シナプス可塑性を指標としたパーキンソン氏病モデル動物の作成と改善薬の開発研究

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研究成果

高齢化社会に伴い老人性疾患が増大してきており社会的な問題にまでなっている。その中でもパーキンソン氏病は、老化に伴って多発する筋強剛、無動、振戦、姿勢保持障害等の症状を呈する神経変性疾患である。しかしながら、本疾患の病因、治療法ともに解明されていないために、パーキンソン氏病は現在、難病指定を受けている疾病の１つである。（１）この疾病は黒質－線条体のドーパミン神経系が変性を受け、ドーパミン欠乏が起こることに由来すると思われる。ところで、運動系をつかさどる小脳では、シナプスの可塑的変化を伴う長期抑制現象（long-term depression; LTD）が発生し、このＬＴＤ　は運動の学習、記憶の形成に重要であると考えられている。一方、線条体も錐体外路系の運動発現に非常に重要な働きを担っている脳部位であるにもかかわらず、この部位で運動の学習、記憶の可塑性に焦点をあてた研究は見当たらない。そこで著者は、パーキンソン氏病の運動疾患をこのような観点から研究した。（２）パーキンソン氏病を神経変性疾患と考え、ドーパミンの終末部位が豊富な線状体、海馬を含め種々の脳部位の細胞死のメカニズムに焦点を当てた研究を行った。（３）パーキンソン氏病では運動活動性のリズムの振幅が低下するのみならず、サーカディアンリズムそのものも変化する可能性が臨床上指摘されている。そこで、パーキンソン氏病モデル動物の作成をサーカディアンリズムのシナプス可塑性の変化から追究した。

学習、記憶の基礎過程であるシナプス部位の長期増強現象（long-term potentiation; LTP）あるいは　LTDが発生することが、空間認知の学習記憶系に重要な脳部位の１つである海馬で見いだされている。また運動系の学習、記憶に関連して小脳部位でもＬＴＤが見られているが、錐体外路系の学習をつかさどっている線条体においては不明である。そこで著者は、ＬＴＰあるいはＬＴＤが線条体神経でも発生するか否かにつき、線条体からのドーパミンやグルタミン酸遊離を指標として、その遊離にＬＴＰあるいはＬＴＤ現象が見られるか否かにつき調べた。その結果、線状体の一過性の高頻度電気刺激により、ドーパミン遊離が長時間（約２時間）持続することが明らかになった。学習、記憶の基礎過程であるＬＴＰあるいはＬＴＤ現象を、ドーパミン遊離という生化学的手法で世界で初めて捉えることができた（1-1）。ハロペリドールなどの抗精神病薬はその副作用としてパーキンソン氏病症状、動物等ではカタレプシーが出現することが知られている。そこで、このような副作用発生時に線条体のＬＴＰあるいはＬＴＤ形成も障害されているかどうかについて

調べた。錐体外路症状が強く発現するハロペリドール投与は、ＬＴＰ現象を抑制することが明らかとなった。また、線状体の神経変性を引き起こす脳虚血をラットに施すと、その後ドーパミン遊離の可塑的変化がドーパミンの含量などより敏感に障害され易いことが明らかとなった（1-6）。メタンフェタミンを慢性投与し、その後４－７日間休薬し、再びメタンフェタミンを投与すると作用が強力に出現するという逆耐性現象がみられるが、この作用がNO合成阻害薬により拮抗されることを発見した（1-19）。これらの研究からドパミン神経系のシナプス可塑性を調べる有用な方法であると共に、この可塑性に細胞内情報系としてNOが関与していることが明らかとなった。

次に脳虚血による細胞脱落のより詳細なメカニズムを調べるために、インビトロの脳虚血モデルを作成し、CA1の電気的活動性やグルコース取り込みを指標として種々の化合物について検討した。その結果、グルタチオン、メチルコバラミン、シグマ受容体刺激薬などがインビトロ脳虚血に対して保護的に作用することが明らかとなった（1-2.　1-5.　1-14）。さらに、NO合成阻害薬、カルシュウムチャンネル拮抗薬も保護的に作用することが分かった（1-3.　1-7）。これらの結果より、伝達物質、受容体、細胞内情報伝達系などのシナプス伝達ステップの何れにおいても、虚血性神経細胞死はコントロールできうることが明らかとなった。

今までシナップス可塑性を指標としたパーキンソン氏病モデル動物の作成を手掛けてきた。パーキンソン氏病では運動活性のリズムが低下し、夜間の睡眠も低下するといった報告、すなわちパーキンソン氏病がサーカディアンリズムに係わっている可能性が考えられている。ところでサーカディアンリズムは視床下部の視交差上核に起源があり、外界の光入力は体内時計をリセットし、この興奮性光入力は履歴性（可塑性）を有することも明らかとなっている。そこでパーキンソン氏病モデル動物の作成をサーカディアンリズムのシナップス可塑性の関点から追究した。視神経から視交差上核への興奮性入力にシナップス可塑性が生じるか否かについて調べた結果、視神経の高頻度刺激により、時刻依存的に視交差上核のシナップス電位が増大し、この作用は１時間以上持続した（いわゆる長期増強現象）。また、グルタメートやメチルコバラミンを灌流応用しても同様な現象が観察された（1-12.　1-17）。したがって、光同調に長期増強現象が係わっているものと考えられた。

パーキンソン氏病モデル動物はサルにMTPTを投与したり、ラットに6-OHDAやレセルピンを投与し、脳内のカテコールアミン減少させることによって運動失調を引き起こすことを指標としている。そこで、サーカディアンリズムに対するレセルピン投与の影響を検討し、新規なパーキンソン氏病モデル動物を作成することとした。ハムスターの輪回し行動の輪回し数はレセルピン（３mg/kg）の投与により低下し、さらに輪回し開始時刻も不安定となり、前進する例も見られた。ハムスター輪回し行動リズムのレセルピンによる障害は新規なパーキンソン氏病モデル動物になることが判明した。また、レセルピン投与により位相関係が不安定になったのは、光同調のシナップス可塑性がレセルピンによって障害された可能性も考えられた。

メタンフェタミンの飲水投与により明暗条件飼育にも係わらず、ラットはフリーランリズム行動を示した。このフリーランリズムの周期は２４時間成分と長周期（３０時間以上）成分の２成分からなり、実際の行動のアクトグラムはこれらの成分が重層した形で現れた。このようなメタンフェタミン依存性のリズムが出現したラットの視交差上核切片を作成し、神経放電活動を記録した。その結果、行動上はフリーランリズムを示したラット

の視交差上核神経活動リズムはフリーランしていなかった（1-21）。すなわち行動上活動期でも、非活動期でも視交差上核の放電頻度は昼間高く、夜間低かった。しかしながら、放電活動の昼夜差は対照の水飲水群に比較して小さい傾向が認められた。明暗条件にも係わらずメタンフェタミン飲水ラットはフリーランリズムを示すことから、メタンフェタミンが光同調に係わっている可能性が考えられる。メタンフェタミンは光照射によるハムスター輪回し行動リズムの位相前進や位相後退を用量依存的に抑制した（1-15. 1-18）。メタンフェタミンによるこの抑制効果がセロトニン神経を介する可能性があるので、セロトニン神経を破壊した動物を用いた実験を行った。ハムスターにセロトニン神経破壊薬をあらかじめ投与し、セロトニン神経を破壊した動物にメタンフェタミンを投与しても光照射による位相変化を抑制しなかった。また、メタンフェタミンによる光同調抑制作用は5-HT1A受容体拮抗薬のNAN190の併用投与によって減弱した。

次に光照射によるハムスターの視交差上核内FOSタンパク発現に対するメタンフェタミンの作用について調べた。ハムスターの輪回し行動実験の場合と同様に、メタンフェタミンは光照射により視交差上核内に誘発されるFOSタンパクの発現を用量依存的に抑制した。また、このメタンフェタミンによるこの抑制作用も5-HT1A受容体拮抗薬のNAN190の併用投与によって減弱した。光照射によるハムスター輪回し行動リズムの同調、視交差上核内FOSタンパクの発現は何れもメタンフェタミン投与により用量依存的に抑制された。このようにメタンフェタミンが光同調を阻害することが、メタンフェタミン飲水によるフリーランリズム形成を容易にさせている原因の一つであると考えられる。また、メタンフェタミンによる光同調の抑制作用がセロトニン神経破壊や、セロトニン神経の5HT1A受容体拮抗薬により消失したことにより、メタンフェタミンの抑制作用はこの薬物によるセロトニン神経促進作用に基ずくものと考えられた。

明暗条件下に飼育したラットに１日４時間の制限給餌を施行すると、ラットの運動量は夜間に増大するのみならず、昼間の給餌時間前より制限給餌時間帯にかけて運動量が増大した。さらに、翌日絶食を行ってもラットは給餌時間を覚えており、以前の制限給餌時間帯にのみ活動量が増大した。いわゆる時刻予知行動が出現するようになった（1-8. 1-9. 1-10. 1-11. 1-23.）。次にこのリズム形成に対するNMDA受容体の役割を明らかにするために、NMDA受容体拮抗薬のMK801の作用について調べた。MK801は制限給餌の終了時刻に合わせて、毎日一回６日間行った。その後、７日目に薬物投与は行わず絶食条件下に行動量を測定した。その結果、MK801の投与により時刻予知行動の出現は抑制された。しかしながら、MK801は制限給餌時間内の餌の摂取量には全く影響しなかった。したがって、MK801が餌摂食に作用して時刻予知行動を抑制した可能性は否定された。このことは時刻を認知し記憶する神経機構にNMDA受容体の活性化が重要な役割を担っているものと考えられる。次に、MK801投与や老化動物による時刻予知行動の障害は種々の薬物の投与により改善されることが明らかとなった。

以上の研究を通じて、パーキンソン氏病モデルの開発と評価の多面的展開ができたものと考えられる。今後これらのモデル動物を使用してより有用性のある抗パーキンソン氏病の開発に寄与したい。

新規抗パーキンソン病薬 Talipexole の鎮静作用に関する脳波学的検討

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Electroencephalographic Study on Sedative Effect of Talipexole, a Novel Antiparkinsonian Drug, in Rats with Chronic Electrode Implants

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Talipexole (B-HT 920 CL_2) is expected to be a novel antiparkinsonian drug with higher efficacy and lesser gastrointestinal side effects as compared with conventional dopamine receptor agonists. On the other hand, because of D_2- and α_2-autoreceptor stimulation by talipexole, it produces more marked sedative effects in clinical trial and animal behavioral studies. In the present experiments, electroencephalographic (EEG) effects of talipexole were investigated in unanesthetized rats with chronic electrode implants. The changes in the spontaneous EEG were evaluated during dark time with rats which had been housed in a room maintained on a reversed light-dark cycle (lights on from 19 : 00 to 7 : 00) for 7 days. Talipexole at 10 μg/kg, s.c. induced a slight drowsy pattern: high voltage slow waves in the frontal cortex and disappearance of hippocampal theta rhythm. At doses of 32 and 100 μg/kg, s.c., talipexole caused a more marked drowsy pattern in the spontaneous EEG and significant decreases in the sedative score assessed by simultaneous observations of the EEG and behavioral motor activities. On the other hand, bromocriptine at 1 and 10 mg/kg, s.c. caused only a slight and temporal drowsy pattern in the spontaneous EEG without changing the arousal score. Then, the EEG arousal response induced by electrical stimulation of the mesencephalic reticular formation was studied during light time in rats which had been housed in a room maintained on a normal light-dark cycle (lights on from 7 : 00 to 19 : 00). Both talipexole and bromocriptine slightly increased the threshold voltage to induce arousal response; however, the drug-induced changes were not dose-dependent. These results indicate that talipexole exhibits a sedative effect at much lower doses than bromocriptine, and that its sedative effect seems to be relatively weak and quite different from those noted with sleep inducers.

Key words: Talipexole / Antiparkinsonian drug / EEG /(rat).

緒　　言

talipexole (B-HT 920 CL_2) はドイツの Boehringer Ingelheim 社で合成された azepine 誘導体の非麦角系 dopamine (DA) 作用薬である (Fig 1). talipexole は, D_1 受容体にほとんど作用しない選択的 D_2 作用薬で, 初めての非麦角系パーキンソン病治療薬として有用性が期待されている.

DA 作用薬の作用点は, 低用量では感受性の高い synapse 前 D_2 自己受容体 (presynaptic D_2-autoreceptor) に, 高用量では postsynaptic DA 受容体に, それぞれ作用することが知られている. 正常動物では, talipexole は主として D_2 自己受容体に作用し, 用量を増加しても常同行動や運動量の亢進など postsynaptic DA 受容体に対する作用をほとんど示さない (Hinzen *et al.*, 1986). 一方, reserpine で脳内 DA を枯渇させた動物 (Hinzen *et al.*, 1986), 黒質線条体路を 6-OH-DA により破壊した動物 (Hinzen *et al.*, 1986) あるいは D_1 作用薬との併用時 (Hjorth and Carlsson, 1987) では, talipexole は postsynaptic D_2 受容体に作用を示し, 黒質 DA 神経細胞毒, 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) によって誘発されるサルの Parkinsonism に対する改善作用が

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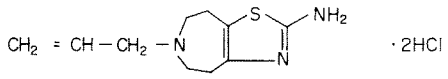

6-allyl-2-amino-5,6,7,8-tetrahydro-4*H*-thiazolo[4,5-*d*] azepine dihydrochloride

FIG 1 Chemical structure of talipexole.
Talipexole is a water-soluble compound. Its molecular weight is 282.24.

報告されている (Hinzen *et al*., 1986; Irifune *et al*., 1993). さらに, talipexole には D_2 agonist 作用の他に α_2 agonist 作用も認められている (Hammer *et al*., 1980).

日本で実施された臨床試験において, talipexole はパーキンソン病に対する優れた有効性とともに, 悪心, 嘔吐, 食欲不振などの消化器系副作用の少ないことが報告されている. 一方, talipexole を投与された患者では, 眠気の発現率が高かった (水野ら, 1992; 柳沢ら, 1992; 中西ら, 1993).

一般に DA 作用薬は, D_2 自己受容体刺激により DA 遊離を抑制するため, 動物の自発運動を減少させ鎮静作用を示す. また, α_2 作用薬は noradrenaline (NA) neurone の α_2 自己受容体刺激による NA 遊離抑制を介して動物を鎮静状態にすることが知られている. 事実, α_2 作用薬である clonidine もヒトで眠気を起こすことが知られている (Michocki, 1992).

以上のことから, talipexole は D_2 自己受容体や α_2 自己受容体に作用して鎮静作用を惹起すると推察されるが, 中枢の睡眠—覚醒系に対するより直接的な検討はなされていない.

そこで, 著者らは talipexole による眠気の特徴を把握するため, 同種のパーキンソン病治療薬 bromocriptine と脳波学的な比較を行った.

脳波の測定は, 動物の行動観察も並行してできるように慢性植え込み電極法によって無麻酔の状態で実施した. 自発脳波および中脳網様体電気刺激による覚醒反応について薬理作用を検討した.

実験方法

1. 動物と飼育条件

体重 250～350 g の雄性 Wistar 系ラット (クロダ実験動物より購入) を用いた. 温度 (23±2°C), 湿度 (60±5%), 明暗サイクル (7:00～19:00 点灯) を一定にして, 飼料 (クレア固型飼料) と水道水は自由摂取で飼育した.

ラットは夜行性動物であり, 特に傾眠を惹起するような薬物の作用を調べるには, ラットが覚醒する夜間に実験を行った方がよいと考えられる. しかし, 夜間実験は研究室の管理などいろいろと問題が多い. そこで, 自発脳波の実験には, 電極植え込み後に昼夜逆転条件 (19:00～7:00 点灯) で 1 週間以上飼育したラットを供した. 実験は部屋を暗くし, 13:00～17:00 に行った.

覚醒反応の実験では, 電気刺激による覚醒反応を指標とするために, 自発脳波は傾眠状態の方が薬物作用の評価に適する. したがって, 脳波覚醒反応の実験は, 正常明暗条件 (7:00～19:00 点灯) で飼育した動物を使用し, 13:00～18:00 に実施した.

2. 脳波測定用電極の植え込み

pentobarbital sodium (30 mg/kg, i.v.) 麻酔下でラットの頭部を東大脳研型脳定位固定装置に固定し, Paxinos and Watson (1982) の脳図譜に従って, ステンレススチール線製の双極電極を慢性的に植え込んだ. 電極植え込み部位は前頭葉皮膚, 背側海馬 (A: −5.2, L: 2.8, H: 2.3) および中脳網様体 (A: −10.8, L: 2.0, H: 8.5) である.

電極は直接 0.2 mm の絶縁ステンレススチール線製双極電極で, 0.5 mm だけ絶縁を除去し, 両極間の距離は 0.5 mm～1.0 mm とした. 脳波の誘導にも電気刺激にも同一の電極を使用した. 皮質脳波誘導用の電極は 1.5 mm ほど絶縁を除去して, 前頭葉 (frontal cortex) の硬膜下皮質表面に密着するように植え込んだ. 各電極は頭蓋骨の穿孔部に歯科用セメントで固定した後, それぞれのコネクターソケットにハンダ付けし, ソケットそのものも, あらかじめ頭蓋骨上にたてたネジ釘とともにセメントで固定し, 電極およびソケットのハンダ付け部分の露出したところは, すべてセメントで被った. 術後 1 週間以上を経過して, 手術創が完全に治癒し, 安定した脳波が記録できるようになった時点で実験を開始した.

3. 脳波の測定と検討項目

1) 自発脳波と自発運動量の測定方法

脳波の測定は, 電極を植え込んだ動物を無麻酔のままで内部を暗くした防音シールドケージ内に入れ, 脳波と自発運動を自動的に計測した. 脳波はポリグラフ (RM-85M 日本光電製) を用いて双極誘導で記録した. 自発運動は, 脳波測定用ケージの真上に置いた焦電型赤外線センサー (オムロン F5B) を用い, ラットの水平, 垂直方向の運動を測定した (Shibata *et al*., 1994).

2) 自発脳波と自発運動による自発脳波 score の取り方

自発脳波と自発運動をもとに, 睡眠—覚醒レベルの指標として自発脳波 score を用いた. すなわち, 脳波上でも自発運動上でも覚醒がみられる場合を+2 点, 脳波上のみ覚醒がみられる場合を+1 点, 脳波上傾眠がみられる場合を 0 点とした. 個々のラットについて, 薬物投与前と投与 150 min 後までの score を 5 min 間隔で記録した. そのうち, 投与後 60 min までの自発脳波 score について個々のラットの平均値を求めた (parametric data に変換). この値を群間で統計的に比較した.

3) 中脳網様体電気刺激による脳波覚醒反応

従来より用いている方法（植木ら，1985）に従って，中脳網様体刺激による脳波覚醒反応閾値の変化を検討した．すなわち脳内電気刺激には，電子管刺激装置（MSE-40 日本光電製）を用い，脳波覚醒反応を測定するための中脳網様体刺激には，100 Hz pulse 幅 0.2 msec の矩形波を用い，刺激時間は 5 sec 間とした．

薬物投与後 180 min まで 30 min 間隔で覚醒反応の発現および持続時間を測定して，薬物無処置時のそれと比較した．薬物投与前の刺激閾値を 100 として，薬物投与後の各覚醒閾値の変化を示した．

実験終了後に，pentobarbital sodium 麻酔下に脳内各部の植え込み電極に 3 mA の直流電流を 10 sec 間加え，1% potassium ferrocyanide 生理食塩液および 10% formalin 生理食塩液にて頭部の血管灌流を行い，脳を摘出固定し，凍結切片を作製して各電極の局在を組織学的に確認した．

4. 使用薬物と被験薬の用量設定について

talipexole (B-HT 920 CL_2, 6-allyl-2-amino-5, 6, 7, 8-tetrahydro-4*H*-thiazolo[4, 5-*d*]azepine dihydrochloride, Boehringer Ingelheim KG), bromocriptine (bromocriptine mesilate, 2-bromo-α-ergocryptine mesilate, Sandoz Pharma, A.G.) は，日本ベーリンガーインゲルハイム株式会社から提供された．使用時に talipexole は生理食塩液に溶解し，bromocriptine は乳酸酸性液で溶解後に NaOH で中和し，いずれもラットに皮下投与した．

talipexole の検討用量（10～100 μg/kg, s.c.）は，脳波や行動上鎮静作用が出現すること，ならびにパーキンソン病モデル動物で 100 μg/kg, s.c. 前後で改善作用が認められることを考慮して設定した．bromocriptine の検討用量（1, 10 mg/kg, s.c.）は抗パーキンソン病作用の認められる薬理用量が talipexole の 10 倍以上であることを考慮して設定した．

5. 統計学的処理

定量的な結果は mean±S.E. で表し，統計学的検定には Dunnett's multiple-comparison test を用いた．定性的結果についてはその旨記述ないしは代表例を図示した．

実験結果

1. 自発脳波に及ぼす影響（昼夜逆転飼育ラットでの検討）

ラットの活動期にあたる暗期において，対照群（生理食塩液投与），talipexole 投与群，bromocriptine 群の自発脳波と行動に及ぼす影響を比較した．

1) 対照群: 防音測定用ケージに入れて脳波を記録すると，しばらくの間は皮質（前頭葉，FC）では低電圧速波，海馬（HC）では 6～8 Hz の同期した高電圧の海馬覚醒波

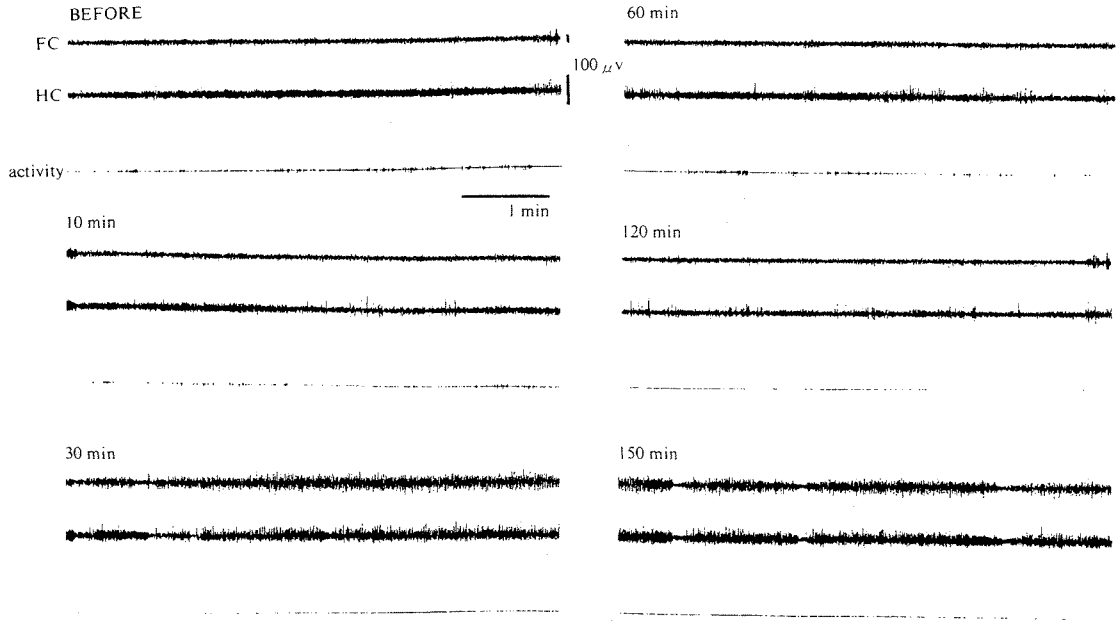

FIG 2 Effect of saline on spontaneous EEG activity in a rat.
All records shown were obtained during dark time from a rat with chronic electrode implants, which had been housed under reversed light-dark condition (19 : 00-7 : 00 light time), then spontaneous EEG were recorded before and 10-150 min after s.c. injection of saline.
FC, Frontal cortex; HC, hippocampus; activity, motor activity indicated by up-down reflection measured by area sensor. The vertical scales, at the right hand bottom in the panel before, indicate 100 μV, and the horizontal scale indicates 1 min. Abbreviations and scales are the same for FIG 2-7.

(theta 波) がみられた．対照群では生理食塩液投与約 10 min 後までは投与前とほとんど同じ脳波が観察された．しかし，30～150 min 後にかけて，皮質では高電圧徐波成分が増加し，海馬では規則正しい theta 波の周期が乱れて不規則となる傾眠パターン (drowzy pattern) が時折出現した．ただし，脳波が傾眠パターンを示している場合でも外部から軽微な刺激を加えると，脳波はただちに覚醒パターン (arousal pattern) に変わった (Fig 2 に代表例を示す)．

2) talipexole 10, 32, 100 μg/kg, s.c.: 投与数 min 後より皮膚と海馬において傾眠パターンが出現した (Fig 3～5 に代表

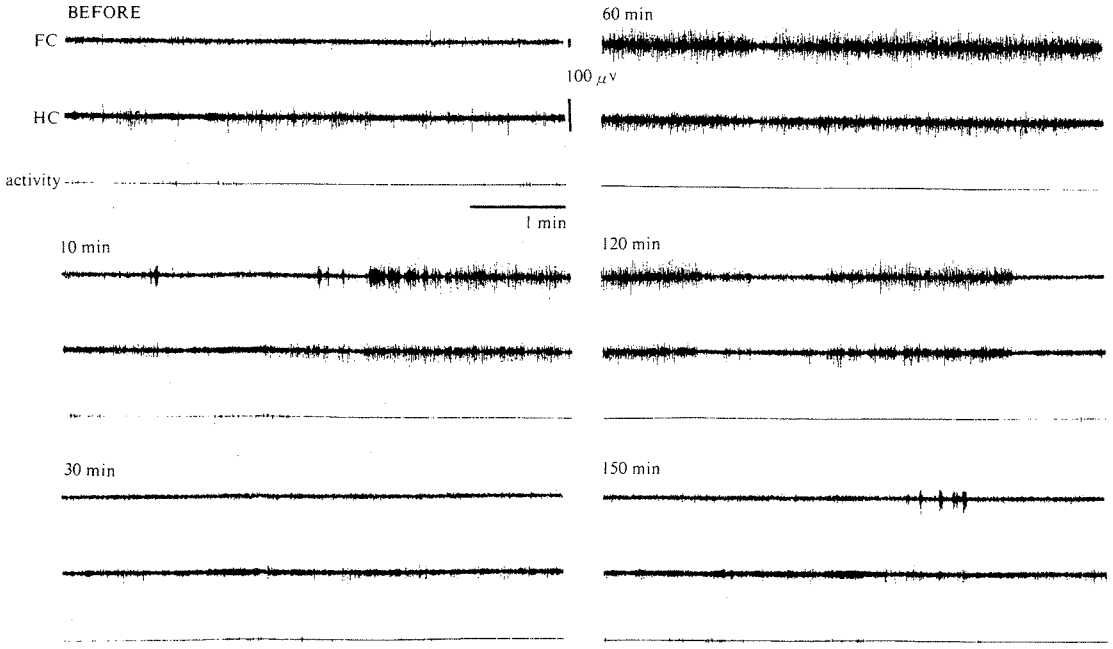

Fig 3 Effect of talipexole (10 μg/kg, s.c.) on spontaneous EEG activity in a rat.
Spontaneous EEG was recorded before (arousal state) and 10-150 min after injection of talipexole at 10 μg/kg, s.c.
Further legends are the same as those described in Fig 2.

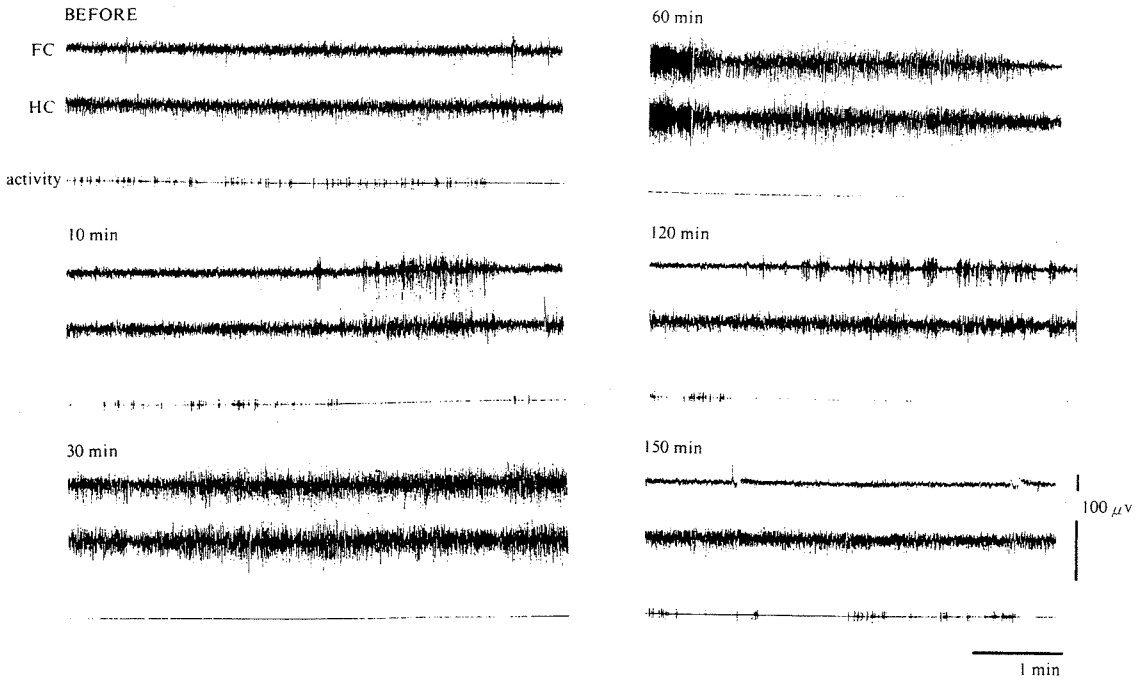

Fig 4 Effect of talipexole (32 μg/kg, s.c.) on spontaneous EEG activity in a rat.
See the legends in Fig 2 and 3, except injection of talipexole at 32 μg/kg, s.c.

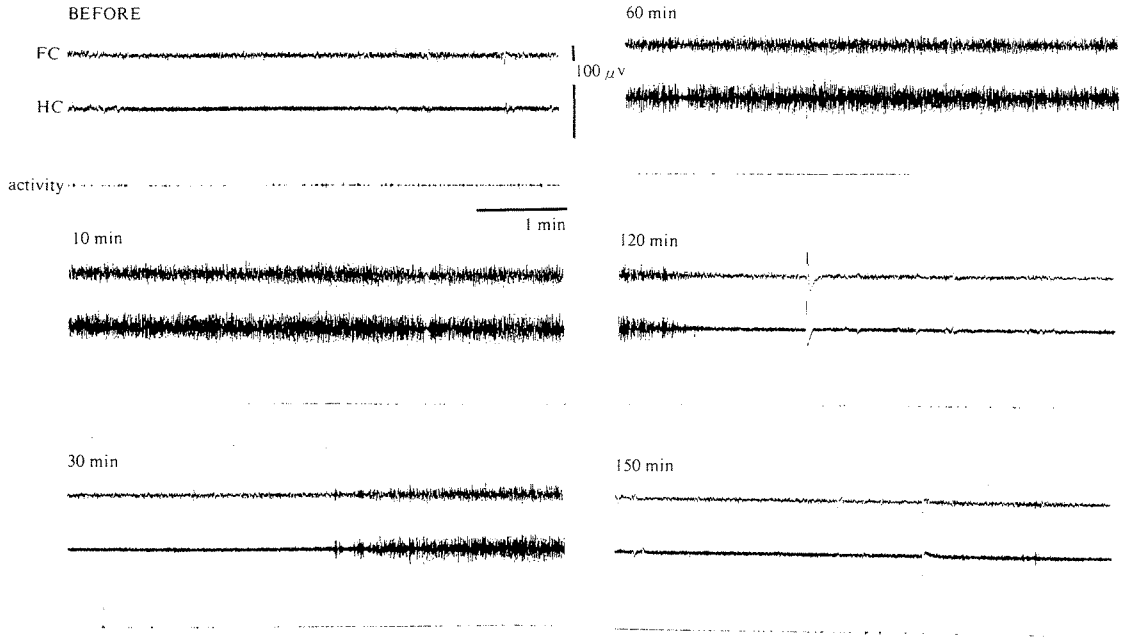

FIG 5 Effect of talipexole (100 μg/kg, s.c.) on spontaneous EEG activity in a rat.
See the legends in FIG 2 and 3, except injection of talipexole at 100 μg/kg, s.c.

例を示す). これらの変化は対照群と比較して 10 μg/kg では大差なく，32 μg/kg 以上で明らかな変化を生じたが，32 μg/kg と 100 μg/kg との間に用量依存的な差は認められなかった．また，脳波の傾眠パターンは，投与 10～60 min 後で最も顕著になり，120 min 後では減弱傾向となり，150 min 後ではほとんど消失した．投与 30～60 min 後において傾眠パターンが顕著に認められる場合でも，途中で時折覚醒パターンが出現した．

3) bromocriptine 1, 10 mg/kg, s.c.: 投与 10～150 min 後にかけて皮質脳波と海馬脳波が幾分傾眠パターンとなる例もみられた (FIG 6 に代表例を示す). しかし，全体的には脳波上の変化は用量依存性も経時的特性もはっきりしない軽度のもの

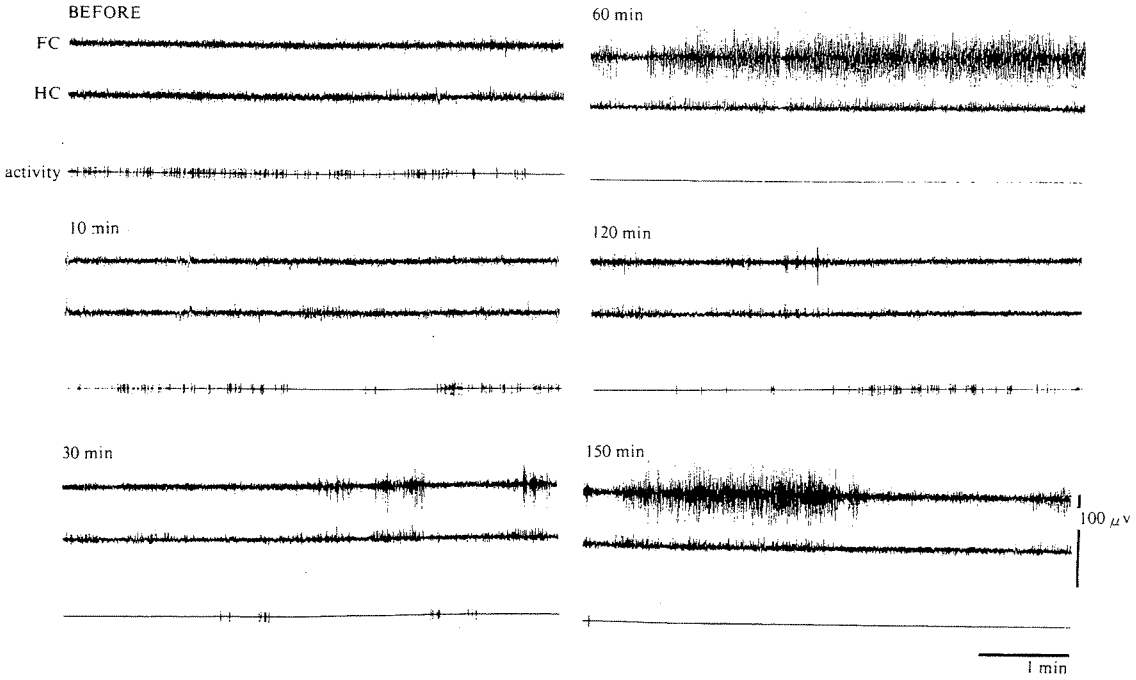

FIG 6 Effect of bromocriptine (10 mg/kg, s.c.) on spontaneous EEG activity in a rat.
See the legends in FIG 2 and 3, except injection of bromocriptine at 10 mg/kg, s.c.

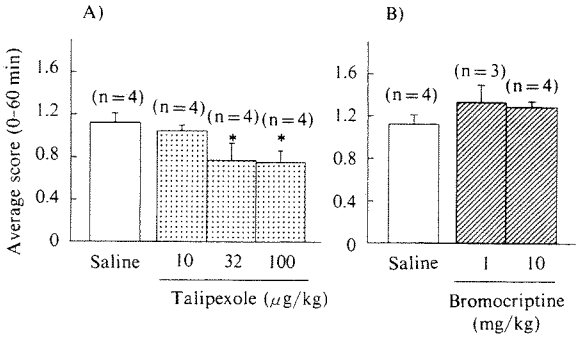

FIG 7 Sedative effects of talipexole (A) and bromocriptine (B) according to sedative score.
Sedative effects were scored by the following rating scale at 5 min intervals for 60 min after drug injections. score +2: rats exhibit arousal state both in behaviorally (motor activity) and EEG observations. score +1: rats exhibit arousal state only in EEG observation. score 0: rats exhibit drowsy pattern in EEG observations.
Values in the figure are mean±SE.
*$P<0.05$, Significant difference from saline-treated animals (Dunnett's multiple-comparison test).

であった.

以上の経時的観察結果から，両薬物の作用特性は投与後 60 min 以内に反映されると考えられた．そこで，両薬物投与 60 min 後までの脳波と自発運動をもとにした自発脳波 score を指標として，両薬物の作用を定量的に比較した．自発脳波 score の 60 min 間の平均値は，talipexole では 32 μg/kg, s.c. 以上で有意に低下したが，bromocriptine では 10 mg/kg, s.c. でも有意の変化はみられなかった (FIG 7A, B).

2. 中脳網様体刺激による脳波覚醒反応閾値に及ぼす影響 (正常明暗条件下飼育ラットでの検討)

1) 対照群: ラットの安静期にあたる明期において，中脳網様体電気刺激によって誘発される脳波覚醒反応に対する talipexole と bromocriptine の作用を比較した．各群 2 匹のラットを用い，薬物投与前に毎回確実に一定の覚醒反応が得られる閾値電圧を選定し，続いて薬物投与 180 min 後まで閾値電圧の変化を測定した．

ラットが行動上安静状態にあり，皮質脳波が高電圧徐波，海馬では規則正しい海馬覚醒波の同期がくずれて不規則となった drowzy pattern を示す時期に，中脳網様体に約 2 V の電気刺激を加えると，皮質の脳波はただちに低電圧速波とな

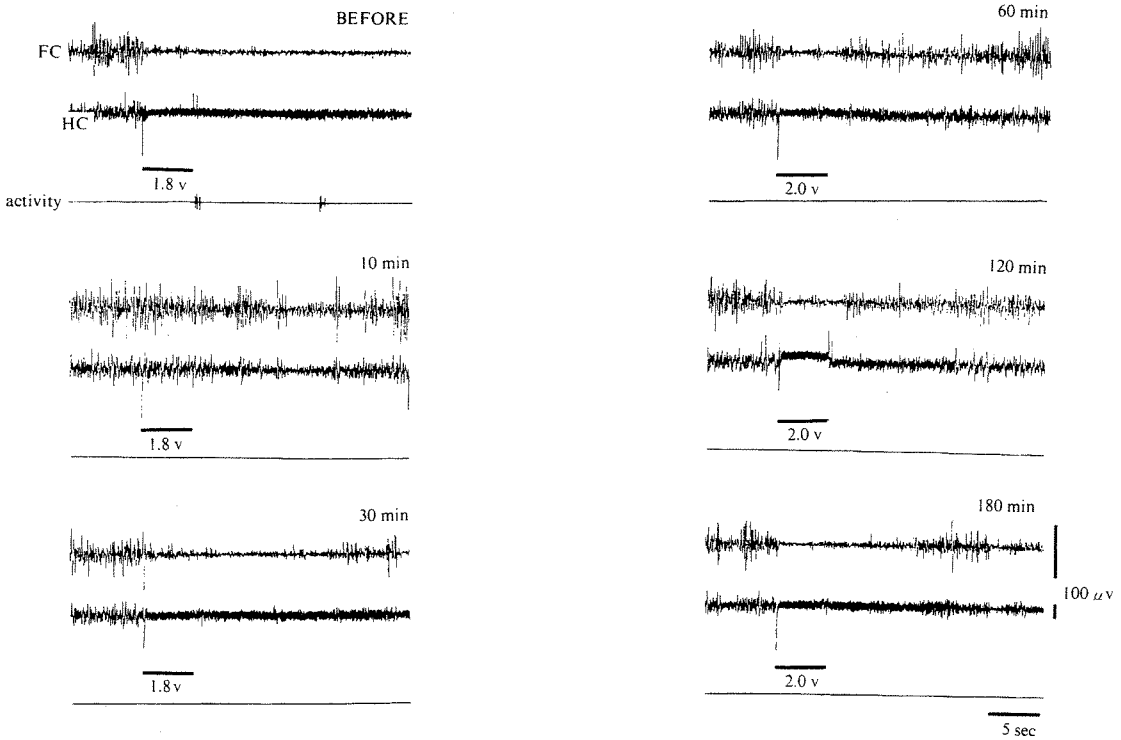

FIG 8 Effect of talipexole (32 μg/kg, s.c.) on EEG arousal response to mesencephalic reticular stimulation in a rat.
All records shown were obtained during light time from a rat with chronic electrode implants, which had been housed under normal light-dark condition (7 : 00-19 : 00 light time).
The mesencephalic reticular formation was stimulated electrically (100 Hz, 0.2 msec duration) for 5 sec, and the threshold voltage inducing arousal response was measured at respective testing time before and 10-180 min after injection of talipexole.

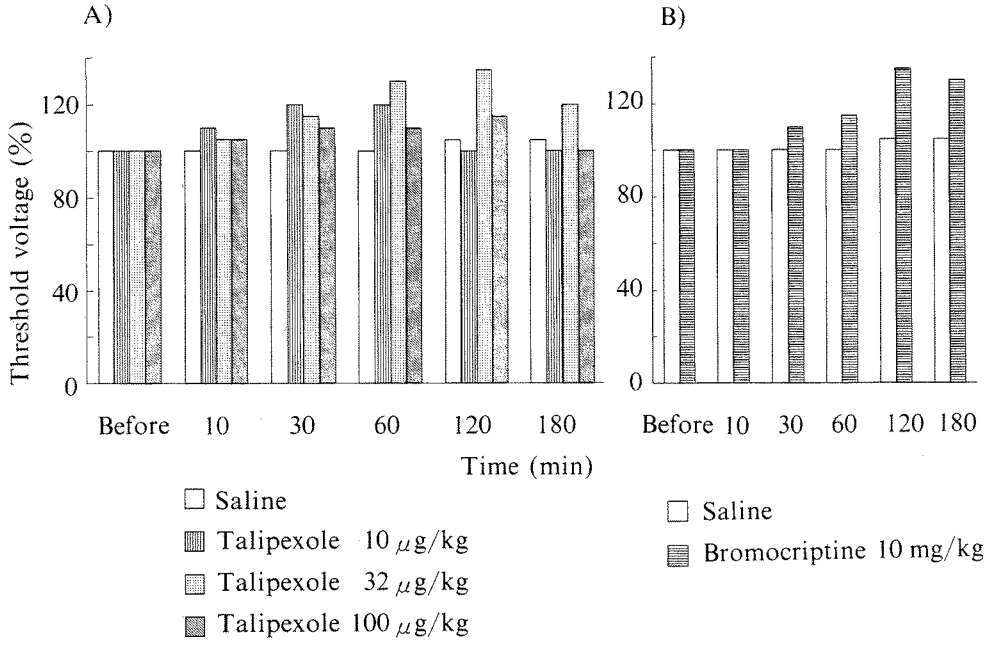

FIG 9 Comparison of the effects of talipexole and bromocriptine on EEG arousal response to stimulation of mesencephalic reticular formation.
Values are shown as the mean of 2 rats. Threshold voltage inducing arousal response was measured as described in the legend in FIG 8, and expressed as percentage of voltage obtained before drug administration.

り，海馬の脳波は同期したtheta波に変わる脳波覚醒反応(EEG arousal response)が誘発された．正常時には，いずれの刺激による覚醒反応も，刺激終了後数min間は持続した(FIG 8, BEFORE).

2) 自発脳波scoreを有意に低下させたtalipexole 32 μg/kg, s.c.の刺激閾値は投与前1.8 Vであったのに対し，薬物投与後に2.0 Vに上昇する例があった (FIG 8).

3) 上記のtalipexoleと同程度の閾値電圧の変動は，自発脳波scoreを変化させないbromocriptine 10 mg/kg, s.c.投与後にも認められた．

以上より，薬物投与180 min後まで平均閾値電圧の推移は，talipexole 10, 32, 100 μg/kgで明確な用量依存性は認められず，閾値の上昇範囲もbromocriptine 10 mg/kg投与後と同様であった (FIG 9).

考　　察

ラットを昼夜逆転条件で飼育した後，活動期に自発運動と脳波を同時に測定し，自発脳波scoreを指標にtalipexoleとbromocriptineの鎮静作用強度を調べた．

生理食塩液を投与した対照群に比較して，自発脳波scoreはtalipexole 10 μg/kg, s.c.では有意な変化を生じず，32～100 μg/kg, s.c.で有意に低下した．しかし，scoreの低下は32～100 μg/kgで同程度であった．皮質脳波，海馬脳波とも，傾眠パターンはtalipexole投与10～60 min後に顕著に出現したが，150 min以内にほとんど消失した．

talipexoleは32～100 μg/kg, s.c.では行動上も脳波上も覚醒レベルを低下させるが，32 μg/kg, s.c.で作用はほぼ頭打ちとなり，途中で覚醒パターンに戻る時間帯が出現するなど，中枢抑制の度合いは比較的軽度であると考えられる．このことは正常明暗条件で飼育したラットの安静期について，中脳網様体電気刺激による脳波覚醒反応を検討した結果からも支持される．

自発脳波scoreを有意に低下させるtalipexole 32～100 μg/kg, s.c.では，刺激閾値が多少上昇する傾向が認められたものの，自発脳波scoreを変化させない10 μg/kg, s.c.と同程度にとどまり，用量依存性も示さなかった．

今回認められたtalipexoleの脳波学的作用は，用量を増すと脳覚醒系を強く抑制する睡眠薬や麻酔薬の作用とは全く異なるものであった．

bromocriptine 1, 10 mg/kg, s.c.では，自発脳波scoreにも中脳網様体の刺激による覚醒反応閾値にも明確な影響を及ぼさなかった．

今回の実験を通じ，bromocriptineに比較してtalipexoleは低用量で覚醒レベルを低下させる作用が強いことが示された．これらの結果は臨床試験におけるtalipexoleとbromocriptineの眠気の発現率の差 (水野ら，1992; 柳沢ら，1992; 中西ら，1993) を説明するものと考えられる．

talipexole投与によって覚醒レベルが低下する機序につい

ては，主として本薬の D_2 作用 (Hinzen *et al.*, 1986) と α_2 作用 (Hammer *et al.*, 1980) が関与していると考えられる．D_2 作用薬である talipexole は側坐核の DA 神経の自己受容体に作用することにより自発運動量を減少させる (廣中直行，社内資料)．また，α_2 作用薬は脳内 NA 神経の起始核である青斑核部の α_2 自己受容体を刺激して神経発火を減少させ，広汎な脳部位の NA 神経活動を低下させることが知られている．talipexole もラットにおける青斑核の神経発火抑制作用が認められている (Seutin *et al.*, 1990)．脳内の覚醒レベルは，多くの神経系や神経活性物質によって調節されているが，脳 NA 神経機能の活性化は覚醒をもたらす．したがって，今回の実験結果と併せると，talipexole の D_2 自己受容体刺激作用に，α_2 自己受容体刺激作用を介する NA 神経機能抑制が加わることにより，被刺激性が保持される範囲内で覚醒レベルを軽度に低下させると推察される．

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Abbreviations B-HT 920 CL_2, talipexole; DA, dopamine; MPTP, 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine; NA, noradrenaline; Bromocriptine, bromocriptine mesilate; EEG, electroencephalographic; FC, frontal cortex; HC, hippocampus.

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N^G-Nitro-L-Arginine Methyl Ester Attenuates the Maintenance and Expression of Methamphetamine-Induced Behavioral Sensitization and Enhancement of Striatal Dopamine Release

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ABSTRACT

We examined the roles of nitric oxide (NO) in methamphetamine (MAP)-induced behavioral sensitization and enhancement of striatal dopamine (DA) release using both *in vivo* and *in vitro* methods. Repeated administration of MAP produced augmentation of MAP-induced locomotor activity after 3-day withdrawal of MAP and an enhancement of MAP-evoked DA release from striatal slices after 6-day withdrawal. When the NO synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) was administered only during the period of MAP withdrawal, the behavioral sensitization and enhancement of DA release were attenuated significantly. In contrast, N^G-nitro-D-arginine methyl ester, an inactive isomer of L-NAME, exhibited no such effect. When L-NAME was administered acutely before the challenge injection of MAP, behavioral sensitization was also attenuated only when the dose of L-NAME was high. Coadministration of L-NAME with MAP did not block the development of sensitization to MAP. We also examined whether MAP-induced behavioral sensitization and enhancement of DA release could be observed *in vivo* in a microdialysis experiment. Challenge injection of MAP caused marked enhancement of DA release in MAP-sensitized rats compared with saline-treated controls corresponding to robust augmentation of locomotor activity. When L-NAME was injected during the MAP withdrawal period, the enhancement of DA release and locomotor activity induced by challenge injection of MAP were attenuated. These results suggest that NO production plays a role in the maintenance (expression) of MAP-induced behavioral sensitization and enhancement of DA release but not in the development of these effects.

Repeated administration of psychostimulant drugs such as AMP, MAP and cocaine produces a behavioral sensitization referred to as augmentation of locomotor activity and stereotyped behavior (Hirabayashi and Alam, 1981; Kuczemski and Segal, 1989; Roy *et al.*, 1978; Tilson and Rech, 1973), even after their long-term withdrawal. Biochemical studies have shown that activation of nigrostriatal or mesoaccumbal DA systems accompanies enhancement of DA release in both the striatum (Castaneda *et al.*, 1988; Kolta *et al.*, 1985; Patrick *et al.*, 1991) and NAc (Robinson *et al.*, 1988; Wolf *et al.*, 1993).

LTP in the hippocampus and LTD in the cerebellum are well-established models of synaptic plasticity. It is well known that LTP requires the activation of EAA receptors, especially NMDA receptor, for its induction but not for its expression, because the NMDA receptor antagonist MK801 markedly blocks the induction phase of LTP (Cocan *et al.*, 1987; Swartzwelder *et al.*, 1989). Moreover, it has been postulated that LTD in the striatum is required for the activation of glutamate- and DA (D_1 and D_2) receptors (Calabresi *et al.*, 1992a, 1992b), and we demonstrated that LTE of striatal DA release required activation of NMDA receptors (Ochi *et al.*, 1994). Therefore, corticostriatal glutamate- and nigrostriatal DA neurons are thought to interact closely and exhibited LTD or LTE.

Similar to LTP, LTD and LTE, the development of behavioral sensitization has been reported to be blocked not only by D_1 and D_2 antagonists (Hamamura *et al.*, 1991; Ujike *et al.*, 1989) but also by NMDA receptor antagonists (Pudiak and Bozarth, 1993; Wolf and Khansa, 1991; Wolf *et al.*, 1994) and AMPA receptor antagonists (Karlar *et al.*, 1991). Thus, the mechanism of sensitization may resemble that of striatal LTD or LTE in that both require the activation of DA and/or EAA receptors.

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ABBREVIATIONS: AMP, amphetamine; NO, nitric oxide; MAP, methamphetamine; DA, dopamine; NOS, nitric oxide synthase; L-NAME, N^G-nitro-L-arginine methyl ester; D-NAME, N^G-nitro-D-arginine methyl ester; NAc, nucleus accumbens; LTE, long-term elevation; LTP, long-term potentiation; LTD, long-term depression; EAA, amino acid; MK801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo-[*a,d*]cycloheptan-5,10-imine hydrogen maleate; NMDA, N-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; SAL, saline; HPLC, high-performance liquid chromatography; ECD, electrochemical detector; i.p., intraperitoneally; L-NA, N-nitro-L-arginine.

It has been reported that the production of NO may be involved in both the maintenance and expression of LTP in the hippocampus (Haley *et al.*, 1992; Iga *et al.*, 1993; Mizutani *et al.*, 1993). These reports support the idea that NO production may be responsible for both the maintenance and the expression of sensitization induced by repeated administration of psychostimulants. Recently, it was demonstrated that the development of cocaine-induced behavioral sensitization was blocked by coadministration of the NOS inhibitor L-NAME (Pudiak and Bozarth, 1993). Contrary to this report, coadministration of L-NAME with AMP failed to block the development of AMP-induced behavioral sensitization as well as enhancement of DA release in the NAc determined using a microdialysis method (Stewart *et al.*, 1994). These reports suggest that NO production is involved in the development of cocaine- but not AMP-induced sensitization. However, the role of NO in the maintenance or expression of MAP-induced sensitization has not been demonstrated. Therefore, in this study, we examined whether L-NAME attenuates the development and/or maintenance (expression) of MAP-induced enhancement of DA release from the striatum.

In the first experiment, we investigated whether repeated administration of MAP caused behavioral sensitization expressed as an increase in locomotor activity after 3-day withdrawal of MAP, and then we examined whether MAP perfusion produced enhancement of DA release from striatal slices after 6-day withdrawal of MAP. In the second experiment, L-NAME was administered during the withdrawal period or acutely injected before the challenge injection. Furthermore, we confirmed *in vitro* results in an *in vivo* microdialysis study. In the last experiment, we examined the effects on behavioral sensitization of coadministration of L-NAME with repeated injection of MAP.

Methods

Animals. Adult Wistar male 12- to 16-week-old rats (Seiwa, Animal Co., Fukuoka, Japan) were used in this study. They were housed under a 12-hr light/dark cycle, and experiments were performed between 1:00 and 5:00 P.M. in the light phase of the cycle to avoid any circadian effects on locomotor activity and/or DA release.

Drugs. L-NAME (Funakosi, Tokyo, Japan), D-NAME (Funakosi, Tokyo, Japan) and MAP hydrochloride (Dainippon, Japan) were used. All chemicals were dissolved in physiological SAL for *in vivo* experiments and in distilled water for *in vitro* analysis.

Drug treatment regimen. All rats were assigned to the following groups (table 1): group A, repeated SAL or repeated MAP; group B, repeated SAL or MAP and subsequent injection of L-NAME or D-NAME during withdrawal period; group C, repeated SAL or MAP followed by acute injection of L-NAME or D-NAME before challenge injection; group D, coadministration of L-NAME with SAL or MAP; or group E, repeated SAL or MAP and subsequent injection of L-NAME during withdrawal period.

TABLE 1
Experimental schedule

Treatment	Repeated administration (days 1–6)	Withdrawal (days 7–9)	MAP challenge[a]	
			Day 10	Day 13
A	SAL or MAP	SAL	MAP	MAP
B	SAL or MAP	NAME	MAP	MAP
C	SAL or MAP	SAL	NAME/MAP	No test
D	NAME/SAL or MAP	SAL	MAP	No test
E	SAL or MAP	NAME	MAP	No test

[a] MAP challenge was performed on day 10 *in vivo* and on day 13 *in vitro*.

Rats received SAL (groups A, B and C) or MAP (1 mg/kg i.p.) (groups A, B, C and E) once daily for 6 days. On day 10 (3-day withdrawal), rats were challenged with MAP (0.5 mg/kg i.p.) (groups A, B, D and E) or L-NAME and MAP (group C). In groups B and E, rats received injection of L-NAME or D-NAME only during the withdrawal period (3 days) after chronic administration of MAP. MAP challenge on day 10 was performed at least 24 hr after the last injection to avoid the acute effects of this drug. In group C, L-NAME or D-NAME was administered 30 min before challenge injection of MAP. In group D, L-NAME (75 mg/kg i.p.) was administered 30 min before daily SAL or MAP injection. In group E, rats were used for microdialysis study at day 10.

Motor activity measurements. Activity counts (number of horizontal and vertical movements such as locomotion and rearing) were determined using an area sensor (Omuron, F5B, Tokyo, Japan) as described in our previous report (Shibata *et al.*, 1994). All animals were injected in their home cages on days 1 through 6. On days 8 and 9, rats were placed in the recording chamber for 80 min of habituation time. Locomotor activity was recorded for 80 min after injection of MAP and printed out on an Intelligence Printer (Muromachi Kikai, Tokyo, Japan).

MAP-evoked DA release from striatal slices. Three days after challenge injection of MAP, rats were decapitated, and the brains were rapidly removed into ice-cold Krebs-Ringer's solution (118.0 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 25.0 mM $NaHCO_3$ and 11.0 mM D-glucose), pH 7.4, equilibrated with a 95% O_2/5% CO_2 gas mixture. Brains were then cut sagittally into slices 450-μm thick with a Sorvall tissue slicer. The striatal part, including the white matter between the neocortex and neostriatum of the slices, was dissected in ice-cold Krebs-Ringer's solution. In this way, we could obtain 8 to 10 slices from each rat. After a 1-hr preincubation period, striatal slices were placed on a dish in 1 ml of MAP-containing solution and incubated for 5 min at 37°C. At the end of the incubation in MAP-containing solution, the solution was transferred immediately to a sample cup containing 100 μl of 0.5 M $HClO_4$ for quantification of DA release. The striatal tissue containing DA was extracted with 200 μl of 0.5 M HClO4 containing 0.1% Na_2S_2O5 and 0.1% EDTA through sonication in sample cups on ice. After centrifugation at 10,000 $\times$ *g* for 10 min at 4°C, supernatants of both the incubation and sonicated solutions were collected for measurement of DA content. DA content was determined with an HPLC-ECD system, including a reverse-phase HPLC column (ODS1181, 3 μm, 6.0 mm $\times$ 250 mm Erma, Tokyo, Japan) and ECD (E-100, Eicom, Kyoto, Japan). The electrode potential was set at +0.65 V against the Ag/AgCl reference electrode. The mobile phase consisted of 0.5 g/l heptane sulfonic acid (Tokyo Kasei Inc., Tokyo, Japan), 1 mM EDTA (Sigma) and 8% acetonitrile (Hayashi Pure Chemical, Osaka, Japan) and was adjusted to pH 3.0 with orthophosphoric acid (Merck). The flow rate was 1.0 ml/min. Data were analyzed with a chromatographic integrator (model 730, Waters). Percent release was calculated using the values obtained from the DA content in incubation medium (*A*) and the DA content remaining in the tissue (*B*). Percent release of DA was calculated with the following equation: $100 \times A/(A + B)$.

Striatal tissue was pooled for the MAP-induced DA release *in vitro*, and the results were averaged across all SAL- or MAP-treated slices. MAP-induced DA release *in vivo* was determined by microdialysis for each individual rat.

Microdialysis study. For the surgical procedure, rats were anesthetized with sodium pentobarbital (35 mg/kg i.p.) and mounted in a stereotactic frame. A burr hole was drilled in the skull, and a guide cannula was placed in the striatum (A 0.7 mm, L 2.6 mm, H 3.2 mm from skull surface) following the brain atlas of Paxinos and Watson (1982). After a 2-day recovery period, rats were injected with SAL, MAP or L-NAME according to the drug treatment regimen. For the dialysis procedure, microdialysis probes extended 3 mm beyond the

guide cannula. Probes were perfused at 2 μl/min with Krebs-Ringer's solution (147 mM NaCl, 2.3 mM $CaCl_2$, 4.0 mM KCl, pH 7.4). Samples collected over periods of 20 min (40 μl) were injected immediately into an HPLC-ECD system (Eicom). The ECD electrode potential was set at 0.60 V against the Ag/AgCl reference electrode. The mobile phase consisted of 7.3 g/l citric acid, 4.0 g/l sodium acetate, 130 mg/l octane sulfonic acid, 5.0 mg/l EDTA and 15% MeOH, and flow rate was set at 1.0 ml/min. In addition, a microdialysis study was performed with simultaneous measurement of the locomotor activity using the area sensor.

Data analysis. Data were analyzed with the use of one- or two-way analysis of variance, followed by *t* test with Bonferroni's correction for each comparison.

Results

Enhancement of DA release from striatal slices in MAP-sensitized rats. Rats were injected daily with SAL or MAP (1 mg/kg i.p.) for 6 days. On day 13 (6-day withdrawal), rats were killed and MAP-evoked (10^{-4} to 10^{-7} M) striatal DA release was measured (table 1, group A). The number of slices used in this experiment ranged from 12 to 35 corresponding to 4 to 11 rats. MAP revealed significant enhancement of DA release in a concentration-dependent manner [$F(3,141) = 96.0$, $P < .001$]. Statistical significance was determined with two-way analysis of variance [$F(3,141) = 3.1$, $P < .05$], and post-hoc analysis showed significant enhancement of DA release between SAL-treated and MAP-sensitized rats at both 10^{-5} M ($P < .001$, *t* test) and 10^{-6} M ($P < .05$, *t* test) (fig. 1). Therefore, the concentration of MAP was fixed at 10^{-5} M in the following experiments.

Effects of L-NAME on maintenance of sensitization. There were significant differences between groups [$(F(7,37)=7.4$, $P < .001$], and a significantly greater increase in locomotor activity in response to MAP (0.5 mg/kg i.p.) challenge at day 10 was observed in MAP-sensitized rats compared with those treated with SAL ($P < .001$, *t* test) (table 1, group A). L-NAME (1–75 mg/kg) was administered during the 3-day withdrawal after 6 days of injection of MAP (table 1, group B). Repeated administration of L-NAME significantly attenuated the maintenance of MAP-induced sensitization of locomotor activity ($P < .05$; fig. 2A), whereas D-NAME had no such effect ($P > .05$, *t* test). The rats treated with SAL for 6 days and subsequent injection of L-NAME for 3 days did not show alteration in their basal response to MAP challenge 24 hr after the last L-NAME injection.

There were significant differences between groups [$F(7,106) = 5.9$, $P < .001$], and enhancement of DA release by MAP (10^{-5} M) perfusion was observed at day 13 between SAL-treated and MAP-sensitized rats ($P < .001$, *t* test) (table

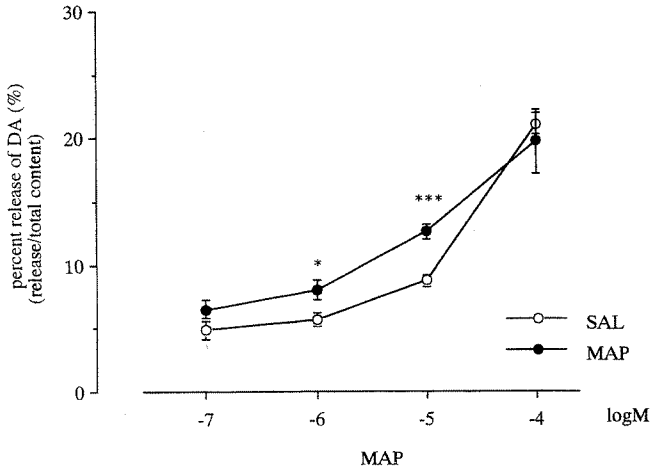

Fig. 1. Concentration-response curve of MAP-evoked DA release from striatal slices in MAP-sensitized rats. Rats were injected daily with SAL or MAP for 6 days. On day 13, rats were killed, and MAP-evoked striatal DA release was measured. The *Y* axis indicates the percentage of release of DA evoked by MAP perfusion. Data points indicate mean ± S.E. Data were analyzed by two-way analysis of variance followed by *t* test with Bonferroni's correction for comparison of each concentration. Points are from 14 to 35 slices for SAL ($n = 4$–11) and 12 to 20 slices for MAP ($n = 4$–6). ***$P < .001$, *$P < .05$ *vs.* SAL-treated group (*t* test).

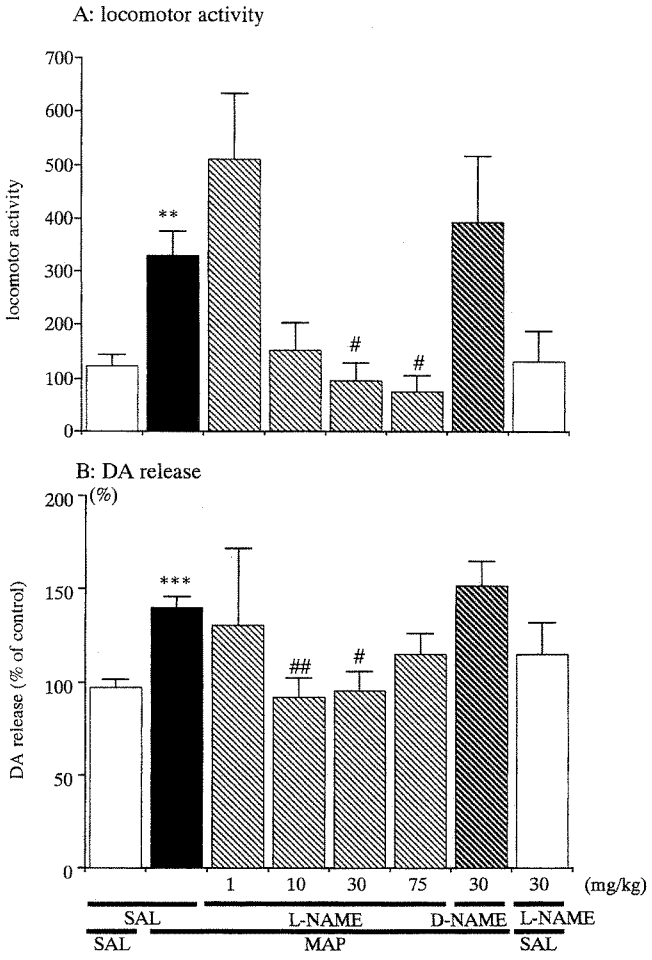

Fig. 2. Effects of L-NAME treatment during MAP withdrawal on challenge injection of MAP-stimulated locomotor activity (A) and challenge perfusion of MAP-evoked DA release from striatal slices (B). Rats were injected daily with SAL or MAP for 6 days. SAL, L-NAME or D-NAME was administered on days 7 through 9. On day 10, MAP-stimulated locomotor activity was observed. On day 13, rats were killed, and MAP-evoked striatal DA release was measured. The data are shown as mean ± S.E. A, Locomotor activity was measured for 80 min. Columns are from SAL/SAL ($n = 13$), MAP/SAL($n = 8$), MAP/L-NAME ($n = 4$ for each dose), MAP/D-NAME ($n = 4$) and SAL/L-NAME ($n = 4$). B, MAP-evoked (10^{-5} M) DA release from control striatal slices in figure 1 was regarded as 100%. The number of slices used in this experiment ranged from 8 to 42 corresponding to 4 to 13 rats. The data were analyzed by one-way analysis of variance followed by *t* test with Bonferroni's correction. ***$P < .001$, **$P < .01$ *vs.* SAL/SAL group (*t* test). ##$P < .01$, #$P < .05$ *vs.* MAP/SAL group (*t* test).

1, group A). In L-NAME-treated rats, the enhancement of DA release by MAP at day 13 was abolished ($P < .01$ for L-NAME 10 mg/kg, $P < .05$ for L-NAME 30 mg/kg, t test; fig. 2B) (table 1, group B). In contrast, D-NAME had no inhibitory effect against MAP-evoked enhancement of DA release. After repeated SAL administration for 6 days followed by repeated L-NAME injection for 3 days, MAP challenge was applied *in vivo* at day 10 and *in vitro* at day 13. L-NAME did not affect MAP-induced locomotor activity or MAP-evoked (10^{-5} M) DA release.

Microdialysis study. *In vivo* DA release was measured using microdialysis in freely moving rats. In this study, we measured locomotor activity simultaneously. Challenge injection of MAP (0.5 mg/kg i.p.) induced a marked increase in DA efflux in MAP-sensitized rats compared with SAL-treated controls. This enhancement lasted for ≥80 min and returned to base line between 160 and 200 min after injection. The dose of L-NAME was set at 30 mg/kg because this dose showed an adequate inhibitory effect on MAP-sensitization, as shown in figure 2. Statistically significant differences were observed by using two-way analysis of variance for both locomotor activity [between groups, $F(2,8) = 30.1$, $P < .001$; between time points, $F(5,40) = 25.5$, $P < .01$; time × group interaction, $F(10,40) = 9.7$, $P < .01$] (fig. 3A, left) and DA release [between groups, $F(2,8) = 22.0$, $P < .001$; between time points, $F(5,40) = 34.3$, $P < .001$; time × group interaction, $F(10,40) = 2.5$, $P < .05$] (fig. 3B, left). Significant differences in locomotor activity at each time point were observed at 40 to 80 min after challenge injection ($P < .001$ for 40 min, $P < .01$ for 60 and 80 min, t test) and DA release at 40 and 80 min ($P < .05$, t test).

When these data were analyzed for total locomotor activity and total DA release for 80 min, significant differences were observed in both locomotor activity [$F(2,8) = 30.1$, $P < .001$] (fig. 3A, right) and DA release [$F(2,8) = 22.0$, $P < .001$) (fig. 3B, right), and post-hoc analysis showed significant differences between SAL-treated and MAP-sensitized rats ($P < .001$, t test), and between MAP and MAP/L-NAME-treated rats ($P < .001$, $P < .01$, t test).

Effects of L-NAME on expression of sensitization. L-NAME and D-NAME were administered 30 min before challenge injection of MAP (table 1, group C). Statistically significant differences were observed using one-way analysis of variance [$F(7,22) = 6.2$, $P < .001$]. Acute injection of L-NAME produced a dose-dependent suppression of the MAP-induced increase in locomotion (fig. 4). Although behavioral sensitization completely disappeared after application of L-NAME (75 mg/kg) ($P < .01$, t test), D-NAME (75 mg/kg) failed to inhibit MAP-induced sensitization.

Effect of L-NAME on development of sensitization. Statistically significant differences were observed using one-way analysis of variance [$F(3,18) = 8.7$, $P < .01$]. Challenge injection of MAP (0.5 mg/kg i.p.) showed marked sensitization of locomotor activity in MAP-sensitized rats ($P < .01$, t test) (table 1, group D). L-NAME administered 30 min before each daily MAP injection did not block the development of MAP-induced sensitization of locomotor activity ($P < .05$, t test) (fig. 5). With this treatment schedule, locomotor activity was slightly enhanced by L-NAME, but the difference did not reach the level of significance.

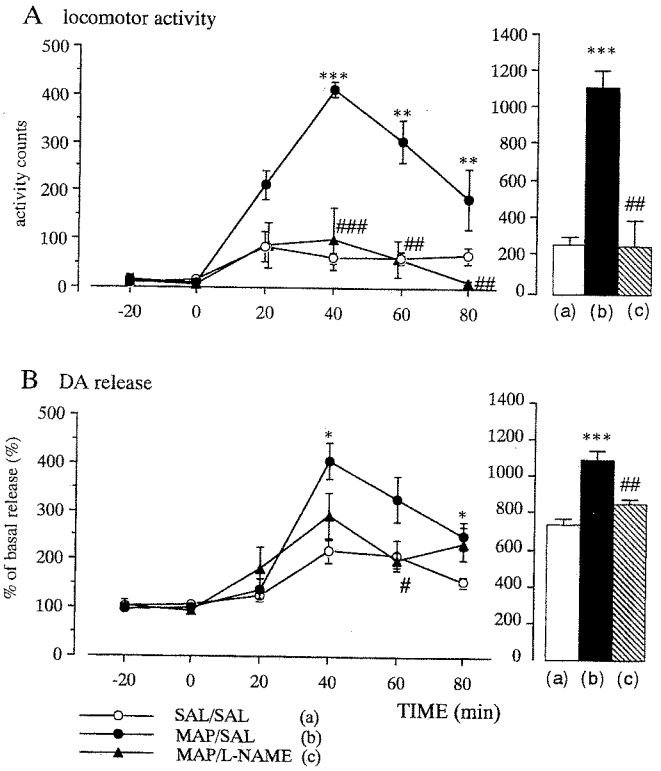

Fig. 3. Effects of L-NAME treatment during MAP withdrawal on challenge injection of MAP-induced (0.5 mg/kg i.p.) locomotor activity (A) and DA release in an *in vivo* microdialysis study (B). Rats were injected daily with SAL or MAP for 6 days. SAL or L-NAME was administered on days 7 through 9. On day 10, MAP-stimulated locomotor activity, and MAP-evoked striatal DA release was observed. The number of animals was 4 for SAL/SAL, 4 for MAP/SAL and 3 for MAP/L-NAME groups. A, Time course of locomotor activity counts induced by MAP challenge (left) and total activity counts for 80 min induced by MAP challenge (right). All rats received MAP at time 0, and locomotor activity counts were measured for 80 min. B, Time course of striatal DA release induced by MAP challenge (left) and total DA release for 80 min induced by MAP challenge (right). Basal DA release for 40 min (2 fractions) before challenge injection were SAL/SAL rats, 106.17 ± 12.18 fmol/40 μl; MAP/SAL rats, 105.87 ± 25.5 fmol/40 μl; and MAP/L-NAME rats, 124.5 ± 16.4 fmol/40 μl. No significant differences in basal DA release were observed between groups; therefore, the basal DA release in each group was set as 100%. The data were analyzed by two-way analysis of variance followed by t test with Bonferroni's correction. ***$P < .001$, **$P < .01$, *$P < .05$ *vs.* SAL/SAL group (t test). ###$P < .001$, ##$P < .01$, #$P < .05$ *vs.* MAP/SAL group (t test).

Discussion

In the present study, we examined the role of NO in MAP-induced sensitization of locomotor activity and striatal DA release. Repeated administration of MAP for 6 days produced augmentation of locomotor activity when rats were challenged with MAP (0.5 mg/kg i.p.) after 3-day withdrawal and enhancement of MAP-induced (10^{-5} M) DA release from striatal slices after 6-day withdrawal. Previous studies demonstrated enhancement of DA release from striatal slices after 3- to 14-day withdrawal (Kolta *et al.*, 1985; Robinson and Becker, 1982), in agreement with our present results. In addition, we demonstrated in a microdialysis experiment that striatal DA release was significantly enhanced, corresponding with increased locomotor activity in MAP-sensitized rats challenged with MAP. Enhancement of *in vivo* DA release by AMP challenge was not observed in NAc after

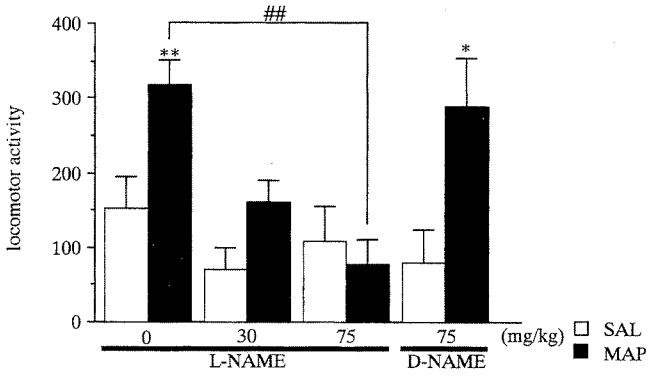

Fig. 4. Effect of acute treatment of L-NAME on MAP-induced sensitization of locomotor activity. Rats were injected daily with SAL or MAP for 6 days. On days 7 through 9, SAL was administered. On day 10, MAP-stimulated locomotor activity was observed 30 min after injections of L-NAME and D-NAME. Columns are from SAL (n = 5), MAP (n = 5), L-NAME (n = 3 for 30 mg/kg, 5 for 75 mg/kg) and D-NAME (n = 4). L-NAME or D-NAME was administered 30 min before MAP challenge. The data were analyzed by one-way analysis of variance followed by t test with Bonferroni's correction. **P < .01, *P < .05 *vs.* SAL-treated group (t test). ##P < .01 *vs.* MAP-treated group (t test).

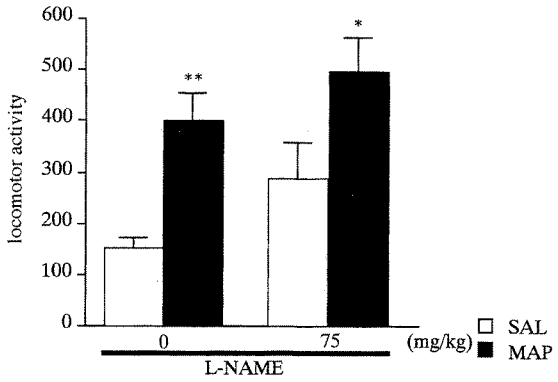

Fig. 5. Effect of L-NAME on the development of MAP sensitization. L-NAME (75 mg/kg) was administered with daily MAP injection for 6 days. On days 7 through 9, SAL was administered. On day 10, MAP-stimulated locomotor activity was observed. Columns are from SAL (n = 6), MAP (n = 8) and L-NAME (n = 4). The data were analyzed by one-way analysis of variance followed by t test with Bonferroni's correction. **P < .01, *P < .05 *vs.* SAL-treated group (t test).

3-day withdrawal, suggesting that the autoreceptor subsensitivity in VTA is predominant in this period (Wolf *et al.*, 1993). This discrepancy may be accounted for by the differences between the two brain regions analyzed (striatum and NAc) and by efficacy differences between AMP and MAP.

When L-NAME, an inhibitor of NOS, was administered during the MAP withdrawal period, the sensitization of locomotor activity and striatal DA release were abolished, whereas D-NAME, an inactive isomer of L-NAME, had no effect. L-NAME administered during the withdrawal period did not alter basal response to MAP challenge. We also observed this effect using an *in vivo* microdialysis method; furthermore, when L-arginine (300 mg/kg i.p.), a precursor of NO, was administered with L-NAME during the MAP withdrawal period, inhibition of sensitization of locomotor activity by L-NAME 75 mg/kg (75 ± 30.3, n = 4) was partially reversed (250.8 ± 56.3, n = 4). Therefore, the effect of L-NAME observed in this study may be related to its inhibition of brain NOS. In the present experiment, the last injection of L-NAME was performed ≥24 hr before the challenge injection of MAP. It was demonstrated previously that single or repeated injection of L-NAME (10 or 75 mg/kg i.p.) greatly reduced NOS activity in various brain regions (Bannerman *et al.*, 1994; Salter *et al.*, 1995), that NOS inhibition by L-NA was still apparent 24 hr after the last injection and that this inhibition was caused by the long biological half-life of L-NA, not by irreversible inhibition of NOS (Tabrizi-Fard and Fung, 1994). Although we do not know whether L-NAME is still present on day 10 for *in vivo* experiments, L-NAME should be gone on day 13 for *in vitro* experiments. Acute injection of L-NAME reduced the response to MAP in both SAL- and MAP-treated rats, and the same results were observed in a previous report (Abekawa *et al.*, 1994). Chronic coadministration of L-NAME with MAP did not block the MAP-induced development of the sensitization, and this result is in agreement with those of previous study (Stewart *et al.*, 1994), although it is unclear at present why repeated administration of L-NAME for 6 days seemed to enhance the locomotor stimulation by MAP challenge on day 10. Thus, the present results indicated that L-NAME blocked the maintenance and expression but not the development of MAP-induced sensitization.

It is unclear at present which trigger chemicals stimulate NOS activation. It is well known that NOS activation is dependent on the elevation of Ca^{2+} concentration in postsynaptic cells and on subsequent activation of the calcium/calmodulin second messenger system. The major pathway of NOS activation is thought to be mediated *via* glutamate release, glutamate receptor activation, Ca^{2+} influx and NOS activation (Bredt and Snyder, 1989, 1992). It is possible that DA efflux stimulates glutamate release *via* a corticostriatal/thalamocortical negative feedback loop (Carlsson and Carlsson, 1990). Therefore, NO production may occur as a result of glutamate release. One possible candidate is the increase of calmodulin content, which is known to activate NOS, after withdrawal of MAP. It has been demonstrated that a low dose of AMP (1 mg/kg) induces calmodulin translocation after repeated AMP administration, and this phenomenon occurred during the drug withdrawal period (Gnegy *et al.*, 1991; Popov and Matthies, 1989; Roberts-Lewis *et al.*, 1986). These reports support the idea that elevation of calmodulin content is induced by MAP withdrawal and that challenge injection induces translocation of calmodulin, although the drug treatment regimen and withdrawal period in the present study was different than those of the above reports.

There have been several reports suggesting interaction between NO production and DA release in the central nervous system. L-Arginine and sodium nitroprusside have been reported to increase the DA release from the medial preoptic area (Lorrain and Hull, 1993) and striatum (Hanbauer *et al.*, 1992; Zhu and Luo, 1992) and inhibit DA uptake (Sakire *et al.*, 1994). Furthermore, it has been reported that NMDA-evoked DA release from striatal slices is dependent on NO production (Hanbauer *et al.*, 1992). An immunohistochemical mapping study revealed that NOS is rich in the striatum, especially in medium spiny neurons but not giant cholinergic neurons (Vincent and Kimura, 1992). This report suggests that NO may be produced postsynaptically in medium spiny neurons and affect the presynaptic dopaminergic nerve terminals as a retrograde messenger.

Several studies have demonstrated that NO plays an important role in maintenance and expression of some kinds of

synaptic plasticity, such as LTP, in the hippocampus (Haley *et al.*, 1992; Iga *et al.*, 1993; Mizutani *et al.*, 1993) and induction of LTD in the cerebellum (Daniel *et al.*, 1993). Recently, it was reported that the timing of NO release is important for LTP because treatment with NMDA before tetanic stimulation, which stimulates NO production, failed to induce hippocampal LTP (Izumi *et al.*, 1992). Therefore, it is suggested that the mechanism of MAP-induced sensitization is similar to that of LTP and that the timing of NO release (NOS activation) may be important for sensitization. The effects of NOS inhibitors on drug-induced synaptic plasticity have been investigated previously. Cocaine, a DA reuptake blocker, induces behavioral sensitization (Angrist., 1983; Segal and Schuckit, 1983). It was demonstrated that coadministration of L-NAME with cocaine blocked the sensitization of locomotor activity (Pudiak and Bozarth, 1993). Moreover, it was reported that L-NAME attenuated the morphine-induced tolerance (Adams *et al.*, 1993; Babey *et al.*, 1994). On the other hand, L-NAME was reported to have no effect on the development of AMP-induced sensitization (Stewart *et al.*, 1994). Therefore, our findings in conjunction with those of these previous studies suggest that NO does not play a role in the development of AMP or MAP sensitization.

In this study, we examined the effects of challenge injection of MAP on locomotor activity and DA release in the rat striatum. Several studies have suggested that there is no association between augmented behavioral responses to AMP and enhancement of DA release in the striatum or NAc (Segal and Kuczenski, 1992; Wolf *et al.*, 1994). In contrast, other reports have revealed a close correlation between these two phenomena under certain conditions in both the striatum (Hamamura *et al.*, 1991; Patrick *et al.*, 1991) and NAc (Robinson *et al.*, 1988; Wolf *et al.*, 1993). In the present study, locomotor activity and enhancement of striatal DA release exhibited a positive correlation in each animal ($r = .713$, $P < .05$).

The striatum is known to play an important role in the control of motor performance in basal ganglia. Recently, it was demonstrated that LTD (Calabresi *et al.*, 1992a, 1992b) and LTE of DA release (Ochi *et al.*, 1995) occur in the striatum. These findings strongly suggested that both DA and EAA receptor activation are required for plasticity of striatal neurons. Thus, synaptic plasticity of striatal DA neurons may be involved in the acquisition of motor learning in basal ganglia.

In conclusion, behavioral sensitization and enhancement of DA release were observed in the present study, and L-NAME attenuated both maintenance and/or expression of sensitization but not its development. These results suggested that NO production may regulate the plastic changes in striatal DA neurons.

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Short communication

Enhancement of dopamine release from the striatum through metabotropic glutamate receptor activation in methamphetamine sensitized rats

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Abstract

An intracerebral microdialysis technique was applied to study the effect of metabotropic glutamate receptor (mGluR) agonist on dopamine release in the striatum of methamphetamine (MAP)-sensitized rats. Rats were treated with MAP (1 mg/kg, i.p.) once daily for 6 consecutive days, followed by a 6-day withdrawal. Perfusion of 0.1 mM (1S,3R)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid through a microdialysis probe placed in the striatum enhanced the extracellular dopamine level, and induced stereotyped behavior in MAP-sensitized rats. The enhancement of dopamine release and the stereotyped behavior were attenuated by co-perfusion of 0.4 mM RS-α-methyl-4-carboxyphenyl-glycine, a mGluR antagonist. The present results suggest that mGluRs may be involved in the expression of MAP-induced sensitization.

Keywords: Methamphetamine sensitization; Expression; Dopamine release; Striatum; Metabotropic glutamate receptor; Microdialysis

Work on the mechanism of behavioral sensitization to amphetamine (AMP) or methamphetamine (MAP) has focused on possible alterations in the dopamine system. The potential role of glutamate receptors has only recently been assessed [12]. Although antagonists of both the *N*-methyl-D-aspartate receptor (NMDAR) and DL-α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) block the behavioral sensitization to amphetamine [11,13,23,36], there are some differences between these receptor mechanisms. NMDAR antagonists can block the induction but not the expression of behavioral sensitization, while AMPAR antagonists can block both [13].

Recently, glutamate receptors (GluRs) have been categorized into two general groups, ionotropic receptors (iGluRs) and metabotropic receptors (mGluRs) [21,31]. It has been reported that not only iGluRs but also mGluRs play important roles in such synaptic plasticities as long-term potentiation (LTP) [1,2,19,20], and long-term depression (LTD) [5,6,17]. However, little is known about the role of mGluR in AMP- and/or MAP-induced sensitization. Therefore, the purpose of the present study was to investigate the role of mGluRs in dopamine release from the striatum in MAP sensitized-rats using a brain microdialysis method.

The animals used in this experiment were male rats of the Wistar strain (Seiwa, Animal Co., Fukuoka, Japan), weighing between 300 and 400 g. The rats were housed under a constant temperature (23 ± 2°C) and a 12-h light/dark cycle (light period: 07.00–19.00 h). The rats were allowed free access to food and water throughout the experiment. The rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.), and were fixed on a stereotaxic instrument. A guide cannula (0.5-mm outer diameter; AG-8, Eicom Co., Kyoto, Japan) was placed just above the striatum, (0.7 mm anterior to the bregma, 2.6 mm lateral to the midline, 3.2 mm ventral to the surface of the skull measured at the bregma), according to the brain atlas of Paxinos and Watson [25].

After a recovery period of at least 2 days, rats were injected with saline (SAL) or MAP (Dainippon Pharmaceuticals Ltd., Japan). SAL or MAP (1.0 mg/kg, i.p.) was administered once daily for 6 consecutive days. Six days

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after the last injection, the rats were used for microdialysis studies.

Brain microdialysis was carried out in unanesthetized, freely moving rats. A concentric dialysis probe (3.0 mm active membrane length and 0.2 mm outer diameter; A-I-8-03, Eicom Co.) was inserted into the striatum through the guide cannula so that the tip of the probe was located at 6.2 mm ventral to the skull surface. During the dialysis experiment, the probe was connected to an infusion pump (EP-60, Eicom Co.) and was perfused with Ringer solution (147 mM NaCl, 4 mM KCl, 2.3 mM $CaCl_2$) at a rate of 2.0 μl/min. Perfusate samples collected at 20-min intervals (40 μl) were injected directly into an HPLC-ECD system for quantification of dopamine. The HPLC-ECD consisted of a pump (EP-10, Eicom Co.) coupled to a reversed-phase column (5 μm, 4.6 × 150 mm; Develosil ODS-HG-5, Nomura Chemical Co., Ltd., Aichi, Japan) and an ECD (ECD-100, Eicom Co.). A graphite working electrode (WE-3G, Eicom Co.) was set at +0.60 V against the Ag/AgCl reference electrode. The mobile phase was composed of 36.8 mM citric acid, 52.6 mM sodium acetate, 0.6 mM sodium 1-octanesulfonate, 14 μM EDTA and 11% methanol. Flow rate was 1.0 ml/min.

Solutions of 0.1 mM (1S,3R)-1-aminocyclopentane-*trans*-1,3-di-carboxylic acid (1S,3R-ACPD; Tocris Cookson Ltd., UK) and 0.4 mM RS-α-methyl-4-carboxyphenylglycine (RS-MCPG; Tocris Cookson Ltd., UK) were made in the Ringer solution. After the basal dopamine level was stabilized (at least 2 h after the probe insertion), drugs were applied directly into the striatum through the microdialysis probe during a 20-min period, and dopamine release was measured for up to 3 h. Data were expressed as a percentage of baseline levels, and significance was analyzed with a two-way repeated measures analysis of variance (ANOVA). The baseline value of the extracellular concentration of dopamine in dialysates from the striatum was 17.7 ± 1.0 pg/20 min (mean ± S.E.M., $n = 24$) in SAL-treated rats. This value was not different from that in MAP-sensitized rats (17.8 ± 0.7 pg/20 min, $n = 27$).

Fig. 1A shows the dopamine level as a function of time after application of 1S,3R-ACPD directly into the striatum in freely moving SAL-treated rat. The dopamine level was significantly higher in 1S,3R-ACPD-treated rats than in vehicle-treated animals (time course 0–180 min × 1S,3R-ACPD or vehicle, $F(9,45) = 2.96$, $P < 0.01$). Fig. 1B shows the effect of 1S,3R-ACPD on the dopamine level in MAP-sensitized rats. Application of 1S,3R-ACPD produced a statistically significantly greater increase in dopamine level in MAP-sensitized rats than in SAL-treated rats (time course 0–180 min × SAL or MAP, $F(9,63) = 2.17$, $P < 0.05$). Interestingly, when 1S,3R-ACPD was applied into the striatum, the MAP-sensitized rats exhibited stereotyped behavior such as grooming, chewing and/or face washing. Fig. 1B also shows the time course of the dopamine level after application of either RS-MCPG or a 1S,3R-ACPD-RS-MCPG mixture into the striatum in

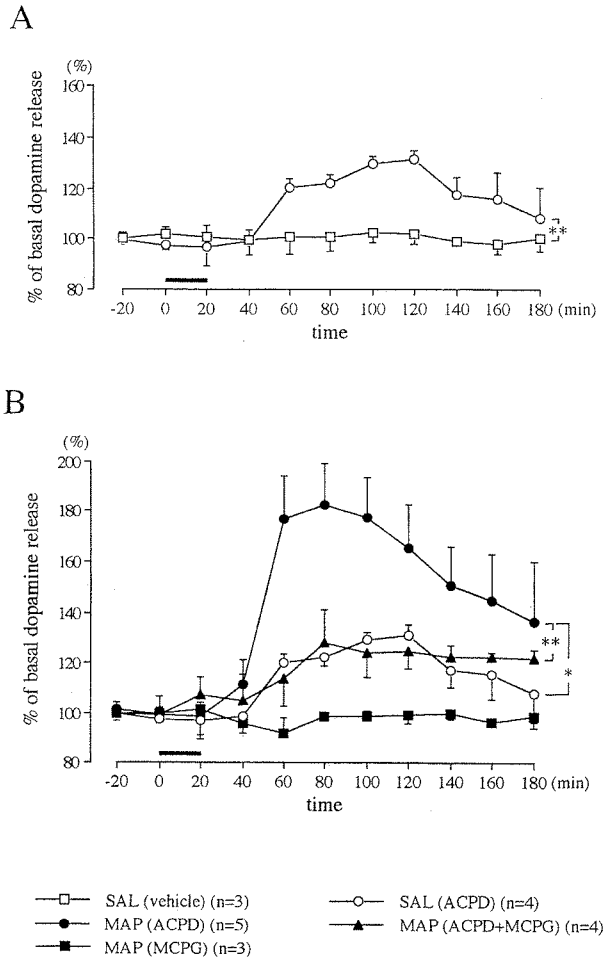

Fig. 1. Time course of dopamine release from the striatum after perfusion of 1S,3R-ACPD in SAL- or MAP-treated rats through microdialysis probe for 20 min. A: effect of 1S,3R-ACPD on dopamine release in SAL-treated rat. B: effect of 1S,3R-ACPD or 1S,3R-ACPD/RS-MCPG on dopamine release in MAP-sensitized rat. The rats were administered saline or 1.0 mg/kg methamphetamine once daily for 6 consecutive days, and after 6-day withdrawal, dialysis experiments were carried out. Each point is the mean ± S.E.M. of the basal dopamine level. The significance of differences was determined by means of two-way ANOVA (* $P < 0.05$, ** $P < 0.01$).

MAP-sensitized rats. Application of RS-MCPG alone did not produce either a dopamine increase or stereotyped behavior in MAP-sensitizedrats. The enhancement of dopamine release by 1S,3R-ACPD was significantly attenuated by the application of RS-MCPG in MAP-sensitized rats (time course 0–180 min × 1S,3R-ACPD or 1S,3R-ACPD/RS-MCPG, $F(9,63) = 3.23$, $P < 0.01$). This level was not significant compared to that of RS-MCPG application. In addition, the stereotyped behavior induced by 1S,3R-ACPD was also attenuated by co-administration of RS-MCPG in MAP-sensitized rats.

Two of the main afferent pathways to the striatum are the dopaminergic and glutamatergic inputs from the substantia nigra and the cortex, respectively. There are many studies indicating that the corticostriatal glutamatergic pro-

jections have a regulatory function of dopamine release from terminals of nigrostriatal dopaminergic neurons. Both in vivo and in vitro studies have shown that Glu stimulates the release of dopamine from the striatum [7,8,14,22]. These studies have suggested that the facilitatory effect of glutamatergic input on dopamine release is, in part, mediated by the activation of iGluRs of the NMDAR and/or AMPAR type which are located on dopaminergic nerve terminals in the striatum. Recently, we demonstrated that long-term enhancement of striatal dopamine release was maintained after the activation of NMDAR and AMPAR [22]. Moreover, it appears that an iGluR-dependent mechanism is involved in AMP and MAP-induced behavioral sensitization, since that is blocked by NMDAR or AMPAR antagonists block these behavioral sensitization [11,13,23,36]. However, an NMDAR antagonist did not attenuate AMP-induced dopamine release in the nucleus accumbens [9], and recent studies have also demonstrated that MK-801 does not prevent MAP-stimulated dopamine release from striatal slices [4]. The role of AMPARs in the MAP-induced increase of dopamine release has not yet been clarified.

Although mGluRs are present in the striatum in widely varying relative densities and cellular patterns [34], little is known as to whether mGluR-dependent mechanism is involved in Glu-stimulated dopamine release. In the nucleus accumbens, it was reported that microdialysis application of the mGluR agonist, 1S,3R-ACPD, caused an increase in dopamine release at a high concentration (1 mM) [24], but a small decrease at a low concentration (0.1 mM) [32]. The present result demonstrated that 0.1 mM 1S,3R-ACPD caused a small release of dopamine from the striatum in SAL-treated rats. The 1S,3R-ACPD-induced dopamine release was, however, significantly enhanced in MAP-sensitized-rats. At present, we do not know the possible mechanism of such augmentation of dopamine release in MAP-sensitized rats. However, these in vivo dialysis results are in good agreement with our previous data that the activation of mGluRs caused an augmentation of dopamine release from MAP-sensitized striatal slices (data not shown).

Trans-ACPD, the racemic mixture of the 1S,3R- and 1R,3S-isomers, has been reported to possess a selective agonistic effect on mGluRs which are coupled to adenylate cyclase [28,33,35]. Application of 0.1 mM *trans*-ACPD also stimulates the inositol phosphate accumulation in striatal neurons [18,30], due to the activation of phospholipase C. On the other hand, 1S,3R-ACPD is reported to inhibit the stimulation of cyclic AMP synthesis by forskolin, and to stimulate brain phosphoinositide hydrolysis, although these effects are blocked by co-administration of RS-MCPG [10,15]. These reports have suggested that 1S,3R-ACPD activates various type of mGluRs, and activation of mGluRs may affect dopamine release through modification of second messenger formation [3,18,28–30,33,35]. Moreover, mGluRs are known to play important roles in such central nervous system plasticities as long-term potentiation in the hippocampus [1,2,19] and long-term depression in the cerebellum [17] and striatum [5,6]. Thus, long-term changes in striatal synaptic function may require the activation of mGluRs. Therefore, it is suggested that mGluRs may have a facilitatory role in the expression of MAP-induced sensitization.

Recently, it was reported that unilateral intrastriatal injection of 1S,3R-ACPD induces rotational behavior [26,27], as well as face washing and scratching [16]. These studies have suggested that activation of mGluRs is involved in the induction of motor and stereotyped behaviors. The present results, showing that 1S,3R-ACPD caused an enhancement of stereotyped behavior and/or locomotor activity in MAP-sensitized rats support this.

In summary, this brain microdialysis study demonstrated that the mGluR agonist, 1S,3R-ACPD, produced a significant enhancement of dopamine release from the striatum in MAP-sensitized rats.

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Methamphetamine-induced sensitization of dopamine release via a metabotropic glutamate receptor mediated pathway in rat striatal slices

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Abstract

We studied the roles of metabotropic glutamate receptors in methamphetamine (MAP)-induced sensitization of dopamine (DA) release from striatal slices. Rats were treated with MAP (1 mg/kg, i.p.) once daily for 6 consecutive days, and after 6-day withdrawal, DA release from striatal slices evoked by 7-1-aminocyclopentane-trans-1,3-dicarboxylic acid (trans-ACPD) was measured. Then, trans-ACPD-induced DA release was significantly enhanced in MAP-sensitized rats, and the inactive form of trans-ACPD (1R,3S-ACPD) did not enhance DA release. The active form of trans-ACPD (1S,3R-ACPD) (0.1 mM)-evoked DA release was attenuated by treatment with 0.4 mM RS-a-methyl-4-carboxyphenylglycine (MCPG), a metabotropic glutamate receptor antagonist. The present results suggest that metabotropic glutamate receptors play an important role in expression of MAP-induced sensitization.

Key Words: methamphetamine-induced sensitization, striatal dopamine release, metabotropic glutamate receptor

Glutamate receptors (GluRs) mediate excitatory neurotransmission in the brain and are important in neural plasticity such as long-term potentiation (LTP) and long-term depression (LTD). There are both ionotropic and metabotropic GluRs.

Ionotropic GluRs are known to mediate the majority of conventional fast excitatory transmission in the central nervous system. Recently, we demonstrated that long-term enhancement (LTE) of striatal DA release occurred after activation of NMDARs and AMPARs [1]. On the other hand, metabotropic glutamate receptors (mGluRs) are known to be coupled to alterations in cAMP formation and phosphatidylinositol turnover, activation of phospholipase D. mGluRs have also been implicated in LTD in the cerebellum [2] and striatum [3]. LTD in the striatum requires the activation of both mGluR and DA receptors [3]. In the striatum, mGluRs are present in widely varying relative densities and cellular patterns [4]. Corticostriatal Glu and nigrostriatal DA neurons are thought to interact closely and to play important roles in neural plasticity in the striatum. In our previous report using microdialysis technique, we suggested that mGluRs are involved in the expression of MAP-induced sensitization [5].

The purpose of the present study was to investigate the role of mGluRs in DA release from striatal slices of MAP sensitized rats.

The animals used in the present experiment were male rats of the Wistar strain (Seiwa Animal Co., Fukuoka, Japan), weighing between 300-400 g. The rats were housed under a constant temperature (23 ± 2 °C) and a 12-h light/dark cycle (light period: 07.00-19.00 h). The rats were allowed free access to food and water throughout the experiment.

MAP (Dainippon Pharmaceuticals Ltd.) (1.0 mg/kg, i.p.) or saline (SAL) was administered once daily for 6 consecutive days. On the 6th day following last injection of MAP, rats were anesthetized with ether and decapitated. The brains were rapidly removed in ice-cold Krebs-Ringer solution (118.0 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl2, 1.2 mM MgCl2, 1.0 mM NaH2PO4, 25.0 mM NaHCO3 and 11.0 mM D-glucose) at pH 7.4, equilibrated with a 95 % O2/5 % CO2 gas mixture and cut sagittally into 450-μM- thick slices using a Sorvall tissue slicer. The striatal part including the white matter between the neocortex and neostriatum of the slices was dissected out in ice-cold Krebs-Ringer solution. After 1 hr preincubation period, striatal slice were placed on a dish in 1 ml of trans-ACPD, 1S,3R-ACPD, 1R,3S-ACPD

and/or MCPG (Tocris Cookson Ltd.) containing solution and incubated for 10 min at 37 □. NMDA was resolved into Mg2+ free Ringer solution. MCPG was pre-treated 10 min before 1S,3R-ACPD application. At the end of the incubation time the solution was transferred immediately to a sample cup containing 100 ㎕ of 0.5 M HClO4 for quantification of DA release into the medium. The striatal tissue containing DA was extracted with 200 ㎕ of 0.5 M HClO4 containing 0.1 % Na2S2O5 and 0.1 % EDTA by solution in sample cups on ice. After centrifugation at 10000 rpm for 10 min at 4 □, supernatants of both the incubation and sonicated solutions were collected for measurement of DA content. DA content was determined with an HPLC-ECD system including a reverse-phase HPLC column (ODS1181, 3 ㎛M, 6.0 mm × 250 mm, Erma) and ECD (E-100, Eicom), The electrode potential was set at +0.65 V against the Ag/AgCl reference electrode. The mobile phase consisted of 0.5 g/l sodium 1-heptanesulfonate (Tokyo Kasei Inc.), 1mM EDTA (Sigma), 8 % acetonitrile (Hayashi Pure Chemical), and was adjusted to pH 3.0 with ortho-phosphoric acid (Merck). Flow rate was 1.0 ml/min (Waters model 510, Waters). Percentage of release was calculated using the values obtained for the DA content in the incubation medium (A) and the DA content remaining in the tissue (B) using the following equation: % of total DA = 100 × A/(A + B).

Data were expressed as the means ± SEM. Significant differences between groups were determined using a one-way ANOVA followed by Student's t-test for individual comparisons.

Basal DA release from striatal slices in MAP sensitized rats was 2.93 ± 0.42 % of total DA content, and this value was not different from SAL-treated rats (2.73 ± 0.46 % of total DA content). In addition, the total contents of DA in the striatum were not different between two groups (data not shown).

Fig.1A shows trans-ACPD-evoked DA release from striatal slices in SAL-treated or MAP-sensitized rats. Trans-ACPD did not cause DA release at any concentration in SAL-treated rats. Whereas the DA release in MAP-sensitized rats was significantly and dose-dependently enhanced by trans-ACPD ($p<0.05$, $p<0.01$). 1R,3S-ACPD, the inactive form of trans-ACPD, failed to enhance DA release from striatal slices in MAP-sensitized rats (Fig. 1A). DA release induced by 10-4 M 1S,3R-ACPD, the active form of trans-ACPD, was 5.70 ± 0.74 % in SAL-treated rats. On the other hand, that was

8.82 ± 0.61 % of total DA content in MAP sensitized rats ($P<0.001$). The enhancement of DA release by 1S,3R-ACPD was significantly attenuated by MCPG (6.27 ± 0.41 % of total DA content, $P<0.001$)(Fig. 1B).

The present results indicate that the mGluR agonist trans-ACPD can enhance DA release from striatal slices of MAP-sensitized rats. Trans-ACPD is a selective agonist for the mGluR [6], but it is a relatively poor agonist for phosphoinositide-linked mGluRs. Trans-ACPD is a racemic mixture of 1S,3R- and 1R,3S- isomers. Of these isomers, 1S,3R-ACPD is responsible for the mGluR agonist activity, because, in contrast to 1R,3S-ACPD, it is a highly selective and efficacious stimulator of brain phosphoinositide hydrolysis. Furthermore the depressant effects of 1S,3R-ACPD on forskolin-stimulated cyclic AMP synthesis in rat cerebral cortical slices are antagonized by MCPG [7]. In addition, MCPG also antagonizes the effect on phosphoinositide-linked mGluRs in rat cerebral cortex [8]. Therefore, this study suggests that the activation of phosphoinositide-linked mGluRs on dopaminergic terminals induce the enhancement of DA release from striatal slices in MAP-sensitized rats. Recently, we reported mGluRs play inportant roles in MAP-sensitization using microdialysis technique[5]. Our in vitro data could support the result.

Since the initial reports which demonstrated that L-Glu stimulates the release of [3H]DA from rat striatal slices [9], numerous studies have been devoted to this presynaptic regulation. In addition, important role of retrograde messengers like nitric oxide (NO) in synaptic transmission and synaptic plasticity is reported. Actually we recently demonstrated that NO synthase inhibitors attenuated MAP-induced sensitization [10]. mGluR expressed in striatal neuron cell body is thought to mediate striatal LTD through a postsynaptic receptor mechanisms [3]. mGluR are also reported to regulate DA release directly or indirectly in the nucleus accumbens[11]. Moreover, we reported that DA release is induced by the activation of mGluR in the nucleus accumbens by microdialysis technique[12]. Thus, it is likely that activation of mGluR may enhance striatal DA release in MAP-sensitized rats. Therefore, the present results support an interaction between the metabotropic glutaminergic and dopaminergic systems in the striatum. The activation of striatal mGluRs by 1S,3R-ACPD produces rotational behavior which is mediated via a functional interaction with striatal DA neurons and their receptors [13]. In addition, it has been

reported that 1S,3R-ACPD causes behavioral changes such as face washing and scratching [14]. The activation of mGluRs may influence MAP-induced behavioral sensitization on locomotor activity and the stereotyped behaviors mentioned above striatum through enhancement of DA release or directly.

In summary, the present study demonstrates that mGluRs may be involved the expression of MAP-induced sensitization.

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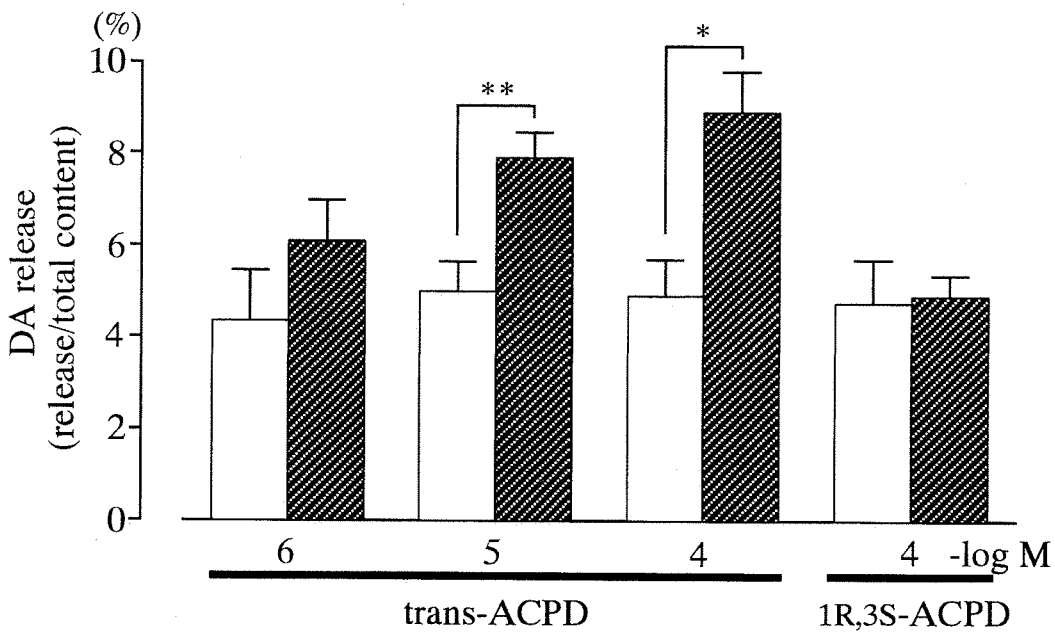
Legends for figures

Fig.1 Effect of metabotropic glutamate receptor agonist and antagonist on dopamine release in MAP-sensitized rats. Fig. 1A shows the effect of active and inactive form of trans-ACPD on dopamine release from striatal slices in MAP-sensitized rats. The rat was received saline or 1.0 mg/kg MAP once daily for 6 consecutive days, and after 6-day withdrawal striatal slices were prepared. Trans-ACPD-induced dopamine release for 10 min was measured using HPLC. The data were shown as means ± SEM. The data were analyzed by one-way ANOVA followed by Student's t-test for individual comparisons. (** $p<0.01$, * $p<0.05$ vs. SAL-treated group). Fig. 1B shows the effect of metabotropic glutamate receptor antagonist on enhancement of active form of trans-ACPD-induced dopamine release in MAP-sensitized rats. The rat was received saline or 1.0 mg/kg MAP once daily for 6 consecutive days, and after 6-day withdrawal striatal slices were prepared. Trans-ACPD-evoked dopamine release for 10 min was measured. MCPG was pre-treated 10 min before 1S,3R-ACPD application. The data were shown as means ± SEM. (** $p<0.01$ by Student's t-test).

A



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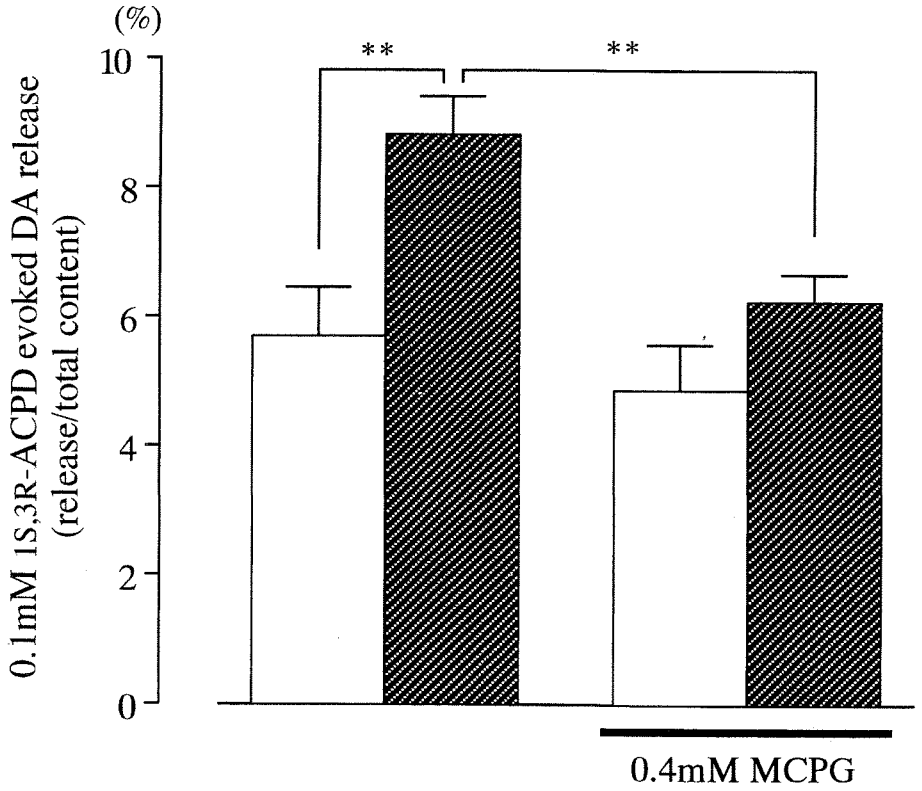

□ SAL (n=5) ▨ MAP (n=6)

Fig. 1 Arai *et al.*

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BRAIN RESEARCH

Research report

Involvement of 5-HT_{1A} receptor mechanisms in the inhibitory effects of methamphetamine on photic responses in the rodent suprachiasmatic nucleus

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Abstract

We examined the role of serotonin 1A (5-HT_{1A}) receptors in the inhibitory effects of methamphetamine (MA) on photic entrainment to the circadian pacemaker in the suprachiasmatic nucleus (SCN) of rodents. MA inhibited optic nerve stimulation-evoked field potential in the SCN, light-induced Fos expression in the SCN and light-induced phase shift of hamster wheel-running rhythm. NAN-190, a 5-HT_{1A} receptor antagonist, eliminated the inhibitory effects of MA. NAN-190 has also been reported to antagonize α_1 adrenergic receptors. However, prazosin, which selectively antagonizes α_1 adrenergic receptors, did not affect the inhibitory action of MA on light-induced Fos expression. In addition, parachloroamphetamine, which is known to be a 5-HT releaser, dose-dependently inhibited light-induced phase shift of wheel-running rhythm. These findings suggest that elevation of endogenous 5-HT levels by MA inhibits the photic entraining responses of the circadian pacemaker in the SCN via 5-HT_{1A} receptor stimulation of the 5-HT released by MA.

Keywords: Circadian rhythm; Suprachiasmatic nucleus; Methamphetamine; NAN-190; 5-HT_{1A} receptor; Serotonin

1. Introduction

The mammalian suprachiasmatic nucleus (SCN) is widely known as a circadian pacemaker, and controls various physiological rhythms such as feeding, drinking, locomotor activity, sleep–wakefulness, plasma adrenal corticosterone level and body temperature (see [8,25] for reviews). However, various findings have indicated that at least two circadian oscillators are located outside the SCN: a feeding-entrainable oscillator and a methamphetamine (MA)-induced oscillator. Also, under chronic administration of MA, a robust free-running rhythm of locomotor activity appeared in SCN-lesioned rodents (see [7] for review). These findings suggest that MA can produce a MA-induced oscillation.

However, MA delays the phase angle in both the rhythm of SCN multiple unit activity and locomotor activity in golden hamsters [14]. Recently, we reported that MA inhibited optic nerve stimulation-evoked vasoactive intestinal polypeptide (VIP) release from the SCN, optic nerve stimulation-induced field potential in the SCN, light-induced Fos expression, and phase shift of free-running rhythm [15]. These findings suggest that MA affects photic entrainment to the circadian pacemaker in the SCN.

In addition, a microdialysis study demonstrated that MA infusion into the SCN increases extracellular serotonin (5-HT) concentration [16], while another biochemical study showed that bath application of MA increases 5-HT concentration in incubated solution [15]. Administration of 5-HT or 5-HT_{1A} receptor agonists to the SCN, blocks both light-induced phase shifts and Fos protein induction during the subjective night [5,24,27]. During the subjective day, these same pharmacological agents induce circadian rhythm phase advances [4,12,17–20,30]. In addition, electrophysiological data indicates that 5-HT or 5-HT_{1A} receptor agonists inhibit the excitatory effects of light in the SCN [13,31]. Thus, 5-HT has been known to modulate the various photic responses in the SCN, via 5-HT_{1A} receptors [5,6,11,24,29,32].

We examined the role of 5-HT_{1A} receptor mechanisms in the inhibitory effect of MA on photic entraining responses in the SCN.

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2. Materials and methods

2.1. Subjects

Male Wistar rats (Seiwa Experimental Animal Co., Yoshitomi-Cho, Japan) weighing 250–350 g were used for the field potential study. Male Syrian hamsters (*Mesocricetus auratus*, Japan SLC, Shizuoka, Japan) weighing 100–150 g were used for studies of Fos expression and phase shift of free-running rhythm. All animals were housed in temperature-controlled animal quarters (23 ± 2°C) in either 12:12 h light/dark cycle (field potential study and Fos expression study) or in constant darkness (phase shift of free-running rhythm study). Food and water were given ad libitum.

2.2. Drugs

The drugs used in this study were: D-methamphetamine hydrochloride (MA) (Dainippon, Osaka, Japan), 1-(2-methoxyphenyl)-4-[4-(2-phthalimido)butyl]piperazine hydrobromide (NAN-190) (Research Biochemicals, Natick, MA, USA), prazosin hydrochloride and DL-parachloroamphetamine (Sigma Chemical, St Louis, MO, USA).

2.3. Optic nerve stimulation-evoked field potential

Rats were kept in constant darkness for 1–2 cycles. Each rat was then decapitated around circadian time (CT; CT 12 = activity onset time) 12 under ether anesthesia, and the brain quickly removed from the skull. Only one horizontal hypothalamic slice, that included both SCN and optic nerve (0.45 mm thickness) was taken from each animal brain. Preparation of the samples was done using a vibratome as reported previously [15]. Each slice was preincubated with Krebs-Ringer solution equilibrated with 95% O_2/5% CO_2 at room temperature. The composition of the control Krebs-Ringer solution was (in mM): NaCl, 129; $MgSO_4$, 1.3; $NaHCO_3$, 22.4; KH_2PO_4, 1.2; KCl, 4.2; D-glucose, 10.0 and $CaCl_2$, 2.5. This buffer was maintained at pH 7.3–7.4. The slice was transferred to a recording chamber and kept in a constant flow medium (35 ± 1°C)(4 ml/min). Optic nerve stimulation-evoked postsynaptic field potential [28] corresponding to EPSP [9,28] was recorded in the ventrolateral part of the SCN using a glass microelectrode. Insulated stainless wires (diameter 0.1 mm) were placed on the optic nerve approximately 1 mm rostral to the optic chiasm. Each of the drugs was added to the Krebs-Ringer solution and perfused for up to 20 min through the experimental chamber. Electrophysiological experiments were conducted during CT 13–CT 0. Five to six slices from identical number of animals were used for each drug treatment.

2.4. Fos immunohistochemistry

Hamsters maintained in a 12:12 h light/dark cycle were injected intraperitoneally with DMSO, NAN-190 or prazosin 45 min before, and saline or MA 30 min before, light exposure. The animals were then exposed to white light (150 lux) at zeitgeber time (ZT; ZT 12 = lights-off time) 20, for 30 min followed by darkness for 30 min. Animals were deeply anesthetized with Nembutal and perfused intracardially with 100 ml of saline (37°C), followed by 100 ml of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). Brains were removed from the skull, fixed with 50 ml of 4% paraformaldehyde, and transferred to 20% sucrose solution in 0.1 M phosphate buffer for 24 h, followed by 30% sucrose solution in 0.1 M phosphate buffer for 48 h. Hypothalamic blocks were cut into sections of thickness 30 μm, from rostral to caudal SCN, on a freezing microtome. The sections were processed for immunohistochemical analysis according to the avidin-biotin-peroxidase complex method. Primary antibody (anti-Fos, Cambridge Research Biochemical, USA, 1:1000) was diluted in 0.1 M phosphate buffer containing 1% normal rabbit serum in 0.3% Triton X-100. To determine the rostral-caudal distribution of Fos-immunoreactive neurons through the SCN, sections at 30-μm intervals were selected for analysis from each animal. The number of cells which expressed Fos immunoreactivity was counted in selected sections covering rostral to caudal SCN. Average cell numbers per SCN were calculated and expressed as the number of cells/SCN. Three to ten animals were used for each treatment.

2.5. Light-induced phase shift of free-running rhythm

Male hamsters were housed individually in transparent plastic cages (20 × 30 × 14 cm), each equipped with a 13 cm diameter running wheel, which closed a microswitch with each evolution. Wheel-running activity was recorded continuously by a computer. Hamsters were kept in constant darkness. After at least 10 days in constant darkness, each hamster received an intraperitoneal injection of NAN-190 60 min before, followed by administration of MA 30 min before exposure to light. Some hamsters (3–5 animals for each dose) were injected with parachloroamphetamine, 30 min before exposure to light. Injections were performed under dim red illumination. Hamsters were transferred to the light stimulation box (30 × 30 × 40 cm) and exposed to white light for 15 min at an average illuminance of 200 lux at CT 20. After light stimulation, hamsters were returned to their respective wheel cages and kept under constant darkness. The onset of the phase of activity was calculated from data for 10 days of stable activity after manipulation. The distance between the two lines on the day of the manipulation was measured and taken as the phase shift value.

2.6. Statistics

The data were expressed as mean ± S.E.M. Whether the difference between groups was significant or not was determined by Student's t-test or one-way ANOVA followed by Dunnett's test.

3. Results

3.1. Optic nerve stimulation-evoked field potential

As shown in Fig. 1A, electric stimulation of the optic nerve caused field potential in the ventrolateral part of the SCN, which corresponds to the synaptic transduction. Bath application of MA (10 μM) for 20 min significantly attenuated the optic nerve stimulation-evoked field potential in the SCN ($n = 6$) ($P < 0.001$ vs. vehicle treatment, $n = 6$; Student's t-test). Whereas, pretreatment of NAN-190 (0.1 μM) with MA abolished the inhibitory effect of MA ($n = 5$) ($P < 0.001$ vs. MA treatment, $n = 6$; Student's t-test) (Fig. 1 and Table 1). However, NAN-190 (0.1 μM) alone did not affect the optic nerve stimulation-evoked field potential in the SCN ($n = 5$) ($P > 0.05$, vs. vehicle treatment).

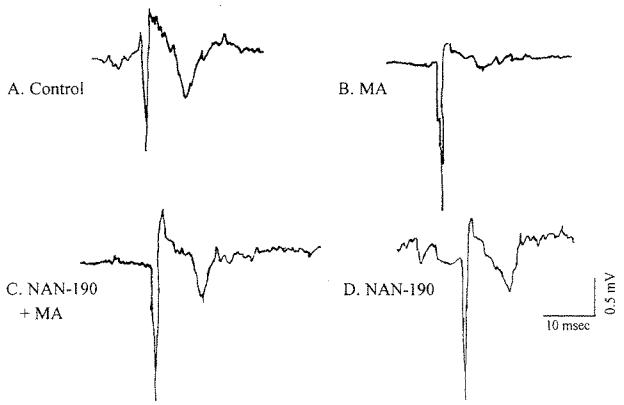

Fig. 1. Representative traces demonstrating the effect of NAN-190 on the inhibitory effect of MA on optic nerve stimulation-evoked field potential in the ventrolateral part of the rat SCN. A: vehicle. B: MA 10 μM, 20 min. C: MA 10 μM+NAN-190 0.1 μM, 20 min. D: NAN-190 0.1 μM, 20 min. Each trace is an average of eight sweeps.

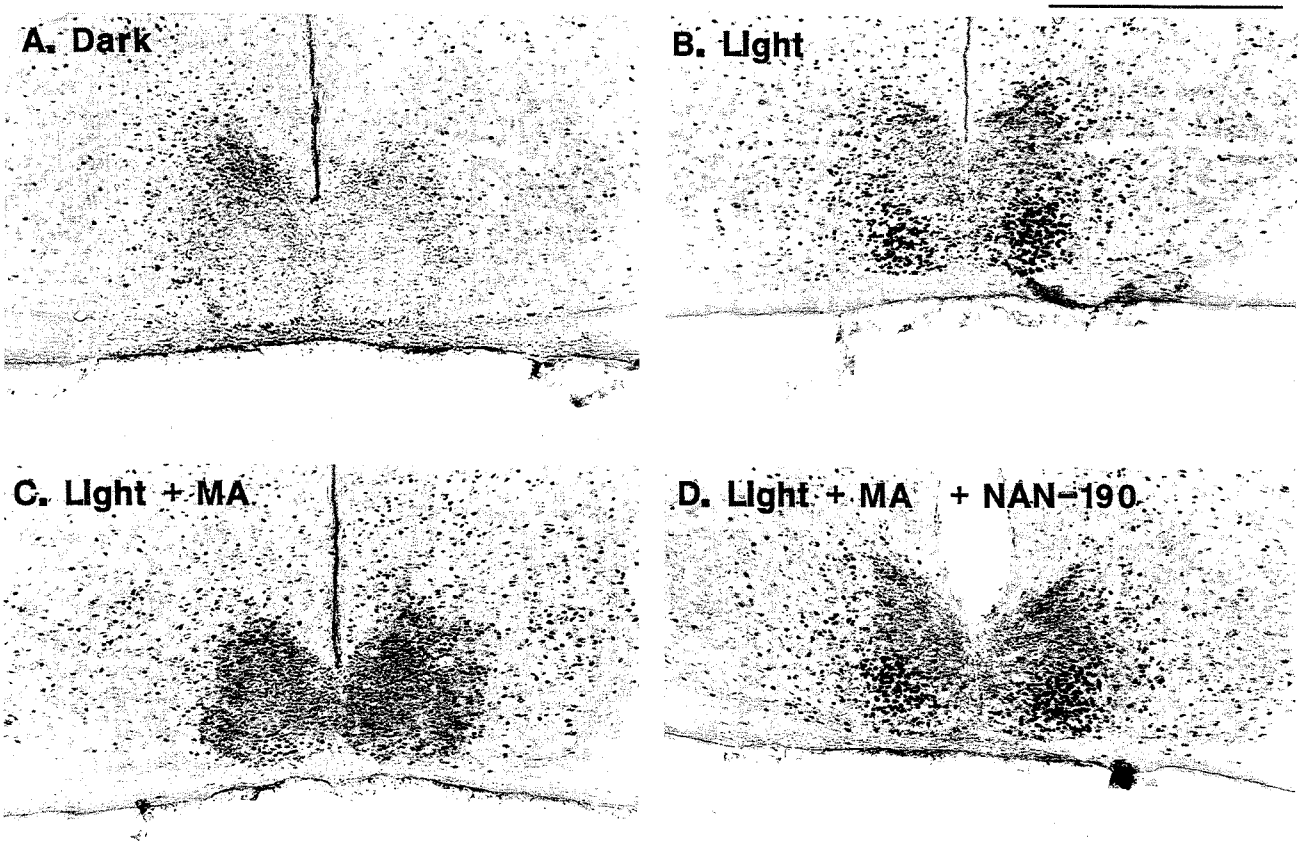

Fig. 2. Representative photomicrographs demonstrating the effect of NAN-190 on the inhibitory effect of MA on light-induced Fos expression in the hamster SCN. Hamsters received an intraperitoneal injection of DMSO (A, B, C) or NAN-190 10 mg/kg (D) 45 min prior to light exposure and then saline (A, B) or MA (C, D) 30 min prior to light exposure (150 lux, 30 min, ZT19.5-20.0) (B, C, D). Hamsters were perfused intracardially with saline 30 min after the end of the light exposure. Scale bar = 0.5 mm.

Table 1
Effect of NAN-190 on MA-induced inhibition of optic nerve stimulation-evoked field potential in rat SCN

Treatment	Peak amplitude [a] (n)	S.E.M.
Vehicle	104.4 (6)	3.0
MA	35.8 (6) ***	3.7
MA+NAN-190	89.9 (5) ###	10.7
NAN-190	82.9 (5)	8.7

[a] The control value before drug application was set to 100%; data represent % of control value. Vehicle indicates DMSO and water treatment. Numbers in parentheses indicate the number of slices examined.
*** $P < 0.001$ vs. Vehicle (Student's t-test).
$P < 0.001$ vs. MA (Student's t-test).

3.2. Light-induced fos expression in the SCN

The exposure to light at ZT 20, induced Fos expression especially within the ventrolateral part of the hamster SCN, which corresponds to the terminal of the retinohypothalamic pathway ($n = 10$) ($P < 0.001$ vs. non-light stimulation, $n = 5$; Student's t-test) (Fig. 2A, B and Fig. 3). Preinjection of MA (10 mg/kg) reduced light-induced Fos expression, especially in the ventrolateral part of the SCN ($n = 8$) ($P < 0.001$ vs. vehicle + light, $n = 10$; Student's t-test) (Fig. 2C and Fig. 3). NAN-190, a 5-HT_{1A} antagonist, significantly attenuated the inhibitory effect of MA on Fos expression ($n = 5$) ($P < 0.001$ vs. MA + light, $n = 8$; Student's t-test). As NAN-190 was reported to antagonize α_1 adrenoceptor [2], we examined the effect of the α_1 adrenoceptor antagonist, prazosin, on MA-induced Fos attenuation. Prazosin (5 mg/kg) did not antagonize the inhibitory action of MA ($n = 4$) ($P > 0.05$, vs. MA + light) (Fig. 2D and Fig. 3).

3.3. Light-induced phase shift of free-running activity rhythm

As shown in Fig. 4A, exposure to pulses of light for 15 min at CT 20, caused a large and stable phase advance of wheel-running rhythm in hamsters ($n = 5$). Preinjection of MA (10 mg/kg) reduced the phase advance induced by the light pulses ($n = 4$) ($P < 0.001$ vs. vehicle + light; Student's t-test) (Fig. 4B and Fig. 5A). NAN-190 attenuated the inhibitory effect of MA on light-induced phase advance in a dose-dependent manner (NAN-190 10 mg/kg; $n = 5$, $P < 0.05$ vs. MA + light; Student's t-test) (Fig. 4C and Fig. 5A). However, NAN-190 (10 mg/kg) alone neither affected light-induced phase advance ($n = 3$) nor caused phase shift ($n = 3$) (Fig. 5D and Fig. 5A).

Parachloroamphetamine, a 5-HT releaser, dose-dependently reduced the phase advance of wheel-running rhythm ($F_{3,14} = 11.63$, $P < 0.001$), with the attenuating effect

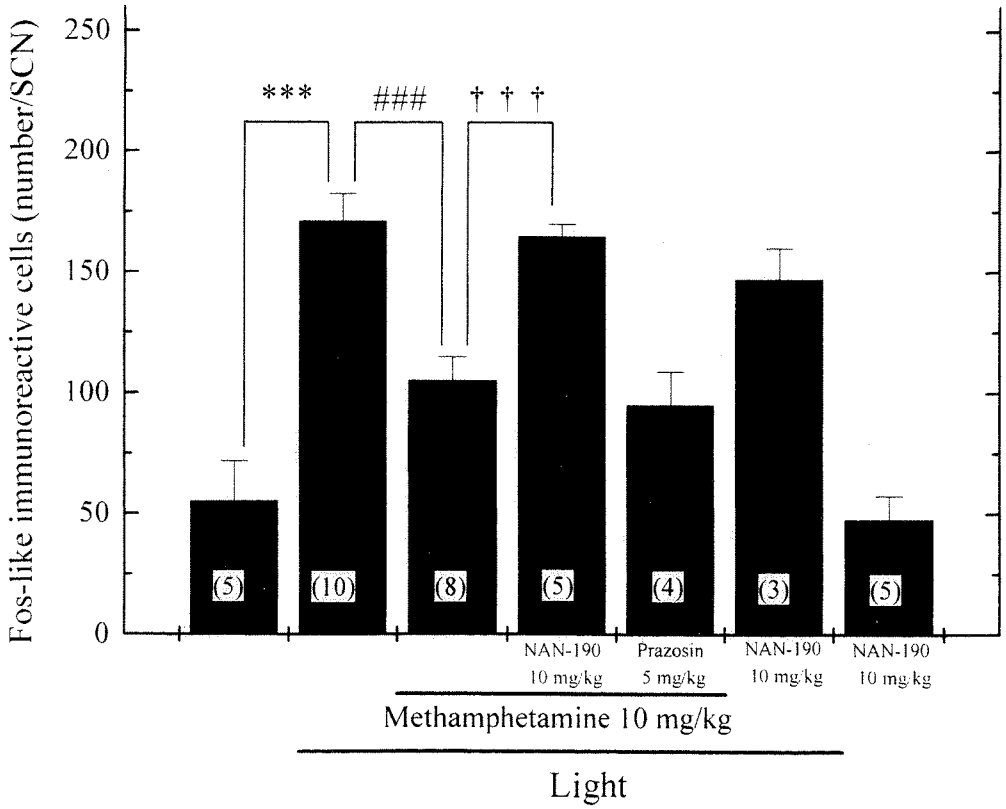

Fig. 3. Effects of NAN-190 and prazosin on the inhibitory effect of MA on light-induced Fos expression in the hamster SCN. Experimental procedure as mentioned in Fig. 2 legend. Data represent a number of Fos-immunoreactive cells per the SCN. Numbers in parentheses indicate the number of animals examined.

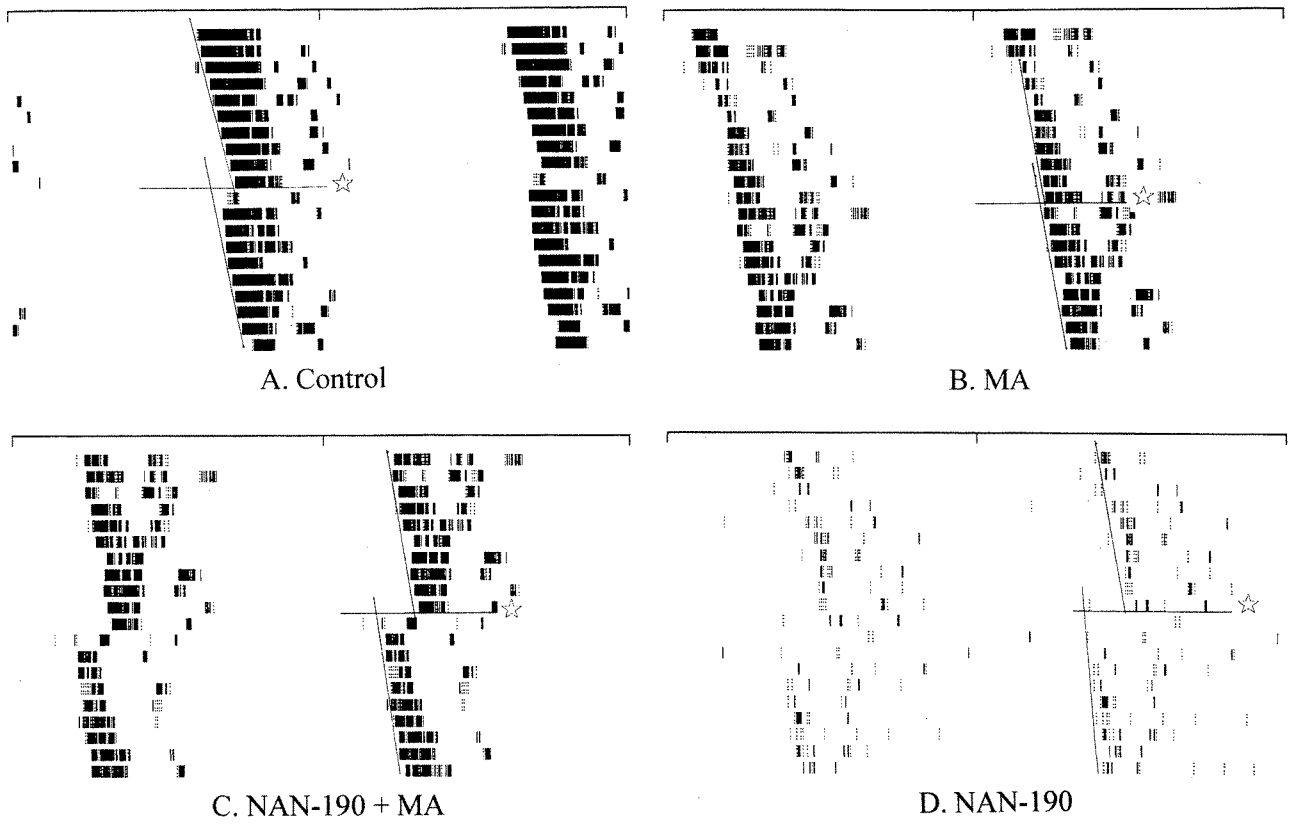

Fig. 4. Representative double plot-actograms demonstrating the effect of NAN-190 on the inhibitory effect of MA on light-induced phase shift of the free-running activity rhythm in the hamsters. Hamsters were maintained in constant darkness and wheel-running behavior monitored by computer. Hamsters were received intraperitoneal injections of DMSO (A, B) or NAN-190 10 mg/kg (C, D) 60 min prior to light exposure, followed by saline (A, D) or MA 10 mg/kg (B, C) 30 min prior to light exposure (200 lux, 15 min) at CT 20. Approximate times of light exposure are indicated by stars.

reaching a significant level at doses of 0.3 ($n = 5$) and 1.0 mg/kg ($n = 5$).

4. Discussion

In the present experiments, we examined the role that 5-HT_{1A} receptor mechanisms may have in the inhibitory effects of MA on the photic entraining responses in rodent circadian rhythm. It has been reported that electrical stimulation of the optic nerve elicits field potential in the ventrolateral part of the SCN, which corresponds to the synaptic transmission at the retinohypothalamic terminal [1,11]. Our present results demonstrated that MA inhibited optic nerve stimulation-evoked field potential and that this inhibitory effect was eliminated by cotreatment with NAN-190, a 5-HT_{1A} receptor antagonist. 5-HT or 5-HT_{1A} receptor agonists reportedly attenuate optic nerve stimulation-evoked field potential in the SCN [11,24]. It is well known that MA releases 5-HT and/or inhibits the uptake of 5-HT. These reports taken together with our findings, suggest that elevation of endogenous 5-HT levels by MA modulates neurotransmission in the retinohypothalamic pathway to the SCN via 5-HT_{1A} receptor mechanisms.

Fos protein, one of the immediate early gene products, is known to increase in the ventrolateral part of the SCN in response to photic entrainment [3,10,21–24,26]. The blocking of Fos expression by antisense oligonucleotides inhibits light-induced phase shift of free-running activity rhythm in rats [33]. These reports have suggested that Fos induction is a useful marker for monitoring photic entrainment to the SCN. We observed that NAN-190 attenuated the inhibitory effect of MA on light-induced Fos expression, especially in the ventrolateral part of the SCN which is the terminal of the retinohypothalamic pathway. As was shown in both our previous report [15] and in the present findings, neither NAN-190 nor MA affected basal Fos expression in the SCN. Thus, the effect of NAN-190 or MA on Fos expression was only effective when photic information was delivered into the SCN.

Finally, we showed that NAN-190 blocked the inhibitory effect of MA on light-induced phase shift of hamster free-running rhythm. NAN-190 completely attenuated the effect of MA on light-induced phase shift at a dose comparable to that which recovered the effect of MA on Fos expression. Parachloroamphetamine, a 5-HT releaser from the nerve terminals, inhibited light-induced phase shift in a dose-dependent manner. 5-HT_{1A} receptor

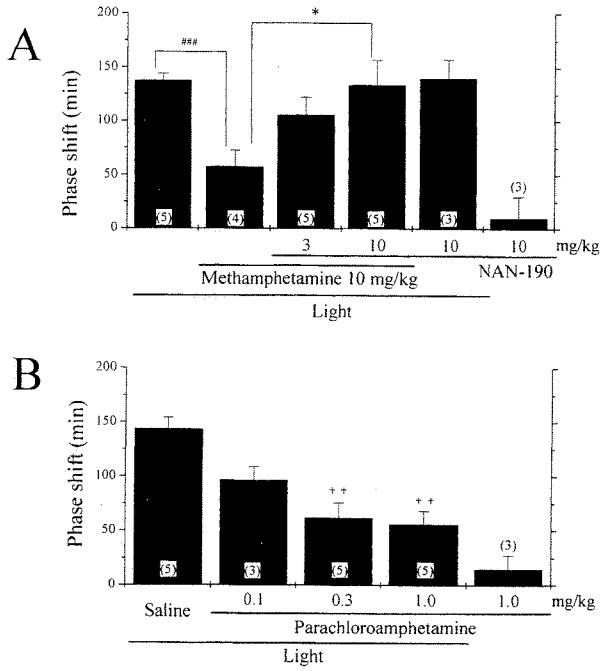

Fig. 5. Effect of NAN-190 on the inhibitory effect of MA on light-induced phase shift of the free-running activity rhythm in the hamsters (A). Dose-dependent effect of parachloroamphetamine on light-induced phase shift of the free-running activity rhythm in the hamsters (B). Experimental procedure as mentioned in Fig. 4 legend. Data represent the phase advance (min) induced by light pulse. $^{###}P < 0.001$ (Student's t-test), $^{*}P < 0.05$ (Student's t-test), $^{††}P < 0.01$ (Dunnett's t-test). Numbers in parentheses indicate the number of animals examined.

agonists and tryptophan-loading reportedly have similar inhibitory actions on photic entrainment [6,24]. Furthermore, microdialysis study has demonstrated that infusion of MA increases the extracellular 5-HT concentration in the SCN [16]. Therefore, 5-HT released by MA in the SCN inhibits light-induced phase shift via 5-HT_{1A} receptor stimulation. Administration of NAN-190 alone did not affect light-induced phase shift nor did it cause a phase shift. These findings suggest that endogenous 5-HT does not have a tonic inhibitory effect via 5-HT_{1A} receptor stimulation on light-induced phase shift.

Since it has been reported that NAN-190 antagonizes α_1 adrenergic receptors [2], and that stimulation of this receptor modulates photic response in the SCN [11], we examined the effect of the α_1 adrenergic receptor antagonist on the inhibitory action of MA on photic responses in the SCN. As shown in Fig. 3, prazosin, a selective α_1 adrenergic receptor antagonist, did not affect the inhibitory effect of MA on light-induced Fos expression in the SCN. Furthermore, we previously reported that noradrenaline depletion by *N*-(chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP-4) did not abolish the inhibitory effect of MA on field potential in the SCN, and that bath application of MA did not release noradrenaline from the SCN in vitro. Based on these findings, we can exclude the possible involvement of α_1 adrenergic receptors in the inhibitory action of MA on photic responses in the SCN.

Neural and molecular mechanisms of inhibitory action of MA on photic responses in the SCN are not clear at present. However, localized SCN administration of 5-HT or intraperitoneal injection of 5-HT_{1A} receptor agonist, reduced the level of extracellular glutamate, which is known to be an important neurotransmitter of the retinohypothalamic pathway, in the SCN as compared to base line levels [27,29]. In addition, these same pharmacological agents inhibited the firing rates of photically responsive cells in the SCN [34]. As previously described, MA inhibited the neurotransmission of the retinohypothalamic pathway in the SCN, therefore endogenous 5-HT released by MA may inhibit photic response in the SCN via 5-HT_{1A} receptor stimulation, resulting in inhibitory action on photic entrainment to the circadian pacemaker in the SCN.

In summary, we demonstrated that MA inhibited optic nerve stimulation-evoked field potential in the ventrolateral part of the SCN in vitro, as well as light-induced Fos expression in the SCN and phase-shift of free-running activity rhythm in vivo. NAN-190, a 5-HT_{1A} receptor antagonist, attenuated and/or eliminated the inhibitory effects of MA. These findings suggest that 5-HT_{1A} receptor stimulation involves the inhibitory effects of MA on photic responses in the circadian pacemaker in the SCN.

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METHAMPHETAMINE MODIFIES THE PHOTIC ENTRAINING RESPONSES IN THE RODENT SUPRACHIASMATIC NUCLEUS VIA SEROTONIN RELEASE

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Abstract—We examined whether methamphetamine modifies the photic entraining responses in the rat suprachiasmatic nucleus. Optic nerve stimulation increased vasoactive intestinal polypeptide release from rat suprachiasmatic nucleus slices, and methamphetamine inhibited this increase in a concentration-dependent manner. Optic nerve stimulation has been reported to evoke field potentials in rat suprachiasmatic nucleus slices. Methamphetamine attenuated this field potential, and maximal inhibition (75.5%) was achieved at a concentration of 100 μM. Systemic administration of methamphetamine (1–5 mg/kg) inhibited light (300 lux, 1 h)-induced Fos expression in the suprachiasmatic nucleus; methamphetamine at a dose of 5 mg/kg, i.p. caused 40% inhibition of light-induced Fos expression. We examined whether the inhibitory effect of methamphetamine on photic entraining responses mediates serotonin release from the suprachiasmatic nucleus. High-performance liquid chromatographic analysis revealed that methamphetamine application increased serotonin release from rat suprachiasmatic nucleus slices in a concentration-dependent manner, but did not affect noradrenaline release. In addition, reduction of serotonin content attenuated the effect of methamphetamine on field potential induced by optic nerve stimulation *in vitro* and also light-induced phase advances of wheel running activity rhythm *in vivo*.

The present results support the idea that methamphetamine produces an inhibitory effect on photic entrainment in the suprachiasmatic nucleus via serotonin release.

Key words: methamphetamine, circadiam rhythm, entrainment, suprachiasmatic nucleus, serotonin, retinohypothalamic tract.

The mammalian suprachiasmatic nucleus (SCN) is widely known as a circadian oscillator (for review see Refs 18 and 47), and the destruction of this nucleus abolishes overt circadian rhythms.[31,61] Various lines of evidence indicate that at least two circadian oscillators are located outside the SCN: a feeding-entrainable oscillator and a methamphetamine-induced oscillator, which are observed in SCN-lesioned rats (for review, see Ref. 16). Under chronic treatment with methamphetamine, a robust free-running rhythm appears in spontaneous locomotor activity.[46] These results suggest that methamphetamine can produce a methamphetamine-dependent oscillation.

In contrast to the above phenomena, there is evidence that methamphetamine may directly affect the circadian oscillator in the SCN. Methamphetamine treatment has been reported to cause a phase delay in the multiple-unit activity rhythms in the SCN in relation to the light–dark cycle as it delays the locomotor rhythm phase.[36] Furthermore, methamphetamine infusion into the SCN was shown to increase extracellular serotonin (5-HT) concentration in a microdialysis study.[37] Recently, it has been demonstrated that 5-HT modulates the photic response of the SCN pacemaker.[44,51,65] These observations have suggested that methamphetamine may affect photic entrainment of the methamphetamine oscillator via methamphetamine-induced 5-HT release. Therefore, we investigated whether methamphetamine affects the photic response in the SCN.

The excitatory amino acid glutamate has been shown to act as a transmitter for the retinohypothalamic tract by electrophysiological, biochemical and behavioral studies.[12,26,29,53] Both *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors have been found to play important roles in the transmission of photic information,[1,2,12] whereas the retinohypothalamic

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Abbreviations: 5,7-DHT, 5,7-dihydroxytryptamine; DSP-4, *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine hydrochloride; EDTA, ethylenediaminetetra-acetate; HPLC, high-performance liquid chromatography; 5-HT, serotonin (5-hydroxytryptamine); NMDA, *N*-methyl-D-aspartate; *p*-CPA, DL-*p*-chlorophenylalanine methyl ester hydrochloride; SCN, suprachiasmatic nucleus; VIP, vasoactive intestinal polypeptide.

tract has been reported to terminate on neurons possessing vasoactive intestinal polypeptide (VIP).[17] VIP and VIP mRNA levels decrease during the light period of the light–dark cycle and increase during the dark period under a light–dark cycle.[3,33,35,62] These observations suggested that VIP might be rhythmically modulated. Previously, we reported that NMDA application during subjective night increased VIP release and produced phase shifts in circadian rhythm.[56] In the present study, we investigated the effect of optic nerve stimulation on VIP release from rat SCN slices, and if methamphetamine influences this on release. Previously, we reported that optic nerve stimulation produced field potentials in the rat SCN.[27] Therefore, in the second experiment, we examined the effects of methamphetamine on optic nerve stimulation-induced field potentials in rat SCN slices.

Photic stimulation can produce the expression of c-*fos*, immediate early genes and Fos protein in the SCN.[11,25,41–43,48] Although the functional significance of this Fos expression is unknown at present, Fos induction has been used as a good marker to investigate the mechanism of entrainment. In fact, the effects of various pharmacologically active drugs have been reported to affect Fos expression.[1,43,44] Therefore, in the third experiment, we investigated the effects of methamphetamine on light-induced expression of Fos-like immunoreactivity in the rat SCN.

Wheel-running rhythm has been used in the hamster as a good method to investigate the circadian rhythm *in vivo*. Therefore, finally we examined the effects of methamphetamine on light-induced phase shifts of free-running rhythm in hamsters.

It is well known that methamphetamine is a strong reuptake inhibitor of monoamines and also a releaser of monoamines in the CNS. In the fourth experiment, to determine the mechanism of methamphetamine-induced inhibitory effect on photic response of the SCN, we examined whether methamphetamine increased 5-HT release from SCN slices by high-performance liquid chromatographic (HPLC) analysis. In addition, we investigated whether the reduction of 5-HT attenuates the inhibitory effect of methamphetamine on the optic nerve stimulation-induced field potential *in vitro* and also light-induced phase advances of wheel-running rhythm *in vivo*.

EXPERIMENTAL PROCEDURES

Vasoactive intestinal polypeptide release induced by optic nerve stimulation

Wistar rats (200–300 g; Seiwa Animals Co., Fukuoka, Japan) were housed under a 12:12 h light–dark cycle with free access to food and water for at least two weeks after being introduced into the laboratory. Each animal was decapitated under ether anesthesia and the brain was quickly removed from the skull. Hypothalamic slices (400–450 μm thick), including half of the SCN and one optic nerve, were cut sagittally using a Vibratome. After 1 h of pre-incubation, the slices were placed in chambers filled with 170 μl warmed Krebs–Ringer solution. The composition of the control Krebs–Ringer solution was (in mM): NaCl, 129; $MgCl_2$, 1.3; $NaHCO_3$, 22.4; KH_2PO_4, 1.2; KCl, 4.2; D-glucose, 10.0; $CaCl_2$, 1.5, equilibrated with 95% O_2/5% CO_2 at 36°C. The medium was replaced and optic nerve stimulation was performed for 10 min at projected Zeitgeber time 13–14. Zeitgeber time 12 was designated as the lights-off time. A single pulse stimulation (80 μs, 0.8 mA and 20 Hz) was used, because this stimulus condition was reported to produce large field potentials in the ventrolateral SCN, and also to increase release of [^{3}H]glutamate from the SCN.[26] Methamphetamine was added to the Krebs–Ringer solution in concentrations ranging from 1 to 100 μm 10 min before stimulation. The medium was heated at 100°C for 10 min in 0.1 N HCl, dried in a vacuum concentrator and stored at 4°C until the assay of VIP. VIP in the medium was determined by the previously described enzymeimmunoassay[57] with minor modifications. The dried samples were reconstituted with 150 μl of assay buffer [0.14 M phosphate buffer containing 25 μM ethylenediaminetetraacetate (EDTA), 0.5% bovine serum albumin and 0.05% Tween 20]. Of these 150-μl reconstituted samples, 100-μl aliquots were used for VIP assay. Each sample was incubated overnight with 50 μl of anti-VIP (1:20,000 in assay buffer; a gift from Dr Mitsusio, Musashi Hospital, Japan) rabbit serum at 4°C in 96 F multi-well plates for enzyme-linked immunosorbent assay, precoated with anti-rabbit immunoglobulin G goat serum (Shibayagi, Japan). Fifty microliters of horseradish peroxidase-conjugated VIP (1:300 in assay buffer) was added to each well. After 3 h of incubation, each well was washed with 0.14 M phosphate-buffered saline, and substrate solution (3.68 mg *o*-phenylenediamine hydrochloride in 0.1 M citrate–phosphate buffer, pH 5.2) was added to each well. After 3 h of incubation, the reaction was stopped by adding 5 N H_2SO_4, and production of VIP was determined using an enzymeimmunoassay reader at 492 nm.

Field potential induced by optic nerve stimulation

Each rat was decapitated under ether anesthesia and the brain was quickly removed from the skull. Hypothalamic slices (400–450 μm thick), including both SCN and optic nerve, were cut horizontally using a Vibratome. After preincubation in Krebs–Ringer solution, the slices were transferred into a recording chamber. Preparations were perfused continuously at 5 ml/min with Krebs–Ringer solution at 36 $\pm$ 1°C. The stimulation electrodes used were described previously.[23,52] Evoked potentials were recorded in the ventrolateral SCN using glass pipette microelectrodes filled with 0.9% NaCl. The drug was added to the Krebs–Ringer solution in concentrations ranging from 1 to 100 μM, and this was perfused for up to 20 min through the experimental chamber.

Light-induced Fos expression

After two days under constant darkness, rats received an intraperitoneal injection of either vehicle or methamphetamine, 30 min before light exposure (300 lux of white light for 1 h) at circadian time 13.5. After light exposure, rats were returned to darkness. Rats were deeply anesthetized with pentobarbital 2 h after the onset of light stimulation and perfused transcardially with 100 ml of saline, followed by 100 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). Brains were removed and fixed with 50 ml of 4% paraformaldehyde and transferred to 30% sucrose solution in 0.1 M phosphate buffer. Hypothalamic blocks were cut into sections at a thickness of 30 μm from rostral to caudal SCN on a freezing microtome. The sections were processed for immunohistochemistry according to the avidin–biotin–peroxidase complex method. Primary antibody (anti-Fos, Cambridge Research Biochemical 1:5000) was diluted in 0.1 M phosphate buffer containing 1% normal goat serum in 0.3% Triton X-100.

To determine the rostral–caudal distribution of Fos-immunoreactive neurons through the SCN, sections at 30 μm intervals were selected for analysis from each animal. Alternate sections were stained with Cresyl Violet to show the localization of the SCN. The number of cells which expressed Fos immunoreactivity was counted on selected sections from rostral to caudal SCN. Average cell numbers per unilateral SCN were calculated and shown as number of cells/SCN.

Methamphetamine-induced monoamine release

Hypothalamic slices (450 μm), including the SCN, were cut coronally using a Sorvall tissue slicer. After 1 h pre-incubation, slices were incubated with methamphetamine (1–100 μM) in 170 μl Krebs–Ringer solution for 20 min. These solutions (100 μl) were transferred immediately to sample wells containing 10 μl of 0.5 M $HClO_4$ for quantification of 5-HT released into the medium. Slices containing monoamines were extracted with 200 μl of 0.5 M $HClO_4$ containing 0.1% $Na_2S_2O_5$ and 0.1% EDTA by sonication in sample wells on ice. After centrifugation at 10,000 × *g* for 10 min at 4°C, supernatants of both the incubation and sonicated solutions were collected for measurement of monoamine content, which was determined with an HPLC–electrochemical detection system, including a reverse-phase HPLC column (ODS1181, 3 μm, 6.0 mm × 250 mm, Erma) and electrochemical detector (E-100, Eicom). The electrode potential was set at +0.65 V against the Ag/AgCl reference electrode. The mobile phase consisted of 0.5 g/l heptane sulfonic acid (Tokyo Kasei Inc., Japan), 1 mM EDTA (Sigma), 8% acetonitrile (Hayashi Pure Chemical), and was adjusted to pH 3.0 with orthophosphoric acid (Merck). The flow rate was 1.0 ml/min. The data were analyzed with a chromatographic integrator (Waters model 730).

Light-induced phase shifts of free running rhythm in hamsters

Adult male golden hamsters (*Mesocricetus auratus*, 90–150 g; SLC Animals Co., Shizuoka, Japan) were housed individually in transparent plastic cages (20 × 30 × 14 cm), each equipped with a 13 cm diameter running wheel, which closed a microswitch with each revolution. Wheel-running activity was recorded continuously by a computer. Hamsters were provided with food and water *ad libitum* and kept under conditions of constant darkness.

After at least 10 days in constant darkness, animals received intraperitoneal injection of saline or methamphetamine 30 min before light exposure. Injections were performed under dim red illumination. Animals were transferred to the light stimulation box (30 × 40 × 40 cm) and exposed to white light for 15 min at an average illuminance of 200 lux at either circadian time 14 or 20. After light stimulation, animals were returned to their respective wheel cages under constant darkness. Animals that received drug injection without light treatment were handled as described above and returned to the cages under constant darkness immediately after injection. The onset of the phase of activity was calculated from data for 10 days of stable activity after manipulation. The distance between the two lines on the day of the manipulation was measured and this value was taken as the phase shift.

p-*Chlorophenylalanine methyl or* N-(2-*chloroethyl*)-N-*ethyl*-2-*bromobenzylamine treated rats and* 5,7-*dihydroxytryptamine-treated hamsters*

p-Chlorophenylalanine methylester hydrochloride (*p*-CPA; 300 mg/kg, concentration 70 mg/ml saline), which causes selective depletion of brain 5-HT,[24] was injected i.p. once daily for three successive days. *p*-CPA-treated rats were killed 24 h after the last injection. *N*-(2-Chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP-4), which causes selective depletion of brain noradrenaline,[45] was injected into rats intraperitoneally at 50 mg/kg. DSP-4-injected rats were then killed two weeks after injection.

Intraventricular infusion of the neurotoxin, 5,7-dihydroxytryptamine creatinine sulfate (5,7-DHT), was reported to result in lasting damage to ascending serotonergic systems in the hamster brain.[34,59] Hamsters were pre-treated by intraperitoneal injection of desipramine hydrochloride (25 mg/kg in saline) 30 min before being anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg) during the light phase of the photoperiod and placed in a stereotactic instrument. Hamsters received a bilateral infusion of 5,7-DHT (75 μg of the free base in 2.5 μl 0.5% ascorbic acid per lateral ventricle) through a Hamilton syringe (coordinates: 2.3 mm lateral, 0.5 mm rostral to bregma and 3.8 mm ventral to the skull surface, with the skull level between lambda and bregma). Each infusion took place over a period of 5 min.

Drugs and data analysis

The drugs used in this study were D-methamphetamine hydrochloride (Dainippon Inc. Osaka, Japan), *p*-CPA (Research Organics Inc. OH, U.S.A.), DSP-4 (Sigma Chemical Co., St. Louis, MO, U.S.A.) and 5,7-DHT (Sigma). Results are expressed as means ± S.E.M. Statistical significance was determined by ANOVA and differences between means were tested for significance using Dunnett's test or Student's *t*-test.

RESULTS

Optic nerve stimulation-induced vasoactive intestinal polypeptide release

Spontaneous release of VIP from an SCN slice in the presence of vehicle of methamphetamine (10 μl water) for 10 min was regarded as 100%. The amount of spontaneous VIP release was 1.9 ± 0.06 fmol/10 min/SCN, and this value decreased to 89.1 ± 4.7% in the absence of stimulation for 10 min. By optic nerve stimulation for 10 min, the amount of spontaneous release increased 135.2 ± 8.6% ($P < 0.01$ vs no stimulation, Student's *t*-test). This increase disappeared when slices were perfused with low-Ca^{2+} Krebs–Ringer solution (before stimulation: 1.85 ± 0.38 fmol/SCN; under stimulation: 1.82 ± 0.03 fmol/SCN). Methamphetamine failed to affect the spontaneous release of VIP for 10 min ($P > 0.05$ vs vehicle treatment, Dunnett's test; Table 1). The release of VIP induced by optic nerve stimulation was attenuated significantly and in a concentration-dependent manner by treatment with methamphetamine in the range from 1 to 100 μM ($P < 0.05$ at 10 and 100 μM methamphetamine, Dunnett's test; Table 1).

Optic nerve stimulation-induced field potentials

Stimulation of the optic nerve induced a field potential in the ventrolateral SCN. Bath application of methamphetamine (10 μM) for 20 min attenuated the negative wave of the SCN field potential (Fig. 1A). However, these effects were completely reversed after 20–60 min of washing with normal Krebs–Ringer solution. Methamphetamine caused significant and concentration-dependent (1–100 μM) inhibition of the SCN field potential ($P < 0.01$ at 1, 10 and 100 μM methamphetamine) (Dunnett's test; Table 2), and maximal inhibition was achieved at a concentration of 100 μM (24.5 ± 1.34%).

Light-induced Fos expression

Exposure of saline-injected rats to light at circadian time 13.5 resulted in Fos expression within the SCN region (Fig. 2c). The majority of cells that displayed Fos-like immunoreactivity were located in the ventrolateral region of the caudal SCN, corresponding with the terminal field of the retinohypothalamic tract. Injection of methamphetamine 1 or 5 mg/kg methamphetamine 30 min before light stimulation reduced the number of Fos-like immunoreactive cells in a dose-dependent manner (Fig. 2d, e). At a dose of 5 mg/kg, methamphetamine reduced the number of Fos-like immunoreactive cells in the SCN region by approximately 70% (85.4 ± 5.2 cells/SCN for saline injection; 59.8 ± 5.6 cells/SCN for methamphetamine injection; $P < 0.05$, Dunnett's test; Table 3). Injection of methamphetamine (5 mg/kg) without light exposure did not induce Fos expression in the SCN (Fig. 2b).

Effects of methamphetamine on light-induced phase shifts of free-running rhythm in hamsters

Light stimulation 30 min after intraperitoneal injection of saline induced large and stable phase shifts of the free-running wheel activity rhythm (Figs 3, 4). Light exposure at circadian time 14 following a saline injection resulted in a significant phase delay of -1.27 ± 0.05 h ($n = 5$) ($P < 0.01$ vs non-light stimulation, -0.16 ± 0.90 h, $n = 3$; Student's t-test), while light stimulation at circadian time 20 caused a significant phase advance of $+2.51 \pm 0.23$ h ($n = 4$) ($P < 0.01$ vs non-light stimulation, $+0.19 \pm 0.063$ h, $n = 9$; Student's t-test; Fig. 3A, C). Injection of methamphetamine (10 mg/kg) 30 min prior to light exposure attenuated both the light-induced phase advances and delays (Fig. 3B, D). Injection of methamphetamine (10 mg/kg) at circadian time 20 caused about 65% inhibition of the light-induced phase advance ($+0.97 \pm 0.08$ h, $n = 4$, $P < 0.01$ vs saline + light), while 5 mg/kg inhibited the phase advance by about 50% ($P < 0.05$ saline + light; Dunnett's test; Fig. 4). Similarly, injection of 10 mg/kg methamphetamine 30 min prior to light stimulation at circadian time 14 inhibited light-induced phase delays (-0.855 ± 0.127 h, $n = 4$, $P < 0.05$ vs saline + light; Dunnett's test). Injection of 10 mg/kg methamphetamine without light exposure failed to alter the phase of the activity rhythm at both circadian times 20 (-0.17 ± 0.09 h) and 14 (-0.09 ± 0.04 h) (Fig. 4).

Methamphetamine-induced monoamine release

Although spontaneous 5-HT release was not detected, methamphetamine application for 20 min increased 5-HT release from the SCN slices in a concentration-dependent manner (Table 4). The absolute value of methamphetamine (100 μM)-induced 5-HT release was 1.84 ± 0.68 ng/SCN, and this value corresponded to 46.6 ± 1.03% of total 5-HT content ($P < 0.01$ vs 1 μM methamphetamine application, 9.13 ± 1.42%, $n = 4$, Dunnett's test).

Spontaneous noradrenaline release was detected at a level of 0.267 ng/SCN, and this value corresponded to 4.19 ± 0.91% of total noradrenaline content. However, methamphetamine application did not affect noradrenaline release (Table 4). Dopamine release was not detected from the SCN using our HPLC system.

Optic nerve stimulation-induced suprachiasmatic nucleus field potential in the p-*CPA- and DSP*-4-*treated rats*

We confirmed that *p*-CPA reduced the content of 5-HT in the cerebral cortex by HPLC (data not shown). In *p*-CPA-treated rats, bath application of 10 μM methamphetamine for 20 min did not inhibit optic nerve stimulation-induced SCN field potentials (Fig. 1B). Even 100 μM methamphetamine caused only slight inhibition of field potential in the *p*-CPA-treated animals (80.8 ± 7.8% for *p*-CPA-treated, 24.5 ± 13.4% for saline-treated, $P < 0.05$; Student's t-test; Table 2). In contrast, 10 μM methamphetamine inhibited the field potential of the SCN in the DSP-4-treated rats (Fig. 1C), similarly to saline-treated rats (Fig. 1A).

Effect of methamphetamine on light-induced phase advance in hamster with 5,7-DHT

In 5,7-DHT-treated hamsters, 10 mg/kg methamphetamine failed to reduce the light-induced phase

Table 1. Effects of methamphetamine on spontaneous and optic nerve stimulation-induced vasoactive intestinal polypeptide release from suprachiasmatic nucleus slices

		Methamphetamine (μM)		
	Vehicle	1	10	100
Non-stimulation	89.1 ± 13.9 (9)	84.7 ± 4.0 (2)	84.6 ± 11.2 (4)	95.2 ± 14.4 (2)
Stimulation	135.2 ± 8.6 (9)	107 ± 10.1 (2)	92.1 ± 17.1 (4)*	84.4 ± 9.0 (4)*

Spontaneous release of VIP from SCN slices in the presence of vehicle of methamphetamine (10 μl water) for 10 min was regarded as 100%. To examine the effects of methamphetamine on spontaneous release of VIP, this drug was applied for 10 min at Zeitgeber time 13 without stimulation of the optic nerve. To examine the effects of methamphetamine on optic nerve stimulation-induced VIP release, this drug was applied for 10 min at Zeitgeber time 13 with stimulation of the optic nerve. Vehicle indicates 10 μl water treatment. Data represent % of pre-release (mean $\pm$ S.E.M.). Numbers in parentheses indicate the numbers of slices examined. *$P < 0.05$ vs vehicle (Dunnett's test).

advance in hamster wheel-running rhythm ($+1.90 \pm 0.21$ h for 5,7-DHT-treated, $+0.97 \pm 0.082$ h for non-treated, $P < 0.01$; Student's t-test, Figs 3F, 4). Treatment with 5,7-DHT produced no effect on the light-induced phase advance of free-running activity rhythm.

DISCUSSION

In the present study, we examined whether methamphetamine modifies the photic entraining responses of the circadian clock in the SCN via 5-HT release. Methamphetamine application *in vitro* attenuated the release of VIP from the rat SCN induced by optic nerve stimulation, and also inhibited optic nerve stimulation-evoked field potentials in rat SCN slices. Furthermore, systemic administration of methamphetamine attenuated light-induced Fos expression in the rat SCN and also light-induced phase shifts in hamster wheel-running rhythm. Methamphetamine application induced 5-HT release from the rat SCN slices. In addition, reduction of 5-HT content attenuated the inhibitory effect of methamphetamine on optic nerve stimulation-induced SCN field potential and light-induced phase advances of hamster wheel-running rhythm. Although methamphetamine-induced rhythms in SCN-lesioned rats are independent of environmental lighting,[16,46] the present results demonstrated that methamphetamine

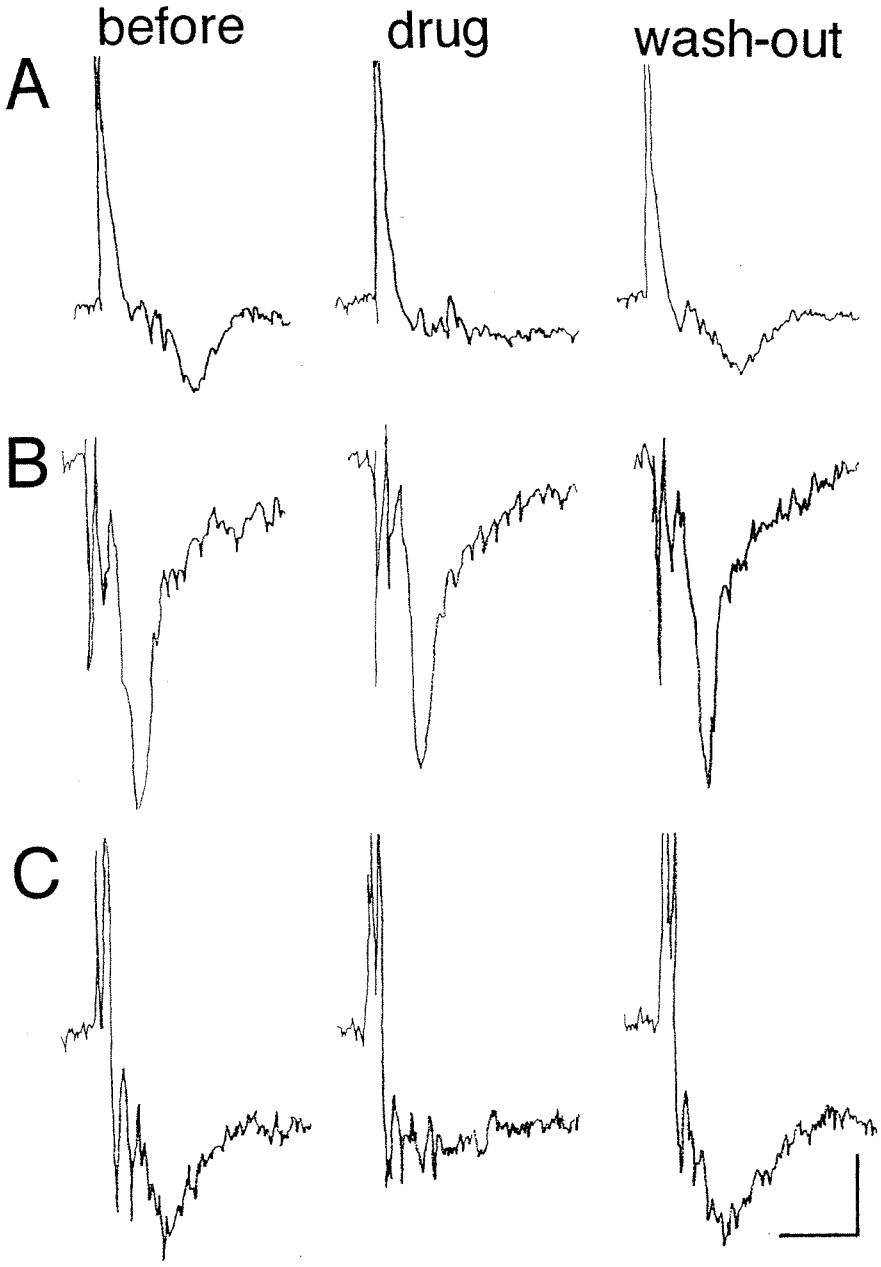

Fig. 1. Effects of methamphetamine on optic nerve stimulation-evoked field potentials in saline-(A), p-CPA-(B) and DSP-4 (C)-treated rat SCN slices. Representative examples. Each trace represents eight superimposed sweeps. Methamphetamine at 10 μM was applied for 20 min followed by 60 min washout. Calibration: 0.5 mV, 10 ms.

Table 2. Dose-dependent effects of methamphetamine on optic nerve stimulation-evoked field potentials in the suprachiasmatic nucleus from saline- or *p*-chlorophenylalanine methyl ester-treated rats

		Methamphetamine (μM)		
	Vehicle	1	10	100
Saline-treated	101 ± 3.0 (5)	54.1 ± 9.3 (4)**	29.6 ± 7.3 (3)**	24.5 ± 13.4 (3)**
p-CPA-treated	101 ± 3.0 (5)	86.4 ± 15.4 (4)	83.5 ± 12.0 (4)†	80.8 ± 7.8 (3)†

The control value before methamphetamine application was set to 100%. Data represent % of control value (mean ± S.E.M.). Vehicle indicates 10 μl water treatment. Numbers in parentheses indicate the numbers of slices examined. **$P < 0.01$ vs vehicle (Dunnett's test). †$P < 0.01$ vs saline-treated rats (Student's *t*-test).

can modify the photic entrainment of the circadian clock in the SCN via 5-HT release.

Exposure to constant light significantly decreases VIP- and peptide histidine isoleucine immunoreactivity in the SCN.[3] SCN levels of VIP and VIP mRNA have been reported to be higher at night than during the day under a light–dark cycle.[3,33,62] Microinjection of VIP, peptide histidine isoleucine and gastrin-releasing peptide into the SCN produces a phase delay when these peptides are administered during

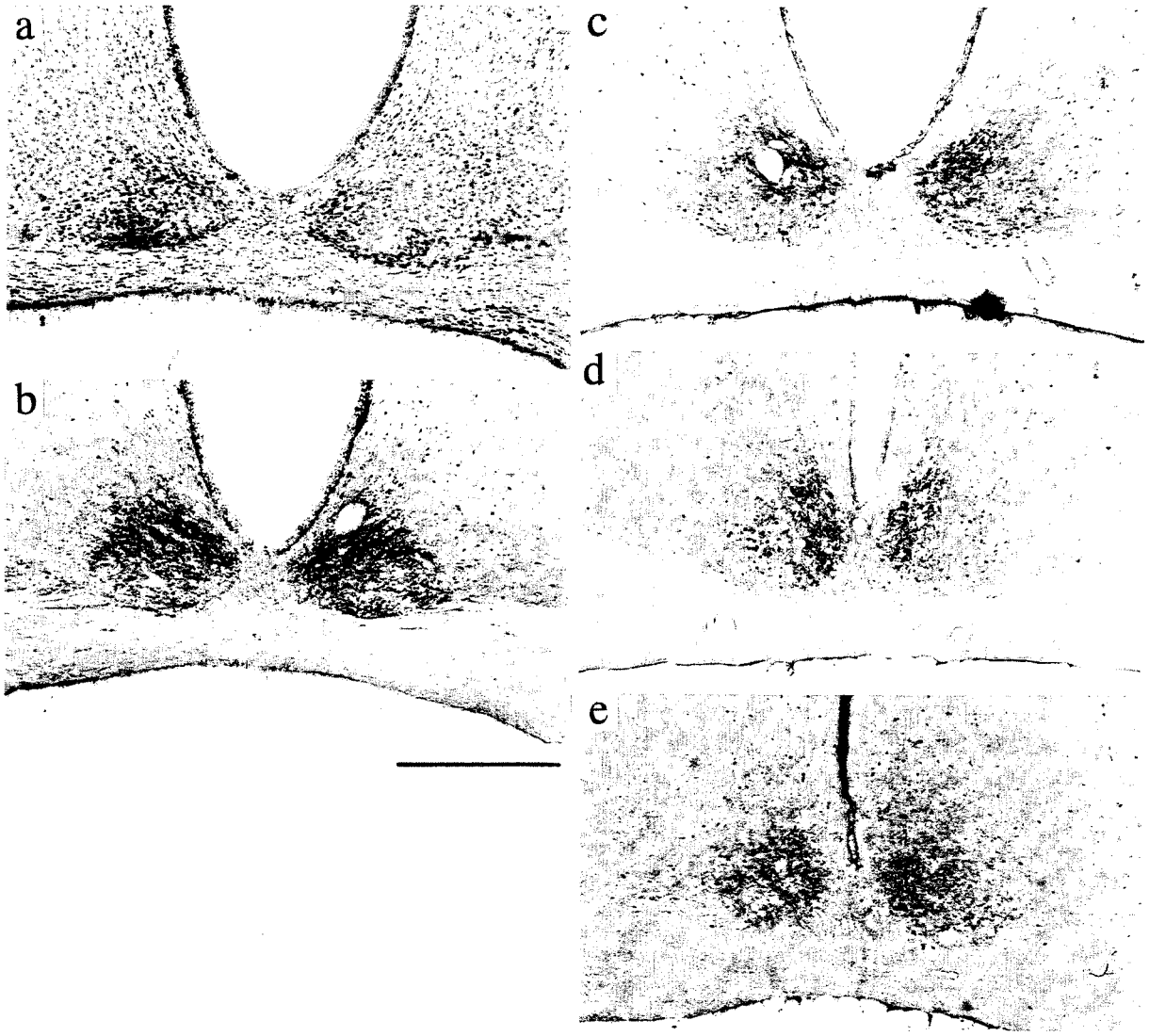

Fig. 2. Representative photomicrographs demonstrating the effect of systemic administration of methamphetamine on light-induced Fos expression in the rat SCN. Animals were maintained under constant darkness for two days, and then received intraperitoneal injection of either saline (c), 1 mg/kg methamphetamine (d) or 5 mg/kg methamphetamine (e) 30 min prior to light exposure (300 lux for 1 h) at circadian time 13.5. Methamphetamine was administered at circadian time 13.5 under constant darkness for two days (a, b). Animals were perfused 150 min after injection of methamphetamine. (a) Cresyl Violet staining; (b–e) Fos immunostaining. a and b were from the same animal. Scale bar = 1 mm.

Table 3. Dose-dependent effects of methamphetamine on light-induced Fos expression in the rat suprachiasmatic nucleus

	Methamphetamine (mg/kg, i.p.)			
	Saline	1	3	5
Number of Fos-like immunoreactive cells/SCN	42.7 ± 2.6 (6)	38.7 ± 4.9 (4)	36.1 ± 2.0 (4)	24.9 ± 2.8 (4)*

Data represent means ± S.E.M. Numbers in parentheses indicated the number of animals examined. Methamphetamine was administered 30 min before light exposure (300 lux for 1 h) at circadian time 13.5. Animals were perfused 120 min after onset of light exposure. *$P < 0.05$ vs saline controls (Dunnett's test).

the early subjective night.[4] Thus, VIP may be an important peptide in processing photic information in the SCN. Recently, we reported that NMDA treatment *in vitro* increased the release of VIP, and co-treatment with VIP and gastrin-releasing peptide during subjective night produced a phase delay of SCN activity rhythm.[56] In the present study, we demonstrated that optic nerve stimulation increased the release of VIP from SCN, and this increase disappeared when the slices were perfused with low-calcium solution. These observations indicate that optic nerve stimulation causes the release of VIP from the rat SCN in a Ca^{2+}-dependent manner. Thus, application of light pulses *in vivo* or optic nerve stimulation *in vitro* may increase the release of VIP, and released VIP may participate in the phase changes of circadian clocks in the SCN. Methamphetamine treatment concentration-dependently attenuated the release of VIP from the rat SCN induced by optic nerve stimulation, but did not affect spontaneous VIP release. This result suggests that methamphetamine dominantly attenuates the VIP release in response to optic nerve stimulation, although the mechanism of this inhibition is uncertain at present.

Electrical stimulation of the optic nerve elicits field potentials in the ventrolateral SCN regions, recorded using rat[27] or mouse[10] hypothalamic slices. Bath application of methamphetamine dose-dependently inhibited the optic nerve-evoked field potential. The present results indicate that methamphetamine may have an inhibitory effect on the regulation of neurotransmission in the retinohypothalamic pathway to the SCN.

Photic regulation of the expression of the immediate early gene c-*fos* in the rodent SCN has been demonstrated and studied extensively.[11,25,41–43,48] Although the functional significance of this regulation is unclear at present, Fos induction does appear to provide a useful marker for monitoring photic entrainment with the SCN. We found that administration of methamphetamine reduced the number of Fos-like immunoreactive cells throughout the ventral SCN. In contrast to the hamster, the ventral SCN is the only region in rats which shows Fos-like immunoreactivity in response to photic stimuli at night.[5,48,49] Administration of methamphetamine without application of a light pulse did not induce Fos-like immunoreactivity in the SCN. It has been reported that administration of methamphetamine induces Fos-like immunoreactive cells through activation of dopaminergic D_1 receptors.[7] As there is almost no D_1 receptor expression in the adult rat SCN,[64] methamphetamine administration alone failed to induce Fos-like immunoreactivity in the SCN.

We demonstrated that methamphetamine application increased 5-HT release but not that of noradrenaline release from rat SCN slices. In addition, reduction of 5-HT content by *p*-CPA attenuated the inhibitory effect of methamphetamine on optic nerve stimulation-induced field potentials. It was reported in a microdialysis study that methamphetamine infusion into the SCN increases extracellular 5-HT.[37] The SCN receives robust serotonergic projection from the midbrain raphe nuclei that terminates predominantly in the retinorecipient region of the SCN.[6,32] 5-HT projection to the SCN appears to preferentially terminate on VIP neurons.[8,20,22] Interruption of 5-HT neurotransmission reduces the VIP immunoreactivity of cell bodies and fibers in the SCN,[19,22] and 5-HT depletion by *p*-CPA decreases VIP mRNA in the SCN.[21] Therefore, these reports and our present results suggest that release of 5-HT by methamphetamine modulates VIP release and field potentials induced by optic nerve stimulation, and also light-induced Fos expression.

It is known that injection of serotonergic agonists during subjective day results in stable phase advances of the circadian rhythms.[28,38–40,54] This effect appears to be mediated through 5-HT_{1A} receptors.[14,53,63] In addition to the direct effect of 5-HT on the circadian oscillation, it has been reported that 5-HT has an inhibitory effect on the response of the SCN oscillator to light: (i) firing rates of light-activated SCN cells,[65] (ii) optic nerve stimulation-induced field potentials,[27,44] (iii) light-induced Fos expression in the SCN[15,44,51] and (iv) light-induced phase shifts of the free-running activity rhythm;[44] all these responses are inhibited by 5-HT_{1A} receptor agonists.[15,44] In the present study, we demonstrated that methamphetamine attenuated the light-induced phase shifts of hamster wheel-running rhythm, and this attenuation was not observed in hamsters with 5-HT depletion

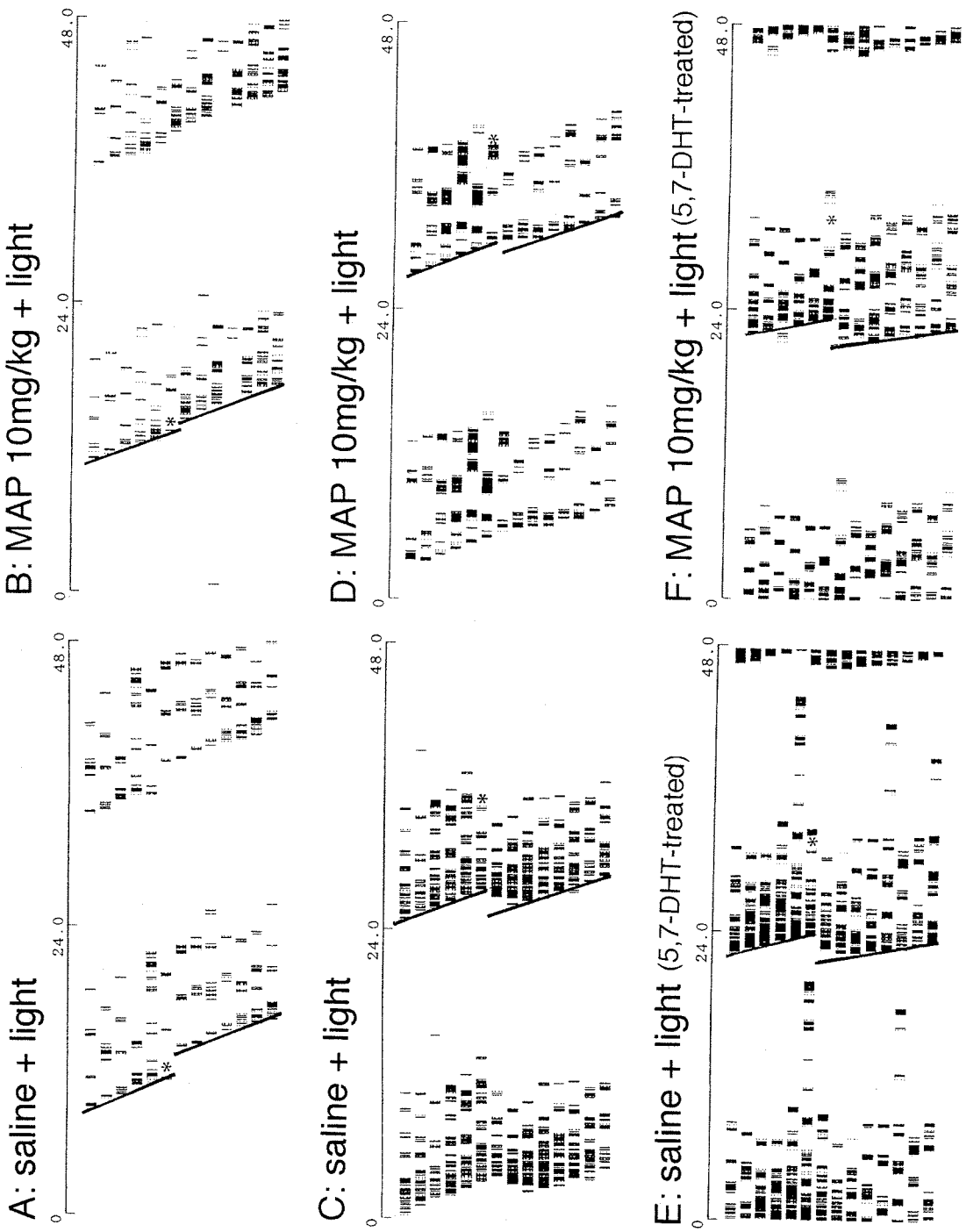

Fig. 3. Representative actograms demonstrating the effect of systemic administration of methamphetamine (MAP) on light-induced phase shift of the free running activity rhythm in non-treated (A–D) and 5,7-DHT-treated (E, F) hamsters. Hamsters were maintained in constant darkness and wheel-running behavior was monitored by computer. Hamsters received intraperitoneal injection of saline or methamphetamine at either circadian time 13.5 or 19.5, followed by light exposure (200 lux, 15 min). Approximate times of light exposure are indicated by the asterisks.

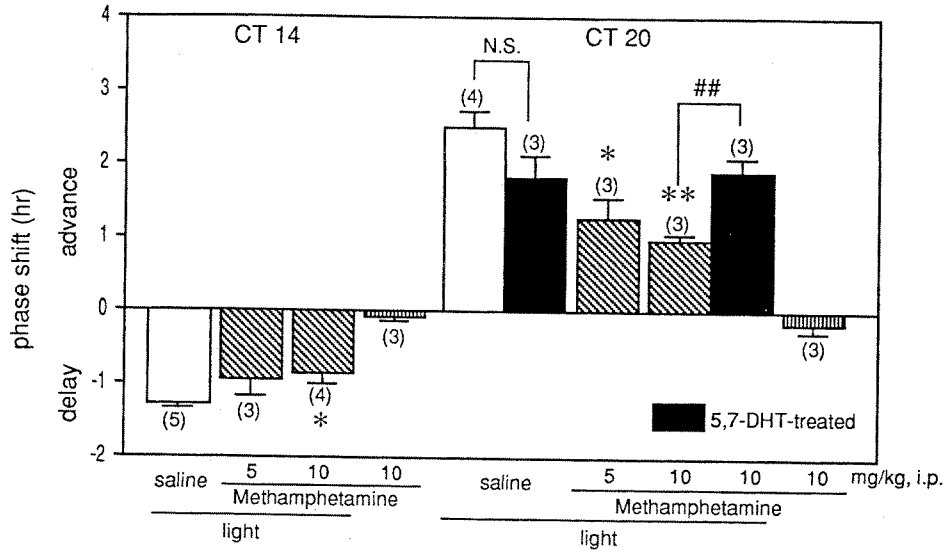

Fig. 4. Dose-dependent effects of methamphetamine on the light-induced phase shift of free-running activity rhythm in 5,7-DHT-treated hamsters. Data represent means ± S.E.M. Numbers in parentheses indicate the number of animals examined. $^{*}P < 0.05$, $^{**}P < 0.01$ vs saline + light (Dunnett's test), $^{**}P < 0.01$ (Student's t-test). N.S., not significant.

Table 4. Dose-dependent effects of methamphetamine on release of serotonin and noradrenaline from rat suprachiasmatic nucleus slices

		Methamphetamine (μM)		
	Control	1	10	100
Noradrenaline	4.2 ± 0.9 (12)	4.2 ± 1.9 (4)	4.5 ± 1.6 (4)	4.4 ± 1.3 (4)
5-HT	N.D. (12)	9.1 ± 1.4 (4)	17.4 ± 3.8 (4)	46.6 ± 1.0 (3)**

Methamphetamine was applied for 20 min. Data are expressed as % release of total content [release/(content + release) × 100] (mean ± S.E.M.). Numbers in parentheses indicate the numbers of slices examined. $^{**}P < 0.01$ vs 1 μM methamphetamine (Dunnett's test). N.D., release was not detected.

by 5,7-DHT. These reports, along with our present results, suggest that methamphetamine increases the release of 5-HT, and this released 5-HT inhibits the photic response via activation of 5-HT_{1A}-like receptors.

Methamphetamine is also known to induce release of noradrenaline in the CNS (for review see Ref. 50). However, in the present study, methamphetamine did not increase noradrenaline release from the rat SCN. This suggests that there are few releasable varicosities in the SCN. It is unlikely that noradrenaline is involved in photic entrainment in the SCN, because tyrosine hydroxylase-immunoreactive fibers are rarely found in the ventrolateral part of the SCN,[9] and noradrenaline content in the SCN of rats kept under light–dark cycle or constant darkness show significant variations over the day.[9] Furthermore, in the present study, reduction of noradrenaline content by DSP-4 did not affect the inhibitory effect of methamphetamine on optic nerve stimulation-induced field potential. This suggests that methamphetamine-induced inhibition of optic nerve–SCN synaptic transmission is unlikely to be involved in the noradrenaline release by methamphetamine. Thus, these observations suggest that the inhibitory effect of methamphetamine on the photic entraining response may rarely mediate noradrenaline release.

There is evidence that glutamate mediates photic information to the SCN through NMDA receptors.[1,26,55,56] Glutamate application has been reported to cause phase shifts of circadian rhythm *in vivo*[30] and *in vitro*.[13,58] Recently, it was reported that microdialysis application of 5-HT_{1A} receptor agonists reduced spontaneous release of glutamate from the SCN.[60] Taken together, these observations suggest that methamphetamine attenuates the photic entrainment via methamphetamine-induced 5-HT release, which may inhibit retinohypothalamic glutamate transmission. Retinohypothalamic transmission has been reported to be inhibited by methamphetamine (present study), 5-HT, a $GABA_B$ receptor agonist (baclofen) and glutamate receptor antagonists.

CONCLUSION

In this study, we made the following observations: (i) methamphetamine inhibits optic nerve

stimulation-induced VIP release and field potentials in SCN slices; (ii) methamphetamine inhibits light-induced Fos expression in the rat SCN and phase shifts in the hamster wheel-running rhythm; and (iii) reduction of 5-HT activity attenuated the effects of methamphetamine. Therefore, the present results suggest that methamphetamine inhibits the photic entraining response in the SCN predominantly by 5-HT release. It will be interesting to determine whether antidepressants and reuptake inhibitors of 5-HT exhibit effects similar to those of methamphetamine.

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