパク質のSUMO-1化は今後重要な課題となるかもしれない。

3．DJ-1のプロテアーゼ活性

DJ-1のX線結晶構造解析を行ったところ、古細菌タンパク質であるプロテアーゼpfiとの構造類似性からDJ-1のプロテアーゼ活性が推測された(Honbou et al. J. Biol. Chem. 2003)。しかしながら、プロテアーゼ基質の結合領域はC末のペプチドでブロックされている。そこで、合成基質を使い組み替え体DJ-1のプロテアーゼ活性を測定したところ、完全長では弱いプロテアーゼ活性しか有しないが、C末のペプチドを欠損したDJ-1はより強いプロテアーゼ活性を示した。次に、すでに明らかにしているDJ-1の種々の結合タンパク質、パーキンソン病関連タンパク質がDJ-1のプロテアーゼ基質である可能性を検討したところ、Parkinのユビキチン化基質であるPael-receptorがヒト293T細胞内でDJ-1により分解された。活性中心であるC106の変異体DJ-1にはこの活性がない。次に、Pael-receptorが誘導するアポトーシスをDJ-1が抑制することがFax解析で明らかとなった。プロテアーゼ阻害剤存在下でもDJ-1のPael-receptorの分解が起こることから、DJ-1はシステインプロテアーゼとして独立に機能すると考えられる(Niki

et al. 投稿中)。また、Pael-receptorのによる ERストレスに対してDJ-1は抵抗性を細胞に与えた (Yokota et al. BBRC, 2003)。細胞内ではDJ-1が他のタンパク質と複合体を形成してこれらのタンパク質がブロックされているC末部分を開くことが考えられるが、この候補としてHsp70をDJ-1結合タンパク質として同定した。

4．DJ-1の転写調節遺伝子、プロテアーゼ基質の同定

siRNAにより定常的にDJ-1発現がノックダウンされているNIH3T3細胞株と、テトラサイクリンによりsiRNA発現が制御できる誘導型DJ-1ノックダウン293細胞株を作成した。これらの細胞、及びL166P導入NIH3T3細胞株を使用してDNAマイクロアレイ、プロテオーム解析を行い親株と比較した。DJ-1ノックダウン細胞では極めて大きな遺伝子発現変動が起こり、特に、ストレス誘導、炎症、更にtau, synphilinといった神経変性疾患関連遺伝子、脳細胞のアポトーシス関連遺伝子が多数含まれていた(Nishinaga et al., in preparation)。

一方、DJ-1ノックダウン細胞ではDJ-1プロテアーゼ基質の発現上昇が見られるはずである。現在までに、神経変性疾患関連タンパク質であるtransthyretin、レチノイン酸結合タンパク質CRABP-1, タンパク質合成の開始因子eIF4Aが候補タンパク質として同定された。Transthyretin (TTR)、CRABP-1は神経変性疾患との関連が示されている。TTRのDJ-1による分解を細胞レベルで明らかにした。更に、FAPモデルマウスにDJ-1タンパク質を直接投与すると肝臓でのTTRのアミロイド化が劇的に阻止され、DJ-1を指標として治療法は考えられた。

5．酸化ストレスとDJ-1

酸化ストレスはパーキンソン病発症と深く関わっていることが以前から報告されている。この酸化ストレスの原因として神経毒を含む環境物質の存在が示唆されている。この中で、環境化学物質であるビスフェノールA (BPB) は胎児期マウスに投与すると成獣マウスになってパーキンソン病症状を示し、また成獣マウス/ラット脳に投与するとドーパミン神経細胞死が起こることが知られている。BPA処理により細胞は活性酸素を発生し、ミトコンドリアcomplex 1活性が低下した。DJ-1はこれに反応し発現上昇しミトコンドリアに移行しcomplex 1活性の維持を図るが、BPA濃度上昇とともに酸化型DJ-1が増加し機能が低下した(Ooe et al. submitted to JBC)。

また、DJ-1はHsp70, CHIPのシャペロンと結合し、酸化ストレスによりその結合が増加した。更に、ミトコンドリアではミトコンドリアに局在するシャペロンであるmtHsp70/Grp75と結合、共局在した (Li et al. submitted to Mol. Brain. Res.)。

6．ミトコンドリアcomplex 1の制御因子としてのDJ-1

酸化ストレスと同時に、ミトコンドリアcomplex 1の機能低下はパーキンソン病発症の本体の１つと考えられている。DJ-1はミトコンドリアcomplex 1サブユニットNDUFA4と結合し、DJ-1ノックダウン細胞では著しくComplex 1の酵素活性が低下し、逆にDJ-1ノックダウン細胞にDJ-1を発現させるとComplex 1の酵素活性が復帰することから<u>complex 1の正の制御因子</u>であることを明らかとした。また、免疫電子顕微鏡観察よりDJ-1はミトコンドリア外膜、マトリックスに加えて、Complex 1が存在する内膜にも存在することが示された。

7．パーキンソン病の創薬ターゲットとしてのDJ-1

6-OHDA投与パーキンソン病モデルラットの中脳黒質にDJ-1タンパク質を直接注入すると、黒室と線条体の<u>ドーパミンニューロン死とが阻止され、ドーパミンが線条体に運搬されることにより、行動異常が劇的に阻止</u>されることを明らかにした。パーキンソン病患者に見られる変異DJ-1であるL166Pにはこの活性がない。線条体では低下し

ていたドーパミン、その代謝物、ドーパミントランスポーター濃度も回復していた。これは6-OHDA投与による活性酸素を注入DJ-1が細胞内に移行し消去していると考えられた。これにより、DJ-1によるパーキンソン病の創薬の可能性が示された(Inden et al. submitted to Science)。

8．ドーパミン合成のキー酵素であるチロシンヒドロキシラーゼとDJ-1が結合することが明らかとなり、ドーパミン合成にDJ-1が関与する可能性が考えられた。

5．DJ-1遺伝子導入マウスの解析

既に野生型DJ-1トランスジェニックマウスは作成していた。今年度、L166P, K130R変異体トランスジェニックマウスを作成した。L166Pトランスジェニックマウスは受精効率が低く増やすことに困難が伴っているがやっと量的に増え解析可能となったので野生型DJ-1トランスジェニックマウス同様に解析予定である。更に、α-シニュクレイントランスジェニックマウスと野生型DJ-1トランスジェニックマウスを掛け合わせており、今後解析の予定である。

考察

DJ-1の基本的な４つの機能―転写調節、抗酸化ストレス、プロテアーゼ、ミトコンドリア complex 1の制御因―が存在することを明らかにした。転写調節因子としては以前からのアンドロゲン受容体の正の転写調節因子であることを明らかにしてきたが、脳で発現する、また脳変性疾患に関する遺伝子との関連は不明である。DJ-1ノックダウン細胞を使用しての DNA マイクロアレイ解析より、tau, synphilin、transthyretin が候補となったので今後解析の必要がある。

２番目の抗酸化ストレス因子としての機能はパーキンソン病との関連で特に注目される。弧発性パーキンソン病が酸化ストレスによって生ずるという考え方は古くから支持されていたが、それを担う遺伝子、タンパク質の同定は行われていなかった。本研究においてDJ-1がその遺伝子の候補であり、実験的に証明した事は極めて意義が高い。弧発性パーキンソン病では実際に非還元型で活性型と考えられるDJ-1の消失と同時に、野生型DJ-1に見られる酸化状態とと異なった DJ-1 が見られることはパーキンソン病発症過程での DJ-1 の抗酸化ストレス機能の重要性が示唆される。

３番目のプロテアーゼ機能もまたパーキンソン病発症と直接関わる現象である。家族性パーキンソン病原因遺伝子として同定された Parkin, UCH-L1 はユビキチンリガーゼ、脱ユビキチン化酵素である。パーキンソン病では tau, α-synuclein、Pael-receptor などのレヴィー体、あるいは小胞体への凝集が起こり、これらが細胞死を誘導することが知られており、ユビキチン化されプロテアソーム系で分解される。一方、DJ-1はシステインプロテアーゼであり、プロテアソーム系と独立してPael-receptor を分解した。このように、生体にとって有害なタンパク質は複数の系により分解される事を示す最初の例である。DJ-1のプロテアーゼ基質は Pael-receptor に加えて、家族性アミロードシスの原因タンパク質トランスサイレチン(TTR)を同定し、細胞内での DJ-1 による分解を示した。家族性アミロードシス患者では、TTRによる凝集体とDJ-1の局在はミラーイメージになっており、パーキンソン病患者のα-synucleinのケースと似ている。現在、α-シニュクレイントランスジェニックマウスと野生型DJ-1トランスジェニックマウスを掛け合わせており、今後解析が期待される。。

パーキンソン病患者に見られる DJ-1 変異体は程度の差はあれ、すべて抗酸化ストレス機能が低下していた。最初に見つかった L166P は極めて異常であり、タンパク質としての構造がとれず、過剰に SUMO-1 化され不溶化されていた。DJ-1 の全ての機能に１３０番目のリジンへの SUMO-1 化は必須である。パーキンソン病患者では健常人と比較して不溶化 DJ-1 の存在が報告されており、これらの DJ-1 が過剰に SUMO-1 化されている可能性が存在し、今後臨床サイドと提携してこの可能性を検討したい。

パーキンソン病発症の１つとしてミトコンドリア complex 1 の機能低下がある。DJ-1 の４番目の機能として、complex 1 の正の制御因子であることが明らかとなった。実際、酸化ストレスを与える薬剤、またビスフェノール A などを細胞に投与するとミトコンドリア、complex 1 の酵素活性が低下

し、同時にDJ-1はミトコンドリアに移動しその機能維持に関与した。DJ-1はDNAに損傷を与える紫外線などの照射によるストレス時には核に、また酸化ストレスではミトコンドリアとそのストレスを受けた場所に移動しストレス除去にあたることが明らかといなった。パーキンソン病患者では既にDJ-1自身の機能低下のため、このストレス防御ができなくなっていると考えられる。

今回、6-OHDAを中脳黒質に投与したパーキンソン病モデルラットにDJ-1タンパク質を直接投与すると黒質と線条体のドーパミンニューロン死の阻害とそれに伴う行動異常が劇的に回復した。投与DJ-1は速やかに細胞内に移行し活性酸素除去にあたったことより、DJ-1が創薬ターゲットになりうることを示したことは大きな成果であった。

C. 結論

本計画はDJ-1の基本的な機能解析を行い、その機能変動がいかにパーキンソン病発症の原因となるかを解明することを第1の目的とし、それらを踏まえて創薬応用を第2の目的とした。

前者においては、DJ-1は転写調節因子、抗酸化ストレス、プロテアーゼ、ミトコンドリアcomplex 1の機能調節の異なる4つの機能を有する事が明らかとなり、これらの機能消失がパーキンソン病の発症原因になると考えられた事は極めて重要である。DJ-1の機能消失が家族性パーキンソン病PARK7に加えて弧発性パーキンソン病の発症に関連する可能性があり次年度以降に臨床分野との共同研究でこの可能性を明らかにしたい。更に、パーキンソン病以外に複数の脳神経変性疾患患者でDJ-1の発現変動、不溶化などが報告され、また、Parkin, tau, α-synucleinとDJ-1との複合体形成も報告され始めたことから、広く脳神経変性疾患患者にDJ-1が関与する可能性がある。事実、DNAマイクロアレイ、プロテオーム解析から神経変性疾患関連因子トランスサイレチンがDJ-1機能のターゲット候補となったので詳細に解析したい。

また、パーキンソン病モデルラット脳にDJ-1タンパク質を直接投与するとドーパミンニューロン死とそれに伴う行動異常が劇的に阻止されることより、DJ-1による創薬が考えられ、今後DJ-1ペプチド、アゴニスト、アンタゴニストの探索を行い、治療薬を模索したい。

D. 健康危険情報

なし

E. 研究発表（本研究に関連するもののみを記す）

1．論文発表

1) Bandopadhyay, R., Kingsbury, A., Cookson, M., Reid, A., Evans, I., Hope, A., Pittman, A., Lashley, T., Canet-Aviles, R., Miller, D., Mc Lendon, C., Strand, C., Leonard, A., Ariga, H., Wood, N., de Silva, R., Hardy, J., Holton, J., Lees' A. and Revesz, T. (2004) The expression of DJ-1 (PARK7) in normal human CNS and idiopathic Parkinson's disease. Brain 127, 420-430.

2) Taira, T., Saito, Y., Niki, T., Iguchi-Ariga, S.M.M., Takahashi, K. and Ariga, H. (2004) DJ-1 plays a role in anti-oxidative stress to prevent cell death. EMBO Rep. 5, 213-218

3) Taira, T., Iguchi-Ariga, S.M.M. and Ariga, H. (2004) Co-localization with DJ-1 is essential for the androgen receptor to exert its transcription activity that has been impaired by androgen-antagonists Biol. Pharm. Bull. 27, 574-577.

4) Kinumi, T.,Kimata, J.,Taira, T.,Ariga, H. and Niki, E. (2004) Cysteine-106 of DJ-1 is the most sensitive cysteine residue to hydrogen peroxide mediated oxidation in vivo in human umbilical vein endothelial cells. Biochem. Biophys. Res. Commun. 317, 722-728.

5) Takahashi-Niki, K., Niki, T., Taira, T., Iguchi-Ariga, S.M.M. and Ariga, H. (2004) Reduced anti-oxidative stress activities of DJ-1 mutants found in Parkinson's disease patients. Biochem. Biophys. Res. Commun. 320, 389-397.

6) Sekito, A., Taira, T., Niki, T., Iguchi-Ariga, S.M.M. and Ariga, H. (2005) Stimulation of transforming activity of DJ-1 by Abstrakt, a DJ-1-binding protein. Int. J. Oncol. 26, 685-690.

7) Shinbo, Y., Taira, T., Niki, T., Iguchi-Ariga, S.M.M. and Ariga, H. (2005) DJ-1 restores p53 transcription activity inhibited by Topors/p53BP3. Int. J. Oncol. 26, 641-648.

2．学会発表

1) 平　敬宏、有賀早苗、有賀寛芳

多機能癌遺伝子産物 DJ-1 の SUMO-1 修飾による機能制御構
第 63 回日本癌学会総会　9/29/04　福岡国際会議場

2) 仁木加寿子、仁木剛史、平　敬宏、有賀早苗、有賀寛芳
パーキンソン病患者に見られる DJ-1 変異体の抗酸化ストレス能の減少
第 77 回日本生化学会大会ワークショップ
10/16/04　パシフィコ横浜

3) 李　紅梅、仁木剛史、平　敬宏、有賀早苗、有賀寛芳
DJ-1-HSP70-Pael receptor 複合体による DJ-1 のプロテアーゼ活性
第 77 回日本生化学会大会ワークショップ
10/16/04　パシフィコ横浜

4) 平　敬宏 (2004)
若年型家族性パーキンソン病 (PARK7)の原因遺伝子 DJ-1 の機能
第 77 回日本薬理学会シンポジウム
3/04　新潟

5) 小出静代、平 敬宏、有賀早苗、有賀寛芳
多機能タンパク質 DJ-1 の神経疾患における機能解析
第２７回日本分子生物学会年会
12/10/04　神戸国際会議場

6) 関戸亜弥、平　敬宏、有賀(井口)早苗、 有賀寛芳
DJ-1 結合タンパク質 HIPK1 の解析
第２７回日本分子生物学会年会
12/10/04　神戸国際会議場

7) 大江洋正、平敬宏、有賀早苗、有賀寛芳
内分泌かく乱化学物質曝露マウスにおけるDJ-1の発現変動と作用機序
第２７回日本分子生物学会年会
12/10/04　神戸国際会議場

8) 平　敬宏、仁木（高橋）加寿子、仁木剛史、有賀（井口）早苗、有賀寛芳
家族性パーキンソン病（PARK7）原因遺伝子 DJ-1 およびその変異体の抗酸化活性と病態
第２７回日本分子生物学会年会
12/10/04　神戸国際会議場

9) 林　拓哉、平　敬宏、有賀寛芳、有賀 早苗
DJ-1 のミトコンドリア complex 1 への結合による活性制御
第２７回日本分子生物学会年会
12/10/04　神戸国際会議場

10) 大江洋正、平敬宏、松本健一、有賀早苗、有賀寛芳
内分泌攪乱化学物質曝露マウスにおける DJ-1 の発現変動と作用機序
CREST「内分泌かく乱物質」終了シンポジウム
9/15/04　コクヨホール、東京

11) 出河千夏，平　敬宏，仁木剛史，有賀早苗，有賀寛芳
多機能タンパク質 DJ-1 の抗酸化ストレスおよび細胞増殖における機能
CREST「内分泌かく乱物質」終了シンポジウム
9/15/04　コクヨホール、東京

12) 平　敬宏、有賀早苗、有賀寛芳
パーキンソン病原因遺伝子 DJ-1 による活性酸素消去機構
第１２５回日本薬学会年会　ワークショップ
3/31/05　東京ビックサイト

H. 知的財産権の出願・登録状況
なし

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Bandopadhyay, R., Kingsbury, A., Cookson, M., Reid, A., Evans, I., Hope, A., Pittman, A., Lashley, T., Canet-Aviles, R., Miller, D., Mc Lendon, C., Strand, C., Leonard, A., Ariga, H., Wood, N., de Silva, R., Hardy, J., Holton, J., Lees' A. and Revesz, T.	The expression of DJ-1 (PARK7) in normal human CNS and idiopathic Parkinson's disease.	Brain	127	420-430	2004
Taira, T., Saito, Y., Niki, T., Iguchi-Ariga, S.M.M., Takahashi, K. and Ariga, H.	DJ-1 plays a role in anti-oxidative stress to prevent cell death	EMBO Rep.	5	213-218	2004
Taira, T., Iguchi-Ariga, S.M.M. and Ariga, H	Co-localization with DJ-1 is essential for the androgen receptor to exert its transcription activity that has been impaired by androgen-antagonists.	Biol. Pharm. Bull.	27	574-577	2004
Kinumi, T.,Kimata, J.,Taira, T.,Ariga, H. and Niki, E	Cysteine-106 of DJ-1 is the most sensitive cysteine residue to hydrogen peroxide mediated oxidation in vivo in human umbilical vein endothelial cells.	Biochem. Biophys. Res. Commun	317	722-772	2004
Takahashi-Niki, K., Niki, T., Taira, T., Iguchi-Ariga, S.M.M. and Ariga, H.	Reduced anti-oxidative stress activities of DJ-1 mutants found in Parkinson's disease patients.	Biochem. Biophys. Res. Commun	320	389-397	2004
Sekito, A., Taira, T., Niki, T., Iguchi-Ariga, S.M.M. and Ariga, H.	Stimulation of transforming activity of DJ-1 by Abstrakt, a DJ-1-binding protein	Int. J. Oncol.	26	685-690	2005
Shinbo, Y., Taira, T., Niki, T., Iguchi-Ariga, S.M.M. and Ariga, H.	DJ-1 restores p53 transcription activity inhibited by Topors/p53BP3	Int. J. Oncol.	26	641-648	2005

DOI: 10.1093/brain/awh054 *Brain* (2004), **127**, 420–430

The expression of DJ-1 (*PARK7*) in normal human CNS and idiopathic Parkinson's disease

Rina Bandopadhyay,[1,*] Ann E. Kingsbury,[1,*] Mark R. Cookson,[4] Andrew R. Reid,[1] Ian M. Evans,[3] Andrew D. Hope,[1] Alan M. Pittman,[1] Tammaryn Lashley,[2] Rosa Canet-Aviles,[4] David W. Miller,[4] Chris McLendon,[4] Catherine Strand,[2] Andrew J. Leonard,[3] Patrick M. Abou-Sleiman,[2] Daniel G. Healy,[2] Hiroyashi Ariga,[5] Nicholas W. Wood,[2] Rohan de Silva,[1] Tamas Revesz,[2] John A. Hardy[4] and Andrew J. Lees[1]

[1]*Reta Lila Weston Institute of Neurological Studies, Royal Free and UCL Medical School,* [2]*Department of Molecular Neuroscience, Institute of Neurology, Queen Square and* [3]*Department of Molecular Endocrinology, University College London, London, UK,* [4]*Laboratory of Neurogenetics, National Institute of Ageing, NIH, Bethesda, Maryland, USA and* [5]*Hokkaido University, Graduate School of Pharmaceutical Sciences, Sapporo and CREST, Japan Science and Technology Corporation, Kawaguchi, Japan*

Correspondence to: Professor Andrew J. Lees MD, FRCP, Reta Lila Weston Institute of Neurological Studies, Royal Free and UCL Medical School, 46 Cleveland Street, London W1T 4JF, UK.
E-mail: a.lees@ion.ucl.ac.uk

These two authors contributed equally to the work

Summary

Two mutations in the DJ-1 gene on chromosome1p36 have been identified recently to cause early-onset, autosomal recessive Parkinson's disease. As no information is available regarding the distribution of DJ-1 protein in the human brain, in this study we used a monoclonal antibody for DJ-1 to map its distribution in frontal cortex and substantia nigra, regions invariably involved in Parkinson's disease. Western blotting of human frontal cortex showed DJ-1 to be an abundant protein in control, idiopathic Parkinson's disease, cases with clinical and pathological phenotypes of Parkinson's disease with R98Q polymorphism for DJ-1, and in progressive supranuclear palsy (PSP) brains. We also showed that DJ-1 immunoreactivity (IR) was particularly prominent in astrocytes and astrocytic processes in both control and Parkinson's disease frontal cortex, whereas neurons showed light or no DJ-1 IR. Only occasional Lewy bodies (LBs), the pathological hallmarks of Parkinson's disease, showed faint DJ-1 IR, localized to the outer halo. In preclinical studies we showed that DJ-1 is expressed in primary hippocampal and astrocyte cultures of mouse brain. By 2D gel analysis we also showed multiple pI isoforms for DJ-1 ranging between 5.5–6.6 in both control and Parkinson's disease brains, whilst exposure of M17 cells to the oxidizing agent paraquat was manifested as a shift in pI of endogenous DJ-1 towards more acidic isoforms. We conclude that DJ-1 is not an essential component of LBs and Lewy neurites, is expressed mainly by astrocytes in human brain tissue and is sensitive to oxidative stress conditions. These results are consistent with the hypothesis that neuronal–glial interactions are important in the pathophysiology of Parkinson's disease.

Keywords: DJ-1; Parkinson's disease; immunohistochemistry; 2D gel electrophoresis; paraquat

Abbreviations: DA = dopaminergic; 2DGE = two-dimensional gel electrophoresis; GFAP = glial fibrillary acidic protein; IR = immunoreactivity, immunoreactive; LB = Lewy body; LN =Lewy neurite; PSP = progressive supranuclear palsy; SN = substantia nigra

Received July 4, 2003. Revised October 6, 2003. Accepted October 11, 2003. Advanced Access publication December 8, 2003

Introduction

Parkinson's disease is an incurable, inexorably progressive neurodegenerative disorder affecting around 2% of the population over the age of 65 (de Rijk *et al.*, 1997). The cardinal presenting clinical features comprise bradykinesia, rigidity and resting tremor, with a therapeutic response to L-dopa (Yahr *et al.*, 1969). Selective and severe loss of dopaminergic (DA) neurons projecting from the substantia nigra (SN) to the striatum is responsible for the major motor handicaps. The pathological hallmark is the accumulation of eosinophilic, proteinaceous, intracytoplasmic inclusions known as Lewy bodies (LBs) in the substantia nigra, locus coeruleus, dorsal nucleus of the vagus, parahippocampal gyrus and other brainstem and cortical regions (Forno, 1996). Currently, three Parkinson's disease genes, two other candidate genes and a further six distinct loci responsible for rare Mendelian forms of Parkinson's disease have been identified (Dawson and Dawson, 2003; Hardy *et al.*, 2003). However, the molecular mechanisms leading to neurodegeneration in Parkinson's disease remain poorly understood.

Two point mutations (A30P, A53T) in the α-synuclein (*PARK1*) gene cause autosomal dominant, early-onset Parkinson's disease in some families (Polymeropoulos *et al.*, 1997), and α-synuclein is a major component of LBs (Spillantini *et al.*, 1997), thus implicating its involvement in the aetiopathology of sporadic Parkinson's disease. The production of α-synuclein in transgenic mice and transgenic fruit flies may induce disturbances of motor behaviour (Masliah *et al.*, 2000) and LB-like inclusions (Feany and Bender, 2000). A variety of mutations (homozygous deletions, multiplications of exons, point-mutations and insertions) in the parkin gene (*PARK2*) cause autosomal recessive juvenile Parkinson's disease (Kitada *et al.*, 1998). Parkin functions as an E3 ubiquitin protein ligase (Shimura *et al.*, 2000) and its levels are low or absent in patients with autosomal recessive juvenile Parkinson's disease (Shimura *et al.*, 1999). Neuropathological examination shows that neuronal death occurs preferentially in the SN and locus coeruleus and LBs are absent in the brains of most parkin-mutated cases (Mori *et al.*, 1998). Recently parkin has been reported to be a component of LBs and Lewy neurites (LNs) (Schlossmacher *et al.*, 2002), suggesting its possible involvement in the pathological processes mediating idiopathic Parkinson's disease. A point mutation in the ubiquitin carboxy terminal hydrolase L1 gene (*UCH-L1*) (Leroy *et al.*, 1998) has been reported to cause autosomal dominant Parkinson's disease in two affected siblings in a German family. Brain pathology is not yet available from these patients but UCH-L1 has been shown to be a component of LBs (Lowe *et al.*, 1990).

Recently, two homozygous mutations in the DJ-1 gene (*PARK7*) have been shown to cause early-onset autosomal recessive Parkinson's disease in families in the Netherlands and Italy (Bonifati *et al.*, 2003). In the Dutch family isolate there was a deletion in exons 1–5 that included the promoter start site of the DJ-1 gene. In the Italian family, a Leu-Pro substitution at position 166 segregated with Parkinson's disease. It is thought that the Dutch patients are unlikely to produce any DJ-1, whereas the point mutation in the Italian family could lead to an impairment of its normal function (Bonifati *et al.*, 2003). The neuropathological changes occurring in individuals affected by these mutations are not known. Other DJ-1 mutations, with homozygous, compound heterozygous and heterozygous genotypes, may confer disease susceptibility in young-onset Parkinson's disease, as shown by further recent genetic studies (Abou-Sleiman *et al.*, 2003; Hague *et al.*, 2003). Some of these mutations may be population-specific (Abou-Sleiman *et al.*, 2003).

DJ-1 is an 189 amino acid protein with multiple functions. It was first identified and cloned as a c-*myc* protein interactor by yeast-two hybrid screening from a HeLa cDNA library (Nagakubo *et al.*, 1997). In association with *ras*, it transformed NIH3T3 cells, suggesting it has oncogenic potential and may be involved in *ras*-mediated signalling pathways (Nagakubo *et al.*, 1997). The rat DJ-1 homologue, CAP1/sP22, was cloned from rat sperm and is important in fertilization (Wagenfeld *et al.*, 1998). An RNA-binding regulatory subunit, RS, purified from rat hepatoma cells (Hod *et al.*, 1999), is almost identical to DJ-1 and has the capacity to bind and inhibit RNA-binding activity. The structure of DJ-1 has some homology to the bacterial proteins ThiJ and Pfp1, which are involved in thiamine synthesis and protease activity respectively (Lee *et al.*, 2003).

DJ-1 may be involved in the regulation of transcription (Takahashi *et al.*, 2001). By binding with protein inhibitor of activated STAT (signal transducers and activators of transcription) (PIAS), a family of (SUMO-1) ligases, DJ-1 can positively regulate transcription of androgen-responsive genes (Takahashi *et al.*, 2001). It can be post-translationally modified by SUMO, a small ubiquitin-like modifier at amino acid position 130 (Takahashi *et al.*, 2001). One DJ-1 molecule interacts with another as seen in yeast-two hybrid system, and L166P mutation down-regulates this interaction (Miller *et al.*, 2003). DJ-1 appears to be sensitive to oxidative stress conditions and undergoes an alteration in its pI (from 6.2 to 5.8) when cultured endothelial cells are treated with paraquat (Mitsumoto *et al.*, 2001). The crystal structure of DJ-1 has been reported, and it suggests that DJ-1 proteins may only function as dimers and that the L166P mutation may disrupt the dimerization of the protein (Honbou *et al.*, 2003; Tao and Tong, 2003).

DJ-1 is expressed in many tissues, including the brain (Nagakubo *et al.*, 1997). DJ-1 mRNA expression is greater in subcortical regions (Bonifati *et al.*, 2003), raising the possibility that it may be important in basal ganglia function. DJ-1 is also expressed by a number of cell lines, is associated with microtubules and localizes to both the nucleus and the cytoplasm (Hod *et al.*, 1999).

Table 1 *Clinical and post-mortem details of cases included in the study*

Case	Sex	Age (years)	Post-mortem delay (h)*	Cause of death
Controls				
C1	F	88	49	COAD
C2	F	88	unknown	Embolism, ischaemia
C3	F	73	28	Bronchopneumonia, bronchial cancer
C4	M	81	40	COAD
C5	F	92	45	MI, IHD
C6	M	85	43	Oesophageal cancer
C7	M	79	56	Prostate cancer
Parkinson's disease				
P1	F	88	37	Parkinson's disease
P2	M	70	46	Bronchopneumonia
P3	M	73	25	Parkinson's disease, chest infection
P4	M	78	72	Bronchopneumonia
P5	F	77	48	Parkinson's disease, chest infection
P6	M	82	27	Bronchopneumonia
P7	M	75	45	Bronchopneumonia
P8	M	88	104	Cerebrovascular accident
P9	M	81	76	Parkinson's disease
P10	M	55	8	Parkinson's disease
P11	F	88	115	Lung cancer
P12	M	80	16	Parkinson's disease
P13	M	66	20	Cerebrovascular accident
P14	M	79	71	Bronchopneumonia
Parkinson's disease with DJ-1 R98Q polymorphism				
P15	M	75	5	Parkinson's disease
P16	F	72	20	MI
P17	F	82	7.3	MI
PSP				
PSP1	M	76	47	PSP
PSP2	M	77	45	PSP
PSP3	F	78	50	Cancer of the colon, parkinsonism

*From death to fixation of brain. Cause of death was as stated on death certificate or hospital post-mortem report. COAD = chronic obstructive airway disease; MI = myocardial infarction; IHD = ischaemic heart disease, PSP = progressive supranuclear palsy.

The function and detailed distribution of DJ-1 in the brain is unknown. It is also not known whether it is associated with LBs and LNs. The aim of this study was to delineate the distribution of endogenous DJ-1 protein in human brain and cultured cells. We also examined the distribution of the protein in normal and pathological states and examined the pI shift of this protein in response to oxidative stress and in Parkinson's disease.

Material and methods

Cases

Brain tissue was obtained from the Queen Square Brain Bank for Neurological Disorders. Human tissue was collected with the informed consent of next of kin and with the permission of the National Hospital for Neurology and Neurosurgery and the Institute of Neurology Joint Research Ethics Committee. All brains were neuropathologically evaluated to confirm the clinical diagnosis and to exclude undiagnosed pathology in the neurologically normal controls. Details of all cases are outlined in Table 1. Three of our cases harbour a heterozygous G to A substitution at position 98 in exon 5, and had the classical clinical and pathological phenotype of Parkinson's disease. This polymorphism results in the substitution of an alanine for a glycine residue (Abou-Sleiman *et al.*, 2003).

Western blotting

Human brain tissue from the frontal cortex of control, Parkinson's disease, Parkinson's disease with DJ-1 heterozygous mutations and progressive supranuclear palsy (PSP) were homogenized in isotonic sucrose (10% homogenates) buffered with HEPES (Sigma, Poole, UK) and spun at 12 000 *g* for 10 min to remove cellular debris. Protein from the supernatants was measured by the bicinchoninic acid method (Biorad, Hemel Hempstead, UK) using BSA as standard. Ten micrograms of protein from each supernatant was

solubilized in NuPAGE (Invitrogen, Paisley, UK) sample buffer and loaded onto 10% Bis-Tris gels and run with the MES (morpholinoethane sulphonic acid) buffer system (Invitrogen). Protein bands were subsequently electroblotted onto Hybond P (Pharmacia Biotech, UK) membrane. Duplicate membranes were blocked with 5% milk (Marvel) in phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween 20 (PBS-T). Western blot analysis was carried out using DJ-1 antibody (clone 3E8; Stressgen, San Diego, CA, USA) at 1 : 5000 dilution or β-tubulin (clone SAP.4G5; Sigma) also diluted 1 : 5000, followed by incubation with horseradish peroxidase-conjugated mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1 : 10 000 dilution. Three 5 min washes with PBS-T were performed between each antibody treatment. The blots were developed by the enhanced chemiluminescence (ECL) method using standard procedures and captured onto Kodak Biomax (Amersham, UK) autoradiography film.

Production of recombinant DJ-1 and antibody preabsorption

DJ-1 cDNA clone was purchased from HGMP (Cambridge, UK; clone No-1309796, GB.Acc – AA770265) and the plasmid DNA was used as the template for the polymerase chain reaction (PCR). The full-length DJ-1 cDNA was constructed using the forward primer 5′-AAAAGAGCTCTGGTCATCCTG-3′ and the reverse primer 5′-GTCTTTAAGAACAAGTGGACG-3′. The PCR reaction was run on a 60–50°C touchdown programme followed by 20 cycles at 95°C for 30 s, 50°C for 30 s and 72°C for 30 s. The correct sequence was verified by sequencing. The PCR product was subcloned into pTrcHis2-topo-cloning/expression vector (Invitrogen) and transformed into one-shot *E.coli* competent cells (Invitrogen). DJ-1 protein was expressed by induction with IPTG (an inducer of β-galactosidase activity in bacteria, Promega, Southampton, UK) (1 mM). The DJ-1 protein was purified using His-select HC nickel affinity gel (Sigma) according to the manufacturer's instructions. The purified fractions were run on SDS–PAGE for analysis, and dialysed against PBS containing 0.1 M PMSF. For preabsorption studies, DJ-1 antibody (1 : 5000, 1 : 3000) was incubated with 50 μg of recombinant DJ-1 fusion protein at 37°C for 1 h. The sample was further incubated sequentially at 4°C overnight. The solution was centrifuged for 10 min at 13 000 r.p.m. and the supernatant was used for blocking specific band on immunoblots and tissue sections.

Immunohistochemistry and immunofluorescence on brain sections

Formalin-fixed sections (6 μm) were first dewaxed in xylene. Endogenous peroxidase was blocked with 0.6% hydrogen peroxide in methanol for 10 min. Following rehydration, antigens were retrieved by autoclaving in citrate buffer (10 mM, pH 6) for 10 min. Sections were blocked with 10% normal goat serum (Sigma) before treating with monoclonal antibody to DJ-1 (Stressgen) and glial fibrillary acidic protein (GFAP; rabbit polyclonal; Dako, UK) at 1 : 3000 dilution overnight at 4°C. Biotinylated secondary anti-mouse antibody was used at 1 : 1000 (Vector Laboratories, Peterborough, UK) for 1 h, followed by avidin-biotin peroxidase complex (ABC) (Vector Laboratories) for 30 min. For visualization, hydrogen peroxide-activated DAB (diamino benzidine) was used. Three 5 min washes in PBS were carried out between each step. Tissue sections were lightly counterstained with haematoxylin, dehydrated through graded alcohols, cleared with xylene and mounted in mounting medium (Merck, UK). For specificity of staining, some sections were stained in the absence of primary antibody or with antibody that had been preabsorbed with recombinant DJ-1.

For double immunolabelling of DJ-1 and GFAP in astrocytes, antigens were retrieved by microwaving for 20 min in citrate buffer (10 mM, pH 6). DJ-1 and GFAP antibodies were used as above. The DJ-1 signal was visualized using the tetramethyl rhodamine and GFAP with the fluorescein signal amplification kit (Perkin Elmer, UK). Sections were washed thoroughly in PBS and mounted in Aquamount (Merck) and scanned using a Leica TCS40 laser confocal microscope.

Immunoelectron microscopy

Small blocks of substantia nigra were obtained from fresh tissue and fixed in 4% paraformaldehyde, 0.1% glutaraldehyde in 0.15 M Sorenson's phosphate buffer (pH 7.4) for 3 h. After fixation, the tissue were washed and stored in 0.15 M phosphate buffer (pH 7.4) overnight at 4°C, followed by dehydration in graded ethanols and embedded in London resin white (Agar Scientific, Stansted, UK). Ultrathin sections mounted on nickel grids were floated on a droplet of ammonium chloride (0.5 M) for 1 h, followed by incubating buffer containing 1.0% BSA, 1.0% normal goat serum, 0.1% sodium azide and 0.1% Tween 20 in PBS (pH 8.2) for 30 min. The sections were incubated in anti-DJ-1 (1 μg/μl, Stressgen) overnight at 4°C, washed and treated with goat anti-mouse gold conjugate (20 nm, BB International, Cardiff, UK) for 4 h at room temperature. After incubation with secondary antibody, the sections were washed in series of droplets of distilled water, stained with 0.5% uranyl acetate and examined in a Philips CM10 electron microscope operating at 80 kV. Control sections were prepared by omitting the primary antibody.

Primary cell culture and staining

Primary neurons and astrocytes from postnatal day 2 mouse pups were dissociated as described previously (Petrucelli *et al.*, 2002). Glial cells were cultured in feeder layers alone for 2–4 weeks and stained for GFAP using a monoclonal antibody G-A-5 (Sigma). These glial-enriched cultures were >95% GFAP-positive. Neuron-enriched cultures were prepared by dissecting and dissociating hippocampi, also from P2 mouse pups, which were plated in serum-free medium for 3 days prior to staining. Neurons were identified by staining with monoclonal antibody to microtubule-associated protein 2 (MAP2) (clone AP-20; Sigma) and represented approximately 75% of the cells in these cultures, the rest being contaminating glial cells. For DJ-1 staining, cells were preincubated with 500 nM Mitotracker CMTMRos (Molecular Probes, Eugene, OR, USA) for 30 min at 37°C prior to staining. Cells were fixed in 4% paraformaldehyde in Dulbecco's PBS (DPBS) for 30 min at room temperature, permeabilized with 0.1% Triton X-100 and quenched with 0.1 M glycine. After washing in DPBS, non-specific immunoreactivity was blocked with DPBS containing 10% foetal bovine serum and 0.1% Triton X-100 and incubated with sheep polyclonal anti-DJ-1 (gift of Gary Klinefelter; 1 : 1000) overnight at 4°C. This antibody was used in preference to the monoclonal antibody used for human tissue, which did not recognize DJ-1 in mouse brain extracts (data not shown). Cells were incubated with

AlexaFluor 488-conjugated donkey anti-sheep IgG conjugated prior to mounting under ProLong Antifade medium (Molecular Probes, Eugene, OR, USA). Slides were examined using a Zeiss LSM510 confocal microscope using independent excitation for both channels. Omission of primary antibody was used to evaluate non-specific fluorescence and in all cases gave no signal.

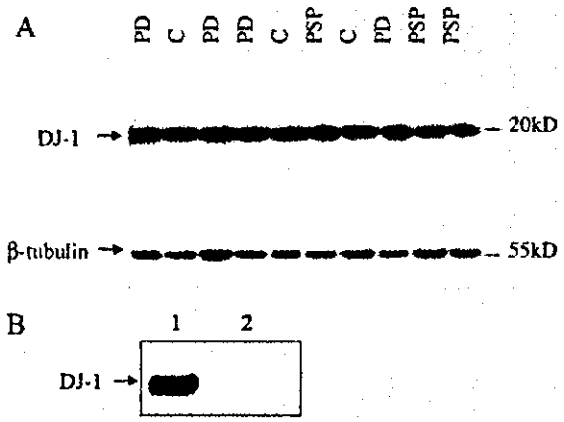

Fig. 1 (A) Immunoblot analysis of DJ-1 in human brain. Lysates of brain tissue of control (C), Parkinson's disease and PSP cases were analysed by immunoblot assay with DJ-1 antibody (*upper panel*). Each lane contained 10 μg of protein. Equivalent loadings were confirmed in replicate blots probed with β-tubulin (*lower panel*). Markers on the right of the blot indicate molecular weights in kilodaltons. (B) Preabsorption of DJ-1 antibody with recombinant DJ-1. The specific signal for DJ-1 (lane 1) shown is blocked by an excess of recombinant DJ1 (lane 2).

2D gel electrophoresis (2DGE)

M17 human dopaminergic neuroblastoma cells were grown in Optimem (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and either left untreated or exposed to 100 μM paraquat (Sigma) for 20 h. Cytosolic protein extracts were made as described previously (Allen *et al.*, 2003). For 2DGE, 500 μg of protein was separated first on 13 cm immobilized pH gradient (IPG; Amersham Biosciences) strips using 3.0–10.0 linear gradients according to the manufacturer's instructions on the IPGPhor system (Amersham Biosciences). The second dimension was resolved on 10–20% SDS–PAGE gels (Jule, Milford, CT, USA). Gels were blotted to Immobilon polyvinylidene difluoride (PVDF) (Amersham Biosciences) membranes and probed with monoclonal antibody to DJ-1 (Stressgen; 1 : 1000). Blots were developed using peroxidase-labelled secondary antibodies (Jackson Immunochemicals; 1 : 5000) and ECL-plus (Amersham Biosciences). pI was calibrated using creatine phosphokinase carbamylated standards (Amersham Biosciences) and molecular weight using Precision prestained markers (Biorad). A similar protocol was used for 2DGE for human extracts as above with some modifications. Briefly, for the first dimension, human brain homogenates (10 μg) from control and Parkinson's disease subjects, including those with heterozygous changes in the DJ-1 gene, were applied to IPG strips and separated as above. Proteins were blotted onto nitrocellulose membranes (Hybond ECL; Amersham Biosciences) which were probed for DJ-1 as detailed in the western blotting section. Protein spots were visualized with an ECL kit (Amersham Biosciences) according to the manufacturer's instructions.

Results

Western blotting

Immunoblotting of homogenized tissue from frontal cortex of control, idiopathic Parkinson's disease and PSP cases with

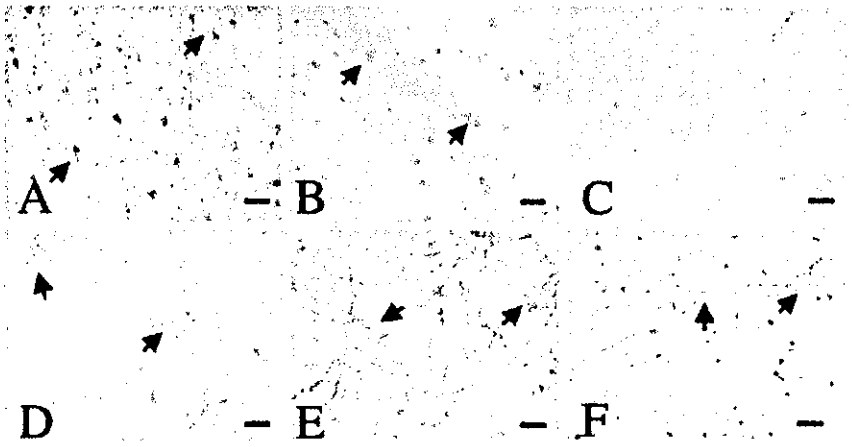

Fig. 2 Immunohistochemistry for DJ-1 in control, and Parkinson's disease tissue. Examples of DJ-1 IR in astrocytes (arrows) in the grey matter from control cortex (**A**) and the white matter in a Parkinson's disease case (**B**). This staining was specific, as a preabsorbed DJ-1 antibody with recombinant DJ-1 shows no positive astrocytic labelling in the white matter from a case of idiopathic Parkinson's disease (**C**). A high-power picture of astrocytes shows both cytoplasmic and nuclear staining for DJ-1 (**D**). Astrocyte morphology was demonstrated with GFAP staining in white matter of a case of idiopathic Parkinson's disease (**E**). In some non-DA neurons from the nigra of a Parkinson's disease case there was cytoplasmic and axonal staining for DJ-1 (**F**). Scale bars = 20 μm for A, B and C, and 10 μm for D, E and F.

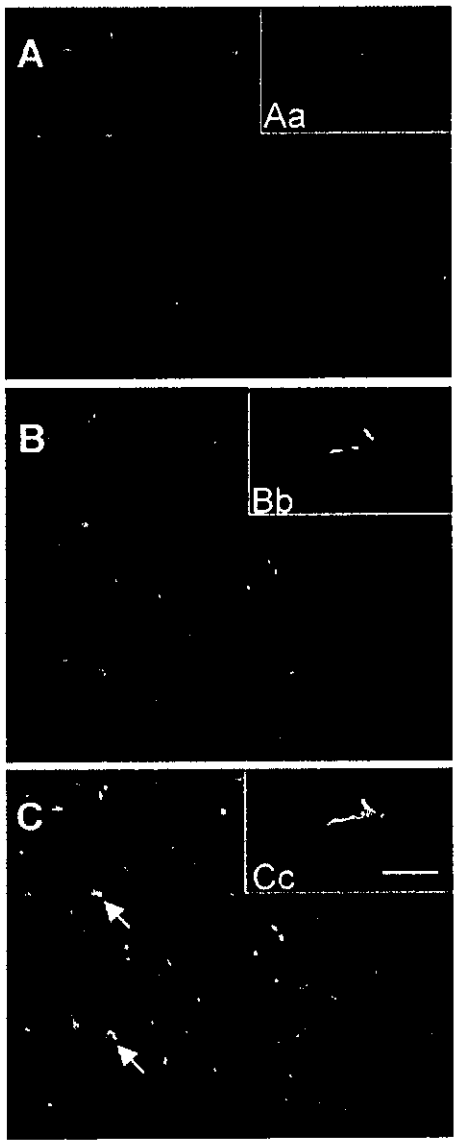

Fig. 3 Confocal images of DJ-1 and GFAP colocalization in astrocytes in frontal cortical white matter in a Parkinson's disease case. (**A**) DJ-1 localization in astrocytes (red). (**B**) GFAP in astrocytes (green). Yellow labelling (arrows) in **C** is true colocalization. Insets **Aa**, **Bb** and **Cc** show enlarged views of a single astrocyte double-labelled for DJ-1 and GFAP. Scale bar = 15 μm.

DJ-1 antibody demonstrated a single band at ~20 kDa (Fig. 1A), which was not detected when the antibody was preabsorbed with excess (50 μg) recombinant DJ-1 (Fig. 1B). Omission of primary antibody also did not give any band for DJ-1 (data not shown). There was no obvious difference in expression levels of DJ-1 in controls, Parkinson's disease or PSP cases. Our Parkinson's disease cases with DJ-1 R98Q polymorphism also gave a similar intensity band (data not shown).

DJ-1 IR in control brain tissue

Frontal cortex

Strong DJ-1 IR was observed in a proportion of glial cells with morphological attributes of astrocytes and DJ-1 IR glia were present in both grey (Fig. 2A) and white matter. In the grey matter, staining for DJ-1 was diffuse throughout the brain parenchyma with some individual glial profiles that were most common in the deep layers, along the white matter border. They were also seen at the cortical surface. DJ-1 IR in glia was present throughout the cell, in cytosol and glial processes. The morphology and distribution of DJ-1 IR cells was similar to that of GFAP IR cells, supporting the view that most DJ-1 IR cells were astrocytes (Fig. 2E), but the dense network of fibres revealed by GFAP staining was not seen with DJ-1 immunohistochemistry (Fig. 2A, B). Glial nuclear staining was seen in both grey and white matter (Fig. 2). There were more astrocytes positive for DJ-1 in the white matter than in the grey matter. Occasional cortical neurons of the deep layers were weakly DJ-1 immunoreactive. Specificity of glial staining by DJ-1 was demonstrated by incubation following pre-absorption of the antibody with recombinant DJ-1 (Fig. 2C) or omission of primary antibody when no staining was seen. Colocalization of DJ-1 and GFAP in astrocytes was also evident by double-labelling immunofluorescence.

Midbrain

Immunopositive cells with astrocyte morphology were observed in the neuropil surrounding DA nigral neurons. Very few were seen among the nigral cell groups. In some cases there was diffuse staining for DJ-1 throughout the nigral neuropil, with no distinct glial morphology; however, in these cases we observed a fair proportion of positively stained glial nuclei. Neuromelanin-containing neurons of the substantia nigra were negative for DJ-1 IR.

DJ-1 IR in Parkinson's disease

Frontal cortex

The pattern of staining for DJ-1 IR in Parkinson's disease frontal cortex was similar to that observed in control brain tissue. DJ-1 IR was present in glial cells in both white matter and grey matter, with the same distribution pattern as described above (Fig. 2B, D). Glial cell processes were positive for DJ-1 and staining was also seen in their nuclei and cytoplasm. DJ-1 and GFAP were found to colocalize in a number of astrocytes, as seen by double- immunofluorescence labelling (Fig. 3A–C). The majority of neuronal perikarya remained negative for DJ-1 IR but occasional neurons in the deep layers were DJ-1-positive. We did not observe any DJ-1-positive cortical LBs or LNs. A similar pattern of staining was seen in brains of Parkinson's disease subjects with *DJ-1* R98Q polymorphisms.

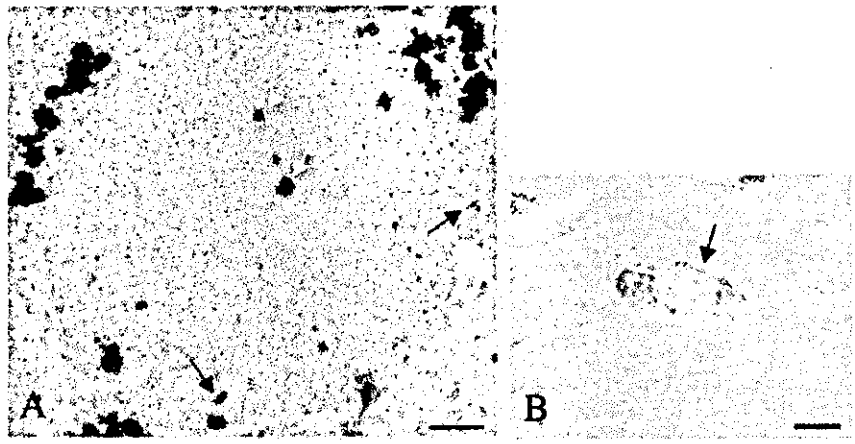

Fig. 4 Electron microscopic (**A**) and immunohistochemical (**B**) localization of DJ-1 in LBs from a case of idiopathic Parkinson's disease. Note that in (**A**), immunogold particles (20 nm) are sparsely scattered throughout the LBs. Some moderate to heavy clustering of gold particles is seen within circular structures (white arrows) in the core of the LB. Some gold conjugates are localized to structures resembling mitochondria at the periphery of the LB (black arrows). Scale bar = 2 nm. (**B**) Using immunohistochemistry, rare LB showed only light immunostaining for DJ-1 at the periphery (arrow) Scale bar = 10 μm.

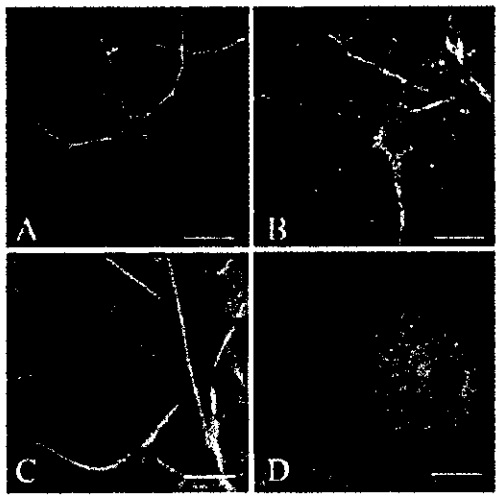

Fig. 5 Expression of DJ-1 in mouse primary neurons and astrocytes *in vitro* and comparison with mitochondrial staining. Cultures enriched in neurons (**A, B**) and glia (**C, D**) were stained for MAP2 (**A**) or GFAP (**C**) to demonstrate the distinct neuronal and glial morphologies respectively. DJ-1 was expressed in both cell types (green staining in **B** and **D**) but showed minimal overlap with mitotracker (red). Scale bar = 20 μm.

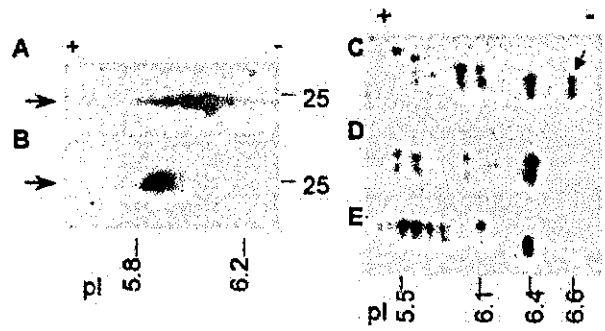

Fig. 6 Multiple isoforms of DJ-1 in neural tissue. (**A, B**) pI shift of endogenous DJ-1 in a human cell line under conditions of oxidative stress. Blots of 2D gels from (**A**) untreated or (**B**) paraquat-treated M17 cells using monoclonal antibody to human DJ-1. The arrow indicates endogenously expressed human DJ-1 (~20 kDa) isoforms, with more acidic isoforms towards the left of the blot (+). Molecular weight markers on the right of each blot are in kDa. The markers below the blot indicate approximate pI values of the different species of DJ-1. (**C–E**). 2D gel electrophoresis of human frontal cortex extracts. Representative blots of 2DGE from control (**C**, $n = 3$), Parkinson's disease (**D**, $n = 3$) and Parkinson's disease tissue with DJ-1 R98Q polymorphisms (**E**, $n = 2$). Multiple pI isoforms were detected for DJ-1 in all the samples. The arrow indicates the 6.6 isoform, which appears to be absent or diminished in Parkinson's disease and Parkinson's disease brains with DJ-1 R98Q polymorphisms (**D, E**) All the spots are ~20 kDa, corresponding to native DJ-1.

Midbrain

In Parkinson's disease nigra, in addition to glial staining, DJ-1 IR was observed only in occasional LBs and a single LN. However the majority of LBs and pale bodies were negative. In the few LBs that were stained, DJ-1 was mainly localized to the periphery (Fig. 4B). The cytoplasm of DA neurons in Parkinson's disease brains was lightly labelled and staining was also seen in neurons of the third nerve nucleus. Some non-melanized neurons and their processes were also lightly labelled (Fig. 2F). In immunoelectron microscope studies, some scattered immunogold labelling of DJ-1 was observed within a small number of nigral LBs. In some, immunogold

particles were found to be concentrated around circular structures (Fig. 4A) In addition, some conjugated gold particles were observed on structures resembling mitochondria around the periphery of the LB (Fig. 4A). No immunogold labelling of the fibrillar material *per se* was detected. No labelling was seen when the primary antibody was omitted (data not shown).

DJ-1 expression in mouse primary astrocytes and hippocampal neurons

Given that DJ-1 expression was found in glial cells *in vivo*, we also cultured cortical astrocytes. DJ-1 was also expressed endogenously in both mouse hippocampal neurons and astrocytes (Fig. 5). Previous results (Bonifati *et al.*, 2003) have shown a possible colocalization of DJ-1, especially the L166P mutant, with mitochondria. However, in both these cultured cell types, endogenous DJ-1 signal (green) did not appear to colocalize with the Mitotracker probe (red) (Fig. 5 B, D).

Multiple isoforms of DJ-1 in oxidative stress models and human brain

Previous results in non-neuronal cell lines have indicated that exposure to oxidative stress produces a shift in pI of DJ-1, but no results in neural tissue have been reported to date. We first replicated previous results showing a paraquat-induced pI shift for DJ-1 in the human dopaminergic neuroblastoma cell line M17. These cells express readily detectable endogenous amounts of DJ-1 with a range of pI isoforms from approximately 6.0 to 6.2 (Fig. 6A). Exposure to 100 μM paraquat resulted in the accumulation of acidic isoforms with a pI of 5.8 (Fig. 6B). We next performed 2DGE of human frontal cortex extracts and found at least six different pI isoforms of DJ-1. In control brain, they ranged from 5.5 to 6.6, the 6.4 pI isoform being the most prominent. Compared with controls (Fig. 6C), in Parkinson's disease samples the 6.6 pI isoform appears to be missing or diminished (Fig. 6D). Additionally, a marked difference in the distribution of the more acidic DJ-1 isoforms was seen in the Parkinson's disease subjects with DJ-1 R98Q polymorphism compared with both control subjects and other Parkinson's disease cases (Fig. 6E).

Discussion

This is the first report on the distribution of DJ-1 protein in the human brain. We have used an antibody for DJ-1 that recognizes a single band corresponding to DJ-1 from human frontal cortex homogenates by western blotting, but not rodent DJ-1 (data not shown). DJ-1 is expressed in high amounts in the frontal cortex, a region of predilection for LB deposition, in control, PSP and Parkinson's disease cases (Fig. 1A). Using this highly specific antibody, we have shown that the major cell type expressing DJ-1 IR in human brain is glial rather than neuronal (Fig. 2A, B, D) We have corroborated the findings on DJ-1 immunohistochemistry by staining with GFAP (Fig. 2E), which produces a similar staining pattern with respect to the morphology and distribution of glial cells. Further confirmation of the localization of DJ-1 in astrocytes has been shown by double-labelling immunofluorescence and confocal microscopy (Fig. 3A–C). The localization of DJ-1 IR in both the cytoplasm and nucleus of glial cells is in keeping with the known distribution of this protein in various cell lines (Nagakubo *et al.*, 1997). In addition, we show that some neurons are weakly DJ-1 immunopositive (Fig. 2F). However, the relative concentrations of DJ-1 protein in neurons may be much lower compared with astrocytes. Staining was specific, as recombinant DJ-1 protein was able to abolish immunoreactivity in both blotting and staining techniques.

The presence of significant amounts of DJ-1 in glial cells in the brain is of interest as the two other Parkinson's disease genes, α-synuclein and parkin, are predominantly neuronal. However, it has been recently shown that parkin expression in glial cells is up-regulated during unfolded protein stress (Ledesma *et al.*, 2002). We have shown that DJ-1 is expressed by mouse primary astrocytes and neurons (Fig. 5). A difference between *in vivo* and *in vitro* results is that cultured mouse hippocampal neurons did express detectable DJ-1, suggesting either that there are species differences or that the tissue culture environment promotes the expression of this stress-responsive protein. As DJ-1 is responsive to oxidative stress (see below), it is possible that DJ-1 is up-regulated in the culture environment due to exposure to free radicals.

The brain regions chosen for this study are known to be vulnerable to pathological damage in Parkinson's disease, including LB and LN accumulation. In both the typical Parkinson's disease cases and in the cases of Parkinson's disease with DJ-1 R98Q polymorphism examined here, DJ-1 IR was localized to only a few nigral LBs, indicating that DJ-1 protein is not an essential component of LBs and is unlikely to be important in their formation in Parkinson's disease. This result is in contrast to the localization of α-synuclein and parkin, which are present in most LBs and LNs (Spillantini *et al.*, 1998; Schlossmacher *et al.*, 2002).

Our 2D gel analysis of human brain extracts and cells exposed to oxidative stress, shows the existence of a range of pI isoforms for DJ-1 protein. This may be specific to brain tissue as only two pI isoforms for DJ-1 have been reported in human endothelial cell cultures and in mouse lung tissue (Mitsumoto and Nakagawa, 2001). In addition, we show that DJ-1 protein responds to the oxidative stressor paraquat by exhibiting more acidic pI isoforms in a neuroblastoma cell line (Fig. 6A), in agreement with a previous study showing DJ-1 sensitivity to paraquat (Mitsumoto *et al.*, 2001). Oxidative stress factors are believed to be implicated in the pathogenesis of Parkinson's disease (Jenner, 2003) and it has been suggested that the shift in pI of DJ-1 is a useful indicator of oxidative stress status both *in vivo* and *in vitro* (Mitsumoto and Nakagawa, 2001). Thus the presence of multiple isoforms

of DJ-1 in control and Parkinson's disease brains and the tendency of the most alkaline pI isoform to be absent from Parkinson's disease cases (Fig. 6D,E) could point to the involvement of oxidative stress in (Abbas *et al.*, 1999) Parkinson's disease. Whether these factors are the cause of the differences in the DJ-1 pI isoform distribution in the brain between cases of Parkinson's disease and cases with DJ-1 R98Q polymorphism remains to be determined, but it is clear that there is greater complexity in DJ-1 expression in the human brain compared with other systems. Our Parkinson's disease cases heterozygous for DJ-1 mutation showed a similar expression pattern for DJ-1 protein compared with idiopathic Parkinson's disease cases and phenotypically they were similar to late-onset Parkinson's disease cases. It is possible that in these individuals, the DJ-1 R98Q polymorphism is non-pathogenic or that the DJ-1 variant together with other yet unknown variants are responsible for the disease phenotype (Abou-Sleiman *et al.*, 2003). In this respect, the DJ-1 R98Q polymorphism may be similar to parkin mutation, for which a single heterozygous mutation may confer disease susceptibility (Abbas *et al.*, 1999) indistinguishable from idiopathic Parkinson's disease. The effect of the G to A heterozygous DJ-1 mutation on the biological function of the protein, however, remains to be studied.

There is increasing interest in the possibility that glial cells may be major contributors to oxidative stress in Parkinson's disease (Czlonkowska *et al.*, 2002; Teismann *et al.*, 2003). The post-mortem Parkinson's disease brain exhibits some increase in astroglia, as seen by GFAP staining (Forno *et al.*, 1992; Mirza *et al.*, 2000). Furthermore, there is an inverse correlation between numbers of GFAP-positive astrocytes and DA cell loss, and areas with a sparse astrocytic response show greater cell loss (Damier *et al.*, 1993). In addition, histological examination of familial Parkinson's disease cases with parkin mutations has also shown gliosis in the nigra (Ishikawa and Takahashi, 1998; Hayashi *et al.*, 2000; Hishikawa *et al.*, 2001). Astrocytes, however, are known for their protective effects on neurons through their capacity to secrete glial cell-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF), and may be protective (Czlonkowska *et al.*, 2002). Addition of astrocytes to neuronal cultures prevents cell death caused by a number of neurotoxic compounds (Hou *et al.*, 1997; Tieu *et al.*, 2001; Mena *et al.*, 2002). Furthermore, astrocytes are rich in glutathione peroxidase, which catalyses the removal of hydrogen peroxide by formation of oxidized glutathione and prevents the formation of hydroxyl radicals by a reaction between hydrogen peroxide and heavy metals. The levels of this important enzyme have been demonstrated to be reduced in homogenates of substantia nigra in Parkinson's disease (Sofic *et al.*, 1992; Sian *et al.*, 1994; Pearce *et al.*, 1997). It is possible that some of this reduction in glutathione peroxidase takes place in nigral glia. Any change in the normal physiological role of astrocytes may therefore contribute to DA cell death by reduced secretion of neurotrophic factors and a reduced capacity of glia to cope with free radical production. The abundance of DJ-1 in astroglia suggests a prominent role for this protein in glial biology and in some neurodegenerative processes, and may also indicate that astrocytes are subjected to oxidative stress in Parkinson's disease.

Present concepts of disease pathogenesis suggest a unifying pathway involving α-synuclein, parkin and UCH-L1, the three genes associated with familial forms of Parkinson's disease, within the ubiquitin–proteasome system (UPS) (Cookson, 2003; Hardy *et al.*, 2003). In this context, reduced proteasome function in Parkinson's disease cases, indicating a defect in the UPS, has been reported (McNaught and Jenner, 2001). How this relates to oxidative stress is unclear, but there is some evidence that the proteasome has a major role in the degradation of oxidatively damaged proteins (Grune *et al.*, 2003; Shringarpure *et al.*, 2003). Furthermore, oxidative stress can inhibit proteasome function (Ding and Keller, 2001) and formation of intracellular protein aggregates is dependent on oxidative events (Demasi and Davies, 2003). Therefore, the pathways involving proteasome function and oxidative stress may intersect, as has been highlighted by others (Chung *et al.*, 2001; Jenner, 2003). It is not yet clear whether DJ-1 will be part of this pathway or whether it will involve new pathways contributing to a common end-point (Cookson, 2003). Interestingly, mutant DJ-1, but not the wild-type protein, is degraded by the UPS in cell culture (Miller *et al.*, 2003). Further work on the cell biology of DJ-1 is needed to establish its precise role in the loss of DA neurons. However, our study provides further data to suggest that glia may be important in the pathogenesis of Parkinson's disease and that interactions between neuronal and glial function should be investigated in greater depth.

Acknowledgements

This study was funded by the Reta Lila Weston Trust. This work was presented at the XVth International Congress of Neuropathology, Turin, September 14–18, 2003.

References

Abbas N, Lucking CB, Ricard S, Durr A, Bonifati V, De Michele G, et al. A wide variety of mutations in the parkin gene are responsible for autosomal recessive parkinsonism in Europe. French Parkinson's Disease Genetics Study Group and the European Consortium on Genetic Susceptibility in Parkinson's Disease. Hum Mol Genet 1999; 8: 567–74.

Abou-Sleiman PM, Healy DG, Quinn NP, Lees AJ, Wood NW. The role of pathogenic DJ-1 mutations in Parkinson's disease. Ann Neurol 2003; 54: 283–6.

Allen S, Heath PR, Kirby J, Wharton SB, Cookson MR, Menzies FM, et al. Analysis of the cytosolic proteome in a cell culture model of familial amyotrophic lateral sclerosis reveals alterations to the proteasome, antioxidant defenses, and nitric oxide synthetic pathways. J Biol Chem 2003; 278: 6371–83.

Bonifati V, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E, et al. Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science 2003; 299: 256–9.

Chung KK, Dawson VL, Dawson TM. The role of the ubiquitin-proteasomal

pathway in Parkinson's disease and other neurodegenerative disorders. Trends Neurosci 2001; 24 (11 Suppl): S7–14.

Cookson MR. Pathways to parkinsonism. Neuron 2003; 37: 7–10.

Czlonkowska A, Kurkowska-Jastrzebska I, Czlonkowski A, Peter D, Stefano GB. Immune processes in the pathogenesis of Parkinson's disease—a potential role for microglia and nitric oxide. Med Sci Monit 2002; 8: RA165–77.

Damier P, Hirsch EC, Zhang P, Agid Y, Javoy-Agid F. Glutathione peroxidase, glial cells and Parkinson's disease. Neuroscience 1993; 52: 1–6.

Dawson TM, Dawson VL. Rare genetic mutations shed light on the pathogenesis of Parkinson disease. J Clin Invest 2003; 111: 145–51.

de Rijk MC, Tzourio C, Breteler MM, Dartigues JF, Amaducci L, Lopez-Pousa S, et al. Prevalence of parkinsonism and Parkinson's disease in Europe: the EUROPARKINSON Collaborative Study. European Community Concerted Action on the Epidemiology of Parkinson's disease. J Neurol Neurosurg Psychiatry 1997; 62: 10–5.

Demasi M, Davies KJ. Proteasome inhibitors induce intracellular protein aggregation and cell death by an oxygen-dependent mechanism. FEBS Lett 2003; 542: 89–94.

Ding Q, Keller JN. Proteasome inhibition in oxidative stress neurotoxicity: implications for heat shock proteins. J Neurochem 2001; 77: 1010–7.

Feany MB, Bender WW. A Drosophila model of Parkinson's disease. Nature 2000; 404: 394–8.

Forno LS. Neuropathology of Parkinson's disease. J Neuropathol Exp Neurol 1996; 55: 259–72.

Forno LS, DeLanney LE, Irwin I, Di Monte D, Langston JW. Astrocytes and Parkinson's disease. Prog Brain Res 1992; 94: 429–36.

Grune T, Merker K, Sandig G, Davies KJ. Selective degradation of oxidatively modified protein substrates by the proteasome. Biochem Biophys Res Commun 2003; 305: 709–18.

Hague S, Rogaeva E, Hernandez D, Gulick C, Singleton A, Hanson M, et al. Early-onset Parkinson's disease caused by a compound heterozygous DJ-1 mutation. Ann Neurol 2003; 54: 271–4.

Hardy J, Cookson MR, Singleton A. genes and parkinsonism. Lancet Neurol 2003; 2: 221–8.

Hayashi S, Wakabayashi K, Ishikawa A, Nagai H, Saito M, Maruyama M, et al. An autopsy case of autosomal-recessive juvenile parkinsonism with a homozygous exon 4 deletion in the parkin gene. Mov Disord 2000; 15: 884–8.

Hishikawa N, Hashizume Y, Yoshida M, Sobue G. Widespread occurrence of argyrophilic glial inclusions in Parkinson's disease. Neuropathol Appl Neurobiol 2001; 27: 362–72.

Hod Y, Pentyala SN, Whyard TC, El-Maghrabi MR. Identification and characterization of a novel protein that regulates RNA-protein interaction. J Cell Biochem 1999; 72: 435–44.

Honbou K, Suzuki NN, Horiuchi M, Niki T, Taira T, Ariga H, et al. The crystal structure of DJ-1, a protein related to male fertility and Parkinson's disease. J Biol Chem 2003; 278: 31380–4.

Hou JG, Cohen G, Mytilineou C. Basic fibroblast growth factor stimulation of glial cells protects dopamine neurons from 6-hydroxydopamine toxicity: involvement of the glutathione system. J Neurochem 1997; 69: 76–83.

Ishikawa A, Takahashi H. Clinical and neuropathological aspects of autosomal recessive juvenile parkinsonism. J Neurol 1998; 245 (11 Suppl 3): P4–9.

Jenner P. Oxidative stress in Parkinson's disease. Ann Neurol 2003; 53 Suppl 3: S26–36; discussion S36–8.

Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 1998; 392: 605–8.

Ledesma MD, Galvan C, Hellias B, Dotti C, Jensen PH. Astrocytic but not neuronal increased expression and redistribution of parkin during unfolded protein stress. J Neurochem 2002; 83: 1431–40.

Lee SJ, Kim SJ, Kim IK, Ko J, Jeong CS, Kim GH, et al. Crystal structures of human DJ-1 and *Escherichia coli* Hsp 31 which share an evolutionarily conserved domain. J Biol Chem 2003; 45: 44552–9.

Leroy E, Boyer R, Auburger G, Leube B, Ulm G, Mezey E, et al. The ubiquitin pathway in Parkinson's disease. Nature 1998; 395: 451–2.

Lowe J, McDermott H, Landon M, Mayer RJ, Wilkinson KD. Ubiquitin carboxyl-terminal hydrolase (PGP 9.5) is selectively present in ubiquitinated inclusion bodies characteristic of human neurodegenerative diseases. J Pathol 1990; 161: 153–60.

Masliah E, Rockenstein E, Veinbergs I, Mallory M, Hashimoto M, Takeda A, et al. Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders. Science 2000; 287: 1265–9.

McNaught KS, Jenner P. Proteasomal function is impaired in substantia nigra in Parkinson's disease. Neurosci Lett 2001; 297: 191–4.

Mena MA, de Bernardo S, Casarejos MJ, Canals S, Rodriguez-Martin E. The role of astroglia on the survival of dopamine neurons. Mol Neurobiol 2002; 25: 245–63.

Miller DW, Ahmad R, Hague S, Baptista M, Canet-Aviles R, McLendon C, et al. L166P mutant DJ-1, causative for recessive Parkinson's disease, is degraded through the ubiquitin-proteasome pathway. J Biol Chem 2003; 278: 36588–95.

Mirza B, Hadberg H, Thomsen P, Moos T. The absence of reactive astrocytosis is indicative of a unique inflammatory process in Parkinson's disease. Neuroscience 2000; 95: 425–32.

Mitsumoto A, Nakagawa Y. DJ-1 is an indicator for endogenous reactive oxygen species elicited by endotoxin. Free Radic Res 2001; 35: 885–93.

Mitsumoto A, Nakagawa Y, Takeuchi A, Okawa K, Iwamatsu A, Takanezawa Y. Oxidized forms of peroxiredoxins and DJ-1 on two-dimensional gels increased in response to sublethal levels of paraquat. Free Radic Res 2001; 35: 301–10.

Mori H, Kondo T, Yokochi M, Matsumine H, Nakagawa-Hattori Y, Miyake T, et al. Pathologic and biochemical studies of juvenile parkinsonism linked to chromosome 6q. Neurology 1998; 51: 890–2.

Nagakubo D, Taira T, Kitaura H, Ikeda M, Tamai K, Iguchi-Ariga SM, et al. DJ-1, a novel oncogene which transforms mouse NIH3T3 cells in cooperation with ras. Biochem Biophys Res Commun 1997; 231: 509–13.

Pearce RK, Owen A, Daniel S, Jenner P, Marsden CD. Alterations in the distribution of glutathione in the substantia nigra in Parkinson's disease. J Neural Transm 1997; 104: 661–77.

Petrucelli L, O'Farrell C, Lockhart PJ, Baptista M, Kehoe K, Vink L, et al. Parkin protects against the toxicity associated with mutant alpha-synuclein: proteasome dysfunction selectively affects catecholaminergic neurons. Neuron 2002; 36: 1007–19.

Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, et al. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 1997; 276: 2045–7.

Schlossmacher MG, Frosch MP, Gai WP, Medina M, Sharma N, Forno L, et al. Parkin localizes to the Lewy bodies of Parkinson disease and dementia with Lewy bodies. Am J Pathol 2002; 160: 1655–67.

Shimura H, Hattori N, Kubo S, Yoshikawa M, Kitada T, Matsumine H, et al. Immunohistochemical and subcellular localization of Parkin protein: absence of protein in autosomal recessive juvenile parkinsonism patients. Ann Neurol 1999; 45: 668–72.

Shimura H, Hattori N, Kubo S, Mizuno Y, Asakawa S, Minoshima S, et al. Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. Nature Genet 2000; 25: 302–5.

Shringarpure R, Grune T, Mehlhase J, Davies KJ. Ubiquitin conjugation is not required for the degradation of oxidized proteins by proteasome. J Biol Chem 2003; 278: 311–8.

Sian J, Dexter DT, Lees AJ, Daniel S, Agid Y, Javoy-Agid F, et al. Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. Ann Neurol 1994; 36: 348–55.

Sofic E, Lange KW, Jellinger K, Riederer P. Reduced and oxidized glutathione in the substantia nigra of patients with Parkinson's disease. Neurosci Lett 1992; 142: 128–30.

Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. Nature 1997; 388: 839–40.