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〔東女医大誌　第83巻　臨時増刊号
頁　E52～E57　　平成25年1月〕

原　著

日本における脊髄性筋萎縮症の臨床実態調査

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（受理　平成24年12月20日）

Clinical Epidemiological Investigation of Spinal Muscular Atrophy in Japan

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No clinical epidemiological investigations of spinal muscular atrophy (SMA) have been carried out in Japan. We performed a population-based study of SMA to survey the number of patients visiting the departments of pediatrics and internal medicine and to clarify clinical features. Simultaneously, we studied the clinical features and laboratory findings of 110 individuals from whom informed consent had been obtained. The number of patients visiting the hospital was estimated to be 0.5-1 per 100,000 people. The male to female ratio was 1 to 1.14. As to the maximum motor functional level, severity varied among SMA subtypes. Eighty-seven percent of patients with type I demonstrated poor head control. More than half of type II patients could sit unsupported at the same point. All patients with type III were able to walk. Patients with all types of SMA showed the same pattern of muscle weakness, with proximal and upper limb dominance. However, there were some patients showing atypical symptoms. Although most SMA patients have homozygous deletion of *SMN1*, the range of clinical severities is broad. We will continue with additional study to elucidate the disease mechanisms in both typical SMA patients and atypical individuals.

Key Words: spinal muscular atrophy (SMA), clinical investigation, maximum motor functional level, muscle weakness, epidemiology

緒　言

脊髄性筋萎縮症（spinal muscular atrophy：SMA）は，脊髄前角細胞，脳神経核の変性・脱落により，進行性の神経原性筋萎縮を示す常染色体劣性遺伝性疾患である．1891年にWerdnigにより最初の臨床例が報告されて以来，現在に至るまで病態解明のために様々な基礎研究が重ねられている．根本的な治療法はいまだに確立されてはいないが，各国で臨床研究が進められている．

SMAの臨床症状は多様であり，近年まで定義が統一されていなかった[1)2)]．1992年，International SMA Consortiumにより，診断基準と分類が確立された[3)]．I型（Werdnig-Hoffmann病）は，生下時から6ヵ月までの発症で坐位保持は不可能，人工呼吸管理をしなければ2歳までにほとんどが亡くなる重症型である．II型は，1歳6ヵ月頃までに発症し，起立または歩行が不可能であるが，2歳以降も生存可能な中間型とされている．III型は，小児期から成人期に発症し，歩行が可能な軽症型である．臨床的重症度は，それぞれの病型のなかでも多様性が認められ[2)]，III型は発症年齢により，IIIa型，IIIb型に分類されることもある[4)]．神経内科では，診断基準を満たすSMAのみならず，下位運動ニューロンが障害される病態を広くSMAとして認識していることが多く，診断が曖昧であることもあった．成人発症で，進行は緩徐であり，呼吸障害や嚥下障害をほとんど

認めない例は脊髄性進行性筋萎縮症と診断していたが，国際的な分類に合わせて2009年より脊髄性筋萎縮症IV型とされた．わが国では，2009年に，厚生労働科学研究費補助金（難治性疾患克服研究事業）神経変性疾患に関する調査研究班において，SMAの認定基準が作成された[5]．同年，特定疾患治療研究事業の対象疾患に指定されたことにより，SMAにおいて医療社会福祉的な環境は改善されつつある．

SMAは，ほぼ全身の臓器に存在する蛋白質であるsurvival motor neuron（SMN）蛋白の欠損あるいは機能障害によって生じる．SMN蛋白は*survival motor neuron*（*SMN*）遺伝子（*SMN1*）によりコードされており，主に*SMN1*の欠失によりSMAが発症する．*SMN*遺伝子（*SMN1*）は5番染色体長腕5q13にあり，向反性に重複したコピー遺伝子（*SMN2*）も存在する[6]．また，*SMN*遺伝子の近傍には，*neuronal apoptosis inhibitory protein*（*NAIP*）遺伝子も存在し，重症度に関与するといわれている．SMAの遺伝子診断は，*SMN1*と*SMN2*のexon 7とexon 8の領域における塩基配列の5塩基の相違を利用して行われる[7]．

欧米では，その発症頻度は約10,000出生に1人とされ，保因者頻度は約50人に1人といわれている．わが国においては，1978年に福山，大澤らが81家系101例について臨床遺伝学的研究を行ったが[8]，それ以降，本格的な臨床調査は実施されたことがなかった．我々は，SMAの特定疾患治療研究事業の対象疾患への認定を目標として，2003年に臨床調査を施行した．今回は，その結果に基づき，患者数，病型別の臨床症状，などについて分析したので報告する．

対象および方法

1. 推定患者数調査

東京女子医科大学倫理委員会の承認のもと，2003年に郵送によるアンケート方式で疫学調査を施行した．対象は，全国の国公立（当時）病院・療養所，大学病院，療育施設および無作為に抽出した全国の主要病院の小児科，内科または神経内科などで，総施設数は2,620であった．病床規模別にみた施設数は，500床以上は720, 200～499床は1,455, 199床以下は283, 無床は162であった．一次調査として，まず，各施設における患者の有無を尋ねた．一次調査で返信のなかった医療機関へは，一次追加調査として同内容のアンケートを送付した．その結果を，橋本らの「難病の全国疫学調査に基づく患者数の区間推定」[9]の方法に基づいて解析し，その時点でのわが国における通院中の患者数を推定した．

2. 臨床症状調査

一次調査，または一次追加調査で「患者あり」と返信のあった施設（科）に対して，二次調査としてアンケート方式の質問票を郵送した．質問票には，臨床病型，診断方法，最高到達運動機能（生涯で獲得し得た最高の運動能力），筋力低下の状態，筋線維束性収縮の有無，中枢神経障害の有無，遺伝子検査所見などの質問項目を設けた．遺伝子検査に関しては，*SMN*遺伝子および*NAIP*遺伝子の欠失について調査した．最高到達運動機能は，大川らの「Werdnig-Hoffmann病における運動機能レベル」[10]を用いて，0（定頸不可能），1（定頸可能），2（坐位保持可能），3（坐位保持可能かつ，その場まわり可能），4（坐位での移動可能），5（立位保持可能），6（介助ありで歩行可能），7（介助なしで歩行可能），8（介助なしで階段昇降可能）と評価した．さらに，国際SMA協会による診断基準[3]では，筋力低下の特徴を左右対称・近位筋優位・下肢優位・体幹筋罹患としていることから，筋力低下の評価についてはこれらを質問項目に入れた．

結　果

1. 推定患者数調査

質問票の発送総数は2,620, 返信数は888であり，無効（廃院，統合などにより返却）の数を除いて計算すると，回答率は34%となった．各施設から報告された患者総数は455例で，診療科別には，小児科365例, 内科81例, 整形外科5例, 不明が4例であった．橋本らの「難病の全国疫学調査に基づく患者数の区間推定」の方法により，患者実数をもとにして，その時点での患者総数を求めた．対象の医療機関を病床数で分類して検討すると，2003年当時，通院中の国内の推定患者数は741～1,391人となった．総務省によると，2003年のわが国の総人口は127,619,000人であることから，SMAの患者数は100,000人当たり0.5～1人と概算できる．

2. 臨床症状調査

二次調査で報告された患者総数455例のうち，臨床調査の項目別アンケートの返信があった110例について検討した．病型別で分類すると，I型は39例，II型は46例，III型は21例，不明は4例であった．男女比は全体では1：1.14で，各臨床病型別ではI型が1：1.43, II型が1：1.14, III型が1：0.75であった．SMAの発症に男女差はないとされているが，今回の調査でも有意な差異は認めなかった．

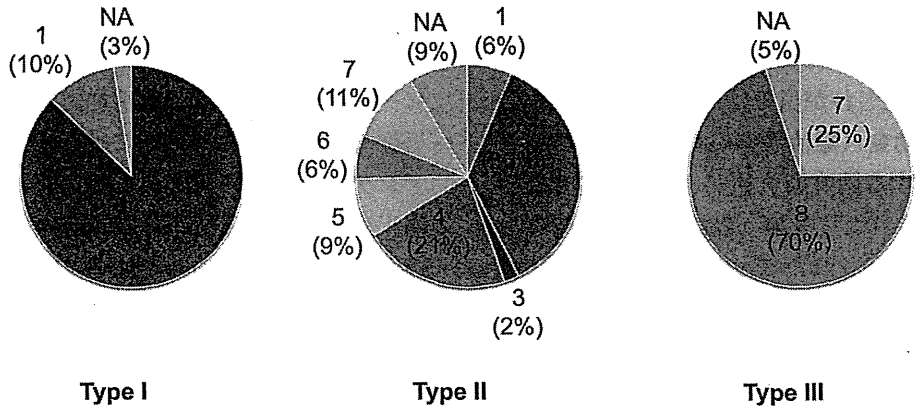

Figure Analysis of maximum motor functional level
The maximum motor functional level represents the patient's peak motor functional abilities. Motor functional level 0: designated as no head control, 1: head control feasible, 2: being able to sit, 3: being able to sit and turn on buttocks, 4: being able to shuffle on buttocks in sitting position, 5: standing with support, 6: walking with support, 7: walking unaided, 8: climbing up stairs without support. NA: (information) not available.

診断方法としては，遺伝子診断が64例（58%），筋生検による病理診断が33例（30%），電気生理学的診断が4例（4%），臨床診断のみが2例（2%），記載なしが6例であった．遺伝子診断は，72例（65%）において施行され，病型別では，I型は31例(74%)，II型は29例(63%)，III型は11例(52%)であり，診療科別では小児科においての実施が多かった．*SMN* 遺伝子はI型の27例(遺伝子検査を施行したI型例の90%)，II型の24例（同II型例の86%），III型の6例(同III型例の75%)に欠失を認めた．*SMN* 遺伝子欠失例のうち，exon7, 8欠失はI型で80%，II型で71%，また，exon7のみ欠失はI型で10%，II型で14%であった．*NAIP* 遺伝子はI型の11例(37%)，II型の2例(7%)に欠失を認め，III型での欠失例はなかった．*NAIP* 遺伝子欠失例は全例 *SMN* 遺伝子欠失を伴っていた．

最高到達運動機能についての結果をFigureに示す．I型では，定頸不可能例は34例(87%)，定頸可能例は4例(10%)であった．II型では，坐位保持可能まで到達した例が16例（36%）と最も多く，介助なしで歩行まで可能であった例は5例(11%)であった．III型では，介助なしで階段昇降まで可能であった例が15例（70%）と多数であり，歩行は全例で可能であった．

筋力低下の状態については，左右の対称性・遠近の優位性・上下肢の優位性・体幹筋罹患の有無を調査した．筋力低下が左右対称であるのは，I型で34例(87%)，II型で37例(80%)，III型で18例(86%)に認めた．筋力低下が左右非対称であるのは，I型で4例(10%)，II型で9例(20%)，III型で3例(14%)に認めた．筋力低下が左右非対称で，かつ，*SMN* 遺伝子欠失のある例は，I型で3例(8%)，II型で5例（11%），III型で2例（10%）存在した．近位筋優位の筋力低下はI型では23例（59%），II型では26例（57%），III型では15例（71%）に認め，遠位筋優位の筋力低下はI型で2例（5%），II型で5例(11%)，III型で4例(19%)存在した．I型，II型では遠近の優位性が不明瞭な例はともに30%程度認めた．遠位筋優位の筋力低下を認め，かつ，*SMN* 遺伝子欠失のある例は，I型で2例(5%)存在した．II,III型で遠位筋優位の筋力低下を認めた例では，筋生検による病理診断で確定診断されていた．筋力低下が下肢優位か否かについても検討したところ，I型は17例（44%），II型は23例（50%），III型は13例（62%）と大多数が下肢優位であり，上肢優位の症例もI型で1例（3%），II型で5例（11%），III型で1例（5%）存在した．上肢優位の筋力低下を認め，かつ，*SMN* 遺伝子欠失のある例は，I型で1例(3%)認めた．体幹筋罹患は，I型で37例(95%)，II型で42例(91%)，III型で12例(57%)に認めた．また，顔面筋の罹患は，I型で17例(44%)，II型で2例（4%）にみられた．顔面筋罹患のある例は，I型では全例，II型では1例が人工呼吸管理を受けていた．舌の筋線維束性収縮は，I型で28例(72%)，II型で25例（54%），III型で2例（10%）に認められた．中枢神経系障害は，I型では8例（21%）に認

められ，低酸素性脳症1例，顔面神経麻痺1例，球麻痺1例，詳細不明が5例であった．II型では中枢神経系障害を1例(2%)に認めたが詳細は不明であり，III型では認められなかった．

考　察

SMAの発症頻度は，欧米では約10,000出生に1人とされ，保因者頻度は約50人に1人といわれている[11]．2003年当時の総人口を基にして検討すると，わが国の推定患者数は100,000人当たり0.5〜1人となった．今回の調査方法では，発症頻度や保因者頻度を算出するのは困難であったため，この結果は概算値にとどまる．諸外国から，発症率あるいは患者数が報告されているが[12)〜18)]，調査方法や対象は統一されておらず，数値にも若干の違いがある．民族による発症率の差はないとされてきたが，近年では，その差異を示唆する報告例も散見される．今後，新しい認定基準を踏まえた上で，再度，わが国における患者数調査を施行することは意義のあることと考える．

SMAの診断方法としては，今回の調査では遺伝子診断が58%，筋生検による病理診断が30%，電気生理学的診断が3.6%，臨床診断のみが1.8%という結果となり，遺伝子診断が多かった．これは，2003年当時の結果であり，2008年に*SMN*遺伝子検査が保険収載されたことを考慮すると，現在では遺伝子診断の比率はさらに増加しているものと考える．遺伝子検査は，確定診断をする上では必須であるが，臨床遺伝専門医による遺伝カウンセリングを行うことが望ましい．

診断基準を満たすSMAは，*SMN*遺伝子の欠失を認めることが多い．わが国では，I型の98%，II型の95%，IIIa型の52%，IIIb型の42%，IV型の15%に*SMN*遺伝子欠失を認めている[19]．本調査では，I型の90%，II型の86%，III型の75%に*SMN*遺伝子の欠失を認めた．前述の*SMN*遺伝子欠失率より，I，II型の欠失率が低いのは，各施設におけるSMAの診断そのものが若干曖昧であった可能性も示唆される．*NAIP*遺伝子はI型の37%，II型の7%に欠失を認め，III型での欠失例はなかった．*NAIP*遺伝子欠失例は全例*SMN*遺伝子欠失を伴っていた．一般に，*SMN*遺伝子とその近傍遺伝子（*NAIP*遺伝子など）の欠失範囲が広いほど，重症であることも明らかになっている[20]．また，本調査において，*SMN*遺伝子exon7のみの欠失を認めた例は，I型では10%，II型では14%存在した．*SMN*遺伝子(*SMN1*)exon7のみの欠失を認める例の中には，*SMN1*からコピー遺伝子である*SMN2*への遺伝子変換を示す例も含まれる可能性もある．*SMN1*から*SMN2*への遺伝子変換を示す例では，臨床症状が軽症になる傾向もあることが示唆されている[21]ことから，今回は未施行であるが，今後，このような*SMN*遺伝子exon7のみの欠失を認めた例において更なる臨床像の分析を進めていくことは有意義である．

SMAの運動機能の評価法として，2003年の調査時は，大川らの「Werdnig-Hoffmann病における運動機能レベル」[10]を用いた．このレベル0からレベル8までの分類により，病型別の大まかな臨床像をみることは可能である．今回の調査でも，各病型の最高到達運動機能を分析し，I型は坐位保持不可能，II型は起立または歩行が不可能，III型は歩行が可能，という診断基準にほぼ合致する結果を得た．現在では，評価者（医師や理学療法士など）による判定の相違を少なくするために，「Hammersmith運動機能評価スケール（Modified Hammersmith Functional Motor Scale)」[22]を用いてSMAの運動機能を評価することが試みられている．

SMAはその臨床病型の範囲が幅広く，前述のI型からIV型のほかに，胎児期発症の最重症例を0型とすることもある．*SMN*遺伝子欠失があっても非典型的な症状を示す例や，*SMN*遺伝子欠失(あるいは同定困難な*SMN*遺伝子変異）がなくてもほぼ典型的な症状を示す例も存在する[20)22)]．今回の調査において，筋力低下という臨床症状に限って検討しただけでも，*SMN*遺伝子欠失があり，かつ，上肢優位，あるいは，遠位筋優位の筋力低下を示す非典型例の存在も明らかとなった．これらの非典型例の存在からも，SMAの病態の複雑さが示唆される．

遺伝子検査では診断できず，臨床診断により確定する例は成人発症例に多い[23]．特定疾患治療研究事業の対象疾患としての認定を受けるためにも，臨床診断は非常に重要といえる．呼吸や嚥下機能障害，側弯症などの合併症へ早期に対応するためにも，早期診断は重要である．現在，SMAの治療法開発に向けて治験の開始準備も進められている．その一環として，希望者が罹患者リストに登録するシステムも構築されつつある．SMAおよびその周縁疾患の臨床像を分析することは非常に重要であり，今後も継続していく予定である．

結　論

2003年当時のわが国におけるSMAの推計通院

患者数は0.5〜1人/100,000人であり，諸外国からの既報告例と概ね同様であった．発症者数，保因者数などについては，引き続き検討を要する．今回の調査では，SMAの確定診断には遺伝子検査を用いた例が半数を占めることが明らかとなった．最高到達運動機能を検討すると，I型では定頸不可能例が，II型では坐位保持可能例が最も多く，III型では歩行は全例で可能であった．臨床症状については多様性が認められた．罹患年齢が幅広いSMAの臨床像の分析は，複数の診療科による協力が不可欠である．診断基準を満たす例のみではなく，SMAの周縁疾患の範疇にある例も含めて，今後も臨床研究を進めていく必要がある．わが国でも統一基準をもって多施設共同研究が可能となるような基盤ができれば，医療的ケアの充実，治療法開発に向けての研究が今後も進展していくと思われる．

本研究は，平成15年度文部省科学研究費基盤研究(課題番号B12470173)の助成によって開始され，平成20年度本学女性医学研究者支援室の助成を受けて進められた．現在は，平成20年度厚生労働科学研究費補助金(難治性疾患克服研究事業)「神経変性疾患に関する調査研究班」(研究代表者　中野今治，分担研究者　斎藤加代子)，および，平成22-24年度厚生労働科学研究費補助金(難治性疾患克服研究事業)「脊髄性筋萎縮症の臨床実態の分析，遺伝子解析，治療法開発の研究」(研究代表者斎藤加代子)において継続して行われている．

本研究の臨床実態調査を施行するに当たり御指導頂きました東京女子医科大学公衆衛生学教室　小島原典子先生に深謝申し上げます．また，アンケートにご回答を頂きました全国の医療機関の先生方にも深謝申し上げます．

開示すべき利益相反状態はない．

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Brain & Development 35 (2013) 165–171

BRAIN & DEVELOPMENT
Official Journal of the Japanese Society of Child Neurology

www.elsevier.com/locate/braindev

Original article

Peripheral nerve abnormalities in pediatric patients with spinal muscular atrophy

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Received 23 November 2011; received in revised form 14 March 2012; accepted 15 March 2012

Abstract

We examined the specific nerve conduction deficits distinguishing spinal muscular atrophy (SMA) subtypes I and II. Five SMA I patients (age, 0.2–1.1 years) and 10 SMA II patients (age, 1.0–2.8 years) were examined. Patients were compared to age-matched controls for motor and sensory conduction velocity (MCV and SCV) changes, compound muscle and sensory nerve action potential amplitudes (CMAP and SNAP), and F-wave occurrence (FO). Slower MCVs were found in three of five SMA I patients; all five exhibited markedly decreased CMAP amplitudes. Tibial nerve CMAP amplitudes significantly reduced in SMA II patients ($p < 0.01$). Slower SCVs and decreased SNAP amplitudes were observed in three of five SMA I patients but not in SMA II patients. Although FOs were reduced in both extremities of SMA I patients, the reduction was prominent in the tibial nerve of SMA II patients ($p = 0.031$). Loss of motor units may be widespread in the early stage of SMA I, while specific to the legs in young SMA II patients. SMA I showed sensory nerve degeneration, especially of large myelinated fibers. SMA II showed no sensory nerve abnormalities.

Keywords: Spinal muscular atrophy; Nerve conduction study; Peripheral nerve abnormality; Sensory nerve degeneration; Wallerian degeneration

1. Introduction

Spinal muscular atrophy (SMA) is a hereditary disease characterized by degeneration and loss of motor neurons in the spinal cord and brain stem. Three clinical types (SMA I–III) are recognized [1,2]. Spinal muscular atrophy type I patients exhibit weakness before 6 months of age and are unable to sit without support, while SMA II patients usually exhibit weakness by 18 months but are able to sit unsupported at some point in their clinical course. Spinal muscular atrophy type III patients generally have a milder course and are able to walk independently. Patients with marked abnormalities in peripheral sensory nerve conduction are excluded by the diagnostic criteria for infantile SMA, but histological studies have shown loss of myelinated fibers, myelin breakdown, and axonal degeneration in sensory as well as motor nerves of SMA I patients [3–6]. For example, sural nerve biopsy in an eight-year-old SMA II patient revealed mild sensory nerve pathology, including myelin breakdown and myelin ovoids (our unpublished case).

Several studies have analyzed nerve conduction in SMA, but samples sizes were small. Furthermore, there

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0387-7604/$ - see front matter © 2012 The Japanese Society of Child Neurology. Published by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.braindev.2012.03.009

was considerable variation in age at assessment, which complicates interpretation because the contribution of different axonal types changes during development. Electrophysiological studies have reported reduced motor conduction velocities (MCVs) in some SMA I patients resulting from loss of large myelinated fibers and smaller compound muscle action potential (CMAP) amplitudes in both SMA I and II patients [7,8]. Conversely, two studies found no reduction in sensory nerve conduction velocity in any type of SMA [9,10], although one reported that sural nerve responses were below detection in all SMA I patients [9].

In light of these contradictory results, the aim of the present study is to assess peripheral nerve conduction abnormalities in pediatric SMA patients in a narrow age range and characterize the nerve and axonal subtypes most affected in the different clinical types.

2. Subjects

Written informed consent was obtained from the parents of all patients in accordance with the Declaration of Helsinki for investigations involving human subjects.

Between September 2001 and January 2011, 15 patients aged 0.2–2.8 years were admitted to National Center Hospital, National Center of Neurology and Psychiatry. All were diagnosed with SMA based on clinical history and typical electromyographic patterns. Peripheral blood samples were drawn for genomic DNA analysis of survival of motor neuron 1 (*SMN1*) and neuronal apoptosis-inhibitory protein (*NAIP*). Patients were diagnosed with SMA I or SMA II according to the criteria established by the International SMA Collaboration Workshop of 1990 [1].

3. Methods

3.1. Electrophysiology

This is a retrospective investigation. On admission to our hospital, all patients are evaluated by nerve conduction study (NCS) under drug-induced sleep to confirm or exclude peripheral neuropathy, while the skin temperature is kept higher than 34 °C. Motor and sensory nerve responses were evoked and recorded using an electromyograph (Neuropack Four, Nihon Kohden Co., Tokyo, Japan).

To evoke CMAPs and the F-waves, supramaximal electrical stimuli (0.2–0.3 ms) were delivered through a two-pronged stimulator placed either over the median and ulnar nerve at the wrist and elbow, respectively, or over the posterior tibial nerve at the ankle and popliteal fossa. The F-wave with the shortest latency (F-wave minimal latency) was selected from 20 consecutive (but clearly identified) F-responses. Surface recording electrodes were placed over the main bulk of the thenar, hypothenar, and abductor hallucis muscles for recording CMAPs and F-waves from the median, ulnar, and tibial nerves, respectively. The latency of the CMAPs and F-waves were measured from the stimulus artifact to the initial negative deflection from baseline. The CMAP amplitudes were measured from the negative to the positive peak. Sensory nerve action potentials were evoked by orthodromic stimulation from a ring electrode placed on the second finger for median nerve recording, on the fifth finger for ulnar nerve recording, or by a two-pronged stimulator placed below the lateral malleolus for sural nerve recording. The SNAPs of the sural nerve were recorded by an electrode positioned at a variable surface position depending on the length of the leg. All SNAPs analyzed were the average of approximately 30 responses evoked using supramaximal stimulus intensity. The latency of sensory conduction was measured from the stimulus artifact to the positive peak of the SNAP, and the SNAP amplitude was measured from the positive to the negative peak.

The nerve conduction parameters from SMA I patients (MCV, CMAP, F-wave minimal latency, F-wave frequency, SCV, and SNAP) were compared to those recorded from non-SMA patients less than 1 year of age, while nerve conduction parameters from SMA II patients were compared to controls between 1 and 3 years of age.

3.2. Control patients

We retrospectively investigated NCSs of nine pediatric patients less than a year old (median: 0.8 years, range: 0.3–0.9 years) and 15 patients between 1 and 3 years old (median: 1.5 years, range: 1.1–2.8 years) examined for different disease conditions over the past 4 years. Neuromuscular disorders were excluded in all but two control patients (one patient aged <1 year with congenital muscular dystrophy and another with Duchenne muscular dystrophy).

3.3. Statistical analyses

The two-tailed unpaired group *t*-test was used to compare the mean MCVs, CMAP amplitudes, sensory conduction velocities (SCVs), SNAP amplitudes, and F-wave minimal latencies between SMA II patients and age-matched controls. The Mann–Whitney *U* test was used to compare the medians of F-wave occurrence (% of evoked responses) of SMA II patients and controls. Differences were considered statistically significant at $p < 0.05$. The MCV, CMAP amplitudes, F-wave latency, SCV, and SNAP amplitudes are expressed as mean ± standard deviation (SD).

4. Results

4.1. Genomic analysis of SMN1 and NAIP genes (Table 1)

Nine boys and six girls with confirmed SMA were recruited for the study (Table 1). The median age at diagnosis was 0.3 years for SMA I (range: 0.2–1.1 years) and 1.9 years for SMA II (range: 1.0–2.8 years). Analyses of *SMN1* and *NAIP* genes confirmed the homozygous absence of *SMN1* exons 7 and 8 in all SMA I patients and in all but one (9/10) SMA II patients. This single SMA II patient (Patient 6) exhibited a single *SMN1* exon 8 deletion. Homozygous absence of *NAIP* exons 5 and 6 was confirmed in three SMA I patients but found in no SMA II patient.

4.2. Motor conduction studies

Motor nerve conduction velocities (MCVs) were slower in three of five SMA I patients (Patients 2, 3, and 5) (Fig. 1a), and all SMA I patients exhibited substantially smaller CMAP amplitudes (Fig. 1b). There was no significant difference between the MCVs of SMA II patients and age-matched controls for any of the three nerves tested (Fig. 1c). In contrast, SMA II patients demonstrated significantly lower CMAP amplitudes of the ulnar and tibial nerves compared to the age-matched controls ($p < 0.000$) and a slightly smaller median nerve CMAP amplitude that did not reach statistical significance ($p = 0.107$) (Fig. 1d).

Table 1
Descriptive characteristics of each patient.

Pt	Age at diagnosis (y)	Sex	Subtype	*SMN1*		*NAIP*	
				Exon 7	Exon 8	Exon 5	Exon 6
1	1.1	F	I	del	del	del	del
2	0.2	M	I	del	del	del	del
3	0.3	F	I	del	del	del	del
4	0.8	F	I	del	del	normal	normal
5	0.3	M	I	del	del	normal	normal
6	2.3	F	II	del	1 copy	1 copy	normal
7	1.5	M	II	del	del	normal	normal
8	2.8	M	II	del	del	1 copy	normal
9	1.3	M	II	del	del	normal	normal
10	1.0	M	II	del	del	normal	normal
11	1.8	F	II	del	del	normal	normal
12	2.0	F	II	del	del	normal	normal
13	1.5	M	II	del	del	normal	normal
14	1.9	M	II	del	del	1 copy	normal
15	2.2	M	II	del	del	normal	normal

del, deletion; *NAIP*, neuronal apoptosis-inhibitory protein; Pt, patients; *SMN1*, survival of motor neuron.

4.3. Sensory conduction studies

The SCVs were slower in the median and sural nerves of three SMA I patients (2, 3, and 5) compared to the control group, while no apparent differences in SNAP amplitudes of either median or ulnar nerve were observed (Fig. 1e and f). A comparison of SCVs and SNAPs from SMA II patients and controls showed no statistical differences in either the ulnar or median nerve (Fig. 1g and h).

The SNAP of the sural nerve decreased with increasing distance between the stimulator cathode and recording electrode; therefore, SNAP values were plotted against this distance. Three SMA I patients (2, 3, and 5) exhibited lower SNAP amplitudes than controls (Fig. 2a), while SMA II patients showed no marked difference in sural nerve SNAP amplitude compared to controls over the same range of inter-electrode distance (Fig. 2b).

4.4. F-wave studies

Prolonged F-wave latency was observed in one SMA I patient (Patient 1) and a decreased F-wave occurrence (% of trials) in two SMA I patients (Patients 1 and 2), (Fig. 3a and b). A comparison of F-wave minimal latencies between SMA II and age-matched controls revealed a statistically significant increase only in the ulnar nerve ($p = 0.017$) (Fig. 3c). The FOs in SMA II patients significantly decreased in both the ulnar ($p = 0.018$) and tibial nerves ($p = 0.034$) (Fig. 3d).

4.5. Nerve conduction studies in the control patients

The average MCVs and CMAP amplitudes of controls aged <1 year (SMA I control group) were 40.5 ± 4.9 m/s and 6.8 ± 3.4 mV in the median nerve ($n = 8$), 43.6 ± 6.0 m/s and 43.6 ± 6.0 mV in the ulnar nerve ($n = 8$), and 36.2 ± 4.3 m/s and 13.3 ± 3.7 mV in the tibial nerve ($n = 9$), respectively. In control patients aged between 1 and 3 years (SMA II control group), these MCV and CMAP values were 50.6 ± 6.1 m/s and 7.4 ± 3.3 mV in the median ($n = 11$), 49.2 ± 4.6 m/s and 10.9 ± 2.3 mV in the ulnar ($n = 12$), and 46.7 ± 7.3 m/s and 14.5 ± 6.1 mV in the tibial nerve ($n = 15$), respectively.

The sensory conduction velocities of controls aged <1 year were 40.4 ± 7.0 m/s in the median ($n = 8$) and 38.7 ± 6.6 m/s in the ulnar nerve ($n = 8$), while SNAP amplitudes in these controls were 10.4 ± 9.1 μV in the median and 9.1 ± 9.2 μV in the ulnar nerve. The SCVs of controls aged 1–3 years were 42.9 ± 5.5 m/s in the median ($n = 9$) and 45.6 ± 6.2 m/s in the ulnar nerve ($n = 12$), while SNAP amplitudes in these patients were 21.1 ± 5.4 μV in the median and 13.7 ± 3.9 μV in the ulnar nerve. The SCV of the sural nerve was $45.5 \pm$

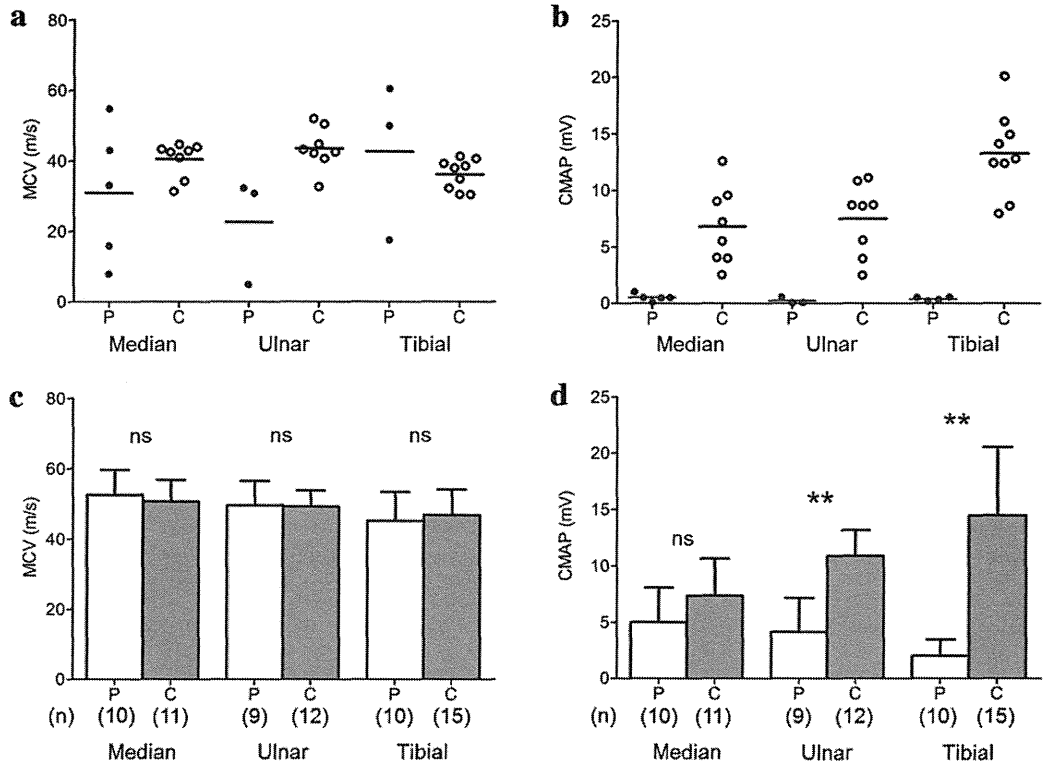

Fig. 1. (a, b) MCV and CMAP values from the ulnar, median, and tibial nerves of SMA I and control patients. Solid lines indicate the means. Three of the SMA I patients show slower MCVs. All five SMA I patients demonstrate decreased CMAP amplitudes in all three nerves. (c, d) Bar graphs comparing MCV (in m/s) and CMAP amplitudes (in mV) between SMA II patients (white) and age-matched control patients (gray). Error bars indicate standard deviations (SDs). (c) There is no statistical differences in mean MCV between SMA II and control patients. (d) CMAPs of the ulnar and tibial nerve were significantly reduced in SMA II patients ($^{**}p < 0.000$). (*n*): sample number, ns: not significant. (e, f) SCV and SNAP values from SMA I (closed circles) and age-matched control patients (open circles). Solid lines indicate the means. (e) Three SMA I patients show slower conduction velocities of the median and sural nerves, while there are no apparent differences in SNAP amplitudes of the median and ulnar nerves between SMA I and the control patients (f). (g, h) Bar graphs comparing the SCVs and SNAPs of SMA II (white) and control patients (gray). Error bars indicate SDs. No statistical difference are seen in any of the tested nerves. (*n*): sample number, ns; not significant.

8.3 m/s in controls less than 1 year of age ($n = 8$) and 50.9 ± 9.1 m/s in those aged between 1 and 3 years ($n = 14$). The SNAP amplitudes of the sural nerve depended on the distance between the stimulating cathode and recording electrodes and are presented in scatter plots (Fig. 2). The *R* values were −0.62 in controls less than 1 year of age ($n = 8$) and −0.47 in those aged between 1 and 3 years ($n = 14$).

The minimum latencies of the F-wave in controls under 1 year of age were 14.4 ± 1.1 ms in the median ($n = 8$), 14.9 ± 0.8 ms in the ulnar ($n = 8$), and 21.5 ± 1.0 ms in the tibial nerves ($n = 9$). The latencies of controls aged between 1 and 3 years were 15.4 ± 1.6 ms in the median ($n = 14$), 15.0 ± 1.5 ms in the ulnar ($n = 13$), and 22.0 ± 2.4 ms in the tibial nerves ($n = 13$). The occurrence of F-waves (FOs, % of trials) in control patients less than 1 year of age and in controls aged between 1 and 3 years are presented in Fig. 3b and d, respectively. Both the mean minimum latency and median occurrence of F-waves were reduced in SMA I patients relative to the controls (Fig. 3a and b); however, the sample size of SMA I patients was small. In SMA II patients, the minimum latency was significantly prolonged in the ulnar nerve. The FO values for each patient were plotted by percentile for F-wave occurrence analysis (Fig. 3d), which revealed that FO was significantly lower in the ulnar and tibial nerves of SMA II patients.

5. Discussion

We assessed the severity of nerve conduction deficits in SMA patients according to clinical type. Patients with SMA I exhibited smaller CMAP amplitudes and decreased F-wave frequencies in all three nerves tested (Figs. 1a and 3b). Similar findings were observed in SMA II, but were especially prominent in the tibial nerve (Figs. 1d and 3d). It is well known that the main pathological changes in SMA involve motor neurons of the anterior spinal horn. The chief electrophysiological findings in patients with motor neuron or axonal degeneration are a decreased maximum CMAP amplitude and but a normal or only minimally reduced MCV [11]. Indeed, SMA II exhibited no significant

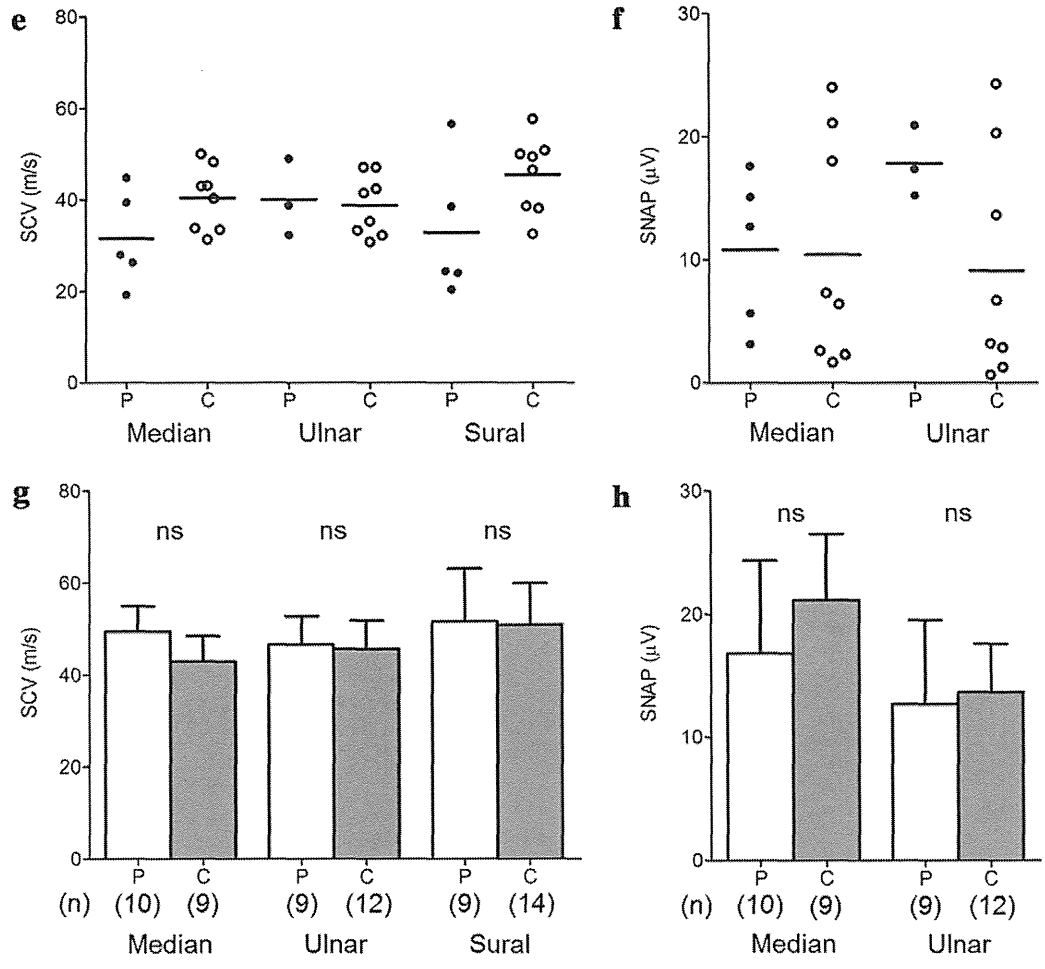

Fig 1. *(continued)*

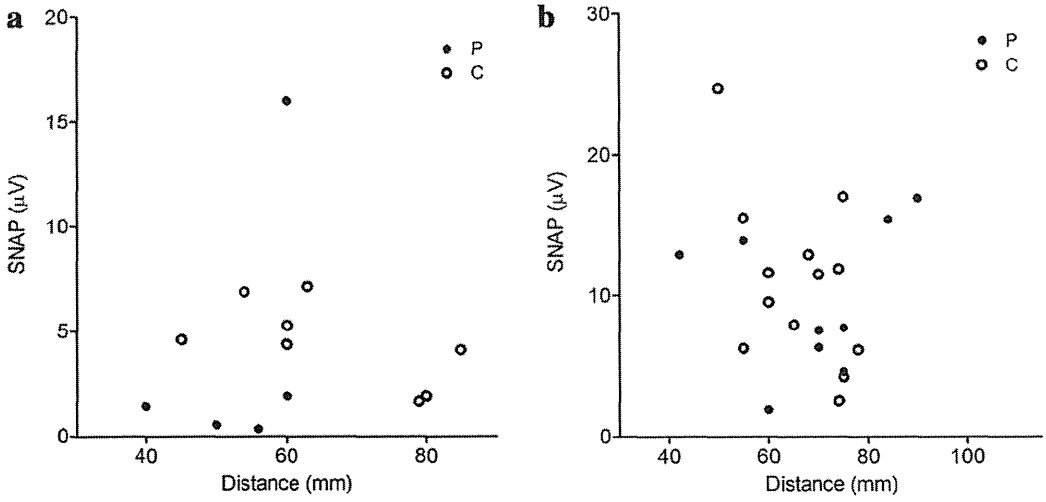

Fig. 2. Scatter plots showing that sural nerve SNAP amplitudes vary with the distance from the stimulating cathode to the distal recording electrode. (a) SMA I and the control patients age <1 year ($R = -0.622$). (b) SMA II and the control patients age 1–3 years ($R = -0.473$). All but one SMA I patient show lower SNAP amplitudes, while SMA II patients exhibit no apparent difference in SNAP distribution compared to controls.

changes in MCV. These results suggest that diffuse loss of ventral horn motor neurons or widespread axonal degeneration occurs at an early stage in SMA I, while these changes occur mainly in the lower extremities of SMA II patients. Slowing of the MCV was also found in three SMA I patients, in accord with several previous

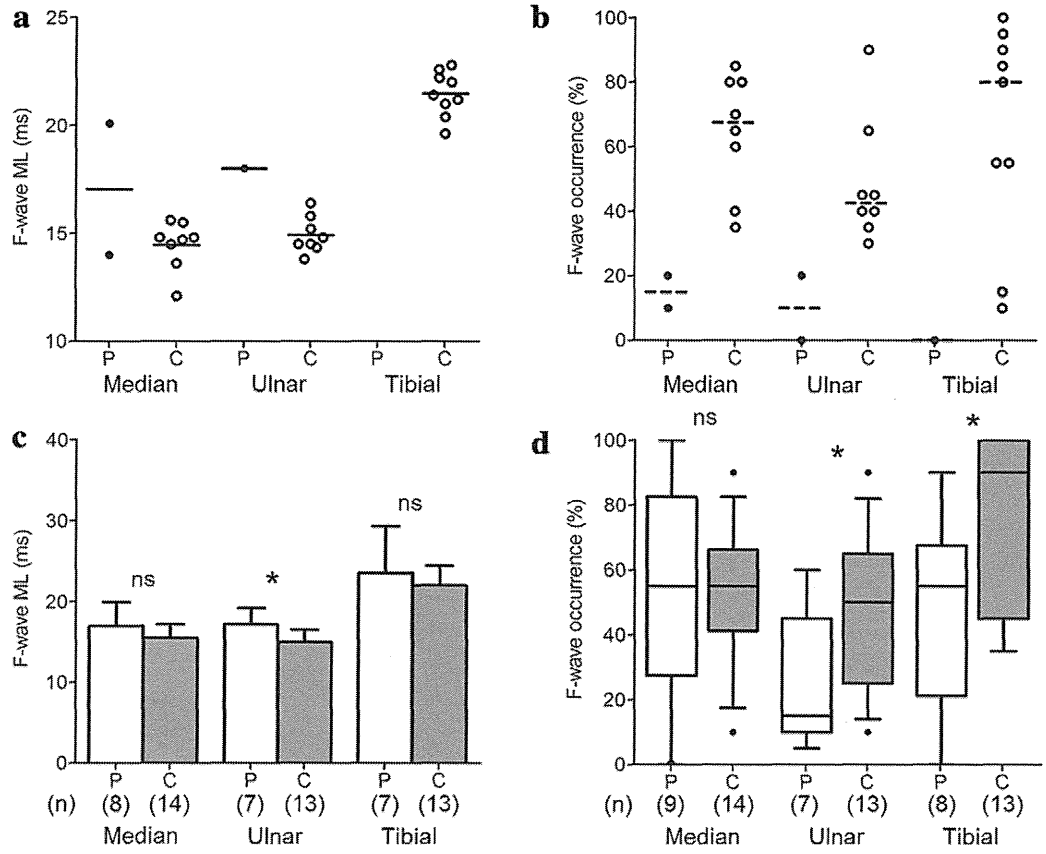

Fig. 3. (a, b) F-wave minimal latency and frequency data of the three nerves in SMA I (filled circles) and control patients (open circles). Solid and dashed lines indicate the means and median. (c) Bar graphs comparing F-wave latencies between SMA II (white) and disease control patients (gray). Error bars indicate SDs. SMA II patients show slow F-wave latencies in the ulnar nerve compared to control patients ($p = 0.013$). (d) Boxplots comparing F-wave frequencies of the three tested nerves between SMA II (white) and control patients (gray). The boxes represent the range from the 25–75th percentile, while the bars span the 10–90th percentile. There is a significant difference (**) between SMA II and the control patients in F-wave frequency of the ulnar ($p = 0.039$) and tibial nerves ($p = 0.031$) by Mann–Whitney U test. (n): sample number, ns; not significant.

studies [7,8,10]. These slower conduction velocities could be caused by demyelination or by loss of fast-conducting myelinated axons, because loss of myelinated peripheral axons can reduce conduction velocity by up to 40% [12]. Chien et al. reported that the number of large myelinated axons was markedly decreased in almost all intramuscular nerve bundles in biopsies of SMA I patients [3]. This reduced MCV in a subpopulation of SMA I patients may reflect loss of spinal motor neurons followed by Wallerian degeneration of axons. In contrast, the normal MCVs in SMA II patients indicate no loss of large diameter myelinated fibers in these patients and suggests that loss is more severe in SMA I patients.

Reduced SNAP amplitude of the sural nerve was observed in all but two SMA I patients (Fig. 2a). Schwartz et al. (1977) did not find measurable sural nerve responses in any of their SMA I patients [9], but the underlying mechanisms were not discussed. Previous reports have shown axonal degeneration of both large and small myelinated fibers of the sural nerve along with signs of Wallerian degeneration [6]. Therefore, it is possible that axonal degeneration can contribute to reduced SNAP amplitudes of the sural nerves. Slower SCVs of the median and sural nerves in some SMA I patients suggest loss of large myelinated fibers in both limbs (Fig. 1e). In contrast, no sensory nerve alterations were reported in histological examinations of sural nerve biopsies [4], and we have encountered only a single case of SMA II with axonal degeneration of the sural nerve (unpublished case). Our SMA II patients showed no apparent difference in SNAP amplitudes compared to control patients (Fig 2b), indicating that SMA II is usually not accompanied by significant degeneration of sensory axons in the sural nerve.

We found significant differences in the pathological progression of SMA I and SMA II. Given that spinal muscular atrophy is a rare genetic disease, this study encompassed a relatively large sample of SMA II patients. In addition, we tested both motor and sensory nerve responses from both the upper and lower extremities and compared these results to well defined age-matched controls. In this study, we included our control patients because they had undergone NCS in the same manner as our SMA patients. However, several limitations should be noted. The sample of SMA I patients was small, which is problematic

because degeneration of large myelinating neurons hinders accurate estimation of conduction parameters. In addition, the distance between recording and stimulating electrodes for sural nerve SNAP amplitude could not be controlled in this retrospective study. Finally, sleep-inducing agents reduce F-wave frequencies, although these agents were applied to both patients and controls. We suggest that the decreased F-wave frequencies in SMA patients reflected axonal degeneration or loss of motor neurons in the anterior horn.

6. Conclusion

In SMA I, the observed reductions in CMAP amplitude and F-wave frequencies in both extremities could reflect diffuse loss of spinal motor neurons. Slower SCVs and reduced SNAP amplitudes suggest that axonal degeneration of sensory nerves, especially large myelinated fibers, can also occur in SMA I. In SMA II, loss of motor neurons was prominent in lower extremities, while no sensory abnormalities were observed.

Acknowledgments

This work was supported by a Grant-in-Aid from the Research Committee of Spinal Muscular Atrophy, the Ministry of Health, Labour and Welfare of Japan, and Intramural Research Grant (23-6) for Neurological and Psychiatric Disorders of NCNP.

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Brain & Development 34 (2012) 213–222

BRAIN & DEVELOPMENT
Official Journal of the Japanese Society of Child Neurology

www.elsevier.com/locate/braindev

Original article

Valproic acid increases *SMN2* expression and modulates SF2/ASF and hnRNPA1 expression in SMA fibroblast cell lines

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Received 1 February 2011; received in revised form 14 April 2011; accepted 14 April 2011

Abstract

Spinal muscular atrophy (SMA) is a common autosomal recessive neuromuscular disorder that is caused by loss of the survival motor neuron gene, *SMN1*. SMA treatment strategies have focused on production of the SMN protein from the almost identical gene, *SMN2*. Valproic acid (VPA) is a histone deacetylase inhibitor that can increase SMN levels in some SMA cells or SMA patients through activation of *SMN2* transcription or splicing correction of *SMN2* exon 7. It remains to be clarified what concentration of VPA is required and by what mechanisms the SMN production from *SMN2* is elicited. We observed that in two fibroblast cell lines from Japanese SMA patients, more than 1 mM of VPA increased *SMN2* expression at both the transcript and protein levels. VPA increased not only full-length (FL) transcript level but also exon 7-excluding (Δ7) transcript level in the cell lines and did not change the ratio of FL/Δ7, suggesting that *SMN2* transcription was mainly activated. We also found that VPA modulated splicing factor expression: VPA increased the expression of splicing factor 2/alternative splicing factor (SF2/ASF) and decreased the expression of heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1). In conclusion, more than 1 mM of VPA activated *SMN2* transcription and modulated the expression of splicing factors in our SMA fibroblast cell lines.

1. Introduction

Spinal muscular atrophy (SMA) is a common autosomal recessive neuromuscular disorder characterized by progressive muscular atrophy of the limbs and trunk, resulting from degeneration of α-motor neurons in the spinal cord. The incidence of the disease is approximately 1 in 6,000 live births, and the carrier frequency is 1/40–1/50 [1]. SMA can be classified into three groups: SMA type I (Werdnig–Hoffman disease; severe form), SMA type II (intermediate form) and SMA type III (Kugelberg–Welander disease; mild form) [2]. This classification is based on the age of onset and the achievement of motor milestones. The gene responsible for SMA is the survival motor neuron (*SMN*), which is present as two highly homologous copies within the SMA gene region on chromosome 5q11.2–13.3: telomeric *SMN* (*SMN1*) and centromeric *SMN* (*SMN2*) [3–6].

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doi:10.1016/j.braindev.2011.04.010

SMN1 and *SMN2* are identical apart from several nucleotide differences. There is a single nucleotide change in the coding region: nucleotide +6 in exon 7 is C in *SMN1* and T in *SMN2*. However, *SMN1* and *SMN2* encode the same protein, because the nucleotide change is synonymous. *SMN1* is homozygously deleted or interrupted in more than 95% of SMA patients [7,8], and deleteriously mutated in the remaining patients [9–11]. Although it encodes the same protein, *SMN2* does not fully compensate for the loss or dysfunction of *SMN1*. In addition, *SMN2* is deleted in approximately 5% of normal individuals [6]. Based on these findings, *SMN1*, but not *SMN2*, has been recognized as the SMA-causing gene.

Interestingly, *SMN2* has never been reported as absent in SMA patients. In addition, the *SMN2* copy number correlates inversely with the disease severity: a higher *SMN2* copy number may ameliorate the clinical phenotype [1,12]. Accordingly, a study using SMA model mice reported that increased copies of *SMN2* could rescue embryonic lethality in mice, indicating modulation of phenotypic severity [13]. Thus, it is thought that *SMN2* may compensate for the loss of *SMN1* to some degree by modifying the severity of the disease through the production of a small amount of functional SMN protein. Increased expression of *SMN2* thus may provide a treatment strategy for SMA, for which there is currently no effective therapy.

SMN1 and *SMN2* show different splicing patterns. All *SMN1*-derived transcripts contain exon 7, i.e., the full-length *SMN* transcript (FL-*SMN* transcript), while the majority of *SMN2*-derived transcripts lack exon 7 (Δ7-*SMN* transcript), because the C–T change in *SMN2* at nucleotide position +6 in exon 7 induces exon skipping [14,15]. In *SMN1*, a heptamer sequence motif including the C at nucleotide position +6 in exon 7 forms a splicing factor 2/alternative splicing factor (SF2/ASF) binding site as an exonic splicing enhancer (ESE) leading to exon 7 inclusion [16–18]. Meanwhile, the corresponding T nucleotide in *SMN2* disrupts the ESE motif and forms a heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) binding site as an exonic splicing silencer (ESS), leading to exclusion of exon 7 [19].

Two SMA treatment strategies targeting *SMN2* have been proposed. The first strategy involves splicing correction which would prevent exon 7 skipping in *SMN2*, thus facilitating the inclusion of exon 7 in the *SMN2* mRNA. Several pharmacological compounds and synthetic nucleotides have been reported as suitable for the first strategy. These pharmacological compounds include aclarubicin (known as an anthracycline antibiotic) [20], sodium vanadate (a phosphatase inhibitor) [21], hydroxyurea (a cell cycle inhibitor) [22] and salbutamol (a β2-adrenoceptor agonist) [23–25]. The synthetic nucleotides are termed ESSENCE (exon-specific silencing enhancement by small chimeric effectors) [16], TOES (targeted oligonucleotide enhancers of splicing) [26] and antisense oligonucleotides masking exonic splicing suppressors [27–29]. Recently, a tetracycline-like compound, PTK-SMA I, has been identified as an alternative to synthetic nucleotides in stimulating splicing of exon 7 [30]. The second strategy is through the activation of *SMN2* transcription. Drugs known to activate *SMN2* transcription are interferons [31] and histone deacetylase (HDAC) inhibitors. HDAC inhibitors which have been reported to activate *SMN2* transcription include sodium butyrate [32], valproic acid (VPA) [33,34], phenylbutyrate [35], benzamide M344 [36], suberoylanilide hydroxamic acid (SAHA) [37], Trichostatin A (TSA) [38] and hydroxamic acid LBH589 [39]. Among these HDAC inhibitors, sodium butyrate, benzamide M344 and VPA are also able to indirectly correct the splicing abnormality, mainly through the upregulation of splicing factors.

VPA has been approved by the U.S. Food and Drug Administration and is already widely used for the treatment of epileptic patients. Some studies have shown that VPA increases the expression of FL-*SMN2* transcript [33,34]. According to Brichta et al., VPA may activate the *SMN2* promoter and correct abnormal splicing of *SMN2* exon 7 in SMA fibroblasts [33]. However, some patients did not respond to VPA treatment at the normal dose given to epileptic patients [40,41]. In this study, we determined the effect of VPA dose on *SMN2* expression in fibroblasts from two Japanese SMA patients. In addition, we also demonstrated changes in two trans-acting splicing factors, SF2/ASF and hnRNPA1, which regulate the splicing of *SMN1* and *SMN2* exon 7.

2. Materials and methods

2.1. Cell culture and VPA treatment

Fibroblast cell lines from a type I SMA patient (OK11, skin biopsy at 10 months old, passage 3–10), a type II SMA patient (AM21, skin biopsy at 25 years old, passage 3–10) were used in this study. Both SMA cell lines are deleted for *SMN1*. OK11 cells carry two copies of *SMN2*, and AM21 cells carried three copies of *SMN2*. In the control cells (CO31), two copies of *SMN1* and *SMN2* are present respectively. These fibroblast cell lines were maintained in Dulbecco's Modified Eagle's medium (Sigma–Aldrich, St.Louis, MO) containing 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal bovine serum (Biological Industries, Haemek, Israel) in a 5% CO_2 atmosphere at 37 °C. VPA (Sigma–Aldrich, St. Louis, MO) was dissolved in aqua dest and was freshly prepared before each use [37]. For the dose-dependency experiment, the fibroblasts were incubated with VPA

at final concentrations of 0 (mock), 0.05, 0.5, 1 and 10 mM for 16 h in a 5% CO_2 atmosphere at 37 °C. This optimal time was established in previous experiment [33].

This study was approved by the ethical committee of Kobe University, and inform consent was obtained from the patients and/or their parents.

2.2. Cell viability assay

To evaluate the cell viability and cytotoxicity of VPA, the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was performed. The cytotoxic effect of 10 mM VPA was studied using OK11 fibroblasts. One hundred microliters of the cell suspension (1×10^5 cells/ml) was placed in the wells of a 96-well culture dish (Iwaki, Chiba, Japan) and incubated in a 5% CO_2 atmosphere at 37 °C. When the cells reached 90% confluency, VPA was added to each well. The final concentration of VPA in the assay was 10 mM. The cell viabilities 2, 4, 8, 16 and 24 h after the addition of VPA were determined using a CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI) which measures the conversion of MTS to violet formazan by dehydrogenases in metabolically active, proliferating cells.

2.3. RNA extraction and cDNA synthesis

Total RNA was isolated from fibroblast cultures in 6-well plates using Sepasol RNA I reagent (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocols. After DNase treatment with DNaseI Amplification Grade (Invitrogen, Carlsbad, CA), total RNA was denatured for 10 min at 65 °C and chilled on ice. Reverse transcription was performed at 55 °C for 30 min in a total volume of 20 μl containing 1 μg of total RNA, 60 μM of random hexamer primers, 1 mM dNTPs, 50 mM Tris/HCl, 30 mM KCl, 8 mM $MgCl_2$ pH 8.5, 20 U of protector RNase inhibitor and 10 U of Transcriptor reverse transcriptase (Roche Diagnostics GmbH, Mannheim, Germany).

2.4. Development of a method for quantification of SMN and splicing factor gene transcript levels

To assess whether VPA is able to influence SMN transcript and protein expression, we treated three passages from each of the two *SMN1*-deleted fibroblast cell lines and the control cell line (OK11, AM21 and CO31, respectively) for 16 h with different concentrations of VPA ranging from 0.05 to 10 mM. After reverse transcription of RNA extracted from the fibroblasts, *SMN* and splicing factor gene transcript levels were determined by quantitative real-time PCR (qRT-PCR).

2.5. Quantitative real-time PCR

qRT-PCR was performed on a LightCycler 1.5 instrument (Roche Diagnostics GmbH, Mannheim, Germany) using FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany). To evaluate the total transcript levels of the SMN genes, we amplified cDNA fragments encompassing SMN exons 1, 2a and 2b. The FL-SMN and Δ7-SMN transcript levels were quantitated from the levels of the products encompassing SMN exons 7 and 8, and SMN exons 5, 6 and 8, respectively. We used glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an endogenous reference gene, and the levels of *SMN* were expressed relative to those of *GAPDH*.

The primers for the FL-*SMN* and Δ7-*SMN* transcripts have been described previously [36]. For the amplification of total-*SMN* transcripts, the primers were designed to bind in *SMN* exon 1 (5′-GCT ATG GCG ATG AGC AGC GGC-3′) and *SMN* exon 2b (5′-GTT GTA AGG AAG CTG CAG TA-3′). *GAPDH* was amplified using primers in exon 2/3 (5′-GAG TCA ACG GAT TTG GTC GT-3′) and exon 4 (5′-GAC AAG CTT CCC GTT CTC AG-3′).

Conditions for all PCRs were optimized regarding the primer concentration, $MgCl_2$ concentration and annealing temperatures. A mastermix of the following reaction components was prepared at the indicated final concentration: 9.4 μl of water, 1.6 μl of $MgCl_2$ (3 mM), 1 μl of forward primer (0.5 μM), 1 μl of reverse primer (0.5 μM), 2 μl of Fast Start DNA Master SYBR Green I and 5 μl of cDNA (equivalent to 40 ng of total RNA). The LightCycler experimental run protocol was as follows: denaturation (95 °C for 10 min), 45 cycles of amplification (95 °C for 15 s, 58–64 °C for 10 s, 72 °C for 25 s with a single fluorescence measurement). Quantitation of the PCR products was performed with the second derivative maximum method of the LightCycler software, using the external standard curve method. qRT-PCR products levels, which correspond to the transcript levels were normalized to those of *GAPDH*. All sample measurements were repeated at least three times and the results were expressed as the mean ± SD.

2.6. Protein extraction and western blotting

Proteins were extracted from the fibroblast cultures by homogenization in lysis buffer containing 1 mM sodium orthovanadate, 1% sodium dodecyl sulfate (SDS) and 10 mM Tris (pH 7.4). Subsequently, the homogenized protein samples were subjected to western blotting. The protein samples were electrophoresed on 10% SDS polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA) by wet blotting. Then, the membranes

were blocked in TBS-T buffer containing 5% dry-milk (ECL™ Blocking Agent; GE Healthcare, Little Chalfont, UK) overnight at 4 °C. Immunostaining of the membranes was performed using several antibodies, each according to the manufacturer's instructions. Detection of the signals with Amersham™ ECL Plus Western Blotting Detection Reagents (GE Healthcare) was carried out using an LAS mini 3000 (Perkin-Elmer Life Sciences, Oak Brook, IL).

The following antibodies were used: mouse anti-SMN (BD Transduction Laboratories™, Franklin Lakes, NJ; 1:1000), mouse anti-splicing factor-2 (SF2/ASF) (Invitrogen Camarillo, CA; 1 μg/ml), mouse monoclonal anti-hnRNPA1 [4B10] (Abcam®, Cambridge, MA; 1:1000), mouse monoclonal anti-beta-actin (Abcam® Cambridge, MA; 1:1000) and peroxidase-linked sheep anti-mouse IgG (ECL, Amersham Biosciences; 1:5000). The intensity of the signals was determined using ImageJ (National Institutes of Health, Bethesda, MD; downloaded from http://rsbweb.nih.gov/ij/). The protein levels were normalized to those of beta-actin. All protein measurements were repeated at least three times and the results were expressed as the mean ± SD.

2.7. Statistical analysis

Statistical analysis of the data was performed using Microsoft Excel 2003 software and Statistical Package for the Social Sciences (SPSS Inc, Chicago, USA). Student's *t* test was conducted to evaluate the differences between groups. A probability of less than 0.05 was considered statistically significant. All data were expressed as the mean ± SD. Analysis of variance test was used to examine the differences between data obtained from the mock and VPA-induced cell cultures.

3. Results

3.1. Cytotoxicity analysis of VPA using a fibroblast cell line

We used the MTS assay to analyze the cytotoxicity of 10 mM VPA in the OK11 cell line after 0, 2, 4, 8, 16 and 24 h incubation with 10 mM VPA. The MTS assay measures survival and/or proliferation of cells [42].

As shown in Fig. 1, the 16 h and 24 h incubation with 10 mM VPA decreased cell viability by 14% and 17%, respectively. This suggests that the 16 h incubation with VPA at this concentration was not particularly toxic to the cell line. Based on these data, we incubated the cells for 16 h in all subsequent experiments.

3.2. VPA induces SMN expression

To confirm the effect of VPA on *SMN* gene expression [33,34], we compared the levels of VPA-induced

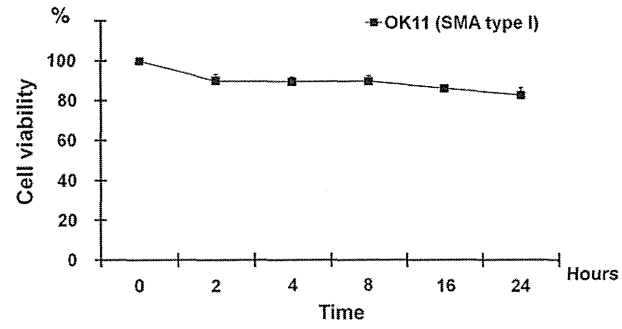

Fig. 1. Cytotoxicity analysis of VPA using the MTS assay. The cell viability of SMA type I fibroblast cell line (OK11) was measured after 0, 2, 4, 8, 16 and 24 h incubation with 10 mM VPA. The cell numbers decreased by only 14% and 17% after 16 h and 24 h incubation with VPA, respectively.

SMN transcripts and SMN protein levels with their respective baseline (mock status) levels in the cell lines, OK11, AM21 and CO31. The culture medium contained VPA at 0.05–10 mM. We measured the total-*SMN*, FL-*SMN* and Δ7-SMN transcript levels and SMN protein levels. Transcript levels were normalized to *GAPDH*, and protein levels were normalized to beta-actin.

The highest *VPA* concentration used in this study, 10 mM, induced the highest total-*SMN* and FL-*SMN* transcript levels in all three cell lines (Fig. 2a and b). The changes in total-SMN transcript levels from 0 to 10 mM of VPA were all statistically significant, at 0.29–0.75 in OK11, 0.44–0.78 in AM21 and 0.68–1.2 in CO31 (arbitrary units relative to GAPDH). The changes in FL-*SMN* transcript levels from 0 to 10 mM of VPA were also all statistically significant, at 0.21–0.51 in OK11, 0.38–0.83 in AM21 and 0.75–1.6 in CO31. Notably, the VPA-induced total-*SMN* and FL-*SMN* levels were similar between OK11 (SMA type I) and AM21 (SMA type II), but that the VPA-induced total-*SMN* and FL-*SMN* levels in CO31 (control) were significantly higher than those in the SMA cell lines. Δ7-*SMN* transcript levels were also significantly increased with 10 mM VPA treatment in cell lines AM21 and C031 (Fig. 2c). However, no change in the ratio of FL-*SMN* to Δ7-*SMN* (FL/Δ7 ratio) was observed in any cell lines (Fig. 2d).

The baseline (mock status) levels of the total-*SMN*, FL-*SMN* and Δ7-*SMN* transcripts in the SMA type I fibroblast cell line, OK11, were significantly lower than those in the SMA type II fibroblast cell line, AM21 (Fig. 2). These baseline *SMN* levels may reflect the differing copy number of the *SMN*2 gene among the cell lines. However, during VPA treatment, the increases in the transcript levels in OK11 and AM21 were similar to each other, which does not reflect the copy number.

To determine the SMN protein levels, we performed western blotting with a monoclonal antibody directed against the amino-terminus of the SMN protein.

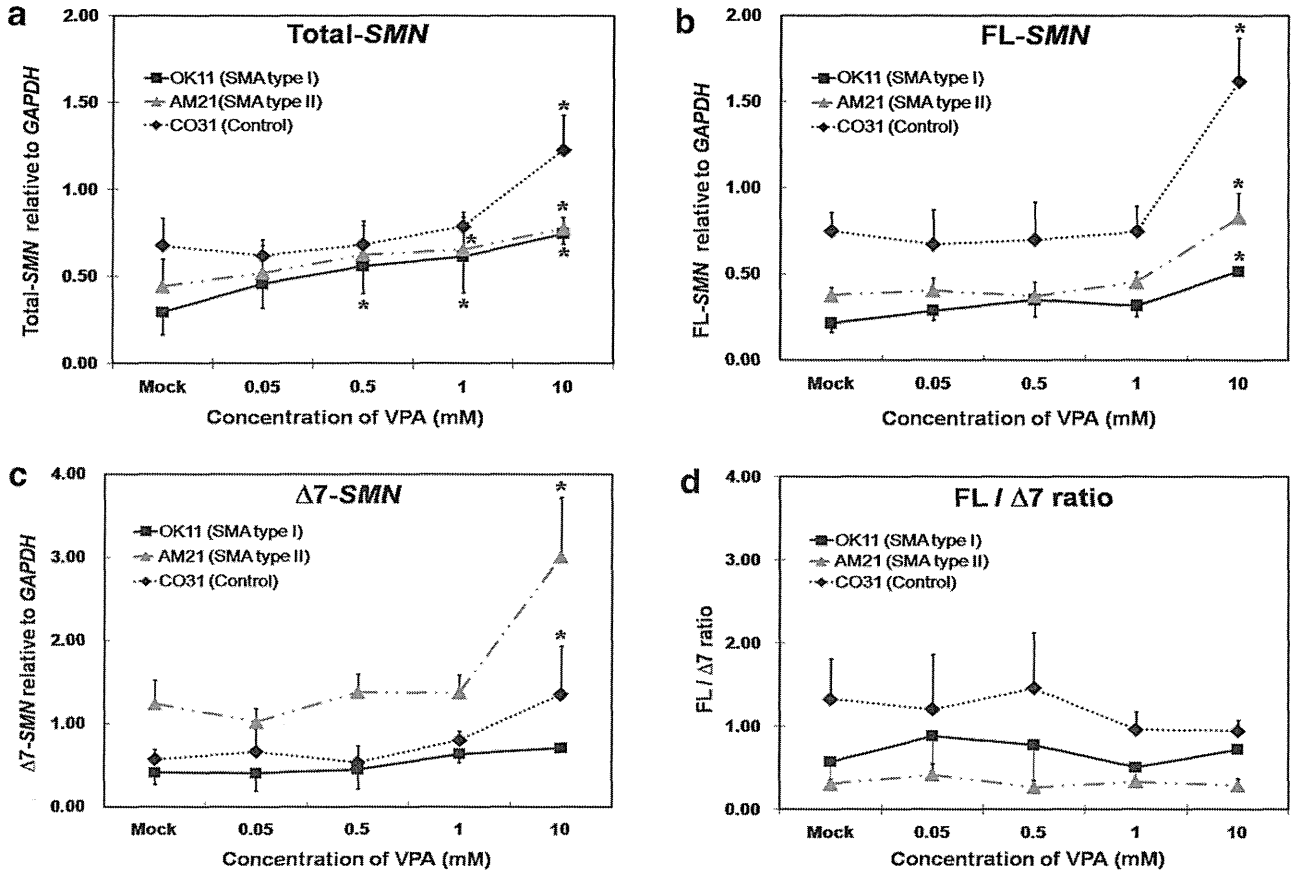

Fig. 2. Quantitative analysis of *SMN* transcripts. Upregulation of *SMN* transcript levels determined by quantitative real-time PCR in the cell lines, incubated for 16 h with different concentrations of VPA. All data are expressed as mean ± SD in arbitrary units relative to *GAPDH*. (a) Total-*SMN*. (b) FL-*SMN*. (c) Δ7-*SMN*. All SMN transcript levels reached their maximum at the highest VPA concentration, 10 mM. $^{*}p < 0.05$ *vs.* the baseline (mock). (d) The calculated FL/Δ7 ratios showed nearly unchanged levels, suggesting no effect on *SMN2* splicing after treatment with up to 10 mM VPA for 16 h.

Because the Δ7-SMN protein is essentially undetectable by western blotting [43], the SMN protein that we detected in this study was mainly FL-SMN protein. As shown in Fig. 3a, western-blotting revealed a slight increase in SMN protein expression in all three cell lines. The changes in SMN protein levels upon treatment with

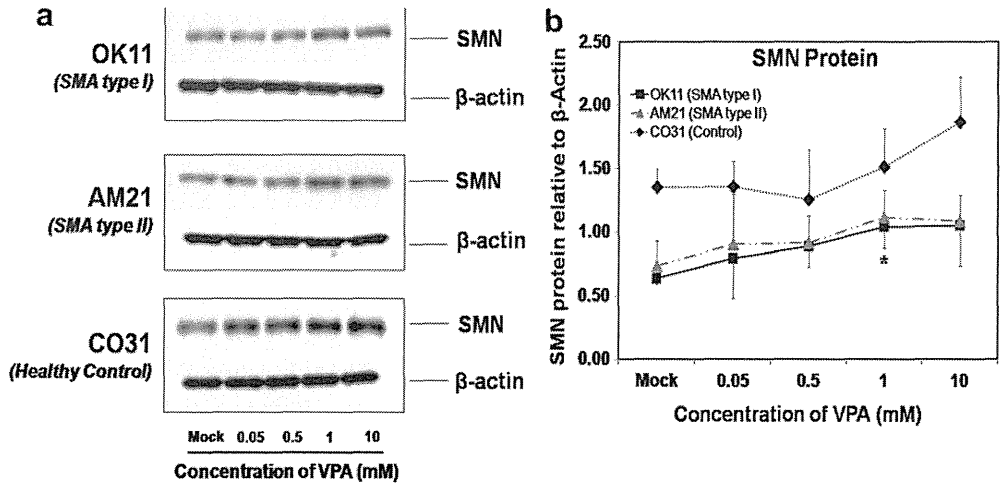

Fig. 3. Quantitative analysis of SMN protein. (a) Representative western blotting data illustrating the increase in SMN protein levels with VPA concentration. (b) Upregulation of SMN protein levels determined by western blotting in the cell lines, incubated for 16 h with different concentrations of VPA. The data are expressed as mean ± SD in arbitrary units relative to beta-actin. Densitometry revealed a 1.5-fold increase in SMN protein at 1 and 10 mM VPA relative to the baseline (mock) levels. $^{*}p < 0.05$ *vs.* the baseline (mock).

0–10 mM of VPA were 0.63–1.05 in OK11, 0.73–1.08 in AM21 and 1.3–1.86 in CO31 (arbitrary units relative to beta-actin; Fig. 3b). The change from the baseline only reached significance for the OK11 line at 1 mM VPA. Compared with the baseline levels of SMN protein (0 mM), we observed an approximately 1.5-fold increase in SMN protein expression in SMA cell lines treated with 1–10 mM.

The baseline SMN protein levels in the SMA type II cells, AM21, were slightly higher than those in the SMA type I cells, OK11. However, during VPA treatment, the SMN protein levels were almost identical. *SMN2* copy number did not contribute to the increase in SMN protein levels in both of our SMA cell lines.

3.3. VPA modulates splicing factor expression

Brichta et al. reported that VPA increased the levels of the splicing factors SF2/ASF, Htra2-β1 and SRp20 [33]. However, they did not describe the effect of VPA on other splicing factors, such as hnRNPA1. Thus, we studied whether VPA treatment changes SF2/ASF or hnRNPA1 protein levels relative to the baseline (mock status) levels in cultured fibroblasts.

Transcript levels of the splicing factors, SF2/ASF or hnRNPA1, upon treatment with 0–10 mM of VPA were fluctuated in our fibroblast cell lines, showing no tendency to increase or decrease with VPA amount (data not shown). However, western blotting revealed an increase in SF2/ASF protein expression in all three cell lines with 1–10 mM VPA (Fig. 4a). The maximum changes in SF2/ASF protein levels were 0.59–0.91 in OK11, 0.65–1.25 in AM21 and 0.96–1.56 in CO31 (arbitrary units relative to beta-actin). Compared with the baseline (mock status), we observed a 1.5–2-fold increase in SF2/ASF protein expression in all cell lines treated with 1 and/or 10 mM VPA (Fig. 4a). On the other hand, treatment with 1–10 mM VPA led to a significant decrease in hnRNPA1 protein expression in all three cell lines (Fig. 4b). The changes in hnRNPA1 protein levels were 0.56–0.29 in OK11, 0.77–0.29 in AM21 and 0.85–0.51 in CO31. Compared with the baseline (mock status), we observed an approximately 2-fold decrease in hnRNPA1 protein expression in all cell lines treated with 10 mM VPA. It should be noted that the ratio of SF2/ASF to hnRNPA1 also increased with increase in the VPA concentration (Fig. 4c).

4. Discussion

4.1. VPA induction of FL-SMN transcript and SMN protein

We showed in this study that VPA treatment increased total-*SMN* transcripts, FL-*SMN* transcripts and SMN protein levels in SMA fibroblast cell lines. VPA is an HDAC inhibitor [44,45]. Treatment with HDAC inhibitors increases acetylated histone levels and relaxes chromatin structure, resulting in the activation of many genes including *SMN2* [33–39,46]. Our SMA cell lines demonstrated an approximately 2-fold increase in FL-*SMN* transcripts and a 1.5-fold increase in SMN protein levels after 16 h incubation with 10 mM VPA. These results are consistent with previous studies of the effect of VPA on *SMN* expression [33,34].

Brichta et al. reported that a 16 h incubation with VPA at concentrations less than 0.5 mM increased FL-*SMN* transcripts in SMA fibroblast cell lines [33]. These authors also reported that 0.5 nM–0.5 mM VPA increased the production of FL-*SMN2* transcripts and SMN protein levels 2 to 4-fold. They also predicted that a considerable increase in SMN protein could be obtained at even lower VPA concentrations than those used in epilepsy treatment, because the serum VPA level required for epilepsy therapy is 0.48–0.7 mM [47].

In contrast, Sumner et al. reported that a 24 h incubation with 1 mM VPA did not increase FL-*SMN* transcripts in SMA fibroblast cell lines [34]. This agrees with our data showing that a 16 h incubation with 1 mM VPA did not increase FL-*SMN* expression in our SMA fibroblast cell lines. The effective concentration of VPA may depend on the responsiveness of *SMN* to VPA in the cell line.

With regards to *SMN2* copy number, our study show that, before VPA treatment, the OK11 fibroblast cell lines with zero *SMN1* and two *SMN2* copies showed significantly lower baseline levels of FL-*SMN* transcripts and SMN protein than those of the AM21 fibroblast cell lines with zero *SMN1* and three *SMN2* copies (Figs. 2b and 3). However, during VPA treatment, both fibroblast cell lines produced similar levels of FL-*SMN* transcript and SMN protein. Contrary to our expectations, our SMA fibroblast cell lines responded similarly to the VPA treatment, regardless of their different *SMN2* copy numbers. This suggests that the *SMN2* copies are not equivalent in response to VPA treatment.

4.2. Modulation of splicing factor expression by VPA

Brichta et al. and Sumner et al. reported that, in SMA fibroblasts, the FL/Δ7 ratio increased 1.5–2-fold after VPA treatment [33,34]. Both of these reports concluded that the increase in VPA-induced FL-*SMN* could be explained not only by *SMN2* transcriptional activation but also *SMN2* exon 7 splicing correction. The upregulation of the FL/Δ7 ratio suggested modulated production of splicing factors in the cells.

VPA activates not only *SMN2*, but also many other genes, including those encoding splicing factors. Brichta et al. showed that VPA induced overexpression of the splicing factor, Htra2-β1, in the SMA cells, and these authors speculated that overexpressed Htra2-β1 plays