表1 メタンフェタミン投与による中脳部位における遺伝子発現の変化

Gene	Accession No.	S-METH	M-METH
Cytokine inducible SH2-containing protein	D31943	↑	=
Mouse gene for beta-1-globin	V00722	↑	=
Upstream transcription factor 1	X95316	↑	=
DNA segment, Chr5	AW413033	↑	=
Murine Glvr-1	M73696	↑	=
Raf-related oncogene	D00024	↑	=
Mus musculus tbc1	U33005	=	↑
ATP-binding cassette, sub-family G(WHITE), member 1	U34920	=	↑
Zinc-finger protein 57	D21850	↑	↑
Glucocorticoid-induced leucine zipper	NM010286	↑	↑
Inositol polyphosphate-5-phosphatase, 145 kDa	U52044	=	↑
ESTs	AI326878	↑	=
ESTs	AI528727	↑	=
ESTs	AI116931	↑	=
ESTs	AA764080	↑	=
ESTs	AI324037	↑	=

メタンフェタミン(METH, 2 mg/kg)は1日1回7日間にわたって投与した。METH急性投与群(S-METH)はMETH投与24時間後に中脳部を分画した。METH慢性投与群(M-METH)はMETHを7日間投与24時間後に中脳部を分画した。1.5倍以上の増加を示したものを抜粋した。(文献14より引用)

ター社)を用いて検討した。その結果，METH(2 mg/kg)急性投与により，有意な自発運動亢進作用が認められた(図2B)。また，METH慢性投与群ではMETH(2 mg/kg)投与による自発運動亢進作用が認められ，この効果はMETH急性投与群に比較して有意に増加していた。今回用いたMETH投与スケジュールによりMETH精神依存が形成され，さらに運動活性における逆耐性も形成されることを確認した。このMETH慢性投与動物を用い，薬物依存形成に関与する脳内遺伝子発現を検討した。

遺伝子発現の網羅的解析はcDNAマイクロアレイIntelliGene(Mouse CHIP Set I，宝酒造：http://bio.takara.co.jp/catalog)を用いて行った。METH(2 mg/kg)投与24時間後およびMETH慢性投与(7日間)24時間後にマウス全脳を摘出し，中脳辺縁系ドパミン神経系の細胞体であるVTAを含有する中脳部を分画し，mRNAの抽出を行った。前述のように，ポリA RNAをCy 3-dUTPおよびCy5-dUTPでラベル化した。データの解析は，内部標準物質(lambdaおよびβ-アクチン)遺伝子の発現蛍光強度に対する変化率を算出し，1.5倍以上の変動が認められた遺伝子群を抽出した。その結果，METH急性投与および慢性投与で変動が維持されている遺伝子としてGILZを見出した(表1)。

そこで，GILZの役割を解析する目的でGILZのS化アンチセンスを作製し，METH逆耐性形成に対する影響を検討した。METH慢性投与時にGILZアンチセンスを前処置したところ，METH逆耐性形成が有意に抑制された。このことから，GILZ mRNAの誘導はMETH逆耐性の形成に関与することが明らかになった。GILZはグルココルチコイドによりその発現が誘導される遺伝子群として同定された経緯があり[8]，その発現はグルココルチコイド量の変動により調節されている。一方，METHの逆耐性形成において中脳辺縁ドパミン神経系が重要な役割を果していることが明らかになっている[7,9]。さらに，生体内のコルチコステロン量の変動は中脳辺縁ドパミン神経系に影響を与えることが報告されていることから[9]，METH慢性投与によって生じる下垂体副腎系の異常がドパミン神経系に影響を与えているものと考えられる。

最近，GILZがRaf-1結合能を有することが報告され，GILZの作用点としてRaf-1が注目されている[8]。Raf-1はMAPキナーゼスーパーファミリーの一員であり，ERK1/2などのMAP上流に位置するMAPKKKである。すでに，METH逆耐性の発現にMAPKが関与することが報告され

ていることから[10]，GILZによるRaf-1の制御はMAPKの機能に影響を与え，METH逆耐性形成に関わっていると考えられる。一方，以前の研究では，METHが転写因子であるAP-1の活性を増加させること[11]，さらに依存性薬物の慢性投与によりc-FosとAP-1の複合体機能が長期間にわたり変化していることが明らかにされている[12]。また，GILZの役割としてAP-1の転写機能を調節することが報告されている[13]。したがって，METH慢性投与によるGILZの増加は，METH精神依存形成およびその維持に関与するAP-1などの各種転写過程の適応を引き起こす重要な因子であると考えられる。

おわりに

マイクロアレイ法は，多数の遺伝子群から薬物の急性投与および慢性投与で特異的変動を示す遺伝子を同定し，行動や細胞内情報伝達系の変化を含めて解析すべき遺伝子の決定に利用可能である。本法をより効果的に利用するためには，薬物の投与スケジュールや標的とする脳部位などを注意深く検討し，サンプル採取のための実験デザインを工夫することが重要である。また，mRNAの詳細な発現量については，ノーザンブロット法やRT-PCR法で再度検討する必要がある。

薬物依存の研究におけるマイクロアレイ法の利用目的は，薬物の依存形成能を予測するマーカー(mRNA)同定および依存形成の神経ネットワークの解明である。薬物の依存形成能を評価するためには，動物の行動解析が必須であることはいうまでもないが，METHおよびMDMAの双方で同一の発現変動を示す遺伝子群を解析し，マーカーとして利用できれば「脱法ドラッグ」の迅速な規制が可能になると考えられる。そのためには，薬物による行動変化を基準として，投与後の適切な時間における脳内mRNA変化のスクリーニングシステムの構築が必要である。さらに，薬物処置後のmRNA経時変化を脳部位ごとに詳細に検討することにより，薬物依存形成の脳内ネットワークが明らかになると考えられる。今後は，マイクロアレイ法による検討から解析すべき遺伝子を決定し，タンパク質の網羅的解析などの情報を組み合わせて，その変動の意義を考える必要がある。

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条件付け場所嗜好性試験による薬物報酬効果の評価：基礎と応用

舩田　正彦

要約：薬物依存症の治療法の確立および治療薬の開発のために，依存性薬物による精神依存形成機構の解明が必要である．このためには，精神依存動物モデルを確実に，かつ安定して獲得する方法論を確立することが必須となる．条件付け場所嗜好性試験（Conditioned place preference，CPP法）は，薬物の精神依存形成能を報酬効果から予測する方法として注目されている．CPP法はパブロフ型条件付けの原理に基づいており，動物に薬物を投与した時，その薬物が引き起こす感覚効果（中枢神経作用）と装置の環境刺激（視覚，触覚，嗅覚刺激など）を結びつける方法として開発された．CPP法は薬物の報酬効果を簡便な装置を利用することで，短期間で評価できることが最大の特徴である．また，短期間での評価が可能であることから，薬物の脳内微量注入による条件付けにより，精神依存形成における責任脳部位の同定が可能になった．一方，揮発性有機溶剤は“吸入”により乱用されることから，依存形成メカニズム解明のためには，薬物吸入により精神依存性を評価する装置の開発が必須であった．そこで，薬物吸入による揮発性有機溶剤用CPP装置の開発を試みた．その結果，トルエン吸入により報酬効果の発現が確認された．このCPP装置は簡便な操作で，一定量の揮発性有機化合物を動物に吸入させることができ，トルエン以外の揮発性有機化合物の報酬効果の評価にも応用できると考えられる．CPP法は装置を工夫することで薬物吸入による依存モデルの作製も可能であり，さまざまな薬物の精神依存形成能の一次的評価方法として非常に有用である．また，操作が簡便であり，評価に要する時間も短期間であることから，薬物の精神依存形成機構の解明に大きく貢献する評価法の一つである．

現在，わが国は第三次覚せい剤乱用期にあり，薬物乱用が大きな社会問題となっている．特に，覚せい剤，コカインおよび大麻などの違法性薬物の入手の可能性がこれまでになく高まり，薬物乱用の若年層への拡大が表面化している．また，こうした薬物の慢性的な使用により，精神疾患を発症することが知られている．医療施設における薬物関連精神疾患に関する調査から，その発病に至る薬物として覚せい剤が50%，有機溶剤は30%を占め主要な原因薬物になっているのが現状である(1,2)．こうした薬物関連精神疾患，薬物依存症の治療法の確立およびその治療薬の開発のために，依存性薬物による精神依存形成機構の解明が必要である．

さらに，法的規制を受けていない化学物質である通称“脱法ドラッグ”の乱用は若年層を中心に浸透しているのが現状である．こうした化学物質は，強力な精神依存形成能を有する危険性や未知の毒性などが発現する危険性を有する．事実，幾つかの化学物質は乱用され重大な社会問題となっている．したがって，化学物質の薬物依存性を，迅速に評価できる動物実験の必要性が高まっている．

こうした背景から，薬物の依存形成能を迅速に評価し，さらに精神依存動物モデルを確実かつ安定して獲得する方法論を確立することが重要である．国内および海外の研究施設において，条件付け場所嗜好性試験（Conditioned place preference，CPP法）は，薬物の精神依存形成能を報酬効果から予測する方法として注目されている(3,4)．海外では1980年代に，ラットを使用した研究からCPP法が確立されてきた(3,4)．国内では1990年代に世界に先駆けて，鈴木らにより遺伝子改変マウスの利用を視野に入れたマウスを使用し

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表1　条件付け場所嗜好性試験および薬物自己投与法による薬物効果の比較

薬物	CPP	Self-administration
Amphetamine	+	+
Methamphetamine	+	+
Cocaine	+	+
Morphine	+	+
Heroin	+	+
Nicotine	+	+
Ethanol	+	+
Diazepam	+	+
Δ^9-THC	+	+
Naloxone	-	0
U-69593	-	0
Pentobarbital	0	+
LSD	+	0

+：Positive effect, -：Aversion, 0：No effect, THC：tetrahydrocannabinol, LSD：lysergic acid diethylamide

たCPP法が確立された(5)．その後，マウスを利用したCPP法に関する研究報告が飛躍的に増えている．CPP法に関する詳細な実験技術に関しては，既に鈴木らのグループにより紹介されている(5,6)．本総説では，こうした報告を踏まえCPP法の基礎として，実際の実験方法と実験を実施する際の留意点に関して総括した．また，CPP法は薬物の報酬効果を，短期間で評価できることが最大の特徴および有用性である．すなわち，動物の維持が短期間で済むため「薬物の脳内微量注入による条件付け」の実施が可能になった．そこで，こうした技術とCPP法を利用した薬物の報酬効果発現の解析を通じ，明確になりつつある薬物精神依存形成における責任脳部位に関する代表的な知見をまとめてみた．さらに，CPP法の応用例として，当研究部で確立に成功した揮発性有機溶剤であるトルエン吸入による報酬効果評価の実例を紹介する．

1. CPP法の基礎

CPP法の原理と特徴：薬物の精神依存形成能は，薬物自己投与法による評価がもっとも信頼性が高いとされている．すなわち，薬物自己投与法により評価される強化効果（reinforcing effect）の有無により，薬物の精神依存形成能が予測される．CPP法によって評価される薬物の報酬効果と自己投与法における薬物の強化効果がよく対応することから，薬物の報酬効果から薬物の精神依存形成能を予知することが可能であると考えられている（表1）．

CPP法はパブロフ型条件付けに基づいており，動物に依存性薬物を投与した時，薬物が引き起こす感覚効果（中枢神経作用）と装置の環境刺激（視覚，触覚，嗅覚刺激など）を結びつける方法として開発された(5,7,8)．条件刺激はCPPボックスの環境の差異に基づく視覚，触覚および嗅覚刺激である．条件反応は各CPPボックス滞在の選択行動である．無条件刺激は薬物であり，無条件反応は薬物によって発現する中枢神経作用と考えられる．

実験方法：CPP法で使用する装置は，白・黒2コンパートメントボックスや白・黒ボックスの中間もしくは連結部分に試験時に利用する第3のニュートラルボックスで構成される3コンパートメントボックスなどが使用されている(7,8)．我々は，主にマウス用の白・黒2コンパートメントボックス（マウス用：15 × 30 × 15 cm，幅×全長×高さ）を使用している．ボックスの特徴としては，黒のボックスは平らな床面，白のボックスは凹凸のある床面で構成されており，ボックスの視覚（実験装置壁面の色：白および黒）および触覚（実験装置床面の材質：凹凸）の違いが条件刺激となっている．薬物の条件付けの方法は，様々なバリエーションがあるが基本的には，前試験法もしくはカウンターバランス法が採用されている(5,7,8)．(1)前試験法は，薬物の条件付け開始前に白・黒2コンパートメントボックスの滞在時間（5～20分間）を測定し，嗜好性を示すボックスを選定し非嗜好性側を薬物処置ボックスとして決定し，薬物の条件付けを行う方法である．(2)カウンターバランス法では，試験する群の中で薬物と溶媒の投与と各ボックスの組み合わせ（薬物-白，薬物-黒，溶媒-白，溶媒-黒）の偏りをなくした条件付けの方法である．どちらの方法を採用する場合も，条件付けは以下のように行う．動物に薬物あるいはその溶媒を投与して白あるいは黒のボックスに一定時間（20～60分間）閉じ込める．次に，6～24時間の間隔を置き，薬物および溶媒の投与とボックスの組み合わせを入れ替え，再度ボックスに一定時間閉じ込める．これらの操作を1セッションとし，この条件付けを数セッション繰り返す．条件付け終了24時間後に試験試行を行う．試験試行では，動物に薬物および溶媒ともに投与せず，白・黒ボックスの滞在時間（5～20分間）を測定する．データの解析は，薬物処置側ボックスの滞在時間から溶媒処置側ボックスの滞在時間を差し引いた値をCPPスコアとする(5,7,8)．前試験法では薬物処置側のボックスにおける，条件付けの前と後の滞在時間の変化を計算しCPPスコアとする．これらの値がプラス（+）であれば，条件付けした薬物の報酬効果が発現し，マイナス（-）であれば薬物処置側のボックスからの回避行動すなわち嫌悪

効果の発現として評価する．

CPP 法を実施する上での留意点：CPP 法では，薬物の条件付けを行う白・黒2コンパートメントボックスの環境条件に注意する必要がある．非嗜好性側を薬物処置ボックスとして，条件付けを行う前試験法を採用する場合，各ボックスの滞在時間に極端な差が認められると，明確な報酬効果の評価が困難になるとされる(4,9)．これは，極端な偏りがある場合，嗜好性側のボックスに薬物を処置して条件付けを行っても，天井効果で報酬効果の測定が不可能になるからである．さらに，非嗜好性側を薬物処置ボックスとすると，非嗜好性側の「環境に存在する嫌悪刺激の軽減」によりCPP スコアがプラスの値を示す可能性を否定できなくなる(9)．例えば，ある環境に視覚による嫌悪刺激がある場合，薬物が視覚を遮断する作用を有していれば，この嫌悪刺激は遮断され非嗜好性側の滞在時間は延長する訳である．したがって，この方法を採用して報酬効果を評価する際には，条件付け前の動物の白・黒2コンパートメントボックスにおける滞在時間の差を，できる限り小さくする工夫が必要である(8,9)．CPP ボックスの床面の材質や装置内の照度などを調整して，ニュートラルに近づける必要がある．薬物の条件付けにおいては，ボックスに閉じこめておく時間の設定に注意が必要である(5,7,8)．報酬効果を示す薬物を例にすると，閉じ込めておく時間は報酬効果が発現し，そのピークを過ぎない時間設定が必要である．薬物によって若干の差があるが，条件付けの時間は20～60分間が一般的である．また，短時間作用型の薬物であれば，条件付けの時間は短くなるので，訓練のセッション数を増やすのが妥当である．また，用量設定も重要であり，催眠作用を有する薬物を評価する場合などは，睡眠が誘発されない用量設定が必要である．ボックスに閉じ込めている時間内は，感覚効果を認知できる状況に保つことが重要である．依存性薬物の効果を検討するのであれば，既報論文を参考に条件設定を行うことが望ましい．CPP 法で報酬効果を評価した場合，他の中枢作用が発現する用量よりも低用量の条件付けで，報酬効果が発現する傾向がある．したがって，新規薬物の評価を行う場合は，薬物による中枢作用発現の用量を参考に低用量からの試行が効果的である(5,7,8)．

薬物の精神依存形成能は，薬物自己投与法による評価がもっとも信頼性が高いとされている．CPP 法によって評価される薬物の報酬効果と自己投与法における薬物の強化効果がよく対応することから，薬物の報酬効果から薬物の精神依存形成能を予知することが可能であると考えられている（表1）．CPP 法および薬物自己投与法による薬物効果の評価結果を比較すると，覚せい剤，コカインおよびニコチンなどの中枢興奮薬においては，CPP すなわち報酬効果の発現と薬物自己投与の発現は，非常に良好な相関性が認められている．同様に，中枢抑制薬のうちオピオイド系薬物のモルヒネおよびヘロイン，ベンゾジアゼピン系薬物のジアゼパム，エタノールにおいても報酬効果の発現と薬物自己投与の発現はよく一致することが示されている(4)．一方，中枢抑制薬のうち，ペントバルビタールでは薬物自己投与の発現は認められるものの，CPP の発現に関しては確認されていない(4)．ペントバルビタールはヒトで乱用される薬物であり，CPP の発現が予想される．薬物によっては，条件付けを開始する前に，特殊な処置をすることで，明確な CPP 発現を評価できる場合がある．例えば，フェンサイクリジンにおいては事前にフェンサイクリジンを処置した動物において，その後の条件付けにより明確な CPP の発現を確認している(10)．また，エタノールではストレス暴露後の条件付けにより，感度良く CPP の発現を確認している(11)．おそらく，オピオイド系薬物以外の中枢抑制薬や幻覚薬などの報酬効果を評価する場合には，こうした全体の実験環境を精査した上での検討が必要であろう．また，薬物によっては，報酬効果発現の用量範囲が狭い場合もあり，薬物の用量設定および実験条件に留意した検討が必要である(5,7,8)．さらに，記憶障害を引き起こす薬物では，条件付け自体が不可能であり，薬物の報酬効果や嫌悪効果の正当な評価が困難である．医薬品の開発などで CPP 法を利用する場合には，別途，記憶に関する試験が必要になる可能性もある．こうした問題点を留意して，既に評価が進んでいる依存性薬物をスタンダードとして，薬物の用量および実験条件を整え CPP 法による実験系を確立していくことが重要である．

CPP 法の有用性：CPP 法の有用性として，薬物の報酬効果の発現強度を用量反応性から検討できる点がある(5,7,8)．実験操作が簡便であることから，短期間で多くの用量群の検討が可能であり，迅速に報酬効果発現における用量反応性の解析ができる．また，条件付け終了後に試験試行を行う時は，基本的には薬物を処置しない（drug-free state）ので，動物の運動機能に影響を与える薬物であっても，その報酬効果は単一の用量反応曲線として評価が容易にできる(4)．さらに，これまでの研究から，薬物の報酬効果は他の中枢作用が発現する用量より低用量で発現することが示されている．例えば，モルヒネ条件付けによる報酬効

表2 薬物報酬効果の発現と脳部位

a) 薬物微量注入による条件付け

Drug	脳部位	効果
Amphetamine	NAC	CPP 発現
	VP	CPP 発現
	Striatum	No effect
Cocaine	NAC	CPP 発現
	PFC	CPP 発現
Morphine	NAC	No effect
	VTA	CPP 発現
	PAG	CPP 発現
	Striatum	No effect
	PFC	No effect

b) 薬物報酬効果の発現に対する拮抗薬注入の影響

Drug-induced CPP	脳部位	効果
Amphetamine	NAC	
	DA：α-flupenthixol	抑制
	D1：SCH23390	抑制
	D2/3：Sulpiride	抑制
	Striatum	
	D1：SCH23390	CPP 発現
	D2/3：Sulpiride	CPP 発現
Cocaine	NAC	
	DA：α-flupenthixol	抑制
	D2/3：Sulpiride	CPP 発現
	Striatum	
	D2/3：Sulpiride	CPP 発現
Morphine	NAC	
	D1：SCH23390	抑制
	D2/3：Sulpiride	CPP 発現
	VTA	
	Naloxone methiodide	抑制

CPP：conditioned plca preference，DA：ドパミン受容体拮抗薬，D1：ドパミンD1受容体拮抗薬，D2/3：ドパミンD2/3受容体拮抗薬，VP：ventral pallidum，NAC：nucleus accumbens，PFC：prefrontal cortex，PAG：periaqueductal gray，VTA：ventral tegmental area

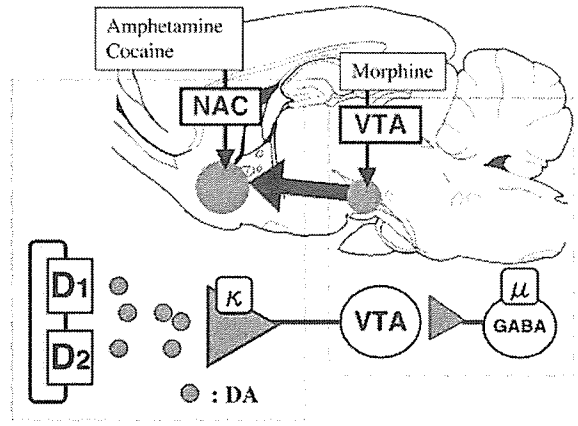

図1 報酬効果と嫌悪効果発現における中脳辺縁ドパミン神経系の役割

（報酬効果）オピオイド受容体作用薬は，中脳辺縁ドパミン（DA）神経系の細胞体である腹側被蓋野（VTA）におけるμオピオイド受容体に作用して，GABA神経系が抑制（脱抑制機序）され，側坐核（NAC）においてドパミン遊離が増加する．中枢興奮薬はNACのドパミン神経シナプス終末部に作用して，ドパミン遊離を増加させる．こうした，ドパミン遊離の増加が報酬効果の発現に関与する．（嫌悪効果）κオピオイド受容体作用薬は，NACでドパミン遊離を抑制することにより嫌悪効果を示す．さらに，ドパミンD1受容体拮抗薬により，NACのドパミンD1受容体が遮断されることで嫌悪効果が発現する．また，オピオイド受容体拮抗薬のナロキソンなどは，VTAのμオピオイド受容体を遮断することにより，GABA神経系が増強されドパミン神経系が抑制されることで，嫌悪効果が発現する．

果の発現は，自発運動促進作用が発現する用量の1/2程度で確認される(7,8)．中枢作用のなかでも報酬効果の発現は，感度良く検出できるようである．したがって，CPP法は薬物の精神依存形成能の一次的評価方法として非常に有用であると考えられる．

CPP法は薬物の報酬効果を短期間で評価できるため，動物の維持期間も短期間ですむ．したがって，薬物の脳内微量注入による条件付けが可能であり，この技術を利用して精神依存形成における責任脳部位の同定に関する研究が進展している(12,13)．代表的な方法として，(a)特定の脳部位に薬物を直接微量注入して条件付けを行い報酬効果の発現を検討する方法と，(b)選択的な受容体拮抗薬などを前処置して，薬物末梢投与による報酬効果発現に対する影響を検討する方法が利用されている(12,13)．現在までに報告された薬物の報酬効果発現と，その責任脳部位に関する知見を表2にまとめた．こうした研究を通じて，薬物の精神依存形成および報酬効果の発現には，脳内ドパミン神経系が関与していることが明らかになっている(14)．脳内のドパミン神経系は黒質（A9）-線条体系と腹側被蓋野（Ventral Tegmental Area: VTA，A10）から側坐核に投射している中脳辺縁系の2つに分類されている．このうち，薬物の報酬効果の発現および精神依存形成には，中脳辺縁ドパミン神経系の活性化が重要な役割を果たしている(6,15)（図1）．モルヒネやヘロインなどのオピオイド受容体作用薬は中脳辺縁ドパミン神経系の細胞体である腹側被蓋野に高密度に存在するμオピオイド受容体を介し，介在ニューロンである抑制性のγ-アミノ酪酸（GABA）神経系を抑制して（脱抑制），中脳辺縁ドパミン神経系の活性化を引き起こす(6,16)．したがって，モルヒネやヘロインを全身投与あるいは腹側被蓋野に微量注入した場合，中脳辺縁ドパミン神経系の投射先である側坐核においてドパミン遊離は著明に増加し，これがモルヒネ報酬効果の発現および精神依存形成に関与しているとされる(6,17)．さらに，側坐核内にドパミンD1受容体拮抗薬を前処

表3 薬物嫌悪効果の発現と脳部位
薬物微量注入による条件付け

Drug	脳部位	効果
U-50, 488H	NAC	CPA 発現
	VTA	CPA 発現
	PFC	CPA 発現
	SN	No effect
	Striatum	No effect
Naloxone	VTA	CPA 発現
	NAC	CPA 発現
	PFC	No effect
	Striatum	No effect
CTOP	VTA	CPA 発現
	NAC	CPA 発現

CPA : conditioned place aversion, NAC : nucleus accumbens, PFC : prefrontal cortex, PAG : periaqueductal gray, VTA : ventral tegmental area, SN : substantia nigra
CTOP : D-Pen-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2

置すると，モルヒネの報酬効果発現は抑制されることから，側坐核のドパミンD1受容体が重要な役割を果たしていると考えられる(18)．また，アンフェタミンやコカインなどの中枢興奮薬を側坐核に微量注入し，条件付けをすると報酬効果が発現する(12, 19)．これらの効果はドパミンD1およびD2/3受容体拮抗薬の併用で抑制されることが示されている(12, 20)．中枢興奮薬の報酬効果発現においては，側坐核のドパミンD1およびD2/3受容体の双方が関与していると考えられる．中脳辺縁ドパミン神経系の主要投射先にあたる側坐核でのドパミン遊離の増加は，薬物の報酬効果の発現すなわち，薬物の精神依存形成に重要な役割を果たしている．

一方，CPP法では薬物の嫌悪効果の評価も可能である．CPP法による評価により，オピオイド受容体拮抗薬であるナロキソン，κオピオイド受容体作用薬U-50, 488HおよびE-2078で条件付けを行うと嫌悪効果が発現することが明らかになっている(13)．薬物自己投与法による検討から，κオピオイド受容体作用薬の精神依存形成は非常にまれであり，むしろ嫌悪作用が発現することが知られている．脳内微量注入による条件付けの実験から，κオピオイド受容体作用薬およびオピオイド受容体拮抗薬による嫌悪効果発現と，その責任脳部位が明らかになっている（表3）．薬物の嫌悪効果の発現においても，脳内ドパミン神経系が関与していると考えられている．中脳辺縁ドパミン神経系の側坐核にκオピオイド受容体作用薬を微量注入して条件付けを行うと，強力な嫌悪効果が発現することが報告されている(21)．側坐核においてκオピオイド受容体は高密度に存在しており，活性化されると側坐核におけるドパミン遊離は，著明に抑制されることが明らかになっている(22)（図1）．また，オピオイド受容体拮抗薬であるナロキソンなどは，中脳辺縁ドパミン神経系の細胞体である腹側被蓋野に存在するμオピオイド受容体を遮断することによりGABA神経系が増強され，結果的にドパミン神経系が抑制されることで強力な嫌悪効果が発現することが証明されている(23)．さらに，ドパミンD1受容体拮抗薬により嫌悪効果が発現するが，この効果の発現には側坐核におけるドパミンD1受容体遮断作用が重要な役割を果たしている(24)．先にも述べたが，覚せい剤，コカインおよびモルヒネなどの依存性薬物は，側坐核においてドパミン遊離量の増加を引き起こし，報酬効果の発現に関与することが知られている．したがって，中脳辺縁ドパミン神経系が活性化されると報酬効果が発現し，一方，その活動が抑制されると嫌悪効果が発現すると考えられる．薬物の嫌悪効果の発現においても，中脳辺縁ドパミン神経系が関与する知見は興味深い．CPP法は，薬物の報酬効果に加え薬物による嫌悪効果の評価も可能であり，医薬品の開発における薬物の有害作用の予測などにその応用が期待される．

2. CPP法の応用

揮発性有機溶剤の評価：有機溶剤は揮発性に富み，低極性および脂溶性の性質を有する有機化合物の総称である．現在までに，頻繁に乱用されてきたシンナーとは塗料，接着剤などの希釈剤として使用される混合溶剤の一般名称であり，トルエンなどが使用される場合もある．我が国における有機溶剤の乱用は昭和30年代後半，「シンナー遊び」として出現し，以後10年間で全国に乱用が拡大した．ここ10年間の有機溶剤の乱用は減少傾向にある．しかしながら，平成15年に実施された中学生および高校生を対象とした薬物乱用に関する実態調査によれば，有機溶剤の生涯経験率は大麻や覚せい剤をしのぎ，高い割合を示している(25)．有機溶剤の乱用は依然として，若年層に深く根づいていると考えられる．トルエンなどの有機溶剤は安価であり，入手は比較的容易である．さらに，有機溶剤を含有する製品は生活に密着している点でも，他の依存性薬物と比較して乱用される危険性が高いと考えられる(25)．

トルエンなどの揮発性有機溶剤は“吸入”経路で乱用されているが，同一経路の薬物摂取による依存性の評価系は十分な検討がなされていなかった．したがって，揮発性有機溶剤の精神依存形成メカニズムは明ら

かにされていないのが現状である．揮発性有機溶剤の精神依存の評価に関するこれまでの報告では，オペラント条件付けを利用し，サルがレバーを押すと鼻腔内に装着したカテーテルから一定量の揮発性有機溶剤（シンナー：主要成分はトルエンと酢酸エチル）含有ガスが供給され連続吸入による自己投与実験法で証明されている(26,27)．薬物自己投与法による精神依存の評価は最も信頼性の高い方法であるが，使用する装置，動物の維持および評価に要する時間などを考慮すると有機溶剤の依存形成メカニズム解明のためには，より簡便かつ迅速な評価方法が必要であると考えられる．さらに，揮発性有機溶剤は“吸入”により乱用されることから，依存形成メカニズム解明のためには，薬物吸入により精神依存性を評価する装置の開発が必須であった．そこで，我々は操作が簡便なCPP法に着目し，薬物吸入による精神依存性評価のための装置，「揮発性有機溶剤用CPP装置」の開発を試みた(28)．既存のマウス用CPP装置（白・黒2コンパーメントボックス）を改良し，密閉性を高めた揮発性有機溶剤用の装置を作製した．トルエンの暴露方法は，実験毎にガス洗浄ビンにトルエンをいれ，洗浄ビン内に空気を送り込みトルエンを気化させた．流量計でトルエン含有ガスの流量を調整し，一定濃度のトルエンをCPP装置内に充満させた．装置内のトルエン濃度はガスクロマトグラフ法により解析した．その結果，トルエン含有ガスの流量を調節することで，装置内のトルエン濃度は流量依存性に制御できることが明らかになった．さらに，この装置を利用してマウスにトルエンを吸入させ，脳内トルエン濃度を測定したところ，トルエン濃度に依存して脳内にトルエンが移行していることが確認された．このような条件下にて，トルエン吸入による条件付け（トルエン1日1回吸入で5日間）を行った．トルエンと空気の吸入の組み合わせは，カウンターバランスの実験デザインとした．条件付け終了後，試験試行を行ったところトルエン吸入ボックスにおける滞在時間の延長，すなわち報酬効果の発現を確認した．揮発性有機溶剤であるトルエンは吸入により乱用されることから，同一の摂取方法で報酬効果を評価することはその依存形成機構を解明するために重要である．この装置は簡便な操作で，一定量の揮発性有機化合物を動物に吸入させることが可能であり，条件付けを実施できることから，吸入モデルによる精神依存形成メカニズムの解明に利用できる．さらに，本装置はトルエン以外の揮発性有機化合物の報酬効果の評価にも応用できると考えられる．

揮発性有機化合物，煙草および大麻などヒトで吸引および吸煙形式で乱用される薬物において，同様な経路で動物に薬物を与えることにより，依存モデルを作製することは非常に重要である．CPP法はその装置を工夫することで，こうした吸入や吸煙という特殊な経路による報酬効果の評価も可能になり，応用範囲が広い利便性の高い実験方法であると考えられる．

CPP法による報酬効果再発の評価：CPP法を使用した報酬効果に関する研究から，薬物の精神依存形成メカニズムの解明が進んでいる．一方，薬物依存症の最大の問題は退薬によって生じる薬物に対する渇望（craving）や再発（relapse）である．薬物依存症の治療法および治療薬の開発のためには，この渇望や再発の動物モデルの作製と解析が重要である．再発を反映するモデルとして薬物自己投与法では，自己投与を獲得した動物を利用して，薬物の再投与（drug priming injection）により，自己投与が惹起される薬物探索行動モデルが利用される．この薬物探索行動モデルを利用して，薬物に対する渇望や薬物摂取の再発に関する脳内神経メカニズムの解析が進んでいる(29)．近年，CPP法においても，こうした薬物探索行動モデルに類似した実験系が確立されつつある．CPP法による再発モデルの実験は，(1)薬物による条件付け，(2)消去および(3)薬物の再投与による試験の3相から構成される．実験の概要を以下に示す．(1)CPP法に従い薬物と溶媒の組み合わせで条件付けを行う．条件付け終了後に試験試行を行い，報酬効果の発現を確認する．(2)その後，この動物に溶媒と溶媒の組み合わせで再度の条件付けを行い，獲得した報酬効果を消去させる．(3)drug priming injectionとして低用量の薬物を投与して，試験試行を行い，報酬効果の発現を確認する．現在までのCPP法を使用した報告では，コカインやモルヒネなどにおいて，drug priming injectionおよびストレス負荷によりCPPの再発現（再発）現象が確認されている(30,31)．コカインに関する研究では，コカイン報酬効果を獲得した動物の内側前頭葉におけるドパミンD1受容体を遮断すると，コカイン再投与によるCPPの再発現が抑制されるとの報告がある(32)．また，コカイン報酬効果を獲得した動物において，ストレス負荷によりCPPの発現が認められるが，この再発現においてはコレシストキニンB受容体が関与していることが報告されている(31)．一方，薬物自己投与法による解析において，コカイン依存動物の再発には，側坐核のドパミン受容体は関与せず，興奮性アミノ酸（AMPA/kinate）受容体の重要性が示唆されている(33)．薬物自己投与法による評価に加え，最近のCPP法を使用した結果から，報酬効果の獲得過程と

再発の脳内神経メカニズムは異なることが示されている(29)．CPP法を利用した渇望および再発のモデルの確立は，短期間での評価が可能であり非常に有用である．現時点では，条件付け終了後の消去の手続きにおいて，溶媒の条件付けをする場合と条件付けはせずに一定の期間をへて消去させる場合の2つの方法がとられており，研究者間で一定していない(30,32)．この部分の十分な検討が必要であろう．CPP法を利用した渇望および再発のモデルの妥当性は更なる検討が必要であると思われるが，薬物依存症の渇望および再発の発症における脳内神経メカニズム解明のために，CPP法を応用した評価系の導入が期待されている．

3. CPP法の今後

CPP法はその装置の改変により，ラット，マウスなどさまざまな動物種を利用して薬物依存の研究ができる有用性をもっている．近年，様々な遺伝子改変マウスが作出されている．これらの動物を用いて，CPP法による薬物報酬効果の検討がなされており，薬物依存形成メカニズムが明らかになってきた．遺伝子改変動物とCPP法での評価を組み合わせることにより，薬物依存形成メカニズムの研究は飛躍的に進むものと期待される．今後は，薬物依存症の渇望および再発モデルに関する研究が重要になると思われる．

CPP法の特徴を最大限に生かし，更なる工夫改良がなされ，「薬物依存症を評価する適切かつ安定したモデル作製の方法」として広く利用されることを期待する．

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Barium potentiates the conditioned aversion to, but not the somatic signs of, morphine withdrawal in mice

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Abstract

The effect of barium, a putative blocker of G-protein-activated inwardly rectifying potassium (GIRK) channels, on naltrexone-precipitated withdrawal signs in morphine-dependent mice was investigated. Mice were chronically treated with morphine (8–45 mg/kg) for 6 days. The morphine-dependent mice were then given naltrexone (1 and 3 mg/kg), after which they showed several somatic signs of withdrawal, as well as conditioned aversion, increased cortical noradrenaline turnover, and decreased dopamine turnover in the limbic forebrain. Pretreatment with barium (1.25 and 2.5 nmol) significantly potentiated the naltrexone-precipitated conditioned aversion and augmented the decrease in dopamine turnover in the limbic forebrain. However, barium pretreatment did not affect the naltrexone-precipitated somatic signs of withdrawal and increased cortical noradrenaline turnover. These findings suggest that modification of GIRK channels may be involved in the expression of aversion to morphine withdrawal mediated through the dopaminergic system but it is not involved in the somatic signs of morphine withdrawal mediated through the noradrenergic system.

Keywords: Conditioned place aversion; Dopamine; G-protein-activated inwardly rectifying potassium channel; Morphine; Withdrawal

1. Introduction

G-protein-activated inwardly rectifying potassium (GIRK) channels are involved in diverse cellular functions, including the maintenance of resting conductance, K^+ homeostasis, pacemaker activity, synaptic inhibition, and neuronal firing rates (Isomoto et al., 1997). The GIRK-type subfamily (GIRK 1, 2, 3, and 4) is comprised of four channel subunits that are subject to G-protein activation (Kubo et al., 1993; Krapivinsky et al., 1995; Lesage et al., 1994). GIRK channels are regulated by a variety of G_i/G_o-coupled inhibitory neurotransmitter receptors such as opioid (Chen and Yu, 1994; Lesage et al., 1994; Kovoor et al., 1995), M_2-muscarinic (Kubo et al., 1993; Krapivinsky et al., 1995), and gamma aminobutyric acid type B ($GABA_B$) receptors (Lewohl et al., 1999; Slesinger et al., 1997). It has been reported that weaver mutant mice with mutant GIRK channels display significantly lower analgesia after the administration of an opiate, suggesting that GIRK channel activation is important for opiate-induced analgesia (Ikeda et al., 2000).

Chronic use of opiates such as morphine is known to lead to physical and psychological dependence, which is characterized by a withdrawal syndrome when drug administration stops. This syndrome, which includes both somatic and affective components, is caused by adaptations in specific brain neurons after repeated exposure to the drug (Nestler and Aghajanian, 1997). It is well established that the noradrenergic system, which originates in the locus ceruleus and projects to the prefrontal cortex, plays an important role in physical dependence on opiates (Funada et al., 1993; Suzuki et al., 1995). Furthermore, the mesolimbic dopaminergic system, which originates in the ventral tegmental area and projects to the nucleus accumbens,

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plays an essential role in mediating drug reinforcement (Devine and Wise, 1994; Phillips and Le-Piane, 1980; Druhan et al., 1993).

It has been reported that ethanol, a frequently abused drug, enhances the function of GIRK channels coupled to $GABA_B$ receptors (Lewohl et al., 1999; Kobayashi et al., 1999). Recently, it was reported that GIRK2 channel null mutant mice showed weaker conditioned taste aversion and weaker conditioned place preference than did wild-type mice (Hill et al., 2003). Blednov et al. (2001) have demonstrated that in GIRK2 mutant mice ethanol neither induces motor activation nor reduces anxiety, and that the effects of ethanol on both sleep time and performance on the rotarod test were the same for GIRK2 mutant mice and wild-type mice. These reports suggest that GIRK channels may be involved in the motivational effect of an abused drug. However, the involvement of GIRK channels in the interaction between morphine withdrawal and changes in the central nervous system has not been documented.

Barium chloride is frequently used as a tool for studying GIRK channels. Barium has been shown to block the GIRK channel current induced by opioid and $GABA_B$ receptor agonists (Kobayashi et al., 1999; Slesinger et al., 1997; Svoboda and Lupica, 1998). To investigate the role of GIRK channels in naltrexone-precipitated behavioral and biochemical changes, here we evaluated the effects of barium pretreatment on naltrexone-precipitated withdrawal signs and conditioned place aversion, and on naltrexone-induced neurochemical changes in morphine-dependent mice.

2. Materials and methods

2.1. Animals

Male ICR mice (20–25 g) were obtained from Tokyo Animal Laboratories (Tokyo, Japan). The mice were maintained on a 12-h light, 12-h dark schedule, and laboratory mouse chow and water were provided ad libitum. All procedures were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, approved by the Japanese Pharmacological Society.

2.2. Chronic morphine treatment

Morphine was injected s.c. twice daily, at 9 AM and 7 PM. According to the schedule described by Funada et al. (1993, 2001), the morphine dose was increased progressively from 8 to 45 mg/kg over a period of 6 days as follows (in mg/kg for AM and PM): 1st day (8, 15), 2nd day (20, 25), 3rd day (30, 35), 4th day (40, 45), 5th day (45, 45), and 6th day (45 at 9 AM only). The control mice were chronically treated with saline (10 ml/kg, s.c.) for 6 days.

2.3. Locomotor activity

The locomotor activity of the mice was measured with an activity monitoring system (NS-AS01, Neuroscience Co., Ltd., Tokyo, Japan) according to Narita et al. (2002). Briefly, the activity monitor is composed of an infrared ray sensor placed over a box ($18.2 \times 26 \times 12.8$ cm, $w \times l \times h$), a signal amplification circuit, and a control circuit. The sensor can detect the movement of animals, based on released infrared rays associated with their temperature. Locomotor activity was counted for 80 min before treatment for habituation and for 2 h after the administration of barium (2.5 nmol, i.c.v.) or saline in a volume 10 μl. The dose of barium (2.5 nmol) did not significantly affect on morphine analgesia (Harris et al., 1975). The data were processed by a computerized analytical system (Multidigital 16-port Counter System, Neuroscience Co., Ltd., Tokyo, Japan).

2.4. Effect of barium on naltrexone-induced conditioned place aversion

The experimental apparatus consisted of a shuttle box ($15 \times 30 \times 15$ cm, $w \times l \times h$, Neuroscience Co., Ltd., Tokyo, Japan), which was divided into two compartments of equal size. One compartment was white with a textured floor while the other was black with a smooth floor. Place conditioning was conducted as previously described (Watanabe et al., 2003). Barium chloride (1.25 and 2.5 nmol, i.c.v.) was administered in a volume 10 μl 2 h after the final injection of morphine (45 mg/kg, s.c.). Control animals were given an equal volume of saline. Fifteen minutes after barium or saline was administered, the mice were injected with naltrexone (1 and 3 mg/kg, s.c.) and immediately confined to either the black or white compartment of the test apparatus for 30 min. After at least 5 h, in the afternoon, the mice were administered saline (10 μl, i.c.v.). Fifteen minutes after the saline injection, the mice were treated with saline (10 ml/kg, s.c.) and confined to the other compartment for 30 min. The sequence of compartment assignation (from white to black or vice versa) was counterbalanced across the subjects overall. For the test session, the center wall used for the conditioning session was replaced by a partial wall in the shape of a T. The mice were allowed free access to both compartments (each was 6×6 cm, $w \times h$) on either side of the partial wall. The test session was carried out 1 day after the final conditioning session, with the mice in a drug-free state. The mice were placed in the center of the shuttle box and allowed free access to both compartments. The time a mouse spent in each compartment during a 900-s session was measured automatically in a blind fashion, using an infrared beam sensor (Neuroscience Co., Ltd., Tokyo, Japan). All sessions were conducted under the conditions of dim illumination (18 lx) and white noise.

2.5. Effect of barium chloride on naltrexone-precipitated withdrawal signs

Barium chloride (2.5 nmol, i.c.v.) was administered in a volume of 10 μl 2 h after the final injection of morphine (45 mg/kg, s.c.). Control animals were given an equal volume of saline. Fifteen minutes after barium or saline was administered, the mice were treated with naltrexone (1 and 3 mg/kg, s.c.) and placed in a clear plastic cylinder (12×30 cm, diameter × height). Naltrexone-precipitated behavioral changes were recorded using a digital video camera (DCR-PC100, Sony) for 30 min. Body weight loss was measured 5 min before and at 15, 30, 45, and 60 min after the administration of naltrexone. The naltrexone-precipitated jumps and body shakes were counted for 30 min. The number of mice that expressed withdrawal signs, such as jumping, shaking,

diarrhea, and ptosis, were counted according to Funada et al. (2001).

2.6. Effect of barium on naltrexone-induced monoamine turnover in the limbic forebrain and cerebral cortex

Using high-performance liquid chromatography (HPLC) with electrochemical detection (ECD), the concentrations of noradrenaline, 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG), dopamine, 3,4-dihydroxyphenyl-acetic acid (DOPAC), homovanillic acid, serotonin (5-hydroxytryptamine, 5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) were determined as reported previously (Funada et al., 1993, 2001; Suzuki et al., 1995). The morphine-dependent mice were administered barium chloride (1.25 and 2.5 nmol, i.c.v.) or saline (10 μl) 15 min prior to injection with naltrexone (3 mg/kg, s.c.) or saline. The mice were killed 60 min after the administration of naltrexone and then immersed in a dry ice-ethanol solution. The brain was quickly removed, and the limbic forebrain (including the nucleus accumbens) and the cerebral cortex were removed onto an ice-cold plate, as described by Funada et al. (1993, 2001). The tissues were frozen to −80 °C and stored until analysis. Each frozen sample was homogenized in 250 μl of 0.2 M perchloric acid containing 100 mM EDTA-2Na and 100 ng isoproterenol (as an internal standard). The homogenates were centrifuged at 10,180 × *g* for 20 min at 4 °C, and the supernatants were maintained at pH 3.0 using sodium acetate. Each sample (10 μl) was analyzed by HPLC and ECD. HPLC consisted of a delivery system (EP-300, Eicom, Kyoto, Japan), an analytical column (SC-5ODS, Eicom, Kyoto, Japan), and a guard column. The ECD system (EC-300, Eicom, Kyoto, Japan) had a graphite electrode and was used at a voltage of +0.75 V, with Ag/AgCl as a reference electrode. The mobile phase consisted of a 0.1 M sodium acetate/0.1 M citric acid buffer, pH 3.9, containing 14% methanol, sodium 1-octanesulfonate, and EDTA-2Na. The flow rate was set to 0.23 ml/min with a column temperature of 25 °C (ATC-300, Eicom, Kyoto, Japan).

2.7. Drugs

The drugs used in the present study were morphine hydrochloride (Sankyo Co., Tokyo, Japan), naltrexone hydrochloride (Sigma Chemical Co., St. Louis, MO, USA), and barium chloride (Wako Pure Chemical Industries Ltd., Osaka, Japan) dissolved in sterile saline.

2.8. Data analysis

Statistical analysis was performed using commercially available software (StatView 4.5 for Macintosh, SAS Institute, Cary, NC, USA). Data are expressed as the means ± S.E.M. Total locomotor activity was statistically evaluated using a one-way random factorial analysis of variance (ANOVA), followed by Fisher's least significant difference test to determine whether or not individual treatment produced a significant difference. Conditioning scores represent the time the mouse spent in the compartment where it had been injected with the drug minus the time spent in the compartment where it had been injected with saline. These conditioning scores were statistically evaluated using one-way ANOVA, followed by Fisher's least significant difference test to determine whether individual treatment produced significant conditioning. For the data on the changes in naltrexone-precipitated body weight loss, two-way ANOVA with time as the repeated measures was used to compare the barium-treated and saline-treated groups. The incidence of withdrawal signs was statistically evaluated using the chi-square test. Biochemical data were statistically evaluated with one-way ANOVA followed by the Fisher's least significant difference test. The turnover of dopamine, noradrenaline, and 5-HT was determined as a ratio: dopamine ratio = (DOPAC + homovanillic acid) (ng/mg of tissue)/dopamine (ng/mg of tissue); noradrenaline ratio = MHPG (ng/mg of tissue)/noradrenaline (ng/mg of tissue); and 5-HT ratio = 5HIAA (ng/mg of tissue)/5-HT (ng/mg of tissue). The percentage change in these ratios was calculated by taking the mean dopamine, noradrenaline, and 5-HT ratios for the saline–saline challenge group as 100%.

3. Results

3.1. Gross behavior in the barium-treated mice

The barium (2.5 nmol, i.c.v.)-treated mice did not exhibit abnormal behavior such as convulsions or wild running. After 80 min of habituation, the barium-treated mice showed no difference in locomotion as compared to the saline-treated mice; the mean total activity count for 120 min was 931.0 ± 370.4 (saline) and 605.9 ± 229.7 (barium) ($F[1, 19] = 0.58$, $P < 0.46$). After chronic treatment with morphine for 6 days, the gross behavior of the barium (2.5 nmol)-treated mice did not differ significantly from that of the saline-treated mice.

3.2. Effect of barium on naltrexone-induced conditioned place aversion

Using this schedule, the administration of naltrexone (3 mg/kg) to mice chronically administered saline, did not produce significant place aversion (47.5 ± 46.7, $P = 0.76$).

As shown in Fig. 1, naltrexone (1 and 3 mg/kg) given to morphine-dependent mice produced significant place aversion when compared with the effect of saline (1 mg/kg: −129.2 ± −80.3, $P = 0.03$; 3 mg/kg: −121.5 ± −43.5, $P = 0.02$). In the barium (2.5 nmol, i.c.v.)-pretreatment group, naltrexone (1 and 3 mg/kg) given to morphine-dependent mice also produced significant place aversion when compared with the effect of saline (1 mg/kg: −197.3 ± −78.9, $P = 0.02$; 3 mg/kg: −261.6 ± −23.7, $P < 0.001$). Barium had no significant effect on the morphine-dependent saline-challenge mice (−3.2 ± 56.5, $P = 0.35$). However, pretreatment with barium (1.25 and 2.5 nmol) potentiated the place aversion induced by the administration of naltrexone (3 mg/kg) in the morphine-dependent mice in a dose-dependent manner ($F[2, 36] = 3.49$, $P = 0.04$), and pretreatment with barium (2.5 nmol) significantly potentiated place aversion induced by the administration of naltrexone ($P = 0.01$, Fisher's test).

3.3. Effect of pretreatment with barium on naltrexone-precipitated withdrawal signs

Pretreatment with barium (2.5 nmol) had no effect on naltrexone (1 and 3 mg/kg)-precipitated body weight loss. Two-way ANOVA indicated that time had a significant effect (1 mg/kg; $F[4, 88] = 139.31$, $P < 0.0001$, 3 mg/kg; $F[4, 96] = 139.70$, $P < 0.0001$), unlike either barium (1 mg/kg; $F[1, 22] = 1.05$, $P = 0.32$, 3 mg/kg; $F[1, 24] = 0.00025$, $P = 0.99$) or the interaction

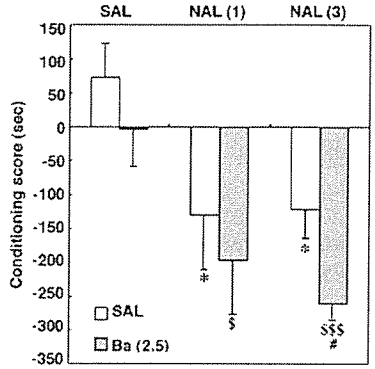

Fig. 1. Effect of barium on naltrexone-induced conditioned place aversion. Barium chloride (Ba, 2.5 nmol, i.c.v.) or saline (SAL, i.c.v.) was administered 2 h after the final injection of morphine (45 mg/kg, s.c.). Fifteen minutes after barium or saline was administered, the mice were injected with naltrexone (NAL, 1 and 3 mg/kg, s.c.). Each column represents the conditioning scores, expressed as means±S.E.M. for 7–15 animals. *$P<0.05$ as compared with the SAL-SAL group of the morphine-dependent mice. $$P<0.05$, $$$P<0.001$ as compared with the SAL-Ba group of the morphine-dependent mice. #$P<0.05$ as compared with the NAL-SAL group of the morphine-dependent mice.

(1 mg/kg; $F[4, 88]=0.52$, $P=0.72$, 3 mg/kg; $F[4, 96]=0.11$, $P=0.98$). Changes in the incidence of each withdrawal sign are shown in Table 1. Pretreatment with barium did not modify the incidence of jumping, body shakes, diarrhea or ptosis, when compared to the effect of pretreatment with saline (chi-square, ns). Pretreatment with barium did not modify the number of withdrawal jumping and body shakes precipitated by naltrexone (3 mg/kg) (Jumping; 65.8±14.7 vs. 72.2±20.6 ns., Body shakes; 5.3±1.1 vs. 4.2±0.6 ns.)

3.4. Effect of barium on naltrexone-induced monoamine turnover in limbic forebrain

As shown in Fig. 2A, naltrexone (1 and 3 mg/kg) given to morphine-dependent mice significantly, and dose dependently, decreased dopamine turnover in the limbic forebrain when compared with the effect of saline ($F[2, 26]=5.61$, $P=0.0094$). Fisher's least significant difference test demonstrated a significant effect at both 1 mg/kg ($P=0.017$) and 3 mg/kg ($P=0.0041$). In the barium (2.5 nmol, i.c.v.)-pretreatment group, naltrexone (1 and 3 mg/kg) given to morphine-dependent mice also significantly and dose dependently decreased dopamine turnover ($F[2, 19]=18.00$, $P<0.0001$). Fisher's least significant difference test demonstrated a

Table 1
Effect of barium on naltrexone-precipitated withdrawal signs

Withdrawal signs		Positive mice/Total mice			
Challenge	Pretreatment	Jumping	Body shakes	Diarrhea	Ptosis
Naltrexone (1)	Saline	11/12	11/12	8/12	5/12
	Barium	12/12	12/12	10/12	6/12
Naltrexone (3)	Saline	12/12	12/12	10/12	1/12
	Barium	12/12	12/12	11/12	5/12

Mice were treated with morphine twice a day for 6 days. Barium chloride (2.5 nmol) or saline was administered intracerebroventricularly 2 h after the final injection of morphine (45 mg/kg, s.c.). Withdrawal was precipitated by naltrexone (1 and 3 mg/kg, s.c.) 15 min after barium or saline administration.

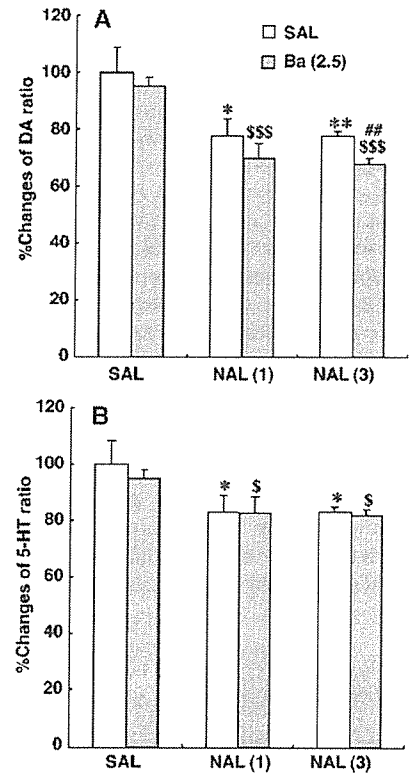

Fig. 2. Effects of barium on decreased dopamine (DA, A) and serotonin (5-HT, B) ratios in the limbic forebrain of morphine-dependent mice. Barium chloride (Ba, 2.5 nmol, i.c.v.) or saline (SAL, i.c.v.) was administered 2 h after the final injection of morphine (45 mg/kg, s.c.). Fifteen minutes after barium or saline was administered, the mice were injected with naltrexone (NAL, 1 and 3 mg/kg, s.c.) or saline (SAL, 10 ml/kg, s.c.). The mice were killed 60 min later. Each column represents the percent change in the dopamine and 5-HT ratios, expressed as the mean±S.E.M. for 6–14 animals. *$P<0.05$, **$P<0.01$ as compared with the SAL-SAL group of the morphine-dependent mice. $$P<0.05$, $$$P<0.001$ as compared with the SAL-Ba group of the morphine-dependent mice. ##$P<0.01$ as compared with the NAL-SAL group of the morphine-dependent mice.

significant effect at both 1 mg/kg ($P=0.0002$) and 3 mg/kg ($P<0.0001$). Pretreatment with barium (1.25 and 2.5 nmol) significantly, and dose dependently, augmented the decrease in dopamine turnover produced by naltrexone (3 mg/kg) in the limbic forebrain ($F[2, 28]=7.12$, $P=0.0032$, 2.5 nmol; $P=0.0012$, Fisher's test). Barium had no significant effect on dopamine turnover in the saline administration group ($F[1, 13]=0.19$, $P=0.67$).

As shown in Fig. 2B, naltrexone (1 and 3 mg/kg) given to morphine-dependent mice significantly and dose dependently decreased 5-HT turnover in the limbic forebrain when compared with the effect of saline ($F[2, 26]=4.22$, $P=0.026$) Fisher's least significant difference test demonstrated a significant effect at both 1 mg/kg ($P=0.036$) and 3 mg/kg ($P=0.011$). In the barium (2.5 nmol, i.c.v.)-pretreatment group, naltrexone (1 and 3 mg/kg) given to morphine-dependent mice also significantly and dose dependently decreased 5-HT turnover ($F[2, 19]=3.97$, $P<0.036$). Fisher's least significant difference test demonstrated significant effect at both 1 mg/kg ($P=0.041$) and 3 mg/kg ($P=0.015$). However, pretreatment with barium did not modify 5-HT turnover in the limbic forebrain in the saline administration group or in the

naltrexone administration group (saline administration group; $F[1, 13]=0.29$, $P=0.60$; naltrexone 1 mg/kg; $F[1, 10]=0.001$, $P=0.98$, naltrexone 3 mg/kg; $F[2, 28]=2.80$, $P=0.078$).

3.5. Effect of barium on naltrexone-induced monoamine turnover in cerebral cortex

As shown in Fig. 3A, naltrexone (3 mg/kg) given to morphine-dependent mice significantly elevated cortical noradrenaline turnover when compared with the effect of saline ($F[1, 23]=15.3$, $P=0.0007$). Pretreatment with barium did not modify noradrenaline turnover in the cerebral cortex in the saline administration group or in the naltrexone administration group (saline administration group: $F[1, 19]=0.49$, $P=0.49$; naltrexone administration group: $F[2, 30]=0.18$, $P=0.83$).

As shown in Fig. 3B, naltrexone (3 mg/kg) given to morphine-dependent mice had no effect on cortical 5-HT turnover when compared with the effect of saline ($F[1, 23]=0.09$, $P=0.77$). Pretreatment with barium did not modify 5-HT turnover in the cerebral cortex in the saline administration group or in the naltrexone administration group (saline administration group: $F[1, 19]=0.47$, $P=0.50$; naltrexone administration group: $F[2, 30]=0.37$, $P=0.69$).

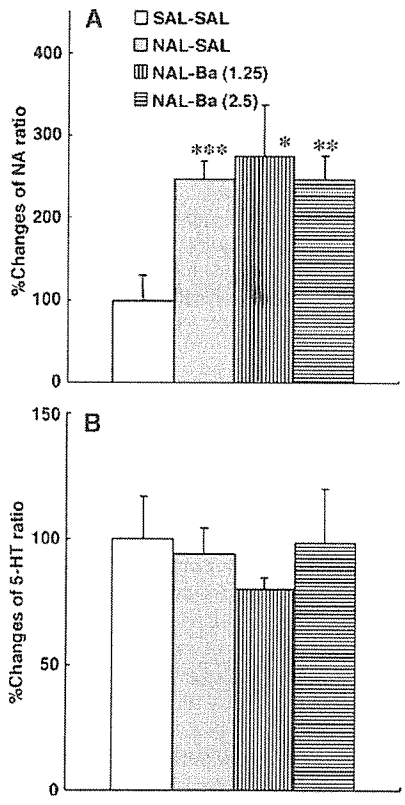

Fig. 3. Effects of barium on cortical noradrenaline (NA, A) and serotonin (5-HT, B) ratios in morphine-dependent mice. Barium chloride (Ba, 1.25 and 2.5 nmol, i.c.v.) or saline (SAL, i.c.v.) was administered 2 h after the final injection of morphine (45 mg/kg, s.c.). Fifteen minutes after barium or saline was administered, the mice were injected with naltrexone (NAL, 3 mg/kg, s.c.) or saline (SAL, 10 ml/kg, s.c.). The mice were killed 60 min later. Each column represents the percent change in the noradrenaline and 5-HT ratios, expressed as the mean ± S.E.M. for 8–15 animals. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ as compared with the SAL-SAL group of the morphine-dependent mice.

4. Discussion

This study demonstrates that barium significantly potentiates naltrexone-precipitated morphine withdrawal aversion in morphine-dependent mice. Furthermore, the neurochemical experiments show that barium significantly augments the naltrexone-precipitated decrease in dopamine turnover in the limbic forebrain in morphine-dependent mice under the same conditions.

It was previously demonstrated that the systemic administration of the opioid receptor antagonist naloxone to morphine-dependent rats produced conditioned place aversion (Watanabe et al., 2002, 2003). Our findings are consistent with these findings in that the injection of naltrexone, another opioid receptor antagonist, into morphine-dependent mice produced significant conditioned place aversion.

The nucleus accumbens is implicated in the aversive stimulus properties of morphine withdrawal but not in the expression of the somatic signs of morphine withdrawal. It has been reported that the administration of naloxone reduces the extracellular dopamine concentration in the nucleus accumbens of morphine-dependent animals (Pothos et al., 1991). The administration of naloxone to morphine-dependent rats causes a decrease in mesolimbic dopaminergic neuronal activity (Diana et al., 1995). In addition, it has been reported that dopamine turnover in the limbic forebrain, including the nucleus accumbens and olfactory tubercle, significantly decreases during naloxone-precipitated morphine withdrawal, indicating that dopaminergic activity in the nucleus accumbens decreases during naloxone-precipitated morphine withdrawal (Suzuki et al., 1995). The present results are consistent with these earlier findings in that the administration of naltrexone to morphine-dependent mice significantly decreased dopamine turnover in the limbic forebrain.

In the present study, we found that pretreatment with barium significantly augmented the naltrexone-precipitated decrease in dopamine turnover in the limbic forebrain of morphine-dependent mice. Moreover, we found that pretreatment with barium in morphine-dependent mice potentiated naltrexone-induced conditioned aversion to withdrawal under the same conditions. These results suggest that the enhancement by barium of the decrease in limbic dopamine turnover can mediate the potentiation of morphine withdrawal aversion. Thus, pretreatment with barium may potentiate naltrexone-induced morphine withdrawal aversion.

Caille et al. (2003) reported that a rat bearing a 6-hydroxydopamine-induced lesion of the nucleus accumbens displayed naloxone-precipitated conditioned place aversion, similar to a sham-operated rat, and that apomorphine, a

dopamine agonist, did not reduce naloxone-precipitated conditioned place aversion. The findings of present study are not consistent with the findings of the previous study in that dopamine turnover in the nucleus accumbens altered naltrexone-precipitated conditioned place aversion. However, there is a report that naloxone-precipitated place aversion becomes increasingly intense as the number of conditioning sessions is increased (Mucha and Iversen, 1984). In the present study, we used only one conditioning session, as compared with Caille et al. who used three. Also, the induction of morphine dependence (pellets vs. injection) and the state of morphine dependence in conditioning and test sessions (dependent state vs. drug-free state) were different in that study and the present study, which may account for the different results. Likewise, as Caille et al. suggested that multivariable mechanisms, such as the dopaminergic, noradrenergic, GABA, glutamatergic and cholinergic systems, are involved in morphine withdrawal aversion, it is possible that these systems were also changed in present study.

In the present study, the administration of naltrexone to morphine-dependent mice significantly decreased 5-HT turnover in the limbic forebrain. Several items of evidence suggest that a change in serotoninergic neurons may be involved in opioid dependence and withdrawal (Carboni et al., 1989; Harris and Aston-Jones, 2001). Acute morphine administration increases 5-HT transmission within the nucleus accumbens and dorsal raphe magnus (Tao and Auerbach, 2002). In contrast, 5-HT transmission is depressed during withdrawal from chronic morphine treatment (Tao et al., 1998; San-Martin-Clark et al., 1996). The results of the present study are consistent with these previous reports in that they provide evidence for reduced 5-HT transmission during morphine withdrawal. Interestingly, pretreatment with barium did not affect 5-HT transmission in the limbic forebrain in morphine-dependent mice or in morphine withdrawal mice. These results indicate that the potentiation by barium of morphine withdrawal aversion may involve the dopaminergic system but apparently not the serotonergic system.

In this study, we used barium to block the GIRK channel. Another type of inwardly rectifying K+ channel (IRK1-3), which is expressed in the brain, is also blocked by extracellular barium (Karschin et al., 1996; Isomoto et al., 1997). In addition, it has been reported elsewhere that barium blocks voltage-dependent K+ channels (Svoboda and Lupica, 1998). To our knowledge, no selective blockers of GIRK channels have been found, and we are unable to confirm whether or not GIRK channels play an important role in morphine withdrawal aversion and in the decrease in dopamine turnover as compared with other types of K+ channels. However, it has been shown that opioids do not activate IRK1-3 or voltage-dependent K+ channels (Isomoto et al., 1997). Thus, the blockade by barium of GIRK channels may be involved in both morphine withdrawal aversion and the decrease in dopamine transmission. However, another experiment using a selective ligand of GIRK channels would be necessary to confirm this.

It has been hypothesized that the central noradrenergic system is involved in the development of morphine dependence and the expression of withdrawal signs (Rasmussen et al., 1990; Lane-Ladd et al., 1997). The systemic administration of morphine inhibits the firing rate of locus ceruleus neurons, and tolerance to the inhibitory effects of morphine develops after chronic morphine treatment (Aghajanian, 1978). Moreover, the firing rate of locus ceruleus neurons increases during morphine withdrawal precipitated by naltrexone (Rasmussen et al., 1990). In previous neurochemical experiments, a naloxone-precipitated increase in the level of noradrenaline turnover was found in the cerebral cortex innervated by the locus ceruleus in morphine-dependent mice (Funada et al., 1993, 2001; Suzuki et al., 1995). The results of the present study are consistent with these previous reports in that naltrexone injection in morphine-dependent mice markedly increased noradrenaline turnover in the cerebral cortex, without altering 5-HT turnover. These findings suggest that the naltrexone-precipitated activation of the noradrenergic system, but not the serotonergic system, in the cerebral cortex is involved in the expression of somatic signs of morphine withdrawal.

In the present study, we found that pretreatment with barium of morphine-dependent mice did not affect the naltrexone-precipitated increase in cortical noradrenaline turnover or the expression of somatic signs of morphine withdrawal induced by naltrexone. It has been reported elsewhere that morphine withdrawal signs, such as jumping and body shakes, are elicited after microinjection of the hydrophilic opioid antagonist methylnaloxonium into the locus ceruleus (Maldonado et al., 1992). Because of the high incidence of the morphine withdrawal signs of jumping and body shakes, they may have been limited by a possible ceiling effect. However, barium did not modify the number of jumping and body shakes. Under the same conditions, pretreatment with barium did not affect the naltrexone-precipitated increase in cortical noradrenaline turnover. These results suggest that the lack of effect of barium on the increase in cortical noradrenaline turnover may be associated with the lack of effect of barium on the expression of morphine withdrawal signs, such as jumping and body shakes.

The diarrhea or body weight loss caused by morphine withdrawal did not appear after the injection of methylnaloxonium intracerebroventricularly, suggesting that peripheral mechanisms are important in the expression of these signs (Stinus et al., 1990; Maldonado et al., 1992). Moreover, morphine withdrawal ptosis was noted after the microinjection of methylnaloxonium into the amygdala, anterior hypothalamus and nucleus raphe magnus (Maldonado et al., 1992). These results suggest that not only the noradrenergic system projecting from the locus coeruleus

but also another system in another brain area are important in the expression of some morphine withdrawal signs. The blockade of GIRK channels in the brain with barium seems not to affect the expression of morphine withdrawal signs.

In conclusion, we found that the blockade of GIRK channels by barium potentiated naltrexone-induced conditioned aversion in morphine-dependent mice but not the expression of naltrexone-precipitated withdrawal signs. Furthermore, the GIRK channel blockade augmented the decrease in naltrexone-induced dopamine turnover in the limbic forebrain without any effects on 5-HT or noradrenaline turnover. These results suggest that the reduction in dopamine transmission due to GIRK channel blockade may be involved in the expression of morphine withdrawal aversion in mice. Our results suggest that the GIRK channel activator might be of value in the treatment of morphine withdrawal.

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ROTENONE INDUCES AGGREGATION OF γ-TUBULIN PROTEIN AND SUBSEQUENT DISORGANIZATION OF THE CENTROSOME: RELEVANCE TO FORMATION OF INCLUSION BODIES AND NEURODEGENERATION

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Abstract—Neurodegenerative disorders are characterized by progressive loss of specific neurons in the central nervous system. Although they have different etiologies and clinical manifestations, most of them share similar histopathologic characteristics such as the presence of inclusion bodies in both neurons and glial cells, which represent intracellular aggregation of misfolded or aberrant proteins. In Parkinson's disease, formation of inclusion bodies has been associated with the aggresome-related process and consequently with the centrosome. However, the significance of the centrosome in the neurodegenerative process remains obscure. In the present study, the morphological and functional changes in the centrosome induced by rotenone, a common insecticide used to produce experimental Parkinsonism, were examined both *in vitro* and *in vivo*. Aggregation of γ-tubulin protein, which is a component of the centrosome matrix and recently identified in Lewy bodies of Parkinson's disease, was observed in primary cultures of mesencephalic cells treated with rotenone. Rotenone-treated neurons and astrocytes showed enlarged and multiple centrosomes. These centrosomes also displayed multiple aggregates of α-synuclein protein. Neurons with disorganized centrosomes exhibited neurite retraction and microtubule destabilization, and astrocytes showed disturbances of mitotic spindles. The Golgi apparatus, which is closely related to the centrosome, was dispersed in both rotenone-treated neuronal cells and the substantia nigra of rotenone-treated rats. Our findings suggested that recruitment of abnormal proteins in the centrosome contributed to the formation of inclusion bodies, and that rotenone markedly affected the structure and function of the centrosome with consequent induction of cytoskeleton disturbances, disassembly of the Golgi apparatus and collapse of neuronal cells.

Key words: aggresome, α-synuclein, Golgi apparatus, Lewy bodies, microtubules, Parkinson's disease.

Rotenone is a naturally occurring organic insecticide obtained from the roots of several tropical and subtropical plant species belonging to the genus *Lonchocarpus* or *Derris* (Fukami and Nakajima, 1971). This insecticide is also a suitable tool for manipulation of fish populations and eradication of harmful exotic fish. The biological effects of rotenone are mainly related to the specific and reversible inhibition of the complex I mitochondrial respiratory chain (Fukami and Nakajima, 1971). Both exposure to environmental toxins and inhibition of the mitochondrial complex I are associated with the etiology and pathogenesis of Parkinson's disease (PD) and other neurodegenerative diseases (Olanow and Tatton, 1999). For those reasons, rotenone has been used to study the neurodegenerative process of the nigrostriatal dopaminergic neurons in Parkinsonism (Betarbet et al., 2000; Hoglinger et al., 2003).

The cell loss of dopamine-containing neurons in the substantia nigra (SN) pars compacta and the presence of hyaline-intracytoplasmic inclusions known as Lewy bodies (LBs) are the pathological hallmarks of PD (Forno, 1996; Olanow and Tatton, 1999). LBs are usually single or multiple spherical perinuclear bodies with a dense core and a peripheral halo, but the elongated forms in the nerve cell processes known as Lewy neurites also have a common appearance (Braak et al., 1995; Forno, 1996). The major components of LBs are aggregated proteins including α-synuclein, ubiquitin and neurofilament subunits (Gai et al., 1995; Spillantini et al., 1997; Irizarry et al., 1998). LBs-like cytoplasmic eosinophilic inclusions containing α-synuclein and ubiquitin have been observed in the SN of rats chronically treated with rotenone (Betarbet et al., 2000; Hoglinger et al., 2003; Sherer et al., 2003b). Moreover, rotenone-exposed cells in culture also show accumulation and aggregation of α-synuclein and ubiquitin proteins (Lee et al., 2002; Sherer et al., 2002). Therefore, the experimental model of Parkinsonism using the insecticide rotenone has been considered as an invaluable tool for the study of the mechanisms involved in the formation of LBs (Perier et al., 2003).

Although inclusion bodies are a common histopathological feature of various neurodegenerative diseases, the mechanisms involved in the formation of these protein aggregates are still unknown (Jellinger, 2003; Olanow et al., 2004). The aggregation of proteins and formation of

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Abbreviations: ANOVA, analysis of variance; AraC, cytosine-β-D-arabinofuranoside; Cdk, cyclin-dependent kinase; DAB, 3,3′- diaminobenzidine; DLB, dementia with Lewy bodies; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; GA, Golgi apparatus; GFAP, glial-fibrillary acid protein; HD, Huntington's disease; LBs, Lewy bodies; MAP2, microtubule-associated protein 2; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MSA, multiple system atrophy; PB, phosphate buffer; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with Triton X-100; PD, Parkinson's disease; PEG, polyethylene glycol; PFA, paraformaldehyde; SDS, sodium dodecyl sulfate; SN, substantia nigra; TBS-T, Tris-buffered saline with Tween-20; TH, tyrosine hydroxylase.

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inclusion bodies in several neurodegenerative disorders, such as amyotrophic lateral sclerosis , Huntington's disease (HD), dementia with LB (DLB) and PD, have been associated with the aggresome-related process and consequently with the centrosome (Waelter et al., 2001; McNaught et al., 2002b; Corcoran et al., 2004). The aggresomes are microtubule-dependent pericentriolar inclusion bodies, which can be a common cellular response to large amount of misfolded or aberrant proteins (Johnston et al., 1998; Kopito, 2000). Formation of the aggresomes is considered to occur when the degradation capacity of the ubiquitin-proteasome system is exceeded either by increased production or by inadequate proteolysis of aberrant proteins (Johnston et al., 1998; McNaught et al., 2002b). All these non-degraded proteins that are dispersed in the cytoplasm are then retrogradely transported along microtubules to the centrosome, where they form an enlarged inclusion of aggregated-ubiquitinated proteins surrounded by cytoskeleton elements known as aggresome (Johnston et al., 1998; Kopito, 2000; McNaught et al., 2002b; Olanow et al., 2004). Thus, the centrosome seems to play an important role in the formation of these complex inclusion bodies during the neurodegenerative processes.

The centrosome, also termed the microtubule-organizing center, is a small perinuclear organelle composed of a pair of centrioles surrounded by the centrosome matrix or pericentriolar material (Sato et al., 2000). The centrosome matrix contains a variety of proteins, such as proteins responsible for the proteasome–proteolytic pathway, cell-cycle regulatory system, and for the nucleation of microtubules (Stearns et al., 1991; Pockwinse et al., 1997; Wigley et al., 1999). The γ-tubulin protein, a member of the tubulin superfamily identified in different eukaryotic cells, is one of the components of the pericentriolar material and is required for the structure and normal function of the centrosome (Oakley and Oakley, 1989; Zheng et al., 1991; Sunkel et al., 1995). This centrosomal protein is directly related to the nucleation and polarity of microtubules, formation of bipolar spindles, duplication of centrioles and cell-cycle progression (Fuller et al., 1995; Draber and Draberova, 2003).

Recent studies reported that LBs in brain samples of PD patients are immunoreactive for γ-tubulin (McNaught et al., 2002b), suggesting that aberrant proteins of the LBs are probably recruited in the centrosome. However, it is still obscure whether the aggregation of proteins in the centrosome can directly affect the structure or function of this organelle. In the present study, we induced the formation of protein aggregates in the centrosome by rotenone treatment in primary cultures of mesencephalic neurons and astrocytes, as well as in dopaminergic neurons of the SN of Lewis rats. Furthermore, we studied the distribution of the microtubules and morphology of the Golgi apparatus (GA), a cytoplasmic organelle embedded in the pericentriolar material of the centrosome and maintained in this position by complex interactions with the microtubules (Rogalski and Singer, 1984; Letourneau and Wire, 1995; Zmuda and Rivas, 1998). The results showed that rotenone induced γ-tubulin protein aggregation, and that such process affected the structure and normal function of the centrosome.

EXPERIMENTAL PROCEDURES

Animals

All animal procedures described in our experiments were in strict accordance with the Guideline for Animal Experiments of Okayama University Advanced Science Research Center and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Special interest was taken to minimize the number and suffering of animals used in this research. Timed pregnant Sprague–Dawley rats and male Lewis rats were purchased from Charles River Japan, Inc. (Yokohama, Japan).

Cell culture

Primary mesencephalic cell cultures were prepared as descried previously (Iwata-Ichikawa et al., 1999) with minor modifications. Briefly, the ventral mesencephalon was dissected in Sprague–Dawley rat embryos (15 days of gestation). After treatment with trypsin, the cells from the mesencephalon were incubated with 0.004% deoxyribonuclease I with 0.03% trypsin inhibitor (Sigma-Aldrich Corporation, St. Louis, MO, USA) at 37 °C for 7 min. After centrifugation (420×*g*×5 min), the cell pellet was gently resuspended in 4 ml of Dulbecco's modified Eagle medium (Gibco BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum, 4 mM L-glutamine, and 60 μg/ml kanamycin sulfate. Dissociated cells were plated at a density of 2×10^5 cell/cm^2 onto six-well culture plates and eight-chamber glass culture slides coated with poly-D-lysine (Becton Dickinson Labware, Bedford, MA, USA). Cells were maintained in the culture medium at 37 °C in a 5% to 95% CO_2–air gas mixture. After 24 h of incubation, the medium was replaced with fresh medium containing 2 μM cytosine-β-D-arabinofuranoside (AraC; Sigma-Aldrich) to inhibit the replication of non-neuronal cells and to obtain neuronal-enriched mixed cell cultures. On the fifth day, the medium was replaced with fresh medium containing rotenone (Sigma-Aldrich; see below for the concentration of rotenone). In order to obtain primary glial-enriched cell cultures, mesencephalic-dissociated cells were incubated in the same culture medium without AraC for 7 days. Then, cells were subcultured and plated at a density 1×10^5 cell/cm^2. Exposure to rotenone was initiated after 5 days of incubation.

Mesencephalic neuronal- and glial-enriched mixed cell cultures were exposed to 5, 25 and 50 nM rotenone diluted in dimethyl sulfoxide (DMSO) for 48 h (the final concentration of DMSO was 0.01%). Then, samples for immunocytochemistry and Western blot analyses were prepared. Cell viability was determined by the Trypan Blue exclusion assay. In addition, cells were immunostained for microtubule-associated protein 2 (MAP2), tyrosine hydroxylase (TH) and glial-fibrillary acid protein (GFAP) and positively stained cells were counted to determine the total number of neurons, dopaminergic neurons and astrocytes, respectively. Four independent experiments were conducted, and five random pictures in each experiment were taken of either the control or rotenone-treated groups. Each picture was taken at a magnification of ×400 corresponding to an area measuring 0.07 mm^2.

Animal experiments

Studies using rotenone infusion were conducted in male Lewis rats weighing approximately 200 g at the time of surgery (7-week-old). An osmotic pump was implanted s.c. in each rat as descried previously (Sherer et al., 2003b). The Alzet osmotic pump (2ML2 or 2ML4; Durect Corporation, Cupertino, CA, USA) was filled with

2 ml rotenone solution dissolved in equal volumes of DMSO and polyethylene glycol (PEG). Under deep anesthesia with pentobarbital, the osmotic pump was implanted under the skin of the back. The flow rate of these osmotic pumps is approximately 5 μl/h (2ML2) or 2.5 μl/h (2ML4). The estimated dose of rotenone per animal was 3 mg/kg/day. Control rats were also implanted with the osmotic pumps but were treated with the vehicle only [DMSO:PEG (1:1)].

Spontaneous locomotor activity was observed in a circular open-field measuring 60 cm in diameter with a surrounding 50-cm high wall. The interior surface was painted in dark gray color and the bottom was divided into two equal concentric zones with 18 areas of equal extension. Each animal was placed in the middle of the open-field at the day before, 1, 2, 3 and 4 weeks after pump implantation, and then the number of line crossing per minute were counted. All observations were performed during the daytime within the same time (from 8:30–11:30 a.m.).

Control (n=8) and rotenone-treated rats (n=20) were killed 2 weeks or 4 weeks (n=14 in each group) after initiation of chronic rotenone infusion. The rats were deeply anesthetized and transcardially perfused with ice-cold saline followed by a fixative containing 4% paraformaldehyde (PFA), 0.35% glutaraldehyde in 0.1 M phosphate buffer (PB; pH=7.4). The brains were quickly removed from the skull and post-fixed for 24 h in a fixative containing 4% PFA in 0.1 M PB. After cryoprotection for 72 h in 15% sucrose in 0.1 M PB with 0.1% sodium azide, the brains were frozen and cut coronally into 20-μm sections on a cryostat. Finally, the brain sections containing the mid-striatum (+2.20 to +0.26 mm from the bregma) and SN (−4.52 to −6.04 mm from the bregma) were stored in 10 mM phosphate-buffered saline (PBS) with 0.1% sodium azide at 4 °C until staining.

Immunocytochemistry

Mesencephalic neuronal- and glial-enriched mixed cell cultures on the chamber slides were washed twice with 10 mM PBS and then fixed with 4% PFA in 0.1 M PB for 10 min. In single or double immunofluorescence staining, the cell culture slides or the brain sections were incubated for 20 min at room temperature in 2.5% normal donkey serum diluted in 10 mM PBS with Triton X-100 (PBST; 0.1%) for cell samples or PBST (0.2%) for brain slices, and then incubated overnight at 4 °C with the primary antibodies diluted in PBST. The following diluted primary antibodies were used: mouse monoclonal anti-γ-tubulin (diluted 1:5000; Sigma-Aldrich) and rabbit polyclonal anti-pericentrin (diluted 1:150; Covance Research Products Inc., Berkeley, CA, USA) to visualize the centrosome, goat polyclonal anti-β-tubulin (diluted 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) to stain microtubules, rabbit polyclonal anti-γ-adaptin (diluted 1:200; Santa Cruz Biotechnology) to detect the GA, sheep polyclonal anti-α-synuclein (diluted 1:1000; Chemicon International, Temecula, CA, USA), rabbit polyclonal anti-TH (diluted 1:2000; Protos Biotech Corporation, New York, NY, USA), rabbit polyclonal anti-MAP2 (diluted 1:1000; Chemicon) and rabbit polyclonal anti-GFAP (diluted 1:1000; Chemicon). After washing with PBS (3×5 min), the cell samples or brain sections were then reacted for 2 h with fluorescein isothiocyanate (FITC)- or rhodamine-conjugated secondary antibodies (diluted 1:200; Chemicon). Finally, the cells were stained with 10 μg/ml Hoechst 33342 (Molecular Probes, Eugene, OR, USA) for 2 min to visualize cell nuclei.

In immunohistochemistry with chromogen, the culture slides and brain sections were kept in 0.5% H_2O_2 in PBST (0.2%) for 30 min at room temperature. The samples were incubated in 1% goat serum in PBST (0.2%) for 30 min. Culture slices or brain sections were then exposed overnight at 4 °C to a rabbit polyclonal antibody for TH (diluted 1:2000) or γ-adaptin (diluted 1:200). After washing with PBST (5×5 min), the samples were incubated with biotinylated goat anti-rabbit IgG antibody (diluted 1:1000; Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature. Following washes in PBST (5×5 min), the slices or sections were reacted for 1 h in the avidin–biotin–peroxidase complex (diluted 1:2000; ABC system; Vector Laboratories). The immunoreactive signals were visualized by 0.02% 3,3′- diaminobenzidine (DAB), 0.4% nickel ammonium sulfate and 0.005% H_2O_2 in 50 nM Tris–HCl buffer. Standard hematoxylin and eosin staining was performed to observe cell morphology in brain samples.

All slides prepared for immunofluorescence studies were analyzed under a fluorescence microscope (Olympus BX50-FLA, Tokyo, Japan) using a mercury lamp through a 470–490 nm, 530–550 nm, or 360–370 nm band-pass filter to excite FITC, rhodamine or Hoechst dye, respectively. Light emitted from FITC, rhodamine or Hoechst was collected through 515–550 nm band-pass filter, 590 nm long-pass filter or 420 nm long-pass filter, respectively. Photos of the immunostained cells were taken at a magnification of ×400. Immunostained brain sections were observed by confocal laser-scanning microscopy (Zeiss LSM 510, Germany). The 488 nm line of an argon-ion laser attenuated to 5% of the maximal intensity with a neutral density was used to excite FITC. The 543 nm line of a helium–neon laser without attenuation was used to excite rhodamine. Light emitted from FITC or rhodamine was collected through a 505–530 nm band-pass filter or 560 nm long-pass filter, respectively. Images were taken at a magnification of ×200 and recorded using the Windows-based Zeiss LSM program. Adobe Photoshop 5.0 software was used for digital amplification of the images.

Western blot analysis

Western blot analysis was performed as reported previously (Asanuma et al., 1995). Mesencephalic neuronal-enriched cell cultures on the six-well culture plates were washed with 10 mM PBS, and then lysed in 150 μl of ice-cold RIPA buffer [1 mM PBS, pH 7.4, 1% Nonident P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)] plus a protease inhibitor (0.1 mg/ml phenylmethylsulfonyl fluoride in isopropanol). After incubation on ice for 60 min, the homogenates were centrifuged (19,200×g×20 min at 4 °C), and then the supernatants (detergent-soluble fraction) of each sample were collected. The pellet (detergent-insoluble fraction) was resuspended in 60 mM Tris–HCl, 2% SDS, and 2.5% 2-mercaptoethanol and sonicated for 20 min. Total protein concentrations of cell lysates from both fractions were determined by Branford method (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Samples of the soluble or insoluble fractions were mixed with the sampling buffer (4% SDS, 0.02% Bromophenol Blue, 20% glycerol, 10% 2-mercaptoethanol in 125 mM Tris–HCl, pH 6.8), and then boiled for 2 min. The proteins were separated through a 12.5% SDS–polyacrylamide gel (Bio-Rad Laboratories), and then electrophoretically transferred onto nitrocellulose membranes (Hybond-ECL; Amersham Biosciences, Buckinghamshire, UK).

The membranes were blocked in Tris-buffered saline with 0.1% Tween-20 (TBS-T) containing 5% non-fat milk powder at room temperature for 1 h. Then the blots were incubated with mouse monoclonal anti-γ-tubulin antibody (diluted 1:5000) or goat polyclonal anti-actin antibody (1:250; Santa Cruz Biotechnology) at room temperature for 1 h. After washing with TBS-T (2×5 min), the blots were reacted with goat anti-mouse IgG or rabbit anti-goat IgG secondary antibody conjugated with horseradish peroxidase (diluted 1:5000; Santa Cruz Biotechnology) at room temperature for 1 h. Specific signals of proteins were visualized by chemiluminescence using the ECL Western blotting detection system (Amersham Biosciences). Finally, the relative protein concentrations were determined by densitometric analysis using the National Institutes of Health image analysis software version 1.56 in four independent experiments. The ratio γ-tubulin/actin was calculated to normalize for loading and transfer of artifacts introduced in Western blotting.