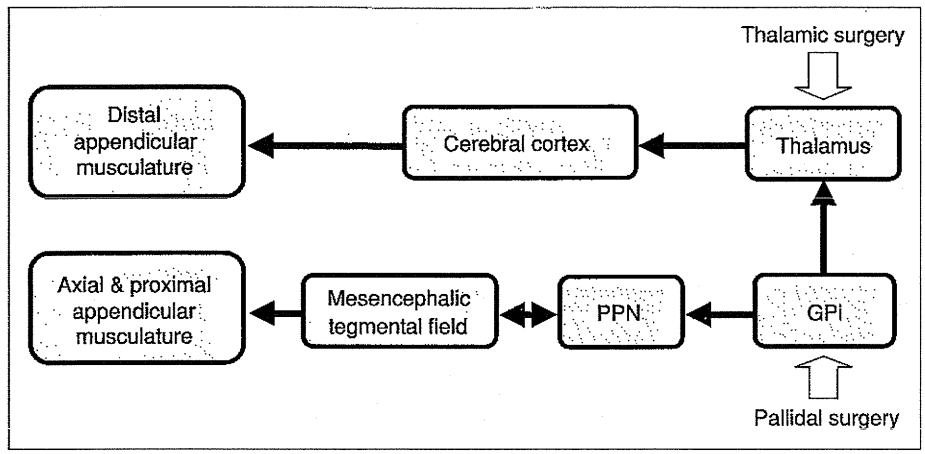

図1　大脳基底核を中心とした神経回路機能モデル
GPiからの出力系は上行性に視床へ投射し，大脳皮質を介して対側の上下肢末梢の運動を調節するとともに，下降性にPPNを中心とする脳幹の領域へも投射し，体幹や下肢の運動やバランスを制御している．したがって，上肢などの末梢性のジストニアは視床手術で治療可能であるが，体軸を含む広範な分布を示すジストニアには淡蒼球手術が推奨される．

される．実際，全身性，分節性，局所性問わず上肢末梢を除く一次性ジストニアの多くはGPiの刺激が効果を示し，上肢にみられる局所性ジストニアの代表であるfocal hand dystonia(FHD)では視床外腹側核(n. ventralis lateralis：VL)の刺激が有効である．視床VL核はVo-complex(Voa＋Vop)と相同である．

逆に，視床手術で体軸のジストニアを改善するのは一般に困難である．Grenoble大学のグループの経験では，12例の全身性ジストニア患者に対して視床中間腹側核(n. ventralis intermedius：Vim)刺激術を行ったところ，わずか5例にのみ有効であり，それも体肢ジストニアだけに軽度から中等度の改善がみられるものの体軸症状にはまったく効果がなかったとされている[7]．同様のことは視床凝固術を用いた1976年のCooperの報告[4]にすでに記載されている．視床下核(subthalamic nucleus：STN)のDBSが全身性ジストニアに対して有効であったという症例報告が散見される．STN-DBSがParkinson病(PD)に付随するオフ期ジストニアを改善しうること[8]を考慮すれば理解できるが，いまだ経験が乏しく，STN-DBSのジストニア治療への応用については慎重であるべきであろう．以下，ジストニアに対する定位脳手術の現在の主役であるGPi-DBSを中心に詳述する．

GPi-DBSの刺激部位および刺激条件

GPiにはDBSに関連した機能分画が存在するが，これはPD治療で最初に見出されたものである(図2)．PD症例では(図2-左)，後腹側部の刺激で固縮(rigidity)の著明な改善とレボドパ誘発性ジスキネジアの完全抑制が得られるが，レボドパの無動(akinesia)に対する効果は阻害され患者は著明な無動状態に陥るとされ，一方，前背側部の刺激ではオフ期無動の中等度の改善とジスキネジアの誘発がみられると報告されている[9]．筆者は頸部ジストニアや他のタイプのジストニア症例において類似した経験をしている(図2-右)．つまり，後腹側部の刺激ではジストニア症状の改善をみるが，前背側部の刺激では逆にジストニアの増悪および舞踏運動を主体とするジスキネジアの誘発がみられた．したがって，ジストニア治療では視索に近接した後腹側部の刺激がもっとも効果的であると考えられる．

DBSの効果を十分に得るためには上記の適切な部位に電極を留置することは当然であるが，その後の刺激条件調節もその手術手技と同等の意義をもつ．

刺激条件に関しては絶対的な指針などはないが，これまでの報告とわれわれの施設の経験をまとめると，一次性全身性・分節性ジストニア

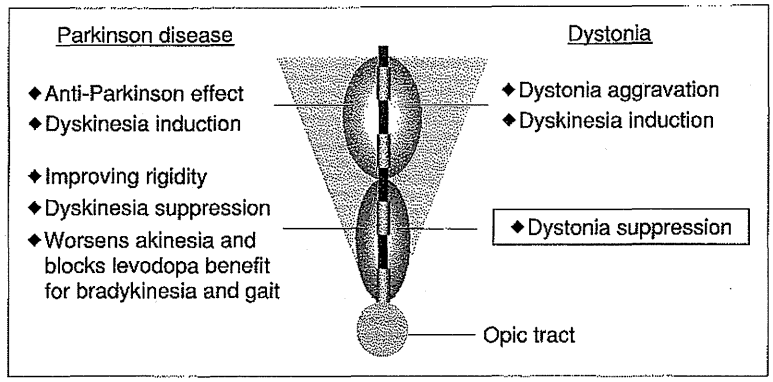

図2　GPi-DBSに関連するGPiの機能分画
PDでは後腹側部の刺激で固縮(rigidity)の著明な改善とレボドパ誘発性ジスキネジアの完全抑制が得られるが，レボドパの無動(akinesia)に対する効果は阻害され患者は著明な無動状態に陥るとされ，一方，前背側部の刺激ではオフ期無動の中等度の改善とジスキネジアの誘発がみられると報告されている．ジストニアでは，後腹側部の刺激ではジストニア症状の改善をみるが，前背側部の刺激では逆にジストニアの増悪および舞踏運動を主体とするジスキネジアの誘発がみられる．

では1～2個の電極を用いた単極かつ，刺激頻度130～185Hz，電圧0.8～3.8V，刺激幅120～450μsecの間の刺激が用いられている．一方でMeige症候群では，60～145Hzの比較的低頻度刺激，1.0～5.2V，120～450μsecで時には双極刺激も使用されうる．

二次性ジストニアも概ね上記刺激範囲内であることが多いが，pantothenate kinase-associated neurodegeneration(PKAN)に代表される脳内鉄蓄積性神経変性疾患(neurodegeneration with brain iron accumulation：NBIA)は60～215Hz，1.0～5.0Vとより低頻度，高電圧が用いられることもあり，ジストニア-舞踏アテトーゼ型脳性麻痺60～150μsecと刺激幅はより小さく，刺激頻度は130Hzが使用されている．

痙性斜頸に関しては刺激条件の検討を目的とした系統的な研究が行われている．Moroらの報告によると，60Hzを超える高頻度刺激と高電圧が症状の改善と有意な関連があり，130Hzの刺激がもっともよい効果をもたらしたが，刺激幅を上げても有意な改善は得られなかった[10]．

すべてのタイプのジストニアにおいていえることであるが，症例によってはジストニア症状の刺激頻度依存性反応がみられる場合があるので注意を要する．刺激強度は最大のジストニア抑制効果が得られ，刺激による耐えがたい副作用が出現しない程度に設定する．個々の症例ごとの刺激条件の最適化は当然必要であるが，最後腹側部を中心とした単極刺激，130Hz，210μsec，2.0～3.0Vほどがもっとも汎用性が高い刺激設定かもしれない．

一次性ジストニア

1．一次性全身性・分節性ジストニア

*DYT1*遺伝子異常に基づくジストニアは最初にGPi-DBSの有効性が示された一次性全身性ジストニアであり[11]，その原型と考えられている．Vidailhetらは，22人のDYT1を含む一次性全身性ジストニアにGPi-DBSを施行し，1年後には，Burke-Fahn-Marsden Dystonia Scale(BFMDRS)のMovement Score(MS)，Disability Score(DS)がそれぞれ術前の55%，44%の改善率であったと報告した[12]．翌年にはKupschらにより，DYT1を含む一次性全身性・分節性ジストニアに対するGPi-DBSのランダム化試験の報告がなされ，シャム刺激群に比べ神経刺激群に有意な症状改善が認められた[13]．さらにVidailhetらは，上記の症例群をさらに追跡し，運動症状改善率は1年後に51%であったが，3年後には58%まで上昇したことを示し[14]，GPi-DBSの一次性全身性・分節性ジストニアに対する有効性はほぼ確立された．また，術後10年経過しても効果が持続している症

例の報告もあり[15]，長期予後も良好と考えられる．

2．Meige症候群

Meige症候群は両側性の顔面攣縮を主体とするジストニアと考えられている[16]．1例報告が主であるが，6例のMeige症候群に対してGPi-DBSが少なくとも6カ月間にわたり70%以上の症状改善をもたらしたという報告もある[17]．われわれの経験では，最長10年にわたり十分な効果が持続している症例もあり[18]，Meige症候群もボツリヌス毒素注射などの保存的加療に抵抗性の場合は両側GPi-DBSのよい適応と考えている．

3．痙性斜頸

痙性斜頸は頻度の高い一次性局所性ジストニアの一つであり，14%もの患者がボツリヌス毒素治療抵抗性を呈する[19]．Kraussらは，6カ月間の経過観察でGPi-DBSが3例の痙性斜頸に対してModified Toronto Western Spasmodic Torticollis Rating Scale (TWSTRS)の50%前後の改善をもたらしたことを報告した[20]．その後，相次いで同様の症状改善を示した報告がなされたが，平均31.9カ月にわたりGPi-DBS後の10例の痙性斜頸を経過観察し，50%を超えるTWSTRSの改善を得られたとの結果がHungらにより示され[21]，長期予後に関しても期待されている．しかしながら，痙性斜頸に関しては，異常収縮を呈する胸鎖乳突筋対側の片側GPi-DBSでも有効であるとの報告もあり[22]，片側のみで十分なのか，あるいは両側刺激のほうがより効果的であるのかさらなる検討が必要である．

Focal hand dystonia

FHDは手・前腕の主動筋・拮抗筋群の過剰な共収縮に起因する一次性ジストニアである[23]．文字を書くときのみジストニアが出現する場合はsimple writer's crampと呼ばれ，他の動作時にも症状が出現するようになるとfocal dystonic writer's cramp，症状が持続性となるとfocal arm dystonia，頸部，体幹，顔面など，ほかの部位も巻き込まれればsegmental dystoniaと進行度に応じて名称が変化していく[23]．

FHDのうち，simple writer's cramp, dystonic writer's crampに対してはVo-complexの視床破壊術が有効であると報告されている[24]．また，Vo-complexとVimの両方の刺激が5例のwriter's crampに対して有効性を示したとの報告もある[25]．われわれの施設では，さらに進行したfocal arm dystoniaにVo-complexとGPiのDBSのいずれもが効果的であった症例も経験しており[26]，今後それぞれのターゲットの刺激術のみならず破壊術のいずれがどの段階のFHDに対してより有効であるかさらに検討していく必要がある．

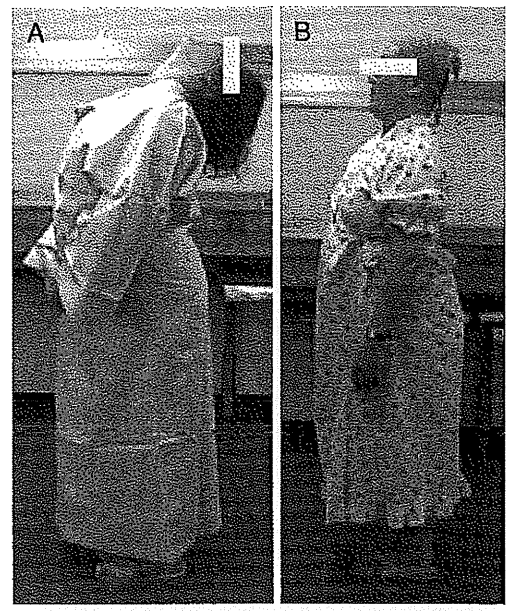

図3　遅発性ジストニアに対するDBSの効果
術前には体幹の高度の後屈を示していたが(A)，両側GPi-DBSにより上記の症状は改善した(B)．

二次性ジストニア

明確な発症要因が存在する二次性ジストニアはDBSに抵抗性であることが多い[27]．難治性である二次性ジストニアの中でも，ジストニア-舞踏アテトーゼ型脳性麻痺[28]，NBIA[29]などにはGPi-DBSが若干の効果を示すが，とくに抗精神病薬の副作用により生ずる遅発性ジストニアはGPi-DBSによく反応し，その改善率は80%を超える(図3)[30]．また，線条体における病理が明確な遺伝性ジストニアであるDYT3に対してもGPi-DBSが行われており，良好な結果が得られている[31]．その他にも1例報告のレベルでは種々の二次性ジストニアに対してDBSが有効であったという論文が多数存在するが，今後どういった型の二次性ジス

トニアがよりよい手術適応となるか，また，いずれの核がターゲットとなりうるか，さらなる報告の蓄積・検証が必要である．

おわりに

ジストニアに対する脳深部刺激(DBS)術を中心に論じてきた．ジストニアは生活の質を大きく左右する疾患の一つであるが，その病態生理の大部分は依然として闇に包まれており，根治的治療法も未だに存在しない．

そのような中でDBSはボツリヌス毒素治療と並ぶジストニアに対する有用な治療法であるが，それによる十分な治療効果が得られている症例はまだ一部である．より多くの患者がDBSの恩恵に与れるよう今後新たな刺激ターゲットの探索，DBSの作用機序の解明を含めさらなる基礎・臨床研究が望まれる．この総説がジストニアとジストニアに対するDBSに関する理解の一助となれば幸いである．

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Short communication

Quantification of potency of neutralizing antibodies to botulinum toxin using compound muscle action potential (CMAP)

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ABSTRACT

We evaluated a method for quantifying botulinum toxin-neutralizing antibodies which utilizes the CMAP. This method can be used just one day after administration, and the detection sensitivity was higher than that of the mouse neutralization test. The CMAP neutralization test detected neutralizing antibodies in patients who were resistant to treatment with the botulinum LL toxin. These results indicate that the CMAP neutralization test is useful for detecting low levels of antitoxin.

Botulinum toxins have recently been developed and used in the treatment of blepharospasm, spasmodic torticollis, dystonia, pain, and urological disorders (Jankovic, 2004; Truong and Jost, 2006; Casale and Tugnoli, 2008). The toxins show a high-level efficacy at very low doses, and are widely used in medical treatment. Resistance to the toxin was reported in some patients who received repeat high-dose (>100 mouse ip LD_{50} per injection cycle) toxin therapy over a long period of time (Borodic et al., 1996; Sesardic et al., 2004; Dressler, 2004). This reduced therapeutic response was reported to lead to the development of neutralizing antibodies in the patients. The potency of neutralizing antibodies in the serum of patients is measured using the mouse neutralization test (Hatheway et al., 1984). This method is based on the LD_{50} assay, which, in turn, is based on the number of surviving mice typically 96 h after the intraperitoneal (ip) injection of a fixed lethal dose of toxin premixed with different amounts of antitoxin. The antitoxin titer in the sample is expressed relative to that of the standard botulinum antitoxin. The detection limit of this method is reported to be about 10–100 mU/mL (Sesardic et al., 2004; Byrne et al., 1998). A reduced therapeutic response in certain patients was reported to be caused by minute amounts of antibodies which could not be detected by the mouse neutralization test (Sesardic et al., 2004). To detect neutralizing antibody presence in patients, a highly sensitive assay for neutralizing antibodies is needed. We reported quantitative of biological activity of botulinum toxin using the compound muscle action potential (CMAP) (Torii et al., submitted for

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publication). CMAP measurement is utilized by the extensor digitorum brevis (EDB) test, which qualitatively assesses the response to the toxin before treatment in patients who might possess antibodies against the botulinum toxin (Kessler and Benecke, 1997). Based on the EDB test, we investigated a highly sensitive quantification method for botulinum toxin-neutralizing antibodies in animal and human sera. In addition, we investigated whether the CMAP neutralization test was able to detect antitoxin in patient's serum, and we compared detection capability of this method, mouse neutralization test and ELISA.

Botulinum neurotoxin types A, B, E, and F (150 kDa, NTX) were cultured and purified using a previously reported method (Sakaguchi et al., 1981; Torii et al., submitted for publication). Equine-derived Japanese standard botulinum antitoxin types A, B, E, and F (National Institute of Infectious Diseases, Tokyo, Japan) were each used as a standard. One unit (U) of corresponding type of standard botulinum antitoxin neutralizes 10,000 mouse intraperitoneally (ip) LD_{50} of toxin types A, B, F or 1000 mouse ip LD_{50} of toxin type E (Jones et al., 2006). The sera of seven patients resistant to treatment with the botulinum LL toxin (BOTOX®, Allergan, Irvine, U.S.) were collected after informed consent was obtained. For the negative control, sera were collected from one volunteer who did not receive any treatment of botulinum toxin. For the positive control, sera were collected from one volunteer who had been immunized three times with the botulinum tetravalent (A, B, E, and F) toxoid. CMAP neutralization test was performed using a modification of a previously report method of CMAP test using female S/D rats (8 weeks of age, Charles River Laboratories Japan, Yokohama, Japan) (Torii et al., submitted for publication). Modification was using mixture which botulinum antitoxins and test toxins in place of toxins. Mixtures were prepared as follows: the standard botulinum antitoxin and various sera were serially diluted with physiological saline containing 0.5% human serum albumin. The test toxins comprised NTX of each type, at quantities whereby the CMAP amplitudes on day 1 after injection were decreased to one quarter of those before administration (type A: 10 mouse ip LD_{50}/mL, B: 60,000 mouse ip LD_{50}/mL, E: 60 mouse ip LD_{50}/mL, F: 600 mouse ip LD_{50}/mL). The type A of test toxin dose also was set at 1 mouse ip LD_{50}/mL to increase the measurement sensitivity. Equal volumes of the antitoxin or serum and test toxin were mixed and reacted for 1 h at room temperature. The anesthetized rats were injected 0.1 mL of a mixture into the left gastrocnemius muscle. The CMAP amplitude of the left hind leg was measured before (0) and 24 h after injection. The mouse neutralization test was performed using a previously reported method using female ICR/CD-1 mice (4 weeks of age, Charles River Laboratories Japan, Yokohama, Japan) (Torii et al., 2002). ELISA was performed using a modification of a previously reported method (Torii et al., 2002). Modifications were buffer using Tris Buffer containing 0.15 M NaCl, secondary antibody using peroxidase-conjugated goat anti-human IgG, IgA and IgM (Sigma, Tokyo, Japan) and substrate using TMB Microwell Peroxidase Substrate (Kirkegaard and Perry Laboratories Inc, Gaithersburg, U.S.). ELISA titers were expressed in multiples of absorbance of the negative control, and antibodies were considered to be detected by ELISA when the absorbance of the sample was more than twice that of the negative control. To determine whether the neutralizing antibody of each type was quantifiable, antibody potencies were plotted versus CMAP amplitudes, and the linearity of the regression line was confirmed by regression analysis using Statistical Analysis for Neurotoxin (SAN, ver. 2.1, self made soft). To determine the antibody titers of patients' sera, the amplitude data of standard botulinum antitoxin were calculated by regression analysis, and the regression line was used as the calibration curve using SAN.

The CMAP amplitude of each mixture of antitoxin and test toxin (types A, B, E, and F) decreased along with the antitoxin titer. For types A and E, regression analysis was performed by plotting the logarithmic values of the CMAP amplitude and antitoxin titer on the vertical and horizontal axes, respectively, and linearity was noted within a range of 3–100 mU/mL in type A ($R^2 = 0.983$) and 1–50 mU/mL in type E ($R^2 = 0.989$). For types B and F, the CMAP amplitudes were plotted on the vertical axis, and the log values of the antitoxin titer on the horizontal axis, and linearity was noted within a range of 25–100 mU/mL in type B ($R^2 = 0.953$) and 3–50 mU/mL in type F ($R^2 = 0.974$) (Fig. 1). To increase the measurement sensitivity, the test toxin dose was set at 1 mouse ip LD_{50}/mL in type A, and linearity was noted within a range of 1–6 mU/mL (data not shown). This method can be used to measure a broad range of neutralizing antibodies titers the day after administration. In this study, the CMAP neutralization test demonstrated six advantages over the standard technique. 1) The CMAP is more sensitive than the mouse neutralization test. 2) The CMAP neutralization test incorporates a concise procedure. 3) The CMAP neutralization test can determine the neutralizing antibody titer within 24 h; whereas, the mouse neutralization test requires 4 days to obtain the same results. 4) The CMAP neutralization test is highly reproducible. 5) Only 20–30 animals are used in the CMAP neutralization test, whereas more than 100 animals are necessary in a single mouse neutralization test. In addition, the rats are anesthetized during the test, and the amounts of injected test toxin do not completely block neuromuscular transmission nor paralyze the muscles. 6) The CMAP amplitude values obtained by the CMAP neutralization test are a continuous quantity. Taken together, these advantages indicate that this method is a useful substitute for the mouse neutralization test.

We then investigated whether minute amounts of neutralizing antitoxin present in patients' sera could be detected by the CMAP neutralization test. The neutralization antibodies in seven patients who showed a reduced therapeutic effect after repeated treatment with type A botulinum LL toxin were determined by the CMAP neutralization test, mouse neutralization test, and ELISA. Using the CMAP neutralization test, all sera showed a neutralizing antibody level of 3–50 mU/mL (Table 1). This suggests that the cause of the reduced therapeutic effect was the production of neutralizing antibody against botulinum LL toxin. In contrast, the mouse neutralization test detected neutralizing antibodies in sera from 1

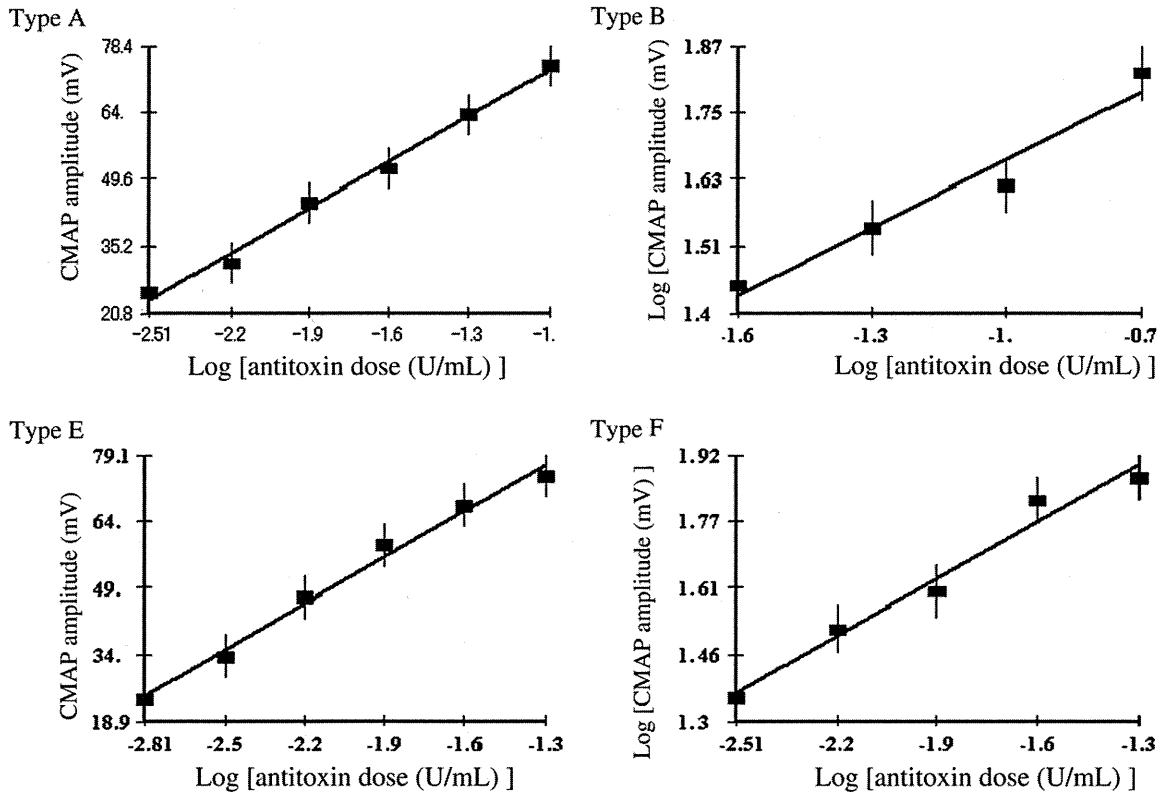

Fig. 1. Dose–response of CMAP amplitude of the test toxin mixed with standard botulinum antitoxin of each type. Rats received mixture of the antitoxin and test toxin into left gastrocnemius muscle. CMAP amplitude was measured for the left hind leg of each rat on day 1 after administration. Each point is the mean ± 95% confidence intervals, $n = 5$.

patient, showing a lower sensitivity than the CMAP neutralization test. ELISA detected neutralizing antibody presence in sera from 2 patients; however, ELISA titers were not correlated with the potency of neutralizing antibodies. This was because ELISA detected all antibodies (including non-neutralizing antibodies) against type A toxin. No antibodies were detected by these methods in the negative control. The antibody titers in serum No. 7 and the positive control detected using the CMAP and mouse neutralization tests showed similar values. Serum No. 7 was also antibody-positive on ELISA (Table 1). Comparing ELISA and the CMAP neutralization test, the correlation coefficient between the two assays was $R^2 = 0.056$ in all sera. No correlation could be identified between these titers.

Table 1
Antibody titers of sera from patients and volunteers measured using the mouse neutralization test, CMAP neutralization test, and ELISA.

Serum No.	Mouse neutralization test (mU/mL)	CMAP neutralization test (mU/mL)	ELISA[a]
1	<100	3	<2
2	<100	4	6
3	<100	4	<2
4	<100	4	<2
5	<100	4	<2
6	<100	5	<2
7	ca)100	50	3
8 (Positive control)	200	190	13
9 (Negative control)	<100	<1	<2

[a] ELISA titer expressed in multiples of the measurement from the negative control value.

As mentioned above, the CMAP neutralization test can be used for the detection of neutralizing antibodies in patients who have received treatment with type A botulinum toxin. Botulinum toxin preparations for treatment are used for various diseases in many patients. Since the therapeutic dose of botulinum toxin is very low, its therapeutic effect may be lost by only minute amounts of antibodies. For patients who show antibody presence, it may be necessary to treat them with increasing toxin doses or to change toxin types. The CMAP neutralization test may be useful to assist in such a diagnosis.

The CMAP neutralization test is capable of detecting minute amounts of neutralizing antibodies, not only against type A toxin, but also against types B, E, and F. Type A and B toxins have already been approved as formulations for the treatment of various disorders, and are currently being used in clinics. However, the effects of type E and F toxins have only recently begun to be investigated in clinical studies, and, in the future, these toxins may therefore be approved as new drugs (Mezaki et al., 1995; Eleopra et al., 1998, 2004). Thus, the ability of the CMAP test to also detect small amounts of neutralizing antibodies against type E and F toxins may be potentially useful for such toxin formulations to be developed in the future. This method is clinically applicable and useful as the measurement is

simple and straight forward, using electromyographs installed at clinical sites.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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Quantitative determination of biological activity of botulinum toxins utilizing compound muscle action potentials (CMAP), and comparison of neuromuscular transmission blockage and muscle flaccidity among toxins

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ABSTRACT

The biological activity of various types of botulinum toxin has been evaluated using the mouse intraperitoneal LD_{50} test (ip LD_{50}). This method requires a large number of mice to precisely determine toxin activity, and so has posed a problem with regard to animal welfare. We have used a direct measure of neuromuscular transmission, the compound muscle action potential (CMAP), to evaluate the effect of different types of botulinum neurotoxin (NTX), and we compared the effects of these toxins to evaluate muscle relaxation by employing the digit abduction scoring (DAS) assay.

This method can be used to measure a broad range of toxin activities the day after administration. Types A, C, C/D, and E NTX reduced the CMAP amplitude one day after administration at below 1 ip LD_{50}, an effect that cannot be detected using the mouse ip LD_{50} assay. The method is useful not only for measuring toxin activity, but also for evaluating the characteristics of different types of NTX. The rat CMAP test is straightforward, highly reproducible, and can directly determine the efficacy of toxin preparations through their inhibition of neuromuscular transmission. Thus, this method may be suitable for pharmacology studies and the quality control of toxin preparations.

1. Introduction

Clostridium botulinum produces toxins that have been classified into 7 serotypes, A–G, based on their immunological characteristics (Sakaguchi, 1983). The toxins act on neuromuscular junctions and induce muscle relaxation by inhibiting acetylcholine release (Sakaguchi, 1983; Jahn and Niemann, 1994). The toxins cause muscle flaccidity, and have recently been utilized to treat spasm in myotonus and dystonia (Jankovic, 2004). Type A and B toxins were approved as drugs for treatment, and are clinically applied. Type C, E, and F toxins have been used in clinical studies (Mezaki et al., 1995; Eleopra et al., 1997, 1998, 2004). The toxins cleave SNARE proteins (i.e., SNAP-25, synaptobrevin, and syntaxin), which fuse to the synaptic vesicle and nerve cell membrane, blocking neuromuscular transmission by

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inhibiting the release of acetylcholine from synaptic vesicles. The different types of toxin are selective for specific SNARE proteins. Type A and E toxins cleave SNAP-25, type B toxin cleaves synaptobrevin II, type C toxin cleaves syntaxin and SNAP-25, and type D, F, and G toxins cleave synaptobrevin I and II. The cleavage sites for each toxin differ, even when toxins cleave the same protein (Montecucco and Schiavo, 1994; Schiavo et al., 2000).

The biological activity of botulinum toxins has been evaluated using the mouse intraperitoneal (ip) LD_{50} test (Pearce et al., 1994). This method is not an assessment of toxin efficacy, which is the inhibition of neuromuscular transmission, but of lethality due to respiratory muscle paralysis caused by the toxin. In this method, the results vary due to the individual sensitivity of mice against toxins, and many mice are required to ensure sufficient accuracy levels for the quality control of preparations. Therefore, the method has posed a problem with regard to animal welfare. International meetings on alternative methods for animal testing have been held, and replacements for the mouse ip LD_{50} test have been discussed (Straughan, 2006). The 3 Rs (refinement, reduction, and replacement) have been proposed for alternative methods, and alternative *in-vitro*, *ex-vivo*, and *in-vivo* test systems have been investigated. The *in-vitro* test system, ELISA, determining the endopeptidase activity, does not use animals, but the sensitivity is lower than the mouse bioassay (Hallis et al., 1996; Wictome et al., 1999). This method could determine only light-chain activity in many cases, and the inaccurate determination of toxin function has been reported. The *ex-vivo* test system using the mouse phrenic nerve-hemidiaphragm is sensitive, but it requires a skilled technique, and has a low reproducibility (Bigalke et al., 2001; Yoneda et al., 2005). The *in-vivo* test systems, such as the digit abduction scoring (DAS) and local flaccid paralysis assays, use scores for evaluation (Aoki, 2001; Takahashi et al., 1990; Sesardic et al., 1996). The methods of evaluation involved scoring, and so they have a disadvantage in that the obtained data are discrete quantities. As each test systems has disadvantages; no alternative method has been established.

We attached a greater importance to the following point in devising an alternative method to determine the activity of botulinum toxin: The potency of botulinum toxin should be evaluated based on their pharmacological effect of inhibiting neuromuscular transmission, and not based on their lethal activity, as in the mouse ip LD_{50} assay. There are several test systems to evaluate the inhibition of neuromuscular transmission, and we focused on the measurement of the compound muscle action potential (CMAP) used for the diagnosis of various nervous disorders. The CMAP is generated by the contraction of muscle fibers; the microcurrent generated by muscle contraction is amplified and recorded. Botulinum toxin affects nerve endings to suppress neurotransmission. Therefore, by determining the CMAP amplitude, the action of the toxin suppressing the transmission of electric stimulation to the muscle can be shown numerically. CMAP measurement is utilized by the extensor digitorum brevis (EDB) test, which checks the response to the toxin before treatment in patients who might have antibodies to the botulinum toxin (Kessler and Benecke, 1997). It was reported that the CMAP amplitude was measured in the rat gastrocnemius muscle which was injected several times with botulinum toxin (Cichon et al., 1995). The CMAP amplitude decreased as the toxin activity increased, but quantitative determination of the toxin was not carried out.

In this study, based on this previous CMAP study, we investigated the quantification of biological activity (effect of neuromuscular transmission blockage) by different types of botulinum toxin. We also compared toxins with an inhibitory effect on neuromuscular transmission. In addition, we investigated the muscle flaccidity-inducing effect of the toxins, compared CMAP data, and the overall effect of the toxins.

2. Materials and methods

2.1. Preparation of toxin

Botulinum neurotoxin type A, B, C, C/D, D, E, and F (150 kDa, NTX) were prepared using modified culture and purification methods, as previously reported (Sakaguchi et al., 1981). *C. botulinum* type A A2, type B Okra, type C CB-19, type C/D 003-9, type D 1873, type E 35396, and type F Langeland were used. For type A, B, E, and F organisms, PYG medium containing 2% peptone, 0.05% yeast extract, 0.5% glucose, and 0.025% sodium thioglycolate was used. For type C, C/D, and D organisms, a basic medium containing 0.8% glucose, 0.5% starch, 1.0% yeast extract, 1.0% ammonium sulfate, and 0.1% cysteine hydrochloride salt was used, and cooked meat (6 g) and 0.5% calcium carbonate were added to 100 mL of the basic medium. The organisms were cultured by allowing them to stand at 30 or 37 °C for 2–3 days. Culture fluid was purified by acid precipitation, protamine treatment, ion-exchange chromatography, and gel filtration of M toxin. M toxin was adsorbed to a DEAE Sepharose column equilibrated with 10 mM phosphate buffer, and eluted with a 0–0.3 M NaCl gradient buffer for NTX and non-toxic protein separation. Each NTX was stored at −70 °C until use.

2.2. Experimental animals

Female ICR/CD-1 mice (4 weeks of age, about 20 g, Charles River Laboratories Japan, Yokohama, Japan) and female S/D rats (8 weeks of age, about 200 g, Charles River Laboratories Japan, Yokohama, Japan) were used for the LD_{50} and CMAP tests, respectively. Animals were maintained under controlled light/dark conditions and had free access to food and water. This study was performed in accordance with the guidelines concerning experimental animals established by the Japanese Pharmacological Society, and was approved by the Animal Ethics Committee of our institute.

2.3. Toxin activity (mouse ip LD_{50}) measurements

The LD_{50} of each NTX was determined following ip injection into mice (Pearce et al., 1994). Seven doses with a dilution factor of 1.25 were used to determine the LD_{50}, and 20 animals were used per dose. Mice were evaluated for the first 96 h after administration, and the LD_{50} was calculated by the probit method.

2.4. CMAP measurements

The different types of NTX were serially diluted with physiological saline containing 0.5% human serum albumin. The following dilutions were prepared: 0.1–300 ip LD_{50}/mL type A, 100–1 × 10^5 ip LD_{50}/mL type B, 1–10,000 ip LD_{50}/mL type C, 0.3–100 ip LD_{50}/mL type C/D, 300–1 × 10^5 ip LD_{50}/mL type D, 1–3000 ip LD_{50}/mL type E, and 10–10,000 ip LD_{50}/mL type F. Rats were anesthetized with an ip injection of pentobarbital sodium (Kyoritsu Seiyaku, Tokyo, Japan). After the eyelid reflex disappeared, the left hind leg was shaved, and 0.1 mL of an NTX dilution was injected into the gastrocnemius muscle of five animals.

The CMAP was measured using a Nicolet Viking Quest (Viasys Healthcare, Tokyo, Japan). Rats were anesthetized and fixed in the prone position. The electrode employed was an alligator clip lead wire (Viasys Healthcare, Tokyo, Japan). The stimulating electrode was placed on the root of the spinal cord, the recording electrode was positioned on the belly muscle of the left hind gastrocnemius muscle, the reference recording electrode was placed on the left hind gastrocnemius tendon, and the earth electrode was positioned on the tail root. Electric stimulation was given at 25 mA for 0.2 ms. The CMAP of anesthetized rats was measured before (0) and 1, 2, 4, 7, and 14 days after administration.

To investigate whether the CMAP amplitude can accurately reflect the inhibition of neuromuscular transmission by botulinum toxin, we evaluated muscle relaxants with different mechanisms of action, nondepolarizing neuromuscular blocking drug, d-tubocurarine (d-Tc, Wako, Osaka, Japan), and a depolarizing neuromuscular blocking drug, succinylcholine (SCC, Wako, Osaka, Japan). d-Tc is an antagonist of nicotinic neuromuscular acetylcholine receptors, and provides muscle relaxation by competitive inhibition of acetylcholine. SCC is binding to the nicotinic acetylcholine receptor, and is opening of the receptor's nicotinic sodium channel; a disorganized depolarization of the motor end plate occurs. SCC is not hydrolyzed by acetylcholinesterase, and occurs to persistent depolarization. The receptor's nicotinic sodium channel is inactivated. When acetylcholine binds to an already depolarized receptor it cannot cause further depolarization. As a result, SCC provides muscle relaxation. So, d-Tc and SCC are relaxed muscle by neuromuscular transmission inhibitory as botulinum toxin. d-Tc and SCC were serially diluted 3-fold to yield 0.1–0.9 and 1–9 mg/mL, respectively. Each drug dilution (0.1 mL) was injected into caudal vein of five animals. Electrodes were attached as described above, and the CMAP amplitude was measured for the left hind leg of each rat at 3 and 2 min after d-Tc and SCC administration, respectively. The rats underwent the insertion of a tracheal tube to maintain respiration after drug administration. The respirator was delivered by SN-480-7 (Shinano manufacturing Co., Tokyo, Japan). Tidal volume was set on the respirator at 2 mL and respiratory frequency at 70 breaths/min.

2.5. Digit abduction scoring assay (DAS assay)

The different types of NTX were compared using the DAS assay (Aoki, 2001), which has been reported to assess the muscle flaccidity-inducing effect of botulinum toxin preparations. In the assay, mice received toxin injection into the gastrocnemius muscle, and the muscle flaccidity-inducing effect of the toxin was determined by the degree of digit abduction. The peak DAS responses were observed on Day 2 or 3 post-injection. The DAS assay was modified for rats, and carried out two days after NTX administration. For negative control, rats were injected with dilution solution (physiological saline containing 0.5% human serum albumin). Rats were suspended by the tail, and the degree of digit abduction in the toxin-treated leg was scored on a 5-point scale by an observer who was masked as to the treatment: score 0 = normal leg extension, and digit abduction equivalent to the contralateral side; score 1 = normal leg extension, but digit abduction differed from the contralateral side or two digits in contact while the other digits completely abducted; score 2 = leg extended, but weak abduction of all digits or three digits in contact; score 3 = leg extended without digit abduction, or leg bent with four digits in contact; score 4 = leg bent with no digit abduction.

2.6. Statistical analysis

The waveforms of a single CMAP were converted to 2000 dots using electromyograph software, and the coordinates of the dots were converted to numbers. The distance between the top and bottom of the waveform was measured as the CMAP amplitude. Statistical analysis for neurotoxin (SAN, ver. 1.07, self made soft) was used to analyze the CMAP amplitude. SAN was created to store raw data of the CMAP amplitude and perform various statistical analyses (i.e., *t*-test, regression analysis, parallel line analysis, and correlation coefficient).

For time course of CMAP amplitude, the MULTTEST procedure of contrast statement was performed using SAS (ver. 9.1).

To determine whether the inhibitory effect on the neuromuscular transmission of each NTX was quantifiable, ip LD_{50} data were plotted versus CMAP amplitudes, and the linearity of the regression line was confirmed by regression analysis.

To evaluate the efficacy of each NTX, the regression line of each NTX was calculated for the peak of the effect, as identified above. Regression lines were used to calculate the doses causing 50% (injection site) and 20% (contralateral site) reductions in the CMAP amplitude, and these values were termed the Effective Dose 50 (ED_{50}) and Toxic Dose 20 (TD_{20}), respectively.

For d-Tc and SCh of the data, the Jonckheere–Terpstra trend test was performed using SAS (ver. 9.1).

3. Results

3.1. Dose-dependent effects of the different NTX types on the CMAP amplitude

The CMAP amplitude was measured to compare the inhibition of neuromuscular transmission at the site of toxin administration. In all types of NTX, the CMAP amplitude decreased depending on the concentration of the ip LD_{50}. Following the administration of type B, C, C/D,

and D NTX, the CMAP amplitude in the left hind leg was reduced for four days at all dose, and was recovered thereafter at type B of or more 30 ip LD_{50}, type C and type C/D of all dose and type D of 100–10,000 ip LD_{50}. Following type A, E, and F NTX administration, the CMAP amplitude was reduced for two days and recovered thereafter at type A of 0.03–3 ip LD_{50}, type E of or more 1 ip LD_{50}, and type F of or more 10 ip LD_{50} (Fig. 1). After the administration of type A, B, C, C/D, D, and F NTX, the CMAP amplitude in the contralateral leg was reduced for four days at type A of more 3 ip LD_{50}, type B of 10,000 ip LD_{50}, type C of or more 30 ip LD_{50}, type C/D of or more 3 ip LD_{50}, type D of 100,000 ip LD_{50}, and type F of or more 3000 ip LD_{50}, and subsequently recovered at type A of 10 ip LD_{50}, type B of or more 3000 ip LD_{50}, type C of 300 ip LD_{50}, type C/D of or more 3 ip LD_{50}, type D of 100,000 ip LD_{50}, and type F of 10,000 ip LD_{50}, respectively. Following type E NTX administration, the CMAP amplitude was reduced for two days at or more 100 ip LD_{50} and recovered thereafter at 300 ip LD_{50} (Fig. 2).

3.2. Analysis of the CMAP amplitude after the different NTX types were administered

Type A, C, C/D, and E NTX reduced the CMAP amplitude in the toxin-injected limb below 1 ip LD_{50} one day after administration. Regression analysis revealed that the CMAP amplitude for type A, B, C, C/D, and D of NTX on days 1, 2, 4, 7, and 14 and type E and F of NTX on days 1, 2, and 4 following administration was related to the LD_{50} concentrations dose-dependently, and linearity was noted when the logit-transformed CMAP amplitude was plotted against the log ip LD_{50}. Although each NTX varied regarding its LD_{50} value for the reduction of the CMAP amplitude, the linearity range of all types of NTX was from the minimum ip LD_{50} to about 10^3 fold (Table 1). Parallel line analysis of the regression lines was performed for each NTX on days 1, 2 and 4, followed by Tukey's test. The regression lines for all types of NTX showed parallelism, except for type C NTX (data not shown).

3.3. Inhibitory effect of the different NTX types on neuromuscular transmission

To compare the inhibition of neuromuscular transmission by the different types of NTX, the ED_{50} was defined as the dose that decreased the CMAP amplitude to 50% of the pre-toxin level, and was calculated for the peak effect at each dose. The ED_{50} rank order of NTX was type D > B > F > E > C > C/D > A. Type A NTX was most potent to reduce the CMAP amplitude, and type D NTX was lowest potent (Table 2).

3.4. Comparison of diffusion to the contralateral site and safety index of the different types of NTX

To compare diffusion to the contralateral site of the different types of NTX, the CMAP amplitude was measured in the right hind leg. The TD_{20} was defined as the dose that decreased the CMAP amplitude to 20% of the pre-toxin level and was calculated to assess the peak effect at each dose. The TD_{20} value rank order of NTX was type D > F > B > C > E > A > C/D. Type C/D NTX was most potent to diffuse to the contralateral site, and type D NTX the lowest potent. The ratio of TD_{20}/ED_{50} was calculated for each NTX, and is expressed as the safety index. The safety index rank order of NTX was type F > C > D > E > A > C/D > B. The results revealed that type F NTX showed the widest safety index and was hard to diffuse, and type B NTX the narrowest index and was prone to diffuse (Table 2).

3.5. Changes in CMAP amplitude induced by muscle relaxants (d-Tc and SCh)

To investigate whether the CMAP amplitude can accurately reflect the inhibition of neuromuscular transmission by botulinum toxin, we evaluated muscle relaxants with different mechanisms of action. The average CMAP amplitudes in groups treated with 0.01, 0.03, and 0.09 mg d-Tc were reduced to about 78, 15, and 2% of that in the vehicle group, whereas those in the groups treated with 0.1, 0.3, and 0.9 mg SCh were reduced to about 68, 12, and 1%, respectively. Differences were significant for both d-Tc and SCh ($p < 0.0001$; Jonckheere–Terpstra trend test). The muscle relaxants induced a dose-related decrease in the CMAP amplitude, similar to botulinum toxin, indicating that the CMAP test can be used to evaluate the inhibition of neuromuscular transmission.

3.6. Comparison of muscle flaccidity induced by the different types of NTX

To compare the muscle flaccidity-inducing effects of the different types of NTX, we evaluated them using the DAS assay. When the log of the ip LD_{50} and median DAS score were plotted on the horizontal and vertical axes, respectively, the reaction curves for each NTX showed dose-dependent linearity. The required toxin value rank order of NTX for muscle flaccidity was type B = D > F > E > A = C = C/D. Type A, C, and C/D NTX were most potent to exhibit a muscle flaccidity-inducing effect, and type B NTX was lowest potent (Fig. 3). The ip LD_{50} dose necessary to achieve a score of 1 in the DAS assay was greater than that required to reduce the CMAP amplitude.

4. Discussion

The neuromuscular transmission inhibitor (d-Tc and SCC) with different mechanisms of action to the botulinum toxin induced a dose-related decrease in the CMAP amplitude. This result indicated that the CMAP amplitude reflected the inhibition of neuromuscular transmission caused by the muscle flaccidly-inducing effect of the drug. The results generated by the CMAP test are continuous data, whereas those of the mouse ip LD_{50} method and scores are discrete data.

We used the rat CMAP test to determine the toxin activity of different NTX types. Type A and C/D NTX reduced the CMAP amplitude one day after administration at 0.01 and 0.03 ip LD_{50}, respectively. In contrast, 10 ip LD_{50} of type B NTX were needed to reduce the CMAP amplitude. Type A, C, C/D, and E NTX required a dose of 1 ip LD_{50} or below, and the CMAP method was more sensitive than the mouse ip

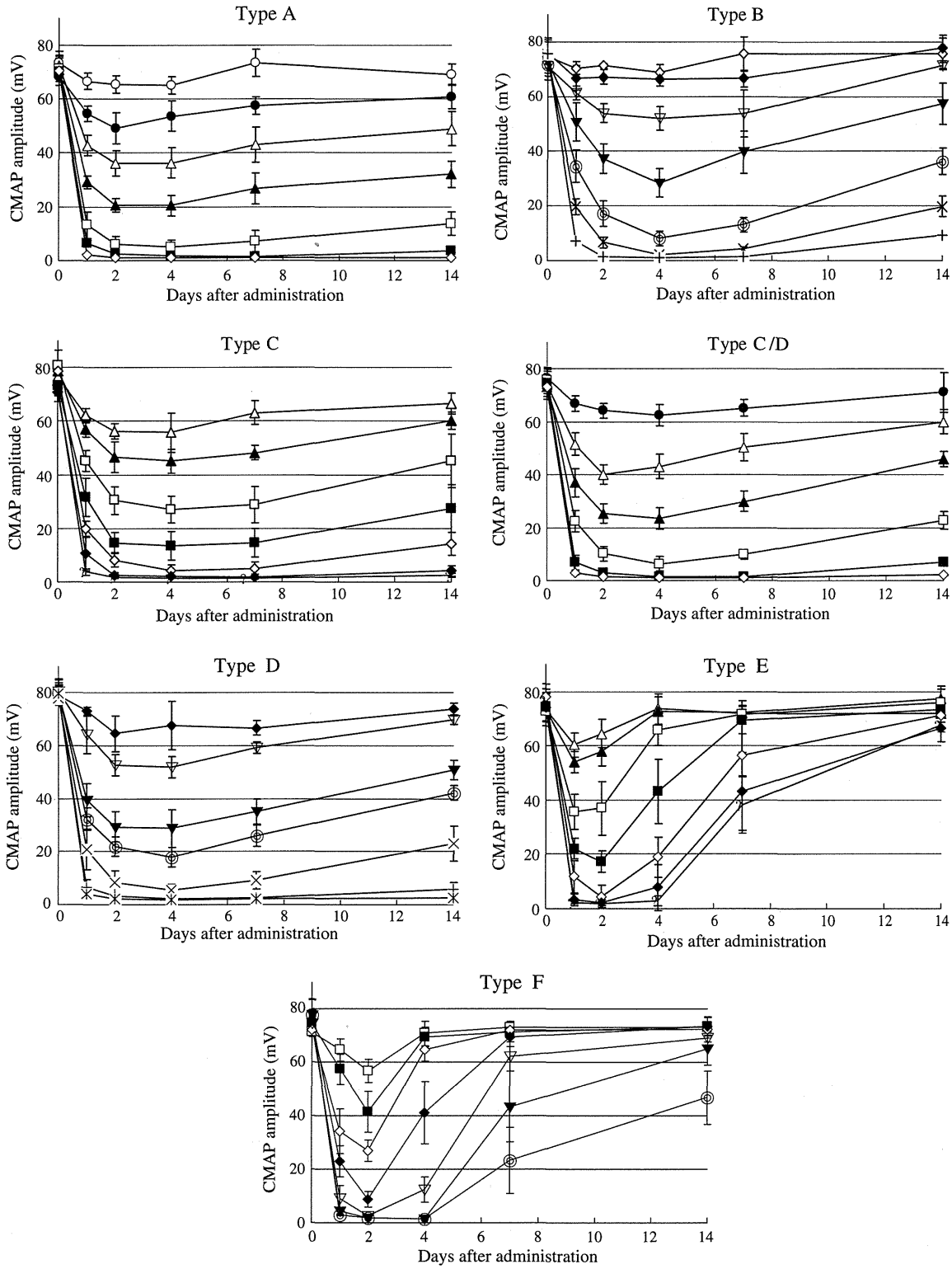

Fig. 1. Dose–response of the CMAP amplitude with the different NTX types at the injection site. Rats received botulinum toxin into left gastrocnemius muscle (○: 0.01 ip LD_{50}; ●: 0.03 ip LD_{50}; △: 0.1 ip LD_{50}; ▲: 0.3 ip LD_{50}; □: 1 ip LD_{50}; ■: 3 ip LD_{50}; ◇: 10 ip LD_{50}; ◆: 30 ip LD_{50}; ▽: 100 ip LD_{50}; ▼: 300 ip LD_{50}; ⊙: 1000 ip LD_{50}; ×: 3000 ip LD_{50}; +: 10,000 ip LD_{50} and ⋆: 30,000 ip LD_{50}). CMAP amplitude was measured for the left hind leg of each rat at before and 1, 2, 4, 7, and 14 days after administration. Each point is the mean ± SD, $n = 5$.

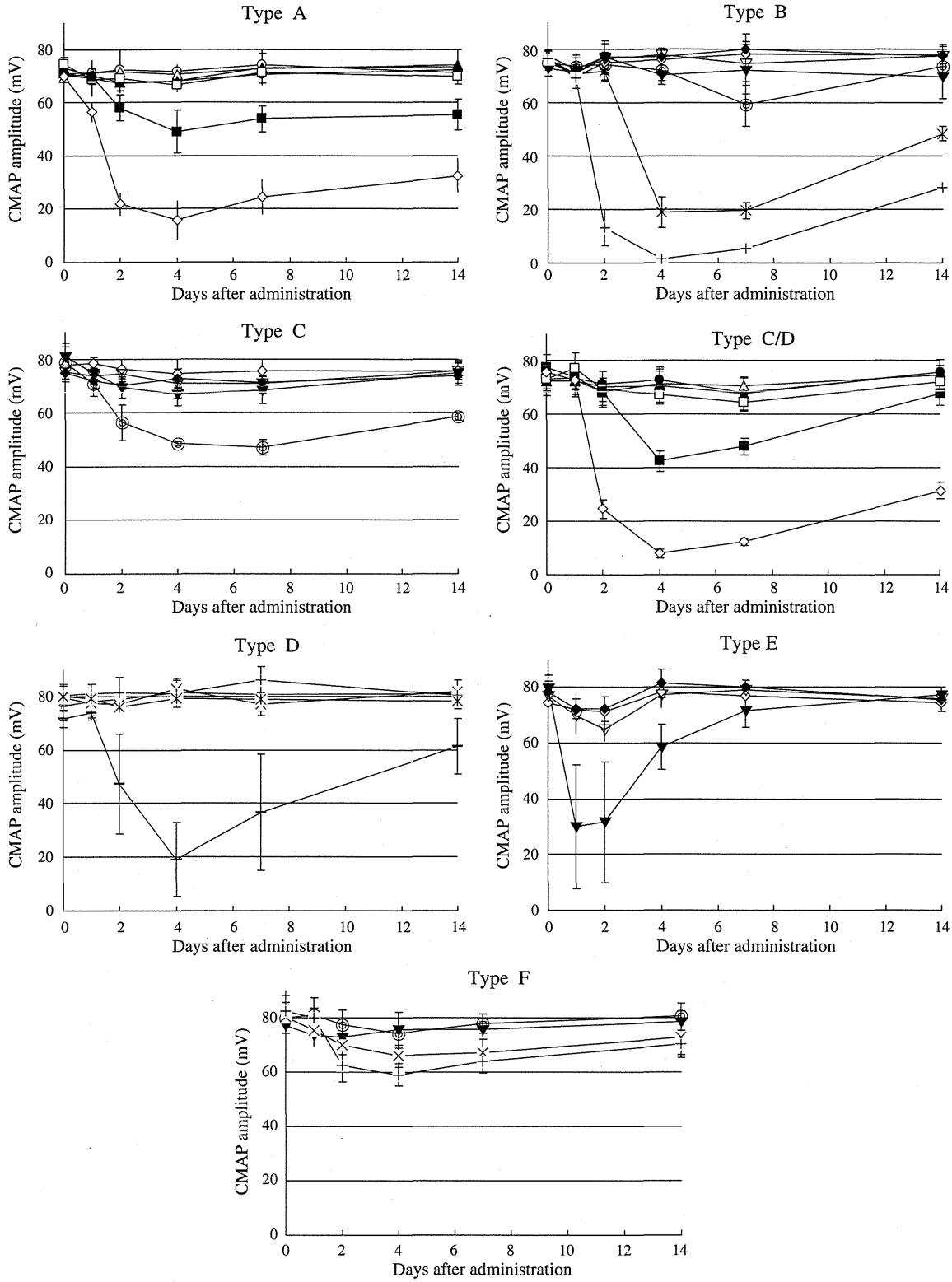

Fig. 2. Dose–response of the CMAP amplitude with the different NTX types at the contralateral site. Rats received botulinum toxin into left gastrocnemius muscle (○: 0.01 ip LD_{50}; ●: 0.03 ip LD_{50}; △: 0.1 ip LD_{50}; ▲: 0.3 ip LD_{50}; □: 1 ip LD_{50}; ■: 3 ip LD_{50}; ◇: 10 ip LD_{50}; ◆: 30 ip LD_{50}; ▽: 100 ip LD_{50}; ▼: 300 ip LD_{50}; ⊙: 1000 ip LD_{50}; ×: 3000 ip LD_{50}; +: 10,000 ip LD_{50}; *: 30,000 ip LD_{50} and –: 100,000 ip LD_{50}). CMAP amplitude was measured for the right hind leg of each rat at before and 1, 2, 4, 7, and 14 days after administration. Each point is the mean ± SD, $n = 5$.

Table 1
Linearity ranges on regression analysis of the CMAP amplitude after the administration of type A to F NTX.

Type	Linearity range (ip LD_{50})				
	1 day (R^2)[a]	2 day (R^2)	4 day (R^2)	7 day (R^2)	14 day (R^2)
A	0.01–30 (0.979)	0.01–10 (0.971)	0.01–10 (0.966)	0.01–10 (0.954)	0.01–10 (0.959)
B	10–10,000 (0.932)	10–10,000 (0.954)	10–10,000 (0.949)	10–10,000 (0.950)	10–10,000 (0.915)
C	0.1–100 (0.957)	0.1–30 (0.957)	0.1–30 (0.955)	0.1–30 (0.968)	0.1–100 (0.946)
C/D	0.03–10 (0.964)	0.03–10 (0.980)	0.03–3 (0.968)	0.03–3 (0.962)	0.03–10 (0.963)
D	30–30,000 (0.942)	30–30,000 (0.953)	30–10,000 (0.958)	30–10,000 (0.949)	30–30,000 (0.937)
E	0.1–30 (0.930)	0.1–30 (0.928)	0.3–100 (0.915)	–	–
F	1–300 (09.37)	1–300 (0.954)	10–300 (0.956)	–	–

[a] R^2: multiple correlation coefficient.

Table 2
ED_{50}, TD_{20}, and safety index values for type A to F NTX.

Type	ED_{50}[a] (ip LD_{50})	TD_{20}[b] (ip LD_{50})	Safety index (TD_{20}/ED_{50})
A	0.09	1.57	18
B	167	1226	7
C	0.54	385	718
C/D	0.13	1.38	11
D	206	36,433	177
E	0.85	50	59
F	4.67	3772	808

[a] ED_{50}, dose which caused a 50% reduction of the CMAP amplitude.
[b] TD_{20}, dose which caused a 20% reduction of the CMAP amplitude.

LD_{50} assay. However, in type B, D, and F NTX, the CMAP method was less sensitive. These results suggest that mice and rats show a different sensitivity to toxins. The results indicated an advantage whereby the method can be used to measure a broad range of toxin activities on the day following administration.

The rat CMAP test is useful not only for measuring toxin activity, but also for evaluating the characteristics of the neuromuscular transmission-inhibitory effect of different NTX types. To assess whether the neuromuscular transmission-inhibitory effect was correlated with the muscle flaccidity-inducing effect, the latter effect of the different NTX types was compared using the DAS assay. The neuromuscular transmission-inhibitory effect (ED_{50}) using the CMAP test was compared to that using the DAS assay, and all types of NTX showed a correlation between the effect of the inhibition of neuromuscular transmission and the potency of muscular flaccidly, except type C NTX. The ED_{50} of type C NTX showed a higher dose than that of type A and C/D NTX; however, the effect of flaccid muscle paralysis was the same. The toxins cleave SNARE proteins (i.e., SNAP-25, synaptobrevin, and syntaxin), which fuse to the synaptic vesicle and nerve cell membrane, blocking neuromuscular transmission by inhibiting the release of acetylcholine from synaptic vesicles. This suggested the possibility that the muscle-relaxing effect of type C NTX is caused not only by the inhibition of neuromuscular transmission through the cleavage of SNARE proteins, but also by other action mechanisms (i.e., the effect on the muscle).

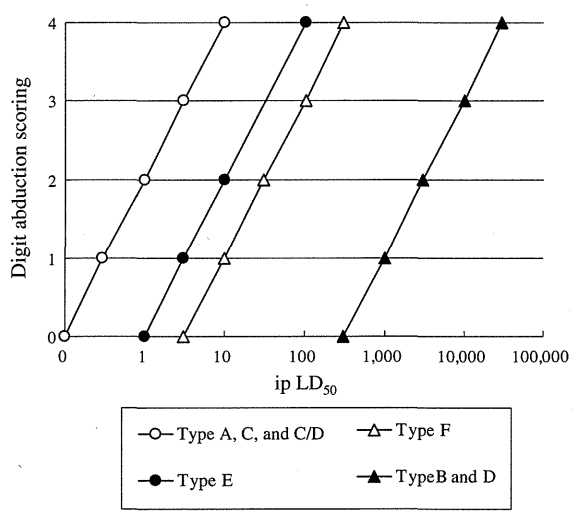

Fig. 3. Comparison of the muscle flaccidity-inducing effect of type A to F NTX on digit abduction in rats two days after administration. The scores indicate the median of each dose, $n = 5$.

Assuming that the findings can be extrapolated to humans, type A and C NTX might show a higher efficacy and safety than other types of NTX as muscle relaxants. Type A NTX showed the strongest effect on the inhibition of neuromuscular transmission and muscle flaccidity, having longer-lasting effects than type B NTX. Type A NTX showed a higher sensitivity than the other types of NTX in humans, and so might be the most suitable as a muscle relaxant. However, the results suggested that type A NTX has the disadvantage that it is prone to diffuse compared to the other types, except for type B and C/D NTX. Type C NTX might be the most suitable for relaxing a particular muscle, because it showed a potent muscle flaccidity-inducing effect and diffuses less than the other types. A dose inhalation toxicity study in monkeys showed equivalent effects between type A and C NTX (LeClaire and Pitt, 2005). In a clinical study, it was reported that type C had a muscle-relaxing effect equivalent to that of type A NTX (Eleopra et al., 1997; Eleopra et al. 2004).

Recently, type C, E, and F toxins have been tried as treatments for various diseases. The inhibitory effect of type E NTX on neuromuscular transmission was the fourth strongest after type A, C, and C/D NTX. The safety index of type E NTX was ranked fourth after type F, C, and D, and the duration of the effect of type E NTX was the shortest. It was reported that the muscular flaccidity-inducing effect of type E and F toxins was of a short duration in humans, similar to the result in this study. Since an effect of type E toxin on the central nervous system was reported (Bozzi

et al., 2006), the toxin may be of use in diseases of the central nervous system. In the future, the other NTX except types A and B may be applied to treatment, and they might be approved as new drugs.

In this study, the rat CMAP test was able to quantify the toxin activity of types A–F toxin. This method is applicable to different types of botulinum toxin preparation which might be marketed, and aids in quality control. This method is useful for evaluating the pharmacological effects of muscle relaxants.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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Editorial

Introduction

Keywords:
Botulinum toxin
Asian
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This supplement is a collection of articles arising from discussions of the Asian Botulinum Toxin Consortium, which consists of Asian movement disorder neurologists (from India, Korea, Japan, the Philippines, Taiwan, Thailand and Singapore) with a research interest in botulinum toxin (BTX). Although Asians account for approximately 30% of the world's population, the clinical data on Asian patients with movement disorders is not as well represented in the literature, except for conditions known to have an Asian preponderance, such as Lubag (sex-linked recessive dystonia parkinsonism of Panay) [1–3]. Intuitively, such information would be interesting. Although the incidence and prevalence of Parkinson's disease in Asia appear to be similar to, or lower than those in Western patients [4,5], Asians are over-represented among patients with hemifacial spasm [6], whereas they appear to be under-represented among patients with dystonia [7]. Asian patients with stroke behave differently from those of Caucasian or African ethnicity [8,9]; yet the presence, or absence, of an 'Asian phenotype' in movement disorders has yet to be established. Interestingly, some authors have reported that Asian patients require lower doses of BTX for the treatment of hemifacial spasm [10] and cerebral palsy [11] than has been reported in Caucasian patients. Clearly, formal clinical trials, comparing patients of different ethnicities, matched for age, gender and weight and using established clinical scales, are required.

BTX, which is one of the most lethal toxins known to man, has emerged in the past few decades as a potent therapeutic agent, used in the treatment of overactive smooth and skeletal muscles, glandular overactivity, as well as in the treatment of painful conditions. Ryuji Kaji has summarised the clinical indications of BTX to date and highlighted new and emerging indications of BTX, such as epilepsy and urgency in the overactive bladder. Roger Aoki examines how BTX ameliorates painful conditions, summarising clinical and non-clinical results to support the hypothesis that BTX reduces the symptoms associated with chronic pain through a two-step process, that is, reduction of local-pain nerve sensitisation through the local inhibition of neuropeptide release, resulting in an indirect reduction of central sensitisation. The benefits derived from the injection of BTX may be negated by spread of the toxin after injection, leading to unintended weakness. This may be reduced by accurate guidance techniques using electromyography (EMG), ultrasonography, endoscopy or imaging. Erle Lim et al. summarise these techniques and discuss why different techniques are more suited to specific muscle groups or conditions. An important point, previously raised by Simpson et al. [12] in an evidence-based review of BTX in the treatment of spasticity, is reiterated by Lim et al. [13] that is, that though intuitively attractive, localisation techniques, such as EMG or ultrasound-guidance techniques, have not been proven to be more effective than surface localisation techniques. This is partly attributable to the different injection techniques (comprising different formulations, dilution volumes and localisation techniques), lack of uniform rating scales, lack of well-designed multicentre studies and small patient numbers that are a feature of current studies. Raymond Rosales et al. review the use of BTX in the treatment of upper-limb post-stroke spasticity, commencing with the view of spasticity as a continuum of changes, spanning early to late neural alterations, as well as biomechanical modifications to muscle. They describe how BTX may be used to improve the functional outcomes.

Three articles in this supplement focus on dystonia. Roongroj Bhidayasiri examines the clinical features of complex cervical dystonia and discusses the prevalence of dystonia in Thailand. Finally, Petr Kaňovský and Raymond Rosales discuss how BTX, when used to treat dystonia, can modulate brain plasticity, thence resulting in long-term alleviation of the symptoms of dystonia.

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Letters to the Editor Related to New Topics

Assessment of Impairment or Monitoring Change in Friedreich Ataxia

Singh et al.[1] reported an experimental study highlighting the oral motor and motor speech deficits present in individuals with Friedreich ataxia (FA). In the abstract and discussion sections of their article, they suggest that this information provides evidence for the use of their assessment protocol for monitoring change in patient functioning.

We believe that the conclusion cannot be supported on the basis of the analytical techniques presented. Importantly, the assessment tools used in the study are either not designed for use in repeated assessment protocols and/or their stability and reproducibility has not been evaluated in an empirical context. Diadochokinetic tasks (or PATA examinations) are notoriously variant in nature, difficult to assess,[2] and their stability over different test–retest intervals is unknown. The "oral motor examination" derived from the Boston Diagnostic Aphasia Examination is unsuitable for objectively monitoring change in oral motor function as it suffers from several psychometric limitations. No measures of test–retest reliability are provided; it is designed for diagnostic decision making and for making an estimate of severity of impairment; thus, its validity as a marker of change is questionable; it has limited intra/inter-rater reliability[3]; and grading of performance is difficult to quantify as it is based on a finite rating scale that relies on clinical expertise and subjective judgment. Perhaps, these reasons account for the authors not conducting reliability investigations on this aspect of their assessment protocol. The Assessment of Intelligibility of Dysarthric Speech (ASSIDS) also provides a useful index of the severity of dysarthric speech by quantifying both single-word and sentence intelligibility, however, perhaps incorporating the other metrics typically used within the ASSIDS [e.g., speaking rate (words per min) and rate of intelligible speech (number of intelligible words per min)] may have yielded more tangible quantitative findings. Finally, it is not surprising that findings from the picture description task (cookie theft) were poorly correlated with measures of disease severity. The gross measure of quantity (i.e., total number of intelligible words) is intrinsically linked to the number of words produced in a sample. Given that the number of words produced by participants is dependent on their expressive language skill and descriptive vocabulary and that no set word limit was applied, monitoring change in patient function using this method may be inappropriate.

Sophisticated methods exist for the application of standardized speech and oral motor assessments where the data generated in response to various challenges are compared to established normative data. Similarly, patterns of strengths and weaknesses on performance measures can be interpreted and aid the process of differential diagnosis by skilled clinicians. Data provided by Singh et al.[1] fulfill this purpose; however, there is not the same sophistication for assessment of speech in monitoring change in patient function. In related fields (e.g., cognitive testing), it is argued that the assessment of behavior for supporting classifications of CNS impairment has a different practical, methodological, and statistical framework for the assessment of behavior to guide decisions about change in the CNS.[4] Singh et al.[1] acknowledge the need for testing that can be "reproduced without excessive technical expertise," yet they fail to recognize that several other criteria need to be met before an assessment protocol is appropriate for monitoring change. Tasks designed to monitor change should be brief, easy to complete, suitably motivating, and preferably have alternate forms (all of which are designed to limit the impact of practice/familiarity). In addition, monitoring intrasubject variation requires a different statistical framework that examines change from baseline rather than differences from controls or normative data.[5] This requires that assessments satisfy assumptions of normality or can be corrected to normal and that they utilize continuous variables that are not restricted by range, floor, or ceiling effects.

Singh et al.[1] rightly state that "long examination times are not ideal for use in FA because of the highly fatigable nature of the patient population" and that some of their assessments are limited by the use of discrete rather than continuous variables. Given the potential implications of conclusions based on assessments that fail to acknowledge the different methodological requirements for monitoring change in patient functioning, it is important that appropriately conservative analyses are undertaken in studies addressing this issue.

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