村山繁雄	一般臨床のためのパーキンソン病の病理	Modern Physician	25	918-920	2005
村山繁雄、齊藤祐子	高齢者痴呆患者の病理	Dementia Japan	19	67-76	2005
村山繁雄, 齊藤祐子	もの忘れがひどい	診断と治療	93	1277-1280	2005
村山繁雄、齊藤祐子	嗜銀顆粒性痴呆	Clinical Neuroscience	23	297-299	2005
村山繁雄、齊藤祐子、金丸和富、徳丸阿耶、石井賢二、沢辺元司、愛敬尚雄、原田三枝子、直井信子	ブレインバンクの構築と課題	日本老年医学会雑誌	42	483-489	2005
石井賢二	ＰＥＴ利用の手引き-Alzheimer病	臨床医	31	1609-1614	2005
村山繁雄、齊藤祐子	脳卒中専門医のための神経機能解剖学　大脳白質	分子脳血管病	4	70-75	2005
金丸和富	高齢者ケアのガイドライン　歩行障害	ジェロントロジー　ニューホライズン	18	62-65	2006
初田裕幸; 村山繁雄	プリオン病の病理学的診断	神経内科	63	452-458	2006
村山繁雄	末梢神経・筋疾患の病理のみかた.	病理と臨床	24	1143	2006
村山繁雄;:	序論：病理の中での神経・筋の位置づけ.	病理と臨床	24	1144-1155	2006
村山繁雄	進行性多巣性白質脳症.	日内会誌	95	46- 50	2006

村山繁雄	認知症の動的神経病理	臨床検査	50	1083-1084	2006
村山繁雄, 齊藤祐子	ブレインバンク	脳と神経	58	1075-1084	2006
村山繁雄, 齊藤祐子	老化と認知症の臨床・画像・病理連関（動的神経病理）による解明-ブレインバンクプロジェクト.	臨床検査	50	1085-1089	2006
齊藤祐子	神経・筋疾患、剖検の取り扱い.	病理と臨床	24	1160-1165	2006
齊藤祐子	タウ蛋白蓄積症＝タウオパチー	臨床検査	50	1121-1129	2006
齊藤祐子	αシヌクレイン病変は錐体外路症状・知的機能低下の責任病巣とならない	PD Today		12-13	2006
齊藤祐子	パーキンソン病の補助診断としてのMIBG心筋シンチグラフィー検査・特に高齢者における擬陽性例について	PD Today		12-13	2006
齊藤祐子	パーキンソン病における知的機能低下と死亡のリスクの予後・大規模前方視的研究から	PD Today		12-13	2006
徳丸阿耶	認知症　：動的神経病理としてのMRI	臨床検査	50	1090-1098	2006
大場洋　徳丸阿耶ら	パーキンソン病の画像診断—ＭＲＩ	Clinical Neuroscienc	25	55-58	2006
石井賢二	アルツハイマー病の早期診断に有効なのはアミロイドイメージングか、脳代謝・血流画像か　アミロイドイメージングであるとの立場	Cognition and	6(1)	68-73	2007
藤原佳典、西真理子、渡辺直紀、李相侖、井上かず子、吉田裕人、佐久間尚子、呉田陽一、石井賢二、内田勇人、角野文彦、新開省二.	都市部高齢者による世代間交流型ヘルスプロモーションプログラム"REPRINTS"の1年間の歩みと短期的効果.	日本公衆衛生学会誌	53(9)	702-714	2006

石井賢二.	脳疾患におけるPETの現状と展望.	映像情報 Medical	38(11)	1044-1053	2006
石井賢二.	動的神経病理としてのPET.	臨床検査	50(10)	1099-1105	2006
金丸和富	動的神経病理としての髄液バイオマーカー	臨床検査	50	1107-1110	2006
村山繁雄、齊藤祐子	【シヌクレイノパチーの臨床と基礎研究】α-シヌクレイノパチーの病理.	Dementia Japan	21	15-24	2007
村山繁雄, 齊藤祐子	PMLの神経病理	BRAIN and NERVE	59	119-124	2007
大場洋, 徳丸阿耶, 村山繁雄, 齊藤祐子, 松田博史	パーキンソン病の画像診断　MRI.	Clinical Neuroscience	25	55-58	2007
徳丸阿耶, 齊藤佑子, 村山繁雄, 金丸和富	クロイツフェルト・ヤコブ病の画像所見	感染症	37	97-99, 109-112	2007
徳丸阿耶, 齊藤佑子, 村山繁雄, 金丸和富	高齢者の中枢神経感染症	感染症	37	100, 113-115	2007
徳丸　阿耶	プロトン密度強調画像の特徴はなんですか。どのような病変に有用ですか。	小児内科	39	37-39	2007
徳丸　阿耶	拡散強調画像の特徴はなんですか。どのような病変に有用ですか。	小児内科	39	40-43	2007
徳丸阿耶、齊藤祐子、村山繁雄	高齢者における画像診断～高齢者に見られる画像変化を中心に	日獨医報	52	444-459	2007
齊藤祐子, 福田覚, 村山繁雄	末梢神経の基礎　解剖学的側面	Clinical Neuroscience	25	746-749	2007

村山繁雄, 齊藤祐子, 初田裕幸, 崎山快夫	プリオン病の病理診断	日本臨床	25	1401-1406	2007
村山繁雄, 齊藤祐子	神経障害を来す中毒物質　有機溶媒.	Clinical Neuroscience	25	894-895	2007
石井賢二	高齢者医療におけるPETの役割	日本老年医学会雑誌	44	143-153	2007
石井賢二	アルツハイマー病の早期診断に有効なのはアミロイドイメージングか、脳代謝・血流画像か　アミロイドイメージングであるとの立場から	Cognition and Dementia	6	68-73	2007
石井賢二	アミロイドイメージング	臨床神経学	47	915-917	2007
三品雅洋、石井賢二、石渡喜一	アデノシン受容体　-ドパミンとの関連-	臨床神経学	47	835-837	2007
石井賢二	アミロイドイメージング -Alzheimer病の超早期診断と根本治療に向けて-	医学のあゆみ	224	239-240	2008

邦文単行本

著　者　名	論　文　題　名	書　名	編集者	発行社	発行地
村山繁雄・齊藤祐子	PDD(認知症を伴うパーキンソン病)とDLB(レヴィー小体型認知症)の臨床と病理。	Annual Review神経2007		中外医学社	東京
石井賢二	パーキンソン病	臨床医のためのクリニカルPET - 病期・病態診断のためのガイドブック -	クリニカルPET編集委員会	先端医療研究所	東京

石井賢二	脳PET、SPECT	自律神経機能検査（第4版）	日本自律神経学会	文光堂	東京

ブレインバンクの構築と課題

村山　繁雄　　齊藤　祐子　　金丸　和富
徳丸　阿耶　　石井　賢二　　沢辺　元司
高齢者ブレインバンクチーム

日本老年医学会雑誌　第42巻　第５号　別刷

老年医学の展望

ブレインバンクの構築と課題

村山 繁雄[1] 齊藤 祐子[2] 金丸 和富[3]
徳丸 阿耶[4] 石井 賢二[5] 沢辺 元司[2]
高齢者ブレインバンクチーム

〈要 約〉 老化・痴呆の克服を目指し，在宅高齢者支援病院と併設研究所が共同で，ブレインバンクシステムを構築した．法的基盤としては，死体解剖保存法18条と，病院剖検承諾書をもとに行う，共同研究を前提とした．共同研究申し込みの内容に対しては，論文審査と同様の守秘義務のもと，外部委員による事前審査を行うこととした．共同研究者の適格性については審査の上，研究所協力研究員に委嘱するかたちをとった．倫理面では，病院・研究所及び，共同研究先の倫理委員会の承認を前提とした．その上で，バンク管理者，神経病理診断責任者，臨床情報提供者が，共同研究者となることを条件に，共同研究を開始した．標本採取には，神経病理担当医が，開頭剖検例全例に対し，臨床・画像を判断の上，採取法を決定した．凍結側の脳については，割面を含む肉眼所見を正確に写真に残し，代表部位6箇所を採取，神経病理学的診断を行った．凍結については，ドライアイスパウダー法を採用した．反対脳については，既報通り（Saito Y, et al：2004）検討した．現在までの蓄積は，脳パラフィンブロック6,500例以上，凍結脳（部分）1,500例以上，凍結半脳450例以上で，30件以上の共同研究を実行中である．欧米のブレインバンクとはシステムは異なるが，その哲学である，「篤志によるものは公共のドメインに属し，公共の福祉に貢献しなければならない」を共有する点で，ブレインバンクの名称を用いることとした．依然として，大多数の日本の研究者が，欧米のブレインバンクに依存している事態の打開のためには，このシステムが市民権を得るよう，努力していく必要がある．そのためには，同様の哲学を有するもので，ネットワーク構築を行うことにより，公的研究費を得る環境作りが必要である．ブレインバンクの重要性が人口に膾炙された上で，患者団体との提携をめざすことが，現実的と思われる．

Key words：老化，痴呆，アルツハイマー病，パーキンソン病，タウオパチー

（日老医誌 2005；42：483—489）

背 景

ヒトと動物の最も異なる点は脳であり，いかなる脳疾患の研究成果も，ヒト脳を用い，最終確認を行う必要がある．欧米においては，剖検疾患脳とコントロール脳の，病理組織標本と，凍結標本を，保存・管理・提供するブレインバンクが，脳研究の基盤となっている．この構築に，患者団体・研究者が協力しているのが，英国のパーキンソン病協会ブレインバンク（http://www.parkinsonstissuebank.org.uk/），米国の自閉症ブレインバンク（http://medschool.umaryland.edu/BTBank/），米国進行性核上性麻痺バンク（http://mayoresearch.mayo.edu/mayo/research/mcj/BrainBank.cfm）等である．一方，行政サイドと研究者が協力し構築しているものに，欧米のプリオン病ブレインバンク，後天性免疫不全症候群ブレインバンク等がある．

一方我が国において，公的なブレインバンクはこれまで存在していなかった．その結果，日本の脳研究者の多くが欧米のブレインバンクに依存してきた．しかし疾患には人種差があること，海外のブレインバンクでは共同研究を組む点で，臨床病理学的情報が十分に得られない等の問題がある．また，臓器移植と同様で，日本の中で体制を組まず，海外の研究資源を使用することには非難があった．さらに最近は，バンク側が知的所有権を主張し，パテント申請をバンク側で行うことが一般的になり

1）S. Murayama：東京都老人総合研究所老年病ゲノム（神経病理）
2）Y. Saito, M. Sawabe：東京都老人医療センター剖検病理科
3）K. Kanemaru：同 神経内科
4）A. Tokumaru：同 放射線科
5）K. Ishii：東京都老人総合研究所附属診療所（ポジトロン研究施設）

表1　高齢者ブレインバンクの法的基盤と哲学

- 死体解剖保存法18条に基づく保管と研究使用
- 老人医療センター解剖承諾書に基づく研究（共同研究）
- 欧米のブレインバンクと哲学を共有
「篤志によるものは公的ドメインに属し，公共の福祉に役立てなければならない」
- 旧養育院の哲学：高齢者の運動・認知機能障害の予防・改善につながる研究に協力する
- 患者サイドからの献脳の伝統
- 後に続くものの育成：若手研究者の重視

表2　高齢者ブレインバンクの運用

- 共同研究者より，ブレインバンク責任者宛に申請書の提出
- 事前審査:ブレインバンク責任者，ブレインバンク執行医，老人総合研究所自然科学系副所長，センター剖検病理部長，センター神経内科責任医長よりなる委員会（持ち回り）
- 外部委員に諮問（守秘義務下）
- 研究所・医療センター・申請元の倫理委員会で承認
- 研究所研究推進委員会で，ブレインバンクを用いた共同研究内容を前提に，申請者を，研究所協力研究員として委嘱することを，承認
- 共同研究として研究開始

つつあり，結果として，海外の知的所有権のために，我が国の研究者が研究している状況が出現してきた．

東京都老人医療センター（以下センター）と，老人総合研究所（以下研究所）は建物を共有し，1972年の研究所開設以来，センター医師が研究所を兼務し，研究所の医師研究者が，医療センターを兼職する相互協力体制をとってきた．我々のグループも，センター神経内科と病理科を兼務し，センター神経内科が我々と兼務研究を行うかたちで，毎週のブレインカッティングカンファランス，スライドカンファランスによる，高齢者脳の専門神経病理学的検索を行ってきた．旧養育院という，東京都の高齢者医療行政の一翼を担い，地域の在宅高齢者医療に貢献してきた伝統から，剖検とは死因の解明だけでなく，その根底にある，老化・認知症（痴呆）の克服を目指さなければならないという哲学が，存在する(表1)．

元来我が国の死体解剖保存法には，公共の福祉のための，教育・研究への貢献がうたわれている．欧米のブレインバンクの哲学は,「篤志により提供された資源は公共のドメインに属し，公共の福祉のために用いなければならない」ということである．この哲学を共有し（表1），共同研究を前提とし（表2），老化・認知症（痴呆）の克服に向けた，高齢者ブレインバンクの構築の現状と，今後の課題について，以下に述べる．

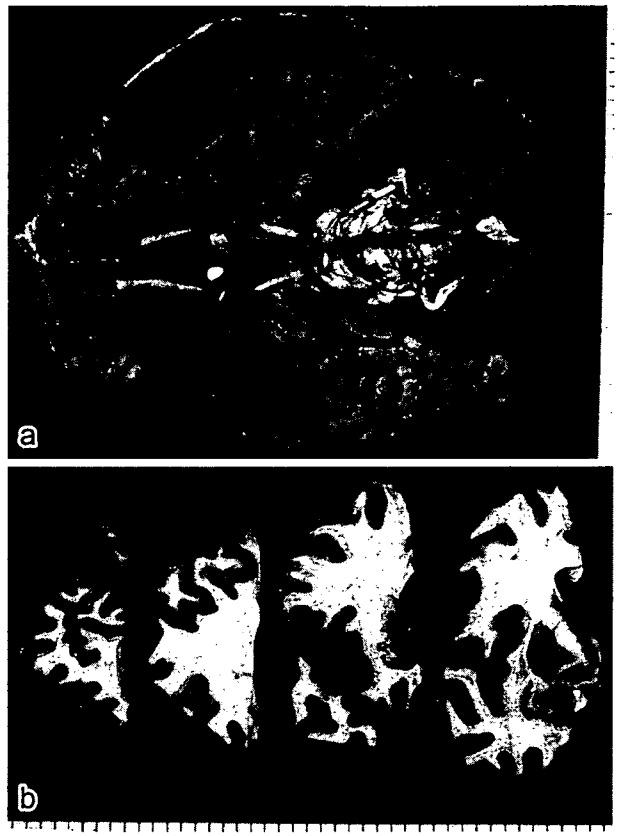

図1　新鮮脳肉眼評価．剖検全例に神経病理担当医が立ち会い，生で半脳を7mm厚にスライスし，肉眼診断，写真撮影の上，迅速凍結を行う．脳は固定前でも評価は可能であり，また画像所見とは，この状態が最もよく相関する．

高齢者ブレインバンクの構築

1972年研究所開設より，神経病理学的診断は，研究所神経病理部門が責任を負い，標本及びブロックの保管・管理を行っていた．さらにセンター剖検病理科で，1995年より，来るべきゲノム研究において，凍結組織の保存は不可欠との認識で，後頭極の凍結保存が開始されていた．1999年6月筆頭著者赴任時，欧米型半脳凍結ブレインバンク構築の提案は，神経病理学的検索がおろそかになるという，臨床側からの反対にあった．

そのため，アルツハイマー病ゲノム研究プロジェクトのかたちで,センター・研究所倫理委員会の承認を受け，海馬の凍結材料を，筋生検と同様のティッシューコンパウンドに固めることを開始した．続いて，パーキンソン病のゲノム研究プロジェクトを，同様の手続きの上立ち上げ，中脳黒質の凍結保存を開始した．この間，側頭葉内側面を侵す，嗜銀顆粒性痴呆が，当施設でアルツハイマー病に継ぐ頻度を持つことが明らかとなり，高齢者タ

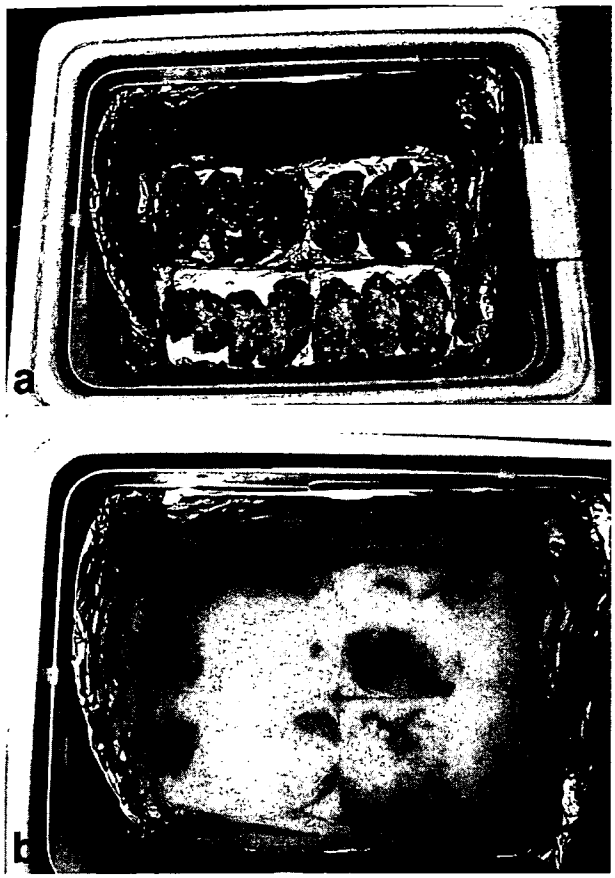

図2　脳迅速凍結法
a. −20度の携帯用冷凍庫に，ドライアイスの板を引き，超低温槽内で冷やした銅板をその上におき，アルミホイルをしき，その上にスライス脳を並べる．
b. その上に細かく砕いたドライアイスを上にかけ，迅速凍結する．

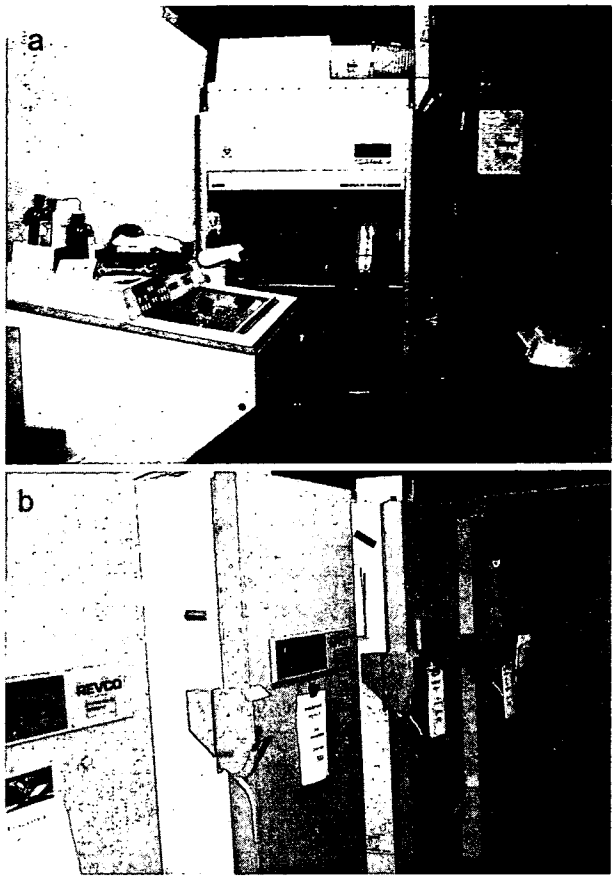

図3　ブレインバンクルームと超低温槽資源庫
a. ブレインバンクルーム．バイオハザードエリアに指定，脳凍結用安全キャビネット，凍結脳切出用クリオスタット，凍結材料重量測定スケールは必需品である．
b. ブレインバンク凍結資源庫
現在11台の超低温槽を保有できるスペースを確保し，熱量計算を行い，室内を一定の温度に保つよう，空調を整備している．超低温槽自体は，故障した時の損害を最低限にするため，縦型としては最小タイプを使用．緊急時のドライアイス10kgが入るスペースを確保しているため，一台での脳の保有数は81例分である．1台は予備機として，ドライアイスを貯蔵，緊急時には入れ替えることで対応予定である．

ウオパチープロジェクトとして，ゲノム研究のため右前頭・側頭極の凍結を，さらに，蛋白化学研究のため，右側頭葉全体の凍結を開始した．続いて，老人班の最初の蓄積部位の解明研究の目的で，頭頂葉皮質を，パーキンソン病のプロテオーム解析のために，線条体の採取を開始した．

これらの過程で，半脳を凍結保存する体制でも，病変部位を肉眼診断し，必要部位を組織学的に検索すれば，神経病理学的検索は可能であるというコンセンサスを，二年かけて築くことができた（図1）．以上の背景の元，2001年7月より，半脳凍結を開始した．

凍結法として，当初は液体窒素で瞬間凍結することを行っていたが，白質と灰白質の間に亀裂が入ること，それらが鋭利であり，共同研究者から危険という指摘を受け，解剖学教室でインシチューハイブリダイゼーション用に，動物脳を凍結する際に用いている方法である，ドライアイスパウダーを用いる方法に移行した（図2）．一方この過程で，未診断粟粒結核・結核性髄膜炎の患者脳を凍結したことによる汚染事故が起き，P2レベルのブレインバンクルームが，研究者予算で整備された（図3a）．また，我々のグループの備品を捨て，超低温槽を置く場所を確保するかたちで対応してきたことが，設置箇所の加熱を招く結果となり，2004年に，空調を整備した超低温槽資源庫を，外部研究費の援助を受けて，整備することが出来た（図3b）．

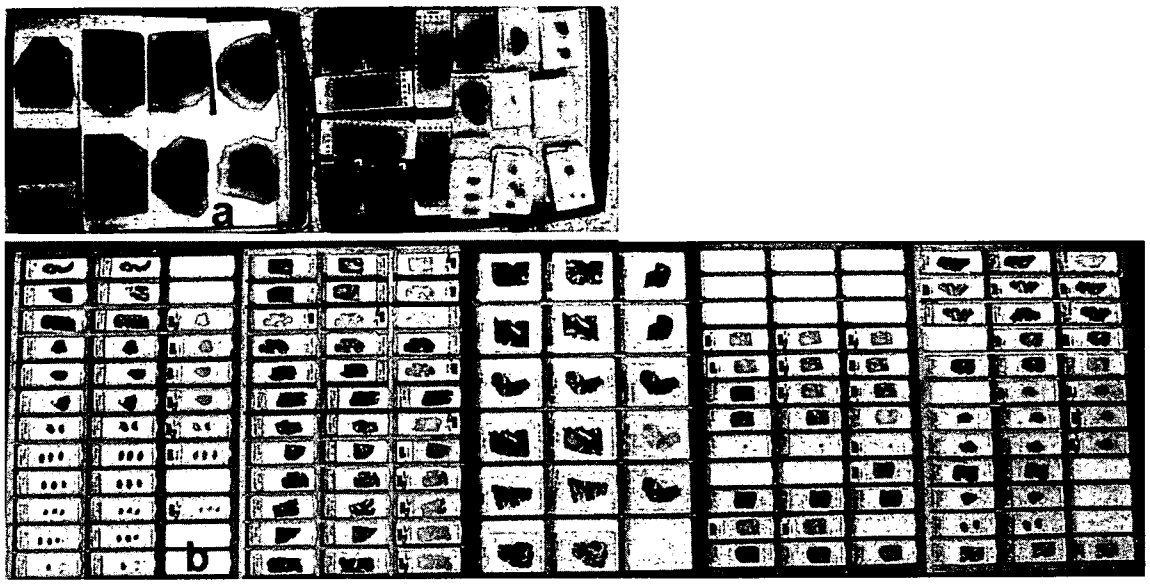

図4　標準プロトコールによる標本管理
a．パラフィンブロックは，6,711例分（脳）が参照可能である．現在は，CERAD（Consotium to establish registry for Alzheimer disease），レヴィー小体型痴呆コンセンサスガイドライン，Braakらの推奨部位を網羅するかたちで作成，部位を公表している．
b．自動免疫染色装置を用いた免疫染色を含め，全例に対し，同一部位に同一染色を施している．疾患毎に，これら標準部位に加え，固有部位を標本化し，評価している．

表3　神経病理学的検索法

1. Hematoxyline and Eosin，Kluever Barrera染色を通常
2. 代表的部位を改良メセナミン銀，Gallyas-Braak，Bielshowsky，Congo赤，Elastica-Massonで染色
3. 免疫組織化学（一次抗体）
   リン酸化タウ
   アミロイドβ蛋白，
   リン酸化・非リン酸化αシヌクレイン，
   ユビキチン
   老化構造物を半定量化
4. ApoE遺伝子多型
   PCR
   ApoE4特異抗体免疫組織化学

これと平行し，これまで大切片を中心とした神経病理学的検索を行っていた体制を，どこでも誰でもが行える体制をモットーに，一般病理で作れる大きさの標本を用い（図4），原理的にはどこでも同じ標本が作れる，市販抗体と（表3），自動免疫染色装置による検索に主軸を移した（図5a）．全国展開している検査会社が，同様の装置を採用しているので，必要な一時抗体を渡し，染色費用さえ払えば，同様の結果が得られるはずである．2003年に外部研究費を受け，1972年からの蓄積剖検例について，地域住民のpaid volunteerの協力を受け，ブロックと標本の一貫した整備を行い，高齢者ブレインバンクリゾースセンターを構築できた（図5b）．またこれら症例の臨床所見・神経病理学的所見をデータベース化し，高齢者臨床神経病理データベースを構築した．データベース構築にあたっては，研究所旧臨床病理部門からセンター剖検病理科に引き継がれていた剖検データベース，センター神経内科，循環器内科で蓄積されてきた臨床データベースを取込み，総合化を図った．以上より，蓄積脳の全てが利用可能な状態を構築することができた（表4）．

今後の課題

標本採取に関して，当施設では医師がon　call体制で対応している．この結果，ある程度の人員が対応できる体制を組まないと，担当医が部署を離れられなくなる．欧米では，この部分は技術員が対応していることが多いが，新鮮脳の病理所見の検討が不確実となる．

ブレインバンク超低温槽の管理については，故障による融解事故が最も問題となる．輸血製剤と同様の危機管理体制を，2005年度に神経内科当直と組み合わせるかたちで，構築することが出来たが（表5），実際事故が起きた時に機能するかどうかが問題である．

標本の供給先の決定においても，米国では，ブレイン

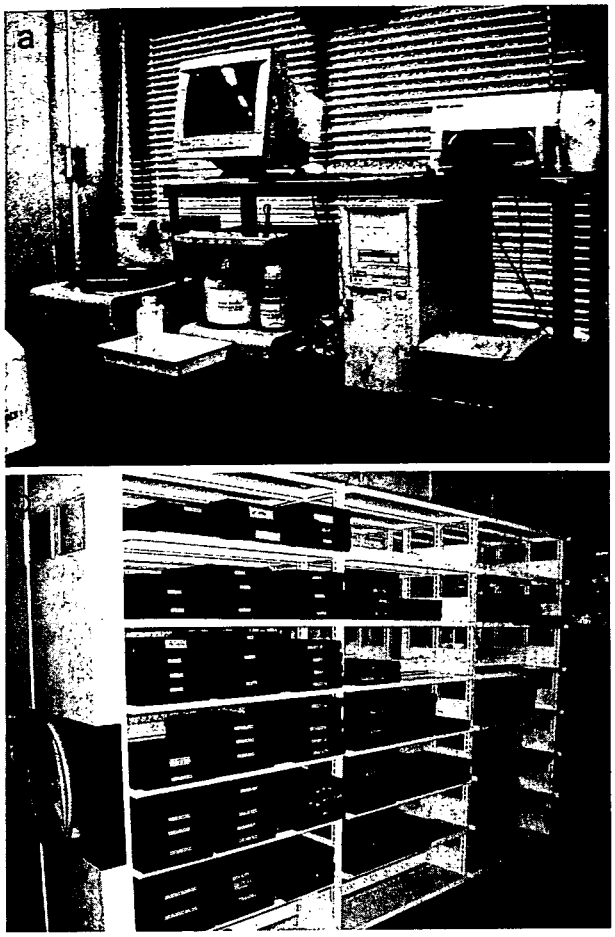

図5　自動免疫染色装置と高齢者ブレインバンクリゾースセンター
a. 自動免疫染色装置
染色プロトコールを公表，国際学会での発表，国際的視察による国際標準化を継続．国内最大大手検査会社が同じ装置を用いており，日本国内での染色結果の共有は，原理的に可能である．
b. 高齢者ブレインバンクリゾースセンター
耐震性を考え，研究所の一階に，移動書架を入れ，ブロックと標本を番号順に整理した．剖検例の病歴・画像は病歴室により全て保存されている．症例の剖検番号と臨床所見，神経病理学的所見をデータベース化してあり，図書館のように，必要な標本とブロックが直ちに取り出せる状態となっている．

表4　高齢者ブレインバンクの内訳

東京都老人総合研究所・老人医療センターが共同で構築中の，在宅高齢者の脳研究資源
1. 高齢者臨床神経病理リゾース
　連続剖検例（1972. 5～）：6,711脳（ブロック）
　　臨床・画像・病理所見
　　都市型老化の基礎データ
2. 高齢者DNAリゾース
　DNA保存例（1995. 1～）：1,530脳（後頭極）
　　老年病ゲノム研究の基礎資源
3. 高齢者凍結半脳リゾース
　半脳凍結保存例（2001. 7～）：425脳
　　あらゆるヒト脳研究の基礎資源

2005. 7. 5現在

表5　高齢者ブレインバンクの危機管理

- 超低温槽資源庫を整備，室温を一定に保つよう空調整備，9：00，16：00，22：00に毎日定時チェック
- アラームシステムを中央監視に連結，緊急時，神経内科常勤医とブレインバンク医師とで構成する，ブレインバンク当番が対応
- 予備機とドライアイス保存，各超低温槽に10kgのドライアイススペースを空けておくことで対応
- 超低温槽日本総代理店が，公的還元として，営業時間帯は365日無休で緊急修理対応

バンクの資源を使った場合，NIH grant 3年間の成果発表までに，何の結果も出さないことは許されない．一方日本の場合，提供しても結果を出す義務を有さない体制であるため，ある疾患の特定部位の脳を，こちらでも蓄積したいので送って欲しいという依頼，ゲノム研究をやりたいので特定の疾患のゲノムを多数欲しいという依頼，あるいは，私のアイデアで基礎研究者に研究させるので，脳を提供して欲しいという依頼等，予想外の依頼への対応に，苦慮することとなった．そのため，外部委員を含めた，委員会体制で対応する形をとることとなった（表2）．この点は，コンセンサス作りが今後必要である．

さらに，研究申請についても，ヒトの脳を常に使える環境にある欧米の研究者と異なり，日本の基礎研究者の場合，動物脳と同じ発想で申請を出してくるため，ブレインバンク側が，より積極的に共同研究に関与して行かないと，実りある結果が期待出来ない状況が明らかとなり，これについても今後の検討が必要である．

ブレインバンク事業において，最も大変なのは，供給であると言われている．供給部位の同定には，固定前の脳の割面（図1）をマークすることで部位を明示し，切り出しはブレインバンクルーム内のクリオスタットの中で（図2），ドライアイスを敷き，厳密な対応をとりながら，彫刻刀と木槌を使いながら，行っている．この際，mRNAの保存を考え，温度をできるかぎり上げない環境で行っている．米国ではこの部分を技術員が対応しているが，詳細な解剖部位の同定と切り出しには，神経病理専門医が行う体制が必要である．

在宅を基本とし，死因のほとんどが一般内科的疾患で

あることより，当施設の脳には正常コントロール，並びに変性疾患とした場合には早期病変が多い点が特徴的であり，欧米のブレインバンクと相補性をなす．しかし，重度痴呆例は少ない．より深刻なのは，剖検数の減少である．この問題の解決のため，同様の哲学を持つ施設と共同で，ブレインバンクネットワークを構築する試みをスタートさせた．

欧米型のブレインバンクは連結不可能匿名化が原則だが，本施設では剖検症例の病歴が全て保管されており，連結可能匿名化が，成果還元の点でより有用である．この点も個人情報保護との問題で，継続的に検討していく必要がある．

本バンクの資源を用いた，いわゆるパワー神経病理（疫学神経病理）の成果も，徐々に上がってきており[2)~15)]，共同研究の成果もやっと出てくるようになった[16)~26)]．若手研究者にいかに興味を持ってもらい，資源の有効活用を行うかが，今後の大きな課題である．

ブレインバンクが人口に膾炙し，その重要性が市民権を獲得した上で，患者団体が主体的に関わっている，欧米型のブレインバンクへの移行について働きかけることが，現段階では現実的と考えられる．

高齢者ブレインバンクチーム（著者を除く）：東京都老人総合研究所老年病ゲノム（神経病理）医師：崎山快夫，仙石錬平，初田裕幸，池村雅子，技師：愛敬直雄，原田三枝子，直井信子；東京都老人医療センター神経内科：三谷和子，吉野正俊，小宮正，椎名盟子，仁科裕史，村上喜生，砂川昌子，広吉祐子，畠中将；同リハビリテーション科：加藤貴行；同病理：新井冨生，笠原一郎

参考文献

1) 村山繁雄，齊藤祐子，文村優一，愛敬直雄，原田三枝子，直井信子：東京都高齢者ブレインバンクの創設．Dementia Jpn 2004；18：54—63.
2) 村山繁雄，齊藤祐子，笠畑尚喜：軽度認知機能障害の神経病理．神経研究の進歩　2004；48：441—449.
3) 村山繁雄：高齢者連続剖検例における頸椎・頸髄病変3年間566例の経験．脊椎脊髄ジャーナル　2002；15：531—536.
4) 小山俊一，齊藤祐子，山之内博，名倉博史，千田宏司，新井冨生ほか：高齢者における頭蓋内—脳動脈硬化の時代的推移に関する病理学的研究．日老会誌　2003；40：267—273.
5) Saito Y, Murayama S: Tau immunoreactivity in spinal anterior horn cells in Alzheimer's disease. Neurology 2000；55：1727—1730.
6) Nakahara H, Yamada S, Mizutani T, Murayama S: Identification of the primary auditory field in archival human brain tissue via immunocytochemistry of parvalbumin. Neurosci Lett 2000；286：29—32.
7) Saito Y, Yamazaki M, Kanazawa I, Murayama S: Severe involvement of the ambient gyrus in a case of dementia with argyrophilic grain disease. J Neurol Sci 2002；196：71—75.
8) Saito Y, Nakahara K, Yamanouchi H, Murayama S: Severe Involvement of ambient gyrus in dementia with grains. J Neuropath Exp Neurol 2002；61：789—796.
9) Saito Y, Kawashima A, Fujiwara H, Ruberu NN, Hasegawa M, Iwatsubo T, et al.: Accumulation of phosphorylated a-synuclein in aging human brain. J Neuropath Exp Neurol 2003；62：644—654.
10) Saito Y, Ruberu NN, Harada M, Arai T, Sawabe M, Nukina N, et al.: In-situ detection of apolipoprotein E e4 in archival human brain. Neuroreport 2004；15：1113—1115.
11) Ruberu NN, Saito Y, Honma N, Sawabe M, Yamanouchi H, Murayama S: Granulomatous meningitis as a late complication of iodized oil myelography. Neuropathology 2004；24：144—148.
12) Kazama H, Ruberu NN, Murayama S, Saito Y, Nakahara K, Kanemaru K, et al.: Association of estrogen receptor alpha-gene polymorphisms with neurofibrillary tangles. Dement Geriatr Cog Diso 2004；18（2）：145—150.
13) Saito Y, Ruberu NN, Sawabe M, Arai T, Kazama H, Hosoi T, et al.: Lewy body-related alpha-synucleinopathy in aging. J Neuropath Exp Neurol 2004；63：742—749.
14) Saito Y, Ruberu NN, Sawabe M, Arai T, Tanaka N, Kakuta Y, et al.: Staging of argyrophilic grains, an age-associated tauopathy. J Neuropath Exp Neurol 2004；63：911—918.
15) Murayama S, Saito Y: Neuropathological diagnostic criteria for Alzheimer disease. Neuropathology 2004；24：254—260.
16) Ikeda K, Akiyama H, Arai T, Kondo H, Haga C, Tsuchiya K, et al.: Neurons containing Alz-50-immunoreactive granules around the cerebral infarction: evidence for the lysosomal degradation of altered tau in human brain? Neurosci Lett 2000；284：187—189.
17) Taniguchi S, Fujita Y, Hayashi S, Kakita A, Takahashi H, Murayama S, et al.: Degradation of p35 to p25 in postmortem human and rat brains. FEBS Letters 2001；489：46—50.
18) Forno LS, J Langston W, Herrick MK, Wilson JD, Murayama S: Ubiquitin-positive neuronal and *tau* 2-positive glial inclusions in frontotemporal dementia of motor neuron type. Acta Neuropath 2002；103：599—606.
19) Kazama H, Hosoi T, Nakahara K, Murayama S, Saito Y, Kanemaru K, et al.: Association beween a promoter polymorphism of the paraoxonase PON1 gene and pathologically verified idiopathic Parkinson's disease. Geratrics and Gerontology International 2002；2：91—96.
20) Shimizu T, Fukuda H, Murayama S, Izumiyama N, Shirasawa T: Isoaspartate formation at position 23 of amyloid beta peptide enhanced fibril foration and deposited onto senile plaques and vascular amyloids in Alzheimer's disease. J Neurosci Res 2002；70：451—461.
21) Yokota O, Terada S, Ishizu H, Nakashima H, Kugo A, Tsuchiya K, et al.: Increased expression of neuronal cyclooxygenase-2 in the hippocampus in amyotrophic

lateral sclerosis both with and without dementia. Acta Neuropath (Berl) 2004;107:399—405.
22) Yokota O, Terada S, Ishihara T, Nakashima H, Kugo A, Ujike H, et al.: Neuronal expression of cyclooxygenase-2, a pro-inflammatory protein, in the hippocampus of patients with schizophrenia. Prog Neuropsychopharmacol Biol Psychiatry 2004;28:715—721.
23) Katsuno T, Morishima-Kawashima M, Saito Y, Yamanouchi H, Ishiura S, Murayama S, et al.: Independent accumulations of tau and amyloid β-protein in the human entorhinal cortex. Neurology 2004;64:687—692.
24) Ishigami A, Ohsawa T, Hiratsuka M, Taguchi H, Kobayashi S, Saito Y, et al.: Abnormal accumulation of citrullinated proteins catalyzed by peptidylarginine deiminase in hippocampal extracts from patients with Alzheimer's disease. Neurosci Res 2005;80:120—128.
25) Tsuchiya K, Murayama S, Mitani M, Oda T, Arima K, Mimura M, et al.: Constant and severe involvement of Betz cells in corticobasal degeneration is not consistent with pyramidal signs: a clinicopathological study of ten autopsy cases. Acta Neuropathol in press.
26) Yoshimi K, Ren Yong-Ri, Seki T, Yamada M, Ooizumi H, Onodera M, et al.: Possibility for neurogenesis in the substantia nigra of Parkinsonian brain. Ann Neurol in press.
27) Mirra SS, Heyman A, McKeel D, et al.: The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. Neurology 1991;41 (4):479—486.
28) McKeith IG, Galasko D, Kosaka K, et al.: Consensus guidelines for the clinical and pathologic diagnosis of dementia with Lewy bodies (DLB): report of the consortium on DLB international workshop. Neurology 1996;47 (5):1113—1124.
29) Braak H, Braak E: Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol (Berl) 1991;82 (4):239—259.

Abstract

Establishment of brain bank for aging research

Shigeo Murayama[1], Yuko Saito[2], Kazutomi Kanemaru[3], Aya Tokumaru[4], Kenji Ishii[5] and Motoji Sawabe[2]

We have established a brain bank for the prevention and treatment of aging-related movement and cognitive disturbances, as a joint project between a rural care hospital and a research institute. The resources of the bank are to be used for collaborative studies approved by the bank's committee. The collaborative investigators should also be qualified by the institute to conduct the research jointly. The collaborative studies require authorization by the institutional review board (IRB) of the institute, the hospital and each facility involved in collaborative studies. The bank continues to have the responsibility for the resources, after the transfer of the resources to the facilities of collaborative investigators, pursuant to Article 18 of the Cadaver Autopsy and Preservation Act. Thus, the status of resource utilization and outcomes from their use in studies will be monitored periodically (every 6 months). We shared the philosophy with the brain banks in the United States that the resources of the bank, donated on the basis of a charitable spirit, belong to the public domain and are regarded as public resources to be used to contribute to promoting public welfare.

Key words: *Aging, Dementia, Alzheimer disease, Parkinson disease, Tauopathy*
(Jpn J Geriat 2005;42:483—489)

1) Department of Neuropathology, Tokyo Metropolitan Institute of Gerontology
2) Department of Pathology, 3) Neurology and 4) Radiology, Tokyo Metropolitan Geriatric Hospital
5) Positron Medical Center, Tokyo Metropolitan Institute of Gerontology

Possibility for Neurogenesis in Substantia Nigra of Parkinsonian Brain

Kenji Yoshimi, PhD,[1] Yong-Ri Ren, MD,[2] Tatsunori Seki, PhD,[1,3] Masanori Yamada, PhD,[1] Hideki Ooizumi, MD,[2] Masafumi Onodera, MD, PhD,[4] Yuko Saito, MD, PhD,[5] Shigeo Murayama, MD, PhD,[5] Hideyuki Okano, MD, PhD,[6] Yoshikuni Mizuno, MD, PhD,[1,2] and Hideki Mochizuki, MD, PhD[1,2]

Recent studies of enhanced hippocampal neurogenesis by antidepressants suggest enhancement of neurogenesis is a potentially effective therapy in neurodegenerative diseases. In this study, we evaluated nigral neurogenesis in animals and autopsy brains including patients with Parkinson's disease (PD). First, proliferating cells in substantia nigra were labeled with retroviral transduction of green fluorescent protein, which is an efficient method to label neuronal stem cells. Subsequent differentiation of labeled cells was followed; many transduced cells became microglia, but no differentiation into tyrosine hydroxylase–positive neurons was detected at 4 weeks after injection, in both intact rodents and those treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Second, polysialic acid (PSA)–like immunoreactivity, indicative of newly differentiated neurons, was detected in the substantia nigra of rodent, primate, and human midbrains. A large number of PSA-positive cells were detected in the substantia nigra pars reticulata of some patients with PD. In rats and a macaque monkey, the dopamine-depleted hemispheres showed more PSA staining than the intact side. A small number of tyrosine hydroxylase–positive cells were PSA-positive. Our results suggest enhanced neural reconstruction in PD, which may be important in the design of new therapies against the progression of PD.

Ann Neurol 2005;58:31–40

Neurogenesis in the hippocampus is essential for the therapeutic effect of antidepressants.[1–3] Enhancement of neurogenesis may open a new therapeutic potential in other central nervous system diseases, especially neurodegenerative diseases.[4] The potential of neurogenesis has been reported in Huntington's disease and Alzheimer's disease.[5–7] In Parkinson's disease (PD), the loss of dopaminergic neurons in the substantia nigra (SN) is the major pathological change.[8] Surgical replacement of dopaminergic neurons was reported to be effective in some patients, but the induction of severe uncontrolled off-medication dyskinesia limits its therapeutic usefulness.[9] If intrinsic dopaminergic neurons could be regenerated in SN of patients with PD, the enhancement of such process should be the primary therapeutic target.

The potential of neurogenesis in the SN has been studied by labeling proliferative neural precursor cells with bromodeoxyuridine (BrdU).[10–13] Because a compensatory enhancement of neurogenesis in the hippocampus has been reported after brain injury,[14–16] the brains have been examined for neurogenesis after dopaminergic cell deprivation, as well as in the intact brains. Kay and Blum[10] report the presence of BrdU-positive proliferative cells in the SN; a part of such cells were microglia, but none of them differentiated into dopaminergic neurons. In another study,[11] neuronal progenitor cells isolated from the SN of rats differentiated to neurons in the hippocampus but not in the midbrain. Using confocal laser scanning microscope, Zhao and colleagues found dopaminergic neurons with BrdU-positive nuclei in the SN, which were considered to have migrated from the midbrain aqueduct,[12] although a different conclusion was reported in another study.[13] The discrepancy between the two studies[12,13] was due to the uncertainty of whether BrdU-positive nuclei were located in or out of the tyrosine hydroxylase (TH)–positive cytoplasm. Another problem was that at a high dose, BrdU could be incorporated into repairing, as well as duplicating, DNA.[17,18]

From the [1]Research Institute for Diseases of Old Ages, Juntendo University; Departments of [2]Neurology and [3]Anatomy, Juntendo University School of Medicine, Bunkyo-ku, Tokyo; [4]Department of Hematology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki; [5]Department of Neuropathology, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku; and [6]Department of Physiology, Keio University School of Medicine, Shinjuku, Tokyo, Japan.

Received Jan 18, 2005, and in revised form Apr 4. Accepted for publication Apr 5, 2005.

Published online May 23, 2005, in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/ana.20506

Address correspondence to Dr Mochizuki, Department of Neurology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan.
E-mail: hideki@med.juntendo.ac.jp

Published by Wiley-Liss, Inc., through Wiley Subscription Services

BrdU is not the only tool to label DNA synthesis in proliferating cells. Retroviral labeling is another method, which has several advantages to BrdU labeling.[19,20] Specific marker genes can be transducted into duplicating chromosomes by retroviral vectors. The expression requires following protein synthesis; thus, the expression of green fluorescent protein (GFP) is highly specific to the cells that proliferate at the time of infection. We efficiently labeled neuronal stem cells with GFP by retroviral transduction, both in vitro and in vivo.[21–23] In these studies, GFP filled the cytoplasm of the cells and expressed a clear Golgi-like morphology of the infected cells. Moreover, local injection in the brain tissue allowed a clear mapping of the migration route.[19,22]

Labeling of proliferating cells with BrdU and retrovirus may not be the only methods to detect neurogenesis. Intrinsic molecules unique to young neurons, such as polysialic acid (PSA) and doublecortin, can be used as tools to detect neurogenesis.[24,25] This is especially important in human subjects, where experimental markers such as BrdU or retroviral vectors are not applicable.

In the first part of this study, we observed the fate of endogenous proliferating cells in rodent SN by examining the morphology of cells after retroviral transduction of GFP. Retroviral injection close to the midbrain aqueduct also was performed to confirm the possible migration of cells derived from neural stem/progenitor cells from this area to the SN. In the second part of this study, we analyzed immunostaining of PSA in human SN tissues of patients with PD and dopamine-deficit animals.

Materials and Methods

Animals and Drug Administration

Adult C57BL/6 mice (10-week-old female) and Sprague–Dawley rats (10-week-old male) were obtained from Charles River Laboratories (Yokohama, Japan). They were housed two to six per cage and maintained on a 12-hour light–dark cycle at constant temperature and humidity. Food and water were provided ad libitum. The animals injected with retrovirus vector (see later) were kept in air-isolated cages with Hepa filter ventilation system. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Juntendo University School of Medicine. Mice were injected with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Sigma Labs, St. Louis, MO) or saline in acute (20mg/kg body weight intraperitoneally (IP) four times a day at 2-hour intervals) or chronic (30mg/kg once a day for 5 days) protocols(Table 1).[26] MPTP was handled carefully, and the remaining solutions were inactivated with bleach.[27]

Preparation of Modified Retroviral Vector

The retroviral vector GCDN sap carrying enhanced GFP gene was packaged with vesicular stomatitis virus G protein (VSV-G) by transduction into 293gpg as described previously.[20–22] The virus producer clone (293gpg/DNEGFP) was harvested in Dulbecco's modified eagle medium with 10% fetal bovine serum (FBS) and tetracycline. When 10 flasks (900ml) became 70% confluent, tetracycline was removed to start the production of the virus under the control of tet-off system. Two days later, the medium was centrifuged at 6,000× *g* and 4°C, followed by resuspension of the viral pellet in phosphate-buffered saline (PBS). Finally, the viral vector was centrifuged to 1,000-fold concentration and stored at −80°C. All containers and tools used to handle retroviral vectors were rinsed with ethanol or disposed after autoclaving.

Stereotaxic Injection of Retroviral Vector

Concentrated viral solution was injected into the mouse SN bilaterally, 1μl into each side. Mice were anesthetized with pentobarbital (60mg/kg body weight IP), then held on a stereotaxic frame, drilled on the skull, and a 31-gauge needle was inserted into the SN (anteroposterior [AP], −2.8 mm; mediolateral [ML], 1.3mm from bregma; dorsoventral [DV],

Table 1. MPTP Administration Schedule for Retroviral Labeling in Mice and Rats

	Target	MPTP	Days to surgery	Survival	Group	n
Mice	Nigra	Sal × 4	2	4 weeks	Sal2d4w	7
		20 × 4	2	4 weeks	A2d4w	8
		20 × 4	7	4 weeks	A7d4w	6
		30/d × 5d	7	4 weeks	C7d4w	4
		Sal × 4	2	16 weeks	Sal2d16w	4
		20 × 4	2	16 weeks	A2d16w	4
	Aqueduct	Sal × 4	7	4 weeks	Sal7dAq4w	3
		20 × 4	7	4 weeks	A7dAq4w	6
Rats	Nigra			2 days	R-SN2d	5
				2 weeks	R-SN2w	3
	Aqueduct			2 days	R-Aq2d	2
				2 weeks	R-Aq2w	6
				4 weeks	R-Aq4w	7

MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

4.5mm below the dural surface; at tooth bar position 0.0mm). The retroviral vector solution was suctioned through the 31-gauge injection needle into a Hamilton microsyringe (Hamilton, Reno, NV) just before injection, and the trace viral solution on the surface of the needle was wiped out to avoid contamination. The needle was slowly placed into the target, and the vector solution was ejected at the rate of 1μl/min and was left steady for 5 minutes. Injection into the rat SN (AP, 5.3mm; ML, 2.4mm from bregma; DV, 7. mm, at tooth bar position −4.0 mm) was made using a similar procedure, but the injection volume was 2μl. To inject the vector into the area ventral to the midbrain aqueduct of mice (AP, −3.5mm; from bregma; DV, −3.5mm) and rats (AP, −5.3mm; from bregma; DV, 7.0mm from bregma), we angled the needle 15 degrees to the right to avoid the sinus. To compare the labeling with BrdU and retroviral vector, we injected some animals with BrdU (50mg/kg IP) just after retroviral injection.

Hemi-Parkinsonian Model

To evaluate the effect of dopamine deprivation on one section, we injected 1-methyl-4-phenylpyridinium salt into the left medial forebrain bundle of rats.[28] 1-Methyl-4-phenylpyridinium salt solution (5μg/μl × 2μl) was injected to the left (AP, −3.6mm; ML, 2.0mm; DV, −7.6mm from bregma) and 2μl saline was injected into the right medial forebrain bundle. A midbrain section of a hemi-parkinsonian macaque monkey[29,30] was stained for PSA. The monkey received slow infusion of MPTP (4mg) into the left caudate nucleus using an osmotic minipump and survived for 6 months after the infusion.

Brain Tissue Sections

Animals were deeply anesthetized with pentobarbital and perfused transcardially with PBS followed by perfusion with 10mM phosphate-buffered 4% formaldehyde solution (pH 7.4). Brains were postfixed in the same fixative for 2 days and allowed to sink in sucrose-PBS (30% sucrose in PBS containing 0.05% sodium azide). The brain tissue was frozen quickly in crushed dry-ice powder; coronal sections were sliced 25μm in thickness on a cryostat, and then stored in sucrose-PBS at 4°C until use.

The site of injection and expression of GFP was confirmed, and only brains showing the location of the injection were subjected to further studies. To identify the distribution of GFP expression, we stained every sixth section with anti-GFP antibody for light microscopy by streptavidin-biotin-peroxidase complex (ABC) and 3,3-diaminobenzidine (DAB). The remaining sections were subjected to double-immunofluorescence staining of TH and a glial marker and visualized using Cy3- and Cy5-conjugated secondary antibodies. At least 12 sections rostral and 12 caudal to the center of injection were stained and examined in each mouse.

Human brain tissue was obtained at Juntendo University Hospital, with the full consent of the family at the time of autopsy. The study protocol was approved by the Human Ethics Review Committee of Juntendo University School of Medicine. Midbrains of six patients with PD were studied, and those of six other neurological diseases (one with Alzheimer's disease, one myasthenia gravis, one muscular dystrophy, one vascular parkinsonism, and two cerebral hemorrhage) were included as the disease controls. The hemisphere of midbrain tissue was cut into blocks and fixed in buffered 4% formaldehyde solution for 2 days, and then moved to sucrose PBS until sink. The blocks were sectioned in the coronal plane (30μm in thickness) by a cryostat and further stored in sucrose PBS at 4°C. Because some sections were friable and easily torn off, human sections were incubated overnight in buffered 4% formaldehyde solution before starting immunostaining.

Double-Immunofluorescence Staining

The primary antibodies used in this study were anti-PSA (clone 12E3 mouse IgM)[24] at a working dilution of 1:500 to 1:2,000, goat anti-TH (1:2,000; Calbiochem, San Diego, CA), rabbit anti-ionized calcium-binding adaptor protein Iba-1 (1:2,000; Wako, Osaka, Japan),[31] rabbit anti-NG2 (1:200; Chemicon, Temecula, CA),[32] rabbit anti–glial fibrillary acidic protein (1:5,000; generous gift from Dr H. Akiyama, Psychiatric Research Institute of Tokyo, Tokyo, Japan), rabbit anti-Pi class glutathione-*S*-transferase–pi (1:10,000; MBL, Nagoya, Japan),[33] rabbit anti-GFP (1:1,000, Chemicon), mouse anti–rat cd11b (1:200; clone OX-42, Immunotech, Marseille, France), and rat anti-BrdU (1:400, clone BU1/75; OBT, Oxford, United Kingdom). Secondary antibodies of fluorescein isothiocyanate, Cy3, Cy5, or biotin-conjugated donkey IgG of minimal cross-species grade (Jackson Laboratories, West Grove, PA) were used at 1:500 dilution. Arexa-594–conjugated donkey anti–goat antibody (Molecular Probes, Eugene, OR) was used in some cases.

Fluorescent microscopic staining was performed as described previously[22,23] with minor modifications. PBS with 0.05% Triton X-100 (Sigma) was used throughout the incubation. Antibodies were diluted in blocking solution of 2% block ace protein solution (Yukijirusi, Sapporo, Japan) in PBS with 0.05% Triton X-100. All incubations were performed at the room temperature, except for anti-BrdU, which was incubated at 4°C. Free-floating sections stored in sucrose PBS were rinsed with PBS and treated with chilled methanol for 10 minutes at −20°C to improve permeability of the antibodies. Then, the sections were incubated in blocking solution for 1 hour followed by overnight incubation in the primary antibody diluted in the blocking solution at room temperature, rinsed in PBS with 0.05% Triton X-100, and then incubated in secondary antibodies. For BrdU staining, the sections were first incubated in 2N HCl (Wako) at 37°C for 30 minutes, neutralized with borate buffer (100mM, pH 8.5) for 10 minutes and PBS for 10 minutes, and incubated in anti-BrdU overnight at 4°C, then Cy3-conjugated anti–rat IgG for 1 hour. As HCl faded the green fluorescence of GFP, the BrdU-stained sections were further immunostained with anti-GFP and fluorescein isothiocyanate–conjugated anti–rabbit IgG.

Immunohistochemistry for Light Microscopy by Streptavidin-Biotin-Peroxidase Complex Method

For light microscopic examination, the tissue sections were stained with elite avidin-biotin complex kit (Vector Laboratories, Burlingame, CA), DAB tablet (Sigma), and Ni-Cl solution (Funakoshi, Tokyo, Japan), as described previous-

ly[26,34] with minor modifications. After treatment with the primary antibodies, the sections were incubated in biotin-conjugated secondary antibodies for 1 hour, and then treated with 3% H_2O_2 in 10% methanol for 10 minutes, treated streptavidin-biotin-peroxidase complex for 1 hour, and visualized with DAB solution for 10 minutes. To distinguish neuromelanin from immunostaining, we added 0.0008% NiCl to DAB solution in human midbrain sections.

Image Analysis and Quantification

The distribution of individual GFP-positive cells was plotted on a brain map. Objects with autofluorescence were discarded (Fig 1, I–M). GFP fluorescence possibly colocalized with TH was recorded on the map and was further confirmed by confocal microscopy (model LSM510 laser scanning microscope; Carl Zeiss Jena, Germany).

In the mouse study, double staining of TH was helpful to determine the location in SN. In human sections, the area with scattered neuromelanin was regarded as substantia nigra pars compacta (SNc), and the area between SNc and the cerebral peduncle was regarded as substantia nigra pars reticulata (SNr). PSA-stained human brain sections were evaluated by an observer blinded to the study protocol. The location of PSA-positive cells was plotted on a low-power photograph of the section. The density of DAB staining of PSA and TH of rat sections was determined using a proce-

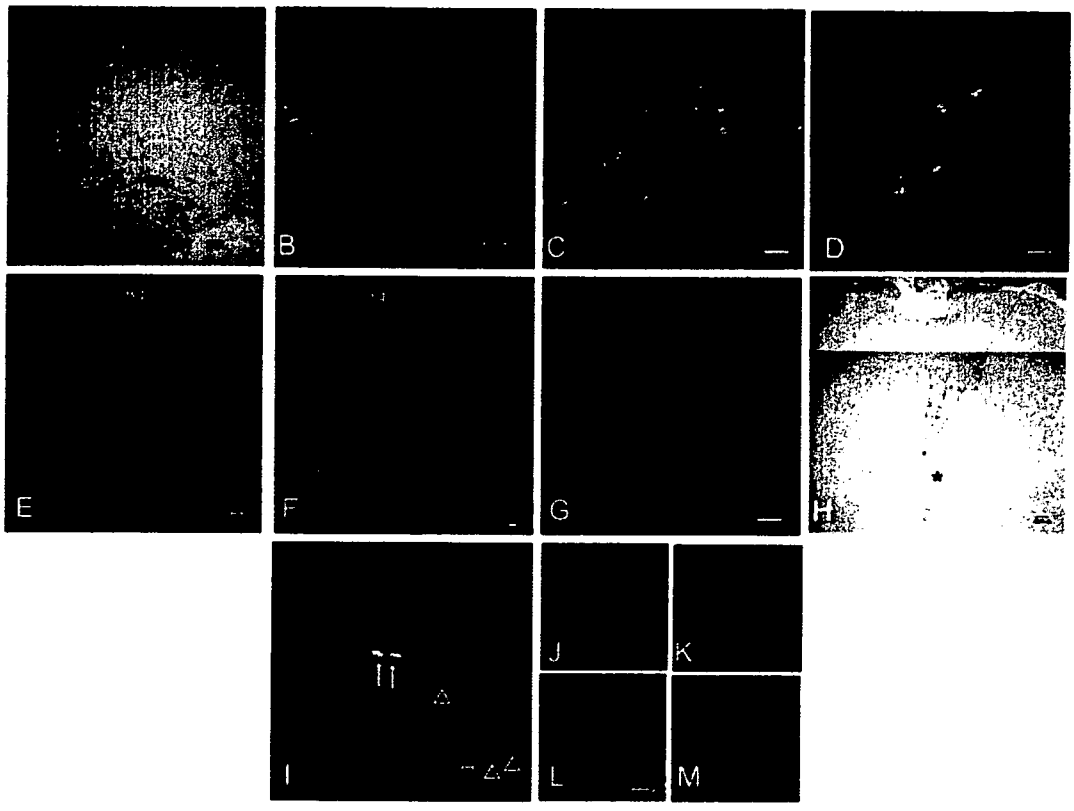

Fig 1. Retroviral green fluorescent protein (GFP) transduction in rodent substantia nigra and lack of colocalization with tyrosine hydroxylase (TH). (A) Low-power view of the injection site in a representative mouse 16 weeks after retroviral injection into the substantia nigra (Sal2d16w). Immunostained with anti-GFP followed by 3,3-diaminobenzidine. Several GFP-positive cells are present in the substantia nigra pars compacta (SNc), substantia nigra pars reticulata (SNr), and the cerebral peduncle. Aq = midbrain aqueduct; Cp = cerebral peduncle; Hi = hippocampus. (B) SN of an intact mouse 2 days after retroviral injection. GFP-positive cells (green) are present beside TH-positive cells (red). Examples of the substantia nigra of (C) a mouse A2d4w and a (D) rat-SN2w. (E) Injection site of an A7dAq4w mouse. Only one GFP-positive cell is present in this section. There are several TH-positive cells (red), but the GFP-cell is negative to TH. (F) Rat aqueduct area Rat-SN2d. (G) One GFP-positive cell (green) was present ventral to the midbrain aqueduct, but no such cells were present in the SN. (H) Low-power view of retroviral injection close to the midbrain aqueduct of a Rat-SN2d rat. An adjacent section to that shown in G and the approximate location of the cell in G is indicated with an asterisk. *(I–M) Example of a false-positive image found in the SN of a C7d4w mouse. (I) The TH-cell–like objects at the lateral end of the SNc indicated by* arrows *exhibit green and red fluorescence and appear yellow in the merged view. Three TH-positive cells on the right (red) are indicated by* arrowheads. *(J, K) Serial confocal images show that these two yellow figures in I are parts of one U-shaped object. (L, M) Nonlaser fluorescent view with red (L) and ultraviolet (UV) (M) filters of the same object in I. Blood vessels occasionally can cause autofluorescence and can be detected under UV excitation (M). (A, F, H)* Red lines *indicate the position of the injection needle. Bars = 20μm (B–G, I–M); 200μm (A, H).*

dure similar to that described previously[34] with LAS-1000 image analyzer (Fujifilm, Tokyo, Japan). The density unit of the corpus callosum of each section was subtracted as the background. Four coronal sections of the middle part of the rat SN (approximately 5.0, 5.18, 5.36 and 5.54mm posterior to bregma) were evaluated with densitometry.

Statistical Analysis

Cell colocalization data (Table 2) were analyzed by χ^2 test. Differences in the number of PSA-stained cells (see Fig 3) was analyzed with two-tailed U-test and density ratio of hemilesioned rat (see Fig 4) was analyzed by two-tailed *U*-test. $p < 0.05$ denoted a statistically significant difference.

Results

Retroviral Expression of Green Fluorescent Protein in Substantia Nigra

GFP was expressed in several cells around the injection site of SN of mice and rats (see Fig 1A–D). Expression of GFP was already evident 2 days after injection (see Fig 1B) and lasted for at least 16 weeks (see Fig 1A). The cell bodies of GFP-expressing cells were less than 10μm in diameter and had several fine processes. Typical TH-positive cells were larger and had bipolar shape. Coexpression of TH and GFP was not identified in mice treated with or without MPTP (see Tables 1 and 2). The results were similar in rats (see Fig 1D).

To study the possible migration of cells derived from neural stem/progenitor cells that are located in the periaqueductal area to SN, we injected the retroviral vector in an area adjacent to the midbrain aqueduct in mice (see Fig 1E) and rats (see Fig 1F, H). In mice, the number of labeled cells was small even at the center of the injection site (see Fig 1E), and no such cells were observed in the SN. They were more frequent in rats than in mice (see Fig 1F, H), but the distribution of GFP cells did not suggest their migration from the aqueduct area in a ventral direction. A few GFP-labeled cells were found in the ventral tegmental area (see Fig 1G), but none were identified in the SN.

Some autofluorescent objects were carefully discarded. The example shown in Figure 1I exhibits green and red fluorescence, but fine focusing on laser scanning microscope showed the image was two parts of one tubular structure (see Fig 1J, K). Examination under ultraviolet excitation light (see Fig 1M) was convenient for detecting autofluorescence. After fine analysis of morphology and autofluorescence, we could not identify TH-immunostained, GFP-expressing cells.

Cell Typing of Proliferating Cells in Substantia Nigra

These GFP-positive cells were characterized by staining with glial markers (Fig 2; see Table 2). GFP was colocalized with marker molecules of microglia Iba-1,[31] oligodendrocyte precursor NG2,[32] and oligodendrocyte (glutathione-*S*-transferase–pi, see Fig 2).[33] The number of GFP-labeled microglia was significantly larger than other protocols in mice of the acute MPTP treatment protocol 2 days before (see Table 2). This is consistent with our earlier observation of microglial activation 2 days after acute MPTP treatment.[26] The relative number of NG2 was reduced 2 days after MPTP and glutathione-*S*-transferase–pi–positive cells were reduced in all of MPTP treatments. Interestingly, no GFP-expressing cells colocalized with glial fibrillary acidic protein (see Fig 2C and Table 2).

To clarify whether the two labeling methods of DNA duplication, BrdU and retroviral vector, label the same cell population, we administered BrdU to some animals after retroviral injection into the SN. The nuclei of some GFP-labeled cells were BrdU-positive (see Fig 2F). The twin cells shown in Figure 2F are probably just after cell division. Both are retroviral vector- and BrdU-labeled proliferating cells, but the morphology of the cell was presented only by retroviral GFP expression.

Polysialic Acid Staining in Substantia Nigra of Humans, Monkeys, and Rodents

In the hippocampus of rodents and human, young neurons were immunostained with PSA (Fig 3A, C), as

Table 2. Number of GFP-Positive Cells Colocalized with Cell Typing Markers in the Ventral Tegmentum

Group	n	Iba-1 (microglia)	NG2 (oligoprecursor)	GFAP (astrocytes)	GST-pi (oligodendrocytes)	TH (DA neurons)
Sal2d4w	4	2/23	12/24	0/19	5/28	0/94
A2d4w	4	94/197[a]	16/127[a]	0/130	5/137[b]	0/591
A7d4w	4	8/85	31/56	0/52	1/51[b]	0/244
C7d4w	3	9/73	40/74	0/70	3/88[b]	0/305

The number of total GFP-positive cells detected in the ventral tegmentum (SNc, SNr, Cp, and VTA) is the denominator and the number of colocalized cells with one of the cell type markers is the numerator. Sections were double-immunostained with tyrosine hydroxylase and a glial marker, as shown in Figure 2. Two sections of each animal were stained, and the section closer to the center of retroviral injection was subjected to quantitative analysis. Each value represents the total cell number in sections of three to four animals.

[a]$p < 0.01$; [b]$p < 0.05$, compared with the other groups, by χ^2 test. Note the high colocalization of Iba-1 and low colocalization of NG2 in group A2d4w.

GFP = green fluorescent protein

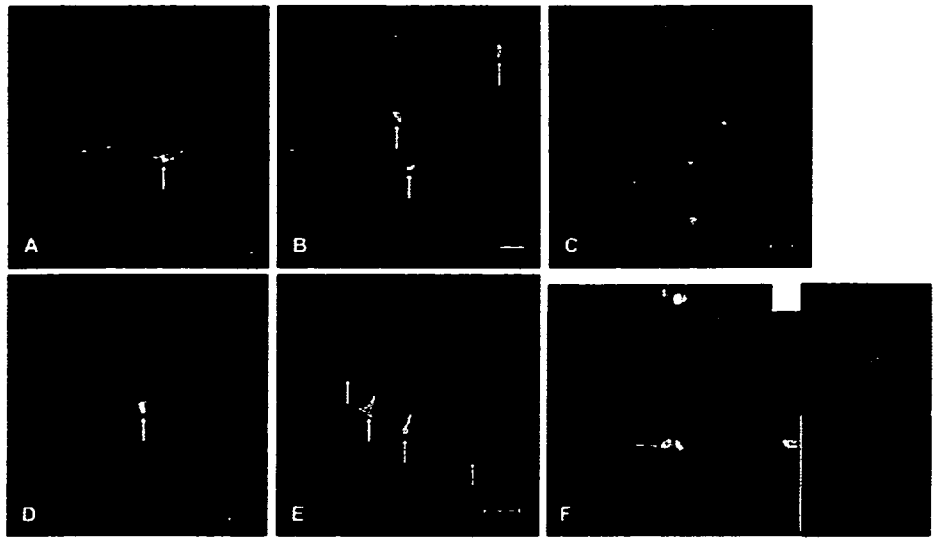

Fig 2. Colocalization of markers with green fluorescent protein (GFP)–expressing cells in the substantia nigra. Nigral sections of mice (A–D) and a rat (E) are immunostained with tyrosine hydroxylase (TH; blue) and a glial marker (red). (A) Iba-1 staining of an A2d4w mouse. Arrow *indicates a GFP-positive microglial cell expressing Iba-1 antigen. (B) NG2 staining of an A7d4w mouse.* Arrows *indicate GFP-positive cells covered with NG2 antigen on the cell surface. (C) Glial fibrillary acidic protein (GFAP) staining of an A2d4w mouse. No expression of GFAP is present among GFP-positive cells. (D) Glutathione-S-transferase–pi (GST-pi) staining of an A7d4w mouse.* Arrow *indicates GFP-positive oligodendrocyte expressing GST-pi. (E) OX-42 staining of a Rat-SN2d rat.* Arrows *indicate GFP-positive, microglia-expressing OX-42 antigen (CD11b) on the cell surface. (F) Comparison of two labeling methods of proliferating cells. These cells are positive for both retroviral transduction of GFP and bromodeoxyuridine (BrdU) incorporation to DNA and are probably just after cell division. Fine morphology of these cells is clearly drawn by GFP, whereas BrdU indicates only the nucleus. BrdU (50mg/kg intraperitoneally) was administered after local retroviral injection into the substantia nigra of a C7d4w mouse. This section was treated with HCl, followed by immunostaining with anti-BrdU and Cy3. The section was further immunostained with anti-GFP and fluorescein isothiocyanate, because HCl treatment reduced green fluorescence of GFP protein. Bars = 20μm.*

reported previously.[24,25] PSA-positive cells were found in the SN (see Fig 3B, D). Occasionally, PSA and TH double-positive cells were found in the SN of rats (see Fig 3B), although this was rare. There was considerable variance in the frequency of PSA-positive cells in human SN. There was no difference in the number of PSA-positive cells in the SNc (see Fig 3G) of patients with different conditions, but some PD sections contained many PSA-positive cells in the SNr (Fig 3H and Table 3). The cell numbers in PD tended to be different from those of disease control patients albeit insignificantly ($p < 0.06$, two-tailed t test). No such difference was noted in the SNc, which could be because of the dense PSA-positive fibers in the area just dorsal to the SNc. Similar results were noted in the monkey six months after MPTP infusion into the left caudate, with less TH staining and larger number of PSA-positive neurons in the ipsilateral SN (Fig 4).

Among the disease control cases, more than 100 PSA-positive melanized neurons were noted in the SNc of one cerebral hemorrhage case. PSA staining of melanized neurons was not often noted in the other brains. This interesting case indicates that human SNc neurons can express PSA in some conditions.

In addition to these 12 samples of free-floating sections, paraffin-embedded sections of human midbrain were also subjected to PSA immunostaining, but the staining was poor and further analysis was performed using only the floating sections. Staining for other intrinsic markers of neurogenesis was attempted, but double cortin was not clear in the SN and nestin gave intense staining of blood vessels but no staining of neurons in the SN, although it was evident in the hippocampus.

Human and monkey staining patterns of the SNr showed increased PSA-positive cells, but the results were not conclusive. The difference in the cell number was not significant, and the human samples were uneven about the cause of death and the midbrain level of the section.

We also examined the changes in PSA immunostaining after dopaminergic deprivation in rats. To evaluate the changes in hemilesioned rats by quantitative and objective measures, we compared the optic density of PSA immunostaining in the left and right SNr. The relative optic density of the lesioned SN to the intact SN was determined in the same section. In rats 2 months after 1-methyl-4-phenylpyridinium salt injec-

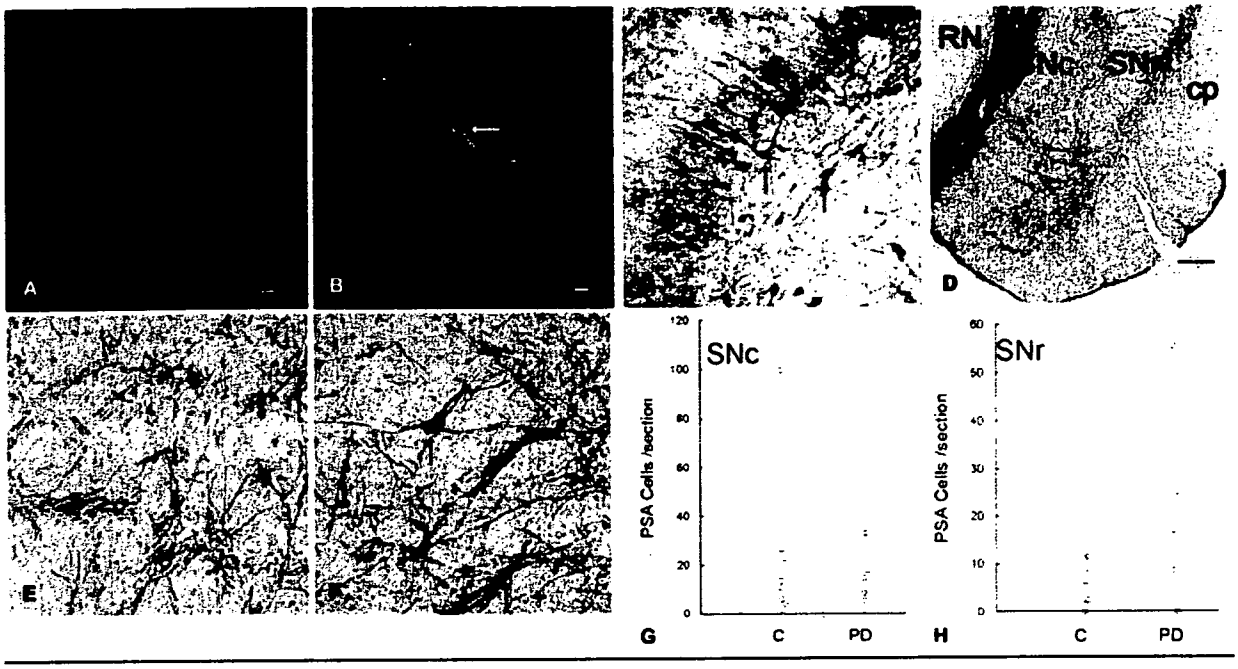

Fig 3. Polysialic acid (PSA)–positive cells in the substantia nigra. Sections of rats, a monkey, and humans are immunostained with monoclonal anti-PSA (clone 12E3). (A, B) Double immunofluorescence of PSA (green) and tyrosine hydroxylase (TH; red). (A) The subgranular zone of the rat hippocampal dentate gyrus is rich in PSA-positive young neurons. (B) The medial area of the substantia nigra of the rat. The surface of a substantia nigra pars compacta (SNc) TH-positive cell is covered with PSA (arrow). *(C, D) Immunostaining of human brain sections with anti-PSA and 3,3-diaminobenzidine. (C) Several cells of the subgranular zone of human hippocampus are PSA-positive* (arrows). *(D) Low-power view of human midbrain of a patient with Parkinson's disease. The area with melanin-containing neurons is regarded as SNc. PSA-positive fiber is dense in the area between SNc and red nucleus (RN). PSA-positive cells and fibers are scattered in substantia nigra pars reticulata (SNr). CP = cerebral peduncle. (E, F) PSA-positive cells in human substantia nigra* (arrows). *SNr of a control (E, muscular dystrophy) and Parkinson's disease (F) brain. (G, H) Number of PSA-positive cells in the SNc (G) and SNr (H) of human hemisphere sections. The average of duplicated counting of each sample by blinded observer is shown. Some nigral samples of Parkinson's disease showed the presence of large numbers of PSA-positive cells especially in the SNr, although the difference from the control brains was not statistically significant* ($p < 0.06$, *two-tailed* t *test). Scale bars = 20μm (A–C, E, F); 1mm (D). (C–F) Sections are counterstained with methyl green.*

tion into the left medial forebrain bundle, TH staining in the left SNc was reduced, whereas PSA staining in the SNr was increased (see Fig 4).

Discussion

Retroviral labeling of proliferating cells in rodents indicated lack of neurogenesis of TH-positive neurons from proliferative stem cells in the SN. In contrast, PSA-positive cells, candidates of newly differentiated young neurons, were present in the SN and increased in number after dopamine deprivation. Although we first intended to analyze dopaminergic neurons in the SNc, marked changes in PSA staining was detected in nondopaminergic neurons in SNr. Although PSA immunoreactivity is not conclusive evidence of neurogenesis,[35] the result suggests compensatory neuronal differentiation from mitotically silent cells.

Retrovirally Green Fluorescent Protein–Labeled Cells Did Not Differentiate into Dopaminergic Neurons

No GFP-positive cells after retroviral injection in the SN were found to express TH. This result is in agreement with some earlier studies[10,11,13] but not with one.[12] Double immunostaining in our study showed that the increased proliferative cells were mainly microglia, which was well in accordance with previous reports.[10,26] We also tried retroviral injection in close proximity to the midbrain aqueduct, but only a small number of cells were labeled and no migration to SN was observed. Because midbrain dopaminergic neurons originally migrate from the neural tube during development,[36] and TH-positive neurons distribute along the midline of the ventral tegmentum of adult animals,[37] it is quite an attractive idea that this area supplies dopaminergic neurons that ultimately migrate to reach the SN.[12] Local injection of a retroviral vector can give conclusive evidence of migration because it