かった（Mann-Whitney-U-test）．

考　察　今後の定位・機能神経外科手術がフレームレスへの方向性をもつか否かは，大いに関心のもたれるところである．NeXframe フレームレスシステムは Henderson らによって報告された．彼らは，ファントムを用いた研究および臨床の場での検討にて，その精度には問題はないと報告している[9-11]．一方，Bjartmarz ら[1]は 14 症例の本態性振戦に従来型のフレームベースド定位脳手術と NeXframe を用いた手術を行って比較し，フレームベースドのほうが精度が高かったと報告している．この検討では，視床中間腹側核（Vim）を標的部位として一側をフレームベースド，他側をフレームレスで電極留置を行っている．最終的な位置確認はわれわれの研究と同様に定位 X 線装置を用いている．

ただし，DBS リードの留置位置が妥当であったかは，定位装置の精度だけの問題ではなく，MRI のゆがみや髄液流出によるブレインシフトなどによる影響も大きい．特に髄液流出によるブレインシフトは高頻度に起こり，留置位置に誤差を生じる．われわれの施設で Obuchi ら[17]が 50 症例をもとにまとめた結果においては，MC point は髄液流出により x 軸上で 0.02±0.39 mm，y 軸上で 1.27±0.7 mm，z 軸上で 0.11±0.34 と主に前後方向で大きなシフトが認められた．以上のことから，画像誘導下に決定された標的部位と，神経生理学的に同定された理想的な刺激部位との間には誤差が生じることを銘記する必要がある．

本研究においても標的部位の座標に向けた中心部位の trajectory に実際の DBS リードを挿入・留置したのは 50％以下であった．したがって，より適切な部位に電極を留置し治療効果を高めるためには，神経生理学的方法による再確認，特にベンガン法を用いた神経生理学的同定は不可欠といっても過言ではないであろう．

NeXframe フレームレスシステムはベンガン法との併用が可能となれば，その有用性をいっそう増すと考えられる．Eljamel ら[2]は multi-tract microrecording が可能なフレームレスシステムを独自に開発し報告しているが，これらはまだ商業ベースには乗っておらず，その精度や安全性についても未知である．われわれは NeXframe に特殊なアダプターを装着し，ベンガン法を可能としたシステムを使用し，その経験を報告した．本システムの精度は従来から用いられているフレームベースドと変わらない程度といえる．システム自体に由来するトラブルもなく，その安全性と精度は今までの報告どおり高いものと考えられた．ただし，ベンガン法を用いるための十分な術野が確保できない点は，今後改善されるべき課題である．従来のフレームレスシステムのように single-tract の記録に限れば，電極カニューレ挿入部の凝固止血はさほど困難ではないが，複数本の電極カニューレの挿入が必要なベンガン法では少なからずリスクを伴うと考えられた．

VI. 終わりに

定位・機能神経外科手術は，最近のニューロモデュレーション治療の発展に伴い重要性を増している．いかに安全に正確に標的組織に電極を留置するかが最も重要な課題であるが，いかに患者のストレスを少なくし，簡便に手術手技が行えるかということも重要である．

今後この領域はより多くの適応症例を扱うことになるであろう．そうした中にあって，多くが局所麻酔下に行われる本手術は，手術利益と手術合併症のバランスに加え，患者ストレスの軽減ということについても，いっそうの検討が必要になると思われる．

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Post-stroke pain に対する大脳皮質運動野の反復経頭蓋磁気刺激による効果

－ドラッグチャレンジテストの結果との比較から－

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要　旨

Post-stroke pain に対する反復経頭蓋磁気刺激療法（rTMS）の除痛効果とドラッグチャレンジテストの結果を比較し，rTMS におけるドラッグチャレンジテストの意義について検討した．rTMS は疼痛部位に相当する大脳皮質運動野を刺激し，sham 刺激の効果との比較も行った．ドラッグチャレンジテストには，ケタミン，モルヒネ，チオペンタールを用いた．rTMS は sham 刺激と比べ，刺激直後，刺激後 180 分，300 分，24 時間後に VAS の有意な低下を認めた．また，ドラッグチャレンジテストでは，ケタミン，モルヒネ，チオオペンタールによる VAS の最大減少率と rTMS による VAS の最大減少率に相関を認めた．rTMS による大脳皮質運動野刺激は post-stroke pain の除痛に有効である．また，ドラッグチャレンジテストは rTMS の有効率を高めるとともに，併用療法の選択にも有用である．（ペインクリニック 32：1857-1865，2011）

キーワード：post-stroke pain，反復経頭蓋的磁気刺激療法，ドラッグチャレンジテスト

はじめに

Post-stroke pain は，体性感覚系の知覚求心路が損傷を受けた後，二次的に出現する神経障害性疼痛であり，脳実質に生じた血管障害を病巣とする．Dejerine と Roussy の報告[1)]以来，責任病巣は視床といわれてきたが，内包や視床皮質間線維などの障害でも出現し，脳卒中の 1～8％に発症すると報告されている[2,3)]．一度発症すると自然緩解することはなく，薬物療法や神経ブロック療法などが奏効しづらく，治療に難渋する症例が多い．

Post-stroke pain に対しては，大脳皮質運動野刺激療法（motor cortex stimulation：MCS）が有効であることが報告されている[4,5)]．その臨床経験を基に，非侵襲的な反復経頭蓋磁気刺激療法（repetitive transcranial magnetic stimulation：rTMS）による大脳皮質運動野刺激が考案され，有効例が報告されている[6,7,8)]．また，治療が困難なことが多い post-stroke pain にお

〈Original Article〉
Motor cortex rTMS for post-stroke pain : Comparison with the results of drug challenge test
Yuhei Matsumura, et al
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表1　Post-stroke pain 症例の概要

症例	性別	年齢（歳）	原因疾患	疼痛部位	疼痛罹病期間（カ月）
1	女	68	右被殻出血	左側上下肢	36
2	女	71	左視床梗塞	右側上下肢	36
3	男	74	左視床梗塞	右側上下肢	7
4	男	59	右視床出血	左側上下肢	12
5	男	85	左延髄外側梗塞	左側顔面	10
6	男	54	右被殻出血	左側上下肢	21
7	女	68	左視床出血	右側顔面	6
8	女	59	右視床梗塞	左側顔面	60
9	男	61	右視床出血	左側上下肢	102
10	男	64	左延髄外側梗塞	右側下肢	25
11	女	79	左被殻出血	右側下肢	74
12	女	67	右脳幹出血	左側下肢	21
13	女	60	左視床出血	右側上下肢	45
14	男	54	右視床出血	左側上下肢	72
15	男	60	左被殻出血	右側上下肢	180
16	女	61	左視床出血	右側上下肢	12

いて，ドラッグチャレンジテスト（drug challenge test）によって痛みの薬理学的背景を明らかにすることは，治療方針を決定するのに有用である[9]．MCSの除痛効果とドラッグチャレンジテストの結果との比較については報告されているが[10]，rTMSを用いた大脳皮質運動野刺激による除痛効果とドラッグチャレンジテストの結果との比較については明らかでない．

そこで，本研究では，post-stroke painにおけるrTMSを用いた大脳皮質運動野刺激による除痛効果と，ドラッグチャレンジテストの結果との比較を行った．

Ⅰ．対象と方法

1）対　　象

日本大学医学部附属板橋病院脳神経外科に，2010年4月から11月までの間にpost-stroke painの診断で紹介され，疼痛の出現領域に対応した脳血管障害を認める16症例（男性8症例，女性8症例，年齢54～85歳，平均年齢65±8.5歳）を対象とした．原因疾患は脳出血11症例，脳梗塞5症例であった．全症例が，疼痛発症から6カ月以上経過しており，平均疼痛罹病期間は44.75カ月であった（表1）．

2）反復経頭蓋磁気刺激（rTMS）

rTMSはプラセボ効果などを確認するために，各症例に対してsham刺激と本刺激を行った．患者をヘッドレスト付きリクライニングチェアに座らせ，頭部顔面を固定して刺激を行った．rTMSの方法は，リアルタイムにコイルの位置ならびに脳表に対するコイルの角度をモニターすることができる光学式TMS用ナビゲーションシステム（Brainsight™ Frameless Navigation System：Rogue Research社）を用いた．このシステムは，患者MRIの脳表画像を用いて，解剖学的に正確な位置に経頭蓋的磁気刺激を行うことができる．このため，全症例に，あらかじめ頭部MRI撮影を行っている．なお，てんかんの既往や心臓ペースメーカなどの体内に植え込み装置が留置されている症例はあらかじめ除外している．

刺激部位は，疼痛部位に対応する大脳皮質運動野（疼痛側と対側）に定め，コイルの刺激点が中心になるように70mmの8の字コイル（PN9925-00：Magstim Company, Whitland社）を患者頭部に専用固定具で固定し，Magstim Super Rapid（Magstim Company, Whitland社）で，反復刺激を行った．刺激条件は，日本臨床神経生理学会が推奨するWasser-

mann のガイドライン[11]にしたがって，50 μV 以上の motor evoked potential（MEP）を刺激 10 回中 5 回以上誘発できる最低刺激強度を求め，これを安静時運動閾値（resting motor threshold）とした．安静時運動閾値と同じ刺激強度（100％の刺激強度）を用いて，5 Hz の周波数で，50 発の刺激（10 秒の刺激時間）を 1 分ごとに 10 回（計 500 発）施行した．

Sham 刺激では，頭蓋骨に対してコイルを 45 度傾けながら離し，本刺激と同条件で刺激を行った．Sham 刺激と本刺激の間隔は刺激の影響が消失すると予想される約 48 時間以上の期間を置いて施行した[8]．

3）ドラッグチャレンジテスト

ドラッグチャレンジテストには，ケタミン，モルヒネ，チオペンタールの 3 種類の薬物を使用した．静脈路確保後，プラセボ効果を判定するため生理食塩水を 5 分間隔で 2 回投与し，ケタミンテストは，塩酸ケタミン 5 mg を 5 分間隔で 5 回，合計 25 mg まで静脈内投与した．モルヒネテストは，同様に塩酸モルヒネ 3 mg を 5 分間隔で 6 回，合計 18 mg まで静脈内投与した．チオペンタールテストは，同様にチオペンタールナトリウム 50 mg を 5 分間隔で 5 回，合計 250 mg まで静脈内投与し，途中で入眠した場合は，その時点で中止とした．

4）効果判定

疼痛の評価には，visual analogue scale（VAS）を用い，副作用についても検討した．rTMS では，本刺激と sham 刺激ともに，刺激開始前，刺激直後，刺激後 60 分，120 分，180 分，240 分，300 分，24 時間後（前日の刺激を行った同一時間）に測定し，

（刺激前 VAS－刺激後 VAS）

÷刺激前 VAS×100％＝％ VAS

として VAS 減少率を算定した．刺激前と比較して 30％以上 VAS が減少したものを rTMS の有効症例とした．また，patient global impression of change（PGIC）を刺激後に評価した（Rank 1：very much improved，2：much improved，3：minimally improved，4：no change，5：minimally worse，6：much worse，7：very much worse）．

ドラッグチャレンジテストでは，薬物投与開始から 60 分後まで 5 分間隔で VAS を連続的に測定し，

（薬物投与前 VAS－薬物投与後 VAS）

÷薬物投与前 VAS×100％＝％ VAS

として VAS 減少率を算定した．薬物投与前と比較して，VAS が 40％以上減少した症例を sensitive 症例，40％以下の症例を resistant 症例とした．本研究は，日本大学医学部倫理委員会の承認を得ており，すべての患者に対して研究の目的と意義を十分に説明し，書面での同意を得た．

5）統計処理について

rTMS による除痛効果の解析では，本刺激と sham 刺激で差があるかどうか Wilcoxon の符号付き順位検定を用いて，$p<0.05$ を有意とした．rTMS とドラッグチャレンジテストによる最大 VAS 減少率の比較には Spearman の順位検定を用いて，両者の相関関係について検討した．また，ドラッグチャレンジテストにおいて，モルヒネ，ケタミン，チオペンタールの少なくとも 1 種類のテスト薬物に sensitive 症例と，すべての薬物に resistant 症例との比較において，刺激による除痛効果を Mann-Whitney の U 検定で解析を行い，$p<0.05$ を有意とした．

Ⅱ．結　果

rTMS による全症例での平均 VAS 減少率は，sham 刺激 3.37％，本刺激 17.58％，最大 VAS 減少率は，sham 刺激 19.30％，本刺激 36.77％であり，ともに本刺激は sham 刺激と比べ優位に VAS の減少がみられた（$p<0.05$）（**図 1**）．刺激直後，刺激後 60 分，120 分，180 分，240 分，300 分，24 時間後の各時点で平均すると，本刺激において刺激直後に最も高い除痛効果が認められた．また，sham 刺激と比べ，刺激直後，刺激後 180 分，300 分，24 時間後に有意に VAS

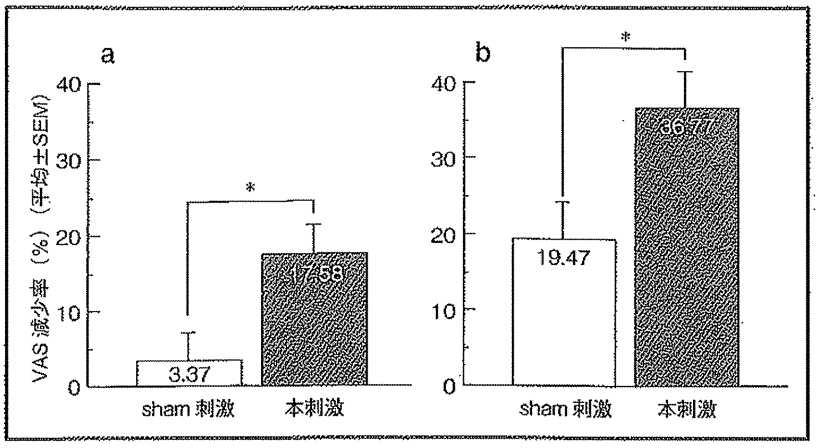

図1　Sham 刺激と本刺激の除痛効果の比較
Sham 刺激と本刺激の除痛効果を全症例比較したところ，平均 VAS 減少率（a），最大 VAS 減少率（b）ともに本刺激の方が優位に VAS の減少がみられた．*：$p<0.05$（Wilcoxon の符号付き順位検定）

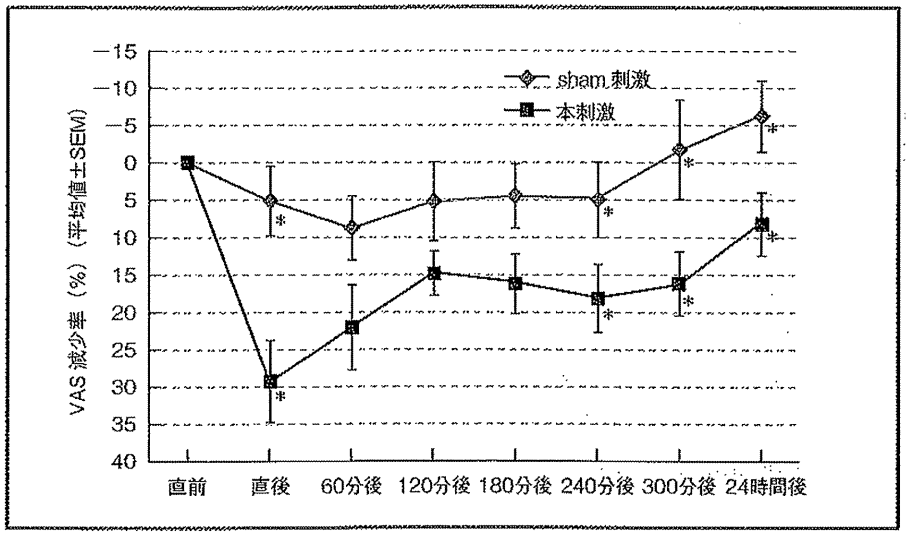

図2　rTMS による大脳皮質運動野刺激後の経時的除痛効果
本刺激が sham 刺激と比べ，刺激直後，刺激後 180 分，300 分，1 日後に有意な VAS の低下を示した．*：$p<0.05$（Wilcoxon の符号付き順位検定）

の減少を示した（$p<0.05$）（**図2**）．rTMS 有効症例は，9/16 症例（56.25%）であった．PGIC scale では，sham 刺激で 4 症例が rank 3（minimally improved），12 症例が rank 4（no change）であったが，本刺激では 1 症例が rank 1（very much improved），1 症例が rank 2（much improved），8 症例が rank 3（minimally improved），6 症例が rank 4（no change）であった（**図3**）．その他，プロトコールを逸脱した症例，痙攣などの rTMS と因果関係が考えられる有害事象は認めなかった．

ドラッグチャレンジテストで ketamine-sensitive 症例は 8/16 症例（50%），morphine-sensitive 症例は 3/16 症例（18.75%），thiopental-sensitive 症例は 5/16 症例（31.25%）であり，ketamine-sensitive 症例が最も多く認められた．すべての薬物に resistant 症例は，6/16 症例（37.5%）存在した．

全症例におけるドラッグチャレンジテストと rTMS による最大 VAS 減少率との比較では，ケタミンテスト（$R=0.629$，$p<0.01$），モルヒネテスト（$R=0.622$，$p<0.01$），チオオペンタールテスト（$R=0.545$，$p<0.05$）であり，rTMS の除痛効果 VAS の減少率はケタミン，モルヒ

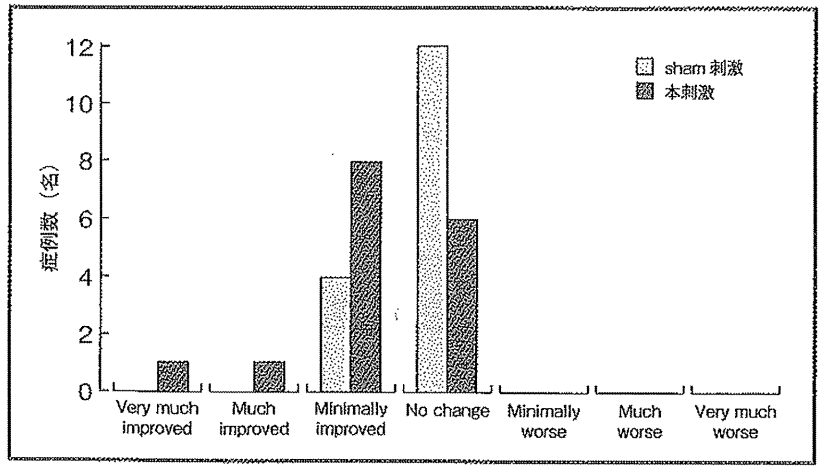

図3　rTMS による大脳皮質運動野刺激後の PGIC
Sham 刺激で 4 症例が rank 3（minimally improved），12 症例が rank 4（no change）であったが，本刺激では 1 症例が rank 1（very much improved），1 症例が rank 2（much improved），8 症例が rank 3（minimally improved），6 症例が rank 4（no change）であった

ネ，チオペンタールのいずれのテストとも正の相関を認めた（**図4**）．1 種類以上の薬物における sensitive 症例と，すべての薬物における resistant 症例において，rTMS での刺激による除痛効果を各時点ごとで平均すると，いずれかの薬物に sensitive 症例では，どの薬物にも resistant 症例と比較して，刺激後 60 分後まで有意に VAS の減少を認めた（$p<0.05$）（**図5**）．

Ⅲ．考　察

Post-stroke pain に対する刺激療法で，明らかな効果が報告されているのは大脳皮質運動野刺激[4,5]である．MCS は開頭術で電極を留置する必要があり，外科的手術を要する．その臨床経験を踏まえ，Migita[12]らが非侵襲である rTMS を用いた大脳皮質運動野刺激を行い，神経障害性疼痛に有効であることを最初に報告した．その後，世界中で臨床研究が進み，今までに多数の報告がなされている[6-8,13-15]．rTMS では，刺激頻度が 1 Hz を超えるものが高頻度 rTMS（fast rTMS），1 Hz 以下のものが低頻度 rTMS（slow rTMS）と定義されている[16]．5 Hz 以上の高頻度 rTMS の刺激は皮質に対して興奮性に作用し，1 Hz 以下の低頻度 rTMS の刺激は抑制性に作用すると考えられている[17]．

Post-stroke pain には，5～20 Hz の刺激で明らかな除痛効果が 50％以上の症例で認められることが報告されている[13]．本研究においても，post-stroke pain に対する rTMS の大脳皮質運動野刺激が疼痛の軽減に有効であることが示された．**図2**で示すように，sham 刺激と比較して，刺激直後，刺激後 180 分，300 分，24 時間後において統計学的に有意な VAS の低下を認めた．しかし，他の報告と同様に，今回用いた単回の刺激では除痛効果は一時的であった[6]．Khedr ら[14]は，連続 5 日間の刺激を行うことで，2 週間持続する除痛効果が得られたと報告しており，連日の刺激を行うことで持続的な除痛効果が得られるものと期待される．

本研究のプロトコールを逸脱した症例，および痙攣などの rTMS に因果関係のある副作用が認められない結果から，単回の刺激での安静時運動閾値（100％の刺激強度），5 Hz の周波数，計 500 発の刺激は安全であることが確認された．疼痛部位では，顔面痛を有する 2 症例において最も除痛効果が高く，ともに PGIC が rank 2 以上であった．Lefaucheur ら[6]は，60 症例の解析で顔面の疼痛で除痛効果が高いと報告しており，本研究でも同様の結果が得られた．

大脳皮質運動野刺激の除痛機序に関しては，脊髄後角内で侵害受容ニューロンを直接に抑制

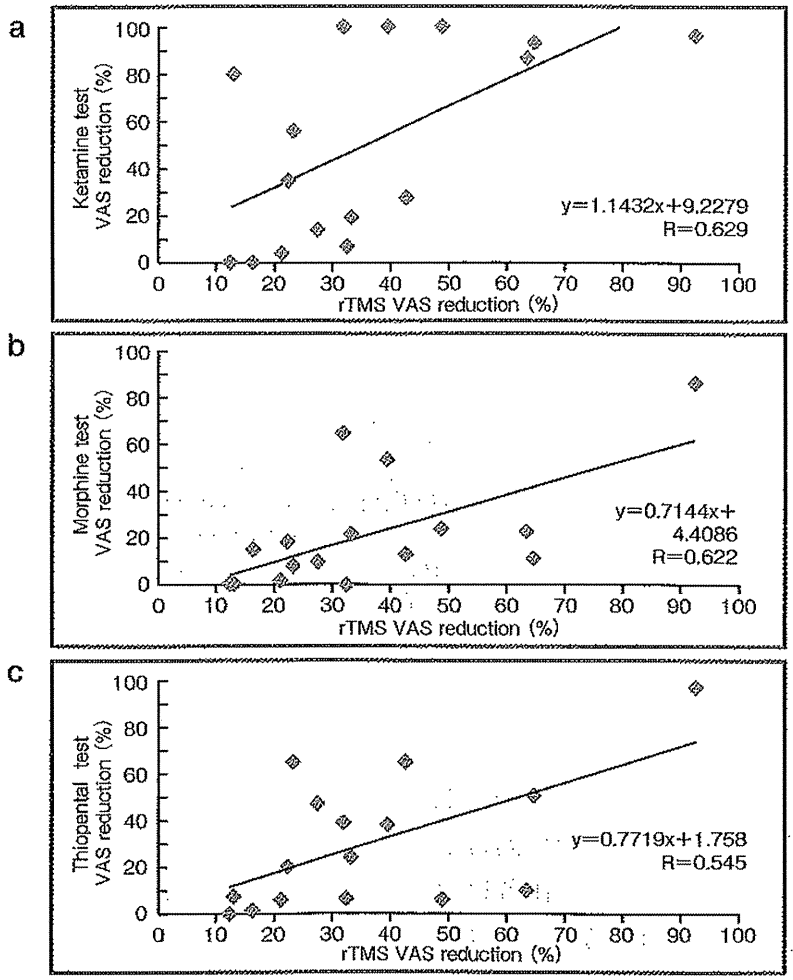

図4　ドラッグチャレンジテストとrTMSの除痛効果の比較
a：ケタミンテスト，b：モルヒネテスト，c：チオペンタールテスト．
ケタミン，モルヒネ，チオペンタールとrTMSによる最大VASの減少率において相関を認めた（ケタミンテスト：$p<0.01$，モルヒネテスト：$p<0.01$，チオペンタールテスト：$p<0.05$）

する事実[18]に加えて，視床，前帯状回，前頭眼窩野，島，脳幹上部などの血流増加を認めることが報告されている[19]．Functional MRIを用いた大脳皮質運動野刺激の研究でも，情動系や下行性疼痛抑制系の賦活による除痛効果が報告されており[20]，rTMSによる除痛には，大脳皮質，視床，脊髄後角などが深く関与しているものと考えられる．また，大脳皮質運動野刺激によって，内因性オピオイドの増加が認められることから，rTMSとMCSによる除痛効果の持続には内因性オピオイドの増加も関与するものと考えられている[21,22]．さらに，神経障害性疼痛では，障害された部位だけではなく，高位の中枢神経系にも機能的な可塑性変化が生じているといわれており，神経刺激療法は神経系を修飾して可塑性変化を起こす機序が想定される[15]．

ドラッグチャレンジテストは，個々の症例における疼痛の薬理学的背景を理解するのに有用である．この評価方法の特徴は，プラセボ投与から始め，少量ずつ段階的に薬物を投与するので，少量から連続的に多量投与までの効果を確認できることである[23]．脳脊髄刺激療法の適応決定のみではなく，テストの結果を基にした薬物の併用療法を加えることができる．

1997年に，山本らは，MCSの除痛効果とケタミンテストとチオペンタールテストに相関関係があり，ケタミンテスト，チオペンタールテストでsensitive症例で，モルヒネテストでre-

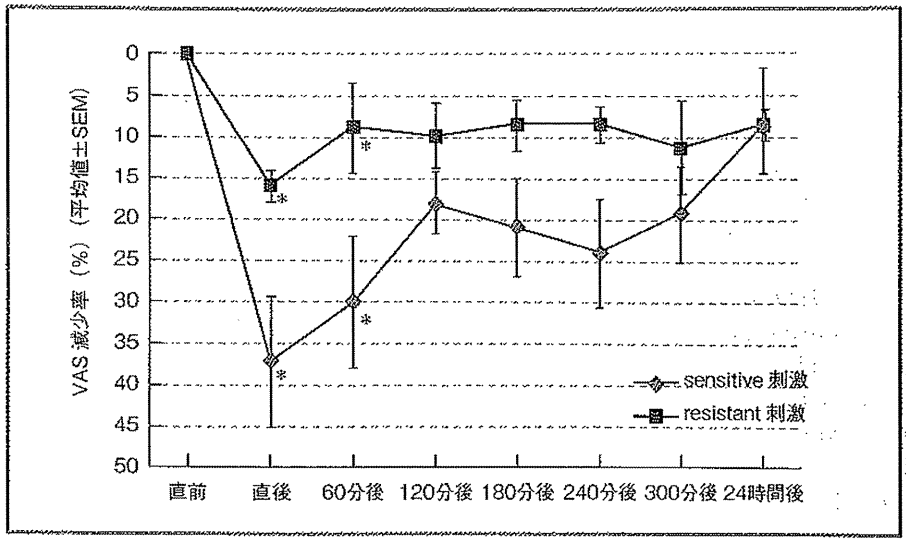

図5　ドラッグチャレンジテストで1つ以上の薬物が sensitive 症例とすべての薬物が resistant 症例の rTMS による除痛効果
刺激後 60 分後まで resistant 症例に対し，sensitive 症例が有意な VAS の低下を認めた．*：$p<0.05$（Mann-Whitney の U 検定）

sistant であった症例には，10/14 症例（71%）において MCS で除痛効果を認めたが，どの薬物にも resistant 症例では，除痛効果を認めた症例はわずか 1/8（12.5%）のみであったと報告している[10]．本研究においては，rTMS の除痛効果がケタミンテスト，モルヒネテスト，チオペンタールテストのいずれのテストとも相関を認めた．ドラッグチャレンジテストで用いた3種類の薬物のうち，1つ以上が sensitive 症例は，どの薬物も resistant 症例と比較して，rTMS の刺激による VAS の低下が刺激後 60 分後まで低下した．また，rTMS の刺激による VAS の低下が，3種類の薬物のいずれとも相関を認めた事実は，post-stroke pain の背景の多様性を示しているものと考えられる．さらに，rTMS の除痛効果は MCS の短期の疼痛軽減効果と相関することが報告[24]されており，ドラッグチャレンジテスト同様に脳脊髄刺激療法の手術適応を決定するに有用である．

Loezer ら[25]の報告を始め，知覚求心路の損傷によって視床，大脳皮質感覚野，脊髄後角などでニューロンの過剰活動が出現することが確認されている[26,27]．このニューロンの過剰活動には興奮性アミノ酸の関与が報告されており[28]，*N*-methyl-*D*-aspartate（NMDA）受容体ブロッカーであるケタミンならびに興奮性アミノ酸のシナプス伝達を抑制するバルビタール系薬物の効果が確認されている[29]．その中でも，ケタミンは central sensitization を改善させることが報告されている[30]．本研究において，ケタミンが rTMS による除痛効果の相関が著明であった．除痛効果が一過性である rTMS に低用量ケタミン点滴療法[23]を併用することで除痛効果を増強できると示唆される．

本研究の結果から，ドラッグチャレンジテストは，post-stroke pain の治療において，rTMS による除痛効果予測や併用療法を決定するのにも有用であり，重要なテストであると考えられる．rTMS は非侵襲で簡便かつ安全性が高く，今後，更なる発展と治療法の確立が望まれる．

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Corticospinal Descending Direct Wave Elicited by Subcortical Stimulation

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Summary: Recent studies have indicated the importance of subcortical mapping of the corticospinal tract (CT) during tumor resection close to the primary motor area. It is substantial evidence that the corticospinal descending direct wave (D-wave) can be used as a guide for mapping of the primary motor cortex (M1) and for monitoring of the CT functional integrity. In the present study, the authors investigated the feasibility of D-wave recordings after subcortical stimulation. The authors examined 14 patients with brain tumors close to the M1 and/or CT, who exhibited no obvious motor deficit before surgery. Subcortical white matter was electrically stimulated in monopolar or bipolar fashion by recording the descending wave (D-wave) from the spinal epidural space using a catheter-type electrode. Subcortical D-wave was more clearly recorded after monopolar stimulation than after bipolar stimulation. The features of the subcortical D-wave, including its waveform, conduction velocity, and latency, were nearly identical to those of the corticospinal D-wave recorded after M1 stimulation. Subcortical D-wave amplitude was prone to change depending on the distance from the stimulation points to the CT. Changes in parameters of subcortical D-wave may provide valuable information to prevent postoperative motor deficit. Further studies are required to clarify the relationship between the distance from the stimulating point to the CT and the amplitude of the subcortically elicited D-wave.

Key Words: D-wave, Motor evoked potential, Intraoperative mapping, Subcortical stimulation, Primary motor cortex.

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For tumor resection close to the motor pathway, several types of functional mapping, such as preoperative neuroimaging and intraoperative electrophysiologic techniques, have been extensively used over the past few decades to decrease intraoperative morbidity. In particular, motor evoked potential measurement after direct electrical stimulation of the motor cortex has been used as an intraoperative mapping and monitoring method during surgery (Duffau et al., 2003; Fujiki et al., 2006; Katayama et al., 1988; Kombos et al., 1999; Yamamoto et al., 2004). Until recently, attention has been mainly paid to functional mapping of the motor cortex. However, brain tumors, such as glial tumors, commonly invade subcortical structures and the cortices, and thus, permanent deficits may occur because of surgical impairments to eloquent pathways running through the subcortical structures.

Recently, subcortical mapping has been regarded as a new methodology to achieve successful neurosurgical operations. For the intraoperative neurophysiologic mapping of subcortical structures, a stimulation method using the same procedures as those used for cortical mapping and recording responses from muscles has been reported (Duffau et al., 2003; Keles et al., 2004).

However, the corticospinal direct wave (D-wave) has been shown as a useful indicator to locate the primary motor cortex (M1) and for mapping and monitoring of the corticospinal tract (CT) functional integrity during surgery (Katayama et al., 1988; Yamamoto et al., 2004). More than 50 years ago, Patton and Amassian (1954) recorded D-wave from medulla after M1 stimulation in experimental animals. Following their report, it has been repeatedly demonstrated that the D-wave can be recorded from the lateral column of the spinal cord or the spinal epidural space in experimental animals (Amassian et al., 1987; Levy et al., 1984).

From previous reports, the D-wave can be recorded only when M1 is stimulated (Amassian et al., 1987; Levy et al., 1984; Patton and Amassian, 1954). Furthermore, unlike the muscle response to motor cortex stimulation, the D-wave is resistant to general anesthesia and relaxant because no interpolating synapses exist between stimulating and recording points. Therefore, recording of the D-wave is very convenient for identification of M1 and consequently reducing the neurologic complications resulting from surgical damage to CT.

The corticospinal motor evoked potential response consists of an initial D-wave and a later sequence of volleys termed "I-waves." The corticospinal D-wave represents activity generated from the direct activation of CT axons, whereas the I-wave reflects those generated from the indirect activation of CT neurons *via* synaptic activity (Katayama et al., 1988; Patton and Amassian, 1954; Yamamoto et al., 2004).

These statements are supported by the following evidences. First, it has been shown that the D-wave can be elicited by double-pulse stimulation at interstimulus intervals of 2 milliseconds (Deletis et al., 2001; Katayama et al., 1988, 1994). Second, the D-wave can be evoked only by the stimulation of restricted areas corresponding to the M1 location (Katayama et al., 1988, 1994; Yamamoto et al., 2004). Furthermore, the disappearance of the response is observed when surgical damage occurs in these areas (Fujiki et al., 2006; Katayama et al., 1988, 1994).

However, the methodology for eliciting and recording cortical D-wave cannot prevent injury to the subcortical part of CT because it cannot indicate its location. In the present study, the methodology of subcortical stimulation with D-wave recording from the spinal epidural space was presented.

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METHODS

Patients

We examined 14 patients with brain tumors around the M1 and/or CT and who did not have obvious motor deficits before surgery. The patients' demographic, clinical, and neurophysiologic data are summarized in Table 1. This study was approved by the Committee for Clinical Trials and Research in Humans at our Nihon University School of Medicine.

Stimulation Method

The first part of the procedure for stimulation and recording of subcortical D-wave was carried out by identification of M1 and recording D-wave. Before brain tumor resection, the cortices were stimulated directly with nearly same type of standard bipolar cortical probe as one used for subcortical stimulation (Haglund et al., 1993) to identify M1. This bipolar cortical probe had 2 contacts of 1.5 mm in diameter, spaced by 5 mm (bipolar stimulation electrode TN202-134; Unique Medical Co, Ltd, Tokyo, Japan). After M1 identification, a multicontact plate electrode was placed over M1 for stimulation and monitoring functional integrity of the M1 and CT during tumor resection.

The multicontact plate electrode had 4 contact points of 3 mm in diameter, spaced 10 mm apart. The plate electrodes were embedded in a thin and soft silicone material to maintain good contact with the cerebral tissue.

In addition, we used a monopolar stimulation for subcortical mapping. This monopolar electrode had a silver ball contact of 2.3 mm in diameter at the tip. The length of the electrode was 20.0 cm, and 10.0 cm apart from the tip electrode is flexible for easy adjustment for subcortical stimulation (monopolar stimulation electrode TF208-063; Unique Medical Co, Ltd). We applied both bipolar and monopolar stimulations for subcortical mapping and compared the responses evoked by both methods.

We attempted to evoke responses by bipolar stimulation of M1 and by monopolar and bipolar stimulations of CT running in the subcortical white matter. After exposure of the cerebral cortex, mapping for M1 identification was started, and then, monitoring of the corticospinal D-wave and subcortical mapping were performed.

For each mapping and monitoring procedure, the stimulations were usually applied as anodal/cathodal biphasic square pulses of 0.2- to 0.4-millisecond duration delivered at a stimulation rate 2 to 5 Hz. The stimulation intensity was usually lower than 25 mA for cortical mapping and for monitoring of the corticospinal D-wave. For subcortical mapping, stimulation intensity was 25 mA. When we used monopolar stimulation, referenced electrode was fixed at the forehead.

Recording Method

D-wave was recorded from the electrode placed in the epidural space. For this purpose, we used a flexible five-contact platinum catheter-type electrode (spinal epidural catheter-type recording electrode TF 209-092; Unique Medical Co, Ltd) inserted into the epidural space of cervical spine, usually from the C-2 to C-3 levels, the day before surgery (Fig. 1). For the placement of recording electrode, patient was placed in prone position, and an 18-gauge epidural Tuohy needle was inserted into the midline epidural space at the cervicothoracic junctions under radiographic control. The catheter-type electrode was inserted into the epidural space with a stylet in the epidural needle and advanced to the appropriate position. The spinal epidural space was identified by changing the resistance of injected saline through the Tuohy needle. The electrode was fixed with adhesive tape, and a drape was placed over the skin.

The 5 contact points of catheter electrode were numbered from 1 to 5 with rostral electrode number 1. Each contact was 3 mm long and 10 mm apart from each other. D-waves were recorded in bipolar fashion between contacts 1 and 2, and contacts 3 and 4 simultaneously, after M1 or subcortical stimulation.

The signals from the electrodes were fed into an amplifier, with a band-pass range from 5 to 5 kHz, and 16 to 32 sweeps were averaged using the NeuroPack machine (Nihon Koden Co, Ltd, Tokyo, Japan). The differences in the peak latency of the D-waves recorded between contacts 1 and 2 and between contacts 3 and 4 were used to calculate D-wave conduction velocity.

TABLE 1. Patients' Characteristic and Essentials of Subcortical Mapping

Case Number	Age (Years)/Sex	Tumor Location	Diagnosis	Postoperative		D-Wave		
				Removal	Deficit	Monopolar Stimulation	Bipolar Stimulation	Maximum Intensity for Stimulation (mA)
1	72/M	Left frontal	Glioma 4	Partial	No deterioration	Present	Present	10
2	49/F	Left frontal	Glioma 2	Total	No deterioration	Present	Absent	15
3	40/M	Left frontal	Glioma 2	Total	No deterioration	Absent	Absent	25
4	63/F	Right parietal	Glioma 2	Total	No deterioration	Present	Absent	25
5	62/M	Left frontal	Glioma 2	Total	No deterioration	Present	Absent	25
6	48/F	Right parietal	Glioma 2	Total	No deterioration	Absent	Absent	25
7	40/F	Right parietal	Glioma 3	Total	No deterioration	Present	Absent	25
8	57/M	Right frontal	Glioma 3	Subtotal	No deterioration	Present	Present	12
9	50/M	Right frontal	Glioma 2	Subtotal	No deterioration	Present	Present	15
10	30/F	Right frontal	Glioma 2	Total	No deterioration	Present	Absent	25
11	42/M	Right frontal	Glioma 2	Total	No deterioration	Present	Absent	25
12	37/M	Left frontal	Glioma 3	Total	No deterioration	Present	Absent	25
13	50/F	Left frontal	Glioma 4	Subtotal	No deterioration	Present	Absent	25
14	40/M	Right frontal	Glioma 2	Total	No deterioration	Present	Absent	25

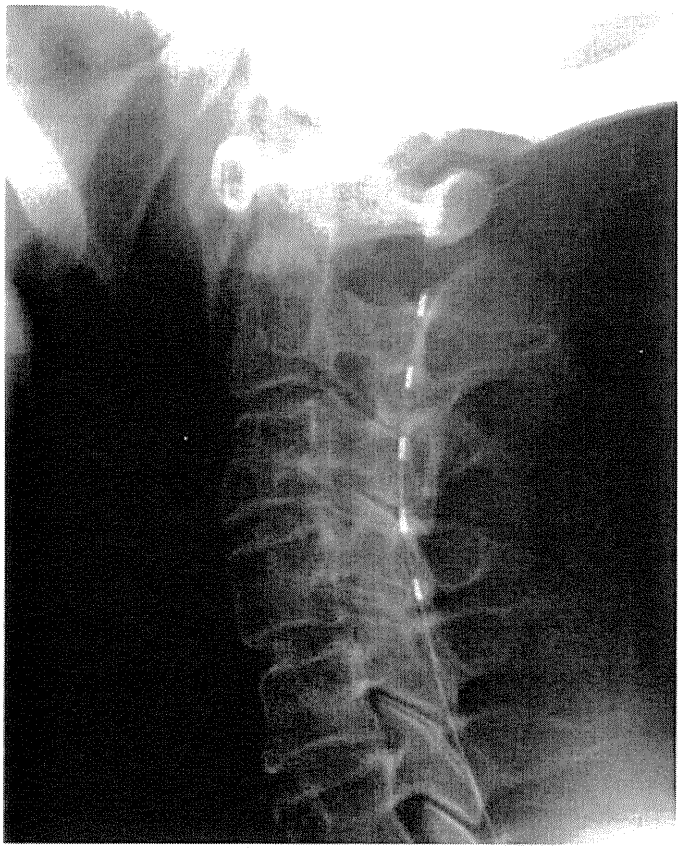

FIG. 1. Catheter-type electrode for D-wave recording from the spinal epidural space. This flexible five-contact platinum catheter-type electrode was inserted into the epidural space of cervical spine, usually from the C-2 to C-3 levels, the day before surgery.

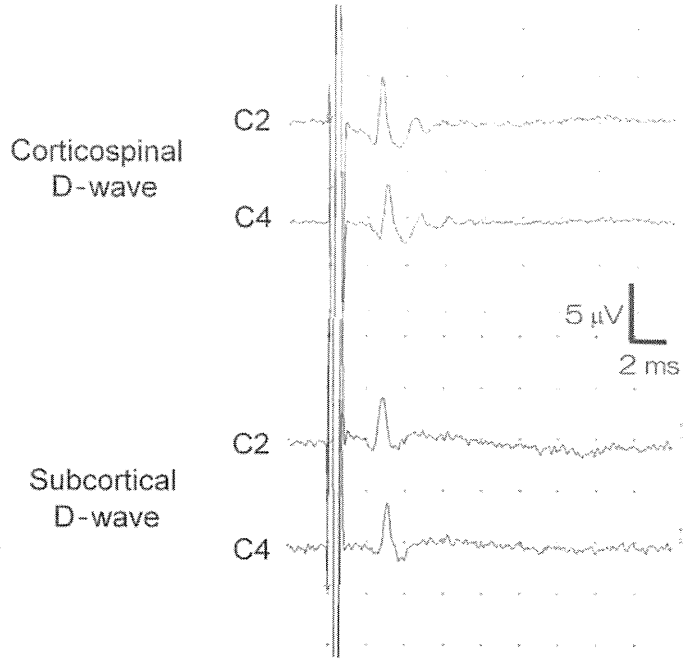

FIG. 2. The waveform of the subcortical D-wave was nearly identical to that of the corticospinal D-wave. The conduction velocity of the subcortical D-wave was nearly the same as that of the corticospinal D-wave. The latency of the subcortical D-wave was slightly shorter than that of the corticospinal D-wave. Such a difference was probably caused by the difference in the distance of the stimulation points from the cortical surface.

Anesthesia Regimen

The mapping and monitoring of the corticospinal D-wave were carried out under general anesthesia with a muscle relaxant and under completely controlled ventilation. No special regimen of the anesthesia was required.

RESULTS

A corticospinal D-wave evoked by M1 stimulation was easily and successfully recorded in all patients. Subcortical stimulation also elicited D-wave with very similar features as corticospinal D-wave. We named this wave the "subcortical D-wave" and observed its characteristics.

Subcortical D-wave was recorded in 12 of 14 patients after monopolar stimulation. Only in three patients, subcortical D-wave was recorded using bipolar stimulation. Moreover, during bipolar stimulation, D-wave was unstable, with change in its amplitude most likely because of the geometry between stimulating electrode and CT fibers.

The waveform of the subcortical D-wave was almost identical to that of the corticospinal D-wave (Fig. 2). The conduction velocity of the subcortical D-wave, calculated from the latencies measured at the 2 contacts, was 65 ± 9.5 m/second, which was almost identical to that of the corticospinal D-wave (62.1 ± 3.5 m/second) (Yamamoto et al., 2004). The latency of the subcortical D-wave was slightly (0.2 to 0.7 milliseconds) shorter than that of the corticospinal D-wave. It is possible that the difference in latency was because of the difference in distance of the subcortical stimulation points from the cortical surface.

According to previous reports, the corticospinal D-wave can be elicited by double-train stimulation at a frequency of 500 Hz (interstimulus interval of 2 milliseconds) (Katayama et al., 1988, 1994). Because subcortical D-wave is conducted through the CT without synaptic connection, we give evidence that the subcortical D-wave has the same feature (Fig. 3).

After tumor resection, in some cases, we had an opportunity to observe the subcortical D-wave evoked by monopolar stimulation at various points in the tumor cavity. The amplitude of the subcortical D-wave changed significantly depending on the distance of the stimulation point from M1 and/or CT (Fig. 4).

DISCUSSION

The reliability of the corticospinal D-wave monitoring has been confirmed in several studies (Fujiki et al., 2006; Katayama et al., 1988, 1994; Yamamoto et al., 2004). Our data showed that the features of subcortical D-wave are nearly identical to those of the corticospinal D-wave. Moreover, we gave evidence that the 2 subcortical D-waves can be elicited by double stimuli with interstimulus intervals of 2 milliseconds. Based on these findings, we believe that it is appropriate to regard the subcortical D-wave as being identical

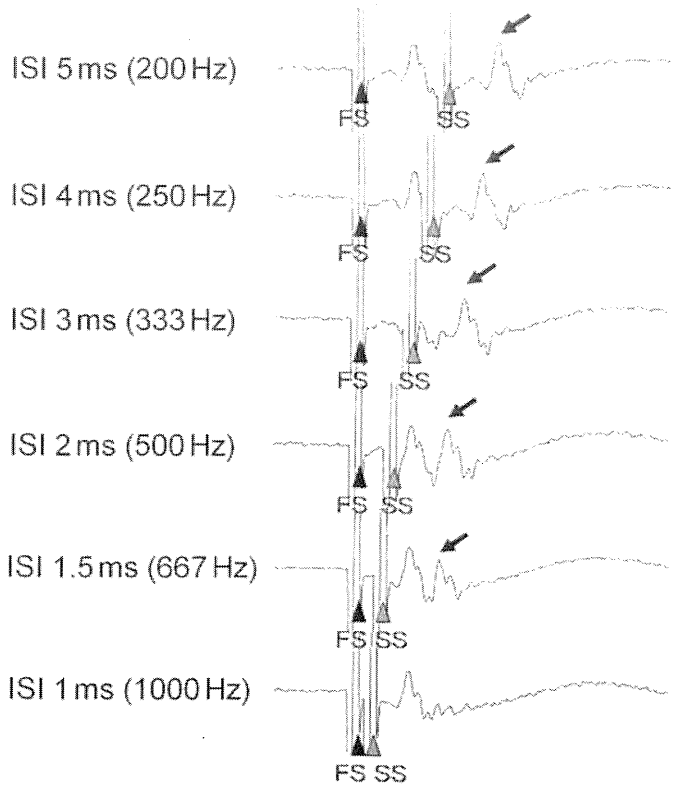

FIG. 3. The subcortical D-wave can be elicited by double-train stimulation at a frequency of more than 500 Hz (ISI of 2 milliseconds). Because subcortical D-wave through CT without synaptic connection as same as the corticospinal D-wave. Black arrows show the response to SS. FS, first stimulation; SS, second stimulation; ISI, interstimulus interval.

to the corticospinal D-wave and that these waves are conducted through the CT.

It was more difficult to elicit the subcortical D-wave by bipolar stimulation than by monopolar stimulation. This could be attributed to differences in geometry of electrical field generated by these two kinds of stimulation. Jayakar (1993) reported that the spatial extent of monopolar stimulation roughly depends on stimulation intensity, whereas that of bipolar stimulation is limited near the neighborhood of the stimulation point regardless of the stimulation intensity. According to an optical imaging study of neural activity, bipolar stimulation evokes only local activation of the cortex immediately beneath the electrode without any significant spread of current (Haglund et al., 1993).

Almost all the previously reported subcortical mapping studies used the electromyogram (motor evoked potential) as an indicator of motor pathway activation. However, as reported previously, responses from muscles can be evoked by stimulation of not only M1 and CT but also the supplementary motor area (SMA), premotor cortex (PM), and probably their descending fibers (Kombos et al., 1999; Usui et al., 2008). This feature is unfavorable for achieving maximum tumor resection with minimum postoperative deficits because of functional impairments of SMA and PM. Deficits because of the lesions of these structures are considerably different from those because of M1 impairments (Fukaya et al., 2003).

Motor deficits caused by damage to SMA and/or PM are reversible in nature, and patients often recover fully within a few months (Zentner et al., 1996). From observations of patients whose SMA and/or PM were removed together with their descending fibers, we know that similar principle can be adopted only for fibers descending from the SMA and/or PM (Fukaya et al., 2003).

As already mentioned, the corticospinal D-wave can be recorded only when M1 is stimulated (Katayama et al., 1988, 1994; Yamamoto et al., 2004). Likewise, the subcortical D-wave, which is identical to the corticospinal D-wave, can be recorded only when CT is stimulated. Indeed, the subcortical D-wave parameters would be the appropriate indicator for subcortical mapping.

The relationship between the distance of the stimulation points from the CT and the amplitude of the subcortical D-wave is worth studying. Further studies are required to verify these points. Monopolar stimulation with epidural D-wave recording for subcortical mapping might provide useful information for preventing postoperative motor deficit.

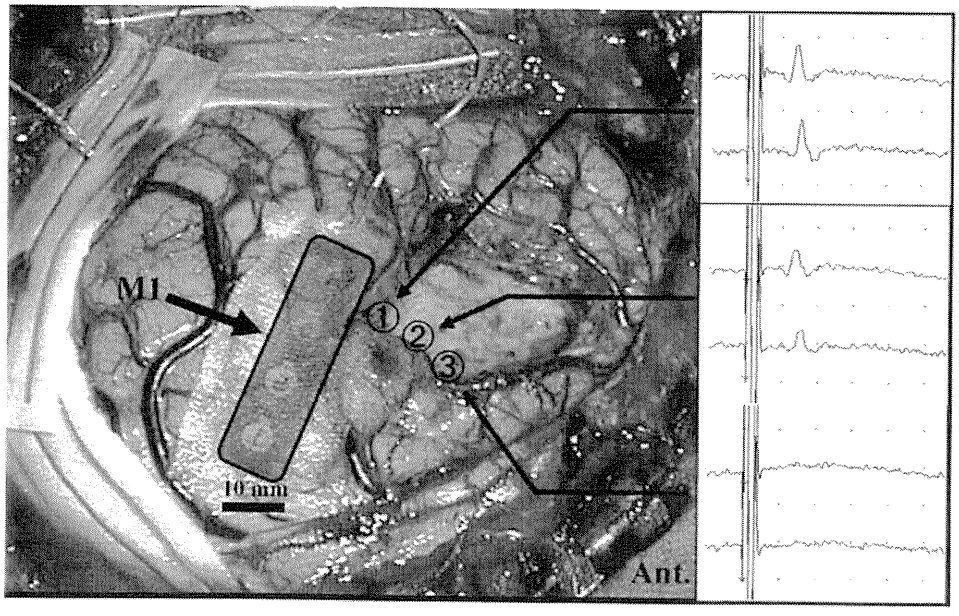

FIG. 4. Intraoperative photograph shows that subcortical D-wave amplitude changes significantly depending on the distance between the stimulating point and M1 and/or CT.

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Decrease in doublecortin expression without neuronal cell death in rat retrosplenial cortex after stress exposure

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Exposure to acute stress by forced swim impairs spatial learning and memory in rats. The retrosplenial cortex plays an important role in spatial learning and memory. A cell population that expresses immature neuronal markers, including doublecortin (DCX), plays a key role in plasticity of the adult brain through formation of new neurons. Here, we aimed to determine whether rats exposed to acute stress showed changes in DCX expression in retrosplenial cortex cells. Twelve male Sprague–Dawley rats were used. Six were subjected to acute stress by forced swim (group S), and the remaining six served as controls (group C). Immunohistochemical staining was performed for DCX, neuron-specific nuclear protein, parvalbumin, calbindin, calretinin, and somatostatin. Newly generated cells were immunohistochemically detected by daily administration of 5-bromo-2′-deoxyuridine for 1 week. Fluoro-Jade B staining was performed to detect cell death. Group S showed lower number of DCX-expressing cells than group C ($P<0.001$). The proportion of DCX-expressing cells showing neuron-specific nuclear protein co-localization (24% in group S; 27% in group C) or parvalbumin co-localization (65% in group S; 61% in group C) remained unchanged after acute stress exposure. Neither 5-bromo-2′-deoxyuridine-positive nor Fluoro-Jade B-positive cells were found in the retrosplenial cortex of groups S and C. DCX-expressing cells in the retrosplenial cortex decreases markedly without cell death after acute stress exposure. Neuronal differentiation of these cells toward gamma aminobutyric acidergic interneurons appears to be unaltered. The decrease in DCX expression may reduce plasticity potential within the retrosplenial cortex and attenuate spatial learning and memory function. *NeuroReport* 23:211–215 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The forced swim paradigm is commonly adopted as a model of acute stress exposure, which induces depression-like symptoms in rats [1,2]. Previous studies have reported an association between the depression-like behavior and impairment of spatial learning and memory [3–5]. However, little is yet known regarding the molecular mechanisms involved in these effects of acute stress exposure.

Learning and memory represent a complex process based on functional and structural changes at the synaptic, neuronal, and circuitry levels [6]. Recent studies have indicated that formation of new neurons is an important mechanism in the plasticity of the adult brain, which contributes to learning and memory. A population of cells that express immature neuronal markers, including doublecortin (DCX), plays a key role in the formation of new neurons [6–8]. DCX-expressing cells exist within the cerebral neocortex and allocortex of the adult brain, and are apparently associated with the plasticity of the brain [9–11].

The retrosplenial cortex plays an important role in spatial learning and memory [12,13]. The information associated with spatial navigation appears to be unified in the retrosplenial cortex. Previous studies have suggested that information for body movement is transferred into the space reference frame in the retrosplenial cortex [14,15]. Neurons exist within the retrosplenial cortex, which produce signals responding immediately to body position and movement as well as cephalic presentation [14,16,17].

In adult animals, DCX-expressing cells are found in the retrosplenial cortex [13], and we have confirmed their existence in adult rats. The aim of the present study was to determine whether rats exposed to acute stress by forced swim demonstrate changes in DCX expression in the cells of the retrosplenial cortex. If alterations in DCX expression are detected, such changes could represent an altered plastic potential of the retrosplenial cortex, and explain part of the mechanisms underlying the impairment of spatial learning and memory that occurs after acute stress exposure.

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Materials and methods

Animals and stress exposure

Twelve male Sprague–Dawley rats were used for the study. Among them, six were exposed to acute stress by forced swim (group S), and the remaining six were used as controls (group C). They were purchased from Charles River Laboratories (Saitama, Japan), and bred at the animal housing facility of Nihon University School of Medicine. The colony was maintained at 22–23°C on a 12 h light/dark cycle (lights on at 08:00 h). All experimental procedures were designed in accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council, National Academy Press, Washington, DC, USA; 2003) and approved by the Animal Care and Use Committee of Nihon University.

Forced swimming is used to induce acute stress exposure [18]. Rats were individually placed into a water tank (22.5 cm in diameter, 45.5 cm in height) containing a water (25°C) depth of 15 cm and forced to swim. After a 15-min induction period in the water, the rats were removed and dried off with a towel before being returned to their home cages. Twenty-four hours later, they were placed in the tank for a second test of 5-min duration. We considered that animals, which demonstrated immobility for more than 150 s, were exposed to acute stress [19] and included them in group S.

Tissue preparation and immunohistochemistry

The rats were anaesthetized with pentobarbital (Somnopentyl, Kyoritsu Seiyaku, Tokyo, Japan; 30 mg/kg body weight intraperitoneally) and transcardially perfusion-fixed with lactated Ringer's solution, followed by perfusion of 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The brains were removed from the skull, kept in the fixative at 4°C for 24 h, and then transferred into 0.1 M PBS. Coronal sections (50 μm) were cut using a vibratome.

Free-floating sections were placed in 0.9% H_2O_2 for 5 min. Sections were then washed once in Tris-buffered saline (TBS) (pH 7.4) for 15 min followed by two consecutive 15 min washes in 1% Triton X-100/TBS solution (TBS/TX) (pH 7.4). AntiDCX antibodies (polyclonal goat, 1:2000; Santa Cruz Biotechnology, Santa Cruz, California, USA) were diluted in TBS/TX containing 3% normal rabbit serum. Sections were incubated with primary antibodies at 4°C for 24 h, and rinsed once in TBS/TX for 15 min followed by two consecutive 15 min washes in TBS.

The secondary antibodies were biotin-labeled rabbit anti-goat IgG antibodies diluted in TBS/TX containing 1% normal rabbit serum (1:400, Vector Laboratories, Burlingame, California, USA). Sections were agitated with secondary antibodies at room temperature for 2 h followed by one 15 min wash in TBS/TX and two in TBS. Sections were incubated with ABC solution (1:200, Vectastain ABC Elite kit, Vector Laboratories) in TBS/TX at room temperature for 1 h, followed by one 15 min wash in TBS/TX and twice in TBS. For color development, the sections were incubated with 0.005% 3,3′-diaminobenzidine (DAB), 3% H_2O_2, and 0.4% $NiCl_2$ in TBS at room temperature for 1–5 min, yielding a brownish color, and this was followed by three 15 min washes with TBS. The sections were placed on gelatin-coated slides, dehydrated in ethanol, cleared in xylene, and mounted with coverslips using Mount-Quick (Daido Sangyo, Tokyo, Japan).

We identified the retrosplenial cortex by referring to the rat atlas of Paxinos and Watson [20]. We counted the numbers of DCX-expressing cells on DAB-developed sections and compared the number per unit area between the controls (group C, $n = 6$) and acute stress models (group S, $n = 6$) to examine any differences in expression related to acute stress exposure.

We used Biozero (BZ-8000; KEYENCE, Osaka, Japan) and a BZ-Analyzer (KEYENCE) to prepare the microphotographs, and employed a VH-Analyzer (KEYENCE) equipped with three-dimensional stereological software to count the number of immunostain-positive cells or Fluoro-Jade B (FJB)-positive cells. NEUROLUCIDA (Version 3; MicroBrightField, Williston, Vermont, USA) was used to analyze the area and all cells count without double counting of positive cells in the retrosplenial cortex (bregma posterior, 2.80 and 3.30 mm), and we then calculated the cell count per unit area. SPSS STATISTICS 17.0 (Japan IBM, Tokyo, Japan) was employed as the statistics software for data analysis. We performed the Mann–Whitney test to compare unrelated groups.

We performed double staining by fluorescent immunohistochemistry. Preliminary treatment was undertaken with 100% methanol for 5 min. The primary antibodies used for this study are listed in Table 1, and secondary antibodies are listed in Table 2. Sections were agitated with secondary antibodies at room temperature in a dark room for 2 h, followed by six 15 min washes with TBS. Fluorescence double staining was performed as described above employing an avidin/biotin blocking kit (Vector) after first secondary antibody sensitization.

In the fluorescent double staining, we investigated the co-localization of DCX with neuron-specific nuclear protein (NeuN), parvalbumin (PV), calbindin (CB), calretinin (CR), or somatostatin (SOM) under a high-power field. It was difficult to determine the precise number of neurons with co-localization, since some neurons were overlapped, especially in layer II. We judged DCX to be co-localized with NeuN, PV, CB, CR, or SOM when more than two-thirds of the cell body was double stained. Although many overlapping neurons were, therefore, omitted from the count, the comparison of co-localization between the two groups appeared to be meaningful.

Table 1 Primary antibodies

Antigen	Class of antibody	Dilution	Manufacturer
Doublecortin (DCX)	Polyclonal goat	1:2000	Santa Cruz Biotechnology
Neuron-specific nuclear protein (NeuN)	Monoclonal mouse IgG	1:500	Chemicon
Parvalbumin (PV)	Monoclonal mouse IgG	1:1000	Sigma
Calbindin (CB)	Polyclonal rabbit	1:1000	Chemicon
Calretinin (CR)	Polyclonal goat	1:1000	Chemicon
Somatostatin (SOM)	Polyclonal rabbit	1:500	Chemicon
Bromodeoxyuridine (BrdU)	Monoclonal mouse IgG	1:1000	Chemicon

Chemicon: Temecula, California, USA.

Table 2 Secondary antibodies used in fluorescent immunohistochemistry

Secondary antibody	Antigen(s)	Dilution	Manufacturer
Fluorescein-labeled rabbit anti-goat IgG	Antidoublecortin (DCX) antibody	1:400	Vector
Alexa Fluor 488-labeled rabbit anti-goat IgG	AntiDCX antibody	1:400	Invitrogen
Texas red-labeled rabbit anti-mouse IgG	Anti-neuron-specific nuclear protein (NeuN) antibody Antiparvalbumin (PV) antibody Antipolysialic acid-neural cell adhesion molecule (PSA-NCAM) antibody	1:400	Vector
Texas red-labeled donkey anti-goat IgG	Anticalretinin (CR) antibody	1:400	Vector
Texas red-labeled donkey anti-rabbit IgG	Anticalbindin (CB) antibody Antisomatostatin (SOM) antibody	1:400	Vector

Invitrogen: Carlsbad, California, USA.

Bromodeoxyuridine-immunohistochemistry and Fluoro-Jade B staining

Six rats were administered 5-bromo-2′-deoxyuridine (bromodeoxyuridine, BrdU) (Sigma, St Louis, Missouri, USA; 50 mg/kg body weight) intraperitoneally daily for a week after stress exposure. After the BrdU injection, they were perfusion-fixed, and free-floating sections were used as described above. For the detection of incorporated BrdU, the sections were incubated in 2 N HCl at 37°C for 30 min. The sections were then washed once in TBS for 15 min followed by two consecutive 15 min washes in TBS/TX. We used monoclonal mouse anti-BrdU antibodies as the primary antibodies (Table 1), and the secondary antibodies, biotin-labeled horse anti-mouse IgG antibodies, were mixed with TBS/TX containing 1% horse serum (1:400, Vector). The DAB reaction and section preparation were carried out as described above.

The FJB staining procedure was performed as reported previously [21]. The sections were mounted on gelatin-coated slides and then air-dried on a slide warmer at 50°C for more than 30 min. The slides were immersed in 80% alcohol containing 1% sodium hydroxide for 5 min. This was followed by 2 min rinse in 70% alcohol and 2 min rinse in distilled water. The slides were subsequently immersed in 0.06% potassium permanganate for 10 min on a rotary shaker. The slides were then rinsed in distilled water for 2 min. The staining solution was prepared from a 0.01% stock solution of FJB prepared by adding 10 μg of the dye powder to 100 ml of distilled water. To prepare 100 ml of staining solution, 4 ml of the stock solution was added to 96 ml of 0.1% acetic acid vehicle. After incubation in the staining solution for 20 min, the slides were rinsed for 1 min in each of three distilled water washes. The slides were then placed on a slide warmer, set at approximately 50°C, until they were fully dry (e.g. 5–10 min). The dry slides were cleared using xylene and coverslipped with DPX mountant for microscopy (Merck, Tokyo, Japan).

Results

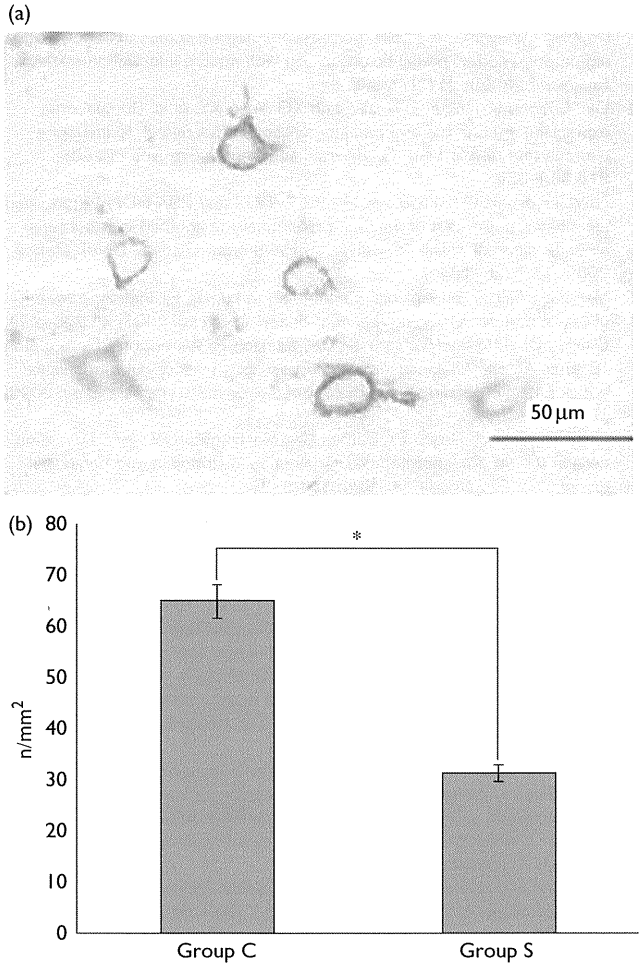
The DCX-expressing cells measured 10–25 μm in diameter (Fig. 1a). In the retrosplenial cortex, we counted 1204 cells in group S and 1956 cells in group C, from the right and left hemispheres separately on two sections for a total of four regions per animal ($n = 24$).

The number of DCX-expressing cells per unit area (mean ± SD) was 31.2 ± 10.9 cells/mm^2 (range, 18.8–56.6) in group S and 64.2 ± 15.2 cells/mm^2 (range, 41.3–88.4) in group C. The number of DCX-expressing cells was clearly lower in group S than in group C (Mann–Whitney test, $P < 0.001$; Fig. 1b), indicating that the DCX expression in the cells of the retrosplenial cortex was clearly decreased after acute stress exposure.

We examined the co-localization of NeuN, PV, CB, CR, or SOM in 100 DCX-expressing cells in each case. The DCX-expressing cells in the retrosplenial cortex demonstrated NeuN co-localization in 24% in group S and 27% in group C (Fig. 2a), suggesting that the proportion of NeuN co-localization in the DCX-expressing cells was unchanged by acute stress exposure.

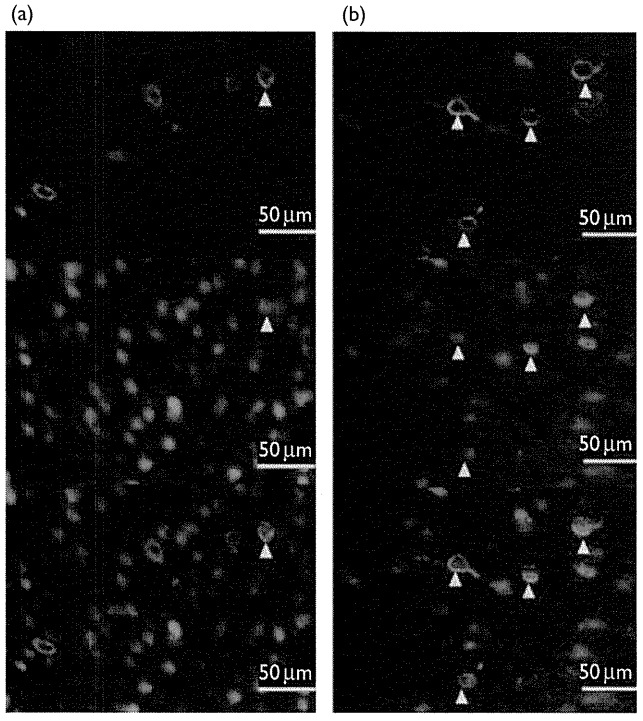
The DCX-expressing cells in the retrosplenial cortex demonstrated PV co-localization in 65% in group S and 61% in group C (Fig. 2b). Clearly, DCX-expressing cells often showed PV co-localization, and no change in the proportion of PV co-localization was induced by acute stress exposure. No DCX-expressing cells demonstrated co-localization with CB, CR, or SOM.

Fig. 1



(a) Doublecortin-expressing cells in the retrosplenial cortex of group C. Scale bar, 50 μm; layer III/IV; 2.80 mm posterior to the bregma. (b) Number of doublecortin-expressing cells per unit area in the retrosplenial cortex. $n=24$. Mann–Whitney test, $^{*}P$ less than 0.001.

Fig. 2



(a) Doublecortin-expressing cells (green) with co-localization of neuron-specific nuclear protein (red) in the retrosplenial cortex of group S (indicated by yellow arrowheads). Scale bar: 50 μm; layer III/IV. (b) Doublecortin-expressing cells (green) with co-localization of PV (red) in the retrosplenial cortex of group S (indicated by yellow arrowheads). Scale bar, 50 μm; layer III/IV.

No BrdU-positive cells were found in the retrosplenial cortex of group S and group C. In contrast, many BrdU-positive cells were present in the subgranular zone of the hippocampus in group S. No FJB-positive cells were found in the retrosplenial cortex of group S and group C. In contrast, many FJB-positive cells were present in the CA1 area of the hippocampus in group S.

Discussion

The present results demonstrate a clear decrease in the number of DCX-expressing cells in the retrosplenial cortex after acute stress exposure by forced swimming. Many of the DCX-expressing cells in the retrosplenial cortex often show co-localization with PV [22], indicating that these cells are gamma aminobutyric acidergic interneurons [23].

The present study also revealed that the proportion of NeuN or PV co-localization among DCX-expressing cells does not change after acute stress exposure. These findings suggest that neuronal differentiation of DCX-expressing cells towards gamma aminobutyric acidergic interneurons may be unaltered by acute stress.

There were neither BrdU-positive cells nor FJB-positive cells in the retrosplenial cortex after acute stress exposure, indicating that the decrease in the number of DCX-expressing cells is unrelated to changes in neurogenesis or cell death. One possible mechanism for the decrease in number of DCX-expressing cells may be accelerated differentiation in the immature undifferentiated cell pool. Recent studies have reported that neuronal differentiation-related genes were up-regulated after stress exposure [24,25].

As mentioned above, cells that express immature neuronal markers, including DCX-expressing cells, play a key role in the formation of new neurons [6–8] and, thereby, the plasticity of the brain, which contributes to learning and memory. We suggest that the decrease in DCX expression in cells of the retrosplenial cortex represents a reduced plastic potential in the retrosplenial cortex, and may be part of the mechanisms underlying the impairment of spatial learning and memory function that occurs after acute stress exposure.